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THE ROLE OF THE RETINOBLASTOMA/E2F1 TUMOR SUPPRESSOR PATHWAY IN THE LESION RECOGNITION STEP OF NUCLEOTIDE EXCISION REPAIR

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Abstract

The retinoblastoma Rb/E2F tumor suppressor pathway plays a major role in the regulation of mammalian cell cycle progression. The pRb protein, along with closely related proteins p107 and p130, exerts its anti-proliferative effects by binding to the E2F family of transcription factors known to regulate essential genes throughout the cell cycle. We sought to investigate the role of the Rb/E2F1 pathway in the lesion recognition step of nucleotide excision repair (NER) in mouse embryonic fibroblasts (MEFs). Rb^{-/-};p107^{-/-};p130^{-/-} MEFs repaired both cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PPs) at higher efficiency than did wildtype cells following UV-C irradiation. The expression of damaged DNA binding gene DDB2 involved in the DNA lesion recognition step was elevated in the Rb family-deficient MEFs. To determine if the enhanced DNA repair in the absence of the Rb gene family is due to the derepression of E2F1, we assayed the ability of E2F1-deficient cells to repair damaged DNA and demonstrated that E2F1^{-/-} MEFs are impaired for the removal of both CPDs and 6-4PPs. Furthermore, wildtype cells induced a higher expression of DDB2 and xeroderma pigmentosum gene XPC transcript levels than did E2F1^{-/-} cells following UV-C irradiation. Using an E2F SiteScan algorithm, we uncovered a putative E2F-responsive element in the XPC promoter upstream of the transcription start site. We showed with chromatin immunoprecipitation assays the binding of E2F1 to the XPC promoter in a UV-dependent manner, suggesting that E2F1 is a transcriptional regulator of XPC. Our study identifies a novel E2F1 gene target and further supports the growing body of evidence that the Rb/E2F1 tumor suppressor pathway is involved in the regulation of the DNA lesion recognition step of nucleotide excision repair.

Introduction

Nucleotide excision repair (NER) provides a primary line of defense against a variety of DNA adducts induced by environmental and endogenous sources [1]. The most relevant UV-induced

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Conflict of Interest Statement

The authors declare that there is no conflict of interest.

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DNA adducts are cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidone photoproducts (6-4PPs). These DNA photoproducts, if left unrepaired, may contribute to mutagenesis, oncogenesis, developmental abnormalities, and even cell death. In humans, three diseases are associated with defects in NER: xeroderma pigmentosum (XP), Cockayne's syndrome (CS), and trichothiodystrophy (TTD) [2]. Patients with XP are predisposed to a high incidence of skin cancer, whereas CS and TTD patients are not cancer-prone, but exhibit developmental and neurological disorders. Based on studies from XP and CS patients, several complementation groups have been identified, representing distinct NER genes XP-A through -G and CSA and CSB. The NER pathway involves all of these gene products and proceeds by a series of discrete enzymatic steps that include DNA lesion recognition, incisions on both sides of the lesion, removal of the damaged strand containing the lesion, and DNA resynthesis and ligation [3,4]. Two distinct pathways exist in NER. Transcription-coupled repair (TCR) refers to the preferential repair of transcribed strands in active genes whereas global genomic repair (GGR) refers to repair throughout the genome, including that in the non-transcribed strands of active genes [5–7].

The DNA lesion recognition step is thought to be the rate limiting step for initiating NER [8, 9]. In GGR, the lesion recognition step involves gene products belonging to xeroderma pigmentosum groups C (XPC) and E (XPE). XPC is found *in vivo* in tight association with its coactivator protein hHR23B. XPE is a heterodimer comprised of damage DNA binding gene products DDB1 and DDB2. XPC binds 6-4PPs directly whereas its binding to CPDs requires preliminary binding of DDB2, which is followed by proteasomal degradation of DDB2 [8–13].

An important regulatory function for NER has been ascribed to the p53 tumor suppressor gene product. Transcriptional activation of target genes by p53 is the primary means by which p53 regulates cell cycle checkpoints and apoptosis, and growing evidence now suggests a transcriptional regulatory role for p53 in NER [12,14]. We demonstrated that p53 transcriptionally regulates the expression of the DDB2 gene [15] and that overexpression of DDB2 enhances GGR in the absence of functional p53 [16,17]. Furthermore, p53 can directly activate DDB2 through a region in the human DDB2 gene that binds and responds transcriptionally to p53 [18]. Similarly, we demonstrated that p53 transcriptionally regulates the XPC gene, and that mRNA and protein products of XPC increase in a DNA damage- and p53-dependent manner [19].

Much like p53, the E2F transcription factors regulate an array of genes involved in cell cycle checkpoint, cell cycle progression, and apoptosis [20]. These E2F factors associate and work intimately with the pocket proteins of the Rb gene family (pRb, p107, p130) in regulating cellular functions [21]. Among the eight E2F family of transcription factors, only E2F1 has been associated with a prominent role in both DNA damage response and apoptosis [22]. In response to γ -irradiation, E2F1 is directly phosphorylated and stabilized by ataxia-telangiectasia mutated kinase (ATM) and Rad3-related kinase (ATR) [23]. The current model suggests that the overall role of E2F1 in the DNA damage response varies greatly and depends on cellular context in directing either pro-apoptotic or anti-apoptotic outcomes [24,25]. Given that E2F1 displays the properties of both an oncogene [26–28] and a tumor suppressor [29–31], and can affect the expression of both pro-apoptotic and anti-apoptotic genes [32,33], the strength of the E2F1 response may be a decisive factor in the cellular response to DNA damage.

While the above-mentioned studies firmly establish E2F1 as having a critical function in mediating the physiological outcomes of DNA damage response, more recent studies suggest that E2F1 may be directly involved in this response by regulating DNA repair. Microarray analyses of mRNAs induced following the induction of activator E2Fs show that E2Fs can regulate a diversity of genes involved in additional biological processes beyond cell cycle

progression [32,34]. For example, E2F1 has been implicated in the transcriptional regulation of genes involved in known and diverse DNA repair pathways, such as mismatch repair (MSH2, MLH1), base excision repair (UNG), nucleotide excision repair (DDB2, RPA), homologous recombination repair (RAD51, RAD54, RECQL), and non-homologous end-joining (DNA-dependent protein kinases) [33–37]. More importantly, a number of functional studies confirmed E2F1 as a transcriptional regulator of essential genes involved in BER [38] and NER [39]. Specifically, Prost and colleagues established a novel role of E2F1 in regulating DDB2 expression and NER activity in mouse hepatocytes. In the current study, we examined the role of the Rb/E2F1 tumor suppressor pathway in regulating NER function in MEFs, and provide mechanistic evidence for an E2F1-dependent activation of not only DDB2 but also XPC in response to UV-induced DNA damage. Our study revealed a putative novel function of the Rb/E2F1 tumor suppressor pathway in regulating the DNA lesion recognition step of NER.

Materials and Methods

Cell Culture

Rb^{-/-} single mutant and Rb^{-/-};p107^{-/-};p130^{-/-} triple knockout (TKO) mouse embryonic fibroblasts (MEFs) were harvested from 12.5–14.5 day-old embryos obtained after injection of mutant embryonic stem (ES) cells into blastocysts and reimplantation into foster mothers [40]. Wildtype (WT) and E2F1^{-/-} MEFs [30] were generously provided by Drs. Rosalie Sears and Charles Lopez (Oregon Health and Science University). MEFs were grown in DMEM (Mediatech) supplemented with 10% FBS, 2 mM L-glutamine, and penicillin/streptomycin/fungizone, and maintained in 37°C incubator with 5% CO₂.

Global Genomic Repair

Repair of CPDs and 6-4PPs in the various cell lines at different times following UV-C (254_{nm}) irradiation was measured using an enzyme-linked immunosorbent assay (ELISA). Briefly, exponentially growing cells were prelabeled with [³H]-thymidine for ~48 hours, washed with PBS, and UV-irradiated with 10 J/m² using a germicidal lamp calibrated to deliver a dose of 1 J/m² per second. The cells were lysed either immediately (0 hr) or after incubation in nonradioactive growth medium for various times over a 24-hour period following UV exposure to allow repair of DNA lesions. The lysis solution contained 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5% SDS, 0.1 mg/mL proteinase K, and 0.1 mg/mL RNase. Genomic DNA was isolated by phenol/chloroform extraction, followed by ethanol precipitation. DNA concentration and specific radioactivity were determined by Hoechst staining and scintillation counting, respectively. Genomic DNA was denatured and distributed in triplicate (20 ng of DNA for the detection of CPDs per well and 400 ng of DNA for the detection of 6-4PPs per well) onto 96-well microtiter plates precoated with 0.003% protamine sulfate. The amount of DNA loaded for each sample was determined by normalizing to scintillation counts to account for any DNA replication that may have occurred, as previously described [17]. The plates were blocked in 0.05% PBS-Tween 20 containing 2% FBS. The fixed DNA were incubated with 1:5000 TDM-2 (for CPDs) or 1:5000 64M-2 (for 6-4PPs) (a generous gift provided by Dr. Toshio Mori) [41]. The signals were amplified by 1:2000 Biotin-F(ab')₂ fragment of anti-mouse IgG (H+L) (Zymed), followed by 1:10000 Peroxidase-Streptavidin (Zymed), and developed with the substrate solution (0.1 M citrate phosphate buffer pH 5.0, 100 µg/mL 3,5,3',5'-tetramethylbenzidine (TMB), and 0.003% H₂O₂). All of the above-mentioned incubations were each carried out at 37°C for 30 min followed by 5X washes of 0.05% PBS-Tween 20. The reactions were stopped by 2 M H₂SO₄ and quantitated at 450_{nm} on the VERSAmax microplate reader (Molecular Devices). The decrease of immunofluorescence signal from one time point to another directly measures the rate of repair over time.

Quantitative RT-PCR

Real time RT-PCR was used to evaluate the basal and inducible transcript levels of specific NER genes in Rb family-deficient and E2F1-deficient MEFs. Briefly, total RNA was isolated and purified using RNeasy Protect Mini Kit (Qiagen) with the following modifications. Cells were initially homogenized using the QIAshredder column (Qiagen) and the resulting lysates were treated with RNase-Free DNase (Qiagen) to remove genomic DNA. 5 µg of total RNA from each sample was reverse transcribed using SuperScript™ III First-Strand Synthesis System (Invitrogen) to create various cDNA libraries. The Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) was used for PCR of cDNA samples in a protocol consisting of 50 cycles of denaturation (95°C for 15 sec), primer annealing (57°C for 30 sec), and primer extension (72°C for 30 sec) using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). For calibration and generation of standard curves, we used MEF cDNA pooled from all of the samples and made reference standards. The transcript level of each gene was normalized to that of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene and expressed as either fold activation over WT level (to examine basal transcripts) or fold induction over 0-hour reference level (to examine DNA damage-inducible transcripts). The mouse DDB2 primers used were 5'-GCCGATACCCAGATCCTAATCTT-3' and 5'-ACACATCATCTTCCCTGAGCTTC-3'. The mouse XPC primers used were 5'-ATCATTTCCAATTGCTTTACCAA-3' and 5'-GTTCCGATGAACCACTTTACCAG-3'. The mouse PCNA primers used were 5'-CACGTATATGCCGAGACCTTAGC-3' and 5'-CTCCACTTGCAGAAACTTCACC-3'. The mouse GAPDH primers used were 5'-GGAGAAACCTGCCAAGTATGATG-3' and 5'-GACAACCTGGTCCTCAGTGTAGC-3'.

Promoter Analysis for Putative E2F Binding Sites

Putative E2F1 binding sites were determined using a rigorous algorithm and weight-substitution matrix from the following site: <http://compel.bionet.nsc.ru/FunSite/SiteScan.html> (based on the work of Kel and colleagues) [42]. Genomic mouse DNA sequence from a portion of chromosome 6, which contains the XPC gene, was evaluated as the input sequence (NCBI accession number NT_039353). The search method was carried out using default parameters (weight matrix + forbidden nucleotides, 0.8 cut-off E2F score value).

Chromatin Immunoprecipitation (ChIP) Assay

The E2F1 ChIP assays were performed using the Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology) according to manufacturer's protocol. Briefly, cells were initially cross-linked with 1% formaldehyde, washed in PBS, and lysed in SDS lysis buffer containing 1 mM PMSF and protease inhibitor cocktail (Sigma). The lysates were sonicated to shear the chromatin to fragments of approximately 500–1000 base pairs in length. The lysates were pre-cleared with Protein A agarose (PAA) beads and subsequently incubated with C-20 E2F1 antibody (Santa Cruz Biotechnology) overnight at 4°C on a rotating platform. The antibody-antigen immune complexes were precipitated by incubation with PAA beads for 1 hour. Immunocomplexes were washed sequentially with low and high salt buffers, LiCl buffer, and TE buffer. The protein-antibody complexes were eluted with elution buffer (1% SDS, 0.1 M NaHCO₃). The cross-links were reversed by the addition of NaCl at 65°C for 4 hours. DNA was purified using the QIAquick PCR Purification Kit (Qiagen) and PCR-amplified via ABI PRISM 7900 Sequence Detection System (Applied Biosystems). All signals were corrected and normalized to baseline levels found in a control site 5000+ base pairs upstream of the putative binding site. The mouse XPC primers flanking the site of interest used were 5'-CACGCACTGAAGGCGAACTAT-3' and 5'-GTAGGACCACGCCCTCGAA-3'. The mouse XPC primers flanking a control site 5000+ base pairs upstream of the putative binding

site used were 5'-TCAACAGGGCAGTACAAAAATGA-3' and 5'-TAGTTGGGCTTAAAAGCATCTGG-3'.

Results

Rb family-deficient MEFs exhibit increased global genomic DNA repair

In our initial experiment, we examined the ability of MEFs deficient in the Rb gene family to repair DNA photoproducts following UV-C irradiation. Given that the Rb pocket proteins are strong inhibitors of E2F functions, we chose these Rb family-deficient cell lines to mimic E2F activation in the absence of its negative regulators. The use of cells that are genetically deficient for pRb, p107, and p130 allowed us to eliminate functional compensation among the Rb family members, and therefore permitted us to more clearly observe the repair phenotype in response to E2F activation that might otherwise be disguised. Rb^{-/-};p107^{-/-};p130^{-/-} TKO MEFs repaired both 6-4PPs and CPDs more efficiently than did WT MEFs (Figs. 1a and 1b). The difference in the rate of repair between WT and TKO MEFs can be seen as early as 2 hours following UV irradiation and is maximal at 8–24 hours. For 6-4PPs, the Rb^{-/-} single mutant MEFs showed a trend toward increased repair at 24 hours. For CPDs, the loss of all three Rb proteins was required to achieve significant enhancement of DNA repair, suggesting functional redundancy among the Rb family members in response to this type of DNA lesion. In summary, our results demonstrate that genetic loss of Rb function enhances NER activity.

Rb family-deficient MEFs express higher basal levels of DDB2

Given that DNA lesion recognition is the rate limiting step in NER, an increase in DDB2 or XPC levels may explain the enhanced GGR in Rb family-deficient cells. Prost and colleagues recently showed an increase in DDB2 expression in Rb-deficient mouse hepatocytes [39]. We sought to evaluate if this observation could be extended to include MEFs, which are more commonly studied in rodent DNA repair. Accordingly, we carried out real time RT-PCR experiments to assess DDB2 transcript level in WT, Rb^{-/-}, and Rb TKO MEFs. We observed a 3.3-fold increase in basal DDB2 transcript levels in Rb TKO MEFs compared to WT MEFs (Fig. 2). We also measured XPC levels but found that they did not differ significantly between WT, Rb^{-/-}, and TKO MEFs. As a positive control for E2F activation, mRNA expression of proliferating cell nuclear antigen (PCNA), a well-established E2F target gene, was also shown to be elevated in Rb^{-/-} and Rb TKO MEFs.

E2F1-deficient MEFs exhibit decreased global genomic DNA repair

Since most of the known Rb functions in the cell are intimately linked to its association with the E2F transcription factors, we sought to determine whether the increased repair observed in Rb family-deficient MEFs is mediated by E2F1. Accordingly, GGR assays were performed with MEFs containing homozygous knockout of E2F1. E2F1^{-/-} MEFs were impaired for the removal of 6-4PPs (Fig. 3a) and CPDs (Fig. 3b) by 15–25%, suggesting a role for E2F1 in NER.

E2F1-deficient MEFs exhibit decreased DNA damage-inducible expression of DDB2 and XPC following UV-C irradiation

Given that DDB2 transcript levels were elevated in Rb family-deficient MEFs (Fig. 2), we were interested to determine if DDB2 transcript levels were correspondingly decreased in E2F1-deficient MEFs. No significant difference was observed in the basal transcript level of DDB2 between WT and E2F1^{-/-} MEFs. However, we observed that DDB2 expression is UV-inducible in WT MEFs whereas this response was largely abrogated in E2F1^{-/-} MEFs (Fig. 4a). This result is consistent with the previous identification of a putative E2F binding site in the proximal promoter of the mouse DDB2 gene [39,43]. The transcript level of XPC was

highly inducible in WT cells as compared to E2F1^{-/-} cells, exhibiting a 4-fold increase 16 hours following UV-C irradiation (Fig. 4b). We did not observe any significant difference in the transcript level of another NER gene, XPA, between WT and E2F1^{-/-} cells (data not shown). These observations suggest a specific activation of DDB2 and XPC rather than a global upregulation of repair genes in the presence of E2F1. All of the transcription profiles showed a drop in mRNA levels 4 hours after UV-C irradiation, likely indicating a decrease in overall transcription immediately following UV-induced genotoxic stress.

Proximal promoter region of the mouse XPC gene contains a putative E2F binding site

To further analyze and evaluate the putative transcriptional regulation of XPC by E2F1, we examined the promoter-proximal region of mouse XPC gene for E2F1 binding sites. We used a computer algorithm to search for consensus E2F1 responsive elements previously determined from a collection of 45 known E2F1 sites present in 33 genes [42]. We evaluated the XPC gene from 4851 nucleotides upstream of the transcription start site to the end of the gene itself. Although a few putative E2F1 sites were uncovered, one site, located from -73 to -62, was characterized by a high score value of 0.91 (Fig. 5). This score indicates that the probability of such a prediction resulting in a false positive is less than 5% [42]. Both a schematic representation of the relative position of this binding site and the DNA sequence context in which it exists are shown in Figure 5.

As a biological reference point, we also used the same algorithm to search for E2F1 sites in the DDB2 gene. A previous study has already shown that at least one such site exists in the 5'-untranslated region (UTR) of human and mouse DDB2 [39,43]. We confirmed this finding in both human and mouse DDB2, which presented score values of 0.93 and 1.00, respectively.

E2F1 binds the mouse XPC promoter in a DNA damage-dependent manner

To confirm the putative E2F1 binding site in the XPC promoter (Fig. 5), we carried out ChIP assays in WT and E2F1^{-/-} MEFs (Fig. 6). In WT MEFs, E2F1 binds to the XPC promoter in a UV-dependent manner (Fig. 6). E2F1 binding occurs during basal metabolism and increases significantly above baseline following UV-C irradiation. This event is completely abrogated in the absence of E2F1. Prost and colleagues recently demonstrated that E2F1 binds the DDB2 promoter in mouse hepatocytes [39]. However, we did not conclusively observe E2F1 binding to the DDB2 gene in mouse fibroblasts likely due in part to the fact the DDB2 levels are characteristically low in MEFs. Nevertheless, our data confirms that E2F1 binds the mouse XPC promoter in the native chromatin structure. We show that E2F1 is a transcriptional regulator of XPC and may play a fundamental role in the regulation of the DNA lesion recognition step of mouse NER.

Discussion

The E2F transcription factors are downstream effectors of the retinoblastoma tumor suppressor pathway required for timely regulation of essential genes involved in DNA replication and cell cycle progression. A number of experiments involving DNA microarrays and computer-assisted promoter analyses strongly suggest that E2F may be involved in biological processes that extend beyond cell cycle regulation. For example, recent data have revealed putative E2F target genes that are involved in several DNA repair pathways, including those in NER [33–37]. Among the eight members of the E2F family (E2F1–E2F8), only E2F1 is phosphorylated and stabilized upon DNA damage and in response to other cellular stresses [23,44–47]. Furthermore, protein-protein interactions between E2F1 and a number of repair factors have been established [48–52]. Although the functional significance of these independent findings is not well understood, the collective body of evidence strongly implicates a role for E2F1 in DNA damage repair.

Berton and colleagues initially established a function of E2F1 in promoting DNA repair [53]. Mice containing homozygous knockout of E2F1 exhibited enhanced keratinocyte apoptosis following UV-B irradiation whereas mice with transgenic overexpression of E2F1 displayed decreased epidermal apoptosis. Furthermore, E2F1^{-/-} mice were deficient for the removal of DNA photoproducts while transgenic mice exhibited an enhanced level of repair. Therefore, the suppression of apoptosis by E2F1 is related to the activation in DNA repair. Prost and colleagues confirmed this initial observation by demonstrating that E2F1 is a transcriptional regulator of DDB2 in mouse hepatocytes [39]. Since most of our understanding of murine DNA repair is based on previous experiments with MEFs, we sought to see if these preliminary observations in hepatocytes could be extrapolated to include MEFs.

Given functional redundancy and compensation among the Rb family members, we used primary MEFs chronically deficient in functional pRb, p107, p130 to more precisely observe subtle repair phenotypes that might otherwise be disguised. TKO MEFs repaired DNA lesions more efficiently than did WT MEFs. This observation correlates with the previous finding that TKO MEFs consistently exhibited the most profound phenotype among the Rb family-deficient cell lines with respect to overall cell size, cell cycle time, population doubling time, and the degree of cell cycle arrest following DNA-damaging agents [40]. Whereas the unscheduled DNA synthesis (UDS) carried out by Prost and colleagues could not discriminate between repair of CPDs and 6-4PPs, our sensitive ELISA-based DNA repair assay demonstrates that Rb family-deficient MEFs are capable of removing both types of DNA lesions (Fig. 1). Consistent with data showing that transient elimination of Rb results in gene induction of DDB2 [37,39], our study shows that the genetic loss of Rb is correlated with an increase in DDB2 transcript level in MEFs. Given that PCNA is an established E2F1 target gene, the increase in PCNA transcripts in Rb-deficient MEFs suggests that the concomitant increase in DDB2 transcripts is likely E2F1-mediated.

MEFs containing homozygous knockout of E2F1 exhibited a decrease in the repair of both 6-4PPs and CPDs. Interestingly, we did not observe any difference in basal DDB2 transcript levels between WT and E2F1^{-/-} MEFs. However, upon treatment with 10 J/m² UV-C irradiation, WT cells induced DDB2 expression to a higher level as compared to E2F1-deficient cells. This finding suggests that E2F1 is involved in the DNA-damage inducibility of DDB2. Nichols and colleagues investigated the proximal promoter of the DDB2 gene and identified an E2F element near the putative transcription start site [43]. Mutation of this conserved E2F consensus sequence decreased DDB2 promoter activity. Furthermore, ChIP analysis established E2F1 binding to the DDB2 promoter while siRNA knockdown of E2F1 resulted in decreased DDB2 expression in hepatocytes [39]. The fact that we did not conclusively observe E2F1 binding to the DDB2 promoter in MEFs may be attributed to the difference in cell type. Hepatocytes characteristically express much higher level of DDB2 than do MEFs and may differ fundamentally in the regulation of this gene [54]. We cannot rule out the possibility that DDB2 induction in our MEFs may be a secondary effect of E2F1 activation rather than direct promoter binding. More experiments are necessary to evaluate the fundamental differences between mouse hepatocytes and MEFs in the context of DNA repair, so that one can more appropriately compare murine and human repair processes.

XPC transcript levels were likewise enhanced following UV-C irradiation in the presence of E2F1 whereas XPC activation was attenuated in the absence of E2F1. Unlike DDB2 activation, XPC upregulation in response to DNA damage was unexpected since there was no previously reported link between E2F1 and XPC. Interestingly, XPC was even more inducible in response to DNA damage than was DDB2. Accordingly, we examined the proximal promoter region of the mouse XPC gene for putative E2F responsive elements using the stringent contextual E2F SiteScan algorithm (<http://compel.bionet.nsc.ru/FunSite/SiteScan.html>) [42]. We found a putative E2F1 binding site upstream (-73 to -62) of the XPC transcription start site. The score

assigned to this putative site by the weighted matrix was 0.91, which according to Kel and colleagues is a score of high confidence that will produce less than 5% false positives. Our ChIP analysis confirms that this putative site is a bonafide E2F1 binding site and supports the idea that E2F1 is a transcriptional regulator of XPC. Furthermore, E2F1-dependent XPC activation following UV irradiation is likely in part responsible for the enhanced DNA repair observed in the mouse fibroblasts.

Whereas the role of p53 in human NER is well defined [12], the role of p53 in rodent NER has been a source of long-standing debate in the DNA repair field [1,55]. Some rodent cells characteristically do not repair damaged DNA to any appreciable extent and may be deficient in p53-regulated NER [18]. It is possible that E2F1, not p53, is the dominant factor that regulates the DNA lesion recognition step of NER in mouse. There are a number of intriguing observations that are consistent with this idea. For example, ATM and ATR kinases phosphorylate both p53 and E2F1 in response to DNA damage [23]. Furthermore, the kinetics of E2F1 induction in response to various damaging agents is similar to the kinetics of p53 stabilization [44,56,57]. These lines of evidence suggest that E2F1 and p53 may have overlapping functions in response to DNA damage. However, the relative importance of either p53 or E2F1 in species-specific response to DNA damage remains to be established.

While transcriptional regulation of the DNA lesion recognition step of NER by the Rb/E2F1 tumor suppressor pathway is an attractive model, we cannot rule out the possibility that the Rb/E2F1 pathway likewise participates in DNA damage repair via transcription-independent mechanisms. Studies have shown that the UV-DDB complex (DDB1-DDB2) interacts with E2F1 and can stimulate E2F1-activated transcription [48,58]. An appealing model suggests that under normal cell cycle metabolism, the UV-DDB complex serves as a transcriptional cofactor of E2F1 to facilitate the expression of genes required for cell cycle progression. However, during genotoxic stress, the UV-DDB complex becomes involved in DNA lesion recognition. This decrease in the level of available UV-DDB in turn reduces E2F1 transcriptional activity and leads to cell cycle arrest required as a window of opportunity for DNA repair. Therefore, the association and functional coordination between UV-DDB and E2F1 may serve as a possible mechanism by which a cell facilitates cell cycle progression while ensuring genomic stability.

Since the loss of Rb function is commonly associated with various types of cancer, it appears surprising that the loss of Rb gene family members results in enhanced DNA repair. There is a growing body of evidence which suggest that DNA lesion recognition factors are tightly regulated and that constitutive expression of DDB2 and XPC may actually lead to mutagenic events [59]. Since UV-DDB recognizes conformational changes in DNA structure rather than a specific type of lesion, abnormally high DDB levels may lead to unwarranted binding to natural variations of the secondary DNA structure, and may initiate gratuitous repair that could interrupt normal DNA metabolism and threaten genomic stability [60]. Therefore, high and non-regulated levels of DDB2 and XPC may lead to mutagenesis that ultimately can increase the incidence of cancer. This may explain why in the human system, repair factors are p53- and damage-inducible, and in the case of mice, E2F1- and damage-inducible to maintain lesion recognition factors at appropriate levels.

In conclusion, the discovery of a regulatory function of E2F1 in the NER pathway demonstrates a novel role for E2F1 in DNA repair in addition to its well established functions in cell cycle checkpoint, cell cycle progression, and apoptosis. More importantly, our study demonstrates that E2F1 is involved in the regulation of DNA lesion recognition factors DDB2 and XPC in mouse NER. Uncovering new E2F1 target genes and protein partners will be invaluable in paving the way for future investigations of E2F1 not only as a responder of DNA damage but also as an effector of DNA repair. Most importantly, a thorough understanding of the

mechanism by which the Rb/E2F1 tumor suppressor pathway regulates NER will provide us valuable insights into potential targets for therapeutic intervention and treatment against cancer.

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Abbreviations

Rb	retinoblastoma
MEFs	mouse embryonic fibroblasts
TKO	triple knockout
NER	nucleotide excision repair
BER	base excision repair
GGR	global genomic repair
TCR	transcription-coupled repair
CPDs	cyclobutane pyrimidine dimers
6-4PPs	6-4 pyrimidine–pyrimidone photoproducts
DDB2	damaged DNA binding gene 2
XPC	xeroderma pigmentosum gene C
PCNA	proliferating cell nuclear antigen
ELISA	enzyme-linked immunosorbent assay
RT-PCR	reverse transcriptase-polymerase chain reaction
ChIP	chromatin immunoprecipitation

PMSF

phenylmethylsulphonyl fluoride

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Figure 1.

Global genomic repair assay of wildtype and Rb family-deficient MEFs following 10 J/m² UV-C irradiation. (a) Repair of 6-4PPs by WT, Rb^{-/-} single mutant, and Rb^{-/-};p107^{-/-};p130^{-/-} TKO MEFs. (b) Repair of CPDs by WT, Rb^{-/-} single mutant, and Rb^{-/-};p107^{-/-};p130^{-/-} TKO MEFs. Repair curves are represented as percent of DNA repair (y-axis) as a function

of time following UV-C irradiation (x-axis). All data points and corresponding standard errors are the result of triplicate experiments (n=3).

Figure 2.

XPC, DDB2, and PCNA transcript levels in wildtype and Rb family-deficient MEFs. Basal, XPC, DDB2, and PCNA transcript levels in WT, Rb^{-/-} single mutant, and Rb^{-/-};p107^{-/-};p130^{-/-} TKO MEFs as measured by real time RT-PCR. XPC, DDB2, and PCNA transcript levels are normalized to that of GAPDH and expressed as fold activation over WT level, which is arbitrarily given the value of 1.0. All data points and corresponding standard errors are the result of triplicate experiments (n=3).

Figure 3. Global genomic repair assay of wildtype and E2F1^{-/-} MEFs following 10 J/m² UV-C irradiation. (a) Repair of 6-4PPs by WT and E2F1^{-/-} MEFs. (b) Repair of CPDs by WT and E2F1^{-/-} MEFs. Repair curves are represented as percent of DNA repair (y-axis) as a function of time following UV-C irradiation (x-axis). All data points and corresponding standard errors are the result of triplicate experiments (n=3).

Figure 4.

DDB2 and XPC transcript levels in wildtype and E2F1^{-/-} MEFs following 10 J/m² UV-C irradiation. DNA damage-inducible (a) DDB2 and (b) XPC transcript levels in WT and E2F1^{-/-} MEFs as measured by real time RT-PCR. DDB2 and XPC transcript levels are normalized to that of GAPDH and expressed as fold activation over basal level (0 hour time point), which is arbitrarily given the value of 1.0. All data points and corresponding standard errors are the result of triplicate experiments (n=3).

Figure 5.

A putative E2F1 binding site in the proximal promoter of mouse XPC gene. Schematic diagram showing a putative E2F1 binding site in the mouse XPC gene and the corresponding nucleotide sequence flanking the predicted site (underlined). +1 denotes the transcription start site.

Figure 6.

Chromatin immunoprecipitation assay of wildtype and E2F1^{-/-} MEFs following 10 J/m² UV-C irradiation. The binding of E2F1 to the putative XPC promoter site as measured by real time RT-PCR. All signals are corrected and normalized to levels found in a control site 5000+ base pairs upstream of the putative binding site. All data points and corresponding standard errors are the result of triplicate experiments (n=3).