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ATM Promotes Apoptosis and Suppresses Tumorigenesis in Response to Myc

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ATM PROMOTES APOPTOSIS AND SUPPRESSES TUMORIGENESIS IN RESPONSE TO MYC

by

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Dedication

To

My Dearest Tatayya

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ATM PROMOTES APOPTOSIS AND SUPPRESSES TUMORIGENESIS IN RESPONSE TO MYC

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Precancerous lesions from a variety of human tissues display markers of DNA damage suggesting that genetic instability occurs early during the process of carcinogenesis. Consistent with this, several oncogenes can activate ATM and other components of the DNA damage response pathway when expressed in cultured cells. Here we demonstrate that preneoplastic epithelial tissues from four different transgenic mouse models expressing the oncogenes *c-myc*, SV40 T antigen, human papilloma virus (HPV) E7, or *E2F3a* display γ-H2AX foci and other markers of DNA damage. Moreover, transgenic expression of these oncogenes leads to increased levels of damaged DNA as measured by the comet assay. In at least the Myc transgenic model, the formation of γ-H2AX foci is dependent on functional ATM. Inactivation of *Atm* also impairs p53 activation and reduces the level of apoptosis observed in transgenic tissue overexpressing Myc. This correlates with accelerated tumor development in Myc transgenic mice lacking ATM. To understand the mechanism by which oncogenes induce DNA damage, we employed an adenoviral overexpression system. Under conditions in which Myc or E2F3a induced replication is inhibited, we see a reduction in the DNA

damage induced by these oncogenes both by comet assay and levels of γ-H2AX. Moreover, Myc and E2F3a induced increased levels of the Cdt1 protein, a replication origin- licensing factor implicated in aberrant DNA replication. Taken together, these findings suggest that deregulated oncogenes induce unscheduled DNA replication leading to DNA damage and activation of the ATM DNA damage response pathway, which is important for the activation of p53, induction of apoptosis and the suppression of tumorigenesis.

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Chapter I: Introduction

1.1 DNA DAMAGE AND TUMORIGENESIS

Cancer is a disease caused by defects in the genetic information of the cell. The defects or mutations in oncogenes or tumor suppressor genes lay the foundation for the initiation of deregulated cell proliferation, a hallmark of cancer (Hanahan and Weinberg 2000). During the course of cancer development, which is a multistep process, the sequential accumulation of genetic alterations cause the cell to acquire additional hallmarks of cancer ultimately leading to tumorigenesis. The resultant tumors exhibit loss or gain of defined chromosomal regions leading to allelic imbalance at the molecular level. Recently Vogelstein's group have modeled genetic progression and the waiting time to cancer in colorectal cancers (Beerenwinkel, Antal et al. 2007). As per their model, one or two initial mutations that give rise to a benign tumor (adenoma) will progress to metastatis over a period of five to 20 years by acquiring mutations in about 20 cancer driver genes. The occurrence of genetic instability, at early stages of cancer development, driving the acquisition of critical mutations in cancer genes is implicit in their argument.

Evidence for genetic instability at an early stage of cancer development is shown in colorectal tumorigenesis (Shih, Zhou et al. 2001). The authors argue that if genetic instability is essential to human neoplasia, it is expected to begin early during the tumorigenic process and thereby accelerate the acquisition of growth-promoting mutations. Their results show that allelic imbalance, a molecular measure of chromosomal instability, of at least one chromosomal region is present in 90% of the adenomas of average size of 2 mm (representing early neoplastic growth). However, the

authors have not probed for genetic instability at very early stages of cancer development such as in hyperplasias, which characterize pre-neoplastic stages in the evolution of cancer.

Evidence that DNA damage occurs in preneoplastic lesions was reported in human urinary bladder, lung and skin hyperplasias (Bartkova, Horejsi et al. 2005; Gorgoulis, Vassiliou et al. 2005). The authors assayed for the presence of activated ATM DNA damage response pathway markers in normal, hyperplastic, dysplastic and malignant tissues. Their results show that the ATM DNA damage response (DDR) is absent in normal tissues, strongest in hyperplasias and becomes progressively attenuated and is lost in carcinomas suggesting that defects in ATM DDR pathway are crucial for cancer progression. The authors conclude that the ATM –mediated DNA damage response, by suppressing genetic instability, acts as a barrier to cancer progression.

To account for the DNA damage in precancerous lesions, the authors overexpressed oncogenes like cyclin E and Cdc25A in U2-OS cells and observed ATM DDR activation suggesting that deregulated oncogenes can induce DNA damage at least *in vitro*. Therefore, it is imperative to understand *in vivo*, the role of oncogenes in inducing DNA damage, genetic instability and its contribution to tumorigenesis. Such a critical understanding of the role of oncogenes in DNA damage and cancer will contribute to the development of effective molecularly targeted therapies against cancers. In addition, this understanding will assist in the better management of cancer with the existing chemotherapeutic drugs whose mode of action relies on inducing DNA damage to activate the DDR response to induce apoptosis and senescence in cells. Therefore this dissertation project broadly aims at understanding the role of the c-Myc oncogene in the

induction of DNA damage and the role of the ATM- mediated DNA damage response in the suppression of both Myc –induced DNA damage and tumorigenesis.

1.2 ATM AND THE DNA DAMAGE RESPONSE (DDR) PATHWAY

1.2.1 AT Disease

Ataxia- telangiectasia (AT) is a rare human autosomal recessive disorder which was first described by Elena Boder as having early onset progressive cerebellar ataxia, oculocutaneous telangiectasia, (dilated blood vessels), susceptibility to bronchopulmonary disease, and lymphoid tumors (Boder 1985). Other abnormalities associated with this disorder include severe radiation sensitivity, immunodeficiency, cell cycle checkpoint defects and chromosomal instability (Lavin and Shiloh 1997). AT patients are highly tumor prone. They exhibit a roughly 100- fold increase in the incidence of B and T cell lymphomas and lymphoid leukemias (Taylor, Metcalfe et al. 1996). Within this general increase in lymphoid tumor incidence, there is a four to fivefold higher incidence of T-cell based tumors as compared to B cell-based tumors. The higher than normal degree of V (D) J chromosomal rearrangements in the T lymphocytes from AT patients may account for the higher incidence of T cell based tumors (Taylor, Metcalfe et al. 1996). Cells derived from AT patients are defective for a variety of cellular responses induced by DNA damaging agents such as ionizing radiation (IR), radiomimetic drugs, and topoisomerase inhibitors. For example, AT cells have an abnormal checkpoint response throughout the cell cycle in response to DNA damage (Pandita, Lieberman et al. 2000). This phenotype results in another cellular AT hallmark, radioresistant DNA synthesis (Painter and Young 1980; Kastan, Zhan et al. 1992). T cells

and neurons from AT patients are also defective for the induction of apoptosis in response to DNA damage (Xu and Baltimore 1996; Herzog, Chong et al. 1998).

1.2.2 *ATM* Gene, ATM Protein and Related Family Members

The gene that is defective in AT, AT Mutated (*ATM*) was localized to chromosome 11q22-23 and cloned by positional cloning (Gatti, Berkel et al. 1988; Savitsky, Bar-Shira et al. 1995; Savitsky, Sfez et al. 1995; Uziel, Savitsky et al. 1996). The *ATM* gene covers 160kb of genomic DNA, contains 66 exons, and encodes a 13kb transcript. The protein product of this transcript is about 350 KDa and contains 3056 amino acids (Savitsky, Sfez et al. 1995; Uziel, Savitsky et al. 1996).

AT is a member of the phospho-inositide 3-kinase (PI3K)- related protein kinase (PIKK) family of serine/threonine kinases. Members of this family are very large proteins (300–500 KDa) which in addition to ATM also include the ATM and Rad3-related protein (ATR), DNA-dependent protein kinase (DNA-PKcs), ATX/SMG1, mammalian target of rapamycin (mTOR), and transformation/transcription domain-associated protein (TRRAP), the only member with an inactive kinase domain (Shiloh 2003). Underscoring the importance of genomic stability, four of the PIKK family members (ATM, ATR, DNA PKcs and SMG1) have vital functions in DNA damage response and repair. SMG1 also plays an important role in mRNA surveillance, contributing to the destruction of mRNAs with premature termination codons, thereby reducing the production of truncated proteins (Maquat and Carmichael 2001). DNA PKcs plays a key role in non-homologous end-joining repair of DNA double strand breaks (Smith and Jackson 1999). ATR, an essential gene unlike ATM, is classically associated with the response to UV radiation-

induced photoproducts and stalled replication forks (Tibbetts, Brumbaugh et al. 1999; Shiloh 2001).

1.2.3 ATM Activation and Functions

1.2.3.1 Upstream signals for ATM activation

Events in the DNA damage response (DDR) pathway, from the recognition of the DNA damage to the final cellular physiological outcome(s), are under the control of numerous proteins which can be categorized into sensor (MRN complex), transducer (ATM, ATR) and effector (BRCA1, p53, SMC1) proteins based on the stage and function in the pathway. ATM is a transducer protein that receives signals from upstream sensor proteins like the MRN (MRE11 11, NBS1 and RAD 50) complex, p53 binding protein 1 (53BP1) and the mediator of DNA damage checkpoint protein-1 (MDC1) adaptor protein that are initially recruited to the sites of DNA damage. DNA double strand breaks (DSBs) are initially recognized by the MRN complex, which rapidly accumulates at the DSBs followed by MDC1 and 53BP1. The retention of MRN complex and 53BP1 on the chromatin is dependent on MDC1 (Shiloh 2006; You, Bailis et al. 2007; Lavin 2008). NBS and ATLD cells, which lack functional NBS1 and MRE11 components of the MRN complex respectively, are defective in ATM activation upon DNA DSBs. Tanya Paull's group has shown that MRN is essential for activation of ATM *in vitro* and further demonstrated that MRN binds tightly to both DNA and ATM (Lee and Paull 2004; Lee and Paull 2005). The MRN component that binds to ATM has been identified as NBS1 (Falck, Coates et al. 2005; You, Chahwan et al. 2005). MDC 1 through its interaction with both NBS1 and ATM mediates the MRN-dependent

recruitment of ATM to the double strand breaks (Lou, Minter-Dykhouse et al. 2006; Wu, Luo et al. 2008). ATM molecules remain dormant as inactive homodimers prior to DNA damage (Bakkenist and Kastan 2003). However, upon recruitment to the DSB by the MRN sensor, ATM rapidly undergoes autophosphorylation, becomes monomerized and activated to phosphorylate its numerous effector proteins. One of the first proteins to be phosphorylated by activated ATM is a histone H2 variant, H2AX, which upon phosphorylation at serine 139 is referred to as γH2AX. MDC1, which binds to phosphorylated H2AX, is required for sustained interaction of ATM with chromatin and the further recruitment of ATM. A single molecule of MDC1 can simultaneously bind both γH2AX and ATM through its BRCT and FHA domains respectively (Stucki, Clapperton et al. 2005; Lou, Minter-Dykhouse et al. 2006). ATM, from the MDC1-ATM pair that is recruited to the site of damage, phosphorylates H2AX, which subsequently binds MDC1, thereby stabilizing the attachment of MDC1-ATM to the DSB site. ATM then phosphorylates additional H2AX molecules in the vicinity thereby facilitating the binding of more MDC1 molecules (Lou, Minter-Dykhouse et al. 2006). This cyclic process leads to the expansion of phosphorylated H2AX molecules over mega- bases of DNA flanking DSBs (Figure 1). This amplification of ATM- mediated DNA damage response signals creates an expanding platform for the recruitment of additional DNA damage response proteins. One of them is the multifunctional tumor suppressor protein BRCA1, which is phosphorylated by ATM and acts as a scaffolding protein that makes some non- chromatin- associated substrates accessible to the activated ATM protein

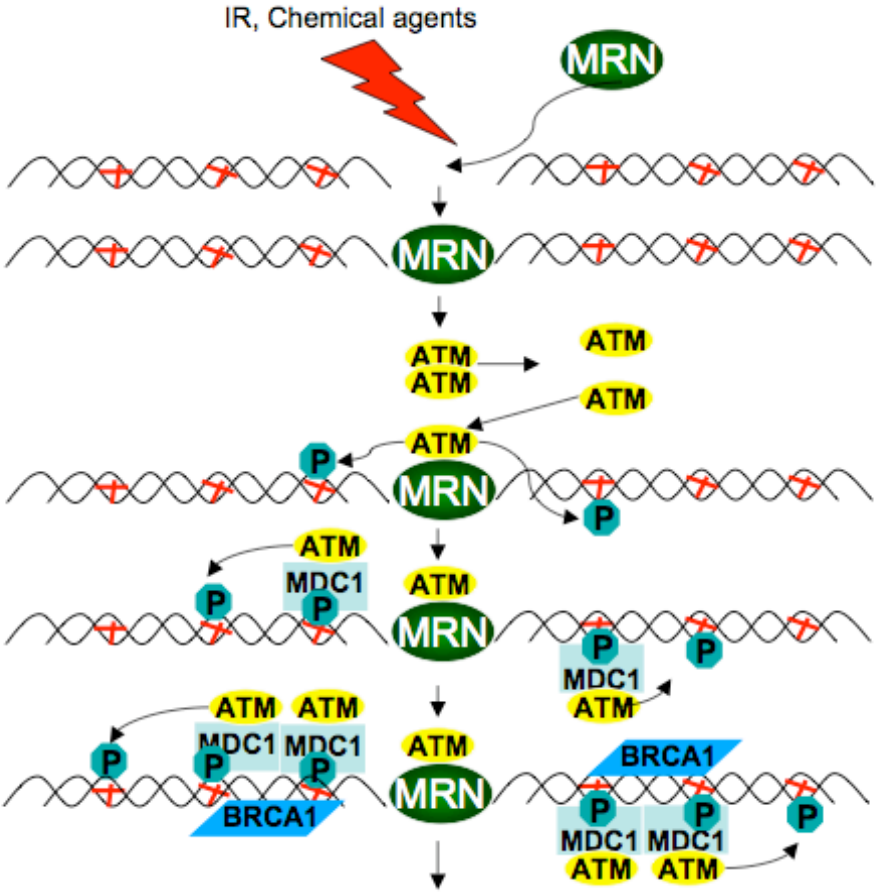


Figure 1: Early events in the DNA double- strand break (DSB) response (Modified from Shiloh, Y, Trends in biochemical Sciences, 2006).

DNA DSBs are initially recognized by the MRN complex through mechanisms, which are not clearly understood. Although not shown here, the retention of the MRN complex on the chromatin is dependent on the interaction between MDC1 and NBS1. Chromatin bound MRN complex recruits ATM to DSBs. On being recruited to chromatin, inactive ATM dimer autophosphorylate its partner, monomerizes and is activated to phosphorylate H2AX ('X' on chromatin denotes H2AX molecules). MDC1 protein binds to phosphorylated H2AX (γH2AX) and recruits more ATM to the chromatin to further phosphorylate H2AX molecules in the vicinity, which now can bind more MDC1 molecules, and this cyclic process leads to the expansion of phosphorylated H2AX molecules over mega- bases of DNA flanking DSBs. This expanding platform enables the recruitment of additional DNA damage response proteins like BRCA1 that acts as a scaffolding protein for ATM substrates.

(Foray, Marot et al. 2003; Kurz and Lees-Miller 2004). Now the stage is set for the ATM- dependent phosphorylation of its downstream effector proteins.

1.2.3.2 Downstream ATM- dependent signaling cascade

Genotoxic stress, being a life-threatening event, calls for a rapid, extensive and specialized response involving multiple mechanisms to activate DNA repair processes, transcription, cell cycle control and if needed, apoptosis or senescence. Therefore, it is not surprising that eukaryotic cells have evolved complex and elaborate networks to coordinate these crucial processes for cell survival and at its core are ATM and ATR. The DNA damage dependent activation of ATM leads to the rapid phosphorylation of a multitude of proteins involved in DNA repair, cell cycle checkpoint activation and transcription. A large- scale proteomic analysis of proteins phosphorylated in response to DNA damage on ATM and ATR consensus sites identified more than 900 phosphorylation sites in over 700 proteins (Matsuoka, Ballif et al. 2007). These proteins were arranged in interconnecting modules that are involved in DNA repair, DNA replication and cell cycle control. A description of these networks is beyond the scope of the present study. Therefore, only protein targets of ATM that are relevant to the present study will be discussed.

ATM's response to DSBs is very diverse (Figure 2). A peak into the downstream effector pathways that ATM regulates upon DNA damage illustrates three recurring aspects of its signaling:

- ATM phosphorylates protein kinases, like c-Abl, Chk1 and Chk2, which are capable of targeting several downstream effectors (like p53, BRCA1)

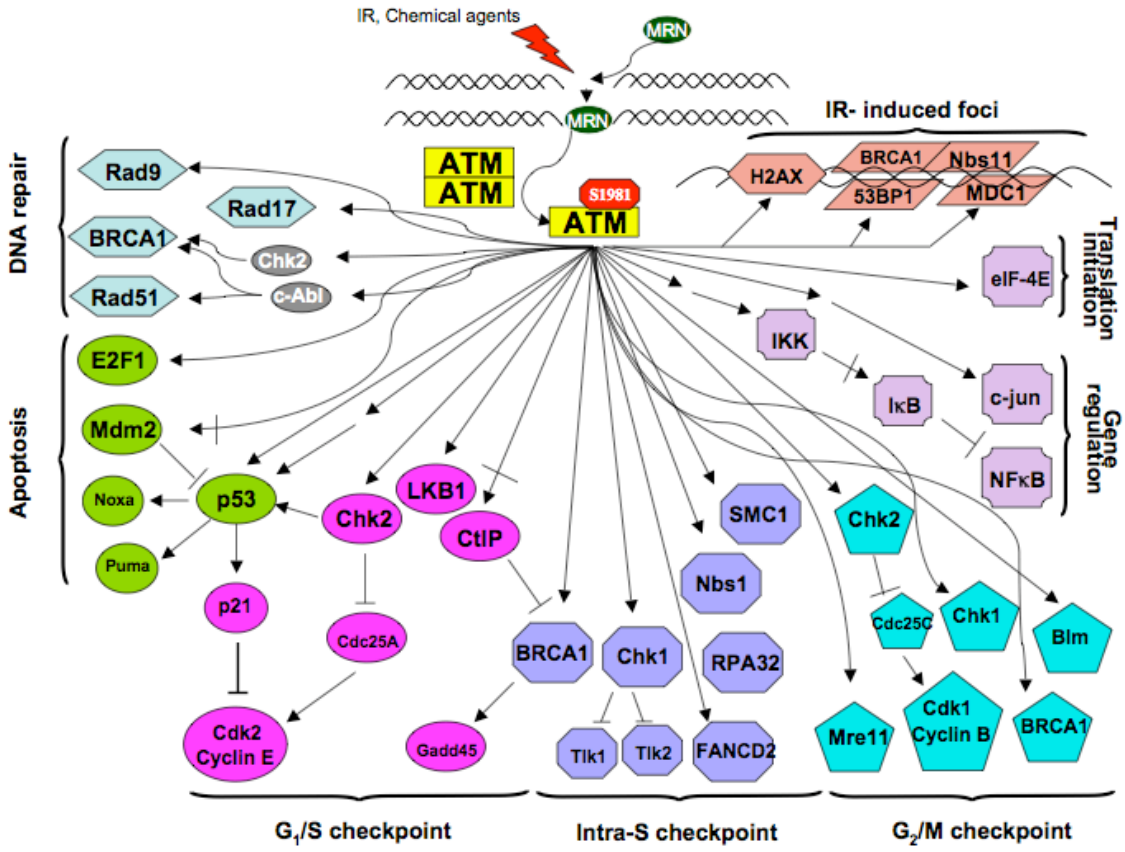


Figure 2: ATM mediated DNA Damage Response Pathway. (Modified from Kurz and Lees-Miller DNA Repair, 2004)

Various ATM substrates that mediate DNA damage focus formation, DNA repair, apoptosis, cell cycle checkpoints, gene regulation and translation. Please refer text for a brief description of these pathways. → indicates direct phosphorylation of substrate by ATM. →→ indicates indirect phosphorylation events mediated by ATM through an intermediary substrate.
⊥ indicates inhibition. ⊣→ indicates ATM- dependent phosphorylation events that relieve downstream events. For example, ATM's phosphorylation of Mdm2 on serine 395 relieves p53 from Mdm2 mediated inhibition.

simultaneously, and as such, control subsets of pathways. This ensures rapid and high amplification of the signal.

- ATM regulates the same endpoint by using different pathways. For example, ATM mediates the intra-S checkpoint through multiple pathways each of which is governed by its targets like SMC1, NBS1, FANCD2, RPA32 and Chk1.
- ATM approaches the same effector through several different mechanisms. For example, ATM mediates the activation and stabilization of p53 in at least three different ways: by directly phosphorylating on serine 9 and stabilizing it, indirectly through Chk2 phosphorylation on serine 20 and by relieving Mdm2 inhibition of p53 (Figure 2).

ATM, through its numerous substrates, mediates DNA repair, apoptosis, cell cycle checkpoints, gene regulation and translation, all of which attempt to ensure the integrity of the genome. Therefore it is not surprising that the disruption of the ATM DDR pathway is selected for during the process of tumorigenesis to facilitate the expansion of activating mutations that drive cancer progression. *ATM* has been ranked at number three among all protein kinases based on the probability of carrying at least one driver mutation, indicating that somatic mutations in the *ATM* gene itself can be a driving force for cancer (Greenman, Stephens et al. 2007). Moreover, the expression of the *ATM* gene is silenced by promoter hypermethylation in head and neck squamous cell carcinoma, non-small cell lung carcinoma and breast cancer (Ai, Vo et al. 2004; Vo, Kim et al. 2004; Safar, Spencer et al. 2005). The importance of the ATM/ATR DDR pathway in the maintenance of genomic integrity and the suppression of cancer becomes evident by the fact that p53, an effector protein in the ATM/ATR DDR pathway, is mutated in 50% of human cancers (Hollstein, Sidransky et al. 1991; Levine, Momand et al. 1991).

1.2.4 Oncogenic Stress and the Activation of the ATM DDR Pathway

It is known that deregulated oncogenes like E2F1, Myc and E1A can induce apoptosis through the Arf-Mdm2-p53 pathway. However, previous results from my lab showed that deregulated E2F1 expression can induce the stabilization and phosphorylation of p53 at serine 15 and other residues in the absence of ARF (Russell, Powers et al. 2002). This original finding has been confirmed by others who have also extended it to show that many oncogenic factors, including Myc, cyclin E, and Cdc25, can also induce the phosphorylation of p53 and other ATM targets (Rogoff, Pickering et al. 2002; Vafa, Wade et al. 2002; Lindstrom and Wiman 2003; Bartkova, Horejsi et al. 2005; Bartkova, Rezaei et al. 2006). In addition it has been shown that ATM is also required for the induction of senescence by the Ras oncogene (Di Micco, Fumagalli et al. 2006). However, none of these studies were conducted *in vivo* to show the physiological relevance of oncogene activation in the induction of DNA damage and the role of the ATM DDR pathway in the suppression of oncogene- induced DNA damage and tumorigenesis. In addition, the nature of the oncogenic stress sensed by ATM is not clear. To address these concerns, c-Myc is employed in this thesis as the model oncogene to test the concept of oncogene-induced DNA damage.

1.3 c-MYC

1.3.1 The History of c-Myc

The *c-myc* gene, which was identified as the cellular homolog of the retroviral *v-myc* oncogene 30 years ago, was the first nuclear proto-oncogene to be discovered

(Roussel, Saule et al. 1979; Sheiness and Bishop 1979). c-Myc (from henceforth referred to as Myc unless otherwise stated) is a transcription factor that regulates the expression of a number of genes involved in diverse cellular functions. Myc, a member of the basic region helix-loop-helix leucine zipper (bHLH-LZ) family of transcription factors, heterodimerizes with another bHLH-LZ containing protein, Max, to bind CACA/GTG consensus core sequence (E-box elements) in the promoters of its target genes and activate their transcription (Blackwell, Kretzner et al. 1990; Blackwood and Eisenman 1991; Blackwood, Luscher et al. 1992). Myc also represses genes via Inr (initiator)-dependent as well as Inr- independent mechanisms (Claassen and Hann 1999; Gartel and Shchors 2003). Targeted homozygous deletion of the murine c-*myc* gene results in embryonic lethality, thereby indicating that it has a critical role in development (Davis, Wims et al. 1993). Inactivation of c-*myc* in rat fibroblasts led to a substantial increase in the cell doubling time, indicating a crucial role for Myc in cell proliferation (Mateyak, Obaya et al. 1997). Myc transactivates the *htert* promoter leading to the expression of telomerase, which protects telomeres and extends the life span of primary human cells, which in many cases become immortalized (Greenberg, O'Hagan et al. 1999; Lutz, Leon et al. 2002). Myc can also induce apoptosis under certain circumstances. Myc's ability to induce apoptosis was first observed in 32D.3 myeloid progenitor cell lines in the absence of IL-3 and later in serum-deprived Rat1 fibroblasts (Askew, Ashmun et al. 1991; Evan, Wyllie et al. 1992).

1.3.2 Myc Biology and Function

The human c-Myc gene consists of three exons: an untranslated exon I, and exons II and III encompassing the protein coding sequences. Transcription initiates primarily

from two major promoters, P1 and P2, located within exon I and when translated results in Myc1 and Myc2 having molecular masses of 67 KDa and 64 KDa respectively with the 64KDa Myc2 species being the major isoform. Unless otherwise indicated, Myc protein refers to the more abundant, growth promoting 64KDa Myc2 molecule (Facchini and Penn 1998).

The Myc protein is highly conserved among different species. It is organized into three domains: a globular N-terminal domain (NTD; amino acids 1 to 203), a central unstructured domain (amino acids 204 to 237) and an α–helical carboxy-terminal domain (CTD; amino acids 238 to 439) [Figure 3]. The NTD harbors conserved Myc boxes, MBI and MBII, which are essential for the transactivation of Myc target genes. The CTD harbors a basic region (BR), helix-loop-helix (HLH) and leucine zipper (LZ) motifs for dimerization with its partner Max and subsequent DNA binding of Myc-Max heterodimers. Contiguous BR-HLH-LZ motifs, a characteristic of DNA binding transcription factors, help Myc to undergo protein-protein interactions, bind to specific DNA sequences and activate or repress gene transcription.

In normal cells, Myc functions to integrate extracellular signals and acts as a central switch for a wide range of signaling pathways that regulate proliferation or differentiation. Myc converts the pro-proliferative environmental cues into specific gene-regulatory programs for cell growth control. The denouement of Myc's modulation of transcription of its target genes is cell proliferation, cell growth, inhibition of differentiation and tumorigenesis. Therefore, at the right time, at the right place and in the right amount, Myc is essential for normal cell function, but its deregulation is extremely dangerous. This dualism requires a tight control of Myc expression entailing a very tight

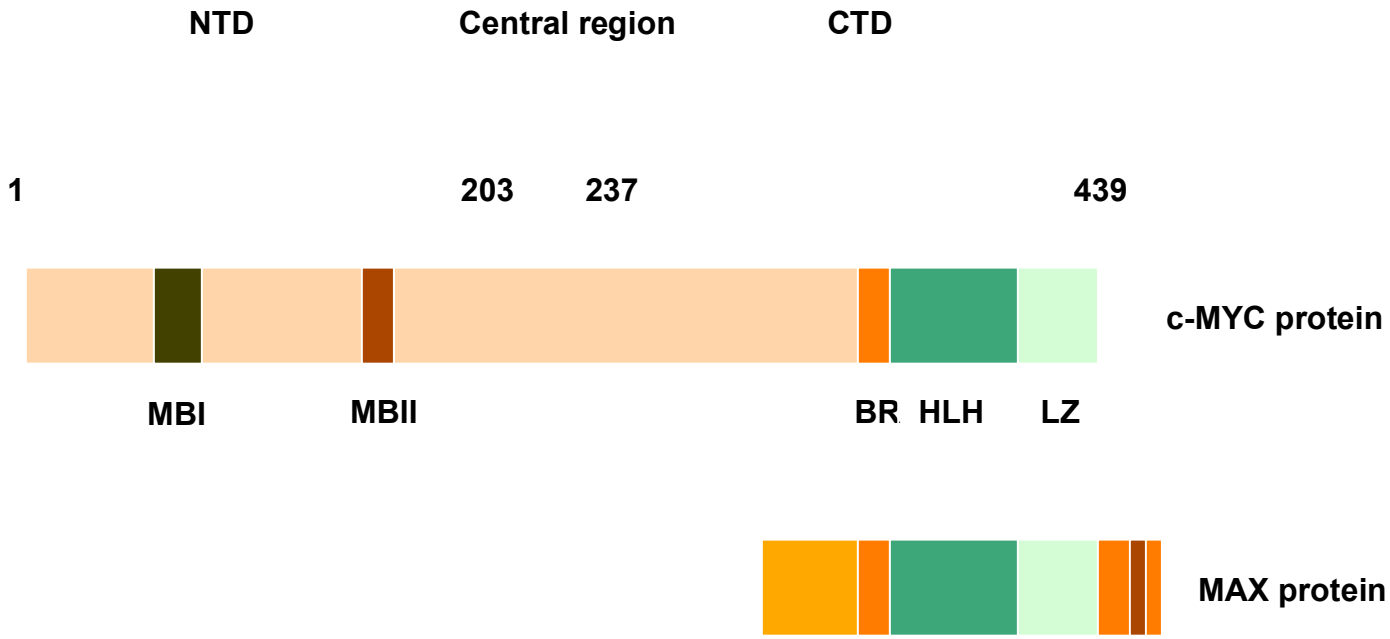


Figure 3: Functional domains of human c-Myc protein

MBI and MBII in the amino terminal domain (NTD) refer to the conserved Myc boxes I and II, which are essential for the transactivation of Myc target genes. The carboxy terminal domain (CTD) harbors the basic (BR) helix-loop-helix (HLH) leucine zipper (LZ) motifs that mediate DNA binding and heterodimerization with other BR-HLH-LZ proteins, like MAX or MIZ-1, to activate or repress transcription of its target genes respectively.

control of the *Myc* promoter. Regulation of the *Myc* promoter is very complex and reflects Myc's complex biological properties as a potent and essential, but also dangerous key factor for cell growth control (Wierstra and Alves 2008).

An extensive array of signaling molecules encompassing every major signal transduction pathway bearing proliferative or antiproliferative cues impacts the *Myc* promoter either directly or indirectly (Wierstra and Alves 2008). Growth factors, mitogens, cytokines, hormones, vitamins and ligands impact the *Myc* promoter positively. Myc promoter is activated by most major proliferative pathways like Wnt, Notch, PI3K/Akt, Ras/Raf, JAK/STAT, Src, interleukins (IL-2, IL-3, IL-6, IL-12), cytokines, lymphokines and growth factors (PDGF, EGF, CSF-1) (Figure 4). To ensure normal cellular homeostasis by the maintenance of physiological Myc levels, the *Myc* promoter is negatively impacted by differentiation and antiproliferation factors like C/EBPα, C/EBPβ, Blimp-1, GATA-1, KLF11, IFN-γ, p21, p53, and TGF- β (Figure 4). However, in a normal cell, the specific signal, the actual pathway and the outcome of a particular signal depends upon the cell type and cellular context as well as on the developmental or physiological state of the cell.

The *Myc* promoter integrates diverse and dynamic inputs to output Myc mRNA. Consequently, the Myc protein, by both positively and negatively regulating its target genes, has a positive effect on cell cycle progression and cell growth. There is a feedback coupling of the *Myc* promoter to Myc regulated genes (Wierstra and Alves 2008) [Figure 5]. Factors that both regulate *Myc* promoter and are later regulated by Myc fall into four different groups of feedback mechanisms (Wierstra and Alves 2008).

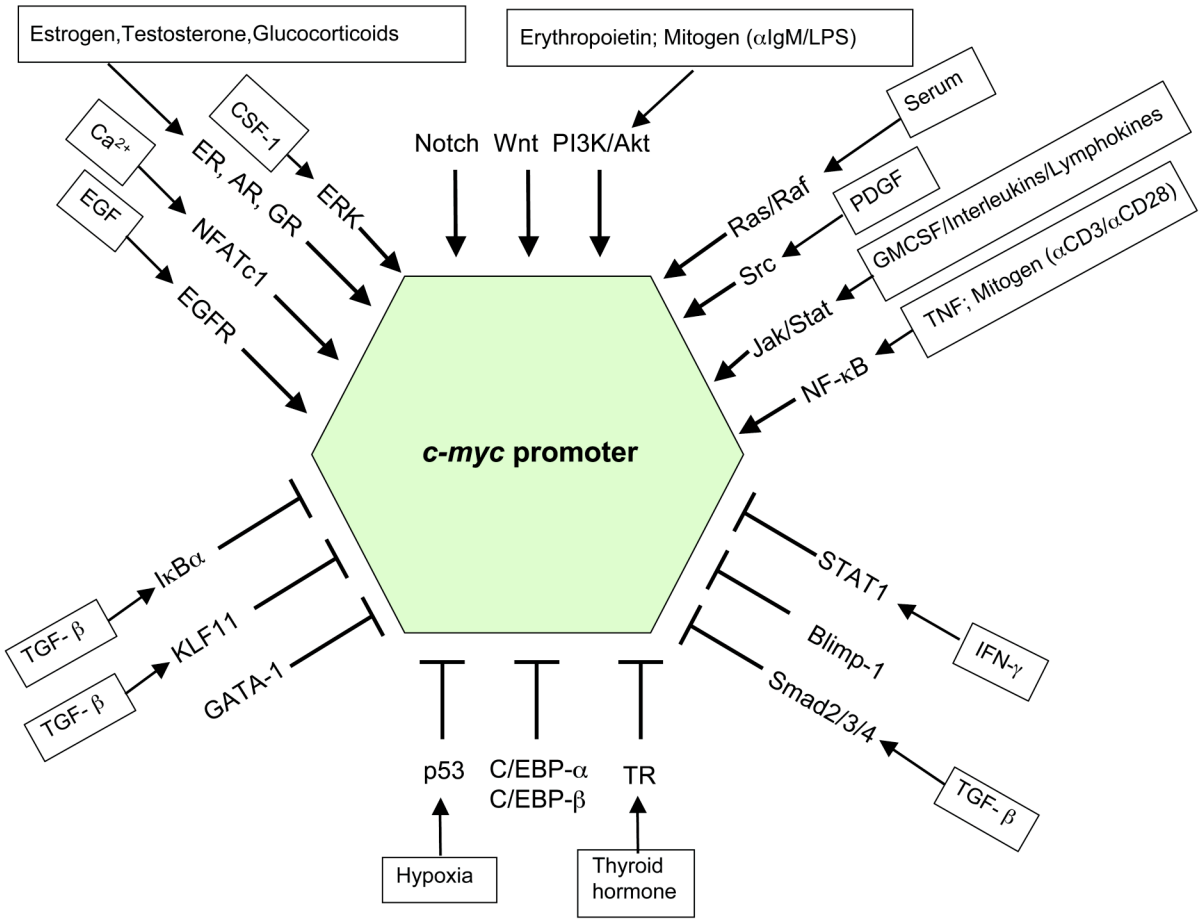


Figure 4: A summary of the major signaling pathways controlling the human and/or murine *c-myc* promoter

↓- Arrow represents pathways that activate and ⊥ represents pathways that repress the *c-myc* promoter. It should be noted that cross talk and redundancy exists between different signal transduction pathways in the regulation of the *c-myc* promoter. For example, cross talk is seen between the Wnt pathway and signaling by TGF-β family members (not shown). Smad 3 activated through the TGF-β signaling pathway can potentially antagonize the transactivation of the *c-myc* promoter by β-catenin/TCF-4 transcription factor complex of the Wnt pathway. Redundancy for signaling through the TGF-β signaling pathway is shown here. TGF-β antagonizes the transactivation of the *c-myc* promoter by targeting several different transcription factors (like KLF11, Smad2/3 and NF-κB as shown here) that bind to the *c-Myc* promoter.

Group 1: Factors that activate *Myc* promoter and are in turn activated by Myc. Prominent proliferation genes like E2Fs fall in this group. This positive feedback mechanism promotes cell proliferation and provides robustness against competing antiproliferative signals.

Group 2: Factors that repress *Myc* promoter and are in turn repressed by Myc. Prominent antiproliferation and differentiation genes like TGF-β are in this group. This negative feedback regulation is important for normal tissue homeostasis. Myc protein represses its own promoter in a concentration-dependent manner and this autosuppression is seen disrupted in many cancers and contributes to high Myc levels seen is such cancers.

Group 3: Factors that activate *Myc* promoter but are repressed by Myc. Prominent proliferation genes like STAT3 represent this group. Such negative feedback contributes to tight control of cell cycle progression and limits the time window of a particular mitogenic response and serves to maintain normal homeostasis.

Group 4: Factors that repress *Myc* promoter but are activated by Myc. Prominent tumor suppressors like p53 are in this group. This feedback mechanism ensures proper security against inappropriate hyperproliferative signaling by Myc.

Tight and complex regulation of *Myc* promoter coupled with the various feedback mechanisms underscores the importance of maintaining physiological levels of Myc in quiescent and growing cells. This is not surprising considering that in almost all cancers where Myc is oncogenic, its levels are very highly elevated. Therefore it is logical that the cell has evolved such extensive and complex mechanisms, which are invariably

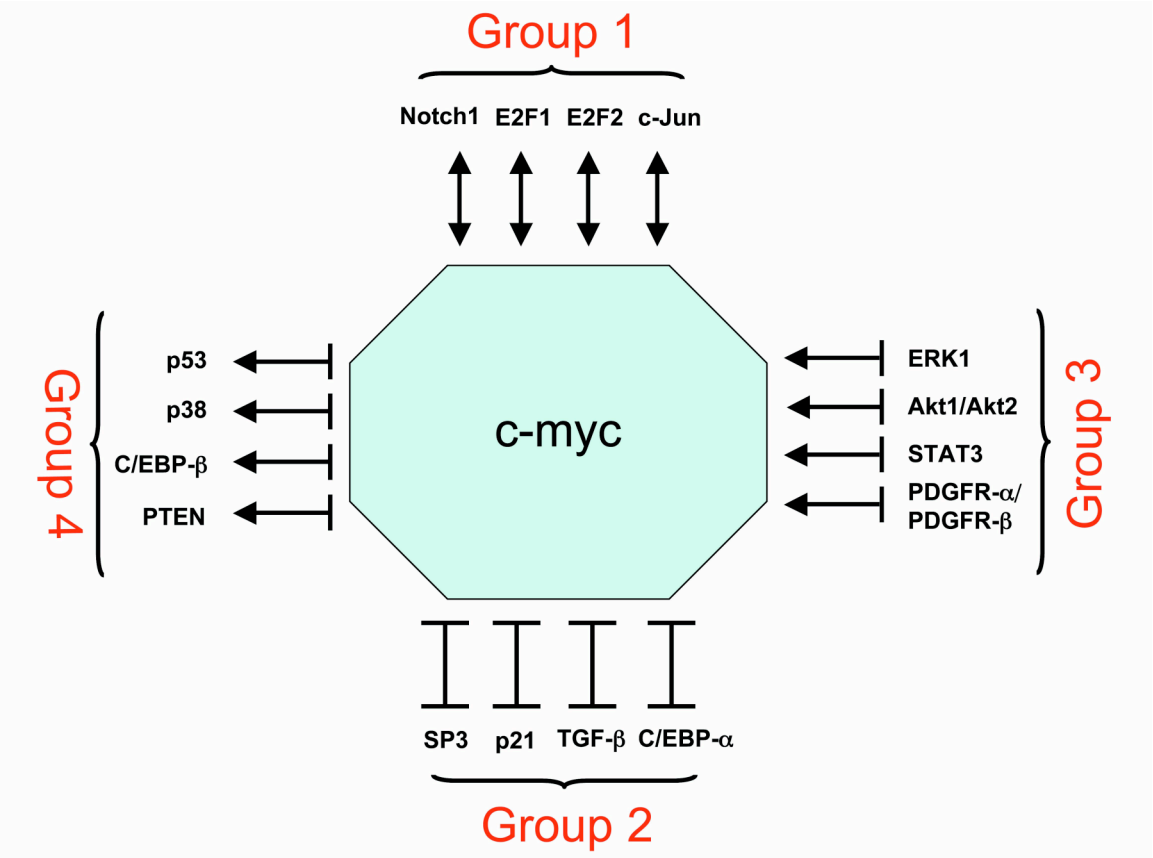


Figure 5: Feedback loops between *c-myc* and its regulators.
Please refer text for description.

*Note: Only subsets of Myc's feedback loops are represented. The number of feedback loops represented here does not constitute all feedback loops between Myc and its regulators.

↕ Represents feedback loops that activate *Myc* promoter and are in turn activated by Myc **(Group 1)**

⊥ Represents feedback loops that repress *Myc* promoter and are in turn repressed by Myc **(Group 2)**

⟝ Represent feedback loops that activate *Myc* promoter but are repressed by Myc **(Group 3)**; Feedback loops that repress *Myc* promoter but are activated by Myc **(Group 4)**

disrupted in cancers, to regulate its amount to safe and acceptable levels in both quiescent and proliferating cells.

1.3.3 Myc and Oncogenesis

Gerard Evan aptly refers to Myc as "the oncogene from Hell" (Soucek and Evan 2002). This puts into perspective the potent oncogenic ability of deregulated Myc, which is considered as one of the most insidious of all the known oncogenes.

The Myc oncogene family encodes a group of three nuclear phosphoprotein transcription factors (c-Myc, L-Myc and N-Myc) which, when deregulated, induce the development of a large number of human tumors including cancers of lymphoid, mesenchymal and epithelial origin. Expression or repression of c-Myc is normally tightly controlled by a flux of mitogenic and anti-mitogenic signals in the cell. However, when Myc is deregulated, it does not respect the anti-growth and anti-proliferative signals being transduced and makes the cell refractory to anti-mitogenic signals. Such cells start proliferating and may in due course be transformed.

Considering Myc's central role in cell growth control, even slight changes in the amount of its protein may have dramatic consequences for cell proliferation and cell fate. Therefore a tight control of its expression and activity is achieved at multiple levels, viz., transcription initiation and elongation, translation, and stability of its mRNA and protein (both with extremely short half-lives of less than 30 minutes). These multiple levels of regulation ensure that Myc activity is correlated strictly with cell proliferation. As mentioned above, initiation of transcription at *Myc* promoter ensues only upon the

integration of multiple proliferative and antiproliferative signals that also includes positive and negative feedback mechanisms. In addition, Myc's transcription is also controlled at elongation by way of blockage of transcription elongation through promoter proximal pausing of Pol II. *Myc* mRNA translation is dependent upon the activation of Ras and PI3K pathways which enhance ribosomal recruitment of *Myc* mRNAs. Myc's protein stability can be increased or decreased upon phosphorylation at Ser-62 (by ERK) and Thr-58 (by GSK-3) respectively. The short half-lives of both *Myc* mRNA and Myc protein ensures that to achieve functional Myc activity within the cell, active and continuous synthesis of both of its RNA and protein is required, which again is subject to tight regulation at multiple levels (Wierstra and Alves 2008). An additional important mechanism for control of Myc expression is Myc's repression of its own promoter as was discussed above. Myc's expression is frequently deregulated in cancers leading to an inappropriate, non-physiological increase in Myc activity. Myc's deregulation can be due to mutations in *Myc* locus (translocation of the Myc allele as in Burkitt's lymphoma) or in the upstream signal transduction pathways that regulate *Myc* expression or in exon 1 and intron 1 leading to the loss of the block to transcriptional elongation or point mutations in Myc protein coding sequences that increase its stability. It should not be difficult to anticipate the extent of mayhem that can result from the deregulation of oncoproteins like Myc, which have essential and crucial roles in positively affecting cell growth and for the very reason are extremely tightly regulated.

Myc's potent oncogenic ability is related to its multifaceted role in the cell. The majority of the diverse cellular roles of Myc is dependent upon its transcriptional activity. Using high-density oligonucleotide arrays, Cawley and colleagues mapped the binding sites for Myc *in vivo* on human chromosomes 21 and 22 and extrapolated the

transcription factor binding site (TFBS) regions to the full genome (Cawley, Bekiranov et al. 2004). They found an unexpectedly large TFBS with a minimal estimation of 25,000 for Myc. Interestingly, only a minority of sites to which Myc binds correspond to protein-coding genes and the majority are correlated with non-coding RNAs, whose role in tumorigenesis will be discussed in subsequent paragraphs. Potentially, therefore, Myc regulates a significant number of all genes in the genome of an organism, some of which could account for the diverse roles of Myc both in normal and cancerous cells. Myc's known target genes can be functionally categorized and their contribution to transformation and oncogenesis evaluated.

First, Myc either transactivates or represses several genes that control cell cycle progression. Myc exerts its proliferative effects by modulating the actions of a number of positive and negative regulators of cell cycle progression. Myc, an immediate early gene whose levels get upregulated upon mitogenic activation, heterodimerizes with its partner Max and induces, in addition to others, the expression of cyclin D2 and CDK4 (cyclin dependent kinase 4) which can promote advance through early G1. Myc also increases the expression of Cul1 protein, which degrades p27Kip1, an inhibitor of CDKs. At the same time, Myc partners with Miz-1 to repress the expression of p15INK4B and p21Cip1 CDK inhibitors, which shut down the actions of CDK4/6 and CDK2 respectively. The now activated cyclin D-CDK4/6 and cyclin E-CDK2 complexes hyperphosphorylate and inactivates the tumor suppressor retinoblastoma protein (Rb) to release the S phase inducing E2F transcription factors (E2F1, E2F2 and E2F3). In addition, Myc's transcriptional activity increases the expression of E2Fs, which can transcribe genes required for DNA replication, thereby causing the cell to enter S phase **(**Weinberg, 2006**).** Aberrantly proliferating cells with deregulated Myc have greatly reduced dependence on

external growth factors. Quiescent serum starved cells, expressing conditional MycER fusion protein, entered cell cycle (G1), progressed through S phase and completed mitosis in the absence of external growth factors upon Myc's activation (Eilers, Schirm et al. 1991). In addition, deregulated Myc blocks differentiation as it is refractory to anti-proliferative signals from TGF-β, whose growth repressive effects are enforced partly by the p15INK4B and p21Cip1 CDK inhibitors (Feng, Liang et al. 2002; Wu, Cetinkaya et al. 2003)**.** Deregulated Myc can also override checkpoint mechanisms that control chromosome number and ploidy leading to the accumulation of polyploid cell populations and chromosomal translocations that can potentially cause mutations in other genes (Felsher and Bishop 1999; Lutz, Leon et al. 2002; Vafa, Wade et al. 2002; Karlsson, Deb-Basu et al. 2003)**.**

A second mechanism by which Myc contributes to transformation is by transactivating genes that cause deregulation of specific metabolic pathways. For example, Myc enhances glucose uptake and glycolysis by upregulating lactate dehydrogenase A (LDH-A). Reduction in Myc expression reduces the levels of LDH-A, whose levels are frequently increased in human cancers and are required for Myc-induced transformation of Rat1a fibroblasts, human lymphoblastoid cells, and Burkitt lymphoma cells (Shim, Dolde et al. 1997). LDH-A has an important role in the induction of Warburg effect, wherein tumor cells under hypoxic conditions consume glucose as an energy source without oxygen and overproduce lactic acid aerobically. Myc upregulates serine hydroxymethyltransferase, a metabolic enzyme that generates one-carbon units, which are required for folate metabolism and consequent cell growth and proliferation (Nikiforov, Chandriani et al. 2002). Other important Myc responsive genes that play a crucial role in cellular metabolism and transformation are ornithine decarboxylase

(ODC), dihydrofolate reductase (DHFR) and thymidine kinase (Bello-Fernandez, Packham et al. 1993; Mai and Jalava 1994; Pusch, Soucek et al. 1997).

A third mechanism accounting for Myc's transformative ability is its role as a central regulator of cell growth and ribosome biogenesis in mammalian cells. Myc activates several genes that encode ribosomal proteins, translation factors, and proteins involved in the biogenesis and processing of ribosomal RNAs (Rosenwald, Rhoads et al. 1993; Greasley, Bonnard et al. 2000; Boon, Caron et al. 2001; Adhikary and Eilers 2005). Myc also directly activates the transcription of ribosomal genes and of RNA pol III dependent genes (Gomez-Roman, Grandori et al. 2003; Grandori, Gomez-Roman et al. 2005).

Finally, Myc contributes to oncogenesis by facilitating dedifferentiation, angiogenesis and loss of cell adhesion, all of which promote invasion and metastasis. In a transgenic mouse model of pancreatic β cell carcinogenesis, Myc induced the dedifferentiation of insulin producing mature β cells to non-insulin producing β cell lineage (Pelengaris, Khan et al. 2002). Myc is known to upregulate the inhibitor of differentiation gene, Id2, which by antagonizing members of the Rb family of proteins have a role in regulating differentiation (Lasorella, Noseda et al. 2000). In the above mentioned mouse pancreatic β cell carcinogenesis model, Myc also elicited an angiogenic response leading to the generation of an extensive network of blood vessels permeating every islet. Others have shown that deregulated Myc abets angiogenesis by upregulating VEGF and angiopoietin-2 while inhibiting anti-angiogenic factors like thrombospondin-1 (Tikhonenko, Black et al. 1996; Baudino, McKay et al. 2002). In addition, Myc induced the loss of E cadherin, the principal intercellular adhesion

molecule in epithelia, resulting in the withdrawal of β cell –cell contacts throughout all pancreatic islets. About 30% of all downregulated genes in a transgenic mouse model where Myc is targeted to the basal layer of interfollicular epidermis were involved in cellular adhesion (Frye, Gardner et al. 2003). Myc's repression of these genes brought about profound changes in the adhesive and motile behavior of keratinocytes.

Considering the multiple pleiotropic effects of Myc which encompasses all the aspects of cellular metabolism, it is no wonder that Myc along with Oct3/4, Sox and Klf4 transcription factors could induce reprogramming of differentiated mouse and human somatic cells to pluripotent stem cells. Takahashi and Yamanaka could reprogram mouse embryonic and adult fibroblasts, and human dermal fibroblasts to pluripotent stem cells just by transducing the four defined transcription factors (Takahashi and Yamanaka 2006; Takahashi, Tanabe et al. 2007). The resultant induced pluripotent stem cells (iPS) were similar to embryonic stem cells in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes and telomerase activity. *In vitro,* these cells could also differentiate into cell types of the three germ layers, and when subcutaneously transplanted into nude mice, they could produce tumors containing a variety of tissues from all three germ layers. Cartwright and colleagues showed that murine ES cells could be maintained in a pluripotent self-renewing state by stably expressing Myc which inhibited differentiation (Cartwright, McLean et al. 2005)**.**

Myc achieves its pro-tumorigenic effects not only through transcriptional activation but also by transcriptional repression of genes involved in growth arrest and differentiation. In addition to the CDK inhibitors, p15INK4B and p21Cip1 as discussed above, Myc also represses genes involved in growth arrest (Gadd45 and H-ferritin), and

differentiation (c/EBP-α) (Freytag and Geddes 1992; Marhin, Chen et al. 1997; Wu, Polack et al. 1999).

Cawley and colleagues have shown that Myc binds to about 25,000 transcription factor binding site regions of which only 22% are located at the 5′ termini of protein-coding genes while 36% lie within or immediately 3′ to well-characterized genes and are significantly correlated to noncoding RNAs (Cawley, Bekiranov et al. 2004). Since then evidence is accumulating that Myc regulates a vast repertoire of microRNAs (miRNAs), a set of noncoding RNAs that regulate gene expression. Myc transcriptionally upregulates and represses miRNAs involved in promoting and suppressing tumorigenesis respectively. Myc directly upregulates the pro-tumorigenic 17-92 miRNA cluster while it represses the anti-tumorigenic miRNAs like let-7, miR15-a, miR-16-1, miR-22, miR-26a, miR-29c, miR-34a, miR150 and miR-195 (O'Donnell, Wentzel et al. 2005; Chang, Wentzel et al. 2007). While let-7 targets Ras and is frequently downregulated in lung cancer, miR-15a and miR-16-1 target Bcl-2 and are deleted or downregulated in a majority of individuals with chronic lymphocyte leukemia; and miR-34a, a component of the p53 tumor suppressor network, which has a potent anti-proliferative and pro-apoptotic activity is mapped to 1p36, a locus frequently deleted in cancers (Calin, Dumitru et al. 2002; Cimmino, Calin et al. 2005; Johnson, Grosshans et al. 2005; He, He et al. 2007). miR-26a, miR-150 and miR-195 show a marked anti-tumorigenic activity in a mouse model of B-cell lymphoma (Chang, Wentzel et al. 2007). The enforced expression of Myc repressed miRNAs diminished the tumorigenic potential of the lymphoma cells. Therefore, one of the mechanisms by which Myc promotes tumorigenesis is by extensively reprogramming the miRNA transcriptome.

All the activities of deregulated Myc are geared towards tumorigenesis except for its ability to induce apoptosis and autophagy, which are anti-tumorigenic. Although the ability of Myc to induce apoptosis is well characterized, only recently is evidence provided that Myc can induce autophagy, also referred to as type II cell death. Tsuneoka and colleagues reported that overexpressed Myc induces autophagy in rat 3Y1 fibroblasts independent of apoptosis and oncogenic transformation (Tsuneoka, Umata et al. 2003). Along with apoptosis, autophagy could represent a security mechanism against hyperproliferative signaling by Myc. Therefore the suppression of Myc- induced apoptosis and probably autophagy could be important for tumorigenesis. In the pancreatic transgenic mouse model discussed above, activation of Myc in adult, mature β cells induces uniform β cell proliferation but is accompanied by overwhelming apoptosis leading to reduction in β cell mass. Rapid progression to tumorigenesis could only be achieved by the suppression of Myc- induced β cell apoptosis by overexpressing Bcl-XL (Pelengaris, Khan et al. 2002). Many of the cancers in which Myc is deregulated also show the disruption of apoptotic pathways. Therefore it is important to understand all the pathways and mechanisms underlying Myc- induced apoptosis.

1.3.4 Myc and Apoptosis

Anticancer agents induce apoptosis in tumors as well as in normal tissues thereby causing loss of normal cells, in addition to tumor cells, and contributes to the side effects of cancer therapy. Apoptotic programs can be manipulated to produce massive changes in cell death; therefore the proteins controlling apoptosis are potential drug targets (Lowe and Lin 2000). Deregulated Myc is a potent inducer of apoptosis (Pelengaris, Khan et al. 2002). While all the other functions of deregulated Myc promote oncogenesis, its

apoptosis inducing ability suppresses oncogenesis. Therefore Myc's ability to induce apoptosis can be specifically taken advantage of in designing novel therapeutic strategies that eliminate Myc sensitized cells while sparing normal cells. Although chemotherapeutic drugs are being used to treat cancers in which Myc is overexpressed, they are not particularly effective as deregulated Myc disrupts multiple control mechanisms in a cell to induce cancer. In addition these drugs are mutagenic and toxic to normal cells. In the past few years several attempts have been made, although *in vitro*, to take advantage of Myc's apoptotic ability to specifically kill cancer cells. Drugs vinblastine and nocodazole were selectively cytotoxic to Rat1 cells overexpressing Myc while being cytostatic to the same cells without deregulated Myc (Yu, Ravera et al. 1997)**.** Myc synergized with doxorubicin to induce apoptosis in a neuroblastoma cell line where N-Myc is overexpressed using an inducible system (Fulda, Lutz et al. 1999)**.** Neither enforced expression of N-Myc nor doxorubicin treatment alone could trigger apoptosis in these cells. Therefore, understanding Myc induced apoptosis and delineating the multiple pathways involved is a prerequisite for taking full advantage of the potential therapeutic opportunities that exist to specifically target Myc overexpressing cells in cancer. My research also aims at understanding the pathways involved in Myc- induced apoptosis.

Deregulated Myc induces apoptosis in a p53-dependent and -independent manner. p53-independent pathways involve apoptosis mediated by CD95/Fas ligand and TNF-alpha through their respective death receptors (Janicke, Lee et al. 1994; Klefstrom, Vastrik et al. 1994; Hueber, Zornig et al. 1997; Wang, Brunner et al. 1998)**.** Cytochrome c, a major player in mitochondrial apoptosis, is a direct transcriptional target of c-Myc, which also mediates its release from mitochondria (Juin, Hueber et al. 1999; Morrish,

Giedt et al. 2003). Myc sensitizes cells to apoptosis by causing the release of cytochrome c, but the ability of cytochrome c to activate apoptosis is critically dependent on signals from CD95/Fas or p53 apoptotic pathways. One mechanism by which Myc induces apoptosis in a p53-dependent manner is through the Arf-Mdm2- p53 pathway (Zindy, Eischen et al. 1998). Arf regulates p53 by binding to and inhibiting the action of Mdm2, a negative regulator of p53 (Sherr 2001). Although Myc activation leads to increased Arf expression, there is no evidence to show that Myc directly upregulates Arf.

p53 can act independently of Arf. In normal human fibroblasts and WI38 lung embryonic fibroblasts, E2F1 or Myc overexpression induces p53 accumulation independent of Arf (Lindstrom and Wiman 2003). Mice that lack both *Arf* and *p53* develop cancers at a faster rate, and the tumor spectrum is much broader as compared to the tumor spectrum in mice lacking either *Arf* or *p53* alone (Weber, Jeffers et al. 2000). Also, cells lacking both *Arf* and *p53* proliferate more rapidly than those lacking either gene alone (Eischen, Weber et al. 1999). This conclusive genetic evidence indicates that both Arf and p53 have tumor suppressive functions independent of each other. It is known that the p53 tumor suppressor can be regulated in an Arf- independent manner in response to DNA damage, by posttranslational modifications, including phosphorylation, which results in its stabilization (Giaccia and Kastan 1998). Previously, we have shown in a mouse skin transgenic model that Myc's ability to induce apoptosis is largely dependent upon p53 but only partly dependent upon Arf (Rounbehler, Schneider-Broussard et al. 2001; Russell, Powers et al. 2002) [Figure 6]. This indicates that additional players may modulate p53- dependent apoptosis in response to Myc.

Although we have sufficient understanding of the pathways that Myc mediates to

effect its multitude of biological activities, it is just the tip of the iceberg considering the lack of understanding on the role played by thousands of potential Myc target genes in modulating the diverse functions of Myc. Some of these unknown pathways are very much likely to impact Myc-induced apoptosis. This is further complicated by the discovery of a major role for Myc in the regulation of a wide variety of miRNAs that regulate Myc-induced apoptosis in addition to other cellular functions. An initial clue of how oncogenes can induce apoptosis in a p53-dependent but Arf-independent manner came from studies from our lab on E2F1. E2F1, an oncogenic transcription factor like Myc, signals to p53 through ATM. In primary human fibroblasts lacking functional *ATM*, the ability of E2F1 to induce p53 phosphorylation and apoptosis is impaired (Powers, Hong et al. 2004). Whether Myc similarly employs the ATM DNA damage response pathway to signal to p53 and induce apoptosis has not been determined. It has been suggested that the activation of the ATM DNA damage checkpoint response by oncogenic stresses inhibits genomic instability and subsequently the formation of cancer (Bartkova, Horejsi et al. 2005). There is evidence that Myc oncogenic pressure can induce DNA damage, at least *in vitro*. In normal human fibroblasts and Rat1A cells, deregulated Myc produces various kinds of chromosomal abnormalities including deletions and translocations (Felsher and Bishop 1999; Vafa, Wade et al. 2002; Karlsson, Deb-Basu et al. 2003). However, whether Myc induces DNA damage and activates the ATM DNA damage response pathway *in vivo* needs to be established. The main thrust of this dissertation is based on the pursuit to understand the *in vivo* roles played by tumor suppressors like ATM and p53 in Myc-induced apoptosis and tumorigenesis.

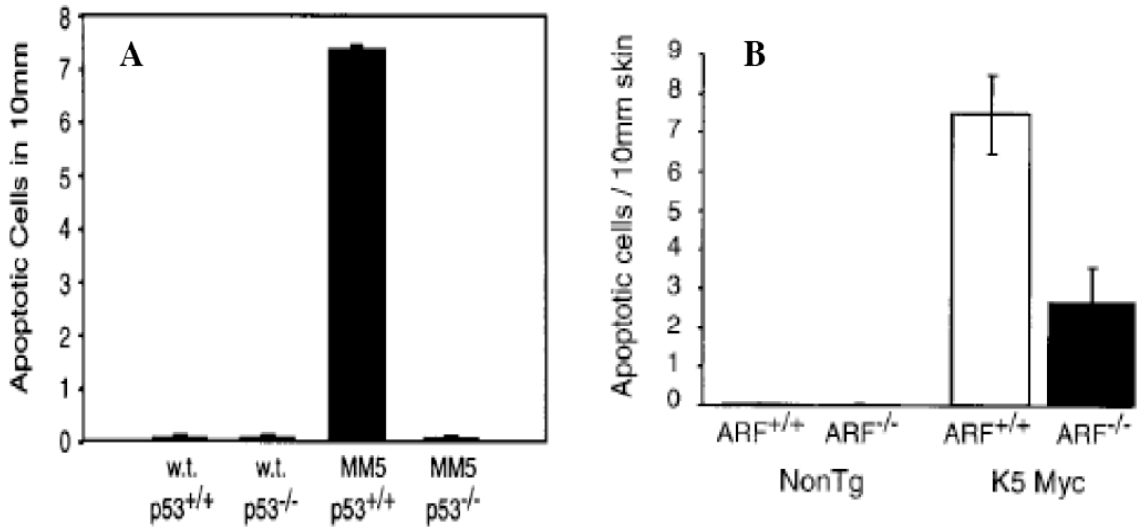


Figure 6: Apoptosis in K5Myc transgenic epidermis is largely dependent on p53 but only partly dependent on Arf. (Adapted from Rounbehler RJ et al., Oncogene, 2001; Russell JL, et al., Mol. Cell Biol., 2002).

The average number of activated-caspase 3 positive keratinocytes per 10mm of epidermis is presented for; **(A)** nontransgenic p53+/+, nontransgenic p53-/-, K5Myc (line MM5), p53+/+, and K5Myc (line MM5), p53-/- mice and **(B)** nontransgenic (w.t.) ARF+/+, nontransgenic (w.t.) ARF-/-, K5Myc ARF+/+ and K5Myc, ARF-/- mice.

1.4 HYPOTHESIS AND SPECIFIC AIMS

Deregulated c-Myc induces DNA damage in vivo leading to the activation of the ATM DNA damage response pathway to induce apoptosis and suppress tumorigenesis.

To test my hypothesis, I am proposing three specific aims:

Specific Aim 1: Determine if Myc and related oncogenes induce DNA damage *in vivo*.

Specific Aim 2: Assess the *in vivo* roles of ATM in Myc- induced apoptosis and tumorigenesis.

Specific Aim 3: Determine the mechanism by which Myc and E2F3a induce DNA damage.

To address my specific aims, I have employed the K5Myc and K5E2F3a transgenic mouse models, which are briefly described below.

1.5 K5MYC TRANSGENIC MOUSE MODEL

1.5.1 Keratin (K5) Promoter Driven Transgenes

Expression of keratin 5 (K5) occurs predominantly in the basal layer of proliferating keratinocytes in the epidermis (Ramirez, Bravo et al. 1994). As keratinocytes differentiate, they move out of the basal layer towards the epithelial surface. During this process of terminal differentiation, the expression of K5 is downregulated in suprabasal cells. Therefore *K5* promoter driven transgenes will show expression only in the basal layer of the epidermis. K5 is expressed from day 13.5

postcoitum embryos in the precursors of most epithelia and organs that express K5 at adult stages. The K5Myc and the K5E2F3a transgenes were generated as previously described (Rounbehler, Schneider-Broussard et al. 2001; Paulson, McArthur et al. 2006) and their expression is directed specifically to the squamous epithelia.

1.5.2 K5Myc Transgenic Mouse Model

Myc promotes the development of hyperplasia and hyperproliferation in the epidermis of K5Myc transgenic mice. The level of hyperplasia correlated with the percent of interfollicular basal keratinocytes in S phase. 6% of basal keratinocytes are in S phase in K5Myc transgenic mice as compared to 2% in wild type mice. Myc also induced a fivefold increase in apoptosis in the epidermis of transgenic mice as compared to the basal levels of apoptosis in wild type mice. Apoptosis in K5Myc transgenic mice is largely dependent upon p53. The K5Myc transgenic mice also develop spontaneous tumors in the skin and oral epithelium (K5- expressing tissues). The earliest tumors start appearing between 25 to 30 weeks of age in K5Myc mice and about 40% develop tumors by one year of age. However, tumor development in K5Myc mice is accelerated in a p53 heterozygous background. All K5Myc mice heterozygous for p53 develop tumors by 41 weeks of age (Rounbehler, Schneider-Broussard et al. 2001). A majority of the tumors in K5Myc mice are tumors of the oral cavity followed by skin squamous cell carcinomas and papillomas.

1.5.3 K5E2F3a Transgenic Mouse Model

E2F3a is a member of the E2F family of transcription factors. Like Myc, overexpression of E2F3a can induce quiescent cells to enter cell cycle and can also induce apoptosis. *E2F3* gene amplification and overexpression is seen in several types of human cancers (Veltman, Fridlyand et al. 2003; Feber, Clark et al. 2004; Oeggerli, Tomovska et al. 2004; Grasemann, Gratias et al. 2005; Orlic, Spencer et al. 2006). Like K5Myc mice, K5E2F3a transgenic mice develop hyperplasia, and hyperproliferation and show apoptosis in the transgenic epidermis (Paulson, McArthur et al. 2006). However, unlike Myc, apoptosis induced by E2F3a in the transgenic epidermis is independent of p53. Like Myc, E2F3a also induces spontaneous tumor development in K5 tissues, albeit with much longer latency. Only 20% of K5E2F3a mice develop tumors in K5 expressing tissues by 2 years of age. Also the tumor spectrum in these mice differs from that of K5Myc mice. Although these mice develop papillomas and squamous cell carcinomas of the skin, they do not develop oral tumors, which form the majority of tumors in K5Myc mice.

Chapter II: Materials and methods

2.1 MICE AND TREATMENTS

The generation of K5Myc transgenic mice has been previously described (Rounbehler, Schneider-Broussard et al. 2001). Briefly, the transgene contains the bovine K5 promoter (Ramirez, Bravo et al. 1994), the rabbit β-globin intron 2, the simian virus 40 polyadenylation signal, and a murine *c-myc* genomic fragment. K5Myc transgenic mice (line MM5) were bred to mice containing an inactivated *Atm* allele (Barlow, Hirotsune et al. 1996) to generate K5Myc mice heterozygous for *Atm*. Male K5Myc mice heterozygous for *Atm* were then bred to *Atm* heterozygous female mice to generate K5Myc and nontransgenic mice that were homozygous, heterozygous, or nullizygous for *Atm*. K5Myc mice were originally in the SSIN strain background. $Atm^{+/-}$ mice were obtained from The Jackson Laboratory and were in the C57BL/6J strain background. The genetic background of mice in this study was, therefore, a mixture of SSIN and C57BL/6J strains. Sibling mice were used for comparisons in all experimental procedures.

The generation of the K5E2F3a transgenic mice, K14HPVE7 and TRAMP mice has been described (Greenberg, DeMayo et al. 1995; Herber, Liem et al. 1996; Paulson, McArthur et al. 2006). K5E2F3a transgenic previously generated in our lab are maintained in the FVB background. TRAMP mice were obtained from Dean Tang's lab at Science Park Research Division of UT MD Anderson Cancer Center and were maintained in the C57BL background. K14HPVE7 mice were obtained from Paul Lambert's lab and were maintained by backcrossing to the FVB strain.

Caffeine has been shown to inhibit the catalytic activities of both ATM and ATR kinases (Sarkaria, Busby et al. 1999). Caffeine treated lung carcinoma cell lines treated with radiation showed a defective ATM-dependent checkpoint response and fail to arrest in G2 phase and progressed to mitosis while the S phase cells exhibit radioresistant DNA synthesis (Sarkaria, Busby et al. 1999). To inhibit the ATM and ATR DDR pathway in E2F3a transgenic mice, we have employed caffeine sourced from Sigma Chemical Co. and dissolved in deionized autoclaved water at a concentration of 0.4 mg/ml. This dose is similar to the amount consumed by a heavy coffeee drinker (approximately 15 mg/kg/day) and is therefore physiologically relevant (Bartkova, Rezaei et al. 2006). Mice were given free access to water with caffeine shortly after weaning and treatment continued for up to 6 months, with weekly water changes.

All experiments with mice were performed in accordance with national guidelines and regulations and approved by the UT MD Anderson Cancer Center Institutional Animal Care and Use Committee.

2.2 CELLS AND VIRUSES

NHFs and primary AT fibroblasts (Coriell Cell Repositories, Camden, NJ) were maintained in MEM with 2 mM glutamine, nonessential amino acids, and 15% FBS.. Recombinant adenoviruses expressing human *c-Myc*, *GFP*, *E2f3a*, *Cdt1* and *Geminin* have been previously described (Vaziri, Saxena et al. 2003; Powers, Hong et al. 2004; Paulson, Pusapati et al. 2008). While AdMyc, AdGFP and AdE2F3a were generated in our lab, AdCdt1 and AdGeminin were a kind gift from Anindya Dutta of University of Virginia. For infections, cells were washed, counted and cultured in 10 cm plates in 3 ml of serum-free medium containing adenovirus vectors at a multiplicity of infection of 100.

After 1 h of infection, 7 ml of media with 0.5% fetal bovine serum was added and cells incubated for an additional 24 h for the comet assay or 48 h for the apoptosis assay and immunoblotting. To inhibit DNA replication in cultured cells, aphidicolin (Sigma Chemical Co., St Louis, MO, USA) was added to cell media at a final concentration of 5 g/ml at the time of infections. Aphidicolin is a selective inhibitor of DNA polymerase α and therefore potently inhibits DNA synthesis. (Ikegami, Taguchi et al. 1978; Krokan, Wist et al. 1981).

2.3 IMMUNOBLOTTING AND ANTIBODIES

Epidermal protein lysate was collected by scraping dorsal skin and resuspending epidermal tissue in modified RIPA lysis buffer (50 mM Hepes, pH 7.4/1% IGEPAL/0.25% Na deoxycholate/150 mM NaCl/1 mM EDTA/1 mM PMSF/1 mM NaF/1 mM Na_3VO_4) containing protease (Sigma, P-8340) and phosphatase (Sigma, P-2850) inhibitor mixtures. Protein extract was prepared by freeze thawing followed by supernatant collection after centrifugation. Cells were harvested and lysed in RIPA lysis buffer with protease and phosphatase inhibitors, and proteins were extracted as described above.

Protein samples (50 μg) were separated on 6-10% SDS/PAGE gels depending on the protein size and transferred to a poly(vinylidene difluoride) membrane. After transferring, the membranes were blocked in 5% milk in TBST (TBS and 0.01% Tween 20) for 30 min and probed with primary antisera or antibodies for 1 h in 5% milk in TBST). The following rabbit polyclonal antisera were acquired: p53 pS15 (catalog no. 9284; 1:1,000), p53 (catalog no. 9282; 1:1,000), and cleaved caspase-3 (catalog no. 9661; 1:1,000) from Cell Signaling Technology (Beverly, MA). c-Myc (catalog no. sc-788;

1:1,000); E2F3 [C-18] (catalog no. sc-878; 1:3,000); Geminin (catalog no. 13015; 1:5000); Cdt1 [H-300] (catalog no. sc-28262; 1:1000); and β-tubulin (catalog no. sc-9104; 1:2,000) from Santa Cruz Biotechnology. GAPDH (catalog no. ab9483; 1:10000), a goat polyclonal antibody is obtained from Abcam. The following mouse monoclonal antibodies were obtained: ATM pS1981 (catalog no. 200-301-400; 1:1,000) from Rockland, ATM (catalog no. GTX70103; 1:1,000) from Genetex, and β-actin (catalog no. sc-8342; 1:2,000) from Santa Cruz Biotechnology. Bands were visualized by using enhanced chemiluminescence reagent (Amersham Pharmacia).

2.4 SINGLE-CELL GEL ELECTROPHORESIS (COMET) ASSAY

Primary keratinocytes were isolated from adult mouse epidermis as previously described (Wu and Morris 2005). Briefly, dorsal skin samples were incubated in trypsin for 2 h at 32°C, and then the epidermal layer was separated from the dermis. The scraped epidermis was stirred at 100 rpm on a magnetic stirrer for 20 min at room temperature. The cells were then pelleted and resuspended in PBS. Primary keratinocytes at a concentration of 2×10^5 cells per ml were then embedded in low-melting-point agarose on a glass slide by using the CometAssay kit and the manufacturer's protocol (Trevigen, catalog no. 4250-050-K). Briefly, the embedded cells were incubated overnight at 4°C in lysis solution (2.5 M NaCl/100 mM EDTA, pH 10/10 mM Tris/1% sodium lauryl sarcosinate/0.01% Triton X-100) followed by incubation in alkaline solution (300 mM NaOH/EDTA 1 mM) for 40 min to denature the DNA. The samples were then washed twice in TBE buffer and electrophoresed at 19 V for 10 min in 1× TBE buffer. The samples were stained with 50 μl of SYBR Green, and nuclei were visualized by fluorescent microscopy. Tail length and olive tail moment of 70 nuclei per slide were scored by using cometscore software (TriTek). Student's *t* test was used to derive *P*

values. Tail moment is the product of the tail length and the fraction of total DNA in the tail, which gives a measure of the extent of DNA damage.

2.5 CYTOGENETIC STUDIES

Metaphase chromosome spreads were generated from primary keratinocytes isolated from K5Myc transgenic and wild type sibling controls. Metaphase spreads can be generated by treating cells with colcemid, a commonly used microtubule depolymerizer, which causes dividing cells to arrest at metaphase. Cells were treated with colcemid (at a concentration of 0.02 μg/ml) for 2.5 hours to arrest cells in metaphase. Cells were collected after colcemid treatment, gently lysed in hypotonic solution and fixed in methanol/acetic acid. Trained professionals in the Cytogenetics Core Facility at MD Anderson Cancer Center, Houston analyzed at least hundred chromosome spreads from each genotype for chromosomal aberrations.

2.6 IMMUNOFLUORESCENCE

Formalin-fixed, paraffin-embedded mouse skin sections were deparaffinized, boiled in 10 mM sodium citrate for 10 min, and blocked in 50% goat serum for 30 min. Sections were then incubated overnight at 4°C with antibodies against histone H2AX pS139 (γH2AX) (catalog no. 05-636, Upstate Biotechnology; 1:300), ATM pS1981 (catalog no. 600-401-400, Rockland; 1:200) and SMC1 pS957 (catalog no. 200-301-397, Rockland; 1:300). Alexa Fluor 488-conjugated goat anti-rabbit or anti-mouse secondary antibodies from Molecular Probes were used, and the sections were imaged by using an Olympus laser confocal microscope or an Olympus BX60 fluorescent microscope.

2.7 ACTIVATED CASPASE-3 IMMUNOHISTOCHEMISTRY

Formalin-fixed, paraffin-embedded skin sections were immunohistochemically stained with an antibody specific for the activated form of caspase-3 (R & D Systems, 1:2,000 dilution) by using the Histostain-Plus kit (Zymed). Histological specimens from intestine and skin, which were earlier confirmed to have extensive apoptosis, were used as positive controls. The stained slides were examined microscopically to determine the average number of positive epidermal keratinocytes per 10 mm of linear skin.

2.8 STATISTICAL CONSIDERATIONS

To obtain the appropriate sample size of adequate power for the tumor incidence study reported in figure 14, power analysis was done by two- group Chisquare test. A 0.05 two-sided significance level will have 80% power to detect the difference between K5Myc transgenic mice wild type and null for *Atm* when the sample size in each group is at least 14. The proportion of tumor incidence in transgenic mice wild type and null for *Atm* is estimated as 0.4 and 0.9 respectively.

Fisher's exact test was used to test for relationship between genotype and tumor incidence (Figure 14). To compare the Olive moments between samples in various comet assay experiments presented under figures 7, 20, 21 and 22 and Table 1, two- sample independent t test was employed. χ^2 test, using a 2x2 contingency table, was employed to compare the differences in the percent chromosomal fusions in wild type and K5Myc transgenic keratinocytes (Table 2). Olive moments of at least 70 cells in each sample were subjected to statistical analysis. Unpaired t-test was employed to compare the number of caspase-3 positive cells in the epidermis of K5Myc transgenic mice wild type

and null for *Atm* (Figure 13). At least four independent mice in each group were used to obtain the data. For all statistical tests employed in the present study, the levels of significance associated with P values are: P value < 0.001 is considered as highly significant; P value between 0.001 and 0.05 is considered significant and P value > 0.05 is considered not significant.

Chapter III: Oncogenes induce DNA damage *in vivo*

3.1 RATIONALE AND SIGNIFICANCE

As early as in 1994, it has been shown that the selective activation of the Ha-*ras*-gene in NIH 3T3 cells can lead to chromosomal damage and genomic instability (Denko, Giaccia et al. 1994). Later, several groups demonstrated that overexpressed Myc can also induce DNA damage and genomic instability in Rat1A fibroblasts and normal human fibroblasts (Felsher and Bishop 1999; Vafa, Wade et al. 2002; Karlsson, Deb-Basu et al. 2003). Recently Bartkova *et al* showed that the overexpression of several oncogenes, including Cyclin E, Cdc25a and E2F1, resulted in a DNA damage response (activation of DNA damage response markers γH2AX, pT-Chk2, pS-p53 and pS-SMC1) mediated by the ATM signaling pathway (Bartkova, Horejsi et al. 2005). Whether such DNA damage and genome destabilizing affects of the deregulated oncogenes are relevant to cancer causation and if so at what stages in the development of cancer do they initiate is a matter of debate as all these results were obtained in cultured cells and not *in vivo* and therefore may not be physiologically relevant.

Bert Vogelstein's group reported the presence of genomic instability at early stages of cancer development in colorectal cancers and concluded that chromosomal instability (CIN- losses or gains of defined chromosomal regions) occurs at early stages in colorectal neoplasia (Shih, Zhou et al. 2001). Allelic imbalance, a measure of CIN, of at least one chromosome was observed in 90% of colorectal adenomas of average diameter 2mm (representing an early neoplastic stage and having only low grade dysplasia without any evidence of high- grade dysplasia or carcinoma). Allelic imbalance

of chromosome 5q region that harbors the *adenomatous polyposis coli* (APC) tumor suppressor gene, which is lost in majority of colon cancers, is seen in 55% of adenomas. The authors conclude that CIN is a common event in colorectal tumors and occurs very early during tumorigenesis. Whether DNA damage and genomic instability is a common feature during the early stages of development of all cancers or is more restricted to a few cancer types because of their molecular mode of origin (for instance the loss of the *APC* gene in colonic cells may selectively predispose them to genomic instability) needs to be addressed. Also to what extent CIN, or genomic instability, is an early event and consequently a driving force for the inactivation of tumor suppressor genes thereby contributing to tumor progression is a major question. More specifically, if genetic instability is a common feature of early neoplastic growth, as in colorectal adenomas, one might expect it to begin even earlier (during hyperplastic stages itself) during the tumorigenic process. Studies in the past three years have provided conclusive evidence that DNA damage occurs even during preneoplastic stages of human carcinogenesis.

Hyperplastic human urinary, and lung epithelia, colorectal adenomas and pre-invasive carcinoma *in situ* lesions of the breast exhibit activated markers of the ATM DDR pathway establishing that the activation of this pathway is a common event during pre-invasive stages of several human cancers (Bartkova, Horejsi et al. 2005). As cancer progressed, the activation of the DDR markers was progressively suppressed in some of these tumors indicating that the ATM DDR pathway may be disrupted in later stages. This led to the hypothesis that ATM DDR acts as a barrier to early human tumorigenesis. However, the nature and cause of the DNA damage in these precancerous lesions of either tissue is not accounted for. It is an established fact that oncogene activation is a prerequisite for tumorigenesis. Therefore, the extent to which oncogene activation

contributes to the DNA damage phenotype in hyperplastic and neoplastic tissues is an important question to be addressed.

Taken together, these studies raise some critical questions. Do oncogenes induce DNA damage *in vivo?* If so, how early in the development of cancer do oncogenes induce DNA damage? Is DNA damage induction restricted to a few oncogenes or is it common to majority of the oncogenes? To address these pertinent questions, I have employed several transgenic mouse models of human cancer that overexpress c-Myc, E2F3a, SV40 T antigen and human papilloma virus (HPV) E7 oncogenes.

3.2 RESULTS

3.2.1 Myc Induces DNA Damage *in Vivo*

To determine if transgenic expression of Myc caused DNA damage, primary keratinocytes were isolated from K5Myc mice and immediately used in the single-cell gel electrophoresis (comet) assay (Figure 7). Keratinocytes isolated from mice exposed to ionizing radiation (IR), a potent and acute inducer of DNA damage, were used as a positive control for DNA damage. In the absence of IR, only a few wild-type keratinocytes had a detectable comet tail (Table 1). After IR treatment, the majority of keratinocytes had comet tails, indicative of DNA damage. Many keratinocytes isolated from K5Myc mice also had detectable comet tails, although the number of positive cells and the length of the comet tails was lower than in the IR-treated keratinocytes. Nonetheless, measurement of the comet tail moment confirmed that K5Myc keratinocytes had significantly more DNA damage compared with untreated wild-type keratinocytes (Table 1).

To further confirm Myc's role in the induction of DNA damage and to see its effects at the level of chromosomes, primary keratinocytes isolated from 1-2 day old wild-type and K5Myc transgenic pups were cultured to 70% confluency, treated with colcemid and subjected to cytogenetic analysis (Figure 8). Analysis of at least 125 metaphase spreads in each case revealed a two-fold increase in both the percentage of aberrant metaphases and percentage of cells with chromosomal breaks from Myc transgenic keratinocytes over wild type keratinocytes (Table 2). However, the striking difference is in the percentage of cells with chromosomal fusions, a measure of gross

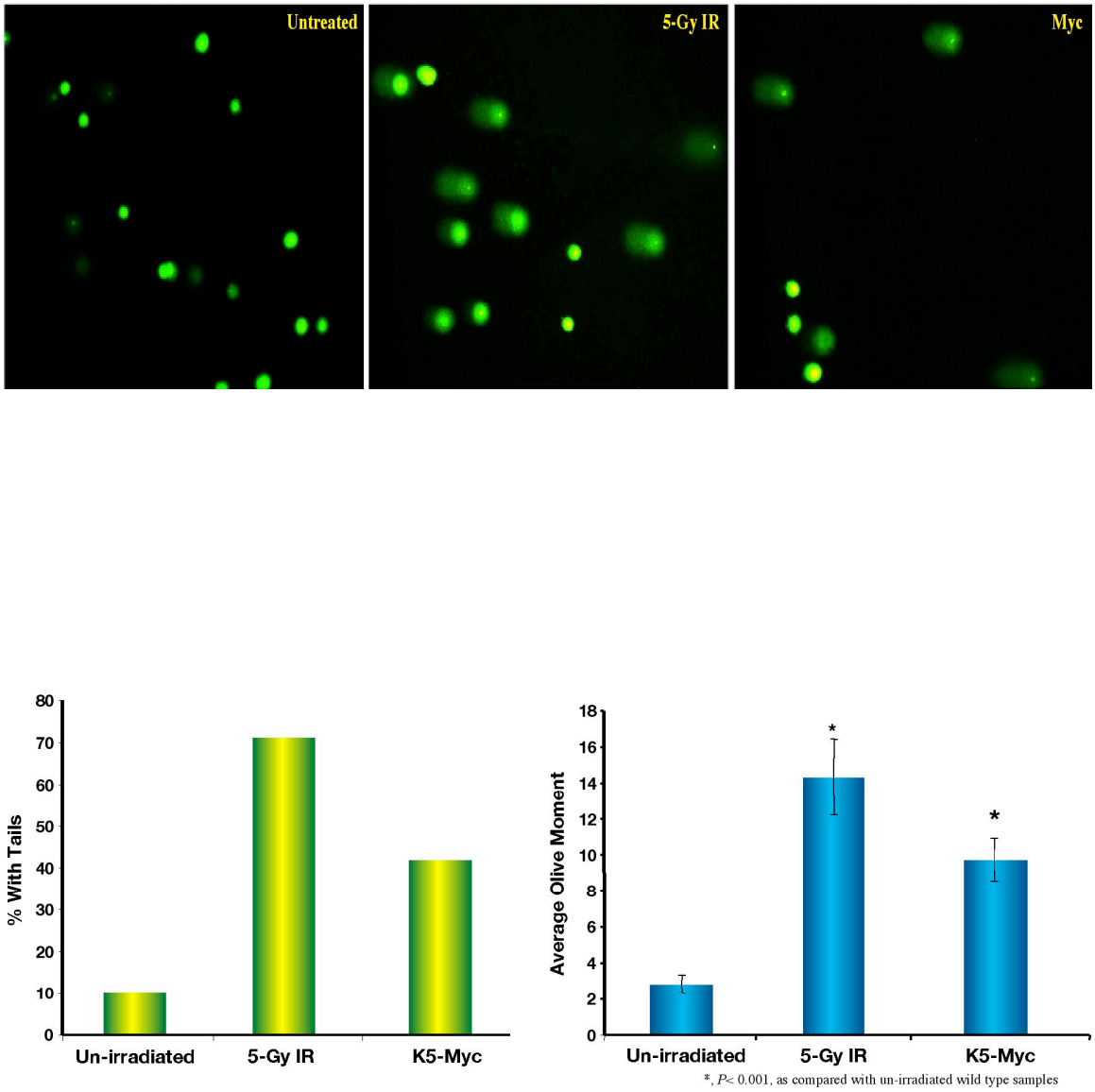


Figure 7: Myc induces DNA damage in adult mouse epidermal keratinocytes.

Single- cell gel electrophoresis (comet) assay on adult mouse epidermal primary keratinocytes from unirradiated and irradiated (5 Gy) wild type and K5Myc mice. Tail length and Olive moment of 70 nuclei from each of the three genotypes were scored. *P < 0.001, as compared with unirradiated wild type keratinocytes by two-sample independent t test.

Table 1: DNA damage in primary keratinocytes as measured by comet assay

Cells	% with tails	Average olive moment
Untreated	10.3	2.8±0.5
5 Gy IR	71.1	14.3±2.1*
K5Myc	41.8	9.7±1.2*

* $P<0.001$, as compared with untreated wild-type samples by two-sample independent t test.

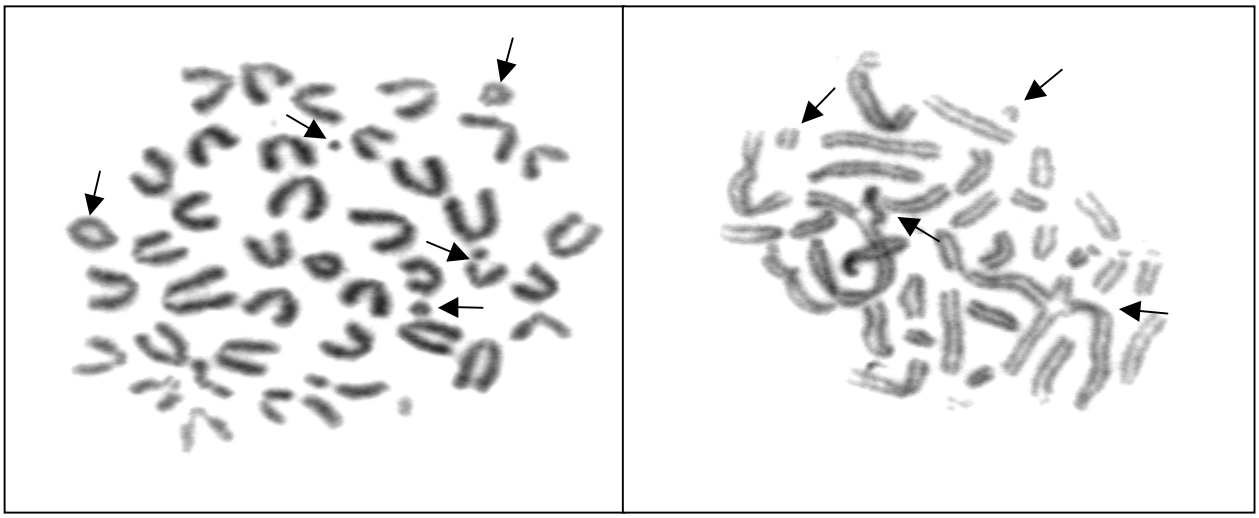
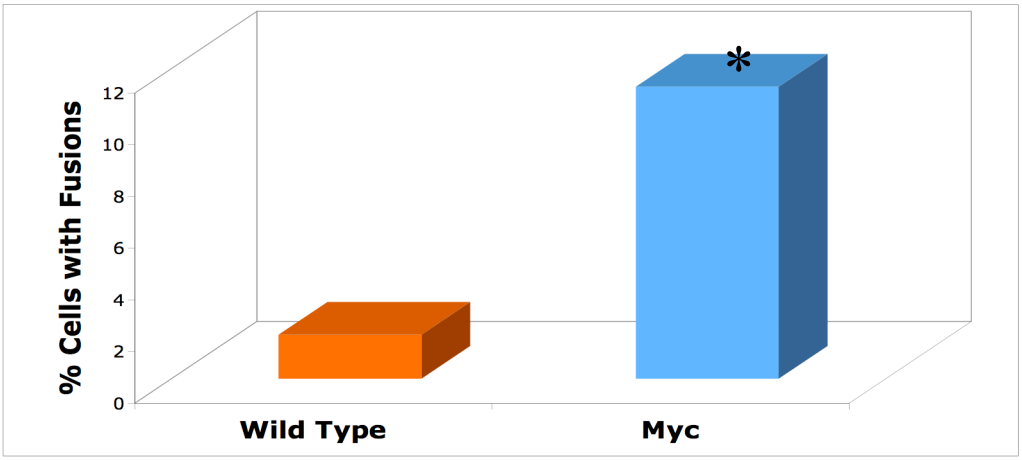


Figure 8: Gross chromosomal abnormalities in Myc transgenic keratinocytes.

Metaphase spreads from wild type and K5Myc transgenic primary keratinocytes were scored for chromosomal abnormalities. Both metaphase spreads depicted above are from transgenic keratinocytes. The arrows show chromosome breaks, fragments and fusions.

Table 2: Cytogenetic analysis of metaphase spreads from wild type and K5Myc transgenic keratinocytes

	Number of Metaphases	% Aberrant Metaphases	% Cells with Breaks	% Cells with Fusions
K5Myc	128 (4 mice)	23.6	14.5	11.3
Wild type	176 (5 mice)	13.1	8.0	1.7



*P= 0.001 as compared by χ^2 test.

chromosomal instability. Myc transgenic keratinocytes show a sevenfold increase in chromosomal fusions (11.3%) as compared to the basal level of fusions (1.7%) in wild-type keratinocytes. These results indicate that Myc induces DNA damage that is translated it into gross chromosomal abnormalities.

3.2.2 Myc Activates Components of the ATM DDR Pathway

To determine if Myc- induced DNA damage activates the ATM DDR pathway, K5Myc transgenic mice were bred into an *Atm* null background. Mice treated with IR, a well-characterized inducer of ATM autophosphorylation at serine 1981, a marker of ATM activation, were used as positive control. ATM phosphorylated at serine-1981 could be detected in primary tissue from K5Myc mice by immunofluorescence (Figure 9). Phosphorylated ATM localizes to subnuclear foci that are sites of DNA double-strand breaks (Bakkenist and Kastan 2003). The staining pattern of phospho-ATM in Myc transgenic epidermis was similar to the focal pattern of staining in mouse epidermis exposed to IR (Figure 9). This finding indicates that Myc- induced DNA DSBs activate ATM**.** One of the most commonly used and sensitive markers for detecting DNA double-strand breaks is the formation of subnuclear foci containing γH2AX (Rogakou, Pilch et al. 1998; Paull, Rogakou et al. 2000). As expected, exposure of wild-type mice to IR led to the formation of subnuclear γH2AX foci (Figure 9). In unirradiated K5Myc epidermis, γH2AX foci were also observed in most nuclei, and this depended on the presence of ATM (Figure 9). K5Myc epidermis also stained positive for other markers of DNA double-strand breaks, including phospho-SMC1 (Figure 9). SMC1 is phosphorylated by

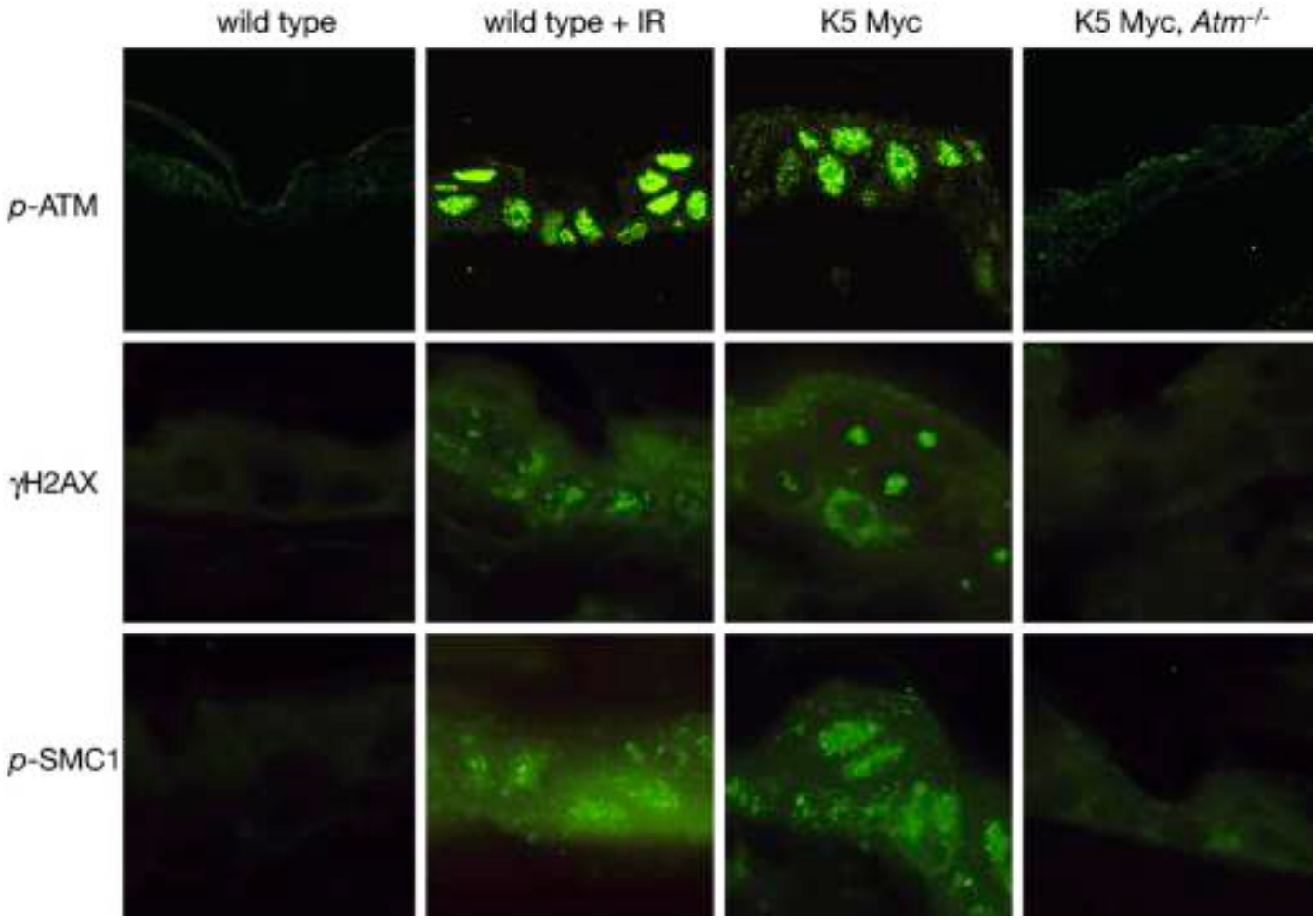


Figure 9: K5Myc transgenic tissue stains for markers of DNA double-strand breaks.

Immunofluorescent staining was performed on skin sections from untreated wild-type mice, wild-type mice exposed to 3 Gy of IR 20 min before killing, K5 Myc mice, and K5 Myc, *Atm*$^{-/-}$ mice by using antibodies specific for ATM phosphorylated at serine-1981 (*p*-ATM), γH2AX, or SMC1 phosphorylated at serine-957 (*p*-SMC1).

ATM and, like γH2AX, localizes to sites of DNA double- strand breaks (Kim, Xu et al. 2002; Yazdi, Wang et al. 2002; Kitagawa, Bakkenist et al. 2004). As with γH2AX, phospho-SMC1 displayed a focal pattern of staining in K5Myc epidermis, similar to the pattern observed in IR-treated epidermis, and this depended on the presence of ATM (Figure 9). Thus, transgenic expression of Myc in the epidermis results in the ATM-dependent formation of γH2AX and phospho-SMC1 foci that represent sites of double-strand breaks. Taken together, these findings further confirm that the transgenic expression of Myc induces DNA damage *in vivo,* which is then recognized by the ATM DDR pathway.

3.2.3 Human Papilloma Virus E7 (HPVE7) Oncoprotein Induces γH2AX Focus Formation *In Vivo*

Human papillomaviruses (HPVs) are small DNA viruses that are implicated in human cancer. A subset of HPVs, the high- risk HPV types 16 and 18 (HPV-16 and HPV-18), are associated with more than 90% of cervical carcinomas, a leading cause of death by cancer among women worldwide (zur Hausen and de Villiers 1994). The cell transforming capability of HPV E7 oncoprotein is due to its ability to disrupt the function of the retinoblastoma (Rb) tumor suppressor protein. In the K14HPV16E7 transgenic mouse model, the E7 oncogene induces hyperplasia of the squamous epithelia of the skin, ear and the forestomach (Herber, Liem et al. 1996). E7 also induces a fivefold increase in apoptosis in transgenic skin as compared to the nontransgenic skin. K14HPV16E7 mice develop skin tumors late in life, beginning from 9 months of age. Some of these tumors are low- grade squamous cell carcinomas, which were highly differentiated while the high- grade tumors were poorly differentiated.

To determine whether the induction of DNA damage *in vivo* by oncogenes is specific for Myc or is a more general phenomenon, K14HPV16E7 transgenic skin was stained for the activated form of H2AX. E7 expressing transgenic skin stained positive for γH2AX and the pattern of focal staining was similar to the subnuclear foci seen in K5Myc transgenic skin (Figure 10).

All experiments that I have conducted in the K5Myc and K14HPV16E7 transgenic mice, to address the question whether oncogenes induce DNA damage *in vivo* at very early stages of tumorigenesis, are done in mice aged between 8 to 12 weeks. At this early age, these different transgenic mice exhibit only hyperplasia with no evidence of any neoplastic growth in the transgenic skin.

3.2.4 SV40 TAg Activates Components of the ATM DDR Pathway in the Transgenic Prostate of TRAMP Mice

The prostate- specific rat probasin promoter drives the expression of the simian virus 40 large tumor antigen (SV40 TAg) oncogene in the mouse prostate. The transforming ability of TAg is due in large part to its perturbation of the Rb and p53 tumor suppressor proteins. In this highly penetrant model of prostate cancer, the TAg oncoprotein gets expressed from 8 weeks of age. TRAMP male mice between 8 to 12 weeks of age, a time corresponding to sexual maturity, develop mild epithelial hyperplasias. The earliest tumors start developing from around 12-15 weeks of age in the

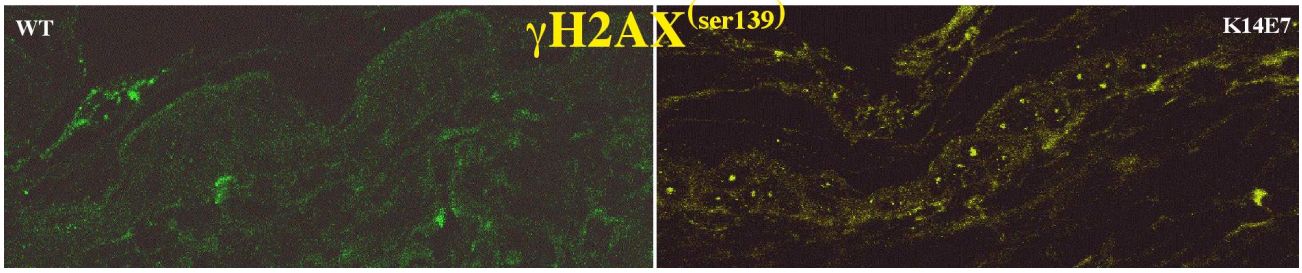


Figure 10: **HPVK14E7 epidermis stains positive for γH2AX focus formation.**

Immunofluorescent staining was performed on skin sections from wild-type and transgenic mice using antibody specific for γH2AX.

epithelium of the dorsolateral prostate. By 28 weeks of age, 100% of TRAMP mice develop metastatic prostate cancer in the lymph nodes or lungs (Greenberg, DeMayo et al. 1995; Gingrich, Barrios et al. 1996). To assay for the presence of oncogene- induced DNA damage, I have employed transgenic prostate exhibiting only hyperplasia with no evidence of neoplastic growth.

To confirm that DNA damage induction by oncogenes is a more general phenomenon and is not restricted by tissue type, prostate from both wild type and TRAMP mice was stained for γH2AX and phospho-SMC1. While the TAg expressing prostate epithelium of the TRAMP mice showed extensive focal staining of both γH2AX and phospho-SMC1, the wild-type prostate epithelia were negative for these markers of DNA damage (Figure 11). We have also found that in the K5E2F3a transgenic mouse model, the deregulated expression of E2F3a causes activation of the ATM DDR pathway. E2F3a, like Myc, induced DNA damage and activated the ATM DDR pathway as evidenced by ATM autophosphorylation at serine 1981 and ATM- dependent subnuclear γH2AX focus formation (Paulson, Pusapati et al. 2008). Our experiments with different oncogenes suggest that the ability to induce DNA damage may be a common feature and is not restricted to Myc alone.

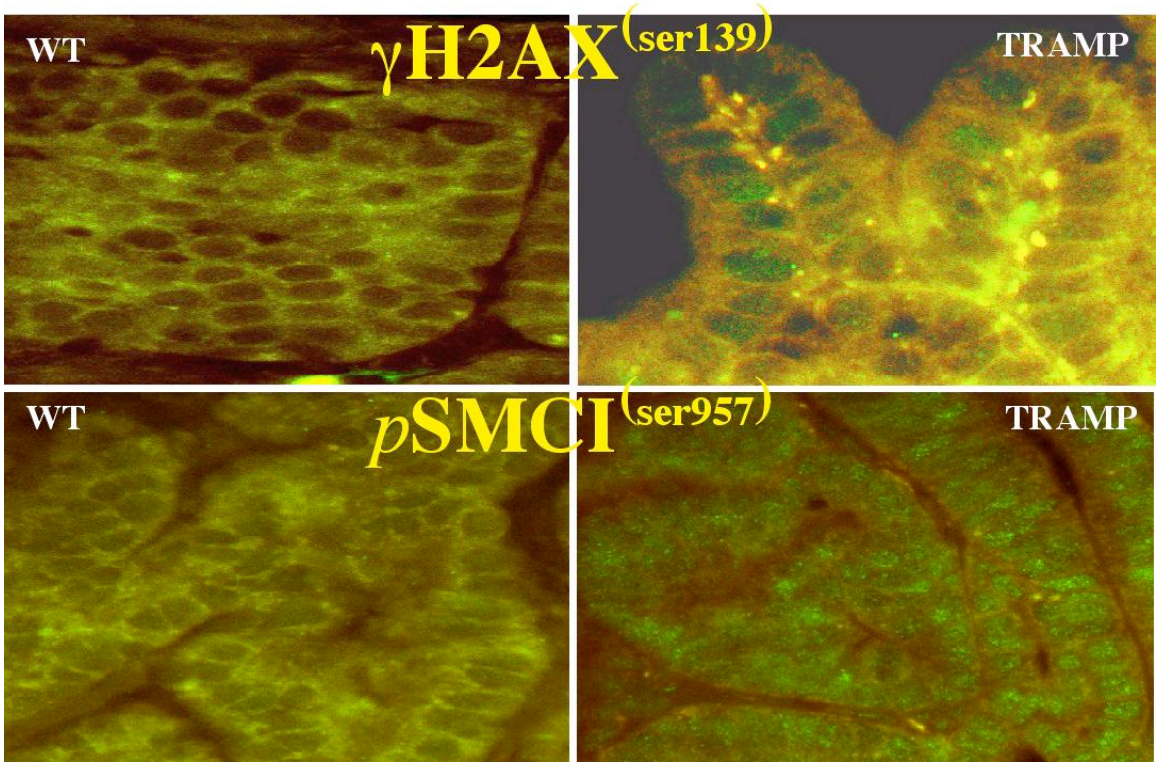


Figure 11: TRAMP prostate exhibits γH2AX and phospho-SMC1 focus formation. Formalin-fixed and paraffin-embedded prostate tissue from wild type and TRAMP mice were immunostained with antibodies specific for γH2AX and phospho-SMC1^{ser957}.

3.3 DISCUSSION

Phosphorylation of several DNA damage response proteins, including ATM, Chk2, p53, and H2AX, can be observed in precursor stage cancers of the breast, colon, lung, skin, testes, and urinary bladder (Bartkova, Horejsi et al. 2005). This has led to the suggestion that, in many cancers, DNA damage occurs during the earliest stages of tumor development, before genomic instability and the loss of wild-type p53 function. In agreement with this idea, forced expression of oncogenic growth factors, cyclin E, Cdc25A, or E2F1 in cultured cells can induce the ATM signaling pathway (Powers, Hong et al. 2004; Rogoff, Pickering et al. 2004; Bartkova, Horejsi et al. 2005; Gorgoulis, Vassiliou et al. 2005). Overexpression of Myc was also shown to induce DNA damage *in vitro* (Felsher and Bishop 1999; Vafa, Wade et al. 2002), but whether this occurs *in vivo* has been a matter of debate (Soucek and Evan 2002). It has been reported that deregulated expression of Myc in the liver of transgenic mice leads to chromosomal damage, but only when coexpressed with a TGFα transgene (Sargent, Sanderson et al. 1996).

The data presented here shows that transgenic expression of Myc on its own induces the ATM DNA damage response pathway, as evidenced by ATM phosphorylation and the formation of γH2AX and phospho-SMC1 foci. An increase in DNA damage over background levels could also be detected in primary keratinocytes isolated from K5Myc mice. This finding indicates that Myc overexpression, a common event in human cancers, causes DNA damage *in vivo* and could contribute to the activation of the ATM signaling pathway that is observed in early-stage clinical specimens.

Myc's ability to induce chromosomal breaks in keratinocytes confirms previous observations by Vafa *et al* who reported a significant increase in the breakage frequency (number of breaks/cell) in Myc overexpressing normal human fibroblasts. However, the most striking observation of our study is that Myc induces a significant amount of chromosomal fusions (a sevenfold increase over basal level) (Figure 8). The presence of gross chromosomal aberrations in Myc transgenic keratinocytes from 1-2 day old pups indicates that DNA damage and genomic instability can occur very early upon oncogene activation and during the process of tumorigenesis. Our findings correlate with those of Karlsson *et al* who showed in an *in vitro* inducible model that activation of Myc within one cell division cycle induced chromosomal breaks, deletions and translocations (Karlsson, Deb-Basu et al. 2003).

The presence of markers of DNA damage in different transgenic tissues expressing other oncogenes like SV40 TAg, HPV E7 and E2F3a suggests that several oncogenes can induce the accumulation of DNA damage before cancer development. Therefore the induction of DNA damage is not restricted to any one oncogene or tissue but seems to be a common feature to the process of oncogenesis. The deregulation of cell cycle is a common feature to the process of oncogenesis mediated by these four oncogenes. Whether this is of any relevance to their induction of DNA damage is an interesting question, which will be partly addressed under chapter 5.

It has been hypothesized that the ATM checkpoint acts as a barrier to tumorigenesis (Bartkova, Horejsi et al. 2005). Recent genomic analyses of a variety of human cancers found that somatic mutations in ATM are relatively common and appear to be a driving force in tumorigenesis (Greenman, Stephens et al. 2007). Whether the activated ATM DDR pathway has a role in suppressing Myc and E2F3a- induced DNA

damage and tumorigenesis will be addressed in the next section.

Chapter IV: ATM induces apoptosis and suppresses tumorigenesis in response to Myc and E2F3a

4.1 RATIONALE AND SIGNIFICANCE

It is becoming increasingly evident that the ATM DDR pathway plays a major role in the suppression of tumorigenesis. Recently, it was found that the *ATM* gene itself is mutated in different cancers and that these mutations can be a driving force in cancer development (Davies, Hunter et al. 2005; Greenman, Stephens et al. 2007). There is also evidence that *ATM* expression is suppressed through promoter hypermethylation in several cancers (Ai, Vo et al. 2004; Vo, Kim et al. 2004; Safar, Spencer et al. 2005). The observation that activation of the ATM DDR pathway occurs in early precancerous lesions of several types of human cancers underscores the importance of this pathway in suppressing cancer progression. Cancers at advanced stages exhibiting activation of the ATM pathway show mutations in the p53 tumor suppressor, a downstream effector in the ATM pathway, and loss of apoptosis. (Gorgoulis, Vassiliou et al. 2005). However, other advanced cancers having a wild type p53 show loss in ATM pathway activation upstream of p53, and also loss of apoptosis. Ultimately the ability of the ATM DDR pathway to induce apoptosis rests on conveying proper DNA damage signals to wild-type p53.

Myc- induced apoptosis in K5Myc transgenic epidermis is dependent partly on Arf and largely on p53 (Figure 6). Considering that Myc is inducing DNA damage in the K5Myc transgenic tissue (Figures 7 and 8) and activating the ATM DDR pathway (Figure 9), it can be hypothesized that Myc is signaling to p53, in an Arf-independent

pathway, and inducing apoptosis through ATM, which is acting to suppress Myc-induced tumorigenesis.

4.2 RESULTS

4.2.1 ATM Is Required for p53 Accumulation and Phosphorylation in Response to Myc

K5Myc mice exhibit aberrant apoptosis in their epidermis that depends largely on functional p53 (Rounbehler, Schneider-Broussard et al. 2001). Consistent with these findings, K5Myc-transgenic epidermis contains elevated levels of p53 protein compared with nontransgenic epidermis (Figure 12). To determine whether this elevated p53 in K5Myc tissue is phosphorylated, antisera specific for the serine-18 phosphorylated form of murine p53 was used in a Western blot of epidermal protein isolated from K5Myc mice. As a positive control for phosphorylated p53, epidermal protein from a nontransgenic mouse irradiated with 10 Gy of IR was used (Figure 12, lane 1). K5Myc mice contained elevated levels of total and phosphorylated p53 that were similar to those of IR-treated mice whereas phosphorylated p53 was barely detectable in epidermal extracts from an untreated, nontransgenic sibling mouse (Figure 12). This finding that Myc overexpression leads to p53 phosphorylation in K5Myc tissue *in vivo* agrees with previous results by others on the ability of Myc to stimulate p53 phosphorylation in cultured fibroblasts (Vafa, Wade et al. 2002; Lindstrom and Wiman 2003).

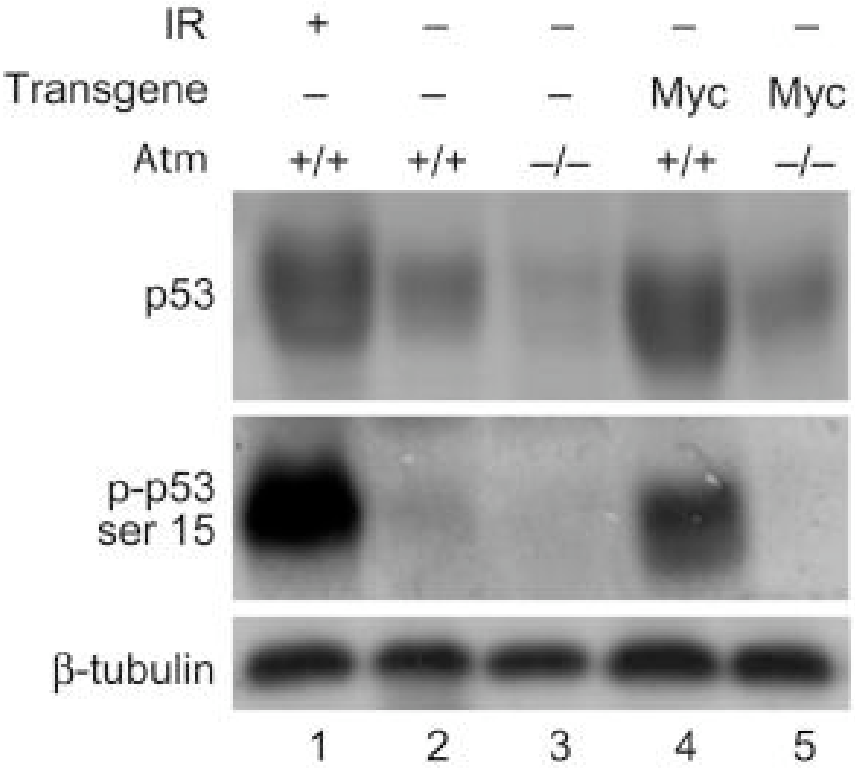


Figure 12: ATM-dependent accumulation and phosphorylation of p53 in response to Myc.

Western blot analysis was performed on epidermal protein lysates from nontransgenic (lanes 1-3) and K5 Myc-transgenic (lanes 4 and 5) mice that were wild-type (lanes 1, 2, and 4) or nullizygous (lanes 3 and 5) for *Atm*. The wild-type mouse in lane 1 was treated with 10 Gy of IR 30 min before it was killed. Antisera specific to total p53 (*Top*), phospho-serine-15 p53 (*Middle*), or β-tubulin (*Bottom*) were used as indicated.

Human p53 serine-15 and the homologous murine p53 serine-18 are phosphorylated by ATM and ATR (Banin, Moyal et al. 1998; Canman, Lim et al. 1998; Tibbetts, Brumbaugh et al. 1999). To determine whether ATM is involved in p53 stabilization and serine-18 phosphorylation in the K5Myc model, these mice were bred into an *Atm*- null background. The total level of p53 protein in K5Myc tissue was significantly reduced and serine-18 phosphorylation was undetectable in the absence of ATM (Figure 12).

To further examine the requirement for ATM in mediating p53 accumulation and phosphorylation induced by Myc, normal human fibroblasts (NHFs) and primary fibroblasts from a patient with ataxia-telangiectasia (AT) were obtained. These cells were infected with a recombinant adenovirus expressing Myc (AdMyc) or a control adenovirus expressing GFP (AdGFP). As a positive control for ATM-dependent phosphorylation, these cell cultures were also irradiated with IR. Exposure to IR induced the phosphorylation of ATM on serine-1981, a marker of ATM activation. Phosphorylation of ATM also occurred in NHFs infected with AdMyc but not AdGFP (Pusapati, Rounbehler et al. 2006). As expected, IR induced p53 phosphorylation at serine-15 in NHFs, and this was significantly reduced in the AT cells (Pusapati, Rounbehler et al. 2006). Phosphorylation of p53 also occurred in NHFs infected with AdMyc but not AdGFP. In AT cells, however, overexpression of Myc did not induce significant levels of phosphorylated p53 (Pusapati, Rounbehler et al. 2006). Thus, ATM is required for p53 accumulation and phosphorylation in response to Myc overexpression in both primary transgenic epidermal tissue and cultured primary human fibroblasts.

4.2.2 ATM Promotes Apoptosis Induced by Myc

Activation of p53 in response to Myc overexpression leads to the induction of apoptosis. Consistent with this, infection of NHFs with AdMyc, but not AdGFP, resulted in the formation of the activated, cleaved form of caspase-3 (Pusapati, Rounbehler et al. 2006). In contrast, Myc overexpression did not increase the level of activated caspase-3 in AT cells. This finding suggests that ATM participates in Myc-induced apoptosis. To determine whether this is the case *in vivo*, the effect of *Atm* status on apoptosis in K5Myc mice was examined by measuring the number of epidermal keratinocytes staining for the activated form of caspase-3. Inactivation of *Atm* resulted in a significant decrease in the number of apoptotic cells observed in K5Myc transgenic epidermis (Figure 13). In contrast to the impaired apoptosis observed in response to Myc overexpression, $Atm^{-/-}$ mice had normal levels of apoptosis in the epidermis after exposure to UV radiation (Pusapati, Rounbehler et al. 2006). This finding demonstrates that the absence of ATM does not cause a general impairment of apoptosis in mouse keratinocytes, but rather inactivation of *Atm* specifically inhibits apoptosis in response to Myc overexpression.

4.2.3 ATM Inactivation Cooperates with Myc Overexpression in Tumorigenesis

Suppressing Myc-induced apoptosis, through the inactivation of *p53* or *Arf* or the overexpression of Bcl-2, has been shown to accelerate tumorigenesis in a number of experimental systems (Fanidi, Harrington et al. 1992; Blyth, Terry et al. 1995; Elson, Deng et al. 1995; Eischen, Weber et al. 1999; Schmitt, Fridman et al. 2002). To

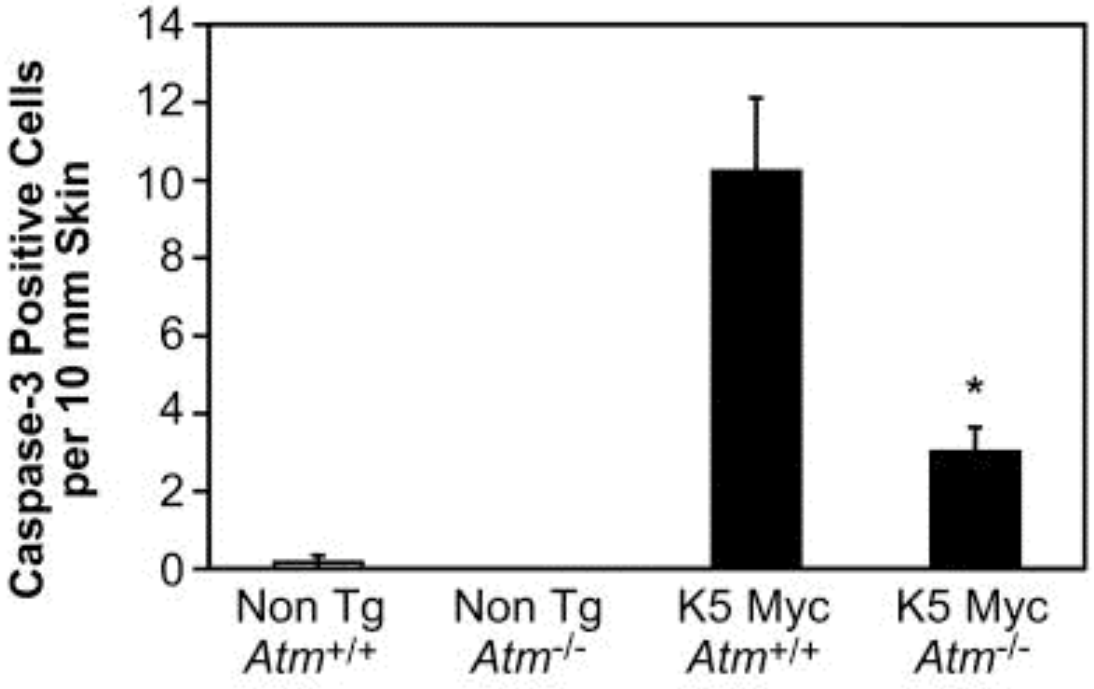


Figure 13: Inactivation of *Atm* reduces apoptosis in K5 Myc-transgenic mice.

Skin sections taken from mice with the indicated genotypes were immunohistochemically stained with an antibody specific for the activated form of caspase-3. The average number of positive epidermal cells per 10 mm of skin was determined microscopically from at least four independent mice in each group. The number of caspase-3-positive cells in K5 Myc-transgenic mice null for *Atm* is statistically different from the number in K5 Myc-transgenic mice wild-type for *Atm* by unpaired *t* test ($P = 0.0175$).

determine whether *Atm* inactivation would also promote Myc-driven tumor development, K5Myc mice that were wild-type, hemizygous, or nullizygous for *Atm* were generated and maintained. K5Myc mice develop spontaneous tumors in several K5-expressing tissues, including the skin and oral epithelium, starting at ≈30 weeks of age (Rounbehler, Schneider-Broussard et al. 2001; Rounbehler, Rogers et al. 2002). $Atm^{-/-}$ mice normally develop lymphomas by 6 months of age but very rarely develop tumors in epithelial tissues (Barlow, Hirotsune et al. 1996; Elson, Wang et al. 1996; Xu, Ashley et al. 1996). Consistent with previous results from my lab, no K5Myc mouse wild-type for *Atm* developed a tumor before 30 weeks of age (Figure 14). Inactivation of one *Atm* allele had no significant effect on the rate of tumor development in K5Myc mice. In contrast, 67% of K5Myc mice null for *Atm* developed an epithelial tumor by 30 weeks of age (Figure 14). These findings demonstrate that ATM, like p53, promotes Myc- dependent apoptosis and suppresses Myc- induced tumorigenesis.

Similar to $Atm^{-/-}$ mice, more than half of the K5Myc mice null for *Atm* (15 of 26) developed a lymphoma before or concurrent with the development of an epithelial tumor. However, all mice that developed a lymphoma before developing an epithelial tumor were removed from the study shown in Figure 14 and Table 3. By 49 weeks of age, 100% of K5Myc, $Atm^{-/-}$ mice that did not develop a lymphoma had developed an epithelial tumor. In contrast, only 40% of the transgenic mice wild-type or hemizygous for *Atm* developed a tumor by 1 year of age (Figure 14 and Table 3). All of the epithelial tumors that arose in the K5Myc mice were in the skin or oral epithelium (Table 3).

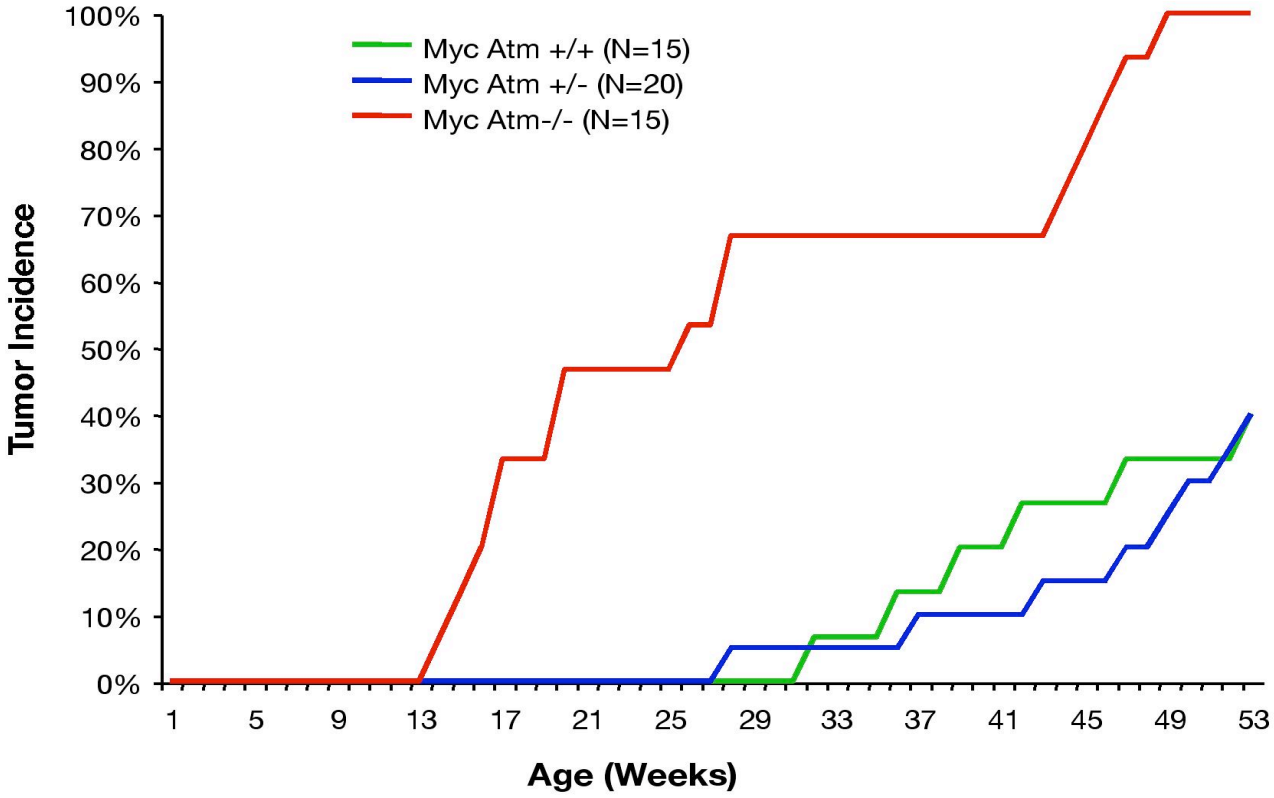


Figure 14: Inactivation of *Atm* accelerates epithelial tumorigenesis in K5Myc transgenic mice.

K5 Myc-transgenic mice wild-type (+/+), hemizygous (+/-), or nullizygous (-/-) for *Atm* were monitored for spontaneous tumor development for 1 year. Only tumors from squamous epithelial tissues are included. Tumor incidence in K5 Myc-transgenic mice that are $Atm^{-/-}$ is statistically highly significant from transgenic mice that are $Atm^{+/+}$ or $Atm^{+/-}$ by Fisher's exact test ($P < 0.001$).

Table 3: Epithelial tumors in K5Myc transgenic mice

Genotype	*n*	Tumor incidence at 1 year, %	Average age of onset, weeks	Tumor types
$Atm^{+/+}$	15 (8 F, 7 M)	40	42.0	Two squamous papillomas; Four SCC of skin
$Atm^{+/-}$	20 (12 F, 8 M)	40	44.8	Three squamous papillomas; One SCC of skin; Four SCC of oral cavity
$Atm^{-/-}$	15 (7 F, 8 M)	100	29.2	Four squamous papillomas; Three SCC of skin; Nine SCC of oral cavity

F, female; M, male; SCC, squamous cell carcinoma.

4.2.4 ATM/ATR Pathway Inactivation Promotes Skin Tumorigenesis in E2F3a Transgenic Mice

Deregulated E2F3a, like Myc, induces DNA damage in K5E2F3a transgenic tissue and activates ATM, which induces apoptosis (Paulson, Pusapati et al. 2008). To directly test whether ATM suppresses E2F3a-driven tumorigenesis, as it does with Myc-driven tumorigenesis, K5E2F3a transgenic mice were generated and maintained in an $Atm^{-/-}$ background. However, all $Atm^{-/-}$ mice developed thymic lymphomas before developing an epithelial tumor and therefore it was not possible to observe cooperation between ATM loss and E2F3a overexpression in this experiment.

As an alternative approach to inactivating ATM, caffeine was given to K5E2F3a transgenic mice to inhibit ATM and ATR kinase activities. This approach was recently used by Bartkova *et al.* (Bartkova, Rezaei et al. 2006) to inhibit the DDR in Ras-expressing cells, which led to larger and more invasive tumors in a xenograft mouse model. K5E2F3a transgenic and wild- type sibling mice were given caffeine in their drinking water at a final concentration of 0.4 mg/ml beginning shortly after weaning.

Treatment of K5E2F3a mice with caffeine suppressed the DDR as indicated by reduced γH2AX foci formation in the nuclei of transgenic epidermal keratinocytes (Figure 15). Approximately half of the K5E2F3a mice given caffeine developed visible skin lesions after several months of treatment, with several mice developing multiple lesions (Table 4). Skin lesions did not develop in untreated K5E2F3a transgenic mice or in wild-type mice given caffeine. Histopathological analysis of several of these lesions

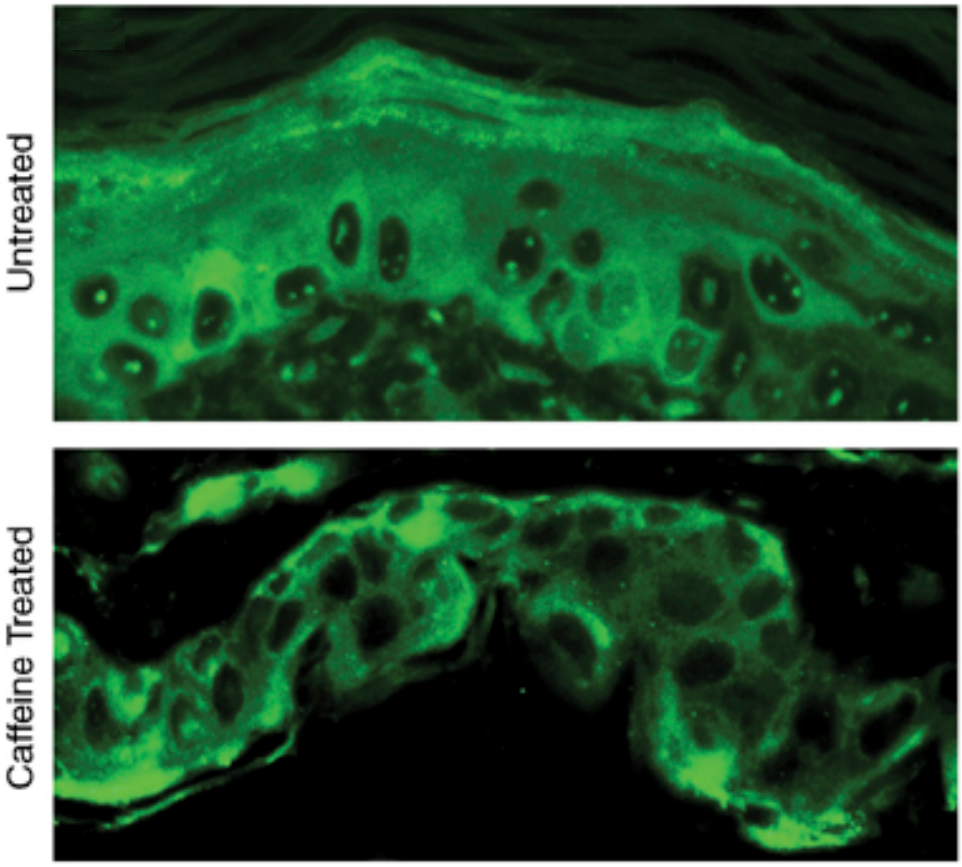


Figure 15: Caffeine treatment inhibits the DDR and promotes skin tumorigenesis in K5E2F3a transgenic mice.

Skin sections from untreated (top) and caffeine-treated K5E2F3a transgenic mice were immunofluorescently stained for γH2AX. Note reduction of nuclear foci in transgenic mouse treated with caffeine.

Table 4: Caffeine promotes skin tumor development in K5E2F3a mice

Treatment	Genotype	Total number of mice (N)	Percentage of tumor incidence	Average time to tumor formation (days)
Untreated	Wild type	21	0	-
Untreated	K5E2F3a	15	0	-
Caffeine	Wild type	25	0	-
Caffeine	K5E2F3a	23	47.8	56.2

showed them to be well-differentiated tumors probably arising from the infundibular region of the hair follicle. These tumors have several characteristics of human keratoacanthoma, including large keratin cysts and in some cases signs of invasiveness (Figure 16). Also like keratoacanthomas, these tumors grew rapidly up to a point and then stopped, often spontaneously regressing after several weeks. Taken together, these findings provide additional support for the idea that ATM can suppress tumorigenesis at an early stage by responding to oncogene-induced DNA damage.

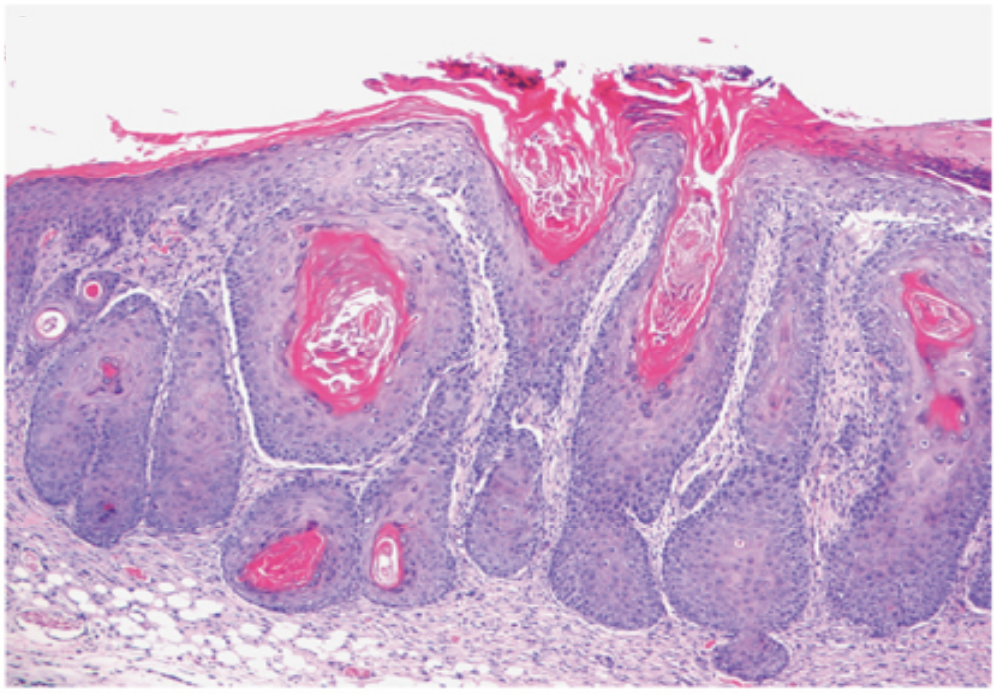


Figure 16: **Skin lesion from a K5E2F3a transgenic mouse given caffeine (0.4 mg/ml) in drinking water stained with hematoxylin and eosin**.

Note keratin accumulation and apparent involvement of hair follicles.

4.3 DISCUSSION

4.3.1 ATM Phosphorylates p53, Induces Apoptosis and Suppresses Tumorigenesis in Response to Myc

In addition to promoting cell proliferation, Myc also induces apoptosis, and this functions to suppress tumor development. A number of mediators have been shown to be important for Myc-induced apoptosis, with one of the most critical being p53 (Felsher and Bishop 1999). It has been proposed that oncogenes such as Myc and DNA damage activate p53 through distinct mechanisms. Specifically, ARF is believed to be involved in the activation of p53 in response to oncogenes whereas kinases such as ATM are thought to be important for p53 activation in response to DNA damage (de Stanchina, McCurrach et al. 1998; Harris and Levine 2005). Indeed, in the K5Myc mice used in this study, inactivation of *Arf* reduced the level of apoptosis observed in the epidermis (Russell, Powers et al. 2002).

However, the findings presented here demonstrate that ATM is also critical for p53 activation and the induction of apoptosis in response to Myc. In the absence of ATM, p53 accumulation and phosphorylation in response to Myc are greatly reduced. Moreover, the spontaneous apoptosis observed in the epidermis of K5Myc mice is significantly decreased by *Atm* inactivation. *Atm* inactivation reduced apoptosis in K5Myc transgenic tissue to a level that is similar to that observed when *Arf* was inactivated, but not to the near-complete inhibition of apoptosis observed in the absence of p53 (Rounbehler, Rogers et al. 2002; Russell, Powers et al. 2002). This finding suggests that, in at least some cases, the ARF and ATM pathways cooperate to activate

p53 and promote apoptosis. ATM phosphorylates additional targets, including Mdm2 and Chk2 that could also potentially contribute to p53 activation and apoptosis. Whether Myc promotes the ATM-dependent phosphorylation of these other targets remains to be determined.

Inhibition of Myc-induced apoptosis has been shown to accelerate tumor development in a number of experimental systems (Fanidi, Harrington et al. 1992; Blyth, Terry et al. 1995; Elson, Deng et al. 1995; Rounbehler, Schneider-Broussard et al. 2001; Schmitt, Fridman et al. 2002). Consistent with this finding, *Atm* inactivation not only impairs apoptosis but also accelerates epithelial tumor development in K5Myc mice. This finding provides experimental evidence to support the idea that the ATM DNA damage response pathway functions to suppress the emergence of tumors from precancerous lesions. In addition to impairing apoptosis, the absence of ATM may also promote tumorigenesis by causing genetic instability. Cells from humans and mice lacking ATM have defective telomere maintenance, end-to-end chromosome fusions, and an increased frequency of spontaneous translocations that could contribute to cancer development (Smilenov, Morgan et al. 1997; Liyanage, Weaver et al. 2000; Hande, Balajee et al. 2001; Stumm, Neubauer et al. 2001; Undarmaa, Kodama et al. 2004).

4.3.2 ATM Suppresses E2F3a- Induced Tumorigenesis

Like Myc, E2F3a overexpression causes DNA damage and results in the activation of ATM, which is important for the induction of apoptosis. E2F3a-induced apoptosis is significantly reduced by the absence of ATM in transgenic mouse tissue and in primary human fibroblasts (Paulson, Pusapati et al. 2008). These results are similar to

our findings with Myc as well as a previous study from my lab demonstrating that ATM is critical for apoptosis induced by E2F1 (Powers, Hong et al. 2004). Other groups have recently shown that cellular senescence induced by the deregulated expression of oncogenes such as Ras is also dependent on ATM (Bartkova, Rezaei et al. 2006; Di Micco, Fumagalli et al. 2006; Mallette, Gaumont-Leclerc et al. 2007). Thus, ATM appears to be critical for responding to oncogenic stress generated by a variety of oncogenes and can promote either apoptosis or senescence depending on the context.

Bartkova and coworkers (Bartkova, Rezaei et al. 2006) recently demonstrated that caffeine in the drinking water can inhibit ATM activation and stimulate the development of larger and more invasive tumors using a Ras-driven xenograft mouse model. Interestingly, the dose of caffeine (approximately 15 mg/kg/day) used in that experiment is physiologically relevant and similar to the amount consumed by a heavy coffee drinker (Mandel 2002). Similar to those findings, we found that the same concentration of caffeine also promoted *de novo* tumorigenesis in K5E2F3a transgenic mice. These skin tumors appeared to arise from the infundibular portion of the hair follicle and had a number of similarities to human keratoacanthomas. Some of these lesions had features of well-differentiated squamous cell carcinoma with signs of invasion, while others had features of sessile (broad based) skin papillomas. However, in all cases, there was abundant keratin accumulation and most lesions appeared connected with hair follicles. The rapid appearance, lack of progression and frequent spontaneous regression of these lesions are also behaviors reminiscent of human keratoacanthomas (Karaa and Khachemoune 2007). The fact that these tumors in caffeine-treated transgenic mice do not progress and often regress suggests that there are additional, backup tumor

suppressive mechanisms that respond to increased E2F3a activity in addition to ATM.

4.3.3 ATM: A Caretaker as well as a Gatekeeper Cancer Susceptibility Gene

Kinzler and Vogelstein defined gatekeeper genes as those that directly regulate the growth of tumors by inhibiting growth or promoting death and caretaker genes as those that maintain the integrity of the genome, thus preventing the accumulation of cancer-driving mutations (Kinzler and Vogelstein 1997). Based on this definition, it has been widely assumed that ATM functions as a caretaker as it mediates DDR and prevents genomic instability. However, findings from the present study and those from others suggest that ATM may also function as a gatekeeper by directly responding to oncogene-induced DNA damage and inducing either apoptosis or senescence in oncogene-expressing cells. Therefore, like *p53*, *ATM* can be reclassified as both a caretaker and gatekeeper having dual roles in the suppression of tumorigenesis.

4.3.4 Dual Role of ATM in Tumor Suppression

A dual role for ATM in tumor suppression can be proposed based on our findings (Figure 17). My finding that ATM promotes p53-dependent apoptosis in response to Myc to suppress tumorigenesis establishes the gatekeeper role for ATM. We have also shown in *Atm-/-* thymic lymphoma cell lines, lacking the K5Myc transgene, that Myc was highly overexpressed compared with the levels of Myc observed in primary T cells from wild-type or *Atm-/-* mice (Pusapati, Rounbehler et al. 2006). This result, which establishes the caretaker role of ATM in tumor suppression, is consistent with the observation that the

Myc gene translocation or amplification is a common event in lymphomas from *Atm-/-* mice (Liyanage, Weaver et al. 2000). Therefore ATM has a dual role in tumor suppression; as a caretaker it prevents genetic instability that can potentially lead to the activation of cancer driver mutations and as a gatekeeper inhibits tumor growth by ensuring apoptosis or senescence (Figure 17).

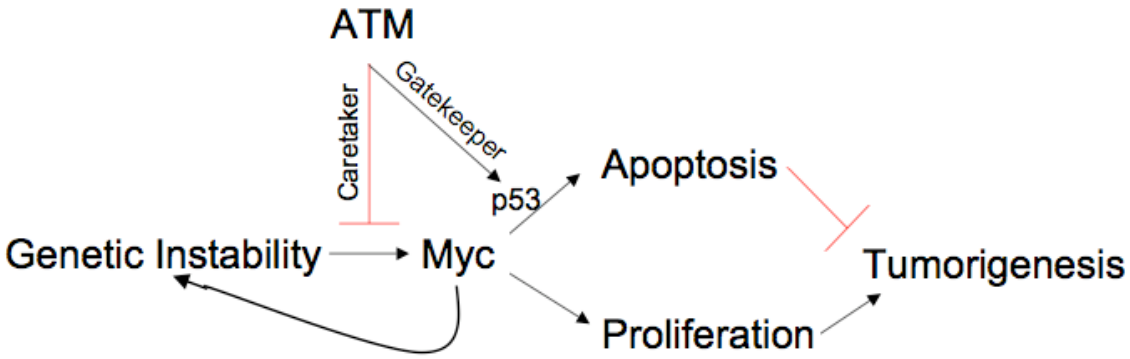


Figure 17: A model for dual role of ATM in tumor suppression

Chapter V: Aberrant DNA replication may be responsible for Myc- and E2F3a- induced DNA damage

5.1 RATIONALE AND SIGNIFICANCE

Our studies have conclusively linked DNA damage lesions in preneoplastic stages to oncogene activation (Myc and E2F3a) using genetically defined transgenic and knockout mouse models, which was subsequently confirmed *in vivo* by at least one other study (Reimann, Loddenkemper et al. 2007), albeit in a different transgenic mouse model (Eμ- Myc B-cell lymphoma model). Others have shown the activation of the ATM DDR pathway in Ras and Myc-induced tumors in genetically defined mouse models expressing these activated/deregulated oncogenes (Di Micco, Fumagalli et al. 2006; Shreeram, Hee et al. 2006). Although now there is conclusive evidence that Myc can induce DNA damage *in vivo*, there is no consensus on the mechanism (s) by which DNA damage arises or accumulates. Various studies have proposed several mechanisms, some of which are enumerated below.

5.2 MECHANISMS FOR ONCOGENE- INDUCED DNA DAMAGE

5.2.1 Myc Induces an Increase in Intracellular Reactive Oxygen Species (ROS) Levels Which Cause DNA Damage

Deregulated expression of Myc in quiescent cells leads to entry into S-phase, which is associated with a metabolic burst, a potential source of intracellular ROS. High levels of ROS can cause oxidative base modifications and single- or double stranded

breaks (Helbock, Beckman et al. 1999). Myc elicited increases of cellular ROS production has been implicated in DNA damage induced by Myc (Vafa, Wade et al. 2002; KC, Carcamo et al. 2005; Reimann, Loddenkemper et al. 2007). Vafa *et al* and Sagun *et al* showed that deregulated expression of Myc in serum/growth factor deprived cells increases ROS and active cell cycling is not required for the induction of DNA damage. Another study reported that ROS induced by Myc in NIH 3T3 cells is cytotoxic only under serum- deprived conditions (Tanaka, Matsumura et al. 2002). One common theme in all the three studies reported here is that antioxidants reduced both the levels of ROS induced by Myc and the consequent DNA damage indicating that ROS induction by Myc could be a possible mechanism by which it induces DNA damage.

Two studies reported *in vivo* ROS generation by Myc. However, Myc requires the co-expression of TGF-α to induce ROS as well as chromosomal and mitochondrial genome instability in a hepatocellular carcinoma mouse model (Factor, Kiss et al. 1998; Factor, Laskowska et al. 2000). Another study reported that Myc induces DNA damage via ROS production in cells freshly isolated from lymphomas (Reimann, Loddenkemper et al. 2007). However, the metabolic state of tumor cells is likely to be very different from non-transformed cells harboring an activated oncogene and the presence of ROS in cells derived from advanced tumors may very well be the consequence of tumorigenesis and not a causative factor for oncogene- induced DNA damage induction seen at preneoplastic stages of tumorigenesis. Recent studies countered oxidative stress as a major mechanism for oncogene-induced DNA damage (Bartkova, Horejsi et al. 2005; Ray, Atkuri et al. 2006). Ray *et al* showed that Myc overexpression in NHFs induces ROS only under some conditions like low serum or ambient supraphysiologic oxygen saturation (20%), but generally induces widespread DSBs *in vivo* and *in vitro*

independent of ROS production. Bartkova *et al* found that the bulk of the DNA damage caused by Cyclin E, Cdc25A and E2F1 oncogenic stimulus was independent of oxidative stress. Taken together, these studies indicate that oxidative stress is unlikely to be the major causative factor for Myc-induced DNA damage under physiological conditions.

5.2.2 Deregulated Myc Induces DNA Damage by Interfering with DNA DSB repair

Karlsson *et al* showed that Myc overexpression globally suppresses the repair of DNA DSBs (Karlsson, Deb-Basu et al. 2003). They postulate two mechanisms by which Myc might be interfering with the repair of DNA DSBs that occur spontaneously in normal cells. Firstly, Myc might be indirectly interfering with factors that mediate homology- directed recombination, nonhomologous end joining or single-strand annealing, all of which are responsible for repairing DNA breaks and preserving chromosomal integrity in mammalian cells. However, evidence for this mechanism is presently lacking. Secondly, Myc might be interfering with cell cycle checkpoints that are required for appropriate DNA repair. There is substantial evidence that Myc- induced abrogation of cell cycle checkpoints could induce DNA damage.

5.2.3 Abrogation of Cell Cycle Checkpoint(s) by Myc Leads to DNA Damage

Several studies have shown that the abrogation of at least one of the cell cycle checkpoints (G1/S, intra-S or G2/M checkpoints) by Myc could account for Myc-induced DNA damage. Deregulated Myc induced the acceleration of Rat1A cells and NHFs through the cell cycle even under DNA damage promoting conditions that normally lead to arrest of cells in the G1 and S phases (Felsher and Bishop 1999). In NHFs, Myc

induced aneuploidy with no chromosomal aberrations. However in Rat1A cells, which have a defective p53 pathway, Myc in addition to aneuploidy also induced extensive karyotypic abnormalities like chromosome breaks, double minute and polycentric chromosomes. This suggests that additional mutational events that disrupt the G1/S and S phase checkpoints are needed to cooperate with Myc- induced oncogenic stress to induce DNA damage. K5Myc transgenic keratinocytes in the present study that were shown to harbor Myc-induced DNA damage at preneoplastic stages have an intact ATM-p53 pathway (Figures 9, 12 and 13). Therefore, the abrogation of G1/S and intra-S phase checkpoints is unlikely to be the major mechanism accounting for Myc-induced DNA damage and genomic instability observed in the K5Myc transgenic keratinocytes.

5.2.4 Oncogene-Induced Replication Stress Preferentially Targets Common Fragile Sites in Preneoplastic Lesions to Induce Genomic Instability

Replication stress refers to replication fork stalling or collapse due to various reasons, including depletion of nucleotides, DNA polymerase inhibition and DNA lesions that topologically constrain the progression of the fork. Common fragile sites are chromosomal regions that are late replicating and prone to breakage under conditions of replication stress (Albertson 2006). Recently, several groups have reported increased frequency of chromosomal aberrations at common fragile sites in preneoplastic lesions (Bartkova, Horejsi et al. 2005; Di Micco, Fumagalli et al. 2006; Tsantoulis, Kotsinas et al. 2008). They have presented evidence that in preneoplastic lesions, allelic imbalance and loss of heterozygosity (a measure of genomic instability) is more frequent at common fragile sites than in the rest of the genome. Two of these studies implicated Ras- induced replication stress in increased chromosomal breakages at certain common fragile sites (Di

Micco, Fumagalli et al. 2006; Tsantoulis, Kotsinas et al. 2008). Whether Myc similarly induces replication stress leading to increased chromosomal breakage at common fragile sites and if so, how it induces replication stress need to be investigated.

5.2.5 Rereplication- As a Mechanism for Oncogene- Induced DNA Damage

Rereplication refers to the process of initiation of a second round of DNA replication in one cell cycle. Evidence for rereplication- induced chromosomal breakage has been previously reported (Vaziri, Saxena et al. 2003; Melixetian, Ballabeni et al. 2004; Di Micco, Fumagalli et al. 2006). It has also been reported that deregulated Myc abrogated G2/M arrest in colcemid treated Rat1A cells by enabling rereplication to induce polyploidy (Li and Dang 1999). Myc-dependent cell division kinase 2 (CDK2) activity has been implicated in Myc's induction of DNA rereplication in G2/M arrested cells (Li and Dang 1999). Therefore cyclin/cdk complexes, and other Myc targets of replication origin licensing and S phase initiation may contribute to Myc-induced DNA rereplication. A basic understanding of the events at eukaryotic origins of replication is a prerequisite to understanding how oncogenes can potentially mediate rereplication.

5.3 DNA REPLICATION: EVENTS AT THE EUKARYOTIC ORIGINS OF REPLICATION

Eukaryotic genomes, being vast unlike bacterial genomes, need to replicate within a reasonable amount of time and hence initiate replication at thousands of origins (~300,000) in each cell cycle. To ensure the integrity of the genome, each of the origins

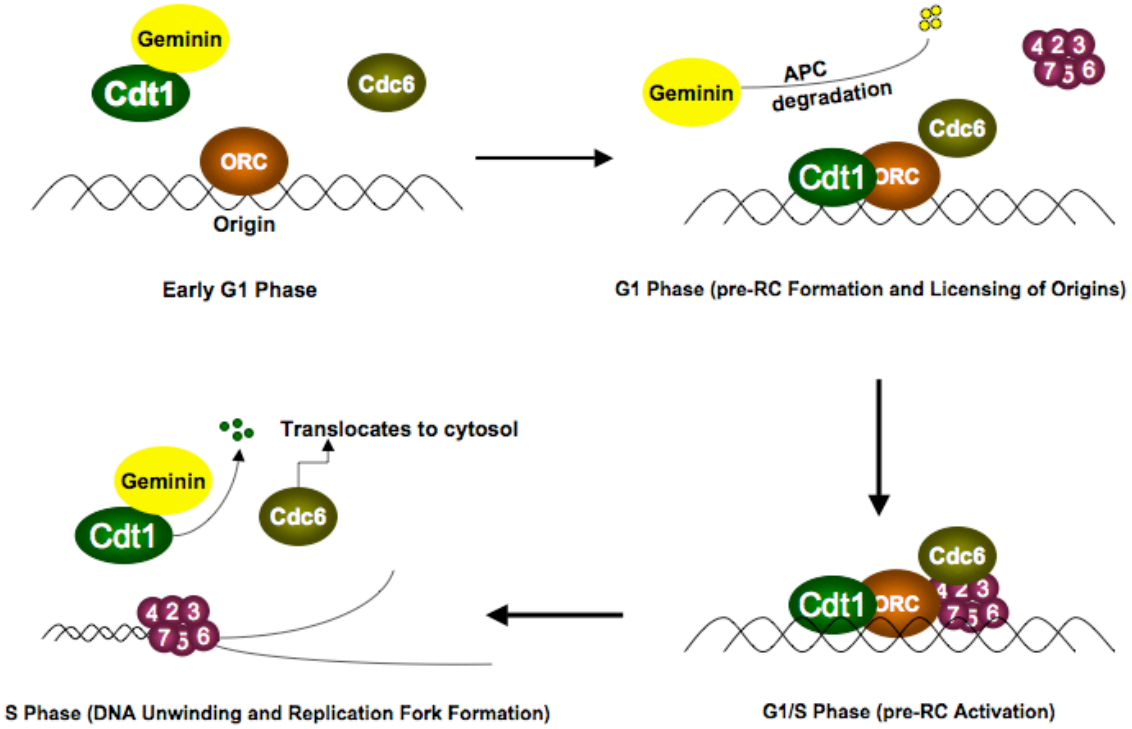


Figure 18: **Schematic of events at the eukaryotic origins of replication: from licensing of origins (pre-RC formation) to initiation of DNA replication.**

undergoes only one initiation (firing) event in each S phase so that the genome is duplicated precisely once in each cell cycle. This tight regulation of origin firing is enforced by a complex interplay of numerous replicative factors at the origins. The events, leading to replication fork initiation, controlled by these replicative factors can be grouped into two discrete steps: origin licensing and origin unwinding or replication initiation (Arias and Walter 2007). During origin licensing, which occurs in the G1 phase, prereplicative complexes (pre-RCs) sequentially assemble at origins, starting with origin recognition complex (ORC), Cdc6, Cdt1 and the MCM2-7 complex in that order. The primary function of ORC, Cdc6 and Cdt1 is to deliver the replicative DNA helicase, the MCM2-7 complex, to origins of replication (Figure 18). In the second step, which occurs at the G1/S transition, the MCM2-7 complex is activated to initiate DNA unwinding at the licensed origins. Licensed origins do not initiate DNA synthesis until signals are transduced to them by the S-phase promoting kinases, CDKs (cyclin-dependent kinases) and DDK (Dbf4 and Drf1-dependent kinase). The cooperative actions of these kinases cumulatively lead to the loading of MCM10, Cdc45 and GINS (MCM/Cdc45 associate proteins) onto the MCM2-7 complex, which triggers origin firing and DNA unwinding in S phase. Upon origin unwinding, replication protein A (RPA) binds single-stranded DNA; DNA polymerase α/primase is recruited to synthesize a RNA primer, which is recognized by replication factor C (RFC) which in turn recruits the DNA polymerase δ processivity factor, PCNA, to initiate replication elongation.

The cell has evolved several regulatory mechanisms to prevent origin 'relicensing' during the same cell cycle. At the G1/S transition when loaded MCM2-7 complex is ready to be activated, the CDKs not only phosphorylate proteins required for origin firing but also phosphorylate ORC, Cdc6 and Cdt1, which results in their

inactivation and/or dissociation from the origin, nuclear export, and proteosomal degradation (Figure 18). In addition, DNA replication inhibitor, geminin binds to Cdt 1 and targets it for proteosomal degradation during S, G2 and M phases. Geminin is inactivated by anaphase promoting complex (APC) during early G1 where Cdt1 functions to load the MCM2-7 complex onto origins (Arias and Walter 2007). Therefore these multiple levels of regulation ensure that origins fire once and only once per cell cycle thereby safeguarding the integrity of the genome.

Some of the proposed mechanisms accounting for oncogene-induced DNA damage, like generation of ROS by Myc, telomere erosion/attrition and induction of aberrant DNA replication have been tested by others and us. Results from our lab indicate that Myc does increase ROS levels in primary human fibroblasts, which are grown in relatively high oxygen conditions (Powers, Hong et al. 2004). However, Myc failed to induce oxidative DNA damage in K5Myc transgenic tissue (unpublished data). Our results were later confirmed by other findings, which showed that Myc can induce DNA breaks independent of ROS production (Ray, Atkuri et al. 2006). The occurrence of gross chromosomal abnormalities (Figure 8 and Table 2) in keratinocytes from 2 day- old pups precludes the possibility that telomere erosion or attrition contributes to Myc- induced DNA damage as it is highly unlikely that the relatively long telomeres of mice would be eroded at such an early stage in the life-cycle of an organism. Furthermore, protecting human telomeres from eroding by expressing telomerase did not suppress oncogene-induced DNA damage (Di Micco, Fumagalli et al. 2006). However, our preliminary studies do indicate a role for DNA replication in Myc and E2F3a- induced DNA damage.

Based on our findings and from others, we hypothesize that Myc and E2F3a induce chromosomal instability by deregulating CDK activity, upregulating replication licensing factors and stimulating illegitimate origin firing.

5.4 RESULTS

5.4.1 Inhibition of Replication by Aphidicolin and Geminin Suppresses Myc and E2F3a- induced DNA Damage

Testing for DNA damage under conditions that inhibit DNA replication will give insights into the contribution made by oncogene- induced replication to DNA damage. To test whether DNA synthesis was required for the DNA damage caused by Myc and E2F3a, aphidicolin, a DNA polymerase inhibitor, and geminin, a negative regulator of Cdt1, were employed. While aphidicolin was directly added to cells, geminin is overexpressed in cells through adenoviral infection. Neither aphidicolin nor geminin affected the expression of Myc or E2F3a (Figure 19). However, both these inhibitors dramatically reduced the DNA damage induced by AdMyc and AdE2F3a infections as measured by the comet assay (Figure 20, 21, and 22). Our lab has previously shown that another E2F family member, E2F1, which like Myc and E2F3a activates ATM, does not induce DNA damage in cells infected with AdE2F1 (Powers, Hong et al. 2004; Hong, Paulson et al. 2008). To further confirm our results, NHFs infected separately with adenoviruses expressing green fluorescent protein (GFP), E2F1, E2F3a and Myc alone and also co-infected with adenovirus expressing geminin were subjected to the comet assay 48 hours post-infection. As expected, E2F1 did not induce DNA damage over and above the basal level of DNA damage seen in GFP expressing cells and geminin did not have any affect on these levels. However, Myc- and E2F3a- induced DNA damage was substantially reduced by geminin (Figure 22). Moreover, aphidicolin and geminin also inhibited E2F3a- induced phosphorylation of H2AX, a marker for DNA DSBs, in NHFs infected with AdE2F3a (Figure 23).

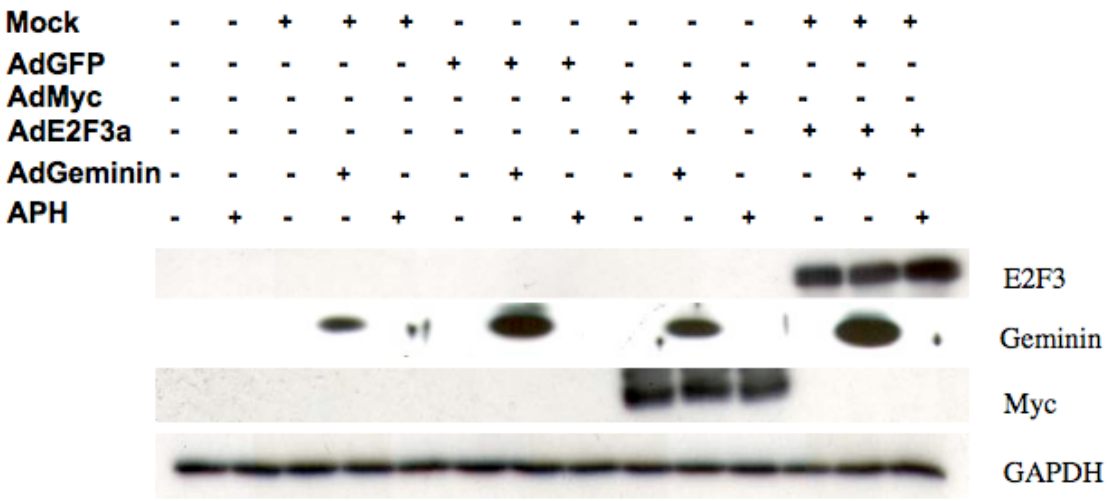


Figure 19: Replication inhibitors, aphidicolin and geminin, do not affect the transgenic expression of either Myc or E2F3a.

Western blot analysis of NHFs either mock infected or infected with adenoviruses expressing either GFP or Myc or E2F3a were either co-infected with AdGeminin or treated with aphidicolin (5μg/ml) at the time of infection. Antibodies specific for E2F3, Geminin, Myc and GAPDH were used as indicated.

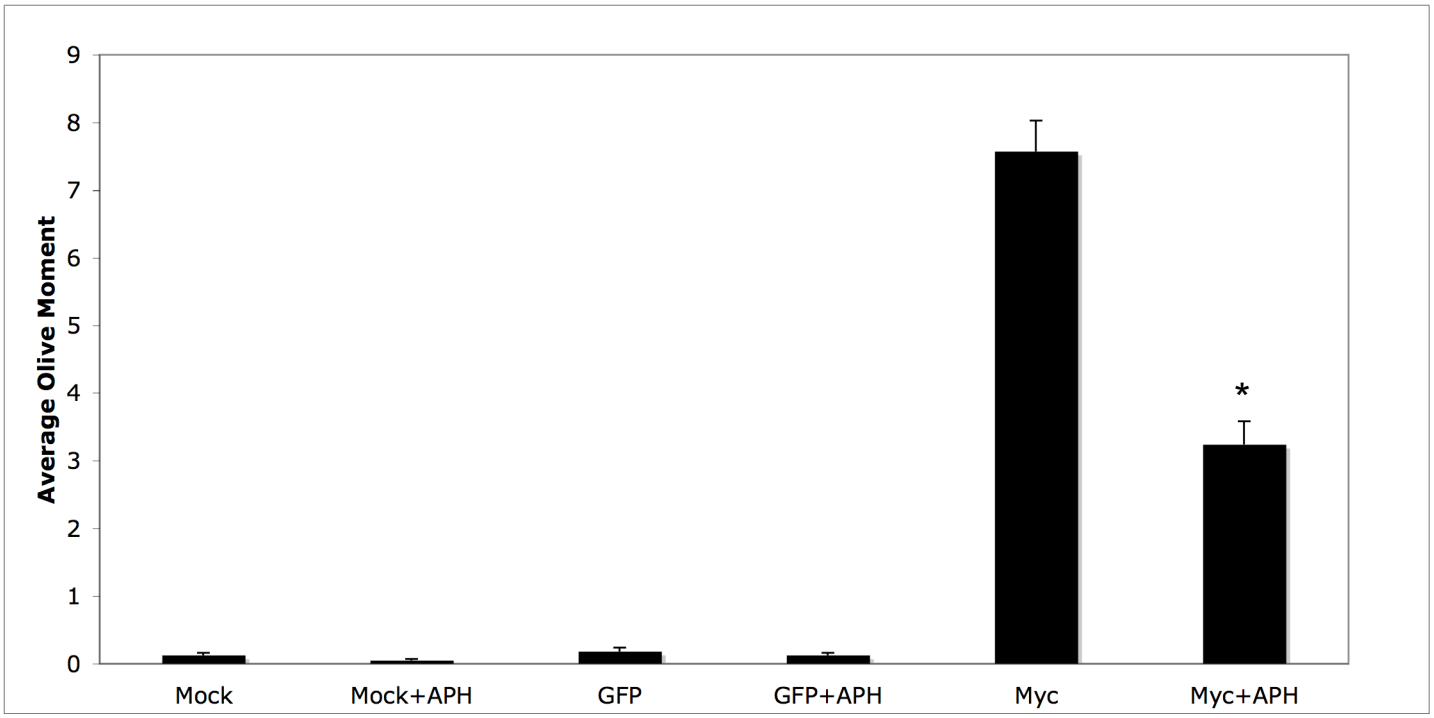


Figure 20: Aphidicolin suppresses Myc-induced DNA damage.

NHFs infected with AdGFP control virus or AdMyc were untreated or exposed to aphidicolin (5 g/ml) at the time of infection. 48 h post-infection, cells were subjected to the comet assay and the average Olive tail moment was calculated as previously described. *P < 0.001 as compared by two-sample independent t test.

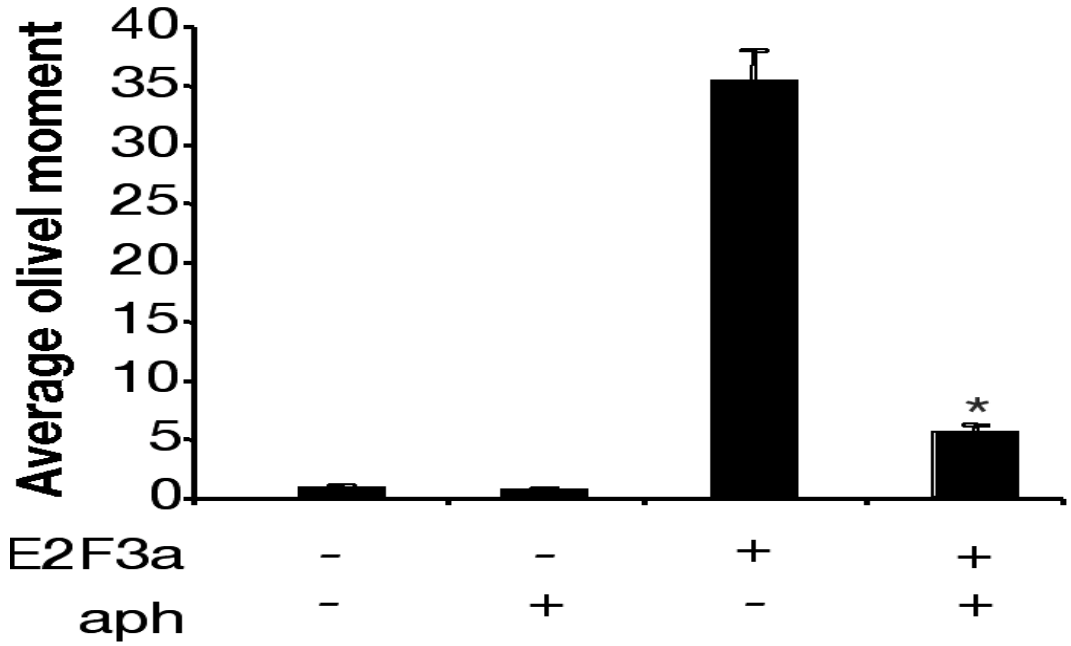


Figure 21: Aphidicolin suppresses E2F3a-induced DNA damage.

NHFs either mock infected or infected with AdE2F3a were untreated or exposed to aphidicolin (5 g/ml) at the time of infection. 24 h post-infection, cells were subjected to the comet assay and the average Olive tail moment was calculated as previously described. *P < 0.01 as compared by two-sample independent t test.

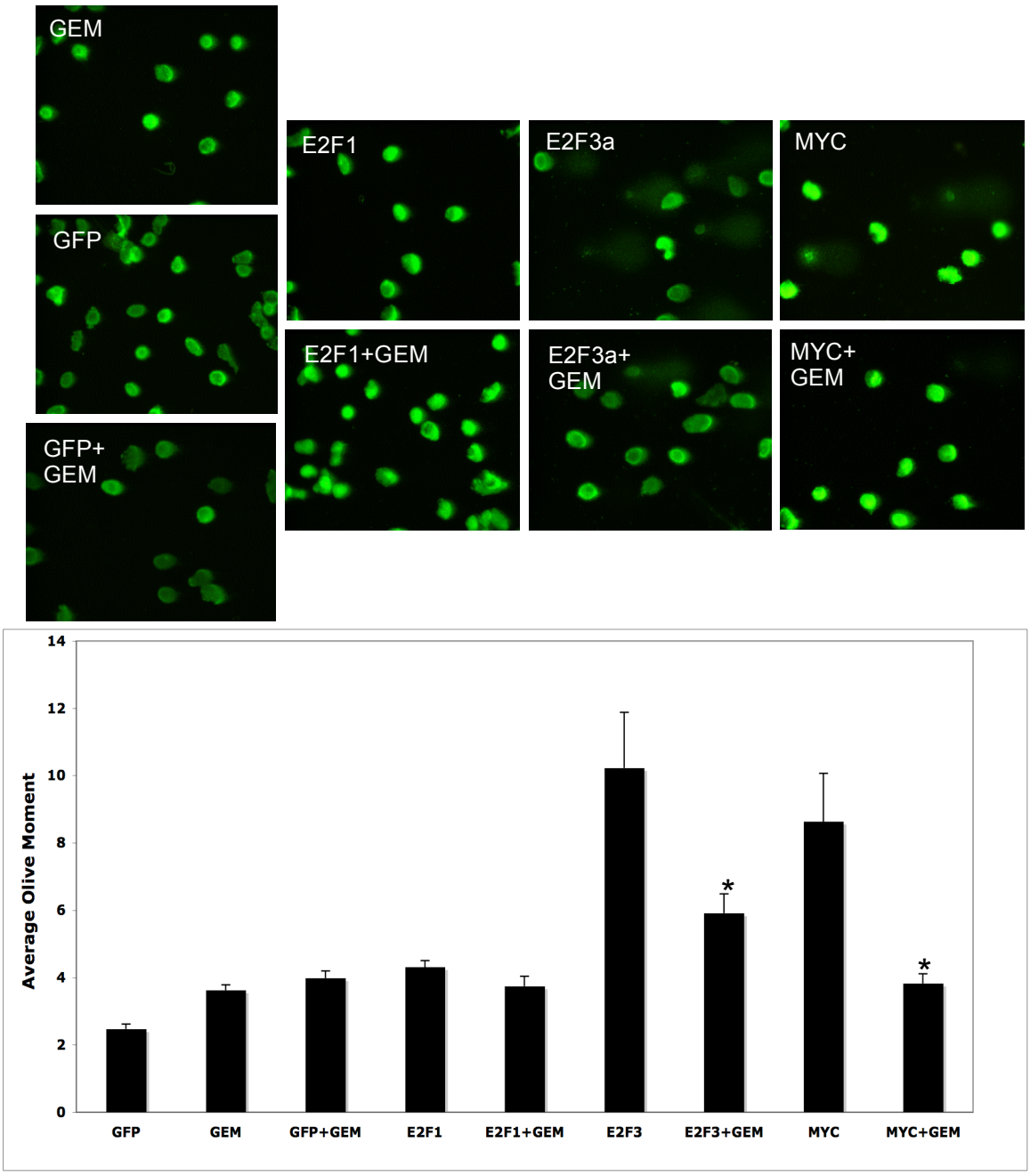


Figure 22: Aberrant DNA replication caused by Myc and E2F3a but not E2F1 is implicated in Myc and E2F3a- induced DNA damage.

NHFs were infected with AdGFP or AdMyc or AdE2F3a or AdE2F1 alone or were co-infected with AdGeminin. 48 h post-infection, cells were subjected to the comet assay and the average Olive tail moment was calculated as previously described. *P < 0.01 (E2F3a+Gem); *P< 0.001 (Myc+Gem) as compared with AdE2F3a and AdMyc infected samples respectively by two-sample independent t test.

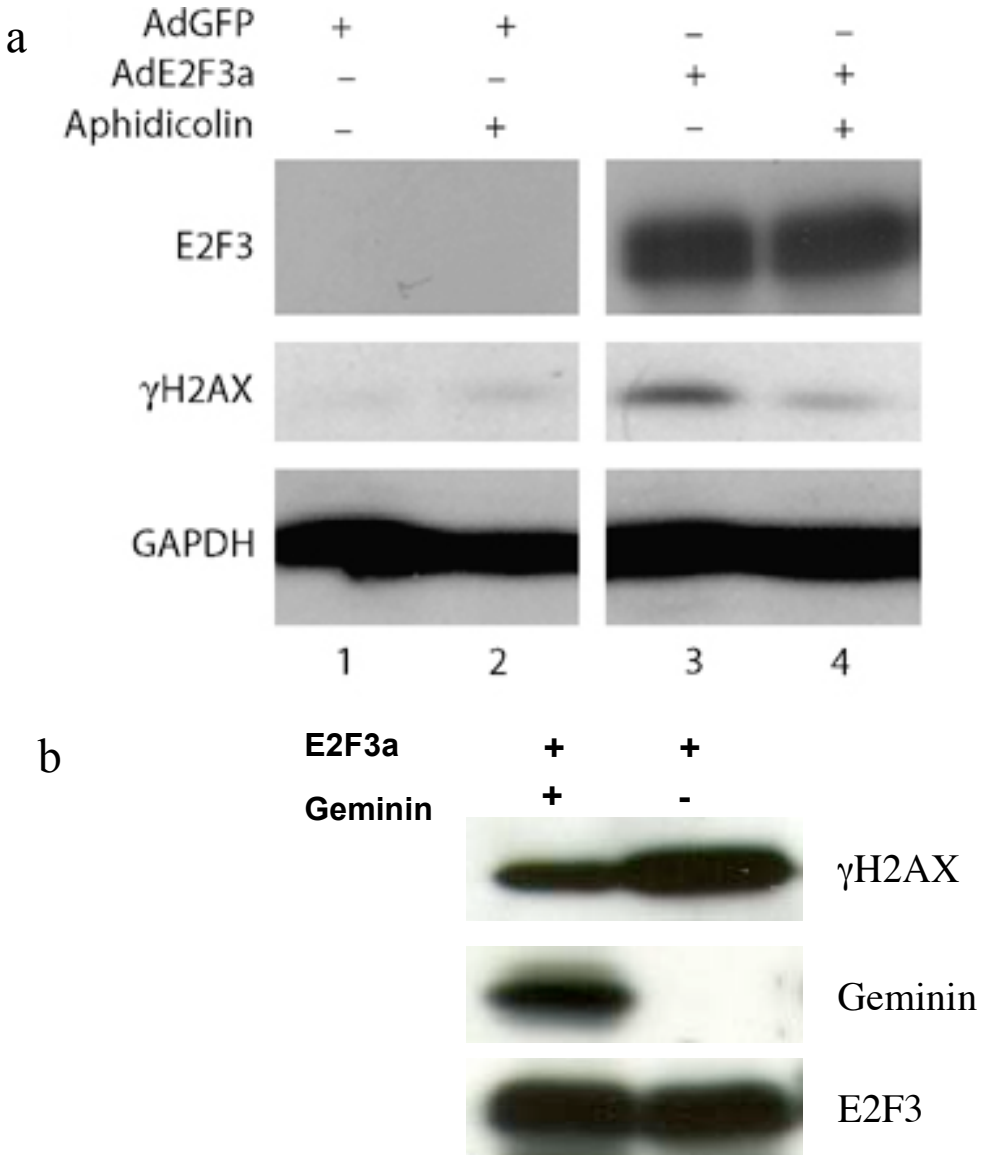


Figure 23: Inhibition of DNA replication by aphidicolin and geminin suppresses E2F3a- induced γH2AX phosphorylation.

a) Western blot analysis of NHFs infected with AdGFP (green fluorescent protein) as a control (lanes 1 and 2) or AdE2F3a (lanes 3 and 4) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of aphidicolin (5 μg/ml). Antibodies specific for E2F3, γH2AX, and glyceraldehyde 3 phosphate dehydrogenase (GAPDH) were used as indicated. Images for each primary antibody are from the same blot and exposure with irrelevant, internal lanes removed. **b)** Western blot analysis of NHFs infected with AdE2F3a alone or co-infected with AdGeminin. Antibodies specific for E2F3, γH2AX and geminin were used as indicated.

Taken together, these results strongly suggest that at least some DNA replication is involved in the generation of DNA damage by Myc and E2F3a deregulation.

5.4.2 Cyclin-dependent kinase 4 (Cdk4) Does Not Contribute to Myc- Induced DNA Damage

Upregulation of critical cell cycle regulators like cyclin E and CDK4 is one of the ways by which Myc promotes DNA replication. Deregulated expression of these crucial cell cycle regulators is seen in several cancers (He, Allen et al. 1994; Ekholm-Reed, Mendez et al. 2004; Ekholm-Reed, Spruck et al. 2004). CDK4 is a known Myc target gene and a strong correlation exists between Myc deregulation and the aberrant expression of CDK4 (Hermeking, Rago et al. 2000). Previous studies from our lab have shown that Cdk4 is required for the proliferative and oncogenic activities of Myc in K5Myc transgenic mice. Cdk4, by sequestering p21 and p27 CDK inhibitors, indirectly increases the Cdk2 kinase activity, which is crucial for cells to transit from G1 to S phase (Miliani de Marval, Macias et al. 2004). A role for Cdk2 has been proposed in Myc-induced rereplication in Rat1A cells arrested at G2/M phase (Li and Dang 1999). To ascertain whether Cdk4 contributes to Myc- induced aberrant replication and DNA damage, skin from wild-type, Cdk4 null, K5Myc transgenic and K5Myc transgenic Cdk4 null mice was stained for γH2AX (Figure 24). As expected, wild-type and Cdk4 null epidermis stained negative and K5Myc transgenic tissue stained positive for γH2AX foci. However, the absence of Cdk4 did not prevent the formation of γH2AX foci in K5Myc Cdk4 null transgenic tissue. This suggests that Cdk4 does not contribute to Myc- induced DNA damage or conversely, Myc does not mediate induction of DNA damage through Cdk4-dependent DNA replication. Therefore, DNA replication activities downstream of

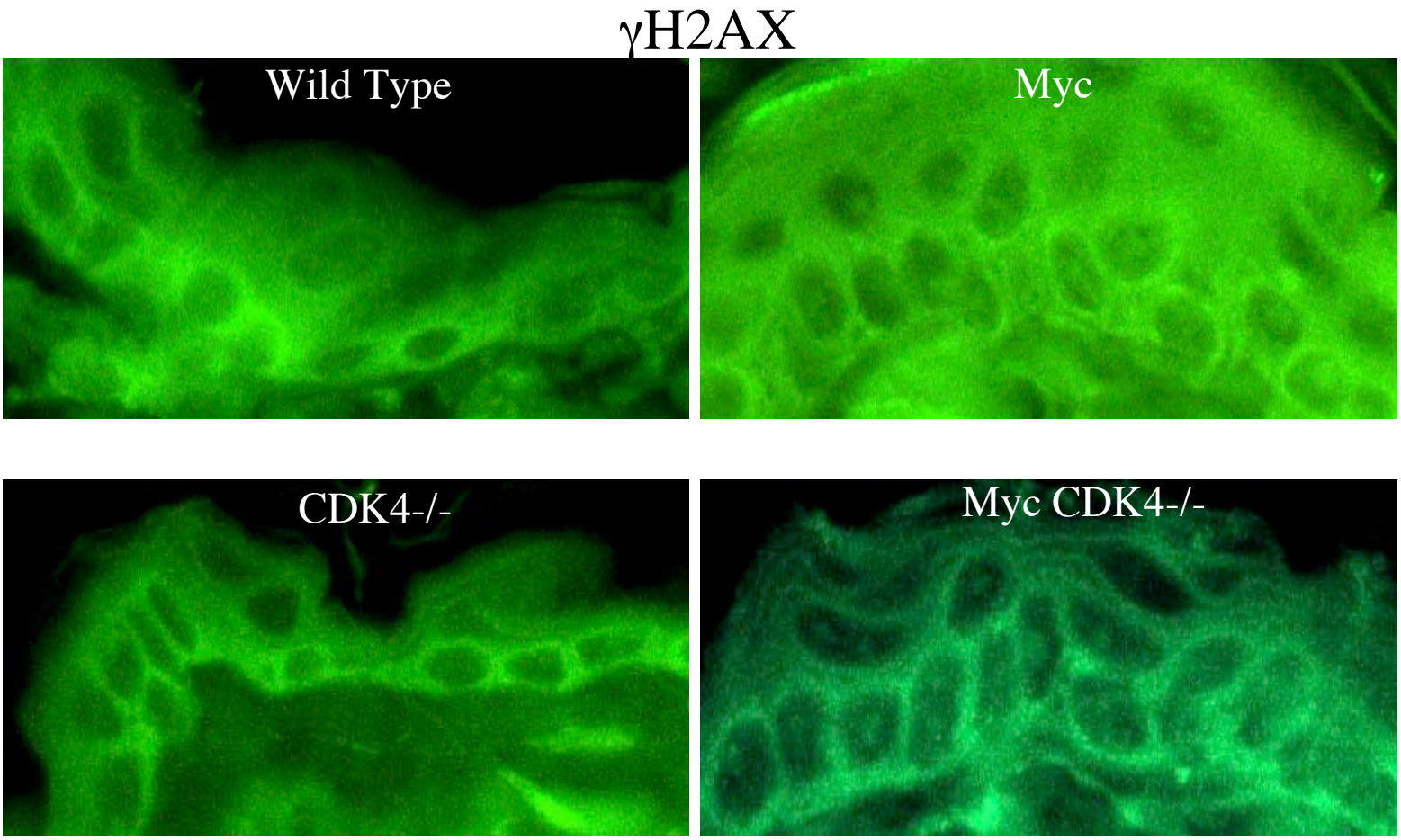


Figure 24: Loss of Cdk 4 does not suppress Myc- induced γH2AX phosphorylation.

Immunofluorescent staining was performed on skin sections from wild-type mice, *Cdk4*-/- mice, K5Myc mice, and K5Myc *Cdk4*$^{-/-}$ mice by using antibodies specific for γH2AX.

Cdk4, which are mediated by Myc, need to be probed for their potential role in promoting DNA damage.

5.4.3 Myc and E2F3a Upregulate Cdt1, a Crucial Component of the Pre-Replicative Complex (pre-RC)

Previously it was reported that Myc-dependent origin refiring specifically at the *DHFR* locus caused *DHFR* gene amplification in cell lines as well as in a mouse model (Mai, Hanley-Hyde et al. 1996; Taylor and Mai 1998). Preliminary results from my lab show that geminin, the negative regulator of Cdt1 and a potent inhibitor of origin firing (Vaziri, Saxena et al. 2003; Saxena and Dutta 2005) suppresses Myc and E2F3a- induced DNA damage (Figures 22 and 23b). Our results therefore suggest a role for Cdt1 in Myc and E2F3a- induced DNA damage. Based on these findings, we hypothesize that rereplication at origins might contribute to oncogene-induced DNA damage. It has been shown that the deregulation of Cdt1 by itself can cause origins to fire inappropriately and activate the ATM-Chk2-p53 pathway. Recently, some elegant experiments by Davidson et al., in cell free Xenopus egg extracts showed that overexpression of Cdt1 causes uncontrolled rereplication in G2 phase leading to the generation of small fragments of double-stranded DNA and a strong checkpoint activation (Davidson, Li et al. 2006). In fact Cdt1 overexpression is observed in several human cancers including non-small-cell lung cancers and colon cancers (Karakaidos, Taraviras et al. 2004; Xouri, Lygerou et al. 2004; Bravou, Nishitani et al. 2005).

To determine whether Myc and E2F3a upregulate Cdt1 levels, cell lysates from NHFs infected with AdGFP, AdMyc and AdE2F3a were blotted for Cdt1 protein levels

(Figure 25). Both Myc and E2F3a upregulated Cdt1 levels compared to the GFP control. Putting our results together (Figures 22, 23 and 25), it can be hypothesized that Myc and E2F3a upregulation of replication licensing factors like Cdt1 stimulates illegitimate origin firing leading to the induction of DNA damage and consequent activation of the ATM DDR pathway.

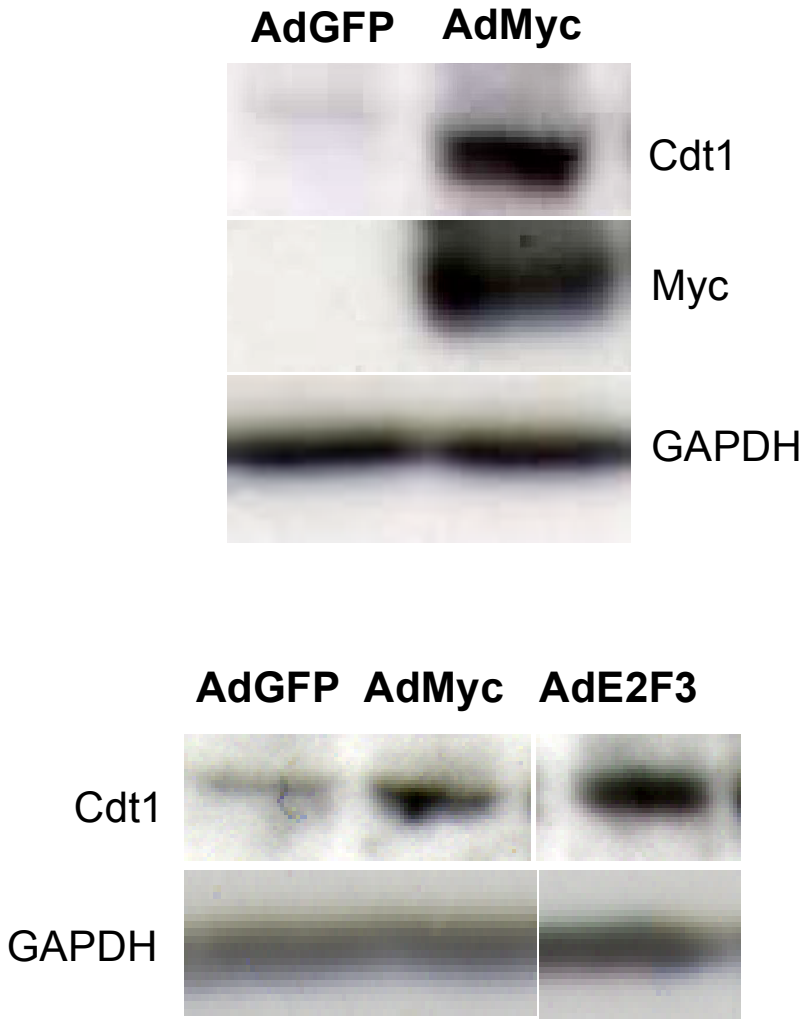


Figure 25: Myc and E2F3a upregulate Cdt1, a critical component of the pre-replicative complex.

Western blot analysis of NHFs infected with AdGFP as a control or AdE2F3a or Myc. Antibodies specific for Myc, Cdt1 and GAPDH were used as indicated. Images for each primary antibody are from the same blot and exposure with irrelevant, internal lanes removed.

5.5 DISCUSSION

Results presented here show evidence that Myc and E2F3a- induced aberrant DNA replication, probably due to illegitimate firing of origins, contributes to the induction of DNA damage. Others have also implicated aberrant DNA replication mediated by inappropriate origin firing in the generation of Ras- induced DNA damage and subsequent ATM DDR pathway activation (Di Micco, Fumagalli et al. 2006). Therefore, there is a common underlying mechanism that contributes to DNA damage induced by oncogenes that activate the ATM DDR pathway.

The coordinated and sequential functions of cyclin-dependent kinases, CDK4/6, and CDK2 are required for proper entry of cells into S phase. Previously our lab has shown that Myc upregulates Cdk4 protein levels, which subsequently mediates Myc-induced hyperproliferation and tumor formation in K5Myc transgenic mice (Miliani de Marval, Macias et al. 2004). However, our studies indicate that Cdk4 may be dispensable for Myc-induced DNA damage in K5Myc transgenic tissue (Figure 24). In the context of our results that implicate aberrant DNA replication induced by Myc as a causative factor for DNA damage, it is intriguing that Cdk4, which is required for Myc- induced proliferation, is dispensable for Myc to induce DNA damage. Probably redundancy in Cdk functions would have led to Cdk4 functions being taken over by Cdk6. One other possibility is that cyclin D, which normally interacts with Cdk4/6 might be interacting with Cdk2 when Cdk4 is absent. Evidence for such interactions were reported by Malumbres et al., in the tissues of *Cdk4*$^{-/-}$ *Cdk6*$^{-/-}$ mice, where the D- cyclins showed enhanced interaction with Cdk2, which could account for the relatively normal proliferation rates seen in *Cdk4*$^{-/-}$ *Cdk6*$^{-/-}$ fibroblasts in culture (Malumbres, Sotillo et al.

2004). Cdk4/6 loss of function did not prevent phosphorylation of pRb by Cdk2, which led to E2F target induction and almost normal proliferation of fibroblasts (Kozar, Ciemerych et al. 2004). To lend support to our data, unpublished work from Bruno Amati's group demonstrates that the absence of Cdk4 does not suppress Myc- induced DNA damage whereas the absence of Cdk2 suppresses ATM DDR pathway activation and DNA damage induction in mouse embryo fibroblasts. Therefore it is highly likely that in K5Myc transgenic tissue, DNA damage could be mediated in a Cdk4- independent manner through Cdk6 redundant functions, and/or through interactions of D-cyclins with Cdk2 or through some unknown mechanisms. Such reorientation of Cdk6 and Cdk2 functions in the absence of Cdk4 may not be sufficient to induce hyperreplication in the Myc transgenic tissue but might be sufficient to phosphorylate and activate origin-licensing factors that are implicated in inappropriate origin firing leading to DNA damage.

The evidence presented here for implicating illegitimate firing of origins as a mechanism for oncogene- induced DNA damage is only preliminary in nature and is by no means conclusive. Experiments to confirm these preliminary results are underway in our lab. In addition our experiments are directed at understanding whether Myc preferentially targets certain origins for re-firing like origins at or near fragile sites, or whether it is more random. Answers to such questions will help in understanding the nature of oncogene- induced genomic instability, which might have future therapeutic implications.

Chapter VI: Conclusions, perspectives and future directions

6.1 CONCLUSIONS

We have shown through different routes that the oncogenes c-Myc and E2F3a induce DNA damage *in vivo*. We have shown in each case by employing the comet assay, which directly measures DNA damage, that keratinocytes from Myc and E2F3a overexpressing mice harbor substantial DNA damage (Figure 7 and Paulson, Pusapati et al. 2008). We have confirmed our results by staining the transgenic tissue with markers of DNA damage, like phospho-ATM^{ser1981}, γH2AX and phospho-SMC1^{ser957} (Figure 9 and Paulson, Pusapati et al. 2008). With each of these markers we have observed clear focus formation, which unequivocally indicates the occurrence of DNA damage in oncogene-deregulated transgenic tissue. To directly visualize DNA damage at the level of chromosomes, metaphase spreads from wild type and Myc transgenic keratinocytes were subjected to cytogenetic analysis. Although chromosomes from transgenic keratinocytes exhibited various kinds of abnormalities, the most striking finding is a sevenfold increase in the occurrence of chromosomal fusions in transgenic keratinocytes over the basal level.

Chromosomal fusions are hallmarks of translocations, which can potentially activate more oncogenes or disrupt tumor suppressors. It is striking that the gross chromosomal abnormalities are observed in transgenic keratinocytes from as early as 2 day- old mice. The average time to tumor initiation in Myc transgenic mice is about 40 weeks. Putting together our data and the genetic progression and the waiting time to cancer model proposed by Beerenwinkel, Antal *et al*., it can be hypothesized that genetic

instability seen at the very early stage in K5Myc transgenic tissue facilitates the acquisition of critical mutations in cancer genes that finally drive tumor progression. Based on this hypothesis, acceleration in the acquisition of these critical mutations should ideally decrease tumor latency. Our data supports this argument. Average tumor latency is reduced by almost half, to 20 weeks, in K5Myc transgenic mice that lack *Atm* (Figure 14). Therefore it can be hypothesized that ATM, by inducing apoptosis in response to oncogenic stress by Myc, is eliminating cells harboring genomic instability, which otherwise would have acquired critical mutations that drive cancer. K5Myc mice that eventually develop tumors (approximately 40%) may ultimately override these tumor suppressive mechanisms imposed by ATM and others, and acquire driver mutations that propel them towards cancer. By the same reasoning, it can be said that the majority of K5Myc mice that do not develop tumors by one year of age (about 60%), may not be acquiring critical driver mutations that are necessary for tumor development. However, the loss of ATM may accelerate the acquisition of driver mutations, ultimately leading to tumor development in 100% of K5Myc transgenic mice null for *Atm* by one year of age (Figure 14).

We have also shown that DNA damage induction is not restricted solely to cellular oncogenes like Myc and E2F3a. SV40 T antigen and HPV E7 oncogenes induced γH2AX and phospho-SMC1 foci formation in the respective transgenic tissues. Although we have not further confirmed DNA damage induction by these oncogenes by different methods like comet assay and cytogenetics, the very presence of nuclear foci is now routinely considered as a valid and strong indicator of the occurrence of DNA damage. Our results suggest that the ability to induce DNA damage may be a common feature of oncogenes. Deregulation of the cell cycle is also a common feature to the process of

oncogenesis mediated by these four oncogenes. Whether other oncogenes that mediate their oncogenic potential independent of cell cycle deregulation induce DNA damage (for example, the anti-apoptotic factor, Bcl-2) is an interesting question. Findings from our lab show that the deregulation of cell cycle by overexpressed E2F1 does not induce DNA damage at least *in vitro*. Therefore all oncogenes that deregulate the cell cycle may not necessarily induce DNA damage.

Our preliminary results strongly implicate aberrant DNA replication induced by Myc and E2F3a in the generation of DNA DSBs. We hypothesized that aberrant DNA replication induced by oncogenes leads to DNA damage. Employing both chemical (aphidicolin) and physiologically relevant protein inhibitors (geminin) of replication, we have observed a reduction in Myc and E2F3a- induced DNA damage directly by the comet assay as well as a reduction in the levels of activated markers of DNA damage, like γH2AX (Figures 20, 21 and 23). We further validate both our assays and results by showing that under the same conditions overexpressed E2F1 does not induce DNA damage, a result that has been repeatedly shown in our lab (Figure 22). To tease out the mechanism(s) underlying oncogene-induced DNA damage, we initially investigated aspects of the cell-cycle that are deregulated by Myc. However we found that the cyclin-dependent kinase Cdk4, which is required for Myc's hyperproliferative and oncogenic capabilities in K5Myc transgenic tissue, does not mediate Myc- induced DNA damage in K5Myc transgenic epidermis (Figure 24). Based on others findings we predict that redundancy in the functions of cyclin dependent kinases, Cdk4, Cdk6 and Cdk2, contributes to Myc-induced DNA damage in the absence of Cdk4, which we intend to test in the future.

To test whether other events in addition to deregulated CDK activity contribute to oncogene-induced DNA damage, we probed for Myc- and E2F3a-mediated upregulation of Cdt1, a critical component of the pre-replicative complex (pre-RC) that has been previously implicated in rereplication and is deregulated in several human cancers. We found that both overexpressed Myc and E2F3a upregulate Cdt1 levels in NHFs (Figure 25). Based on our results, we hypothesize that Myc- and E2F3a- mediated upregulation of replication licensing factors like Cdt1 stimulates illegitimate origin firing leading to the induction of DNA damage and consequent activation of the ATM DDR pathway. Our findings are summarized in a model in figure 26.

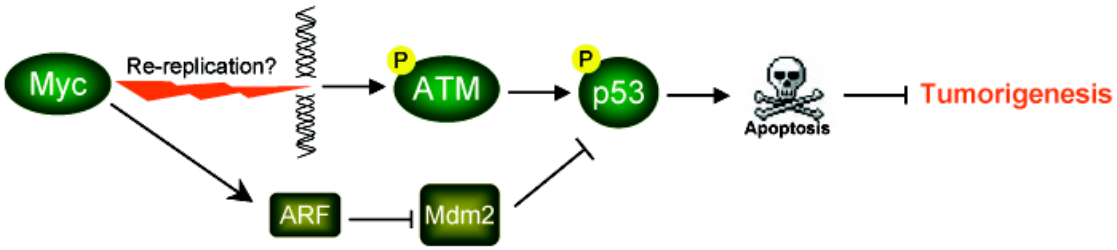


Figure 26: Evolving model for Myc- induced DNA damage and the activation of the ATM DDR pathway.

Myc, probably by causing re-replication, induces DNA damage that is recognized by the ATM DDR pathway to induce p53-dependent apoptosis. Myc also signals to p53 through the ARF-Mdm2-p53 axis to induce apoptosis and suppress tumorigenesis. The integrity of both the pathways is essential for the induction of apoptosis that is relevant for tumor suppression.

6.2 FUTURE DIRECTIONS

Evidence for deregulated Cdt1-dependent rereplication in the generation of strand breaks has been recently reported (Davidson, Li et al. 2006). Based on their results, the authors proposed a model to explain how Cdt1-induced rereplication may potentially induce DSBs, which we also favor. Briefly, Cdt1-dependent illegitimate origin firing or a second initiation of replication within the same round of cell cycle creates a second replication bubble whose forks are chasing the forks of the first replication bubble. Collisions between these replication bubbles lead to stalling of replication forks that cause the displacement of replication fork proteins from the DNA and the release of a small DNA fragment (representing newly synthesized DNA from the second replication bubble) from the chromosomal DNA. We are presently conducting experiments in our lab to test oncogene-induced DNA damage at origins of replication. Mai and coworkers have demonstrated that Myc overexpression leads to the specific refiring of the DNA replication origin located at the *DHFR* locus leading to *DHFR* gene amplification in rodent and human cells (Taylor and Mai 1998). Other findings have shown that *β-globin* and *lamin B2* genes similarly located near origins of replication are amplified by activated Ras (Di Micco, Fumagalli et al. 2006). Our experiments are addressing whether Myc-induced DNA damage occurring in or near origins close to *DHFR*, *β-globin* and *lamin B2* loci is leading to their amplification. Using ChIP, we are selecting for γH2AX bound nucleo-protein complexes in AdMyc infected cells. Analysis of γH2AX bound DNA fragments by solexa sequencing would identify whether Myc induces DNA damage at or near origins of replcation or at fragile sites or in origins preferentially located in fragile sites or in telomeric regions or randomly in not so well defined regions of DNA. Eventually our findings will hopefully throw light on the nature of oncogene-induced

DNA damage, the mechanisms underlying it and also provide insight into the genes that are likely to be more frequently amplified upon Myc deregulation. The genes thus identified in our *in vitro* assays can be compared with those found to be frequently amplified in human cancers where Myc's deregulation is observed. If a correlation exists, our results identifying the mechanism of Myc-induced DNA damage and gene amplification will be validated *in vivo*.

6.3 CLINICAL IMPLICATIONS OF ONCOGENE-INDUCED DNA DAMAGE

Our findings that Myc's oncogenicity is enhanced when ATM DDR pathway is disrupted predicts that cancer pathways in which Myc is deregulated selects for mutations in *Atm* or its downstream effectors like p53. Evidence for both exists as previously discussed above. Most of the conventional chemotherapy drugs currently employed in cancer treatment rely on the induction of DNA damage and the integrity of DDR pathways to induce apoptosis and thus eliminate cancerous cells. In view of our recent findings, it will be interesting to investigate whether the failure of chemotherapy treatments in some patients is due to compromised DDR pathways that no longer can induce drug-dependent apoptosis to eliminate cancer cells. Recently Reimann and colleagues presented evidence to this effect (Reimann, Loddenkemper et al. 2007). Previously it has been shown that ATM pathway interferes with cell death by enhancing DNA repair and cell survival in cells treated with DNA damaging drugs. Contrary to these reports, Reimann *et al* showed that the DNA damaging drug adriamycin (ADR) induced substantial apoptosis in lymphomas arising in *Eμ-myc* transgenic mice that have an intact ATM DDR pathway. However, ADR induced apoptosis was abrogated in an *Atm* null background. The loss of apoptosis in DDR compromised lymphomas also

translated to shorter relapse rates when mice harboring transplanted lymphomas were treated with radiation. Therefore the authors conclude that "the preservation of an intact DDR at diagnosis is critical for the execution of an apoptotic response and long-term outcome after DNA-damaging therapy *in vivo"* (Reimann, Loddenkemper et al. 2007).

One of our collaborators at the MD Anderson Cancer Center in Houston reported that non-cancerous tissue surrounding breast tumors in some patients stains for γH2AX marker of DNA damage. This suggests that apparently normal surrounding tissue probably harbors activated oncogenes or other oncogenic mutations, which may account for the observed DNA damage, and an intact DDR pathway. Knowledge on the integrity of the DDR pathway in tissues surrounding tumors may have diagnostic value and may potentially predict treatment outcomes with DNA damaging drugs. Therefore studies addressing such concerns need to be conducted as they have therapeutic implications.

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