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The Role Of E2F1 In The Response To Dna Double Strand Breaks

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THE ROLE OF E2F1 IN THE RESPONSE TO DNA DOUBLE STRAND BREAKS

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DEDICATION

I dedicate this dissertation to my dear husband Ruifeng, for being the greatest support in my life, and for all the happiness and suffering we have been through to reach this point.

To my daughter Claire, for bring sunshine and happiness to my life.

To my parents, for their incomparable love, and for showing me the way of being myself.

To my sister, for taking my responsibility at home to allow me the freedom to pursue my career.

To my friends, for providing me the best place to rest my mind and the most sincere encouragement.

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THE ROLE OF E2F1 IN THE RESPONSE TO DNA DOUBLE STRAND BREAKS

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The importance of E2F transcription factors in the processes of proliferation and apoptosis are well established. E2F1, but not other E2F family members, is also phosphorylated and stabilized in response to various forms of DNA damage to regulate the expression of cell cycle and pro-apoptotic genes. E2F1 also relocalizes and forms foci at sites of DNA double-strand breaks but the function of E2F1 at sites of damage is still unknown. Here I reveal that E2F1 deficiency leads to increased spontaneous DNA break and impaired recovery following exposure to ionizing radiation. In response to DNA double-strand breaks, NBS1 phosphorylation and foci formation are defective in cells lacking E2F1, but NBS1 expression levels are unaffected. Moreover, it was observed that an association between NBS1 and E2F1 is increased in response to DNA damage, suggesting that E2F1 may promote NBS1 foci formation through a direct or indirect interaction at sites of DNA breaks. E2F1 deficient cells also display impaired foci formation of RPA and Rad51, which suggests a defect in DNA end resection and formation of single-stranded DNA at DNA double-strand breaks. I also found E2F1 status affects foci formation of the histone acetyltransferase GCN5 in response to DNA double-strand breaks. E2F1 is phosphorylated at serine 31

(serine 29 in mouse) by the ATM kinase as part of the DNA damage response. To investigate the importance of this event, our lab developed an E2F1 serine 29 mutant mouse model. I find that E2F1 serine 29 mutant cells show loss of E2F1 foci formation in response to DNA double-strand breaks. Furthermore, DNA repair and NBS1 foci formation are impaired in *E2f1*$^{S29A/S29A}$ cells. Taken together, my results indicate novel roles for E2F1 in the DNA damage response, which may directly promote DNA repair and genome maintenance.

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Abbreviations

6-4 PP: Pyrimidine (6-4)pyrimidone Photoproducts

ARF: Alternative reading frame protein

ATM: Ataxia-telangiectasia mutated kinase

ATR: Ataxia- telangiectasia and Rad3 related

CAF1: Chromatin assembly factor 1

CDK: cyclin-dependent kinase

ChIP: Chromatin immunoprecipitation

CPD: Cyclobutane pyrimidine dimer

DP: E2F1 differentiation regulated transcription factor proteins

H3K16Ac: Acetylated Histone H4 at lysine 16

H3K9Ac: Acetylated Histone H3 at lysine 9

HAT: Histone Acetyltransferase

HR: Homologous recombination

HU: hydroxyurea

IRIF: IR-induced foci

K5: keratin 5

MAF: Primary mouse adult fibroblasts

MEF: Primary mouse embryo fibroblasts

MRN: Mre11-Rad50-NBS1

NCS: Neocarzinostatin

NER: Nucleotide excision repair

NHEJ: Non-homologous end-joining

NHF: Normal human fibroblast

RB: Retinoblastoma protein

RFC: Replication factor C

ROS: reactive oxygen species

RPA: replication protein A

siRNA: short interfering RNA

SSA: single-strand annealing

SSB: single-strand break

STAGA complex: SPT3-TAFII31-GCN5-L acetylase complex

TopBp1: Topoisomerase binding protein 1

uH2A: Ubiquitylated Histone H2A

UV: Ultraviolet

Chapter 1. Background and Introduction

1.1 E2F family and E2F1

1.1.1 E2F family

The E2F transcription factor family is composed of eight E2F members and three related differentiation regulated transcription factor proteins (DP), which regulate the expression of genes involved in different process such as cell proliferation, cell cycle progression, DNA replication, DNA repair and apoptosis (1-5). All E2F members contain a DNA binding domain that binds to the promoter of its target genes (Figure 1.1). E2F1-5 also have transactivation domains located at the carboxyl terminus. E2Fs require these two domains as essential structural components for their transcriptional regulatory function. Within the transactivation domain there is a region involved in pocket protein (RB, p107 and p130) binding, which regulates transcriptional activity and in turn cell proliferation. Each E2F member has individual as well as overlapping functions. E2F1-3 are thought to function primarily as the “activating” E2Fs. In contrast, E2F4 and 5 are reported to possess transcriptionally repressive activity (6, 7). Furthermore, E2F6, E2F7 and E2F8 are also considered as transcription repressors in an RB family-independent manner (7).

DP proteins bind to several E2F members through their dimerization domain to form functional E2F-DP heterodimers. The marked box domain which is embedded between the transactivation domain and dimerization domain is involved in multiple protein-protein interactions and is important for the apoptosis induction function of E2F1 (8) (Figure 1.2).

The role of the E2Fs in regulating the cell cycle has been well investigated (9, 10). When a cell is quiescent, E2F family members bind with pocket proteins to form complexes that repress the transcription of cell cycle and cell proliferation genes. Co-factors, such as DNMTs, HDACs, and SWI/SNF complexes, are recruited to E2F target gene promoters by pocket proteins to alter chromatin structure and inhibit access to the basal transcriptional machinery (11). Following mitogenic stimulation by growth factors, cyclin D is expressed and associates with cyclin-dependent kinase (CDK) 4/6 to form an activated complex that phosphorylates RB family proteins. E2F transcription factors are released from RB/E2F complexes when RB is hyperphosphorylated and in turn E2F transcription factors are free to activate the expression of target genes for proliferation, cell cycle progression, apoptosis induction, DNA replication and repair. Among those E2F target genes, the major cell cycle regulators cyclin E and A also facilitate cell cycle progression and G1/S phase transition by further promoting phosphorylation of RB family proteins and other key cell cycle regulators (6, 7).

1.1.2 E2F1 induces apoptosis

Among the E2F family members, E2F1 is special not only because it was the first E2F family member to be identified but also it has a unique apoptosis induction function in the presence of cell stress or when over-expressed (12-16). Although recent research has reported that E2F3 can also function in the process of apoptosis induction it may still mainly depend on the presence of E2F1 (17-19).

The mechanisms of apoptosis regulation by E2F1 are still not well understood. In general, E2F1 induces apoptosis in either a p53 dependent (15, 16, 20, 21) or independent manner (22-25). It was shown that the p53 protein is stabilized when E2F1 is over-expressed and this leads the cell to undergo apoptosis. One mechanism by which E2F1 can induce p53 is through the transcriptional regulation of the alternative reading frame protein (ARF), which is a p53 activator, by repressing p53 ubiquitination and degradation via MDM2 (26-28). Furthermore, it was also found that over-expression of E2F1 can activate the PI3 kinase family member Ataxia-telangiectasia mutated (ATM), which is a main regulator of the DNA damage response (DDR). Activation of ATM will induce phosphorylation of its many downstream targets to regulate college processes, such as DNA repair, cell cycle checkpoints, cell senescence and cell death. The p53 tumor suppressor is one of most important targets of ATM and it is phosphorylated at serine 15, which leads to further modifications, p53 stabilization and activation of p53 transcriptional capacity (29-31).

In cells lacking p53 or with mutant p53, E2F1 can still induce apoptosis through p53-independent mechanisms, especially apoptosis induced in response to DNA damage. E2F1 can transcriptionally promote expression of the p73 protein to induce apoptosis (22, 25). Recent research also showed that E2F1 uses the death receptor pathway to suppress cell survival (32). Other targets of E2F1 that can induce apoptosis include Apaf1 and Caspase 3 (23).

1.1.3 Dual role of E2F1 in cancer development

Research based on E2F1 null mice and a transgenic mouse model in which E2F1 is over-expressed in epithelial tissue via a keratin 5 (K5) promoter demonstrates that E2F1 displays paradoxical dual activities as both an oncogene and a tumor suppressor in different experimental contexts (33-36). Spontaneous tumors arise primarily in the skin epithelial tissue of K5 E2F1 mice and tumors develop earlier when on a p53-deficient background (20, 36). These results confirm an oncogenic role of E2F1 *in vivo* as suggested by earlier *in vitro* studies (37, 38). Findings based on other E2F1 transgenic models also confirm the oncogenic activity of E2F1 (39-41). Conversely, the same K5 E2F1 transgenic mouse model is resistant to skin tumor development when used in the two-stage (DMBA/TPA) carcinogenesis assay (36). In this assay, Ras mutation is the initiating event to produce skin tumors and it was found that E2F1 over-expression inhibited carcinogenesis at the promotion stage, which required functional p53 and ARF proteins (42).

Aside from the above observations, the earliest evidence for a tumor suppressive function of E2F1 were found using an *E2f1* knockout mouse model (43, 44). T cells of mice lacking E2F1 have a moderate defect in their development, but cells without E2F1 still display regular cell cycle kinetics. The normal cell cycle progression in cells lacking E2F1 is possibly related to compensation by other E2F family factors (45). Nevertheless, E2F1 null mice develop spontaneous tumors. Dysplasia and tumors such as lymphomas and reproductive tract sarcomas were found in these mice as they aged (43, 44). In addition, a study from our laboratory using a Myc transgenic mouse model

demonstrated that loss of E2F1 can accelerate the development of Myc-induced epithelial tumors (46).

While the mechanism by which E2F1 functions as a tumor suppressor is not very clear, there are several possible explanations that could help us understand its role in tumor suppression. E2F1 can be considered as a transcriptional repressor when it is bound with RB, with the RB-E2F1 complex turning off the expression of genes important for proliferation. Based on this model, loss of E2F1 would have the same effects as loss of RB. However, there is no significant evidence to support this model and it is now clear that other E2F family members have the primary function of transcriptional repression (47).

It is natural to connect apoptosis induction function of E2F1 to its tumor suppression ability. As discussed previously, E2F1 is a pro-apoptosis factor and this function could inhibit tumor development under the stress of oncogenes. It is also reported that apoptosis induced by Retinoblastoma (RB) protein inactivation requires both E2F1 and p53 in some tissues. However, in *Rb* knockout mice, tumor development was impaired or not affected by the absence E2F1 (12, 35). Based on this result, E2F1 does not seem to act as a tumor suppressor in response to RB deficiency. On the other hand, E2F1 appears to suppress tumor development when Myc is over expressed in certain tissues. It is interesting that decreased levels of apoptosis were not observed in this model but in fact increased levels of apoptosis were found in Myc transgenic tissues lacking E2F1. Taken together, these findings strongly suggest that E2F1 suppresses tumor development through a non-apoptosis mechanism.

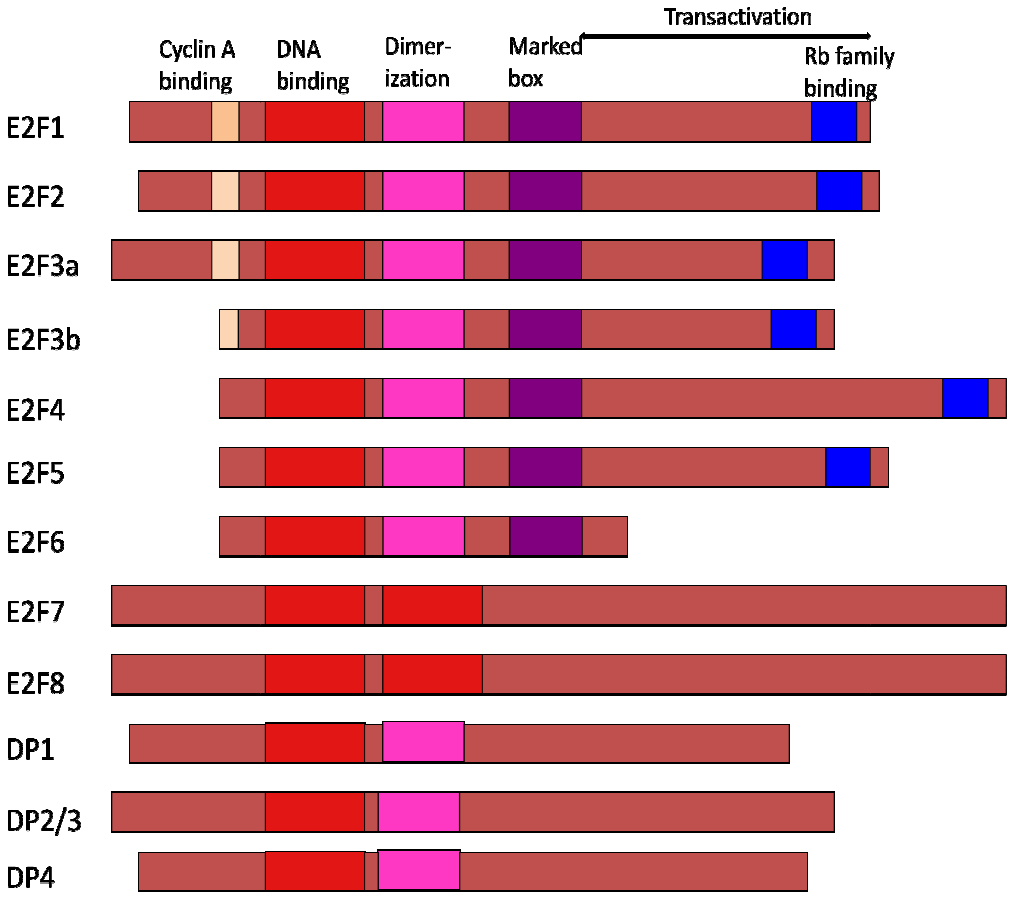


Figure 1.1 E2F family

The cyclin A-binding domain is indicated by yellow box and it only exists in E2F1-3b. The DNA-binding domain is indicated by red color box and the DP protein dimerization domain is indicated by pink box. Marked box domain, which is next to the dimerization domain, is indicated by purple color. The transactivation domain is contained in the C-terminus and the RB family protein-binding motif is indicated by blue color. DP proteins are binding partners of E2Fs, containing a related dimerization domain and DNA binding domain. E2F7 and 8 do not require dimerization with DP proteins and have two DNA binding domains. Reproduced from (DeGregori, J., and D.G. Johnson. 2006. Current molecular medicine 6: 739-748), with permission from Bentham Science Publishers Ltd.

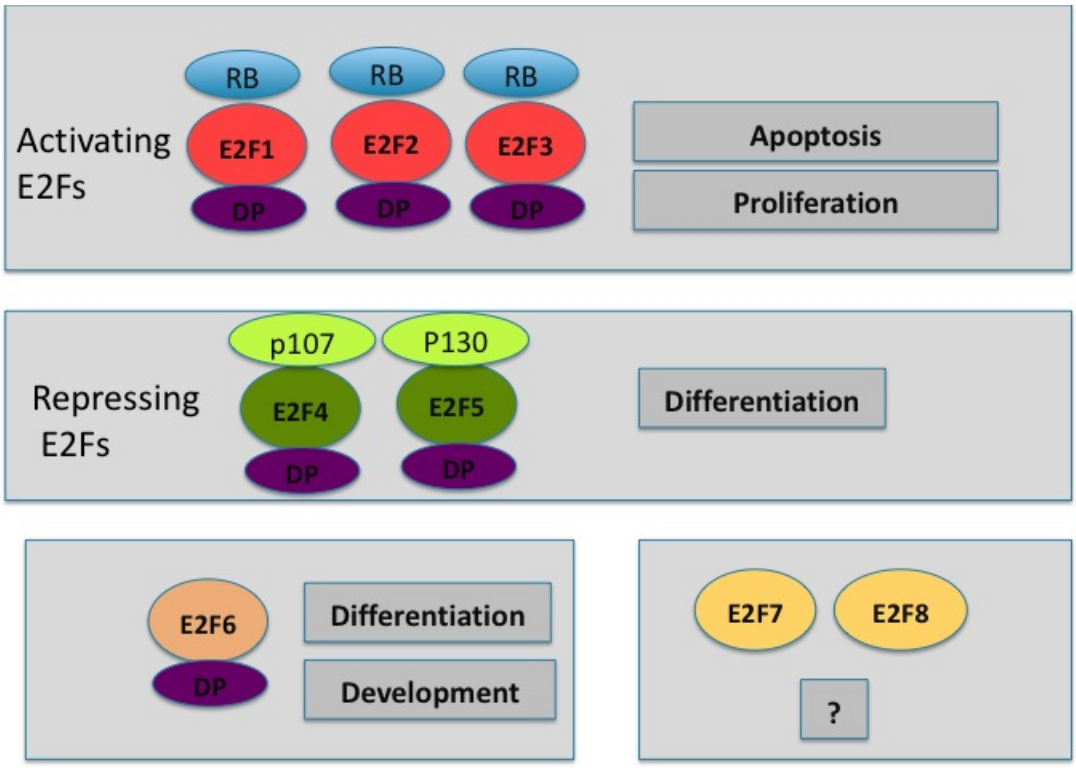


Figure 1.2 Subgroups of E2Fs and their functions.

Based on their biological activities, binding partners and co-factors, the E2F family can separated into at least four subgroups. Among them, only E2F1-3 binds to RB, while E2F4 and 5 also bind to p107 and p130. E2F1-3 mainly activate downstream target genes for proliferation and apoptosis when E2F-RB interactions are dissociated. On the other hand, E2F4-5 are thought to function primarily as transcriptional repressors and be important in the process of differentiation. E2F6 acts as a transcriptional repressor independent of the RB family and functions in organ differentiation and development. The interactions and functions of E2F7 and E2F8 have not been well explored but it is suggested that E2F7/8 also repress transcription and respond to DNA damage.

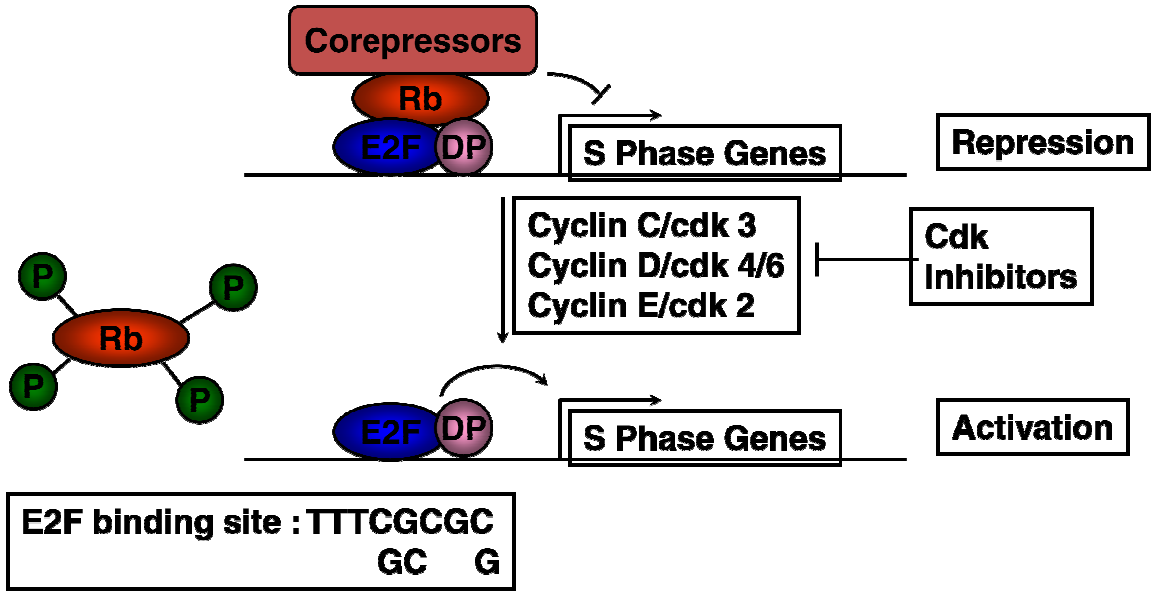


Figure 1.3 Working model for E2F1 in regulating cell cycle progression.

In quiescent cells, E2F1 is bound to and repressed by RB with recruitment of co-repressors (HDAC, etc). Follow mitotic stimulation by growth factors, elevated cyclin D levels promote phosphorylation of RB by forming active cyclinD/CDK4 or 6 complexes. Consequently, the RB-E2F1 complex is dissociated and now free E2F1 activates expression of its target genes. Those target genes encode proteins involved in DNA replication and cell cycle progression.

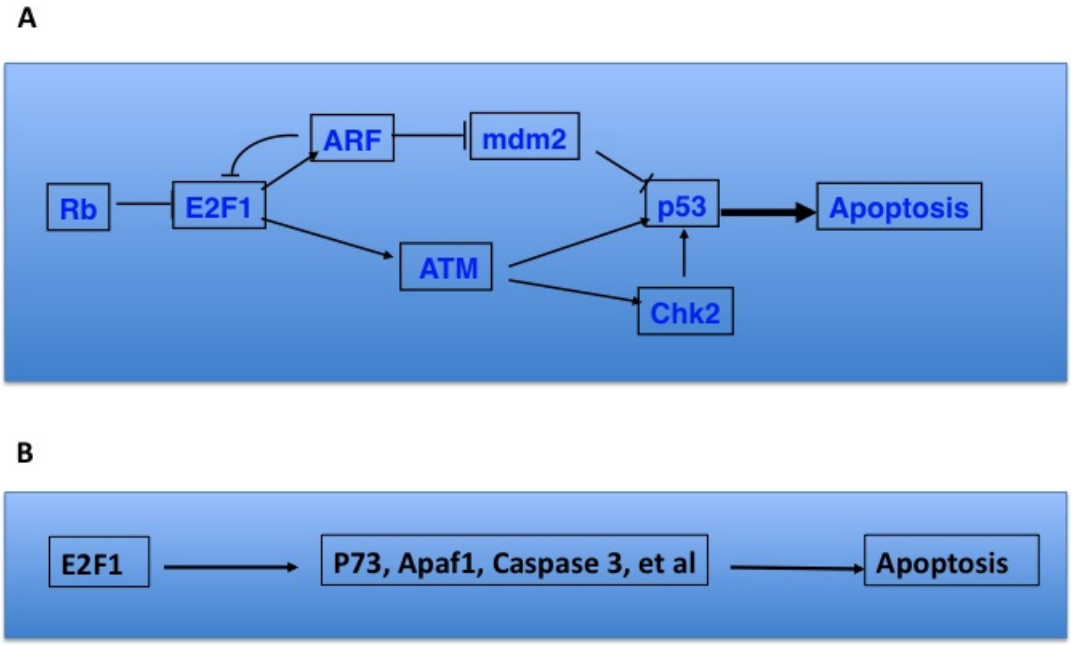


Figure 1.4 Working model for E2F1 regulation of apoptosis.

E2F1 can mediate apoptosis through both p53 dependent and independent mechanisms.

A. E2F1 signaling to p53 through ARF, a gene that is transcriptionally activated by E2F1, stabilizes p53 by repressing MDM2. E2F1 can also induce phosphorylation of p53 at serine 15 by activating the ATM kinase and in this way, trigger and activate p53.
B. For E2F1-induced, p53-independent apoptosis, E2F1 can transcriptionally induce pro-apoptotic genes including APAF1, p73 and some caspases.

1.2. Background on the DNA damage response

1.2.1 General background of DNA damage response

Each cell of the human body is confronted with around 10^4-10^5 DNA lesions per day (48). This DNA damage needs to be repaired in a timely and efficiently manner to maintain genomic stability. If not repaired, the accumulation of DNA damage will block gene transcription and DNA replication and lead to mutations that can cause cancer and other life threatening diseases.

Generally speaking, DNA is attacked by two different sources of harmful agents: endogenous agents generated during metabolism and exogenous agents from the environment. Ultraviolet (UV) light is one of the most common environmental DNA damaging agents. UV-induced DNA damage is mainly composed of cyclobutane pyrimidine dimers (CPD) and pyrimidine-pyrimidone adducts (6-4 PP), which produce stalled DNA replication forks and DNA strand distortion. Another prototypical DNA damaging agent is ionizing radiation that can generate various forms of DNA lesions, with double-strand break as the most toxic type. There are also numerous genotoxic chemicals, such as alkalyating agents, that can cause several types of DNA damage (49).

To deal with these DNA lesions, an elaborate network of DNA damage response systems is initiated. These include various DNA repair mechanisms depending on the different types of DNA damage, damage tolerance processes and cell cycle checkpoint pathways. The DNA damage response has important functions and defects in DNA damage response factors can cause severe

diseases, including neurological degeneration, premature aging, immune deficiency and cancer susceptibility (50, 51).

1.2.2 ATM/ATR mediated DNA damage response pathways

The DNA damage response is a signal transduction network that senses and responds to DNA damage to protect the cell and organism. This network consists of sensors, transducers and effectors (Figure 1.5). The key signal transducers of DNA damage response signaling are several members of the PI3 kinase family: ATM kinase and Ataxia-telangiectasia and Rad3 related (ATR) kinase (52-54). ATM is activated in response to DNA double-strand breaks and ATR is activated in response to stalled replication and transcription forks. ATM/ATR target two well studied downstream protein kinases that execute functions of the DNA damage response: Chk1 and Chk2. Chk1 and Chk2 reduce CDK activity through various mechanisms such as activation of p53 and induction of the cyclin-dependent kinase inhibitor p21 (53, 55, 56). Inactive CDK slows down or stops cell cycle progression at different cell cycle checkpoints (G1-S, intra-S and G2-M). This arrest of the cell cycle gives the cell more time for DNA repair before the cell progresses to DNA replication or mitosis. In general, ATM/ATR improves DNA repair by several mechanisms: by activating transcription factors to induce the expression of DNA repair proteins, by modifying chromatin structure to facilitate the recruitment of DNA repair factors to damage sites, and by regulating the activity of DNA repair proteins by direct phosphorylation or indirectly through ubiquitylation, acetylation or SUMOylation (57). After the above events, if there is effective DNA repair, the DNA damage response will be inactivated to allow the

cell to resume its normal function. If the damage is too severe, chronic DNA damage response signaling can trigger cell death by apoptosis or senescence, both of which can have potential antitumor functions (58, 59).

It is well known that ATM is activated in response to DNA double-strand break but the mechanism of ATM initiation is not yet very clear. Recent research has shown that ATM is recruited to broken DNA molecules by a DNA double strand break sensor, the Mre11-Rad50-Nbs1 (MRN) complex. Usually, ATM exists as inactive homodimers but when ATM is recruited to a DNA double-strand break site, it autophosphorylates itself on multiple residues and the dimers dissociate into active monomers. The MRN complex itself is one of the substrates of ATM and is also important for downstream signaling by recruiting other substrates to ATM and DNA double-strand break sites. Beside the MRN complex, there are many other mediators, such as 53BP1 and BRCA1 that are also ATM kinase substrates that play various roles in recruiting additional substrates and checkpoint signaling. At the site of DNA damage, the variant histone H2AX is phosphorylated on serine 139 by ATM/ATR and it is referred to as γH2AX (60). Mediator of DNA damage checkpoint protein 1 (MDC1) is recruited directly to γH2AX through a phospho-specific interaction and MDC1 in turn recruits many additional factors to DNA damage sites (61). The accumulation of these factors at sites of DNA damage leads to the formation of IR-induced foci (IRIF). MDC1 binds to Nijmegen breakage syndrome 1 (NBS1) through another phospho-specific interaction to recruit the MRN complex (62). Although the MRN complex can directly bind broken DNA ends, the majority of

MRN is recruited by γH2AX-MDC1, resulting in microscopically visible foci (Figure1.5). H2A ubiquitination at DNA double-strand break is also dependent on γH2AX and MDC1 and involves yet another phosphorylation-dependent interaction between MDC1 and the RNF8 E3 ubiquitin ligase. RNF8-mediated ubiquitination of H2A in turn recruits another E3 ligase, RNF168, which further amplifies the ubiquitin signal (63). RNF168 is important for the recruitment of BRCA1 through the ubiquitin-binding protein Rap80 and ABRA1 (64-66). 53BP1, an important mediator in DNA recombination and Chk2 activation, is also recruited to IRIFs in an MDC1-H2AX dependent manner. The role of IRIF in DNA damage response signal transduction and DNA damage repair is not yet well understood. The manner of recruitment of mediator proteins in the DNA damage response will be an active area of future study.

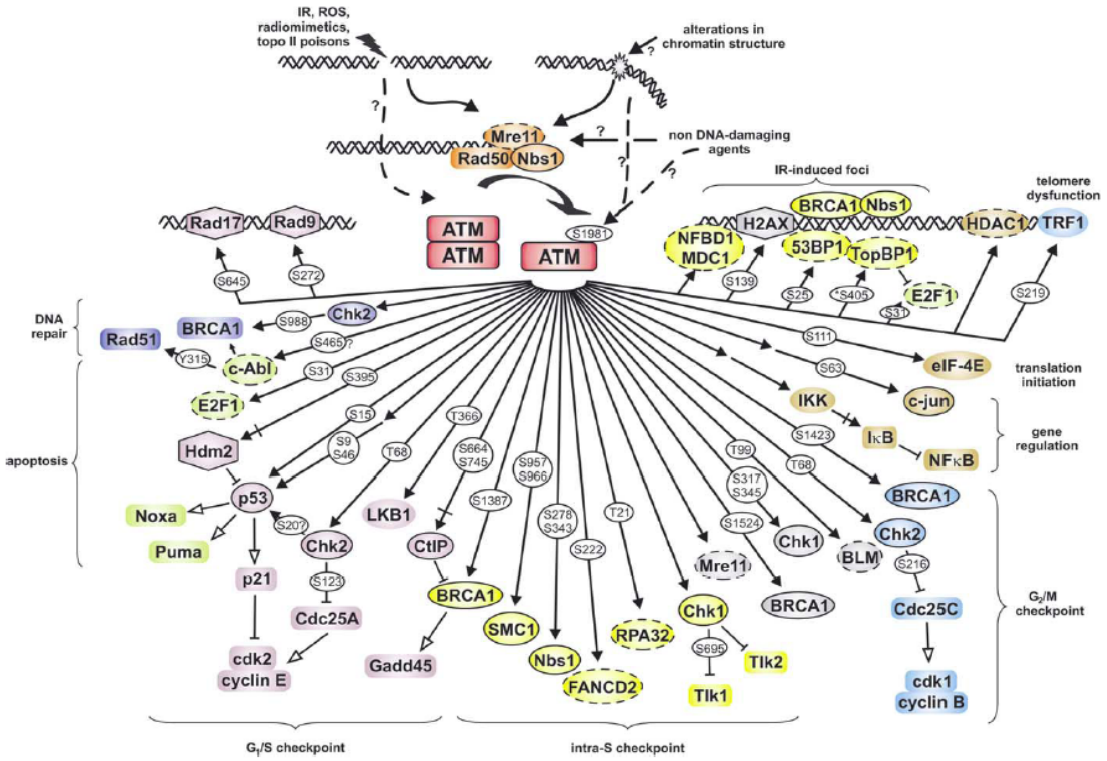


Figure 1.5 ATM signaling pathway

In response to DNA double-strand breaks, inactive ATM dimer is autophosphorylated at serine 1981, as well as other sites, and becomes an active monomer. The active ATM kinase phosphorylates hundreds of down targets to regulate a variety of important cell process, such as DNA repair, induction of checkpoints and apoptosis. A brief description can be found in the Chapter 1. Reproduced from (Kurz, E. U., and S. P. Lees-Miller. 2004. DNA repair 3:889-900), with permission from Elsevier Ltd.

1.2.3 Genomic instability and endogenous DNA damage

It has been estimated that up to 10^5 spontaneous DNA lesions per cell per day are generated by normal cellular metabolism (51). Some endogenous DNA damage arises during physiological processes such as occasional base mismatch during replication or DNA strand break due to faulty topoisomerase activity. In addition, non-enzymatic methylations and hydrolytic reactions produce thousands of DNA base lesions in the cell each day. Furthermore, oxidized DNA base and DNA break are generated by another endogenous source of DNA damage: reactive oxygen species (ROS), which is derived from normal cellular metabolism. These reactive oxygen and nitrogen compounds could also be generated by macrophages and neutrophils at sites of infections and inflammation (67). Such endogenous source chemicals could attack DNA and lead to adducts that impair DNA base pairing, block DNA replication and transcription, cause base deletion or single-strand break (SSB). DNA double-strand break can be formed when two SSBs are generated in close proximity or when the DNA replication apparatus meet a SSB or some other lesions (49). To maintain genomic integrity, DNA damage must be repaired effectively. A defect in DNA damage response factors can lead to accumulations of various type of DNA damage and lead to various diseases, such as cancer.

1.2.4 Double-strand break repair

Although DNA double-strand break do not occur as frequently as some other types of DNA damage, they are quite toxic and difficult to repair. DNA double-strand break repair is performed by two principal repair mechanisms: nonhomologous end joining (NHEJ) and homologous recombination (HR). Besides these, alternative-NHEJ and single-strand annealing (SSA) are other repair pathways.

In mammals, NHEJ is the predominant DNA double-strand break repair pathway and functions in all phases of the cell cycle. In NHEJ, the initiating step is binding of a heterodimer of Ku protein to a double-stranded DNA end. This step senses the DNA double-strand break by Ku protein which then interacts with the protein kinase DNA-PK resulting in assembly of the DNA-PK complex and activation of its kinase activity. DNA-PK activates and recruits essential repair factors such as end-processing enzymes, polymerases and DNA ligase IV to load to DNA break sites to facilitate repair. Classic NHEJ requires limited DNA end resection and almost always results in sequence deletions, so it is an error-prone repair mechanism (68).

Homologous recombination repair uses sister-chromatid sequences as the template to perform faithful repair. It only occurs in S and G2 phase and it is an error free repair mechanism. The initiating step of homologous recombination repair is strand resection and coating with the replication protein A (RPA) complex. The RAD51 protein is then recruited and loaded onto single-stranded DNA with the assistance of BRCA2 protein (69). Following this step, strand

invasion creates a D-loop and a Holiday junction. Finally, strand extension is performed through DNA synthesis using the sister chromatid as a template. Holiday junction resolution terminates the homologous recombination repair process. According to the cell cycle phase and the nature of the DNA double-strand break, different subpathways of homologous recombination repair exist (70) (Figure 1.6).

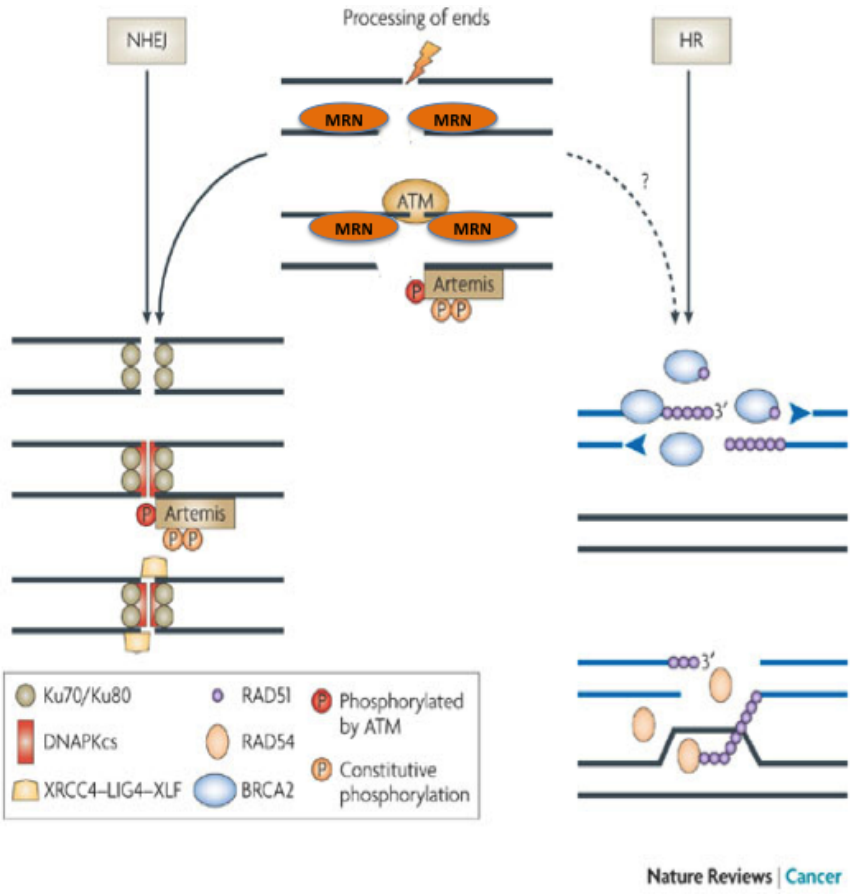


Figure 1.6 An overview of DNA double-strand break repair pathway responding to IR damage

In response to DNA double-strand breaks, Mre11-Rad50-Nbs1 (MRN) complex senses the DNA damage and recruits ATM to the site of DNA damage. The activated ATM further phosphorylates NBS1 and other substrates factors to amplify the DNA damage signaling transduction and facility DNA repair. Homologous recombination repair and NHEJ represent two important DNA double-strand break repair pathways. Brief descriptions can be found in Chapter 1. Reproduced from (Lobrich M., and P.A. Jeggo. 2007. Nature reviews 7:861-869.), with permission from Nature publishing group.

1.3 DNA damage repair and chromatin modifications

In eukaryotic cells, DNA is packaged into a compact chromatin structure that is mainly constructed with histones. The nucleosomes are formed by core histones including two H2A-H2B dimers and a H3-H4 tetramer, which are connected by the linker histone H1. The length of DNA strand wrapping around this structure is about 146 base pairs. The structure is then condensed into a higher order structure, which is a fiber around 30 nm in diameter, and then this fiber is further condensed into chromatin which is visible with conventional microscopes (71). At the same time, the maintenance of chromatin structure requires some non-histone proteins, including DNA polymerase, scaffold protein, heterochromatin protein 1 and polycomb, which can serve as chaperons or scaffolds (72).

Chromatin structure plays an important role in all biological processes involving DNA, such as mitosis, meiosis, DNA damage response, gene expression and DNA replication. Chromatin provides reliable protection and a more stable environment for DNA and the chromatin structure changes lead to delicate regulation of DNA activities. Chromatin structure functions as a barrier to processes involving DNA, such as DNA replication and transcription, and appropriate chromatin modification machinery is necessary for further pursuit of these activities.

This principle also works in the process of DNA repair. It is reported that chromatin structure inhibits DNA repair both *in vitro* and *in vivo* (73, 74). After DNA damage, the damaged site first needs to be recognized and sensed by

specific proteins, but their accessibility is prevented by the chromatin structure at the sites of damage (75, 76). To facilitate DNA repair, histone modifications and chromatin remodeling are executed at the damaged area. These processes include acetylation and methylation, as well as phosphorylation, ubiquitylation and ribosylation,

In response to DNA double-strand breaks, the earliest event of histone modification is phosphorylation of the histone variant H2AX, called γH2AX. It appears quickly soon after damage occurs and distributes out up to 50kb flaking the damaged site (77). γH2AX provides a docking site for MDC1 via BRCT domains in MDC1, which mediates many of the downstream pathways for checkpoint induction and DNA double-strand break repair. MDC1 is also be phosphorylated by Casein kinase 2 (CK2) and this event promotes the interaction of MDC1 with NBS1 protein through the FHA domain of NBS1. NBS1 is a component of the Mre11-Rad50-NBS1 (MRN) complex and functions to recruit ATM to sites of DNA double-strand break. Activated ATM phosphorylates H2AX to amplify the DNA damage response signal. These events promote transduction of the ATM-mediated DNA damage response signal to diverse downstream proteins, which are essential for DNA repair, apoptosis, checkpoint establishment, and senescence induction (78-80).

Following γH2AX formation, further chromatin modifications also occur in response to DNA double-strand breaks. MDC1 also recruits RNF8 protein by a phospho-specific binding site. RNF8 is a specific E3 ubiquitin ligase in conjunction with the UBC13 E2 conjugating enzyme to add mono- or poly-

ubiquitin to H2A and H2AX at sites of DNA double-strand break (64). Then another E3 ligase RNF168 further amplifies the ubiquitylation signal, to produce more ubiquitylated H2A (uH2A) to increase the recruitment of downstream DNA repair factors. Formation of uH2A at sites of DNA double-strand break promotes dimethylation of histone H4 at lysine 20, although the mechanism is not known. This event supplies a binding site for 53BP1 protein, which plays an important role in DNA double-strand break repair and checkpoint induction (81-85). The BRCA1 localization to sites of DNA double-strand break is also essential for DNA double-strand break repair and checkpoint induction (86-89). In this process, some other chromatin modifications also coordinate with ubiquitylation to recruit downstream repair proteins. For example, a histone acetyltransferase (HAT), TIP60, can acetylate H2A and other core histones at sites of DNA double-strand break to facility recruitment of DNA damage response proteins (90-95) (Figure 1.7).

Beside these, chromatin remodeling plays an important role in the DNA damage response and needs specific ATP-dependent chromatin remodeling complexes. Among chromatin remodeler families, a SWI/SNF family member Ino80 is well known to be involved in the DNA double-strand break response (96, 97). All these events link chromatin modifications and remodeling with repair of DNA double-strand break (98-100).

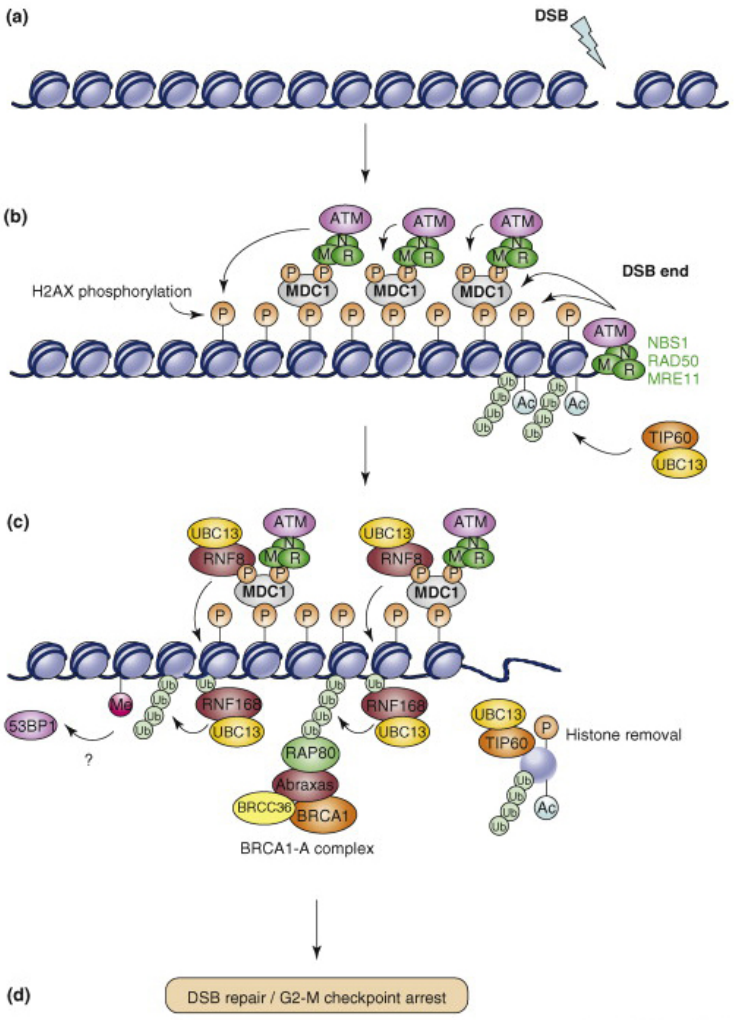

(a)
DSB
(b)
ATM
MDC1
H2AX phosphorylation
DSB end
NBS1
RAD50
MRE11
TIP60
UBC13
(c)
RNF8
RNF168
53BP1
Me
?
RAP80
Abraxas
BRCC36
BRCA1
BRCA1-A complex
Histone removal
(d)
DSB repair / G2-M checkpoint arrest

Figure 1.7 DNA repair in the context of chromatin modification.

When a DNA double-strand break happens in chromatin, γH2AX is formed at the sites of DNA lesions, and this event leads to the recruitment of MDC1. MDC1 recruits the MRN complex and ATM to amplify the DNA damage response signal. At the same time, Tip60 HAT acetylates γH2AX and recruits E2 ubiquitin-conjugating enzyme UBC13, which adds ubiquitin to H2A and γH2AX. This modified γH2AX will be removed from chromatin surrounding broken ends of DNA double-strand break. Furthermore, ATM- phosphorylated MDC1 can attract the RNF8 E3 ubiquitin ligase-UBC13 complex to promote ubiquitylation of H2A and γH2AX. These events cause subsequent binding of RNF168, which is another E3 ubiquitin ligase, to the polyubiquitylated histones resulting in the amplification of the ubiquitylation cascade. These histone modifications promote recruitment of 53BP1 through recognition of dimethylated H4 at lysine 20, and Rap80-ABRA1-BRCA1 complex, through recognition of polyubiquitin. These downstream events can directly play a role in checkpoint induction and DNA repair. Reproduced from (Van Attikum, H., and S.M. Gasser. 2009. Trends in cell biology 19:207-217.), with permission from Elsevier Ltd.

1.4 E2F1 plays a role in the DNA damage response

A number of E2F family members are now known to respond to DNA damage. E2F1 is the first reported and the best characterized E2F family member shown to respond to DNA damage. E2F1 protein is stabilized and accumulates in response to various forms of DNA damage through an increase in its half-life (101, 102). This involves phosphorylation by ATM or ATR at amino acid serine 31, which is a unique site for E2F1 not found in other E2F family members (103). This phosphorylation event creates a binding site for 14-3-3τ and interaction between E2F1 and 14-3-3τ prevents E2F1 ubiquitination and degradation (104). Besides ATM and ATR, it was reported that the Chk2 kinase can also phosphorylate human E2F1 at another site (not conserved in mouse E2F1) and this phosphorylation event also contributes to stabilization of E2F1, although the mechanism is unclear (105).

It was assumed that E2F1 stabilization in response to DNA damage would transcriptionally activate pro-apoptotic targets to induce cell death. Indeed, in some contexts apoptosis in response to DNA damage does involve E2F1 and the transcriptional induction of the *p73* gene (103, 104, 106). Specific targeting of E2F1 to the *p73* promoter in response to DNA damage involves the acetylation of E2F1 at multiple residues near its DNA-binding domain, which is stimulated by serine 31 phosphorylation (73). It is reported that E2F1 acetylation occurs only in response to DNA double-strand breaks but not in response to UV irradiation. The enzyme that acetylates E2F1 in response to DNA double-strand breaks is still

unclear with different groups proposing that either P/CAF or p300 acetyltransferases are responsible for this modification of E2F1 (106, 107).

DNA damage also increases the interaction between E2F1 and the RB tumor suppressor protein and this leads to the transcriptional repression of many cell-cycle-related genes such as *cyclin A* (108, 109). Surprisingly, an E2F1-RB complex may also contribute to the transcriptional activation of pro-apoptotic genes like *p73* and *caspase 7* (108). However, there are other reports that indicate RB is released from E2F1 in response to DNA damage (110, 111). In fact, an E2F1 transcriptional repression complex not containing RB but containing TopBP1 was demonstrated to form following DNA damage (112, 113). It was previously reported that TopBP1 interacts with RAD9 and could activate ATR at sites of DNA damage (114-116). TopBP1 contains nine BRCA1 C-terminal (BRCT) domains and the sixth BRCT domain specifically binds E2F1 when phosphorylated on serine 31 (113).

TopBP1 specifically represses the transcriptional activity of E2F1, but not other E2F family members, following E2F1 phosphorylation by ATM/ATR (112). Following DNA damage, regulation of E2F1 by TopBP1 is important for inhibiting cell cycle progression, repressing apoptosis and promoting cell survival (113). It is reported the PI3K/AKT kinase can phosphorylate TopBP1 at serine 1159, which creates a binding site for a BRCT domain in another TopBP1 molecule (112). So AKT regulates the formation of homodimer of TopBP1, which is required to bind E2F1. Thus, AKT activity determines whether TopBP1 will inhibit E2F1-dependent transcription of cell cycle and pro-apoptotic genes in response

to DNA damage or whether E2F1 stabilization will activate the expression of these genes to induce apoptosis. This model is consistent with Nevin's report that identified a subset of E2F1 target genes that is specifically repressed by PI3K/AKT signaling (117). This signaling may allow TopBP1 to integrate both survival and DNA damage response signals to modulate cell fate through E2F1 regulation.

TopBP1 binding not only inhibits E2F1 transcriptional activity, but also localizes E2F1 to sites of DNA double-strand break (112). A discussed in the next section, findings from our lab suggest that E2F1 has a direct function in promoting repair of UV radiation-induced DNA damage (118). Furthermore, E2F1 was shown to associate with the MRN complex through the N terminus of NBS1 (119). Taken together, evidence suggests that E2F1 could play a direct role in promoting the repair of DNA double-strand break and maintaining genome stability.

1.5 E2F1 plays a role in UV induced DNA damage.

In previous studies, I have shown that loss of E2F1 leads to significantly delayed UV damage repair both *in vitro* and *in vivo* (120). To elucidate the mechanism, I irradiated the cells with filtered UV light through small pores that creates localized DNA damage to areas of 3 μm to 8 μm in diameter in the nuclei. I then stained the cells with specific antibodies against CPD or 6-4PP photoproducts to visualize the local UV damage by immunofluorescence. By co-staining with antibody against E2F1, I observed clear co-localization of E2F1 with UV damage, which requires its serine 31 site and the ATR kinase. In addition, I demonstrated that knockdown of E2F1 with RNA interference does not affect the expression levels of UV damage repair proteins, including XPA, XPC, DDB1, DDB2, p62 of TFIIH complex, XPF and RPA2. However, E2F1 knockdown does cause significantly decreased recruitment of XPA, XPC and p62 proteins to sites of UV damage, which can well explain the impaired DNA repair in the absence of E2F1. These findings indicate that E2F1 impacts UV damage repair by affecting DNA repair protein accessibility in a transcription-independent manner. Further mutation analysis also confirmed that serine 31 and the dimerization domain, but not the DNA binding domain or transactivation domain, which is essential for transcription, are required for localization of E2F1 to UV damage sites and subsequent efficient DNA repair. It was interesting to observe that the Marked box domain of E2F1 is not required for its recruitment, but indispensible for its functions in DNA repair. Because this domain is responsible for protein-protein

interactions, further investigations focused on this domain will be of great help in understanding the function of E2F1 in DNA repair (118).

Following the above study, I further dissected the mechanisms by which E2F1 affects repair protein accessibility. Among all possibilities, it is likely that E2F1 affects chromatin accessibility of the repair machinery. Chromatin structure functions as a barrier for the recruitment of DNA repair proteins to damaged DNA areas, while E2F1 is intrinsically able to bind to chromatin modifiers as demonstrated by its functions as a transcription factor under other settings, such as cell cycle regulation. In my study I found that E2F1 binds to the GCN5 histone acetyltransferase and brings GCN5 to sites of UV damage, without affecting the level of GCN5. Because GCN5 mostly uses histone H3 and H4 as substrates, I examined a battery of acetylated H3 and H4 sites using specific antibodies and found that acetylated H3 at lysine 9 (H3K9Ac) and acetylated H4 at lysine 16 (H4K16Ac) increase at sites of DNA damage. After I knocked down either E2F1 or GCN5, both the total level and the localization of H3K9Ac, but not H4K16Ac, were significantly impaired. These findings indicate that E2F1 uses GCN5 to induce local histone H3 acetylation at lysine 9, which probably contributes to changes in the local structure of the chromatin and facilitates recruitment of DNA repair proteins. To confirm this hypothesis, I also knocked down GCN5 with RNA interference assay and demonstrated that localization of XPA, XPC and p62 to sites of DNA damage is also significantly diminished. Our DNA repair assays also showed that UV damage repair is significantly impaired by the knockdown of GCN5, similar to E2F1. It should be pointed out that H4K16Ac also appears to be

a specific histone modification to UV damage, but apparently it is not regulated by E2F1 or GCN5 (121).

1.6 Hypothesis

Both endogenous and exogenous agents constantly damage cellular DNA constantly. Accumulation of DNA damage causes genomic mutations and even cancer development. In contrast, DNA damage is also capable of inducing cell death, which makes many DNA damaging agents potent cancer therapeutic modalities, such as ionizing irradiation and some DNA double-strand break inducing drugs. Thus, it is essential to study the cellular response to DNA damage to further investigate cancer development mechanisms and the molecular biology related to cancer therapy. ATM and ATR kinases are the two key players in various DNA damage response pathways and their functions and downstream targets have been well investigated (122).

E2F1 is the first indentified member in the E2F family and regulates the expression of multiple genes critical for cell cycle progression, apoptosis, DNA replication and DNA repair (1-5). It was found that E2F1 is phosphorylated by ATM/ATR at serine 31, which is unique for E2F1 in the E2F family. The phosphorylation of serine 31 is required for E2F1 protein stabilization in response to DNA damage and creates a binding site for the sixth BRCT domain of TopBP1. The transcriptional activity of E2F1 is repressed by interaction with TopBP1 independent of RB. Furthermore, TopBP1 also localizes E2F1 to sites of DNA double-strand break, although the function of E2F1 at sites of break remains unclear (101-104, 112).

It was reported by our group that E2F1 functions to facilitate repair of UV induced DNA damage through a non-transcriptional mechanism involving

enhanced recruitment of DNA repair enzymes to sites of damage (118, 120, 121). **For this project, I hypothesize that E2F1 has important transcription-independent functions for the recruitment and/or retention of some DNA repair factors to sites of double-strand break.**

Chapter 2. Absence of E2F1 causes accumulation of spontaneous DNA damage and genomic instability

2.1 Introduction

The development of cancer significantly relies on the acquisition of genomic instability, which is created by defective surveillance and repair mechanisms for DNA damage caused by endogenous and exogenous sources (123). Newly developed concepts in cancer biology have emphasized the importance of genomic instability in the establishment of cancer hallmarks, and add it in the category of “Enabling Characteristics” (124). With the advance of technologies and knowledge, it has been realized that genetic alterations are truly indispensible for tumorigenesis and function as the predominant determining factor. When genomic instability creates growth or survival advantages, cells with this abnormality are frequently selectively expanded. This process can serve as the starting point of carcinogenesis. Genomic instability can also influence subsequent steps during carcinogenesis such as invasion and metastasis.

The significance of genome stability maintenance and DNA damage response mechanisms cannot be over-emphasized. In fact, numerous important tumor suppressors are themselves critical DNA repair factors or DNA damage response regulators with p53, BRCA1, and ATM as prototypical examples (48). Loss of these factors in cells leads to genomic instability followed by tumor development. There are also various pathway-specific DNA repair factors whose loss or deficiency can cause specific forms of genomic instability and tissue-specific tumorigenesis. For example, defective XPC or XPA proteins in the nucleotide excision pathway are strongly associated with UV radiation-specific DNA damage, mutations and skin cancers.

In addition to DNA repair factors, proteins that regulate orderly cell cycle progression and death are also important in the maintenance of genomic stability in a relatively indirect manner. Loss of these proteins can cause uncontrolled DNA replication and its related complications. Over-replication of DNA inevitably results in errors that may be beyond the capacity of DNA repair and genomic instability is therefore initiated. For example, the RB protein is critical in cell cycle regulation and loss of RB has been shown to be associated with dramatic genomic abnormalities (125, 126).

The E2F family has been considered as the primary players in cell cycle regulation downstream of RB. However, it is now realized that the E2F family regulates an extremely wide diversity of biological processes beyond promoting cell cycle progression. Among them, E2F1 was clearly shown to have dual roles in tumor development depending on different circumstances. E2F1 is a potent apoptosis inducer and can activate p53 and transcriptionally regulate a number of pro-apoptotic genes. However, those functions failed to show a clear correlation with a role for E2F1 in tumor suppression. In contrast, there are accumulating data suggesting that E2F1 functions as an important chromatin modifier to facilitate recruitment of nucleotide excision repair factors for UV-induced DNA damage in cell culture. Therefore, a role for E2F1 as a tumor suppressor could largely depend on its functions in DNA repair.

In addition to UV-induced DNA damage, E2F1 also accumulates at sites of DNA double-strand break, but whether and how E2F1 functions to promote DNA double-strand break repair is at present unknown. Based on these findings, I

hypothesized that E2F1 has transcription-independent functions for the recruitment and/or retention of DNA repair factors to sites of double-strand break.

To test this hypothesis, I investigated whether the loss of E2F1 causes genomic instability as demonstrated for other well-established tumor suppressors with critical DNA repair functions, as well as examining if E2F1 plays an important role in the response of DNA double-stranded breaks (127).

2.2 Materials and methods

2.2.1 Cell culture

Primary mouse adult fibroblasts (MAFs) were isolated from *E2f1* knockout and wild type mice. Briefly, the peritoneal fascia of mice at least 5 weeks old was excised, minced into small pieces, and dissociated by agitation in 0.2% mg/ml collagenase supplemented with 100 units DNase I. After washing in PBS, tissue was incubated in 25% trypsin for 20 minutes. Isolated cells are then washed twice and plated in tissue culture dishes. MAFs were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The HCT116 colon carcinoma cell line was obtained from ATCC and cultured in McCoy's 5A medium (Gibco) with 10% FBS.

2.2.2 DNA double-strand break treatments and agents

Neocarzinostatin (NCS) is a radiomimetic drug obtained from Sigma. During treatment, normal 10% FBS DMEM medium was changed to 10% FBS DMEM with NCS (50 ng/ml). At the appropriate time point, cells were fixed for immunofluorescence experiments or harvested for Western blot.

For ionizing radiation (IR) cells were cultured in 10 cm plates or 4 well chamber slides and treated with the indicated Gy dose using a RS-2000 Biological Irradiator (Rad Source).

2.2.3 Immunofluorescence staining

Briefly, cells grown on chamber slides (Nunc.) were washed with PBS and fixed using 3.7% formaldehyde. Fixed cells were incubated with 3% BSA for an hour, washed twice with washing buffer (0.5% BSA in PBST or PBST), incubated

with appropriate primary antibodies (e.g., specific for γH2AX or E2F1), and followed by incubation with fluorescently tagged secondary antibody (Alexa 488 or Alexa 594, Invitrogen). Cells were then stained with DAPI to identify the nucleus and sealed in mounting media (Vector Lab) with cover slips. Appropriate fluorescent images were captured using a Nikon eclipse 80i microscope equipped with an X-cite 120 fluorescence illumination system and Metamorph image analyzed software. The foci numbers were analyzed and counted using the Focicounter software program on randomly selected pictures captured for each experimental group (128).

2.2.4 Cytogenetic analysis

Primary mouse keratinocytes isolated from 1-2 day old pups were treated with colcemid for 2.5 hours and metaphase spreads counted. At least 40 metaphases were counted for each genotype. The values for each genotype represent the average from 3 different mice.

2.2.5 Western blot analysis

E2f1 knockout or wild type MAFs were cultured on 10 cm plates in starvation medium (0.5% FBS in DMEM) for 24 h then cultured in normal medium (10% FBS in DMEM) for at least more than 24 h before treatment. Whole cell lysates were harvested at different time points using 1X lysis buffer (Cell signaling). Antibodies were obtained from the following sources: E2F1 (C-20), β-tubulin, Santa Cruz; γH2AX, Millipore.

2.2.6 Comet assay

The single cell gel electrophoresis or comet assay was performed using the comet assay kit from Trevigen (17, 129, 130). Briefly, after different treatments (IR or mock), cells are harvested and embedded in low melting agarose on a glass slide and incubated overnight at 4^{o}C in lysis buffer. After washing, samples were electrophoresed at 19 V for 5-20 minutes in TBE and stained with SYBR Green. Nuclei were visualized and images captured using a fluorescent microscope. Tail length and Olive moment of at least 70 nuclei per slide were calculated using COMETSCORE software (Tritek).

2.3 Results

2.3.1 E2F1 accumulates at sites of NCS-induced DNA double-strand break

To confirm that E2F1 can form foci by accumulating at sites of DNA double-strand break, I applied the radiomemitic drug neocarzinostatin (NCS) to HCT116 cells to induce double-strand break then used immunofluorescence (IF) to detect E2F1 localization. Phosphorylation of the histone variant H2AX (referred to as γH2AX), a widely used marker of DNA double-strand break, was used as a positive control to detect co-localization of E2F1 with sites of DNA double-strand break.

E2F1 staining in mock treated cells was relatively even throughout the nucleus. In contrast, NCS treated cells showed increased staining intensity and foci formation that partially co-localized with γH2AX foci (Figure 2.1). This confirms a previous report that E2F1 accumulates at sites of DNA double-strand break to form foci (112) and indicates potential direct involvement of E2F1 in the repair of DNA double-strand breaks.

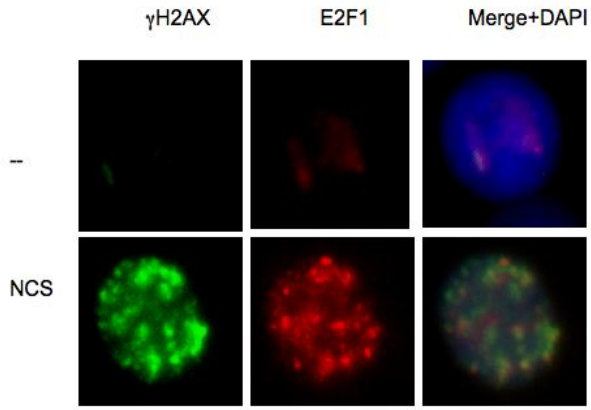


Figure 2.1 E2F1 forms foci that partially co-localizes with γH2AX in response to DNA damage.

HCT116 cells were mock treated or treated with neocarzinostatin (NCS 50ng/ml) for 15 min then incubated for an additional 1 h in fresh media. Immunofluorescence was performed to examine co-localization of E2F1 with γH2AX. Reproduced from (Chen,J, Cell Cycle 10:1287-1294.), with permission from Landes Bioscience.

2.3.2 E2F1 deficiency leads to genomic instability

Since E2F1 protein stability and subcellular localization are regulated in response to DNA damage, we wonder whether the absence of E2F1 would affect genome stability. A previous graduate student, Raju Pusapati performed cytogenetic analysis on primary mouse keratinocytes lacking E2F1. This analysis showed increased numbers of chromosomal breaks, fragments, and fusions in cells lacking E2F1 compared to wild type cells. This result indicates that E2F1 is involved in maintaining genomic integrity.

Genotype	% aberrant met.	% with breaks	% with fragments	% with fusions
Wild type	13.0	10.3	0	4.6
E2f1$^{-/-}$	31.5	18.6	4.6	17.4

Figure 2.2 Genomic instability in the absence of E2F1.

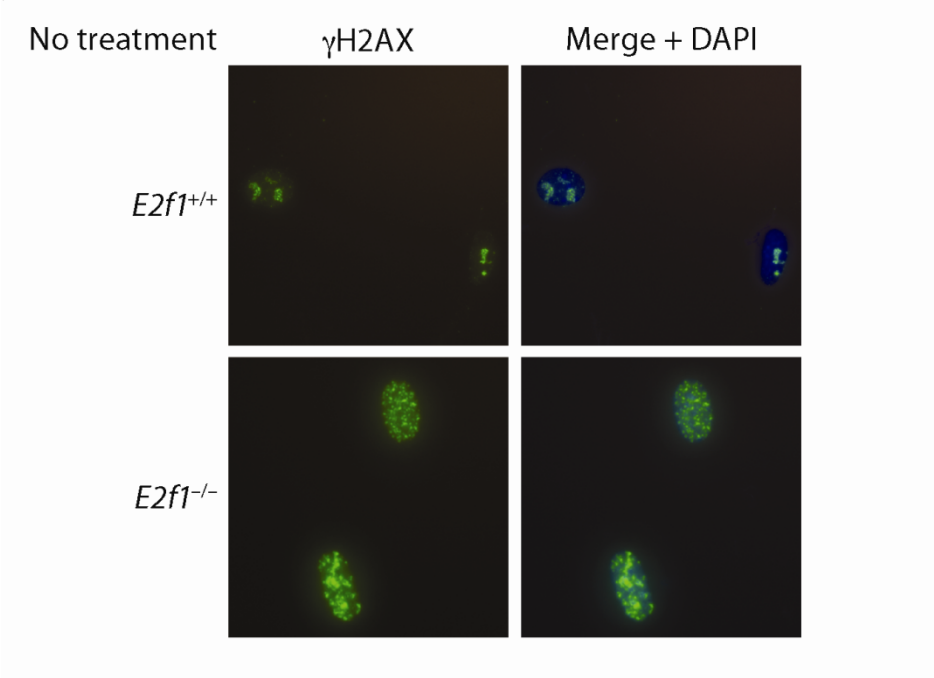
Primary mouse keratinocytes isolated from 1-2 day old pups were treated with colcemid for 2.5 h and metaphase spreads counted. At least 40 metaphases were counted for each genotype. The values for each genotype represent the average from 3 different mice. Arrows indicate chromosomal breaks, fragments and fusions (Experiment performed by Raju Pusapati).

2.3.3 Absence of E2F1 leads to the accumulation of endogenous DNA damage.

It is well known that γH2AX is a rapid and sensitive marker for DNA double-strand break (131). Immunofluorescence (IF) staining for γH2AX foci was used to estimate the amount of DNA damage in cells from *E2f1* knockout and wild type mice. Untreated primary mouse adult fibroblasts (MAFs) were immunofluorescently stained for γH2AX and fluorescent images were captured randomly. γH2AX foci were counted using the image analysis software Focicounter (128).

I observed that γH2AX foci numbers are increased in *E2f1* knockout cells, even in the absence of an exogenous DNA damaging agent. Only 35% of *E2f1*$^{-/-}$ MAFs showed no visible γH2AX foci while 60% of wild type MAFs lacked visible γH2AX foci. Furthermore, approximately 25% of primary *E2f1*$^{-/-}$ MAFs displayed more than 6 γH2AX foci per cell while only 5% of wild type MAFs had 6 or more foci (Figure 2.3). This result strongly suggests that cells lacking E2F1 display significantly more spontaneous DNA damage, in particular DNA double-strand breaks.

A


No treatment
γH2AX
Merge + DAPI
E2f1+/+
E2f1−/−

B

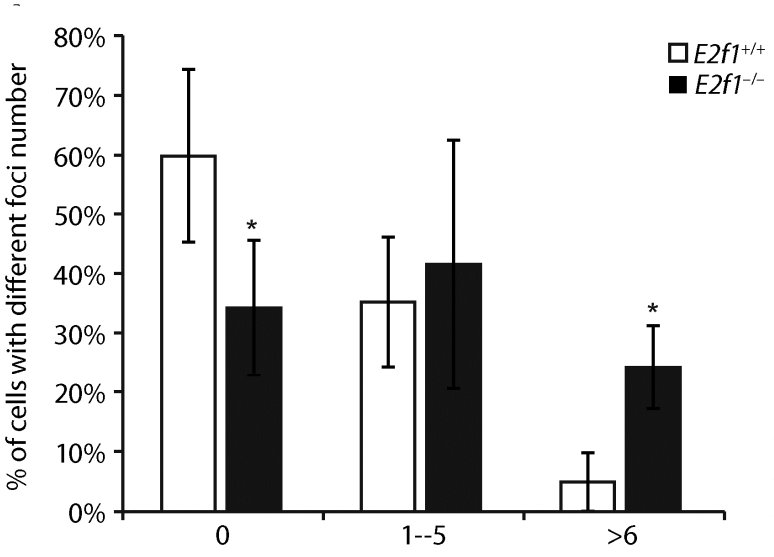

% of cells with different foci number
80%
70%
60%
50%
40%
30%
20%
10%
0%
E2f1+/+
E2f1−/−
*
*
0
1--5
>6

Figure 2.3 The absence of E2F1 leads to spontaneous DNA damage.

A. Untreated primary wild type and *E2f1* knockout MAFs were immunofluorescently stained for γH2AX. DAPI was used as a counterstain.

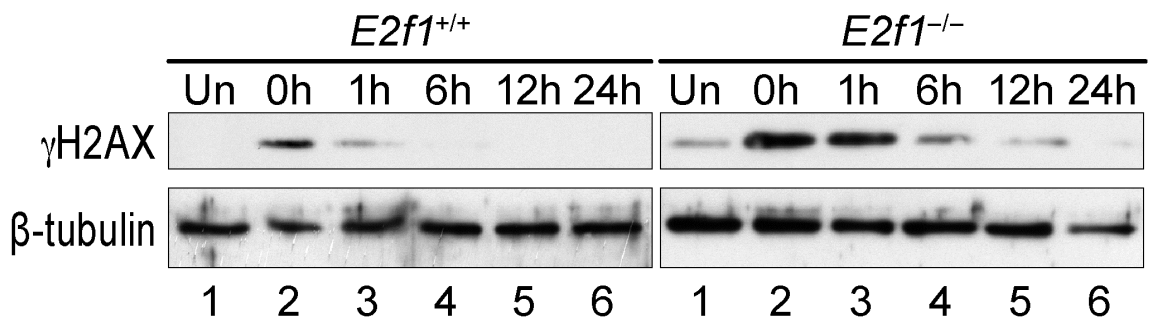
B. Images were analyzed using the FociCounter software program. The percentage of cells presenting different foci number was calculated after scoring more than 85 cells for each genotype in three independent experiments. *indicates statistically significant difference ($p<0.05$). Reproduced from (Chen,J, Cell Cycle 10:1287-1294.), with permission from Landes Bioscience.

2.3.4 E2F1 deficiency impairs recovery from IR

I found that E2F1 is also important for cells to recover from ionizing radiation (IR) treatment. Western blot analysis for γH2AX was performed to indirectly evaluate and monitor DNA damage production and recovery over time. Consistent with our previous results, MAFs without E2F1 showed increased levels of γH2AX even before IR treatment (Figure 2.4A). After IR treatment, γH2AX was generated in both wild type and *E2f1*$^{-/-}$ MAFs but cells without E2F1 displayed higher levels of γH2AX compared to wild type MAFs. Moreover, while γH2AX disappears in wild type cells at the 6h time point after irradiation, cells lacking E2F1 showed persistent γH2AX after irradiation up to the 12 hours time point.

The single cell gel electrophoresis assay (also referred to as comet assay) is widely used to measure DNA breaks. A similar phenomenon was observed when I used the comet assay to monitor DNA damage in wild type and *E2f1*$^{-/-}$ MAFs. In harmony with the finding in the above western blot, primary MAFs lacking E2F1 showed significantly increased DNA damage, compared to wild type MAFs without any treatment. The absence of E2F1 also results in anomalously higher levels of DNA breaks at one hour and persistence of DNA damage at 24 hours post-IR (Figure 2.4B) All in all, these findings suggest the absence of E2F1 causes increased levels of endogenous DNA damage and impaired repair of IR induced DNA double-strand break.

A


E2f1+/+
E2f1−/−
Un 0h 1h 6h 12h 24h Un 0h 1h 6h 12h 24h
γH2AX
β-tubulin
1 2 3 4 5 6 1 2 3 4 5 6

B

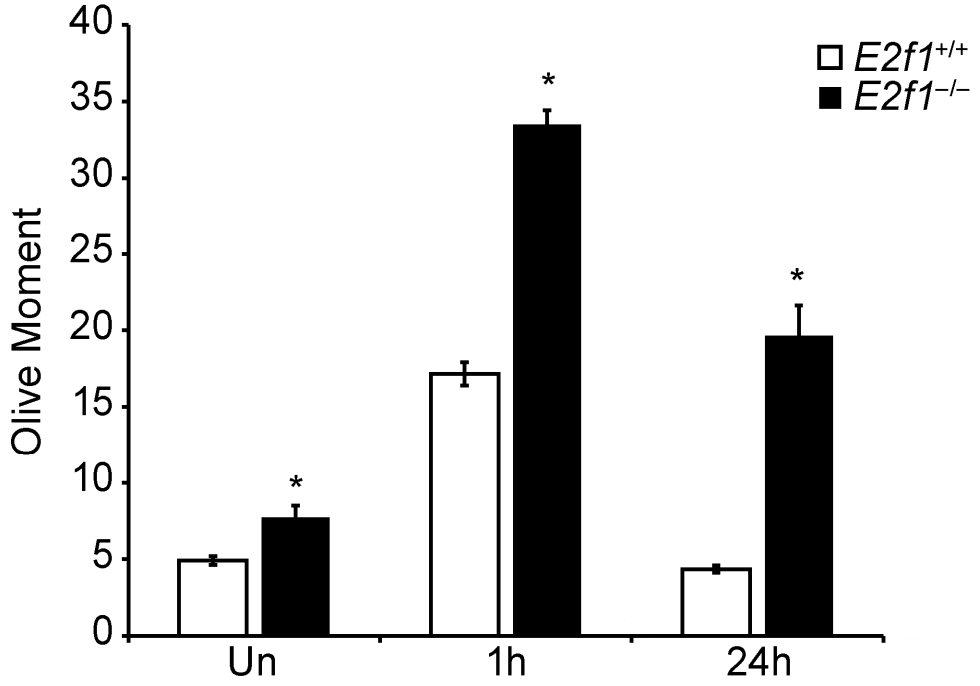

Olive Moment
40
35
30
25
20
15
10
5
0
E2f1+/+
E2f1−/−
*
*
*
Un
1h
24h

Figure 2.4 The absence of E2F1 impairs cell recovery following IR exposure.

A. Primary wild type (lanes 1-6) and *E2f1* knockout (lanes 7-12) MAFs were untreated (Un, lanes 1 and 7) or treated with 5 Gy of IR and harvested at different time points: Immediately (0h, lanes 2 and 8); 1 h (1h, lanes 3 and 9); 6 h (6h, lanes 4 and 10); 12 h (12h, lanes 5 and 11); and 24 h (24h, lanes 6 and 12). Western blot analysis was performed using antibody to γH2AX and β-tubulin. Reproduced from (Chen,J, Cell Cycle 10:1287-1294.), with permission from Landes Bioscience.

B. Primary wild type and *E2f1* knockout MAFs were untreated or treated with 10 Gy of IR and incubated for 1 h and 24 h. Cells were harvested and subjected to the comet assay. Images of nuclei and comet tails were taken and the Olive moment of each tail was calculated using cometscore software. The average Olive moment was determined for 50 cells per each genotype. *indicates statistically significant difference ($p<0.05$). Reproduced from (Chen,J, Cell Cycle 10:1287-1294.), with permission from Landes Bioscience.

2.4 Discussion

It is well documented that E2F1 has dual roles in tumorigenesis and that *E2f1* inactivation predisposes mice to spontaneous tumorigenesis. However, the mechanisms underlying its functions in tumor suppression have not been clearly elucidated. Our previously published data have clearly shown that E2F1 is critical in UV-induced DNA damage repair (118, 120, 121). E2F1 may also be involved in DNA double-strand break repair as it was shown that E2F1 localizes to DNA double-strand break in a TopBP1- and ATM-dependent manner (113, 132). In our current study, I confirm that endogenous E2F1 localizes to sites of DNA double-strand breaks, indicating a direct function for E2F1 in double-strand break repair.

A previous graduate student in our lab observed significantly increased chromosomal aberrations in primary keratinocytes from *E2f1* knockout mice, including chromosomal breaks, fragments and fusions. To further test the validity of the those findings, I also examined the presence of spontaneous DNA damage, which is universally present in tumors with prominent genomic instability (133). I found that primary MAFs lacking E2F1 had significantly more DNA damage foci as indicated by γH2AX staining. Consistently, the level of γH2AX and numbers of foci in primary *E2f1* knockout fibroblasts was significantly higher than in wild type cells. Moreover, after treatment with IR, primary *E2f1* knockout fibroblasts showed a significantly delayed ability to resolve the DNA damage compared to wild type cells as indicated by persistent γH2AX and comet tails. This suggests an important role for E2F1 in DNA double-strand break repair.

Overall, it appears that E2F1 plays a direct role in DNA double-strand break repair and loss of E2F1 causes significant genomic instability. This role for E2F1 in maintaining genomic stability may be important for suppressing the cancerous transformation of cells. Therefore, elucidation of the mechanisms underlying the functions of E2F1 in the response to DNA double-strand breaks may shed light on the mystery of E2F1 in tumor suppression.

Chapter 3. E2F1 promotes the recruitment of DNA repair factors to DNA double-strand break.

3.1 Introduction

I have demonstrated that E2F1 is important for maintaining genomic stability, which may reasonably explain its function in tumor suppression. However, the mechanism by which E2F1 functions in DNA repair and genome maintenance is at present unclear. In 2001 it was found that E2F1 is phosphorylated at serine 31 by the ATM/ATR kinases (103), which raises a question of role of E2F1 in the DNA damage response. The same group demonstrated later that E2F1 binds to TopBP1 when phosphorylated at serine 31 and recruits the Brg1/Brm containing SWI/SNF chromatin remodeling complex to repress transcription. This TopBP1 mechanism of transcriptional repression is important for preventing E2F1-induced cell cycle progression and apoptosis in response to DNA damage.

Previous results from our group also showed that E2F1 suppresses apoptosis in response to UV-induced DNA damage as opposed to its traditionally perceived role in apoptosis induction (120, 134). In addition, our group found that E2F1 significantly contributes to the removal of UV-induced DNA damage, especially during the early stages post-damage, which was demonstrated *in vivo* in mouse skin and *in vitro* in cultured primary mouse fibroblasts (120). Further study did not reveal any changes in nucleotide excision repair protein levels in the absence of E2F1, indicating a transcription-independent mechanism for its functions in DNA repair. More interestingly, further exploration revealed that E2F1 recruits histone acetyltransferase GCN5 to modify the local chromatin structure at sites of DNA damage, specifically promoting acetylation of histone H3 at lysine 9. This modification may contribute an increase in accessibility of

DNA repair proteins to sites of UV-induced DNA damage.

These findings inspired us to expand our understanding of functions of E2F1 in the DNA damage response by investigating role of E2F1 in repairing other types of DNA damage. DNA double-strand breaks are the most detrimental type of DNA damage and can arise due to both endogenous and exogenous damaging sources. DNA double-strand breaks also serve as the prototypical model in exploration of the DNA damage response network and our understanding of ATM-mediated signaling pathways is largely derived from studies of this type of DNA damage.

As mentioned previously, the DNA double-strand break response pathway is composed of damage sensing, transduction and effectors proteins with concurrent chromatin modifications to either promote the accessibility of the DNA damage or to form a platform for signal amplification and transduction. There are branches of error-free homologous recombination repair and error-prone pathways, including non-homologous end joining. As for DNA double-strand break repair, Mre11-Rad50-Nbs1 complex recruitment, ATM activation and foci formation are the earliest and rate limiting steps. RPA foci formation with recruitment of CtIP and ATR signaling factors is the point where DNA double-strand breaks end resection occurs that marks the point for following the homologous recombination pathway. In addition, recruitment of Rad51 recombinase would also be a critical event to examine in the absence of E2F1.

Chromatin modification has been recognized as an essential component in the process of DNA double-strand break repair. Indeed, γH2AX foci formation

is one of the earliest identified events signaling the presence of DNA damage and is also critical for the amplification of the damage signal. The presence of γH2AX provides a binding module for the MDC1 protein, which in turn recruits NBS1, a component of the MRN complex. In addition, some reports have shown that acetylation of histone H3 by Tip60 is important for ATM activation, while the identification of histone ubiquitylation at sites of DNA breaks revealed a complex cascade, sequentially performed by RNF8, RNF168, and perhaps other ubiquitin ligases. It is now clear that ubiquitylated H2A and H2AX provide a key chromatin context for DNA repair protein recruitment, with the best example being a BRCA1 containing complex (135). Other studies also suggested the involvement of histone methylation in the process of DNA damage recognition and processing, which needs further investigation (136, 137).

Interestingly, previous work from our group has demonstrated that E2F1 uses the histone acetyltransferase GCN5 to facilitate nucleotide excision repair for UV damage involving acetylation of histone H3 at lysine 9. Therefore I speculated that E2F1 may use a similar mechanism to promote DNA double-strand break repair. The potential relationship between E2F1 and the GCN5 complex, including the TRRAP protein that was reported to interact with E2F1 in other settings (138), will be specifically examined in the DNA double-strand break response. In addition, other potential partners of E2F1 in chromatin modification will also be investigated with a focus on the ubiquitin ligase RNF8.

I are also interested in the possibility that phosphorylation of E2F1 at serine 31 by the ATM kinase regulates function of E2F1 in DNA double-strand

break repair. Our hypothesis is that this phosphorylation event turns E2F1 into DNA repair protein as well as a transcriptional regulator in response to DNA damage. As a matter of fact, our lab previous data already demonstrated that serine 31 phosphorylation is required for ability of E2F1 to repair UV-induced DNA damage. On the other hand, the DNA-binding domain and transactivation domain of E2F1 are dispensable for DNA repair despite the fact that they are essential components for transcription. Inspired by the these findings, the role of E2F1 serine 31 in maintaining genomic stability and promoting double-strand break repair will also be explored. Our lab generated a knock-in mouse model in which *E2F1* sequences encoding serine 31 (29 in mouse) were altered to encode alanine (S29A). This will be a very helpful tool for studying the role of phosphorylation of serine31 of E2F1 in the DNA damage.

3.2 Materials and methods

3.2.1 Cell culture

MAFs were isolated from *E2f1* knockout and wild type mice and cultured as described in chapter 2. Primary mouse embryos fibroblasts (MEFs) were isolated from 12.5 to 13.5 postcoitum (p.c.) mouse embryos. The embryos are dissociated in sterile PBS then head and internal organs of embryos were removed for genotyping. The embryo carcasses were minced with curved scissors and then trypsinized to produce single-cell suspensions. Each cellular suspension was pipetted and passed through a cell strainer (70 micron) to be transferred into a 100 mm plate. MEFs were incubated in DMEM supplemented with 10% FBS. Normal human fibroblasts (NHFs) were obtained from Coriell Institute were maintained in DMEM supplemented with 10% FBS. The HCT116 colon carcinoma cell line was obtained from ATCC and cultured in McCoy's 5A medium (Gibco) with 10% FBS.

3.2.2 Small interference RNA

siRNAs for E2F1 and control are obtained from Santa Cruz. Transfection was performed by standard methods using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

3.2.3 Western blot

The antibodies used for western blot analysis are total NBS1, pNBS1 ser343, SMC1 (Cell signaling), pATM ser1981 (Rockland), γH2AX (Millipore), total Chk1 (Santa Cruz), pChk1 ser345 (Santa Cruz), β-tubulin (Santa Cruz), and Rad51 (Santa Cruz).

3.2.4 Immunofluorescence staining

Co-localization of proteins in response to DNA double-strands breaks and foci formation detection were performed as described in Chapter 2.

3.2.5 Quantitative RT-PCR

MAFs isolated from *E2f1* knockout and wild type mice were treated or untreated with 5 Gy IR then harvested post one h of treatment. Total RNA of each sample was isolated using the RNeasy kit (Qiagen, Inc., Valencia, CA, USA). The RNA was quantified with Nanodrop and 20 ng of total RNA were applied for the one-step real time reverse-transcriptase PCR assays with an ABI Prism 7700 (Applied Biosystems, Foster City, CA) instrument. Probes and primers were adopted from the mouse Rad51 assay provided by the Applied Biosystems (Mm01337943_m1). The reactions were designed according to the manufacturer's instructions, with a 30-minute of reverse transcription step followed by amplification with annealing temperature at 60℃. The PCR product is 106 bps. The level of Rad51 is calculated with comparative CT methods, with normalization to the housekeeping gene GAPDH level. The untreated controls serve as the reference group (designated as 1.0). All groups were assayed in triplicate.

3.3 Results

3.3.1 NBS1 foci formation in response to DNA double-strand breaks is dependent on E2F1

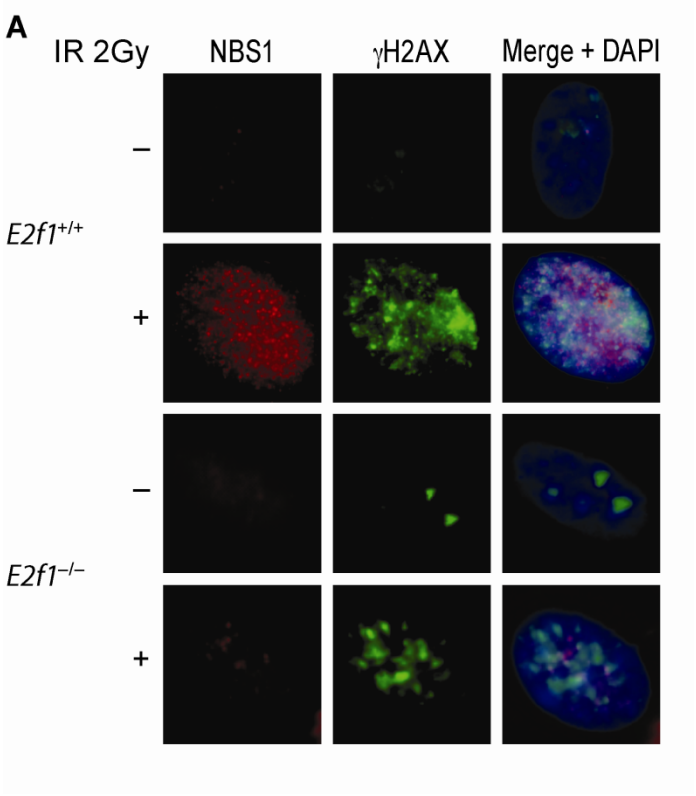
The MRN complex is involved in ATM activation in response to double-strands break as well as DNA end resection, which is important for both NHEJ and HR. MRN components, such as NBS1, are some of the earliest proteins recruited to the sites of DNA double-strand breaks. To investigate whether E2F1 status would affect NBS1 recruitment to sites of DNA double-strand breaks, NBS1 foci formation was examined by IF in *E2f1* wild type and knockout MAFs. The result showed that in response to IR- induced DNA double-strand breaks, NBS1 formed multiple foci in wild type cells but foci formation was impaired significantly in cells lacking E2F1 (Figure 3.1A). Approximately 60% of wild type cells showed NBS1 foci formation while only 20% cells displayed NBS1 foci formation in the absence of E2F1 (Figure 3.1B). Consistent with previous result, γH2AX foci formation appeared to increase in the absence of E2F1.

Western blot analysis was also performed to determine if NBS1 expression levels are affected by the absence of E2F1. The total protein level of NBS1 was the same in MAFs with or without E2F1 and NBS1 protein levels showed no change in response to DNA damage (Figure 3.2). As I speculated, NCS treatment, which induces double-strand breaks, lead to autophosphorylation and activation of ATM in both wild type and *E2f1*$^{-/-}$ MAFs. In contrast, hydroxyurea (HU) treatment, which induces replication stress and activates ATR, did not cause autophosphorylation of ATM. Consistent with this, activated ATM

phosphorylated NBS1 on its target site in response to NCS treatment but HU treatment did not in wild type MAFs. Interestingly, I observed that NBS1 phosphorylation in response to NCS treatment was impaired in MAFs lacking E2F1.

To further confirm these findings, I used short interfering RNA (siRNA) to deplete E2F1 in normal human fibroblasts (NHFs) and then examined NBS1 foci formation and protein expression levels in these cells. Control or E2F1 siRNA was applied to NHFs for 48 hours, then cells were treated with IR or NCS to induce DNA double-strand breaks. IF results showed that either IR or NCS treatment produced bright foci formation in NHFs transfected with control siRNA but not NHFs transfected with E2F1 siRNA (Figure 3.3A). NBS1 foci formation decreased from around 80~90% to 20~30% in cells with E2F1 compared to cells lacking E2F1 (Figure 3.3B). This observation is similar to the finding in MAFs lacking E2F1. Cells knocked down for E2F1 displayed enhanced γH2AX foci formation consistent with our previous finding in chapter 2 that E2F1 absence causes enhanced γH2AX foci.

Western Blot analysis indicated that E2F1 depletion did not affect total NBS1 protein levels either with or without IR treatment (Figure 3.4). However, consistent with what was observed in *E2f1*$^{-/-}$ MAFs, E2F1 knock down impaired NBS1 phosphorylation after IR treatment in NHFs.


A
IR 2Gy
NBS1
γH2AX
Merge + DAPI
–
E2f1+/+
+
–
E2f1–/–
+

B

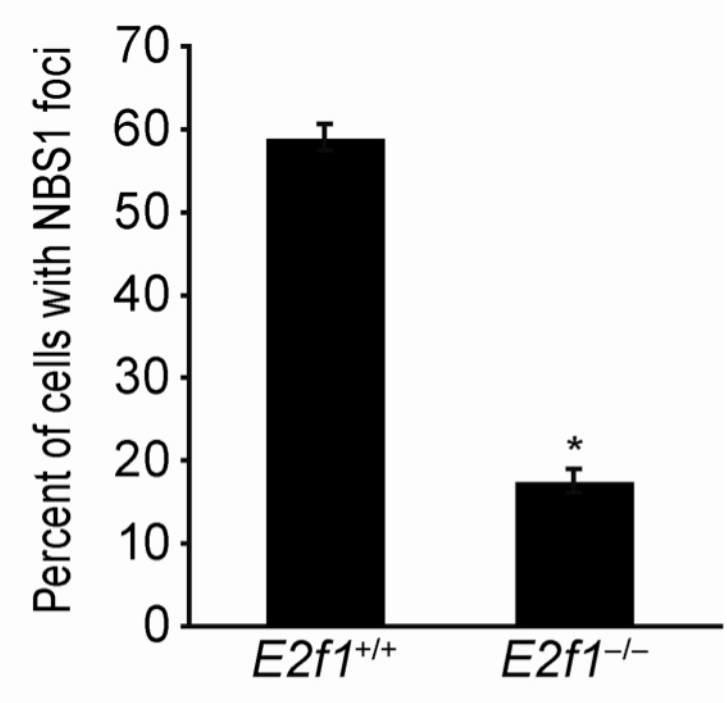

Percent of cells with NBS1 foci
70
60
50
40
30
20
10
0
*
E2f1+/+
E2f1–/–

Figure 3.1 Lack of E2F1 impairs NBS1 foci formation in response to DNA damage.

A. Primary wild type and *E2f1* knockout MAFs were untreated or treated with 2 Gy of IR. After exposure to IR, cells were incubated for one h before performing IF staining with NBS1 and γH2AX antibodies. DAPI was used as a counterstain. Reproduced from (Chen,J, Cell Cycle 10:1287-1294.), with permission from Landes Bioscience.

B. The percentage of cells displaying NBS1 foci was determined by counting 100 cells for each genotype using IF images from three independent experiments. *indicates statistically significant difference ($p<0.05$). Reproduced from (Chen,J, Cell Cycle 10:1287-1294.), with permission from Landes Bioscience.

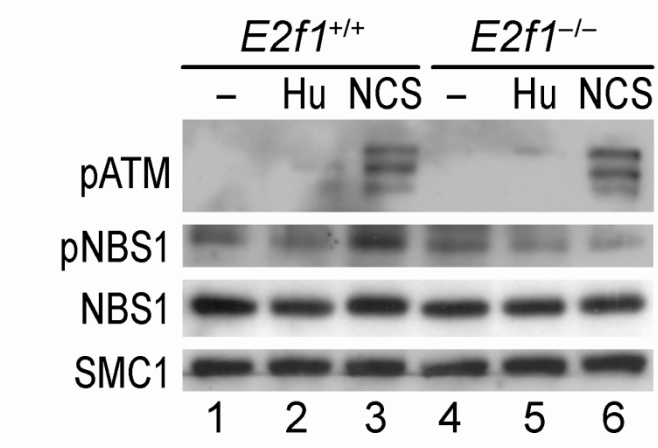
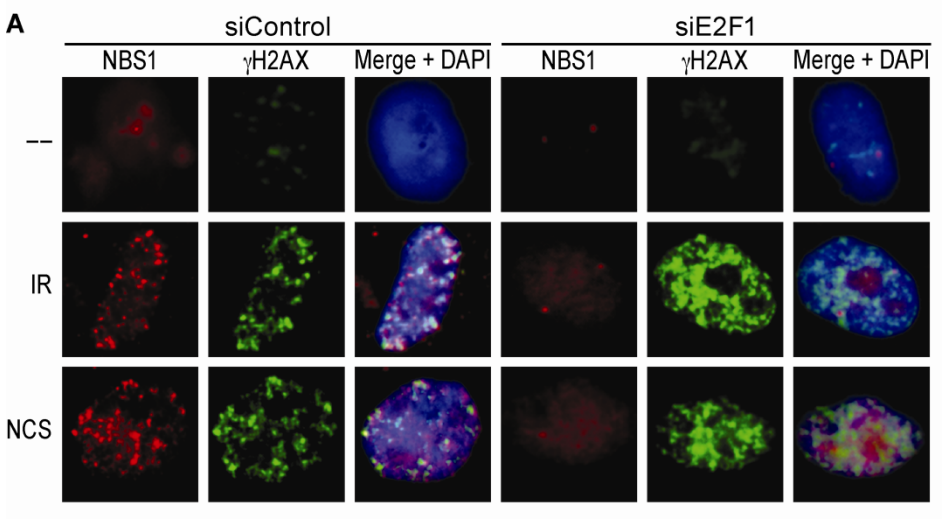


Figure 3.2 Lack of E2F1 impairs NBS1 phosphorylation in response to DNA damage.

Primary wild type and *E2f1* knockout MAFs were untreated or treated with HU (1.5 ng/ml) or NCS (50 ng/ml) for one hour. Whole cell extract was harvested for western blot analysis. The antibodies used in this experiment are: phospho-ATM antibody at serine 1981 site, total NBS1 antibody, phospho-NBS1 antibody at serine 343 site, and total SMC1 antibody as loading control. Reproduced from (Chen,J, Cell Cycle 10:1287-1294.), with permission from Landes Bioscience.


A
siControl
siE2F1
NBS1
γH2AX
Merge + DAPI
NBS1
γH2AX
Merge + DAPI
--
IR
NCS

B

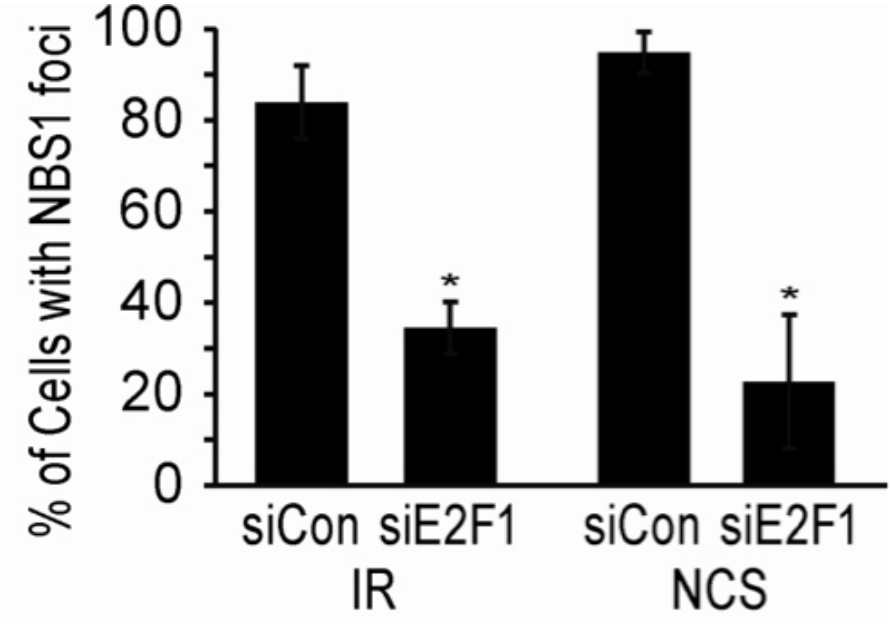

% of Cells with NBS1 foci
100
80
60
40
20
0
*
*
siCon
siE2F1
siCon
siE2F1
IR
NCS

Figure 3.3 E2F1 deficiency impairs NBS1 foci formation in response to DNA damage in human cells.

A. NHFs were transfected with E2F1 siRNA or control siRNA. 48 h after transfection, cells were untreated or exposed to 10 Gy of IR or NCS (50 ng/ml) for 15 min. After incubation for one h, cells were used to perform IF staining with NBS1 and γH2AX antibodies. DAPI was used as a counterstain. Reproduced from (Chen,J, Cell Cycle 10:1287-1294.), with permission from Landes Bioscience.

B. The percentage of cells displaying NBS1 foci was determined by counting 100 cells for each genotype using IF images from three independent experiments. *indicates statistically significant difference ($p<0.05$). Reproduced from (Chen,J, Cell Cycle 10:1287-1294.), with permission from Landes Bioscience.

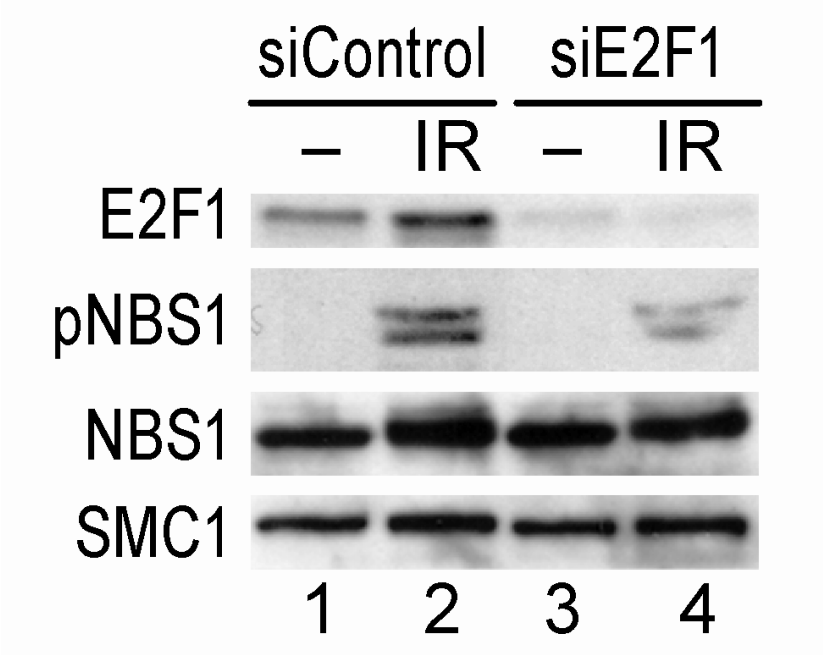


Figure 3.4. E2F1 deficiency impairs NBS1 phosphorylation in response to DNA damage in human cells.

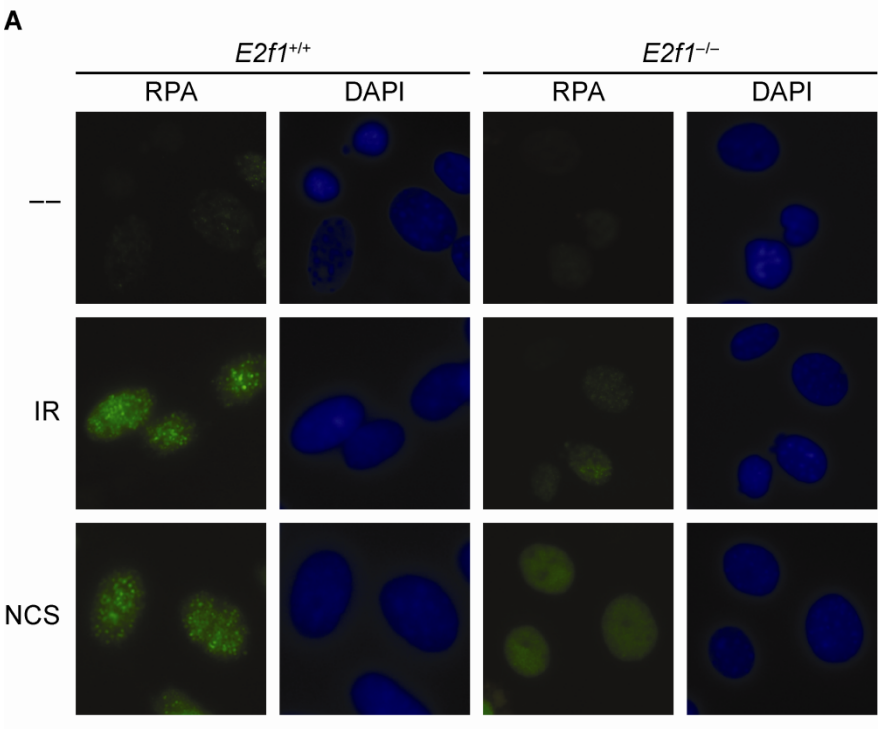
NHFs were transfected with E2F1 siRNA or control siRNA. 48 h after transfection, cells were untreated or exposed to 10 Gy of IR and then incubated for one h. Whole cell extract was used for western blot analysis using antibodies for: total E2F1, total NBS1, phospho-NBS1 at serine 343 site and total SMC1. Reproduced from (Chen,J, Cell Cycle 10:1287-1294.), with permission from Landes Bioscience.

3.3.2 E2F1 promotes foci formation of RPA at sites of double-strand break

Single-strand DNA is created at sites of DNA double-strand break by MRN-dependent end processing, which is required for both microhomology-mediated end joining and homologous recombination (139, 140). This single stranded DNA is stabilized by binding of the three subunits factor replication protein A (RPA). Since E2F1 absence could impair NBS1 foci formation at DNA double-strand break sites, I wondered whether E2F1 could also affect MRN-mediated DNA end processing and the generation of single- stranded DNA. To determine this, IF staining was performed in wild type and *E2f1*$^{-/-}$ MAFs treated with IR and NCS to generate DNA double-strand break. I observed that RPA formed robust foci in wild type MAFs but RPA foci formation was significantly impaired in cells lacking E2F1 (Figure 3.5A). Image analysis showed that while most wild type cells treated with IR generate greater than 10 RPA foci per cell, around 70% of *E2f1*$^{-/-}$ MAFs displayed no RPA foci formation after IR treatment (Figure 3.5 A).

Western blot analysis was performed to determine if E2F1 affected RPA protein levels. Like NBS1, E2F1 absence does not affect RPA protein levels, before or after damage. This indicates that E2F1 affects RPA localization to sites of DNA double-strand break and suggests E2F1 is important for MRN-dependent DNA end processing.

A


E2f1+/+
E2f1−/−
RPA
DAPI
RPA
DAPI
−−
IR
NCS

B

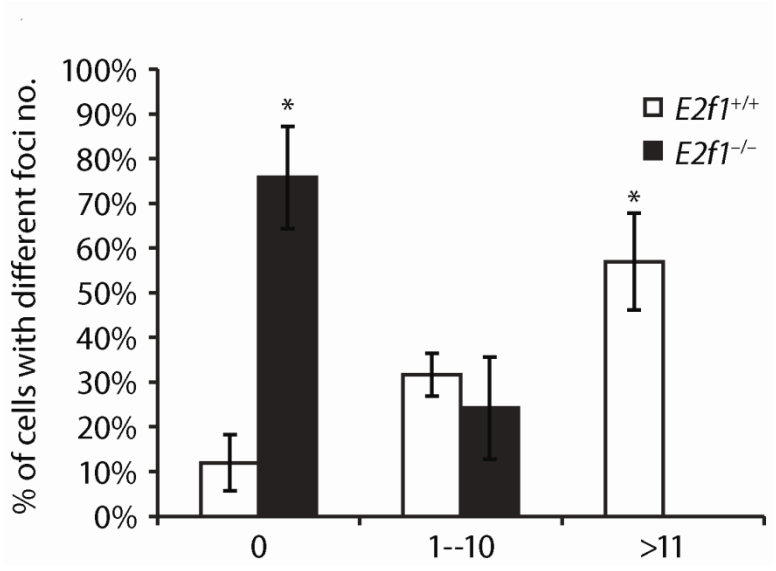

% of cells with different foci no.
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
*
*
E2f1+/+
E2f1−/−
0
1--10
>11

Figure 3.5 The absence of E2F1 impairs RPA foci formation in response to DNA damage.

A. Primary wild type and *E2f1* knockout MAFs were untreated or treated with 10 Gy of IR or NCS (50 ng/ml) then incubated for one h. After treatment, cells were immunofluorescently stained for the RPA2 protein. DAPI was used as a counterstain. Reproduced from (Chen,J, Cell Cycle 10:1287-1294.), with permission from Landes Bioscience.

B. IF images were analyzed using the FociCounter software program. The percentage of cells presenting different foci number was calculated after scoring more than 55 cells for each genotype in three independent experiments. *indicates statistically significant difference ($p<0.05$). Reproduced from (Chen,J, Cell Cycle 10:1287-1294.), with permission from Landes Bioscience.

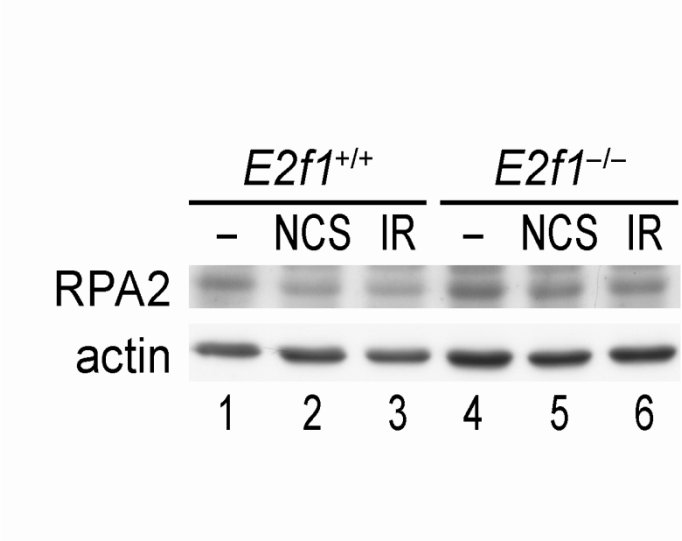


Figure 3.6 The absence of E2F1 does not affect RPA protein levels.

Primary wild type and *E2f1* knockout MAFs were untreated or treated with 10 Gy of IR or NCS (50 ng/ml) then incubated for one h. Whole cell extracts were harvested for western blot analysis using RPA2 antibody and actin antibody as a loading control. Reproduced from (Chen,J, Cell Cycle 10:1287-1294.), with permission from Landes Bioscience.

3.3.3 E2F1 promotes Rad51 foci formation at the sites of double-strand breaks.

Rad51 is the main recombinase in homologous recombination (HR) repair of DNA double-strand breaks. Rad51 is involved in the homology searching and DNA strand pairing stages of the homologous recombination process. Rad51 interacts with the single-strand DNA-binding RPA factor and displaces it on single-stranded DNA in a process requiring BRCA2 (141, 142).

Using IF I observed that Rad51 foci brightness and foci number in each cell was reduced significantly in cells lacking E2F1 compared to wild type cells following IR treatment. In contrast, foci formation of another DNA double-strand break response factor, 53BP1 was unchanged without E2F1 (Figure 3.7). Western blot analysis showed Rad51 protein levels increased in response to IR or NCS treatment, and this was impaired in cells lacking E2F1 (Figure 3.8). However, Rad51 mRNA levels did not change following DNA damage with or without E2F1 (Figure 3.9). This indicates that the E2F1-dependent increase Rad51 protein levels in response to DNA damage is not due to transcriptional up-regulation.

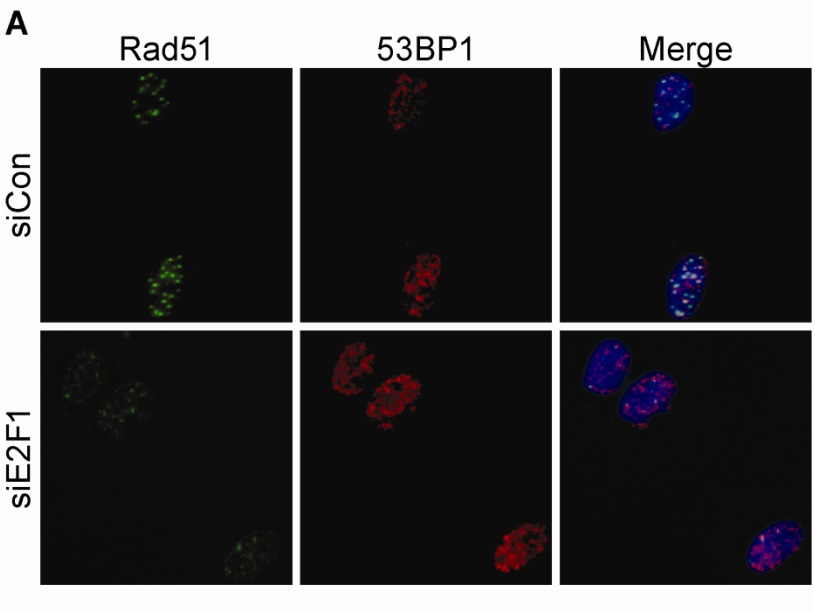


Figure 3.7 Knock down of E2F1 impairs Rad51 foci formation in response to DNA damage.

NHFs were transfected with E2F1 siRNA or control siRNA. 48 hours after transfection, cells were treated with 10 Gy of IR and then incubated for one h. IF staining was performed using Rad51 and 53BP1 antibodies. DAPI was used as a counterstain. Reproduced from (Chen,J, Cell Cycle 10:1287-1294.), with permission from Landes Bioscience.

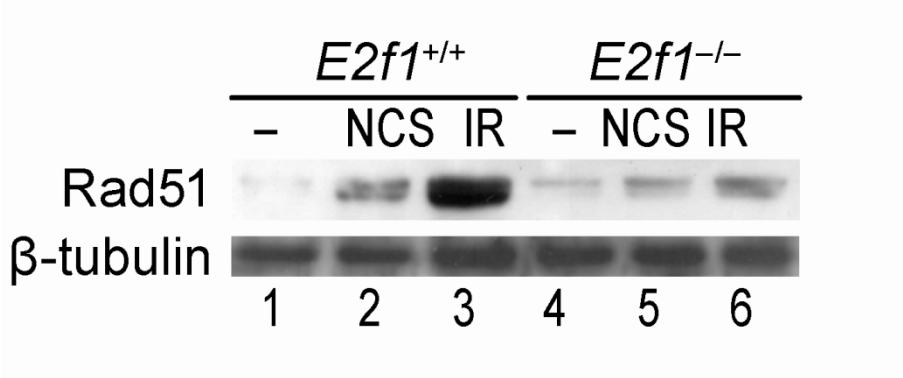


Figure 3.8 The absence of E2F1 impairs the increase in Rad51 protein levels in response to DNA damage.

Primary wild type and *E2f1* knockout MAFs were untreated or treated with 5 Gy of IR or NCS (50 ng/ml) and then incubated for one h. Whole cell extracts were used for western blot analysis using Rad51 antibody and β-tubulin antibody as a loading control. Reproduced from (Chen,J, Cell Cycle 10:1287-1294.), with permission from Landes Bioscience.

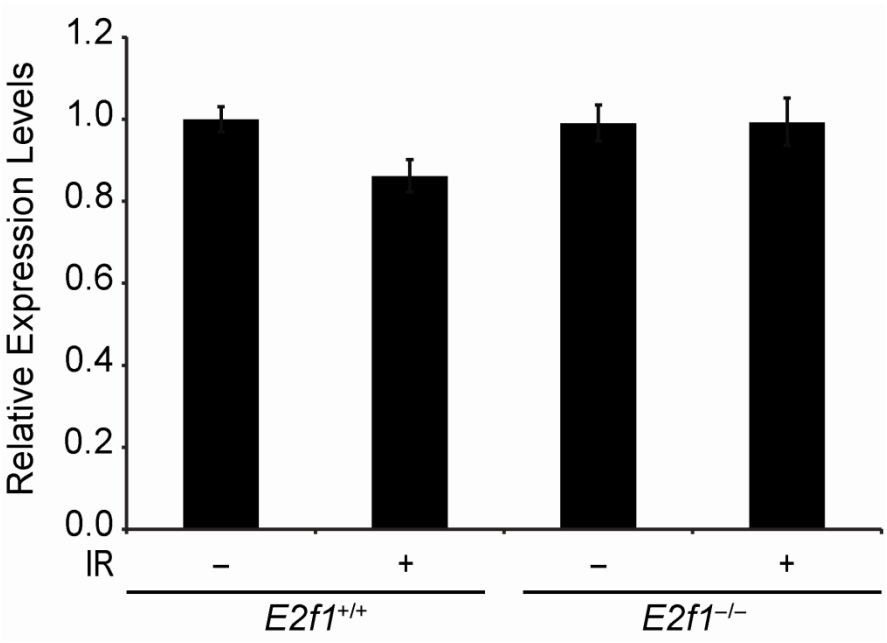


Figure 3.9 The absence of E2F1 does not affect Rad51 mRNA levels.

Primary wild type and *E2f1* knockout MAFs were untreated or treated with 10 Gy of IR and then incubated for one h. Cells were harvested, RNA extracted, and the mRNA level for Rad51 was determined by real-time PCR. Reproduced from (Chen,J, Cell Cycle 10:1287-1294.), with permission from Landes Bioscience.

3.3.4 Absence of E2F1 affects phosphorylation of Chk1 in response to DNA double-strand breaks.

It is reported that RPA-covered single-stranded DNA (RPA-ssDNA) generated at sites of DNA damage is involved in the activation of ATR-Chk1 signaling (143). To further confirm our findings that the absence of E2F1 impairs DNA end resection in response to double-strand breaks, I checked phosphorylation of Chk1 by western blot. I used siRNA to deplete E2F1 in NHFs and then examined Chk1 protein expression levels and phosphorylation in these cells. Western Blot analysis indicated that E2F1 depletion did not affect total Chk1 and SMC1 protein levels either with or without IR treatment (Figure 3.10). However, E2F1 knock down impaired Chk1 phosphorylation at serine 345 site after IR treatment in NHFs. This finding is consistent with our conclusion that E2F1 is important for DNA end resection and formation of single-stranded DNA at site of double-strand breaks.

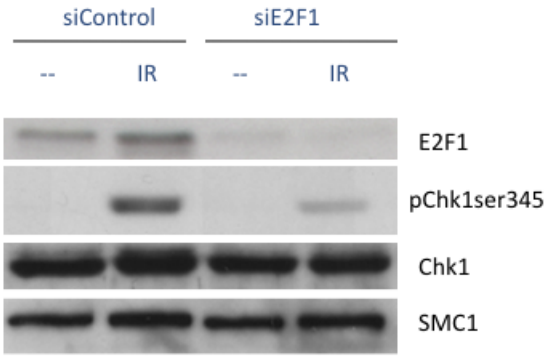


Figure 3.10 The absence of E2F1 affects phosphorylation of Chk1.

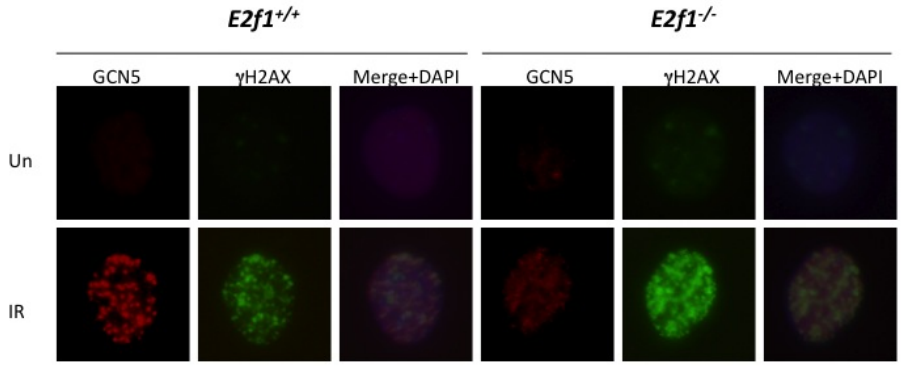
NHFs were transfected with E2F1 siRNA or control siRNA. 48 h after transfection, cells were untreated or exposed to 10 Gy of IR and then incubated for one h. Whole cell extract was used for western blot analysis using antibodies for: total E2F1, total Chk1, phospho-Chk1 at serine 345 site and total SMC1.

3.3.5 The absence of E2F1 impairs GCN5 foci formation in response to DNA damage.

Our previous work demonstrated that E2F1 associates with GCN5 and recruits GCN5 to sites of UV-induced DNA damage (144) E2F1-dependent recruitment of GCN5 is associated with increased H3K9 acetylation at sites of UV damage and enhanced recruitment of DNA repair factors, including XPC and XPA.

To investigate whether E2F1 recruits GCN5 to sites of DNA double-strand breaks, the ability of GCN5 to form foci was examined by IF in wild type and *E2f1* knockout MAFs. The result showed that in response to IR-induced DNA double-strand breaks, GCN5 formed multiple foci in wild type cells. However, GCN5 foci formation was significantly impaired in cells lacking E2F1 (Figure 3.10A). Around 70% of wild type cells showed GCN5 foci formation while fewer than 20% cells lacking E2F1 displayed GCN5 foci (Figure 3.10B). In contrast, γH2AX foci formation increased in the absence of E2F1 as previously observed.

A


E2f1+/+
E2f1-/-
GCN5
γH2AX
Merge+DAPI
GCN5
γH2AX
Merge+DAPI
Un
IR

B

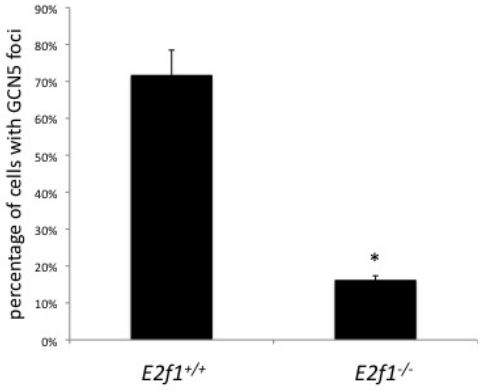

percentage of cells with GCN5 foci
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
*
E2f1+/+
E2f1-/-

Figure 3.11 The absence of E2F1 impairs GCN5 foci formation in response to DNA damage.

A. Primary wild type and *E2f1* knockout MAFs were untreated or treated with 5 Gy of IR and then incubated for one h. Cells were IF stained for GCN5 and γH2AX. DAPI was used as a counterstain.

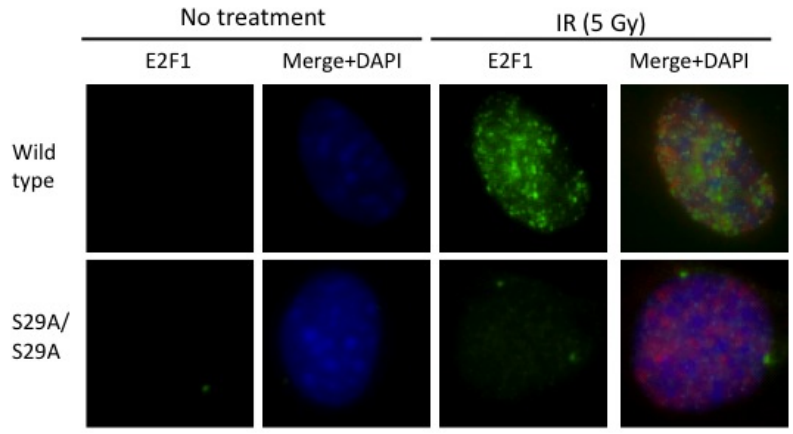
B. Images were analyzed by foci counting. The percentage of cells presenting different foci number was calculated after scoring more than 100 cells for each genotype under microscope. *indicates statistically significant difference ($p<0.05$).

3.3.6 Mouse E2F1 serine 29 is required for the accumulation of E2F1 at sites of DNA double-strand breaks.

As a tool for studing the role of E2F1 in the DNA damage response, Ihave generated a knock-in mouse model in which E2F1 sequences encoding serine 29, which is equivlent to human E2F1 serine 31, were altered to encode alanine (S29A). Lines from two independent ES cell clones have been generated and heterozygous mice were crosed to generate *E2f1*$^{S29A/S29A}$ homozygous knock-in mice.

MEFs were generated from *E2f1*$^{S29A/S29A}$ and wild type sibling embryos and were used to examine E2F1 expression and localization in response to IR. As predicted, expression of E2F1 was decreased in *E2f1*$^{S29A/S29A}$ cells compared to wild type cells following IR treatment (data not shown). IF staining showed that E2F1 foci formation in response to IR was impaired in S29A mutant cells (Figure 3.11). Moreover, primary keratinocytes from *E2f1*$^{S29A/S29A}$ mice displayed increased levels of spontaneous breaks, similar to *E2f1*$^{-/-}$ mice (Figure 3.12). In addtion, NBS1 foci formation was significantly impaired in *E2f1*$^{S29A/S29A}$ MEFs just like in *E2f1*$^{-/-}$ MEFs (Figure 3.13). Taken together, these findings indicate the S29A knock-in mutation leads to many of the same cellular phenotypes observed as when *E2f1* is knocked out.

A


No treatment
IR (5 Gy)
E2F1
Merge+DAPI
E2F1
Merge+DAPI
Wild type
S29A/ S29A

B

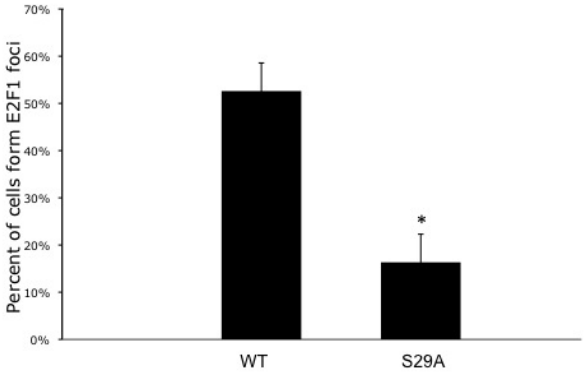

Percent of cells form E2F1 foci
70%
60%
50%
40%
30%
20%
10%
0%
*
WT
S29A

Figure 3.12 S29A mutantion of mouse E2F1 impaires its localization to DNA double-strand break sites.

A. Primary wild type and E2F1 S29A mutant MEFs were untreated or treated with 5 Gy of IR and then incubated for one h. Cells were IF stained using E2F1 antibody. DAPI was used as a counterstain.

B. Images were analyzed by foci counting. The percentage of cells presenting different foci number was calculated after scoring more than 100 cells for each genotype.

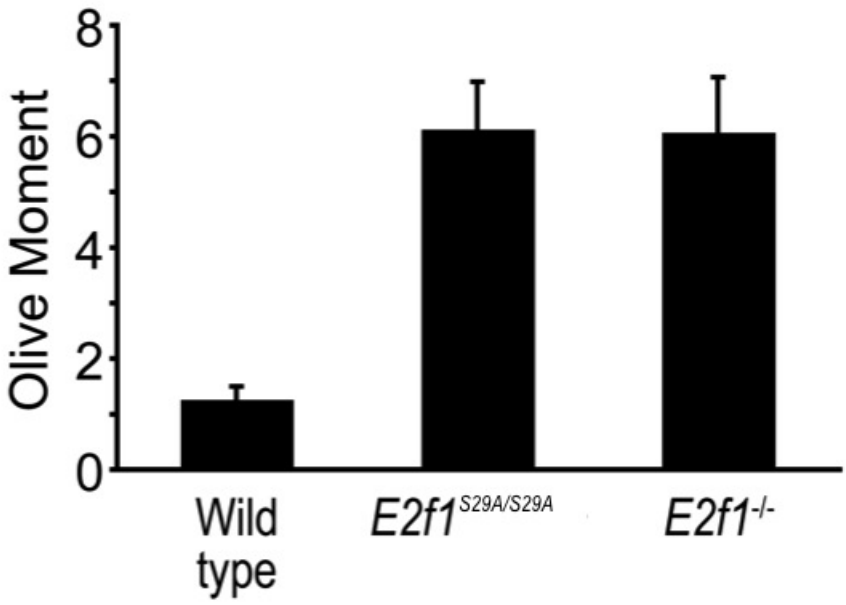
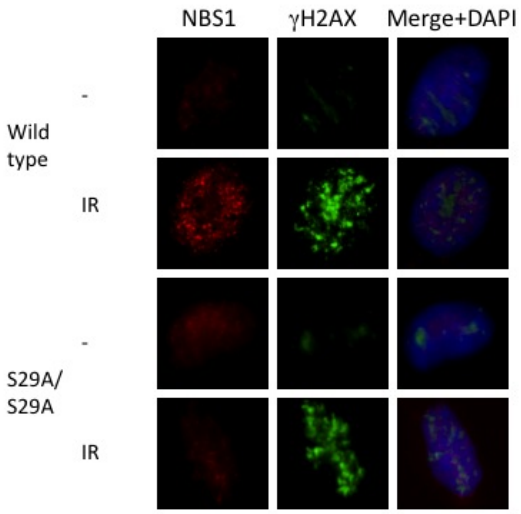


Figure 3.13 S29A mutantion of E2F1 leads to accumulation of endogenous DNA damage.

Primary keratinocytes were isolated from wild type, *E2f1*$^{S29A/S29A}$ and *E2f1*$^{-/-}$ mice and subjected to the comet assay. The average Olive moment was calculated from 70 cells per genotype in three independent experiments.

A


NBS1
γH2AX
Merge+DAPI
-
Wild type
IR
-
S29A/ S29A
IR

B

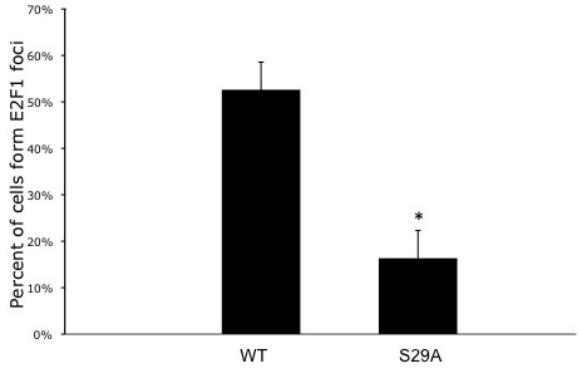

Percent of cells form E2F1 foci
70%
60%
50%
40%
30%
20%
10%
0%
*
WT
S29A

Figure 3.14 The E2F1 S29A mutation impairs NBS1 foci formation in response to IR

A. Primary wild type and *E2f1*$^{S29A/S29A}$ MEFs were untreated or treated with 5 Gy of IR. After exposure to IR, cells were incubated for one h and used to perform IF staining with NBS1 and γH2AX antibodies. DAPI was used as a counterstain.

B. The percentage of NBS1 cells displaying foci was calculated by analyzing IF images as in A. The result was determined for 100 cells for each genotype from three independent experiments. *indicates statistically significant difference ($p<0.05$).

3.4 Discussion

The E2F1 protein is now known to play important roles in the response to DNA damage. Other reports demonstrated that E2F3, E2F7 and E2F8 are also involved in the DNA damage response, but E2F1 is indispensible for their functions (145, 146). The mechanisms underlying a role for E2F1 in the DNA damage response, which may be related to its function in tumor suppression, are controversial and require detailed investigation.

Here I have clearly demonstrated that E2F1 contributes to genome stability and DNA double-strand break repair by monitoring γH2AX levels and DNA breaks by the comet assay. However, the mechanism by which E2F1 regulates DNA repair has been debated. Some groups found that E2F1 transcriptionally up-regulates the DDB2 protein and/or XPC protein in response to UV-induced DNA damage, while other groups have demonstrated that E2F1 is also responsible for transcription of genes for homologous recombination repair, mismatch repair and base excision repair (147-151). While the significance of transcriptional regulation by E2F1 cannot be underestimated for apoptosis induction, the role of E2F1-mediated transcriptional regulation in DNA repair is unclear. Most of the genes for DNA repair mentioned above are apparently regulated by other E2F family members as well as other transcription factors, such as p53, and E2F1 has not been shown to be the dominant player in their regulation network (152). In addition, the modest upregulation of these DNA repair genes in response to damage is delayed compared to the more rapid effects of E2F1 status on NER factor recruitment and repair, which occur within

one hour. More importantly, I also demonstrated that mutation of the DNA binding domain or deletion of transactivation domain of E2F1 does not affect its ability to stimulate repair of UV-induced damage (118). Therefore, I examined potential non-transcriptional functions of E2F1 in DNA double-strand break repair.

First, I investigated which portion of the DNA damage response pathway is affected by the absence of E2F1. Although the level of phospho-ATM is not changed in *E2f1* knockout primary fibroblasts, NBS1, an essential component of the MRN complex, requires E2F1 for its ability to form foci at sites of DNA double-strand breaks. This phenomenon was confirmed in normal human fibroblasts depleted of E2F1 by specific small interference RNA. I also observed that phosphorylation of NBS1 is significantly decreased in the absence of E2F1. Previous studies have shown that NBS1 was phosphorylated by the ATM kinase (77, 153). It should be noted that total levels of NBS1 are not affected by the absence or knockdown of E2F1, indicating that E2F1 is involved in DNA double-strand break repair in a transcription-independent manner.

I then examined how E2F1 affects the recruitment of other DNA repair proteins in addition to NBS1. I found that RPA2 foci formation in response to DNA double-strand breaks is also significantly impaired in the absence of E2F1. As the coating protein for exposed single-stranded DNA, RPA foci formation serves as a marker for DNA end resection at DNA double-strand breaks. At the same time, I did not observe any change in total RPA2 protein level by western

blot in the presence or absence of E2F1, which indicates that E2F1 affects RPA foci formation independent of its transcriptional regulation.

I also studied Rad51, the DNA recombinase that is essential for the final steps of homologous recombination. Localization of Rad51 to sites of DNA damage is one of the most important indicators of functional homologous recombination repair (154). In *E2f1* knockout cells, Rad51 foci formation is significantly impaired. However, 53BP1, another common marker of DNA double-strand breaks, still forms foci in response to DNA damage despite the absence of E2F1. I also observed that the protein levels of Rad51 are dramatically increased in response to DNA damage in wild type cells, possibly indicating DNA damage related protein stabilization, which is significantly impaired in the *E2f1* knockout cells. However, it should be noted that the basal levels of Rad51 before induction by DNA damage are very low in both wild type and *E2f1* knockout cells, indicating that E2F1 is not involved in the basal transcription of Rad51. Although another report suggested that E2F1 might be the major transcription factor for Rad51 gene expression (155), our finding with quantitative RT-PCR analysis of wild type and *E2f1* knockout cells did not reveal any difference in the levels of Rad51 mRNA, both before and after DNA double-strand break induction. The impaired increase in Rad51 protein levels following DNA double-strand break induction in cells lacking E2F1 is most likely due to an unknown mechanism that affects the protein stability of Rad51. Further investigation will be necessary to answer this intriguing question.

To further confirm that depletion of E2F1 affects DNA end resection, I also examined Chk1 phosphorylation, which is a downstream event of ATR signaling activated by RPA-covered single-stranded DNA. The phosphorylation level of Chk1 at serine 345 decreased significantly in E2F1 knockdown NHFs compared to control NHFs after IR treatment while the total Chk1 level did not change. This result further supports that E2F1 status affects recruitment of DNA repair proteins, especially DNA end resection factors, to sites of DNA double-strand breaks.

Since I have observed that several key steps of DNA double-strand break repair are regulated by E2F1, the underlying mechanism for this regulation is very meaningful. As mentioned previously, chromatin modification plays critical roles in the signal transduction of the DNA damage response, and our previous study has shown a role for E2F1 in histone acetylation following UV irradiation. With this in mind, I examined the localization of several chromatin modifiers that may serve as partners of E2F1 in chromatin modification at sites of DNA double-strand breaks, including Tip60, TRRAP, RNF8 (data not shown) and GCN5. Among these proteins, only localization of GCN5 to sites of DNA damage was found to be significantly impaired in the absence of E2F1, which was decreased by more than 3-fold. This is consistent with our findings in the UV damage response, where I found that E2F1 recruits GCN5 leading to histone H3 lysine 9 acetylation at local areas of DNA damage. However, I was unable to detect localization of acetylated H3 lysine 9 at sites of DNA double-strand breaks by

immunostaining. Further studies will be necessary to investigate the specific chromatin changes at sites of DNA double-strand breaks regulated by E2F1.

How protein modifications regulate the functions of E2F1 in response to DNA double-strand breaks is also a critical question to address. It has been well documented that E2F1 is phosphorylated at serine 31 by ATM or ATR in response to different types of DNA damage. In response to UV damage, it appears that ATR is the major player, while ATM is more important in the response to DNA double-strand breaks. Under either circumstance. E2F1 can be recruited to sites of DNA damage. While the role of E2F1 in UV damage repair has been well demonstrated, its function at sites of double-strand breaks is still not elucidated. Another group showed that E2F1 is recruited to sites of DNA double-strand breaks through a phospho-specific interaction with TopBP1. Here I confirmed that E2F1 serine 29 in mouse, which is equivalent to human serine 31, is indeed required for localization of E2F1 to sites of DNA double-strand breaks as mutation of serine 29 leads to a significant decrease in E2F1 foci formation. In addition, S29A knock-in primary mouse fibroblasts displayed significantly impaired NBS1 foci formation compared to the wild type cells, indicating that serine 29 phosphorylation is important for the function of E2F1 in DNA repair. This is consistent with our results that serine 29 knock-in primary mouse fibroblasts showed significantly increased levels of spontaneous DNA breaks as demonstrated by the comet assay. These findings support the hypothesis that ATM-mediated phosphorylation of E2F1 is required for its localization to sites of DNA double-strand breaks as well as its functions in DNA repair.

Overall, I have shown that E2F1 is involved in the DNA double-strand break response and important for maintaining genome stability. E2F1 status affects NBS1, RPA and Rad51 foci formation, the phosphorylation of NBS1 and Rad51 protein stabilization in response to DNA damage. This appears to be achieved in a transcription-independent manner and may involve GCN5 as a partner of E2F1. The role of chromatin modifications, such as histone acetylation, requires further investigation. In addition, I provide evidence to support the hypothesis that the phosphorylation of E2F1 serine 31 (29 in mouse) by the ATM kinase is a critical event that brings E2F1 to sites of DNA damage and is required for proper regulation of DNA repair.

Chapter 4. Summary and future directions

4.1 Summary

E2F1 possesses the ability to promote cell cycle progression and cell survival, as well as apoptosis induction and DNA repair. The role of E2F1 in tumor development is complex and context-dependent. In this thesis, I reveal novel roles for E2F1 in response to DNA double-strand breaks that may have important implications for tumor suppression. It was demonstrated that E2F1 protein levels increase after DNA damage, which involves serine 31 phosphorylation event by the ATM or ATR kinase. Furthermore, it was also shown that this phosphorylation is required for E2F1 binding to TopBP1, which suppresses apoptosis by inhibiting E2F1 transcriptional activity. TopBP1 binding also recruits E2F1 to sites of DNA double-strand breaks to form foci that co-localize with other DNA double-strand break markers such as γH2AX. Moreover, in my previous project, I showed that E2F1 is recruited to UV-induced DNA damage and directly affects the local chromatin structure by recruiting GCN5 and promoting histone H3 acetylation at lysine 9 specifically, which may regulate the accessibility of the DNA repair machinery. This function in UV damage repair is entirely transcription-independent and serine 31 phosphorylation is critical for this repair activity. Based on the data above, I hypothesize that E2F1 may also promote DNA double-strand break repair through a transcription-independent mechanism.

To test the efficacy of the above hypothesis, I examined the role of E2F1 in the maintenance of genomic integrity and DNA double-strand break repair. In support of our hypothesis, I observed significant chromosomal abnormalities in

primary keratinocytes and spontaneous DNA damage in primary fibroblasts from *E2f1* knockout mice, indicated by γH2AX immunofluorescence staining and the comet assay. This is a common feature of cells lacking “caretaker” genes, which are important for genomic stability and play critical roles in DNA repair (156). I confirmed this assumption using both the comet assay for DNA breaks removal and examination of γH2AX levels after ionizing radiation, which indicated significantly delayed repair in *E2f1* knockout primary fibroblasts. Therefore, I speculate that E2F1 functions in the repair of DNA double-strand breaks to maintain genomic stability, which may relate to its role in tumor suppression.

Since I have found that E2F1 contributes to DNA double-strand break repair, I wanted to explore the underlying mechanism. NBS1, as part of the MRN complex, is one of the first proteins to be recruited to sites of double-strand breaks and participates in both NHEJ and HR. I identified that phosphorylation and foci formation of NBS1 in response to DNA double-strand breaks is significantly impaired in E2F1 knock down or knockout cells, while the total protein levels of NBS1 are not affected. Likewise, RPA foci formation but not RPA protein levels, was also impaired by E2F1 deficiency. To examine a further downstream component of DNA double-strand break repair, I looked at Rad51 foci formation, since Rad51 is the major recombinase for the process of homologous recombination. I found that Rad51 foci formation was also impaired in the absence of E2F1. Interestingly, I observed decreased Rad51 protein levels in *E2f1* knockout cells but quantitative RT-PCR did not reveal any difference in *Rad51* mRNA levels between wild type and *E2f1* knockout cells, suggesting that

E2F1 affects post-translational regulation of the Rad51 protein. I also found E2F1 deficiency affects phosphorylation of Chk1 by ATR, which requires recruitment of the ATR-ATRIP complex to RPA coated single-stranded DNA. All of these results point to a role for E2F1 in DNA end resection and the formation of single-stranded DNA at sites of double-strand breaks.

To further investigate the mechanism how E2F1 affects these DNA damage response factors, I demonstrated that the histone acetyltransferase GCN5 is recruited to sites of DNA double-strand breaks and loss of E2F1 significantly decreases GCN5 foci formation, which is similar to what was observed in the response to UV damage. However, further studies are required to reveal the role of GCN5 in E2F1-mediated repair of DNA double-strand breaks.

I have also hypothesized that serine 31 phosphorylation of E2F1 is the major signal for E2F1 to act as a DNA repair protein instead of a transcription factor. While this theory was confirmed for UV-induced DNA damage repair (134) and suggested for double-strand break repair by other studies (113). I performed a more thorough investigation and found that phosphorylation of E2F1 serine 31 (serine 29 in mice) is required for both its recruitment to sites of DNA double-strand breaks and its functions in DNA repair using cells from a novel *E2f1* S29A knock-in mouse model. In particular, I find that *E2f1* S29A knock-in cells display a similar extent of decreased NBS1 foci formation as well as genomic stability, comparable to *E2f1* knockout cells.

Taken together, I conclude that E2F1 regulates the recruitment and/or retention of several proteins at sites of double-strand breaks to contribute to DNA repair. This may involve the modification of chromatin structure by GCN5 or other chromatin modifiers. In addition, serine 31/29 phosphorylation of E2F1 is a critical upstream regulatory event for E2F1 to function in the DNA damage response. Overall, this novel function in directly regulating DNA double-strand break repair is likely important to maintain genomic stability, which may explain how E2F1 functions as a tumor suppressor.

4.2 Future directions

Various cancer therapeutic agents function through DNA damage induction especially causing DNA double-strand breaks. Comprehensive understanding of the underlying mechanisms of the response to these agents can help us tailor more effective treatment strategies. Therefore it will be of great significance to further uncover the novel role of E2F1 in the DNA damage response. I propose that the following areas of research will benefit further advances of this project.

- Detailed mechanisms underlying regulation of NBS1 by E2F1 in the DNA double-strand break response
- Functions of E2F1 in end resection and homologous recombination repair
- Detailed mechanisms underlying regulation of GCN5 by E2F1 in the DNA double-strand break response
- *In vivo* Investigations of the S29A knock-in mouse model

These areas will be elaborated in details as follows.

4.2.1 Detailed mechanisms underlying regulation of NBS1 by E2F1 in the DNA double-strand break response

As discussed in Chapter 1, MDC1 is recruited to DNA double-strand break sites by directly binding to γH2AX. This event recruits the E3 ligase RNF8, which in turn recruits RNF168, another E3 ligase that further amplifies ubiquitylation of the histone H2A to generate uH2A for the accessibility of DNA repair machinery. In particular, RNF168 is important for the recruitment of BRCA1 through the ubiquitin-binding protein Rap80 and ABRA1(85). Therefore, it will be interesting to know whether E2F1 deficiency affects recruitment of MDC1, RNF8 and RNF168 to DNA double-strand breaks. An important control for these experiments is to determine if E2F1 deficiency affects the expression level of these proteins by western blot analysis. If MDC1 levels are unaffected by E2F1 deficiency but recruitment to sites of DNA double-strand breaks is impaired, this could indicate that MDC1 is unable to gain access to γH2AX since H2AX phosphorylation is normal or even higher in E2F1 deficient cells. If MDC1 is recruited to sites of DNA double-strand breaks but NBS1 and RNF8 are not (even through they are expressed normally), then this would imply that NBS1 and RNF8 associations with MDC1 are regulated by E2F1.

It will also be necessary to examine whether these factors affect the interaction between E2F1 and NBS1 if they function upstream of the pathway. The potential impact of E2F1 on MDC1-NBS1 and MDC1-RNF8 interactions should be investigated as well. Whether E2F1 status affects the formation of uH2A will provide direct evidence that E2F1 plays a role in chromatin

modification in response to DNA damage. The results provided from the above experiments can potentially provide the crucial information for our findings of impaired DNA double-strand break repair in cells lacking E2F1.

Primary cells isolated from *E2f1* $^{S29A/S29A}$ mice can be used to examine whether the serine 29 mutation causes the same effects as E2F1 knockout in response to IR or other DNA double-strand break inducing agents. Our preliminary data has already shown that NBS1 foci formation is impaired in *E2f1* $^{S29A/S29A}$ cells similar to *E2f1* knockout cells. Future experiments can investigate whether E2F1 serine 29 mutation also affects Rad51 and RPA recruitment to DNA double-strand break sites by both IF staining and a well-controlled DNA double-strand break generating system developed by the Kastan group (77). Briefly, this group developed an ER-I-Ppol/ChIP assay, which introduces the inducible expression of a rare cutting endonuclease in cells to create DNA double-strand break at specific loci in the genome. A ChIP assay can then identify histone modifications and proteins that are recruited to sites of double-strands break.

Recently, Komatsu's group reported NBS1 could recruit Rad18 and regulates translesion DNA synthesis (TLS) (157). This raises the possibility that E2F1 affects NBS1 and Rad18 in TLS besides homologous recombination repair to facilitate maintenance of genome stability. It will therefore be interesting to examine whether E2F1 status also affects recruitment of Rad18 and TLS to sites of double-strand breaks.

4.2.2 Functions of E2F1 in end resection and homologous recombination repair

In my study, I presented evidence that E2F1 affects l DNA end resection at DNA double-strand break sites. To further understand this pathway, it will be beneficial to examine effects of E2F1 on the subcellular localization and activation of other essential homologous recombination repair factors, such as BRCA1, BRCA2 and Rad52. In addition, it will be critical to know whether the status of E2F1 eventually affects homologous recombination repair efficiency. Southern blotting is a traditional way to evaluate homologous recombination repair efficiency. An I-Scel-based homologous recombination assay measured by random-plasmid integration will also be a good tool to detect homologous recombination repair (158).

If the above results are confirmed, further study in experimental animal should be applied. A C57BL/6J $p^{un/un}$ mouse model was established for measuring the frequency of homologous recombination *in vivo* (159). The murine pigmentation (*p*) gene is normally transcribed in melanocytes and cells of the RPE. The duplication allele, called pink-eyed unstable (p^{un}), interrupts the *p* gene and leads to pink eyes and visible fur-spots in the mice. Homologous recombination events are required for accurate *p* gene reconstitution. Bishop et al has successfully investigated the roles of *BRCA1* and *Blm*, which are two homologous recombination repair factors, in Cre-C57BL/6J $p^{un/un}$ mice (160). Therefore, I can also utilize this mouse model to detect the role of E2F1 in homologous recombination repair in vivo in a similar manner by crossing it with wild type, *E2f1*

$^{-/-}$ and *E2f1* $^{S29A/S29A}$ mice.

The evaluation of the function of E2F1 in homologous recombination repair will shed a light on therapeutic strategies in tumors with abnormally low E2F1 expression.

4.2.3 Detailed mechanisms underlying regulation of GCN5 by E2F1 in the DNA double-strand break response

I proposed that E2F1 promotes H3K9 acetylation at sites of DNA damage by recruiting GCN5 through direct protein-protein interaction, which probably leads to chromatin relaxation for the recruitment of DNA repair proteins. Similar to the cellular response to UV, IR and other agents that induce DNA double-strand break cause a general relaxation of chromatin that peaks at about 1 hour after exposure (161, 162). Therefore, it will be interesting to examine a possible role for E2F1 in modifying chromatin structure in response to DNA double-strand break.

We could perform an assay to detect a possible interaction between E2F1 and GCN5 in response to DNA double-strand breaks. Following this, we could examine the possible effects of E2F1 and GCN5 on histone modifications in the ER-I-PpoI/ChIP assay described previously. By introducing this system into cells from *E2f1*$^{-/-}$, *E2f1* $^{S29A/S29A}$, and *Gcn5*$^{hat/hat}$ (cells without GCN5 HAT activity) mice, I will confirm the recruitment of E2F1 and GCN5 to sites of DNA double-strand break, as well as the regulation of GCN5 by E2F1. Following that, we can determine if H3K9 acetylation at sites of DNA double-strand break is dependent on E2F1 and the HAT activity of GCN5. These cells can also be used to directly

determine the role of E2F1 and GCN5 in chromatin relaxation in response to IR by the micrococcal nuclease assay.

To further examine if GCN5 is important for E2F1-dependent processes at DNA double-stand break, we can examine NBS1, RPA and Rad51 foci formation and H2A ubiquitination in cells deficient for GCN5. DNA damage repair efficiency can also be examined in cells lacking GCN5.

4.2.4 *In vivo* Investigations of the S29A knock-in mouse model

As discussed in the previous chapter, our lab developed an *E2f1* serine 29 mutant mouse model to further investigate the significance of E2F1 phosphorylation by ATM in response to DNA double-strand breaks. With this model, we can determine whether serine 29 phosphorylation is required for the function of E2F1 in response to IR or other DNA double-strand break inducing agents *in vivo*.

First of all, an IR sensitivity experiment can be performed on these mice to examine the importance of E2F1 in the resistance to DNA damage. Briefly, wild type and *E2f1* $^{S29A/S29A}$ mice can be exposed to different doses of IR and monitored for survival as well as tumorigenesis. Some of the mice can be sacrificed after IR treatment to examine effects on the internal organs. Other mice can be exposed to low dose IR and observed for lymphomagenesis and development of other cancers.

In addition, *in vivo* experiments to study the functions of E2F1 in S29A mutant in response to oncogenic stress will be of great significance as well. For this *E2f1* $^{S29A/S29A}$ mice can be crossed with Myc transgenic mice to investigate

the effects of E2F1 serine 29 mutation on the oncogenic stress response induced by Myc overexpression. Previous studies in our lab have shown that transgenic expression of Myc induces DNA damage and activates ATM (129). Our Previous studies have also demonstrated that the absence of E2F1 accelerated tumor development in the K5 Myc transgenic model. Repeating this experiment with the E2F1 S29A knock-in model will give more definitive clues about the significance of E2F1 in the DNA damage response with respect to tumor suppression.

Reference:

1. Polager, S., Y. Kalma, E. Berkovich, and D. Ginsberg. 2002. E2Fs up-regulate expression of genes involved in DNA replication, DNA repair and mitosis. Oncogene 21:437-446.
2. Ishida, S., E. Huang, H. Zuzan, R. Spang, G. Leone, M. West, and J. R. Nevins. 2001. Role for E2F in control of both DNA replication and mitotic functions as revealed from DNA microarray analysis. Molecular and cellular biology 21:4684-4699.
3. Ma, Y., R. Croxton, R. L. Moorer, Jr., and W. D. Cress. 2002. Identification of novel E2F1-regulated genes by microarray. Archives of biochemistry and biophysics 399:212-224.
4. Muller, H., A. P. Bracken, R. Vernell, M. C. Moroni, F. Christians, E. Grassilli, E. Prosperini, E. Vigo, J. D. Oliner, and K. Helin. 2001. E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. Genes & development 15:267-285.
5. Ren, B., H. Cam, Y. Takahashi, T. Volkert, J. Terragni, R. A. Young, and B. D. Dynlacht. 2002. E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. Genes & development 16:245-256.
6. DeGregori, J., and D. G. Johnson. 2006. Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis. Current molecular medicine 6:739-748.

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

8. Hallstrom, T. C., and J. R. Nevins. 2003. Specificity in the activation and control of transcription factor E2F-dependent apoptosis. Proc Natl Acad Sci U S A 100:10848-10853.

9. DeGregori, J., G. Leone, A. Miron, L. Jakoi, and J. R. Nevins. 1997. Distinct roles for E2F proteins in cell growth control and apoptosis. Proceedings of the National Academy of Sciences of the United States of America 94:7245-7250.

10. Johnson, D. G., J. K. Schwarz, W. D. Cress, and J. R. Nevins. 1993. Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature 365:349-352.

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

12. Pan, H., C. Yin, N. J. Dyson, E. Harlow, L. Yamasaki, and T. V. Dyke. 1998. Key roles for E2F1 in signaling p53-dependent apoptosis and in cell division within developing tumors. Mol. Cell Biol. 2:283-292.

13. Tsai, K. Y., Y. Hu, K. F. Macleod, D. Crowley, L. Yamasaki, and T. Jacks. 1998. Mutation of *E2f-1* suppresses apoptosis and inappropriate S phase entry and extends survival of *Rb*-deficient mouse embryos. Mol. Cell 2:293-304.

14. Shan, B., and W. H. Lee. 1994. Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. Molecular and cellular biology 14:8166-8173.

15. Qin, X. Q., D. M. Livingston, W. G. Kaelin, Jr., and P. D. Adams. 1994. Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proceedings of the National Academy of Sciences of the United States of America 91:10918-10922.

16. Wu, X., and A. J. Levine. 1994. p53 and E2F-1 cooperate to mediate apoptosis. Proceedings of the National Academy of Sciences of the United States of America 91:3602-3606.

17. Paulson, Q. X., M. J. McArthur, and D. G. Johnson. 2006. E2F3a stimulates proliferation, p53-independent apoptosis and carcinogenesis in a transgenic mouse model. Cell cycle (Georgetown, Tex 5:184-190.

18. Martinez, L. A., E. Goluszko, H. Z. Chen, G. Leone, S. Post, G. Lozano, Z. Chen, and A. Chauchereau. E2F3 is a mediator of DNA damage-induced apoptosis. Molecular and cellular biology 30:524-536.

19. Lazzerini Denchi, E., and K. Helin. 2005. E2F1 is crucial for E2F-dependent apoptosis. EMBO reports 6:661-668.

20. Pierce, A. M., I. B. Gimenez-Conti, R. Schneider-Broussard, L. A. Martinez, C. J. Conti, and D. G. Johnson. 1998. Increased E2F1 activity induces skin tumors in mice heterozygous and nullizygous for p53. Proc. Natl. Acad. Sci. USA 95:8858-8863.

21. Kowalik, T. F., J. DeGregori, J. K. Schwarz, and J. R. Nevins. 1995. E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. Journal of virology 69:2491-2500.

22. Irwin, M., M. C. Marin, A. C. Phillips, R. S. Seelan, D. I. Smith, W. Liu, E. R. Flores, K. Y. Tsai, T. Jacks, K. H. Vousden, and W. G. Kaelin, Jr. 2000. Role for the p53 homologue p73 in E2F-1-induced apoptosis. Nature 407:645-648.

23. Moroni, M. C., E. S. Hickman, E. Lazzerini Denchi, G. Caprara, E. Colli, F. Cecconi, H. Muller, and K. Helin. 2001. Apaf-1 is a transcriptional target for E2F and p53. Nature cell biology 3:552-558.

24. Vorburger, S. A., A. Pataer, K. Yoshida, G. N. Barber, W. Xia, P. Chiao, L. M. Ellis, M. C. Hung, S. G. Swisher, and K. K. Hunt. 2002. Role for the double-stranded RNA activated protein kinase PKR in E2F-1-induced apoptosis. Oncogene 21:6278-6288.

25. Stiewe, T., and B. M. Putzer. 2000. Role of the p53-homologue p73 in E2F1-induced apoptosis. Nature genetics 26:464-469.

26. Bates, S., A. C. Phillips, P. A. Clark, F. Stott, G. Peters, R. L. Ludwig, and K. H. Vousden. 1998. p14ARF links the tumour suppressors RB and p53. Nature 395:124-125.

27. Hershko, T., M. Chaussepied, M. Oren, and D. Ginsberg. 2005. Novel link between E2F and p53: proapoptotic cofactors of p53 are transcriptionally upregulated by E2F. Cell death and differentiation 12:377-383.

28. Sherr, C. J. 2006. Divorcing ARF and p53: an unsettled case. Nature reviews 6:663-673.

29. Powers, J. T., S. Hong, C. N. Mayhew, P. M. Rogers, E. S. Knudsen, and D. G. Johnson. 2004. E2F1 uses the ATM signaling pathway to induce p53 and Chk2 phosphorylation and apoptosis. Molecular Cancer Research 2:203-214.

30. Rogoff, H. A., M. T. Pickering, F. M. Frame, M. E. Debatis, Y. Sanchez, S. Jones, and T. F. Kowalik. 2004. Apoptosis associated with deregulated E2F activity is dependent on E2F1 and Atm/Nbs1/Chk2. Molecular and cellular biology 24:2968-2977.

31. Kurz, E. U., and S. P. Lees-Miller. 2004. DNA damage-induced activation of ATM and ATM-dependent signaling pathways. DNA repair 3:889-900.

32. Phillips, A. C., M. K. Ernst, S. Bates, N. R. Rice, and K. H. Vousden. 1999. E2F-1 potentiates cell death by blocking antiapoptotic signaling pathways. Mol Cell 4:771-781.

33. Pierce, A. M., S. M. Fisher, C. J. Conti, and D. G. Johnson. 1998. Deregulated expression of E2F1 induces hyperplasia and cooperates with ras in skin tumor development. Oncogene 16:1267-1276.

34. Johnson, D. G., and J. DeGregori. 2006. Putting the oncogenic and tumor suppressive activities of E2F into context. Current molecular medicine 6:731-738.

35. Yamasaki, L., R. Bronson, B. O. Williams, N. J. Dyson, E. Harlow, and T. Jacks. 1998. Loss of E2F-1 reduces tumorigenesis and extends the lifespan of Rb1(+/-) mice. Nat. Genet. 18:360-364.

36. Pierce, A. M., R. Schneider-Broussard, I. B. Gimenez-Conti, J. L. Russell, C. J. Conti, and D. G. Johnson. 1999. E2F1 has both oncogenic and tumor-suppressive properties in a transgenic model. Mol Cell Biol 19:6408-6414.

37. Johnson, D. G., W. D. Cress, L. Jakoi, and J. R. Nevins. 1994. Oncogenic capacity of the E2F1 gene. Proc. Natl. Acad. Sci. U S A 91:12823-12827.

38. Xu, G., D. M. Livingston, and W. Krek. 1995. Multiple members of the E2F transcription factor family are the products of oncogenes. Proc. Natl. Acad. Sci. USA 92:1357-1361.

39. Olson, M. V., D. G. Johnson, H. Jiang, J. Xu, M. M. Alonso, K. D. Aldape, G. N. Fuller, B. N. Bekele, W. K. Yung, C. Gomez-Manzano, and J. Fueyo. 2007. Transgenic E2F1 expression in the mouse brain induces a human-like bimodal pattern of tumors. Cancer research 67:4005-4009.

40. Agger, K., E. Santoni-Rugiu, C. Holmberg, O. Karlstrom, and K. Helin. 2005. Conditional E2F1 activation in transgenic mice causes testicular atrophy and dysplasia mimicking human CIS. Oncogene 24:780-789.

41. Conner, E. A., E. R. Lemmer, M. Omori, P. J. Wirth, V. M. Factor, and S. S. Thorgeirsson. 2000. Dual functions of E2F-1 in a transgenic mouse model of liver carcinogenesis. Oncogene 19:5054-5062.

42. Russell, J. L., R. L. Weaks, T. R. Berton, and D. G. Johnson. 2006. E2F1 suppresses skin carcinogenesis via the ARF-p53 pathway. Oncogene 25:867-876.

43. Weinberg, R. A. 1996. E2F and cell proliferation: a world turned upside down. Cell 85:457-459.

44. Yamasaki, L., T. Jacks, R. Bronson, E. Goillot, E. Harlow, and N. J. Dyson. 1996. Tumor induction and tissue atrophy in mice lacking E2F-1. Cell 85:

537-548.

45. Field, S. J., F.-Y. Tsai, F. Kuo, A. M. Zubiaga, W. G. Kaelin, Jr., D. M. Livingston, S. H. Orkin, and M. E. Greenberg. 1996. E2F-1 functions in mice to promote apoptosis and suppress proliferation. Cell 85:549-561.

46. Rounbehler, R. J., P. M. Rogers, C. J. Conti, and D. G. Johnson. 2002. Inactivation of E2f1 enhances tumorigenesis in a Myc transgenic model. Cancer research 62:3276-3281.

47. Takahashi, Y., J. B. Rayman, and B. D. Dynlacht. 2000. Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. Genes & development 14:804-816.

48. Harper, J. W., and S. J. Elledge. 2007. The DNA damage response: ten years after. Molecular cell 28:739-745.

49. Jackson, S. P., and J. Bartek. 2009. The DNA-damage response in human biology and disease. Nature 461:1071-1078.

50. Hoeijmakers, J. H. 2001. DNA repair mechanisms. Maturitas 38:17-22; discussion 22-13.

51. Hoeijmakers, J. H. 2009. DNA damage, aging, and cancer. The New England journal of medicine 361:1475-1485.

52. Cimprich, K. A., and D. Cortez. 2008. ATR: an essential regulator of genome integrity. Nat Rev Mol Cell Biol 9:616-627.

53. Bartek, J., and J. Lukas. 2007. DNA damage checkpoints: from initiation to recovery or adaptation. Current opinion in cell biology 19:238-245.

54. Shiloh, Y. 2003. ATM and related protein kinases: safeguarding genome integrity. Nature reviews 3:155-168.

55. Riley, T., E. Sontag, P. Chen, and A. Levine. 2008. Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol 9:402-412.

56. Kastan, M. B., and J. Bartek. 2004. Cell-cycle checkpoints and cancer. Nature 432:316-323.

57. Huen, M. S., and J. Chen. 2008. The DNA damage response pathways: at the crossroad of protein modifications. Cell research 18:8-16.

58. Campisi, J., and F. d'Adda di Fagagna. 2007. Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol 8:729-740.

59. Halazonetis, T. D., V. G. Gorgoulis, and J. Bartek. 2008. An oncogene-induced DNA damage model for cancer development. Science (New York, N.Y 319:1352-1355.

60. Rogakou, E. P., D. R. Pilch, A. H. Orr, V. S. Ivanova, and W. M. Bonner. 1998. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. The Journal of biological chemistry 273:5858-5868.

61. Stucki, M., and S. P. Jackson. 2006. gammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes. DNA repair 5:534-543.

62. Giunta, S., R. Belotserkovskaya, and S. P. Jackson. DNA damage signaling in response to double-strand breaks during mitosis. The Journal of cell biology 190:197-207.

63. Al-Hakim, A., C. Escribano-Diaz, M. C. Landry, L. O'Donnell, S. Panier, R. K. Szilard, and D. Durocher. The ubiquitous role of ubiquitin in the DNA damage response. DNA repair 9:1229-1240.

64. Huen, M. S., R. Grant, I. Manke, K. Minn, X. Yu, M. B. Yaffe, and J. Chen. 2007. RNF8 transduces the DNA-damage signal via histone ubiquitylation and checkpoint protein assembly. Cell 131:901-914.

65. Kolas, N. K., J. R. Chapman, S. Nakada, J. Ylanko, R. Chahwan, F. D. Sweeney, S. Panier, M. Mendez, J. Wildenhain, T. M. Thomson, L. Pelletier, S. P. Jackson, and D. Durocher. 2007. Orchestration of the DNA-damage response by the RNF8 ubiquitin ligase. Science (New York, N.Y 318:1637-1640.

66. Mailand, N., S. Bekker-Jensen, H. Faustrup, F. Melander, J. Bartek, C. Lukas, and J. Lukas. 2007. RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins. Cell 131:887-900.

67. Kawanishi, S., Y. Hiraku, S. Pinlaor, and N. Ma. 2006. Oxidative and nitrative DNA damage in animals and patients with inflammatory diseases in relation to inflammation-related carcinogenesis. Biological chemistry 387:365-372.

68. Hartlerode, A. J., and R. Scully. 2009. Mechanisms of double-strand break repair in somatic mammalian cells. The Biochemical journal 423:157-168.

69. West, S. C. 2003. Molecular views of recombination proteins and their control. Nat Rev Mol Cell Biol 4:435-445.

70. Lobrich, M., and P. A. Jeggo. 2007. The impact of a negligent G2/M checkpoint on genomic instability and cancer induction. Nature reviews 7:861-869.

71. Luger, K., A. W. Mader, R. K. Richmond, D. F. Sargent, and T. J. Richmond. 1997. Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 389:251-260.

72. Reeves, R. 2009. HMG nuclear proteins: Linking chromatin structure to cellular phenotype. Biochimica et biophysica acta.

73. Ozaki, T., R. Okoshi, M. Sang, N. Kubo, and A. Nakagawara. 2009. Acetylation status of E2F-1 has an important role in the regulation of E2F-1-mediated transactivation of tumor suppressor p73. Biochemical and biophysical research communications 386:207-211.

74. Smerdon, M. J. 1991. DNA repair and the role of chromatin structure. Current opinion in cell biology 3:422-428.

75. Hara, R., J. Mo, and A. Sancar. 2000. DNA damage in the nucleosome core is refractory to repair by human excision nuclease. Molecular and cellular biology 20:9173-9181.

76. Wang, Z. G., X. H. Wu, and E. C. Friedberg. 1991. Nucleotide excision repair of DNA by human cell extracts is suppressed in reconstituted nucleosomes. The Journal of biological chemistry 266:22472-22478.

77. Berkovich, E., R. J. Monnat, Jr., and M. B. Kastan. 2007. Roles of ATM and NBS1 in chromatin structure modulation and DNA double-strand break repair. Nature cell biology 9:683-690.

78. Chapman, J. R., and S. P. Jackson. 2008. Phospho-dependent interactions between NBS1 and MDC1 mediate chromatin retention of the MRN complex at sites of DNA damage. EMBO reports 9:795-801.

79. Lou, Z., K. Minter-Dykhouse, S. Franco, M. Gostissa, M. A. Rivera, A. Celeste, J. P. Manis, J. van Deursen, A. Nussenzweig, T. T. Paull, F. W. Alt, and J. Chen.

2006. MDC1 maintains genomic stability by participating in the amplification of ATM-dependent DNA damage signals. Molecular cell 21:187-200.

80. Wu, L., K. Luo, Z. Lou, and J. Chen. 2008. MDC1 regulates intra-S-phase checkpoint by targeting NBS1 to DNA double-strand breaks. Proceedings of the National Academy of Sciences of the United States of America 105:11200-11205.

81. Doil, C., N. Mailand, S. Bekker-Jensen, P. Menard, D. H. Larsen, R. Pepperkok, J. Ellenberg, S. Panier, D. Durocher, J. Bartek, J. Lukas, and C. Lukas. 2009. RNF168 binds and amplifies ubiquitin conjugates on damaged chromosomes to allow accumulation of repair proteins. Cell 136:435-446.

82. Panier, S., and D. Durocher. 2009. Regulatory ubiquitylation in response to DNA double-strand breaks. DNA repair 8:436-443.

83. Stewart, G. S. 2009. Solving the RIDDLE of 53BP1 recruitment to sites of damage. Cell cycle (Georgetown, Tex 8:1532-1538.

84. Stewart, G. S., S. Panier, K. Townsend, A. K. Al-Hakim, N. K. Kolas, E. S. Miller, S. Nakada, J. Ylanko, S. Olivarius, M. Mendez, C. Oldreive, J. Wildenhain, A. Tagliaferro, L. Pelletier, N. Taubenheim, A. Durandy, P. J. Byrd, T. Stankovic, A. M. Taylor, and D. Durocher. 2009. The RIDDLE syndrome protein mediates a ubiquitin-dependent signaling cascade at sites of DNA damage. Cell 136:420-434.

85. van Attikum, H., and S. M. Gasser. 2009. Crosstalk between histone modifications during the DNA damage response. Trends in cell biology 19:207-217.

86. Kim, H., J. Chen, and X. Yu. 2007. Ubiquitin-binding protein RAP80 mediates BRCA1-dependent DNA damage response. Science (New York, N.Y 316:1202-1205.

87. Sobhian, B., G. Shao, D. R. Lilli, A. C. Culhane, L. A. Moreau, B. Xia, D. M. Livingston, and R. A. Greenberg. 2007. RAP80 targets BRCA1 to specific ubiquitin structures at DNA damage sites. Science (New York, N.Y 316:1198-1202.

88. Wang, B., and S. J. Elledge. 2007. Ubc13/Rnf8 ubiquitin ligases control foci formation of the Rap80/Abraxas/Brca1/Brcc36 complex in response to DNA damage. Proceedings of the National Academy of Sciences of the United States of America 104:20759-20763.

89. Wang, B., S. Matsuoka, B. A. Ballif, D. Zhang, A. Smogorzewska, S. P. Gygi, and S. J. Elledge. 2007. Abraxas and RAP80 form a BRCA1 protein complex required for the DNA damage response. Science (New York, N.Y 316:1194-1198.

90. Ikura, T., V. V. Ogryzko, M. Grigoriev, R. Groisman, J. Wang, M. Horikoshi, R. Scully, J. Qin, and Y. Nakatani. 2000. Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. Cell 102:463-473.

91. Ikura, T., S. Tashiro, A. Kakino, H. Shima, N. Jacob, R. Amunugama, K. Yoder, S. Izumi, I. Kuraoka, K. Tanaka, H. Kimura, M. Ikura, S. Nishikubo, T. Ito, A. Muto, K. Miyagawa, S. Takeda, R. Fishel, K. Igarashi, and K. Kamiya. 2007. DNA damage-dependent acetylation and ubiquitination of H2AX enhances chromatin dynamics. Molecular and cellular biology 27:7028-7040.

92. Kusch, T., L. Florens, W. H. Macdonald, S. K. Swanson, R. L. Glaser, J. R. Yates, 3rd, S. M. Abmayr, M. P. Washburn, and J. L. Workman. 2004. Acetylation by Tip60 is required for selective histone variant exchange at DNA lesions. Science (New York, N.Y 306:2084-2087.

93. Squatrito, M., C. Gorrini, and B. Amati. 2006. Tip60 in DNA damage response and growth control: many tricks in one HAT. Trends in cell biology 16:433-442.

94. Keogh, M. C., J. A. Kim, M. Downey, J. Fillingham, D. Chowdhury, J. C. Harrison, M. Onishi, N. Datta, S. Galicia, A. Emili, J. Lieberman, X. Shen, S. Buratowski, J. E. Haber, D. Durocher, J. F. Greenblatt, and N. J. Krogan. 2006. A phosphatase complex that dephosphorylates gammaH2AX regulates DNA damage checkpoint recovery. Nature 439:497-501.

95. Doyon, Y., and J. Cote. 2004. The highly conserved and multifunctional NuA4 HAT complex. Curr Opin Genet Dev 14:147-154.

96. Thiriet, C., and J. J. Hayes. 2005. Chromatin in need of a fix: phosphorylation of H2AX connects chromatin to DNA repair. Molecular cell 18:617-622.

97. Morrison, A. J., and X. Shen. 2005. DNA repair in the context of chromatin. Cell cycle (Georgetown, Tex 4:568-571.

98. Morrison, A. J., J. Highland, N. J. Krogan, A. Arbel-Eden, J. F. Greenblatt, J. E. Haber, and X. Shen. 2004. INO80 and gamma-H2AX interaction links ATP-dependent chromatin remodeling to DNA damage repair. Cell 119:767-775.

99. Morrison, A. J., J. A. Kim, M. D. Person, J. Highland, J. Xiao, T. S. Wehr, S. Hensley, Y. Bao, J. Shen, S. R. Collins, J. S. Weissman, J. Delrow, N. J. Krogan, J.

E. Haber, and X. Shen. 2007. Mec1/Tel1 phosphorylation of the INO80 chromatin remodeling complex influences DNA damage checkpoint responses. Cell 130:499-511.

100. Morrison, A. J., and X. Shen. 2009. Chromatin remodelling beyond transcription: the INO80 and SWR1 complexes. Nat Rev Mol Cell Biol 10:373-384.

101. Blattner, C., A. Sparks, and D. Lane. 1999. Transcription factor E2F-1 is upregulated in response to DNA damage in a manner analogous to that of p53. Molecular Cellular Biololgy 19:3704-3713.

102. Hofferer, M., C. Wirbelauer, B. Humar, and W. Krek. 1999. Increased levels of E2F-1-dependent DNA binding activity after UV- or gamma-irradiation. Nucleic acids research 27:491-495.

103. Lin, W. C., F. T. Lin, and J. R. Nevins. 2001. Selective induction of E2F1 in response to DNA damage, mediated by ATM- dependent phosphorylation. Genes & development 15:1833-1844.

104. Wang, B., K. Liu, F. T. Lin, and W. C. Lin. 2004. A role for 14-3-3 tau in E2F1 stabilization and DNA damage-induced apoptosis. The Journal of biological chemistry 279:54140-54152.

105. Stevens, C., L. Smith, and N. B. La Thangue. 2003. Chk2 activates E2F-1 in response to DNA damage. Nature cell biology 5:401-409.

106. Pediconi, N., A. Ianari, A. Costanzo, L. Belloni, R. Gallo, L. Cimino, A. Porcellini, I. Screpanti, C. Balsano, E. Alesse, A. Gulino, and M. Levrero. 2003. Differential

regulation of E2F1 apoptotic target genes in response to DNA damage. Nature cell biology 5:552-558.

107. Ianari, A., R. Gallo, M. Palma, E. Alesse, and A. Gulino. 2004. Specific role for p300/CREB-binding protein-associated factor activity in E2F1 stabilization in response to DNA damage. The Journal of biological chemistry 279:30830-30835.

108. Ianari, A., T. Natale, E. Calo, E. Ferretti, E. Alesse, I. Screpanti, K. Haigis, A. Gulino, and J. A. Lees. 2009. Proapoptotic function of the retinoblastoma tumor suppressor protein. Cancer cell 15:184-194.

109. Inoue, Y., M. Kitagawa, and Y. Taya. 2007. Phosphorylation of pRB at Ser612 by Chk1/2 leads to a complex between pRB and E2F-1 after DNA damage. The EMBO journal 26:2083-2093.

110. Dick, F. A., and N. Dyson. 2003. pRB contains an E2F1-specific binding domain that allows E2F1-induced apoptosis to be regulated separately from other E2F activities. Molecular cell 12:639-649.

111. Markham, D., S. Munro, J. Soloway, D. P. O'Connor, and N. B. La Thangue. 2006. DNA-damage-responsive acetylation of pRb regulates binding to E2F-1. EMBO reports 7:192-198.

112. Liu, K., F. T. Lin, J. M. Ruppert, and W. C. Lin. 2003. Regulation of E2F1 by BRCT domain-containing protein TopBP1. Molecular and cellular biology 23:3287-3304.

113. Liu, K., Y. Luo, F. T. Lin, and W. C. Lin. 2004. TopBP1 recruits Brg1/Brm to repress E2F1-induced apoptosis, a novel pRb-independent and E2F1-specific control for cell survival. Genes & development 18:673-686.

114. Greer, D. A., B. D. Besley, K. B. Kennedy, and S. Davey. 2003. hRad9 rapidly binds DNA containing double-strand breaks and is required for damage-dependent topoisomerase II beta binding protein 1 focus formation. Cancer research 63:4829-4835.

115. Kumagai, A., J. Lee, H. Y. Yoo, and W. G. Dunphy. 2006. TopBP1 activates the ATR-ATRIP complex. Cell 124:943-955.

116. Yamane, K., X. Wu, and J. Chen. 2002. A DNA damage-regulated BRCT-containing protein, TopBP1, is required for cell survival. Molecular and cellular biology 22:555-566.

117. Hallstrom, T. C., S. Mori, and J. R. Nevins. 2008. An E2F1-dependent gene expression program that determines the balance between proliferation and cell death. Cancer cell 13:11-22.

118. Guo, R., J. Chen, F. Zhu, A. K. Biswas, T. R. Berton, D. L. Mitchell, and D. G. Johnson. E2F1 localizes to sites of UV-induced DNA damage to enhance nucleotide excision repair. The Journal of biological chemistry 285:19308-19315.

119. Maser, R. S., O. K. Mirzoeva, J. Wells, H. Olivares, B. R. Williams, R. A. Zinkel, P. J. Farnham, and J. H. Petrini. 2001. Mre11 complex and DNA replication: linkage to E2F and sites of DNA synthesis. Molecular and cellular biology 21:6006-6016.

120. Berton, T. R., D. L. Mitchell, R. Guo, and D. G. Johnson. 2005. Regulation of epidermal apoptosis and DNA repair by E2F1 in response to ultraviolet B radiation. Oncogene 24:2449-2460.

121. Guo, R., J. Chen, D. L. Mitchell, and D. G. Johnson. GCN5 and E2F1 stimulate nucleotide excision repair by promoting H3K9 acetylation at sites of damage. Nucleic acids research 39:1390-1397.

122. Matsuoka, S., B. A. Ballif, A. Smogorzewska, E. R. McDonald, 3rd, K. E. Hurov, J. Luo, C. E. Bakalarski, Z. Zhao, N. Solimini, Y. Lerenthal, Y. Shiloh, S. P. Gygi, and S. J. Elledge. 2007. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science (New York, N.Y 316:1160-1166.

123. Hanawalt, P. C. 1998. Genomic instability: environmental invasion and the enemies within. Mutation research 400:117-125.

124. Hanahan, D., and R. A. Weinberg. Hallmarks of cancer: the next generation. Cell 144:646-674.

125. Hernando, E., Z. Nahle, G. Juan, E. Diaz-Rodriguez, M. Alaminos, M. Hemann, L. Michel, V. Mittal, W. Gerald, R. Benezra, S. W. Lowe, and C. Cordon-Cardo. 2004. Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control. Nature 430:797-802.

126. van Harn, T., F. Foijer, M. van Vugt, R. Banerjee, F. Yang, A. Oostra, H. Joenje, and H. te Riele. Loss of Rb proteins causes genomic instability in the absence of mitogenic signaling. Genes & development 24:1377-1388.

127. Chen, J., F. Zhu, R. L. Weaks, A. K. Biswas, R. Guo, Y. Li, and D. G. Johnson. 2011. E2F1 promotes the recruitment of DNA repair factors to sites of DNA double-strand breaks. Cell Cycle 10:1287-1294.

128. Jucha, A., A. Wegierek-Ciuk, Z. Koza, H. Lisowska, A. Wojcik, M. Wojewodzka, and A. Lankoff. FociCounter: a freely available PC programme for quantitative and qualitative analysis of gamma-H2AX foci. Mutation research 696:16-20.

129. Pusapati, R. V., R. J. Rounbehler, S. Hong, J. T. Powers, M. Yan, K. Kiguchi, M. J. McArthur, P. K. Wong, and D. G. Johnson. 2006. ATM promotes apoptosis and suppresses tumorigenesis in response to Myc. Proc Natl Acad Sci U S A 103:1446-1451.

130. Hong, S., Q. X. Paulson, and D. G. Johnson. 2008. E2F1 and E2F3 activate ATM through distinct mechanisms to promote E1A-induced apoptosis. Cell cycle (Georgetown, Tex 7:391-400.

131. Bonner, W. M., C. E. Redon, J. S. Dickey, A. J. Nakamura, O. A. Sedelnikova, S. Solier, and Y. Pommier. 2008. GammaH2AX and cancer. Nature reviews 8:957-967.

132. Liu, K., J. C. Paik, B. Wang, F. T. Lin, and W. C. Lin. 2006. Regulation of TopBP1 oligomerization by Akt/PKB for cell survival. The EMBO journal 25:4795-4807.

133. Marnett, L. J., and J. P. Plastaras. 2001. Endogenous DNA damage and mutation. Trends Genet 17:214-221.

134. Guo, R., J. Chen, F. Zhu, A. K. Biswas, T. R. Berton, D. L. Mitchell, and D. G. Johnson. 2010. E2F1 localizes to sites of UV-induced DNA damage to enhance nucleotide excision repair. J Biol Chem 285:19308-19315.

135. Stewart, G. S., B. Wang, C. R. Bignell, A. M. Taylor, and S. J. Elledge. 2003. MDC1 is a mediator of the mammalian DNA damage checkpoint. Nature 421:961-966.

136. Chaudhuri, S., J. J. Wyrick, and M. J. Smerdon. 2009. Histone H3 Lys79 methylation is required for efficient nucleotide excision repair in a silenced locus of Saccharomyces cerevisiae. Nucleic Acids Res 37:1690-1700.

137. Faucher, D., and R. J. Wellinger. Methylated H3K4, a transcription-associated histone modification, is involved in the DNA damage response pathway. PLoS Genet 6.

138. McMahon, S. B., H. A. Van Buskirk, K. A. Dugan, T. D. Copeland, and M. D. Cole. 1998. The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. Cell 94:363-374.

139. Niu, H., S. Raynard, and P. Sung. 2009. Multiplicity of DNA end resection machineries in chromosome break repair. Genes Dev 23:1481-1486.

140. Symington, L. S., and J. Gautier. Double-Strand Break End Resection and Repair Pathway Choice. Annu Rev Genet.

141. Carreira, A., J. Hilario, I. Amitani, R. J. Baskin, M. K. Shivji, A. R. Venkitaraman, and S. C. Kowalczykowski. 2009. The BRC repeats of BRCA2 modulate the DNA-binding selectivity of RAD51. Cell 136:1032-1043.

142. Sung, P., L. Krejci, S. Van Komen, and M. G. Sehorn. 2003. Rad51 recombinase and recombination mediators. The Journal of biological chemistry 278:42729-42732.

143. Choi, J. H., L. A. Lindsey-Boltz, M. Kemp, A. C. Mason, M. S. Wold, and A. Sancar. Reconstitution of RPA-covered single-stranded DNA-activated ATR-Chk1 signaling. Proc Natl Acad Sci U S A 107:13660-13665.

144. Guo, R., J. Chen, D. L. Mitchell, and D. G. Johnson. 2011. GCN5 and E2F1 stimulate nucleotide excision repair by promoting H3K9 acetylation at sites of damage. Nucleic Acids Res 39:1390-1397.

145. Moon, N. S., and N. Dyson. 2008. E2F7 and E2F8 keep the E2F family in balance. Developmental cell 14:1-3.

146. Martinez, L. A., E. Goluszko, H. Z. Chen, G. Leone, S. Post, G. Lozano, Z. Chen, and A. Chauchereau. 2010. E2F3 is a mediator of DNA damage-induced apoptosis. Mol Cell Biol 30:524-536.

147. Lin, P. S., L. A. McPherson, A. Y. Chen, J. Sage, and J. M. Ford. 2009. The role of the retinoblastoma/E2F1 tumor suppressor pathway in the lesion recognition step of nucleotide excision repair. DNA repair 8:795-802.

148. Prost, S., P. Lu, H. Caldwell, and D. Harrison. 2007. E2F regulates DDB2: consequences for DNA repair in Rb-deficient cells. Oncogene 26:3572-3581.

149. Chen, D., Z. Yu, Z. Zhu, and C. D. Lopez. 2008. E2F1 regulates the base excision repair gene XRCC1 and promotes DNA repair. J Biol Chem 283:15381-15389.

150. Kachhap, S. K., N. Rosmus, S. J. Collis, M. S. Kortenhorst, M. D. Wissing, M. Hedayati, S. Shabbeer, J. Mendonca, J. Deangelis, L. Marchionni, J. Lin, N. Hoti,

J. W. Nortier, T. L. DeWeese, H. Hammers, and M. A. Carducci. Downregulation of homologous recombination DNA repair genes by HDAC inhibition in prostate cancer is mediated through the E2F1 transcription factor. PLoS One 5:e11208.

151. Wang, A., R. Schneider-Broussard, A. P. Kumar, M. C. MacLeod, and D. G. Johnson. 2000. Regulation of BRCA1 expression by the Rb-E2F pathway. J Biol Chem 275:4532-4536.

152. Liu, Y., and M. Kulesz-Martin. 2001. p53 protein at the hub of cellular DNA damage response pathways through sequence-specific and non-sequence-specific DNA binding. Carcinogenesis 22:851-860.

153. Lim, D. S., S. T. Kim, B. Xu, R. S. Maser, J. Lin, J. H. Petrini, and M. B. Kastan. 2000. ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway. Nature 404:613-617.

154. Suwaki, N., K. Klare, and M. Tarsounas. RAD51 paralogs: Roles in DNA damage signalling, recombinational repair and tumorigenesis. Seminars in cell & developmental biology.

155. Shen, W. H., A. S. Balajee, J. Wang, H. Wu, C. Eng, P. P. Pandolfi, and Y. Yin. 2007. Essential role for nuclear PTEN in maintaining chromosomal integrity. Cell 128:157-170.

156. Levitt, N. C., and I. D. Hickson. 2002. Caretaker tumour suppressor genes that defend genome integrity. Trends in molecular medicine 8:179-186.

157. Yanagihara, H., J. Kobayashi, S. Tateishi, A. Kato, S. Matsuura, H. Tauchi, K. Yamada, J. Takezawa, K. Sugasawa, C. Masutani, F. Hanaoka, C. M. Weemaes,

T. Mori, L. Zou, and K. Komatsu. NBS1 recruits RAD18 via a RAD6-like domain and regulates Pol eta-dependent translesion DNA synthesis. Mol Cell 43:788-797.

158. Miller, K. M., J. V. Tjeertes, J. Coates, G. Legube, S. E. Polo, S. Britton, and S. P. Jackson. Human HDAC1 and HDAC2 function in the DNA-damage response to promote DNA nonhomologous end-joining. Nat Struct Mol Biol 17:1144-1151.

159. Bishop, A. J., M. C. Hollander, B. Kosaras, R. L. Sidman, A. J. Fornace, Jr., and R. H. Schiestl. 2003. Atm-, p53-, and Gadd45a-deficient mice show an increased frequency of homologous recombination at different stages during development. Cancer Res 63:5335-5343.

160. Brown, A. D., A. B. Claybon, and A. J. Bishop. A conditional mouse model for measuring the frequency of homologous recombination events in vivo in the absence of essential genes. Mol Cell Biol 31:3593-3602.

161. Murr, R., J. I. Loizou, Y. G. Yang, C. Cuenin, H. Li, Z. Q. Wang, and Z. Herceg. 2006. Histone acetylation by Trrap-Tip60 modulates loading of repair proteins and repair of DNA double-strand breaks. Nature cell biology 8:91-99.

162. Ziv, Y., D. Bielopolski, Y. Galanty, C. Lukas, Y. Taya, D. C. Schultz, J. Lukas, S. Bekker-Jensen, J. Bartek, and Y. Shiloh. 2006. Chromatin relaxation in response to DNA double-strand breaks is modulated by a novel ATM- and KAP-1 dependent pathway. Nature cell biology 8:870-876.

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