

INTRODUCTION

DNA damage and types of repair

DNA is subject to various types of damage that can impair cellular function leading to cell death or carcinogenesis. DNA damage blocks normal cellular processes such as replication and transcription [1, 2]. Should DNA damage persist it can have catastrophic consequences for the cell and for the organism including mutagenesis, cancer, and cell death (i.e. in neurons). DNA damage may come from internal as well as external sources and is associated with various types of genetic diseases and cancers. Therefore DNA repair provides a mechanism to restore DNA nucleotide sequences to the native state. Several DNA repair pathways have been identified including base excision repair (BER), mismatch repair (MMR) and nucleotide excision repair (NER). The type of lesion will dictate which pathway is utilized for repair. For example an N7-methyl guanine residue can only be removed by BER, while a benzopyrene guanine adduct residue can only be removed by NER [3].

Nucleotide Excision Repair

Nucleotide Excision Repair (NER) is a versatile DNA repair pathway because of its ability to repair a wide range of nucleotide adducts [4]. NER is responsible for the repair of bulky DNA damage lesions including cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine pyrimidone photoproducts (6-4PPs) caused by UV radiation [3, 5]. NER repairs platinum DNA adducts that are formed by cancer chemotherapeutic drugs such as cisplatin and carboplatin [1, 6, 7]. UV radiation and platinum compounds cause covalent linkage of adjacent thymine or guanine residues respectively. Aromatic

hydrocarbons, as found in cigarette smoke, cause guanine adducts that also require NER for their removal. The steps involved in NER include recognition of DNA damage, excising the lesion as a 25-30 single stranded oligomer, resynthesizing the excised region of DNA, and finally ligating the newly repaired DNA strand [5, 8]. NER is divided into two distinct sub-pathways: transcription-coupled repair (TC-NER), which affects the transcribed strand in genes that are expressed, and global genomic repair (G-NER), which is responsible for removing DNA damage from the overall genome. These pathways differ in the early recognition step of DNA damage, but utilize the same proteins in physically excising the lesion and restoring the DNA sequence.

TC-NER is the repair of lesions on DNA that is simultaneously undergoing transcription. While TC-NER only occurs in a small percent of the genome it is nonetheless critical to cell survival. DNA damage recognition in TC-NER occurs when transcription is stalled by the arrest of RNA polymerase II as it encounters the site of DNA damage [9, 10]. The stalled polymerase is thought to trigger the recruitment of repair proteins including Cockayne syndrome complementation group A and B (CSA and CSB) [11]. Defects in TC-NER result in genetic diseases such as Cockayne Syndrome (CS). CS is a rare autosomal recessive genetic disorder that is characterized by severe sunlight (UV) sensitivity, growth and developmental defects, and neurological disease [12-14]. Patients with CS are not cancer prone but typically die at an early age due to neurological problems [15]. Mutations in either the CSA or CSB gene are seen in over 90% of CS patients and result in defective TC-NER [12, 16]. CS cells are defective in TC-NER and highly sensitive to UV-radiation and typically undergo apoptosis if exposed to UV-radiation.

G-NER repairs damaged DNA throughout the entire genome. Patients that are defective in G-NER suffer from a rare autosomal recessive disease known as *xeroderma pigmentosum* (XP). Patients with XP are unable to carry out G-NER resulting in sunlight sensitivity, predisposition to skin, lung, liver, and bladder cancers and in some cases neurological disorders [17-19]. Seven human genetic complementation groups (XPA through XPG) have been identified for *xeroderma pigmentosum*, all of which are defective in NER. Sugawara *et al.* have proposed a multistep damage recognition mechanism for G-NER that is currently accepted as the mechanism for G-NER [20]. G-NER is initiated by the recognition of the DNA lesion by XPC. XPC then recruits XPA, RPA and XPG to the site of damage. The XPA and XPG proteins help to form a pre-incision complex which is stabilized by RPA. TFIIH, a helicase, unwinds the DNA near the site of DNA damage. XPF and XPG are endonucleases that cut the damaged DNA strand 5' and 3' of the lesion ends respectively. The excision releases a fragment that is about 25-30 nucleotides in length. DNA polymerase δ or pol ϵ fills in the excised gap. The new fragment is then ligated by DNA ligase I completing the repair [20, 21]. **Figure 1** depicts the general mechanism for global genomic nucleotide excision repair (G-NER).

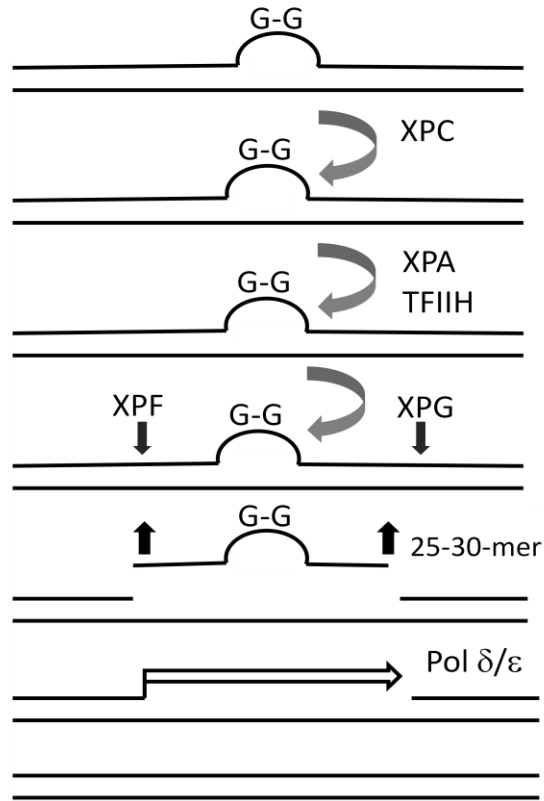


Figure 1. Basic mechanism for nucleotide excision DNA repair. In G-NER XPC is required for early lesion recognition (in TC-NER CSA and CSB substitute for XPC). In either subpathway, after XPA and TFIIH are recruited, the damaged DNA strand is excised by XPF and XPG releasing a 25-30-mer oligonucleotide. The excised gap is filled in by DNA polymerase δ or ϵ and ligated by DNA Ligase I to complete DNA repair. The diagram shows a guanine diadduct such as produced by the chemotherapy drug cisplatin.

XPC

One of the most common complementation groups of XP seen in patients is that of XPC [22]. XPC patients show defective G-NER but normal TC-NER. XPC patients exhibit sensitivity to UV radiation and a dramatically increased risk of skin cancer [19]. In addition, somatically-acquired mutations in XPC have been associated with the poor prognosis of patients with Nonsmall Cell Lung Carcinoma (NSCLC) [23]. Specific allelic variants of XPC are associated with increased risk of colorectal cancer [11]. Decreased XPC expression has also been shown to correlate with bladder cancer malignancy and its resistance to cisplatin treatment [24].

As mentioned, the XPC protein is required for early DNA damage recognition and has been shown to be the initiator of G-NER [25, 26]. The XPC step is the rate limiting step in G-NER. XPC has been shown to exhibit a strong binding affinity for damaged DNA with affinity for UV-damaged DNA and lesions that cause helical distortions [20, 27, 28]. Cells that lack XPC exhibit little or no NER [29, 30] (**Figure 2**). Studies involving XPC knockout mice showed that these mice are viable and develop normally; however, they exhibit increased sensitivity to UV light and are highly susceptible to skin and lung cancer similar to XPC patients [30-35].

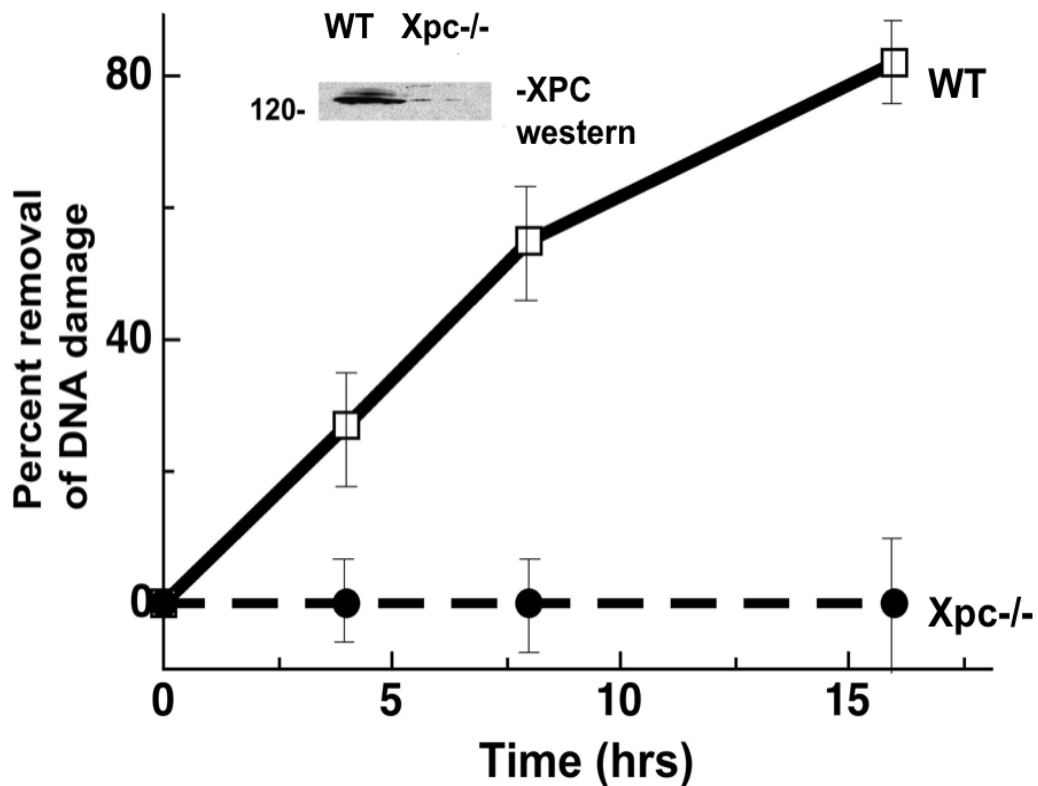


Figure 2. XPC is rate-limiting and is required for NER DNA repair. The purpose of this experiment was to verify the NER defective phenotype of the mice obtained from commercial sources. The mice originated from Allen Bradley’s laboratory prior to their commercial distribution [30]. Bone marrow pooled from three untreated 10-week-old wild type (WT) or knockout Xpc ($-/-$) mice were used to illustrate that XPC is required for DNA repair. XPC protein was below detection by western blot in Xpc $-/-$ mice bone marrow (inset). Removal of DNA damage was determined by culturing WT and Xpc $-/-$ cells in cytokine-containing medium for 15 hours. Cells were irradiated with 20 J/m^2 254 nm UV-radiation in 1mm depth of phosphate-buffered saline and returned to tissue culture. Time points were taken at 0, 4, 8 and 16 hours and assayed using an antibody to

UV-induced 6-4 photoproducts. *Xpc*^{-/-} bone marrow did not remove DNA lesions after 16 hours, whereas, WT cells removed 80% of the lesions by 16 hours ($P < 0.02$ by t-test). The data confirms the known rate-limiting and required role of XPC in G-NER. The assay measures mostly G-NER [36].

Regulation of XPC

Studies have shown that XPC is required for the recognition of DNA damage in G-NER and for the recruitment of DNA repair factors to the site of damage. Given the importance of XPC, relatively little is known about how XPC is regulated. At least one level of regulation occurs at the transcriptional level by transcription factor/tumor suppressor p53 [37]. Studies conducted by Ford *et al.* revealed that Xpc is inducible in a time and dose dependent manner following UV irradiation in cells that expressed WT p53, while induction was not seen in cells that lacked p53. Further investigations revealed a p53 binding element in the Xpc promoter region and showed evidence for transcription regulation of Xpc by p53 [37, 38].

XPC protein regulation was explored in studies by Wang *et al.* where they investigated post translational modifications of XPC. Previous studies showed evidence of higher molecular weight forms of XPC after UV irradiation by western blot. Later XPC was shown to be modified by sumoylation and by ubiquitination [39]. The studies by Wang *et al.* involved mutating XPC at lysine 655 to alanine (K655A). The K655A mutation was shown to block sumoylation, ubiquitination, and degradation of XPC [40]. The data also showed that XPC degradation occurs prior to the recruitment of XPG in the multistep NER process. Other studies provided evidence for the regulation of XPC by XPE (also known as DDB2) which is an ubiquitin ligase. Studies by El-Mahdy *et al.* showed that the E3 ubiquitin ligase cullin 4a (Cul4a) mediates the degradation of DDB2 and that this event is associated with the regulation of XPC damage recognition in NER [41].

Regulation of XPC by ubiquitination

The ubiquitin polypeptide has been linked to the regulation of many proteins. One or more ubiquitin peptides are covalently attached to target proteins typically leading to proteosomal degradation. Ubiquitin is covalently attached to target proteins by activating an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin-protein ligase [42, 43]. The ubiquitin pathway has been implicated in DNA repair and has been found to be required for optimal NER [44-46]. The ubiquitin-proteasome pathway and the NER pathway combine to promote DNA repair in UV damaged cells [46-52]. It is commonly thought that the cell cycle suspends its activity to allow time for DNA repair to occur and that DNA repair machinery is in some way removed prior to entering S-phase. It is feasible to think that upon RB phosphorylation the cyclin E protein is activated and thus acts as a signal for the ubiquitination of XPC. Previous studies involving the CDT1 checkpoint and cyclin E may provide insight into the mechanism of XPC ubiquitination. The CDT1 cell cycle checkpoint is first phosphorylated by cyclin E and then ubiquitinated by Cul4a [53-56]. Studies have also shown that XPC is post-translationally modified by ubiquitination [39]. While CUL4a has been identified as an E3 ubiquitin ligase used to target XPC [41], other ubiquitin ligases may be involved in this process. The MDM2 protein is a known E3 ubiquitin ligase and promotes the ubiquitination of both p53 and RB, however, its role and involvement in DNA repair has not been established.

DNA Repair and the Cell Cycle

It has long been thought that p53 in its native wildtype (WT) form protects cells from DNA damage in part by trans-activating downstream genes whose products are mediators of cell cycle arrest responses. The p21/waf1/cip1 gene (p21) is a prominent example [57]. After DNA damage, p53 induces p21 which is a cyclin dependent kinase inhibitor. Inhibition of cyclin-dependent kinase(s) causes cell cycle arrest. It is widely held that cell cycle arrest(s) protect cells by preventing their S phase entry and thereby prevents replication of damaged DNA templates. However, the findings that Xpc was a p53 regulated gene suggested an active role for repair and not merely a passive protective role that is cell cycle arrest. It would seem therefore, that the relationship between DNA repair and cell cycle arrest would be important to study.

Early studies showed that p53 could mediate protection from UV -radiation at the cell survival level and that p53, via its downstream genes, promoted DNA repair [58, 59]. However as p53 regulates at least 100 genes, the finding by Ford that XPC was p53 regulated was a key finding. Other p53 regulated proteins i.e., Gadd45 also contribute to NER [60, 61]. There are probably additional factors besides p53 that regulate XPC expression and/or function. It is with this in mind that we further investigated XPC's regulation and involvement with the cell cycle.

In line with the idea that p21 and cell cycle arrest could promote DNA repair, a tetracycline-inducible p21 promoted DNA repair of a UV-damage reporter gene [62]. Moreover, cells null for both p21 alleles were defective in repair of UV-damage using similar experimental approaches [62, 63]. The cell lines used in these studies lacked functional p53, thus, p21 was able to bypass a requirement for p53 in promoting DNA

repair. However, as no known biochemical contribution by p21 to DNA repair has been forthcoming, it is likely that any contribution by p21 to DNA repair and protection from DNA damage is a cellular effect and not one that can be demonstrated biochemically. Attempts to show an effect of p21 on NER *in vitro* showed either no effect [64] or an inhibitory effect [65] in contrast to the cell based studies cited above.

Because of the conflicting studies of the possible role of p21 in promoting NER, and because XPC is a required biochemical constituent of NER, we focused on XPC regulation in relation to NER and in relation to the RB-mediated cell cycle checkpoint, independent of p53 and p21. The latter factors have been studied in detail elsewhere and the contrasting nature of these studies suggests that a simplified approach is needed [58, 60, 62, 63].

DNA Repair and Cell Cycle Checkpoints

The cell cycle is composed of four main phases G₀, G₁, S, M and G₂ which are essential to the ordered proliferation of the cell. The process is tightly regulated. Many studies show a delay in the G₁ phase of the cell cycle after DNA damage that allows time for DNA repair prior to S phase where DNA replication occurs [66-71]. DNA damage can cause the cell cycle to arrest or may result in cell death. It is thought that in most instances as DNA damage is encountered, the cell cycle is stalled to complete the repair process before continuing into DNA replication and cell division. Otherwise, continued proliferation of cells with damaged DNA could lead to mutations and carcinogenesis. Therefore, cell cycle checkpoints are critical to responding to DNA damage and to repairing DNA.

Cell cycle checkpoints are crucial in preventing the replication of damaged DNA. In addition to allowing time for DNA repair to take place, cell cycle checkpoints ensure that the cell has carried out the biochemical events needed to proceed to the next stage in the cycle [2, 72-74]. The G1/S cell cycle checkpoint (**Figure 3**) is particularly important because it governs S phase entry [75]. Studies conducted by Marini *et al.* showed that checkpoint activation in G0/G1 was dependent on the recognition and processing of damaged lesions by G-NER [76]. Known checkpoints occur to facilitate the transition into G1/S, S, and G2/M; once the cells pass a checkpoint they are restricted from returning to the previous cell cycle phase.

The regulation of the G1/S restriction point involves several proteins. One of the main proteins involved in G1/S is the retinoblastoma protein (RB). RB exists in an under-phosphorylated state for most of G1, however, as the cycle transitions from G1 to S phase RB is phosphorylated at multiple residues allowing the release of E2F and progression of the cell cycle into S phase. RB acts as a mediator of the G1 to S phase transition and is required to for G1/S arrest response to DNA damage [77]. Cyclin-dependent kinases (CDKs) specifically phosphorylate RB and promote S phase entry. In addition, microarray data exploring the role of XPC in cisplatin treated fibroblasts and XPC defective human cell lines revealed that genes involved in the cell cycle and cell proliferation were induced when cells were treated with cisplatin [78]. These studies revealed that cisplatin DNA damage resulted in cell cycle arrest and in the activation of the p53 pathway.

Regulation of the cell cycle and XPC by p53

DNA damage-induced G1 arrest has been associated with the p53 tumor suppressor protein [79]. In normal cells, the p53 pathway is activated by DNA damage to cause cell cycle arrest. However, most cancer cells are either p53-null or carry p53 mutations meaning that they are defective in mounting a response to DNA damage. As stated before, XPC is also now known to be regulated by p53 [37]. Therefore theoretically a large percentage of human cancers, by lacking a DNA damage response and Xpc induction may be sensitive to DNA damaging agents commonly used in chemotherapy like cisplatin and carboplatin [63]. However, current studies conducted herein indicate that even p53 null cells can regulate XPC in concert with a G1/S RB-mediated checkpoint response.

The finding that p53 regulates the DNA repair protein XPC [37] suggests a more active role of the G1 checkpoint in promoting DNA repair, not merely allowing time for repair but actually increasing the availability of the rate-limiting DNA repair protein XPC. Our data indicates a role for RB in stabilizing XPC even in the absence of p53. MDM2 on the other hand, seems to destabilize XPC, which makes sense after repair is complete (usually 3-4 hours). These data add a new level of regulation of the G1/S checkpoint in relation to DNA repair.

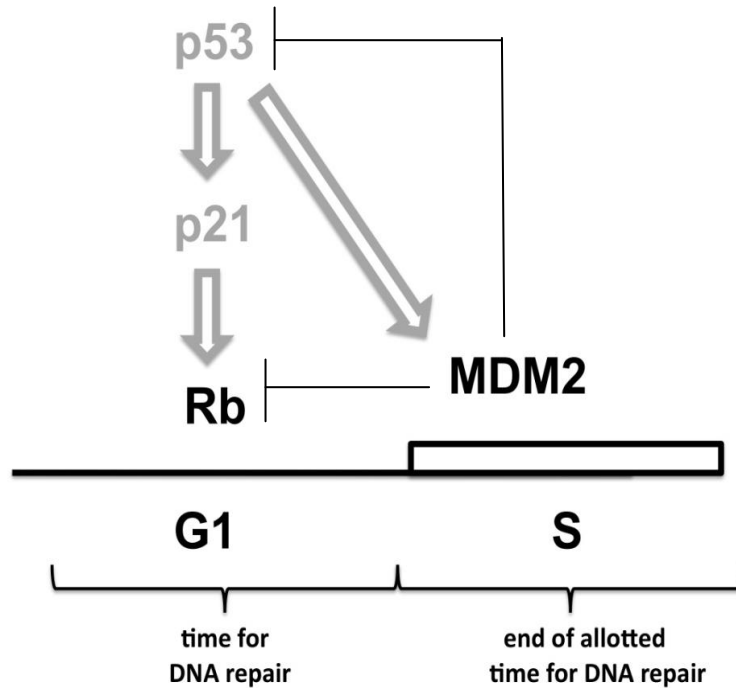


Figure 3. Diagram of the G1/S checkpoint. Normally cycling cells are driven by cyclin dependent kinases (CDKs) which phosphorylate RB and thus move cells into S phase. Rb is thought of as a gatekeeper of the G1/S transition. After DNA damage, Rb mediates p53/p21-mediated cell cycle arrest [57, 58, 80, 81]. p21 (also called Cip1 or Waf1) is an inhibitor of CDKs. Thus, RB phosphorylation is inhibited after DNA damage and cells arrest in G1. It has long been thought that G1 arrest mediated by p53, p21, and Rb, facilitates DNA repair or allows time for repair. After DNA repair is complete, MDM2, also a p53-regulated protein, relieves the G1 arrest and promotes S phase entry and acts as a negative regulator of both RB and p53.

RB and DNA repair

Although the relationship between the RB protein and the cell cycle is known, little is known about RB and DNA repair. In studies conducted by Harrington *et al.*, Rb ^{-/-} mouse embryos were defective in cell cycle arrest after treatment with chemotherapeutic drugs and γ and UV-irradiation, implicating a role for RB in DNA damage response [77]. Studies also suggest that loss or inactivation of RB should sensitize cells to DNA damage and that RB plays a role in determining cell fate after DNA damage [82]. Other investigations have suggested other roles for RB in response to UV damage [83]. Recent studies of the E2F1 transcription factor, a downstream effector of RB, showed that E2F1 accumulates at sites of DNA damage and binds to the XPC gene promoter after UV irradiation [84, 85]. E2F1 may transcriptionally or post-transcriptionally regulate XPC although the effects on XPC may be positive or negative. Further exploration of the relationship between RB and DNA repair is needed to determine its role in the repair process. It is with this in mind that studies herein investigate the DNA repair protein XPC and the involvement of RB and G1/S cyclins in XPC regulation. We hypothesize that key G1/S proteins, including RB and G1/S cyclins, are involved in the regulation of XPC in the context of the G1/S cell cycle checkpoint.

MATERIALS AND METHODS

Cell lines

H1299 human lung carcinoma cells and Saos2 human osteosarcoma cells were cultured in DMEM 4500mg/L glucose supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine and gentamicin. Cells were plated for transfection as described below at sub-confluency. H1299 and Saos2 carry deletions in both p53 alleles and do not express p53 protein [86]. Extensive characterization as part of the National Cancer Institute (NCI) anticancer drug screen showed absence of functional p53 and absence of the G1/S checkpoint in these cell lines [86].

Cell transfection and plasmids

Cells were evenly seeded at 50% confluency onto cell culture dishes and incubated at 37°C overnight to allow adhesion. Cells were transiently transfected using either Fugene HD transfection reagent (Roche Applied Science, Indianapolis, IN, USA) or Turbofect transfection reagent (Fermentas Life Science, Ontario, Canada) per manufacturer's protocols. Transfection mixtures were added to each plate drop-wise. The plate was then gently rocked back and forth by hand to achieve even distribution. The cells were incubated for 48 hours at 37°C before collection. All plasmids, with the exception of shRNA plasmids, were pCMV3 derivatives and encoded full-length cDNAs driven by the CMV promoter. Plasmids used for shRNA studies were pRS derivatives driven by the U6 promoter. Empty pCMV3 or pCMV6 (Origene, Rockville, MD) plasmids were used as vector controls. Full-length cDNA encoding Xpc, Rb, or cyclin E

were in pCMV6 (Origene). RB mutants driven by CMV plasmids were a gift from Dr. Nick Dyson, Massachusetts General Hospital. Additional plasmids used throughout are listed in Table 1.

PLASMID	DETAILS	SOURCE
pCMV6-XL5	Empty Vector	Origene (Rockville, MD)
Xpc	Xpc cDNA in pCMV6-XL5	Origene
Mdm2	Mdm2 cDNA in pCMV6-XL5	Origene
Rb	Rb cDNA in pCMV6-XL5	Origene
pCMV3	Empty Vector	Dr. M.B. Kastan
cyclin E	cyclin E ORF in pCMV3	Dr. M.L. Smith [87]
cyclin D1	cyclin D ORF in pCMV3	Dr. Phil Hinds
pRS	Empty Vector	Origene
Cul4a	shRNA Constructs against Cul4A in pRS plasmid	Origene
Cul4a DN	Cul4a dominant negative in pCMV3	Addgene (Cambridge, MA)
Mdm2	shRNA Constructs against Mdm2 in pRS plasmid	Origene

Table 1. Plasmids used in H1299 and Saos cell transfection experiments. The pCMV3 and pCMV6-XL5 plasmids were used as vector controls. The Xpc cDNA in pCMV6-XL5 and Rb cDNA in pCMV6-XL5 were used to carry out XPC, RB co-transfections

and throughout most experiments. The amount of plasmid used was optimized for each experiment ranging from 0.1-1 μ g.

Immunoblotting

Samples were lysed by directly adding 2x SDS loading dye (1.5g SDS, 2% bromophenol blue, 1.23g DTT, dissolved in 10ml of water) and collected by scraping and transferred into 1.5ml eppendorf tubes. The samples were boiled for approximately 30 minutes and 15-20 μ l of sample were added to precast 4-20% Tris-Glycine gels (Invitrogen, Carlsbad, CA) and run at 100-150 volts. Proteins were transferred to nitrocellulose membranes at 5 volts (<10) amps overnight. Nitrocellulose membranes were blocked with 4% nonfat milk buffer prior to probing with primary and secondary antibodies. Primary antibodies to XPC A-5 mouse monoclonal or XPC H-300 rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at concentrations of 0.1-1 μ g/ml in most experiments. Anti- β actin rabbit polyclonal (Thermo Scientific, Waltham, MA, USA), anti- β galactosidase mouse monoclonal BG-02 (Abcam, Cambridge, MA, USA), and other additional antibodies were used as indicated in figure legends and in Table 2. Secondary antibodies were peroxidase conjugates from Santa Cruz. Immunoblots were detected by ECL (Pierce Thermo Scientific, Rockville, IL, USA) and exposed to Kodak Biomax XAR film (Sigma Aldrich, St. Louis, MO, USA).

Western blot antibodies

PRIMARY ANTIBODY	CLONE	SOURCE
XPC	A-5 mouse monoclonal	Santa Cruz Biotechnology
XPC	H-300 rabbit polyclonal	Santa Cruz Biotechnology
XPC	D10 mouse monoclonal	Santa Cruz Biotechnology
XPC	3.26 mouse monoclonal	Santa Cruz Biotechnology
RB	XZ55 and X2104 mouse monoclonals	Calbiochem
RB	AF11 mouse monoclonal	Calbiochem
cyclin E	HE12 mouse monoclonal	Calbiochem
cullin 4a	Rabbit polyclonal	Abcam
hHR23b	Rabbit polyclonal	Abcam
biotin	BN-34 mouse monoclonal	Sigma-Aldrich
ubiquitin	PD41 mouse monoclonal	Santa Cruz Biotechnology
β -Galactosidase	BG-02 mouse monoclonal	Abcam
β -Actin	Rabbit polyclonal	Thermo Scientific
PCNA	PC10 mouse monoclonal	Calbiochem

Table 2. Western blot antibodies used in immunoblotting H1299 and Saos2 cellular extracts. Primary antibodies in Table 2 were used at various concentrations, generally 0.1-1 μ g/ml to ensure the optimization of Western blots to detect their respective proteins.

Recombinant proteins

The following recombinant proteins were used as controls in several western blot and immunoprecipitation experiments:

RECOMBINANT PROTEIN	DETAILS	SOURCE
XPC	partial protein with GST tag	Abnova
XPC	full length baculovirus construct	Dr. Suk-Hee Lee
MDM	Full length recombinant protein with GST	Novus Biological
MDM2 Ring domain	Mdm2 C-terminal RING domain	Biomol International

Table 3. Recombinant proteins were used as controls for immunoblots and immunoprecipitation experiments. Immunoblots and immunoprecipitation experiments were conducted as described herein.

Immunoprecipitation (IP)

Cells were harvested and lysed in immunoprecipitation (IP) lysis buffer containing 1% Triton X-100 and protease inhibitors. Samples were then transferred to a 1.5ml microcentrifuge tube and incubated on ice 30 minutes and pelleted via microcentrifugation. The supernatant was transferred to a new tube containing 40-50 μ l of protein A plus protein G agarose beads and 1 μ l/ μ g of XPC clone D-10 antibody (Santa Cruz). Rb antibody XZ155 was used in some experiments as indicated. The mixture was then allowed to incubate at 4°C on a rotator overnight. After incubation the samples were

microcentrifuged for 30 seconds to pellet the beads. Beads were washed 4 times with lysis buffer. Beads were resuspended in 50µl of 2X SDS loading buffer and boiled prior to loading immunoprecipitated protein onto 4-20% Tris-Glycine gels and immunoblotting.

Host Cell Reactivation

The host cell reactivation assay (HCR) is used to measure DNA repair in cells (**Figure 4**). HCR is an established assay in use in several laboratories [58, 88, 89]. A plasmid encoding a chloramphenicol acetyltransferase (CAT) reporter gene was damaged by cisplatin to produce 1 to 5 adducted bases per plasmid molecule. NER-defective fibroblasts from XPC patients are defective in “reactivating” and thereby expressing the reporter gene. NER-proficient cells repair the gene and thereby express the reporter [36]. The ability of the host cells to repair the damaged plasmid is reflected in reporter gene expression. Activity encoded by the damaged reporter gene is determined by ELISA, and is a reflection of the DNA repair capacity of the host cell. One advantage of the assay is that only the damaged plasmid and not the cells receive DNA damage. Thus, cellular effects e.g. apoptosis are not an issue.

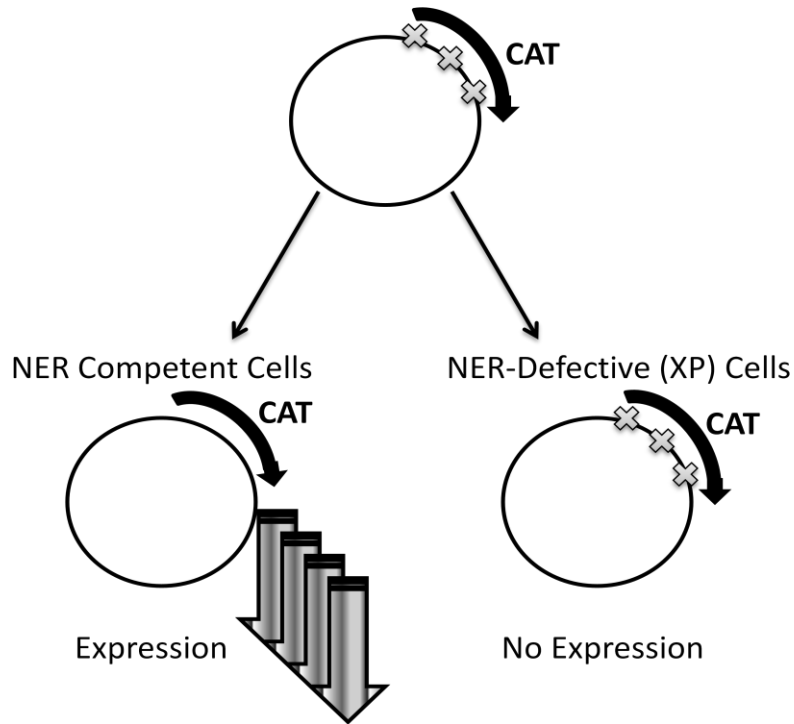


Figure 4. Diagram of host cell reactivation assay. The HCR assay measures DNA repair by restoring a plasmid that encodes a reporter gene (CAT). The reporter gene has been inactivated due to induced damage to the plasmid caused by platinum adducts (indicated by X). The ability of the host cell to repair the damaged plasmid is determined by reporter gene expression. Cells that are defective in NER are unable to repair the damaged plasmid and therefore unable to express the reporter gene. However, NER competent cells are capable of repairing the damage and express the reporter CAT reporter gene indicating the host cells ability to repair DNA damage.

CAT ELISA

The chloramphenicol acetyltransferase enzyme-linked immunosorbent assay (CAT ELISA) immunoassay (Roche Applied Science, Indianapolis, IN, USA) was used for the quantitative determination of the CAT enzyme. Briefly, equivalent protein concentrations of lysed cell extracts were added to an anti-CAT-coated 96-well microplate and refrigerated overnight. Microplate wells were then washed and a digoxigenin-labeled antibody to CAT (anti-CAT-DIG) was added to wells at room temperature for at least 1 hour. Wells were then washed and an antibody to digoxigenin conjugated to peroxidase (anti-DIG-POD) was added for 1 hour. The peroxidase substrate ABTS was added after washing. The plate was then incubated and read at 405 nm in a Tecan Spectra plate reader at multiple time intervals.

Pulse Chase Analysis

After H1299 cells were transfected with Xpc and Rb plasmids, cellular proteins were labeled for 4 hours with an L-methionine analog that can be subsequently labeled with biotin (Invitrogen, Carlsbad, CA, USA). The free label was removed by placing the cells in complete medium and cells were harvested after 0, 4, and 8 hours. XPC was immunoprecipitated as described above and then labeled by biotinylation (Invitrogen). The L-methionine analog has a reactive azide group that is then covalently modified by an alkyne-biotin reagent (Invitrogen) and detected using a mouse monoclonal anti-biotin antibody (Sigma, St. Louis, MO, USA).

Statistical Analysis

All data are presented as mean or relative units \pm standard error. Data were analyzed as indicated in figure legends using one way ANOVA or paired t-test with Microsoft Office Excel 2007 (Seattle, WA) or Graph Pad Prism (San Diego, CA).

RESULTS

XPC protein expression and DNA repair activity in the presence of RB

In an effort to investigate the relationship between the G1/S cell cycle checkpoint and DNA repair, our study focused on the retinoblastoma protein RB, a known mediator of the G1/S checkpoint, and its association with the XPC DNA repair protein. In order to determine the effects of RB on XPC, we chose H1299 human lung cancer cells to carry out most experiments. H1299 cells were chosen because they have low endogenous XPC, thereby making it easier to detect transfected XPC in transient expression experiments. Additionally, these cells are p53 null [86]. To determine the effectiveness of our plasmids and the efficiency of our transfections H1299 cells were transfected with pCMV-Rb, pCMV-Xpc, or empty plasmid and protein expression was observed. The pCMV-Rb and pCMV-Xpc plasmids showed appreciable expression of their respective proteins when introduced to H1299 cells compared to empty CMV plasmid (**Figure 5A**).

We then combined pCMV-Rb and pCMV-Xpc and transfected them into H1299 cells. Empty pCMV plasmid was used to keep the amount of plasmid DNA constant across all the dishes, while pCMV-Rb and pCMV-Xpc plasmids alone were transfected as controls. When combined with pCMV-Rb, the abundance of XPC protein was markedly increased (**Figure 5B**, lane 8) when compared to empty plasmid or the Xpc or Rb plasmid alone. Most importantly, combining pCMV-Rb with pCMV-Xpc markedly enhanced DNA repair of a cisplatin-damaged reporter plasmid, pCMV-CAT (**Figure 5C**, lane 8). Thus, the co-transfection of Rb together with Xpc significantly ($p= 0.0005$)

enhanced the DNA repair activity of XPC as measured by host cell reactivation assay. Interestingly, increasing the amount of XPC plasmid alone did not affect the DNA repair activity of the cells (**Figure 5A**, lane 4 compared to **Figure 5C**, lane 4), indicating that XPC exists in an inactive form that can be activated in combination with RB. Data herein (**Figure 5A** and **C** control lanes) provide evidence that, despite increases in XPC expression, its DNA repair capacity does not increase. Our data shows DNA repair is increased with the addition of RB (**Figure 5C**) implicating a level of regulation that does not rely on transcription.

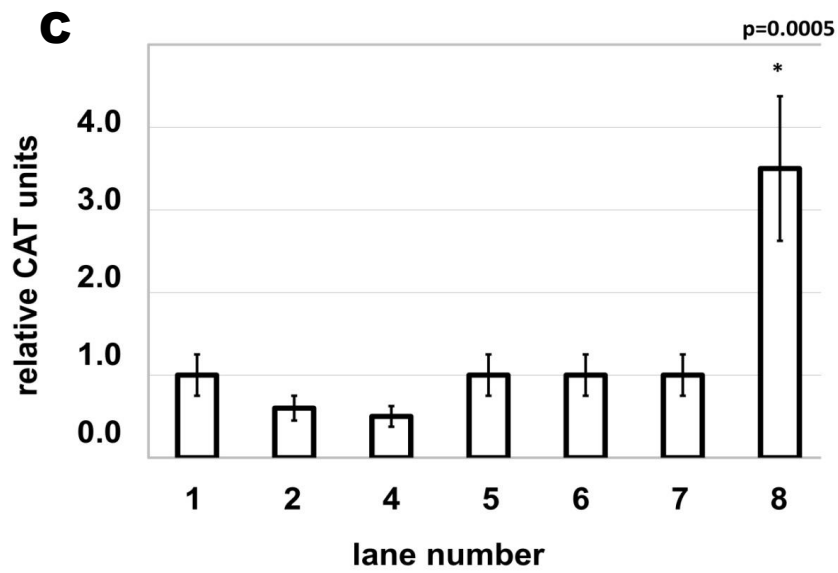
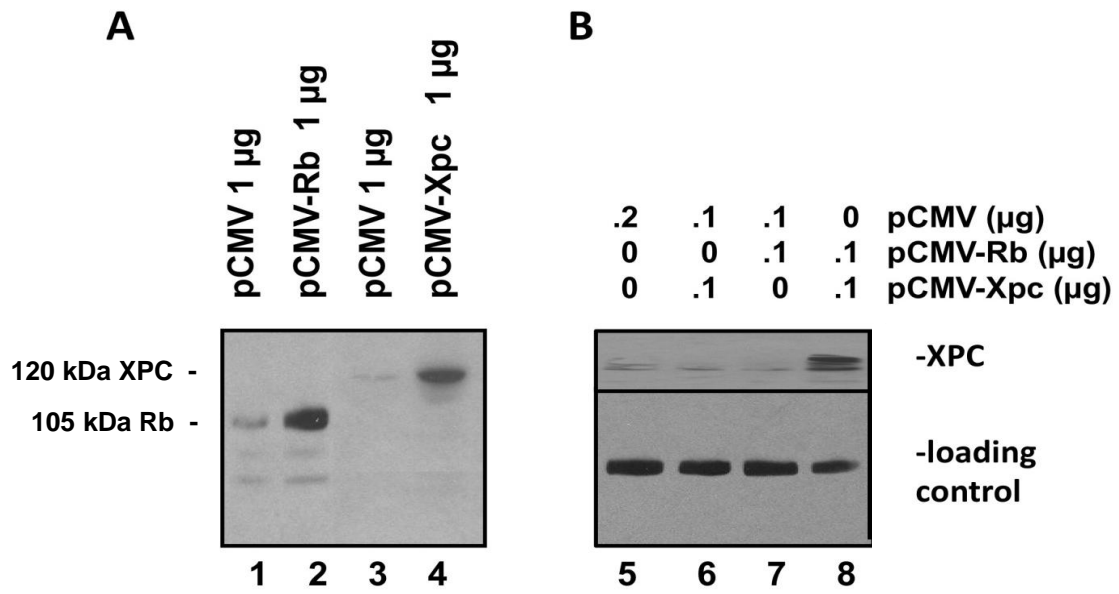


Figure 5. RB enhances XPC-mediated host cell reactivation of a cisplatin-damaged reporter gene. **A)** Plasmids were transfected at indicated amounts. The total amount of plasmid DNA was kept constant by adding empty CMV plasmid where necessary. H1299 cells have low endogenous XPC. RB and XPC were encoded by the respective plasmids and were readily detected by western blot (lanes 2 and 4). **B)** Mixing RB and XPC plasmids resulted in a clear increase in XPC protein (western blot, lane 8). XPC was detected with antibody A5, while RB was detected with antibody AF-11. Actin served as a loading control. **C)** Host cell reactivation (HCR) corresponding to the same lanes as in A and B. Neither RB alone, nor XPC alone (bar graph, lanes 2 and 4 respectively) resulted in increased HCR. When combined (bar graph, lane 8) a significant increase in HCR was observed ($P= 0.0005$). CAT activity was measured by ELISA as previously described in materials and methods.

RB activation of XPC does not require ongoing protein synthesis

It is likely that the observed enhancement of XPC-mediated DNA repair is due to 1) interaction of XPC with RB and/or 2) activating modifications of XPC protein that are triggered by interaction with RB. A third possibility is that RB enhanced the translation of XPC protein. However, if this were the case, it would result in increased activation of the XPC protein observed in **Figure 5C** lane 8. As can be seen in **Figure 5A**, simply increasing the amount of XPC to 1 μ g did not lead to a functional increase in XPC DNA repair activity as indicated and measured by host-cell reactivation (**Figure 5A**, lane 4 compared to **Figure 5C**, lane 4). We conducted experiments with cycloheximide to block protein synthesis to address this possibility and observed that co-transfection with RB led to increased XPC levels in the presence of cycloheximide (**Figure 6A**).

Therefore, the observed enhancement of XPC by RB does not require ongoing protein synthesis. To determine whether Rb affected the half-life of XPC we conducted pulse-chase experiments, in which cells co-transfected with Rb and Xpc were labeled with an L-methionine analog to label methionine residues during a 4-hour “pulse” according to manufacturer’s instructions (Invitrogen). Cells were returned to complete medium with excess unlabeled L-methionine (“chase”) and were harvested 0, 4, or 8 hrs later. The co-transfection of Rb extended the half-life of XPC beyond 8 hrs, compared to <4 hrs in the control transfectants (**Figure 6B**). Thus, RB appeared to increase the half-life of XPC.

XPC and Rb Co-Immunoprecipitation

Given these results, we hypothesized a possible protein-protein interaction between RB and XPC. We used XPC and RB recombinant proteins to detect a possible interaction between RB and XPC, but failed to detect an interaction (results not shown). One possibility was that the interaction required protein folding concurrent with translation. If this were the case, the two proteins would interact when co-expressed in the same cell. When mixed as full-length purified proteins, they would not show an apparent interaction. To investigate this likelihood, H1299 cells were co-transfected with XPC and Rb and immune complexes containing RB were probed for the presence of XPC and vice-versa. XPC was detected in RB immune complexes, and RB was detected in XPC immune complexes (**Figure 7**). Thus, there is a putative interaction between Rb and XPC in H1299 cells.

RB deletion mutants

We attempted to “map” the interaction domain of RB with XPC using a series of RB deletion mutants for co-transfection experiments. The idea was that we could map the domain by determining which mutants did or did not stabilize XPC. RB deletion mutants were transfected into p53null/Rb null Saos2 cells for these experiments (**Figure 8**). Mutant 1 contains a deletion in the N terminus domain of the RB protein from amino acid 303-392. Mutant 2 (Δ 773-909) contains deletions involving the E2F and MDM2 binding domains. While mutant 3 contains a deletion beginning with the cyclin/cdk docking site KXLKXL deleting amino acids 870-909. All mutants were compared to full length RB protein [90].

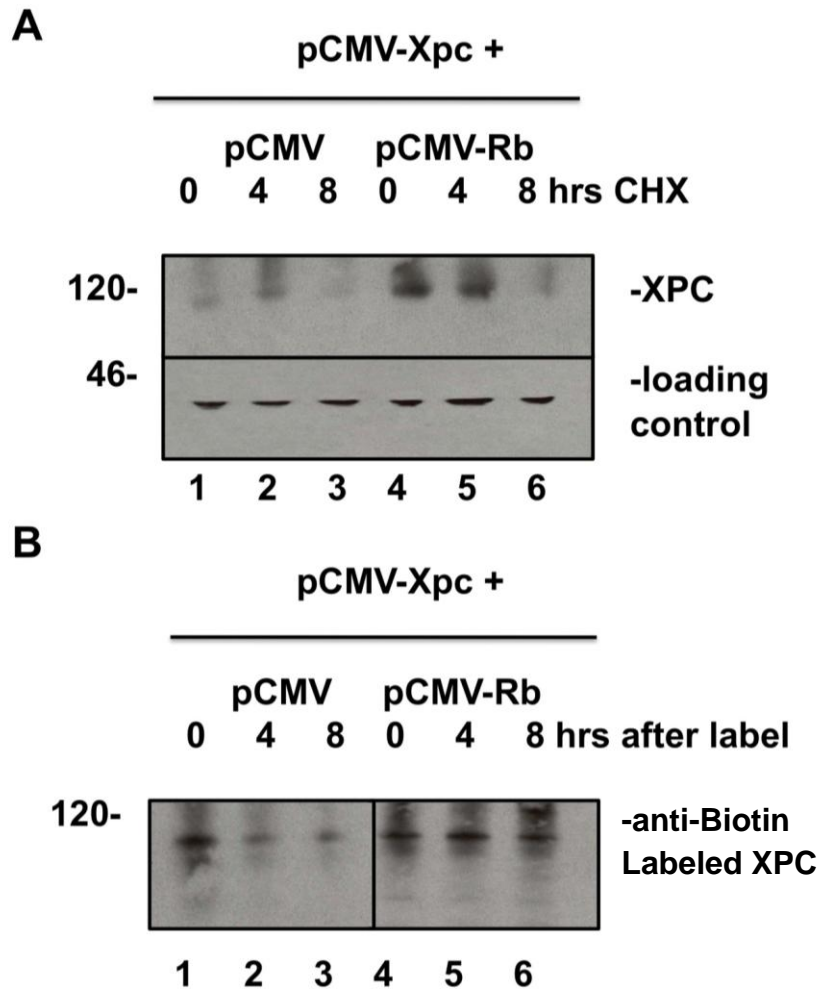


Figure 6. RB stabilizes XPC in the presence of cycloheximide or in pulse-chase experiments. **A)** H1299 cells were transfected as in **Figure 5** with plasmids encoding XPC and RB. After 48 hrs in culture, cycloheximide (CHX; 10 μ M) was added for 15 hrs. Western blots show accumulation of XPC protein where RB was co-transfected with XPC (lanes 4 and 5). Labeled XPC was detected by anti-XPC monoclonal antibody A5. Actin served as a loading control because of its long half life. **B)** Metabolic labeling of XPC with an L-methionine analog. Cells were transfected with XPC and RB plasmids as indicated, then labeled (“pulsed”) for 4 hours after a 48 hr culture incubation. The label

was removed and cells returned to complete medium and “chased” for 0, 4, or 8 hrs.

Immunoprecipitated XPC was detected using an anti-biotin antibody. Accumulated XPC

is evident in lanes that were co-transfected with RB (lanes 5 and 6).

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Surprisingly, each of the three deletion mutants failed to stabilize XPC (**Figure 9**). Each of the mutants are partially defective in G1 cell cycle arrest, thus, perhaps a G1 arrest is required. Alternatively, each of the mutants may be improperly folded such that they cannot interact with XPC. Each of the mutants is expressed similar to wildtype pRb (**Figure 9**) but we cannot exclude a defect in protein folding.

XPC and G1/S cyclins

As RB is a key mediator of the G1/S transition in cells, we tested whether the effect of stabilizing XPC was linked to the cell cycle role of RB, or whether it could be a novel function of RB. To explore this prospect we co-transfected a CMV plasmid encoding the G1/S cyclin, cyclin E, together with RB and XPC-encoding plasmids in to Saos2 cells. Cyclin E serves as the signal for S-phase entry by phosphorylating RB at the G1/S transition. The addition of Cyclin E completely abolished the stabilization of XPC

by RB (**Figure 10**, lane 8). Thus, it is likely that RB in its underphosphorylated G1 form interacts with and stabilizes XPC, and that RB in its cyclin E-phosphorylated form does not contribute to the stabilization of XPC.

The simplest hypothesis to explain the data is that RB protects XPC from degradation and that cyclin E reverses that protection from degradation. The proteasome inhibitor MG132 rescued XPC from cyclin E-mediated turnover in H1299 cells transfected with cyclin E (**Figure 11**). Cyclin E, as expected, decreased DNA repair in host-cell reactivation assays again suggesting that cyclin E plays a negative role in regulating XPC (**Figure 11**). Thus, it is likely that XPC is tightly linked to the G1/S cell cycle checkpoint by proteasome-mediated stabilization and destabilization mechanisms.

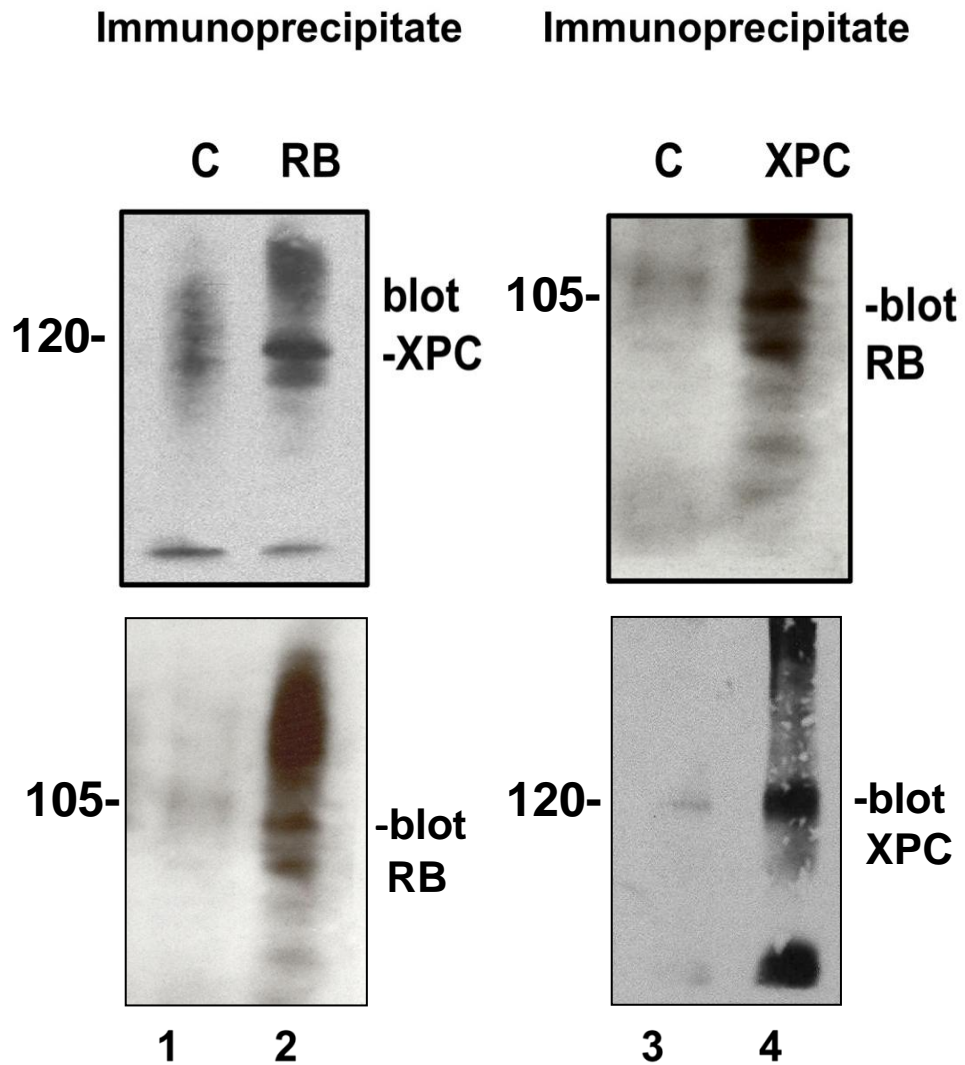
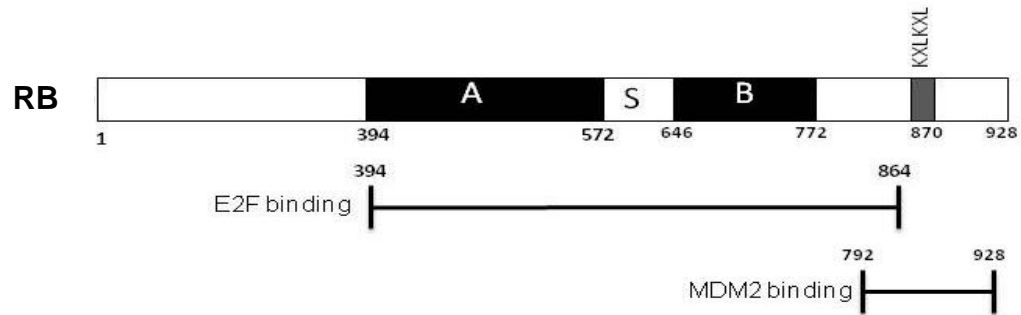


Figure 7. Putative interaction between RB and XPC. H1299 cells were transfected with Rb and Xpc plasmids. Immune complexes containing RB or XPC were probed on Western blots to detect possible interaction. Left panel, immune complexes obtained using a negative control (C), MOPC21 IgG, or anti-RB monoclonal antibodies XZ104 and XZ155; right panel, immune complexes obtained using a negative control,

nonimmune rabbit IgG, or anti-XPC rabbit polyclonal antibody H300. The upper left panel was probed by western blot with polyclonal rabbit anti-XPC. The upper right panel was probed by western blot with monoclonal anti-RB clone AF-11. Lower left and right panels were probed with RB and XPC respectively to serve as additional controls.



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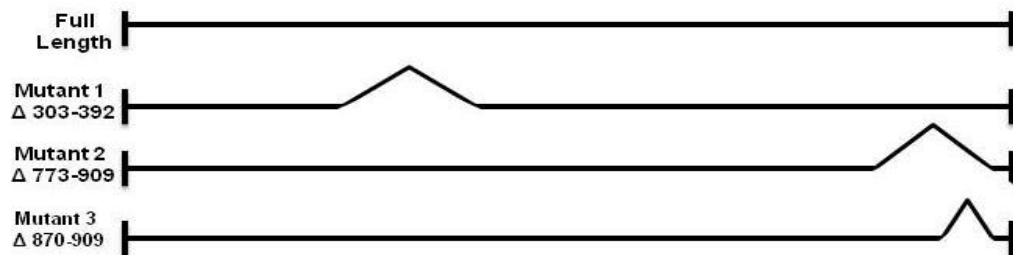


Figure 8. Structure of RB and Rb deletion mutants. Wildtype Rb contains 928 amino acid residues. The LXCXE-binding (pocket) domain begins at amino acid 394 and contains two subdomains (A and B), and an intervening spacer (S). The C-terminus also interacts with E2F, a downstream effector of the RB family, and MDM2, a p53 regulated protein and ubiquitin ligase. A cyclin/cdk docking site (KXLKXL) is also present in the C terminus beginning at amino acid residue 870. Three deletion mutants (a gift from Dr. Nick Dyson) were used in co-transfection experiments. Mutant 1 contains a deletion in the N terminus from amino acid 303-392. Deletion mutant 2 (Δ 773-909) contains deletions involving the E2F and MDM2 binding domains. Mutant 3 contains a deletion beginning with the cyclin/cdk docking site KXLKXL deleting amino acids 870-909.

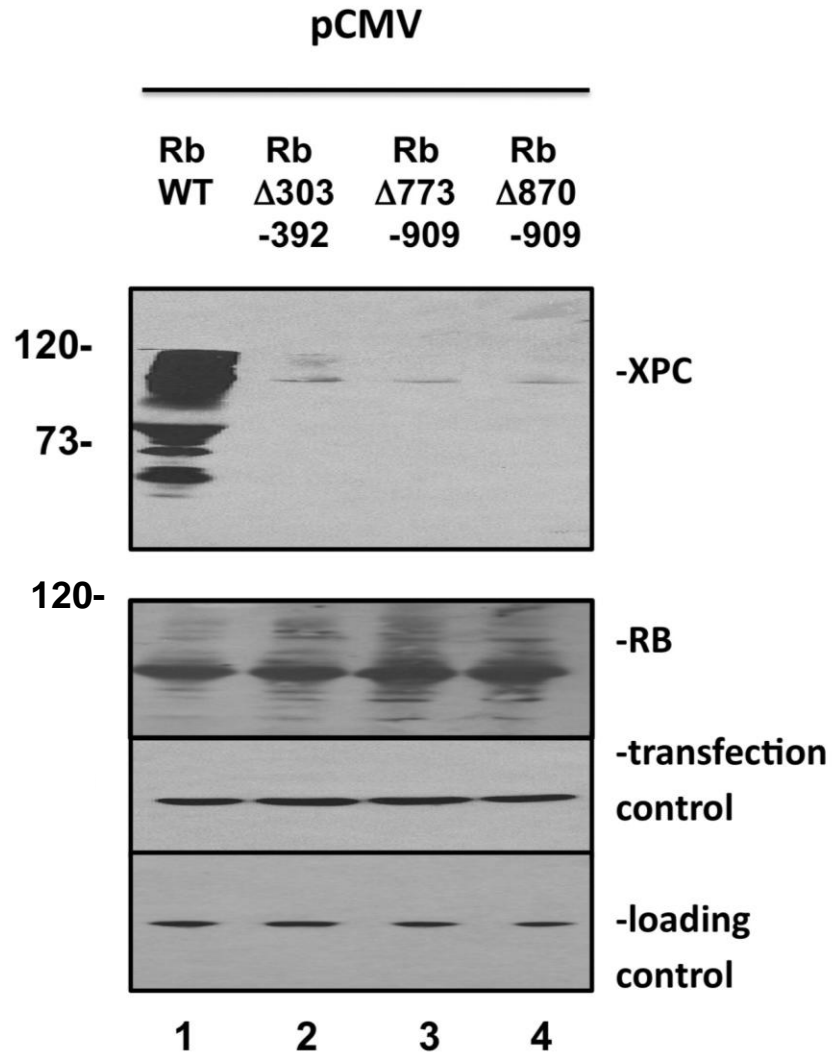


Figure 9. RB mutants fail to stabilize XPC. Wildtype RB or three deletion mutants were co-transfected together with XPC as previously described. Saos2 cells were used. Robust stabilization of XPC was observed by wildtype RB (lane 1). Each of the mutants failed to stabilize XPC (lanes 2, 3, 4). Beta galactosidase served as a co-transfection control. Actin served as a loading control. XPC was detected by anti-XPC monoclonal antibody A5.

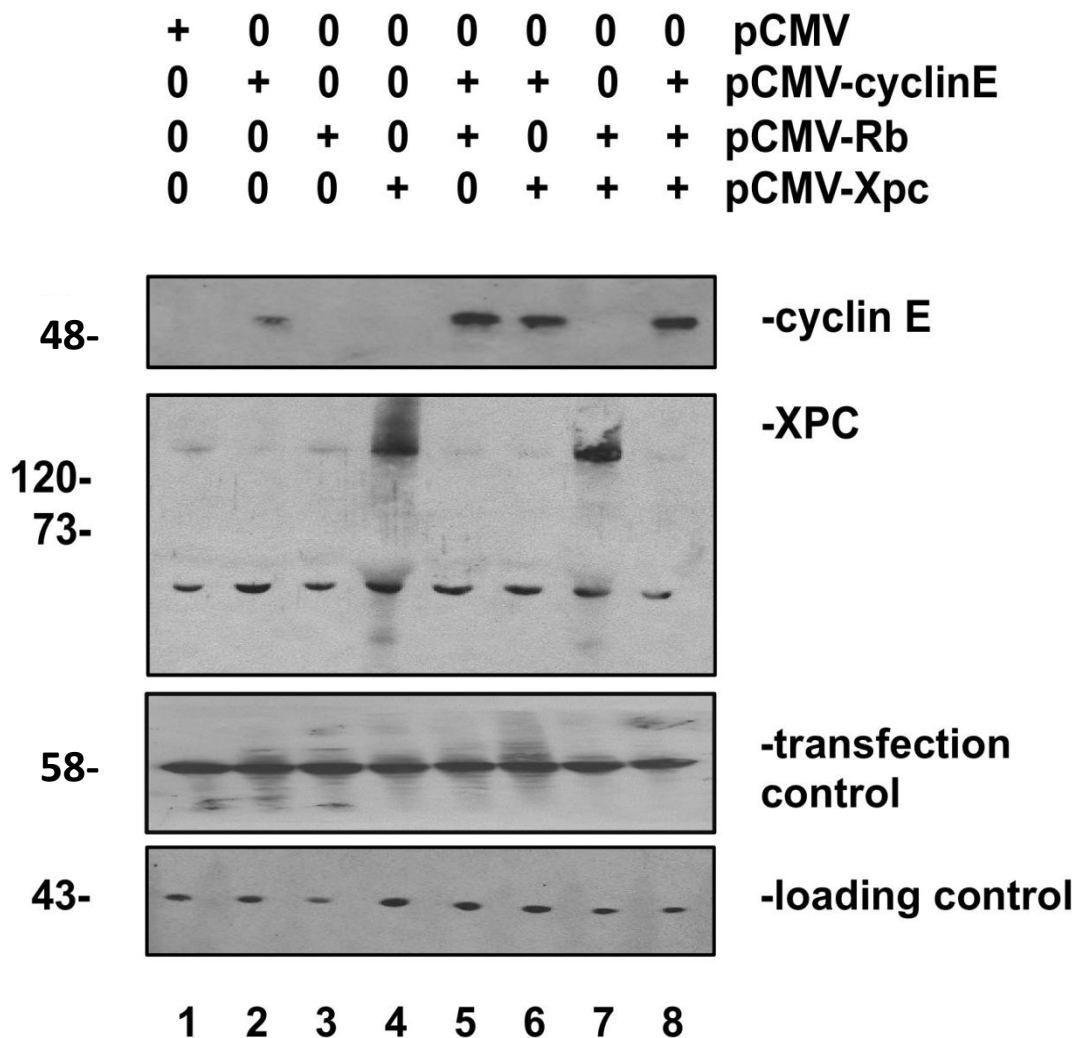


Figure 10. Cyclin E counteracts (wild type) RB stabilization of XPC suggesting that stabilization of XPC is related to the cell cycle role of RB. Saos2 cells were transfected as previously with the indicated plasmids, again keeping the total amount of plasmid DNA constant. Co-transfection of cyclin E alone or in the presence of RB and/or XPC strongly decreased XPC protein expression suggesting that cyclin E counteracts any stabilizing effect of RB on XPC. Beta galactosidase served as a co-transfection control.

Actin served as a loading control. XPC was detected by anti-XPC monoclonal antibody

A5. RB was detected by anti-RB monoclonal anti-RB clone AF-11 (data not shown).

Cyclin E was detected using monoclonal antibody HE-12 (Calbiochem).

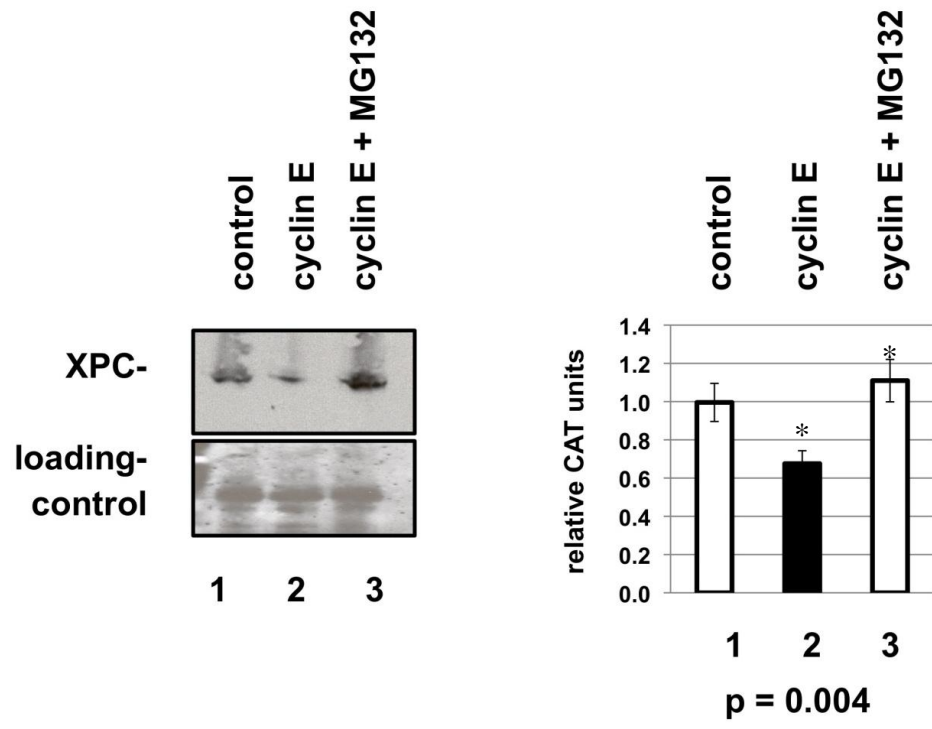


Figure 11. Ubiquitin ligase inhibitor MG-132 rescues XPC from cyclin E-triggered degradation. Cyclin E inhibited XPC expression (left panel) and host-cell reactivation (HCR; right panel; $p=0.004$ comparing lanes 1 and 2 via paired t-test). MG-132 was added ($10\mu\text{M}$) 15 hrs prior to cell lysis. MG-132 restored XPC and HCR to control levels. H1299 cells were utilized for both panels.

XPC ubiquitination

Previous studies have shown evidence for the regulation of XPC by post-translational modifications including ubiquitination [34]. To determine whether Cyclin E is involved in the negative regulation of XPC by serving as a signal for ubiquitination, we transfected H1299 cells with Xpc, Cul4a-dominant negative (DN), cyclin D1 and cyclin E plasmids. Immune complexes were probed on Western blots with anti-XPC and anti-ubiquitin to detect ubiquitination of XPC. Ubiquitinated Xpc is detected in the complex containing cyclin E (**Figure 12**, lane 4). Ubiquitinated Xpc was not observed in lane 2 where Cul4a-DN was used as a control as studies have shown that XPC is regulated by E3 ubiquitin ligase or lane 3 (an additional control) containing the G1/S mediator cyclin D1. These data indicate that Cyclin E may be involved in the negative regulation of XPC and may act as a signal for the ubiquitination and subsequent degradation of XPC upon S-phase entry. It is possible that the phosphorylation of RB by cyclin E upon s-phase entry in turn signals the disassociation of XPC from DNA.

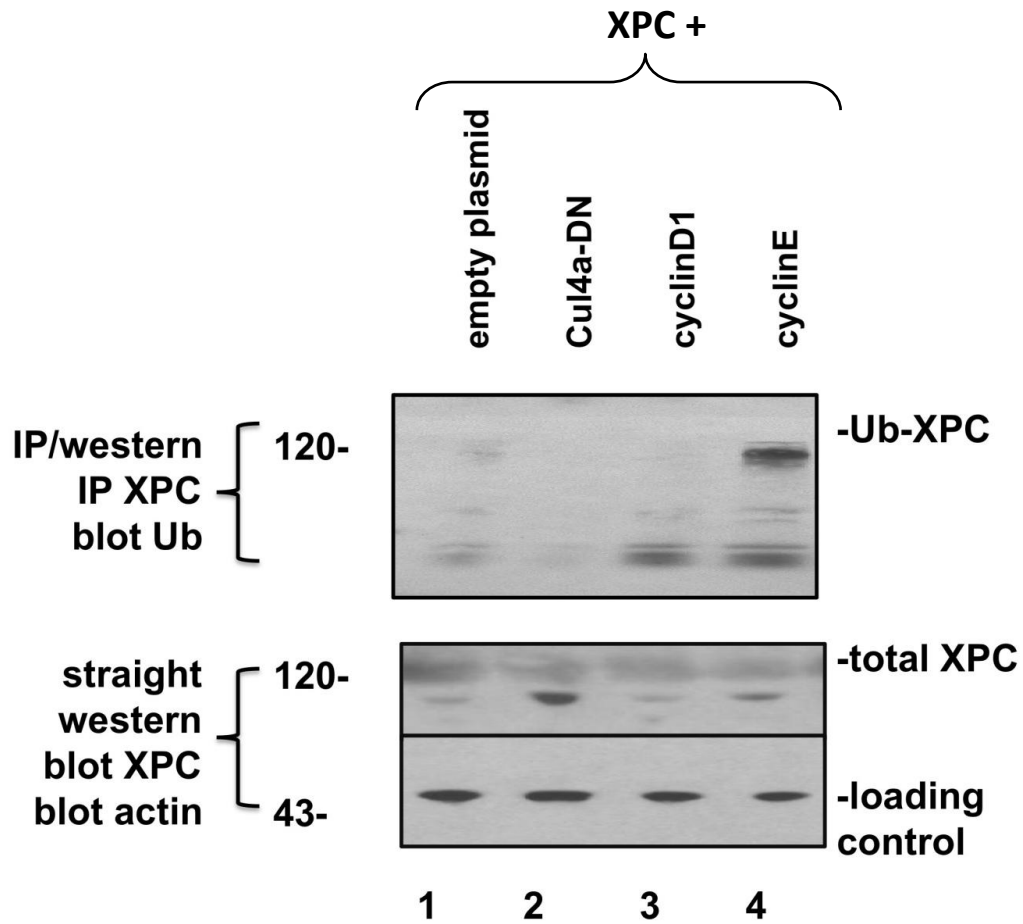


Figure 12. Cyclin E may act as a signal for the ubiquitination of XPC. Immune complexes of transfected cell lysates were probed on Western blots using an anti-ubiquitin mouse monoclonal antibody PD41 (Santa Cruz Biotechnology) to detect possible XPC ubiquitination. Ubiquitinated Xpc is detected in lane 4 in the presence of cyclin E, however, ubiquitinated Xpc was not detected in control lanes.

XPC and MDM2

While it is known that XPC is modified by E3 ubiquitin ligase Cul4a, multiple ubiquitin ligases are probably involved in the negative regulation of XPC. The MDM2 protein is a known E3 ubiquitin ligase. The involvement of MDM2 in p53 protein regulation and the identification of an MDM2 binding site within the structure of the RB protein (see **Figure 8**) have made it a protein of interest in the negative regulation of XPC. However, any role for MDM2 in DNA repair and XPC regulation is not known. To explore the relationship between XPC and MDM2, H1299 cells were transfected with empty plasmids or those containing XPC, MDM2 or both. Western blot analysis revealed that XPC steady state level decreased in the presence of MDM2 (**Figure 13A** lanes 3 and 4) indicating that MDM2 may suppress XPC steady state levels. Conversely, when MDM2 is knocked down by shRNA a modest increase in XPC expression was observed (**Figure 13B**). To determine the functional implications of these data, DNA repair was measured by host-cell reactivation in the presence of XPC, MDM2 or both and also in the presence of Cul4a (positive control) and Mdm2 shRNA (lanes correlate with western blots). When E3 ubiquitin ligases Cul4a and MDM2 were knocked down by shRNA DNA repair activity increased significantly (**Figure 13C**, $p = \leq 0.05$). These preliminary data provide evidence that MDM2 may be involved in the negative regulation of XPC possibly as an E3 ubiquitin ligase targeting XPC for degradation. Further investigations are needed to explore this possibility.

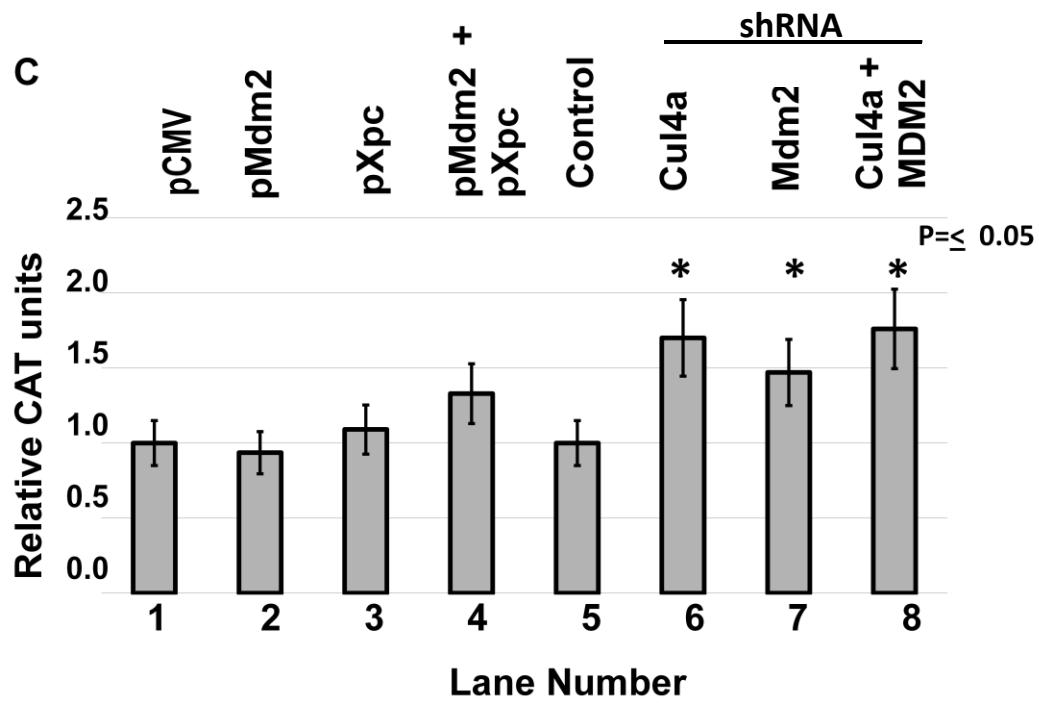
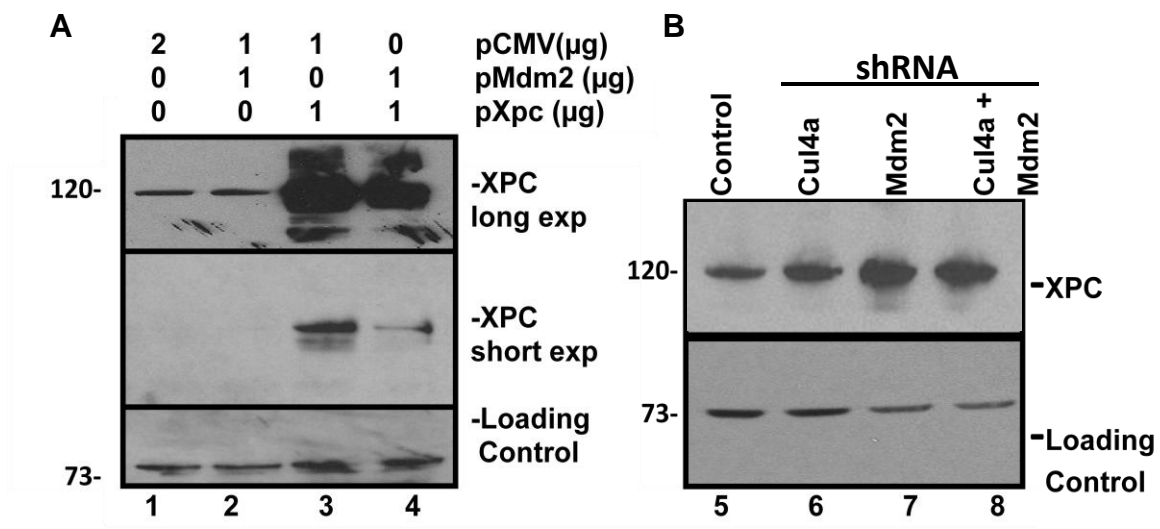


Figure 13. MDM2 may be involved in the negative regulation of XPC. **A)** H1299 human lung carcinoma cells were cultured and transfected with either an empty plasmid or plasmids containing XPC or MDM2 cDNA. Western blots of co-transfected cells reveal that XPC expression decreased in the presence of MDM2. **B)** p53 null H1299 cells were cultured and transfected with either an empty plasmid or a plasmid containing Cul4a or MDM2 shRNA. Western blot analysis showed that XPC expression increased when MDM2 is knocked down. **C)** DNA repair activity is increased when MDM2 is knocked down. DNA repair was measured by chloramphenicol acetyltransferase (CAT) assay in the presence of XPC, MDM2 or both, and also when Cul4a (positive control) and Mdm2 were knocked down by shRNA. When either Cul4a or MDM2 were modestly knocked down by shRNA DNA repair increased significantly $p \leq 0.05$. This increase in XPC expression and DNA repair activity indicate that MDM2 may be involved in the negative regulation of XPC.

DISCUSSION

One important aspect of the G1/S checkpoint is that p53 transcriptionally activates Xpc [37]. This finding suggested that p53 and XPC could actively promote NER, in cells that carry functional p53. However, the data herein suggests an additional level of regulation that does not rely upon p53. We used human lung cancer H1299 cells, which carry deletions in both p53 alleles and have low endogenous levels of XPC and we still observed a significant increase in the cell's DNA repair capacity as measured by host-cell reactivation (HCR) when combined with RB (**Figure 5B** and **C**, lanes 8). One often ignored feature of the HCR assays that we and others have used extensively is that it measures NER of a damaged reporter gene. The cells do not receive direct DNA damage. In this assay, we detected a latent XPC protein that did not promote HCR (**Figure 5A** and **C**, lanes 4). When combined with RB, XPC was in some way activated for HCR (**Figure 5B** and **C**, lanes 8). While most human tumors have defects in the p53 or Rb pathways, the data implies that if either p53 or Rb is intact, NER mechanisms are capable of repairing damaged DNA. Given evidence that RB may interact with XPC (**Figure 7**), it is likely that the interaction of XPC with RB promotes NER at least as measured by HCR. Post-translational modification(s) of XPC in the presence of RB is in evidence (**Figure 5B**, lane 8).

Several studies showed that p21/Waf1/Cip1 promoted NER in p53-defective cell lines although the mechanistic basis was not known [62, 63, 91-93]. The view was that simply arresting the cell cycle by p21 promoted NER. Our data provide a mechanistic insight, that p21 would lead to accumulation of underphosphorylated RB, which would in

turn stabilize XPC in a form that is active for NER (**Figure 5B and C**, lanes 8). Our finding that cyclin E destabilized XPC and led to a decrease in HCR is also consistent with the published studies on p21 (**Figure 11**).

The data represented herein suggests a model for both positive and negative regulation of XPC (**Figure 14**). Our data shows that XPC exists in functionally active and inactive forms. The data shows that upon DNA damage XPC is then stabilized by the underphosphorylated form of RB and correlates with DNA repair events of the G1/S phase of the cell cycle. The data also show that G1/S cyclins abrogate XPC expression and function which likely corresponds to RB phosphorylation and S-phase entry and that cyclin E may provide a signal for XPC ubiquitination. Perhaps a parallel is suggested by the Cdt1 cell cycle checkpoint protein which is first phosphorylated by cyclin E and then ubiquitinated by Cul4a.

The cellular model used for the experiments herein is atypical of DNA repair studies. Most studies involving DNA repair mechanisms center around biochemical and cell free systems. Our use of a cellular model of DNA repair is likely more reflective of the signaling mechanisms that actually occur in the cell. The majority of XPC and Rb experiments used an over expression model, whereas, preliminary data involving MDM2 utilized overexpression and the knockdown of MDM2 as a cellular model. Additional studies utilizing a knock down or inducible system of expression may give added insight into the mechanism of action of XPC regulation in the context of the cell cycle.

In response to DNA damage, XPC protein is phosphorylated, ubiquitinated, and SUMOylated, however, the amino acid residues involved and the net effects on XPC function are not well-known [37, 39, 94]. Presumably, some post-translational

modifications promote XPC-mediated NER while others inhibit XPC-mediated NER.

The data presented in this study supports the long acknowledged theory that NER occurs readily in G1 but cannot occur in S-phase. Therefore, protein modifications and protein-protein interactions likely restrict XPC function to G1 while inactivating XPC upon S-phase entry.

The XPC protein exists in a heterotrimeric complex that includes the hHR23b and centrin 2 proteins. The hHR23b protein acts to stimulate and stabilize XPC, while the centrin 2 protein plays an important role in centrosome duplication [95-97]. The yeast homologue for hHR23b, Rad23, provided a link between DNA repair and the ubiquitin pathway that led to the discovery that XPC is modified by ubiquitination [48]. Western blot analysis collected from this study (data not shown) indicated that transfecting H1299 cells with the hHR23b protein does not have a noticeable effect on XPC expression. While the XPC protein has been shown to be modified by the ubiquitin ligase Cul4a, most proteins can be poly-ubiquitinated and modified by several ubiquitin ligases [98-99]. The relationship between MDM2 and G1/S mediators along with its involvement in the regulation of the p53 and RB tumor suppressor proteins have made it a likely player in the negative regulation of XPC. MDM2 is an ubiquitin ligase for both RB and p53, and may also act as an ubiquitin ligase for XPC. Our data indicates that MDM2 affects XPC expression and that knocking down MDM2 increased the DNA repair capacity of XPC as measured by HCR (**Figure 13**) thereby suggesting that MDM2 may play a role in the negative regulation of XPC and possibly act as an E3 ubiquitin ligase for XPC.

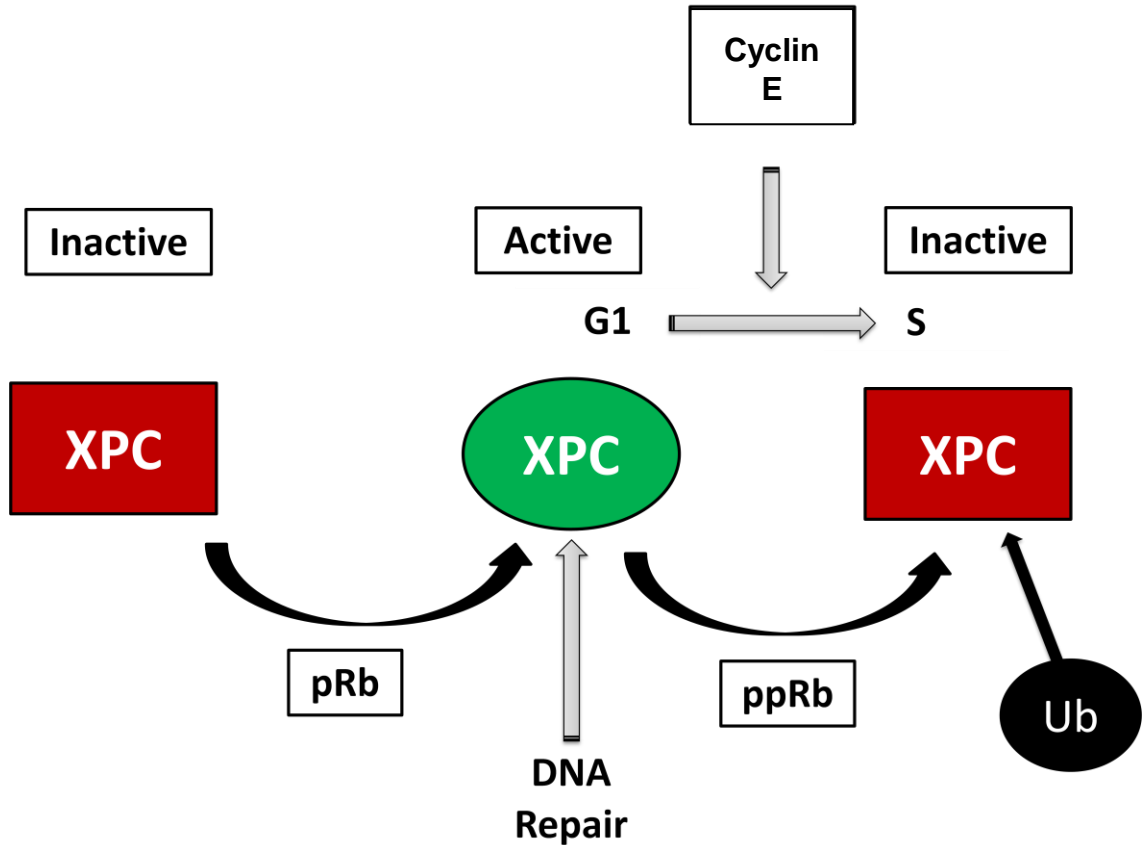


Figure 14. Schematic of a possible mechanism of XPC regulation in the context of the cell cycle. Data herein reveals a functionally inactive (rectangle) and active (oval) form of XPC DNA repair protein. These data suggest that upon DNA damage XPC is then stabilized by RB and that occurrence correlates with the DNA repair events of the G1 phase of the cell cycle. These data also show that G1/S cyclins, primarily cyclin E (cyclin D data not shown) abrogate XPC expression which likely corresponds to RB phosphorylation and S-phase entry. Data also reveals that MDM2 may signal ubiquitination of XPC in some way.

There is evidence that XPC is positively regulated by additional factors including damaged DNA-binding protein 2 (DDB2). DDB2 is a subunit of the damaged DNA-binding protein DDB1/DDB2 and is responsible for the phenotypic features of XP-E patients [98-101]. DDB2 has been shown to positively regulate XPC by promoting the accumulation and activating the recruitment of XPC to damaged DNA [98]. Additionally, the mRNA and protein levels for both XPC and DDB2 increase in response to DNA damage. While the exact role of the DDB1/DDB2 complex is still unclear, it is possible that the complex may also be regulated at the G1/S checkpoint. One reason why the role of DDB1/DDB2 is unknown is that it is not required in purified systems that carry out NER *in vitro*. Such systems have formed the foundation for mechanistic studies of DNA repair proteins [102]. Like XPC, DDB2 is positively regulated at the level of transcription by p53 and is postrationally regulated by poly-ubiquitination. Therefore, it is not unreasonable to think that other NER proteins, eg. XPA, may likewise be tightly regulated by post-translational mechanisms including ubiquitination.

The NER pathway is considered to be an error-free pathway. NER utilizes the nucleotide sequence from intact complementary DNA in order to fill in the 25-30mer gap excised during repair (**Figure 1**). XPC has also been shown to be involved other DNA repair pathways including base excision repair (BER) and error prone DNA repair mechanisms [103-107]. Studies conducted by Uehara *et al.* showed that XPC may be involved in the suppression of multiple mutations generated by error-prone trans-lesional DNA synthesis [104]. XPC deficient mice contain much higher numbers of unrepaired lesions that result in multiple mutations. These mutations are suspected to be generated by trans-lesional DNA polymerases such as pol zeta and pol eta. Pol eta is also involved

in the NER pathway and in error prone DNA repair mechanisms. The regulation of XPC in the context of the G1/S cell cycle may be of particular importance in regards to error prone mechanisms and preventing mutations caused by trans-lesional bypass polymerases.

The studies herein were conducted in a p53-null cancer cell line, H1299, which carries deletions in both p53 alleles [86]. Although it is expected that p53 will contribute to XPC expression and cellular NER in cells with functional p53, our study raises the possibility that p53-defective cancer cells might still retain some degree of XPC regulation and the capacity to regulate cellular NER. We used a cisplatin-damaged reporter gene for the HCR assays in cells that lack functional p53. However, our data indicate that substantial DNA repair occurs; indicating NER DNA repair mechanisms were intact (**Figure 5**, lane 8). These data suggest it may be possible for cancer cells lacking functional p53 to evade or resist cisplatin therapy by repairing the cisplatin DNA damage [108]. From a chemotherapeutic perspective, it may be necessary to target additional components of the G1/S checkpoint, such as RB, in order to maximize cancer cell killing by cisplatin.

Platinum-based chemotherapy is used as the preferred treatment method for patients with advanced NSCLC [109]. Platinum agents (i.e. cisplatin and carboplatin) are widely used in the clinic to treat various types of cancers including lung, bladder, liver, breast, testicular, myeloma, genitourinary and gastrointestinal [110]. Platinum chemotherapy agents cause DNA damage to the cells and inhibit DNA synthesis [111]. The platinum compound forms covalent bonds with purine bases in the DNA strands and primarily forms 1,2 or 1,3-intrastrand crosslinks, as well as, a small percentage of

interstrand crosslinks [111-113]. These crosslinks result in a helical distortion of the DNA which blocks DNA replication and transcription [111]. While initial platinum responsiveness is high, the majority of cancer patients will eventually become cisplatin-resistant [108, 114]. One mechanism of cisplatin resistance that has been proposed involves increased DNA repair [114]. NER typically repairs the DNA damage caused by platinum-based chemotherapy drugs. Studies herein suggest that even in cancer cells that are p53 null XPC is still capable of repairing DNA damage caused by platinum chemotherapy drugs when RB is intact. The data herein suggest that the addition of DNA repair inhibitors may be necessary for the effective treatment of certain cancers that are treated using platinum chemotherapy drugs [115, 116].

FUTURE DIRECTIONS

The Xpc complex

Data herein involving the regulation of Xpc DNA repair protein reveal an additional level of regulation that involve post-translational modification of XPC, however, the extent of these modifications is not known and the involvement of other factors within the XPC complex have not been clearly defined. XPC exists in a heterotrimeric complex including hHR23b and centrin-2. While the XPC component is known to be required for NER, specific components may have additional functions within the complex. Particularly, the hHR23b component has been shown to stimulate and stabilize XPC. Little is known of the relationship between hHR23b and the cell cycle. However, it may be that hHR23b is modified upon the ubiquitination of XPC and is involved in the destabilization of the XPC complex. Also it may be that knocking down the hHR23b protein may adversely affect the stability of XPC and its ability to bind to the site of DNA damage, thus affecting the function of XPC in NER. The possibility of this occurrence should be addressed in order to determine the effects of individual components of the XPC complex on NER and the regulation of XPC in the context of the cell cycle.

NER Co-factors

Nucleotide excision repair occurs in a multistep complex that involves additional co-factors. Other co-factors, i.e. DDB1/DDB2 and XPA, are involved in the regulation of XPC and have been shown to be required for NER in certain cell types. It is possible

that downstream NER factors involved in repairing DNA damage signal the ubiquitination of XPC and its dissociation from the NER complex. The possibility of additional NER co-factors being involved is likely and should be explored.

Chemotherapeutic Implications

Most aspects of cancer chemotherapy involve causing damage to the cellular DNA. High levels of DNA damage may cause cell-cycle arrest or cell death as is the goal of cancer chemotherapy drugs. Platinum chemotherapeutic agents are commonly used to treat various types of cancers but the majority of cancer patients typically develop a resistance to chemotherapeutic drugs. Intrinsic cellular NER DNA repair mechanisms may continue to repair the damage caused by platinum chemotherapy drugs and likely contribute to chemotherapeutic drug resistance and decrease the effectiveness of these treatments. The data herein suggests that XPC and NER cofactors are capable of repairing DNA damage from platinum agents (i.e. cisplatin) commonly used in cancer therapeutics regardless of whether the p53 and pRB proteins are expressed. These data suggest that the addition of DNA repair inhibitors may be necessary for the effective treatment of certain cancers. Several studies have proposed the use of drugs targeting specific DNA repair proteins in addition to traditional chemotherapy [115-117]. The combination of chemotherapy drugs like temozolomide and platinum chemotherapy drugs like cisplatin and carboplatin along with inhibitors of poly(ADP-ribose) polymerase 1 (PARP1) is currently under investigation and involved in several clinical trials. The decreased repair of intermediate DNA lesions induced by temozolomide in the presence of PARP inhibitors, and observations showing that PARP inhibitors

preferentially kill neoplastic cells make it a significant protein to explore [118-120]. Data herein suggests that it is also worthwhile to investigate the effects of inhibiting additional DNA repair proteins (i.e. XPC) to increase the effectiveness of current patient chemotherapy. Should studies persist it would be interesting to investigate the effects of XPC activity in the presence of cisplatin damage and DNA repair inhibitors.

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