UNIVERSITY OF CALIFORNIA, SAN DIEGO

Cyclin D:Cdk4/6 Complexes Activate Rb by Mono-Phosphorylation

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Biology

by

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2013

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DEDICATION

I would like to dedicate this dissertation to my parents, who have supported me in all facets of life. Without their support and encouragement, none of this would be possible.

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ACKNOWLEDGEMENTS

I would like to acknowledge the numerous people that have helped me write this dissertation. First, I would like to thank my mentor, Dr. Steven Dowdy, for his constant encouragement and positive attitude during this whole process. I would like to thank the rest of my committee for their insightful comments and suggestions. I want to acknowledge Dr. Gary Shapiro, who initiated this project; for Dr. Sergei Ezhevsky, who taught me the basics of 2D IEF; for Dr. Manuel Kaulich, who mentored me scientifically; for Dr. Bryan Meade, who was always there to talk science or other unrelated topics; for Dr. Xianshu Cui, who always kept me organized; and for all the members of the Dowdy Lab for their scientific input.

I would like to acknowledge my parents, my brother, and my sister-in-law for their unconditional love and support. I would like to acknowledge my fiancée Vasudha, who was always there for me even in the toughest of times. Also to my peers in the Department of Biology for the occasional hangout.

I would like to acknowledge Dr. Piotr Sicinski from the Dana-Farber Institute for Cyclin D Knockout cells that were used in the manuscript and in this dissertation.

All the chapters (Chapters 1-6), in part, of this dissertation were taken from a prepared manuscript, where I was the primary author/researcher. The manuscript is currently in preparation - Narasimha AM, Kaulich M, Shapiro GS, Sicinski P, Dowdy SF. Activation of RB by Mono-Phosphorylation *(In Preparation)*.

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1. **Narasimha A**, Shi W, Dowdy SF. Cell-Cycle Controls in G1 and G0 *Encyclopedia of Biological Chemistry. Second Edition* 401-406.

2. **Narasimha AM**, Kaulich M, Shapiro GS, Sicinski P, Dowdy SF. Activation of RB by Mono-Phosphorylation. *(In Preparation)*

ABSTRACT OF THE DISSERTATION

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by

Anil Madihally Narasimha

Doctor of Philosophy in Biology

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Professor Steven F. Dowdy, Chair
Professor Tony Hunter, Co-Chair

The prevailing model of G_1 cell cycle progression proposes Cyclin D:Cyclin-dependent kinase 4/6 (Cdk4/6) complexes inactivate the retinoblastoma tumor suppressor protein (Rb) during Early G_1 phase by progressive multi-phosphorylation or "hypo-phosphorylation" to release E2F transcription factors, resulting in the gradual activation of Cyclin E:Cdk2 complexes. However, due to the use of supra-physiologic overexpression studies, absence of quantification of

how many phosphates are present on “hypo-phosphorylated” Rb, and lack of clarity on what is the active isoform of Rb, this model remains largely unproven. Rb contains 16 Cdk phosphorylation sites and is thought to exist in three general isoforms: 1) un-phosphorylated Rb, 2) “hypo-phosphorylated” Rb, and 3) inactive hyper-phosphorylated Rb; however, the un-phosphorylated Rb and “hypo-phosphorylated” Rb isoforms cannot be resolved by 1D SDS-PAGE. Using highly synchronized primary and tumorigenic cells, performing biochemistry on physiologic levels of activities of proteins, and developing 2D isoelectric focusing (2D IEF) of Rb, I show that Rb is exclusively mono-phosphorylated on 14 different sites during Early G_1 phase and that this phosphorylation is mediated by Cyclin D:Cdk4/6 complexes. Mono-phosphorylated Rb functioned to induce a G_1 arrest, bind E2F transcription factors and regulate the global transcriptional profile, whereas un-phosphorylated Rb was non-functional, showing that mono-phosphorylated Rb is the biologically active isoform of Rb. These observations fundamentally change our understanding of G_1 cell cycle regulation and show that Cyclin D:Cdk4/6 complexes *activate* Rb by mono-phosphorylation during Early G_1 phase. Importantly, these observations point to the activation of Cyclin E:Cdk2 complexes as a likely key oncogenic step in the progression of cancer.

CHAPTER 1:

Introduction

Discovery of the Retinoblastoma Protein

The retinoblastoma tumor suppressor gene (Rb) was initially discovered as a gene that was lost during development of retinoblastoma, a pediatric cancer of the eye (Knudsen and Knudsen, 2006). In this type of cancer, malignant tumors grow in the retinal layer of the eye. The cancer itself has two distinct forms – familial and sporadic. The familial or hereditary form of retinoblastoma is an autosomal dominant disorder that can be passed on to subsequent generations (Draper et al., 1992). It is also characterized by early onset and increased multiplicity of tumors that occur in both eyes. Familial retinoblastoma is found in 30 to 40 percent of patients (Draper et al, 1992). The second type of retinoblastoma is known as sporadic retinoblastoma, which involves a single tumor (Knudsen and Knudsen, 2006).

Familial retinoblastoma was first characterized by Dr. Alfred Knudson in the 1970's, when he concluded through the use of extensive statistical analyses that the full-onset of the disease is carried out by two separate genetic events (Horowitz et al., 1990). Known as the "Two-hit hypothesis," Knudson observed that this was consistent with a recessive mutation, but required both alleles being altered (Knudson, 1996). The finding that both alleles required alterations was further substantiated when it was shown that a malignant phenotype was lost when a tumor cell was fused with a normal cell (Stanbridge, 1976). These data became the basis and eventual discovery of tumor suppressor genes and their role in cancer progression. In contrast, oncogenes usually only require one independent genetic event because they are normally gain-of-function mutations. Following the

publication of the "Two-hit hypothesis," many laboratories started researching the genetic basis of retinoblastoma. One of the key findings from this research was the observation that there was genetic inactivation of a locus on chromosome 13 in retinoblastoma lesions (Godbout, 1983; Cavenee et al., 1983). These data resulted in the hypothesis that the loss of the retinoblastoma tumor gene on both alleles was necessary in the formation of this cancer. The Weinberg laboratory was the first laboratory to clone the Rb gene in late 1986, when they isolated a complementary cDNA fragment of about 70 kilobases detecting the chromosomal locus 13q14 (Friend et al., 1986). They also noticed that this cDNA fragment was not normally expressed in retinoblastomas and certain osteosarcomas (Friend et al., 1986). Soon after, multiple laboratories were able to sequence Rb (which they had called the retinoblastoma susceptibility gene) (Lee et al., 1987; Fung et al., 1987; Friend et al., 1986). They noticed that mRNA of Rb was abnormal and mostly undetectable in retinoblastoma tumors compared to normal human fetal retina where mRNA of Rb was present (Lee et al., 1987). The conclusions from this research resulted in the theory that Rb was a tumor suppressor gene that needed to be completely inactivated in order to have cancer formation and subsequent tumorigenesis. Aside from a handful of human malignancies encompassing retinoblastoma and non-small cell lung cancers, Rb itself is infrequently mutated or deleted (Horowitz et al., 1990). Instead, other proteins in the cell cycle pathway are altered that lead to cancer progression. This will be discussed later in this chapter.

Once Rb was identified as a tumor suppressor gene, it was necessary to elucidate the exact function Rb had in a cell. It became clear that Rb was a fairly

large protein – 928 amino acids – and that this protein was localized in the nucleus of the cell (Lee et al., 1987). Furthermore, studies also showed that it inherently had no catalytic activity, and that at best, it weakly bound to DNA (Knudsen and Knudsen, 2006). In 1988, many laboratories found that Rb was able to be sequestered by oncoproteins from DNA tumor viruses, including adenovirus E1A, SV40 large T antigen, and human papilloma virus E7 (Whyte et al., 1988; Munger et al., 1989; Dyson et al., 1989; Dyson et al., 1990). These experiments were done utilizing co-immunoprecipitation studies involving these viral proteins and Rb peptides. The hypothesis then became that Rb could not elicit its function because of this sequestration, and this sequestration could be one event that led to virus-mediated tumor formation (Wang et al., 2004). Thus, Rb was inferred to have critical functions during cell division because preventing the function of Rb could lead to uncontrolled proliferation and eventual tumor formation. It was then discovered that Rb bound to the E2F family of transcription factors, which had been known to regulate cell growth (Kaelin et al., 1992). This was done by copurification of E2F with a fragment of Rb that consisted of the putative functional domain (known as the "pocket domain") (Kaelin et al., 1992). The E2F family of transcription factors, along with their heterodimer DP, are instrumental in regulating cell cycle gene expression and genes necessary for DNA replication (Zhu et al., 2004). Rb can bind to E2F and recruit corepressors that bind to the promoters of E2F-regulated genes and inhibit transcription. These corepressors include HP1, histone de-acetylases (HDACs), and proteins that modulate Histone H3 methylation (Nielsen et al., 2001). Before talking about the importance of Rb-mediated

inhibition of E2F-regulated genes, it is important to talk about the basics of the mammalian cell cycle.

Overview of the Mammalian Cell Cycle

The mammalian cell cycle is divided into 4 phases – two gap phases (G_1 and G_2), S phase, and M phase. Cells not in the cell cycle are in a quiescent, resting state, known as G_0 phase. The start of the cell cycle begins with G_1 phase. During this phase, cells grow in size and synthesize mRNA and proteins necessary for the subsequent phases. The duration of G_1 phase entirely depends on the cell type. For example, fetal hematopoietic stem cells have extremely small G_1 phases, so the time of a given full cell cycle is around 10-14 hours, much shorter than a cell cycle of a wild-type non-tumorigenic cell (Pietras et al., 2011). After G_1 phase, cells enter S phase, where DNA is replicated. Each chromosome will have two sister chromatids, so the amount of DNA effectively doubles. The duration of S phase is fairly consistent regardless of cell type, but a cell has adapted to finish S phase as soon as possible due to exposure of base pairs to external factors that potentially may harm or mutate the DNA (Shermoen et al., 2010). After S phase, cells proceed to another gap phase, known as G_2 phase. This phase allows for cells to correct any mutations or alterations that resulted from DNA replication, including mis-pairing of bases and deletions of small DNA fragments (Al-Khodairy et al., 1992). Once these errors are fixed and the correct signals are sensed, the cell enters M phase, or mitosis. In this phase, the two sister chromatids are segregated, and then through cytokinesis, cells physically divide. Each daughter cell has the same amount of

DNA as the original parent cell. After undergoing cytokinesis, the two daughter cells then proceed into G_1 phase of the next cell cycle.

There also exists a quiescent G_0 phase where cells are not in the cell cycle. Cells in G_0 phase have a smaller cytosol and much lower rate of protein synthesis (Malumbres and Barbacid, 2009). Cells enter G_0 because of two major reasons – they become terminally differentiated, or they cannot enter the cell cycle because of a lack of stimuli. Terminally differentiated cells have exited the cell cycle and are not apt to re-enter unless signals for de-differentiation are sensed. For example, the use of reversine, a purine analog, has been shown to allow fully differentiated myotubes to re-enter the cell cycle (Tsonis, 2004). This process would obviously cause an increase in proliferation and potential tumorigenesis. Proliferative cells that sense a lack of stimuli can enter into G_0 phase, but they can be stimulated to re-enter the cell cycle simply by the addition of growth factors and mitogens. This will cause activation of signaling pathways that allow a cell to enter the cell cycle. If G_0 cells are stimulated to enter the cell cycle by addition of growth factors and mitogens, by the overexpression of an oncogene, or by the inhibition of a tumor suppressor gene, they enter G_1 phase and start the cell cycle.

The key positive regulators involved in cell cycle were discovered by Dr. Leland Hartwell, Dr. Tim Hunt, and Dr. Paul Nurse, who shared the 2001 Nobel Prize for Physiology and Medicine. Dr. Hartwell was the first to start describing the function of the cell cycle by using different *Saccharomyces cerevisiae* deletion mutants to understand the genetic basis of the cell cycle (Hartwell et al., 1974). Dr. Hunt then utilized sea urchin extracts to discover proteins that cycled in expression

during the cell cycle – he appropriately named these proteins "cyclins" (Evans et al., 1983). Finally, using yeast as a model system again, Dr. Nurse discovered the fission yeast homolog of mammalian cyclin-dependent kinases (Cdks). He identified cdc2 as a cell cycle start gene in *Saccharomyces Pombe* through a genetic screen looking for cell division control (cdc) mutants, and found that there were homologs of this protein in higher order organisms (Beach et al., 1982). These proteins are critical regulators of cell cycle regulation, and aberrant expression of these proteins can lead to cancer formation and progression (Knudsen and Knudsen, 2006).

Mammalian Cell Cycle Checkpoints

To prevent uncontrolled proliferation, cells pass through checkpoints during the cell cycle. The first important checkpoint in the mammalian cell cycle occurs in the middle of G_1 phase. Known as the Restriction Point, this checkpoint is the time in the cell cycle where cells make the final decision to either finish the rest of the cycle, or revert back to a quiescent phase. The Restriction Point was first recognized by Dr. Arthur Pardee, where he noted that there was a unique time point in G_1 phase where inhibited cells stopped growing (Pardee, 1974). If a cell is in G_1 phase before the Restriction Point (known as Early G_1 phase), and mitogens and growth factors are withdrawn, the cell has the option of entering a G_0 quiescent phase (Zetterberg et al., 1995). This is also seen when cells respond to stress such as DNA damage, where cells arrest before they enter S phase. If a stress is observed after the cell has passed the Restriction Point, it must proceed through DNA

replication and mitosis (Zetterberg et al., 1995). This could lead to aberrant chromosome segregation or alterations in mitosis and cytokinesis. Furthermore, many tumor cells have mutations, deletions, or altered expressions of certain proteins that prevent this checkpoint from properly functioning. Thus, the Restriction Point checkpoint is an extremely important mode of regulation in the cell cycle.

After cells pass the Restriction Point, there is another checkpoint at the G_1/S phase transition that involves the sensing of reactive oxygen species (ROS) (Havens et al., 2006). As a cell proceeds through G_1 phase, endogenous ROS levels accumulate. When cells are treated with an anti-oxidant to lower ROS levels, cells fail to enter S phase and arrest in Late G_1 phase (Havens et al., 2006). This is due to the lack of Cyclin A protein accumulation, which is a necessary step involved in progression into S phase and initiation of DNA replication.

Another checkpoint in the cell cycle occurs during G_2 phase before a cell goes through mitosis. At this checkpoint, the cell checks for many factors, including making sure DNA replication did not cause any alterations or mutations (Al-Khodairy et al., 1992). This checkpoint was originally discovered in yeast, and subsequently many proteins in DNA damage pathways have been discovered based off of these data in mammalian cells. It is also important to note that select cells arrest at this checkpoint in response to DNA damage to prevent eventual proliferation of mutated or damaged DNA (Zetterberg et al., 1995).

The final major checkpoint of the cell cycle is the spindle checkpoint in mitosis. This occurs during metaphase where all the chromosomes are lined up at

the mitotic plate. This was originally observed by Dr. Ray Zirkle, who used newt cells to observe that there was a delay in anaphase initiation if one chromosome was late in reaching the metaphase plate (Zirkle, 1970). Proteins involved in this checkpoint sense the bipolar tension between the spindle poles, and if the tension is right, the cells proceed on with mitosis and cytokinesis. If there are problems with aligning chromosomes along the metaphase plate, wild-type cells will arrest in metaphase and will not proceed toward finishing mitosis and initiating cytokinesis (Pinsky and Biggins, 2005). The main reason for this checkpoint is for cells to prevent atypical chromosome segregation for the subsequent generation. There are certain pathologies, including Down syndrome, that bypass this checkpoint and continue on with the cell cycle (Pinsky and Biggins, 2005). For the purposes and focus of this study, the proteins involved with the Restriction Point in G_1 phase will be examined further.

Cyclins, Cdks, and CKIs During the Cell Cycle

Progression through the cell cycle is regulated on a number of levels, including assembly of cyclin and Cdk complexes, activating or inhibitory phosphorylations on a number of proteins, and association with cyclin-Cdk complexes with cyclin-dependent kinase inhibitors (CKIs) (Burkhart and Sage, 2008). The main kinases that are involved in cell cycle regulation are Cdks, and these proteins elicit their activity through binding with a cyclin partner. They are responsible for phosphorylating a variety of proteins including histones,

transcriptional activators, cytoskeletal proteins, and tumor suppressor proteins (Malumbres and Barbacid, 2009).

Cdks elicit their activity throughout the cell cycle. In G_1 phase of the cell cycle, Cdk4 and Cdk6 are active, and Cdk2 is activated at the Restriction Point (Haberichter et al., 2007; Ezhevsky et al., 2001). Both Cdk4 and Cdk6 have very redundant functions, and both bind to three D-type cyclins - Cyclin D1, D2, and D3 (Malumbres and Barbacid, 2009). In Late G_1 phase, Cdk2 is activated and paired to Cyclin E to phosphorylate a variety of proteins. These proteins include tumor suppressor genes such as the retinoblastoma protein and the related p107 and p130 pocket proteins, as well as ribosomal proteins such as RL12 (Chi et al., 2008). As cells progress into S phase, Cyclin A is transcribed, at which point it can bind to Cdk2. Cyclin A:Cdk2 complexes also have a number of substrates that are needed to be activated by phosphorylation (Chi et al., 2008). As a cell passes S phase into G_2 phase, Cdk1 gets subsequently activated and becomes the major Cdk during the finish of the cell cycle. Like all Cdks, Cdk1 gets activated by phosphorylation at a region known as the T-loop (T161), as well as de-phosphorylation on inhibitory residues (Y15) by phosphatases (Malumbres and Barbacid, 2009). As mitosis begins, Cyclin B gets transcribed and subsequently can bind Cdk1 to phosphorylate proteins during mitosis. It is important to note that Cyclin B degradation is necessary for cells to exit mitosis (Malumbres and Barbacid, 2009) and enter the next cell cycle.

Some cyclins and Cdks can have compensatory functions. Single knockout mice of Cdk2, Cdk4, or Cdk6 do not result in severe phenotypes, indicating

functional redundancies between Cdks (Berthet et al., 2003; Malumbres et al., 2004; Ortega et al., 2003). This is further evidenced by the fact that inhibiting expression of all cell cycle related Cdks except for Cdk1 does not lead to embryonic lethal mice, and Cdk1 is sufficient to drive cell cycle progression (Santamaria et al., 2007). It is however important to note that these mice are sterile, so Cdk2 is not integral in mitosis, but necessary for meiosis to occur (Santamaria et al., 2007).

There are other Cdks that have cell cycle independent roles. Cdk5, Cdk7, and Cdk9 are involved with transcriptional regulation (Satyanarayana and Kaldis, 2009). Cdk5 has been implicated in sensory pathways, and is integral for brain development (Satyanarayana and Kaldis, 2009). Cdk7 is expressed in a trimeric complex with cyclin H and Mat1, and is an essential component in the TFIIH complex necessary for transcriptional initiation and DNA repair (Larochelle et al., 2007). Cdk9 is an essential component of the positive transcription elongation factor (P-TEFb) (Satyanarayana and Kaldis, 2009). Both Cdk7 and Cdk9 are known to phosphorylate the C-terminal domain of RNA polymerase II (Larochelle et al., 2007). Thus, although some Cdks have cell cycle independent functions, all Cdks still elicit activity through a cyclin binding partner.

Both cyclins and Cdks are regulated through activating and inhibitory phosphorylations, and one of the most intriguing questions in the cell cycle field is the identification of the Cdk-activating kinase (CAK). Activation and regulation of Cdk2 could be the rate-limiting step for cell cycle progression, as this could allow cells to progress past the Restriction Point in G_1 phase (Haberichter et al, 2007). The CAKs have been identified in both budding and fission yeast (Malumbres and

Barbacid, 2009). Cak1 is the CAK in budding yeast, as this phosphorylates the major Cdk – Cdc28p (Kaldis et al., 1996). In fission yeast, the Msc6-Msc2 complex has been shown to have CAK activity, as well as Csk1, which is a functional ortholog of budding yeast Cak1 (Hermand et al., 1998). Cdk7 has been identified as the mammalian CAK (Malumbres and Barbacid, 2009), but this finding has not been fully substantiated in vivo. Recently, the Barbacid laboratory utilized Cdk7 knockout cells and assessed cell cycle progression in these cells. The study showed that deletion of Cdk7 in adult tissues with low proliferative potential had no phenotypic consequences (Ganuza et al., 2012). This would conflict with the theory that Cdk7 is the CAK, because inhibiting CAK activity should prevent cells from passing the Restriction Point in G_1 phase. Identification of the mammalian CAK is essential in understanding G_1 cell cycle progression, as activation of Cdk2 could be the rate-limiting factor in allowing cells to fully complete the cell cycle.

CKIs also play a very important role in cell cycle progression. Based on their sequence homology, there are two classes of CKIs – CIP/KIP proteins and INK4 proteins (Malumbres and Barbacid, 2009). The INK4 proteins, namely p15, p16, p18, and p19, are specific inhibitors of Cdk4 and Cdk6, as they prevent binding of Cdk4 and Cdk6 to the D-type cyclins. The CIP/KIP proteins, namely p21, p27, and p57, inhibit a broader range of Cdks. During Early G_1 phase, p27 can bind to Cyclin E:Cdk2 complexes and inhibit Cdk2 kinase activity (Nakayama et al., 1996). These proteins also are affected and regulated by other pathways in cells. For example, when cells sense DNA damage, the tumor suppressor p53 is activated, which results in the activation of p21 and a cell cycle arrest (Malumbres and

Barbacid, 2009). Another example shows a role for p27 in centrosome assembly. p27 sequesters Cyclin F, which prevents its interaction with a protein called CP110, resulting in centrosome reduplication and mitotic catastrophe (Sharma et al., 2012). The CKI proteins must be regulated in order for a cell to progress through the cell cycle. Deletions or mutations in CKIs are fairly common in many malignancies (Malumbres and Barbacid, 2009). In fact, mice lacking p27 have an increased body size, an increased instance of pituitary tumors, as well as other severe phenotypes (Nakayama et al., 1996).

The Pocket Protein Family

There are many substrates that Cdk-cyclin complexes phosphorylate (Malumbres and Barbacid, 2009), but perhaps the most important proteins that are affected by Cdk phosphorylation during cell cycle progression are the pocket protein family, which includes Rb.

Rb is a member of the pocket protein family, with the other members being p107 and p130. Rb shares approximately 25% sequence identity with both homologies, while p107 and p130 share an approximate 54% sequence homology (Dick and Rubin, 2013). The pocket proteins are so named because they contain a "pocket" domain that consists of an A box, a B box, and a spacer region. The pocket proteins can each bind and repress the function of the E2F family of transcription factors (Cobrinik, 2005). Also, each can be sequestered by viral oncoproteins such as adenoviral E1A and SV40 Large T antigen (Figure 1.1). Furthermore, each protein can be phosphorylated by Cdks on a number of sites on

the molecule (Cobrinik, 2005). Rb has 16 putative Cdk phosphorylation sites, p107 has 17 sites, and p130 has 22 sites (Leng et al., 2002). Though there are similarities between the pocket proteins, there are many differences as well. For example, it is shown that p130 is phosphorylated in quiescent G_0 cells in contrast to p107 and Rb (Canhoto et al, 2000). The hypothesis is that these pocket proteins elicit their function differently based on whether the cells are in the cell cycle (Cobrinik, 2005). It has also been shown that p107 and p130 might prefer binding to certain E2F proteins over others (Hurford et al, 1997). p107 and p130 have shown to preferentially bind to E2F4 and E2F5, while Rb binds E2F1-4 more prominently (Wirt and Sage, 2010). Rb is also different from p107 and p130 in that Rb-null mice are embryonic lethal at E15.5, while p107 and p130 null mutants are viable and have very minor phenotypes (Knudsen and Knudsen, 2006). It is however important to note that mice lacking both p107 and p130 are embryonic lethal because of multiple defects in tissue development (Wirt and Sage, 2010). Most studies have focused on Rb in the last 25 years, because it is the most critical pocket protein, but the literature has started to look at the other pocket proteins more recently to add to the model of G_1 cell cycle progression.

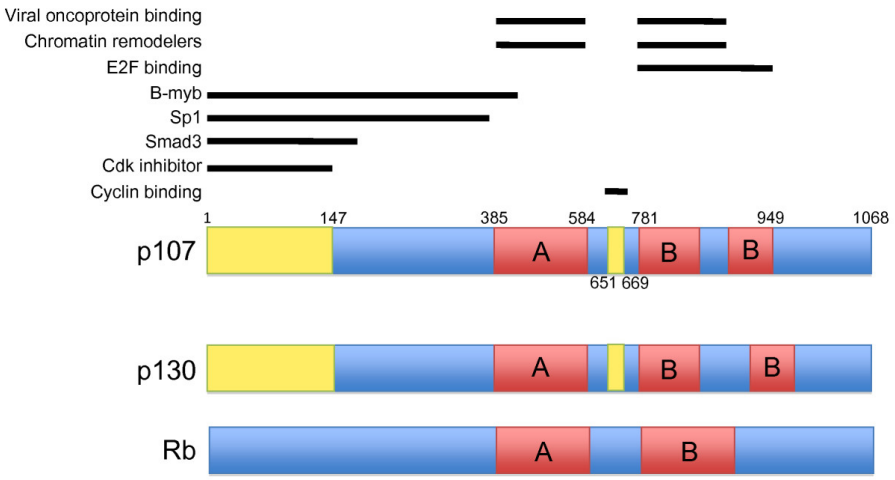

Figure 1.1:
Schematic representation of the pocket protein family and the domains associated with each protein. All proteins have A and B box domains, E2F binding sites, among other domains.
Borrowed from Wirt and Sage, 2010.

The E2F family of Transcription Factors

The E2F family of transcription factors contains 8 different proteins. They regulate a plethora of genes, especially genes necessary for DNA replication, mitochondrial replication, and other genes necessary for cell cycle progression. They usually bind to the motif TTTCCCGC or slight variations of it on target promoter sequences on DNA (Chen et al., 2009). E2F proteins always form a heterodimer with the DP family of proteins to elicit their function. There has been increasing evidence for different functions performed by the different E2F proteins in regulating transcription. It has been concluded that E2F1, E2F2, and E2F3a are transcriptional activators, while the rest of the family are transcriptional repressors (Chen et al., 2009). Knockout studies of E2F proteins have been extensively studied

to elicit the role and function of each family member. Mice with a deletion of a single E2F protein are viable with the exception of E2F4 (Chen et al., 2009). Interestingly, inhibiting E2F4 expression in cells grown in culture had no proliferative effects, but knocking out E2F4 genetically caused embryonic lethality in mice by the increased susceptibility of infections that appeared to be caused by craniofacial defects (Humbert et al., 2000). This would suggest that specific E2F proteins are necessary for proper development. Knocking out all the activator E2Fs (E2F1, E2F2, and E2F3a) also caused embryonic lethality in mice (Chen et al., 2009). Thus, although the E2F family of transcription factors might have redundant roles in proliferative cells, specific E2Fs are necessary for development in mice.

The structure of the proteins in the E2F family is slightly different as well. E2F proteins 1-5 have domains that bind the pocket proteins. E2F proteins 6-8 do not have this domain, and actually have cell cycle independent functions based on the cell type each protein is expressed in (Chen et al, 2009). For example, E2F7 and E2F8, the newest members of the E2F family, have been shown to be important in promoting angiogenesis through transcriptional activation of VEGFA (Weijts et al., 2012). Mice lacking E2F7 and E2F8 have severe vascular defects during development, and thus these proteins are essential in the formation of proper blood vessels (Weijts et al., 2012). The mechanisms of how E2F proteins elicit their function are currently being investigated, and identifying the roles of these proteins could be important in understanding many processes inside a cell. Nevertheless, the most researched function of the E2F family of transcription factors is their role in cell cycle regulation during G_1 phase.

Rb in Other Species

Rb homologs have been found in all sequenced animal genomes, as well as certain plant genomes. The first plant Rb homolog was discovered in maize, and this protein is targeted by RepA protein, which is involved in DNA replication (Liu et al., 1999). This is similar to Rb function in animals, and so plant and animal Rb might have conserved properties. Rb was also found in a single-celled organism – the alga *Chlamydomonas*, where it has been involved in regulating cell size as well as cell cycle progression (Olson et al., 2010).

Budding yeast also have an Rb functional ortholog – Whi5. Whi5 has been shown to be phosphorylated by Cdks (Wagner et al., 2009). These phosphorylations cause the dissociation of Whi5 from SBF complexes that lead to transcriptional derepression of genes necessary for cell cycle progression (de Bruin et al., 2004). Thus, Whi5 acts as a cell cycle inhibitor by repressing transcription and preventing precocious cell cycle entry (de Bruin et al., 2004). However, although Whi5 and Rb play similar roles in regulating cell cycle progression, there is no evidence of any sequence homology (Dick and Rubin, 2013).

Higher order organisms contain more sequence homology to human Rb. In *Drosophila melanogaster*, there are two pocket protein homologs: RBF1 and RBF2, along with two E2Fs: dE2F1, the activator, and dE2F2, the repressor (Lee et al., 2010). A study published in 1996 not only discovered RBF1 as the *Drosophila* Rb homolog, but also showed that RBF1 was repressed by Cyclin E phosphorylation during cell cycle progression (Du et al., 1996).

As mentioned earlier, Mouse Rb (mRb) has the same number of phosphorylation sites as human Rb at the same relative locations on the molecule. However, human Rb has a Cdk phosphorylation site at the extreme N-terminus of the protein (T5), while mRb has an extra Cdk phosphorylation site nearer to the middle of the protein (T364). All the other phosphorylation sites are conserved, and because of this, the function of these sites might be similar.

The Consequence of Rb Loss

As mentioned above, Rb is not lost or mutated in cancer very frequently. This is only seen in retinoblastoma, certain non-small cell lung cancers, and a few colon cancers. Instead, many tumors have overexpression of D-type cyclins, or loss of CKIs, such as p16 (Henley and Dick, 2012; Sherr and McCormick, 2002; Tashiro et al., 2007). Despite this, there has been a lot of published data revolving around the consequences of the loss of Rb.

Rb-null mice are embryonic lethal by E15.5, exhibiting gross neurological and hematopoietic defects (Knudsen and Knudsen, 2006). The apoptosis seen in Rb-null mice was attributed to the fact that there was conflicting growth signals in response to S phase entry in various cell types in the growing embryo. There are certain tumor cell lines that lack Rb, but a lot of the data utilizing these cells might be misleading, as Rb loss can be partially compensated by p107 and p130 expression (Ezhevsky et al., 2001). Thus, many laboratories have utilized Rb floxed murine embryonic fibroblasts (MEFs) to assess the acute loss of Rb and the consequences resulting from it (Sage et al., 2003). The use of RNAi technology has

also resulted in some conclusions regarding the function of Rb. However, siRNAs against Rb do not completely inhibit expression of the protein causing the conclusions made to be misleading.

Rb loss also affects senescent and differentiation pathways. In the context of senescent-induced programs in cells, Rb loss and disruption of the Rb pathway can partially reverse senescence caused by the overexpression of oncogenic Ras in primary human fibroblasts (Serrano et al., 1997). This function could represent a method in which cells bypass a senescent checkpoint by continuing to proliferate when Rb expression is altered. The expression of Rb is also important for differentiation programs. In myogenic differentiation, the absence of Rb prevents differentiation and expression of muscle-specific markers (Novitch et al., 1999; Blais et al., 2007). These Rb-deficient cells have the capacity to re-enter the cell cycle, suggesting the necessity of Rb expression to prevent cells from uncontrolled proliferation. Rb expression is also necessary for osteogenic differentiation (Thomas et al., 2001), showing that Rb is required for differentiation of many cell types. Thus, the absence of Rb alters senescence and differentiation pathways, and this could eventually lead to uncontrolled cell proliferation and tumor formation.

It is still unclear how Rb loss would directly promote tumorigenesis. Onset of tumorigenesis would involve activation of oncogenes along with Rb loss. There are reports that Rb loss could compromise genomic stability and chromosome segregation (Hernando et al., 2004; Zheng and Lee, 2002; Gonzalo et al., 2005). This has been shown through the evidence of elevated Mad-2 levels in Rb-null cells (Hernando et al., 2004). Mad-2 is directly regulated by E2F-dependent

transcription, and so the loss of Rb has shown to delay mitotic entry and to missegregate chromosomes. This would eventually lead to increasing occurrences of aneuploidy, which is one of the hallmarks of cancer formation (Hernando et al., 2004). Furthermore, Rb has been linked to regulate chromatin formation, and removing Rb from cells results in centromere dysfunction and eventual aberrant chromosome segregation (Gonzalo et al., 2005). It is important to state however that the studies mentioned above have only utilized cultured cells. Additionally, although heterozygous mice for Rb are inherently predisposed for the development of pituitary and thyroid tumors, there needs to be another genetic event that would promote complete tumorigenesis, such as the deletion or mutation of p53 or the activation of the oncogenes Ras and Myc (Knudsen and Knudsen, 2006).

The Prevailing Model of G_1 Cell Cycle Progression

With the key proteins introduced, the prevailing model of G_1 cell cycle progression can now be analyzed and evaluated. This progression has been called the "p16-Cyclin D-Rb pathway," identifying some of the major proteins regulating the progression. As cells enter Early G_1 Phase, Rb is "hypo-phosphorylated" (Burkhart and Sage, 2008; Dick and Rubin, 2013). "Hypo-phosphorylation" comes from a term coined in the literature to signify an Rb molecule with minimal phosphorylations (Haberichter et al., 2007, Ezhevsky et al, 1997). This phospho-isoform of Rb is also known as "under-phosphorylated." Unfortunately, this form of Rb is poorly defined in the literature, because there has been no quantitative data addressing the number of phosphates on a given Rb molecule, there has been no

physiologic data addressing the function of "hypo-phosphorylated" Rb, nor has there been any data suggesting the number of phosphates it takes to inactivate the Rb molecule. At the onset of Early G_1 phase, Rb is active. By definition, Rb is active when it binds to the E2F family of transcription factors and represses transcription of E2F-dependent genes necessary for cell cycle progression (Knudsen and Knudsen, 2006). These include genes necessary for DNA replication as well as mitochondrial genes (Chen et al., 2009). It is unclear whether Rb is un-phosphorylated Rb or "hypo-phosphorylated" when active, but it is assumed that as the number of phosphorylations increase on Rb, the less active it becomes. As a cell progresses through Early G_1 Phase, Rb becomes multi-phosphorylated by Cyclin D:Cdk4/6 complexes into a "hypo-phosphorylated" isoform (Ewen et al., 1993; Hinds et al., 1992; Lundberg and Weinberg, 1998; Resnitzky et al., 1994). These phosphorylations are proposed to partially inactivate the protein, causing E2F proteins to slowly release from Rb. The release from Rb causes a gradual increase in the transcription of E2F-dependent genes. One E2F-dependent gene is thought to be Cyclin E (Resnitzky et al., 1994), which is thought to accumulate as a cell progresses through Early G_1 phase. Once Cyclin E reaches a threshold level at the Restriction Point, it binds to Cdk2 and hyper-phosphorylates Rb to render it completely inactive (Burkhart and Sage, 2008; Knudsen and Knudsen, 2006). This allows for complete transcription of E2F-dependent genes. A cell can then pass the Restriction Point and enter Late G_1 and eventual S phase (Figure 1.2 shows a schematic representation of the prevailing model of cell cycle progression).

The implications of this model are three-fold. First, this model involves the D-type cyclins inactivating Rb (Hinds et al., 1992). Second, Rb becomes multi-phosphorylated into a "hypo-phosphorylated" stage by Cyclin D:Cdk4/6 complexes during Early G_1 phase (Knudsen and Knudsen, 1996). Third, the rate-limiting step of the model is the accumulation of Cyclin E, as it must reach a threshold level in order for cells to pass the Restriction Point (Reznitsky et al., 1994). However, some of these implications have been questioned by physiologic data from synchronized cells. For example, Cdk4/6 kinase activity was assayed from cells synchronized and released from contact inhibition or growth arrest. Cdk4/6 kinase activity was constitutively active, which contradicts the notion that Cyclin D:Cdk4/6 complexes inactivate Rb (Ezhevsky et al, 2001; Haberichter et al., 2007). Furthermore, Cdk2 kinase activity was activated right when hyper-phosphorylation of Rb was observed, and there was no gradual ramping up of Cdk2 kinase activity. These data question the theory that Cyclin E accumulation is the critical step in order for cells to pass the Restriction Point (Haberichter et al., 2007). Because some of the implications of the prevailing model have been questioned, it becomes important to elucidate the physiologic mechanism of how cells are regulated in G_1 phase.

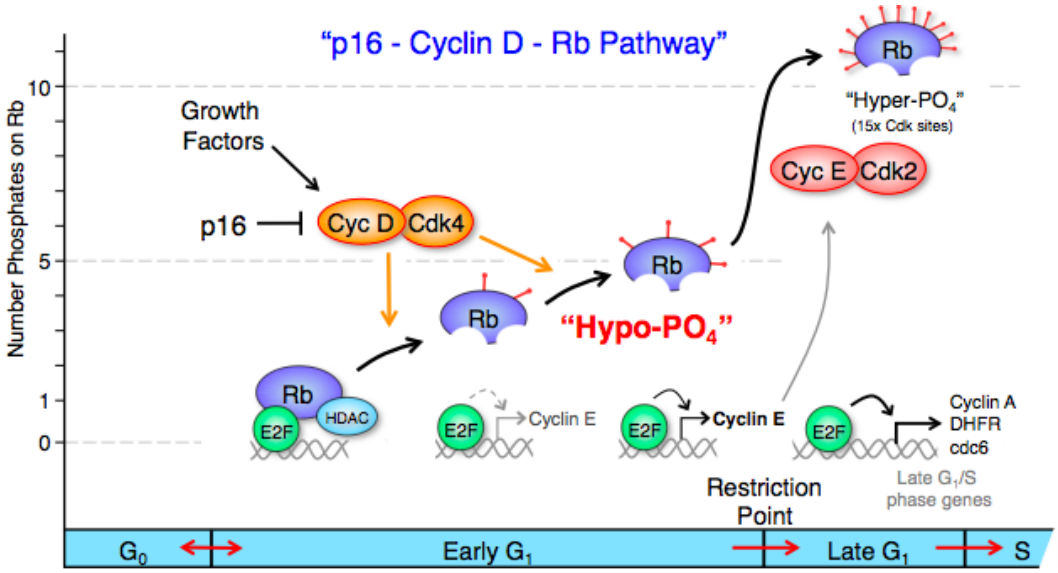

Figure 1.2:
A schematic representation of the prevailing model of G_1 cell cycle progression, the so-called "p16-Cyclin D-Rb pathway." Rb becomes "hypo-phosphorylated" by Cyclin D:Cdk4/6 complexes, which partially inactivates the protein. This causes gradual activation of E2F target genes, including Cyclin E, which can then bind to Cdk2 and complete the inactivation of Rb via hyper-phosphorylation. This allows cells to pass the Restriction Point and transcribe genes needed for S phase.

The Structure of Rb and the Implications of Rb Phosphorylation

Human Rb is a 928 amino acid protein that has a molecular weight of 105 kilodaltons. It contains three main domains. The most prominent domain is the pocket domain, which is necessary for binding of viral oncoproteins as well as the E2F family of proteins (Dick and Rubin, 2013). This is categorized into two subdomains, named the A box and the B box. These subdomains are characterized by a structure that resembles a cyclin fold with three additional helices. The A box consists of helices α3, α4, α6, α7, and α8, while the B box consists of helices α11, α12, α14, α15, and α17 (Lee et al., 1998). The A and B boxes interact non-

covalently such that it folds into a dimeric subunit domain. The C-terminal domain, which comprises the last 150 amino acids, is inherently unstructured, and contains numerous phosphorylation sites (Dick and Rubin, 2013). The spacer region, which connects the A and B boxes, also contains several phosphorylation sites that could be important in the regulation of Rb. The N-terminal domain of Rb also consists of an A' and B' domain, which is similar in structure to the pocket domain, as it also has a globular structure made of two lobes (A' and B') that are each cyclin folds (Hassler et al., 2007). The A' and B' domains are thought to fold over and dimerize with the A and B boxes of the pocket domain (Dick and Rubin, 2013), resulting in a more condensed structure. The N-terminus of Rb also contains many Cdk phosphorylation sites.

Rb is unique in that the protein does not contain typical protein-protein interaction domains (Dick and Rubin, 2013). Rather, it uses the cyclin folds and its pocket domain for interaction. Rb binds to the E2F family of proteins through a highly conserved region of the pocket domain. This contains a motif known as the L-X-C-X-E cleft, where X refers to any amino acid (Dowdy et al., 1993; Hinds et al., 1992). The literature has shown that Rb can bind to viral oncoproteins such as human papilloma virus E7 and SV40 Large T antigen via this motif (Dick and Rubin, 2013). Furthermore, the D-type cyclins use this same L-X-C-X-E motif when binding to Rb, which makes them unique cyclins in that respect (Dowdy et al., 1993). This could affect the robustness and frequency of phosphorylation by Cyclin D:Cdk4/6 complexes. However, Rb is not limited to binding proteins using this motif. For example, Cyclin E:Cdk2 and Cyclin A:Cdk2 complexes bind to Rb via a

C-terminal R-X-L motif, where X is usually a basic amino acid (Adams et al., 1999). These motifs are located downstream of the C-terminal phosphorylation sites on Rb (Adams et al., 1999). Because there are many R-X-L motifs, the robustness of Cdk2 phosphorylation of Rb is much greater compared to Cdk4/6 phosphorylation (Adams et al., 1999). There could be many proteins that Rb regulates by binding via various motifs either in the pocket domain itself, or other places on the protein (Knudsen and Knudsen, 2006).

The human Rb molecule has 16 Cdk putative sites located throughout the molecule (Malumbres and Barbacid, 2009). These sites are serines and threonines immediately followed by a proline, and usually followed by a basic residue (either arginine or lysine) at or around the +3 site (3 sites C-terminal to the acceptor site) (Chang et al., 2007). Mouse Rb also has 16 Cdk phosphorylation sites, but one of them is not conserved (T364). However, one phosphorylation site is buried in the A box of the protein – S567 on human Rb.

As mentioned before, Rb is thought to be "hypo-phosphorylated" in Early G_1 phase, and then is hyper-phosphorylated for the rest of the cell cycle. A 2D phospho-peptide digest was performed comparing "hypo-phosphorylated" and hyper-phosphorylated Rb (Mittnacht et al., 1994). Interestingly, the vast majority of sites were found in both phosphorylated isoforms. This will be important when identifying what is the active form of Rb during this study.

Other Roles for Rb

One of the main functions of Rb is to regulate cell cycle progression in the G_1 phase of the cell cycle. At the end of G_1 phase, Rb is inactive and hyper-phosphorylated. This hyper-phosphorylation is maintained throughout the remainder of the cell cycle, and only at the end of mitosis does Rb get de-phosphorylated by the PP1 phosphatase (Ludlow et al., 1993). There are many reports suggesting that Rb is functional in S phase, specifically DNA replication. For example, it has been suggested that Rb colocalizes with proteins involved in the replication process – some even suggesting that it directly binds to DNA replication factors (Avni et al., 2003). However, it is still unclear whether Rb directly binds to DNA. It is important to note that Rb is inactive during S phase, so more analysis needs to be done to substantiate these findings.

There have also been initial reports of Rb having a role in G_2/M phase of the cell cycle. It has an indirect role for this already, because the Rb/E2F pathway regulates genes necessary for mitosis, including Cdk1, Cyclin B, and Plk1 (Knudsen and Knudsen, 2006). It has also been proposed that Rb has a function at the $_{G2}$/M checkpoint in response to DNA damage. Furthermore, there have been postulations suggesting Rb having a direct role in mitotic control (Jackson et al., 2005). Unfortunately, not a lot of mechanistic data is available, mainly because Rb has always thought to been inactive during these phases of the cell cycle.

It is important to note that Rb has found to have cell cycle regulatory roles independent of the E2F family of transcription factors. For example, Rb has been found to stabilize the cell cycle inhibitor p27 via Skp2-dependent ubiquitylation and

degradation (Ji et al., 2007). This is mediated by the capacity of Rb to bind to the anaphase-promoting complex during cell cycle exit. This would be the first instance of Rb interacting with E3 ligase complexes to mediate eventual degradation (Binne et al., 2007).

Summary

In summary, Rb is an essential tumor suppressor protein that is involved in cell cycle regulation. Rb is regulated by phosphorylation by cyclin-Cdk complexes. How Rb is physiologically regulated is still largely a mystery, mainly because a lot of the previous data have utilized overexpression of key proteins in tumor cell backgrounds. These points will be addressed more in Chapter 3. This study will elucidate the physiologic role and regulation of Rb in G_1 cell cycle progression.

Portions of Chapter 1 were taken from a prepared manuscript, where I was the primary author/researcher. The manuscript is currently in preparation - Narasimha AM, Kaulich M, Shapiro GS, Sicinski P, Dowdy SF. Activation of RB by Mono-Phosphorylation *(In Preparation)*.

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CHAPTER 2:

Materials and Methods

Cell Culture

$Rb^{lox/lox}$ MEFs were obtained from mice given by Anton Berns (Marino et al., 2000). MEFs were isolated from pregnant females at E13.5 and then frozen down once they had stuck to the bottom of the petri dish. MEFs in these experiments were passage 2 MEFs after one trypsinization. 5 μM Tat-Cre protein was added for 1 hour twice to remove endogenous Rb.

TKO D^- MEFs were a gift from Peter Sicinski's laboratory (Dana Farber Cancer Institute; Choi et al., 2012). 5 μM Tat-Cre was added to the cells to delete all of the D-type cyclins. Cells were then serially diluted into single-cell populations to yield a monoclonal population of cells that did not express any D-type cyclins.

HFFs, MEFs, U2OS cells, 293T cells, HeLa cells, H1299 cells, and HCT116 cells were maintained in DMEM high glucose media, plus 10% fetal bovine serum (FBS) (Sigma and/or Omega Biologicals) and 1% penicillin-streptomycin (Invitrogen). HL-60 cells were maintained in 1640 RPMI media with 10% FBS and 1% penicillin-streptomycin. U2OS cells that were under a Tet-responsive system for p16 expression were maintained in DMEM high glucose serum supplemented with 10% FBS, 1% penicillin-streptomycin, and 1 μg/ml of tetracycline to repress p16 expression.

For a G_0 arrest, cells were serum starved for 5 days without any serum added to the media. All cells undergoing serum starvation (U2OS, HFF, MEF) were plated at 40% confluency, and then serum was removed from the media 24 hours later. They were then restimulated to enter the cell cycle via the addition of 10%

FBS to the media. For an Early G_1 phase arrest, cells were contact inhibited by plating them at high density (80% confluency, $6x10^6$ cells/10 cm dish) and letting them grow for 48 hours in the presence of 10% FBS containing media. They were then trypsinized and replated at low density (30% confluency, $1.5x10^6$ cells/10 cm dish) to a new plate in the presence of 10% FBS containing media.

Cells were induced with DNA damage by the addition of 100 ng/ml of DOX (Sigma) for 48 hours. Cells were induced with DNA damage also by the addition of 20 grays of irradiation for 48 hours.

The mitotic index was calculated by looking at cells under a microscope every 20 minutes for 50 hours. The number of mitotic cells was averaged and compared with each sample. A T-test was performed to look for significant values.

Generation of Constructs and Plasmids

Rb constructs were expressed from a pCMV backbone. Human $Rb^{\Delta CDK\text{-}HA}$ was generated by changing all 15 Ser/Thr Cdk acceptor sites to Ala (S567 was left unaltered) and a HA tag was placed on the N-terminus to differentiate between endogenous Rb. $Rb^{\Delta CDK\text{-}HA}$, $Rb^{WT\text{-}HA}$, and T5 constructs were also made with a C-terminal HA tag. Murine $Rb^{\Delta CDK\text{-}HA}$ was similarly generated, with the corresponding S561 left unaltered (buried in the A box of the protein) and a C-terminal HA tag was added. Human Rb single Cdk sites were generated by individually adding back each single Cdk site to $Rb^{\Delta CDK\text{-}HA}$. Rb^{2xCdk} retained T373, S811; Rb^{3xCdk} retained T373, S608, S811; Rb^{6xCdk} retained the N-terminal Cdk sites; Rb^{9xCdk} retained spacer (S608 and S612) and C-terminal Cdk sites.

Rb^{WT-HA} and $Rb^{\Delta Cdk-HA}$ MSCV retroviruses were generated from transfected Bosc 23 cells (Lipofectamine 2000). Transfections of MSCV constructs and pCL-Ampho (5 μg) were done overnight, and the media was then harvested and stored at -80ºC in 1 ml aliquots. The retroviruses were then infected twice via spinfection with different amounts of virus based on the viral titers. Dr. Manuel Kaulich and Dr. Gary Shapiro made most of the constructs shown in this study. His-Cdk2 was purified from BL21 bacterial cells by utilizing a pET24 plasmid. The bacterial cells were then induced with IPTG buffer and incubated for 4 hours. The cells were then sonicated to break up the membranes. The lysate was then washed with a His washing buffer containing 2 mM imidazole. The purified protein was then used as standards for the 2D IEF.

2D IEF

2D IEF were performed similar to Ezhevsky et al., 2001. Cells were lysed using E1A lysis buffer (50 mM HEPES pH 7.0/ 250 mM NaCl/ 0.1% NP-40) supplemented with 1:300 dilution of protease inhibitors (Sigma) and 1:100 dilution of Phosphatase Inhibitor Cocktail 1 (Sigma) (Ezhevsky et al., 2001). 1 mg lysates were then used for immunoprecipitations (IPs). For Rb IPs, C15 rabbit polyclonal antibody (Santa Cruz) was used. For HA IPs, 3F10 rat monoclonal antibody (Roche) was used. For the Rb IPs, Protein A sepharose beads (Pierce) were used. For HA IPs, Protein G beads (Pierce) were used. Beads were washed after the IP and then eluted off the beads with 7 M urea/ 2 M thiourea/ 2% CHAPS buffer

(Sigma) at pH 8.4.

After eluting off the beads, the eluate was then loaded on to the acidic end of a 3-10 pH range immobiline strip from GE healthcare and put on the IPGphor Isoelectric Focusing System (Pharmacia), and covered in mineral oil to prevent desiccation. The voltage was ramped up starting at 200 V for 2 hours, 500 V for 1 hour, 800 V for 1 hour, 1000 V for 30 minutes, 1200 V for 30 minutes, 1400 V for 30 minutes, 1600 V for 30 minutes, 1800 V for 30 minutes, and 2000 V for 2.5 hours. The IPGphor system was hooked up to a water cooler at 16 degrees Celsius to prevent overheating.

After electrophoresis, the immobiline strip was equilibrated and soaked in a solution of 2% SDS/ 6 M urea/ 75 mM Tris pH 8.8/ 29% w/v glycerol supplemented with 1 μM DTT for 1.5 hours, and then 2.5 μM iodoacetamide (Sigma) for another 1.5 hours. The strip was then placed on top of a 6% SDS-PAGE in a large single well for the second dimension. The strip was coated in 0.5% agarose to seal the strip on top of the gel and prevent air bubbles. One small well remained on the side of the gel to place molecular weight markers. The gel was then transferred onto a nitrocellulose membrane (BD biosciences) through wet transfer and blotted for either Rb (BD 554136 mouse monoclonal antibody) or HA (Roche 3F10 rat monoclonal antibody).

FACS Analysis

Cell cycle progression was assayed by DNA content using propidium iodide (PI) and flow cytometry. Cells were washed in PBS and then fixed with 70%

ethanol. A 2x solution of 3x PI, 1% NP-40 (Sigma) in PBS was added 1:1 to cells, filtered, and then assayed through a FL2 channel. Nuclei were counted through a side scatter (SSC) analysis.

Immunoprecipitations, Immunoblotting, and Kinase Assays

Immunoprecipitations (IPs) were done using Protein A or Protein G beads based upon the antibody used. Rb IPs were done with C15 rabbit polyclonal antibody (Santa Cruz). HA IPs were done with 3F10 rat monoclonal antibody (Roche). The IPs were eluted off the beads with 2x Laemmli Sample Buffer and then loaded onto SDS-PAGE gels. IPs generally were incubated with the antibody overnight, and then incubated with beads for an hour before washing and elution. All immunoblotting used 0.01% PBS-Tween for washing. Secondary antibodies (Pierce) were generally diluted 1:5000 in 5% non-fat dry milk.

Primary antibodies used for immunoblotting were anti-Rb 554136 mouse monoclonal (BD Biosciences), anti-HA 3F10 rat monoclonal (Roche), anti-actin C4 mouse monoclonal (Abcam), anti-tubulin mouse monoclonal (company), anti-E1A (13-S5; Santa Cruz), anti-E2F4 (C20; Santa Cruz), anti-Cdk2 (M2; Santa Cruz), anti-Cdk4 (C22; Santa Cruz), anti-Cdk6 (C21; Santa Cruz), anti-Cyclin E (HE12; Santa Cruz), and anti-myogenin (F5D; Santa Cruz). Phospho-specific antibodies used for Rb phosphorylation sites were: T356-PO4 (AB4780, Abcam), S608-PO4 (2181, Cell Signaling), S612-PO4 (OPA1-03891, Thermo Scientific), S795-PO4 (3590, Cell Signaling), S807-PO4/S811-PO4 (9308, Cell Signaling), T821-PO4 (AB4787, Abcam), T826-PO4 (AB4779, Abcam), T821-PO4/T826-PO4 (sc-16669,

Santa Cruz), T373 (AB52975, Abcam), S249-PO4/T252-PO4 (sc-16671, Santa Cruz).

Kinase assays were performed for Cdk4 and Cdk2. Cdk4 kinase assays were performed by immunoprecipitating Cdk4 from lysates using a C22 rabbit polyclonal antibody (Santa Cruz). The IPs were then incubated with 1-2 μCi of ^{32}P, with 10 μM cold ATP, and purified GST-Rb as a substrate. The reaction then proceeded for 1 hour at room temperature, and stopped with the addition of 2x Laemmli sample buffer. The sample was then added to 10% or 12% SDS-PAGE gels and stopped before the dye-front ran off. The gel was stained with Coommassie Blue, destained, and then exposed using film. Cdk2 kinase assays used the same ratios, except Histone H1 was used as a substrate (Haberichter et al., 2007).

qRT-PCR and Microarray Analysis

qRT-PCR was performed as described by using 6-FAM labeled TaqMan probes (Cdc6, 00488573; β2M, 00437762; CcnE, 00438077; Mcm3 Applied Biosystems). Mean values of triplicate samples were normalized to beta-2-microglobulin. Whole-genome microarray analysis was performed as described (Eguchi et al., 2009) using MouseWG-6 v2.0 BeadChips (Illumina) at Biogem core (UCSD). Heat maps were created with Cluster 3.0 and Java TreeView 1.1.3 and gene ontology classifications were based on DAVID Bioinformatics Resources. Dr. Gary Shapiro performed the microarray analysis for this study.

Portions of Chapter 2 were taken from a prepared manuscript, where I was the primary author/researcher. The manuscript is currently in preparation - Narasimha AM, Kaulich M, Shapiro GS, Sicinski P, Dowdy SF. Activation of RB by Mono-Phosphorylation *(In Preparation)*.

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CHAPTER 3:

Aim and Purpose

Aim and Purpose of this Study

There have been numerous studies examining the regulation of Rb in cell cycle progression. Over the last 25 years, the role of Rb in cell cycle regulation has been a highly researched field. Rb is widely regarded as one of the most prominent tumor suppressor proteins, and like many other proteins, it is regulated by phosphorylation. However, the physiologic role of Rb has never been properly assessed, and how it is regulated by phosphorylation remains unknown. Because of this, the physiologic regulation of G_1 cell cycle progression is still largely a mystery. Most of the studies that have been published regarding this topic have utilized over-expression studies of Rb and/or key proteins involved in the pathway (Brown et al., 1999; Chew et al., 1998; Knudsen and Wang, 1997; Leng et al., 1997; Bartek et al., 1997; Sherr and McCormick, 2002; Tashiro et al., 2007). Although the conclusions from these studies are intriguing, the physiologic role of proteins such as Rb and the D-type cyclins are still unknown. Furthermore, a lot of the studies have been done in tumor cell backgrounds that already have many altered biochemical pathways, and reaching conclusions from these data could be misleading.

The physiologic role of how Rb is regulated by phosphorylation has many unanswered questions. Previous experiments have utilized over-expression of Rb constructs with only certain sites that were capable of being phosphorylated (Brown et al., 1999; Chew et al., 1998; Knudsen and Wang, 1997; Leng et al., 1997; Lukas et al., 1997; Sherr and McCormick, 2002; Tashiro et al., 2007). The conclusions reached with these studies are not fully substantiated by physiologic data. The fact that these Rb constructs might cause a cell cycle arrest when overexpressed is

intriguing, but the physiologic implication of Rb phosphorylation is still unclear. Evidence in our laboratory previously showed that the prevailing cell cycle model is problematic and needs to be re-analyzed using better techniques and physiologic data (Ezhevsky et al., 1997; Ezhevsky et al., 2001; Haberichter et al., 2007). For example, HCT116 colon cancer cells were synchronized by contact inhibition, and then released into the cell cycle via trypsinization. Interestingly, Cdk4 kinase activity was constitutively active, while Cdk2 kinase activity was activated concurrently with Rb hyper-phosphorylation (Habericter et al., 2007). Furthermore, Cyclin E levels were analyzed, and they were unchanged during G_1 phase (Haberichter et al., 2007). Thus, two of the three implications of the prevailing model have already been questioned. First, there is no physiologic evidence for the D-type cyclins inactivating Rb. Second, Cyclin E levels do not accumulate during G_1 phase, and so the notion of Cyclin E levels as a rate-limiting step for cells to pass the Restriction Point is not observed in physiologic conditions.

This study will substantiate these results by utilizing physiologic experiments and better techniques to give a more complete understanding of how Rb is regulated during G_1 cell cycle progression. This in turn will give a better understanding of the regulation of key proteins involved in preventing or promoting tumorigenesis.

Portions of Chapter 3 were taken from a prepared manuscript, where I was the primary author/researcher. The manuscript is currently in preparation - Narasimha AM, Kaulich M, Shapiro GS, Sicinski P, Dowdy SF. Activation of RB by Mono-Phosphorylation *(In Preparation)*.

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CHAPTER 4:

Rb is Mono-Phosphorylated During Early G_1 Phase

Introduction

The retinoblastoma tumor suppressor protein (Rb) was discovered to be phosphorylated 25 years ago (Burkhart and Sage, 2008; Knudsen and Knudsen, 2006). Throughout the next decade, many papers were published depicting how and when Rb is phosphorylated during the cell cycle (Lundberg and Weinberg, 1998; Mittnacht et al., 1994; Hinds et al., 1992). However, there has been no clear and quantitative data showing the number and location of phosphates on a given Rb molecule at a given point in the cell cycle. Rb contains 16 cyclin-dependent kinase (Cdk) consensus phosphorylation sites spread throughout the entire molecule (Burkhart and Sage, 2008, Figure 4.3). This motif consists of a serine or threonine residue immediately followed by a proline, with many sites having a basic amino acid residue (either a lysine or arginine) three residues C-terminal to the phospho-acceptor site, yielding an S/T-P-X-B motif (Malumbres and Barbacid, 2009). Prior to this study, Rb was known to exist in three distinct phospho-isoforms – 1) un-phosphorylated, 2) "hypo-phosphorylated" or under-phosphorylated, and 3) hyper-phosphorylated (Burkhart and Sage, 2008; Knudsen and Knudsen, 2005; Paternot et al., 2010). The hyper-phosphorylated form of Rb is considered to be inactive because, with the exception of phosphatases, no proteins can bind to it (Ezhevsky et al., 1997; Ezhevsky et al., 2001; Haberichter et al., 2007). Hyper-phosphorylated Rb releases the E2F family of proteins, and this allows E2F-dependent genes to be transcribed. These genes include factors that are necessary for DNA replication, and other factors promoting cell cycle progression. Hyper-phosphorylated Rb can also be separated from the other two forms by running a low percentage acrylamide

gel. Unfortunately, this technique cannot distinguish between the "un" and the "hypo" forms of Rb, as they migrate as a single band (Ezhevsky et al., 2001, Figure 4.1). Knowing that these phospho-isoforms could be functionally different, it became imperative to use a method to differentiate them to make more insightful conclusions about G_1 cell cycle progression. Thus, a different technique had to be used in order to distinguish and quantify the number of phosphorylations on a given Rb molecule.

Results

Two-Dimensional Isolectric Focusing (2D IEF) is able to Separate the Different Phospho-isoforms of Rb

A SDS-PAGE gel only takes advantage of separating proteins by their molecular weight. Unfortunately, this technique is not sufficient to quantify and separate the three different isoforms of Rb, and only differentiates hyper-phosphorylated from both un-phosphorylated and "hypo-phosphorylated" Rb (Ezhevsky et al., 2001, Figure 4.1). Thus, a different analytical approach was needed, so I utilized 2D IEF. 2D IEF is an approach that separates proteins first by charge, and then by molecular weight. In the first dimension of 2D IEF, proteins are separated by their isoelectric point (pI), which is defined as the pH at which the molecule has no net electrical charge and thus precipitates in the matrix. The pI of un-phosphorylated Rb is ~8.1, and adding just a single phosphate on an Rb molecule lowers the expected pI to 7.4 (ExPASy, scansite.mit.edu). Using the right conditions to separate these isoforms by charge, both un-phosphorylated and mono-phosphorylated Rb can be separated and distinguished. In the second dimension, proteins are separated by molecular weight, utilizing a low percentage SDS-PAGE gel. Using this technique, all phospho-isoforms of Rb, as well as un-phosphorylated Rb, can theoretically be separated and analyzed as a cell progresses through the cell cycle (Figure 4.2 shows a schematic representation of a 2D IEF gel).

In order to have a baseline for where un-phosphorylated Rb would be observed on a 2D IEF gel, 15 out of the 16 sites of Rb were mutated from serines

and threonines to alanines to prevent phosphorylations on those residues (Figure 4.3 shows the phosphorylation sites on the Rb molecule). The lone wild-type Cdk phosphorylation site present in this construct, S567, which had previously been shown to be phosphorylated in a test tube (Harbour et al., 1999), was left unaltered because it is buried in the A box of the protein and is solvent inaccessible. We have termed this construct $Rb^{\Delta Cdk}$, because all but one of the Cdk consensus phosphorylation sites have been mutated. $Rb^{\Delta Cdk}$ was transiently transfected into 293T cells for 48 hours, immunoprecipitated, and loaded onto a 2D IEF. This construct migrated as a single basic species at pI ~8, which was similar to the expected pI of un-phosphorylated Rb, and so S567 is not phosphorylated in cells (Figure 4.3). Starting with the $Rb^{\Delta Cdk}$, varying number of phosphorylation sites were added back to give an indication of how the migration pattern would be affected given the acidic nature of each additional phosphorylation. I was able to separate Rb constructs containing zero, one, or two phospho-acceptor sites on a 2D IEF (Figure 4.4). Three, six, nine, and fifteen phosphorylation sites were then added back in and a progression of species moving toward the acidic end of the gel was observed (Figure 4.4). Interestingly, regardless of the number of phospho-acceptor sites that were added back to the $Rb^{\Delta Cdk}$ construct, there always was a mono-phosphorylated species present. For example, when I added three phospho-acceptor sites back to the $Rb^{\Delta Cdk}$ construct, I observed a mono-phosphorylated species and a tri-phosphorylated species, with no di-phosphorylated species observed (Figure 4.4). The same profile was seen when six, nine, and fifteen phosphorylations were added back - there was the presence of a mono-phosphorylated species along with a more

acidic species. This also could suggest the activity of two distinct kinases – one that mono-phosphorylates Rb, and one that hyper-phosphorylates Rb.

Thus, this was the first time any study quantified the number of phosphates on an Rb molecule. 2D IEF analysis was able to separate specific phospho-isoforms of Rb, including separating un-phosphorylated Rb from mono, di, and tri-phosphorylated versions. With this technique, "hypo-phosphorylated" or under-phosphorylated Rb could now be quantitatively and clearly defined.

After calibrating the 2D IEF to know where each potential phospho-isoform of Rb migrates, the phosphorylation status of endogenous Rb was analyzed. In order to assess the phosphorylation status of Rb at different phases of the cell cycle, three separate conditions were used – cells that were serum starved (G_0 phase), cells that were arrested via contact inhibition or growth arrest (Early G_1 phase), and cells that were growing normally (asynchronous or cycling cells containing cells in all phases of the cell cycle). I initiated these experiments utilizing primary human foreskin fibroblast cells (HFFs), as this was a primary cell line that did not have any cellular pathways altered. In serum starved HFFs, Rb migrated as a single species at pI ~8, which was consistent with the expected pI of un-phosphorylated Rb (Figure 4.5). This is also consistent with previous results in the literature that show no incorporation of ^{32}P during serum starvation (Ezhevsky et al., 2001). In contact inhibited HFFs, where cells are arrested in Early G_1 phase, Rb was exclusively mono-phosphorylated. In an asynchronous population of cells, Rb was mono-phosophorylated and hyper-phosphorylated (Figure 4.5). These data suggest that Rb would be mono-phosphorylated in the Early G_1 phase of the cell cycle, and hyper-

phosphorylated and inactive in the rest of the phases. Samples were premixed in order to corroborate the separation of these phospho-isoforms of Rb. First, a serum starved sample of HFFs was mixed with an $Rb^{\Delta Cdk}$ construct, and this resulted in a co-migration of a single basic species (Figure 4.6). Second, a contact inhibited sample of HFFs was premixed with a construct of Rb that had one phospho-acceptor site, and this also resulted in a co-migration of a single phosphorylation species (Figure 4.6). These data clearly show that the different isoforms of Rb can be separated utilizing 2D IEF. These results also show that cells currently in Early G_1 phase have mono-phosphorylated Rb, and that there is no un-phosphorylated Rb in cycling cells.

Having a standard to compare all the isoforms of Rb would be beneficial in aligning the gels for further experiments. Purified Cdk2 from *E. Coli* was used as a standard. Because there are no post-translational modifications to proteins in bacteria, purified Cdk2 has a predicted pI of 8.2 (scansite.mit.edu). Using this as a standard, HFFs that were serum starved, contact inhibited, or cycling were premixed with purified Cdk2 and loaded onto a 2D IEF gel. The nitrocellulose membrane was cut after the second dimension, and each part was blotted for Rb and Cdk2 respectively. The premixed sample of serum starved HFFs and Cdk2 migrated at the same pI of ~8 (Figure 4.7). The only difference is Rb has a molecular weight of 105 kilodaltons, while Cdk2 has a molecular weight of 35 kilodaltons. The premixed sample of contact inhibited (Early G_1 phase) HFFs and purified Cdk2 showed that contact inhibited HFFs, which contain only mono-phosphorylated Rb, migrates at a more acidic pI, which is consistent with the difference in predicted pIs

between mono-phosphorylated Rb and Cdk2 (Figure 4.7). Finally, the premixed sample of asynchronous HFFs and purified Cdk2 resulted in mono-phosphorylated Rb and a much more acidic hyper-phosphorylated isoform of Rb compared to Cdk2 (Figure 4.7). Thus, a protein standard was established to better distinguish the different phospho-isoforms of Rb.

Rb is Exclusively Mono-Phosphorylated during Early G_1 phase

Previous studies in the literature showed that Rb becomes progressively multi-phosphorylated on different residues as a cell progresses through Early G_1 phase (Knudsen and Knudsen, 2006; Paternot et al., 2010). However, the existing data failed to quantify the number of phosphate groups placed on a given molecule of Rb as a cell progresses through Early G_1 phase. Using 2D IEF, I wanted to analyze the phosphorylation status of Rb quantitatively in different phases of the cell cycle.

To test this, HFFs were synchronized via serum starvation. Interestingly, 3 days of serum starvation, which was the protocol used in many previous studies, was not enough time to completely render Rb un-phosphorylated (Figure 4.8). Three days of serum starvation resulted in a 50-50 mixture of un-phosphorylated and mono-phosphorylated Rb. Four days of serum starvation still resulted in residual mono-phosphorylated Rb, indicating that the half-life of mono-phosphroylated Rb is very long (Figure 4.8). Only at 5 days of serum starvation was Rb completely un-phosphorylated (Figure 4.8).

Thus, HFFs were synchronized by serum starvation for 5 days. This allowed all cells to exit the cycle into a G_0 quiescent phase. Cells were then released into the cell cycle by stimulating them with media containing 10% fetal bovine serum (FBS). As mentioned above, a low percentage acrylamide gel fails to separate the "un" and "hypo" phospho-isoforms of Rb, as it co-migrates as a single band (Figure 4.1). 1D SDS-PAGE analysis resulted in the appearance of hyper-phosphorylated Rb starting at 16 hours post release (Figure 4.9). Cdk4 kinase activity was assessed using GST-Rb as a substrate. In serum starved cells, Cdk4 kinase activity was diminished (Figure 4.9). Once a cell entered the cell cycle, Cdk4 kinase activity was activated by one hour post-release, and remained activated for the duration of Early G_1 phase (Figure 4.9). Cdk2 kinase activity was activated concomitantly with occurrence of Rb hyper-phosphorylation at 16 hours (Figure 4.9). The 2D IEF of a serum starved sample showed that Rb is un-phosphorylated (Figure 4.10). Once the cell entered the cell cycle with the addition of 10% FBS, it took Rb three hours to become completely mono-phosphorylated (Figure 4.10). Mono-phosphorylated Rb remained in cells until 16 hours post-release (Figure 4.10). At that point, Rb became rapidly hyper-phosphorylated by Cyclin E:Cdk2 complexes and subsequently inactivated. These data showed that Rb is exclusively mono-phosphorylated during Early G_1 phase, and there are no intermediate phospho-isoforms observed on a given Rb molecule.

The previous experiment utilized synchronization of HFFs via serum starvation to start them in a quiescent G_0 phase. Another way of synchronization was used - growth arrest or contact inhibition, which arrests cells in Early G_1 phase

(Haberichter et al., 2007). Cells were plated at high density and allowed to grow for 48 hours in the presence of serum-containing media. HFFs, like many primary cells, stop growing and arrest in Early G_1 phase when they are too confluent to grow (Havens et al., 2006). HFFs were synchronized via contact inhibition, trypsinized, and then replated at low density. In this experiment, Rb hyper-phosphorylation was not seen until 8 hours by 1D SDS-PAGE gel analysis (Figure 4.11). Cells progress toward the Restriction Point more rapidly from synchronization via contact inhibition than serum starvation. Cdk4 kinase activity was constitutively on throughout the duration of Early G_1 phase (Figure 4.11). Similar to synchronization via serum starvation, Cdk2 kinase activity turned on at the same time as occurrence of Rb hyper-phosphorylation at 10 hours post-release (Figure 4.11).

One of the hallmarks of the previous model of G_1 cell cycle progression involves partial inactivation of Rb, which allows for leakiness of E2F-dependent transcription (Burkhart and Sage, 2008; Knudsen and Knudsen, 2005). One of the supposed transcriptional targets of E2F is Cyclin E. As Cyclin E levels accumulate more and more, it reaches a threshold level, binds to Cdk2, and completes the inactivation of Rb via hyper-phosphorylation. However, this hypothesis is not observed in HCT116 colon cancer cells (Haberichter et al., 2007), nor is it observed in primary cells. Using the same kinetic analysis, HFFs were lysed and checked for Cyclin E expression both on the protein and mRNA levels. After release from contact inhibition, Cyclin E protein expression did not change by Western Blot analysis (Figure 4.11). Cyclin E mRNA expression also did not alter by qRT-PCR analysis (Figure 4.11). In contrast, cdc6, which is a known E2F target gene (Yan et

al., 1998), was induced at 10 hours post-release. This induction occurred concomitantly with Rb hyper-phosphorylation and Cdk2 activation (Figure 4.11), signifying that once Rb was hyper-phosphorylated and inactivated, E2F target genes were able to be transcribed and induced. Thus, the notion that Cyclin E accumulation causes cells to progress past the Restriction Point is not true in primary cells that are synchronized via contact inhibition.

The 2D IEFs of HFFs synchronized via contact inhibition and released resulted in the presence of only mono-phosphorylated Rb at 0, 1, 2, 3, 4, 5, 6, and 7 hour time points (Figure 4.12). At 8 hours, Rb started to become rapidly hyper-phosphorylated, and this phospho-isoform became the major species by 10 hours post-release (Fgiure 4.12). No intermediate phospho-isoforms were observed, nor was there presence of un-phosphorylated Rb once cells entered the cell cycle. Regardless of how cells are synchronized, Rb is exclusively mono-phosphorylated during Early G_1 phase, and then rapidly hyper-phosphorylated once cells pass the Restriction Point into Late G_1 phase.

Rb is Mono-Phosphorylated in Tumor Cells

The majority of tumors that have wild-type Rb have oncogenic mutations that upregulate Cyclin D:Cdk4/6 activity (Burkhart and Sage, 2008; Sherr and McCormick, 2002). The quantitative phosphorylation status of Rb in these cells has never been ascertained, so four independent tumor cell lines were analyzed for Rb phosphorylation using 2D IEF. All four tumor cell lines (HCT-116 colon carcinoma, H1299 lung adenocarcinoma, U2OS osteosarcoma, HL-60

promyelocytic leukemia) are wild-type for Rb, but have a deletion in the p16 gene. Because p16 is a specific inhibitor of Cdk4/6, Cdk4/6 kinase activity would be at its highest level. Nevertheless, in asynchronous populations of these cells, Rb was mono-phosphorylated and hyper-phosphorylated, with no intermediate phosphorylation species detected by 2D IEF – the exact same phosphorylation pattern seen in asynchronous primary cells (Figure 4.13). Regardless of the cell type, Rb is mono-phosphorylated in Early G_1 phase and hyper-phosphorylated in all other phases of the cell cycle.

Kinetic analyses of tumor cells were next performed to assess the phosphorylation status of Rb as cells progress through Early G_1 phase. The presence of intermediate phospho-isoforms of Rb was highly improbable, because these phosphorylation species would have been detected in an asynchronous population of cells. U2OS osteosarcoma cells were contact inhibited in the presence of serum-containing media and then released by trypsinization and replating at low density. Rb hyper-phosphorylation was not seen until 10 hours by 1D SDS-PAGE analysis (Figure 4.13). Cdk4/6 kinase activity was constitutive throughout the duration of Early G_1 phase (Figure 4.14). 2D IEFs showed that Rb was only mono-phosphorylated and became rapidly hyper-phosphorylated at 10 hours (Figure 4.15). Tumor cells that can be synchronized via contact inhibition resulted in the exact same profile of Rb phosphorylation as primary cells. Regardless of the cell type, mono-phosphorylated Rb is the only form of Rb that exists in Early G_1 phase. Once the cell reaches the Restriction Point, Rb becomes rapidly hyper-phosphorylated.

Even in a p16-deleted tumor cell, with Cdk4/6 kinase activity at its presumed highest level, there are no intermediate phospho-isoforms of Rb observed.

Cells that are Mutated for Rb Are Not Phosphorylated

Rb is not mutated in many cancers – instead, alterations to the pathway usually involve other factors in cell cycle progression, such as overexpression of the D-type cyclins or deletion of tumor suppressors such as p16 (Burkhart and Sage, 2008; Paternot et al., 2010). Nevertheless, cancers such as retinoblastoma and non-small cell lung cancers have deletions or mutations in the Rb protein (Dick and Rubin, 2013). For example, H209 non-small cell lung cancer cells have a point mutation in Rb (C706F) that completely alters the structure of the pocket domain (Knudsen and Wang, 1998). NCI-H436 cells have a deletion of the 21st exon of Rb. NCI-H69 cells have a deletion of the 22nd exon of Rb (Dowdy et al., 1993). All three mutations of Rb yield it to be non-functional due to structural alterations in the pocket domain (Dowdy et al., 1993; Knudsen and Wang, 1998). Because of this, Rb cannot regulate transcription of cell cycle genes because it fails to bind the E2F family of transcription factors.

These cell lines were obtained and analyzed for Rb phosphorylation status through 2D IEF. All three cell lines yielded un-phosphorylated Rb, with no mono-phosphorylation or hyper-phosphorylation observed (Figure 4.16). The hypothesis is that the D-type cyclins cannot bind to Rb via the pocket domain (Dowdy et al., 1993) and thus no phosphorylation is observed.

Un-phosphorylated Rb is Integral for Cell Cycle Exit and Differentiation

If un-phosphorylated Rb was present in cells that have exited the cell cycle via serum withdrawal, there could be a function of this particular isoform in G_0 phase. Previous studies showed that the presence of Rb is necessary for differentiation (Novitch et al., 1999; Blais et al., 2007; Thomas et al., 2001). The hypothesis would be that Rb was un-phosphorylated after differentiation, as this was the form present during cells exiting the cell cycle into a quiescent phase during serum starvation.

The C2C12 mouse myoblast differentiation system was utilized, where with the addition of 2% horse serum, C2C12 myoblasts form into long, multi-nucleated myotubes within 24 to 48 hours (Blais et al., 2007). These myotubes have exited the cell cycle and are considered differentiated. A one-dimensional acrylamide gel showed Rb running at its fastest migrating form once cells were differentiated for 48 hours (Figure 4.17). Myogenin is a marker of myotube differentiation (Blais et al., 2007), and after 48 hours in differentiation media, myogenin expression was induced shown by Western Blot analysis (Figure 4.17). Cdk4/6 kinase activity was analyzed, and after 48 hours, it was abrogated, signifying that these cells had exited the cell cycle and had completely differentiated (Figure 4.17). Rb phosphorylation was next assessed via 2D IEF. In a cycling C2C12 myoblast cell, Rb was mono-phosphorylated and hyper-phosphorylated, similar to any cell type shown previously (Figure 4.18). However, after the addition of horse serum, Rb became un-phosphorylated by 48 hours (Figure 4.18). Thus, Rb is un-phosphorylated as cells exit the cell cycle via differentiation.

Human Leukemia-60 (HL-60) tumor cells were next analyzed to see whether the previous data was consistent in another differentiation system. With the addition of retinoic acid, HL-60 cells differentiate into granulocytes (Breitman et al., 1980). A time course was done to see how long the differentiation process lasted in these cells. At 96 hours after addition of retinoic acid, Rb ran at its fastest migrating form on a one-dimensional acrylamide gel, indicative of cells that have exited the cell cycle via differentiation (Figure 4.19). 2D IEF analyses were next performed and showed that even at 72 hours after retinoic acid addition, Rb still had the characteristic phosphorylation profile seen in asynchronous cells (Figure 4.19). However, at 96 hours after retinoic acid addition, Rb was completely unphosphorylated, showing the presence of this phosphorylation status of Rb during the granulocytic differentiation process (Figure 4.18).

This differentiation process was then further analyzed by performing a more rigorous timecourse in HL-60 cells. After 72 hours of retinoic acid addition, Rb remained in its usual phosphorylation profile – both mono-phosphorylated and hyper-phosphorylated (Figure 4.20). Knowing that Rb became completely un-phosphorylated at 96 hours post retinoic acid addition, samples were taken every 6 hours between 72 and 96 hours to see the process of how Rb became unphosphorylated. After 78 hours, 2D IEF analyses resulted in the presence of un-phosphorylated and hyper-phosphorylated Rb (Figure 4.20). By 84 hours, Rb was completely un-phosphorylated (Figure 4.20). Thus, a more detailed time course showed the phosphorylation profile of how Rb became un-phosphorylated during the differentiation process.

The result of seeing both un-phosphorylated and hyper-phosphorylated Rb yielded two possible explanations. First, there could be an indirect way of Rb becoming un-phosphorylated. This would render cells that passed the Restriction Point to complete the entire cell cycle. Once they exit mitosis, the cells would immediately go into a quiescent phase without proceeding on to G_1 phase of the next cell cycle (Figure 4.21 shows a schematic of the proposed explanations). The second method could be cells that are currently in Early G_1 phase receive differentiation signals and are induced to revert back into a quiescent state to make Rb unphosphorylated (Figure 4.21). Both explanations are possible and not mutually exclusive, but as the next chapter will show, this mono-phosphorylation seen will be dependent on Cyclin D:Cdk4/6 kinase activity.

The next question that was asked was whether un-phosphorylated Rb was functional during cell cycle exit. Dr. Gary Shapiro, a former postdoctoral fellow in the laboratory, performed functional studies utilizing the $Rb^{\Delta Cdk}$ construct in the C2C12 myoblast differentiation system. First, he diminished endogenous Rb expression via transfection of an Rb short hairpin RNA (shRNA). At the same time, he retrovirally infected either the Rb^{WT} or the $Rb^{\Delta Cdk}$ construct into these cells. This method allowed cells undergoing differentiation to be exposed to Rb at all times (Figure 4.22 shows a schematic representation of the experiment). Importantly, exogenous Rb was put back at physiologic levels. This was shown by Western blotting of Rb, Rb-HA, and actin as a loading control (Figure 4.23). The number of nuclei was next quantified via FACS analysis after one day in differentiation medium (2% horse serum). The lower number of nuclei would signify more

differentiation, as there would be a fewer number of cells. When a scrambled shRNA was added, cells underwent differentiation (Figure 4.24). This differentiation was ablated to a certain extent when an shRNA was added against Rb (Figure 4.24). This is consistent with the theory that Rb expression is necessary for differentiation (Blais et al., 2007). When an Rb^{WT} virus was exogenously infected, the cells reverted back to wild-type levels and differentiation was not affected (Figure 4.24). However, when an $Rb^{\Delta Cdk}$ construct was added back, the number of nuclei seen decreased dramatically, indicative of more cells exiting the cell cycle (Figure 4.24).

Next, a transcriptional target, Mcm3, which is a chromosome maintenance protein, was analyzed by qRT-PCR. Transcription of this gene occurs in cells that are currently in the cell cycle (Blais et al., 2007). Consistent with previous results, addition of an Rb shRNA to remove Rb expression resulted in significantly higher expression of Mcm3 (Figure 4.25). Adding back an Rb^{WT} virus resulted in decreased levels of Mcm3, signifying cells exiting the cell cycle. However, adding back an $Rb^{\Delta Cdk}$ construct showed a significant decrease in Mcm3 levels compared to levels observed when the Rb^{WT} was added (Figure 4.25). Thus, not only is Rb expression necessary for differentiation, but the un-phosphorylated isoform of Rb promotes it and eventual cell cycle exit.

Cdk2 Rapidly Hyper-Phosphorylates Rb at the Late G_1 Phase Restriction Point

Previous studies already showed that Cyclin E:Cdk2 complexes phosphorylate Rb and inactivate it via hyper-phosphorylation (Burkhart and Sage,

2008; Knudsen and Knudsen, 2006). However, the fact that Rb became hyper-phosphorylated extremely rapidly in both primary and tumor cells was intriguing. It was then warranted to test whether Cdk2 phosphorylation of Rb, although extremely fast, was processive. The chemical inhibitor Roscovitine was utilized, which is a specific Cdk inhibitor (Malumbres and Barbacid, 2009; Haberichter et al., 2007). HFFs were synchronized via contact inhibition (in the presence of serum-containing media) and then replated at low density after trypsinization. At 4 hours post-release, a control [dimethyl sulfoxide (DMSO)] or 15 μM Roscovitine was added for 6 hours. At 10 hours post-release, most of the cells treated with DMSO were past the Restriction Point, evidenced by activation of Cdk2 and Rb hyper-phosphorylation appearing (Figure 4.26). Cdk4 kinase activity was constitutively on regardless of the treatment (Figure 4.26). The cells treated with Roscovitine failed to induce Cdk2 kinase activity, and Rb hyper-phosphorylation was not observed (Figure 4.26). Thus, inhibiting Cdk2 activity prevented Rb hyper-phosphorylation.

To see whether intermediate phosphorylation species of Rb could be captured, the concentration of Roscovitine was varied between 0 and 15 μM, and then 2D IEFs were performed. With no Roscovitine added, Rb was mostly hyper-phosphorylated. Starting at 4 μM Roscovitine, intermediate phosphorylation species were observed (Figure 4.26). As the concentration of Roscovitine increased, fewer highly acidic species of Rb were observed. At 10 μM Roscovitine, Rb was mono-phosphorylated and di-phosphorylated. Finally, at 15 μM Roscovitine, Rb was completely mono-phosphorylated (Figure 4.27). This was consistent with the fact that Cdk2 kinase activity was completely inhibited (Figure 4.26). Thus,

although the exact quantitation of Rb phosphorylation was hard to assess with each concentration, Cdk2 hyper-phosphorylation of Rb is a processive step and can be slowed down with the addition of specific inhibitors curbing kinase activity.

Discussion

Elucidating the tenets of G_1 cell cycle progression is essential for understanding cancer development and progression. Once cells progress from Early G_1 to Late G_1 past the Restriction Point, they are committed to finishing the entire cell cycle through mitosis and cytokinesis. The prevailing model of G_1 cell cycle progression was based on the theory that Rb becomes partially inactivated by progressive multi-phosphorylation into a poorly defined "hypo-phosphorylated" state (Knudsen and Knudsen, 2006; Paternot et al., 2010). This partial inactivation causes leakiness of E2F-dependent transcription, which leads to gradual activation of target genes such as Cyclin E. Cyclin E levels accumulate and reach a threshold level, at which point it can bind to Cdk2 and phosphorylate Rb to complete the inactivation. This allows for all target genes of the E2F family of transcription factors to be activated, including a number of genes necessary for G_1/S phase transition and cell cycle progression. Using highly synchronized cells and 2D IEF, this study has shown that the prevailing model is not true.

Previous studies on Rb phosphorylation never quantified the number of phosphates on "hypo-phosphorylated Rb," nor used the right techniques to achieve this goal. 2D IEF analysis is perfect for this question because the pKa of phosphate groups is extremely acidic (pKa ~1.0, Wojciechowski et al., 2003). 2D IEF analysis had been used previously to distinguish between phosphorylation species in proteins such as p53 (Heukeshoven et al., 2012) and Cdk2 (Ciarallo et al., 2002). Rb became an ideal candidate for application of this technique because of the different phospho-

isoforms that had already been observed in cell cycle progression. However, because Rb is such a large protein (molecular weight of 105 kilodaltons), it is easy to precipitate and come out of solution during the separation of pIs. With the help of Dr. Sergei Ezhevsky, a former postdoctoral fellow in the laboratory, I was able to focus Rb by developing a stepwise gradient of increasing voltage to prevent Rb from precipitating (details in Chapter 2 – Materials and Methods). Once this technique was mastered, I was able to draw conclusions about G_1 cell cycle progression that were unable to be deduced before.

The constructs of Rb with varying number of phospho-acceptor sites showed that different number of occupied phosphorylation sites could be distinguished on a 2D IEF gel. It was clear to observe the difference between zero and one phosphate groups on an Rb molecule because the predicted pI difference was from 8.1 to 7.4. Furthermore, one, two and three phosphorylations were also able to be distinguished, which would be essential to determine the number of phosphates on "hypo-phosphorylated" Rb. The first hint that Rb could be mono-phosphorylated was that regardless of the amount of phospho-acceptor sites that were present in each construct, mono-phosphorylated Rb was always observed in cycling cells. This presumably were the cells that were currently in Early G_1 phase at the time. The more acidic species were the "hyper-phosphorylated" versions of each construct, occupying most, if not all, of the available phosphorylation sites.

It is important to note that this was the first time a study clearly quantified the number of phosphates on Rb. Previous studies were never able to distinguish between the "un" and "hypo" phospho-isoforms, and so many conclusions inferred

were misinterpreted or ambiguous. With 2D IEF analysis, the difference between specific phosphorylated forms of Rb was clear to distinguish, and so precise conclusions could be made on the phosphorylation status of Rb.

Different isoforms of Rb were detected when endogenous Rb phosphorylation status was analyzed in serum-starved (G_0), contact inhibited (Early G_1), and cycling HFFs. In cycling cells, Rb was mono-phosphorylated and hyper-phosphorylated, with no intermediate phospho-isoforms observed. This was the first evidence that endogenous Rb was only mono-phosphorylated during Early G_1 phase and not multi-phosphorylated. The fact that Rb is completely mono-phosphorylated during contact inhibition showed that this phospho-isoform is the only form present in Early G_1 phase of the cell cycle. This experiment also showed that Rb is completely un-phosphorylated in cells that have exited the cell cycle by withdrawal of mitogens and growth factors. It was necessary to serum starve cells for 5 days, because the half-life of mono-phosphorylated Rb was long, evidenced by the fact that 4 days of serum starvation still had low levels of mono-phosphorylated Rb.

To make sure Rb was exclusively mono-phosphorylated during Early G_1 phase, it was necessary to synchronize cells and check Rb phosphorylation status as a cell passes through the Restriction Point. Whether it was synchronization via serum starvation or via contact inhibition, Rb was exclusively mono-phosphorylated until cells reached the Restriction Point. At that point, Rb became rapidly hyper-phosphorylated by Cyclin E:Cdk2 complexes. When Cyclin E expression was assessed both on the protein and mRNA levels in primary cells, there was no change

during Early G_1 phase, so Cyclin E levels actually do not accumulate and remain constant throughout the duration of Early G_1 phase. Unlike cdc6, which becomes rapidly induced at the Restriction Point, Cyclin E is actually not an E2F-target gene. Instead of Cyclin E accumulation allowing cells to progress past the Restriction Point, it could be a completely disparate factor, such as the activation of Cdk2. This activation could be the rate-limiting step in inactivating Rb and thus allowing cells to progress into Late G_1 phase.

Asynchronous tumor cells show the same distribution pattern of Rb phosphorylation. This is even more surprising, because the tumor cells that were assayed in this study were all p16-negative cell lines yielding Cyclin D:Cdk4/6 (which has been thought to be the "hypo-phosphorylating" kinase of Rb) complex activity at its highest level. Regardless of the high activity, Rb is only mono-phosphorylated and again rapidly hyper-phosphorylated at the Restriction Point. Thus, mono-phosphorylation is a universal phenomenon regardless of cell type. The only tumors that have aberrant Rb phosphorylation are cells that have a deletion or mutation in the Rb gene. This is seen in certain retinoblastoma or non-small cell lung cancer cell lines, where deletions of exons or point mutations completely alter the structure of Rb. In these cell lines, Rb is incapable of binding the E2F family of transcription factors and other proteins and is rendered completely non-functional. Here, Rb is un-phosphorylated, which is consistent with the fact that it cannot bind to the D-type cyclins and induce any kinase activity (Dowdy et al., 1993).

The fact that Rb is un-phosphorylated in quiescent cells was intriguing in that there could be a role for un-phosphorylated Rb outside of the cell cycle.

Previous studies had not definitively shown that Rb was un-phosphorylated in G_0 cells. After 5 days of serum starvation, Rb was completely un-phosphorylated, and Cdk4/6 kinase activity was inhibited. Un-phosphorylated Rb also hastens the differentiation process, as C2C12 myoblast cells differentiated significantly faster when exposed to un-phosphorylated Rb. Although un-phosphorylated Rb is not present in cycling cells, it could have an important role in cells that have exited the cell cycle.

The detailed time course of differentiation in HL-60 cells showed that there was an intermediate time point when Rb was both un-phosphorylated and hyper-phosphorylated during the differentiation process. As the differentiation process begins, it is unknown whether cells immediately exit the cell cycle after mitosis, or whether they enter the next Early G_1 phase and revert back to a quiescent G_0 phase. This is a question that remains unknown, and needs to be answered by elucidating the function of mono-phosphorylated Rb during the differentiation process. Nevertheless, at 96 hours post addition of retinoic acid, Rb is completely un-phosphorylated.

It was interesting to see such a rapid hyper-phosphorylation by Cyclin E:Cdk2 complexes once the cells passed the Restriction Point into Late G_1 phase. Such a quantum leap from 1 to greater than 12 phosphorylations on a single Rb molecule was surprising and showed the robustness of Cdk2 phosphorylation. This rapid phosphorylation is consistent with the fact that Cyclin E and Cyclin A bind to Rb via a C-terminal R-X-L motif and potentially has easier access to all phosphorylation sites (Adams et al., 1999) compared to Cyclin D:Cdk4/6

complexes, which bind Rb via the pocket domain (Dowdy et al., 1993). It was essential to show that this hyper-phosphorylation of Rb was processive although extremely rapid. Roscovitine was used as an inhibitor of Cdk2 (Haberichter et al., 2007), and intermediate phosphorylation species of Rb were able to be isolated with varying concentrations on a 2D IEF. It was however more difficult to quantify the number of phosphates on an Rb molecule in this experiment, either because the pI change of subsequent phosphorylations was much smaller or because the limitations of the technique prevented exact quantification. Nevertheless, it was reassuring to see that Cdk2 phosphorylation of Rb was indeed processive.

With these data, the prevailing model can be amended. Rb is un-phosphorylated in G_0 quiescent cells. As a cell enters the cell cycle, Rb becomes mono-phosphorylated and this mono-phosphorylation persists throughout the duration of Early G_1 phase. Once cells reach the Restriction Point, Rb becomes rapidly hyper-phosphorylated by Cyclin E:Cdk2 complexes. Thus, the activation of Cdk2 could be the threshold step that allows cells to progress past the Restriction Point (Ezhevsky et al., 2001; Haberichter et al., 2007). In order to continue adding to this model, the Rb mono-phosphorylating kinase must be identified.

Portions of Chapter 4 were taken from a prepared manuscript, where I was the primary author/researcher. The manuscript is currently in preparation - Narasimha AM, Kaulich M, Shapiro GS, Sicinski P, Dowdy SF. Activation of RB by Mono-Phosphorylation *(In Preparation)*.

Figures

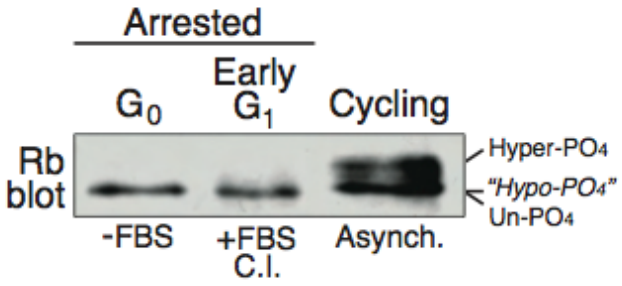

Figure 4.1:
A 6 percent SDS-acrylamide gel with serum starved HFFs (-FBS), contact inhibited or growth arrested HFFs in the presence of serum (+FBS C.I.), and asynchronous or cycling HFFs (Asynch.).
Both serum-starved cells and asynchronous cells run at Rb's fastest migrating form, while the asynchronous sample shows both a slower migrating form (Hyper-PO4) and the fastest migrating form.

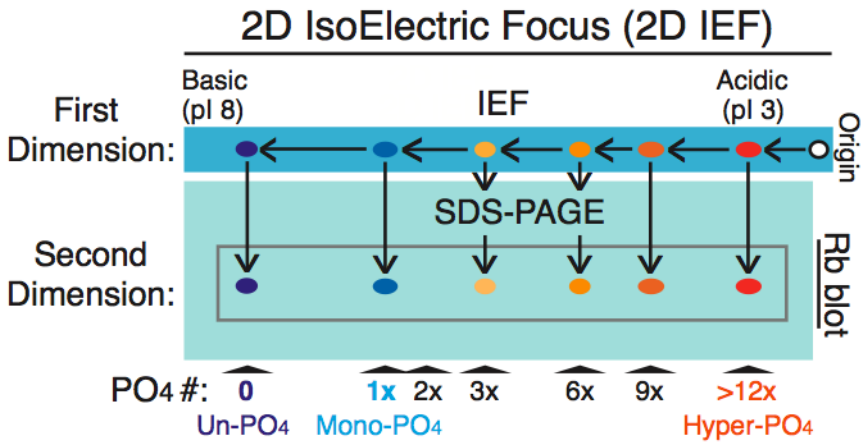

Figure 4.2:
A schematic representation of a 2D-IEF gel.
The samples are loaded on the acidic end of the IEF strip, and then separated by pI. The second dimension is an SDS-PAGE, the gel is then transferred onto a nitrocellulose membrane, and blotted for Rb.

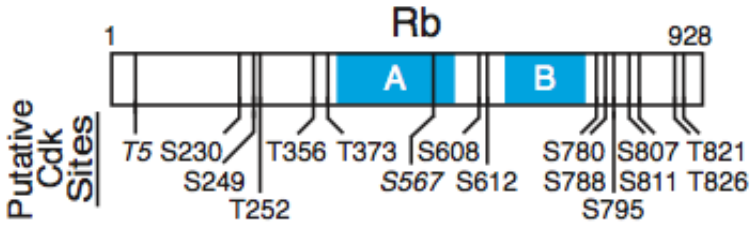

Figure 4.3:
A schematic representation of the Cdk consensus phosphorylation sites on the human Rb molecule.
The A box and B box are the domains (Pocket Domain) on Rb that bind to the E2F family of transcription factors and viral oncoproteins.
T5 and S567 are listed in italics. These sites are not phosphorylated in vivo (data to be shown later).

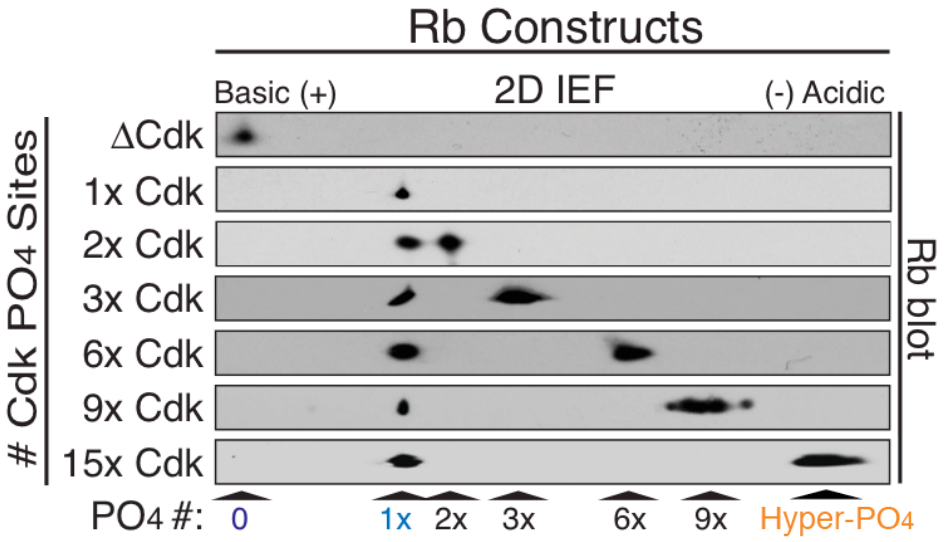

Figure 4.4:
2D-IEFs of HA-tagged Rb constructs transiently transfected into cycling 293T cells for 48 hours.
The number of Cdk phosphorylation sites added back into an $Rb^{\Delta Cdk}$ construct is indicated on the left.

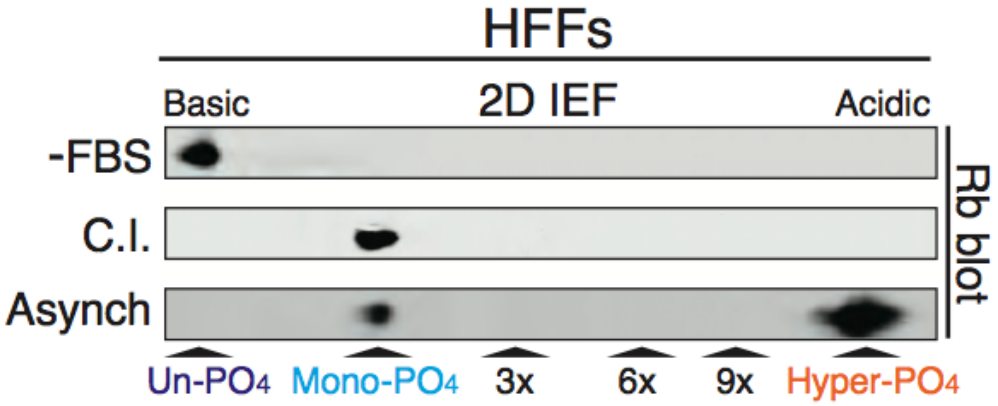

Figure 4.5:
2D IEFs of endogenous Rb in HFFs looking at serum starved (-FBS), contact inhibited (C.I.), and asynchronous (Asynch) conditions.
Samples were loaded on the acidic side.
The presence of mono-phosphorylated Rb is seen in C.I. and Asynch samples.

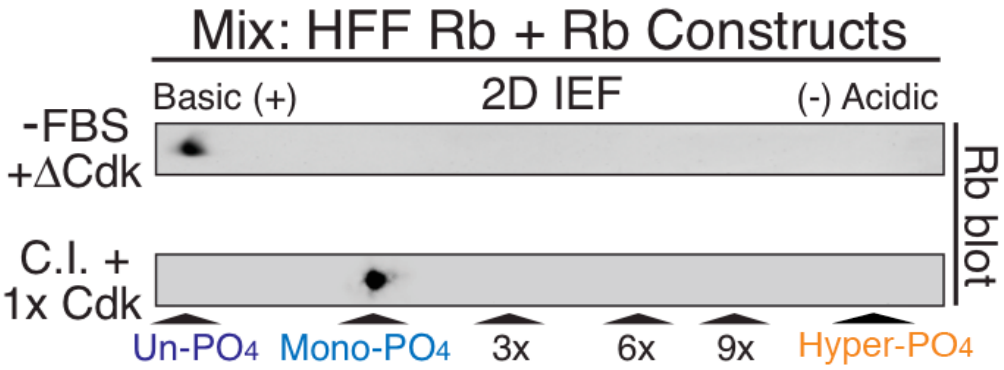

Figure 4.6:
2D IEF immunoblot analysis of Rb from HFFs serum deprived G_0 arrested (-FBS) mixed with ΔCdk Rb construct standard (top panel), and HFFs contact inhibition in early G_1 phase arrested (+FBS) mixed with single Cdk site Rb construct standard (bottom panel).

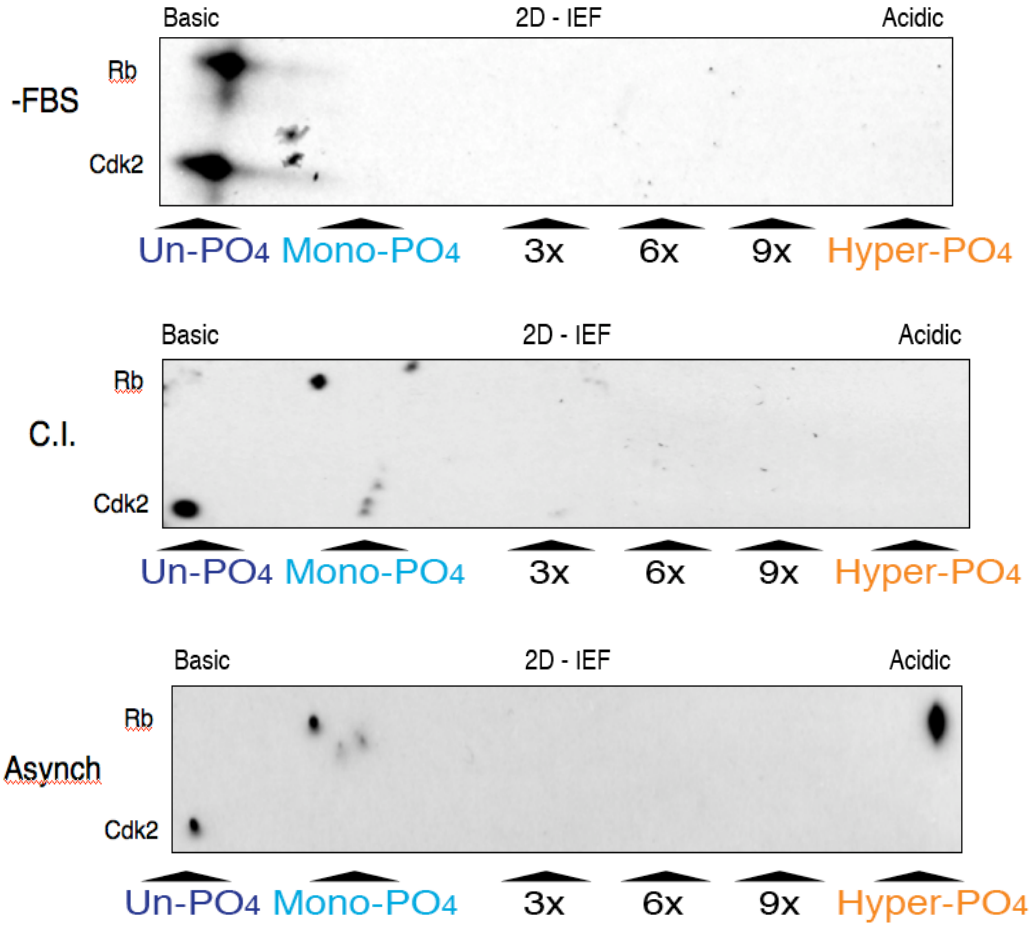

Figure 4.7:
2D IEFs of endogenous Rb from HFFs and purified Cdk2 from *E. coli*.
For the 2nd dimension, a 10% gel was run. After the transfer, the membrane was cut in the middle. The top portion was blotted for Rb, the bottom portion was blotted for Cdk2, and then exposed using the same film. The pI of Cdk2 is 8.2.

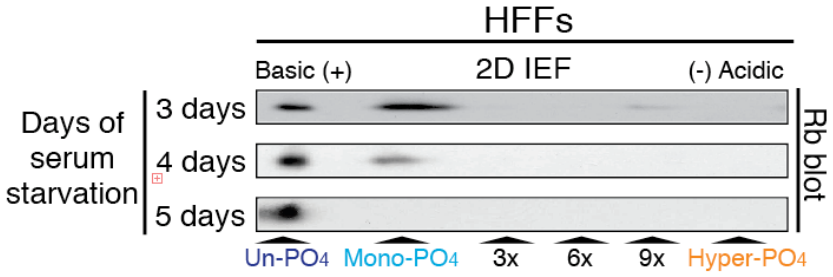

Figure 4.8:
2D IEFs of HFFs were serum starved for the indicated number of days. Rb is completely unphosphorylated after 5 days of serum starvation.

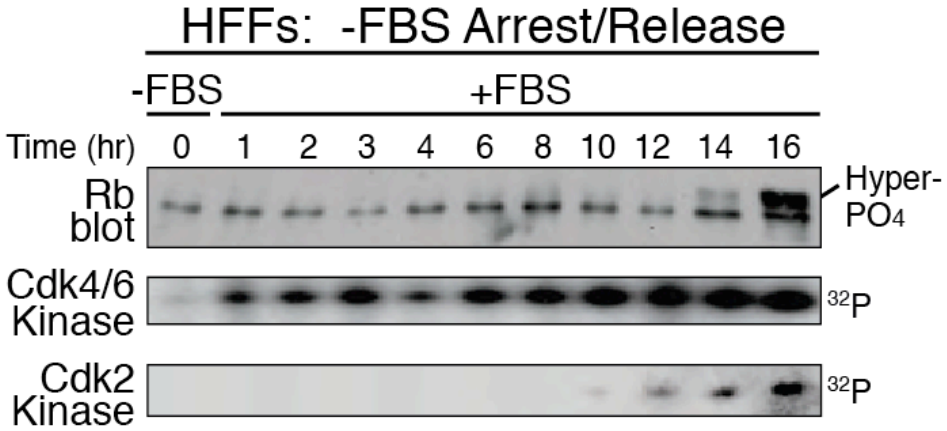

Figure 4.9:
HFFs were serum starved (-FBS) and released (+FBS) for 16 hours.
Rb blot was run on a 6% SDS-PAGE. GST-Rb was used as a substrate for Cdk4/6 kinase assay, while Histone H1 was used for Cdk2 kinase assay.

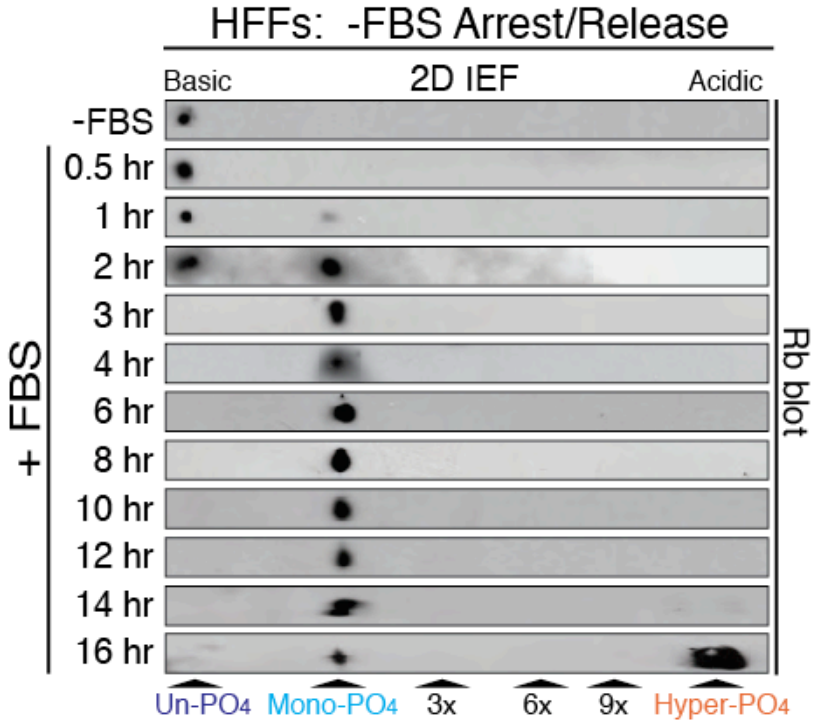

Figure 4.10:
2D IEFs of HFFs that were serum starved (-FBS) and released (+FBS).

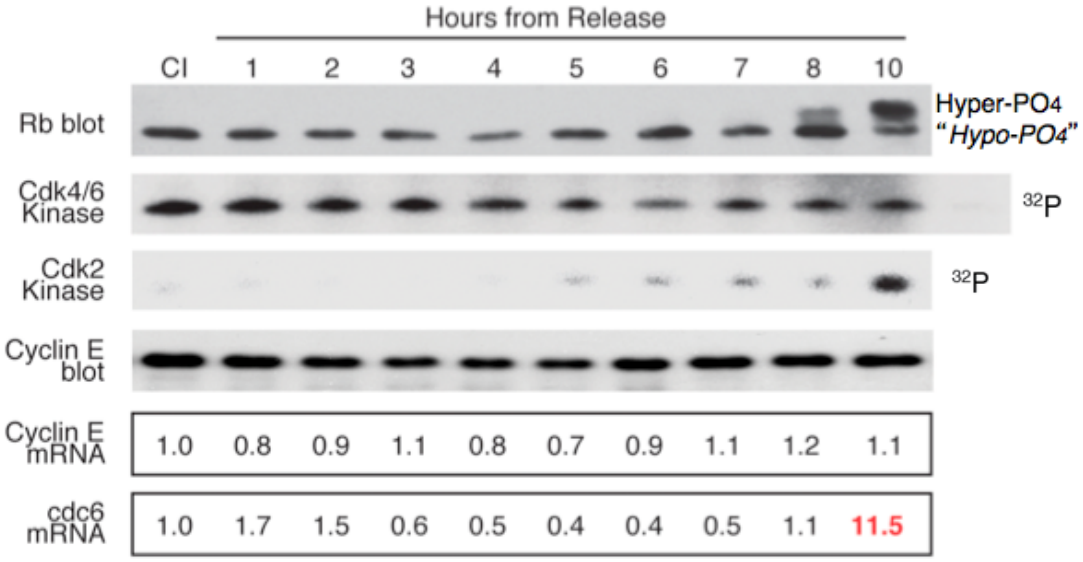

Figure 4.11:
HFFs were contact inhibited (CI) in the presence of serum and released for 10 hours.
Rb blot was a 6% SDS-PAGE, Cyclin E blot was a 10% gel. mRNA levels were analyzed by TAQMAN qRT-PCR relative to CI cells. GST-Rb was used as a substrate for Cdk4/6 kinase assay, Histone H1 for Cdk2 kinase assay.

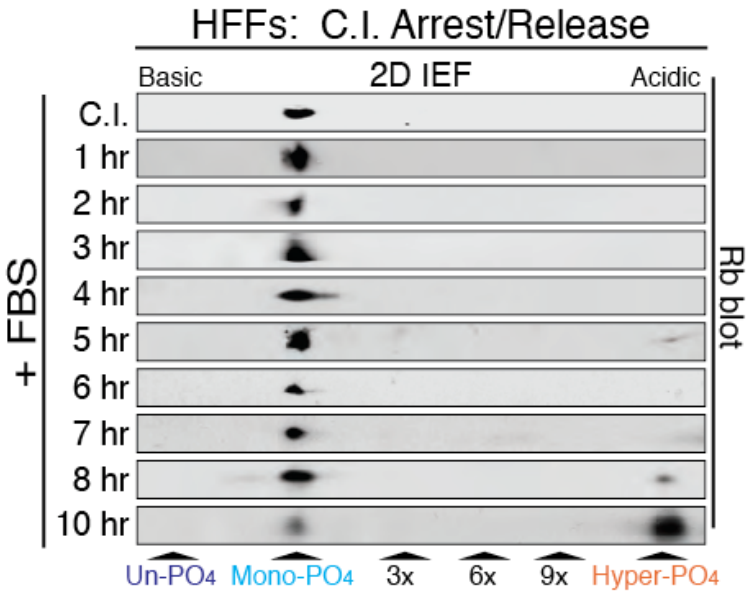

Figure 4.12:
2D IEFs of HFFs that were contact inhibited (C.I.) and released for 10 hours.

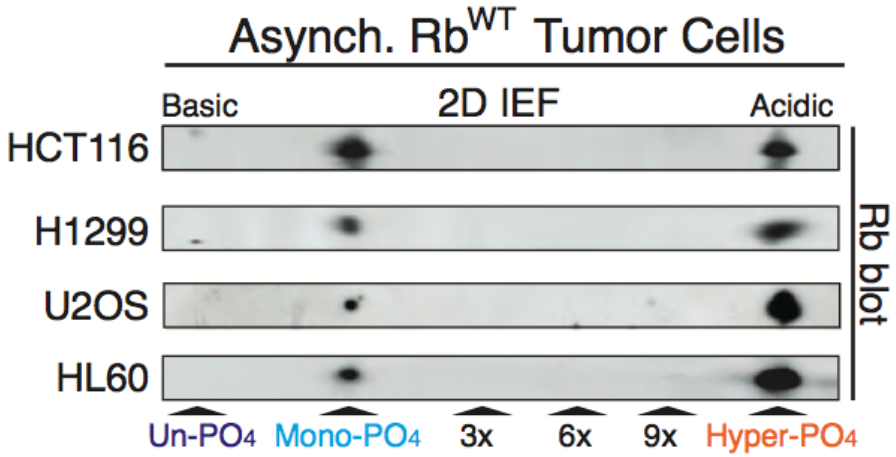

Figure 4.13
2D IEFs of 4 different tumor cell lines containing wild-type Rb (asynchronous). HCT116 colon carcinoma, H1299 lung adenocarcinoma, U2OS osteosarcoma, HL60 human leukemia.

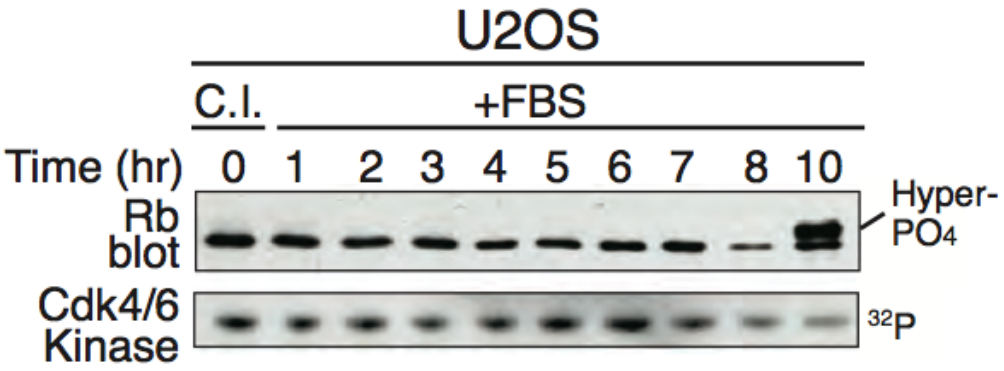

Figure 4.14:
U2OS osteosarcoma cells were contact inhibited (C.I.) and released.
Rb blot is a 6% SDS-PAGE. GST-Rb was used as a substrate for Cdk4/6 kinase assay.

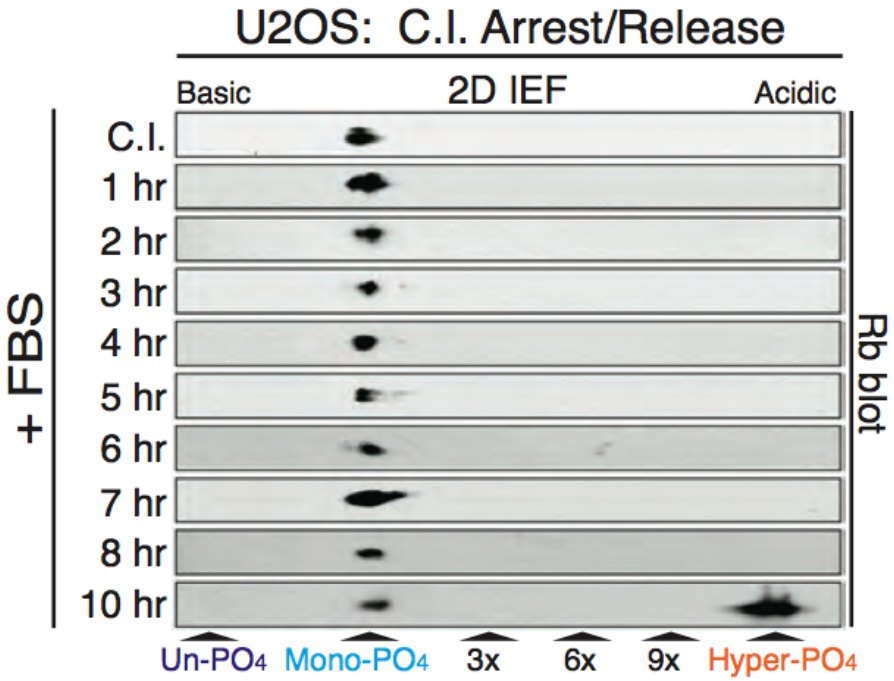

Figure 4.15:
2D IEFs of U2OS osteosarcoma cells that were contact inhibited and released for 10 hours.

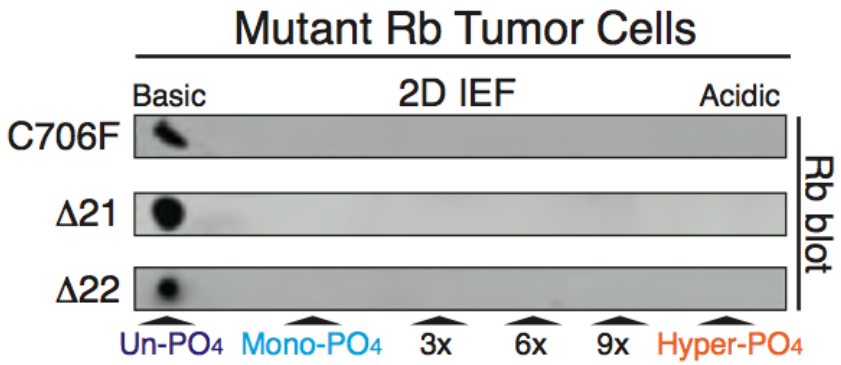

Figure 4.16:
2D IEFs of endogenous Rb in tumor cell lines that contain mutated Rb.

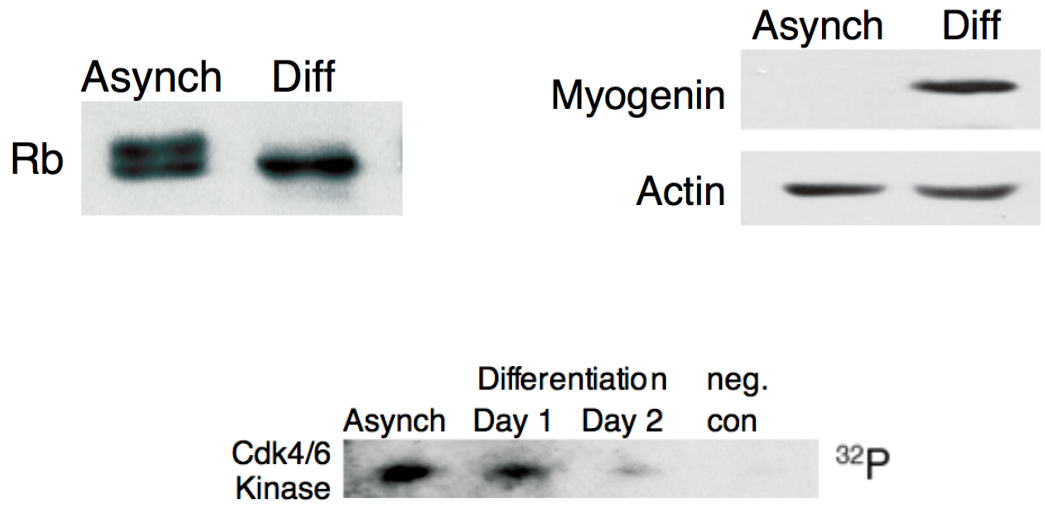

Figure 4.17:
SDS-PAGE showing asynchronous C2C12 cells (Asynch) vs Differentiated C2C12 cells for 48 hours (Diff). Blotted for Rb, myogenin, and actin.
Cdk4/6 kinase assay with cycling C2C12s (Asynch), and C2C12s that were differentiated for 48 hours. GST-Rb was used as a substrate. Neg. con is the negative control (no antibody used).

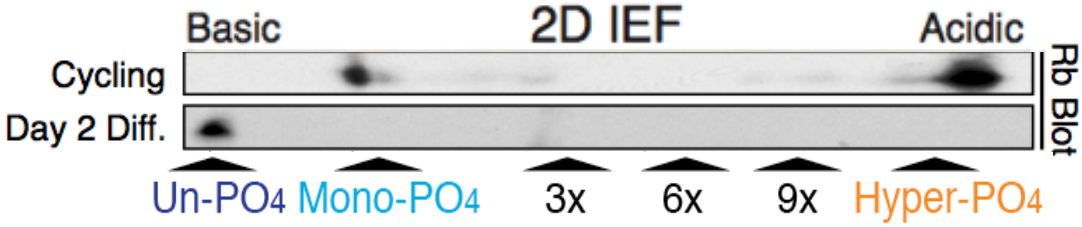

Figure 4.18:
2D IEF of C2C12 cells that are either cycling or differentiated for 48 hours (Day 2 Diff.). The differentiated sample shows the presence of un-phosphorylated Rb.

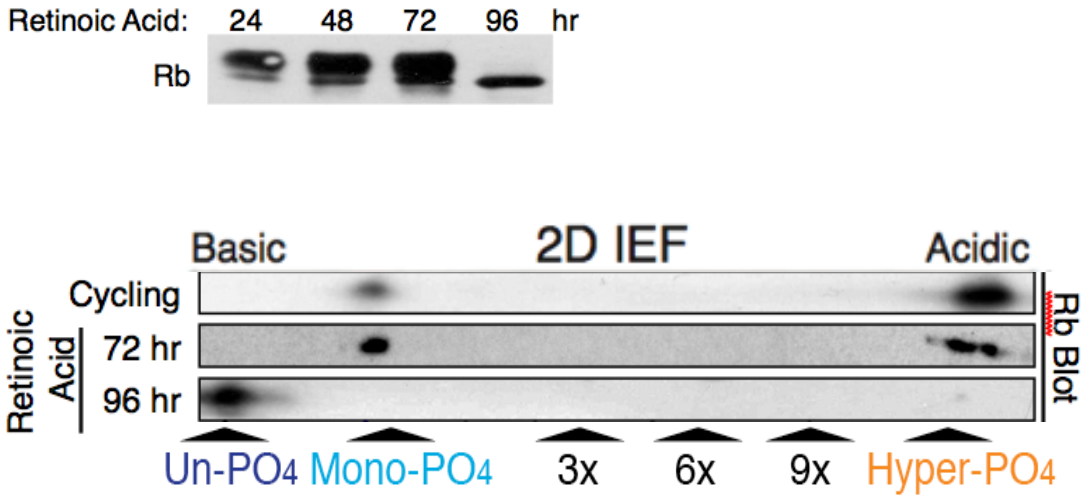

Figure 4.19:
1D SDS-PAGE of Rb showing differentiation of HL-60 cells by 1 μM Retinoic Acid for 96 hours.
2D IEFs of cycling HL-60 cells, 72 and 96 hours of differentiation with 1 μM Retinoic Acid.

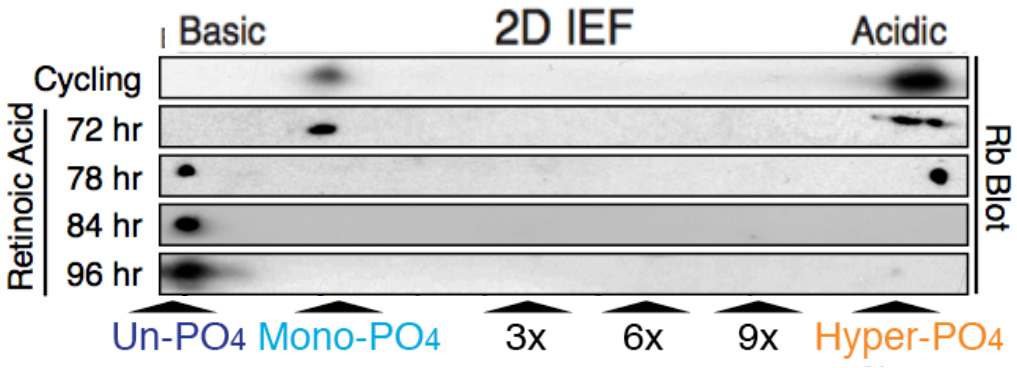

Figure 4.20:
2D IEFs of HL-60 cells undergoing differentiation through the addition of 1 μM Retinoic Acid. Cycling cells shown as a control. Rb is un-phosphorylated and hyper-phosphorylated at 78 hours post addition of Retinoic Acid.

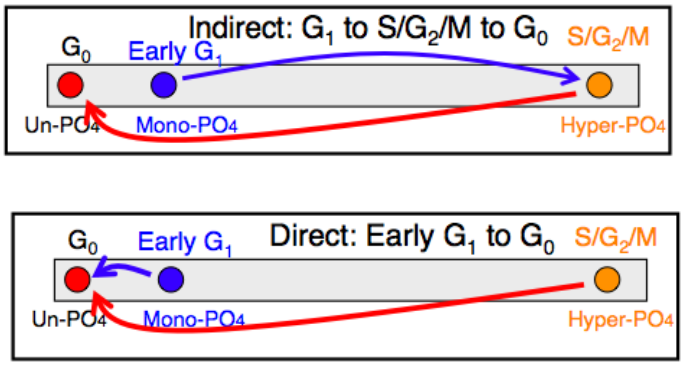

Figure 4.21:
Schematic representation of two theories of differentiation of how Rb becomes un-phosphorylated. The top panel shows an indirect way of cells to get to a quiescent G_0 phase, while the bottom panel shows a direct way.

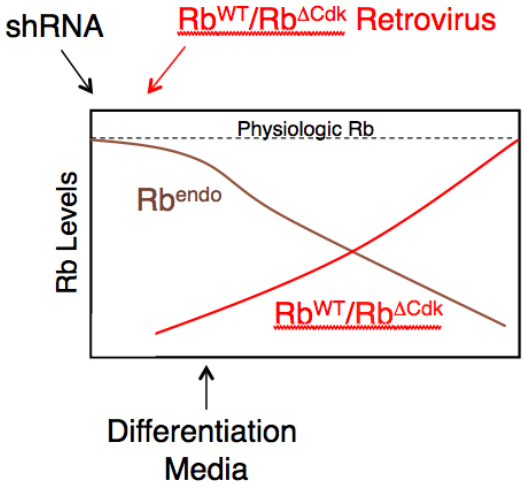

Figure 4.22:
Schematic representation of shRNA and Rb construct infections into C2C12 cells. Differentiation media was added 8 hours after infection.

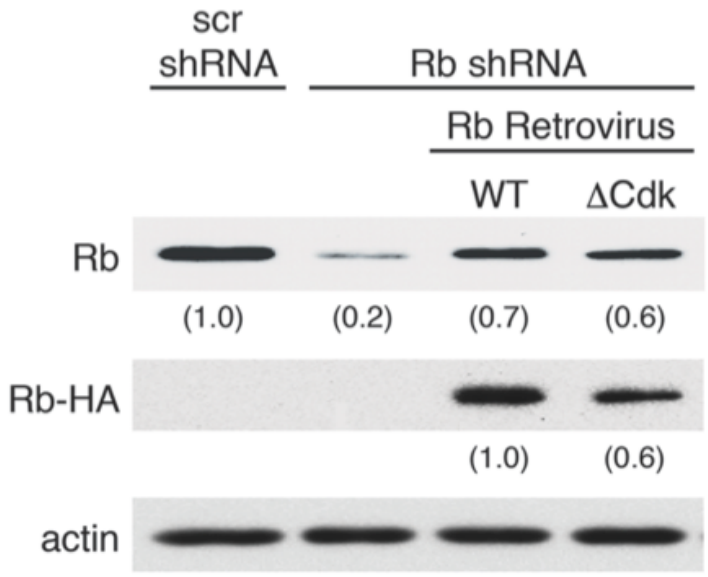

Figure 4.23:
C2C12 cells were transfected with either a scrambled shRNA (scr shRNA) or Rb shRNA. HA-tagged Rb retroviruses were added after – either a WT construct or a ΔCDK construct that cannot be phosphorylated. Relative levels are quantified below. Experiment done by Dr. Gary Shapiro.

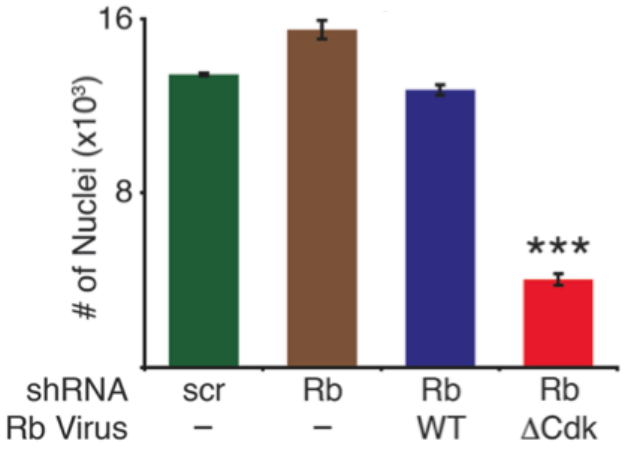

Figure 4.24:
The number of nuclei was quantified via FACS analysis in C2C12 cells during differentiation. Scrambled (scr) or Rb shRNAs were added along with retroviruses for either WT or ΔCDK Rb constructs. Error bars show SEM for three independent experiments. Experiment done by Dr. Gary Shapiro.

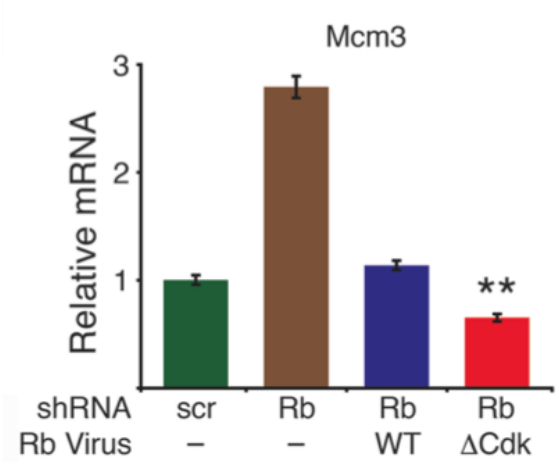

Figure 4.25:
Mcm3 expression levels in C2C12 cells undergoing differentiation were analyzed by TAQMAN qRT-PCR and quantified to scr shRNA levels. Scrambled (scr) or Rb shRNAs were added along with retroviruses for either WT or ΔCDK Rb constructs. Error bars show SEM for three independent experiments. Experiment done by Dr. Gary Shapiro.

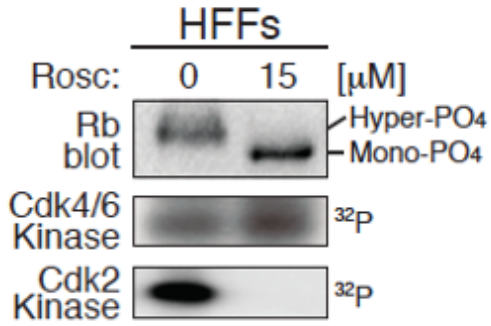

Figure 4.26:
HFFs were contact inhibited and released. At 4 hours post release, either DMSO or 15 μM Roscovitine was added for 6 hours. A 6% SDS-PAGE gel was run for the Rb blot. GST-Rb was used as a substrate for Cdk4/6 kinase assay, while Histone H1 was used for Cdk2 kinase assay.

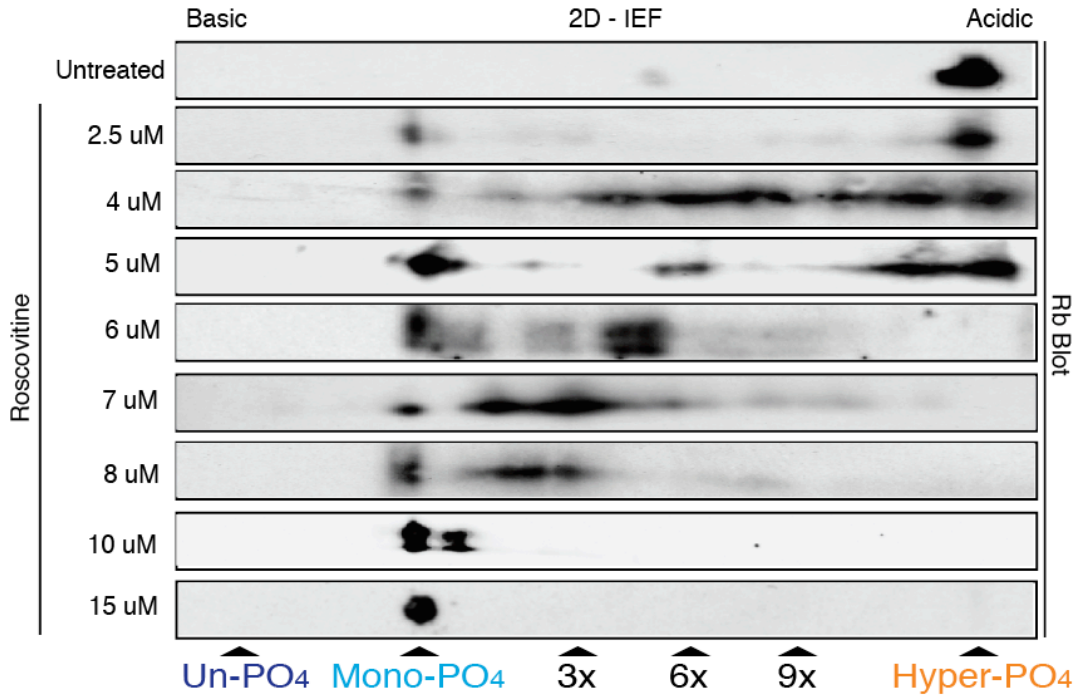

Figure 4.27:
2D IEFs of HFFs that were contact inhibited and released. At 4 hours post release, varying concentrations of Roscovitine were added. Cells were harvested after 10 hours.

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CHAPTER 5:

Cyclin D:Cdk4/6 Complexes Mono-Phosphorylate Rb

Introduction

Rb has been known to be a substrate of two distinct Cdks during G_1 phase of the cell cycle for the past 20 years. This phosphorylation by Cdks happens sequentially – first by Cyclin D:Cdk4/6 complexes, and then by Cyclin E:Cdk2 complexes (Ezhevsky et al., 2001; Haberichter et al., 2007). The prevailing model states that both these kinases inactivate Rb – Cyclin D:Cdk4/6 complexes partially inactivate Rb via "hypo-phosphorylation," and then Cyclin E:Cdk2 complexes complete the inactivation via hyper-phosphorylation (Knudsen and Knudsen, 2006; Paternot et al., 2010; Lundberg and Weinberg, 1998). This hyper-phosphorylation is observed as cells pass the Restriction Point. The previous chapter in this study has already shown that Rb is actually mono-phosphorylated and not multi-phosphorylated into a "hypo-phosphorylated" isoform, and that Cyclin E levels are not the trigger that allows cells to pass the Restriction Point.

There has unfortunately been no physiologic evidence that states that Cyclin D:Cdk4/6 inactivates Rb. Previous studies have utilized semi-phosphorylatable forms of Rb in tumor cell backgrounds (Brown et al., 1999; Chew et al., 1998; Knudsen and Wang, 1997; Leng et al., 1997; Lukas et al., 1997; Sherr and McCormick, 2002; Tashiro et al., 2007). These studies have shown that when D-type cyclins are supraphysiologically overexpressed, Rb is inactivated and cells are driven into S phase (Sherr and McCormick, 2002). They have also shown that when p16 is overexpressed, cells arrest in G_1 phase implying that Rb is active because Cdk4/6 kinase activity is inhibited (Tashiro et al., 2007). These data have been inferred based on utilization of non-physiologic forms of Rb and other key proteins

in G_1 cell cycle progression, and so physiologic functions of Rb and Cyclin D:Cdk4/6 complexes remain unclear.

Based on the literature and the fact that Cdk4/6 kinase activity was constitutively active during Early G_1 phase, Cyclin D:Cdk4/6 became a prime candidate for being the mono-phosphorylating kinase in vivo. From previous work in the literature, Rb was shown to be phosphorylated on Cdk consensus sites, and that these sites were the same sites being phosphorylated during inactivation and hyper-phosphorylation (Mittnacht et al., 1994; Burkhart and Sage, 2008). Identifying the mono-phosphorylating kinase would allow for a better understanding of how Rb is regulated in Early G_1 phase.

Furthermore, the active form of Rb in physiologic conditions remains unknown. By definition, Rb is active when it can bind to E2F proteins and repress E2F-dependent transcription. It is known that Rb is active during Early G_1 phase (Mittnacht et al., 1994), but the phosphorylation status in physiologic conditions remains a mystery. Because mono-phosphorylated Rb is the only form present during Early G_1 phase, the logical hypothesis states that this phospho-isoform is the active form. To test this hypothesis, DNA damage will be induced to simulate a functional response, and then with 2D IEF analysis and physiologic experiments, mono-phosphorylated Rb will be shown to be the active form.

Results

Inhibiting Cdk4/6 Kinase Activity Prevents Rb Mono-Phosphorylation

Cdk4/6 became the prime candiate to be the mono-phosphorylating kinase of Rb in vivo because of its constitutive activity during Early G_1 phase (Haberichter et al., 2007; Figure 4.10). To test this hypothesis, overexpression of p16-INK4 (p16), a specific inhibitor of Cdk4/6, was utilized. This protein binds to Cdk4/6 and prevents binding to the D-type cyclins (Knudsen and Knudsen, 2006). The cyclin-Cdk complex is unable to form, and thus cannot perform any phosphorylating activity (Jiang et al., 1998). Primary human foreskin fibroblast cells (HFFs) were synchronized via serum starvation. Rb was completely un-phosphorylated, consistent with previous results (Figure 5.1). The cells were then re-stimulated with the addition of serum-containing media (10% FBS) for 4 hours. This would allow the cells to fully enter the cell cycle but not reach the Restriction Point, based on the kinetic analysis done in the previous chapter (Figure 4.9). Rb was thus completely mono-phosphorylated (Figure 5.1). The cells were then overexpressed with p16 via adenoviral infection. Even in the presence of serum-containing media, Rb failed to become mono-phosphorylated and remained in an un-phosphorylated state (Figure 5.1). Thus, p16 overexpression prevented Rb from becoming mono-phosphorylated even when cells were stimulated to enter the cell cycle through the addition of mitogens and growth factors.

Similar results were seen when chemical inhibitors were used to restrict kinase activity. PD0332991, a specific chemical inhibitor of Cdk4/6, was utilized

(Fry et al., 2004). The same experiment with HFFs was performed – synchronization via serum starvation and then release into serum-containing media for 4 hours. Without the presence of the inhibitor, Rb was completely mono-phosphorylated (Figure 5.2). However, with the addition of 2.5 μM PD0332991, Rb remained un-phosphorylated even in the presence of serum-containing media (Figure 5.2). Thus, preventing Cdk4/6 kinase activity fails to generate mono-phosphorylated Rb.

The previous results were observed in primary cells, but regardless of cell type, curbing Cdk4/6 kinase activity prevented detection of mono-phosphorylated Rb. To test this in a tumor cell, our laboratory obtained U2OS osteosarcoma cells from Dr. Liang Zhu's laboratory that had a TET-off inducible p16 system (Jiang et al., 1998). With the removal of tetracycline (TET) from the media, p16 expression was induced in these cells. Through serum starvation, Rb was completely un-phosphorylated and when released into serum-containing media, Rb became completely mono-phosphorylated (Figure 5.3). However, when the expression of p16 was induced with the removal of tetracycline from the media, Rb failed to become mono-phosphorylated and remained in an un-phosphorylated state even in the presence of serum-containing media (Figure 5.3). Thus, regardless of cell type, both primary and tumor cells that overexpress or induce the expression of p16 prevent Rb mono-phosphorylation.

Genetic Deletion of D-Type Cyclins Prevent Rb Mono-Phosphorylation

Next, the role of Cdk4/6 was investigated by utilizing genetic conditional triple knockout cyclin D mouse embryonic fibroblasts (TKO D^- cells). These were a gift from Dr. Peter Sicinski's lab at the Dana Farber Cancer Institute. Cells normally contain three D-type cyclins, but in these cells, Cyclin D1 and D3 both contain loxP sites on both alleles and can be deleted with the addition of Cre recombinase. Cyclin D2 is deleted altogether. Utilizing these cells, the presence of mono-phosphorylation was assessed by 2D IEF when the D-type cyclins were acutely removed. 5 μM Tat-Cre (Wadia et al., 2004) was added to these to completely remove Cyclin D1 and D3 from these cells (Figure 5.4). Once the D^- type cyclins were removed, the TKO D^- cells were much slower in progressing through the cell cycle (Figure 5.5). This was assessed both by cell counts experiments as well as mitotic index for 36 hours after plating (Figure 5.6). Rb phosphorylation was then assessed by a 1D SDS-PAGE gel. There was no difference observed on this gel regardless of whether the D-type cyclins were present or not (Figure 5.4). This is consistent with the fact that a one-dimensional acrylamide gel fails to separate the mono-phosphorylated and un-phosphorylated isoforms of Rb (Figure 4.1). Thus, this methodology was not sufficient to assess the differences of Rb phosphorylation. Cycling cells of both the parental ($D1^+/D3^+$) and the TKO D^- cells were analyzed by 2D IEF. The parental cell line had a familiar Rb phosphorylation pattern – the presence of both mono-phosphorylated Rb and hyper-phosphorylated Rb (Figure 5.7). However, the TKO D^- cells showed a pattern of un-phosphorylated Rb and hyper-phosphorylated Rb (Figure 5.7). With the removal

of the D-type cyclins, Rb cannot become mono-phosphorylated. However, this result shows that Rb can become hyper-phosphorylated without the presence of mono-phosphorylation. These cells were then synchronized via contact inhibition in the presence of serum. In the parental cell line with the D-type cyclins present, Rb is completely mono-phosphorylated (Figure 5.8). However, in the TKO D^- cells, Rb is un-phosphorylated during contact inhibition (Figure 5.8). To make sure un-phosphorylated Rb was seen, a sample of serum starved MEFs was premixed with a contacted inhibited TKO D^- sample, and both co-migrated as a single basic species (Figure 5.8). These results show that the Cyclin D:Cdk4/6 is the Rb mono-phosphorylating kinase, and genetically removing the D-type cyclins prevent Rb mono-phosphorylation.

Cyclin D:Cdk4/6 Mono-Phosphorylates Rb after Mitosis

The experiments that have been shown in this study have only accounted for synchronization of cells in either a G_0 quiescent phase or in Early G_1 phase. Rb remains hyper-phosphorylated throughout the rest of the cell cycle once cells have passed the Restriction Point. Once the cells exit mitosis, Rb becomes de-phosphorylated by PP1 phosphatase (Ludlow et al., 1993). It was thus necessary to test whether the phosphatase de-phosphorylates Rb into an un-phosphorylated or a mono-phosphorylated state after mitosis. U2OS cells were synchronized with Nocadozole, an inhibitor that prevents the polymerization of microtubules and arrests cells in G_2/M phase (Knudsen and Wang, 1998). Consistent with the literature, Rb was all hyper-phosphorylated shown by both one-dimensional

acrylamide gel and 2D IEF when 100 ng/ml Nocadozole was added for 12 hours (Figure 5.9, Figure 5.10). Cells were then released for 4 hours into Early G_1 phase of the next cell cycle in the presence of DMSO or 2.5 μM PD0332991, a specific Cdk4/6 chemical inhibitor. After 4 hours, 2D IEF analyses showed that Rb was completely mono-phosphorylated in the presence of DMSO (Figure 5.10). This signified that all cells had completed mitosis and entered into the next Early G_1 phase. However, when cells were exposed to the Cdk4/6 inhibitor, Rb was un-phosphorylated and prevented from becoming mono-phosphorylated (Figure 5.10). Thus, Rb gets completely de-phosphorylated after cells exit mitosis, and then Cyclin D:Cdk4/6 complexes put on one phosphate. The same results were seen in HeLa cells, where Rb was again completely dephosphorylated after mitosis and then mono-phosphorylated once the cells return to Early G_1 phase (Figure 5.11, Figure 5.12). This mono-phosphorylation was mediated by Cyclin D:Cdk4/6 complexes, because if the inhibitor PD0332991 was added to the media, Rb remained un-phosphorylated.

The Cyclin, not the Cdk, is Responsible for Mono-Phosphorylation

Rb is mono-phosphorylated during Early G_1 phase of the cell cycle. Because only one Cdk phosphorylation site is occupied out of a possible 15, the mechanism of how Rb stays mono-phosphorylated became intriguing. There are at least two ways of thinking about how Rb remains mono-phosphorylated. First, the D-type cyclins bind to Rb via the pocket domain (Dowdy et al., 1993), and as soon as one phosphate group is put on Rb, Cyclin D is immediately removed from Rb to

prevent further phosphorylations. Second, Cdk4/6 is not a very robust kinase in terms of Rb phosphorylation, and thus cannot put multiple phosphorylations on a given Rb molecule.

To start the analysis of the mechanism of mono-phosphorylation, our laboratory obtained fusion proteins from Dr. Brian Law (Chytil et al., 2004). These proteins were then expressed as adenoviruses to be infected into TKO D^- MEFs. The proteins were a wild-type Cyclin D1 protein, a Cyclin D1-Cdk2 fusion protein, and a Cyclin D1-$Cdk2^{Mut}$, where the kinase domain of Cdk2 was mutated and prevented from having any kinase activity. The experiment was to infect TKO D^- MEFs (they do not have any endogenous expression of D-type cyclins) with these proteins, and then assess Rb phosphorylation status through 2D IEF. This would answer an important question of the mechanism of mono-phosphorylation – is it the cyclin or Cdk that is important for mono-phosphorylation to occur?

Four constructs expressed as adenoviruses were infected into TKO D^- MEFs – GFP, Cyclin D1, Cyclin D1-Cdk2, and Cyclin D1-$Cdk2^{Mut}$. All these constructs were N-terminally Flag-tagged. It was needed to first look at whether these proteins were able to complex with the endogenous Cdks. The adenovirus expressing Cyclin D1 was able to co-immunoprecipitate with endogenous Cdk4 and Cdk6, but not Cdk2 in TKO D^- MEFs (Figure 5.13). The fusion proteins both blotted for Cdk2, but did not co-immunoprecipitate with endogenous Cdk4 and Cdk6 (Figure 5.13). Inputs using the whole cell lysates are shown for each immunoprecipitation (Figure 5.14). TKO D^- MEFs were then serum starved, infected with the adenoviruses, and then released for 5 hours in the presence of serum-containing media. 2D IEF

analysis of Rb showed cells treated with the GFP adenovirus were un-phosphorylated (Figure 5.15). When the adenovirus for Cyclin D1 was expressed, Rb became mono-phosphorylated, as Cyclin D1 was able to bind to Cdk4 and Cdk6 and mono-phosphosphorylate Rb (Figure 5.15). When the Cyclin D1-Cdk2 fusion protein was expressed, Rb was also mono-phosphorylated, and this mono-phosphorylation was Cdk-dependent, because expression of the Cyclin D1-Cdk2^{Mut} fusion protein resulted in un-phosphorylated Rb (Figure 5.15). Thus, the D-type cyclins are responsible for mono-phosphorylation, because regardless of what Cdk is paired to them, Rb is mono-phosphorylated.

Early G_1 Phase Cells Contain Fourteen Different Mono-Phosphorylated Isoforms

As mentioned above, Rb has 16 Cdk consensus sites throughout the molecule, with one site (S567) buried in the A box domain of the protein. This was already shown not to be phosphorylated (Figure 4.4). After finding Rb mono-phosphorylated throughout the duration of Early G_1 phase, it was necessary to identify which sites were being mono-phosphorylated. This would potentially give insight into how Rb is regulated in Early G_1 phase. Phospho-specific antibodies to specific sites on the Rb molecule were used. Serum starved cells that yield un-phosphorylated Rb (G_0 cells), and contact inhibited cells that yield mono-phosphorylated Rb (Early G_1 cells) were used to make sure these antibodies noticed the phosphorylated form of the protein. I used contact inhibited cells in this

experiment because whatever positive signal was observed implied that the specific site that the antibody recognized was mono-phosphorylated.

HFFs were both serum starved and contact inhibited, and then blotted for specific phosphorylation sites of Rb using a number of antibodies. Not surprisingly, antibodies targeting phosphorylated sites of S249, S252, T373, S608, S612, S795, S807, S811, T821, and T826 had no signal in serum starved cells (Figure 5.16). As another control, HFFs were treated with thymidine, which is an inhibitor of DNA synthesis and arrests cells in S phase of the cell cycle. At this stage, Rb is completely hyper-phosphorylated and should be able to be recognized by each phospho-specific antibody. Consistent with this notion, each site was phosphorylated during a thymidine-induced arrest (Figure 5.16). Importantly, during contact inhibition, each one of those sites was also phosphorylated (Figure 5.16). Thus, antibodies against specific phosphorylation sites on Rb resulted in multiple sites being mono-phosphorylated.

Mono-phosphorylation of various sites could have been a cell-type specific event. To test this, U2OS osteosarcoma cells were analyzed similar to HFF cells. U2OS cells were serum starved (G_0 phase), contact inhibited (Early G_1 phase), or arrested in S phase by the addition of thymidine, and checked for Rb phosphorylation using phospho-specific antibodies. Similar to results seen in HFFs, each phospho-antibody was able to see phosphorylated forms of Rb in both contact inhibited and S-phase arrested U2OS cells (Figure 5.17). As a control, no phosphorylation was seen in serum-starved cells (Figure 5.17). Thus, regardless of cell type, Rb was mono-phosphorylated in Early G_1 phase on at least 8 different

sites. A couple of the antibodies (S249/S252 and S807/S811) recognized both sites, but it was unclear whether a positive signal resulted in both sites being phosphorylated or either site being phosphorylated. Nevertheless, it was seen that Rb could be mono-phosphorylated on a number of sites throughout the entire molecule.

The fact that Rb was mono-phosphorylated also implied that if one phosphorylation site is occupied on a given mono-phosphorylated Rb molecule, no others could be occupied at the same time. To test this, certain phospho-specific antibodies were used for co-immunoprecipitation. HFFs were contact inhibited to render them in Early G_1 phase. Antibodies against S608 and T826 were used for immunoprecipitation, and then the remaining phospho-specific antibodies were used for blotting. Consistent with 2D IEF data that shows that Rb is mono-phosphorylated during contact inhibition, the phospho-specific immunoprecipitation of T826 was only recognized by the T826 antibody, and not by the T373, S608, S612, and S795 antibodies (Figure 5.18). Similarly, the phospho-specific immunoprecipitation of S608 was only recognized by the S608 antibody and not by any of the other phospho-specific antibodies (Figure 5.18). The same experiment was then repeated with HFF cells that were arrested at S phase by thymidine. At this point in the cell cycle, Rb would be completely hyper-phosphorylated, and multiple phosphorylation sites would be occupied. When the antibody against T826 was used for immunoprecipitation, all of the other antibodies were able to recognize it, showing that multiple phosphorylations are occurring on a single Rb molecule (Figure 5.18). The same results were seen when S608 was used for

immunoprecipitation (Figure 5.18). Thus, another independent way besides 2D IEF using phospho-specific antibody immunoprecipitation analysis shows that Rb is mono-phosphorylated during Early G_1 phase.

Antibodies to every phosphorylation site on Rb were not able to be obtained, or they were not specific to that particular site (data not shown). To see whether every site on Rb could be mono-phosphorylated, every possible mono-phosphorylated mutant of Rb was cloned. These constructs would have 14 sites mutated to alanines with one site reverting back to a wild-type serine or threonine. Dr. Manuel Kaulich, a postdoctoral fellow in the laboratory, was able to generate all these constructs in a pCMV vector background. These constructs were then transiently transfected into asynchronous 293T cells and were checked for Rb phosphorylation status by 2D IEF. Out of the 15 constructs that were made, 14 were completely mono-phosphorylated (Figure 5.19). The only site that was not phosphorylated and remained un-phosphorylated was T5 (Figure 5.19). T5 resides on the extreme N-terminus of Rb, and this is the only Cdk consensus site that is not conserved below primates. Thus, there exists 14 different mono-phosphorylated isoforms of Rb in vivo.

To make sure the N-terminal HA tag was not affecting the phosphorylation of the T5 mutant, Dr. Kaulich made a C-terminally HA tagged Rb^{WT}, $Rb^{\Delta Cdk}$, and the T5 mono-phosphorylated mutant. Using 2D IEF analysis, the T5 mutant was observed to be unphosphorylated, while the WT and ΔCDK looked identical to N-terminally HA-tagged versions (Figure 5.20). Thus, regardless of where the tag was placed on the Rb construct, T5 failed to become mono-phosphorylated or even

hyper-phosphorylated. Although this is a Cdk consensus site, it does not get phosphorylated in vivo.

Mono-Phosphorylated Rb is the Active Form of Rb

Rb is a classic tumor suppressor gene – when active, it can induce a cell cycle arrest and prevent cells from progressing through the cell cycle (Burkhart and Sage, 2008; Knudsen and Knudsen, 2006). By definition, Rb is active when it can bind to E2Fs and repress E2F-dependent transcription and thus prevent genes necessary for cell cycle progression from being transcribed (Burkhart and Sage, 2008). Many studies in the literature showed that Rb is in its active form during Early G_1 phase (Brown et al., 1999; Paternot et al., 2010; Knudsen and Knudsen, 2006). However, these studies also mentioned that Rb became more inactive as the phosphorylation sites on Rb became occupied (Burkhart and Sage, 2008; Rubin and Dick, 2013). Because mono-phosphorylated Rb is the only form present in Early G_1 phase, I hypothesized that this phospho-isoform must be the active form of Rb. To test this, it was necessary to figure out a method to see whether mono-phosphorylated Rb was able to participate in a functional response. The functional response that was used for the subsequent experiments was the induction of DNA damage.

To simulate DNA damage, Doxorubicin (DOX), a topoisomerase II-inhibitor that causes an Early G_1 phase cell cycle arrest, was utilized (Attardi et al., 2004). At a sub-optimal dose, these cells will stop cycling completely and arrest in Early G_1 phase. When asynchronous MEFs were treated with DOX for 48 hours, Cdk4/6

kinase activity remained active, but Cdk2 activity was inhibited, indicating that these cells were arrested in Early G_1 phase (Figure 5.21). Consistent with these data, Rb ran at its fastest migrating form via 1D SDS-PAGE analysis (Figure 5.21). When 2D IEF analyses were performed, Rb was mono-phosphorylated and hyper-phosphorylated when DOX was not added, but became exclusively mono-phosphorylated when the drug was added after 48 hours (Figure 5.22). Thus, Rb is mono-phosphorylated during a DNA damage response.

Dr. Gary Shapiro, a former postdoctoral fellow in the laboratory, performed experiments to show that Rb needs to be mono-phosphorylated to function in a DNA damage response. Our laboratory obtained $Rb^{lox/lox}$ MEFs from Anton Berns' lab from the Netherlands Cancer Institute (Marino et al., 2000). With the addition of Tat-Cre (Wadia et al., 2004), Rb was acutely deleted in these cells. Retroviruses were made from HA-tagged Rb^{WT} and $Rb^{\Delta Cdk}$ (a version of Rb that cannot be phosphorylated) constructs to be infected into these cells. A system was created where at the same time Rb was acutely deleted with the addition of Tat-Cre, retroviruses expressing either Rb^{WT} or $Rb^{\Delta Cdk}$ were exogenously added through infection (Figure 5.23 shows a schematic representation of the experiment). Thus, the cell would be exposed to a version of Rb at all times. One of the reasons why there is ambiguity as to what is the active form of Rb is that previous data was assessed in Rb-deleted cells where there could be partial compensation from p107 and p130, the two other members of the pocket protein family (Lundberg and Weinberg, 1998; Knudsen and Wang, 1998). Furthermore, previous data utilized expression of Rb at supra-physiologic levels (Knudsen and Wang, 1998; Paternot et

al., 2010; Mittnacht et al., 1994). It was thus imperative to address questions regarding the active form of Rb by making sure Rb was exogenously put in at physiologic levels, and also making sure that cells were exposed to a form of Rb at all times.

$Rb^{lox/lox}$ MEFs were treated with Tat-Cre, and then infected with retroviruses. Rb levels were checked via Western blotting. The addition of Tat-Cre significantly decreased Rb levels, and exogenous Rb^{WT} or $Rb^{\Delta Cdk}$ were put back at levels similar to that of endogenous Rb (Figure 5.24). These cells were then treated with a sub-optimal dose of DOX (100 ng/mL) and checked for cell cycle profiles by FACS analysis. Control cells arrested in Early G_1 phase, indicated by a high 2n DNA population and a low "greater than 4n" population (Figure 5.25). Cells that were treated with Tat-Cre to acutely remove endogenous Rb but not infected with any viruses had a much higher "greater than 4n" population (Figure 5.25). These cells continued to cycle regardless of whether DNA damage was induced. When a retrovirus containing Rb^{WT} was added back into cells, the 2n DNA population increased while the "greater than 4n" population decreased almost back to wild-type levels (Figure 5.25). However, when the $Rb^{\Delta Cdk}$ was added back in, there was no compensation seen, and "greater than 4n" levels increased significantly (Figure 5.25). The exact same phenotypes were seen if MEFs were treated with 20 grays of irradiation (Figure 5.26). Thus, an Rb construct that cannot be phosphorylated cannot function during a DNA damage response, as cells fail to arrest in Early G_1 phase and continue to cycle. This phenotype also holds true regardless of how DNA damage is administered.

2D IEF analyses were then performed to find out the phosphorylation status of these constructs when DNA damage was induced. $Rb^{lox/lox}$ MEFs were treated with Tat-Cre to acutely remove endogenous Rb, infected with retroviruses containing either an Rb^{WT} or an $Rb^{\Delta Cdk}$ construct, and then synchronized by contact inhibition to induce an Early G_1 phase arrest. They were then released for 48 hours in the presence of serum-containing media with or without the addition of 100 ng/ml DOX. Without the addition of DOX, Rb^{WT} had the same classical phosphorylation profile – the presence of both mono-phosphorylated and hyper-phosphorylated Rb (Figure 5.27). When DOX was added, Rb^{WT} was completely mono-phosphorylated, which was a similar result seen when endogenous Rb was exposed to DNA damage (Figure 5.27). However, $Rb^{\Delta Cdk}$ remained un-phosphorylated even with the addition of DOX (Figure 5.27). This result shows that no other sites on the Rb molecule become phosphorylated when DNA damage is induced in cells.

Endogenous Rb phosphorylation in synchronized cells was then analyzed by 2D IEF during a DNA damage response. MEFs were serum starved for 5 days to yield un-phosphorylated Rb (Figure 5.28). Then, serum-containing media was added to allow the cells to enter the cell cycle. Simultaneously, cells were either untreated or treated with a sub-optimal dose of DOX (100 ng/ml). After 4 hours, Rb phosphorylation was checked by 2D IEF analysis. In both conditions, Rb was mono-phosphorylated showing that this was the phospho-isoform of Rb present in cells undergoing DNA damage as cells entered the cell cycle (Figure 5.28).

There have been some reports in the literature stating that Rb could be phosphorylated by non-Cdk kinases during a DNA damage response, including

Chk1/2 and Aurora B (Inoue et al., 2007; Nair et al., 2009). However, these sites on Rb were all Cdk consensus sites. The hypothesis that Cyclin D:Cdk4/6 was the mono-phosphorylating kinase during a DNA damages response was tested utilizing induction of p16. Rb phosphorylation status was analyzed in U2OS Tet-responsive p16 cells (Jiang et al., 1998) via 2D IEF. U2OS cells were serum starved and then released in the presence of serum-containing media and DOX for 4 hours. In serum starved conditions, Rb was un-phosphorylated (Figure 5.29). When the cells were stimulated with serum-containing media and 100 ng/ml DOX for 4 hours, Rb was completely mono-phosphorylated (Figure 5.29). However, when the expression of p16 was induced with the removal of tetracycline from the media, Rb remained in an un-phosphorylated state even with the exposure to DNA damage (Figure 5.29). Thus, during a DNA damage response, Cyclin D:Cdk4/6 is the only kinase that mono-phosphorylates Rb.

As stated above, Rb is active when it can bind to the E2F family of transcription factors and repress cell cycle progression. Before checking E2F binding, it was necessary to check whether Rb^{WT} or $Rb^{\Delta Cdk}$ bound to adenovirus E1A, which should bind to Rb regardless of phosphorylation status. 293Ts were transfected with these constructs, immunoprecipitated, and then blotted for endogenous E1A. Because the pocket domain is not structurally altered in $Rb^{\Delta Cdk}$, both Rb^{WT} and $Rb^{\Delta Cdk}$ bound to E1A at the same affinity (Figure 5.30). This is consistent with the result that Rb is sequestered by viral oncoproteins (Whyte et al., 1988; Munger et al., 1989; Dyson et al., 1989; Dyson et al., 1990). Dr. Shapiro then assayed whether un-phosphorylated Rb was able to bind E2F4. After the addition of

Tat-Cre to acutely remove endogenous Rb, $Rb^{lox/lox}$ MEFs were infected with either Rb^{WT} or $Rb^{\Delta Cdk}$, and both proteins were immunoprecipitated and blotted for endogenous E2F4. In this case, the Rb^{WT} construct bound to E2F4 at a much higher affinity than $Rb^{\Delta Cdk}$ (Figure 5.31, Figure 5.32). This is seen both in contact inhibited cells, where Rb is mono-phosphorylated, and cells treated with DNA damage, where Rb is also mono-phosphorylated. Thus, un-phosphorylated Rb is not active during Early G_1 phase because it cannot bind to E2F4 during both contact inhibition and DNA damage simulation.

To test this further, qRT-PCR of E2F target genes were analyzed to see whether $Rb^{\Delta Cdk}$ was able to prevent activation of these genes during a DNA damage response. The prediction would be that E2F target genes, such as DHFR, cdc6, and Cyclin A2, would have greater mRNA expression when the $Rb^{\Delta Cdk}$ construct was infected, because these cells had been shown to continue cycling even when DNA damage was sensed (Figure 5.25). When DHFR, cdc6, and Cyclin A2 levels were analyzed by qRT-PCR, deleting Rb with Tat-Cre significantly increased expression of these genes (Figure 5.33). When Rb^{WT} was exogenously added back to the MEFs, the expression of these genes on the mRNA level went down near wild-type levels (Figure 5.33). However, when $Rb^{\Delta Cdk}$ was exogenously added back in, the expression of E2F target genes significantly increased to levels similar to deleting Rb altogether (Figure 5.33). Conversely, p21, which is not an E2F-dependent gene, was at similar levels regardless of Rb expression or Rb phosphorylation (Figure 5.33). Thus, mRNA expression of E2F-depedent genes is significantly higher when

un-phosphorylated Rb is present, signifying that this isoform cannot participate in a functional response.

Finally, microarray analysis was performed by Dr. Gary Shapiro showing a global transcription profile of genes affected by induction of DNA damage. $Rb^{-/-}$ MEFs (cells that were treated with Tat-Cre to remove endogenous Rb), and retrovirally-infected $Rb^{-/-}$ MEFs (either with Rb^{WT} or $Rb^{\Delta Cdk}$) were treated with 100 ng/mL DOX. They were then checked for mRNA expression levels on a global level via microarray analysis after 3 hours. Similar to results that were seen previously, $Rb^{\Delta Cdk}$ MEFs had a very similar expression profile to $Rb^{-/-}$ MEFs (Figure 5.34). This is consistent with the fact that un-phosphorylated Rb is not active and cannot participate in the DNA damage response, so the phenotype should resemble a cell that does not have Rb altogether. Together, these data show that mono-phosphorylated Rb is the active form of Rb.

Mono-Phosphorylated Rb is Selected for during DNA Damage

Mono-phosphorylated Rb is the active form of Rb, as it can participate and function during DNA damage to cause an Early G_1 phase arrest. Knowing this, I was interested in assessing whether cells could select for mono-phosphorylated Rb if that isoform was not present. To test this, MEFs were serum starved for five days to drive all cells out of the cell cycle. These quiescent cells were then treated with DOX without the presence of serum-containing media. Interestingly, Rb became mono-phosphorylated with the addition of 100 ng/ml DOX when 2D IEFs were performed (Figure 5.35). This mono-phosphorylation was also dependent on Cyclin

D:Cdk4/6 complexes, because when p16 was overexpressed in MEFs through addition of an adenovirus, Rb remained un-phosphorylated even with the addition of DOX (Figure 5.35). Cdk4/6 kinase activity, which was blocked during serum starvation, became induced when DOX was added (Figure 5.36). Cdk4/6 kinase activity was off in serum starved cells presumably because the D-type cyclins were not expressed. However, during DNA damage induction, Cyclin D1 expression increased compared to serum starved cells (Figure 5.36). Cdk4 levels remained the same regardless of whether DOX was added or not, but Cdk6 levels were slightly upregulated (Figure 5.36). These data show that not only does Rb become mono-phosphorylated in response to DNA damage, but that this phospho-isoform is selected for.

Seeing that Rb becomes mono-phosphorylated during a DNA damage response, phospho-specific antibodies were used to determine whether the same sites were being phosphorylated compared to contact inhibition. In contrast to contact inhibited cells, where 14 sites were mono-phosphorylated, S249, S252, S608, and T821 failed to become mono-phosphorylated when HFFs were treated with 100 ng/ml DOX without the presence of serum-containing media (Figure 5.37). Thus, certain mono-phosphorylated Rb isoforms are selected for during a DNA damage response. This can lead to an added regulation of Rb, where certain mono-phosphorylated isoforms of Rb might perform different functions either normally or during stress responses.

This added level of regulation is further observed when certain mono-phosphorylated constructs were checked for binding affinities against E2F4.

$Rb^{lox/lox}$ MEFs were treated with Tat-Cre to remove endogenous Rb. They were then exogenously infected with the T373, S608, and S811 mono-phosphorylated mutants. The cells were contact inhibited to render them in Early G_1 phase. These HA-tagged constructs were then immunoprecipitated and checked for endogenous E2F4 binding. As previously observed, exogenous Rb^{WT} bound to E2F4 at high levels, while $Rb^{\Delta Cdk}$ bound to E2F4 at a very low affinity (Figure 5.38). Interestingly, both the T373 and S608 mutants bound at similar affinities as the $Rb^{\Delta Cdk}$, but the S811 construct displayed a stronger affinity toward E2F4 (Figure 5.38). A mutant that contained three wild-type phosphorylation sites (3x – T373, S608, and S811) showed levels of E2F4 bound similar to the Rb^{WT} protein (Figure 5.38). These data reinforce the notion that specific mono-phosphorylation sites can bind to proteins at different affinities, showing another level of regulation of Rb.

In summary, Cdk4/6 is the mono-phosphorylating kinase of Rb during Early G_1 phase. This form is the active form of Rb, because it can participate in a functional response, while un-phosphorylated Rb cannot. The implications of these results will be discussed in the next section.

Discussion

Identifying the mono-phosphorylating kinase of Rb would give a better understanding of regulation and progression of cells during Early G_1 phase of the cell cycle. It was also important to figure out which sites are phosphorylated during mono-phosphorylation. Elucidating when and where Rb was phosphorylated would add to the regulation of how Rb is functioning during Early G_1 phase. This study has shown that Cyclin D:Cdk4/6 is the mono-phosphorylating kinase of Rb, and that the complex is able to mono-phosphorylate Rb on 14 different sites.

Using inhibitors to the function of Cyclin D:Cdk4/6 complexes, the Rb mono-phosphorylating kinase was identified. p16 was used as a specific inhibitor of Cdk4/6. Many studies had utilized this cell-cycle inhibitor because over-expression would lead to a G_1 cell cycle arrest (Jiang et al., 1998; Knudsen and Knudsen, 2006). However, it was important to devise a method where Cyclin D:Cdk4/6 complexes would be the only major Cdk that would potentially be active, so that whatever phenotypic differences were seen was solely because of the regulation of that kinase. Hence, synchronization of cells via serum starvation was performed in order to bring cells to a quiescent G_0 phase. This resulted in un-phosphorylated Rb. From the kinetic analyses done in HFFs, Rb mono-phosphorylation was complete within 3 hours post-release from serum starvation (Figure 4.9). To make sure Rb was completely mono-phosphorylated, cells were released for 4 hours with or without the presence of p16 overexpression via adenovirus. By either using p16 or a chemical inhibitor that specifically inhibited Cdk4/6 kinase activity (PD0332991), Rb failed to become mono-phosphorylated.

Our laboratory was fortunate to obtain conditional Cyclin D knockout cells from Dr. Peter Sicinski's laboratory. This would be a genetic approach to show that Rb mono-phosphorylation was dependent on expression of the D-type cyclins. Thus, the simple experiment was to acutely delete the D-type cyclins with Tat-Cre, and then check Rb phosphorylation via 2D IEF. When they were deleted, the cells cycled much slower and Rb failed to become mono-phosphorylated. Interestingly, in a cycling population of TKO D^- cells, Rb was both un-phosphorylated and hyper-phosphorylated. The initial hypothesis from our laboratory was that Rb mono-phosphorylation acted as a priming step for hyper-phosphorylation. If this were true, a cell would unable to be hyper-phosphorylated unless Rb was mono-phosphorylated first. This would also signify that Cyclin E:Cdk2 complexes would not recognize un-phosphorylated Rb as a substrate. However, deleting the D-type cyclins shows that this hypothesis is not true. Previous studies have showed that in a test tube, Cyclin E:Cdk2 complexes can phosphorylate a construct of unphosphorylated GST-Rb (Jiang et al., 1998), showing that un-phosphorylated Rb can be recognized by the complex. Under the right conditions, such as acutely removing the D-type cyclins, Rb can become hyper-phosphorylated without first being mono-phosphorylated in vivo. In this case, once TKO D^- cells reach the Restriction Point, Cyclin E:Cdk2 complexes hyper-phosphorylate un-phosphorylated Rb. It is important to note this phosphorylation profile has never been seen in physiologic conditions in both normal and tumor cells, which only contain mono-phosphorylated and hyper-phosphorylated Rb in a cycling population. In cells where the structure of Rb is altered (such as in retinoblastoma or in certain

non-small cell lung cancers), Rb is exclusively un-phosphorylated (Figure 4.15), presumably because the D-type cyclins fail to bind Rb, and the interacting domains on the molecule are perturbed.

All the studies that had been done previously utilized the synchronization of cells in either a G_0 or an Early G_1 starting point. It was imperative that the phenomenon of mono-phosphorylation could be seen if cells were synchronized at a different point in the cell cycle. Thus, Nocadozole was used to synchronize cells in G_2/M phase. In this case, the next Early G_1 phase was analyzed. Regardless of where cells were synchronized in the cell cycle, Rb was mono-phosphorylated in Early G_1 phase, and this mono-phosphorylation was mediated by Cyclin D:Cdk4/6 complexes. This was already inferred, because an asynchronous population of cells did not show any other phospho-isoforms besides mono-phosphorylated and hyper-phosphorylated Rb.

This experiment also elucidated the activity of the phosphatase that de-phosphorylates Rb once a cell comes out of mitosis. Previous data showed that PP1 is the phosphatase that is responsible for de-phosphorylating Rb once a cell exited mitosis (Ludlow et al., 1993). However, no one had shown if Rb gets completely de-phosphorylated once a cell starts Early G_1 phase again. With 2D IEF analyses, I showed that Rb is completely de-phosphorylated after mitosis, and then Cyclin D:Cdk4/6 complexes mono-phosphorylate Rb. One facet of this mono-phosporylation that is still unknown is whether Cyclin D:Cdk4/6 complexes put more than one phosphate on Rb while a phosphatase removes all but one phosphate on each molecule, or if Cyclin D:Cdk4/6 complexes put on one and only one

phosphate. Nevertheless, the final result after mitosis is that a cell is mono-phosphorylated, and that this mono-phosphorylation is dependent upon Cyclin D:Cdk4/6 activity.

The mechanism of how Rb only gets mono-phosphorylated when there are 15 potential Cdk phosphorylation sites is unknown. Although elucidating the exact mechanism of how this happens is beyond the scope of this dissertation, it was imperative to determine whether the cyclin or Cdk was important in how mono-phosphorylation occurs. Infecting an adenovirus consisting of a Cyclin D1-Cdk2 fusion protein into a TKO D^- cell background was the perfect experiment to perform, as this would directly address whether the cyclin or Cdk was necessary for mono-phosphorylation. I found that it was the cyclin that was necessary for mono-phosphorylation, because regardless of the Cdk that is bound to it, the D-type cyclins will mono-phosphorylate Rb. It is known that the D-type cyclins bind to Rb via the pocket domain located in the middle of the protein (Dowdy et al., 1993), and if this pocket is altered, Rb fails to become phosphorylated (Figure 4.15). The hypothesis then becomes that the D-type cyclins bind to Rb and then the Cdk phosphorylates Rb on one site during Early G_1 phase. To confirm this hypothesis, a future experiment worth performing is creating a fusion protein consisting of Cyclin E-Cdk4 and assessing Rb phosphorylation in the same background. Observance of hyper-phosphorylation would validate this hypothesis.

As mentioned before, there are 15 Cdk consensus phosphorylation sites on the Rb molecule that are not inside the pocket domain. A previous study had published a 2D phospho-peptide digest of Rb comparing “hypo-phosphorylated” Rb

(which is now known to be mono-phosphorylated Rb) and hyper-phosphorylated Rb, and the conclusion was that the vast majority of the same phosphorylation sites were being phosphorylated – it was just there were more phosphates on the hyper-phosphorylated form in a given Rb molecule (Mittnacht et al., 1994). Although utilizing phospho-specific antibodies would give a sense of how many possible mono-phosphorylated species there were, it was necessary to create all possible mono-phosphorylated constructs of Rb. One of the critiques of the overexpression of these constructs in cycling cells is that the phosphorylation seen could have been a result of Cdk2 or Cdk1 phosphorylation of Rb, because the constructs were transfected into asynchronous 293T cells, which would have all Cdks active. So, the phosphorylation seen on each construct with one phospho-acceptor site could be the equivalent of hyper-phosphorylated Rb, not mono-phosphorylated Rb. However, if a particular site were indeed only phosphorylated by Cdk2 or Cdk1 and not recognized by Cdk4/6, a small fraction of the construct would be un-phosphorylated signifying cells in Early G_1 phase. Because only mono-phosphorylation was seen in the transfection of these constructs, this phosphorylation event was Cyclin D:Cdk4/6 complex dependent.

By definition, Rb is in its active form when it can bind to the E2F family of transcription factors and inhibit cell cycle progression. Previous studies showed that Rb is active at some point in Early G_1 phase, as stressing cells before the Restriction Point can cause a cell cycle arrest (Jiang et al., 1998; Knudsen and Knudsen, 2006; Dick and Rubin, 2013). These studies also showed that any phosphorylation on Rb during G_1 phase inherently inactivated the protein – whether the phosphorylation

was done by Cyclin D:Cdk4/6 complexes or Cyclin E:Cdk2 complexes. However, from the data in this study, Rb is exclusively mono-phosphorylated during Early G_1 phase. The hypothesis then becomes that Rb is in its active state when it is mono-phosphorylated. This seems logical because this is the only form of Rb present in Early G_1 phase. However, this would contradict the theory that any phosphorylation of Rb inherently leads to inactivation of the protein (Dick and Rubin, 2013).

An ideal simulation of a functional response is utilizing DNA damage. Previous studies showed that active Rb is necessary for a cell to arrest in response to DNA damage (Knudsen and Knudsen, 2006; Burkhart and Sage, 2008; Attardi et al., 2004). Doxorubicin, which is a topoisomerase II inhibitor, was used to simulate this. Stressing cells with DOX resulted in the presence of mono-phosphorylated Rb regardless of synchronization. At any point where cells were arrested in Early G_1 phase because of DNA damage, mono-phosphorylated Rb was present. From these data alone, it could be inferred that mono-phosphorylated Rb is the active form.

Dr. Shapiro then developed a genetic system to test whether un-phosphorylated Rb could function during a DNA damage response. The key improvements in this study compared to previous studies were that cells were exposed to a form of Rb at all times, and that exogenous Rb was put back at close to physiologic levels. In these experiments, the $Rb^{\Delta Cdk}$, which cannot be phosphorylated, fails to function during a DNA damage response. First, cells expressing $Rb^{\Delta Cdk}$ continue to cycle regardless of whether DNA damage is induced. There also is a growing population of tetraploid cells signifying possible alterations to cytokinesis. Second, $Rb^{\Delta Cdk}$ fails to bind to E2F4, signifying that un-

phosphorylated Rb is not the active form. $Rb^{\Delta Cdk}$ does bind to E1A, which is consistent with previous results showing that Rb can be sequestered by viral oncoproteins (Knudsen and Knudsen, 2006). This also shows that although 15 phosphorylation sites are mutated from serines and threonines into alanines, the structure of this molecule is still intact, as it can bind to viral oncoproteins. Un-phosphorylated Rb fails to regulate E2F-dependent transcription, as genes that are known to be regulated by the E2F family of transcription factors continue to get expressed even when DNA damage is induced. Finally, $Rb^{\Delta Cdk}$ fails to regulate genes on a global level in response to DNA damage. The phenotype of putting $Rb^{\Delta Cdk}$ back in to cells at physiologic levels closely resembles the phenotype of an Rb-null cell. By contrast, putting an Rb^{WT} construct back into cells at physiologic cells closely resembles wild-type cells that have an endogenous unaltered Rb. Therefore, Rb needs to be mono-phopshorylated in order to participate in a DNA damage response.

DNA damage actually selects for mono-phosphorylated Rb. Rb can be induced to become mono-phosphorylated when a cell senses DNA damage. Serum-starved cells, which normally contain un-phosphorylated Rb, can be induced to express mono-phosphorylated Rb if they sense DNA damage. Regardless of whether a cell is in or out of the cell cycle, Rb needs to be mono-phosphorylated in order to participate in the DNA damage response. This mono-phosphorylation during a DNA damage response is mediated by Cyclin D:Cdk4/6 complexes. These data also potentially raise a concern about treating patients simultaneously with specific Cdk4/6 inhibitors and chemotherapeutic agents that cause DNA damage.

Because this study clearly shows that Rb needs to be mono-phosphorylated in order to participate in a DNA damage response, and that this mono-phosphorylation is mediated by Cyclin D:Cdk4/6 complexes, treating patients with inhibitors to Cdk4/6 might slow or even ablate the response to DNA damage in these cells.

This study also gives a hint that certain mono-phosphorylated isoforms of Rb could perform different functions. This is seen when specific mono-phosphorylated species are not observed when cells are treated with a DNA damaging agent. Furthermore, specific mono-phosphorylated isoforms bind to E2F4 at different affinities. The possibility that each isoform results in binding and recruitment of different factors during different stress responses could add to the intricacies of Rb regulation during Early G_1 phase of the cell cycle. This will be discussed later in Chapter 6.

In conclusion, this chapter has shown that Rb mono-phosphorylation is mediated by Cyclin D:Cdk4/6 complexes. If these complexes are inhibited, Rb fails to become mono-phosphorylated. I have shown this through overexpression or induction of specific cell cycle inhibitors, addition of specific chemical inhibitors, and utilization of genetic knockout cells that acutely remove the D-type cyclins. I also elucidated that the cyclin (specifically the D-type cyclins) is necessary for mono-phosphorylation, and not the Cdk. I have also shown that mono-phosphorylated Rb is the active form of Rb, as it can function in a DNA damage response. On the other hand, a construct of Rb which cannot be mono-phosphorylated fails to respond to DNA damage as cells continue to cycle. Finally, there is the possibility that certain mono-phosphorylated isoforms of Rb perform

different functions based on stress responses. These data fundamentally change the model of G_1 cell cycle progression, and the implications of that will be talked about in the subsequent chapter.

Portions of Chapter 5 were taken from a prepared manuscript, where I was the primary author/researcher. The manuscript is currently in preparation - Narasimha AM, Kaulich M, Shapiro GS, Sicinski P, Dowdy SF. Activation of RB by Mono-Phosphorylation *(In Preparation)*.

Figures

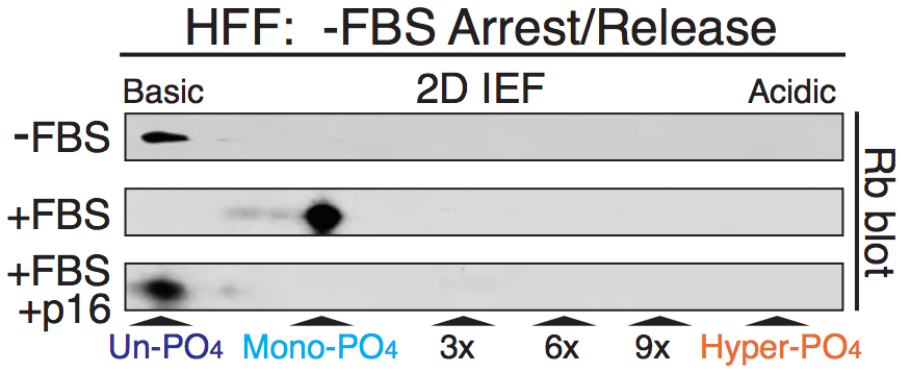

Figure 5.1:
2D IEFs of HFFs that were serum starved (-FBS) and released for 4 hours (+FBS) in the presence or absence of p16 adenovirus.

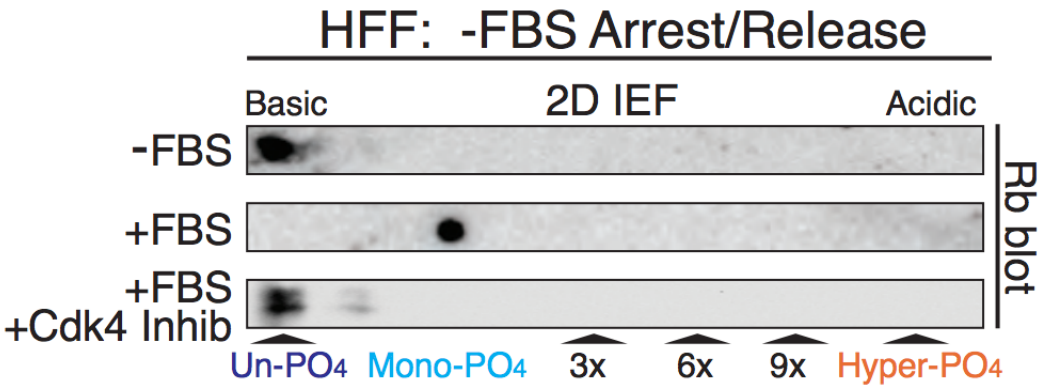

Figure 5.2:
2D IEFs of HFFs that were serum starved (-FBS) and released for 4 hours (+FBS) in the presence or absence of a Cdk4 specific inhibitor, PD0332991 (Cdk4 Inhib).

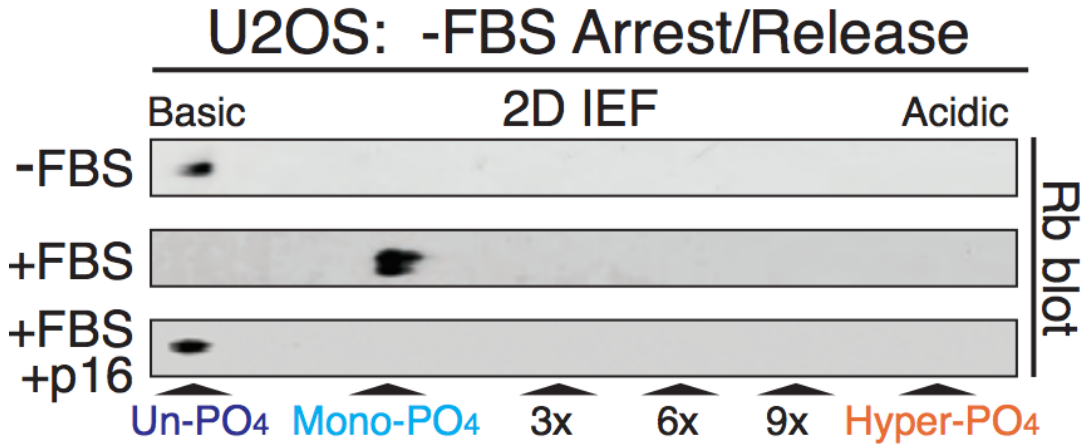

Figure 5.3:
2D IEFs of U2OS osteosarcoma cells (Tet-off inducible p16) that were serum starved (-FBS) and released for 4 hours (+FBS) in the presence or absence of induced p16.

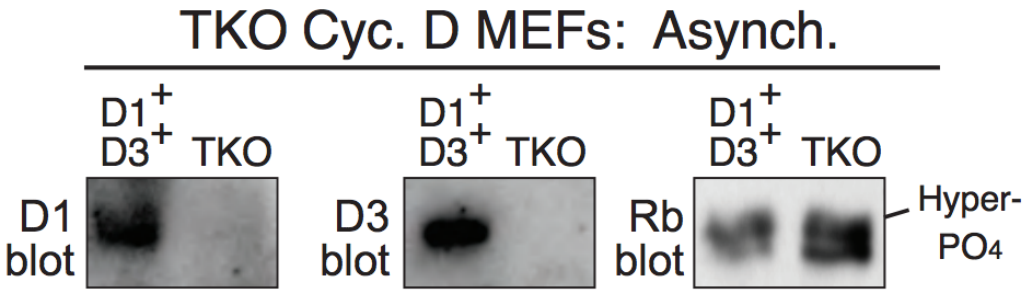

Figure 5.4:
Triple Knockout Cyclin D murine embryonic fibroblasts (TKO Cyc. D MEFs) were treated with 5μM Tat-Cre and then checked for D-type cyclin expression by Western Blotting.
Rb blots were run on a 6% SDS-PAGE.

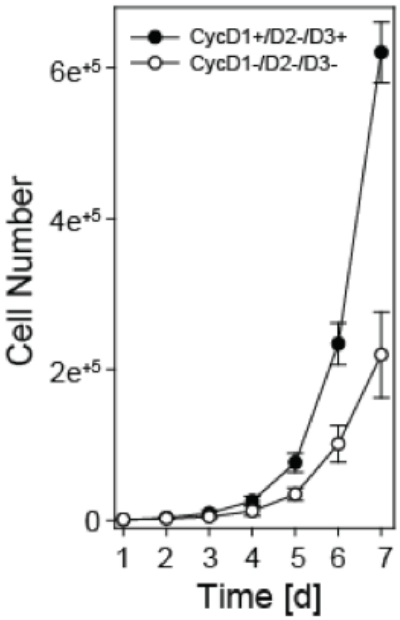

Figure 5.5:
100,000 cells were plated on a 6-well plate and followed through for 6 days (Day 1 = 100,000 cells). The filled in circles are the parental TKO D^- MEFs, while the open circles are TKO D^- MEFs treated with Tat-Cre.

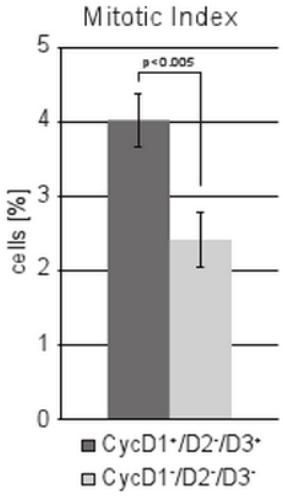

Figure 5.6:
Mitotic index measured by counting mitotic cells every 20 minutes for 50 hours. TKO D^- cells show a significant decrease in mitotic index.

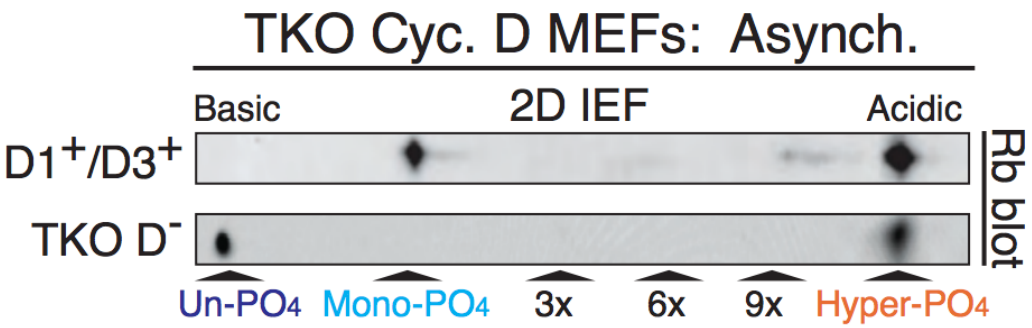

Figure 5.7:
2D IEFs of asynchronous TKO Cyclin D MEFs.
First 2D IEF is the parental cell line with wild-type Cyclin D1 and D3
Second 2D IEF is the cell line treated with Tat-Cre to remove endogenous D-type cyclins.

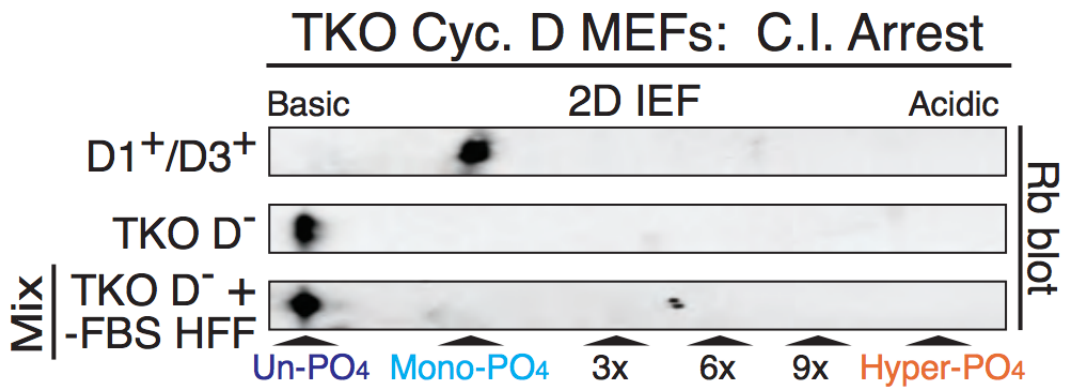

Figure 5.8:
2D IEFs of contact inhibited arrested (C.I. Arrest) TKO Cyclin D MEFs. TKO D- cells do not have any endogenous D-type cyclins when Tat-Cre is added.
Mix is a premixed sample of a serum starved HFF lysate and TKO D- lysate to show co-migration of un-phosphorylated Rb.

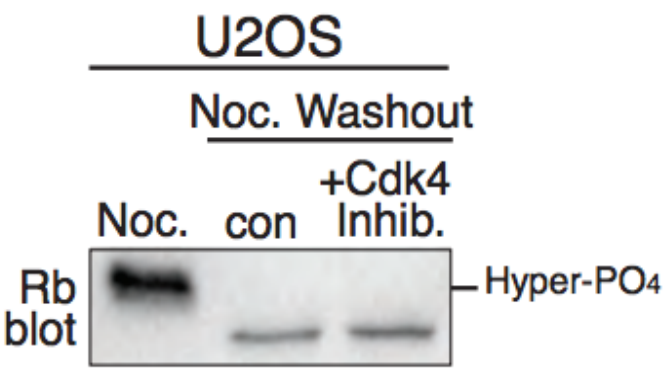

Figure 5.9:
U2OS cells were treated with Nocadozole (Noc.), washed and released for 4 hours in the absence (con) or the presence (Cdk4 inhib.) of PD0332991.

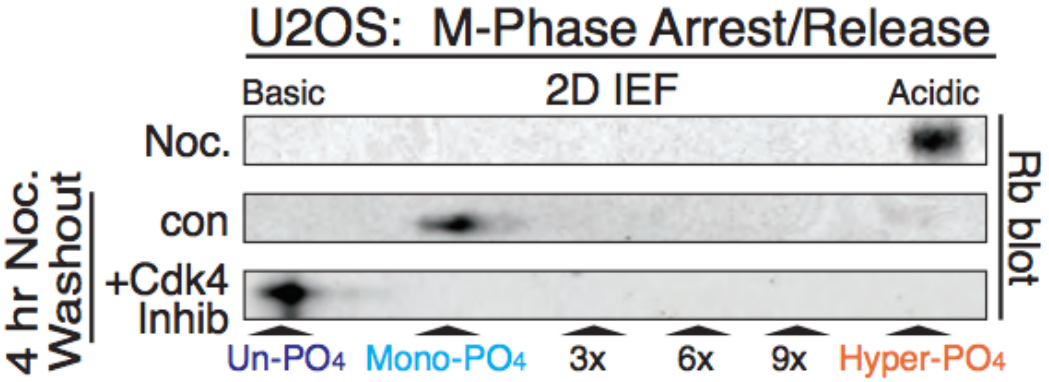

Figure 5.10:
2D IEFs of U2OS cells that were treated with Nocadozole (Noc.), washed and released for 4 hours in the absence (con) or the presence (Cdk4 inhib.) of PD0332991.

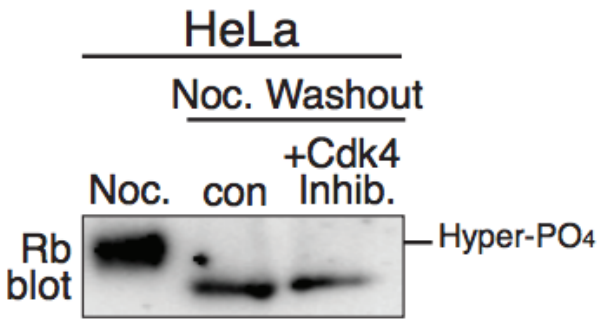

Figure 5.11:
HeLa cells were treated with Nocadozole (Noc.), washed and released for 4 hours in the absence (con) or the presence (Cdk4 inhib.) of PD0332991.

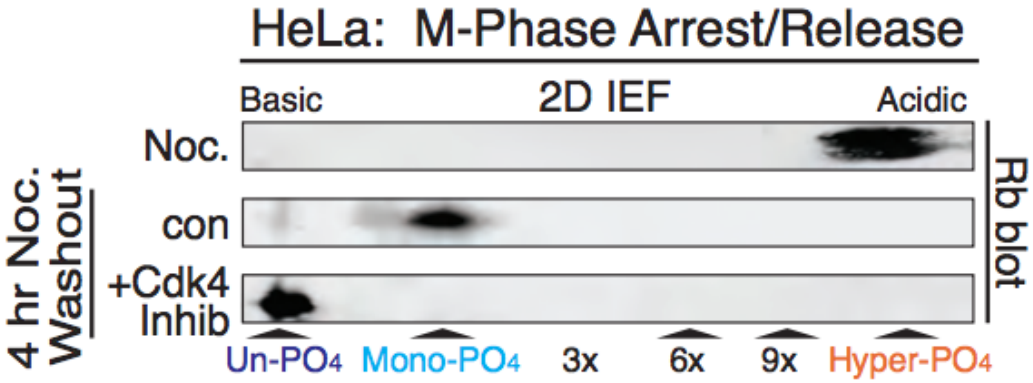

Figure 5.12:
2D IEFs of HeLa cells that were treated with Nocadozole (Noc.), washed and released for 4 hours in the absence (con) or the presence (Cdk4 inhib.) of PD0332991.

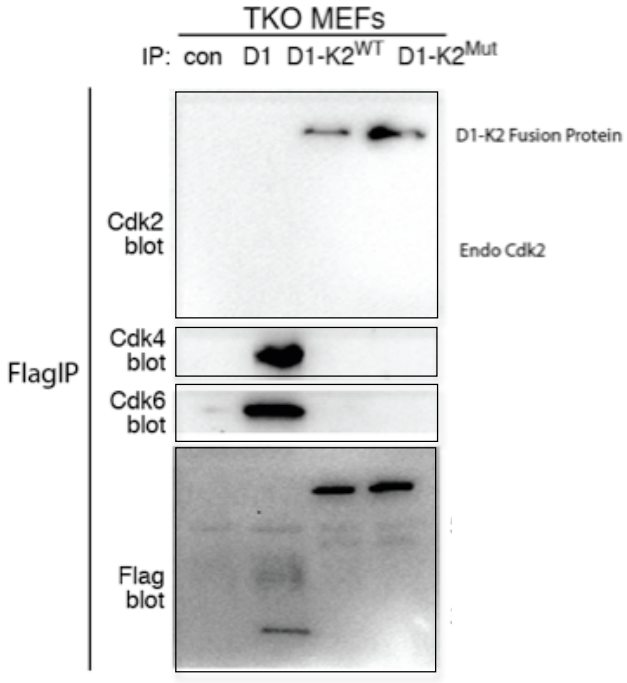

Figure 5.13:
Flag immunoprecipitation of TKO D^- cells adenovirally infected with GFP (con), Cyclin D1 (D1), Cyclin D1-Cdk2 fusion protein ($D1\text{-}K2^{WT}$), or $D1\text{-}K2^{Mut}$. Western blots for Cdk2, Cdk4, Cdk6, and Flag were performed.
The D1-K2 fusion protein has a molecular weight of 70 kDa, while endogenous Cyclin D1 and Cdk2 were each 35 kDa separately.

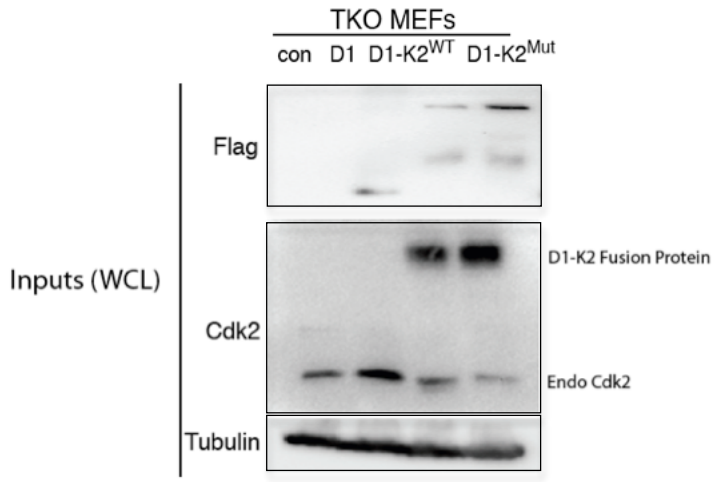

Figure 5.14:
Inputs for the previous experiment (Figure 5.13). Whole cell lysates (WCL) were taken and blotted for Flag, Cdk2, and Tubulin.

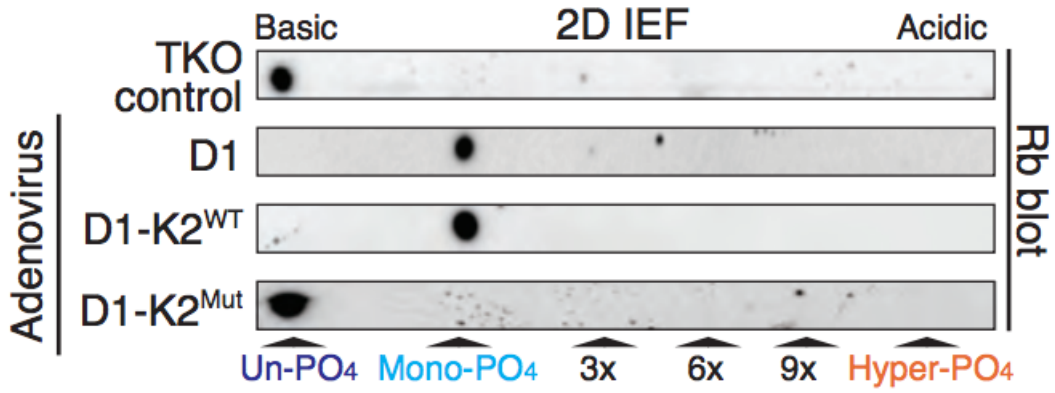

Figure 5.15:
2D IEFs of TKO D$^-$ cells that were serum starved and then released from serum starvation for 5 hours.
TKO Control refers to TKO D$^-$ cells that were not infected with any adenovirus, but do not have any D-type cyclins.

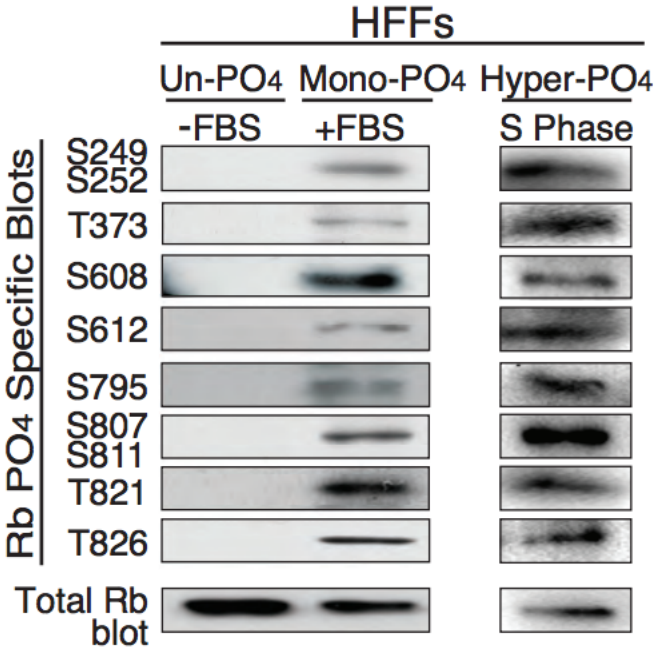

Figure 5.16:
Serum starved (-FBS), Contact inhibited (+FBS), and thymidine arrested (S Phase) HFFs were probed with phospho-specific antibodies for Rb phosphorylation sites.

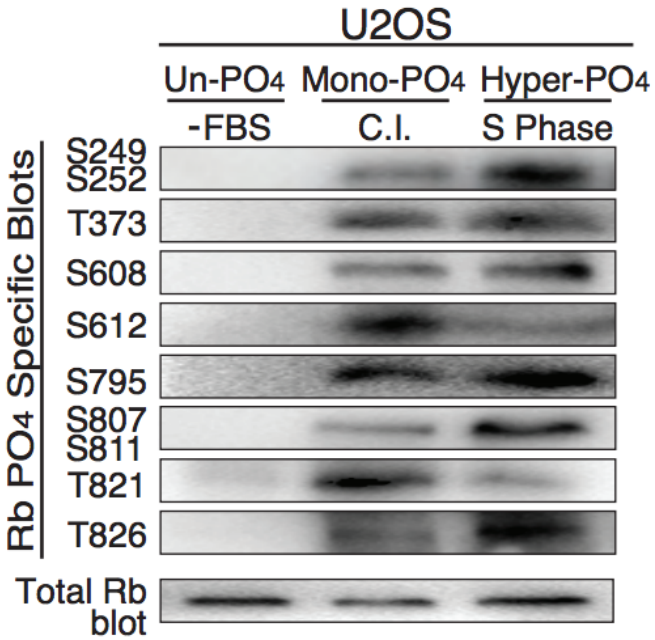

Figure 5.17:
Serum starved (-FBS), Contact inhibited (+FBS), and thymidine arrested (S Phase) U2OS cells were probed with phospho-specific antibodies for Rb phosphorylation sites.

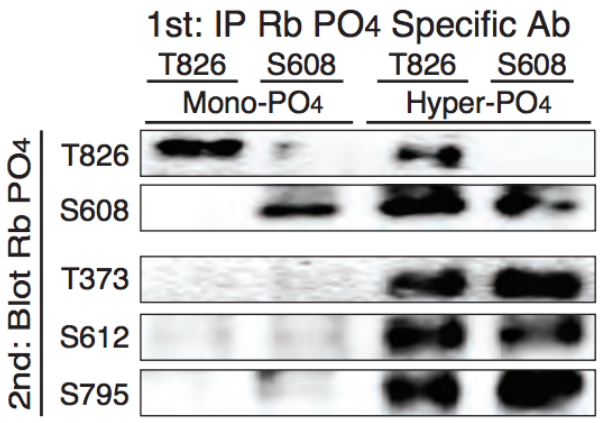

Figure 5.18:
Immunoprecipitations of lysates of HFFs using phospho-specific antibodies for Rb. This was done on either contact inhibited (Mono-PO4) or thymidine arrested (Hyper-PO4) cells.

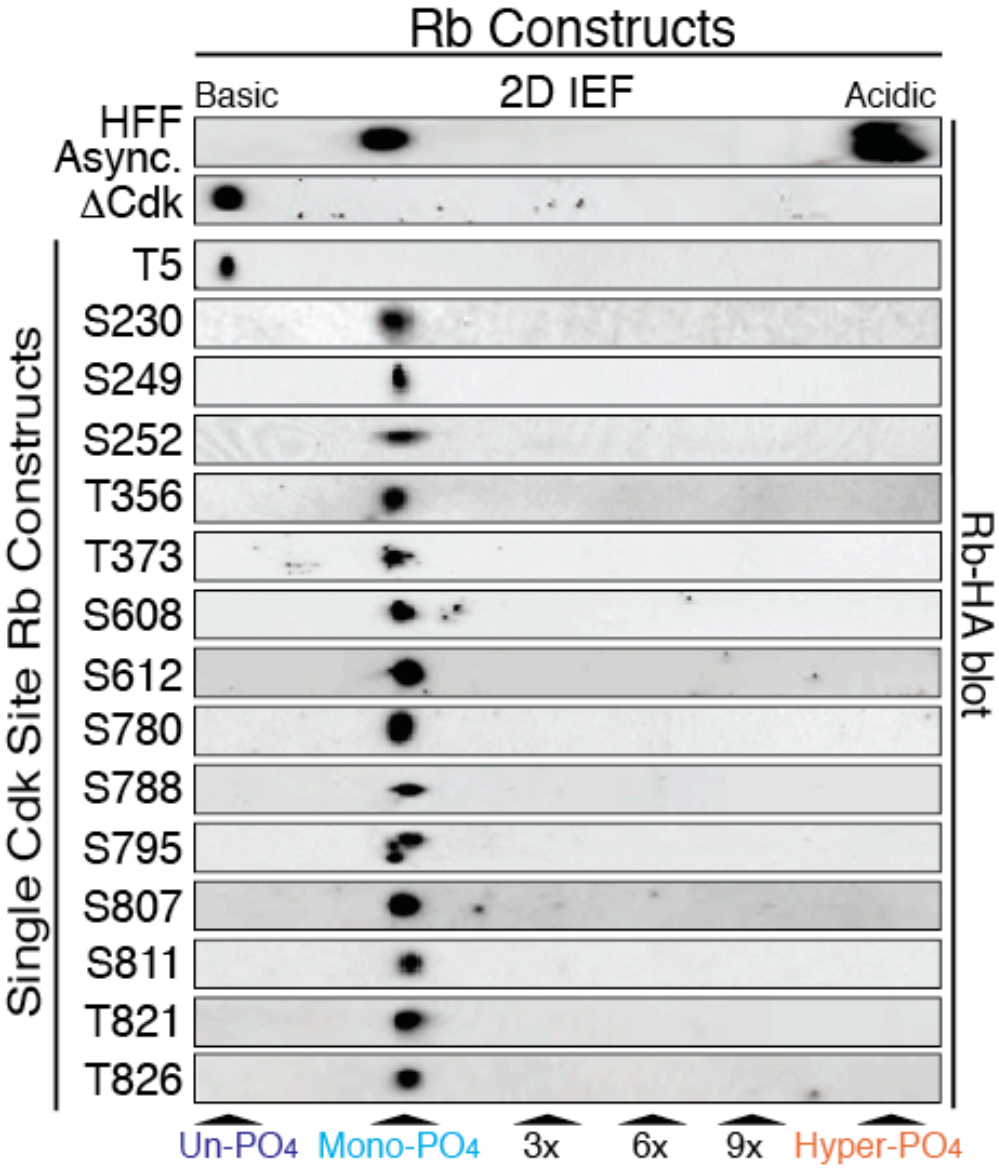

Figure 5.19:
N-terminally HA tagged Rb constructs were transiently transfected into asynchronous 293T cells for 48 hours. 2D IEF analyses were performed blotting for HA. Asynchronous HFFs (HFF Asynch) were run as a control.
Constructs made by Dr. Manuel Kaulich.

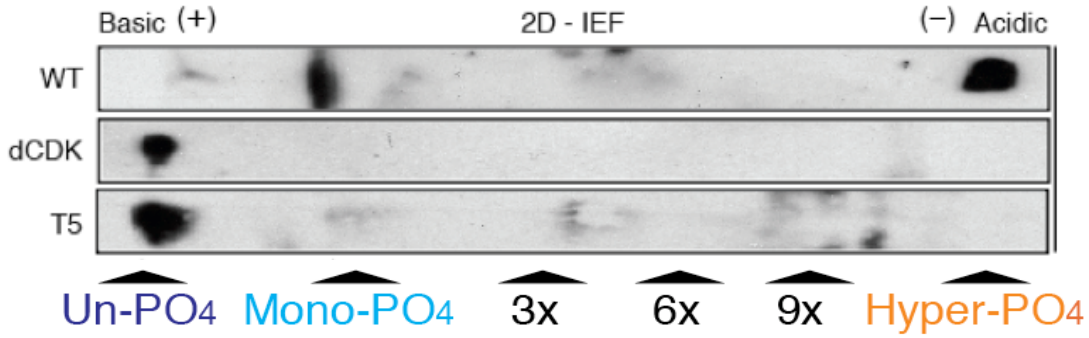

Figure 5.20:
2D IEF analyses of C-terminally tagged Rb constructs transiently transfected into asynchronous 293T cells. The Wild-type Rb (WT) has all phosphorylation sites, $Rb^{\Delta Cdk}$ (dCDK) has all sites mutated into alanines, and T5 has only that site and all 14 other sites made into alanines.
Constructs made by Dr. Manuel Kaulich.

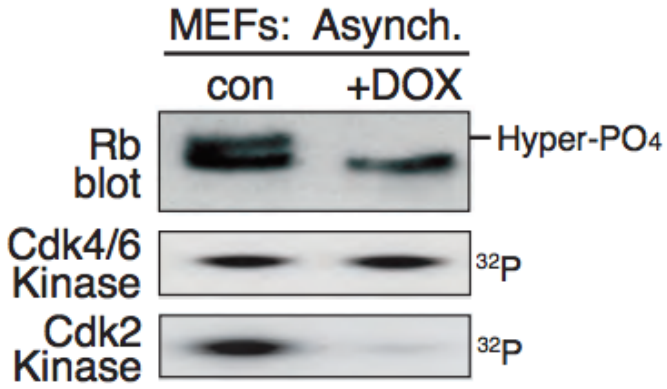

Figure 5.21:
Asynchronous MEFs were either untreated (con) or treated with 100 ng/mL Doxorubicin (+DOX) for 48 hours. GST-Rb was used as a substrate for Cdk4/6 kinase assay, Histone H1 was used as a substrate for Cdk2 kinase assay.

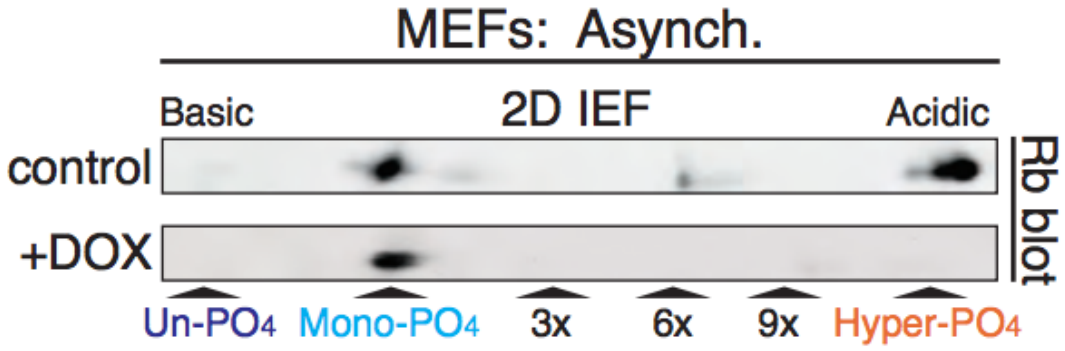

Figure 5.22:
2D IEFs of asynchronous MEFs either untreated (control) or treated with 100 ng/mL Doxorubicin (+DOX) for 48 hours.

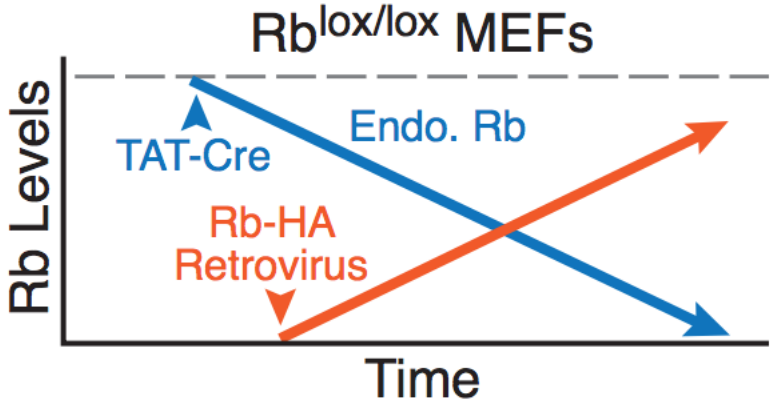

Figure 5.23:
Schematic representation of retroviral infection system putting back an HA-tagged Rb retrovirus into $Rb^{lox/lox}$ MEFs. Endogenous Rb is removed by the addition of Tat-Cre as exogenous Rb is added back in through retroviral infection.

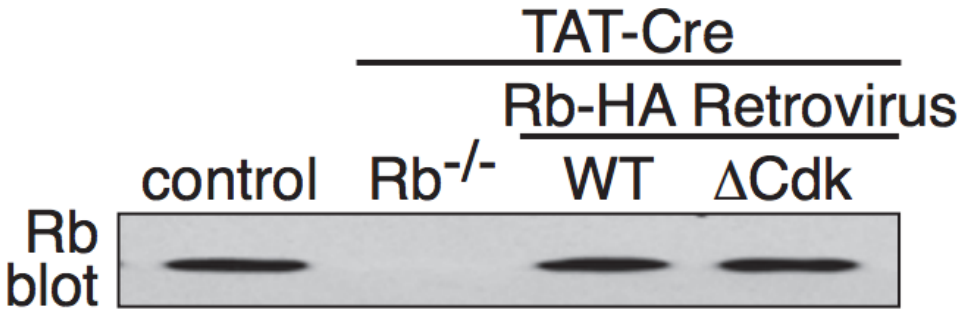

Figure 5.24:
$Rb^{lox/lox}$ MEFs were either untreated (control), treated with Tat-Cre to remove endogenous Rb ($Rb^{-/-}$), and then exogenously infected with Rb^{WT} or $Rb^{\Delta Cdk}$. Rb levels were checked by Western blotting to ensure physiologic conditions. Experiment done by Dr. Gary Shapiro.

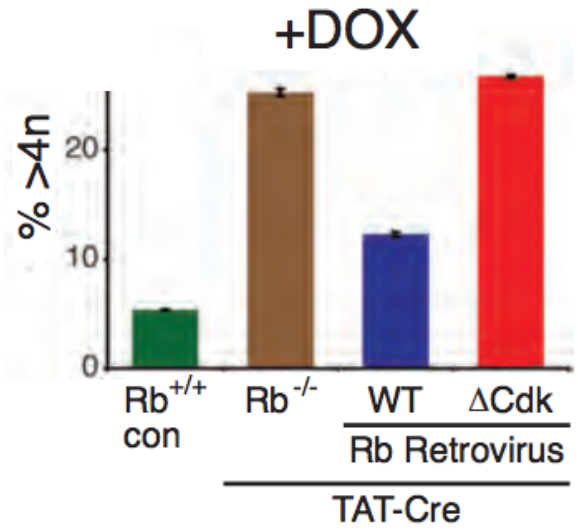

Figure 5.25:
Quantification of >4n DNA content of control MEFs (con), $Rb^{-/-}$ MEFs, retrovirally-expressing Rb^{WT-HA} MEFs and $Rb^{\Delta Cdk-HA}$ MEFs treated with 100 ng/mL Doxorubicin (+DOX) after release from contact inhibition. Error bars indicate SEM from three independent samples. Experiment done by Dr. Gary Shapiro.

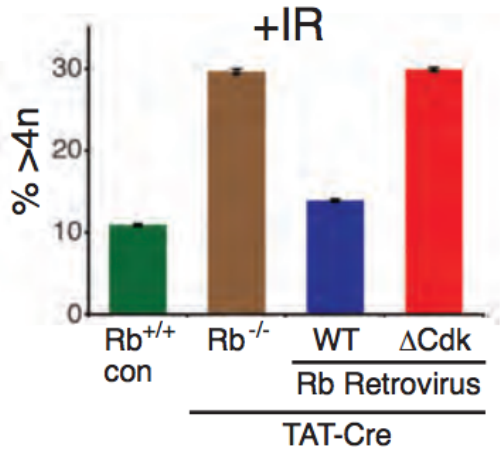

Figure 5.26:
Quantification of >4n DNA content of control MEFs (con), $Rb^{-/-}$ MEFs, retrovirally-expressing Rb^{WT-HA} MEFs and $Rb^{\Delta Cdk-HA}$ MEFs treated with 20 grays irradiation (+IR) after release from contact inhibition. Error bars indicate SEM from three independent samples. Experiment done by Dr. Gary Shapiro.

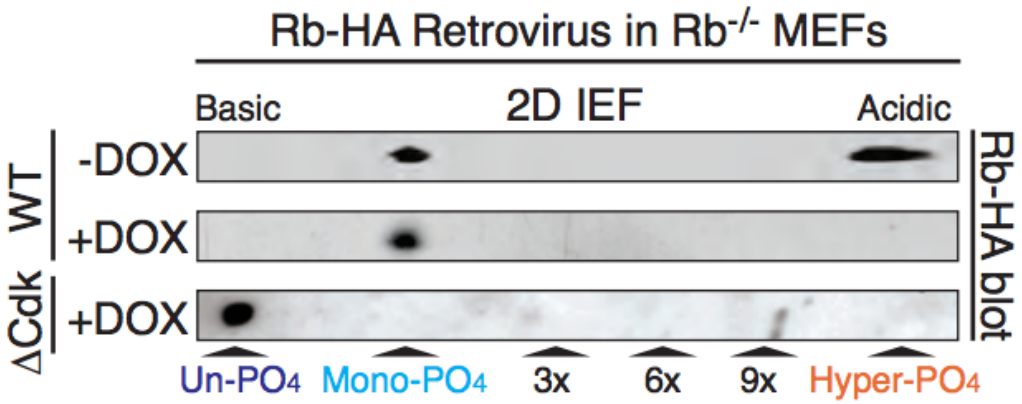

Figure 5.27:
2D IEF analysis of Rb^{WT} from untreated cycling MEFs, and from Doxorubicin (+DOX) treated Rb^{WT} and $Rb^{\Delta Cdk}$ expressing MEFs.

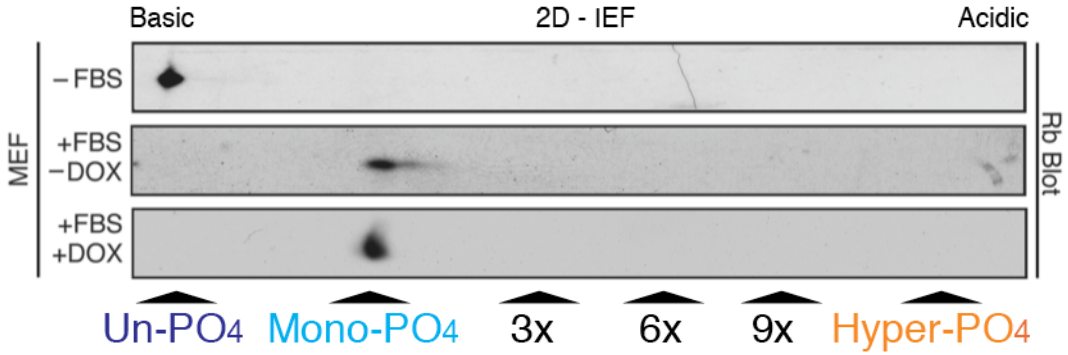

Figure 5.28:
2D IEF analysis of MEFs that were serum starved for 5 days (-FBS). The cells were then released by the addition of serum with or without the presence of 100 ng/ml DOX for 5 hours.

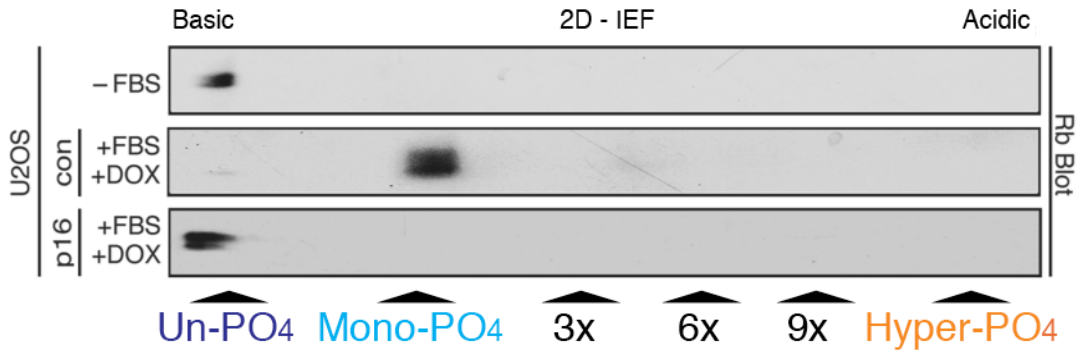

Figure 5.29:
2D IEF analysis of Tet-responsive U2OS cells that were serum starved (-FBS) and released in the presence of serum and treated with 100 ng/ml DOX for 5 hours. p16 was induced by the removal of tetracycline from the media.

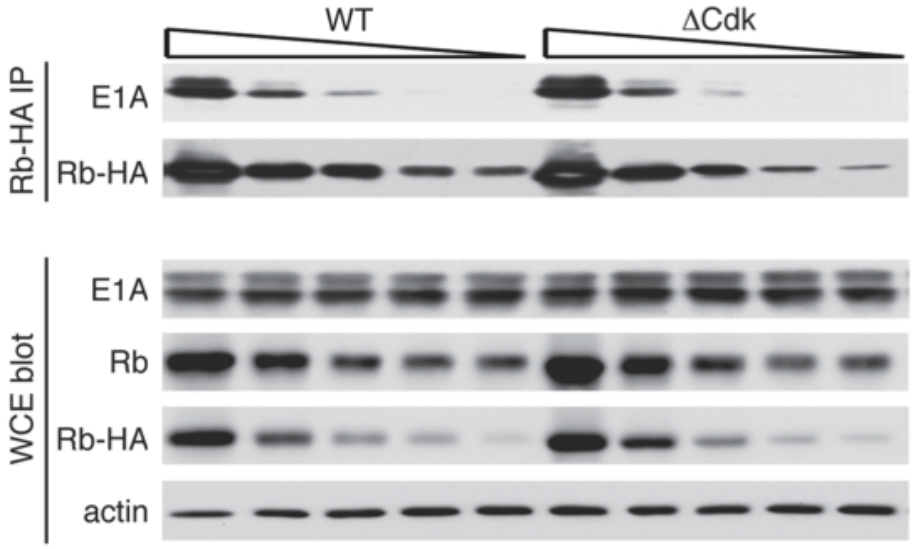

Figure 5.30:
293Ts were transfected with either Rb^{WT} and $Rb^{\Delta Cdk}$ and then immunoprecipitated using an antibody against HA. The IPs were then blotted for E1A. Whole cell extract (WCE) blots are shown for inputs.

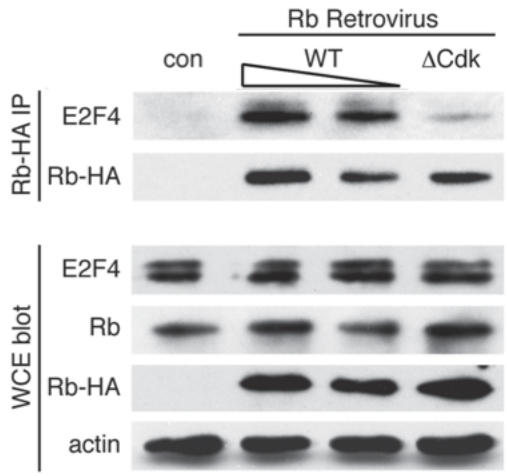

Figure 5.31:
Contact inhibited $Rb^{-/-}$ MEFs were infected with titers of either Rb^{WT} and $Rb^{\Delta Cdk}$ and then immunoprecipitated using an antibody against HA. The IPs were then blotted for E2F4. Whole cell extract (WCE) blots are shown for inputs. Con refers to wild-type MEFs.

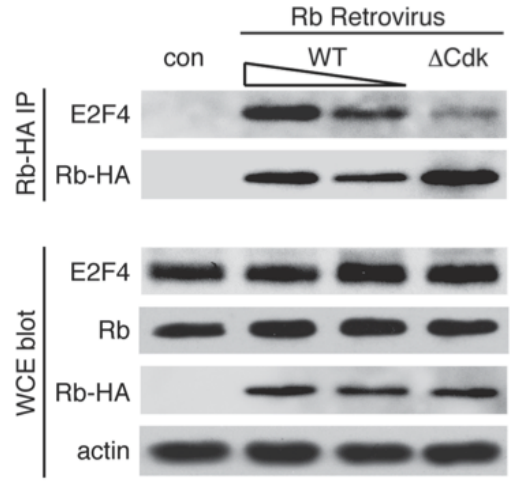

Figure 5.32:
$Rb^{-/-}$ MEFs were treated with 100 ng/mL DOX and were infected with titers of either Rb^{WT} and $Rb^{\Delta Cdk}$ and then immunoprecipitated using an antibody against HA. The IPs were then blotted for E2F4. Whole cell extract (WCE) blots are shown for inputs. Con refers to wild-type MEFs.

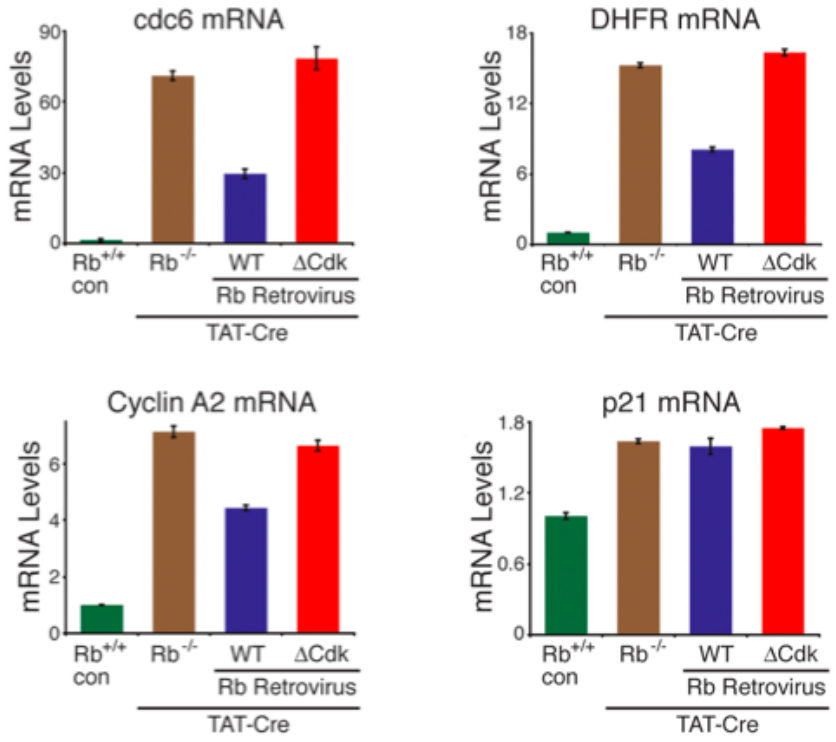

Figure 5.33:
qRT-PCR analysis of E2F-dependent genes in response to 100 ng/ml DOX in MEFs. $Rb^{lox/lox}$ MEFs were either untreated (con), treated with Tat-Cre to remove endogenous Rb ($Rb^{-/-}$), and then retrovirally infected with Rb^{WT} or $Rb^{\Delta Cdk}$. All levels adjusted to control levels. Error bars represent three independent experiments. Experiments done by Dr. Gary Shapiro.

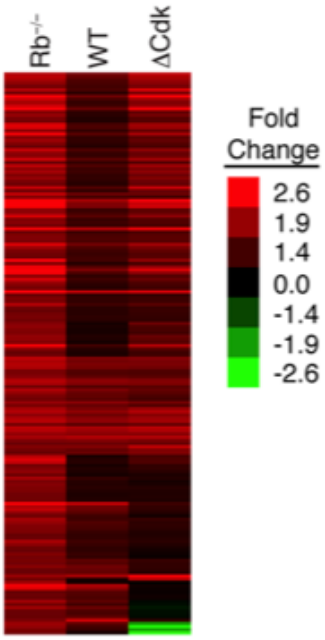

Figure 5.34:
Microarray analysis of MEFs treated with 100 ng/ml DOX.
Heat map of mRNA expression levels of $Rb^{-/-}$ MEFs, $Rb^{WT\text{-}HA}$ MEFs and $Rb^{\Delta Cdk\text{-}HA}$ MEFs compared to the control parental MEFs (endogenous Rb).
Experiment done by Dr. Gary Shapiro.

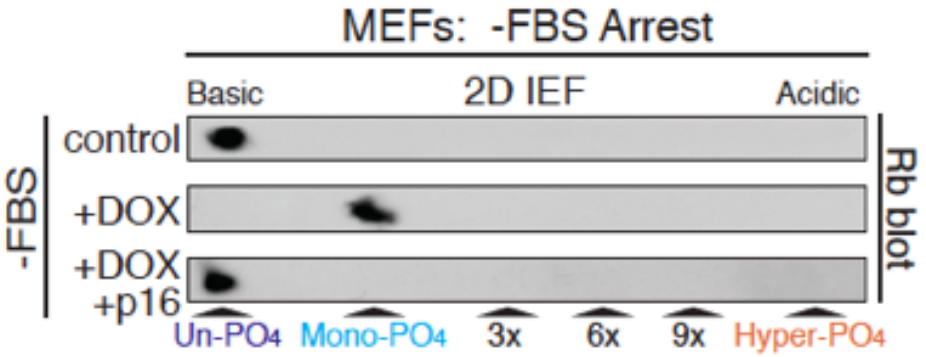

Figure 5.35:
2D IEF analysis of MEFs that were serum starved for 5 days (control), and then treated with 100 ng/ml DOX without the presence of serum for 48 hours in the presence or absence of p16 adenovirus.

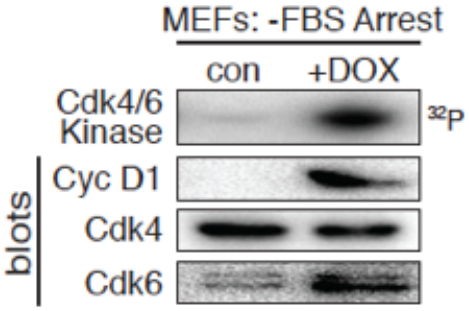

Figure 5.36:
MEFs were serum starved for 5 days (con) and then were treated with 100 ng/ml DOX without the presence of serum for 48 hours. GST-Rb was used as a substrate for Cdk4/6 kinase assay.

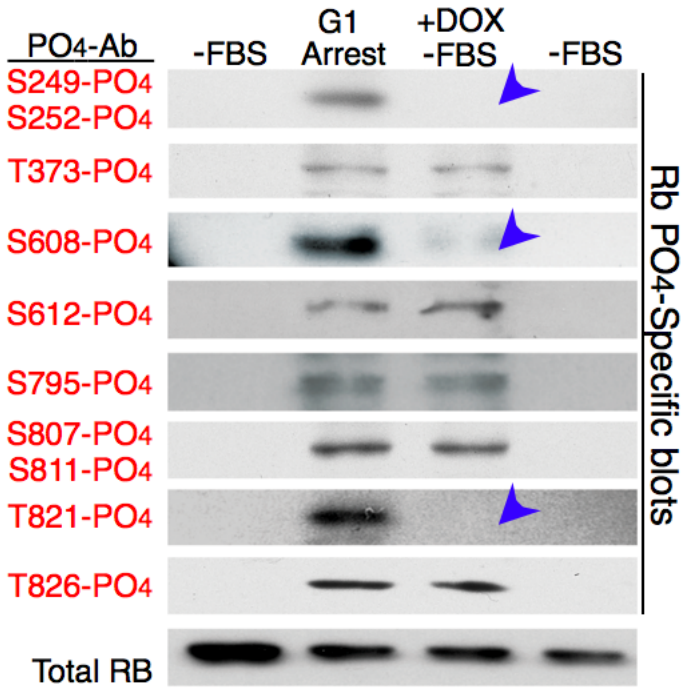

Figure 5.37:
Phospho-specific antibodies against Rb were used on HFFs that were arrested in G_1 phase by contact inhibition (Early G_1 arrest), and HFFs that were treated with 100 ng/ml DOX without the presence of serum. The arrows indicate differences between the two conditions. Serum starved HFFs (-FBS) were put on both sides of the blot as controls.

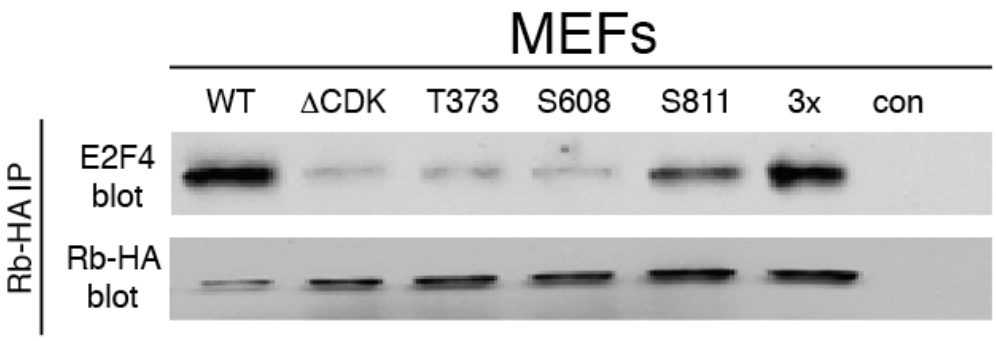

Figure 5.38:
$Rb^{lox/lox}$ MEFs were treated with Tat-Cre, and then exogenously infected with retroviruses expressing the above constructs. Cells were contact inhibited, immunoprecipitated with HA, and then blotted for endogenous E2F4 and HA. Con refers to wild-type MEFs.

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CHAPTER 6:

Summary, Conclusions, and Future Directions

Summary

The prevailing model of G_1 cell cycle progression is based on three major principles. First, Cyclin D:Cdk4/6 complexes inactivate Rb, as previous studies show that any phosphorylation on the Rb molecule lead to inactivation of the protein. Second, as cells progress through Early G_1 phase, Rb becomes multi-phosphorylated into a "hypo-phosphorylated" state, which partially inactivates the protein. This "hypo-phosphorylation" is mediated by Cyclin D:Cdk4/6 complexes. Third, cells pass through the Restriction Point based on accumulation of Cyclin E levels. Once Cyclin E levels reach a threshold, Cyclin E:Cdk2 complexes can form and complete the inactivation of Rb via hyper-phosphorylation. The preceding study has disproved all three principles of the prevailing model by utilizing two-dimensional isoelectric focusing (2D IEF) of highly synchronized cells to clearly quantify and isolate specific phospho-isoforms of Rb during a given cell cycle. This study has also been careful for looking at the physiological function of Rb rather than coming to conclusions based on overexpression studies. This study also identifies the active form of Rb during Early G_1 phase.

With these new data, the prevailing model of how cells progress through G_1 phase can be amended. Rb is now known to be completely un-phosphorylated during a G_0 quiescent state. Un-phosphorylated Rb is the form present during differentiation of cells that are exiting the cell cycle. This form of Rb is active during cell cycle exit, as it promotes differentiation. When a cell enters the cell cycle into Early G_1 phase, Rb becomes mono-phosphorylated. Mono-

phosphorylation can occur on 14 different Cdk consensus phosphorylation sites present throughout the molecule. Mono-phosphorylated mutants were made for each phosphorylation site to demonstrate the diversity of this phenomenon. This mono-phosphorylation is mediated by Cyclin D:Cdk4/6 complexes. If Cdk4/6 kinase activity is specifically inhibited, or if the D-type cyclins are acutely deleted, Rb fails to become mono-phosphorylated. The mechanism of how mono-phosphorylation occurs on Rb is dependent on the cyclin and not the Cdk. This was shown through fusion proteins that mono-phosphorylate Rb even if an aberrant Cdk is artificially tethered to the D-type cyclins. As a cell progresses through Early G_1 phase, Rb remains mono-phosphorylated. This was shown through highly synchronized kinetic analyses of both primary and tumor cells. Higher order or intermediate phosphorylation species of Rb are never seen in a cell progressing through Early G_1 phase. Mono-phosphorylated Rb is the active form of Rb. This has been shown in two ways. First, mono-phosphorylated Rb is the form present during a functional DNA damage response. Surprisingly, cells can also be induced to have mono-phosphorylated Rb even if they are not currently in the cell cycle. Second, mono-phosphorylated Rb binds to the E2F family of transcription factors, while un-phosphorylated Rb does not. By definition, mono-phosphorylated Rb thus becomes the active form. When cells reach the Restriction Point, Rb becomes rapidly hyper-phosphorylated and inactivated by Cyclin E:Cdk2 complexes. Although extremely rapid, this phosphorylation is processive, because intermediate phospho-isoforms of Rb can be captured by curbing Cdk2 kinase activity through use of a chemical inhibitor. This inactivation releases E2F and genes necessary for

cell cycle progression begin to be transcribed. Thus, a cell can proceed towards S phase and DNA replication. Figure 6.1 shows the new model of G_1 cell cycle progression.

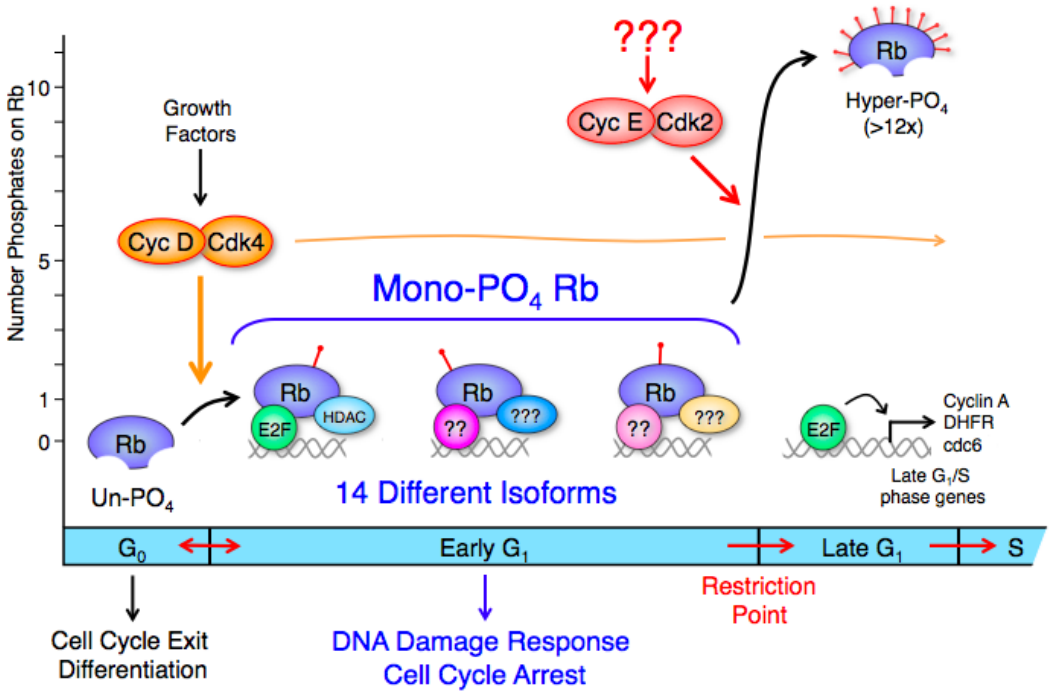

Figure 6.1 Working Model of G_1 Cell Cycle Progression
A schematic representation of G_1 cell cycle progression showing the different mono-phosphorylated isoforms, and how these isoforms are activated by Cyclin D:Cdk4/6 complexes. Mono-phosphorylated Rb can occur on 14 different Cdk sites. Once the cell reaches the Restriction Point, Rb becomes inactive by hyper-phosphorylation. This hyper-phosphorylation is mediated by Cyclin E:Cdk2 complexes.

Conclusions and Unanswered Questions

The Implications of the New Model of G_1 Cell Cycle Progression

The data from this study has fundamentally changed the model of G_1 cell cycle progression. The main protein involved in G_1 cell cycle regulation is Rb, and now there is a better understanding of how Rb is regulated by phosphorylation through the use of physiologic experiments and two-dimensional isoelectric focusing (2D IEF).

Rb is exclusively mono-phosphorylated during Early G_1 phase. The notion of Rb being multi-phosphorylated into a "hypo-phosphorylated" state was drawn from data utilizing supra-physiologic levels of Rb. Furthermore, the constructs of Rb had only certain sites available for phosphorylation, so drawing physiologic conclusions from these overexpressed constructs could be misleading. To answer the basic questions of Rb phosphorylation, utilizing 2D IEF to separate proteins by both charge and molecular weight was essential. It took about a year to adjust the conditions and parameters, but once the 2D IEF was calibrated to focus Rb, I was able to clearly quantify and separate the different isoforms of Rb. Previous studies never utilized this technique before to focus Rb, except for a study done by a former postdoctoral fellow in the laboratory (Ezhevsky et al., 2001). Studies on Rb phosphorylation were able to separate hyper-phosphorylated Rb from un-phosphorylated Rb and "hypo-phosphorylated" Rb on a low percentage acrylamide gel. However, the separation of the latter two isoforms was necessary to draw conclusions about Early G_1 phase cell cycle progression. Being able to distinguish

these two isoforms was essential to come to the conclusion that Rb was indeed mono-phosphorylated.

One of the major points of emphasis of this study was the analysis of Rb at physiologic levels. Most of the 2D IEF experiments shown have utilized the analysis of endogenous Rb in either primary or tumor cells. Regardless of whether a primary cell or tumor cell was analyzed, Rb was mono-phosphorylated throughout the duration of Early G_1 phase. This was the first time that anyone had ever quantified the number of phosphates on a given molecule of Rb during the cell cycle. The reason why studies termed Rb in Early G_1 phase as "hypo-phosphorylated" or under-phosphorylated is because although they identified that Rb was phosphorylated, they did not identify the extent of this phosphorylation or number of phosphates on a given molecule. With this technique, it was clear that Rb was only mono-phosphorylated during Early G_1 phase based on the kinetic analyses of both primary HFF cells and tumorigenic U2OS cells. To make sure that the species that was seen was only mono-phosphorylated and not an amalgam of many different phospho-isoforms, it was important to be able to distinguish between one and two phosphates on a given Rb molecule. Different phosphorylation constructs of Rb were used as standards, and this allowed me to distinguish the various phospho-isoforms by 2D IEF analysis. Given that there was never any time where endogenous Rb showed any intermediate phospho-isoforms, I concluded that Rb was exclusively mono-phosphorylated during Early G_1 phase.

One of the other principles that G_1 cell cycle progression was based on was the fact that continuing phoshorylation of Rb resulted in more inactivation of the

protein. Specifically, Cyclin D:Cdk4/6 complexes inactivated Rb via phosphorylation. This has shown to be untrue – Cyclin D:Cdk4/6 complexes activate Rb through mono-phosphorylation. As mentioned before, mono-phosphorylated Rb is the active form of the protein as it can participate in a functional response and bind to the E2F family of transcription factors. This, however, becomes a little paradoxical: how can proven oncogenes such as the D-type cyclins activate a known tumor suppressor gene such as Rb? Never has there been evidence in other pathways that show an oncogene activating a tumor suppressor gene.

The answer could very well be quite simple. The Restriction Point during the middle of G_1 phase is an extremely important checkpoint, as it is the so-called "point of no return" for the commitment of a cell to complete the cell cycle. Cells make the decision at this time to complete an entire cell cycle or revert back to a quiescent state. However, another point that could be a very important decision in the life of a cell is committing to start the cell cycle – the exit from a quiescent G_0 state into the cell cycle and Early G_1 phase. This is a monumental decision for a cell, as it makes the commitment to enter the cell cycle. Cells in a quiescent state are no threat to become cancerous and uncontrolled. These cells are usually terminally differentiated and have no inherent stimuli to proliferate again. However, cells that start to enter the cell cycle are always at risk for bypassing checkpoints and amassing mutations that can render it into a cancer. A cell predispositioned for tumorigenesis would rather be in the cell cycle containing mono-phosphorylated Rb than in a differentiated state and out of the cell cycle.

This process is dependent upon the activity of the D-type cyclins, still making them very potent oncogenes. Another way of analyzing this issue is by assessing the next cell cycle. As proliferating cells exit mitosis and enter into the next Early G_1 phase, Rb gets completely de-phosphorylated by PP1 phosphatase (Figure 5.10; Ludlow et al., 1993). This is shown through the 2D IEF experiments where cells are synchronized by the microtubule de-polymerizing drug Nocadozole in mitosis and then released into the next Early G_1 phase. The mono-phosphorylation that is necessary for Rb when cells reach the next Early G_1 phase is Cyclin D:Cdk4/6 dependent. This phosphorylation is necessary to occur for cells to progress on to the next cell cycle – another way of putting cells at risk for uncontrolled proliferation. Thus, the D-type cyclins can be considered oncogenes when looking at the contexts of promoting cell cycle entry and promoting cells for continued proliferation.

The notion of D-type cyclins activating Rb is consistent with mouse models in cancer progression. Deletion of p16 or overexpression of the D-type cyclins is common in many cancers (Burkhart and Sage, 2008; Sherr and McCormick, 2002). However, many of the phenotypes seen in mouse models with these alterations are subtle, and the predisposition to cancer is relatively mild and delayed. In contrast, Rb loss has much more of an extreme phenotype. Rb-null mice are embryonic lethal at E15.5, and Rb loss causes more drastic phenotypes in adult mice compared to p16 deletion (Burkhart and Sage, 2008). Although the D-type cyclins have other functions besides phosphorylating Rb, if the D-type cyclins did inactivate Rb, the phenotype of p16-deleted or Cyclin D-overexpressed mice should be similar to a

phenotype seen in Rb-null mice. This is however not true, and so the theory of D-type cyclins inactivating Rb is not observed on the level of mouse modeling.

One of the other major principles that was proven false during this study is the fact that Cyclin E accumulation drives the cell past the Restriction Point in G_1 phase. Primary HFF cells that were synchronized via contact inhibition and released showed no increase in Cyclin E expression both on the protein and mRNA levels, which is consistent with previous data (Haberichter et al., 2007). This had been considered the "rate-limiting" step during G_1 phase, as this was the cause of a cell to move past the Restriction Point. Now that this is not true, what could be the rate-limiting step to push the cells past this checkpoint?

As mentioned in the introduction, the essential step for cell cycle progression could be the activation of Cdk2 by the Cdk-activating kinase (CAK). Instead of Cyclin E accumulation that allow for inactivation of Rb, the key step could be the activation of Cdk2 by the CAK, which would result in the hyper-phosphorylation and inactivation of Rb to move cells pass the Restriction Point. Cdk2 is activated at the T-loop on T160 (Malumbres and Barbacid, 2009). In order for Cdk2 to be fully activated, T160 needs to be phosphorylated, and T14 and Y15 need to be de-phosphorylated (Malumbres and Barbacid, 2009). There have been several studies identifying what the mammalian CAK is in vivo. Some reports specify it to be Cdk7, which is part of the TFIIH transcription complex along with Cyclin H and Mat1 (Larochelle et al., 2007). There are a few problems with this assessment. First, the T160 site is not a Cdk consensus phosphorylation site – T160 is not followed by a proline, which is a necessary motif for Cdk-dependent

phosphorylation. Second, Cdk7 knockout cells cycle and do not arrest in adult tissues (Ganuza et al., 2012). If Cdk7 was indeed the CAK, cells would have no way of progressing past the Restriction Point and would remain arrested in Early G_1 phase. Furthermore, there is a low molecular weight form of the CAK that was identified by a couple of studies (Dowdy et al., 1993; Solomon et al., 1992). Because of this, Cdk7 is, at best, 1 of 2 different CAKs, or, at worst, not part of this pathway at all. Identifying the mammalian CAK will be integral in completing the model of G_1 cell cycle progression and fundamentally understanding how cells pass the Restriction Point.

The Mechanism of Mono-Phosphorylation

Rb has 14 sites located on the molecule that are mono-phosphorylated by Cyclin D:Cdk4/6 complexes in vivo. This study has shown that the cyclin dictates the mono-phosphorylation, and not the Cdk. However, it still remains unknown how Rb actually gets mono-phosphorylated and stays mono-phosphorylated throughout the duration of Early G_1 phase.

There are a couple of theories on how this might occur. One theory would result in Cyclin D:Cdk4/6 complexes putting exactly one phosphate on a given Rb molecule. Another theory would involve Cyclin D:Cdk4/6 complexes putting more than one phosphate on a given Rb molecule, and then a phosphatase de-phosphorylating Rb until there was only one phosphate remaining. The latter theory seems unlikely though, because it would involve two distinct proteins (a kinase and a phosphatase) collaborating on a third molecule rather than just one protein being

highly regulated. Nevertheless, the phosphatase model cannot be ruled out. More likely, Cyclin D:Cdk4/6 complexes put one and only one phosphate on Rb. How would this be possible? The D-type cyclins bind to Rb via the pocket domain (Dowdy et al., 1993), so once Cyclin D binds, Cdk4/6 can mono-phosphorylate Rb. However, it could be that once one phosphate is put on Rb, the structure of Rb conformationally changes, immediately releasing the D-type cyclins and preventing any more phosphorylations from occurring. Cyclin D:Cdk4/6 complexes have a lower affinity for Rb as a phosphorylation susbtrate compared to the other cyclin-Cdk complexes, so once Rb changes conformation, the structural alteration prevents Cyclin D:Cdk4/6 complexes from further phosphorylating Rb. This could be because the pocket domain itself is altered and cannot recognize the D-type cyclins anymore. By contrast, Cyclin E:Cdk2 complexes bind to Rb via an RxL motif (Adams et al., 1998), which is outside of the pocket. These complexes have a much higher affinity for Rb as a substrate for phosphorylation. Thus, a possibility of how Rb stays mono-phosphorylated relies on the conformational change of the pocket domain that occurs when a phosphate group is put on the molecule.

Another possible reason why Rb only becomes mono-phosphorylated is because of charge or steric hindrance. It is possible that once a phosphate group is placed on the molecule, Rb could be extremely difficult to become further phosphorylated because of either the charge or the mass of the group put on. This could definitely make sense for phosphorylation sites that sit next to each other, such as S249 and S252, S608 and S612, S807 and S811, and T821 and T826.

Because some of the phosphorylation sites are very close together, this hindrance could prevent multiple phosphorylations from occurring.

The one facet of mono-phosphorylation that is still unknown is the ratio of sites that are phoshorylated. It is possible that certain sites are preferred over others, and thus are more prevalent in a given cell. This could be due to a number of reasons. The physical interaction of the D-type cyclins and Rb might prevent certain sites from getting phosphorylated very frequently. The D-type cyclins bind to Rb via the pocket domain, which is located between the approximate residues 400 and 750. If Cdk4/6 is the arm that mono-phosphorylates Rb, it might reach S608, S612 (sites in the spacer region between the A and B boxes), or certain C-terminal phosphorylation sites much easier than some N-terminal phosphorylation sites such as S249 or S252. This could also be a theory on why T5 does not get phosphorylated – Cdk4/6 is incapable of reaching it when the D-type cyclins bind via the pocket domain.

The other interesting aspect of mono-phosphorylation is the fact that the same phosphorylation sites activate Rb by mono-phosphorylation, and inactivate Rb through hyper-phosphorylation. This has never been shown in the phospho-regulation of other proteins. It could however occur on the other pocket proteins – p107 and p130. These have multiple Cdk phosphorylation sites on them as well, and the same mechanism of activation by mono-phosphorylation and inactivation by hyper-phosphorylation on the same sites could occur, allowing for a pocket-protein specific mechanism of activation and inactivation.

The Function of Mono-Phosphorylation

As mentioned earlier, Rb is activated by mono-phosphorylation on 14 different phosphorylation sites. Each mono-phosphorylated isoform of Rb could have different functions. Rb is known to have numerous binding partners that include transcription factors and other proteins involved in cell cycle regulation (Dick and Rubin, 2013). Each mono-phosphorylated species of Rb can potentially recruit different binding factors, which would render different functions for each isoform. Each mono-phosphorylated isoform could also have a slightly different conformation, which would help in the recruitment and recognition of distinct factors.

There could be two different sets of proteins that bind to mono-phosphorylated Rb. The first set would be an overlapping group of core targets that would bind to most, if not all, of the 14 mono-phosphorylated isoforms of Rb. These could include the E2F family of transcription factors. However, this is not necessarily true, because the structure of the pocket domain could be slightly altered with a mono-phosphorylation on a specific residue. Because E2F proteins bind within the pocket domain, the alterations in the structure of this could cause certain mono-phosphorylated isoforms to preferentially bind them. Nevertheless, this set would bind to mono-phosphorylated isoforms regardless of where the phosphorylation was present. The second set would cover a distinct subset of factors and binding proteins that are recruited for a specific mono-phosphorylation event. For example, a phosphorylation on S249 could recruit Factors X, Y, Z, while

a phosphorylation on T821 could recruit Factors X, A, and B. Thus, there would be a factor that is recruited to both sites (Factor X), as well as other proteins recruited specifically for certain phosphorylations (Factors Y, Z and Factors A, B). This would allow for another level of regulation for cells progressing through G_1 phase.

This hypothesis is further evidenced by the fact that when cells respond to a stress such as DNA damage, certain sites are mono-phosphorylated, but others are not. When cells were treated with DNA damage without the presence of serum, there were certain sites (S249, S252, S608, and T821) that were not phosphorylated compared to cells undergoing contact inhibition. This leads to the hypothesis that these sites might be necessary for recruiting factors necessary for arresting cells during contact inhibition, but not essential for factors that need to be recruited for a DNA damage response. Identifying these factors could be pivotal in completing the G_1 cell cycle progression model.

Future Directions

One of the most important questions that is concluded from this study is the functional significance of each site on mono-phosphorylated Rb. Because Rb is mono-phosphorylated on 14 different sites during Early G_1 phase, it is possible that each isoform can perform different functions based on the binding of different proteins. Experiments need to be done on a proteomic level to ascertain the binding capacities of each mono-phosphorylated isoform of Rb. It would be beneficial to have a composite of binding partners of each mono-phosphorylated isoform, and then determine what the function of each form is based on what factors it recruits. This would allow a much better and deeper understanding of Rb regulation in G_1 phase of the cell cycle. Now, not only is Rb regulated by phosphorylation, but certain mono-phosphorylated isoforms of Rb might be preferred, adding another level of regulation. It could also be hypothesized that a disproportion of specific mono-phosphorylated species could lead to pathologies. As mentioned before, it is still unknown what the proportion of mono-phosphorylated isoforms of Rb is present during normal cell cycle progression compared to stress responses such as growth arrest or DNA damage.

This also leads to another necessary experiment – elucidating the structure of mono-phosphorylated Rb. Although most of the structure of un-phosphorylated Rb has been solved and postulations have been made about the conformational changes when Rb is phosphorylated at certain sites (Dick and Rubin, 2013), the structure of mono-phosphorylated Rb itself has not been solved. The structure of Rb was first

elucidated by Dr. Seth Rubin's laboratory, but this only encompasses the amino acid residues 40-750 (Dick and Rubin, 2013). What is still unknown is the conformation of the C-terminus of Rb, and this contains many phosphorylation sites that are mono-phosphorylated. Elucidating the entire structure would allow for a structural analysis that could lead to why certain mono-phosphorylated isoforms of Rb bind to certain proteins, but others do not. It is still assumed that the structure of Rb changes when a phosphate group is added. The structural analysis can also give a more thorough view of how Cyclin D:Cdk4/6 complexes are prevented from putting multiple phosphorylations on an Rb molecule. There could be structural changes resulting from mono-phosphorylation could release the cyclin from Rb. There could also be other binding partners of Rb that prevent Cyclin D:Cdk4/6 complexes from multi-phosphorylating Rb in vivo. Thus, it would be beneficial to elucidate the structure of the different mono-phosphorylated isoforms of Rb.

Another aspect of the model relies on the fact that un-phosphorylated Rb is active during cell cycle exit and differentiation. This study shows that un-phosphorylated Rb actually promotes differentiation. To look at this in more depth, cells undergoing differentiation can be analyzed for Rb binding partners. These binding partners can then be compared to Rb binding partners during G_1 phase of the cell cycle. Identifying specific proteins that bind to un-phosphorylated Rb during differentiation would allow for a more complete understanding of the process of cell cyle exit.

One of the basic questions of cell cycle progression is the mechanism of how cells pass the Restriction Point in G_1 phase. As mentioned earlier, activation of

Cdk2 by the CAK could be the pivotal step that allows cells to transverse this checkpoint. Identifying the CAK and elucidating its regulation could be imperative when coming to disease therapies. If this is indeed the "rate-limiting" step, the CAK or proteins regulating CAK activity could be targeted, as this would prevent cells from passing the Restriction Point. Obviously, selective targeting toward cancer cells would be necessary.

In summary, this work has fundamentally changed the view on G_1 cell cycle progression, and how Rb is regulated through phosphorylation. Rb becomes activated by mono-phosphorylation and this is mediated by Cyclin D:Cdk4/6 complexes. Filling in more details about G_1 cell cycle progression can answer fundamental questions of how a cell makes the decision to progress past the Restriction Point and potentially becomes a cancer cell.

Portions of Chapter 6 were taken from a prepared manuscript, where I was the primary author/researcher. The manuscript is currently in preparation - Narasimha AM, Kaulich M, Shapiro GS, Sicinski P, Dowdy SF. Activation of RB by Mono-Phosphorylation *(In Preparation)*.

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