Pathways Linking Deregulated Proliferation to Apoptosis: a Dissertation

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Recommended Citation
A Dissertation Presented

By

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Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

April 29, 2004

Program in Immunology and Virology
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Parts of this dissertation have appeared in the following publications:


Pathways Linking Deregulated Proliferation to Apoptosis

A Dissertation Presented
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April 29, 2004
ACKNOWLEDGMENTS

I would like to thank my mentor Tim Kowalik for his guidance, support, and friendship during my graduate career. Joining Tim’s lab was possibly the best decision I made during my graduate career and provided me the best possible place to grow as a scientist. I would also like to thank the members of my committee, Michelle Kelliher, Stephen Doxsey, Maria Zapp, Michael Brodsky, and Karl Munger for their thoughtful advice on my research projects. I am also grateful to the members of the Kowalik lab for their contributions. Fiona Frame has been a scientific colleague and, most of all, a good friend. Fiona contributed Chapter V Figure 1B and Chapter V Figure 8. Michelle Debatis contributed Chapter III Figure 5. Mary Pickering contributed Chapter III Figure 4 and Chapter III Figure 12A. I would also like to thank Brad Stadler and former lab member Jon Castillo for their helpful scientific advice, for making the Kowalik lab an enjoyable place to work, and for their friendships outside of science.

I would like to thank my parents, Martin and Linda Rogoff, for their support during my years in graduate school. I would also like to thank the four legged members of the Rogoff household; Andy, Bada, Marley, Teddy Bear, and Chloe for bringing love and relaxation to my homelife. Most of all, thank you to my wife Tina, who has provided daily love and encouragement throughout my graduate career.
ABSTRACT

Proper regulation of cellular proliferation is critical for normal development and cancer prevention. Most, if not all, cancers contain mutations in the Rb/E2F pathway, which controls cellular proliferation. Inactivation of the retinoblastoma protein (Rb) can occur through Rb loss, mutation, or inactivation by cellular or viral oncoproteins leading to unrestrained proliferation. This occurs primarily by de-repression and activation of the E2F transcription factors, which promote the transition of cells from the G_{1} to S phase of the cell cycle. In order to protect against loss of growth control, the p53 tumor suppressor is able to induce programmed cell death, or apoptosis, in response to loss of proper Rb cell cycle regulation.

E2F1 serves as the primary link between the Rb growth control pathway and the p53 apoptosis pathway. While the pathway(s) linking E2F1 to p53 activation and apoptosis are unclear, it has been proposed that E2F1 activates p53-dependent apoptosis by transactivation of \( p19^{ARF} \) leading to inhibition of Mdm2-promoted degradation of p53. We tested this hypothesis, and found that \( p19^{ARF} \) is not required for E2F1-induced apoptosis. Instead, we find that expression of \( E2F1 \) leads to covalent modifications of p53 that correlate with p53 activation and are required for apoptosis.

The observation that E2F1 induces covalent modification of p53 is consistent with the p53 modifications observed following DNA damage. We therefore hypothesized that E2F1 may be activating components of the DNA damage response to activate p53 and kill cells. Consistent with the DNA damage response, we find that E2F1-induced
apoptosis is compromised in cells from patients with the related disorders ataxia telangiectasia and Nijmegen breakage syndrome, lacking functional Atm and Nbs1 gene products, respectively. E2F1-induced apoptosis and p53 modification also requires the human checkpoint kinase Chk2, another component of the DNA damage response. We find that the commitment step in E2F1-induced apoptosis is the induction of Chk2.

Having found that E2F1 requires DNA damage kinases to induce apoptosis, we next examined events upstream of kinase activation. To this end, we observe relocalization of the DNA damage repair MRN complex (composed of Mre11, Nbs1, and Rad50) to nuclear foci specifically following expression of E2F1. Expression of E2F1 also induces relocalization of the DNA damage recognition proteins γH2AX and 53BP1 to nuclear foci, consistent with the location of these complexes observed following DNA double strand breaks. As a consequence of activating some or all of these DNA damage signaling proteins, expression of E2F1 blocks cell cycle progression in diploid human fibroblasts. The observed block in cell cycle progression is found to be, in part, due to activation of a p21-dependent cell cycle checkpoint.

The E7 protein from the oncogenic human papillomavirus (HPV) is able to bind to and inactivate members of the Rb family. HPV infects quiescent, non-cycling cells that lack expression of DNA replication machinery that is essential for replication of the viral genome. By expression of the E7 protein, HPV is able to bypass normal Rb-mediated growth control and induce quiescent cells to enter S phase where the host cell DNA replication enzymes are present for viral replication. We find that expression of E7 can also result in apoptosis that is dependent specifically on E2F1. Additionally, E7-induced
apoptosis, like E2F1-induced apoptosis, requires Atm, Nbs1, and Chk2. Expression of E7, like that of E2F1, induces E2F1-dependent covalent modification of p53 that correlates with apoptosis induction.

These findings demonstrate that deregulation of the Rb/E2F growth control pathway leads to activation of an apoptosis program with some similarity to the pathways activated by DNA damage. Our observations suggest that E2F1 not only functions as a sensor for deregulation of Rb, but may also play an important role in regulating cellular growth control in response to other oncogenic stimuli.
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CHAPTER I

INTRODUCTION
A. Cell Cycle

The cell cycle is a tightly controlled sequence of events in which genetic material is precisely copied so that each daughter cell will receive an identical copy of the genetic material following cell division. The eukaryotic cell cycle is divided into mitosis (M phase), the phase in which chromosomes are segregated and cell division occurs, and an interphase period that occurs between subsequent mitosis. Interphase consists of a gap phase that occurs following cell division and prior to DNA replication known as the G1 phase. DNA is then replicated during S phase, and is followed by another gap phase, G2, prior to cells re-entering mitosis. Many cells in the body reach a state of terminal differentiation where they will exit from the cell cycle following mitosis and enter a resting, or G0, state (Figure 1.1). Malfunctions during the cell cycle, including errors in DNA replication, spindle formation, cytokinesis, or DNA damage can result in activation of a cell cycle checkpoint to allow for repair to take place so that mutations are not passed to daughter cells (365).

B. Cell cycle regulation

1. Cyclins and cyclin dependent kinases

Precise control of the cell cycle is necessary for cells to grow and divide at the proper time and to ensure passage of properly replicated DNA to daughter cells. Multiple levels of regulation are present to ensure that the cell cycle will not advance to the next phase if the cellular or environmental conditions are not conducive for cell cycle progression. Progression from one phase of the cell cycle to the next is achieved mainly
by the action of cellular cyclins and cyclin-dependent kinases (Cdk) (Figure 1.2). Cdk are a family of serine-threonine protein kinases that control progression of the cell cycle by phosphorylating target proteins at specific times (272, 318). Cdk protein levels remain constant during the cell cycle, so their activity is regulated by expression of the cyclin family of proteins (281, 318, 319). Timed expression and rapid ubiquitin mediated proteolysis limits cyclin expression to specific intervals of the cell cycle, limiting the activity of the Cdk to certain phases of the cell cycle (102, 126, 281, 282, 330). Beginning in G₁, the cyclin D family (consisting of cyclin D1, D2, and D3) forms a complex with Cdk4 or Cdk6. The cyclin D-Cdk4/6 complex begins the cell's entry into S phase primarily by phosphorylation of the retinoblastoma (Rb) family of proteins (364). This is followed by cyclin E-Cdk2 activity and additional Rb phosphorylation pushing the cell from G₁ into S phase (298). The complex of cyclin A and Cdk2 is required for cells to progress through S phase (121, 422). In the G₂ and M phases of the cell cycle, cyclin A forms a complex with Cdk1, and this complex is required for transition from G₂ to M (446). Mitosis is then further regulated by activity of the cyclin B-Cdk1 complex (11, 200) (Figure 1.1).

2. Cdk inhibitors

Additional layers of Cdk regulation take the form of cyclin dependent kinase inhibitors and protein localization. Cyclin dependent kinase inhibitors play important roles in coordinating proliferation during normal development and differentiation, as well as during cellular stress (63, 223). Cyclin dependent kinase inhibitors can be divided into
the INK4 family and the Cip/Kip family (367). The INK4 family, including p16\textsuperscript{INK4a}, target Cdk4 and Cdk6 and prevent their association with cyclin D (45). Without cyclin D association, Cdk4/6 remain inactive and the cell cycle is unable to progress through G\textsubscript{1} (325, 360). The Cip/Kip family of cyclin dependent kinase inhibitors, including p21 and p27, are able to inactivate multiple cyclin-Cdk complexes and are able to block cell cycle progression in G\textsubscript{1} and, to a lesser extent, S and M phases of the cell cycle (139, 150, 221, 320). Unlike p16, p21 and p27 promote a stable association between cyclin D and their Cdk partners (215). Following cellular growth signals, increased levels of cyclin D-Cdk4 complexes will titrate p21 and p27 from cyclin E-Cdk2 complexes allowing for progression through G1 (32). The localization of both Cdk activators and inhibitors also contributes to proper cell cycle progression. For example, to prevent inappropriate mitosis the cyclin B-Cdk1 inhibitor Wee1 is kept in the nucleus, while the cyclin B-Cdk1 activator Cdc25 is sequestered in the cytoplasm prior to M phase (144, 237, 312, 451).

3. The retinoblastoma protein family

In the late G\textsubscript{1} phase of the cell cycle, cells must pass through the G\textsubscript{1} restriction point in order to enter into S phase and begin DNA replication (307). Passing the G\textsubscript{1} restriction point means that the cell can continue through the remainder of the cell cycle and back into G\textsubscript{1} without additional growth factor requirements (307, 363). The G\textsubscript{1} restriction point is controlled by the retinoblastoma (Rb) family of proteins. The Rb family consists of three related genes; \textit{pRb} (known as Rb), \textit{p107}, and \textit{p130} (387). Rb was originally identified due to homozygous loss of the \textit{Rb} allele that predisposes children to
Figure 1.1. Cyclin/Cdk regulation of the cell cycle.

Eukaryotic cells replicate DNA during S phase and divide during mitosis (M phase). Separating the S and M phases of the cell cycle are two gap phases, G₁ and G₂. Activity of cellular cyclins and cyclin dependent kinases (Cdk) regulate progression from one phase of the cell cycle to the next. Sites of activity of Cyclin-Cdk complexes during the cell cycle are depicted.
development of retinoblastoma, a malignant tumor of the retina (116, 118, 203, 224). p107 and p130 have been identified more recently, and have structural and functional similarities to Rb (59, 105, 120, 135, 355, 414, 456, 461). Together, Rb, p107, and p130 are known as the pocket proteins due to a large, conserved protein-protein interaction domain (58, 220). Studies of Rb family knockout mouse models and cells from those mice reveal some functional overlap between family members in regards to development (62, 222) and cell cycle regulation (67, 345). Rb family members also play important roles in terminal differentiation (67), however, the role of individual Rb family members in differentiation is unclear and may depend on the experimental system analyzed (44, 61, 293, 334, 352, 453).

The Rb family function mainly as negative regulators of cell cycle control. During G₁, the Rb family are in an active, unphosphorylated state (429). In this state, the pocket proteins are able to interact with transcription factors, such as the E2F family, that control the entrance of the cell into S phase (4, 429). While bound to Rb, the E2F family are unable to transactivate the expression of genes required for S phase (4, 429). A major function of the Rb family of proteins is to repress transcription from E2F regulated promoters (5, 89, 109, 114). At the G₁ restriction point, the Rb family becomes sequentially phosphorylated by the cellular cyclin-Cdk complexes (225). D type cyclins complexed to either Cdk4 or Cdk6 phosphorylate Rb during G₁ (104, 193). This is followed by cyclin E-Cdk2 phosphorylation leaving Rb in a hyperphosphorylated state (154, 206, 230). In this state, the Rb family is unable to bind to and inhibit activity of the S phase promoting E2F family members (55) (Figure 1.2).
Figure 1.2. Rb/E2F regulation of the cell cycle.

Rb/E2F complexes regulate entry of cells from the G₁ to S phase of the cell cycle. Prior to the restriction point (R), Rb/E2F complexes repress E2F regulated promoters, preventing entry into S phase. Phosphorylation of Rb at the restriction point by cyclin/Cdk complexes promotes dissociation of Rb and E2F, allowing expression of E2F regulated genes.
4. The E2F family

The Rb family controls cell cycle progression mainly through their ability to interact with the E2F family of transcription factors. E2F was first identified as a cellular transcription factor capable of binding to two consensus E2F “TTCGCGC” sites within the adenovirus E2 promoter (208, 209, 286). Human E2F1 was later cloned by screening cDNA libraries for proteins that interact with the pocket domain of Rb (148, 186, 362). Many cellular genes regulated by the E2F family have been identified including genes involved in controlling cell cycle regulation, DNA synthesis, and DNA replication. These genes include cyclin A, cyclin E, cyclin D1, dihydrofolate reductase (DHFR), thymidine kinase (TK), DNA polymerase α, and ORC1 (382). E2F homologs have been identified in mouse (232), Drosophila melanogaster (297), yeast (249, 418), Xenopus laevis (315, 391), and plants (326, 359).

To date, seven members of the human E2F family have been described (69, 81, 404) (Figure 1.3). The E2F family can be subdivided into categories based on their ability to interact with the Rb family and on their transcriptional activity. E2F1, E2F2, and E2F3a are potent activators of transcription and interact mainly with Rb (3, 148, 149, 174, 183, 226, 227, 324). E2F4, and E2F5 are transcriptional repressors, and interact primarily with the Rb family members p107 and p130 (26, 153, 171, 270, 347, 414). The functions of the remaining members of the E2F family remain somewhat unclear. E2F3b, encoded by an alternate transcript from the E2F3 locus, interacts with Rb and may function as a transcriptional repressor in quiescent cells (227). E2F6 shares little homology with the other E2F family members outside of the DNA binding domain, does
not interact with any of the Rb family members, lacks a transactivation domain, and is reported to repress transcription by interaction with polycomb proteins (275, 403). Likewise, E2F7 is unable to interact with Rb proteins, lacks a transactivation domain, and appears to act as a transcriptional repressor (69, 81).

In cells, E2F proteins act in conjunction with their heterodimeric binding partners, the DP family of proteins. The DP family consists of DP1 and DP2. DP1 was first identified as a ubiquitously expressed binding partner for E2F1 that enhanced the transactivation ability of E2F1 (18, 125, 149, 213). DP2 shares high homology to DP1, and is also acts as an E2F binding partner that can enhance the transactivation ability of E2F family members (299, 435, 458). Unlike DP1, DP2 is expressed as tissue specific splice variants (72, 299, 339, 458). In vitro, all combinations of E2F-DP heterodimers can form and recognize similar E2F consensus binding sites (38, 226, 458). The large number of E2F-DP combinations possible in cells suggests that different heterodimers may differentially regulate genes. While some evidence of this exists (398), the role of the different E2F-DP heterodimers in regulating gene expression remains unclear.

5. Importance of the E2F family in growth control

The important and unique roles for the E2F proteins have become partially realized through genetic studies in mice. Cells from mice lacking E2F1 display limited cell cycle defects except for a prolonged G0 phase, suggesting that E2F1 may have a role in timing S phase entry from quiescence (113, 425, 449), while cells lacking E2F2 show little defect in proliferation (228). Cells from mice lacking E2F3 exhibit a delayed cell
Figure 1.3. Schematic representation of the E2F family.

The E2F family consists of seven members, distinguished by their transactivational ability and preference for association with Rb family members. E2F1, E2F2, and E2F3a are potent activators of transcription, and interact mainly with Rb. E2F4 and E2F5 are transcriptional repressors, and interact preferentially with p107 and p130. E2F6 and E2F7 lack Rb family binding and transactivation function, and may function as transcriptional repressors. Shaded regions represent conserved domains.
cycle, failure to upregulate many E2F responsive genes in response to mitogen stimulation, centriole duplication, and premature centriole separation (167, 343). However, cells from mice lacking all three activator E2Fs show severe proliferative defects and fail to induce E2F target genes that are critical for the transition from G1 to S (438). Cells lacking E2F4 or E2F5, or both, do not display the cell cycle defects or loss of gene expression associated with loss of E2F3 (166, 331). However, loss of E2F4 or E2F5 does result in a failure to properly exit from the cell cycle in response to growth inhibitory signals such as expression of p16\textsuperscript{INK4a} (37, 166, 331).

E2F family members also play a role in the regulation of differentiation, or exit from the cell cycle. Repression of E2F1 regulated promoters is essential to promote and maintain exit from the cell cycle in various cell types (82, 424). Mutation of Rb in some cell types can lead to re-entry into the cell cycle and uncontrolled proliferation and an increase in apoptosis (60, 179, 219). Repression of E2F1 in some cell types is accomplished by the activity of the C/EBP\(\alpha\) transcription factor (322). Active repression of E2F regulated genes by a complex composed of p130 and E2F4 or E2F5 is also essential for maintaining the differentiated state of some cell types (28, 274, 322). Taken together, E2F family members can have different roles depending on the cells state of differentiation.

6. Rb/E2F regulation of cell cycle progression

Cellular growth control by the retinoblastoma protein family occurs primarily through Rb-mediated repression of E2F regulated promoters and active transcriptional
repression by Rb/E2F complexes. While it is unclear which Rb/E2F function is most important in vivo, both mechanisms have been shown to occur in vitro. Rb inhibition of E2F-mediated transactivation occurs by virtue of Rb binding to the E2F transactivation domain and inhibiting transcriptional activation of E2F responsive promoters (Figure 1.4 A) (114, 137, 147, 341). The importance of E2F repression by Rb is demonstrated by the observations that ectopic expression of E2F1, E2F2, or E2F3 is sufficient to drive quiescent cells into S phase (76, 183, 211).

Rb can also actively repress E2F-mediated transcriptional activation by recruitment of chromatin remodeling enzymes, including HDACs, SWI/SNF complexes, and methyl transferases (136, 137, 290, 417, 443). HDACs, or histone deacetylases, are a family of enzymes involved with the removal of acetyl groups from histone tails that promotes chromatin condensation (201, 207) and have been shown to be associated with transcriptional repressor complexes (8, 130, 140, 141, 145). HDAC1 and HDAC2 contain LxCxE motifs, allowing them to bind to Rb at the LxCxE binding site, while HDAC3 lacks the LxCxE motif and binds to Rb at another location (34, 243, 247). E2F family members bind to Rb at a site distinct from the LxCxE motif and HDAC3 binding site, allowing complexes containing HDAC1, HDAC2, or HDAC3, Rb, and E2F to form. Interaction between Rb and HDACs is at least partially responsible for Rb-mediated repression of E2F responsive promoters (34, 216, 243, 247, 457). Additionally, recruitment of HDACs to Rb/E2F repressor complexes serves to inhibit the function of the histone acetyl transferases (HAT) p300/CBP that is recruited to promoters by interaction with E2F1 (405). HATs function by adding acetyl groups to histone tails,
relaxing chromatin structure, and allowing transcriptional machinery access to promoters (201). By recruitment of HDACs to corepressor complexes, Rb is able to actively repress E2F-mediated transcription by altering chromatin structure (Figure 1.4 B). Repressor complexes containing p107 and p130 have not been characterized as thoroughly as Rb repressor complexes, but studies have shown association of p107 and p130 with HDAC proteins (112, 169, 194, 386).

Active repression by Rb also occurs by recruitment of ATP-dependent SWI/SNF complexes. Rb recruits SWI/SNF complexes by interaction with the ATPase components of the complexes, Brg1 and Brm (87, 375, 389). Similar to HDAC complexes, repressor complexes containing Brm, Rb, and E2F can form at promoters containing E2F binding sites (406). Dominant-negative ATPase mutant Brg1 or Brm can block Rb-mediated growth suppression (87, 388) and expression of Brg1 in cells lacking Brg1 and Brm results in a growth arrest that is dependent on interaction with Rb (87). Additionally, expression of p16\textsuperscript{NK4a} induces a Rb-dependent growth arrest that requires functional Brg1 (434, 457).

The formation of multiple Rb/E2F repressor complexes during the G\textsubscript{1} phase of the cell cycle prevents expression of S phase promoting genes and thus prevents entry into S phase. However, different Rb/E2F complexes also form at promoters during the different phases of the cell cycle. Most cell cycle regulated promoters bind both E2F1,2,3/Rb complexes and E2F4,5/p107 or p130 complexes. The use of chromatin immunoprecipitation (ChIP) allows analysis of promoter occupancy during distinct phases of the cell cycle and has provided a picture of the dynamic regulation by Rb/E2F
complexes (329, 395, 431). Regulation of the b-myb gene is an example of expression by derepression. The b-myb promoter is occupied by an E2F4/p107 repression complex during G1. During S phase, the E2F4/p107 complex dissociates and E2F4 no longer binds to the b-myb promoter allowing expression of b-myb during S phase (395, 431). Regulation of the dihydrofolate reductase (DHFR) promoter is an example of transcriptional activation. The DHFR promoter is occupied in G0 and G1 by an E2F4/p107 repression complex. As cells progress from G1 into S phase, the complex is replaced by an E2F1/Rb complex, and following hyperphosphorylation of Rb, free E2F1, allowing expression of DHFR in early S phase (Figure 1.4 C). This complex is quickly replaced in late S phase with an E2F4/p107 repression complex (431). Consistent with their proposed roles as activator E2Fs, E2F1, 2, and 3 occupy many E2F responsive promoters during S phase, while E2F4 is the predominant E2F occupying these same promoters during G0 and G1. During S phase, some of the promoters tested also showed preference to binding by E2F1, E2F2, or E2F3, such as DHFR, TK (thymidine kinase), and Cdc6, while other promoters such as p107, and Cdc2, showed no preference for E2F binding (329, 395, 431).

7. Rb family independent regulation of E2F

In addition to regulation by the Rb family of proteins, the E2F family is also subject to Rb independent regulation. The majority of information regarding E2F regulation has come from studies of the E2F1 family member. The association of E2F1 with Rb during G1 serves to protect E2F1 from degradation. When Rb becomes
Figure 1.4. Models of Rb/E2F regulation of E2F regulated promoters.

(A) Binding of Rb/E2F complexes to E2F regulated promoters represses E2Fs transactivation function. (B) By recruiting chromatin remodeling enzymes such as HDACs or SWI/SNF complexes to promoter regions, Rb is able to actively repress transcription. (C) Dissociation of Rb from E2F allows expression from E2F regulated promoters.
phosphorylated as cells enter into S phase, E2F1 becomes acetylated by p300/CBP, enhancing E2F1 stability and DNA binding activity (253). As cells proceed into mid S phase, the cyclin A/Cdk2 complex physically associates with E2F1 at a conserved N-terminal domain (212, 310, 444). Subsequent phosphorylation of both E2F1 and the DP heterodimeric binding partner by the cyclin A/Cdk2 complex at the S to G2 phase transition reduces E2F1 DNA binding ability and promotes E2F1 degradation by the proteasome (131, 252).

Because of the ability of E2F1 to drive cells into the cell cycle, E2F1 protein levels must be very tightly controlled. When Rb dissociates with E2F1 at the G1 to S transition, E2F1 protein begins to be degraded by the ubiquitin-proteasome pathway (42). The degradation process is initiated by the human p14ARF (mouse p19ARF) protein that binds to the C-terminus of E2F1 and recruits the p45SKP2 ubiquitin protein ligase (25, 251). This leads to poly-ubiquitination of E2F1 and degradation by the proteasome. Additionally, in response to some stimuli, E2F1 can be phosphorylated by the ataxia telangiectasia mutated (Atm) kinase and the human checkpoint kinase, Chk2 (235, 383). These phosphorylation events stabilize E2F1 protein, possibly by interfering with p45SKP2 binding, and can also alter E2F1 DNA binding specificity (383).

8. E2F regulated genes

The role for E2F in regulating genes involved in the G1 to S phase transition, S phase progression, and DNA replication has been confirmed by numerous microarray studies. Genes induced by E2F expression include genes involved in S phase promotion
such as cyclin E, genes required for the synthesis of DNA such as ribonucleotide reductase, thymidine kinase, and DNA polymerase α, and genes involved in assembly of pre-recognition complexes at origins of replication including those encoding Orc proteins, Cdc6, and Mcm proteins (173, 187, 244, 279, 332, 380). Microarray analysis has also identified additional classes of genes that are regulated by E2F proteins. These include genes involved in DNA damage recognition, DNA repair, and apoptosis (279, 332). The findings that E2F proteins regulate genes encoding DNA damage recognition and repair enzymes is not surprising given that DNA replication is an error prone process. Additionally, in response to DNA damaging agents, E2F1 is upregulated, providing a mechanism for increased expression of DNA damage recognition and repair enzymes (279). E2F1 can also downregulate expression of genes encoding anti-apoptotic factors (314), and upregulate expression or pro-apoptotic factors (279). The ability of E2F proteins to regulate such a diverse set of genes suggests that E2F function is crucial to many biological pathways, although it remains unclear as to how E2F regulation may segregate by biological pathway.

C. Deregulation of the cell cycle

1. Deregulation of the cell cycle by small DNA tumor viruses

Much of what is known regarding cell cycle regulation originally came about through studies of the small DNA tumor viruses. Small DNA tumor viruses, including adenoviruses, human papillomaviruses (HPV), and polyomaviruses such as SV40 are dependent on host cell DNA replication machinery for replication of viral genomes.
However, the small DNA tumor viruses replicate in terminally differentiated, non-dividing cells that lack the required DNA replication machinery. In order to replicate, a virus must push cells into S phase where host cell DNA replication machinery is available for replication of viral genomes (39, 65, 78).

The induction of S phase by the small DNA tumor viruses is accomplished by virally encoded proteins that inactivate members of the Rb family of proteins. Each of the small DNA tumor viruses accomplishes this through a conserved LxCxE motif that binds and inactivates Rb family members. The adenovirus E1A protein contains an LxCxE motif and binds with high affinity to all three members of the Rb family of proteins (95, 138, 231, 260). By binding to Rb family members, E1A disrupts the association of Rb/E2F complexes allowing expression of E2F responsive promoters, resulting in induction of S phase (12, 16, 19, 95, 138, 163, 231, 260, 328, 455). HPV encodes the LxCxE motif containing E7 protein that also binds to and inactivates Rb family members resulting in increased E2F activity and deregulation of the cell cycle (54, 80, 302, 436). Expression of E7 also results in degradation of Rb family members by the proteasome, providing another mechanism to bypass cellular growth control (27, 33, 128). The polyomavirus SV40 encodes the large T antigen that is also capable of binding to Rb family members and blocking Rb-mediated G1 growth arrest (74, 90, 103, 432, 454).

2. Cell cycle deregulation and cancer

Long before the mechanisms of cell cycle deregulation by the small DNA tumor viruses were known, these viruses were found to be associated with tumor formation.
Adenovirus was the first human virus identified to induce tumors in an animal model (402). HPV is capable of inducing tumors in humans, and may be the causative agents in most cervical cancers (31, 88, 464, 465). SV40 was first isolated from monkey kidney cells (392) and is capable of transforming some rodent cell types (92, 122-124, 399, 400). There is also some evidence that SV40 may be associated with some human cancers (202). In all cases, the small DNA tumor viruses require the function of the oncogenes E1A, E7, or large T for efficient transformation (79, 93, 94, 180, 313, 344).

In addition to virus-associated transformation, cancer often results from cellular mutations that result in loss of normal growth control and lead to unrestrained proliferation. The high frequency of cell cycle regulators mutated in human cancers demonstrates the importance of proper cellular growth control for the prevention of cancer (363). The cell cycle mutations most often observed in human cancers include overexpression of cellular cyclins, loss of cyclin dependent kinase inhibitors, and loss of Rb either by mutation, deletion, or inactivation (262).

Loss or mutation of cellular Cdk5, although rare, has been associated with some tumors. Cdk4 amplification and Cdk4 and Cdk6 mutations have been identified in melanomas, sarcomas, and gliomas (91, 433). CdkI and Cdk2 overexpression has also been reported in a subset of colon cancers (198, 447). Occurring more frequently in human tumors is the overexpression or deregulation of cellular cyclins. Overexpression of cyclin D1 often occurs as a result of a gene translocation that puts the cyclin D1 gene under the control of the gene encoding the immunoglobulin heavy chain (133, 278, 430). Cyclin D2, cyclin D3, cyclin A, and cyclin E have all been reported to be amplified or
overexpressed in some tumors, and elevated levels of *cyclin E* expression may correlate with shorter survival (85, 170, 197, 218, 357).

In addition to alterations in expression of Cdns and cyclins, mutations in both Cdk activators and inhibitors occur in human cancer. Deregulation or overexpression of the Cdk activating enzyme Cdc25 results in deregulated activation of cyclin-Cdk complexes, entry into S phase, and is associated with tumor formation. Cdc25A and Cdc25B are activated by c-myc, an oncogene that is frequently mutated in tumors, and are often overexpressed in breast cancer (119, 291). Loss or mutation of the cyclin dependent kinase inhibitors *p16, p27,* and *p21* leads to unscheduled inactivation of Rb. Mutations in cyclin dependent kinase inhibitors often correlates with tumor aggressiveness and serves as a marker for poor patient prognosis (100, 188, 369).

The Rb family of tumor suppressors is one of the most frequently studied cell cycle regulators due to its involvement in viral and mutation-induced tumors. As discussed earlier, the Rb protein is the target of many viral oncoproteins that deregulate the cell cycle in order to provide a conducive environment for viral replication. In human tumors, the Rb protein is mutated in retinoblastoma, lung cancer, and acute lymphoblastic leukemia (162, 410). Moreover, it is estimated that greater than 90% of human tumors contain mutations in the Rb growth control pathway (133). While mutations in the Rb growth control pathway are common in human tumors, mutations in E2F family members are rarely found in human tumors. *E2F* expression has been reported to be either reduced or amplified in different tumor types (99, 106, 110, 157, 177). The lack of direct E2F
family mutations in human tumors suggests that cells must maintain a certain level of E2F proteins in order to properly grow and avoid apoptosis (discussed below).

D. Apoptosis

1. Overview of apoptosis

Cell death has long been observed as a function of normal development (248). The observation that cells appeared to be destined to die at specific times during development was proposed in 1965 by Lockshin and Williams and termed programmed cell death (239). The term apoptosis, Greek for falling of leaves, was coined in 1972 to describe the morphological changes associated with programmed cell death (195, 196). Apoptosis is characterized morphologically by cellular shrinking, condensation of the chromatin, nucleosomal DNA fragmentation, convolution of the plasma membrane known as blebbing, and formation of the dying cells into apoptotic bodies that are surrounded by an intact plasma membrane (415, 442). Since the initial characterization of the phenomenon of apoptosis in the 1970s, there has been extensive work done to determine its biological importance and underlying molecular mechanisms. The apoptosis program has been found to be vital for normal development, for maintaining tissue homeostasis, and for proper function of the immune system. Deregulation of the apoptosis program can lead to disastrous consequences such as neurodegeneration, autoimmunity, and in its absence, cancer.

2. The p53 tumor suppressor
The p53 protein plays an essential role in mediating both cellular growth arrest and apoptosis in response to diverse environmental stimuli. The importance of p53 in maintaining genomic stability is suggested by findings that greater than 50% of human tumors contain mutations in the \textit{p53} gene (159). Activation of p53 was first observed in cells exposed to ultraviolet radiation (250). It was then demonstrated that p53 was essential for mediating a cellular growth arrest in response to irradiation or DNA-damaging agents (192). The role of p53 in apoptosis induction was discovered by the introduction of p53 into a p53 deficient cell line (452). The ability of p53 to induce apoptosis was later shown to correlate with the ability of p53 to act as a tumor suppressor (393). Depending on the cellular context or duration or extent of DNA damage, p53 can induce a cellular growth arrest, primarily through transactivation of the \textit{p21} promoter, to allow for repair of damaged DNA or induce apoptosis (168, 192, 229). Consequently, p53 has become known as a sensor of genomic stability due to its ability to respond to damaged DNA, hypoxia, nucleotide depletion, viral oncogene expression, and other genotoxic stresses (323).

3. Apoptotic targets of p53

The apoptosis program has been divided into two major pathways, the intrinsic pathway and the extrinsic pathway (2). Much of the data involving p53-mediated apoptosis have linked p53 to activation of the intrinsic, or cellular stress pathway. In the apoptosis pathway, the p53 protein functions as a sequence specific transcription factor (204). Activation of the apoptosis program by p53 has been linked to its transactivation
function (15, 57, 181, 349). Perhaps the most important proapoptotic transcriptional targets of p53 in the intrinsic pathway are members of the Bcl-2 family. Transactivation by p53 of the Bcl-2 family members Bax, Bid, Puma, and Noxa ultimately leads to permeabilization of the outer mitochondrial membrane, release of proapoptotic factors from the damaged mitochondria, activation of cysteine proteases known as caspases, and apoptosis (2, 214, 269, 284, 295, 350, 368). p53 also transactivates components of the apoptotic machinery, including Apaf1 and the effector caspase, caspase-6. Apaf1 helps to initiate the caspase cascade by activating caspase-9 (190, 276, 336), while an increase in caspase-6 protein levels may sensitize cells to release of cytochrome-c from mitochondria (245).

The extrinsic apoptosis pathway involves activation of cell surface death receptors by their appropriate ligands, resulting in caspase activation and apoptosis (14). The contribution of p53 to apoptosis resulting from activation of the extrinsic pathway remains unclear. p53 may elevate sensitivity to death receptor signaling by transactivation of genes encoding cell surface receptors or ligands. Direct transcriptional targets of p53 include Fas and Fas ligand, and studies using mutant p53 constructs have proposed a role for p53 in upregulation of these receptors as a means of promoting apoptosis in tumor cells (129, 280, 301).

In addition to the two major apoptotic pathways, p53 is able to induce many apoptotic genes that are not directly associated with these pathways. These genes include p53Aip1, which is able to disrupt mitochondrial function and induce apoptosis in some tumor cell lines (257, 296). PTEN is another transcriptional target of p53 that plays an
important role in p53-mediated apoptosis (378). PTEN does not act as a direct inducer of apoptosis, but instead acts to inhibit the antiapoptotic protein Akt. Akt is a kinase that inhibits cell death by phosphorylating numerous targets. PTEN functions as a phosphatase, preventing activation of Akt and the subsequent cellular survival signals (420).

4. p53 activation

In response to cellular stressors, p53 is covalently modified, including phosphorylation at numerous N- and C-terminal serine residues and acetylation on C-terminal lysine residues (10). While it remains unclear as to which posttranslational modifications are required for p53 activation under specific cellular stressors, these modifications play an important role in mediating p53 function. p53 modifications have been shown to increase transactivation function, increase protein stability, alter protein-protein interactions, and even change promoter specificity (10) (Figure 1.5).

Several cellular kinases have been identified that play critical roles in the activation of p53 following DNA damage, the best studied of the stress responses that activates p53. These kinases include Atm, the kinase mutated in ataxia telangiectasia (AT), Atr, the Atm and Rad3 related kinase, and their downstream kinase substrates, checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2) (1, 372). The role of Atr in regulating the p53 response to DNA damage is not well understood due in part to the early embryonic lethality of atr−/− mouse embryos (36, 71). However, it has been proposed that Atr is activated in response to certain types of DNA damage and can
phosphorylate p53 on serine 15 (43, 134, 199, 217). Atr is also able to phosphorylate and activate Chk1 (132, 146, 289), which can subsequently phosphorylate the N-terminus of p53 (370).

The role of Atm in activating p53 following DNA damage is better understood. In response to γ-irradiation or genotoxic drugs that induce DNA double strand breaks, Atm is activated and can directly phosphorylate p53 at serine 15 (191). In cells from AT patients, there is a delay in activation of p53 following γ-irradiation (374). In addition to directly phosphorylating p53, Atm can phosphorylate and activate the human checkpoint kinase Chk2 (35, 52, 258, 259, 264). Chk2 is able to further phosphorylate p53 at additional N-terminal serine residues including serine 15 and serine 20, causing increased p53 stability and transcriptional activation (53, 155, 370). The importance of Chk in this pathway is demonstrated in dominant negative Chk2 expressing cells (53) and Chk2 deficient mice, which exhibit a defect in apoptosis and a decrease in p53 stabilization in response to γ-irradiation (396).

5. Deregulated proliferation and p53

Inactivation of Rb by cellular or viral oncoproteins or loss of Rb will stimulate cells to bypass G0/G1 and enter S phase (96, 101, 273, 286). Ectopic expression of viral oncogenes, such as the high risk HPV-16 E7 protein, can also lead to the induction of apoptosis (164, 304). The ability of E7 to induce apoptosis is dependent on its ability to interact with Rb (304). E7-induced apoptosis can be inhibited by expression of the viral
Figure 1.5. Model of p53 activation following cellular stress.

The Atm and Atr kinases are activated by certain forms of DNA damage or replicative stress. Atm and Atr can directly phosphorylate p53 at serine 15 as well as phosphorylate and activate Chk1 and Chk2. Chk1 and Chk2 can subsequently phosphorylate serine 20. These modifications may stabilize p53 by promoting interaction with p300/CBP and inhibiting interaction with Mdm2. Additional kinases and acetyl transferases (shown in green) are activated in response to certain types of stress and may be important in increasing p53 stabilization and transactivation function.
protein E6 (304). The HPV E6 protein is able to bind to and promote the degradation of p53 (351). The studies with HPV and other small DNA tumor viruses suggest a link between Rb/E2F cell cycle control and p53-mediated apoptosis. There is evidence that ectopic expression of various E2F family members can induce both S phase progression and apoptosis depending on the levels of expressed cDNA or cell type context (56, 152, 211, 324, 419, 440, 462). However, in fibroblasts, exogenous expression of E2F1, but not E2F2 or E2F3, causes apoptosis (76, 210).

The link between E2F1 and p53 dependent apoptosis has been demonstrated by genetic studies in mice. Apoptosis observed in transgenic mouse models expressing a fragment of the SV-40 large T antigen and in the central nervous system of Rb-/- mouse embryos is p53 dependent and associated with E2F1 (246, 305, 408). Expression of an E2F1 transgene in the skin of K5E2F1 transgenic mice results in increased proliferation and p53 dependent apoptosis (316). Additionally, mice deficient for E2F1 have an excess of mature T cells due to a defect in thymocyte apoptosis (113).

Expression of E2F1 leads to an accumulation of p53 protein (152, 211). A potential mechanism for the stabilization of p53 observed following expression of E2F1 is activation of the p19ARF/Mdm2 pathway. Expression of E2F1 has been shown to transactivate the expression of the mouse p19ARF and human p14ARF promoters (25, 172, 335). p19ARF encodes a protein that modulates the activity of Mdm2 (321, 459). Mdm2 is an E3-like ubiquitin ligase that regulates the stability of p53 by promoting its degradation by the proteasome (115, 117, 142, 160). By inhibiting Mdm2 activity, p19ARF allows for stabilization, activation, and accumulation of p53 protein (321, 459). Thus, it has been
hypothesized that p19ARF is a key protein linking the Rb/E2F and p53 pathways (25, 189). One hypothesis for how aberrations in the Rb/E2F pathway are recognized by p53 is that de-regulated E2F expression activates p53 by inducing p19ARF.

D. DNA damage and E2F

1. DNA double strand break recognition

Activation of p53 often occurs as a result of damaged DNA. The p53 activation pathways begin when specialized proteins recognize a DNA break. The DNA damage sensors are sensitive enough to recognize a single double strand break, an important task for maintaining genomic integrity and preventing inactivation of critical genes or chromosomal abnormalities (333, 416). The double strand break repair pathway is divided into two major pathways, non-homologous end joining (NHEJ) and homologous recombination (HR). The NHEJ pathway is the predominant form of repair activated by double strand breaks.

Within thirty minutes of the induction of DNA double strand breaks caused by ionizing radiation or genotoxic drugs, a complex containing Mre11, Nbs1, and Rad50 (MRN complex) relocalizes to sites of the DNA breaks (285). The Mre11 component contains both exonuclease and endonuclease activities, and is thought to process broken DNA ends prior to repair (308, 407). Nbs1 is required for proper localization of the complex to DNA breaks following ionizing radiation (47). The role of Rad50 in the MRN complex is to hold the broken DNA ends in close proximity allowing for Mre11 to process the broken DNA ends, and repair by other DNA repair factors (70, 161). Three
distinct MRN complexes have been observed in cells. Type I foci are thought to be involved in telomere length regulation and can be observed in untreated cells (241, 437). Type II and Type III foci form early and late, respectively, following exposure of cells to ionizing radiation, and have been termed ionizing radiation induced foci (IRIF) (255). Type IV MRN foci are associated with normal cellular DNA replication, and may form in order to repair DNA breaks that arise during S phase (64). Type IV foci are thought to relocalize to sites near origins of DNA replication by virtue of an interaction between E2F1 and the Nbs1 component of the complex (254).

2. Checkpoint signaling

Following relocalization of the MRN complex to sites of DNA double strand breaks, a cell cycle checkpoint is activated resulting in a pause in cell cycle progression or apoptosis. Cells from patients with the rare human disorders Nijmegen breakage syndrome (NBS) and ataxia-telangiectasia-like disease (ATLD), lacking functional Nbs1 and Mre11, respectively, fail to undergo growth arrest in response to ionizing radiation (371, 384, 394). A functional MRN complex is required for activation of Atm (412) that leads to activation of Chk2 and subsequent phosphorylation and activation of p53 as discussed.

In addition to MRN relocalization, other DNA damage signaling proteins localize to sites of double strand breaks, including γH2AX and 53BP1. Following DNA damage, the histone protein H2AX becomes phosphorylated by Atm, and γH2AX rapidly relocalizes to sites of DNA breaks at or near the formation of MRN foci (205, 338).
γH2AX relocalization is though to be important for the recruitment of DNA repair factors to sites of DNA breaks (309). 53BP1 is a DNA damage response protein originally identified due to its binding to p53 and enhancement of p53 transactivation function (175, 176). 53BP1 is recruited to nuclear foci following DNA damage by binding to γH2AX (426). The importance of 53BP1 in cell cycle checkpoint activation has been demonstrated by mouse models and siRNA gene knockdown experiments in human cells. Loss of 53BP1 results in a defect in S and G2/M checkpoint activation, hypersensitivity to ionizing radiation, and the development of thymic lymphomas (84, 111, 423, 427). 53BP1, like H2AX, is phosphorylated by Atm (327) and is required for the Atm-dependent activation of Chk2 (427) (Figure 1.6).

3. E2F1 and DNA damage signaling

Following treatment of cells with DNA damaging agents, E2F1 protein accumulates (30, 158, 165, 235, 266, 294), and is phosphorylated at an N-terminal Atm recognition sequence that is unique to E2F1 among the E2F family members (235). This phosphorylation of E2F1 is largely dependent on Atm and is required for efficient E2F1 stabilization following DNA damage (235). Chk2 has also been shown to phosphorylate and stabilize E2F1 following DNA damage, and this modification has been shown to be required for E2F1 dependent apoptosis following DNA damage by altering its promoter specificity (383). Additionally, DNA damage induced apoptosis is compromised in thymocytes from $E2F1^{-/-}$ mice (235), suggesting that E2F1 has multiple roles in DNA damage signaling.
Figure 1.6. Model of DNA double strand break signaling.

DNA double strand breaks are initially detected by the MRN complex, composed of Mre11, Nbs1, and Rad50. Relocalization of 53BP1 and γH2AX to sites at or near DNA double strand breaks and phosphorylation of these components by Atm acts to transduce, through Chk2, the detection of a DNA break. Phosphorylation and activation of p53 by Atm, Chk2, and possibly other kineses, can lead to activation of a cell cycle checkpoint resulting in growth arrest or apoptosis.
E. Thesis aims

The objective of this dissertation was to determine the pathway(s) by which deregulation of the Rb growth control pathway results in p53-dependent apoptosis. Given that p19\textsuperscript{ARF} can modulate p53 stability through inhibition of Mdm2 activity, it had been proposed that this was the pathway by which E2F1 induces apoptosis. We tested this hypothesis, and the data presented in Chapter III demonstrate that p19\textsuperscript{ARF} is not required for E2F1-induced apoptosis. Instead, apoptosis correlated with the ability of E2F1 to induce covalent modification of p53. The observation that E2F1 could induce p53 modifications similar to those observed following DNA damage, prompted us to examine the role of DNA damage kinases in mediating signaling to p53 and apoptosis. The experiments in Chapter IV demonstrate a role for the DNA damage kinases Atm and Chk2, as well as the DNA damage recognition and repair protein Nbs1 in E2F1-induced apoptosis. Comparing the ability of E2F family members E2F1 and E2F2, we found that the induction of Chk2 expression was specific for E2F1 and correlated with p53 activation and apoptosis.

To determine if ectopic E2F expression properly modeled Rb family inactivation, we confirmed our results obtained by E2F overexpression by expression of HPV-16 E7. In this biologically relevant context, E7 specifically required E2F1 and activation of Atm, Nbs1, and Chk2 to induce apoptosis. The experiments in Chapter V address the role of Nbs1 in this apoptosis pathway. Expression of E2F1 specifically induced the relocalization of the Nbs1 containing MRN complex to nuclear foci that correlated with the formation of 53BP1 and γH2AX containing foci. Due to the role of the MRN
complex in cell cycle checkpoint activation following DNA damage, we hypothesized that a similar cell cycle checkpoint may be activated following expression of E2F1. We found that expression of E2F1 in normal human fibroblasts blocked cell cycle progression that is mediated, in part, by activating a p21-dependent checkpoint. These findings provide further insight into the pathways linking deregulated proliferation control to p53 and apoptosis.
CHAPTER II

MATERIALS AND METHODS
A. Cell culture

Early passage wildtype and genetically matched $p53^+$ and $Mdm2^+/p53^+$ MEFs were isolated from mouse embryos as described (184). MEFs derived from $p19^{ARF-}$ mouse embryos were a generous gift from Charles Sherr (St. Jude Children’s Research Hospital, Memphis, TN). MEFs derived from $p19^{ARF-}/p53^+$ mouse embryos were a generous gift from Gerard Zambetti (University of Tennessee, Memphis, TN). $Ink4a^-$ MEFs were kindly provided by Ronald DePinho (Harvard Medical School, Boston, MA). $atm^-$ and genetically matched wildtype mice were purchased from The Jackson Laboratory, Bar Harbor, ME. 293 cells were generously provided by Joseph Nevins (Duke University Medical Center, Durham, NC). MEFs and 293s were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Primary human dermal fibroblasts GM00316B and GM02270A (Normal), primary human embryonic lung fibroblasts GM01604 (HEL fibroblasts), GM03395C and GM05823C (AT), and GM07166A (NBS) were obtained from Coriell Cell Repositories, Camden, NJ. Human cells were cultured as recommended by Coriell.

B. Adenoviral vectors

Recombinant adenoviruses encoding p53, E2F1, E2F2, and E2F3a have been described previously (76, 211, 354). An adenovirus encoding an E2F1 DNA binding mutant, E2F1_{e132} (183), was created by homologous recombination in 293 cells (211, 288). An adenovirus encoding the E2F1_{1-283} mutant was a generous gift from W. Douglas
Cress (H. Lee Moffitt Comprehensive Cancer Center, Tampa, FL). The p53N/C virus was generated from the pCB6+p53N/C construct generously provided by Karen Vousden (National Cancer Institute, Frederick, MD). The Chk1, DN-Chk1, Chk2, DN-Chk2, and HPV-16 E7 recombinant adenoviruses were created by homologous recombination in *Escherichia coli* (143). DN-Chk1 contains an aspartic acid to alanine substitution at position 330. Plasmids encoding Chk2 and DN-Chk2 constructs were generously provided by David Johnson (M.D. Anderson Cancer Center, Smithville, Texas). DN-Chk2 contains a serine to alanine substitution at position 347. A plasmid encoding HPV-16 E7 was generously provided by Karl Munger (Harvard Medical School, Boston, Massachusetts). Control viruses encode either an empty expression cassette or β-galactosidase (β-gal). Infection with control virus had no effect on parameters tested relative to mock infection (data not shown). Viruses were propagated in 293 cells and purified by centrifugation through cesium-chloride gradients. Virus titers were determined by immunohistochemical staining for the adenovirus hexon protein with an anti-adenovirus antibody (Biodesign International) and visualized using a 3,3'-diaminobenzidine (DAB) substrate kit from Vector Laboratories (50).

C. Virus infection

Cells were washed once with PBS and serum free DMEM containing virus was added to the cells. Infections were carried out at 37°C in 5% carbon dioxide (CO₂) for 1 hour (211). The viral inoculum was then removed and replaced with DMEM containing the appropriate serum concentrations and cultured under the conditions described above.
D. Western blot analysis

Whole cell extracts were harvested from recombinant adenovirus infected cells at 24 hours post infection unless otherwise noted. Cells were washed twice with cold PBS and lysed in whole-cell extract buffer (50 mM HEPES, 2 mM magnesium chloride, 250 mM sodium chloride, 0.1 mM EDTA, 1 mM EGTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1x mammalian protease inhibitor cocktail (Sigma), 1x phosphatase inhibitor cocktails I and II (Sigma)) by incubation for 30 min on ice followed by sonication. Soluble proteins were separated by centrifugation at 13,000 x g in a microcentrifuge and supernatants stored at -70°C. Proteins were separated by SDS-PAGE analysis and transferred to PVDF membrane (Perkin Elmer) by electroblotting. p19ARF was detected using anti-p19ARF polyclonal antisera at 1:500 (Novus), E2F1 was detected using monoclonal antibody KH-95 at 1:500 (Santa Cruz Biotechnology), E2F2 was detected using polyclonal antisera C-20 at 1:500 (Santa Cruz Biotechnology). p53 protein was detected using monoclonal antibody D0-1 at 1:500 or polyclonal antisera Ab-7 at 1:2000 (Oncogene Research Products), phospho-serine 15 and -serine 20 forms of p53, the phospho-threonine 68 form of Chk2, and the phospho-serine 345 form of Chk1 were detected using polyclonal antisera specific for each modification at 1:1000 (Cell Signaling Technologies). Chk1 was detected using polyclonal antisera FL-476 at 1:500 (Santa Cruz Biotechnology). Chk2 was detected using polyclonal antisera H-300 at 1:500 (Santa Cruz Biotechnology) or monoclonal antibody clone #7 at 1:500 (Lab Vision Corporation). Atm was detected using polyclonal antisera Ab-3 at 1:150 (Oncogene
Research Products) and the phospho-serine 1981 form of Atm was detected using polyclonal antisera at 1:600 (Rockland). p21 was detected using monoclonal antibody F-5 at 1:400 (Santa Cruz Biotechnology). Actin was detected using polyclonal antisera I-19 at 1:1000 (Santa Cruz Biotechnology). Immunoreactive proteins were detected with a chemiluminescence kit (Perkin Elmer) according to the manufacturers recommendations. Actin blots are shown as protein loading controls. Relative changes in the levels of p53 were estimated from scanned images of western blots using Multianalyst software (BioRad).

E. Apoptosis analysis by flow cytometry

At the indicated times post infection, cells were trypsinized, combined with any floating cells, pelleted, washed with PBS, repelleted, and resuspended in 400 μl of PBS. All centrifugations were at 500 x g for 5 min at 4°C. Subsequently, cells were fixed in cold ethanol (final concentration, 70%) and stored at 4°C. Cells were processed for propidium iodide (PI) staining as described (211). Flow cytometric analysis was performed by the University of Massachusetts Medical School Flow Cytometry Core Facility. Cells undergoing apoptosis were defined as the population that contains less than a 2N DNA content.

F. Apoptosis analysis by ELISA

Cells were plated in 10 cm dishes at 6000 cells per cm², or 24 well plates at 1 x 10⁴ cells per well. Virus infections were performed 24 hours after plating. At 96 hours
post infection, cells were centrifuged at 500 x g for 10 min at 4°C, lysed, and Cell Death Detection ELISA plus assay performed as described by the manufacturer (Roche). The ordinate axis represents DNA fragmentation relative to control which is defined as 1. Error bars represent standard deviations calculated from experiments performed in triplicate.

G. Immunohistochemical staining for p53 protein accumulation

Cells were infected with the appropriate adenovirus constructs and immunohistochemically stained for p53 protein as described (210). AdCon, AdE2F1, AdE2F1\textsubscript{e132} were infected at an MOI of 100. At the time of harvest (48 hpi), cells were washed three times with PBS and then fixed for 15 min each in 3.7% formaldehyde followed by methanol. The cells were then washed in PBS-0.5% Tween 20. Cells were then incubated with anti-p53 monoclonal antibody pAb421 (Oncogene Research Products) in the presence of 1% BSA in PBS-0.5% Tween-20 for 45 min at room temperature. The cells were washed three times with PBS-0.5% Tween 20, and bound antibody was detected using Vectastain DAB substrate kit as described by the manufacturer.

H. p53 immunofluorescence

Cells were infected with AdCon, AdE2F1, or AdE2F2 at an MOI of 100 and at 24 hpi the cells were fixed in 3.7% formaldehyde followed by methanol. Fixed cells were incubated with anti-p53 monoclonal antibody pAb421 at 1:200 (Oncogene Research
Products) and polyclonal antisera specific for phospho-Serine 15 p53 at 1:200 (Cell Signaling Technologies). Bound antibodies were detected using fluorescein isothiocyanate (FITC) or Rhodamine Red-X conjugated secondary antibodies (Jackson Immunoresearch Laboratories, Inc. and Southern Biotechnology Associates, Inc.).

I. Cell fractionation immunofluorescence

Glass coverslips were pre-treated with 40% hydrochloric acid for 2 min followed by a 5 min wash with 70% ethanol. Cells were plated on treated coverslips at 3000 cells per cm². Cells were infected with recombinant adenoviral vectors and at 24 hpi the cells were washed three times with PBS and incubated for 5 min in cytoskeleton buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100) and 5 min in cytoskeleton stripping buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 1% Tween 40, 0.5% sodium deoxycholate). Cells were then washed 3 times with PBS and fixed in fixing buffer (200 mM sucrose and 4% paraformaldehyde in PBS, pH 7.4) for 30 min at room temperature and permeabilized (0.5% Triton X-100, 20 mM HEPES pH 7.4, 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose) for 15 min at room temperature (267). Fixed cells were then blocked in 10% FBS for 1 hour at room temperature. Cells were then incubated with Mre11 monoclonal antibody 12D7 at 1:1500 (GeneTex), γH2AX polyclonal antisera at 1:600 (Upstate Biotechnology), 53BP1 polyclonal antisera at 1:400 (Novus Biologicals), or Nbs1 polyclonal antisera at 1:400 (Novus Biologicals). Bound antibodies were detected using
FITC or Rhodamine Red-X conjugated secondary antibodies (Jackson Immunoresearch Laboratories, Inc. and Southern Biotechnology Associates, Inc.).

**J. S phase analysis**

Cells were plated at 3000 cells per cm². Cells were infected with recombinant adenoviral vectors and pulsed with 10 mM 5-Bromo-2’-deoxyuridine (BrdU) (Roche) immediately following infection. At 24 hpi, cells were fixed and permeabilized as described above. Following permeabilization, cells were incubated in 50 mM glycine for 10 min at room temperature. DNA was denatured with 4 N HCl with 0.1% Triton X-100 for 10 min at room temperature, and the samples washed extensively with PBS followed by a wash in 50 mM glycine (254). Cells were then blocked in 10% FBS and incubated for 1 hour with polyclonal antisera specific for BrdU at 1:200 (Immunologicals Direct). Bound antibody was detected using a Rhodamine Red-X conjugated secondary antibody (Jackson Immunoresearch Laboratories, Inc).

**K. Northern blot analysis**

Poly-(A) RNA was isolated using the Micro-FastTrack mRNA isolation kit from cells as described by the manufacturer (Invitrogen). Total cellular RNA was isolated using Trizol as described by the manufacturer (Invitrogen). Biotinylated Chk2, Atm, and GAPDH probes were generated by PCR using primers as described by the manufacturer (KPL). Hybridized probes were visualized with a chemiluminescence kit (KPL) as
described by the manufacturer. Blots were sequentially probed and stripped. PCR primers used to make biotinylated probes:

- Chk2F (5'-ATGTCTCGGGAGTCGGATGTTG-3')
- Chk2R (5'-GCACCACTTCCAAGAGTTTTTGAC-3')
- AtmAF (5'-ACGATGCTTACGGAAGTTGC-3')
- AtmAR (5'-GGACAGAGAAGCCAAATACTGGACTG-3')
- GAPDHF (5'-CAAGGTCATCCATGACAAC-3')
- GAPDHR (5'-TGGTCGTTGAGGGCAATG-3')

L. RNA interference

siRNAs used in this study were generated by Qiagen, Valencia, CA. siRNA oligonucleotides were transfected into cells at a concentration of 100 nM using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. Control siRNAs (siCon) recognize either GFP or retrovirus LTR, and had no effect on parameters tested relative to mock transfection. siRNAs used in this study:

- siGFP (5'-CGUAACGGCCACAAGUUUC-3')
- siLTR (5'-GAUCGCAUAUAAGGCAGC-3')
- siE2F1a (5'-GGCAGGAUCAGUUUUCC-3')
- siE2F1b (5'-CUGACCAUGAUACCUGGC-3')
- siE2F1c (5'-GUCACGCUAUGAGACCUCA-3')
- siChk2a (5'-CUCAGCCAGUCCUCAC-3')
siChk2b (5’-GAACCUGAGGACCAAGAAC-3’)
siE2F2a (5’-GUGCAUCAGAGUGGAUGGC-3’)
siE2F2b (5’-CAAGAGGCUGGCCUAUGTG-3’)
siE2F3a (5’-AGCGGUCAUCAGUACCUCU-3’)
siE2F3b (5’-CUGUUAACCGAGGAUUCAG-3’)
sip21a (5’-AGGCCCGCUCUACAUCUU-3’)
sip21b (5’-CUAGGCGGUUGAAUGAGAG-3’)
siAtr (5’-GACGGUGUGCUCAUGCGGC-3’)
siAtm (5’-GCGCCUGAUUCGAGAUCUCC-3’)
siNbs1a (5’-AUCAUGCGUGUUAACUGC-3’)
siNbs1b (5’-AUGUUGAUCUGUCAGGAC-3’)
sip14^{ARF}a (5’-ATCCTAAAGGCCGCGGAGT-3’)
sip14^{ARF}b (5’-AAATAACACCTTGCTGTC-3’)
sip53 (5’-GCAUGAACCAGAGGCCCAU-3’)

CHAPTER III

E2F1 INDUCES PHOSPHORYLATION OF p53
THAT IS COINCIDENT WITH p53
ACCUMULATION AND APOPTOSIS
A. Introduction

During normal cell proliferation, E2F family members modulate the expression of many genes involved in the transition from G₁ to S phase and DNA replication (287). E2F transcriptional activity is regulated by interaction with members of the retinoblastoma (Rb) family of proteins (137). Inactivation of Rb by cellular or viral oncoproteins or loss of Rb will stimulate cells to bypass Go/G₁ and enter S phase (96, 101, 273, 286). S phase induction also occurs when E2F1, E2F2, or E2F3 is exogenously expressed in quiescent cells (76, 183, 211). In fibroblasts, expression of E2F1, but not E2F2 or E2F3, can result in both S phase progression and apoptosis (76, 210).

Apoptosis induction by E2F1 occurs primarily by activation of the p53 apoptotic signaling pathway. The link between E2F1 and p53-dependent apoptosis has been demonstrated in cell culture and by genetic studies in mice (211, 246, 305, 324, 408, 440). It has been proposed that the E2F1 transcription factor serves as a link between the Rb/E2F proliferation pathway and the p53 apoptosis pathway by inducing the expression of p19ARF, a protein that regulates p53 stability. We tested this hypothesis to determine the requirement for p19ARF in the E2F1 apoptosis pathway and to better understand the mechanism of E2F1-induced apoptosis.
B. \( p19^{\text{ARF}} \) is not required for E2F1-mediated apoptosis

Human \( p14^{\text{ARF}} \) and mouse \( p19^{\text{ARF}} \) promoters contain multiple E2F binding sites that are responsive to ectopic expression of E2F1 (25, 172, 335). Because \( p19^{\text{ARF}} \) mRNA levels increase upon E2F1 expression (76), we wanted to determine whether the elevated mRNA levels corresponded to an increase in \( p19^{\text{ARF}} \) protein levels. Indeed, we found that infecting MEFs with an adenovirus encoding E2F1 (AdE2F1) led to increased levels of \( p19^{\text{ARF}} \) protein (Figure 3.1). Given that \( p19^{\text{ARF}} \) expression leads to p53 accumulation via inactivation of Mdm2 functions and that E2F1 expression leads to p53-dependent apoptosis, we determined whether \( p19^{\text{ARF}} \) was required for E2F1-mediated, p53-dependent apoptosis. We find that E2F1 expression induced apoptosis regardless of \( p19^{\text{ARF}} \) status as measured by the percentage of cells with sub-2N DNA content (Figure 3.2). Apoptosis is observed at all multiplicities of infection (MOI) in the wildtype MEFs, but only at the highest dose of E2F1 in the \( p19^{\text{ARF}-/-} \) MEFs, suggesting that while \( p19^{\text{ARF}} \) is not required, it may contribute to E2F1-mediated apoptosis. MEFs infected with an adenovirus encoding an E2F1 DNA binding mutant, E2F1e132, showed little or no increase in apoptosis over controls. The percentage of apoptotic cells was also measured in \( p19^{\text{ARF}-/-} \) and \( p19^{\text{ARF}-/-}/p53^{-/-} \) MEFs to test the requirement for p53 in the apoptosis observed in \( p19^{\text{ARF}-/-} \) MEFs. The \( p19^{\text{ARF}-/-}/p53^{-/-} \) MEFs were less susceptible to apoptosis as compared to MEFs lacking only \( p19^{\text{ARF}} \) (Figure 3.3). In addition, expression of E2F1 also induced apoptosis in \( ink4a^{-/-} \) MEFs (unpublished observations). \( ink4a^{-/-} \) mice do not express \( p19^{\text{ARF}} \) because of the deletion of a shared exon with \( p16^{\text{INK4A}} \), which encodes a
Figure 3.1. E2F1 induces p19^{ARF} protein accumulation.

Wildtype and p19^{ARF/-} MEFs were infected with AdCon, AdE2F1, or AdE2F1_{e132} at MOIs of 25 (E2F1_{low}) or 100 (E2F1_{hi}). Cells were harvested and lysates generated at 24 hours post infection (hpi). Western blot analysis was performed to detect p19^{ARF} protein levels. Actin blot is shown as protein loading control.
Figure 3.2. E2F1 induces apoptosis in the absence of p19ARF.

Wildtype and p19ARF-/ MEFs were infected with AdCon, AdE2F1, or AdE2F1e132 at MOIs of 100, 300, or 500. Cells were harvested for PI staining and flow cytometry at 72 hpi. Percentages represent cells with sub-2N DNA content.
Figure 3.3. E2F1 induces p53-dependent apoptosis in the absence of p19ARF.

p19ARF⁻/⁻ and p19ARF⁻/⁻/p53⁻/⁻ MEFs were infected with AdCon, AdE2F1, or AdE2F1ₑ₁₃₂ at MOIs of 100, 300, or 500. Cells were harvested for PI staining and flow cytometry at 72 hpi. Percentages represent cells with sub-2N DNA content.
cyclin dependent kinase inhibitor (361). Thus, E2F1 can induce apoptosis in p19ARF deficient MEFs that are generated from independent genetic lesions in mice.

C. p19ARF and Mdm2 contribute to p53 accumulation

Given that apoptosis does not require p19ARF, we asked if p19ARF is necessary for E2F1-mediated changes in p53 protein levels. To address this question we expressed E2F1 in p19ARF−/− MEFs and assessed p53 protein levels by immunohistochemistry. As shown in Figure 3.4, E2F1 expression resulted in increased levels of endogenous p53 protein in wildtype MEFs. When E2F1 was expressed in p19ARF−/− MEFs, fewer cells stained positive for p53 protein and more modest changes in p53 protein levels were observed. The changes in endogenous p53 protein levels observed by immunohistochemical staining following E2F1 expression correlate with the changes observed by immunoblotting for endogenous p53 (Figure 3.10). Likewise, Nip et al. observed an increase in the half-life of p53 following E2F1 expression in 32D.3 cells, a tumor cell line that lacks p14ARF expression (292). In both MEF genotypes, expressing the E2F1e132 DNA binding mutant had little or no effect on p53 protein levels. These results are consistent with a model where p19ARF can contribute to E2F1-mediated changes in p53 protein levels.

p19ARF affects p53 protein levels by binding to Mdm2 and preventing it from promoting the degradation of p53 (321, 459). Therefore, we determined if Mdm2 is necessary for E2F1-mediated accumulation of p53. It is not possible to duplicate the experiments described above for Mdm2 because there are no cell lines or MEFs available
Figure 3.4. E2F1 requires p19ARF to induce p53 accumulation.

Wildtype and p19ARF−/− MEFs were infected with AdCon, AdE2F1, or AdE2F1e132 at an MOI of 100. Cells were fixed and immunohistochemically stained for p53 protein levels at 48 hpi.
that lack Mdm2 expression but retain endogenous, wildtype p53. However, MEFs can be derived from Mdm2−/−/p53−/− embryos (184, 263). To analyze the effects of E2F1 on p53 protein levels in these cells, we introduced p53 in trans by infecting cells with an Adp53 recombinant adenovirus, essentially creating a “pseudo” Mdm2−/− MEF. To control for the addition of exogenous p53 transcript, wildtype, p19ARF−/− and p53−/− MEFs were also infected with Adp53 at the same low MOI. As observed in the immunohistochemical and immunoblot analysis for endogenous p53, E2F1 expression resulted in a substantial (>6 fold) increase in exogenous p53 levels in wildtype MEFs in comparison to control infected cells when measured by immunoblot analysis (Figure 3.5 and Figure 3.10). Others have made similar observations in p19ARF−/− MEFs following retroviral gene transfer of E2F1 (463). p53 also accumulated in response to E2F1 in p53−/− MEFs that ectopically expressed p53 (Figure 3.5). Therefore, exogenously expressed p53 protein is affected by E2F1 in a manner similar to the endogenous protein. Infection with AdE2F1 resulted in a modest (~2.4 fold) increase in total p53 protein levels in Adp53-infected p19ARF−/− MEFs as compared to AdCon infection of the same cell type (Figure 3.5). The basal levels of ectopically expressed p53 were higher in p19ARF−/− MEFs relative to wildtype cells. This observation is consistent with the data obtained by immunostaining and immunoblotting for endogenous p53 in p19ARF−/− MEFs (Figure 3.4 and Figure 3.10), although the difference is exaggerated when cells are infected with Adp53 (Figure 3.5). Others have also reported higher basal levels of p53 in p19ARF−/− MEFs (73). E2F1 expression minimally affected p53 protein levels in Adp53-infected Mdm2−/−/p53−/− MEFs. We suspect that the Mdm2 deficiency elevates baseline levels of p53 in these cells due to
Figure 3.5 E2F1 requires p19ARF and Mdm2 to increase p53 protein levels.

Wildtype, p53\textsuperscript{−/−}, p19ARF\textsuperscript{−/−}, or Mdm2\textsuperscript{−/−}/p53\textsuperscript{−/−} MEFs were infected with AdCon, AdE2F1, or AdE2F1\textsubscript{e132} at MOIs of 25 (E2F1\textsubscript{low}) or 100 (E2F1\textsubscript{hi}). Cells were harvested and lysates generated at 24 hpi. Western blot analysis was performed to determine levels of p53 protein. Actin blots are shown as protein loading controls.
an absence or greatly reduced level of p53 ubiquitin ligase activity. E2F1e132 had little or no effect on p53 protein levels in each cell type, consistent with a transcriptional mechanism being at least partly responsible for E2F1-mediated accumulation of p53. The reduced ability of E2F1 to alter p53 levels above controls in p19ARF-/- MEFs, or in MEFs lacking Mdm2 but expressing p53, is consistent with a model where E2F1 modulates p53 protein levels, in large part, through the p19ARF/Mdm2/p53 pathway.

D. E2F expression leads to the phosphorylation of p53

In response to DNA damage, p53 is activated by covalent modifications, including the phosphorylation at certain serines and acetylation of lysine residues. Of these modifications, phosphorylation of serine 15 on human p53, or serine 18 on mouse p53, is commonly observed in response to DNA damage (20, 43). Given that both DNA damage (189) and E2F1 do not necessarily require p19ARF to activate p53, we determined whether E2F1-mediated activation of p53 function is coincident with p53 phosphorylation. To detect phospho-serine 18, we used an antibody specific for this modification. We find that E2F1, and to a lesser extent E2F2, expression in wildtype MEFs results in an increase in endogenous p53 protein levels and the phospho-serine 18 form of p53 as detected by immunofluorescence (Figure 3.6). When merged it is apparent that only a subset of the cells with an increase in total p53 has increased levels of the phospho-serine 18 form of p53. Total p53 protein and the phospho-serine 18 form of p53 were present at low levels in cells infected with control virus (Figure 3.6). This data
suggest that E2F1 may signal p53 accumulation and p53 phosphorylation by separate pathways.

E. Differential abilities of E2F1 and E2F2 to phosphorylate p53 and induce apoptosis

To discriminate between E2F1-specific induction of p53 and increased E2F activity in general, we measured the levels of p53 in extracts of cells infected with an adenovirus encoding E2F2 in comparison to E2F1. E2F2 is functionally similar to E2F1; E2F2 is a potent inducer of S phase when expressed in quiescent cells, is normally induced at the G1 to S transition, specifically interacts with Rb, and induces a similar array of genes when overexpressed (76, 174, 226, 279, 358). However, like other E2F family members, E2F2 is unable to induce apoptosis in REF52 cells (76, 210). We find that E2F2 expression does not lead to apoptosis in MEFs, even though it is capable of inducing the expression of p19ARF protein (Figure 3.8 and Figure 3.9). Ectopic E2F2 expression did result in increased levels of endogenous p53 protein in wildtype MEFs, although not as effectively as E2F1 (Figure 3.6 and Figure 3.10). However, expression of E2F1, but not E2F2, alters p53 protein levels in p19ARF−/− MEFs (Figure 3.7 and Figure 3.10).

We next compared the ability of E2F1 and E2F2 expression to induce a change in p53 phosphorylation. We find that ectopic expression of either E2F1 or E2F2 leads to an increase in the levels of both endogenous p53 and the phospho-serine 18 form of p53 in wildtype MEFs, although the levels of phospho-serine 18 p53 is less in E2F2 expressing
Figure 3.6. Expression of E2F1 or E2F2 results in an increase in p53 protein levels and levels of the phospho-serine 18 form of p53 in wildtype MEFs.

Wildtype MEFs were infected with AdCon, AdE2F1, or AdE2F2 at an MOI of 100. Cells were fixed and stained for total p53 (rhodamine Red-X) and the phospho-serine 18 form of p53 (FITC) at 24 hpi. DAPI staining is shown in blue. Merged images are also shown.
Figure 3.7. E2F1 specifically induces accumulation of p53 and the phospho-serine 18 form of p53 in cells lacking p19ARF.

*p19ARF−/−* MEFs were infected with AdCon, AdE2F1, or AdE2F2 at an MOI of 100. Cells were fixed and stained for total p53 (rhodamine Red-X) and the phospho-serine 18 form of p53 (FITC) at 24 hpi. DAPI staining is shown in blue. Merged images are also shown.
sample (Figure 3.10). Similar results were observed by immunofluorescence staining for p53 and phospho-serine 18 p53 in p19ARF−/− MEFs (Figure 3.7). Expression of E2F1, but not E2F2, leads to increased levels of the endogenous phospho-serine 18 form of p53 in p19ARF−/− MEFs (Figure 3.7 and Figure 3.10). In addition to phosphorylation at serine 15 (the human equivalent to serine 18 in mouse p53), phosphorylation of serine 20 (serine 23 in mouse) on human p53 is commonly observed following DNA damage (53, 155, 370). Since we have had difficulties detecting mouse phospho-serine 23 p53 by immunoblot analysis, cells were coinfected at a low MOI with an adenovirus encoding human p53, which when phosphorylated can be detected by the anti-phospho-serine 20 antibody. Using this approach, we find that E2F1 expression results in an increase in the phospho-serine 20 as well as the phospho-serine 15 forms of p53 in both wildtype and p19ARF−/− MEFs (Figure 3.11 A and Figure 3.11 B).

E2F2 expression results in a slight increase in both the phospho-serine 15 and phospho-serine 20 forms of p53 in wildtype but not in p19ARF−/− MEFs (Figure 3.11 A and Figure 3.11 B). This inability of E2F2 to affect the phosphorylation of p53 in the absence of p19ARF was also observed for the endogenous mouse p53 (Figure 3.7 and Figure 3.10). The ability of E2F2 to affect p53 protein levels was, unlike E2F1, solely dependent on p19ARF, since both endogenous and exogenous p53 protein levels were unchanged when E2F2 was expressed in p19ARF−/− MEFs while E2F1 expression leads to an increase in p53 in both wildtype and p19ARF−/− MEFs (Figure 3.10 and 3.11 C). We note that the differential ability of E2F1 and E2F2 to alter the ectopically expressed p53 in p19ARF−/−
Figure 3.8. E2F2 does not induce apoptosis in wildtype MEFs.

Wildtype MEFs were infected with AdCon, AdE2F1, or AdE2F2 at MOIs of 100, 300, or 500. Cells were harvested for PI staining and flow cytometry at 72 hpi. Percentages represent cells with sub-2N DNA content.
Figure 3.9. E2F1 and E2F2 induce p19ARF protein accumulation.

Wildtype or p19ARF−/− MEFs were infected with AdCon, AdE2F1, AdE2F2, or AdE2F1e132 at an MOI of 100. Cells were harvested and lysates generated at 24 hpi. Western blot analysis was performed to determine levels of p19ARF protein. Actin blot is shown as protein loading control.
Figure 3.10. E2F2 requires p19ARF to induce p53 protein and alter levels of the phospho-serine 15 form of p53.

Wildtype or p19ARF-/- MEFs were infected with AdCon, AdE2F1, AdE2F2, or AdE2F1e132 at an MOI of 100. Cells were harvested and lysates generated at 24 hpi. Western blot analysis was performed to determine levels of total p53 protein and levels of the phospho-serine 18 form of p53. Actin blot is shown as protein loading control.
MEFs appears to be exaggerated relative to the endogenous protein in these experiments. These results suggest that E2F2 utilizes the p19\textsuperscript{ARF}/Mdm2 pathway to affect p53 levels.

**F. p53 phosphorylation contributes to E2F1-mediated apoptosis**

Having found a correlation between E2F1-induced phosphorylation of p53 and apoptosis, we determined if these covalent modifications are required or contribute to E2F1-mediated apoptosis. Studies of the DNA damage response have suggested that it is unlikely that a single p53 modification is responsible for activating p53 dependent apoptosis (48), so we initially addressed this question by using a p53 mutant in which most of the N- and C- terminal amino acid residues known to be phosphorylated upon DNA damage have been changed to alanines. This mutant, p53N/C, has been shown to activate expression of p21, Mdm2 and Bax reporter constructs as effectively as wildtype p53 and has been reported to induce apoptosis in response to certain stimuli (13). Mdm2 has also been shown to regulate the levels of p53N/C (13). Indeed, we find that p53N/C protein accumulates to levels similar to wildtype p53 when expressed by recombinant adenovirus transduction (Figure 3.12 A). To measure apoptosis, p53\textsuperscript{−/−} MEFs were transduced with AdE2F1 or AdE2F2 and either wildtype Adp53 or Adp53N/C (Figure 3.12 B). Without the addition of p53, levels of apoptosis in E2F1 or E2F2 expressing samples were similar to AdCon infected cells. Cells expressing both E2F1 and wildtype p53 underwent apoptosis. In contrast, apoptosis was greatly reduced in cells transduced with E2F1 and p53N/C encoding adenoviruses suggesting a contribution by these
Figure 3.11. E2F1 induces p53 protein and levels of the phospho-serine 15 and phospho-serine 20 forms of p53 in cells lacking p19ARF.

(A) Western blot analysis of the phospho-serine 15 form of p53 in wildtype and p19ARF−/− MEFs infected with AdCon, AdE2F1, or AdE2F2 and an MOI of 100. (B) Western blot analysis of the phospho-serine 20 form of p53 in wildtype and p19ARF−/− MEFs infected with AdCon, AdE2F1, or AdE2F2 and an MOI of 100. (C) Western blot analysis of total p53 protein levels in wildtype and p19ARF−/− MEFs infected with AdCon, AdE2F1, or AdE2F2 and an MOI of 100. Cells were harvested and lysates generated at 24 hpi. Blots were sequentially probed and stripped. Actin blot is shown as protein loading control.
potential phosphorylation sites to apoptosis. As expected, E2F2 did not induce apoptosis when co-expressed with either p53 or p53N/C.

We next examined the role of p53 kinases on apoptosis signaling in response to E2F1 expression by using caffeine, a potent inhibitor of several of these kinases. We find that there is a dose dependent decrease in apoptosis in E2F1 expressing MEFs following treatment with increasing doses of caffeine (Figure 3.13). Treatment of cultured cells with caffeine prior to ionizing radiation results in a delayed and attenuated accumulation of p53 protein (192). This defect in p53 accumulation is due to the inhibition of the ataxia telangiectasia mutated (Atm) and the Atm-Rad3-related (Atr) kinases that phosphorylate p53 on serine 15. Inhibition of Atm and Atr will also block subsequent signals that activate the checkpoint kinases Chk1 and Chk2 and perhaps other kinases that phosphorylate p53 on serine 20 (29, 348). The observations that blocking p53 phosphorylation either by mutating potential phosphorylation sites on p53 or treatment with the p53 kinase inhibitor, caffeine, can significantly compromise apoptosis demonstrates that p53 phosphorylation is a critical step in E2F1 mediated apoptosis.
Figure 3.12. p53 phosphorylation correlates with E2F1-induced apoptosis.

(A) p53−/− MEFs were infected with Adp53 or Adp53N/C at MOIs of 10, 20, or 50. Cells were harvested and lysates generated at 24 hpi. Western blot analysis was performed to detect total p53 protein levels. Actin blot is shown as protein loading control. (B) Analysis of apoptosis in p53−/− MEFs infected with AdCon, AdE2F1, or AdE2F2 at an MOI of 500 and coinfected with Adp53 or Adp53N/C at an MOI of 20. Cells were harvested for PI staining and flow cytometry at 72 hpi. Percentages represent cells with sub-2N DNA content.
Figure 3.13. Caffeine inhibits E2F1-induced apoptosis.

Analysis of apoptosis in wildtype MEFs infected with AdCon or AdE2F1 at an MOI of 500. Caffeine was added at the indicated doses to the culture medium immediately following viral infection. Cells were harvested and apoptosis detected at 72 hpi. Error bars represent standard deviation of experiment performed in triplicate.
G. Discussion

The signaling mechanism by which the RB/E2F pathway in general, and E2F1 specifically, communicates with p53 has been thought to involve the p19ARF/Mdm2 pathway. Indeed, we find that p19ARF and Mdm2 are required for the majority of p53 protein accumulation observed following E2F1 expression. However, contrary to the role of the p19ARF pathway in p53 accumulation, we find that E2F1 induces apoptosis in MEFs lacking p19ARF (see model, Figure 3.14). It is unlikely that E2F1 induction of p73 is directly responsible for the apoptosis observed in p19ARF- MEFs since we show that E2F1 failed to induce apoptosis in MEFs lacking both p19ARF and p53. These results are in contrast to earlier studies which concluded that p19ARF is important for E2F1-mediated apoptosis (73, 463). However, reports published by Tsai et al. (409) and Tolbert et al. (401) show that p19ARF was not required for apoptosis in Rb deficient mouse embryos or following Rb inactivation by transgenic expression of a fragment of the SV-40 large-T antigen, respectively. Likewise, a report published by Russell et al. showed that crossing an E2F1 transgenic mouse into a p19ARF- background does not reduce apoptosis caused by expression of the E2F1 transgene (342). Thus, studies using several different models arrive at the conclusion that the apoptosis associated with deregulated E2F activity does not require p19ARF.

It is conceivable that forcing cells into S phase by ectopic E2F expression can induce the apoptotic response. However, it is not likely to simply be ectopic S phase induction following E2F expression that triggers apoptosis, since both E2F1 and E2F2 can induce S phase at similar efficiencies (76), and we find that E2F2 does not induce
apoptosis in MEFs. Moreover, there does not appear to be a specific phase of the cell cycle in which E2F1 induces apoptosis (75, 211). Our findings that ectopic E2F1, but not E2F2, expression results in increased p53 phosphorylation in the absence of p19ARF, and that this covalent modification of p53 contributes to E2F1-mediated apoptosis, suggests that E2F1 may activate a cellular response similar to DNA damage.

The observation that caffeine inhibits E2F1-mediated apoptosis suggests that the action of one or more p53 kinases is likely to be important for E2F1 signaling. Signaling cascades that activate protein kinases responsible for phosphorylating N-terminal residues on p53 upon DNA damage are well documented (10). Candidate kinases include ataxia telangiectasia mutated (Atm), the Atm-Rad3-related protein (Atr), Chk1 and Chk2. Atm and Atr phosphorylate p53 at serine 15 as well as activate the Chk kinases by phosphorylation (20, 43, 217, 238, 259). Active Chk kinases can then phosphorylate p53 at serine 15 and serine 20 (370).

Recently, Atm/Atr has been shown to phosphorylate the N-terminus of E2F1, but not E2F2 or E2F3 (235). This observation, together with the data presented here leads us to speculate that ectopic E2F1 expression or activation of endogenous E2F1 upon phosphorylation by Atm, leads to increases in the activity and, perhaps, levels of one or more of the p53 kinases, which then phosphorylate p53 and promote apoptosis. Given that the E2F1 DNA binding mutant, E2F1e132, did not induce p53 phosphorylation or apoptosis, transcriptional activation of one or more p53 kinases might provide a mechanism for E2F1 activation of this pathway. However, overexpression of Atm/Atr,
Figure 3.14. Model of E2F1-induced apoptosis and p53 accumulation.

E2F1, E2F2, and to a lesser extent, E2F3 can signal p53 accumulation through the p19ARF/Mdm2 pathway. p53 phosphorylation contributes to E2F1-mediated apoptosis and can occur in the absence of p19ARF. p19ARF may function to attenuate E2F proliferation-promoting activity as shown by the dashed lines. Activation of the p19ARF/Mdm2 pathway may increase levels of p53, contributing substrate to the p53 kinases activated by E2F1, as shown by the dashed arrow.
Chk1, or Chk2 kinases has been found to be insufficient for their activation (238, 259). Therefore, an additional signal(s) may be necessary to stimulate their activity.

It is conceivable that E2F1, E2F2, or E2F3 could signal through $p19^{ARF}/Mdm2$ to increase p53 protein levels and that the increased pools of p53 would provide more substrate for the p53 kinases activated by E2F1. Thus, the $p19^{ARF}/Mdm2$ pathway may act as both an attenuator of proliferation by targeting E2F family members for degradation, and as an amplifier of a DNA damage signal by increasing pools of p53 available for phosphorylation. The decision to undergo growth arrest or apoptosis would then depend on the cellular context or extent of DNA damage.

Our data implicates p53 phosphorylation as a key step in E2F1-mediated p53-dependent apoptosis. These observations raise the possibility that E2F1 signaling and DNA damage response pathways may converge and involve the same or related kinases to activate p53. Alternatively, E2F1 may contribute to or be a component of the DNA damage pathway. Given these possibilities, it is possible that a role of E2F1 may be to amplify DNA damage signals resulting in p53-mediated apoptosis.
CHAPTER IV

APOPTOSIS ASSOCIATED WITH DEREGERULATED E2F ACTIVITY IS DEPENDENT ON E2F1 AND Atm/Nbs1/Chk2
A. Introduction

Having found that E2F1 can induce p53-dependent apoptosis in the absence of p19^{ARF}, we wanted to determine the pathway(s) by which E2F1 activates p53 to induce cell death. The findings presented in chapter III demonstrate a requirement for covalent modification of p53 in the E2F1 apoptosis pathway, and that the p53 kinase(s) activated by E2F1 are sensitive to caffeine. The apoptosis program initiated by DNA damage also correlates with p53 modification, with a subset of DNA damage responsive p53 kinases being sensitive to caffeine (10). Therefore, we hypothesized that E2F1 may activate one or more DNA damage response kinase cascades resulting in p53 activation and apoptosis.

Kinases that have been shown to phosphorylate the N-terminus of p53 in response to DNA damage include ataxia telangiectasia mutated (Atm), the Atm-Rad3-related protein (Atr), and the human checkpoint kinases Chk1 and Chk2. Atm and Atr phosphorylate p53 at serine 15 as well as activate the Chk kinases by phosphorylation (20, 43, 217, 238, 259). Active Chk kinases can then further phosphorylate p53 at serine 15 and serine 20 (370). Activation of downstream kinase activation by Atm can also require Nbs1 (40, 234, 460). To address which kinase(s) may be involved in E2F1-mediated p53 activation and apoptosis, we used primary human fibroblasts from patients with ataxia telangiectasia and Nijmegen breakage syndrome, lacking functional Atm and Nbs1 gene products, respectively. Using cells from these patients, we tested the hypothesis that E2F1 and deregulation of Rb family members by expression of the HPV-16 E7 protein induces apoptosis through activation of a pathway that shares some similarities to the apoptosis program induced by DNA damage.
B. Roles for Atm and Nbs1 in apoptosis induction

Atm kinase activity is often induced in response to cellular stress leading to phosphorylation of many substrates, including serine 15 on p53. Given that ectopic E2F1 expression also results in phosphorylation of serine 15 on p53 (340), we determined whether Atm was required for E2F1-mediated apoptosis and p53 phosphorylation. We found that E2F1-mediated apoptosis was compromised in fibroblasts isolated from an AT patient ectopically expressing E2F1 (Figure 4.1). Similar results were obtained from AT fibroblasts isolated from a different donor (data not shown). In addition, apoptosis was also reduced following expression of E2F1 in atm−/− MEFs (Figure 4.2). The apoptosis observed in fibroblasts ectopically expressing E2F1 appears to be specific to E2F1 because the related E2F family member, E2F2, was unable to induce apoptosis (Figure 4.1).

We next examined the ability of E2F1 and E2F2 to induce the phosphorylation of serine 15 and serine 20 residues on p53 in the absence of Atm. We found that in cells lacking Atm, both E2F1 and E2F2 were still able to induce total p53 protein levels (Figure 4.3) likely due to induction of p14ARF (340). However, phosphorylation of p53 at serine 15 was reduced and phosphorylation at serine 20 was absent in AT cells following E2F1 expression (Figure 4.3). An increase in the phospho-serine 15 form of p53 was also observed following E2F2 expression in normal, but not AT, cells (Figure 4.3). Because E2F2 expression led to lower levels of the phospho-serine 15 form of p53 than E2F1, it was possible that E2F2 was not inducing apoptosis because it is not as efficient at
Figure 4.1. E2F1-induced apoptosis is reduced in human fibroblasts lacking functional Atm or Nbs1.

Apoptosis analysis in normal human dermal fibroblasts, AT fibroblasts, and NBS fibroblasts. Cells were infected with recombinant adenovirus encoding E2F1 (AdE2F1), E2F2 (AdE2F2), or a control virus (AdCon) at an MOI of 1000. Cells were harvested and apoptosis detected at 96 hours post infection (hpi). Error bars represent standard deviation of experiment performed in triplicate.
Figure 4.2. E2F1-induced apoptosis is reduced in mouse embryo fibroblasts lacking \textit{atm}.

Apoptosis induction in wildtype and \textit{atm}^{-/-} MEFs. Cells were infected with AdE2F1 or AdCon at an MOI of 500. Cells were harvested and apoptosis detected at 72 hpi. Error bars represent standard deviation of experiment performed in triplicate.
Figure 4.3. E2F1 and E2F2 require Atm and Nbs1 to efficiently induce phosphorylation of p53.

Western blot analysis of p53 protein levels following infection with AdCon, AdE2F1, AdE2F2 at an MOI of 1000. Cells were harvested and lysates generated at 24 hpi. The phospho-serine 15 and phospho-serine 20 forms of p53 were detected with polyclonal antisera specific for each modification. Actin blots are shown as protein loading controls.
inducing Atm activity as E2F1. To control for the differences in activation by E2F1 and E2F2 of the kinase(s) that phosphorylate p53 on serine 15, we expressed E2F2 at doses that resulted in levels of serine 15 phosphorylation and p53 accumulation that were similar to levels observed following E2F1 expression (Figure 4.6). Even at these elevated doses, E2F2 still did not induce apoptosis (Figure 4.5) or phosphorylation of p53 at serine 20 (Figure 4.6). Because E2F2 can also activate Atm resulting in p53 phosphorylation at serine 15 in the absence of apoptosis, these results suggest that while E2F1 requires Atm to signal apoptosis, Atm activation is not the commitment step for apoptosis induction.

In addition to directly phosphorylating p53 on serine 15 (20, 43), Atm also activates other kinases that lead to p53 phosphorylation on serine 20 (53, 155, 259, 370, 443). Since downstream kinase activation by Atm can require Nbs1 (40, 234, 460), we asked whether functional Nbs1 protein was necessary for E2F1-induced apoptosis. We found that E2F1-induced apoptosis was compromised in fibroblasts from NBS patients, similar to the reduction observed in AT cells (Figure 4.1). Although expression of E2F1 was found to be slightly lower in AT cells than in normal cells (Figure 4.4), increased amounts of E2F1 still did not induce apoptosis in AT cells (data not shown). Multiple forms of both endogenous and exogenously expressed E2F1 are routinely observed by western blot, likely due to posttranslational modifications associated with E2F1 regulation (212, 252, 253, 310). It remains unclear as to what patterns of posttranslational modifications contribute to the observed altered mobility of E2F1. Although E2F1 was able to induce total p53 protein levels in NBS cells (Figure 4.3), we observed a modest
Figure 4.4. AdE2F1 and AdE2F2 are expressed to similar levels in normal, AT, and NBS fibroblasts.

Normal, AT, and NBS fibroblasts were mock infected or infected with a low dose (MOI of 100) of either E2F1 or E2F2. Cells were harvested and lysates generated at 24 hpi and levels of E2F1 or E2F2 were compared by western blot analysis. Actin blots are shown as protein loading controls.
**Figure 4.5. Increased doses of AdE2F2 does not lead to apoptosis.**

Normal human fibroblasts were infected with AdCon, AdE2F1 at an MOI of 1000, or increasing doses of AdE2F2 (MOI of 1000, 1500, 2000, or 2500). Cells were harvested and apoptosis detected at 96 hpi. Error bars represent standard deviation of experiment performed in triplicate.
Figure 4.6. Increased doses of AdE2F2 lead to increased levels of phospho-serine 15 p53 but not phospho-serine 20 p53.

Normal human fibroblasts were infected with AdCon, AdE2F1 at an MOI of 1000, or increasing doses of AdE2F2 (MOI of 1000, 1500, 2000, or 2500). Cells were harvested and lysates generated at 24 hpi. Cellular lysates were analyzed for levels of p53 and the phospho-serine 15 and phospho-serine 20 forms of p53. Actin blots are shown as protein loading controls.
decrease in the levels of the phospho-serine 15 form and a large decrease in the levels of the phospho-serine 20 form of p53 in NBS cells following E2F1 expression (Figure 4.3), demonstrating that functional Nbs1 protein is required for E2F1-mediated apoptosis and for signaling p53 phosphorylation at the serine 20 residue. Ectopic E2F2 expression, found to be similar in all three cell types (Figure 4.4), failed to induce apoptosis in NBS cells (Figure 4.1) but did cause an increase in both total p53 levels and the phospho-serine 15 form of p53 (Figure 4.3). These results are consistent with a mechanism whereby E2F2 alters the phospho-serine 15 form of p53 in NBS cells through its ability to activate Atm.

**C. Chk2 is required for E2F1-mediated apoptosis**

Given that phosphorylation of p53 on serine 20 correlates with apoptosis, we proceeded to use this as a marker to identify any additional kinase(s) that may contribute to E2F1-mediated apoptosis. Among the Atm-induced kinases that require functional Nbs1 protein for activation and that directly phosphorylate p53 on serine 20, is the human checkpoint kinase Chk2 (40, 234, 370). To examine the role of Chk2 in E2F1-induced apoptosis, we coexpressed E2F1 with a kinase defective form of Chk2 (DN-Chk2) to inhibit Chk2 kinase activity in fibroblasts. We observed a reduction in apoptosis when E2F1 was coexpressed with DN-Chk2. Apoptosis levels did not appreciably change with a dominant negative form of Chk1 (DN-Chk1) (Figure 4.7), another DNA damage responsive kinase that is capable of phosphorylating p53 on serine 20 (370). Expression
Figure 4.7. Dominant negative Chk2, but not dominant negative Chk1 reduces E2F1-induced apoptosis.

Normal human fibroblasts were infected with AdCon or AdE2F1 at an MOI of 1000 and coinfectected with a recombinant adenovirus encoding dominant negative Chk1 (DN-Chk1) or dominant negative Chk2 (DN-Chk2) at an MOI of 1000. Cells were harvested and apoptosis detected at 96 hpi. Error bars represent standard deviation of experiment performed in triplicate.
Figure 4.8. siRNA targeting of Chk2 inhibits E2F1-induced apoptosis.

(A) HEL fibroblasts were transfected with siChk2a, siChk2b, or a control siRNA (siCon). Cells were harvested and lysates generated at 48 hours post transfection. Actin blot is shown as protein loading control. (B) HEL fibroblasts were transfected with siChk2b or siCon 24 hours prior to infection with AdCon, AdE2F1, or AdE2F2 at an MOI of 250. Cells were harvested and apoptosis detected at 96 hpi. Error bars represent standard deviation of experiment performed in triplicate.
of DN-Chk1 or DN-Chk2 alone did not alter levels of E2F1 protein (data not shown). To confirm the involvement of Chk2 in E2F1-mediated apoptosis, we used siRNAs to reduce the levels of Chk2 in cells (Figure 4.8 A). We observed a reduction in apoptosis following E2F1 expression in cells transfected with siChk2b (Figure 4.8 B), and no effect of this siRNA following expression of E2F2 (Figure 4.8 B). Similar results were obtained using siChk2a (data not shown).

We next determined the involvement of Chk2 in E2F1-induced p53 accumulation and modification. Since Chk2 directly phosphorylates p53 on serine 20, we asked if DN-Chk2 expression or Chk2 siRNA transfection could block E2F1-induced p53 phosphorylation. We found that DN-Chk2 expression and the Chk2 siRNA reduced the levels of the phospho-serine 20 form of p53 following E2F1 expression but had no effect on either total p53 levels or the levels of the phospho-serine 15 form of p53 (Figure 4.9 and 4.10). Coexpression of DN-Chk1 was unable to inhibit E2F1-induced p53 accumulation and had only a modest effect on p53 phosphorylation (Figure 4.9).

D. E2F1 specifically induces Chk2 expression

We have shown that E2F1 requires Atm, Nbs1, and Chk2 to efficiently induce apoptosis. However, E2F2 is also able to activate Atm resulting in phosphorylation of p53 at serine 15 but does so without inducing apoptosis. We confirmed the activation state of Atm using an antibody that recognizes a modified form of Atm observed following DNA damage that correlates with Atm activation (17). Expression of either E2F1 or E2F2 led to an increase in the levels of the phospho-serine 1981 form of Atm,
Figure 4.9. DN-Chk2, but not DN-Chk1, inhibits phosphorylation of p53 at serine 20.

Normal human fibroblasts were infected with AdCon or AdE2F1 at an MOI of 1000 and coinfected with DN-Chk1 or DN-Chk2 at an MOI of 1000. Cells were harvested and lysates generated at 24 hpi. Western blot analysis was performed to determine levels of p53 protein and levels of the phospho-serine 15 and phospho-serine 20 forms of p53. Actin blots are shown as protein loading controls.
Figure 4.10. siRNA targeting of Chk2 inhibits E2F1-induced phosphorylation of p53 at serine 20.

HEL fibroblasts were transfected with siCon or siChk2. Twenty four hours post transfection, cells were infected with AdCon or AdE2F1 at an MOI of 250. Cells were harvested and lysates generated at 24 hpi. Western blot analysis was performed to determine levels of p53 protein, levels of the phospho-serine 15 and phospho-serine 20 forms of p53, and levels of Chk2 protein. Actin blots are shown as protein loading controls.
Figure 4.11. Expression of E2F1, not E2F2, leads to increased Chk2 protein levels in normal, AT, and NBS cells.

Normal human fibroblasts, AT fibroblasts, and NBS fibroblasts were infected with AdCon, AdE2F1, or AdE2F2 at an MOI of 1000. Cells were harvested and lysates generated at 24 hpi. Western blot analysis was done to determine levels of Chk1 and Chk2 protein. Actin blots are shown as protein loading controls.
Figure 4.12. Expression of E2F1\textsubscript{e132} fails to induce apoptosis, increase Chk2 protein levels or induce phosphorylation of p53.

(A) Normal human fibroblasts were infected with AdCon, AdE2F1, or AdE2F1\textsubscript{e132} at an MOI of 1000. Cells were harvested and apoptosis detected at 96 hpi. Error bars represent standard deviation of experiment performed in triplicate. (B) Normal human fibroblasts were infected with AdCon, AdE2F1, or AdE2F1\textsubscript{e132} at an MOI of 1000. Cells were harvested and lysates generated at 24 hpi. Western blot analysis was done to determine levels of Chk2 protein or levels of (C) p53 protein and the phospho-serine 15 and phospho-serine 20 forms of p53. Actin blots are shown as protein loading controls.
while leaving the total Atm protein levels unchanged (Figure 4.12). The difference between E2F1 and E2F2 signaling appears to be the ability of E2F1 to stimulate Chk2 activity, which results in an increase in the phospho-serine 20 form of p53 and correlates with E2F1-induced apoptosis. Because Atm activation is upstream of Chk2 in signaling to p53, E2F1 expression must have an additional effect(s) downstream or independent of Atm that is specific to E2F1 for apoptosis induction. We found that expression of E2F1, but not E2F2, led to an increase in the levels of Chk2 protein, and this increase occurred in the absence of Atm or functional Nbs1 (Figure 4.11). E2F1 expression also results in accumulation of the phospho-threonine 68 form of Chk2 (data not shown), a modification observed following DNA damage that may be associated with Chk2 activation (259, 264). The phospho-threonine 68 modification of Chk2 may not be a reliable marker of Chk2 activation (6, 356, 439). Instead, we examined the Chk2 substrate p53 serine 20 residue as a marker for Chk2 activation. While expression of E2F1 resulted in an increase in Chk2 protein levels in the absence of functional Atm or Nbs1 (Figure 4.11), we did not observe an increase in the phospho-serine 20 form of p53 in these cells (Figure 4.3). Induction and activation of Chk2 by E2F1 appeared to be specific because E2F1 expression did not result in an increase in Chk1 protein (Figure 4.11) or in an increase in the phospho-serine 345 form of Chk1 (data not shown). Chk2 protein accumulation appeared to result from an increase in Chk2 mRNA levels following E2F1 expression (Figure 4.13 A). E2F2 expression did not lead to an increase in Chk2 protein levels (Figure 4.11) or Chk2 mRNA (Figure 4.13 A). Expression of an E2F1 DNA binding
Figure 4.13. Expression of E2F1 or E2F2 leads to an increase in the phospho-serine 1981 form of Atm while leaving total Atm protein levels unchanged.

Normal human fibroblasts were infected with AdCon, AdE2F1, or AdE2F2 at an MOI of 1000. Cells were harvested and lysates generated at 24 hpi. Western blot analysis was performed to determine levels of Atm and levels of the phospho-serine 1981 form of Atm.
Figure 4.14. E2F1, not E2F2, leads to an increase in Chk2 mRNA levels but not Atm mRNA levels.

(A) Northern blot analysis for Chk2 mRNA isolated from normal human fibroblasts infected with AdCon, AdE2F1, or AdE2F2 at an MOI of 1000. GAPDH shown as loading control. (B) Northern blot analysis for Atm mRNA isolated from normal human fibroblasts and infected as in (A). GAPDH shown as loading control.
mutant, E2F1<sub>e132</sub>, did not lead to an increase in Chk2 protein levels over control cells (Figure 4.12) suggesting that Chk2 may be induced by a transcriptional mechanism. We also did not detect apoptosis, p53 accumulation, or p53 phosphorylation following expression of E2F1<sub>e132</sub> (Figure 4.12). Since neither E2F1 nor E2F2 induced the expression of <i>Atm</i> mRNA (Figure 4.13 B), induction of <i>Chk2</i> expression appears to be specific to E2F1 and correlates with the induction of apoptosis.

**E. Chk2 and E2F cooperate in apoptosis induction**

Given that the induction of <i>Chk2</i> mRNA and protein levels following expression of E2F1 is associated with E2F1-specific apoptosis, we next asked whether Chk2 cooperates with E2F1 to signal apoptosis. We found that coexpression of Chk2 with a reduced amount of <i>E2F1</i> encoding virus resulted in enhanced levels of apoptosis, whereas expression of Chk2 alone had a nominal effect on apoptosis levels (Figure 4.14). Thus, it appears that Chk2 is limiting for E2F1-induced apoptosis. This observation raises the possibility that the different abilities of E2F1 and E2F2 to activate the apoptosis program lies in the capacity of E2F1 to induce <i>Chk2</i> expression. Given this possibility, we determined if Chk2 could cooperate with E2F2 to induce apoptosis if provided <i>in trans</i>. We found that coexpression of Chk2 with E2F2 permitted E2F2 to induce apoptosis (Figure 4.14) and led to an increase in the phospho-serine 20 form of p53 (Figure 4.15). Coexpression of E2F1 or E2F2 with Chk1 had no effect on apoptosis (data not shown). The sudden onset of serine 20 phosphorylation at the point where cells are
Figure 4.15. E2F1 and E2F2 cooperate with Chk2 in apoptosis induction.

Normal human fibroblasts were infected with AdCon, AdE2F1, or AdE2F2 at an MOI of 500 and coinfectected with the indicated doses of AdChk2. Cells were harvested and apoptosis detected at 96 hpi. Error bars represent standard deviation of experiment performed in triplicate.
Figure 4.16. Chk2 cooperates with E2F2 to induce phosphorylation of p53 at serine 20.

Normal human fibroblasts were infected with AdCon, AdE2F1, or AdE2F2 at an MOI of 500 and coinfectected with the indicated dose of AdChk2. Cells were harvested and lysates generated at 24 hpi. Western blot analysis was performed to determine levels of total p53 protein and levels of the phospho-serine 20 form of p53. Actin blots are shown as protein loading controls.
undergoing apoptosis in response to coexpression of E2F2 and Chk2 may mean that additional events are required to lead to phosphorylation of p53 through Chk2. These data suggest that an E2F1-specific increase in Chk2 expression is essential for p53 activation and apoptosis induction.

**F. Apoptosis induction by HPV-16 E7 is dependent on E2F1 and Atm/Nbs1/Chk2**

We next examined the role of Chk2 in apoptosis resulting from deregulation of endogenous E2F activity. The human papilloma virus (HPV) type 16 E7 protein binds to and inactivates Rb family members resulting in the release of Rb-associated factors, including E2F proteins (277). We found that expression of the HPV-16 E7 protein resulted in apoptosis induction in human fibroblasts plated at low density (Figure 4.16). Similar to the apoptosis observed following ectopic E2F1 expression, apoptosis resulting from Rb family inactivation by HPV-16 E7 required functional Atm and Nbs1 proteins (Figure 4.17). Since Rb inactivation by E7 results in release of five E2F family members from Rb proteins, we used siRNAs targeted to E2F1 to address the requirement for E2F1 in E7 induced apoptosis. We screened three siRNAs targeted to E2F1 for their ability to inhibit E2F1 expression (Figure 4.18 A). siE2F1c was used for the remainder of the experiments shown, but similar results were obtained with the other E2F1 siRNAs (data not shown). We found that pre-treatment of cells with siE2F1 blocked the ability of E7 to induce apoptosis (Figure 4.18 B and data not shown). Having observed that E7 induces apoptosis through E2F1, we next determined if E7-induced apoptosis requires Chk2. We found that reducing Chk2 levels with an siRNA decreased the ability of E7 to induce
Figure 4.17. HPV-16 E7 induces apoptosis in human fibroblasts.

HEL fibroblasts were infected with AdE2F1 at an MOI of 250, or AdCon and AdE7 at an MOI of 1000. Cells were harvested and apoptosis detected at 96 hpi. Error bars represent standard deviation of experiment performed in triplicate.
Figure 4.18. HPV-16 E7-induced apoptosis is reduced in cells lacking functional Atm or Nbs1.

Normal, AT, and NBS fibroblasts were infected with AdCon or AdE7 at an MOI of 1000. Cells were harvested and apoptosis detected at 96 hpi. Error bars represent standard deviation of experiment performed in triplicate.
Figure 4.19. siRNA targeting of E2F1 or Chk2 inhibits E7-induced apoptosis.

(A) Western blot analysis of E2F1 protein levels in cells transfected with siCon, siE2F1a, siE2F1b, or siE2F1c. Cells were harvested and lysates generated at 48 hours post transfection. Actin blot is shown as protein loading control. (B) Analysis of apoptosis in cells transfected with the marked siRNA 24 hours prior to infection with AdCon or AdE7 at an MOI of 1000. Cells were harvested and apoptosis detected at 96 hpi. Error bars represent standard deviation of experiment performed in triplicate.
apoptosis (Figure 4.18 B). To confirm the requirement of and determine the specificity for E2F1 in E7-mediated apoptosis, we used siRNAs targeted to E2F1, E2F2 or E2F3 (Figure 4.19). siE2F2a and siE2F3a were used for the remainder of the experiments shown, but similar results were obtained with the other E2F2 and E2F3 siRNAs (data not shown). We found that neither siE2F2 nor siE2F3 were able to block apoptosis induced by E7 expression (Figure 4.20). These data suggest that apoptosis resulting from deregulation of endogenous E2F activity occurs specifically through E2F1 and the Atm/Nbs/Chk2 pathway.

G. HPV-16 E7 induces Chk2 expression and p53 Modification

We next asked if E7 expression induces modifications to p53 similar to E2F1. We found that E7 expression resulted in an increase in p53 levels, and the levels of the phospho-serine 15 and phospho-serine 20 forms of p53 (Figure 4.21). Additionally, Chk2 protein levels were elevated following either E2F1 or E7 expression (Figure 4.22 A). Similar to E2F1, expression of E7 resulted in an increase in the phospho-serine 1981 form of Atm while leaving total Atm protein levels unchanged (Figure 4.22 B). Expression of E7 also resulted in an increase in the phospho-threonine 68 form of Chk2 (data not shown). These observations suggest that E7 is able to activate Atm kinase activity resulting in an increase in active Chk2 kinase.

We next determined whether E2F1 was required for E7 to increase Chk2 and p53 levels, and modifications to p53. We found that E2F1 is not required for much of the observed increase in p53 or the phospho-serine 15 form of p53 following E7 expression
Figure 4.20. siRNA knockdown of E2F2 and E2F3.

siRNAs targeted to E2F2 or E2F3 were transfected into HEL fibroblasts. Cells were harvested and lysates generated at 48 hours post transfection. Western blot analysis was performed to determine efficiency of siRNA targeting of E2F2 or E2F3. Actin blots are shown as protein loading controls.
Figure 4.21. HPV-16 E7 requires E2F1 but not E2F2 or E2F3 to induce apoptosis.

HEL fibroblasts were transfected with the marked siRNA 24 hours prior to infection with AdCon or AdE7 at an MOI of 1000. Cells were harvested and apoptosis detected at 96 hpi. Error bars represent standard deviation of experiment performed in triplicate.
Figure 4.22. HPV-16 E7 expression results in p53 accumulation and modification, similar to those observed following expression of E2F1.

HEL fibroblasts were infected with AdE2F1 at an MOI of 250, or AdCon and AdE7 at an MOI of 1000. Cells were harvested and lysates generated at 24 hpi. Western blot analysis was performed to determine the levels of total p53 protein and the levels of the phospho-serine 15 and phospho-serine 20 forms of p53. Actin blots are shown as protein loading controls.
(Figure 4.23). This increase in p53 protein was likely due to activation of the p14ARF/Mdm2 pathway by E2F2 and/or E2F3, while the increase in the phospho-serine 15 form of p53 is likely due to the ability of E2F2, and possibly other E2Fs, to activate Atm (Figure 4.12 and Figure 4.22 B). However, we found that E2F1 was required for E7 to induce the phosphorylation of p53 at serine 20 and to increase the levels of Chk2 protein (Figure 4.23). Additionally, we found that E7 expression resulted in an increase in Chk2 mRNA levels (Figure 4.24) and this could be attributed to E2F1 (Figure 4.24).

Having found that E2F1 is required for E7 to induce Chk2 expression and phosphorylation of p53 at serine 20, we next determined if Chk2 was required for E7 to induce this modification. We found that an siRNA directed against Chk2 was able to block E7-induced phosphorylation of p53 at serine 20, while total p53 levels and the levels of the phospho-serine 15 form of p53 remained unaffected by addition of this siRNA (Figure 4.23). These results demonstrate a requirement for E2F1 in Chk2 induction and kinase activation following Rb inactivation by HPV-16 E7 as measured by an increase in the phospho-serine 20 form of p53. Taken together, apoptosis resulting from inactivation of Rb family members is dependent specifically on E2F1 and its ability to induce Chk2 expression.
Figure 4.23. HPV-16 E7 expression results in an increase in Chk2 protein levels and levels of the phospho-serine 1981 form of Atm, while leaving total Atm protein levels unchanged.

(A) HEL fibroblasts were infected with AdE2F1 at an MOI of 250, or AdCon and AdE7 at an MOI of 1000. Cells were harvested and lysates generated at 24 hpi. Western blot analysis was performed to determine Chk2 protein levels. Actin blot is shown as protein loading control. (B) HEL fibroblasts were infected as in (A). Cells were harvested and lysates generated at 24 hpi. Western blot analysis was performed to determine Atm protein levels and levels of the phospho-serine 1981 form of Atm.
Figure 4.24. HPV-16 E7 requires E2F1 and Chk2 to increase the levels of the phospho-serine 20 form of p53.

HEL fibroblasts were transfected with the marked siRNA 24 hours prior to infection with AdCon or AdE7 at an MOI of 1000. Cells were harvested and lysates generated at 24 hpi. Western blot analysis was performed to determine the levels of total p53, the levels of the phospho-serine 15 and phospho-serine 20 forms of p53, and levels of Chk2. Actin blots are shown as protein loading controls.
Figure 4.25. HPV-16 E7 requires E2F1 to induce Chk2.

HEL fibroblasts were untransfected or transfected with the marked siRNA 24 hours prior to infection with AdE2F1 at an MOI of 250, or AdCon and AdE7 at an MOI of 1000. RNA was isolated at 24 hpi. Northern blot analysis was done to determine levels of Chk2 RNA. GAPDH shown as loading control.
H. Discussion

In this chapter we describe a pathway involving the E2F1-specific induction of Chk2 expression that links loss of proliferation control to an apoptotic pathway with some similarity to the apoptosis pathway induced by DNA double strand breaks. Unlike the DNA damage signals that activate p53 in the absence of de novo gene expression (178), E2F1-mediated apoptosis requires induction of Chk2 expression and possibly other components of apoptosis signaling (283) to fully activate p53 and kill cells. The results presented here suggest that deregulated E2F1 function induces apoptosis by activation of an Atm/Nbs1/Chk2/p53 pathway following disruptions in the Rb/E2F proliferation pathway (Figure 4.25).

To model deregulation of E2F proteins, we expressed the HPV-16 E7 protein, and determined the requirement for E2F family members and the Atm/Nbs1/Chk2 pathway for signaling to p53 and apoptosis induction. It has been suggested that E2F1 is responsible for a portion of the phenotypes associated with E7 expression, including disruption of normal cell cycle control, interfering with cellular differentiation, and apoptosis induction (54, 286). Supporting the involvement of E2F1 in E7-induced apoptosis, transgenic mice that express E7 in the lens exhibit increased apoptosis in the fiber cell compartment that is dependent on E2F1 (261, 304). The results presented here suggest that apoptosis induction by HPV-16 E7-mediated Rb family inactivation in primary human fibroblasts relies primarily on the ability of E7 to activate E2F1 and the Atm/Nbs1/Chk2/p53 pathway (Figure 4.25).
Figure 4.26. Model of p53 activation following deregulation of E2F1.

Deregulation of the Rb/E2F proliferation pathway results in activation of an apoptotic pathway with some similarities to the apoptosis pathway induced by DNA double strand breaks. Apoptosis induction associated with deregulated E2F activity is dependent on E2F1, and the ability of E2F1 to induce Chk2 and to signal through Atm/Nbs1 resulting in activation of p53 and apoptosis.
In this study, we examined phosphorylation of the serine 20 residue on p53 as a marker for Chk2 activation. In normal, AT, and NBS cells, while we observed an increase in total p53 levels, only in normal cells did we observe an increase in the phospho-serine 20 form of p53, a substrate for active Chk2 kinase. Inhibition of Chk2 activity by a dominant negative construct or by siRNA targeting resulted in a failure to phosphorylate the serine 20 residue following expression of either E2F1 or E7, demonstrating the specificity of this modification by Chk2.

While we describe the involvement of E2F1 in apoptosis resulting from Rb family inactivation, E2F1 also plays a role in apoptosis induction following exposure of cells to DNA damaging agents (235). We speculate that activation of E2F1 by DNA damage leads to increased \( p14^{ARF} \) expression resulting in increased pools of p53 protein. E2F1 is also able to activate Atm kinase activity and induce \( Chk2 \) expression leading to increased p53 activation and E2F1 activity. E2F1 activation following DNA damage would therefore act to amplify DNA damage signals converging at p53 to result in apoptosis.
CHAPTER V

E2F1 INDUCES Mre11 FOCI FORMATION AND BLOCKS CELL CYCLE PROGRESSION IN HUMAN FIBROBLASTS
A. Introduction

Having found that Chk2 induction is specific to E2F1, we next determined what upstream signals are specifically activated by E2F1. Activation of p53 following DNA damage involves both activation of DNA damage response kinases, such as Atm, Atr, and Chk2, as well as relocalization of DNA damage recognition proteins (1, 285, 372). Among the DNA damage recognition proteins that relocalize to sites of DNA breaks are Mre11, Rad50, and Nbs1, components of the MRN DNA damage recognition complex (47, 285). Activation of certain S phase cell cycle checkpoints also requires the activity of Atm (373). The Atm triggered S phase checkpoint requires, among other proteins, functional Nbs1, a component of the MRN complex that is also required for E2F1-induced apoptosis and recognition of certain forms of DNA damage (412). In addition to MRN relocalization following DNA damage, the DNA damage sensors 53BP1 and γH2AX also relocalize to sites at or near DNA breaks (205, 217, 426). Because of the requirement for DNA damage response proteins for E2F1-induced apoptosis, we hypothesized that E2F1 may induce relocalization of some or all of these DNA sensors.
B. E2F1 specifically causes MRN relocalization

The MRN complex functions in checkpoint signaling by relocalization to discrete nuclear foci following certain types of DNA damage (47, 66, 255, 285). Therefore, we examined the cellular localization of the MRN complex following expression of E2F proteins by examining the Mre11 component of the complex. We found that expression of E2F1 induces relocalization of Mre11 to discrete nuclear foci, while no foci were observed following expression of E2F3a (Figure 5.1 A). Mre11 foci were observed in 98% of cells following expression of E2F1, but at levels similar to control following expression of E2F3a (Figure 5.1 B). Formation of Mre11 foci correlated with an increase in the phospho-serine 20 form of p53 (Figure 5.2) and with apoptosis induction (Figure 5.3). Expression of E2F3a resulted in an increase in the phospho-serine 15 form of p53 (Figure 5.2), a target of the Atm kinase, but not with an increase in the phospho-serine 20 (Figure 5.2) form of p53 or with apoptosis induction (Figure 5.3).

C. E2F1 induces irradiation induced foci (IRIF)-like formation

The formation of MRN foci has been described under a number of cellular conditions (241, 254, 255, 285, 437). To determine the type of MRN foci formed following expression of E2F1, we sought to further characterize the Mre11 foci. MRN foci formation requires the Nbs1 component of the complex (47). Similarly, we found that E2F1-induced Mre11 foci fail to form in cells lacking Nbs1 (Figure 5.4). DNA damage foci have also been shown to form independently of Atm (267). Likewise, we
Figure 5.1. E2F1 specifically induces Mre11 foci formation.

(A) Normal human dermal fibroblasts were infected with AdCon, AdE2F1, or AdE2F3a at an MOI of 1000. Cells were fixed and stained for Mre11 at 24 hpi. (B) Normal human dermal fibroblasts were infected with AdCon, AdE2F1, or AdE2F3a at the indicated MOIs. Cells were fixed and stained for Mre11 at 24 hpi. Cells were counted to determine the percentage of cells with Mre11 foci. A minimum of 300 cells per well was counted.
Figure 5.2. Expression of E2F1, but not E2F3a, results in an increase in the phospho-serine 20 form of p53.

Normal human dermal fibroblasts were infected with AdCon or AdE2F1 at an MOI of 1000, or AdE2F3a at MOIs of 1000, 1500, 2000, or 2500. Cells were harvested and lysates generated at 24 hpi. Western blot analysis was done to determine levels of total p53 protein, and levels of the phospho-serine 15 and phospho-serine 20 forms of p53. Actin blots are shown as protein loading controls.
Figure 5.3. E2F1, but not E2F3a, induces apoptosis in human fibroblasts.

Normal human dermal fibroblasts were infected with AdCon or AdE2F1 at an MOI of 1000, or AdE2F3a at MOIs of 1000, 1500, 2000, or 2500. Cells were harvested and apoptosis detected at 96 hpi. Error bars represent standard deviation of experiment performed in triplicate.
**Figure 5.4. E2F1 fails to induce Mre11 foci in NBS cells.**

Normal human dermal fibroblasts or NBS fibroblasts were infected with AdCon or AdE2F1 at an MOI of 1000. Cells were fixed and stained for Mre11 at 24 hpi.
found that E2F1 is able to induce the formation Mre11 foci in cells lacking Atm (Figure 5.5). To confirm the involvement of Nbs1 in the Mre11 foci observed following expression of E2F1, we examined the colocalization of Nbs1 and Mre11. We found that Nbs1 and Mre11 colocalize to nuclear foci following expression of E2F1 (Figure 5.6 A). In addition to relocalization of the MRN complex, DNA damage also induces relocalization of other DNA damage response proteins, including γH2AX and 53BP1 (9, 205, 338, 353, 426). We found that expression of E2F1 induces relocalization of γH2AX and 53BP1 to nuclear foci, and this correlates with the relocalization of Mre11 foci (Figure 5.6 B and Figure 5.6 C). Taken together, these results suggest that ectopic expression of E2F1 induces the formation of foci similar to the irradiation induced foci (IRIF) seen following DNA damage.

MRN complex relocalization has also been observed during normal S phase progression, when no extrinsic DNA damaging agent has been present (254). Since E2F1 and E2F3a have been described to induce S phase in rodent fibroblasts (76, 183, 211), we determined if the observed foci could be attributed to S phase progression. A hallmark of the reported S phase MRN foci is their localization at or near sites of BrdU incorporation. When we examined the subset of cells that incorporate BrdU following expression of E2F1, we found no colocalization of Mre11 and BrdU (Figure 5.7). Indeed, we observed very few BrdU positive cells following expression of E2F1. The rare cells that show BrdU incorporation following expression of E2F1 show no colocalization of Mre11 and BrdU (Figure 5.7 bottom panels).
Figure 5.5. E2F1 induces Mre11 foci formation in AT cells.

Normal human dermal fibroblasts or NBS fibroblasts were infected with AdCon or AdE2F1 at an MOI of 1000. Cells were fixed and stained for Mre11 at 24 hpi.
Figure 5.6. E2F1 induces relocalization of DNA damage response proteins.

Normal human dermal fibroblasts were infected with AdCon or AdE2F1 at an MOI of 1000. Cells were fixed and stained for Mre11 and Nbs1 (A), Mre11 and γH2AX (B), or Mre11 and 53BP1 (C) at 24 hpi. Merged images are also shown.
Figure 5.7. Expression of E2F1 induces Mre11 foci that do not correlate with BrdU incorporation.

Normal human dermal fibroblasts were infected with AdCon or AdE2F1 at an MOI of 1000. Cells were fixed and stained for Mre11 and BrdU at 24 hpi. Merged images are also shown.
Normal human dermal fibroblasts were infected with AdCon, AdE2F1, or AdE2F1\textsubscript{1-283} at an MOI of 1000. Cells were fixed and stained for Mre11 at 24 hpi.
Figure 5.9. $E2F1_{e132}$ induces Mre11 foci formation similar to $E2F1$.

Normal human dermal fibroblasts were infected with AdCon, Ad$E2F1$, or Ad$E2F1_{1-283}$ at an MOI of 1000. Cells were fixed and stained for Mre11 at 24 hpi.
It is possible that an interaction between E2F1 and Nbs1 (254) may provide a mechanism for the observed MRN relocalization following expression of E2F1. The MRN complex has also been shown to relocalize to sites that contain E2F binding elements near origins of DNA replication (254). To examine whether the interaction of E2F1 and Nbs1 is required for MRN relocalization, we used an E2F1 mutant, E2F1<sub>1-283</sub>, that lacks the Nbs1 interaction domain (254). We found that expression of E2F1<sub>1-283</sub> results in MRN relocalization similar to expression of full length E2F1 (Figure 5.8). Additionally, expression of a DNA binding mutant of E2F1, E2F1<sub>e132</sub>, was found to induce relocalization of Mre11 (Figure 5.9), suggesting that E2F1 is not responsible for targeting the MRN complex to specific sites on DNA. Taken together, these results suggest that expression of E2F1 induces foci consistent with IRIF, and not foci correlative of S phase induction.

D. E2F1 blocks cell cycle progression in human fibroblasts, roles for Nbs1 and 53BP1

The observed formation of MRN foci following expression of E2F1 suggests that E2F1 may be activating a cell cycle checkpoint. However, the importance of E2F1 and E2F3a in promoting the transition from $G_1$ to S phase of the cell cycle has been well documented. Ectopic expression of E2F1 or E2F3a in rodent fibroblasts has been shown to promote S phase entry, even under serum deprivation (76, 183, 210). However, ectopic expression of E2F1 in human cells has been found to be insufficient for S phase entry, and can lead to senescence in normal human fibroblasts (83, 240, 265, 379). Following
Figure 5.10. E2F1, but not E2F3a, blocks cell cycle progression.

Serum starved HEL fibroblasts were infected with AdCon, AdE2F1, or AdE2F3a at an MOI of 250. Immediately following infection, cells were returned to normal growth medium. Cells were fixed and stained for BrdU incorporation at 24 hpi. A minimum of 300 cells per well was counted.
Figure 5.11. Expression of E2F1 and E2F3a results in increased levels of the phospho-serine 1981 form of Atm.

HEL fibroblasts were infected with AdCon, AdE2F1, or AdE2F3a at an MOI of 250. Cells were harvested and lysates generated at 24 hpi. Western blot analysis was performed to determine the levels of the phospho-serine 1981 form of Atm.
DNA damage, relocalization of DNA damage response proteins, including the MRN complex and 53BP1, can lead to activation of a cell cycle checkpoint, blocking cell cycle progression (84, 111, 185, 384, 394, 423, 450). We therefore tested the ability of E2F1 to block cell cycle progression. When normal growth medium was added to starved cells at the time of E2F expression, we found that E2F1 blocks cells from entering the cell cycle while expression of E2F3a allowed cell cycle progression as measured by BrdU incorporation (Figure 5.9). A block in cell cycle progression is commonly observed following DNA damage when a cell cycle checkpoint is activated to prevent replication of damaged DNA (49). Firing of an S phase checkpoint can involve activation of Atm and downstream signaling proteins (191). We found that expression of E2F1 or E2F3a is able to activate Atm, as measured by an increase in the phospho-serine 1981 form of Atm (Figure 5.10).

Since Atm activation was not unique to E2F1 and did not correlate with the observed block in cell cycle progression, we examined the roles of DNA damage signaling proteins Nbs1 and 53BP1 in preventing cell cycle progression. Nbs1 and 53BP1 are important in the Atm-dependent S phase checkpoint, and loss of either Nbs1 or 53BP1 hypersensitizes cells to ionizing radiation and abrogates activation of DNA damage induced cell cycle checkpoints (66, 84, 111, 423, 427). To determine the roles of Nbs1 and 53BP1 in blocking cell cycle progression following expression of E2F1, we used siRNAs to reduce the levels of Nbs1 or 53BP1 in cells (Figure 5.11 A). We found that transfection of cells with siNbs1 or si53BP1 prior to expression of E2F1 partially alleviated the block in cell cycle progression (Figure 5.11 B). Transfection of siNbs1 and
Figure 5.12. Roles for Nbs1 and 53BP1 in blocking cell cycle progression.

(A) HEL fibroblasts were transfected with siRNAs targeted to 53BP1 or Nbs1. Cells were harvested and lysates generated at 48 hpi. Western blot analysis was performed to determine levels of 53BP1 or Nbs1 protein. (B) HEL fibroblasts were transfected with marked siRNAs, serum deprived for 48 hours, and infected with AdCon or AdE2F1 at an MOI of 250. Immediately following infection, cells were returned to normal growth medium. Cells were fixed and stained for BrdU incorporation at 24 hpi. A minimum of 300 cells per well was counted.
Figure 5.13. Expression of E2F1 leads to accumulation of p21 protein.

HEL fibroblasts were infected with AdCon or AdE2F1 at an MOI of 250. Cells were harvested and lysates generated at indicated hpi. Western blot analysis was performed to determine levels of p21 protein. Actin blot is shown as protein loading control.
Figure 5.14. Role for p21 in blocking cell cycle progression.

(A) HEL fibroblasts were transfected with siRNAs targeted to p21. Cells were harvested and lysates generated at 48 hpi. Western blot analysis was performed to determine levels of p21 protein. (B) HEL fibroblasts were transfected with marked siRNAs, serum deprived for 48 hours, and infected with AdCon or AdE2F1 at an MOI of 250. Immediately following infection, cells were returned to normal growth medium. Cells were fixed and stained for BrdU incorporation at 24 hpi. A minimum of 300 cells per well was counted.
Figure 5.15. Role for p53 in blocking cell cycle progression and p21 accumulation.

(A) HEL fibroblasts were transfected with an siRNA targeted to p53. Cells were harvested and lysates generated at 48 hpi. Western blot analysis was performed to determine levels of p53 protein. (B) Cells were transfected with marked siRNAs, serum deprived for 48 hours, and infected with AdCon or AdE2F1 at an MOI of 250. Immediately following infection, cells were returned to normal growth medium. Cells were fixed and stained for BrdU incorporation at 24 hpi. A minimum of 300 cells per well was counted. (C) Cells were transfected with the marked siRNA 24 hours prior to infection with AdCon or AdE2F1 at an MOI of 250. Cells were harvested and lysates generated at 24 hpi. Western blot analysis was performed to determine levels of p21 protein.
si53BP1 together showed no effect on cell cycle progression above transfection of the single siRNAs (data not shown). This data suggests that Nbs1 and 53BP1 play a role in blocking cell cycle progression following expression of E2F1.

E. Roles for p53 and p21 in E2F1-induced cell cycle block

In the DNA damage response pathway, Nbs1 and 53BP1 function upstream of p53 activation (185, 423). We therefore determined if p53 played a role in the block in cell cycle progression. We found that treatment of cells with sip53 prior to expression of E2F1 partially alleviated the observed block in cell cycle progression (Figure 5.14 B). Activation of p53 following certain forms DNA damage can result in activation of a p21-dependent cell cycle checkpoint (23, 24, 97, 98). We therefore determined if p21 played a role in the block in cell cycle progression observed following expression of E2F1. We found that expression of E2F1 in randomly cycling cells resulted in a rapid accumulation of p21 protein (Figure 5.12). Additionally, we found that reducing p21 protein levels in cells with an siRNA targeted to p21 (Figure 5.13 A) partially alleviated the block in cell cycle progression observed following expression of E2F1 (Figure 5.13 B). Treatment of cells with sip21 together with sipNbs1 and/or sip53BP1 showed no effect on cell cycle progression above sip21 alone (data not shown). Since p21 is a known transcriptional target of p53 (97, 98), we determined if p53 was responsible for the observed induction of p21. We found that treatment of cells with an siRNA targeted to p53 (Figure 5.14 A) reduced the ability of E2F1 to promote accumulation of p21 (Figure 5.14 C). Treatment of cells with sip53 together with sip21, sipNbs1, or sip53BP1 showed no effect on cell cycle
progression above sip2I alone (data not shown). Together, these observation suggest that expression of E2F1 blocks cell cycle progression in part through its ability of activate p53 resulting in accumulation of p21 protein.

The observation that expression of E2F1 resulted in a rapid accumulation of p21 protein and a block in cell cycle progression suggests that E2F1 is activating a cell cycle checkpoint prior to the induction of apoptosis. To test whether checkpoint activation by E2F1 results in a delay in the apoptotic response, we reduced the levels of 53BP1 or p21 in cells with siRNAs and examined the ability of E2F1 to induce apoptosis. We found that treatment of cells with si53BP1 or sip2I prior to expression of E2F1 resulted in an accelerated induction of apoptosis (Figure 5.15). Taken together, these results suggest that E2F1 is activating a cell cycle checkpoint that delays the induction of apoptosis.
Figure 5.16. Reduced levels of 53BP1 of p21 leads to enhanced E2F1-induced apoptosis.

HEL fibroblasts were transfected with the marked siRNA 24 hours prior to infection with AdCon or AdE2F1 at an MOI of 250. Cells were harvested and apoptosis detected at indicated times following infection. Error bars represent standard deviation of experiment performed in triplicate.
E. Discussion

In this chapter we describe E2F1-induced relocalization of the DNA damage recognition MRN complex into discrete nuclear foci characteristic of foci observed following DNA damage. Mre11 relocalization is found to correlate with relocalization of the DNA damage response proteins γH2AX and 53BP1. We also find that expression of E2F1 results in a block in cell cycle progression, at least partially dependent on activation of a cell cycle checkpoint pathway involving Nbs1, 53BP1, p53, and p21. Expression of E2F1 results in an increase in p21 protein levels that correlates with the observed block in cell cycle progression. These data suggest that initially following expression of E2F1, a growth arrest pathway consisting of Nbs1, 53BP1, and p21 predominates (Figure 5.16 left). The finding that reducing p21 and 53BP1 protein levels accelerates apoptosis suggests that continued expression of E2F1 or inactivation of this cell cycle checkpoint pathway favors activation of the apoptosis pathway (Figure 5.16 right).

Our data show that formation of Mre11-containing foci following expression of E2F1 correlates with relocalization of the DNA damage signaling proteins γH2AX and 53BP1. However, we observe incomplete overlap of these proteins. Colocalization of the DNA damage response proteins Mre11 and γH2AX has been observed following γ-irradiation (51, 309). However, these studies examined foci formation at later time points following γ-irradiation when the majority of lesions have been repaired (377). Using a chromatin extraction protocol, Petrini and colleagues found that Mre11 and γH2AX foci do not colocalize, but remain adjacent throughout the course of DNA repair (267, 268). This is not surprising given that γH2AX foci can be observed not only at sites of
Figure 5.17. Model of E2F1-induced apoptosis and growth arrest.

(Left) Initial expression of E2F1 leads to a block in cell cycle progression through Nbs1, 53BP1, p53, and p21. (Right) Continued expression of E2F1 leads to activation of the apoptosis program. Reduction of 53BP1 or p21 protein levels acts to reduce the ability of E2F1 to promote growth arrest and favors the apoptosis pathway.
damaged DNA, but also over large portions of a chromosome surrounding DNA damage (337). Therefore, our data showing incomplete overlap of E2F1-induced foci is consistent with foci observed following γ-irradiation. The question remains as to why DNA damage-like foci form following expression of E2F1. We speculate that ectopic E2F1 activity may lead to DNA breaks either directly or as a result of slowing or preventing DNA repair. The MRN complex and other DNA damage signaling proteins may then recognize these breaks in a manner similar to the DNA damage response, and initiate a signaling pathway resulting in activation of a cell cycle checkpoint or apoptosis.

Our data describe a partial alleviation of the E2F1-induced block in cell cycle progression by siRNA targeting of the p21 checkpoint pathway. While it appears that the p21 pathway is only partially required for this checkpoint, we cannot rule out the possibility that, due to incomplete knockdown in gene expression, that the observed checkpoint is totally dependent on the p21 pathway. However, this seems unlikely due to the undetectable levels of p21 protein remaining after siRNA treatment, and only a modest alleviation of the cell cycle block. It remains unclear as to what additional components may be required for mediating this block. It is possible that p14ARF may be contributing to the observed block in cell cycle progression. p14ARF has been found to play a role in E2F1-induced senescence (83). However, the role of p14ARF in the E2F1-induced senescence pathway is dependent on p53 function (83). We observe only a partial increase in cell cycle progression following treatment of cells with sip53, suggesting that an additional pathway(s) that are p53 independent may be contributing to the observed growth arrest. One such pathway may involve activation of the cyclin
dependent kinase inhibitor p16\textsuperscript{ink4a}. p16 plays roles in both growth arrest and senescence by inhibiting Rb phosphorylation (7, 189, 381). The potential role of p16 in E2F1-mediated growth arrest remains to be determined.
CHAPTER VI

DISCUSSION
A. Thesis overview

The goal of this study was to determine the pathway(s) by which deregulation of the Rb growth control pathway results in p53-dependent apoptosis. It had been well established that inactivation of Rb by mutation, gene deletion, or binding by viral oncoproteins results in apoptosis that is often p53-dependent. Mouse models and in vitro evidence suggest that E2F1 is the primary mediator linking Rb inactivation to p53 activation and apoptosis (246, 305, 316, 408). The prevailing hypothesis of E2F1-induced apoptosis proposed a central role for the p19ARF/Mdm2 pathway in activation of p53 and apoptosis following deregulation of cellular growth control (25, 189).

The data presented in this thesis demonstrate that p19ARF is not required for p53-dependent apoptosis following expression of E2F1. Instead, we found that E2F1-induced apoptosis requires covalent modifications of p53 by a caffeine sensitive kinase(s). The DNA damage kinases Atm and Chk2, both of which are sensitive to caffeine, as well as the DNA damage response protein Nbs1 are required for E2F1 and HPV-16 E7 to induce apoptosis. Upregulation of Chk2 is specific to E2F1, and correlates with p53 activation and apoptosis. The data also show that E2F1 specifically induces the relocalization of DNA damage recognition and response proteins including Mre11, Nbs1, 53BP1, and γH2AX prior to apoptosis. The relocalization of DNA damage sensors correlates with a block in cell cycle progression that is mediated, in part, by activation of a p21-dependent cell cycle checkpoint. These result suggest that deregulation of E2F1 results in activation of a cell cycle checkpoint prior to the induction of apoptosis.
B. E2F1 is the central mediator of apoptosis following loss of Rb growth control.

E2F1 has been reported to play dual roles as both an oncogene and a tumor suppressor. It has been proposed that the ability of E2F1 to act as a tumor suppressor is mainly due to activation of the apoptosis program (408). The tumor suppressive properties of E2F1 are revealed in mice lacking E2F1 which exhibit defects in apoptosis and display an increase in tumor formation (113, 449). The role of E2F1 in the DNA damage pathway, discussed below, also support the role of E2F1 as a tumor suppressor. The role of E2F1 as an oncogene have been attributed mainly to the ability of E2F1 to promote re-entry into the cell cycle. Overexpression of E2F1 and activated Ras in primary cells can result in transformation (182). Overexpression of E2F1 in vivo can also result in an increase in tumor formation and loss of E2F1 leads to reduction in tumor growth in Rb\textsuperscript{+/−} mice (317, 448).

Our data support the roles of E2F1 as both an oncogene and a tumor suppressor. Overexpression of E2F1 or inactivation of Rb by the HPV-16 E7 protein results in apoptosis, consistent with the role of elevated levels of E2F1 acting to suppress tumorigenesis. Specifically, the data in chapter IV demonstrate that E2F1, and not the other activator E2F family members, acts as the central mediator of apoptosis induction in response of loss of Rb growth control. However, our studies also indicate how E2F1 may also function as an oncogene. Overexpression of E2F1 in human fibroblasts leads to relocalization of DNA damage recognition and response proteins, suggesting that elevated E2F1 activity may lead to an increase in mutation rate and/or genomic instability, possibly by an accumulation of DNA breaks.
It appears as though p53 also plays dual roles in the E2F1 signaling pathways. Accumulation of p21 protein following expression of E2F1 is likely due to transactivation of p21 expression by p53. Indeed, targeting of p53 with an siRNA reduced the ability of E2F1 to increase p21 protein levels. Induction of apoptosis by E2F1 also requires activation of p53 through covalent modification by Atm, Chk2, and possibly additional kinases. It remains to be determined if there are differences in p53 activation by E2F1 that mediate either a growth arrest or apoptosis.

It is conceivable that in normal cells, E2F1 has the ability to respond to loss of proper growth control and induce a block in cell cycle progression associated with redistribution of DNA damage repair molecules. If proper cell cycle regulation is not returned, sustained E2F1 activity would then promote apoptosis. In human cells, the inactivation of Rb by cellular or viral oncoproteins would lead to increased E2F1 activity. E2F1 would then activate p21 resulting in activation of cell cycle checkpoint to prevent inappropriate proliferation and tumorigenesis. Activation of p21 could further inhibit E2F1 activity by blocking cellular cyclin-Cdk activity and additional Rb phosphorylation. However, sustained E2F1 activity may bypass the cell cycle arrest, and promote apoptosis. Therefore, E2F1 may act as a transducer of signals resulting from deregulation of Rb growth control, promoting a block in cell cycle progression to allow for restoration of proper cell cycle regulation or apoptosis to eliminate the potential for subsequent transformation of cells. It remains unclear as to why E2F1 blocks cell cycle progression in human fibroblasts but is able to induce S phase in rodent cells, even in the presence of increased p21 protein levels (77). It is possible that rodent E2F1 fails to activate the p53-
independent checkpoint pathway suggested in Chapter V. This would be consistent with the observations that rodent fibroblasts fail to undergo senescence as efficiently as human fibroblasts (346, 376).

The importance of the E2F family, and E2FI specifically, in responding to loss of appropriate cellular proliferation control suggests that E2F genes may be an attractive target for mutation in tumorogenesis. However, while mutations in the Rb growth control pathway are common in tumors, mutations in E2F family members are rarely found in human tumors. E2F expression has been reported to be either reduced or amplified in some different tumor types (99, 106, 110, 157, 177). However, the infrequency of E2F1 mutations identified in human tumors supports the dual roles of E2F1 demonstrated by our findings. We speculate that mutations resulting in loss of E2F function would be disadvantageous for tumor formation possibly due to a loss in proliferative capacity. Amplification of E2F1 would be similarly disadvantageous due to the ability of E2F1 to induce apoptosis when deregulated.

C. Pathways linking E2F1 to p53

1. Role of ARF in E2F signaling

Our data presented in chapter III clearly show that distinct pathways mediate p53 accumulation and p53-dependent apoptosis following ecotopic expression of E2F1 in rodent fibroblasts. However, in MEFs, mouse models, and human cells, p19ARF is dispensable for apoptosis induction by E2F1 (236, 340, 342, 401, 409). While p19ARF is not required for E2F1-induced apoptosis, it is likely that activation of the p19ARF/Mdm2
pathway influences E2F1 signaling. Evidence points to a role for p19\textsuperscript{ARF} in negative regulation of cellular growth control. Mouse p19\textsuperscript{ARF} and human p14\textsuperscript{ARF} both physically interact with E2F1, inhibit E2F1 transactivation function, and promote its degradation (107, 251, 256). In addition to inhibition of E2F1, expression of p19\textsuperscript{ARF} is able to inhibit deregulated proliferation associated with expression of the cellular oncoproteins Ras and c-Myc as well as the viral oncoproteins E1A and E7 (73, 303, 306, 463). p19\textsuperscript{ARF} also physically interacts with DP1, the heterodimeric E2F binding partner (68). The interaction between p19\textsuperscript{ARF} and DP1 is inhibited by coexpression of DP1 and E2F1, suggesting that DP1-E2F1 complexes that exist during the G1-S boundary are resistant to negative regulation by p19\textsuperscript{ARF} (68). Taken together, induction of p19\textsuperscript{ARF}, either by ectopic expression of E2F or expression of HPV-16 E7, would provide negative feedback on the growth promoting activity of E2F1.

However, there is a species-specific difference in the activity of mouse p19\textsuperscript{ARF} and human p14\textsuperscript{ARF} in regards to p53 stabilization and cellular growth control. The results presented in chapter III describe the majority of p53 accumulation following expression of E2F1 is dependent on p19\textsuperscript{ARF}, while in human fibroblasts, using an siRNA to target p14\textsuperscript{ARF} had little effect on p53 stabilization following expression of E2F1 (236). The human 14\textsuperscript{ARF} protein lacks the C-terminal forty amino acids that are present in the mouse p19\textsuperscript{ARF} protein (25). These C-terminal amino acids have been proposed to be important for an interaction between p19\textsuperscript{ARF} and the peroxisomal targeting chaperone protein Pex19p (390). The interaction of Pex19p with p19\textsuperscript{ARF} may reduce the growth suppressive activity of p19\textsuperscript{ARF} in mouse cells by sequestering p19\textsuperscript{ARF} in the cytoplasm (421). Human
p14^{ARF} lacks the C-terminal Pex19p interaction domain, and so has been proposed to have enhanced growth suppressive properties (421), possibly through mechanisms independent of p53 (46, 428).

The data presented in chapter V describe a block in cell cycle progression following expression of E2F1 in normal human fibroblasts. The block in cell cycle progression is due, in part, to the activation of a Nbs1- and 53BP1-dependent checkpoint pathway. Activation of this pathway results in a rapid induction of p21 protein following expression of E2F1. Due to the enhanced growth suppressive properties of human p14^{ARF}, it is conceivable that induction of p14^{ARF} protein following expression of E2F1 could also contribute to the S phase block that is observed in normal human fibroblasts and not in rodent fibroblasts.

The cyclin-dependent kinase inhibitor p16^{ink4a} may also play a role in the block in cell cycle progression observed following expression of E2F1. p16 prevents activation of Cdk4/6, which is thought to be an essential step in Rb inactivation and crucial for the entry into S phase (108, 156). Upregulation of p16 protein levels by E2F1 would act to maintain Rb in its growth inhibitory state, blocking additional E2F activity and preventing the transition from G1 to S phase. The importance of the p16 pathway in controlling deregulated proliferation is suggested by the finding that most, if not all, human tumors contain mutations in the cyclin D/p16/Rb pathway or in proteins that control this pathway (366). Similar to the function proposed above for ARF, p16 may also provide negative feedback on the growth promoting activity of E2F1.
2. IRIF-like foci formation

Data presented in chapter IV and chapter V suggest that ectopic expression of E2F1 or Rb inactivation by HPV-16 E7 activates an apoptosis program with similarities to the apoptosis pathway activated by DNA double strand breaks. Both expression of E2F1 and DNA double strand breaks result in relocalization of DNA damage repair and checkpoint signaling proteins to discrete nuclear foci. In response to γ-irradiation or certain genotoxic agents, proteins, including γH2AX, 53BP1, and the MRN complex, relocalize to sites of actual DNA breaks. MRN and γH2AX were thought to be responsible for the initial recognition of DNA breaks, with the MRN complex functioning to initiate repair and γH2AX functioning to recruit other DNA repair factors as well as 53BP1 to activate a cell cycle checkpoint. However, recent evidence suggests that the mediator of DNA damage checkpoint protein 1 (Mdc1) is required for MRN, γH2AX, and 53BP1 foci formation (127, 242, 271, 385, 445) as well as for efficient Chk2 activation following γ-irradiation (242, 311, 445). Inhibiting Mdc1 expression with an siRNA results in decreased p53 stabilization and a failure to activate a cell cycle checkpoint following γ-irradiation (127, 242, 385).

The mechanism of MRN, γH2AX, and 53BP1 foci formation following expression of E2F1 remains unclear. It has been proposed that E2F1 may target the MRN complex to DNA by virtue of an interaction with the Nbs1 component of the complex (254). This scenario seems unlikely due to the foci formation observed following expression of E2F1-283, an E2F1 mutant lacking the Nbs1 interaction domain. Additionally, no interactions between E2F1 and either γH2AX or 53BP1 have been
described. It is possible that E2F1 could direct MRN, γH2AX, and 53BP1 foci formation through interaction with Mdc1, if such an interaction exists. Another possibility of how E2F1 induces DNA damage foci is that ectopic expression of E2F1 prevents the repair of DNA damage that may arise during replication or induces DNA damage by an unknown mechanism prior to activation of the apoptosis program. E2F1-induced DNA damage would then recruit DNA damage repair and checkpoint signaling proteins resulting in p53 activation and either apoptosis or the growth arrest described in chapter V.

The possibility exists that expression of E2F1 could result in DNA breaks indirectly through the induction of reactive oxygen species (ROS). Accumulation of ROS can lead to the induction of apoptosis through several mechanisms, including direct caspase activation (41). Expression of the c-Myc oncogene results in ROS accumulation that contributes to the DNA breaks and Mre11 relocalization observed following expression of c-Myc (413). Together with the observations that expression of E2F1 can induce ROS accumulation and that c-Myc induced apoptosis is compromised in E2F1−/− MEFs suggests that c-Myc induced accumulation of ROS, and subsequent DNA damage, may be a consequence of E2F1 activation (228, 397). Therefore, it is possible that ROS-induced DNA damage contributes to the observed relocalization of DNA damage sensors following expression of E2F1. The failure of expression of E2F3a to result in relocalization of DNA damage sensors may then be due to a failure to cause ROS accumulation. The contribution of ROS accumulation to E2F1-induced apoptosis, and E2F signaling, remains to be determined.
The findings in chapter V that expression of E2F1 in human cells results in a block in cell cycle progression coupled with the ability of E2F1 to induce DNA replication in rodent cells, suggests that E2F1 may be activating a DNA replication fork arrest. Replication fork arrest occurs in response to perturbations in many aspects of DNA replication including insufficient pools of dNTPs, polymerase inhibition, DNA modifications, or DNA breaks that may arise during replication (300). It is possible that constitutive expression of E2F1 induces DNA damage as a consequence of stalled replication forks, and results in recruitment of recognition and repair complexes. This seems unlikely, as E2F3a induces inappropriate S phase but fails to induce foci formation or checkpoint activation. Additionally, stalled replication forks activate p53 through an Atr/Chk1-dependent pathway (22), contrary to the Atm/Chk2 pathway required for E2F1 to activate p53.

3. Role of Atm in E2F signaling

Atm serves as the primary link between DNA damage sensors and activation of cell cycle regulatory proteins. In undamaged cells, Atm exists as an inactive dimer. Rapidly following DNA double strand breaks, Atm undergoes intermolecular autophosphorylation at the serine 1981 residue resulting in dimer dissociation and an increase in Atm kinase activity (17). Atm phosphorylation is thought to be triggered by chromatin changes associated with an influx of DNA repair factors to sites of DNA damage (17). It remains unclear as to how Atm is activated following expression of E2F1, E2F2, E2F3a, or HPV-16 E7. We speculate that expression of E2F proteins or E7
results in chromatin changes associated with induction of S phase or activation of DNA damage response proteins. In the case of HPV-16 E7, activation of Atm may be a result of chromosomal structural changes and DNA breaks that occur following E7 expression (86). However, since activation of Atm by E2F1 results in apoptosis, we cannot rule out the possibility that the different E2F family members activate Atm by distinct mechanisms.

Interestingly, Atm was found not to be required for apoptosis resulting from Rb inactivation in murine brain choroid plexus epithelium (233). In this model, apoptosis resulting from Rb inactivation is also dependent on E2F1 and p53, and occurs in the absence of p10^ARF (408, 409). While it is not apparent why this apoptosis is Atm-independent in the choroid plexus epithelium, p53-dependent apoptosis that is Atm-independent has been described in certain cell types (21, 151). Alternatively, these observations suggest that there may be a species-specific bias for Atm in the E2F1-mediated apoptosis pathway. Indeed, the reduction in apoptosis observed in atm^−/− MEFs is not as dramatic as that seen in human dermal fibroblasts following E2F1 expression. We speculate that in the murine system other signaling pathways such as Atr/Chk1 may compensate for the loss of Atm, whereas a more stringent requirement for Atm in human dermal fibroblasts is observed. Therefore, there may be both cell-type and species-specific requirements for Atm in apoptosis induction, and it is possible that other Atm-related kinases may compensate for the loss of Atm function in some cells.
Figure 6.1. Model of E2F signaling network.

Inactivation of Rb during a normal cell cycle leads to activation of E2F1, E2F2, and E2F3a resulting in cell cycle progression (shown in green). Inappropriate Rb inactivation by mutation or viral oncoproteins can lead to activation of two distinct, E2F1-specific pathways. The apoptosis pathway (shown in red) signals to p53 through Nbs1, Atm, and Chk2 resulting in p53 covalent modification and apoptosis. The checkpoint pathway (shown in blue) signals, in part, through Nbs1, 53BP1, p53, and p21. The checkpoint pathway also involves a signaling pathway independent of p53 and p21 through an, as of yet, undefined mediator. ARF may play a role in E2F1 signaling by increasing pools of p53 protein.
4. Activation of p53

We have defined a requirement for covalent modification of p53 for the induction of apoptosis following expression of E2F1. Activation of p53 by covalent modification, specifically phosphorylation of serine 15 (serine 18 in the mouse) and serine 20 in response to DNA damage, has been a topic of great interest due to the location of these residues within the Mdm2 interaction domain of p53. Mutational analysis of p53 sites known to be covalently modified, including serine 15 and serine 20, have been inconsistent regarding the requirements for phosphorylation at any one of these sites for p53 dependent apoptosis in response to DNA damage (13, 411, 441). The observation that expression of E2F1, 2, and 3a results in phosphorylation of p53 at the serine 15 residue suggests that some p53 modifications may occur as a result of ectopic induction of S phase. Chromatin alterations that occur as a consequence of DNA replication may be sufficient to activate Atm, leading to an increase in the phospho-serine 15 form of p53. This initial modification of p53 at the serine 15 residue during the onset of S phase would be insufficient for activation of the apoptosis program, but may prime p53 for additional modifications should errors, including DNA damage or stalled replication forks, occur during DNA replication.

While our study examined the phosphorylation status of two N-terminal residues on p53 known to be phosphorylated following DNA damage, we have not established that either of these modifications are causal in p53 mediated apoptosis, nor have we ruled out the involvement or importance of modification(s) to other residues on p53 following E2F1 expression. However, our finding that apoptosis is reduced when E2F1 is
coexpressed with a p53 mutant lacking many of the known phosphorylation sites demonstrates a contribution by potential p53 phosphorylation targets to E2F1-induced apoptosis.

C. Model of E2F1 apoptosis pathway

Expression of E2F1 leads to relocalization of DNA damage repair and recognition proteins, including the MRN complex and 53BP1, to discrete nuclear foci. The molecular events leading to relocalization of these components remains to be determined. Activation of Atm by autophosphorylation is proposed to occur following relocalization of DNA damage sensors by, as yet, an unknown mechanism (17). Activated Atm is then able to phosphorylate p53 at the serine 15 residue. However, expression of E2F1, E2F2, and E2F3a results in Atm activation and phosphorylation of the serine 15 residue on p53. Unique to E2F1 is the ability to induce Chk2 mRNA and proteins levels. Chk2 is able to phosphorylate the serine 20 residue, and this modification is found to correlate with E2F1-induced apoptosis. Chk2 may be limiting in this pathway, and when provided in trans, Chk2 enhances E2F1-induced apoptosis and allows E2F2 to induce apoptosis. It is possible that E2F1, E2F2, or E2F3a could also signal through p14ARF/Mdm2 to increase p53 protein levels and that the increased pools of p53 would provide more substrate for Atm and Chk2 (Figure 6.1).
D. Summary

The dual functions of E2F1 in checkpoint activation and apoptosis identified in this dissertation provide novel insight into the function of Rb/E2F growth control both in normal cells and in cells containing mutations in the Rb/E2F growth control pathway. The genomic integrity of mammalian cells is subject to constant attack, either by external damaging agents, or internal mutagens such as errors in DNA replication. If mutations arise that compromise proper Rb-mediated growth control, our results suggest that E2F1 may initiate a signaling cascade to activate a cell cycle checkpoint. If proper growth control cannot be restored, continued E2F1 activity may lead to apoptosis, preventing an accumulation of mutations that may lead to transformation. The importance of E2F1 as a signal transducer of loss of proper Rb-mediated proliferation control can be observed in mouse models. Loss of E2F1 in Rb mouse models results in a decrease in S phase and a decrease in apoptosis in the central nervous system and choroid plexus, suggesting an important role for the E2F1 apoptotic pathway in preventing tumor formation (305, 408). The observation that adult mice lacking E2F1 develop tumors (449) also suggests that the E2F1 signaling network is important in suppressing tumor formation.

However, our data may point to a role for an E2F1-specific checkpoint activation in these mouse models. It is possible that loss of Rb function in mice lacking E2F1 fails to activate the E2F1-induced cell cycle checkpoint pathway described in Chapter V. Since E2F3a, and possibly E2F2, cannot compensate for E2F1 in this pathway, cells may fail to properly block cell cycle progression and this may lead to additional mutations. Compounding the loss of the E2F1 growth control checkpoint would be the failure to
activate the E2F1-induced apoptosis pathway. Therefore, loss of E2F1 would lead to an inability to respond to loss of Rb-mediated growth control.

Although we have defined a pathway linking deregulated E2F1 activity to p53 and apoptosis, integration of E2F1 signaling and activation of the Atm/Chk2/p53 pathway also offers a mechanism for the proposed involvement of E2F1 in apoptosis resulting from DNA damage. Following treatment of cells with DNA damaging agents, E2F1 protein accumulates (30, 158, 165, 235, 266, 294), and is phosphorylated at an N-terminal Atm recognition sequence that is unique to E2F1 among the E2F family members (235). This phosphorylation of E2F1 is largely dependent on Atm and is required for efficient E2F1 stabilization following DNA damage (235). Chk2 has also been shown to phosphorylate and stabilize E2F1 following DNA damage, and this modification has been shown to be required for E2F1 dependent apoptosis following DNA damage by altering its promoter specificity (383). Additionally, DNA damage induced apoptosis is compromised in thymocytes from E2F1−/− mice (235), suggesting that E2F1 has multiple roles in DNA damage signaling. We speculate that activation of E2F1 by DNA damage leads to increased p14ARF expression resulting in increased pools of p53 protein. E2F1 is also able to activate Atm kinase activity and induce Chk2 expression leading to increased p53 activation and E2F1 activity. E2F1 activation following DNA damage would therefore act to amplify DNA damage signals converging at p53 to result in either a growth arrest or apoptosis (Figure 6.1).
E. Future directions

While we have made significant advances toward our understanding of E2F1 signaling, a number of questions require further exploration. How does expression of E2F1 result in relocalization of DNA damage response factors? If DNA damage foci form following DNA damage, what is causing the DNA damage following expression of E2F1? The finding that E2F1 activates both a cell cycle checkpoint and an apoptotic response suggests that there may be a molecular switch between the divergent outcomes. How the cell decides which pathway to favor remains unclear. A divergence in E2F1 function was also found between human and rodent cells. It remains to be determined why E2F1 can induce S phase in rodent cells, but blocks cell cycle progression in human fibroblasts. Given the additional mechanisms of proliferation and checkpoint control described in this dissertation, E2F1 may function in multiple pathways in the cellular response to DNA damage.

Lastly, the exact mechanisms that control E2F specificity remain to be determined. The specific induction of Chk2 by E2F1 suggests that transcriptional control of pro-apoptotic, and possibly anti-apoptotic, genes defines the primary difference between the activator E2F family members in the apoptosis pathway. Specificity for E2F1-mediated transactivation of the subset of E2F responsive genes involved in apoptosis would provide an attractive mechanism for the initiation of the apoptosis program. However, transcriptional activation or derepression, while required for apoptosis, does not account for all of the observed consequences of E2F1 expression. The difference between E2F1 and E2F2 or E2F3a in the formation of DNA damage-like
Mre11 foci suggests that additional E2F1-specific domains may be important for mediating this response. Specificity for E2F family members inactivation of the apoptotic pathway would therefore depend on multiple domains that are unique to E2F1 and serve specific functions in activation of components of the pathway.
CHAPTER VII

APPENDIX A
Figure A1. E2F1 induces apoptosis.

HEL fibroblasts were transfected with the indicated amounts of either pCDNA or pCDNA-E2F1. Cells were harvested and apoptosis detected at 96 hpi. Error bars represent standard deviation of experiment performed in triplicate.
Figure A2. siChk2 inhibits E2F1-induced apoptosis.

HEL fibroblasts were cotransfected with the marked siRNA together with either pCDNA or pCDNA-E2F1. Cells were harvested and apoptosis detected at 96 hpi. Error bars represent standard deviation of experiment performed in triplicate.
**Figure A3.** DNChk1 and DNChk2 do not effect E2F1 expression.

Normal human fibroblasts were coinfected with AdDNChk1 or AdDNChk2 at an MOI of 1000 and with AdE2F1 at an MOI of 1000. Cells were harvested and lysates generated at 24 hpi. Western blot analysis was performed to determine levels of E2F1 protein. Actin blots are shown as protein loading controls.
Figure A4. Specific requirement for E2F1 in HPV-16 E7 induced apoptosis.

HEL fibroblasts were transfected with the indicated siRNA 24 hours prior to infection with AdCon or AdE7 at an MOI of 1000. Cells were harvested and apoptosis detected at 96 hpi. Error bars represent standard deviation of experiment performed in triplicate.
CHAPTER VIII

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