E2F1 regulates the base excision repair gene XRCC1 and promotes DNA repair.

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Running Title: E2F1 regulates XRCC1 and promotes DNA repair

The E2F1 transcription factor activates S-phase promoting genes, mediates apoptosis, and stimulates DNA repair through incompletely understood mechanisms. X-ray repair cross complementing group 1 (XRCC1) protein is important for efficient single-strand break/base excision repair (SSB/BER). Although both damage and proliferative signals increase XRCC1 levels, the mechanisms regulating XRCC1 transcription remain unclear. To study these upstream mechanisms, the XRCC1 promoter was cloned into a luciferase reporter. Ectopic expression of wild-type E2F1, but not an inactive mutant E2F1(132E), activated the XRCC1-promoter-luciferase reporter, and deletion of predicted E2F1 binding sites in the promoter attenuated E2F1-induced activation. Endogenous XRCC1 expression increased in cells conditionally expressing wild-type, but not mutant E2F1, and methyl methanesulfonate (MMS)-induced DNA damage stimulated XRCC1 expression in E2F1\textsuperscript{+/+}, but not E2F1\textsuperscript{+-/-}, MEFs. Additionally, E2F1\textsuperscript{+-/-} MEFs displayed attenuated DNA repair after MMS-induced damage compared to E2F1\textsuperscript{+/+} MEFs. Moreover, CHO cells with mutant XRCC1 (EM9) were more sensitive to E2F1-induced apoptosis compared to CHO cells with wild-type XRCC1 (AA8). These results provide new mechanistic insight into the role of the E2F pathway in maintaining genomic stability.

The E2F family of transcription factors plays an important role in promoting both cellular proliferation and cell death. The “activating” E2Fs (E2F1-3) are important for regulating S-phase specific genes as well as promoting apoptosis---particularly in the case of E2F1 (1-8). Mounting evidence implicates E2F1 as an important component of the DNA damage response as E2F1 protein is phosphorylated and stabilized via ATM/ATR and Chk2 dependent pathways (9-12). Additionally, genes that are important in DNA repair, such as homologous recombination and mismatch repair, are bonafide E2F targets (13-17). Many repair genes have also been identified as putative E2F targets during genome wide screens (15,18-21). Moreover, it has also been suggested that E2F1 protein may be a component of repair complexes (22,23). Importantly, E2F1 has a functional in vivo role in promoting DNA repair and suppressing apoptosis after UVB (24). Together, these studies implicate E2F1 in DNA repair, although the actual mechanisms and specific repair pathways remain obscure.
DNA SSBs are one of the most common DNA lesions and pose a major threat to genetic stability and survival through accumulation of mutations or through conversion to double-strand breaks (25). SSBs can arise by direct damage to DNA bases or sugar moieties, or indirectly as intermediates during the process of BER (26). SSBR/BER requires highly coordinated overlapping enzymatic steps dependent on the nature and origin of the SSB lesion (25-27).

XRCC1 is a scaffolding protein that promotes efficient repair by interacting with many of the proteins that catalyze SSBR/BER (26,28-30). Although XRCC1 null mice are not viable (31), mouse and Chinese hamster ovary (CHO) cells with mutant or attenuated XRCC1 demonstrate decreased SSBR and hypersensitivity to many different types of DNA damage (26,32-37). In human cells, XRCC1 is necessary for efficient SSBR, genomic stability and survival (38-40). Furthermore, XRCC1 polymorphisms are associated with variable cancer risk which suggests a possible role in cancer development (41). XRCC1-mediated repair includes an S-phase SSBR pathway that operates at replication forks, and a rapid cell cycle independent SSBR/BER pathway (42-45). Posttranslational mechanisms, such as phosphorylation, also modulate XRCC1 function and promote genetic stability (46,47). Additional mechanisms may also regulate XRCC1 since mRNA and protein levels increase after DNA damage in part through a MAPK signaling pathway (48,49). However, although both cell cycle specific and DNA damage signals are upstream of XRCC1, the mechanisms regulating XRCC1 expression remain incompletely characterized.

In this report, we demonstrate that XRCC1 is a direct E2F target gene and that E2F1 enhances SSBR/BER. These results provide new mechanistic insight into the role of E2F1 in the maintenance of genomic stability and cell survival.

**Experimental Procedures**

**XRCC1 promoter and plasmids.** The 5’-region from -881 to +158, relative to the transcription start site (GI:21624595) (50), was cloned by polymerase chain reaction (PCR) of genomic DNA. Forward primer: 5’-ggagcgagaacctctctttgtg-3’; reverse primer: 5’-accagttcttgctgctgaggac-3’. The PCR product was cloned into the pGL3-Basic luciferase vector (Promega) using standard techniques. Deletions of the XRCC1 promoter region were similarly engineered. All constructs were sequence verified. The E2F expression constructs, DP1 and pRSV β-galactosidase (β-gal) expression vectors were gifts from Drs. William Kaelin (Dana Farber Cancer Institute, Boston, MA, USA), WC Lin (University of Alabama, Birmingham, AL, USA), and Rosalie Sears (Oregon Health & Science University, Portland, OR, USA).

**Luciferase-reporter assays.** Cells at 60% confluence in 6-well plates were transfected with the specified XRCC1 luciferase reporter plasmid, together with indicated amounts of specified E2F expression vector, pRSV β-galactosidase expression vector (0.1 μg), and appropriate amount of empty plasmid vector, for a total of 1.0 μg plasmid DNA per transfection. Luciferase assays were performed 36 hours post-transfection with an AutoLumatB95 and relative luciferase light units were normalized to β-galactosidase activity as described previously (51).

**Western blotting.** Total cellular lysates were prepared, quantitated, resolved on 10% SDS-PAGE, immunblotted with the specified primary and HRP-conjugated secondary antibodies, and visualized with chemiluminescence (Pierce SuperSignal) as previously described (51). Anti-XRCC1 mouse monoclonal antibody was from NeoMarkers, anti-E2F1 antibody was from Santa Cruz, anti-tubulin antibody was from Sigma, and HRP-conjugated secondary antibodies were from Jackson Immunological.

**Northern Blotting.** Total RNA was prepared using the Total RNA Preparation Kit (Promega, Madison, MI) according manufacturer’s instructions. Twenty μg total RNA was resolved by electrophoresis on a 1.0% formaldehyde-agarose gel, transferred and UV cross-linked to a Zeta-Probe®GT membrane (BioRad, Hercules, CA),
Cells were grown in freshly made O

RT-PCR. XRCC1 and GAPDH mRNA levels were determined by semi-quantitative RT-PCR using standard techniques. Briefly, equivalent amounts of total RNA was prepared from the indicated cells at the specified conditions described in the text, and oligo-dT primed first-strand cDNA synthesis performed with Superscript III reverse transcriptase (Invitrogen). PCR was performed on equivalent amounts of RT product with Taq DNA polymerase for 30 cycles (95°C for 15 seconds, 65°C for 20 seconds, 72°C for 40 seconds). XRCC1 forward primer: 5'-gtatgcaggctcagggatgagac-3'; reverse primer: 5'-agctggcgcacccatcctgac-3'. GAPDH forward primer: 5'-aagggcgcctcttttgta-3'; reverse primer: 5'-gtcgaggatgtgctgac-3'.

Chromatin immunoprecipitation (ChIP). ChIP analysis on Saos2 cells was performed as described (51) with minor modifications. Briefly, cells were cross-linked with 1% formaldehyde/PBS, stopped with 0.125 M glycine, lysed (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris pH 8.0, 5 mM EDTA plus fresh protease inhibitors), and sonicated to yield 600-1000 nucleotide chromatin fragments. Extracts centrifuged at 13000g X 10 minutes and pre-cleared with protein A-Sepharose/salmon sperm DNA slurry (1:1) for 30 minutes at 4°C. 2 µg of anti-E2F1 antibody (Santa Cruz) or control antibody (normal rabbit IgG) added and incubated overnight at 4°C followed by protein A/G-agarose for 2 hours. Beads washed twice with ice-cold RIPA buffer, four times with ice-cold IP wash buffer (100 mM Tris pH 8.0, 500 mM LiCl, 1% NP-40, 1% deoxycholic acid), and twice more with ice-cold RIPA buffer. IP elution buffer (50 mM NaHCO3, 1% SDS freshly made) was added for 15 minutes at RT, beads spun out and supernatant collected. This was repeated and supernatants combined. Cross-links were reversed, and then DNA purified by phenol-chloroform extraction and ethanol precipitation. PCR of XRCC1 promoter sequence was performed with 25 cycles (94°C for 15 seconds; 65°C for 45 seconds) and analyzed on a 2% TAE-agarose gel. XRCC1 specific primers: forward 5'-ggacgcgaaccctcttttttgga-3'; reverse 5'-ggctcaggctcgggtcacttct-3'. XRCC1 control primers: forward 5'-ctgggatagaggagttggcagtgtc-3'; reverse 5'-ggctcaggctcgggtcactttc-3'.

Cell culture. Cells were grown in DMEM or McCoy's media supplemented with 10% heat-treated fetal bovine serum (FBS) and 290 µg of L-glutamine, 100U penicillin, and 100 µg of streptomycin per ml at 37° C in 5% CO2. Saos2 cells stably transected with various tetracycline-regulated E2F1 expression vectors (a gift from Dr. Karen Vousden, Beatson Institute, UK) were maintained in tetracycline-free FBS (Clontech). E2F1+/+ and E2F1/-/ mouse embryonic fibroblasts (MEFs) were gifts from Dr. Joseph Nevins, (Duke University, NC, USA). Chinese hamster ovary (CHO) cell lines AA8 and EM9 were obtained from the American Type Culture Collection (ATCC).

Comet assay. Single-cell agarose alkaline gel electrophoresis was performed per standard methods with minor modifications (52). Equivalient numbers of cells (1 X 105) were collected at the indicated time points, mixed with low-melt-temperature agarose (Sigma), and then layered onto agarose-coated glass slides. Slides were maintained at 4°C to solidify and for all subsequent steps. Slides were submerged in lysis buffer (2.5 M NaCl, 0.1M EDTA, 10 mM Tris-Cl (pH 7.0), 1% Triton X-100, 1% DMSO) for one hour, washed with nH2O, and incubated for 45 minutes in alkaline electrophoresis buffer (50 mM NaOH, 1 mM EDTA, 1% DMSO, pH 12.8). After electrophoresis (25 min, 25 V), air-dried and neutralized slides were stained with 2 µg/ml propidium iodide. Average comet tail moment was scored for duplicate slides (50 cells/slide) in randomly selected fields, and automated calculations were performed using Comet Assay II software (Perceptive Instruments; Suffolk, U.K.).

In situ DNA strand break detection. DNA polymerase I-mediated labeling assay was performed as described previously with minor modifications (53). Briefly, cells grown on cover-slips were fixed in 10% formalin for
10 minutes. Cells were rinsed in PBS, permeabilized with 1% Triton X-100 for 20 minutes, and then incubated in a moist-air chamber at 37°C for 90 min in a labeling mixture containing: 10 µM each of dGTP, dATP, dCTP and 7 µM dTTP, 3µM FITC-dUTP, 20 U/ml of E. coli DNA polymerase I (Sigma, St. Louis, MO) in reaction buffer containing 5 mM MgCl$_2$, 10 mM 2-mercaptoethanol, and 20 µg/ml bovine serum albumin (BSA). The reaction was terminated by washing the slides twice in PBS. Non-specific labeling was determined by incubation in the reaction buffer without the enzyme.

Apoptosis assay. Equivalent numbers of indicated cells were plated and allowed to settle for 24 hours at 50% confluency. Adenovirus infections were performed at MOI of $1 \times 10^10$ (Stratagene AdEasy system). E2F1-expressing and GFP-expressing adenoviruses were a gift from Dr. Rosalie Sears (Oregon Health & Science University, Portland, OR, USA). Forty eight hours after infection, cells were harvested and subjected to Annexin V and flow cytometry as previously described (54). Briefly, attached and floating cells were collected and re-suspended in Annexin V Binding Buffer (BD Biosciences) at a concentration of $1 \times 10^6$ cells per ml. APC-conjugated Annexin V was immediately added, incubated for 15 minutes at room temperature, and then analyzed on a Becton Dickinson FACScan. The percentage of apoptotic cells was determined by the increase in Annexin V-APC signal on stained cells over the baseline signal (defined on unstained cells) per standard flow cytometry convention.

Results.

E2F1 expression stimulates endogenous XRCC1 expression. Despite the important role that XRCC1 plays in SSBR/BER (26), little is known about the upstream pathways that regulate its expression. Because DNA damage signals and proliferative signals stimulate XRCC1 expression (26,48,49), we wondered if E2F1 could stimulate endogenous XRCC1 expression (Figure 1). To test this, we utilized a Saos2 stable cell line containing an E2F1 cDNA expression vector under control of a tetracycline-regulated promoter (55). We found that tetracycline-induced E2F1 expression stimulated an increase in XRCC1 protein (Figure 1A, lanes 1 and 2) and mRNA levels (Figure 1B, lanes 1 and 2). To demonstrate that E2F1 transcription function was required to induce endogenous XRCC1 expression, we additionally utilized a tetracycline-regulated Saos2 stable cell line containing a DNA-binding incompetent mutant E2F1 (132E)(55). Conditional expression of mutant E2F1(132E) could not stimulate endogenous XRCC1 protein (Figure 1A, lanes 3 and 4) or mRNA (Figure 1B, lanes 3 and 4). These results suggest that E2F1 stimulates endogenous XRCC1 expression by a transcription-mediated mechanism.

XRCC1 promoter has features suggestive of an E2F target gene. Since transcription competent wild-type E2F1 was capable of stimulating XRCC1 mRNA and protein levels (Figure 1), we examined the genomic sequence encompassing the XRCC1 promoter (Figure 2A). Sequence analysis from –881 to +158 relative to the putative transcription start site (green sequence) revealed a CpG island (boxed sequence) as well as putative E2F binding sites from –819 to –803 (red sequence). To verify that an active promoter was present in this region, we cloned this genomic fragment into the pGL3-Basic luciferase reporter vector. After transfection into Saos2 cells, we found that this genomic region stimulated the luciferase reporter nearly six-fold relative to empty vector (Figure 2B). Sequential deletions from the 5’ end attenuated luciferase activation relative to empty vector. This data suggests that a functional XRCC1 promoter is contained within this region although the possibility of other control elements outside of this region cannot be excluded.

The XRCC1 promoter-luciferase reporter is stimulated by E2F1. Since the XRCC1 promoter sequence was typical for an E2F target gene (Figure 2), we reasoned that the XRCC1 promoter-luciferase reporter would be stimulated by E2F. Co-transfection of increasing amounts of E2F1 expression
vector with the XRCC1 promoter-luciferase reporter caused a dose-dependent increase in luciferase activation (Figure 3A). In contrast, DNA-binding incompetent E2F1(132E) could not activate the XRCC1 promoter-luciferase reporter (Figure 3A). We also found that E2F1 more efficiently stimulated the XRCC1 promoter-reporter relative to other family members (E2F2, E2F3 and E2F4)(Figure 3B). To determine the contribution of the predicted E2F binding sites for their ability to influence XRCC1 promoter activation, we co-transfected E2F1 expression vector with an XRCC1 promoter-luciferase reporter which lacked the E2F binding sites (ΔE2F1-XRCC1) as shown in Figure 3C. We found that in response to E2F1 co-transfection, stimulation of the XRCC1 promoter-luciferase reporter containing the E2F binding sites was over 15-fold higher than the ΔE2F1-XRCC1 promoter-reporter (Figure 3C, left hand). As a further control for specificity, mutant E2F1(132E) did not stimulate either promoter (Figure 3C, right hand). Together, these results suggest that the XRCC1 promoter is stimulated by E2F.

**Endogenous XRCC1 promoter binds endogenous E2F1.** Since XRCC1 transcription is stimulated by E2F overexpression (Figures 1 and 3), we wanted to demonstrate that endogenous E2F1 could bind the endogenous XRCC1 promoter in its native chromatin context. To examine this, we performed a chromatin immunoprecipitation (ChIP) assay on the proximal XRCC1 promoter region (Figure 4). XRCC1 promoter-specific PCR primers amplified this region after chromatin IP with an anti-E2F1-specific antibody, but not non-immune IgG (Figure 4, lanes 1-3). To further demonstrate that this was a specific interaction, XRCC1-specific primers which amplify a region that does not contain E2F-binding sites (control primers) could not generate a PCR product after chromatin IP with anti-E2F1-1-specific antibody (Figure 4, lanes 4-6). These findings demonstrate that the XRCC1 promoter, in its native chromatin context, can specifically bind E2F1 protein and that XRCC1 is a bonafide E2F target gene.

**Endogenous E2F1 stimulates the XRCC1 promoter after DNA damage.** Since XRCC1 is an E2F target gene (Figures 1-4) and E2F1 is a DNA damage-inducible protein (9-12), we wondered whether MMS, which causes heat-labile DNA damage repaired by an XRCC1-mediated BER pathway (56), could stimulate XRCC1 transcription in an E2F1-dependent manner (Figure 5). To investigate this, we transfected E2F1/- and E2F1+/+ mouse embryonic fibroblasts (MEFs) with the XRCC1 promoter-luciferase reporter and then treated them with MMS. Compared to no treatment, we found the XRCC1 promoter-luciferase reporter was stimulated by MMS in E2F1+/+ but not E2F1-/- cells (Figure 5). Consistent with transcriptional activation, an increase in XRCC1 mRNA and protein levels (Figure 5B, left hand and right hand, respectively) was observed after MMS treatment in E2F1+/+, but not E2F1-/-, cells. These results demonstrate that MMS-induced DNA damage stimulates XRCC1 transcription at least in part through an E2F1-dependent pathway.

**E2F1 enhances DNA repair after MMS-induced damage.** Since XRCC1 is a bonafide E2F1 target gene (Figures 1-5), we wished to determine if loss of E2F1 would result in attenuated DNA repair after MMS-induced DNA damage (Figure 6). To investigate this, we treated E2F1-/- and E2F1+/+ MEFs with MMS and then measured strand-break repair by single-cell alkaline agarose gel electrophoresis (comet assay) (52) as shown in Figure 6A. The average tail moment was similar in undamaged cells (left hand bars, control). As expected after incubation in MMS, the average tail moment increased equivalently in both E2F1-/- and E2F1+/+ MEFs (middle bars, 0 hr). However, if cells were allowed to recover in MMS-free media (right hand bars, R), E2F1-/- MEFs had a statistically significant longer average tail moment compared to E2F1+/+ MEFs (33% versus 22% respectively, p = 0.013 unpaired 2-tailed t-test). To further confirm that E2F1-/- MEFs had decreased DNA repair capacity, we utilized an in situ DNA polymerase I-mediated FITC-dUTP labeling assay (53) to detect persistent DNA strand breaks after recovery from MMS treatment (Figure 6B). As expected, immunofluorescence staining
demonstrated reduced XRCC1 staining in E2F1-/- MEFs compared to E2F1+/+ MEFs (top panels). Without damage, no DNA polymerase I-mediated FITC-dUTP labeling was detected (middle panels, C). After MMS exposure, FITC-dUTP labeling was detected in both E2F1+/+ and E2F1-/- MEFs (middle panels, 0h). However, if MMS exposure was followed by recovery with incubation in MMS-free media, less FITC-dUTP labeling was detectable in E2F1+/+ MEFs compared to E2F1-/- MEFs (middle panels, R).

If XRCC1 was downstream of E2F1 and could mediate E2F1 function, we reasoned that loss of XRCC1 would result in increased E2F1-induced apoptosis due to diminished DNA repair. To examine this, we utilized Chinese hamster ovary (CHO) cells containing wild-type XRCC1 (AA8 cell line) and a cell line derived from AA8 cells that is mutant for XRCC1 (EM9 cell line) (26,32). We infected cells with an adenosine virus expressing wild-type E2F1, or a control adenosine virus expressing green fluorescent protein (GFP), and quantified the level of E2F1-induced apoptosis at 48 hours using Annexin V staining and flow cytometry (Figure 6C). Under these conditions, AA8 cells did not have a significant increase in apoptosis after infection with E2F1-expressing adenosine virus compared to control GFP-expressing adenosine virus (left hand graph). In contrast, EM9 cells demonstrated a statistically significant 1.8-fold increase in apoptosis after infection with E2F1-expressing adenosine virus compared to control GFP-expressing adenosine virus (right hand graph) (p = 0.04, unpaired 2-tailed t-test). Together, these results suggest a mechanism by which the E2F1-XRCC1 pathway can stimulate DNA repair and promote genomic stability and cell viability. However, the existence of other E2F1-mediated pathways that stimulate BER independent of XRCC1 remains likely.

Discussion.

The E2F1 pathway is centrally involved in the highly complex networks coupling cellular proliferation and apoptosis (1,5,7,8,12,57). We have shown that XRCC1, which plays a critical role in SSBR/BER (26), is a direct E2F1 target gene. Because E2F1 is a damage-inducible protein (9-12), our data provides novel insight into an important DNA repair function of the E2F pathway and opens new avenues for investigation.

Our finding that E2F1 is upstream of XRCC1 significantly expands on prior observations that E2F plays a role in other repair pathways such as MMR and NER (13-17,19-21,24). Our data is consistent with the report that the BER protein uracil-DNA glycosylase (UDG) is also E2F regulated (58). Intriguingly, although E2F1 is best characterized as a transcription factor, E2F1 protein may have a direct role in DNA repair as suggested by its localization to repair complexes (22,23). Thus, it is likely that multiple E2F regulated mechanisms function in parallel with XRCC1 to stimulate repair.

We found that enforced E2F expression stimulated XRCC1 levels (Figures 1 and 3) and that MMS, which induces predominantly heat-labile DNA damage repaired by an XRCC1-mediated BER pathway (56), causes an E2F1 dependent increase in XRCC1 expression (Figure 5). This is consistent with prior reports demonstrating that cellular stress (such as from γ-irradiation) increases endogenous XRCC1 levels (48,49,59) although this may be cell type specific (60). How MMS-induced stress activates the E2F1-XRCC1 axis remains unknown. Cellular sensitivity to MMS may involve an ATR-dependent pathway and genetic evidence suggests that MMS-induced damage activates the yeast Rad53 (Chk2 human homologue) pathway (61,62). Given that the ATM/ATR and Chk2 pathways phosphorylate and activate E2F1 protein (9-12), it is possible that these kinases stimulate XRCC1 expression through E2F1 activation, although this remains to be demonstrated. Interestingly, Chk2 mediated stabilization of the FoxM1 transcription factor stimulates expression of DNA repair genes including XRCC1 (63). Given that XRCC1 function is complex, it is likely that its control involves multiple levels. Indeed, posttranslational mechanisms modulate XRCC1 function as evidenced by the ability of DNA-PK to phosphorylate XRCC1 (46), as well as for the requirement of protein kinase CK2 to
phosphorylate XRCC1 and enhance SSBR and genetic stability (47). Consistent with the complex control of XRCC1, we found that serum starvation followed by re-feeding stimulated XRCC1 expression (data not shown). This is consistent with cell conditions of high E2F activity, but also suggests that serum/mitogenic factors may be important too. This could be a cell type specific phenomenon since density arrest and release does not alter XRCC1 levels in human T24 cells (64). Nevertheless, our discovery of an E2F1-XRCC1 pathway provides a previously undescribed mechanism for regulating XRCC1 function.

The biological importance of E2F regulation of XRCC1 is suggested by the attenuated in vivo DNA repair in E2F1-/- versus E2F1+/+ MEFs (Figure 6A/B). Two different methods demonstrated reduced DNA repair after MMS-induced DNA damage which correlates with the decreased XRCC1 levels observed in E2F1-/- cells. The repair of MMS-damaged DNA still occurs in E2F1-/- cells suggesting that the E2F1-XRCC1 axis is not an absolute requirement in these systems. This is not surprising given the complex and overlapping repair pathways involved. However, the significance of even a modestly reduced XRCC1-mediated repair function may have important implications for maintaining genomic stability and cell viability. Consistent with this notion of XRCC1 mediating E2F1 activity is the observation that loss of XRCC1 function resulted in an attenuated E2F1-induced apoptotic response in EM9 cells compared to AA8 cells (Figure 6C).

Although E2F1 is a damage-response protein, it also plays an important role in promoting the expression of a large number of genes required for replication and proliferation (15,19-21). Given the intimate relationship between proliferation and replication/repair, the control of XRCC1 by E2F in undamaged cells further integrates SSBR with cell cycle progression as might be expected if enhanced SSBR were necessary to repair SSBs at replication forks (26,42-45,65). Whether, and in what context, the other E2F family members play a role, as well as what specific SSBR pathways are utilized (e.g. long patch BER) remains to be explored.

The p53-E2F network controls and integrates critical functions such as proliferation, cell cycle checkpoints, apoptosis and DNA repair (7,8,12,66,67). In particular, p53 can promote BER (68-72), and our discovery that E2F1 may also promote BER expands our understanding of the p53-E2F1 network in regulating DNA repair. Disruption of these cooperative pathways has profound implications for tumorigenesis as evidenced by enhanced tumor formation in knock-out mouse models for both p53 and E2F1; although intriguingly, both oncogenic and tumor suppressor functions for E2F1 are suggested in compound p53-/-;E2F1-/- mice (12,73-75). Our findings that E2F1 regulates XRCC1 implies that an intact E2F pathway may be important for maintaining genomic stability in response to highly dangerous SSBs. This observation opens new avenues for investigation that may ultimately allow us to develop novel preventive and therapeutic strategies for cancer patients.

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Figure Legends.

**Figure 1. E2F1 expression stimulates endogenous XRCC1.** (A). Western blots and, (B). ethidium bromide-stained agarose gel of semi-quantitative RT-PCR, on Saos2 cell lines conditionally expressing wild-type E2F1 (lanes 1 and 2) or DNA binding incompetent mutant E2F1(132E) (lanes 3 and 4). Prior to induction, cell lines were maintained in 0.5% FBS containing media for 30 hours, induced with 2.0 µg/ml doxycycline, and then harvested 24 hours later.

**Figure 2. XRCC1 promoter fragment sequence and luciferase-reporter assay define an active promoter region.** (A). Genomic sequence from -881 to +158 relative to the predicted transcription start site/cDNA 5’ end indicated as +1. Exon 1 indicated in green type and translation-initiation codon in blue type. Open box indicates CpG island. Underlined sequences in red denote putative E2F-binding sites. (B). (Left hand): Schematic representation of a series of promoter deletion mutants in pGL3-Basic-luciferase reporter. Red boxes denote putative E2F-binding sites. Black box represents exon 1. Open box with [luc] denotes luciferase reporter. (Right hand): Luciferase readout of indicated promoter-reporters after transfection into Saos2 cells. Light units normalized to a β-gal signal. In all, 1.0 µg of indicated promoter-reporter and pRSV β-gal plasmids were used for all transfections. S.D. of triplicate experiments is shown.

**Figure 3. E2F stimulates the XRCC1 promoter-luciferase reporter.** (A). Relative light units, normalized to a β-gal signal, of pGL3-XRCC1(-881 to +158) (0.1 µg) co-
transfected with increasing amounts of wild-type E2F1 or mutant E2F1(132E) expression plasmids (50 to 250 ng), into Saos2 cells. (B). Relative light units, normalized to a β-gal signal, of pGL3-XRCC1(-881 to +158) (0.1 µg) co-transfected with increasing amounts of the indicated E2F expression vectors (50 to 250 ng), into Saos2 cells. (C). Relative light units, normalized to a β-gal signal, of the relative fold-change of the XRCC1(-881 to +158) promoter-reporter versus an E2F binding site deleted XRCC1 promoter-reporter (ΔE2F1-XRCC1), in response to increasing amounts of co-transfected wild-type E2F1 or mutant E2F1(132E) expression plasmids (50 to 250 ng). S.D. of triplicate experiments is shown.

Figure 4. Chromatin immunoprecipitation of E2F1 at the XRCC1 promoter.
Ethidium bromide-stained agarose gel of PCR products performed after ChIP using anti-E2F1 antibody (lanes 1 and 4) or non-immune control IgG (lanes 2 and 5). Input chromatin (lanes 3 and 6) at 1:1000 dilution of PCR products from IP reactions performed with XRCC1 specific primers (lanes 1-3), or control primers (lanes 4-6).

Figure 5. Endogenous E2F1 stimulates the XRCC1 promoter after DNA damage.
(A). Relative light units, normalized to a β-gal signal, of pGL3-XRCC1(-881 to +158) (0.1 µg) luciferase-reporter plasmid transfected into E2F1+/+ or E2F1-/- MEFs, followed by incubation with 10 µg/ml MMS (2nd and 4th column) or 0 µg/ml MMS (first and third column) for 12 hours prior to assay. Standard deviation of triplicate experiments shown. (B). Northern blot (left hand) and Western blot (right hand) on equivalent amounts of
lysates prepared from E2F1+/+ or E2F1-/- MEFs after incubation with 10 µg/ml MMS (second and fourth lanes) or 0 µg/ml MMS (first and third lanes).

**Figure 6. Attenuated DNA repair in E2F1-/- MEFs.** (A): Comet assay expressed as average tail moment on E2F1+/+ and E2F1-/- MEFs untreated (left hand graph, control), after incubation for one hour in media containing 50 µg/ml MMS (middle graph, 0 hr), and after incubation for one hour in media containing 50 µg/ml MMS followed by recovery with incubation in fresh drug-free media for 2 hours (right hand graph, R). Experiments were performed in duplicate and measurement of mean tail moment was from 50 cells per slide from 15-20 randomly selected fields representing the whole area of each slide. Statistical analysis performed using unpaired two-tailed t-test on comet tail moments that were determined using Comet Assay II software (Perceptive Instruments; Suffolk, U.K.). Error bars represent standard deviation from separate experiments. (B): Indirect immunofluorescence microscopy on XRCC1 immunostaining (top panels), DNA polymerase I-mediated FITC-dUTP labeling assay (middle panels), and DAPI staining (bottom panels) performed on E2F1-/- and E2F1+/+ MEFs untreated (panels C), after incubation for one hour in media containing 50 µg/ml MMS (panels 0 hr), and after incubation for one hour in media containing 50 µg/ml MMS followed by recovery with incubation in fresh drug-free media for 6 hours (panels R). (C): Percent of apoptosis as determined by Annexin V staining and flow cytometry 48 hours after infection of indicated cells with adenovirus expressing wild-type E2F1 (Adeno-E2F1) or adenovirus expressing GFP (Adeno-control). Error bars represent standard deviation of triplicate experiments and statistical analysis performed using unpaired two-tailed t-test.
### A.

|       | wtE2F1 | E2F1(132E) |
|-------|--------|------------|
| tet   | -      | +          |
|       | -      | -          |
|       | +      | +          |

- **XRCC1-**
  - 1
  - 2
  - 3
  - 4

- **E2F1-**
  - 1
  - 2
  - 3
  - 4

- **Tubulin-**
  - 1
  - 2
  - 3
  - 4

### B.

- **XRCC1-**
  - 1
  - 2
  - 3
  - 4

- **GAPDH-**
  - 1
  - 2
  - 3
  - 4
A. XRCC1 promoter fragment

B. Chen et. al. Fig.2
Chen et al. Fig 3A/B.
C.

Chen et al. Fig. 3C.
Chen et al Fig 4
A. MEFs

E2F1+/+  E2F1 -/-

Normalized light units (XRCC1 promoter/pGL3)

|          | 0 µg/ml MMS | 10 µg/ml MMS | 0 µg/ml MMS | 10 µg/ml MMS |
|----------|-------------|--------------|-------------|--------------|
| E2F1+/+  | 3 ± 1       | 9 ± 2        | 2 ± 0       | 4 ± 1        |
| E2F1 -/- | 3 ± 1       | 9 ± 2        | 2 ± 0       | 4 ± 1        |

B. Chen et. al Fig. 5

E2F1+/+  E2F1 -/-

| MMS | - | + | - | + |
|-----|---|---|---|---|
| XRCC1- |    |    |     |     |
| 28S   |    |    |     |     |
| 18S   |    |    |     |     |

| E2F1+/+  | E2F1 -/- |
|----------|----------|
| MMS      | - | + | - | + |
| XRCC1-   |    |    |     |     |
| Tubulin- |    |    |     |     |
A. 

![Graph showing tail amount with error bars and p-values](image)

B. 

|        | E2F1+/+ |          | E2F1-/- |          |
|--------|----------|----------|----------|----------|
|        | MMS      |          | MMS      |          |
|        | C        | 0 h      | R        | C        | 0 h      | R        |
| XRCC1  | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) |
| FITC-dUTP | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) |
| DAPI   | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) |
C.

Percent Annexin V

- AA8 (XRCC1+/+)
- EM9 (XRCC1-/-)

p = 0.18

p = 0.04

Adeno-control
Adeno-E2F1
E2F1 regulates the base excision repair gene XRCC1 and promotes DNA repair
Dexi Chen, Zhiyong Yu, Zhiyi Zhu and Charles D. Lopez

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