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Targeting Cyclin Dependent Kinases in Embryonic Stem Cells and
Cancer

by

Noelle Huskey Mullin

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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in the

GRADUATE DIVISION

of the

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By

Noelle Huskey Mullin

Dedication

This dissertation is dedicated to my grandfather, Dr. Harry Douglas Huskey, PhD

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Many people have contributed to the completion of this dissertation. Most importantly I would like to thank my mentor, Dr. Andrei Goga. Andrei has been a continual source of support during my training at UCSF. He has spent countless hours discussing scientific experiments with me, reading drafts of grant applications and papers, and providing general guidance. Andrei has made it clear that he genuinely cares about my happiness and success both in and out of the lab. It has been a real honor to work in Andrei's lab and I look forward to collaborating with him throughout my scientific career.

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Contributions

Chapters 2 and 3 contain unpublished material that is currently being prepared for submission. Expected authorship is as follows:

Noelle E. Huskey, Kimberly Evason, Tingxia Guo, David Pardo, Katelyn J. Creasman, Robert L. Judson, Colin Melton, Nichole A Reyes, Robert Blelloch, Matthias Hebrok, Scott A. Oakes, and Andrei Goga.

Kimberly Evason performed histological analysis of stem cell driven tumors for figures 23 and 24. Katelyn Creasman assisted with western blotting. David Pardo assisted with mRNA expression analysis of p53 targets. Tingxia Guo and Matthias Hebrok carried out experiments in human embryonic stem cells. Robert L. Judson and Robert Blelloch generated iPS cells. Niki Reyes, Colin Melton, Robert Blelloch and Scott Oakes generated Bax/Bak deficient ES cells. I was responsible for all the remaining experimental design, execution and analysis, as well as manuscript preparation. Andrei Goga supervised work, assisted with experimental design and manuscript preparation.

Chapter 4 contains previously published material, reprinted with permission from the Proceedings of the National Academy of Sciences (PNAS):

Dai Horiuchi*, Noelle E. Huskey*, Leonard Kusdra, Lara Wolhbold, Karl A. Merrick, Chao Zhang, Katelyn J. Creasman, Kevan M. Shokat, Robert P. Fisher, and Andrei Goga

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(*authors contributed equally to this work).

Len Kusdra preformed cell proliferation studies and provided Fig 33. Robert P. Fisher, Lara Wolhbold, and Karl A. Merrick generated HCT116 cell lines expressing the Cdk2AS allele. Chao Zhang and Kevan M. Shokat synthesized 1NM-PP1 and provided advice on experimental design. Katelyn J Creasman assisted with western blots. All other experimental design, execution, and analysis, as well as manuscript preparation, was preformed by Dai Horiuchi and I, under the supervision of Andrei Goga.

Abstract

Targeting Cyclin Dependent Kinases in Embryonic Stem Cells and Cancer

Noelle Huskey Mullin

Cyclin dependent kinases (Cdks) are a family of conserved serine/threonine kinases that regulate cell cycle progression in mammalian cells. Activation of individual Cdks at distinct phases of the cell cycle ensures the proper timing and coordination of cell cycle events. Over the past decade, gene knockout models combined with studies using small molecule kinase inhibitors have shown that the effect of individual Cdk inactivation is highly dependent on cellular and genetic context. Here we examine the effect of Cdk1 inhibition on embryonic stem (ES) cells. Additionally, we develop a chemical genetic approach that allows for the selective inhibition of Cdk2 in multiple cell and cancer types.

Embryonic stem (ES) cells are an attractive source for stem cell therapies due to their rapid proliferation and capacity for differentiation. A limitation in the field of regenerative medicine however, is the propensity for ES cells to form teratomas when transplanted *in vivo*. Selective depletion of undifferentiated cells during regeneration therapies could reduce the carcinogenic risks of these procedures. We show that inhibiting Cdk1 results in the activation of a DNA damage response, nuclear p53 stabilization, and induction of pro-apoptotic p53 target genes in ES but not differentiated cells. Furthermore we show that clinically relevant Cdk1 inhibitors prevent formation of

ES cell-derived tumors and inhibit growth of established ES cell-derived teratomas *in vivo*. Our data demonstrate that ES cells are uniquely sensitive to Cdk1 inhibition, and identify Cdk1 as a pharmacological target that could increase the safety of regeneration therapies. In an independent project, we use a chemical-genetic approach to achieve selective inhibition of Cdk2 kinase activity using an analog sensitive (AS) allele. We show that inhibition of Cdk2 kinase activity slows proliferation of non-transformed cells, whereas siRNA knockdown of Cdk2 does not, highlighting the differences between these approaches. We also show that Cdk2 inhibition attenuates anchorage-independent growth of transformed cells. Finally, we develop a Cdk2-AS mouse model that will allow for the acute inhibition of Cdk2 in a variety of cell types and cancer models. Together, these results enhance our understanding of the effects of Cdk inhibition in defined cellular and genetic contexts.

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Chapter 1: Introduction

1.1 Control of the mammalian cell cycle by cyclin dependent kinases

Cell division, the process by which a single parent cell divides into two daughter cells, is a fundamental requirement for all life. In single cell organisms, cell division generates an entire new organism. In multicellular organisms, extensive cell division during development allows for the generation of an entire organism from a single cell, whereas cell division in adult organisms provides replacements for cells that die during natural processes or due to environmental damage. Cell division requires the precise replication and subsequent segregation of chromosomal DNA equally between two daughter cells (Morgan, 2007). These processes occur through a series of highly regulated events known as the cell cycle (Morgan, 2007). The major regulators of the cell cycle are a family of highly conserved proteins known as the cyclin dependent kinases (Cdks) and their activating subunits known as the cyclins (Morgan, 1995).

The basic regulation of the cell cycle by Cdks has been well established through pioneering studies done in yeast (Nurse et al., 1998). Cell cycle progression in yeast is regulated by a single Cdk, Cdk1, originally known as Cdc28 in *Saccharomyces cerevisiae* and Cdc2 in *Schizosaccharomyces pombe* (Forsburg and Nurse, 1991; Russell and Nurse, 1986). The number of Cdks and cyclins has increased considerably throughout evolution. Mammalian cells express at least 11 Cdks, four of which (Cdk1, 2, 4, and 6) are directly involved in cell cycle regulation (Malumbres and Barbacid, 2009). Activated Cdks function as kinases, catalyzing the attachment of a phosphate group, derived from ATP,

to a large array of cellular substrates. Phosphorylation of these substrates initiates the cellular processes that are required for cell cycle events (Kitagawa et al., 1996).

The cell cycle is classically divided into two phases, interphase and M phase. Interphase is further divided in three sub-phases, including S phase and two gap phases, G1 and G2 (Morgan, 2007). During S phase DNA is replicated and chromosomes are duplicated. G1 refers to the initial phase of the cell cycle, immediately proceeding S-phase, while G2 occurs after S phase and before mitosis. The gap phases provide time for cell growth and synthesis of mRNA and proteins required for the events that occur during S and M phases. M phase, during which the physical division of the cell occurs, is composed of two parts: nuclear division (mitosis) and the division of the cytoplasm to form two daughter cells (cytokinesis)(Morgan, 2007) .

According to the classical view of the cell cycle, specific Cdk-cyclin complexes are necessary for driving the various events that occur during each phase of the cell cycle (Morgan, 1997; Satyanarayana and Kaldis, 2009; Schafer, 1998). The role of these individual Cdk-cyclin complexes has been extensively studied in various model organisms and a general model of the cell cycle has evolved (Fig. 1). The decision for somatic cells to enter in G1 phase and thus initiate the cell cycle program is generally dependent on growth factors and mitogenic signaling through the mitogen activated protein kinase (MAPK) pathway (Meloche and Pouyssegur, 2007; Yamamoto et al., 2006). Signaling through the MAPK pathway results in the increased expression of D type cyclins, which bind and activate Cdk4 and 6. Cdk4/6-cyclin D complexes phosphorylate members of the retinoblastoma (Rb) family of proteins, which disrupts their inhibitory action on the E2F family of transcription factors (Sherr and Roberts,

1999; Weinberg, 1995). Subsequent activation of the E2F transcription factors initiates the transcription of multiple cell cycle related genes, including cyclins E and A (Cobrinik, 2005). During late G1 phase, cyclin E forms complexes with Cdk2, which results in further phosphorylation of Rb family members and subsequent activation of E2F transcription factors, thus creating a positive feedback loop (Harbour et al., 1999). At this point cell cycle progression is no longer dependent on mitogenic signaling and cells are fully committed to undergo a complete cell cycle (Malumbres and Barbacid, 2001; Pardee, 1974).

Activated Cdk2-cyclin E regulates the transition into S phase, during which Cdk2-cyclin E complexes initiate a variety of S phase events. As S phase progresses, there is an abrupt decrease in Cdk2/cyclin E complexes as cyclin E is targeted for degradation (Clurman et al., 1996). In addition to cyclin E, Cdk2 also forms complexes with cyclin A. Cdk2-cyclin A activity is first detected at the end of G1, after which it steadily increases as cells begin to replicate their DNA in S-phase and does not decline until cyclin A is degraded in the early phases of mitosis. Cdk2-cyclin A complexes phosphorylate many substrates that are required for both S phase events as well as exit from S phase (Hwang and Clurman, 2005; Mitra and Enders, 2004; Petersen et al., 1999). Towards the end of S phase, cyclin A forms complexes with Cdk1 (Furuno et al., 1999), which are required for the initiation of mitosis (Pagano et al., 1992). As cells transition through G2, cyclin B levels accumulate and Cdk1 forms complexes with cyclin B. Cdk1-B complexes regulate various events during the G2-M transition and throughout mitosis. Finally, the inactivation of Cdk1-cyclin B complexes due to degradation of cyclin B triggers exit from mitosis (Pines, 2006).

A central component of this cell cycle model is that individual Cdk-cyclin complexes are active at distinct phases of the cell cycle. Temporal restriction of Cdk activity ensures that cell cycle events occur in the correct sequence. Multiple regulatory mechanisms exist that are responsible for Cdk activity, including the tightly controlled expression of their regulatory cyclin subunits, endogenously expressed Cdk inhibitor proteins, and inhibitory phosphorylation events (Morgan, 1995). By definition, activation of Cdk kinase activity requires association with a cyclin subunit, which triggers a conformational change at the ATP binding site (Jeffrey et al., 1995). Throughout the cell cycle, cyclins are synthesized and degraded at distinct points. Prior to the start of G1, cyclins D, E, A and B, which activate Cdk2 and Cdk1, are not expressed due to their degradation in the previous cell cycle, ensuring that cell cycle events are not triggered prematurely (Peters, 2006). Cyclins are targeted for degradation by the proteasome by two large multiunit ubiquitin ligases, the SCF-Fbxwf ubiquitin ligase (Koepp et al., 2001; Schwab and Tyers, 2001; Strohmaier et al., 2001), and the anaphase-promoting complex/cyclosome (APC/C)(Harper et al., 2002). Destruction of cyclins by ubiquitin-mediated proteolysis allows for unidirectional and irreversible transitions through the cell cycle. Cyclins are also major determinants of Cdk specificity towards individual substrates (Loog and Morgan, 2005; Roberts, 1999), allowing for an additional level of control during progression of the cell cycle.

A second mechanism of regulation includes the Cdk inhibitory proteins (CKI) that can bind and inactivate Cdks (Sherr and Roberts, 1999). These proteins act primarily during G1 when Cdk activity is low and external regulatory factors regulate entry into the cell cycle. They are important for causing the arrest of cells in G1 in the response to

unfavorable growth conditions or intracellular signals such as DNA damage (Besson et al., 2008). Two CKI gene families have been defined based on their evolutionary origins, structure and function. The INK4 gene family encodes p16^{INK4a}, p17^{INK4b}, p18^{INK4c}, and p19^{INK4d}, all of which bind and inhibit Cdk4 and Cdk6 (Sherr and Roberts, 1999). The Cip/Kip family members p21, p27, and p57 inhibit Cdk2-Cyclin E complexes, but can activate Cdk4 and 6 (Sherr and Roberts, 1999).

Finally, Cdks are also negatively regulated by phosphorylation events. In mammalian cells, phosphorylation at two adjacent residues on Cdk1 (Thr14 and Tyr5 in humans) blocks kinase activity (Lew and Kornbluth, 1996). These residues are located within the ATP binding pocket and their phosphorylation likely interferes with the orientation of the ATP phosphates (Morgan, 2007). The phosphorylation of these residues is controlled by the combination of kinases, Wee1 and Myt1, and a phosphatase, Cdc25 (Perry and Kornbluth, 2007), which together regulate the transition into M phase through control of Cdk1. In general, the regulators of Cdk activity are targets of multiple cellular pathways, including the DNA damage response pathway (Dasika et al., 1999). As a result, checkpoints exist throughout the cell cycle, and progression can be arrested in response to unfavorable events such as DNA damage or issues with DNA replication during S phase (Abraham, 2001; Enoch and Nurse, 1990). Together, these systems of control allow Cdks to precisely regulate the timing and sequence of cell cycle events.

The observation that individual Cdks are activated at distinct phases of the cell cycle led to the assumption that each Cdk is necessary for normal cellular proliferation. For example Cdk2 becomes active during the G1-S transition and has been shown to phosphorylate many substrates involved in S phase events. Therefore it was assumed that

Cdk2 is critical for G1-S transition and initiating DNA synthesis. This has been supported by studies showing that a dominant negative form of Cdk2 attenuates growth of cells in culture (van den Heuvel and Harlow, 1993) and microinjected antibodies against Cdk2 block S-phase initiation in mammalian cells (Pagano et al., 1993). Over the past decade, however, studies with genetic mouse models have challenged the theory that individual Cdks, other than Cdk1, are necessary for cell cycle progression (Satyanarayana and Kaldis, 2009). While Cdk1 deficient mice fail to develop beyond the morula stage of embryogenesis (Santamaria et al., 2007), systematic knockout of individual interphase Cdks resulted in viable mice, demonstrating that Cdk2, Cdk4 and Cdk6 are not essential for general cell proliferation (Berthet et al., 2003; Malumbres et al., 2004; Rane et al., 1999; Tsutsui et al., 1999). Instead, proliferation defects in specialized cell types were observed, suggesting that the requirement for individual Cdks is cell type-dependent. Combinatorial knockouts of multiple Cdks yielded comparable results; Cdk4/6, Cdk4/2, and Cdk2/6 knockout mice each exhibit various developmental defects but general cell cycle progression is not disrupted in most cell types (Barriere et al., 2007; Berthet et al., 2006; Malumbres et al., 2004; Santamaria et al., 2007). Even mice deficient for all three interphase Cdks are able to develop to e12.5, suggesting that cellular proliferation in the single and double knockout mouse models is not simply due to compensation by other interphase Cdks (Santamaria et al., 2007).

Cyclin knockout models have yielded slightly different, although complimentary, results (Malumbres and Barbacid, 2009). Ablation of either cyclin A2 or B1 leads to early embryonic lethality, emphasizing the importance of Cdk1 in cell cycle proliferation (Brandeis et al., 1998; Murphy et al., 1997). Mice lacking cyclins E1 and E2 also die in

early in development, due to defects in the endo-reduplication of trophoblast cells (Geng et al., 2003; Parisi et al., 2003). In accordance with the Cdk2 knockout mice however, cyclin E knockout mice are viable when the placental defect is restored (Parisi et al., 2003), suggesting that most cells are proliferating normally. Genetic ablation of all three D cyclins results in mice with similar developmental defects as Cdk4/6 double knockout mice: these mice die during embryogenesis due to hematopoietic defects but proliferation of other cell types seems unaffected (Kozar et al., 2004; Sicinski et al., 1995). Taken together, the Cdk and cyclin mouse knockout models suggest there is a high degree of redundancy within the mammalian cell cycle regulatory system.

Genetic mouse models have provided valuable insight on the contribution of individual Cdks and cyclins to cell cycle regulation during development. When contemplating functions of a kinase however, it is important to acknowledge the differences between genetic ablation of the kinase versus the acute inactivation of kinase activity. When an individual Cdk is genetically ablated it is possible for other Cdks to form complexes with its cyclin binding partner, allowing for compensatory mechanisms to occur (Satyanarayana and Kaldis, 2009). Indeed, in knockout mouse models lacking all three interphase Cdks, Cdk1 was found to bind to all cyclins, resulting in phosphorylation of Rb and activation of the E2F transcription factors (Santamaria et al., 2007). In contrast, when kinase activity of a Cdk is inhibited, the Cdk remains bound to its cyclin binding partner, which could eliminate compensation by other Cdks. Through the advancement of methods to selectively inhibit individual Cdk kinase activity (discussed in section 1.5) we can expect to gain a more thorough understanding of the complex regulation of the mammalian cell cycle.

1.2 Cyclin dependent kinases as targets for cancer therapeutics

Loss of cell cycle control is a hallmark of human cancer (Hanahan and Weinberg, 2000). As central regulators of the cell cycle, Cdks have been extensively pursued as therapeutic targets for the treatment of cancers and other proliferative diseases (Malumbres and Barbacid, 2009; Malumbres et al., 2008; McInnes, 2008). Indeed, deregulated cyclins, Cdks and Cdk inhibitors have been implicated in a wide variety of human cancers (Malumbres and Barbacid, 2007). Unfortunately, the first generation of Cdk inhibitors, including Flavopiridol and Roscovitine, have had limited success in clinical trials, exhibiting only modest effects and high levels of toxicity (Shapiro, 2006). These initial compounds were broad-spectrum kinase inhibitors, which likely contributed to the high toxicity observed with their use (Shapiro, 2006). The development of second and third generation Cdk inhibitors with increased specificity and improved pharmacokinetics has reopened the possibility that Cdks may have utility as therapeutic targets in cancer.

A key question to consider while moving forward with Cdk inhibitors as therapeutic agents is what are the cellular and genetic contexts that will benefit most from Cdk inhibition. Mouse knockout models of Cdks and cyclins have demonstrated that the requirement for individual Cdks differs greatly depending on the cell type (Barriere et al., 2007; Berthet et al., 2003; Berthet et al., 2006; Rane et al., 1999; Santamaria et al., 2007). Whether or not tumor cells retain the same requirements for individual Cdks as the cells from which they originated remains to be determined. These results could provide

insights as to potential side effects/ toxicities associated with Cdk inhibitors. Future generation of conditional knockout mice will allow for a more detailed analysis of Cdk requirements in adult somatic tissues.

Perhaps even more relevant are numerous recent studies investigating Cdk inhibition in distinct genetic contexts. For example, both D1 and Cdk4 knockout mice are resistant to mammary tumors driven by ErbB-2 suggesting that Cdk4 may be important in certain breast cancers (Yu et al., 2001; Yu et al., 2006). It was also found that Cdk2 inhibition is synthetically lethal in the context of high MYCN expression (Molenaar et al., 2009). This suggests that Cdk2 inhibitors would be effective against tumors exhibiting amplified MYCN, including neuroblastomas, which are the second highest cause of cancer related death in children (Maris et al., 2007). Additionally, Cdk2 inhibition sensitized cells to c-Myc induced cellular senescence (Campaner et al., 2010; Hydbring et al., 2010). Finally, Cdk1 inhibition results in a synthetic lethality in cells that have been transformed with c-Myc (Goga et al., 2007). This suggests that targeting Cdk1 could have utility in cancers with high Myc expression. Work in the Goga lab has identified once such cancer, the hard to treat triple negative subgroup of breast cancers. Pre-clinical studies in the lab have demonstrated that Cdk1 inhibition induces cell death in triple negative breast cancer cell lines and that Cdk1 inhibitors cause tumor regression in triple negative mouse xenograft models (Horiuchi et al., 2012b). The use of Cdk1 inhibitors for the treatment of triple negative breast cancers is currently being evaluated in phase II clinical trials. Together, these results demonstrate that downstream effects of Cdk inhibition are highly dependent on cellular and genetic context. Moving forward, the

identification and characterization of the context dependent-effects of Cdk inhibition could lead to promising therapeutic approaches for the treatment of specific cancers.

1.3 Cell cycle characteristics of embryonic stem cells

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) during the blastocyst stage of development. They are the *in vitro* counterparts to the epiblast of the early developing embryo (Burdon et al., 2002; Pauklin et al., 2011). ES cells retain the pluripotent potential of these early epiblast cells and are able to give rise to all cell types of an adult organism, which is demonstrated by their ability to form viable chimeras and contribute to the germ line when transplanted back into tetraploid blastocysts (Beddington and Robertson, 1989; Martin, 1981). Since the successful isolation of human ES cells in the late 1990s (Thomson et al., 1998) there has been considerable interest in utilizing the pluripotent potential of ES cells in regenerative medicine (Draper and Fox, 2003; Lebkowski et al., 2001).

Both ES cells and their *in vivo* counterparts exhibit highly specialized cell cycles, characterized by rapid proliferation and abbreviated gap phases. In the developing embryo, lack of an early G1 phase of the cell cycle facilitates rapid, mitogen-independent, expansion of the pluripotent cells of the ICM, increasing from 20-25 cells present at 4.5 days post coitum (d.p.c) to 8060 cells by 7.5 (d.p.c)(Rodda et al., 2002). ES cells share the unique proliferative capabilities and altered cell cycle structure seen in early development. The differences in cell cycle structure between ES and differentiated

cells can be largely attributed to altered regulation of Cdk-cyclin activity (Fig. 1) (Orford, 2008 #541).

In somatic cells, cyclin expression is regulated in a cell cycle-dependent manner, which limits Cdk-cyclin activity to precise points in the cell cycle (Morgan, 2007). Cyclin expression is upregulated as the E2F family of transcription factors becomes active during late G1 phase. Subsequently, cyclins are targeted for degradation by two multiunit ubiquitin-protein ligases, the anaphase promoting complex/ cyclosome (APC/C) and SCF-Fbxwf ubiquitin ligase (Koepp et al., 2001; Schwab and Tyers, 2001; Strohmaier et al., 2001). These basic regulatory mechanisms of cell cycle regulation are also present in ES cells, but they operate in a modified way. In somatic cells, APC/C is inactivated just before S-phase by the psuedosubstrate inhibitor, early Emi1 (early mitotic inhibitor-1) {Reimann, 2001 #1678}. In ES cells, APC/C activity is attenuated due to high expression of Emi1 throughout the cell cycle, resulting in reduced fluctuation of cyclin A and B expression (Ballabeni et al., 2011). Activity of the SCF-Fbxwf ubiquitin ligase in ES cells has been less well characterized, but cyclin E is also expressed independent of cell cycle phase (Stead et al., 2002). General expression levels of cyclin E, A and B are also higher in ES cells compared to somatic cells, and endogenous Cdk inhibitors, p21, p27, and Ink4/Arf locus are not expressed (Li et al., 2009; Sabapathy et al., 1997; Stead et al., 2002). The combined effect of elevated and precocious cyclin activity with the lack of endogenous Cdk inhibitors results in an overall increased activity of Cdk1 and 2 in ES cells.

Increased activity of Cdk2 alters the structure of G1 (Orford and Scadden, 2008). Rb is hyperphosphorylated due to sustained Cdk-cyclin E activity, and is therefore

constitutively inactive (Savatier et al., 1994). This effectively omits the early G1 phase and accordingly mouse ES cells express very low levels of D type cyclins and have almost no detectable Cdk4/6 activity (Stead et al., 2002). Due to the lack of early G1 phase and constitutively active Cdk2-cyclin E complexes, ES cells are not dependent on MAPK signaling for cell cycle entry. Interestingly, MAPK signaling induces mES cells to differentiate (Burdon et al., 1999). It has been proposed that the lack of early G1 allows mES cells to avoid the differentiation effects of mitogenic signaling pathways present in early the G1 phase (Burdon et al., 2002). Therefore the unique cell cycle structure of ES cells is thought to not only be critical for their rapid proliferation, but also contribute to their capability for self-renewal.

1.4 Tumorigenicity of pluripotent cells

The two defining characteristics of ES cells are their ability to proliferate indefinitely in culture (self-renewal) and their capacity to differentiate into all the somatic lineages (pluripotency). These characteristics make ES cells an attractive source for stem cell therapies. The discovery of induced pluripotent stem (iPS) cells has made these therapies even more approachable (Takahashi et al., 2007). These same characteristics of ES cells, however, also render them tumorigenic, and prior studies have shown that when even a few undifferentiated cells are transplanted into mice they can give rise to teratomas (Ben-David and Benvenisty, 2011; Knoepfler, 2009); in fact, the ability to give rise to benign teratomas and malignant teratocarcinomas when transplanted *in vivo* has become a hallmark of ES and iPS cells (Shih et al., 2007). The capability of ES cells to give rise to tumors represents a major limitation to the field of regenerative medicine.

The similarities between cancer and stem cells have long been recognized. The first pluripotent mammalian stem cells to be derived in culture were actually embryonic carcinoma cells, derived from teratocarcinomas (Evans and Kaufman, 1981). More recent work has shown that many of the phenotypic similarities observed in ES cells and cancers can be attributed to shared gene expression profiles (Chiou et al., 2008; Sperger et al., 2003; Wong et al., 2008). It has also been demonstrated that a defined set of Myc-target genes are expressed both in poorly differentiated, aggressive tumors and ES cells, and that ectopic expression of Myc can reactivate a stem cell specific transcriptional profile (Ben-Porath et al., 2008; Wong et al., 2008). Interestingly, the transcriptional profile shared by ES cells and poorly differentiated cancers is distinct from that which is found in adult stem cell populations (Wong et al., 2008). These studies provide a molecular basis for the similarities observed between embryonic stem cells and poorly differentiated, aggressive cancers.

Due to the tumorigenicity of pluripotent ESCs and iPSCs, future regeneration therapies will likely focus on transplantation of stem cell-derived progenitors that have been differentiated towards a desired lineage. Nonetheless, the tumorigenic potential of undifferentiated cells remains a critical obstacle to the field of regenerative medicine, as the retention of undifferentiated cells within a population of ESC-derived cell types would put transplant patients at risk for teratocarcinomas. Indeed, many preclinical studies done in animals have shown that even after long-term differentiation protocols, residual undifferentiated cells can form tumors (Bjorklund et al., 2002; Germain et al., 2012; Wernig et al., 2004). Both methods to selectively deplete undifferentiated cells during the differentiation process *in vitro*, as well as the development of therapeutic

strategies to treat teratocarcinomas would greatly increase the safety of regeneration therapies.

1.5 Selective inhibition of individual kinases using a chemical genetic approach

Cdks are part of the large family of protein kinases that make up the human kinome. Protein kinases regulate a wide array of biological processes through the catalytic addition of phosphate from a donor ATP to their protein substrates (Hanks, 2003). Historically, our knowledge of the function of an individual kinase has been attained through identifying its protein substrates as well as determining the downstream effects of its inactivation. Therefore the study of protein kinases has been greatly advanced by the development of small molecule kinase inhibitors (Sedlacek, 2000). Screens of chemical compound libraries have identified protein kinase inhibitors, which generally work through competitive inhibition of the ATP-binding domain to disrupt catalytic function (Gray et al., 1998). The high degree of conservation of the ATP binding domain within the 500+ members of the human kinome, however, makes it extremely difficult to design inhibitors that are completely selective for a single kinase. The lack of specificity of small molecule inhibitors is especially problematic when attempting to identify individual functions of closely related kinases (Karaman et al., 2008).

Researchers often employ complementary approaches to study kinase inactivation in the absence of sufficiently selective kinase inhibitors. Genetic depletion approaches such as siRNA knockdown, antisense oligonucleotides, or animal knockout models,

allow for selective depletion of an individual kinase with high specificity but still have limitations when trying to understand individual kinase function (Thyagarajan et al., 2003). Ablation of an individual kinase potentially allows for cells to compensate for lack of kinase activity through activation of other cellular pathways. Therefore these approaches will likely yield different results than acute kinase inhibition. Another alternative approach is the use of temperature sensitive mutants, which allow for conditional inactivation of a kinase under restrictive temperatures (Tan et al., 2009a). In these systems, however, the restrictive conditions often have major effects on cell physiology, complicating the interpretation of loss of function studies. Thus there is a need for additional methods to study the acute and selective inactivation of individual kinases.

A chemical genetic approach that allows for the sensitization of Src-family tyrosine kinases to engineered small molecules was developed in the late 1990's (Liu et al., 1998). This approach, known as the analog sensitive approach, involves mutating a bulky amino acid residue, such as a phenylalanine, within the ATP binding site of a kinase to a smaller residue, such as a glycine or an alanine, thus creating a unique expanded pocket (Fig. 2). Small molecules can be designed to fit in the newly engineered pocket by the addition of bulky chemical side chains. These engineered small molecules inhibit the modified kinase without affecting other mammalian kinases that lack the analog sensitive mutation. This approach has been since been applied a wide variety kinases, including members of the Cdk family, Cdk1 and Cdk2 (Hochegger et al., 2007; Horiuchi et al., 2012a; Merrick et al., 2011) (Bishop et al., 2000).

The analog sensitive approach is especially beneficial for the study of closely related family members, such as Cdk1 and 2, which exhibit high degrees of homology between their ATP binding sites (Tripathi et al., 2012). Even relatively specific new-generation Cdk inhibitors such as CVT-313 (Cdk2), purvalanol A (Cdk1), and Ro-3306 (Cdk1) only show ~10-100 fold increase in selectivity towards their intended target over closely related family members, based on *in vitro* inhibition studies (IC50s) (Brooks et al., 1997; Gray et al., 1998; Vassilev et al., 2006). Mouse knockout models and biochemical analysis have shown that Cdk1 compensates for Cdk2 when Cdk2 protein is ablated, making it difficult to determine Cdk2 function through genetic depletion models (Barriere et al., 2007; Berthet et al., 2003; Santamaria et al., 2007). Therefore the development of analog sensitive alleles of Cdk1 and 2 will greatly enhance our understanding of individual kinase function. In chapter 4 of this thesis, data demonstrating the use of an analog sensitive allele of Cdk2 to study Cdk2 function in normal and transformed cells will be presented. Furthermore, in chapters 4 and 5, the development of an analog sensitive Cdk2 mouse model will be discussed. This mouse model will provide valuable tools allowing for the study of acute Cdk2 kinase inhibition in a large array of tissue types and cancer models. We believe this approach will greatly contribute to our understanding of individual kinase function and provide direction for the development of Cdk inhibitors as therapeutic targets.

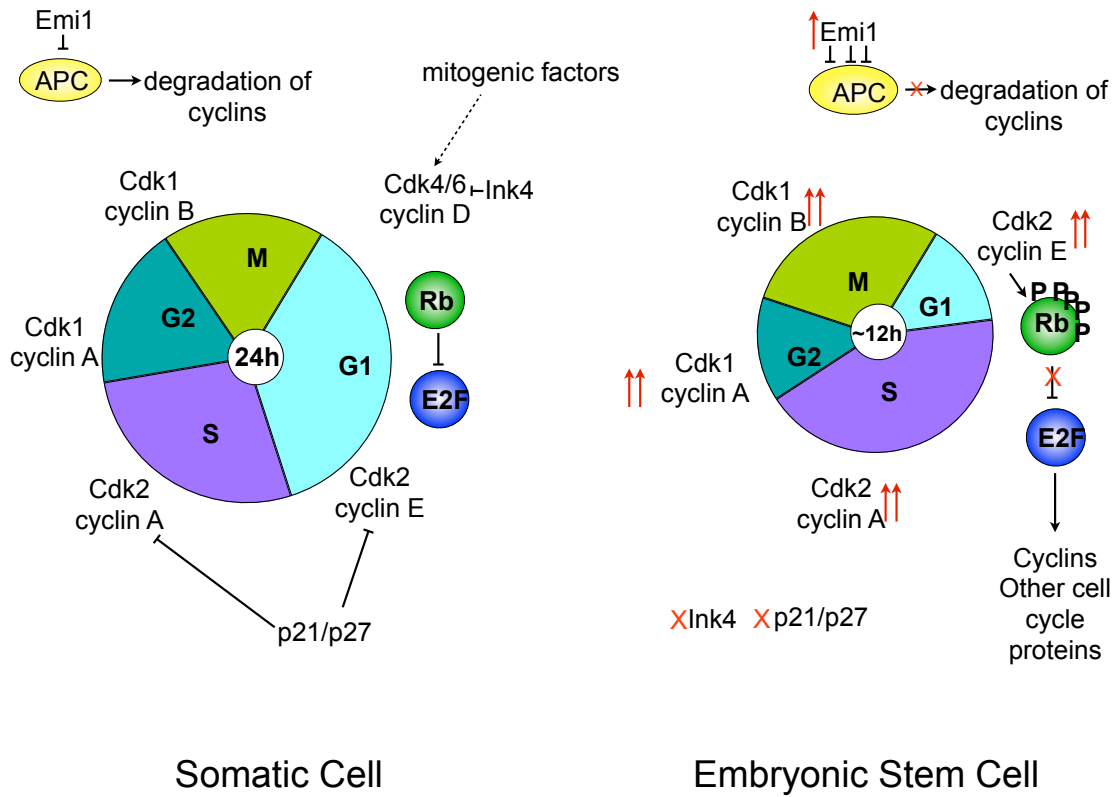


Figure 1. Cell cycle regulation in somatic cells versus embryonic stem cells.

Schematic highlighting key differences in cell cycle of somatic versus embryonic stem cells. In brief, elevated expression of Emi1 causes attenuation of APC in embryonic stem cells. As a result, cyclins are expressed at high levels, with reduced fluctuation as cell cycle progresses. Elevated, constitutive Cdk2-cyclin E activity results in hyperphosphorylation of Rb, leading to constitutive activity of E2F family transcription factors and overexpression of cyclins and other cell cycle proteins. As a result ES cells exhibit an abbreviated cell cycle program with shortened Gap phases.

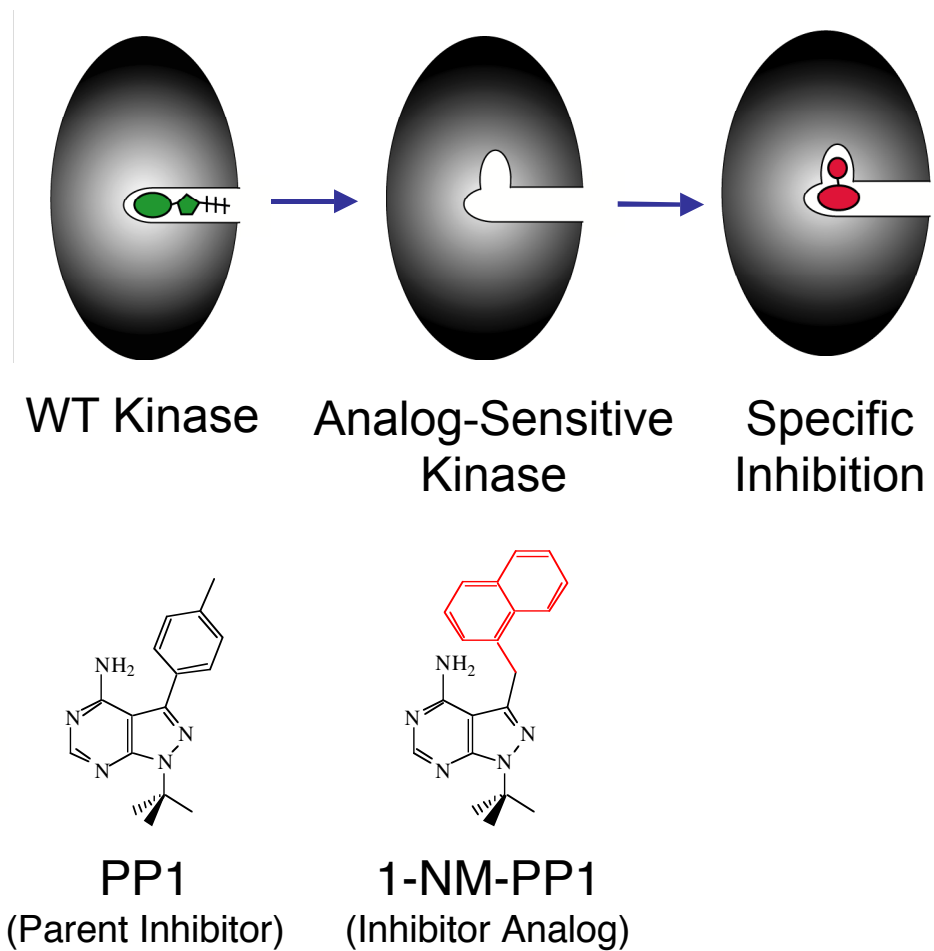


Figure 2. An analog sensitive approach to study kinase inhibition.

Model demonstrating an engineered analog sensitive kinase with the new binding pocket generated by the analog sensitive mutation. Below is an example of a chemical inhibitor, 1NM-PP1, derived from a general parent inhibitor, PP1, and designed to selectively inhibit the analog sensitive kinase.

Chapter 2

Cdk1 Inhibition Selectively Induces Apoptosis in ES but not Differentiated Stem Cells

2.1 Introduction

The principal events of the mammalian cell cycle are controlled by Cdks 1, 2, 4 and 6, which are activated upon binding to various cyclin regulatory subunits (Morgan, 2007). In general, acutely inhibiting Cdk1 or Cdk2 in non-transformed, proliferating somatic cells results in a reversible G2/M or G1/S cell cycle arrest respectively, but does not cause significant amounts of cell death (Gray et al., 1998; Horiuchi et al., 2012a; Pagano et al., 1993; van den Heuvel and Harlow, 1993). In this chapter we examine the effect of acutely inhibiting Cdks in embryonic stem (ES) cells.

ES cells are pluripotent cells derived from the inner cell mass at the blastocyst stage of development. They exhibit a unique cell cycle program, characterized by rapid proliferation rates, shortened gap phases and the absence of a restriction or check point at the G1/S transition (Orford and Scadden, 2008). These differences can be attributed, at least in part, to altered Cdk-cyclin activity. In somatic cells, cyclin expression is regulated in cell cycle-dependent manner, which allows Cdk-cyclin complexes to become activated at precise points in the cell cycle (Morgan, 2007). Cyclins fluctuate with each cell cycle progression in part due to their active degradation at the end of mitosis by the anaphase

promoting complex/ cyclosome (APC/C) together with E2 enzymes. In ES cells, APC/C activity is attenuated, resulting in reduced fluctuation of cyclin expression (Ballabeni et al., 2011). Additionally, ES cells express high levels of cyclins compared to somatic cells, and endogenous Cdk inhibitors, p21, p27, and Ink4/Arf locus are not expressed (Li et al., 2009; Sabapathy et al., 1997; Stead et al., 2002), resulting in increased activity of Cdk2 and Cdk1. Constitutive Cdk2/ cyclin E activity leads to hyperphosphorylated Rb which effectively bypasses early G1 phase and accordingly, mouse ES cells express very low levels of D type cyclins and have almost no detectable Cdk4/6 activity (Stead et al., 2002). These differences in cyclin/ Cdk expression and activity led us to ask whether ES cells might respond differently than somatic cells to the inhibition of individual Cdks, specifically Cdk2 and Cdk1.

Recent studies analyzing the ablation/ inactivation of individual Cdks or cyclins in somatic versus pluripotent cells suggest that ES cells might have differential requirements in terms of Cdk and cyclin expression. In a study by Kalaszczynska *et al.*, acute ablation of Cyclin A, which binds to both Cdk1 and Cdk2, was achieved by using cells from conditional A1 and A2 knockout mice (Kalaszczynska et al., 2009). They found that ablation of both isoforms of cyclin A has no effect on cellular proliferation of fibroblasts but significantly inhibits proliferation of ES and hematopoietic cells. Additionally small molecule inhibition of Cdk1 induces the differentiation of trophoblast cells, a specialized pluripotent cell type that gives rise to the placenta, into non-proliferating giant cells (Ullah et al., 2008). This suggests that pluripotent cells have unique requirements for Cdk1. We hypothesized that, due to their unique cell cycle

program, ES cells might show heightened sensitivity to inactivation of cell cycle proteins. Here we report on the effect of Cdk inhibition in ES cells.

2.2 Results

siRNA knockdown of Cdk1 and cyclins B1/B2 results in apoptosis in embryonic stem cells:

Progression through the ES cell cycle is regulated by Cdk2-cyclin E at the G1 and S transition, Cdk1-cyclin A during late S phase/ early G2, and Cdk1-cyclin B during G2 and M phase (Stead et al., 2002). To evaluate the requirements of individual Cdks and cyclins for ES cell viability, small interfering RNA (siRNAs) were used to selectively deplete Cdks 1 and 2, as well as cyclins D, E1 and E2, A, and B1 and B2 in mouse ES cells. Treatment of ES cells with siRNAs led to significant depletion of each targeted protein, as determined by western blotting (Fig. 3).

We analyzed cell cycle profiles after Cdk and cyclin knockdown using propidium iodide (PI) staining and FACs analysis. Knockdown of Cdk2, cyclin D or cyclins E1/E2 had little affect on cell cycle profiles, consistent with what has previously been reported in somatic cells and mouse knockout models (Fig. 4) (Barriere et al., 2007; Li et al., 2012; Tetsu and McCormick, 2003). Knockdown of cyclin A resulted in an increased fraction of cells in S and G2/M phases and a decreased fraction of cells in G1 phases, in accordance with what has previously been reported in cyclin A deficient ES cells (Fig. 4) (Kalaszczynska et al., 2009). Strikingly, cell cycles profiles of ES cells after siRNA knockdown of either Cdk1, or its activating cyclins B1/B2, were hard to interpret, due to

a significant increase in the sub2N percentage of cells, indicating substantial amounts of cell death (Fig. 4). We chose to more carefully examine this cell death phenotype. siRNA knockdown of Cdk2 or cyclins D or E1/E2 had little or no effect on ES cell viability, as determined by quantification of sub2n levels (Fig. 5A), cell morphology (Fig. 5B), and western blotting for poly (ADP-ribose) polymerase (PARP) cleavage, a marker of caspase-dependent apoptosis (Fig. 5C). In contrast, knockdown of Cdk1 or cyclins B1/B2, which bind exclusively to Cdk1, drastically induced cell death, and knockdown of cyclin A, which binds to both Cdk1 and 2, resulted in intermediate levels of cell death (Fig. 5A-C). Previous reports have suggested that Cdk1 inhibition can induce differentiation of pluripotent trophoblast cells (Ullah et al., 2008). We however found no evidence of differentiation of mES cells after siRNA knockdown of any of the Cdks or cyclins, as determined by staining for ES cell associated marker, alkaline phosphatase (Fig. 6). This is consistent with recent findings that elongation of the ES cell cycle is compatible with pluripotency (Li et al., 2012). These results indicate that siRNA knockdown of either Cdk1 or its cyclin binding partners, but not other Cdks or cyclins, induces cell death in ES cells.

Small molecule inhibition of Cdk1 induces apoptosis in pluripotent but not differentiated cells.

An alternative, more clinically relevant, method to disrupt Cdk activity is through the use of small molecule inhibitors, which target kinase activity by blocking the ATP binding site. In a previous study, we found that siRNA knockdown of Cdks can have minimal effects compared to small molecule inhibition, presumably due to compensation by other

Cdks occurring in the case siRNA knockdown (Horiuchi et al., 2012a). We asked whether inhibition of Cdks using small molecules would yield similar results as siRNA knockdown in mES cells. mES cells were treated with a panel of Cdk inhibitors, including Cdk2 inhibitor CVT313, and two Cdk1 inhibitors, purvalanol A and Ro-3306. These three small molecule inhibitors were chosen because of their relatively high specificities towards their intended target and were used at concentrations that have been shown to effectively inhibit intended kinases in the literature (Brooks et al., 1997; Goga et al., 2007; Gray et al., 1998; Vassilev et al., 2006). CVT-313 inhibition of Cdk2 resulted in a slight increase in the percentage of cells in G1 while causing little if any cell death (Fig. 7A and B). In contrast, purvalanol A and Ro-3306 caused cell cycle arrest at G2/M and extensive levels of cell death within 24 hours of treatment, as determined by PI staining to assess sub2n DNA content (Fig. 7A and B). In subsequent experiments we exclusively used purvalanol A, as we observed a slightly stronger phenotype than with Ro-3306 (Fig 7B). Based on these results, we concluded that ES cells are sensitive to both siRNA knockdown and small molecule inhibition of Cdk1, but not Cdk2.

We next asked whether sensitivity towards Cdk1 inhibition was dependent on a pluripotent state. We induced mES cells to differentiate by removing leukemic inhibitory factor (LIF) from the media and culturing cells with low concentrations of all-*trans* retinoic acid (RA), which down-regulates the LIF receptor (Tighe and Gudas, 2004). After four days of RA treatment, minimal levels of pluripotency associated transcription factors Nanog and Oct 4 could be detected by immunofluorescence, indicating that efficient differentiation had occurred (Fig 8A). We compared the effect of Cdk1 inhibition on differentiated ES cells (mES-diff) and an alternative somatic cell type,

mouse embryonic fibroblasts (MEFs), with mES cells, mouse induced pluripotent stem cells (miPS), and human embryonic stem cells (hES). Differentiated mES cells treated with purvalanol A underwent a G2/M arrest, indicating that Cdk1 was being targeted (Fig 8B). Neither differentiated ES cells, nor MEFs, underwent significant levels of cell death after 24 hours of purvalanol A treatment (9A and B). In contrast mES, miPS and hES cells exhibited significant levels of cell death, ranging from approximately 30% in miPS cells, to 70% in hES cells, as determined by a FACs based viability dye exclusion assay and PARP cleavage (Fig 9A-C). We therefore concluded that Cdk1 inhibition selectively induces cell death in ES cells, while sparing differentiated cells .

To determine whether Cdk1 inhibitor-induced cell death in pluripotent cells is dependent on the intrinsic apoptotic pathway, we utilized mES cells deficient for pro-apoptotic proteins, Bax and Bak. Bax and Bak are two multi-domain Bcl-2 family proteins that are critical for the permeabilization of the mitochondrial outer membrane and release of cytochrome c, a hallmark of the intrinsic apoptotic pathway (Wei et al., 2001). Modified ES cells, deficient for both Bax and Bak, were generously provided by the Oakes laboratory (Huang et al, manuscript in preparation)(Takeuchi et al., 2005). These cells lack expression of Bax and Bak (fig. 10A) but maintain typical ES cell morphology (not shown) and express the pluripotency markers, Oct4 and Nanog (Fig 10B). In contrast to wild type mES cells, which undergo substantial cell death after purvalanol A treatment, we found that treatment of Bax^{-/-}Bak^{-/-} ES cells with purvalanol A induced no cell death or PARP cleavage (Fig 11A and B). These data indicate that Cdk1 inhibitor-induced cell death in pluripotent cells is dependent on the intrinsic apoptotic pathway.

Cdk1 inhibitor treatment induces the DNA damage response in ES but not differentiated cells.

In order to understand why Cdk1 inhibition selectively causes cell death in ES cells, while sparing differentiated cells, we sought to determine which cellular pathways were contributing to Cdk1 inhibitor-induced cell death. We first examined the DNA damage response pathway. Cdks are known to regulate several components of the DNA damage response pathway, and Cdk1 activity is critical during the repair of double stranded breaks (DSB) by homologous recombination (Falck et al., 2012; Johnson et al., 2011; Peterson et al., 2011). Furthermore, ES cells are uniquely dependent on homologous recombination for DSB repair, as they do not utilize non-homologous end joining, an alternative mechanism for DSB repair (Tichy et al., 2010). We therefore hypothesized that Cdk1 inhibition might trigger the DNA damage response in ES cells.

To address our hypothesis, we examined the effect of Cdk1 inhibition on the phosphorylation of histone H2A variant, H2AX, in ES and differentiated cells. Phosphorylation of H2AX at Ser-139 is an early cellular response to DNA damage in mammalian cells (Mah et al., 2010). As a positive control, cells were treated with doxorubicin, an agent known to cause double stranded DNA breaks. As expected, mES, Bax^{-/-} Bak^{-/-} and differentiated mES cells all showed an increase in the phosphorylated form of H2AX (γ -H2AX) after treatment with doxorubicin (Fig 12). Similar to doxorubicin treatment, purvalanol A also induced an increase in γ -H2AX levels in ES and Bax^{-/-} Bak^{-/-} cells, as determined by western blotting (Fig. 12). Bax^{-/-} Bak^{-/-} mES cells do not undergo cell death, and therefore provide a useful tool for identifying cellular effects that are occurring upstream of cell death. Our results show that Cdk1 inhibition

induces the DNA damage response upstream of apoptosis in ES cells. In contrast, when differentiated ES cells were treated with purvalanol A, we observed no change in γ -H2AX levels, suggesting that Cdk1 inhibition only induces DNA damage in pluripotent cells (Fig. 12). To confirm accumulation of γ -H2AX foci, we next examined $Bax^{-/-} Bak^{-/-}$ mES and differentiated ES cells after purvalanol A treatment using immunofluorescence. In accordance with the western blot analysis, doxorubicin treatment resulted in the formation of γ -H2AX foci in both cell types, whereas only the undifferentiated $Bax^{-/-} Bak^{-/-}$ mES showed a significant increase in the percentage of cells containing γ -H2AX foci after treatment with purvalanol A (Fig 13A-C).

To further assess whether Cdk1 inhibition induces DNA damage in undifferentiated cells, we examined levels of phosphorylated ATM (Ser 1981), a marker of double stranded breaks. Similar to γ -H2AX foci, we found that Cdk1 inhibition in $Bax^{-/-} Bak^{-/-}$ mES, but not differentiated mES cells, resulted in a significant increase in the percentage of cells exhibiting phosphorylated ATM (Fig. 14A-C). We therefore concluded that Cdk1 inhibition induces the DNA damage response in ES but not differentiated cells.

Cdk1 inhibitor-induced cell death in ES cells is mediated by p53

Activation of the DNA damage response typically results in either cell cycle arrest and DNA repair or removal of the damaged cell through apoptosis. A major mediator of the DNA damage response in somatic cells is the p53 tumor suppressor protein (Meek, 2009). p53 functions as a transcription factor to positively regulate numerous target genes involved in cell cycle arrest and apoptosis (Vousden and Lu, 2002). Phosphorylation of

p53 by multiple DNA damage effector kinases, including ATM, results in the stabilization and nuclear localization of p53 (Canman et al., 1998). Whether p53 is activated downstream of the DNA damage response in ES cells is less established. It has previously been reported that although ES cells express abundant cytoplasmic levels of p53 after treatment with genotoxic agents, p53 is not efficiently translocated to the nucleus, thus limiting its activity as a transcription factor (Aladjem et al., 1998). We asked whether p53 was mediating Cdk1-inhibitor induced cell death in ES cells. We first asked whether p53 expression was necessary for cell death by treating ES cells deficient for p53 (Sabapathy et al., 1997) with purvalanol A. We found that p53^{-/-} ES cells exhibited reduced sensitivity to Cdk1 inhibition compared to wild type ES cells, undergoing ~20% cell death as opposed to 50%, as determined by cell viability assays and lack of PARP cleavage (Fig. 15A and B). This suggests that p53 mediates Cdk1 inhibitor-induced cell death in ES cells.

To further investigate whether p53 mediates Cdk1-inhibitor-induced cell death in ES cells, we asked whether purvalanol A treatment led to an increased activation of p53. p53 activity is tightly regulated at multiple levels, including protein stability and sub-cellular localization (Xu, 2003). p53 protein is typically rapidly degraded by the proteasome but can undergo several post-translational modifications that increase protein stability including phosphorylation and acetylation (Xu, 2003). Specifically, phosphorylation of Serine 18 (serine 15 in human) of p53 by ATM during the DNA damage response inhibits MDM2 mediated degradation of p53 thus increasing p53 stability (Canman et al., 1998). We examined total p53 protein levels as well as the phosphorylation status of serine 18 after Cdk inhibitor treatment in ES versus

differentiated ES cells. As a positive control, cells were treated with doxorubicin, which triggers the DNA damage response and p53 activation by inducing double stranded breaks. mES, differentiated mES and $Bax^{-/-} Bak^{-/-}$ mES all showed an increase in p53 levels after treatment with doxorubicin (Fig 16A). Strikingly, p53 protein levels were also dramatically increased in mES cells and $Bax^{-/-} Bak^{-/-}$ mES after as little as four hours of purvalanol A treatment, and p53 levels continued to increase after 8 hours (Fig. 16A). Increase in total protein levels of p53 was accompanied by phosphorylation of Serine 18 (Fig. 16A). In contrast, differentiated ES cells did not show an increase in total or phosphorylated p53 levels after purvalanol A treatment (Fig. 16A). This difference in p53 activation likely accounts for the unique sensitivity to Cdk1 inhibitors observed in pluripotent cells. We also observed a similar increase in p53 levels after siRNA knockdown of Cdk1 as well as cyclins B1/B2 (Fig. 16B). Therefore Cdk1 inhibition results in an increase in p53 protein levels in ES but not differentiated cells.

Activity of p53 as a transcription factor is also regulated at the level of cellular localization. Several post-translational modifications at the N-terminal portion of p53 have been described to affect cellular localization of p53, including phosphorylation of serine 18 by ATM, which contributes to nuclear retention by masking a nuclear export signal (Zhang and Xiong, 2001). To determine the cellular localization of p53 in ES cells after Cdk1 inhibitor treatment we looked at p53 levels in cytosolic and nuclear fractions. We found that Cdk1 inhibitor treatment leads to p53 up-regulation in the nuclear fraction of ES cells (Fig. 17). These results suggest that Cdk1 inhibition causes an increase in p53 expression specifically in the nucleus of mES cells.

Finally, in order to understand how p53 induction contributes to apoptosis in ES cells after Cdk1 inhibitor treatment, we asked which, if any, p53 transcriptional targets were up regulated after Cdk1 inhibitor treatment. Using a real time PCR-based array, we examined mRNA expression levels of a panel of established p53 transcriptional targets involved in both apoptosis and cell cycle arrest before and after Cdk1 inhibitor treatment in ES and differentiated cells (Fig 18A). While many established p53 targets involved in apoptosis, including Bax and Bbc3 (Puma), were not significantly up regulated after Cdk1 inhibitor treatment in ES cells, we did observe a significant increase in three pro-apoptotic targets, Pmaip1 (Noxa), Trp73, and Zmat3 (Fig18A). Only one cell cycle associated target, Rb, was significantly up regulated. Interestingly, in addition to its role in cell cycle regulation, Rb has also recently been implicated in apoptosis through the up regulation of pro-apoptotic targets including Trp73 (Ianari et al., 2009). We did not observe a significant increase in any genes after Cdk1 inhibitor treatment of differentiated ES cells. Of the apoptotic-associated targets, up regulation of Noxa was of particular interest. Noxa expression levels have recently been shown to correlate with Oct4 expression (Gutekunst et al., 2013), suggesting that Noxa may play an important role in the regulation of apoptosis in ES cells. To verify these results we examined relative mRNA levels of Puma and Noxa using taqman-based real time PCR, before and after Cdk1 inhibition in mES, differentiated mES and p53-deficient ES cells (Fig 18B). Similar to our array results we observed no change in Puma levels whereas Noxa was significantly up regulated after Cdk1 inhibitor treatment in ES cells. This up regulation was not observed in either the differentiated ES cells or the p53-deficient ES cells indicating that up regulation of Noxa after Cdk1 inhibition is p53-dependent. These data

provide further support that p53 is activated in ES cells after purvalanol A treatment, and suggest a possible mechanism responsible for the activation of the intrinsic apoptotic pathway.

2.3 Discussion

In this study, we compare the effect of acutely inhibiting Cdk1 in ES versus differentiated cells. We report that in contrast to differentiated cells, which exhibit little if any cell death after Cdk1 inhibition, Cdk1 inhibition in ES cells induces a DNA damage response, p53 activation and subsequent cell death. These results suggest a unique requirement for Cdk1 in ES cells and highlight differences in cell cycle regulation and the DNA damage response in pluripotent versus differentiated cells.

The discovery that Cdk1 inhibition induces the DNA damage response in ES cells is, perhaps, not surprising. While Cdk1 inhibition alone has not previously been linked to the induction of a DNA damage response, Cdk1 has been implicated in DNA damage repair pathways, particularly in the repair of double stranded breaks (DSB) by homologous recombination. During mitosis, Cdk1-cyclin B phosphorylation of CtIP is necessary for DNA end resection following double stranded break formation, and Cdk1 is thought to regulate timing of the repair of DSBs (Peterson et al., 2011). Additionally phosphorylation of BRCA1 by Cdk1 is necessary for the efficient formation of BRCA1 foci at sites of DSBs (Johnson et al., 2011), which is critical for homology-directed DSB repair (Moynahan et al., 1999). Interestingly, ES cells have been shown to be dependent on homologous recombination for DSB repair, as they do not utilize non-homologous end joining, an alternative mechanism for DSB repair (Tichy et al., 2010). It is possible that

ES cells are especially sensitive to Cdk1 inhibition due to impaired homologous recombination. Unrepaired damage could trigger the DNA damage response signaling pathway and subsequent activation of downstream apoptotic pathways.

In general, ES cells are thought to be more sensitive to genotoxic stress than somatic cells, lacking a G1 checkpoint and removing damaged cells by apoptosis rather than arresting for DNA repair (Hong and Stambrook, 2004). This observation makes sense from an evolutionary point of view. ES cells differentiate to form all 3 germ layers, generating every cell type in an adult organism. Therefore a mutation in an ES cell could potentially compromise multiple cell lineages and affect subsequent generations. ES cells likely have evolved mechanisms that protect the integrity of their genome. One such mechanism could be to eliminate damaged cells from the population through apoptosis, rather than risk error prone DNA repair. Thus ES cells may be especially susceptible to DNA Damage or impaired DNA damage repair pathways triggered by Cdk1 inhibition.

We found that p53 expression is necessary for Cdk1 inhibitor-induced apoptosis in ES cells. Reports on the role of p53 in ES cells in response to DNA damage have been contradictory, and it is likely dependent on the source of DNA damage. Studies report that p53 is not efficiently relocated to the nucleus after γ -irradiation or treatment with the antimetabolite n-phospho-n-acetyl-L-aspartate (PALA) (Aladjem et al., 1998; Chuykin et al., 2008). In contrast, accumulation of p53 in the nucleus and activation of downstream targets has been reported after doxorubicin treatment (Solozobova et al., 2009), and it is thought that p53 is responsible for the rapid apoptotic response in ES cells after UV exposure (Corbet et al., 1999). Here we report that after treatment of ES cells with either doxorubicin or Cdk1 inhibitor, purvalanol A, p53 is phosphorylated at serine 18 and its

nuclear expression levels are increased. Furthermore we observed p53-dependent induction of a subset of p53 transcriptional target genes, including pro-apoptotic genes Noxa, Zmat3, Tp73 and Rb. These results provide an example of p53 mediation of the DNA damage response in ES cells.

The increase in expression of pro-apoptotic p53 target genes Noxa, Zmat3, Tp73 and Rb provides additional insight on the mechanism by which Cdk1 inhibitors induce apoptosis in pluripotent cells. Specific function of Zmat3, Tp73 or Rb has not been described in ES cells. Future experiments will be needed to determine whether these genes have a unique role in regulating apoptosis in ES cells. Noxa functions as a pro-apoptotic protein primarily through the binding and inactivation of anti-apoptotic protein Mcl-1. It has previously been reported that both Mcl-1 and Noxa expression levels correlate with Oct4 expression (Gutekunst et al., 2013). Furthermore, the sensitivity of germ cell tumors to cisplatin has been attributed to Noxa. Together with our results, this suggests that Noxa and Mcl-1 may play an important role in the balance of anti- and pro-apoptotic factors in embryonic stem cells. Overall, our results highlight several differences between ES and differentiated cells in regards to cell cycle regulation, DNA damage repair and apoptosis.

2.4 Figures

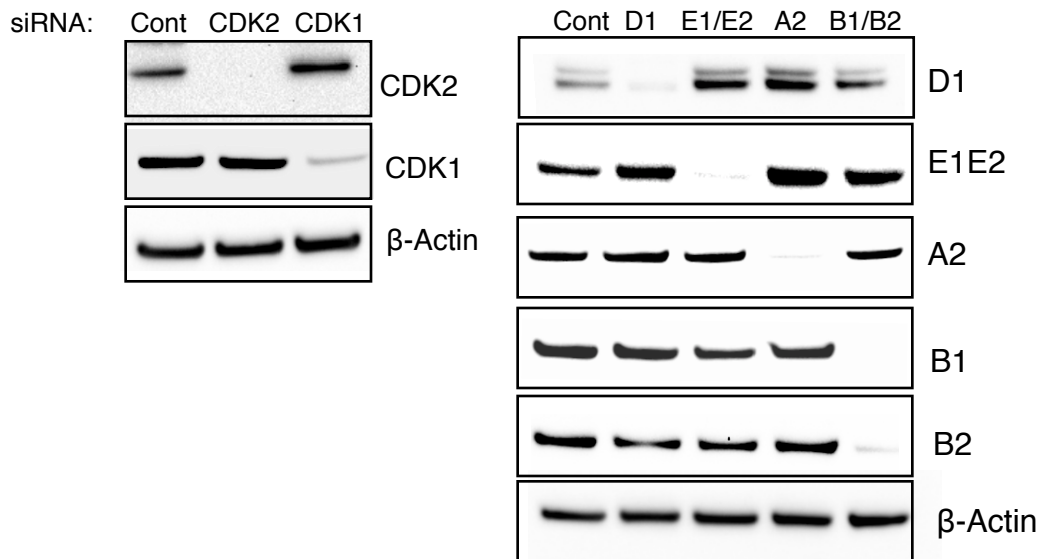


Figure 3. siRNA knockdown of individual Cdks and cyclins in ES cells. Protein levels of indicated Cdks and cyclins after siRNA knockdown in ES cells, determined by western blotting. Cont refers to non-targeting control siRNA.

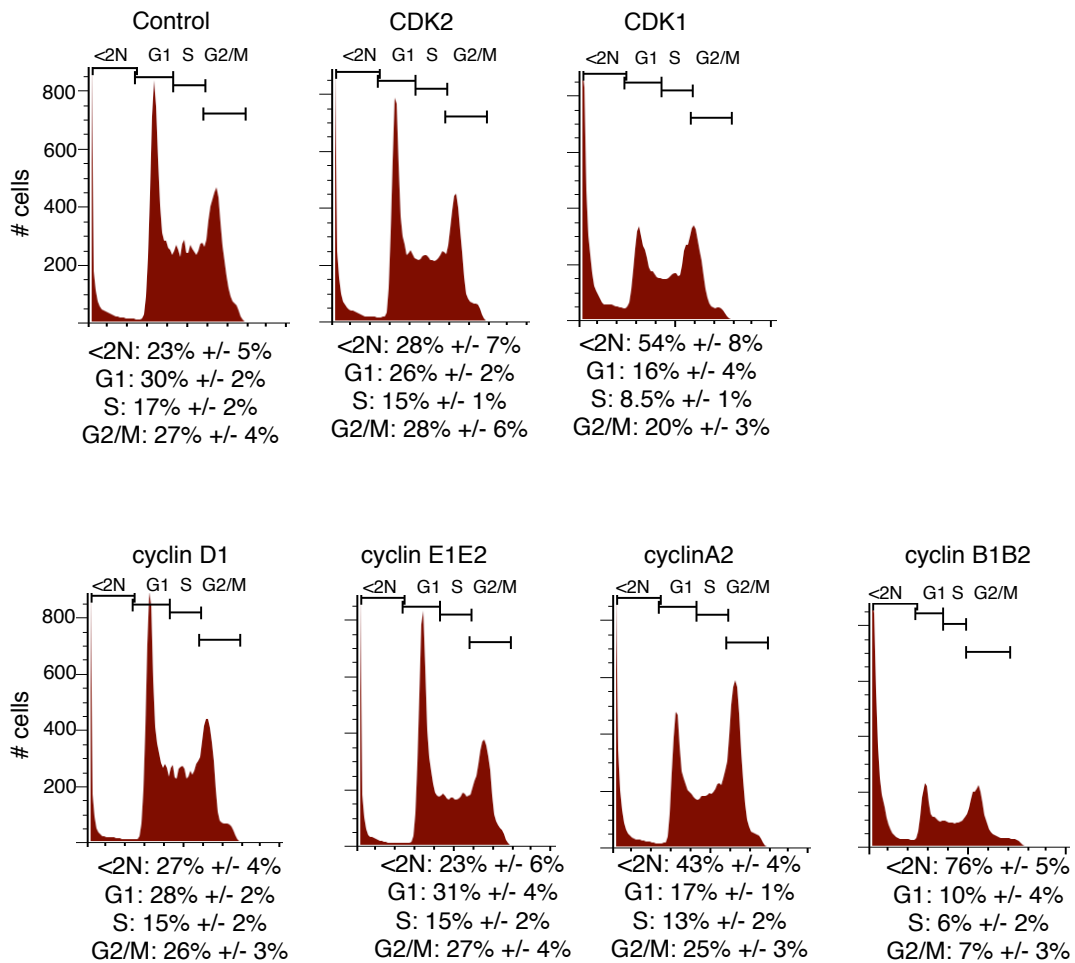


Figure 4. Effect of siRNA knockdown of individual Cdks and cyclins on ES cell cycle. Propidium iodide staining to analyze DNA content after siRNA knockdown of indicated Cdks and cyclins. Control used was a non-targeting siRNA. Gates represent sub 2n (dead), G1, S and G2 populations. Shown are the mean percentages of cells in each gate \pm SEM from three separate experiments.

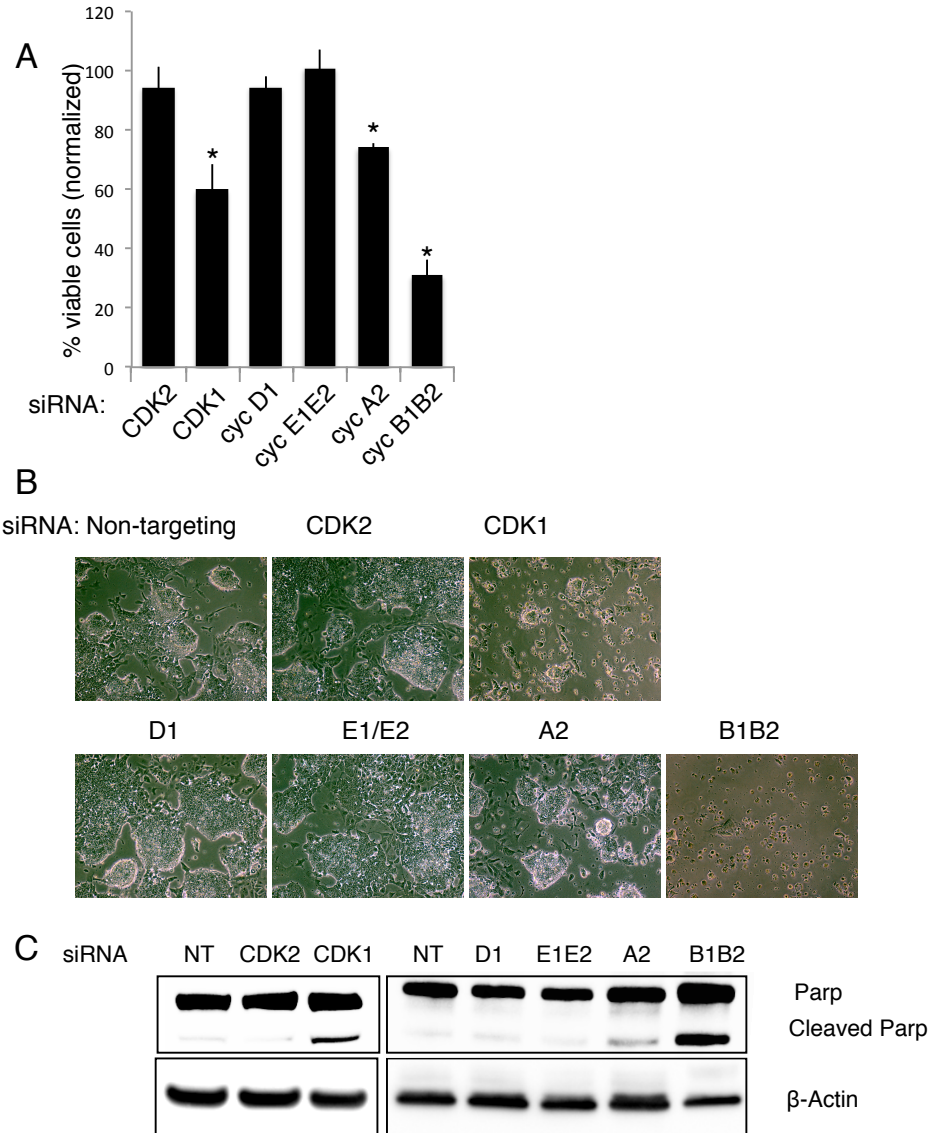


Figure 5. siRNA knockdown of Cdk1 and cyclins B1/B2 or A induces apoptosis in mES cells. (A) Percentage of viable cells after Cdk and cyclin knockdown normalized to non-targeting control siRNA. Cell death was determined by the Sub 2N cell population identified by PI staining and FACS analysis (Fig 3). Shown are mean values \pm SEM from three independent experiments. Asterisk denotes $p < .03$, determined by the t test. (B) Cell morphology after Cdk and cyclin knockdown. (C) Detection of PARP cleavage, a marker of apoptosis, by western blotting.

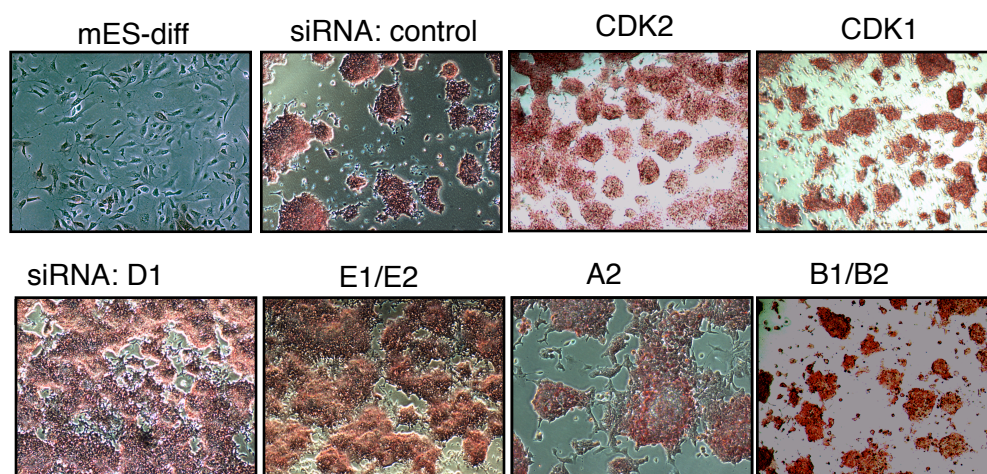


Figure 6. siRNA knockdown of individual Cdks and cyclins does not induce differentiation of mES cells. Alkaline phosphatase staining, as a marker of pluripotency after siRNA knockdown of Cdks and cyclins. Retinoic Acid-differentiated mES cells (mES-diff) used as a negative control.

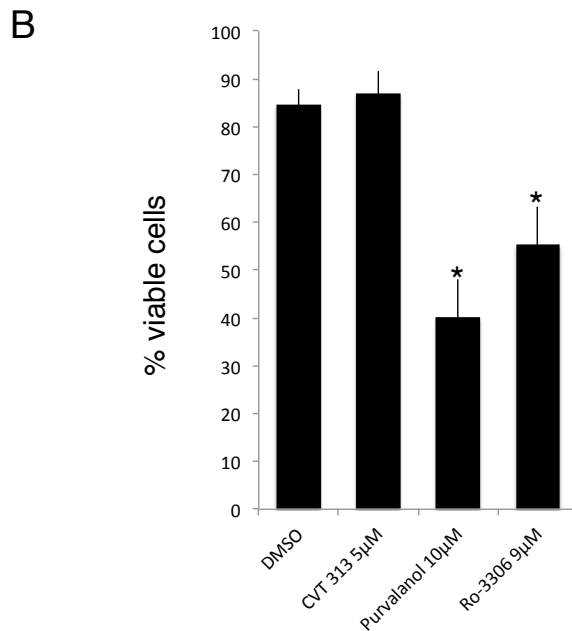
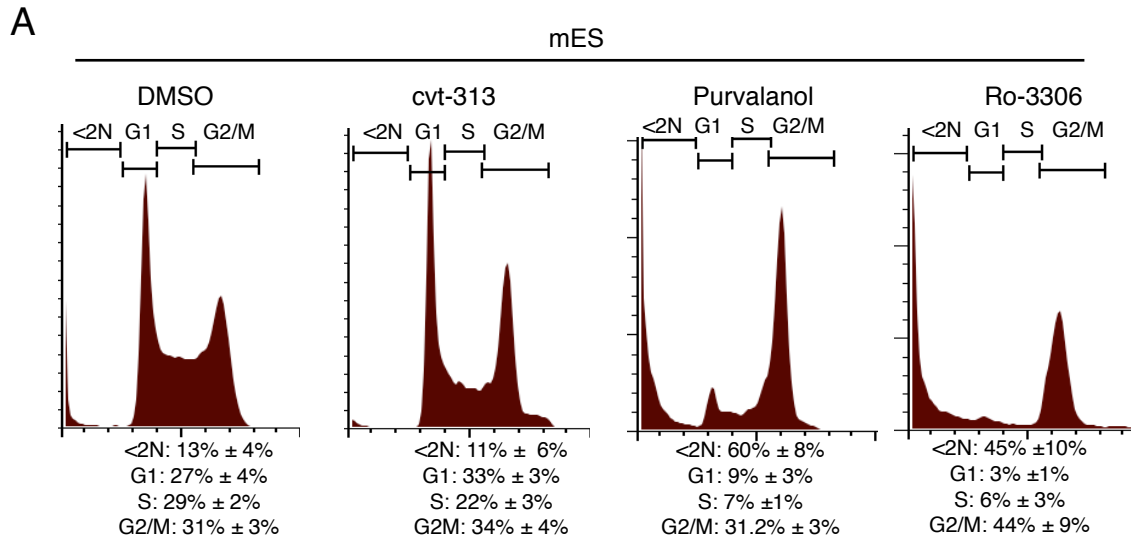


Figure 7. Small molecule inhibition of Cdk1 induces apoptosis in ES cells. (A) Effect of Cdk2 inhibitor, CVT 313, and Cdk1 inhibitors, purvalanol A and Ro-3306 on ES cell DNA content. (B) Cell viability of mES after treatment with CVT-313, purvalanol A and Ro-3306. Cell death was determined by Sub2N DNA content from (A). Shown are mean values ± SEM from at least 3 independent experiments. Asterisk denotes $p < .007$, as determined by the t test.

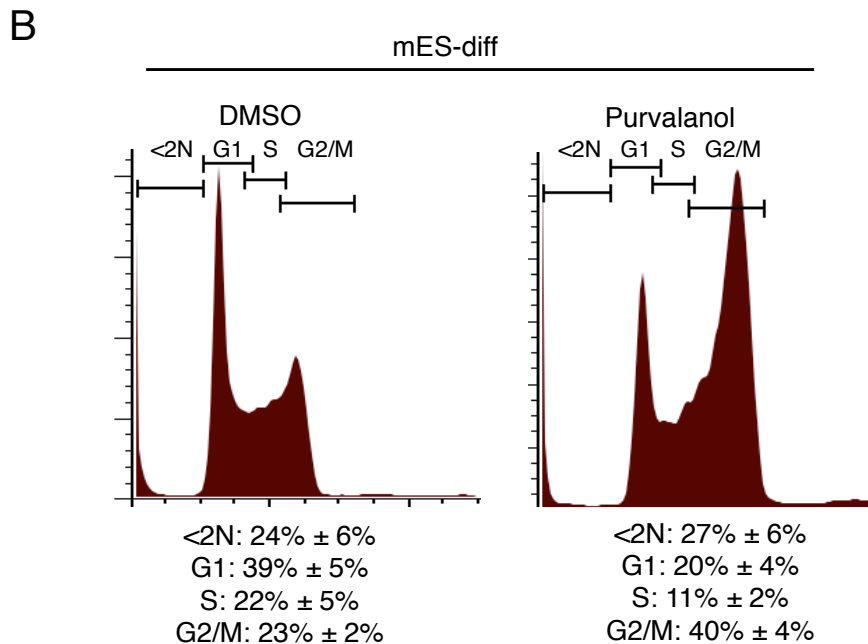
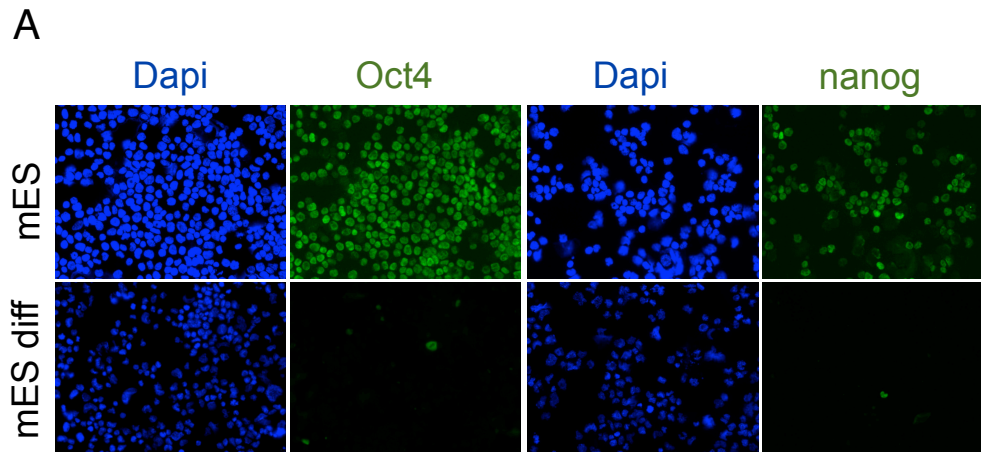


Figure 8. Purvalanol treatment of differentiated mES cells induces a G2/M cell cycle arrest. (A) Oct4 and Nanog expression in mES and differentiated mES (mef-diff) cells. mES cells were differentiated by 4d treatment with 1 μ M retinoic acid. DAPI staining identifies nuclei. (B). Cell cycle analysis bases on DNA content in differentiated mES cells after 24h 10 μ M A treatment. Shown are mean values of cell cycle distribution from three independent experiments.

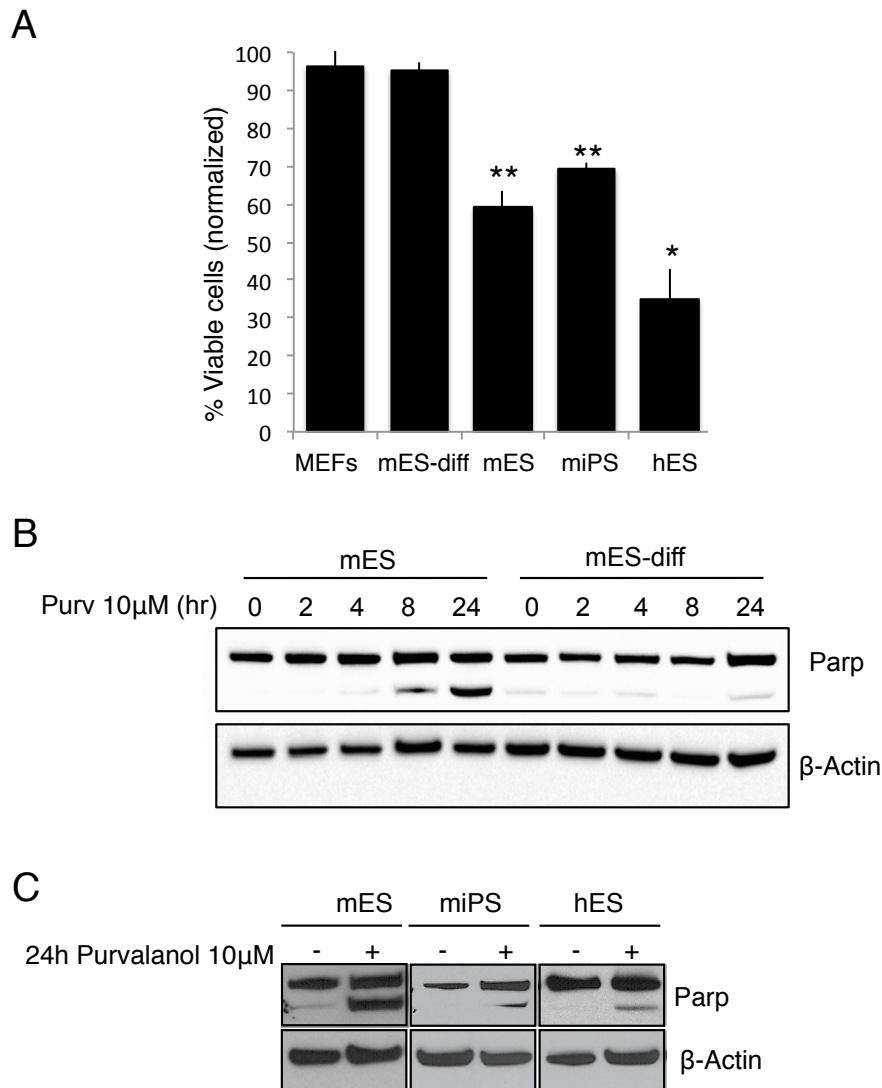


Figure 9. Cdk1 inhibition induces apoptosis in pluripotent but not differentiated cells. (A) Cell viability of indicated cell types after 24h treatment with purvalanol A (10 μ M), as determined by staining with a viability exclusion dye and FACS analysis. Shown are mean values \pm SEM from at least three independent experiments. Double asterisk denotes $p < .005$ and single asterisk denotes $p < .01$ as determined by the t test, compared to DMSO treated control. (B) and (C) Western blots of PARP cleavage after purvalanol A treatment.

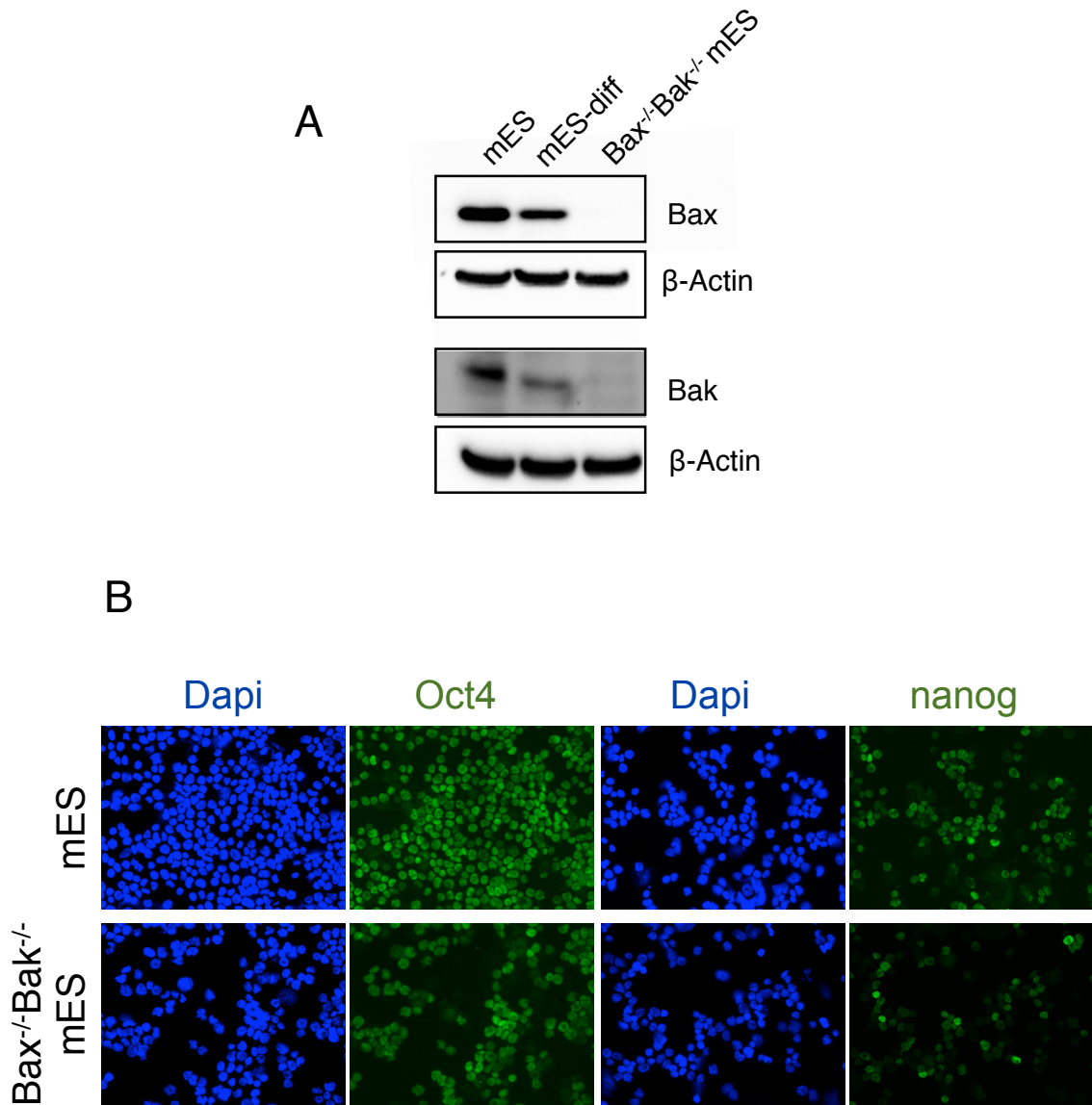
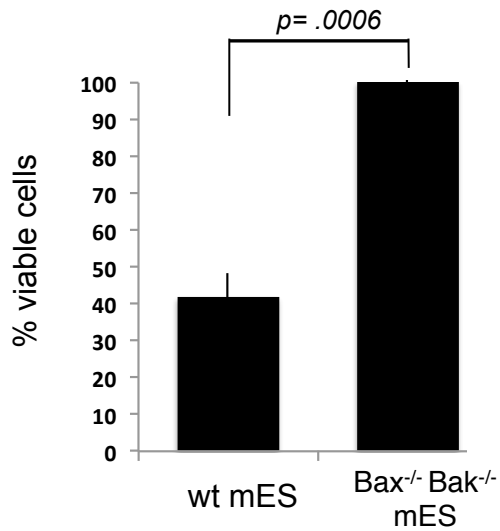


Figure 10. Bax^{-/-}Bak^{-/-} mES lack Bax and Bak but retain expression of pluripotency markers Oct4 and Nanog. (A) Western blot showing Bax and Bak protein levels in mES and Bax^{-/-}Bak^{-/-} mES cells. (B) Immunofluorescence showing Oct4 and Nanog levels in mES and Bax^{-/-}Bak^{-/-} mES cells. DAPI staining (blue) identifies nuclei.

A



B

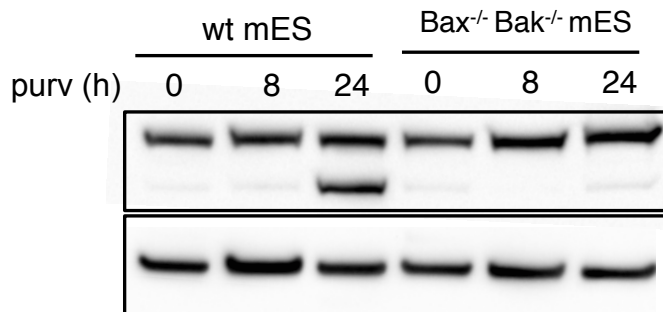


Figure 11. Bax^{-/-}Bak^{-/-} mES are resistant to Cdk1 inhibitor-induced cell death. (A) Cell viability of wild type (wt) and Bax^{-/-}Bak^{-/-} mES cells after 24h treatment with purvalanol A(10µM), as determined by staining with a viability exclusion dye and FACS analysis. Shown are mean values +/- SEM from at five independent experiments. (B) Western blot showing PARP cleavage after purvalanol A treatment (10µM), for the indicated amount of time in hours (h).

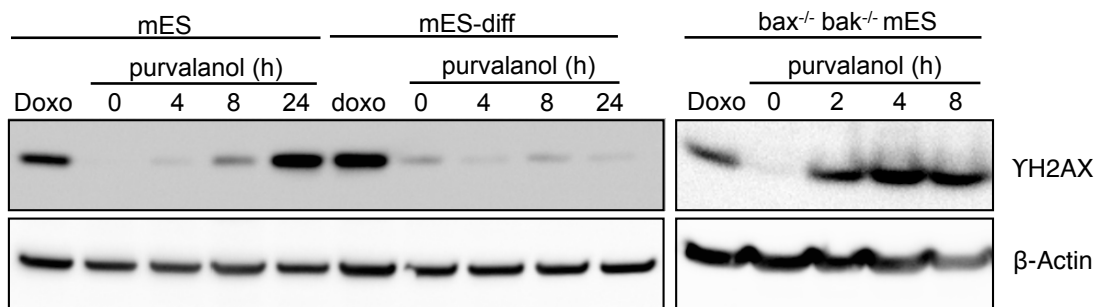


Figure 12. Cdk1 inhibition increases phosphorylation of H2AX in wt and $Bax^{-/-}Bak^{-/-}$ mES, but not differentiated cells. Western blot of the phosphorylated (Ser-139) form of H2AX (γ -H2AX) after purvalanol A treatment ($10\mu\text{M}$) for indicated time in hours(h)

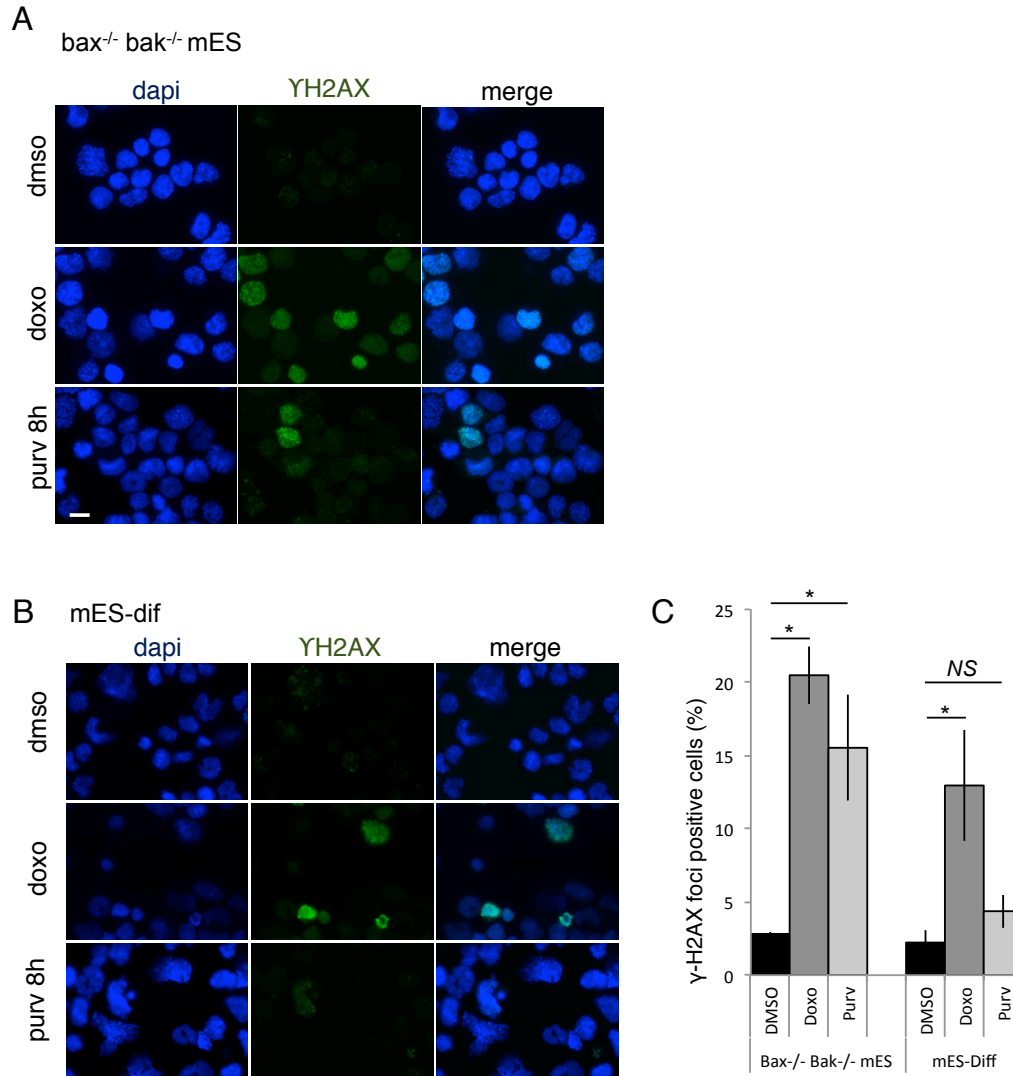


Figure 13. Cdk1 inhibition results in an increase in γ -H2AX foci in $Bax^{-/-} Bak^{-/-}$ mES but not differentiated mES cells. (A) and (B) Detection of γ -H2AX foci by immunofluorescence after purvalanol A treatment (10 μ M) in $Bax^{-/-} Bak^{-/-}$ mES cells and differentiated mES (mES-dif). Cells were treated with DMSO as a negative control, 1 μ M doxorubicin (doxo) as a positive control or Cdk1 inhibitor, 10 μ M purvalanol A (purv) for 8 hours. Scale bars = 20 μ m. (C) Mean \pm SEM number of cells expressing γ -H2AX foci over at least three experiments. Asterisk denotes significance of differences ($p < .05$) as determined by the t test. NS=not significant

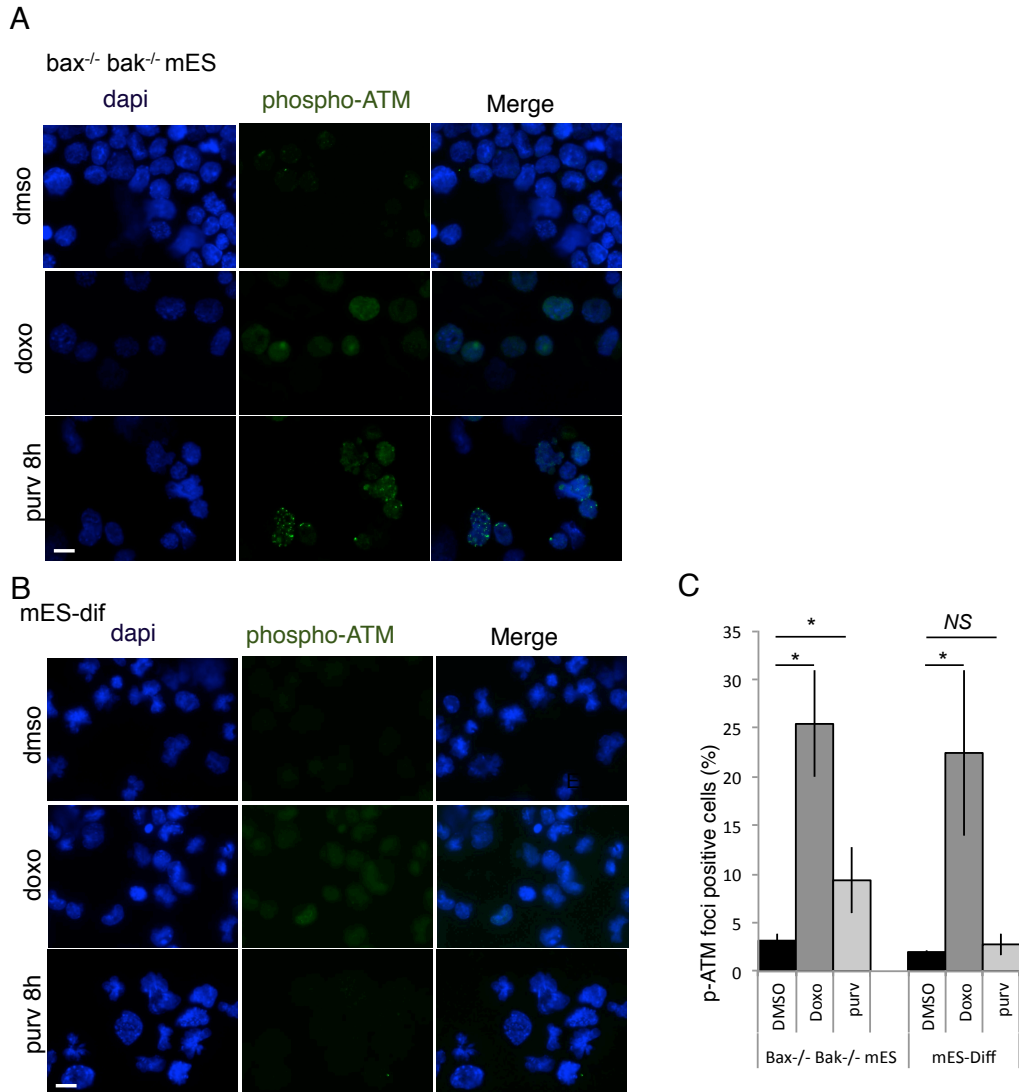


Figure 14. Cdk1 inhibition results in an increase in pATM foci in Bax^{-/-}Bak^{-/-} mES but not differentiated mES cells. (A) and (B) Detection of pATM foci by immunofluorescence after purvalanol A treatment (10 μ M) in Bax^{-/-}Bak^{-/-} mES cells and differentiated mES (mES-dif). Cells were treated with DMSO as a negative control, 1 μ M doxorubicin (doxo) as a positive control or Cdk1 inhibitor, 10 μ M purvalanol A (purv) for 8 hours. Scale bars = 20 μ m. (C) Mean \pm SEM number of cells expressing γ -H2AX foci over at least three experiments. Asterisk denotes significance of differences ($p < .05$) as determined by the t test. NS=not significant.

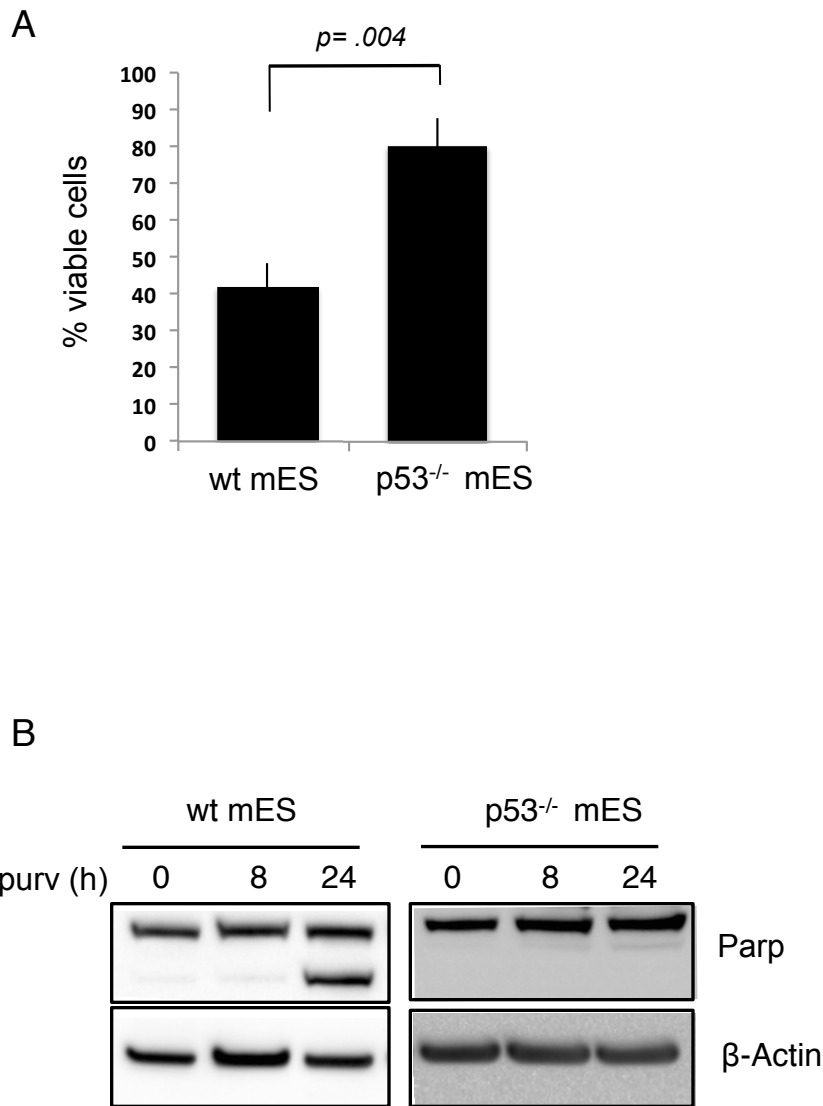
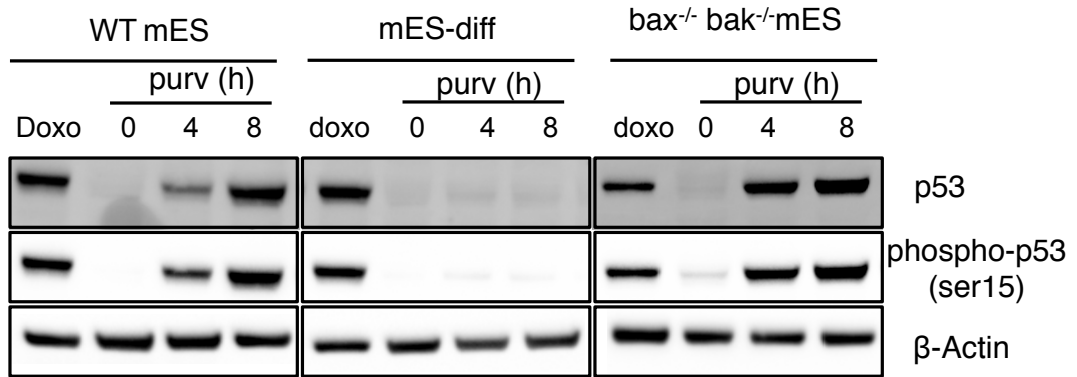


Figure 15. p53 is necessary for Cdk1 inhibitor induced cell death in ES cells (A) Cell viability of wildtype (wt) and p53 deficient ES cells after 24h of treatment with 10µM purvalanol A (purv). Shown are mean values ± SEM from five individual experiments. (B) Western blot showing PARP cleavage in wild type mES and p53^{-/-} mES cells, after purvalanol A treatment for the indicated amount of hours (h).

A



B

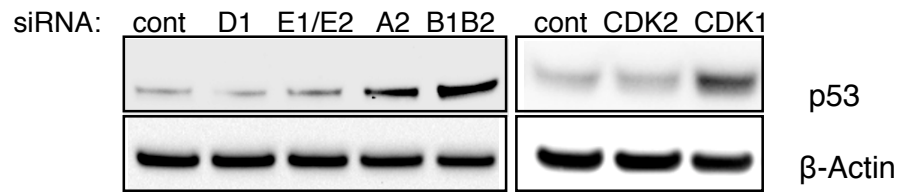


Figure 16. Cdk1 inactivation induces p53 protein expression in mES but not differentiated cells. (A) Western blot showing p53, and phosphorylated p53 at serine 15 protein expression levels. Cells were treated with purvalanol A for the indicated amount of time in hours (h). Cells were treated with 1 uM Doxorubicin (Doxo) for four hours as a positive control. (B) Western blot showing p53 expression levels in mES cells after siRNA knockdown of Cdk1 and cyclins.

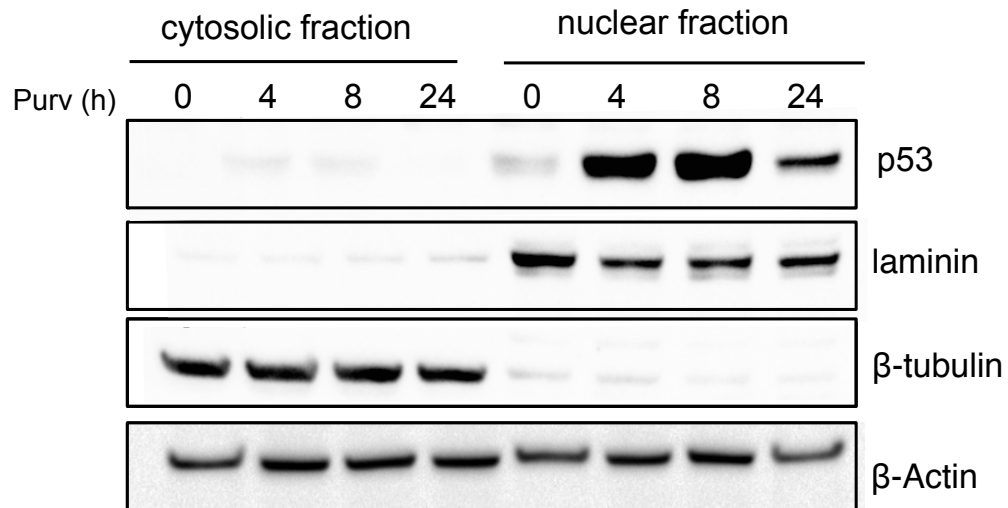


Figure 17. Cdk1 inhibition induces p53 protein expression in the nuclear fraction of mES cells. Western blot showing p53 protein levels in nuclear and cytosolic fractions after 10uM purvalanol A treatment for the indicated time in hours.

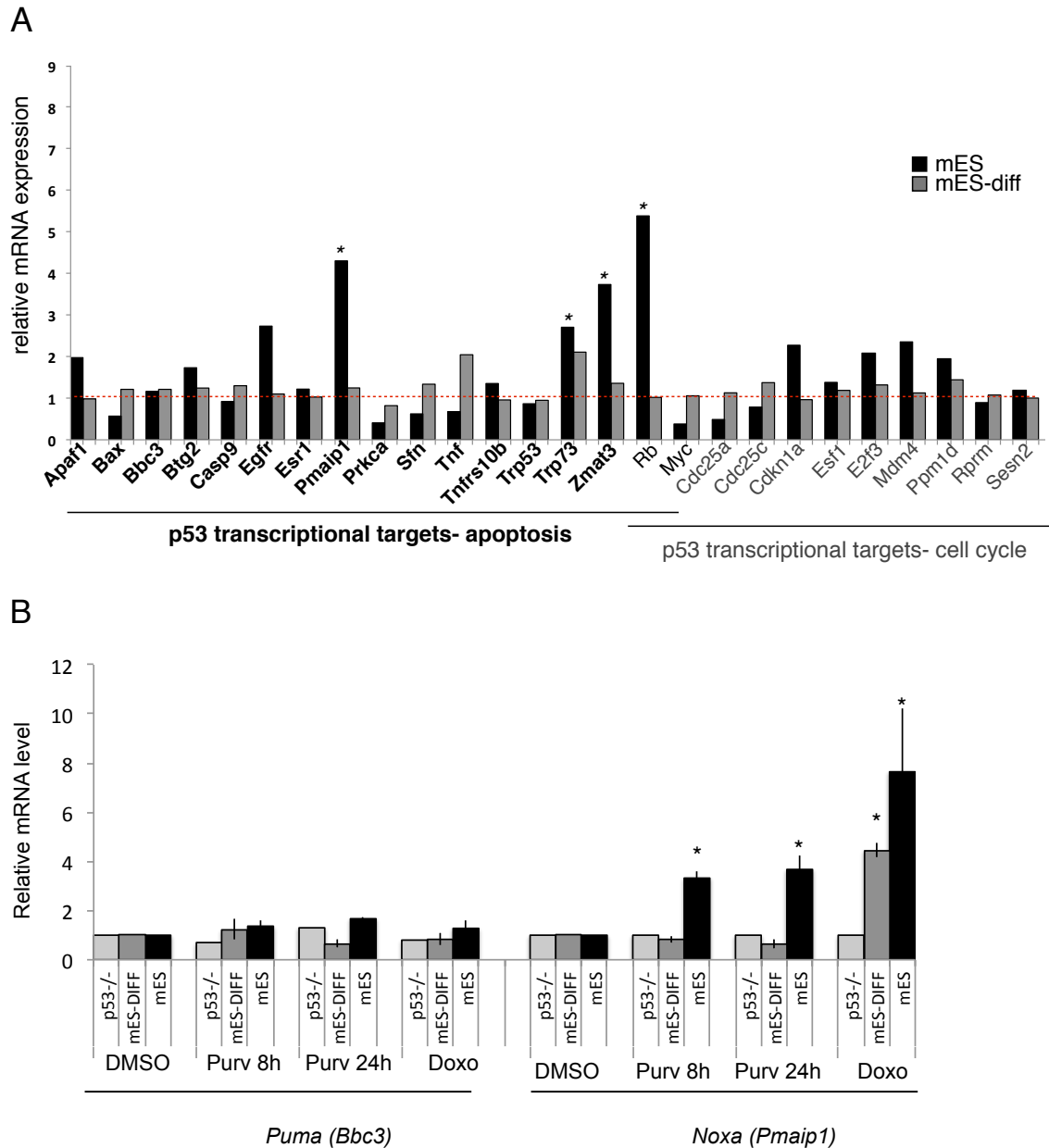


Figure 18. Cdk1 inhibition results in activation of a subset of pro-apoptotic p53 transcriptional targets in mES cells. (A) Realtime PCR screen of known p53 transcriptional targets. Graph indicates relative mRNA expression of purvalanol treated cells normalized to DMSO treated cells (B) Validation of results in (A) using mES, mES-diff, and p53-deficient cells. For both (A) and (B), Asterisk denotes significance of differences ($p < .05$) between treated and untreated samples, determined by the t test.

Chapter 3

Cdk1 Inhibition Prevents Stem Cell Driven Tumors

3.1 Introduction

A potential clinical utilization of pluripotent stem cells is their *in vitro* differentiation towards a specific cell type of interest, prior to transplantation into patients. A possible risk of this approach would be the presence of improperly differentiated pluripotent cells at the time of transplantation, which could subsequently develop into teratomas (Ben-David and Benvenisty, 2011; Knoepfler, 2009). Our data suggest that pluripotent cells are uniquely sensitive to Cdk1 inhibition. We therefore reasoned that Cdk1 inhibitors such as purvalanol A could be used to safely deplete residual undifferentiated cells during stem cell therapies, thus reducing the risk of stem cell derived teratomas.

Several strategies have been proposed to deplete residual undifferentiated cells from differentiated cultures, including cell sorting based on stem cell specific surface antigens (Fong et al., 2009), introduction of a stem cell specific suicide gene (Schuldiner et al., 2003), and the use of stem cell specific cytotoxic antibodies (Choo et al., 2008; Tan et al., 2009b). Introduction of a stem cell specific suicide gene requires the genetic manipulation of the stem cells, which is not optimal for stem cell therapies. Furthermore, cell-sorting techniques require single cell dissociation of ES cells, which could have

negative effects on the health of ES cells or on the differentiation process. Additionally, cell sorting techniques and other antibody-based methods do not completely eliminate undifferentiated cells from a mixed population. Therefore alternative methods are required for the elimination of undifferentiated ES cells from culture.

One potential approach is the development of small molecules that will potently and selectively kill undifferentiated cells. In a recent study published by Ben David *et al*, the authors performed a high-throughput screen to identify potential small molecules for this purpose (Ben-David et al., 2013). Through this screen the authors determined that small molecules targeting the biosynthesis of fatty acids selectively induced ER stress, the unfolded protein response, and apoptosis in hES but not differentiated cells. Based on our results from chapter two, we investigated an alternative but similar approach, using small molecules that selectively target Cdk1. Relatively specific Cdk1 inhibitors with good pharmacokinetics have been developed and are currently being evaluated in clinical trials as treatment for a variety of cancers (Dickson and Schwartz, 2009). Here we report on the use of these small molecule inhibitors for the prevention and treatment of stem cell driven tumors.

3.2 Results

Cdk1 inhibition *in vitro* depletes the tumor-propagating population from a heterogeneous cell population.

Our data, presented in chapter two, suggest that pluripotent cells are uniquely sensitive to Cdk1 inhibition. We therefore reasoned that Cdk1 inhibitors such as purvalanol A could be used to safely deplete residual undifferentiated cells during stem

cell therapies, thus reducing the risk of stem cell derived teratomas. In order to test this hypothesis we partially differentiated mES cells expressing GFP driven by an Oct4 reporter by treating with retinoic acid for two days, resulting in an approximate 80% reduction of Oct4 expression (Fig 18a). Immunofluorescence using antibodies against Oct4 and Nanog, as well as alkaline phosphatase staining, revealed a heterogeneous population of cells, with cells in various states of differentiation (Fig. 18b). Treatment of partially differentiated mES cells with purvalanol A for 24 hours further reduced the level of Oct4 expression, as determined by FACS analysis of GFP expression, suggesting that Cdk1 inhibition selectively depletes pluripotent cell populations (Fig. 19a). We next asked if depletion of Oct4 positive cells from a heterogeneous population would reduce the risk of teratoma formation. Equal numbers of viable partially differentiated ES cells, treated with either purvalanol A or diluent (DMSO) for 24 hours, were subcutaneously transplanted into BALB/c Nu/Nu mice, and mice were monitored daily for tumor formation. Mice that received DMSO treated cells began to develop visual tumors as early as 6 days after transplantation, with 100% of the mice exhibiting tumors by 21 days post-transplantation (Fig. 19b). In contrast, mice that were transplanted with cells pre-treated with purvalanol A exhibited a decreased incidence of tumor formation with only 25% of total mice developing tumors after 60 days post-transplantation (Fig. 19b). Additionally tumor development was delayed after pre-treatment of purvalanol A, with the first visual tumor not appearing until 12 days post-transplantation (Fig. 19b). These results suggest that Cdk1 inhibition depletes the tumor-propagating cell population from a heterogeneous population of cells, and thus could increase the safety of stem cell therapies.

Cdk1 inhibitor treatment *in vivo* prevents formation of stem cell-derived tumors. As an alternative approach, we asked whether *in vivo* treatment with Cdk inhibitors immediately after subcutaneous transplantation with partially differentiated ES cells could prevent the formation of stem cell derived tumors. To address this question we used an alternative Cdk inhibitor which inhibits Cdk1, 2, 5, and 9. This Cdk inhibitor was used instead of purvalanol A due to its improved pharmacokinetic properties and its efficacy at low nanomolar concentrations, which can be achieved *in vivo*. As previously described, mice were transplanted with partially differentiated mES cells that had not been pretreated with Cdk1 inhibitor purvalanol A. 72 hours after transplantation, we began treating the mice with intra-peritoneal (IP) injections of Cdk inhibitor (50mg/kg) or vehicle control. Mice were treated twice weekly for four weeks and monitored daily for tumor formation.

Within the control treated group, mice began to develop tumors as early as 8 days after transplantation and four out of five mice exhibited tumors within 14 days post transplantation (Fig 20). In contrast, out of 8 original mice in the drug-treated group, only one mouse formed a tumor during the 4-week treatment course (Fig 20). One mouse became sick and was sacrificed during the third week of treatment. The 6 remaining mice remained tumor-free for 30 days post-transplantation (Fig 20). Therefore, in addition to depleting the tumor propagating stem cells from heterogeneous population prior to transplantation, *in vivo* treatment with Cdk1 inhibitors immediately after stem cell transplantation also prevents formation of stem cell-driven tumors.

Cdk inhibitor treatment *in vivo* slows growth of previously formed stem cell- driven tumors. Without treatment, the teratomas that formed after mice were transplanted with control treated cells grew aggressively, reaching an ethical endpoint (400 mm²) within 2-3 weeks of detection. We asked whether growth of the established stem cell-driven tumors could be inhibited by treatment with Cdk inhibitors. As described previously, mice were transplanted with partially differentiated mES cells and tumors were allowed to develop. When the tumors reached a size within a range of 64-81mm² in volume, we began treating mice with intra-peritoneal injections of Cdk inhibitor (50mg/kg) or vehicle control. Mice were treated twice weekly for four weeks, or until tumors reached an ethical endpoint in size, and tumor growth was monitored daily.

Within the control treated group, the tumors continued to grow rapidly, with all the tumors reaching an ethical end point within 2 weeks after the start of injections. In contrast, within the group that received Cdk inhibitor treatment, the average tumor growth rate was significantly slower, indicating that the Cdk inhibitors were effective at inhibiting teratoma growth (Fig. 21A and B). We next asked whether drug treatment affected the composition of the tumors. After either 26 days of treatment or the tumor reaching an ethical endpoint, tumors were collected and hematoxylin and eosin (H&E) staining was performed. In a blinded study, 10 fields from each tumor were characterized based on their percent composition of the following categories: necrosis/ apoptosis, embryonal carcinoma tissue, immature teratoma (including undifferentiated epithelium/ neural epithelium and cellular atypical glial tissue), mature teratoma (including differentiated epithelium/ neural epithelium, differentiated mesenchymal and maturing glial tissue), and other (including inflammatory cells, fibroblasts and stromal tissue).

Tumors from the control treated group exhibited characteristics from all three germ layers with a large immature teratoma component (Fig 22). Strikingly tumors from the Cdk inhibitor-treated group showed a significant increase in necrotic tissue (Fig. 22 and 23), suggesting that the stem cell derived tissues were undergoing cell death after Cdk inhibitor treatment. Furthermore there was a significant decrease in the immature teratoma component after Cdk inhibitor treatment (Fig. 22 and 23). These results suggest that Cdk1 inhibition slows tumor growth through induction of necrosis and depletion of the poorly differentiated, immature component within the teratomas.

3.3 Discussion

In this study we investigate the ability of Cdk1 inhibitors to selectively deplete undifferentiated cells. We show that Cdk1 inhibitor, purvalanol A, decreases the percentage of Oct4-positive cells within a partially differentiated, heterogeneous cell population. Furthermore, we demonstrate that the removal of the Oct4-positive cells reduces the tumorigenic potential of a heterogeneous cell culture when used in teratoma xenograft studies. Due to the tumorigenicity of pluripotent ESCs and iPSCs, future regeneration therapies will likely focus on transplantation of stem cell-derived progenitor cells, rather than undifferentiated stem cells. Preclinical studies, however, have shown that even after long-term differentiation protocols, small amounts of undifferentiated cells remaining in culture can form tumors when transplanted into animals (Bjorklund et al., 2002; Germain et al., 2012; Wernig et al., 2004). Our results suggest that Cdk1 inhibitors could be used to remove residual undifferentiated cells from cell populations during differentiation protocols, thus increasing the safety of stem cell therapies.

One potential concern of this approach is the effect that Cdk1 inhibition would have on differentiating progenitor cells. Our data shows that as ES cells differentiate they no longer undergo apoptosis upon Cdk1 inhibition. Even transient cell cycle arrest however, could potentially interfere with proper differentiation to a specific cell type. Effect of Cdk1 inhibition on progenitor cells will need to be determined on a protocol to protocol basis, as Cdk1 requirements are expected to vary in a tissue type dependent manner. Additionally, while incidence of tumor formation was greatly reduced after purvalanol A treatment, some tumors still did grow. As previously discussed, even small amounts of ES and iPS cells give rise to tumors when transplanted into animals (Ben-David and Benvenisty, 2011; Knoepfler, 2009). It is therefore likely that the tumors that formed after Cdk1 inhibitor treatment were a result of residual undifferentiated cells that escaped depletion by purvalanol A. The possibility remains, however, that more differentiated progenitor populations of cells might also retain tumorigenic potential. As we develop stem cell based therapies, it will be important to further investigate the tumor initiating potential of progenitor cell populations.

In addition to depleting a tumor propagating population of cells *in vitro*, we also demonstrate that Cdk inhibitors can inhibit growth of previously established stem cell-derived teratomas. Specifically, we found that Cdk inhibitors both significantly increased levels of necrosis and decreased the percent composition of immature undifferentiated tissue within the teratomas. Histologically, the stem cell-derived tumors that formed in these studies resemble germ cell tumors and teratocarcinomas, in that they exhibit all three germ layers as well as a large embryonal carcinoma component (Ulbricht, 2005). The Cdk inhibitor used for the *in vivo* studies is a pan-Cdk inhibitor, targeting Cdk1, 2, 5

and 9. Based on our studies that show that siRNA knockdown of Cdk1 induces apoptosis in undifferentiated cells, it is likely that this drug targets tumor growth through inactivation of Cdk1. It is possible, however, that inhibition of other individual Cdks, in particular Cdk5 and Cdk9, could also contribute to the observed phenotype (Shapiro, 2006). Additionally combined inhibition of multiple Cdks at once could be necessary for therapeutic benefit. These possibilities will need to be taken into consideration as we develop more selective kinase inhibitors for cancer therapies. Nonetheless, our data suggests that Cdk inhibitors will be useful for the treatment of both germ cell tumors and stem cell-derived tumors arising from stem cell transplant therapies.

3.4 Figures

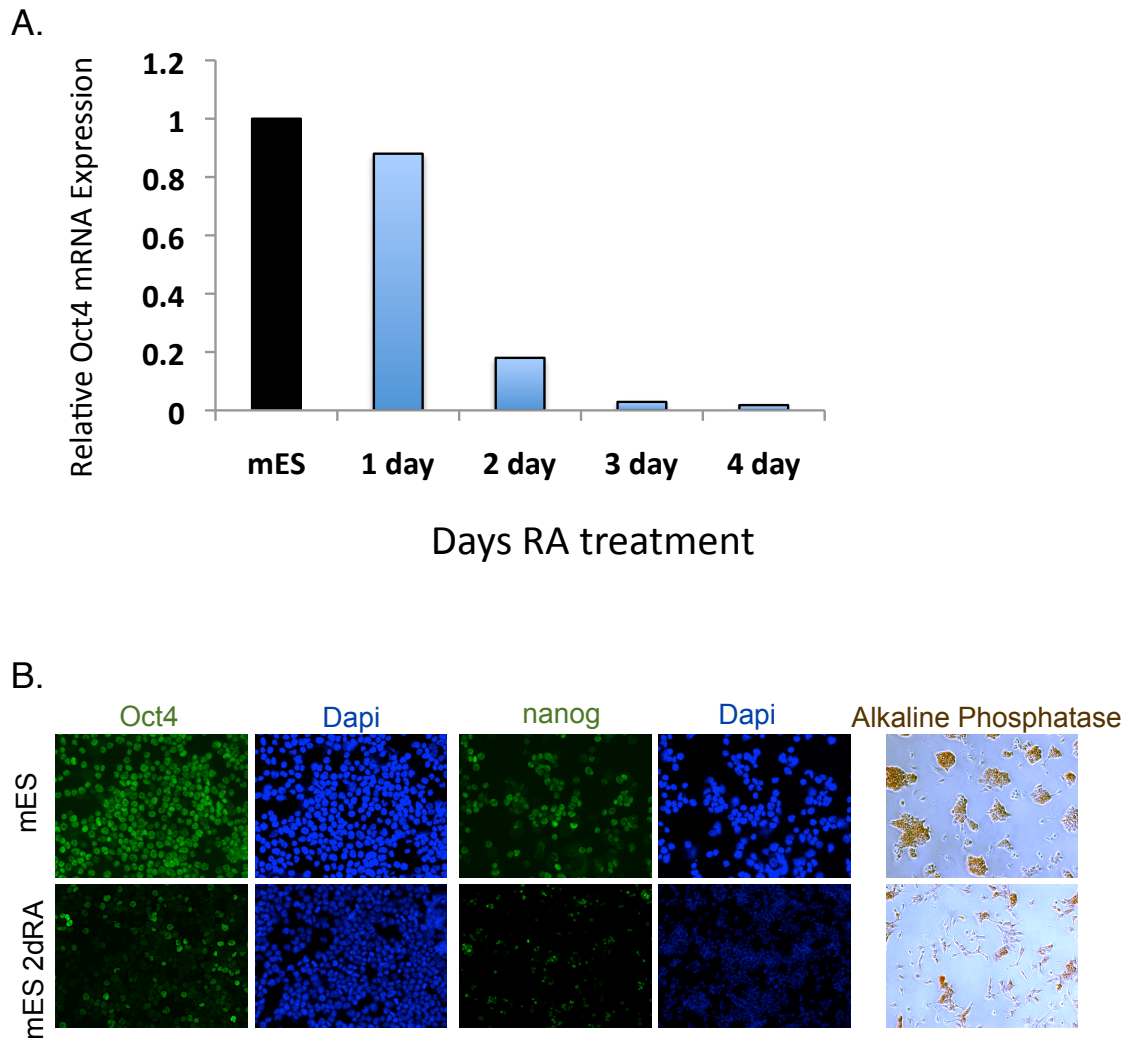


Figure 19 Characterization of pluripotency markers in ES cells after partial differentiation. (A) Relative expression of Oct4 mRNA after treatment with Retinoic Acid (1 μ M) for the indicated time in days. (B) Detection of Oct4 and Nanog by immunofluorescence. DAPI staining (blue) identifies nuclei. Alkaline phosphatase staining as a marker of pluripotency.

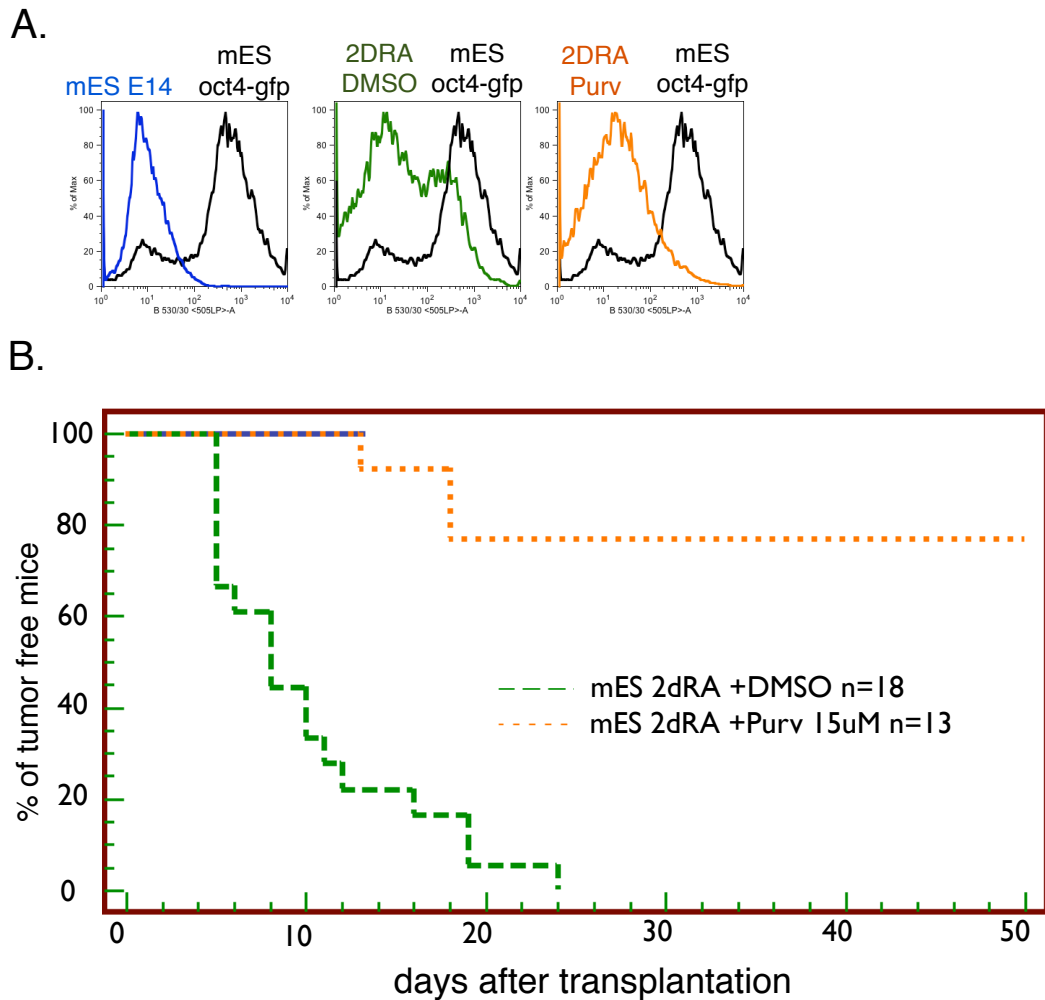


Figure 20. Cdk1 inhibitor treatment reduces the tumor propagating cells *in vitro*

(A) Percentage of GFP positive cells by GACs analysis. mES cells expressing GFP driven by the OCT4 promoter were undifferentiated, differentiated for 2 days and treated with DMSO (2dRA DMSO) or differentiated for 2 days and treated with 15 μ M purvalanol A for 24h (2dRA purv). mES cells without the Oct4 reporter were used as a negative control. **(C)** Kaplan Meier survival curve showing the percent of mice that remained tumor free. Mice were injected with partially differentiated ES cells treated with DMSO (2dRA+ DMSO) or partially differentiated ES cells treated with 15 μ M purvalanol A for 24h.

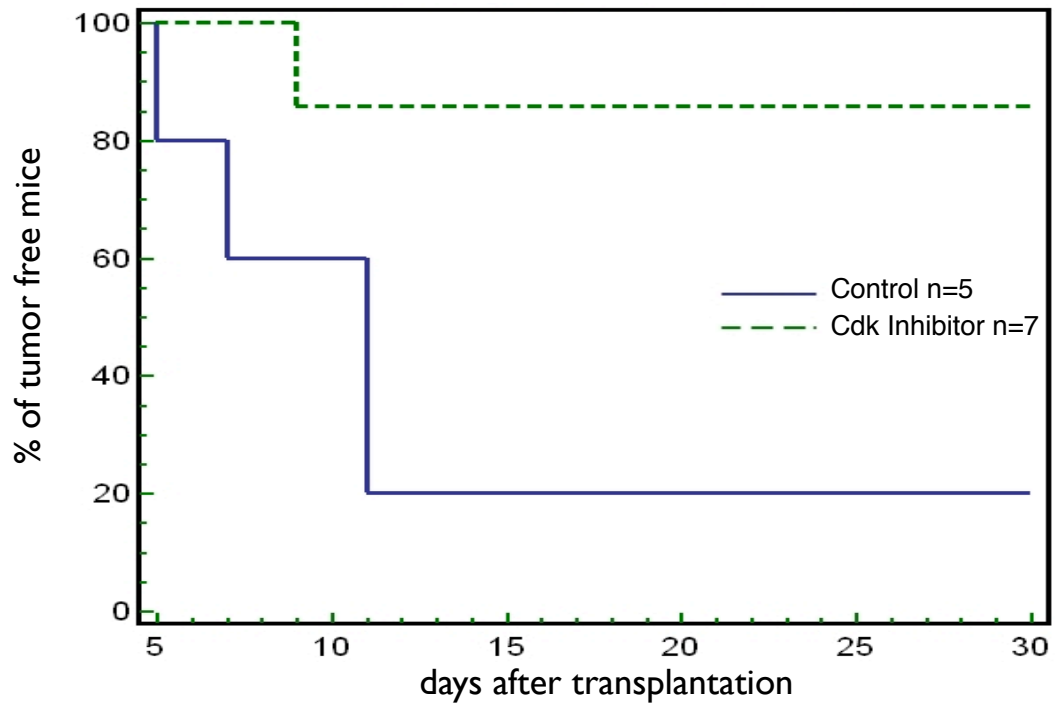


Figure 21. Cdk1 inhibitor treatment *in vivo* prevents formation of stem cell-derived tumors. Kaplan Meier survival curve showing percentage of mice that remained tumor free. Mice received subcutaneous transplantations of partially differentiated stem cells and 72 hours later began a treatment course of Cdk1 inhibitor or a vehicle control.

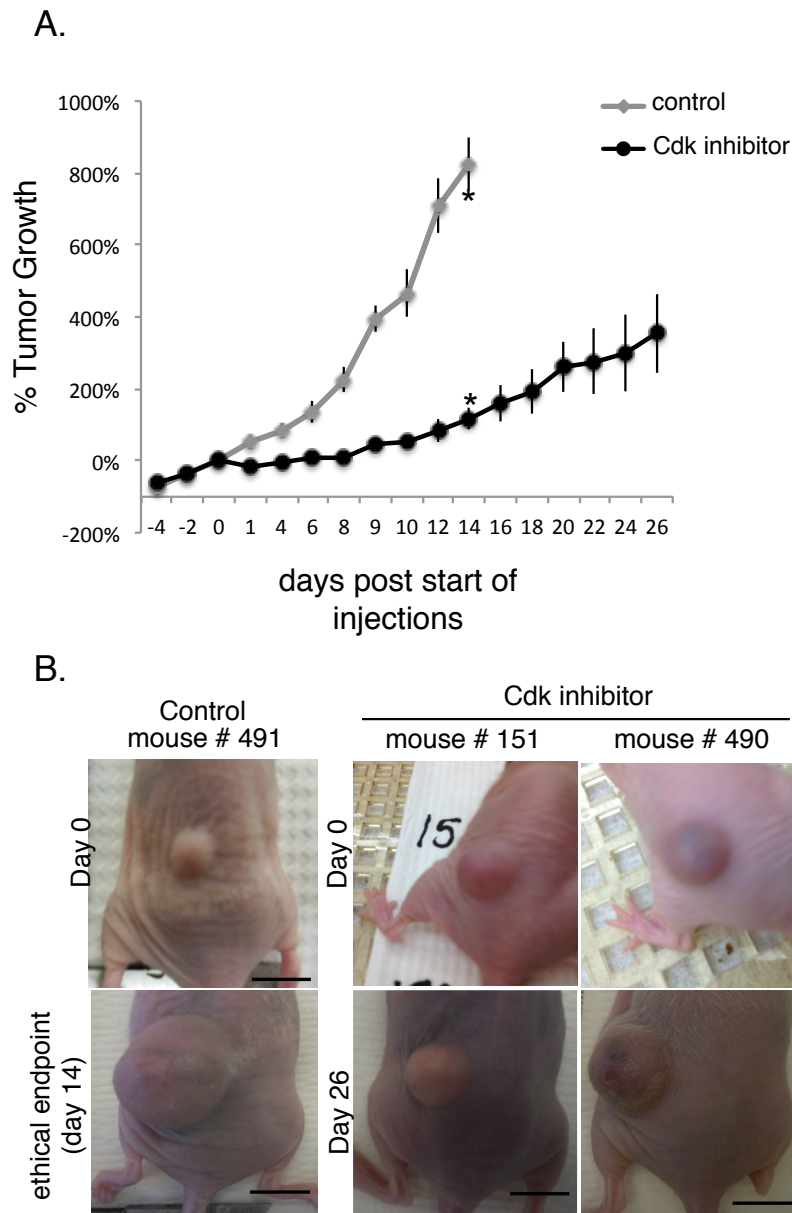


Figure 22 Cdk inhibitor treatment *in vivo* slows growth of previously formed stem cell-driven tumors (A) Percent increase of tumor growth in mice with previously growing tumors that were treated with Cdk1 inhibitor (n=7) versus diluent (control) (n=8). Shown are mean values \pm SEM. Asterisk denotes significance of difference at day 14 ($p=.0001$) as determined by the t test. (B) Representative pictures of tumors after treatment with Cdk inhibitor or diluent (control).

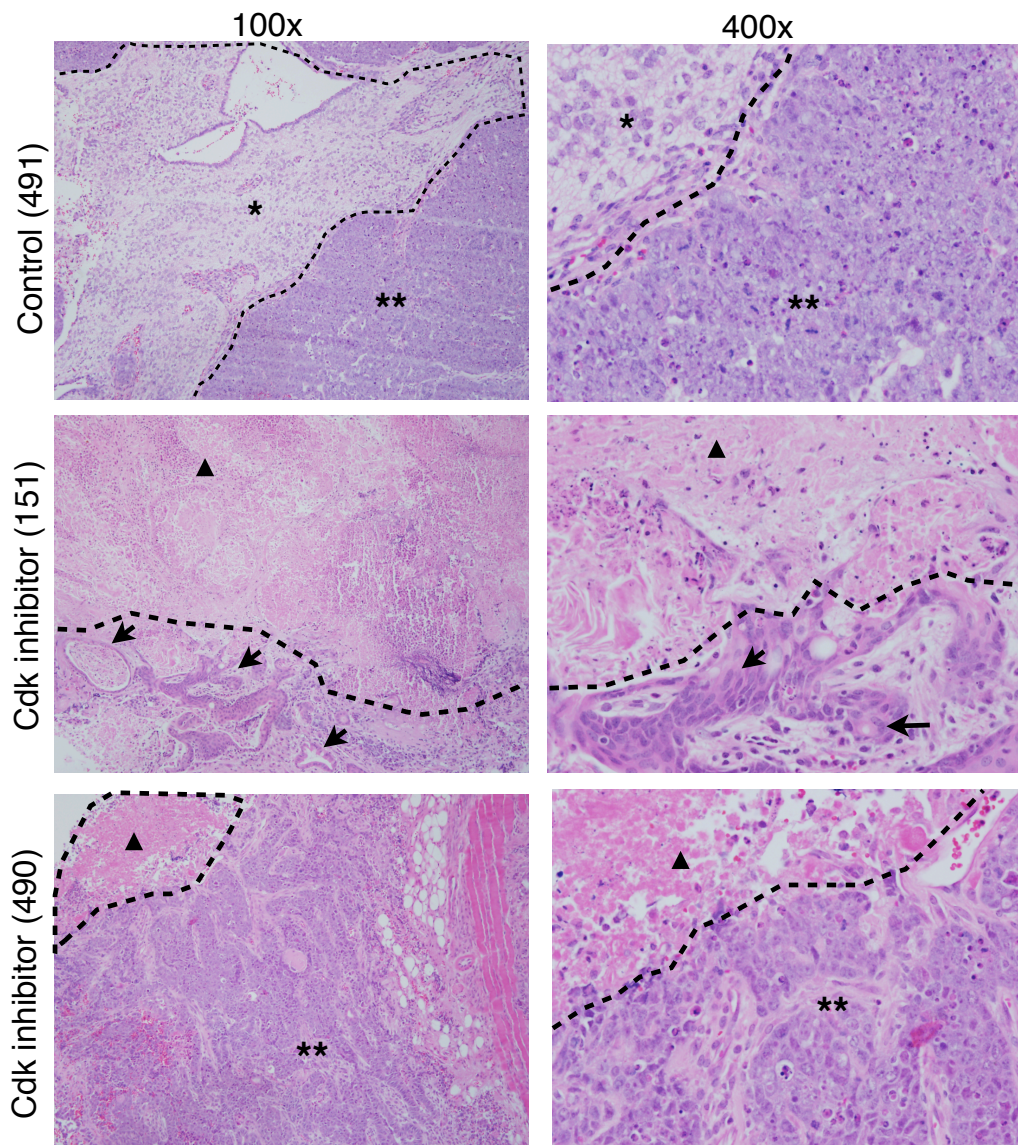


Figure 23. Histology of tumors from control and Cdk inhibitor treated animals.

Representative hematoxylin and eosin staining of tumor sections collected from mice were treated with Cdk inhibitor or diluent (control). Section marked with a single asterisk represents undifferentiated neuroepithelium, classified as immature teratoma. Section marked with a double asterisk represents embryonic carcinoma. Arrows designate sections of mature teratoma. Sections marked with triangles represent areas of necrosis.

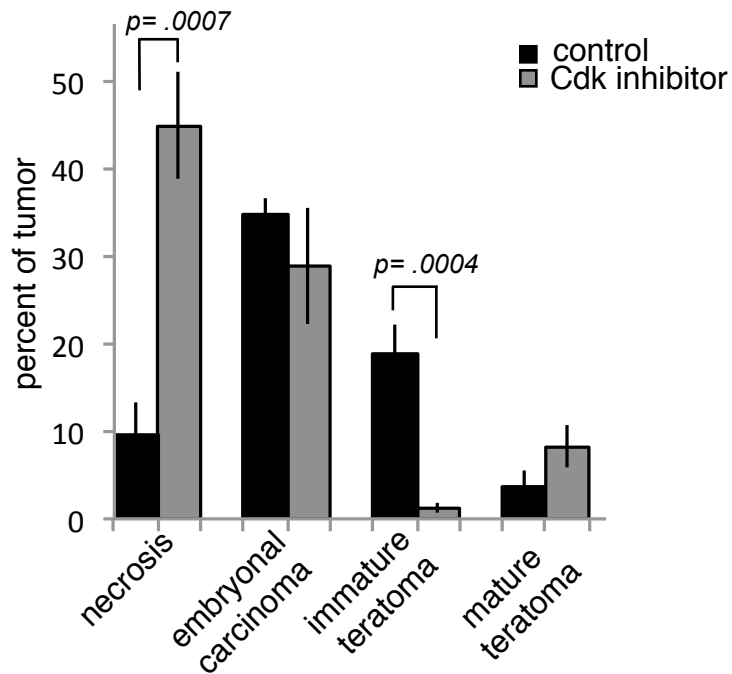


Figure 24. Cdk1 inhibition reduces an immature teratoma component of stem cell-derived tumors. Quantification of percent composition of indicated tissue type control and treated groups. Shown are mean percentages \pm SEM from 8 control tumors and 7 treated tumors. 10 different fields from each tumor were scored in a blinded study.

Chapter 4

Chemical Genetic Analysis Reveals Cdk2 is Indispensable for Cellular Proliferation in Normal and Transformed Cells

4.1 Introduction

During eukaryotic cell division cyclin dependent kinase (Cdk) activity initiates both the DNA synthesis (S) phase and mitosis. Based on the timing of its activation and its cyclin-binding preferences, Cdk2 is suspected to be responsible for facilitating the G1-S transition and initiating DNA synthesis as well as controlling the exit from S phase (Morgan, 2007). This has been supported by studies showing that a dominant negative form of Cdk2 attenuates growth of cells in culture (van den Heuvel and Harlow, 1993) and microinjected antibodies against Cdk2 block S-phase initiation in mammalian cells (Pagano et al., 1993). Due to its role in cell cycle control, Cdk2 has been extensively studied in the context of cancer. Indeed, Cdk2 expression and activity have been implicated in a variety of cancers (Kim et al., 1999; Marone et al., 1998; Yamamoto et al., 1995; Yamamoto et al., 1998). Furthermore, overexpression of Cdk2 binding partners, cyclins E and A, can participate in the transformation of cells (Barrett et al., 1995; Haas et al., 1997). It has also been shown that cyclin E-deficient cells are resistant to oncogenic transformation, and that its overexpression accentuates tumor formation in mice (Bortner and Rosenberg, 1997; Ma et al., 2007). Additionally, elevated cyclin E

expression in several tumor types correlates with a worse prognosis for patients (Keyomarsi et al., 2002; Tissier et al., 2004). Thus, there has been considerable interest in the development of small molecule inhibitors of Cdk2 as a potential therapy for various cancers (Malumbres et al., 2008).

Recent studies, however, have suggested that Cdk2 may have a redundant role in regulating cell cycle progression, challenging whether Cdk2 would be an effective therapeutic target. Genetic ablation of Cdk2 has little effect on cellular proliferation during early murine development (Barriere et al., 2007; Berthet et al., 2003; Ortega et al., 2003). Furthermore, depletion of Cdk2 using siRNAs or antisense oligonucleotides has little effect on the proliferation rates of various colon cancer lines (Tetsu and McCormick, 2003). Thus, it was concluded that Cdk2 is dispensable for cellular proliferation. These approaches, however, result in ablation of Cdk2 protein expression, potentially allowing for compensation by other Cdks (Santamaria et al., 2007), and are therefore likely to have different effects than acute inhibition of Cdk2 kinase activity using small molecules. Prior attempts to inhibit Cdk2 kinase activity *in vivo* have relied on pan-Cdk small molecule inhibitors that are not entirely selective for any single Cdk (Malumbres et al., 2008). Therefore it remains unknown whether specific and acute inhibition of Cdk2 activity can attenuate cellular proliferation or cellular transformation in the context of oncogenic signaling. In this chapter we use a chemical genetic approach in which we replace endogenous WT Cdk2 (Cdk2^{WT}) in transformed mouse embryonic fibroblasts (MEFs) and human colon cancer cells with an analog-sensitive (AS) version that is mutated to allow for acute and selective inhibition using modified ATP analogs. This approach has been recently used to identify a distinct, non-redundant role of Cdk2 in

cellular proliferation of non-transformed cells (Merrick et al., 2011). Here we show that small molecule inhibition of Cdk2 also disrupts cellular growth of transformed MEFs and human colon cancer cells, defining a role for Cdk2 in cellular proliferation under the control of oncogenic signaling.

4.2 Results

Analog sensitive Cdk2 forms active complexes *in vitro* and can be selectively inhibited by modified ATP analogs.

An analog sensitive allele of Cdk2 was previously generated in which a phenylalanine residue within the ATP binding pocket was replaced with a glycine (F80G) (Bishop et al., 2000). Replacing a bulky amino acid with a smaller one creates a unique expanded pocket. Small molecules can be designed to fit in the newly engineered pocket, allowing them to inhibit the modified kinase selectively without affecting other mammalian kinases that lack the analog sensitive mutation. Reconstitution of baculovirus produced and purified analog-sensitive Cdk2 (Cdk2^{AS}) and cyclin A followed by an *in vitro* kinase activity assay revealed that Cdk2^{AS} can be selectively inhibited by a pyrazolopyrimidine derivative 1NM-PP1 (IC₅₀=5nM) while the IC₅₀ of 1NM-PP1 for wild-type Cdk2 is ~ 6,000-fold higher (29 μM) (Bishop et al., 2000).

We asked if Cdk2^{AS} could assemble an active complex with cyclins *in vivo*. The human Cdk2^{AS} or Cdk2^{WT} cDNA, was tagged at the 3' end with a hemagglutinin (HA) epitope sequence and transfected into human embryonic kidney (HEK) cells (Fig. 25A). Cdk2^{WT} or Cdk2^{AS} protein was immunoprecipitated from whole cell lysates with anti-HA

antibodies, and kinase activity toward the histone H1 substrate was tested in the presence of increasing amounts of the 1NM-PP1 inhibitor (Fig. 25B and C). Immunoprecipitated Cdk2^{WT} was not appreciably inhibited by 1 μ M 1NM-PP1 consistent with the purified protein studies (Bishop et al., 2000) (Fig. 25B). In contrast, we found Cdk2^{AS} to be inhibited at low nanomolar (~3-5 nM) 1NM-PP1 concentrations in the presence of 500 μ M ATP (Fig. 25C). Thus, Cdk2^{AS} is an active kinase when isolated from human cells and is extremely sensitive to a selective inhibitor.

Acute inhibition of Cdk2 kinase activity results in decreased proliferation of mouse embryonic fibroblasts, while Cdk2 depletion with siRNA does not.

To study the effects of acute Cdk2 inhibition on cellular growth *in vivo*, we generated MEFs in which the endogenous mouse Cdk2 is functionally replaced with human Cdk2^{AS} or Cdk2^{WT}. We used Cdk2^{Flox/Flox} MEFs [kindly provided by Marcos Malumbres and Mariano Barbacid (Central Nacional de Investigaciones, Madrid, Spain)] and deleted endogenous Cdk2 by Cre-recombinase expression. Human Cdk2^{AS} or Cdk2^{WT} was then introduced by replication-defective retrovirus to generate two matched stable cell lines (Fig26 A and B). The MEFs continued to proliferate when cultured using the standard NIH 3T3 protocol, suggesting they underwent spontaneous immortalization. The p53 pathway appeared to remain intact however, as treatment with DNA damaging reagent doxorubicin induced expression of p21, a transcriptional target of p53 (Fig. 26C).

Using the AS allele of Cdk2, we compared the effects of acute Cdk2 kinase inhibition to siRNA knockdown of Cdk2. MEFs expressing human Cdk2^{WT} and Cdk2^{AS} proliferate at comparable rates, with cell numbers increasing exponentially over a period

of four days (Fig. 27A). Upon treatment of the cells with 1NM-PP1, Cdk2^{AS} MEFs showed a dramatic decrease in proliferation, whereas the treatment of MEFs expressing Cdk2^{WT} had no effect (Fig. 27A, $p < 0.00001$). Thus, 1NM-PP1 only affects the proliferation of cells that express the Cdk2^{AS} allele, demonstrating the specificity of the chemical-genetic approach.

In contrast to treatment with 1NM-PP1, Cdk2 knockdown with siRNAs resulted in an *increase* in proliferation of MEFs (Fig. 27B). We reasoned that this effect might be attributable to functional compensation by another Cdk, namely Cdk1, because Cdk1 has been shown to be capable of replacing all the other interphase Cdks (Santamaria et al., 2007). To investigate this possibility, we examined protein levels of Cdk1 and Cdk2 following treatment with either siRNAs against Cdk2, or 1NM-PP1. We saw dramatic decrease in Cdk2 protein levels upon treatment with Cdk2 siRNA, indicating efficient knockdown had occurred (Fig. 27B). Interestingly, we also saw a greater than 75% increase in Cdk1 protein levels within 48 hours of Cdk2 siRNA treatment, which paralleled the decrease in Cdk2 protein expression. These results suggested that compensation by Cdk1 might be responsible for the increased proliferation rates observed after Cdk2 knockdown. In contrast, treatment of Cdk2^{AS} MEFs with 1NM-PP1 resulted in no appreciable decrease of Cdk2 protein after 48 hours (Fig. 27C). Prolonged inhibitor treatment (6 days), however, did demonstrate a modest decrease in Cdk2 expression (Fig. 27C). Cdk1 levels remained relatively unchanged over the first four days of treatment, but showed an approximate 33% increase, which paralleled the decrease in Cdk2, after 6 days of treatment (Fig. 27C). We therefore conclude that acute inhibition of Cdk2 disrupts MEF cell proliferation, whereas Cdk2 knockdown does not, and this is likely due

to a lack of compensation by Cdk1 when Cdk2 kinase activity is acutely inhibited.

We next asked whether the decrease in proliferation observed after acute Cdk2 inhibition was attributable to decreased and/or delayed transition into S phase. Cdk2^{AS} and Cdk2^{WT} MEFs were treated with either the vehicle (DMSO) or the selective inhibitor (1NM-PP1), and BrdU incorporation was determined (Fig. 28A and B). The Cdk2^{AS} MEFs showed a significantly decreased percentage of cells in S phase and an increased percentage of cells in G1 when treated with 1NM-PP1 (Fig. 28A and B). Additionally, MEFs were synchronized by serum starvation and released into media containing 10% serum (vol/vol) with or without 1NM-PP1. We found that 24 hours after the cells were released, 1NM-PP1 treatment resulted in approximately 55% decreased phosphorylation of the retinoblastoma protein (Rb) (Fig. 28C). We therefore concluded that specific inhibition of Cdk2 kinase activity leads to a delayed transition from G1 to S phase.

Acute inhibition of Cdk2 results in decreased proliferation in both anchorage-dependent and anchorage-independent growth of human colon cancer cells.

We next asked whether acute inhibition of Cdk2 could decrease proliferation of human cancer cells. HCT116 cells, a human colon cancer cell line, harbor a constitutively active mutant KRAS (G13N) (Okumura et al., 1999) and also show a 2-fold increase in MYC expression compared with more differentiated colon cancer cells (Taylor et al., 1992). Importantly, HCT116 cells are amenable to homologous recombination for genetic ablation or replacement of candidate genes (Waldman et al., 1995). We used engineered HCT116 cells, in which the Cdk2^{AS} alleles were inserted into both endogenous Cdk2 loci (HCT116-Cdk2^{AS}), replacing the endogenous WT gene by

the method previously described for human Cdk7 (Larochelle et al., 2007; Merrick et al., 2011). We first asked whether acute inhibition of Cdk2 could decrease proliferation of the HCT116 cells. We found that, in the absence of 1NM-PP1, HCT116-Cdk2^{AS} cells exhibit a modest proliferation defect when compared to WT HCT116 cells (Fig. 29A), which is consistent with what has been previously reported (Merrick et al., 2011). Upon treatment with 1NM-PP1 we found that proliferation of HCT116-Cdk2^{AS} cells was further decreased, whereas we saw no effect in the HCT116-Cdk2^{WT} cells (Fig.29A), suggesting that similar to the MEFs, acute inhibition of Cdk2 significantly decreases proliferation of human cancer cells. Additionally, we found that treatment of HCT116-Cdk2^{AS} cells with 1NM-PP1 resulted in an increase of cells in G1 and a decrease of cells in S phase when subjected to a BrdU incorporation assay (Fig. 29B and C). We therefore concluded, in contrast to what was previously reported, inhibition of Cdk2 disrupts proliferation of a human cancer cell line.

We next determined whether Cdk2 kinase activity is essential for anchorage-independent growth, a hallmark of cellular transformation. Both HCT116-Cdk2^{WT} and HCT116-Cdk2^{AS} cells formed visible colonies within three weeks of growth in soft agar (Fig. 30A). In contrast, when the analog sensitive line was treated with 1NM-PP1, the number and size of colonies that formed were drastically reduced (Fig. 30A and B), thus indicating that Cdk2 is necessary for anchorage-independent growth of a human cancer cell line.

As a second approach to examine anchorage-independent growth we used a tumorsphere formation assay, which examines colony growth under non-adherent culture conditions (Zhang et al., 2010) An advantage of this approach is that cells can be

recovered from tumorspheres for cell cycle analysis and protein extraction. HCT116 cells formed visible tumorspheres within 10 days when grown on ultra low attachment plates, as has been previously reported (Ma et al., 2007). Similar to the soft agar assay, we found that 1NM-PP1 treatment reduced size and number of tumorspheres formed from HCT116-Cdk2^{AS} cells (Fig. 31A). This result could be quantified using a luminescence-based assay to measure the amount of ATP in an individual well, which corresponds to the number of live cells (Fig. 31B). We next asked whether 1NM-PP1 treatment affected cell cycle distribution of the proliferating tumorspheres. When exposed to BrdU for 24 hours, we found that HCT116-Cdk2^{AS} tumorspheres grown in the presence of 1NM-PP1 showed a significant decrease of cells in S phase (Fig 31 C and D). These results confirm that acute Cdk2 kinase inhibition attenuates anchorage-independent growth of an established human cancer cell line.

Cdk2 is required for anchorage-independent growth, but not monolayer growth, in MEFs transformed with a variety of oncogenes.

HCT116 tumor cells harbor multiple oncogenic events, including a KRAS activating mutation and MYC overexpression (Okumura et al., 1999; Taylor et al., 1992). To study the consequence of Cdk2 inhibition in a more defined genetic background, we used MEFs, which are amenable to cellular transformation by diverse oncogenic signals. We infected Cdk2^{WT} and Cdk2^{AS} MEFs with replication-defective retrovirus to express human MYC, activated HRAS, or v-ABL (Fig. 32 A), three well-characterized oncogenes. As with the untransformed MEFs, we observed that the p53 pathway remained intact in these transformed cells, as evidenced by p21 induction following

treatment with the DNA damaging agent doxorubicin (Fig. 32B).

We first sought to determine if overexpression each of the three oncogenes could alter cellular proliferation in the context of acute Cdk2 inhibition. Interestingly, in contrast to non transformed Cdk2^{AS} MEFs and HCT116-Cdk2^{AS} cells, treatment with 1NM-PP1 did not appreciably alter cell proliferation of the transformed MEFs, regardless of which oncogene was overexpressed (Fig 33). These observations suggest that overexpression of individual oncogenes can decrease sensitivity of MEFs to Cdk2 inhibition.

In contrast to the monolayer growth of cultured cells, 3D anchorage-independent growth of transformed cells in soft-agar is known to be critically dependent on the abundance of cyclin E and A expression (Barrett et al., 1995; Carstens et al., 1996; Clurman et al., 1996) the activating subunits of Cdk2. We therefore asked whether growing the transformed MEFs in non-adherent culture conditions would increase their sensitivity to Cdk2 inhibition. When grown in soft agar, all three of the oncogene-transformed cell lines acidified the media and formed visible colonies within three weeks. In contrast, when analog sensitive cell lines were treated with 1NM-PP1, the number and size of colonies that formed were drastically reduced regardless of which oncogene was used to transform the cells (Fig. 34A). Furthermore, when transformed MEFs were grown on ultra low-attachment plates, 1NM-PP1 significantly reduced the amount of tumorspheres that formed (Fig. 34B). These results indicate that although it has little effect on cells grown in a monolayer, acute specific inhibition of Cdk2 can significantly reduce anchorage-independent growth, a hallmark of cancer cells, regardless of which of these oncogenes is driving cellular transformation.

Altered expression of the cell cycle regulators in MEFs cultured in non-adherent condition results in increased sensitivity to Cdk2 kinase inhibition.

We next asked why the transformed MEFs were more sensitive to Cdk2 inhibition when grown in soft agar and non-adherent culture conditions. It has been shown that cells exhibit significant transcriptional alterations, including the expression of cell cycle proteins, when grown in adherent versus non-adherent culture conditions (Barrett et al., 1995; Carstens et al., 1996; Fang et al., 1996; Perou et al., 1999; Sandberg and Ernberg, 2005; Virtanen et al., 2002). Thus, we hypothesized that the observed sensitivity of the oncogene-transformed MEFs to a selective small-molecule inhibitor of Cdk2 could be attributable to altered expression of the cell cycle proteins. We examined the expression of cell cycle proteins in cells grown in adherent versus non-adherent culture conditions. We found that in each of the transformed lines, protein expression of all of the cyclins, with the exception of cyclin E in the MYC-transformed MEFs, was significantly diminished in the non-adherent cells (Fig.35A). Likewise, Cdk1 and Cdk2 expression was also diminished in the non-adherent cells (Fig. 35A). Interestingly, we also found that the endogenous inhibitors of cell cycle progression, p27 and p21, as well as the Rb tumor suppressor protein were markedly up-regulated in the non-adherent cells (Fig. 35B), suggesting that cell cycle control mechanisms are heightened in this context. Corresponding with a decrease in cyclin D1 and E1 expression, we also found a decrease in the inactivating phosphorylation of Rb (Fig. 35B). Thus, both transformed MEFs and human tumor cells attenuate expression of pro-proliferative cell cycle proteins when

grown in non-adherent conditions.

4.3 Discussion

We have generated cell lines expressing an analog sensitive human Cdk2 allele that can be potently, selectively, and acutely inhibited by a small-molecule inhibitor, 1NM-PP1. Using this allele, we have shown that acute small molecule inhibition of Cdk2 kinase activity, but not Cdk2 depletion, dramatically attenuates growth of multiple cell types. These include non-malignant MEFs and a human colon cancer cell line in which both endogenous Cdk2 alleles are replaced with Cdk2^{AS}. Furthermore, abundant overexpression of multiple oncogenes appears to overcome the effects of Cdk2 inhibition on cell proliferation in monolayer culture, but anchorage-independent growth in soft-agar or tumorsphere assays is still dramatically diminished when the oncogenes are overexpressed. These findings contrast with prior studies, which found that Cdk2 was not required for the proliferation of several cancer cell lines (Tetsu and McCormick, 2003). Our results emphasize that a fundamental difference exists between genetic loss or gene knockdown approaches when compared to small molecule inhibition of Cdk2 kinase activity. Furthermore, our findings suggest that Cdk2 inhibition may indeed have utility in the treatment of cancers driven by a wide variety of oncogenic signals.

Genetic ablation of *Cdk2* using engineered knockout (KO) mice shows that the mice are viable and embryonic fibroblasts derived from these mice exhibit relatively normal proliferation (Berthet et al., 2003; Ortega et al., 2003). The modest effects observed in the *Cdk2* KO mice are likely due to compensation by other Cdks such as Cdk4/6 at the G1/S transition or Cdk1 within the G1/S and G2 phases (Santamaria et al.,

2007). For example Cdk1/cyclin E complexes are not detected until Cdk2 expression is lost, indicating a switch whereby cyclin E preferentially binds to Cdk2, in the absence of Cdk2, cyclin E can be associated instead with Cdk1 (Santamaria et al., 2007). In contrast to genetic loss, specific inhibition of Cdk2 kinase activity by small molecule inhibitors does not immediately allow the cell to compensate for the missing protein. The presence of an inhibitor-bound and inactive Cdk2 protein prevents cyclin “switching” to other Cdks, such as Cdk1.

We find that acute and selective inhibition of Cdk2 can attenuate anchorage-independent growth of cells transformed by a variety of different oncogenes as well as human HCT116 tumor cells. Several prior studies found that expression of cyclins E and A, the activating subunits of Cdk2, is rapidly down-regulated in cells grown in an anchorage-independent manner (Barrett et al., 1995; Carstens et al., 1996; Fang et al., 1996). In contrast cyclin A overexpression in Rat1a cells is sufficient to induce anchorage-independent growth (Barrett et al., 1995). Thus, Cdk2 activity may be limiting in transformed cells grown in an anchorage-independent manner. Our results support this hypothesis, because the ability of three potent oncogenes to elicit anchorage-independent growth is substantially diminished following selective Cdk2 inhibition (Fig. 4C).

Transformed cells lose contact inhibition, can adhere to one another and form 3D colonies when grown in soft-agar. Several prior studies have found that many conventional chemotherapeutics are less potent against tumor cells when they are grown in 3D cultures (Frankel et al., 1997; Green et al., 1999). In contrast, we find that selective Cdk2 inhibition preferentially blocks the proliferation of tumor cells in soft-agar. Inhibition of Cdk2 may therefore define a unique type of therapy that preferentially

affects the anchorage-independent growth of tumor cells in 3D culture.

Prior attempts to inhibit Cdk2 acutely *in vivo* have relied on small molecules that are not entirely selective for one Cdk. Our chemical-genetic approach allows for truly selective inhibition of a kinase of interest with unparalleled specificity. We now show that acute inhibition of Cdk2 alone is sufficient to diminish proliferation of normal and malignant cells. Our study challenges the notion that Cdk2 is dispensable and instead identifies it as a potentially useful therapeutic target for arresting the anchorage-independent proliferation of tumor cells driven by a variety of oncogenic signals.

The analog sensitive approach highlights the differences between acute kinase inhibition and genetic ablation. Moving forward, this approach will be incredibly useful for identifying specific Cdk2 function. Using the Cdk2 analog sensitive allele over expressed in these studies, we have generated a targeting construct for the generation of an analog sensitive knockin mice (Fig. 36). These mice have been generated and bred to homozygosity. We are currently characterizing MEFs derived from mice. We believe these mice will be a useful tool for studying Cdk2 function in a variety of cell types as well as different cancer models.

4.4 Figures

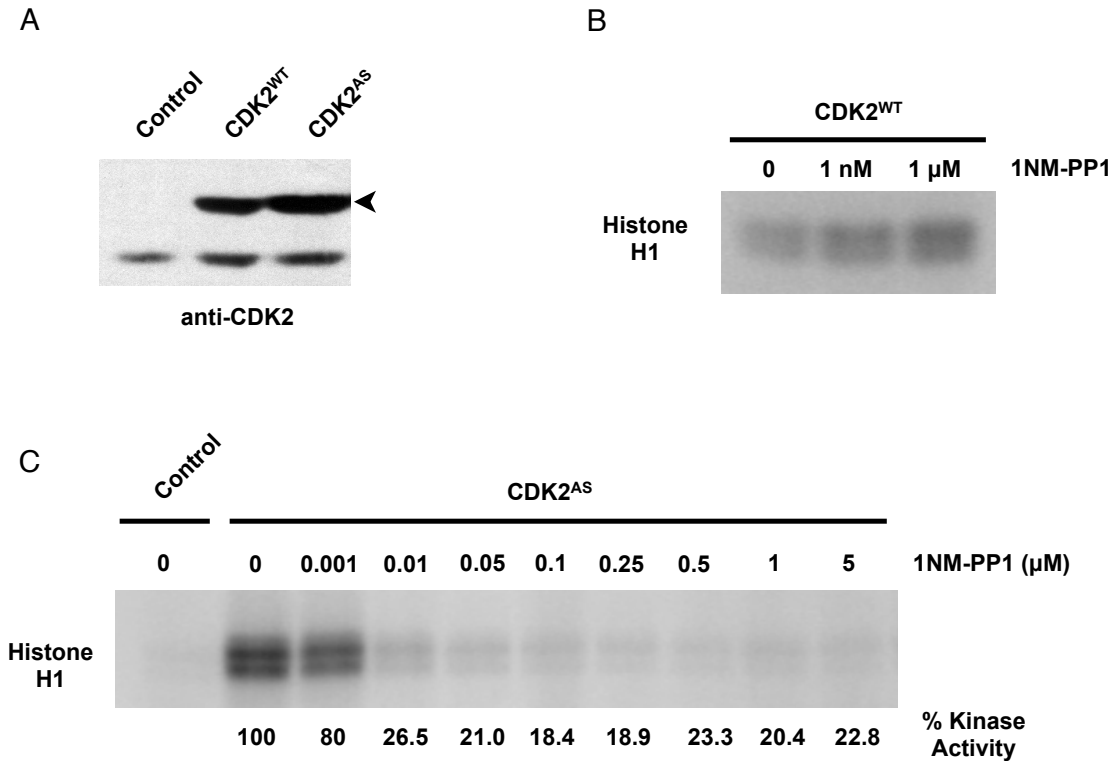
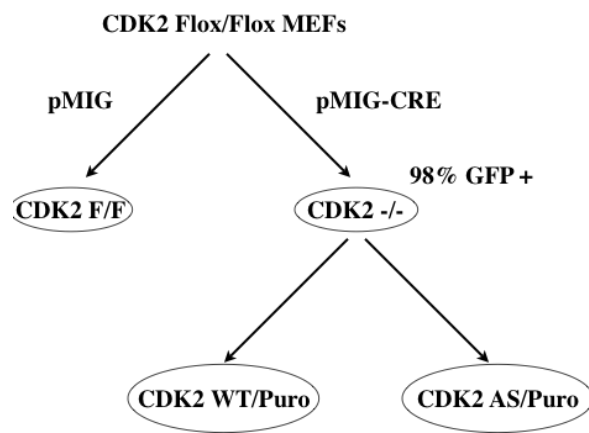
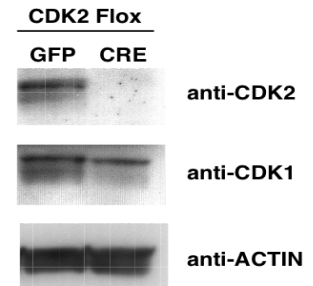


Figure 25. Analogue Sensitive Cdk2 is Catalytically Active and can be Potently Inhibited by the Selective Inhibitor 1NM-PP1. (A) Expression of hemagglutinin-tagged Cdk2^{WT} or Cdk2^{AS} proteins in HEK293T cells. Western blot shows expression of tagged (arrow) and endogenous Cdk2 proteins. (B) Cdk^{WT} was immunoprecipitated (IP) from HEK293T cell lysates, with anti-HA antibodies, and kinase activity towards the histone H1 substrate were performed in the presence of increasing amounts of 1NM-PP1. (C) Cdk^{AS} was immunoprecipitated (IP) from HEK293T cell lysates, with anti-HA antibodies, and kinase activity towards the histone H1 substrate were performed in the presence of increasing amounts of 1NM-PP1.

A.



B.



C.

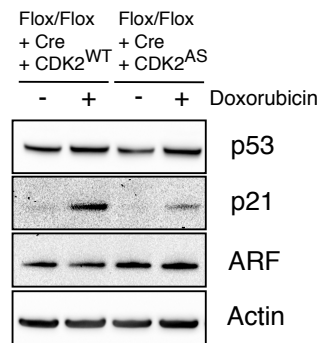


Figure 26. Endogenous Cdk2 is Deleted in Cdk2^{flox/flox} MEFs and replaced with Cdk2^{AS} or Cdk2^{WT} Alleles. (A) Schematic of the generation of MEFs expressing the WT or AS allele of Cdk2. (B) Cre-recombinase was retrovirally expressed in Cdk2^{flox/flox} MEFs and depletion of Cdk2 protein expression is shown by western blotting. (C) Western blot analysis of the expression of p53, p21, and ARF in the MEFs with Cdk2^{WT} or Cdk2^{AS} in the presence or absence of doxorubicin treatment.

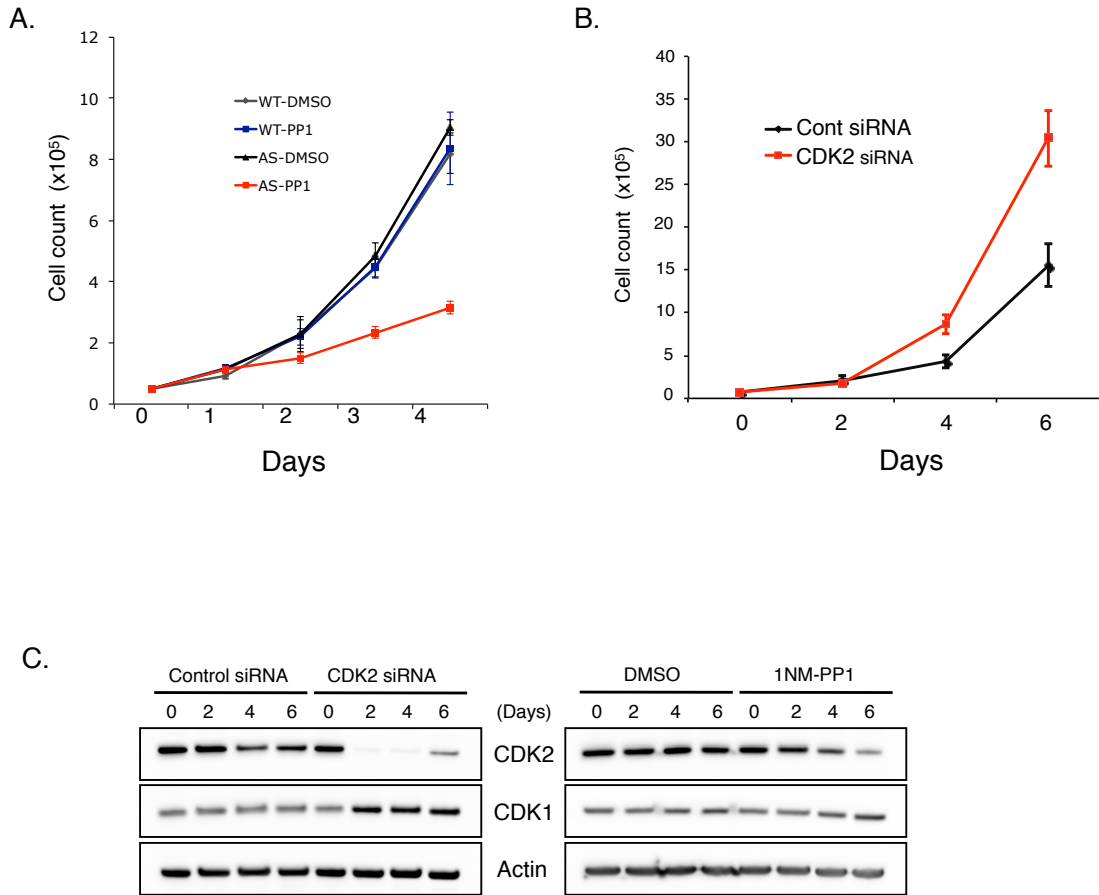


Figure 27. Small Molecule Inhibition But Not siRNA Mediated Knock-Down of Cdk2 in MEFs Decreases Cellular Proliferation. (A) Growth curve of MEFs carrying $Cdk2^{AS}$ or $Cdk2^{WT}$ Alleles. Cells were treated with either 1NM-PP1 or DMSO. Growth curves represent the average of 5 independent experiments. The error bars represent means \pm S.E.M. Proliferation of the $CKD2^{AS}$ cells in the presence or absence of 1NM-PP1 on both Days 3 and 4 ($p < 0.00001$; two-tailed t -test). (B) Growth curve of MEF- $Cdk2^{AS}$ cells treated with either non-targeting control siRNA or Cdk2 specific siRNA. Growth curves represent the average of 3 independent experiments. The error bars represent means \pm S.E.M. The statistical significance applies to both days 4 and 6 ($p < 0.00001$; two-tailed t -test). (C) Western analysis of Cdk expression in MEFs treated with either control/Cdk2 siRNA or with DMSO/1NM-PP1.

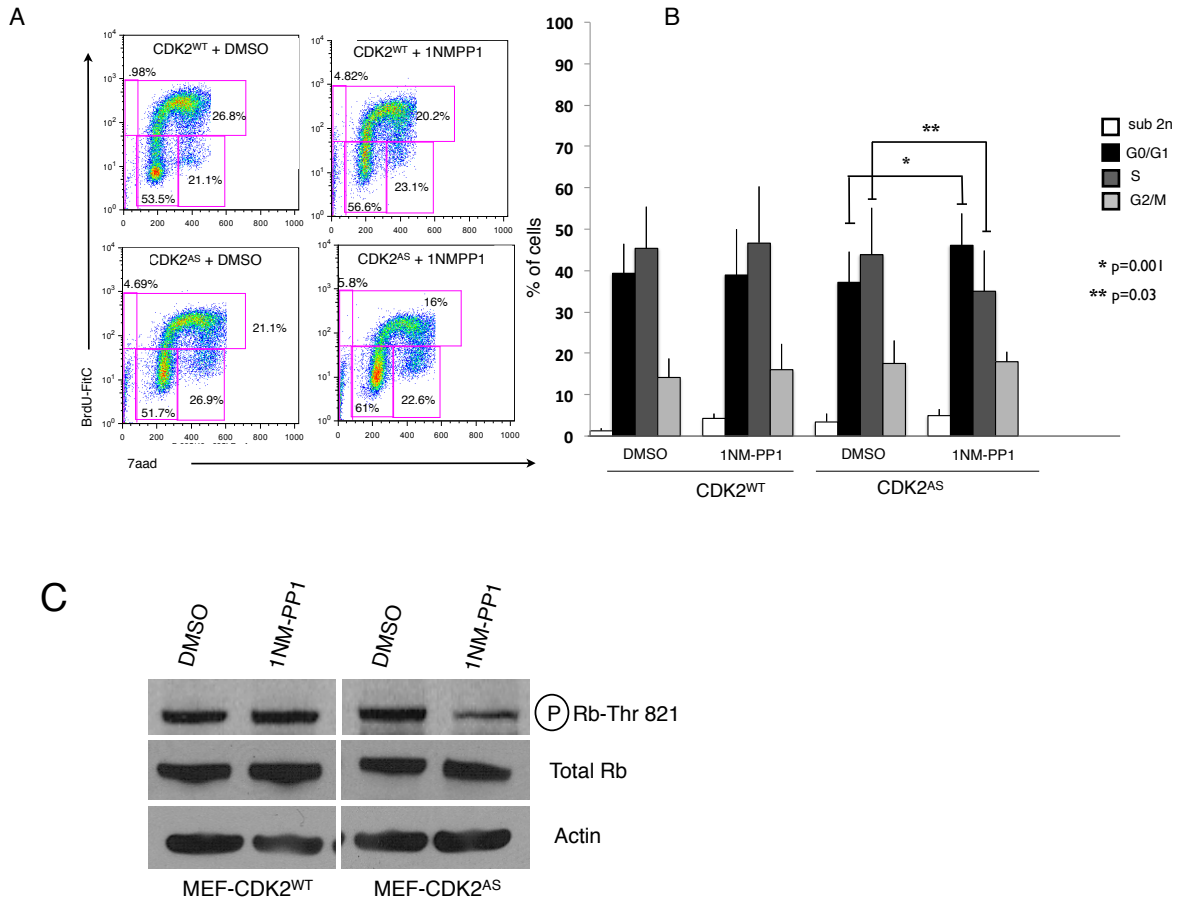


Figure 28. Small Molecule Inhibition of Cdk2 delays entry in S phase: (A) Cell cycle analysis of asynchronously growing Cdk2^{WT} and Cdk2^{AS} MEFs. Cells were treated with DMSO or 5 μ M 1NM-PP1 for 72h, pulsed with BrdU for 45min and then stained with anti BrdU antibodies and 7aad followed by FACs analysis. Gated populations represent dead (sub2n), G0/G1, S and G2/M cell populations. (B) Histogram representation of data shown in (A). The experiment was repeated 4 times. Shown here are the mean values +/- SEM. Significance of differences determined by the t-test. (C) Rb phospho-Thr821 abundance following 36 hr 1NM-PP1 treatment cells. The cells were first serum-starved for 90 hours in the presence of 0.1% serum and subsequently released into medium containing 10% serum and DMSO or 1NM-PP1, and harvested for analysis after 36h.

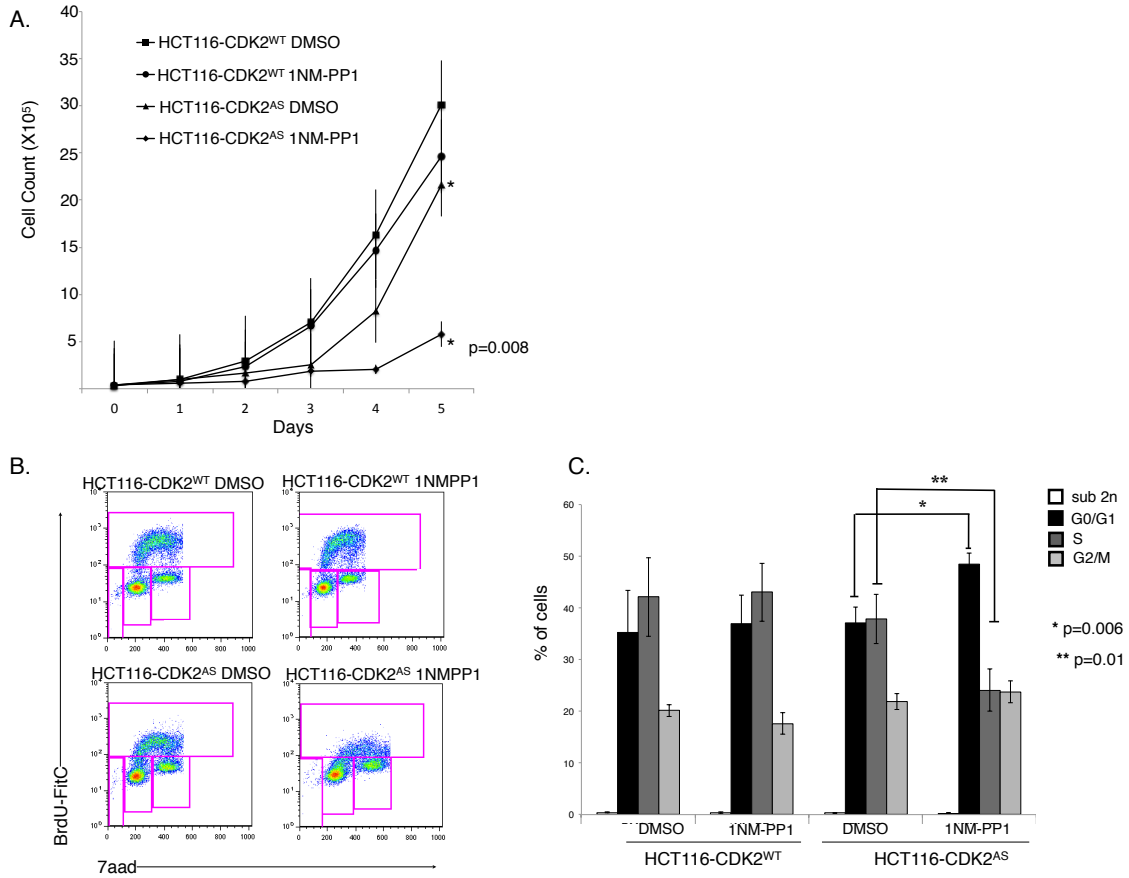


Figure 29. Diminished Proliferation of HCT116 Colon Cancer Cells Following Acute Cdk2 Inhibition. (A) Growth curve of HCT116-Cdk2^{WT} or HCT116-Cdk2^{AS} cells treated with DMSO or 1NM-PP1. The experiment was repeated 4 times. Shown are mean values +/- SEM. Significance of differences determined by the t test. (B) Cell cycle analysis of asynchronously growing Cdk2^{WT} and Cdk2^{AS} HCT116 cells. Cells were treated with DMSO or 5 μ M 1NM-PP1 for 72h, pulsed with BrdU for 45min and then stained with anti BrdU antibodies and 7aad followed by FACs analysis. Gated populations represent dead (sub2n), G0/G1, S and G2/M cell populations. (C) Histogram representation of data shown in (A). The experiment was repeated 4 times. Shown here are the mean values +/- SEM. Significance of differences determined by the t-test.

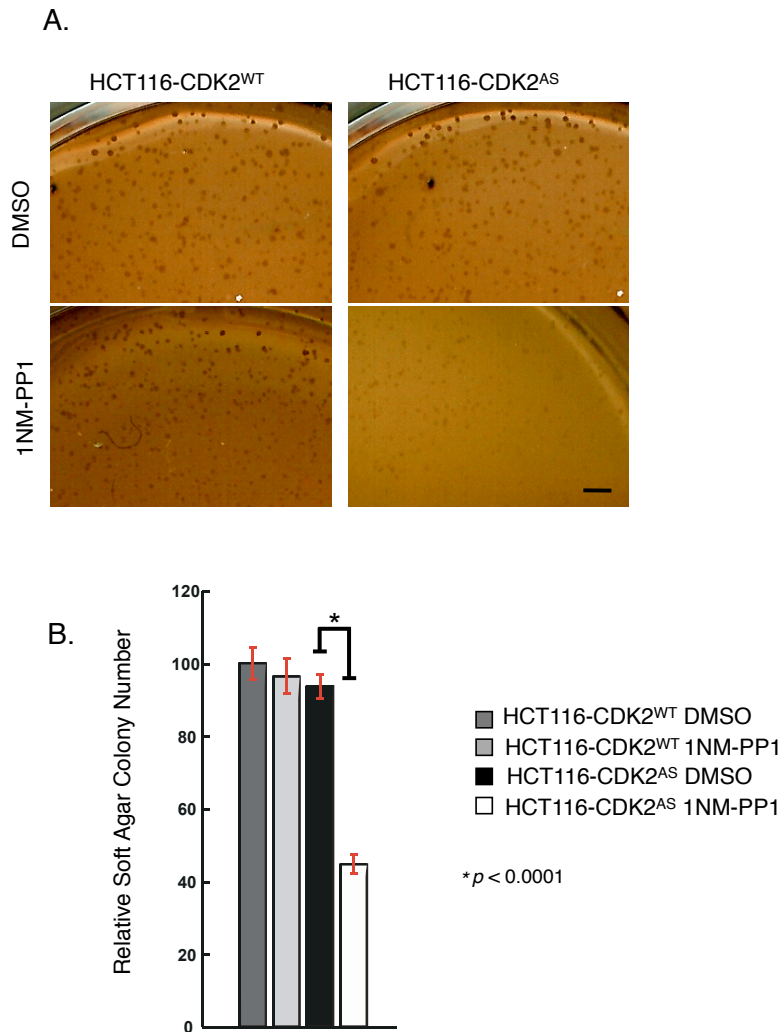


Figure 30. Decreased anchorage-independent growth in soft agar of HCT116 colon cancer cells following acute Cdk2 inhibition. (A) Anchorage independent growth of HCT116 cells in soft agar. HCT116 cells were pretreated with DMSO or 5 μ M 1NM-PP1 for 72 hours. Following pretreatment, cells were plated in 0.32% soft agar containing DMSO or 5 μ M 1NM-PP1. Scale bar = 5 mm. (B) Quantification of colony growth in soft agar after 21 days. The experiment was repeated 3 times. Shown are mean values \pm SEM. Significance of differences determined by the t-test.

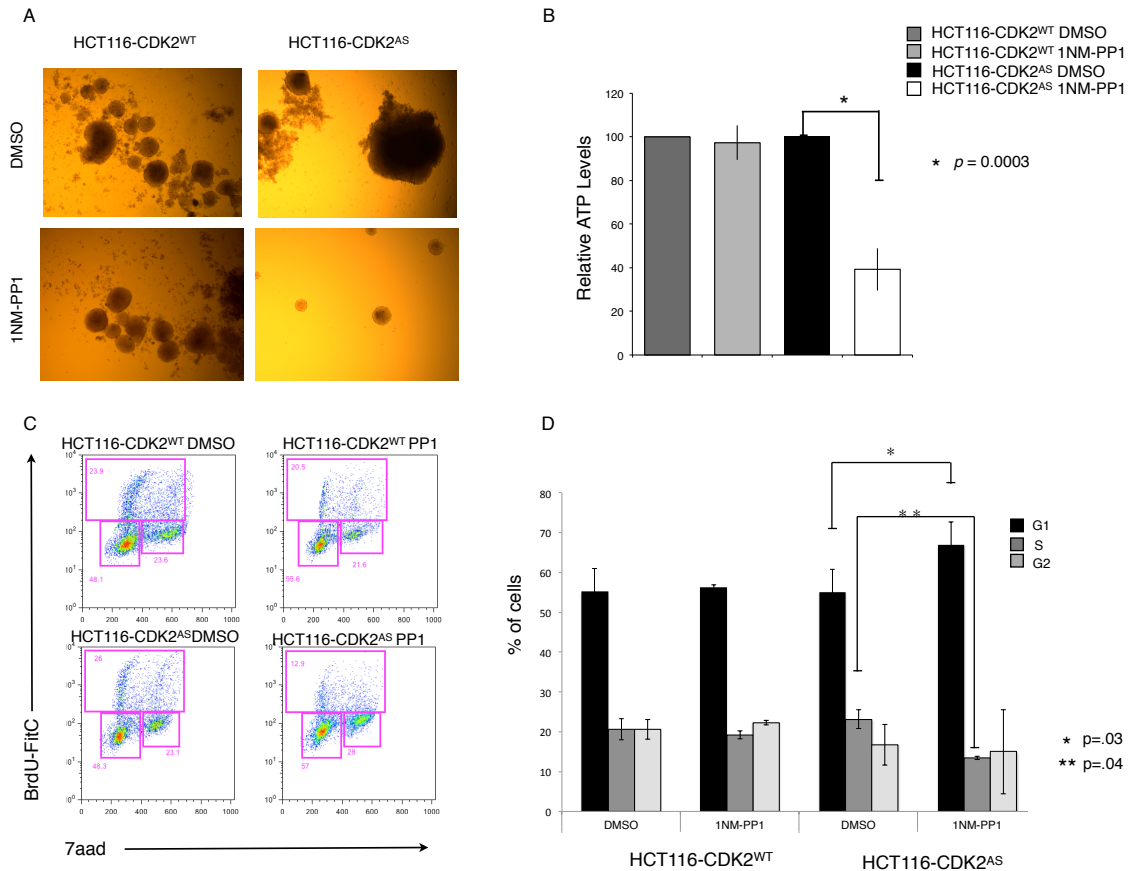


Figure 31. Decreased anchorage-independent growth of HCT116 colon cancer cells following acute Cdk2 inhibition. (A) Tumorsphere formation of HCT116 cells. HCT116 cells were seeded into ultra-low adherent plates in media containing DMSO or 5 μ M 1NM-PP1. Magnification = 40x. (B) Amount of live cells per well after 14d, quantified using a luminescence-based assay to determine ATP levels. The experiment was repeated 5 times. Shown here are mean values +/- SEM. (C) Cell cycle analysis of tumorspheres formed from Cdk2^{WT} and Cdk2^{AS} HCT116 cells. Tumorspheres were grown for 2 wks with DMSO or 1NM-PP1. Tumorspheres pulsed with BrdU for 24h and then stained with anti BrdU antibodies and 7aad followed by FACs analysis. Gated populations represent dead (sub2n), G0/G1, S and G2/M cell populations. (D) Histogram representation of data shown in (C). The experiment was repeated 4 times. Shown are the mean values +/- SEM. Significance of differences determined by the t-test.

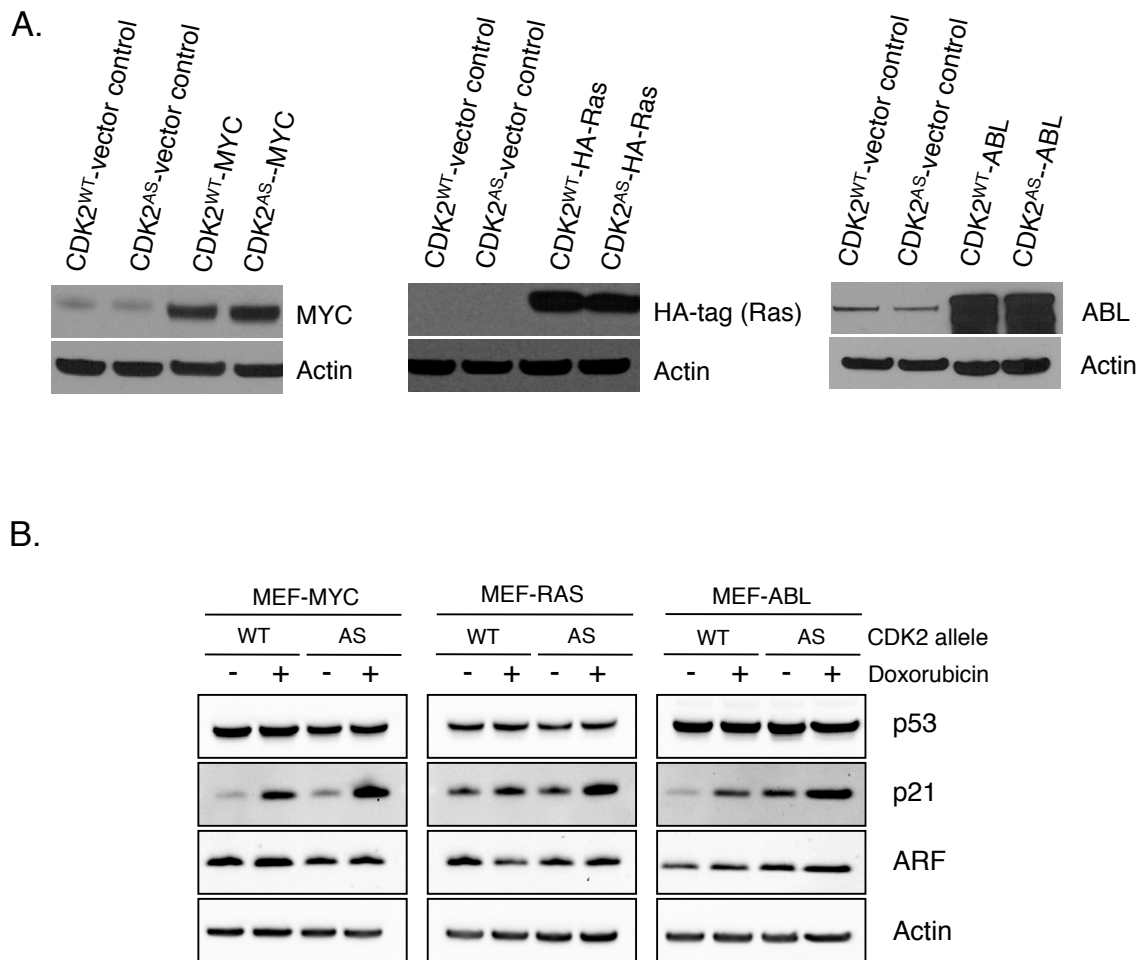


Figure 32. Generation of MEFs stably expressing a variety of oncogenes (A) Western blot showing retroviral overexpression of the oncogenes MYC, RAS, and ABL in MEF- $Cdk2^{WT}$ or $-Cdk2^{AS}$ cells. (B) Western blot analysis of the expression of p53, p21, and ARF in the oncogene-transformed MEFs.

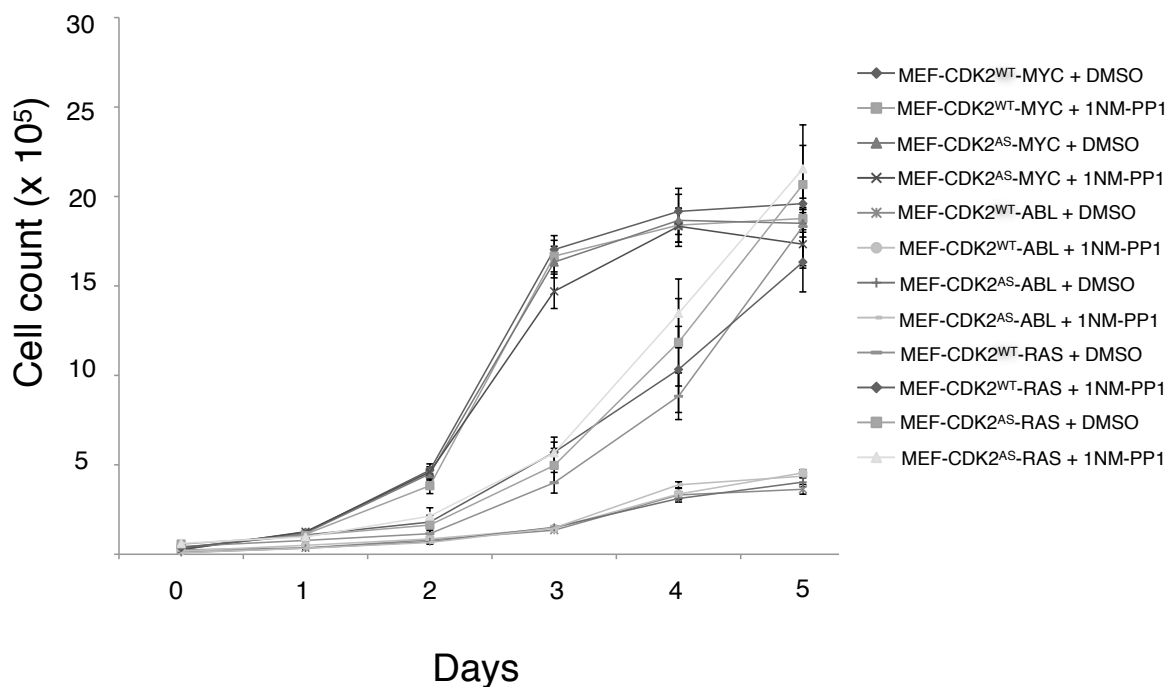


Figure 33. Acute Cdk2 inhibition does not effect proliferation of oncogene transformed MEFs. Growth curve of the oncogene-transformed MEFs carrying either the CKD2^{WT} or CKD2^{AS} allele, treated with DMSO or 1NM-PP1. The experiment was repeated 4 times. Shown are mean values +/- SEM.

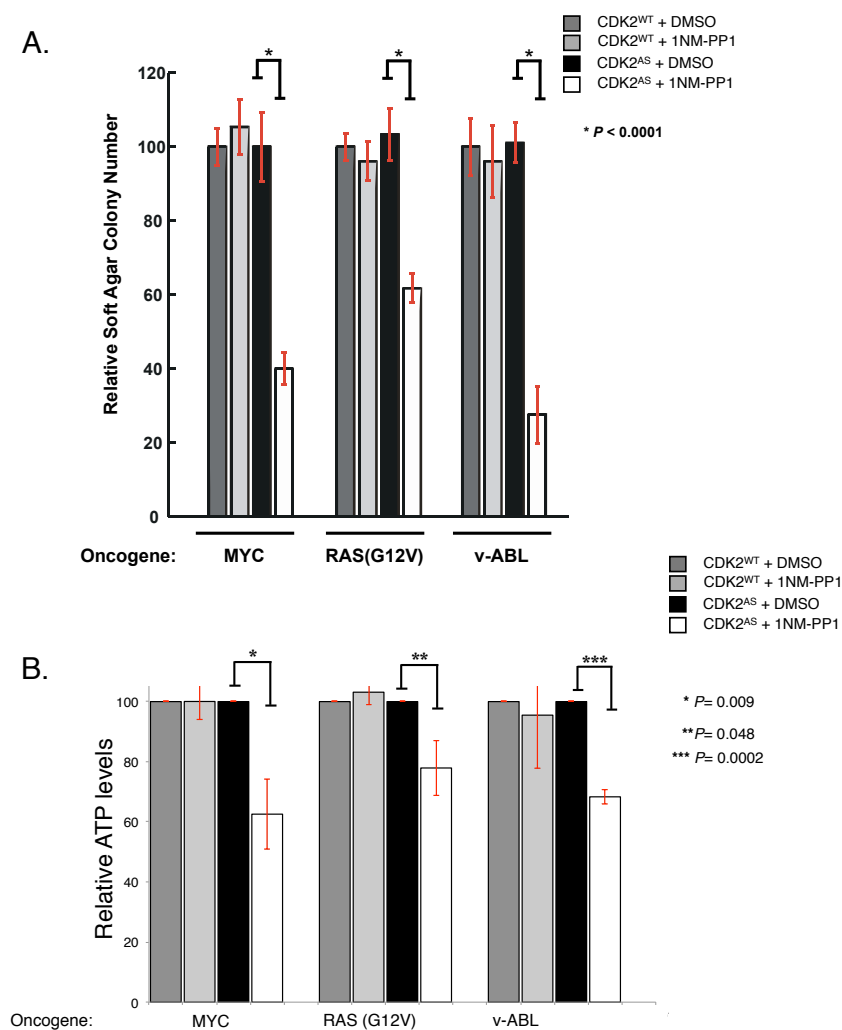


Figure 34. Acute Cdk2 inhibits anchorage-independent growth of oncogene transformed MEFs (A) Quantification of anchorage-independent colony formation in soft agar of oncogene transformed MEFs after 3wks. The experiment was repeated 3 times. Shown are mean values +/- SEM. Significance of differences determined by the t-test. (B) Tumorsphere formation from oncogene transformed MEFs grown on low attachment plates, quantified by ATP abundance after 10-14 days of growth. 1NM-PP1 wells were normalized to DMSO treated wells for each individual cell line. Experiment was repeated 3 times with Abl-overexpressing cells and 5 times each with MYC and RAS overexpressing cells. Shown are mean values +/- SEM. Significance of differences determined by the t-test.

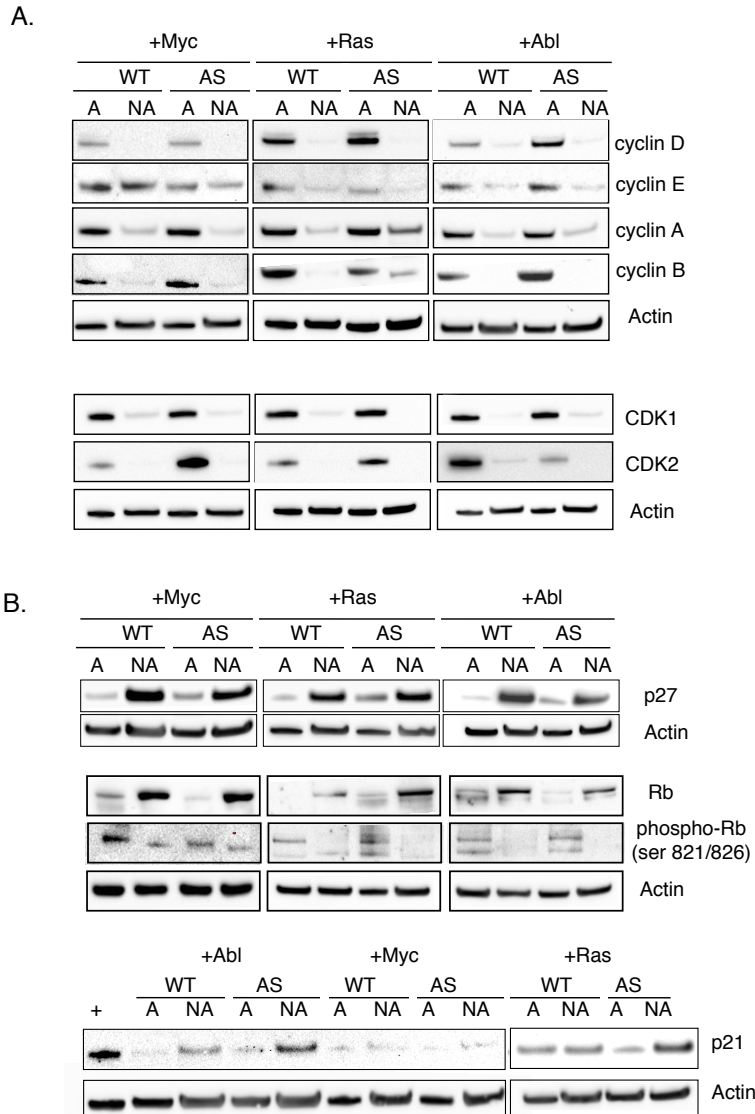


Figure 35. Non-Adherent Growth of Oncogene-Transformed MEFs Results in the Altered Expression of Cell Cycle Proteins (A) Western blot analysis of cyclins, Cdk1 and Cdk2 expression in the oncogene-transformed MEFs grown either in adherent or non-adherent conditions (A: adherent; NA: non-adherent). (B) Western blot analysis of tumor suppressors p27, p21, and Rb in the oncogene-transformed MEFs grown either in adherent or non-adherent conditions (A: adherent; NA: non-adherent).

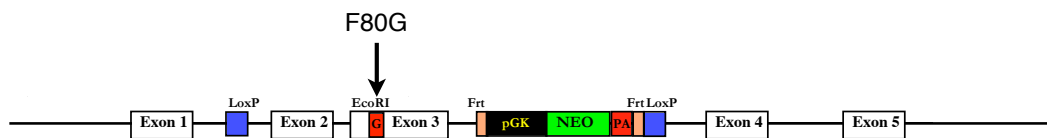


Figure 36. Cdk2AS Targeting construct: Schematic of the construct used to generate Cdk2AS knockin mice. A phenylalanine was mutated to a glycine at position 80 (within the ATP binding site) to allow for inhibition by an engineered small molecule, 1NMPP1.

Chapter 5

Conclusions and Future Directions

Cdks are an evolutionarily conserved family of kinases that regulate the complex series of events known as the cell cycle. From the moment of their discovery as cell cycle regulators in the early 1980's there has been considerable effort to develop small molecule Cdk inhibitors for therapeutic use against cancers and other proliferative diseases (Malumbres and Barbacid, 2009). Extensive analysis of animal knockout models has suggested, however, that many Cdk-cyclin complexes function in a cell type specific manner (Satyanarayana and Kaldis, 2009). In this thesis we investigate the effect of inhibiting Cdks in one such specialized cell type, pluripotent embryonic stem cells. Additionally, we develop a model that allows for the analysis of Cdk2 function in a wide variety of cell types and disease models. This body of work contributes to our current understanding of the regulation of the mammalian cell cycle.

We first investigated the requirement of individual Cdks and cyclins for normal cell proliferation and viability in mES cells. We found that siRNA knockdown of Cdk1 or Cdk1 binding partners, cyclins B1/B2 and cyclin A, but not other Cdks or cyclins, induced cell death in mES cells. Additionally small molecule inhibition of Cdk1 but not Cdk2, rapidly induced cell death in mES, hES and miPS cells. In contrast, inhibition of Cdk1 in MEFs or differentiated ES cells resulted in reversible G2/M cell cycle arrest but little if any cell death. These results raised the question as to whether there are specific

cellular characteristics that render ES cells sensitive to Cdk1 inhibition. Mouse ES cells proliferate rapidly, in part due to an abbreviated cell cycle program with truncated gap phases (Orford and Scadden, 2008). It is possible that the sensitivity of ES cells to cell cycle inhibitors is simply due to their rapid proliferation rather than intrinsic cellular characteristics/ pathways related to pluripotency. This is unlikely, as the characterization of cell viability after Cdk1 inhibition within other cellular systems, such as panels of triple negative and receptor positive breast cancer cell lines, did not identify a correlation between sensitivity to Cdk1 inhibitors and cell proliferation rates (Horiuchi et al., 2012b). Furthermore, mouse embryonic stem cells deficient for the miRNA processor DGCR8 exhibit a slower cell cycle program, characteristic of differentiated cells with elongated gap phases (Wang et al., 2007), but remain sensitive to Cdk1 inhibitors (data not shown). Nonetheless, it will be interesting to further investigate if and how cell cycle program affects ES sensitivity to Cdk1 inhibitors. Loss of miRNA expression in DGCR8^{-/-} ES cells alters many cellular signaling pathways and likely affects general sensitivity to apoptotic factors (Jovanovic and Hengartner, 2006), complicating the analysis of cell viability after Cdk1 inhibition. It will therefore be useful to assess sensitivity to Cdk1 inhibition in alternative pluripotent cell types. Ectopic expression of individual Cdk inhibitor proteins p21 and p27 results in an elongated G1 phase in ES cells but does not induce differentiation (Li et al., 2012; Wang et al., 2008). Additionally epiblast stem cells, which are derived later in development than mES cells from post implantation embryos, retain pluripotency but exhibit a slower cell cycle program (Pauklin et al., 2011). Either of these cell types would be useful for studying the ES cell cycle program's contribution to Cdk1 inhibitor-induced cell death.

We found that Cdk1 inhibition induces the DNA damage response in ES but not differentiated cells. This likely contributes to the selective effect that Cdk1 inhibitors have in ES cells. Currently, neither the mechanism of Cdk1 inhibitor-induced activation of the DNA damage response, nor the type of DNA damage incurred in ES cells is known. Cdk1 inhibition in ES cells increases the percentage of cells containing foci of phosphorylated ATM. Phosphorylation of ATM occurs primarily as a response to double stranded DNA breaks (DSB) (Shiloh, 2003), suggesting that Cdk1 inhibition causes an increase in DSBs in ES cells. DSBs can occur naturally in the cell during meiosis (Keeney and Neale, 2006), as a result of fork collapse during DNA replication (Shrivastav et al., 2008), or from physical stress when dicentric chromosomes are pulled apart during mitosis (Murnane, 2006). Cdks regulate both DNA replication as well as chromosome segregation, and it is plausible that Cdk1 inhibition could result in an increase in DSBs through interference with either of these processes. Cells generally use two independent mechanisms for DSB repair, non-homologous end joining, or a higher fidelity option, homologous recombination (Shrivastav et al., 2008). Cdk1 specifically has been implicated in several steps required for repair of DSBs by homologous recombination (Johnson et al., 2011; Moynahan et al., 1999; Peterson et al., 2011). Notably, ES cells have been shown to predominately use homologous recombination for the repair of DSBs (Tichy et al., 2010). Therefore, it is possible that Cdk1 inhibition induces the DNA damage response in ES cells through both increased incidence of DSBs as well as reduced capacity for DSB repair. Differentiated cells theoretically would be less sensitive to Cdk1 inhibition due to their increased ability to repair DBSs using non-homologous end joining, therefore explaining the selective effect Cdk1 inhibition has

towards ES cells. As a future direction it will be interesting to examine markers of homologous recombination to determine whether DSB repair pathways are compromised in ES cells after Cdk1 inhibition. It will also be important to examine markers of other types of DNA damage, to determine whether additional factors are responsible for the selective induction of the DNA damage response after Cdk1 inhibition in ES cells.

Our results show that p53 is necessary for Cdk1 inhibitor-induced cell death in ES cells. Upon Cdk1 inhibition, nuclear expression of p53 increases, and p53 is phosphorylated at serine 15, a verified target of ATM kinase activity, further confirming that Cdk1 inhibition induces DNA damage in ES cells. Coinciding with p53 up-regulation, we observe a p53-dependent increase in mRNA expression of pro-apoptotic p53 target genes Noxa, Zmat3, Tp73 and Rb, providing additional insight on the mechanism of Cdk1 inhibitor-induced apoptosis in pluripotent cells. Interestingly several canonical p53 targets associated with cell cycle arrest and cell death, including Bax, Puma, and p21 (Vousden and Lu, 2002), were not significantly increased. These results suggest that ES cells might utilize unique components of intrinsic apoptotic pathway when compared to somatic cells.

The observation that Cdk1 inhibitors can selectively induce apoptosis in ES cells has several potential therapeutic implications. One example is the use of Cdk1 inhibitors to deplete undifferentiated stem cells during stem cell therapies, thus reducing the risk of teratomas/teratocarcinoma development in transplant patients. This concept is discussed in chapter 3 of this thesis. We show that Cdk1 inhibitors are able to reduce the Oct4 positive population of cells within a partially differentiated culture and decrease the incidence of stem cell driven tumors. Furthermore we demonstrate that administration of

Cdk1 inhibitors after transplantation of stem cells can both prevent the formation of stem cell-driven tumors, as well as inhibit the growth of tumors that have already formed. More research will be required to determine the effect of Cdk1 inhibition on the differentiated progenitor cells. Nonetheless, this suggests that Cdk1 inhibitors could be used to both increase the safety of stem cell therapies and treat patients with germ cell tumors.

A second potential use of Cdk1 inhibitors would be to target tumor initiating cells, or cancer stem cells, within adult cancers. Tumor initiating cells are defined as a subpopulation of cells within the tumor bulk that are responsible for tumor recurrence after chemotherapies and thought to exhibit many of the hallmark characteristics of ES cells. Recently, the phenotypic similarities between ES cells and poorly differentiated, aggressive cancers have been attributed to a common gene expression profile that is distinct from that of adult stem cells (Ben-Porath et al., 2008; Wong et al., 2008). Furthermore this gene expression profile can be reactivated by ectopic expression of Myc (Wong et al., 2008), an oncogene that is known to regulate important stem cell pathways and is often correlated with poorly differentiated cancers (Cartwright et al., 2005; Dang, 2012). It has therefore been hypothesized that embryonic stem cells might provide a useful model for the study of aggressive cancers, and more specifically, populations of stem cell-like tumor initiating cells (Kim and Orkin, 2011; Orkin, 2011).

The extent of the similarities between embryonic stem cells and tumor initiating cells is still unknown. Nonetheless, a study from our lab demonstrated that, similar to ES cells, Cdk1 inhibitors induce apoptosis in at least one subtype of poorly differentiated, high Myc expressing cancers, the triple negative (TN) breast cancers (Horiuchi et al.,

2012b). TN breast cancers are characterized by a lack of estrogen and progesterone receptors and low HER2 expression (Tan et al., 2008). TN breast cancers also exhibit a relatively high proportion of putative breast cancer stem cells, defined by a CD44⁺CD24^{-/low}ESA⁺ expression pattern of cell surface markers (Fillmore and Kuperwasser, 2008). We found that Cdk1 inhibition both induces cell death in triple negative breast cancer cell lines and causes tumor regression in mouse xenograft models of triple negative tumors (Horiuchi et al., 2012b). In future experiments we plan on testing whether inhibition of Cdk1 or other cell cycle kinases can selectively deplete the CD44⁺CD24^{-/low}ESA⁺ population of cells within triple negative cell lines.

In addition to studying the effect of Cdk inhibition in embryonic stem cells, we have also utilized an analog sensitive human Cdk2 allele to investigate the effect of inhibiting Cdk2 in both normal cells and cells transformed by a variety of oncogenes. Use of the analog sensitive approach allows for the acute inhibition of Cdk2 kinase activity with unparalleled selectivity. We found that, in contrast to Cdk2 knockout models or depletion of Cdk2 using siRNAs, acute inhibition of Cdk2 kinase activity significantly slows the proliferation of multiple cell types. Additionally, using this approach, we were able to establish that Cdk2 kinase activity is necessary for the anchorage-independent growth both HCT116 colon cancer cells as well as MEFs transformed by a variety of oncogenes.

These results highlight the differences between acute inhibition of kinase activity and genetic ablation. One caveat pertaining to these experiments, however, was that the analog sensitive form of Cdk2 was over expressed in MEFs using retroviruses. This resulted in expression levels of Cdk2 that were beyond the normal physiological range. It

is generally believed that cyclin levels, rather than Cdk levels, are the rate-limiting factor for Cdk-cyclin complex activity (Morgan, 1995); subsequently we do not expect the overexpression of Cdk2 to have a significant impact on our results. An alternative approach, however, is to generate knock-in cell lines that express the Cdk2-AS allele at the endogenous promoter. We have used this approach with HCT116 colon cancer cell lines. Additionally, we have taken this approach to generate Cdk2-AS knockin mouse models, which we are currently breeding to homozygosity. The Cdk2-AS mice will facilitate studies to determine the effect of acute Cdk2 inhibition in a variety of cell types, including germ cells. Additionally, Cdk2-AS mice could be crossed with various cancer models and the effect of Cdk2 inhibition on tumor progression and maintenance could be determined. We believe that the Cdk2-AS knockin model, along with analog sensitive mouse models of other Cdks will greatly enhance our understanding of Cdk regulation of the mammalian cell cycle.

Materials and Methods

Cell Culture: Mouse embryonic stem cell lines, E14, oct4-GFP E14, p1.2 (p53^{-/-}), and Bax^{-/-} Bak^{-/-} were maintained on plates coated with .1% gelatin in knockout DMEM (gibco) supplemented with 15% FBS, 1X nonessential amino acids, 1X glutaMAX-1, 100 units/mL penicillin-streptomycin, .1 μ M 2-mercaptoethanol and recombinant leukemic inhibitory factor. miPS cells were generated as previously described (reference). Media was changed daily. mES cells were differentiated by culturing for four days in Lif-free mES media with 1 μ M retinoic acid. UCSF4 human embryonic stem cells (NIH registry No.is 0044) were maintained on non-proliferative MEFs in standard DMEM/F12 medium supplemented with 10ng/ml FGF2. Mouse embryonic fibroblasts were grown in DMEM supplemented with 10% FBS and 100 units/mL penicillin-streptomycin. HCT116 colon cancer cells were grown in RPMI-1640 media supplemented with 10% FBS and 100 units/ mL penicillin-streptomycin. All cell line were maintained in 5% CO₂ at 37C. Alkaline phosphatase staining was carried out using the Vector [®] Red Alkaline Phosphatase Substrate Kit (Vector Laboratories), according to manufacturer's instructions.

Transfections: mES were reverse-transfected using Lipofectamine RNAi MAX (Invitrogen), according to manufacturers instructions. siRNA against mouse Cdk1, Cdk2, Cyclin D1, Cyclin E1 and E2, Cyclin A, Cyclin B1 and B2 and a pool of nontargeting

control siRNA were purchased from Dharmacon (siGENOME SMART pool siRNA; Dharmacon).

Cell cycle/ cell death assays Mouse ES cell lines were treated with DMSO, 10 μ M purvalanol A, 9 μ M Ro-3306, 5 μ M Cvt-313 for 24 hours. After treatment cells were collected using trypsin. UCSF4 hES cells were dissociated with Accutase into single cells and plated onto matrigel-coated tissue culture plate at 125,000 cells per 1cm². Cells were allowed to grow to confluent with daily medium change. 42 hours after seeding, cells were treated with 10 μ M purvalanol A for 24 hours. For all cell types, cell viability was determined using a flow cytometry-based Guava ViaCount viability assay (Millipore, performed according to the manufacturer's instruction unless otherwise specified. For cell cycle analysis, treated cells were trypsinized, resuspended in mL of PBS, and subsequently fixed by drop wise addition of ice cold 70% EtOH and storage at -20C. Fixed cells were stained with propidium iodide to measure DNA content using standard protocols. Samples were analyzed on a LSRII flow cytometer (BD biosciences). Cell populations were gated to exclude doublets. Cell cycle distribution and sub 2N levels were determined using FlowJo (Tree Star Inc.) analysis software.

For bromodeoxyuridine (BrdU) incorporation experiments in adherent growth conditions, cells were treated with 5 μ M 1NM-PP1 or diluent (DMSO) for 72h, and then incubated with 10 μ M BrdU for 45 min. Cells were harvested using .05% trypsin , and fixed, DNase treated and dual stained with a FITC conjugated BrdU antibody and 7-amino-actinomycin D (7-AAD) (to determine DNA content), using the BrdU Flow Kit from BD Pharmingen™. Tumorspheres grown on ultra low adherent plates were exposed

to 10 μ M BrdU for 24 hours to account for a slower proliferation rate and dissociated to single cell suspension using .05% trypsin followed by mechanical dissociation using a 24 gauge syringe. Cells were fixed and stained using the same protocol for adherent cells. Cells were analyzed using an LSRII flow cytometer (BD biosciences), and percentages in G1, S, and G2/M were determined using FlowJo (Tree Star Inc.) analysis software. Cell death was accounted for by using 7-AAD staining to identify cells with less than 2N DNA content. The two-tailed paired Student t test was used to determine the differences between groups.

Microscopy/ immunofluorescence: Standard glass slides were prepared by centrifuging re-suspended using a cytopspin (Thermoscientific), at 790RPM for 5 minutes. Cells were fixed with 4% paraformaldehyde (Electron Microscopy Services) in phosphate-buffered saline (PBS) for 30 minutes, permeabilized with .05% triton x-100, blocked in 5% normal goat serum for 60 minutes and immunostained overnight at 4C for γ -H2AX (rabbit **polyclonal**, Sigma), phospho-ATM ser 1971(mouse monoclonal, Rockland), Oct4 (Rabbit polyclonal, Santa Cruz Biotechnology), or Nanog (Rabbit). Cells were incubated with AlexaFluor488 and AlexaFluor55 (Invitrogen) secondary antibodies. ProLong gold-containing DAPI (Invitrogen) was used to mount slides for image acquisition. Images were obtained using a Zeiss Axio Imager2 and images were quantified using MetaMorph[®] Microscopy Automation and Image Analysis Software.

***In vivo* Xenograft Studies:** For *in vitro* ES cell depletion studies, ES cells were differentiated using 1 μ M retinoic acid in Lif free media for 2 days. Cells were then treated for 24 hours with DMSO or 10 μ M purvalanol. After treatment, 10⁶ viable cells

cells in 200 μ l PBS were subcutaneously injected into immunodeficient female mice (BALB/c nude/nude) aged ~6–8 wk. Viable cell counts were determined using trypan blue staining and counting with a hemacytometer. Animals were monitored for tumor formation daily. For *in vivo* tumor prevention studies, immunodeficient female mice were subcutaneously injected with 2 day differentiated/ DMSO treated ES cells as described above. 72 hours after subcutaneous injections, animals were treated with either Cdk inhibitor at 50 mg/kg or vehicle alone (20% (2-Hydroxypropyl)-beta-cyclodextrin) via intra-peritoneal (IP) injection for four weeks. Animals were monitored for tumor formation daily. For tumor regression studies, immunodeficient female mice were subcutaneously injected with 2 day differentiated/ DMSO treated ES cells as described above. The tumors were allowed to grow to ~50–100 mm³ in volume before the animals were treated with either Cdk inhibitor at 50 mg/kg or vehicle alone via IP injection. After 4 weeks of treatment, tumors were collected, fixed in 4% paraformaldehyde (Electron Microscopy Services) and slides were prepared for hematoxylin and eosin staining. Slides were analyzed in a blinded study by a trained pathologist. All animal experiments were approved by the University of California San Francisco institutional animal care and use committee

Analysis of mRNA levels using quantitative PCR: Total RNA was isolated from cells using mirVana (Ambion) and digested with DNaseI to remove contaminating DNA (Ambion). For p53 pathway analysis array, cDNA was prepared from 400ng of total RNA using RT² First Strand Kit (SAB), and used for real time polymerase chain reaction (PCR) with a RT² Mouse p53 Signaling Pathway Profiler PCR Array (PAMM-027z).

Arrays were run on a Roche LightCycler 480II. Analysis was performed using SAB provided data analysis programs, according to manufacturer instruction. For all other mRNA analysis, cDNA was prepared from 500ng of total RNA using iscript reverse transcription kit (Bio-rad). Real-time PCR was performed using probes specific for mouse *Noxa*, *Puma* and *GapDH*(ABI), according to the manufacturer's instructions. Samples were run in triplicate on a Real-Time Thermal Cycler (Bio-Rad Laboratories). Variation of *Noxa* and *Puma* expression was calculated using the $\Delta\Delta CT$ method (Schmittgen, 2001) with *GapDH* mRNA as an internal control.

***In vitro* kinase assays:** Wild-type Cdk2 (Cdk2^{WT}) and analog-sensitive Cdk2 (Cdk2^{AS}) cDNAs were tagged at the 3' end with a hemagglutinin (HA) epitope and transfected into human embryonic kidney (HEK) cells. Equal amounts of Cdk2^{WT} or Cdk2^{AS} proteins were immunoprecipitated (IP) from whole cell lysates, with anti-HA antibodies, and kinase activity toward the histone H1 substrate assessed in the presence of increasing amounts of the 1NM-PP1 inhibitor as previously described (Bishop et al., 2000; Ubersax et al., 2003).

Generation of Cdk2^{WT} and Cdk2^{AS} cell lines: MEFs in which the Cdk2 allele is flanked by Lox-P recombination sites were provided by Dr. Mariano Barbacid's laboratory. Endogenous Cdk2 was deleted using recombinant retrovirus expressing Cre-recombinase. Human Cdk2^{WT} or Cdk2^{AS} alleles were introduced into Cdk2^{-/-} MEFs using retroviral transduction and selected for puromycin resistance. These cells were subsequently transformed by oncogenes c-MYC, HRASG12V, and v-ABL, via retroviral

transduction. HCT-116 Cdk2^{WT} and Cdk2^{AS} were generated by introducing human Cdk2^{AS} alleles into the Cdk2 locus using a recombinant adeno associated virus (AAV) homologous recombination strategy that is described in detail elsewhere (Merrick et al., 2011)

Cell proliferation assays: A total of 5×10^4 MEFs were plated onto six-well plates in triplicate and the time-course experiment was repeated five times. The cells were harvested at each time point and the cell number was counted using Guava ViaCount reagent (Millipore) according to the manufacturer's instruction.

Anchorage independent cellular proliferation assays: Anchorage independent proliferation was determined by soft agar colony growth and tumorsphere formation assays. For soft agar growth, MEFs and HCT116 cells were treated with 5 μ M 1NM-PP1 or DMSO for 72 hours. After 72 hours pretreatment, cells were seeded at a density of 5×10^5 cells per well in a standard 6-well culture dish in 0.32 % agar containing 5 μ M 1NM-PP1 or DMSO. Cells were cultured for 21 days at which point colonies were counted. The two-tailed student t-test was used to determine significant differences between groups. For tumorsphere formation assays, MEFs and HCT116 cells were seeded at a density of 3×10^3 or 1.2×10^4 cells per well of 24 well or 6 well ultra low-adherent plates (Corning), respectively. Cells were cultured between 10 and 14 days at which point cell quantification was performed for each well using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's instruction.

Protein lysates and western blotting analysis: Cultured cells were washed with ice-cold phosphate buffered saline (PBS) and harvested directly into radioimmunoprecipitation assay (RIPA) buffer (50mM Tris, 150mM NaCl, 0.5% sodium-deoxycholate, 1% Nonidet P-40, 0.1% SDS, 2mM EDTA, pH 7.5) containing COMPLETE protease inhibitor mixture (Roche) and phosphatase inhibitors (Santa Cruz Biotechnology). Protein concentrations were determined by performing Detergent-Compatible Protein Assay (Bio-Rad) using bovine serum albumin (BSA) as standard. Quantification of western blots was done using ImageJ (National Institutes of Health) densitometry analysis or a Bio-Rad ChemiDoc XRS+ Molecular Imager equipped with Image Lab software. The following antibodies were used for western analyses: β -tubulin (Bd Biosciences), laminin (Epitomics), p53-ser-18 (Cell Signaling, listed as p53-ser-15), Bax (Cell Signaling), Bak (Santa Cruz Biotechnology, Inc.), PP1- α (Epitomics), PP1- α (pT320; Epitomics), γ -H2AX (Bethyl), phospho ATM (Rockland) Cyclin D (cell signaling), MYC (Epitomics), β -Actin (Sigma), PARP (Cell Signaling), Rb (Clone 4.1., University of Iowa Hybridoma Bank), Oct3/4 (Santa Cruz Biotechnology), Nanog (Abcam), Phospho-Thr821 Rb (Invitrogen), Phospho-Thr821/826 Rb (E-10, Santa Cruz Biotechnology), Phospho-Ser 807/811 Rb (Cell Signaling), Cdk2 (D-12, Santa Cruz Biotechnology), Cdk1 (Santa Cruz Biotechnology), cyclin D1 (DCS6, Cell Signaling), cyclin E (Millipore), cyclin A (C-19, Santa Cruz Biotechnology), cyclin B1 (GNS1, Thermo Scientific), p21 (BD Pharmingen), p27 (BD Transduction Laboratory), p19ARF (Clone 5-C3-1, Millipore), p53 (CM5, Leica Microsystems), HA-tag (6E2, Cell Signaling), and ABL (BD Pharmingen). Nuclear and cytoplasmic fractionation was performed using a NE-PER Nuclear Protein Extraction Kit (Pierce).

Statistical Analysis

Data are expressed as the mean \pm standard error (S.E.M) from at least 3 separate experiments performed in triplicate, unless otherwise noted. Differences in groups were analyzed using a double-sided student's t test.

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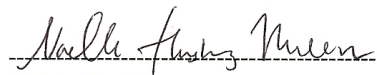
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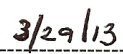
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