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POST-TRANSLATIONAL REGULATION OF XPC IN REPAIR OF UV-INDUCED DNA DAMAGE

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To my parents Dr. Nita Shah and Dr. Mahesh Shah

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ABSTRACT

Ultraviolet (UV) radiation from sunlight is a major etiologic factor for skin cancer, the most prevalent cancer in the U.S., as well as premature skin aging. In particular, UVB radiation causes formation of specific DNA damage photoproducts between pyrimidine bases. These DNA damage photoproducts are repaired by a process called nucleotide excision repair, also known as UV-induced DNA repair. When left unrepaired UVB-induced DNA damage leads to accumulation of mutations, predisposing people to carcinogenesis and to premature aging. Although the core NER proteins have been identified and characterized, molecular regulation of NER remains poorly understood.

Here, I show that ubiquitin-specific peptidase 11 (USP11) positively regulates NER by deubiquitinating xeroderma pigmentosum complementation group C (XPC) and promoting its retention at the DNA damage sites. In addition, UV irradiation induces both USP11 recruitment to the chromatin and USP11 interaction with XPC in an XPC-ubiquitination-dependent manner. Furthermore, we found that USP11 is down-regulated in chronically UV-exposed mouse skin and in skin tumors from mice and humans. Our findings indicate that USP11 plays an important role in maintaining NER capacity, and suggest that USP11 acts as a tumor suppressor *via* its role in DNA repair.

I also found that phosphorylation of XPC acts as a novel post-translational regulatory mechanism of the NER pathway. We show that XPC is phosphorylated at serine 94. Moreover, after UVB irradiation, XPC phosphorylation regulates recruitment of ubiquitinated XPC and its downstream NER factors to the chromatin. In addition, upon evaluating the predicted kinases for XPC phosphorylation, we found that casein kinase II (CK2) promotes NER. Furthermore, CK2 kinase mediates XPC phosphorylation at serine 94, and also promotes recruitment of

ubiquitinated XPC to the chromatin after UVB irradiation. Our findings have identified XPC phosphorylation as a new mechanism for regulating NER following UV-induced DNA damage.

Our findings have uncovered USP11 and XPC phosphorylation at S94 as novel post-translational regulators of XPC activity in NER, and have significantly increased our understanding of the molecular regulatory mechanisms of UV-induced DNA damage repair.

CHAPTER 1: INTRODUCTION

UV irradiation causes DNA damage

Ultraviolet (UV) radiation from sunlight is a major etiologic factor for skin cancer, the most prevalent cancer in the U.S. [1-6], as well as premature skin aging. UV radiation is classified into 3 types based on the wavelength- UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm) [1, 7]. All UVC is blocked by the ozone layer, preventing it from reaching the surface of the earth [1]. UVB forms only about 5% of all UV radiation reaching the earth's surface, which effectively causes DNA damage [2, 8, 9]. UVA forms about 95% of all UV radiation entering the earth, but is weaker than UVB in terms of causing DNA damage [2, 8, 10, 11].[1]

UVB and UVC are absorbed directly by DNA, causing the formation of thymine dimers, mainly cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP) [2, 5]. UVA exposure also causes thymine dimers; in addition, it leads to generation of reactive oxygen species (ROS) *via* photosensitizing reactions, and thus indirectly causes oxidative DNA damage lesions [2, 11, 12].

[1]All tables, figures and most of the text in this thesis have been published in the following papers. The content has been reproduced with permission and with some changes limited to reorganization for lucidity.

1. Shah, P. and He, Y. *Molecular Regulation of UV-Induced DNA Repair.* Photochem Photobiol, 2015. **91**: 254-264.

2. Shah, P., Qiang, L., Yang, S., Soltani, K., & He, Y.-Y. *Regulation of XPC deubiquitination by USP11 in repair of UV-induced DNA damage.* Oncotarget, 2017. **8**(57), 96522–96535.

3. Shah, P., Zhao, B., Qiang, L., He; Y.-Y. *Phosphorylation of xeroderma pigmentosum group C regulates ultraviolet-induced DNA damage repair*, Nucleic Acids Research, 2018. gky239, https://doi.org/10.1093/nar/gky239

Repair of UV-induced DNA damage and associated disease conditions

In humans and mice UV-induced CPD and 6-4PP lesions are repaired by nucleotide excision repair (NER), the most versatile DNA repair system. NER eliminates a wide variety of helix-distorting base lesions induced by environmental carcinogenic sources, including UV and air pollutants [13-20]. Even though a primitive, more efficient DNA repair mechanism involving photolyases has been identified, it is absent in humans [20, 21]. When NER is defective and the damage is left unrepaired it leads to various disorders including xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD) (Table 1) [16, 17, 22]. These disorders are characterized by increased carcinogenesis in various organs, developmental and immunological defects, neuronal and retinal degeneration, and aging (Table 1) [16, 17, 22]. Defective NER predisposes affected individuals to carcinogenesis in the skin, brain, and lungs, and sensitizes mice to carcinogenesis in the skin, lungs, and liver [17, 23-27]. Even though the versatile NER pathway can correct bulky nucleotide adducts distorting the DNA structure from a variety of environmental carcinogens, it is crucial for correction of UV-induced DNA photoproducts in the skin, since NER defective patients have high propensity to develop sunlight exposure induced skin cancer [27]. Patients with defective NER manifest a 2,000-10,000 fold increase in risk of skin cancer, have a significantly lower age of onset of skin cancer compared to the general population, and have skin cancer as the most common cause of death as compared to other internal cancers [27]. This establishes the most significant association of NER defects with UV-associated skin cancer amongst all cancers. Essential NER factors have been identified, including xeroderma pigmentosum complementation group A-G (XPA-XPG) and cockayne syndrome group A (CSA) and B (CSB) [16, 17, 22].

Biochemical characterization of the nucleotide excision repair pathway

There are two main types of NER: global genome nucleotide excision repair (GG-NER) and transcription coupled nucleotide excision repair (TC-NER) [16, 17]. GG-NER is mainly responsible for removing most of the CPD and 6-4PP damage in non-transcribed regions, whereas TC-NER does the same in regions under active transcription in the genome [16, 17]. These two pathways differ in their damage recognition, but the following steps are the same in both pathways (Figure 1). In GG-NER, XPE (also known as DNA damage binding protein 2, or DDB2) and XPC first bind to the damage site and are responsible for UV-induced DNA damage recognition, in the heterodimeric complex with DDB1 (DNA damage binding protein 1) and HR23B, respectively [16, 17]. For TC-NER, CSA and CSB proteins mediate recognition of UV-induced DNA damage in actively transcribed regions by relieving the stalled RNA polymerase II (RNA pol II) at these sites [16, 17].

Table 1: Disorders associated with defective NER (See Ref [17]).

Disease due to defective NER	**Genes causing disorder**	**Characteristics**
Xeroderma pigmentosum(XP)	*XPA, XPB, XPC, XPD, XPE, XPF, XPG, XPV (Xeroderma Pigmentosum Variant)*	Sunlight exposure predisposes to various cancers, especially squamous cell carcinoma, basal cell carcinoma and melanoma skin cancer.
Trichothiodystrophy (TTD)	*XPB, XPD, TTDN1 (TTD non-photosensitive 1 protein or M-phase-specific PLK1-interacting protein) and TTDA (general transcription factor IIH, polypeptide 5 (GTF2H5))*	Brittle, sulfur-deficient hair and ichthyosis, mental retardation
Cockayne syndrome (CS)	*CSA, CSB, XPD* and *XPG*	Developmental and neurological disorders, decreased lifespan

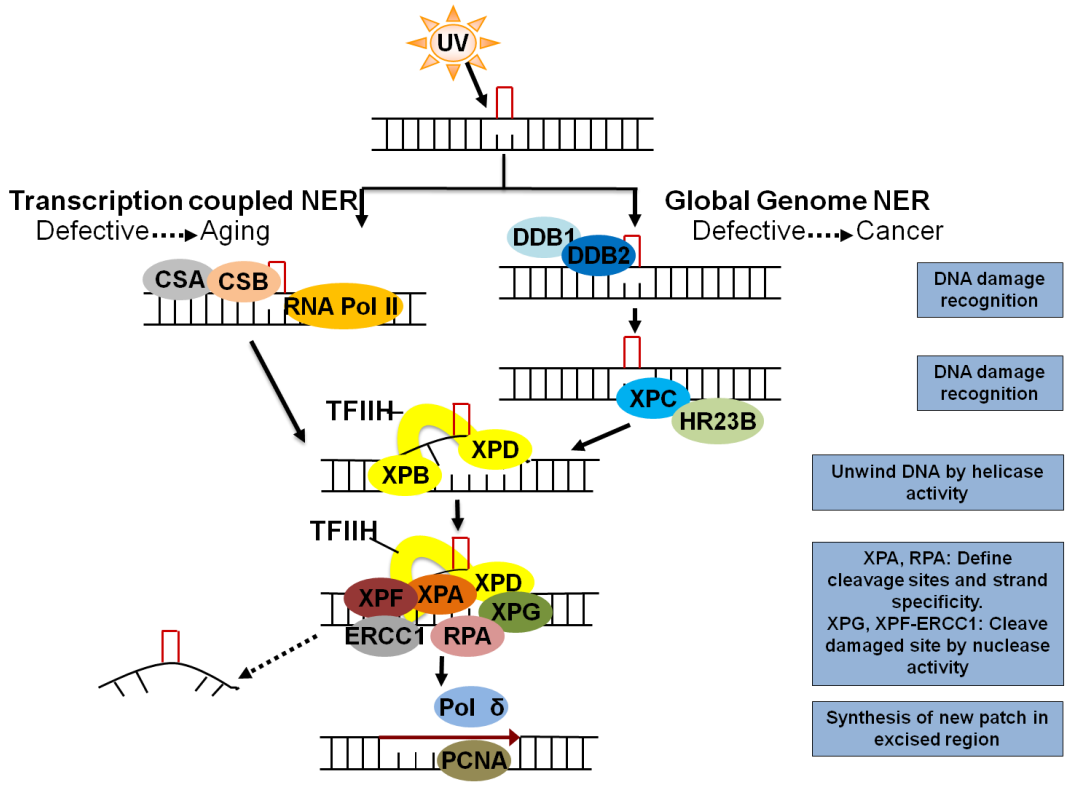

Figure 1: Sequential assembly of molecular players to remove UV-induced CPD and 6-4PP DNA damage lesions in global genome nucleotide excision repair (GG-NER) and transcription coupled nucleotide excision repair (TC-NER).

Following recognition, the rest of the NER pathway is the same for both the GG-NER and the TC-NER pathways [16, 17] (Figure 1). Upon recognition of the DNA damage, XPB and XPD, which form part of the transcription factor II H (TFIIH) complex, unwind the DNA through their helicase activity [16, 17] (Figure 1). XPA and RPA (replication protein) define the cleavage sites and strand specificity, to which XPG (also known as excision repair cross-complementation group 5, ERCC5) and the nuclease complex XPF-ERCC1 (excision repair cross-complementation group 1) bind to cleave the damaged site, followed by its excision [16, 17]. The excised portion is replaced with a newly synthesized patch with the help of proliferating-cell nuclear antigen (PCNA) and replicative polymerase (Pol) δ [16, 17] (Figure 1).

Alternate mechanisms to nucleotide excision repair

Unrepaired DNA damage leads to replication fork breakdown and subsequent genomic instability during cell division, since regular high fidelity DNA polymerases cannot synthesize past DNA lesions [28-31]. Translesion synthesis (TLS) polymerases can bypass the DNA damage and allow DNA replication to continue [28, 32-38]. Even though TLS polymerases were originally considered low fidelity polymerases contributing to mutagenesis, recent advances suggest that TLS could be error-prone or error-free in a damage-specific and polymerase-specific manner [28, 31, 39-43].

Translesion synthesis across unrepaired CPD lesions is mediated by Pol η (initiation and extension) in an error-free manner [28, 41, 43-46]. When Pol η is absent, for example in XP-V patients, a two-step process involving initiation (TLS Pol ι,κ and/or an unknown polymerase) and extension (TLS Pol ζ or κ) mediates translesion synthesis across the CPD lesion in an error-prone manner [28, 47, 48]. TLS across 6-4PP lesions is carried out by Pol ζ in an error-free manner, and alternatively by Pol η or Pol ι in an error-prone manner [42, 49]. Since TLS is potentially mutagenic, regulating the comparatively error-free NER would be more desirable [31, 50].

CHAPTER 2: MOLECULAR REGULATION OF NUCLEOTIDE EXCISION REPAIR

Although the major players of the NER pathway have been identified and their functions characterized, the molecular regulation of these factors is less well understood. It is important to understand how NER is regulated, which could be exploited to prevent or ameliorate pathologies associated with defective NER. Recent advances regarding the regulators of individual factors involved in NER are summarized as follows.

XPC regulation

XPC is an indispensable factor for initial recognition of bulky DNA damage in non-transcribed regions [16, 17, 22]. Loss of XPC function inhibits UV-induced CPD and 6-4PP DNA lesion repair, leading to accumulation of mutations upon replication, and increased cancer risk with UV exposure [16, 17, 22]. Being such an important protein, XPC is regulated at multiple levels: genetic (mutations and polymorphisms), transcriptional, and post-translational, in addition to regulation under specific conditions like immunosuppression.

XPC polymorphisms

In spite of having normal NER, the Lys939Gln polymorphism in XPC has been associated with various cancers, indicating that NER-independent function of XPC is also important for cancer [51, 52]. The XPC intron 11-5C/A SNP causes a reduction in DNA repair capacity due to a change in the frequency of alternatively spliced XPC mRNA.[53]

Promoter methylation of XPC

Recent studies have shown that XPC promoter methylation is increased by $BRAF^{V600E}$

(V600E mutant V-Raf Murine Sarcoma Viral Oncogene Homolog B), leading to decreased XPC mRNA levels and reduced DNA repair capacity [54] (Figure 2). This may play an important role in promoting spontaneous as well as UVB-induced melanomagenesis [54]. XPC promoter hypermethylation is also associated with reduced XPC expression in lung tumors from patients [55]. In lung cancer cell lines, the XPC promoter region, which consists of 17 CpG islands, was shown to be associated with XPC hypermethylation, leading to XPC repression [55].

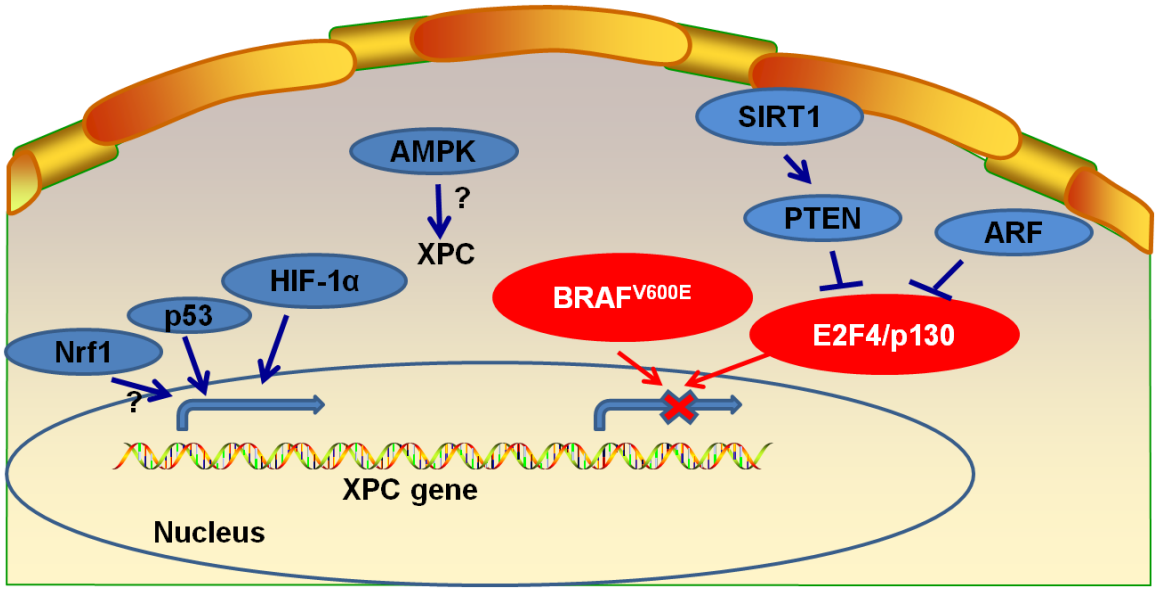

Figure 2: Transcriptional Regulation of XPC.

Transcriptional regulation of XPC

Various distinct transcription factors, such as p53, nuclear factor erythroid 2-related factor 1 (Nrf1, also called NFE2L1), Hypoxia-inducible factor-1 alpha (HIF-1α), AMP-activated protein kinase (AMPK), E2F transcription factor 4 (E2F4), and 130 kDa retinoblastoma-associated protein (p130), have been found to regulate XPC expression, and non-transcription factors also have been found to act *via* some of these transcription factors to modulate XPC

expression (Figure 2).

The p53 tumor suppressor signaling is well known to regulate XPC to enhance NER [56]. In HaCaT keratinocytes, Nrf1 promotes CPD repair by increasing XPC expression [57]. Nrf1 increases XPC expression via increasing glutathione availability [57]. HIF-1α also contributes to increased XPC transcription after UVB [58]. In mouse skin and normal human epidermal keratinocytes, the AMPK pathway promotes UVB-induced DNA repair by increasing XPC expression [59]. Under growth arrest conditions, E2F4 and p130 repressors were found to bind the XPC promoter region in a genome-wide binding screen [60]. In MEF (mouse embryonic fibroblast) cells, ARF was shown to reduce the binding of the E2F4-p130 repressor complex to XPC promoter, thus leading to increased expression of XPC [61, 62]. ARF (Alternative reading frame) was shown to be required for efficient NER of UVC-induced CPD and 6-4PP lesions, due to its function of regulating XPC expression [62].

Loss of phosphatase and tensin homolog (PTEN), an important tumor suppressor, was shown to inhibit CPD repair and to a lesser extent 6-4PP repair *in vitro* (in HaCaT cells) and *in vivo* (in mouse epidermis) *via* decreasing XPC protein levels, by regulating XPC transcription [63]. In *in vitro* cell culture models (MEFs and human keratinocytes), sirtuin 1 (SIRT1) inhibition impairs CPD and 6-4PP repair by increasing XPC transcription [64]. SIRT1 increases XPC transcription by activating PTEN through its deacetylase activity, which inhibits phosphorylation of AKT and impairs the nuclear localization of p130 transcriptional repressor [64].

XPC regulation by post-translational modifications and protein-protein interactions

XPC activity can be regulated by two types of post-translational modifications:

ubiquitylation and sumoylation [65]. XPC can be polyubiquitinated by the UV-DDB E3 ligase complex consisting of DDB2, DDB1 (DNA damage binding protein 1), Cul4A, and several other proteins [66-69] (Figure 3). Ubiquitination of XPC increases XPC binding to DNA [68]. Other studies also report that the UV-DDB complex mediates targeting of the XPC-HR23B complex to the site of CPD DNA damage [70, 71]. Current experimental evidence suggests that ubiquitination of XPC is not associated with XPC degradation [72]. Additionally, it was shown that DDB2 is necessary for degradation of XPC after UV-induced DNA photoproduct formation, but DDB1 and Cul4A, which are members of the same UV-DDB complex, inhibit XPC degradation upon UVC-induced DNA damage [73]. After UV-induced DNA photoproduct formation, degradation of XPC was shown to be necessary for recruiting XPG at DNA damage sites and thus for efficient NER [73]. Ubiquitin-specific protease 7 (USP7) promotes XPC deubiquitination as well as prevents XPC degradation in order to enhance NER [74]. XPC was shown to be sumoylated after UVC-induced DNA damage, which could promote stabilization of XPC [65]. Another study demonstrated that sumoylation of XPC subsequent to UV-DDB induced ubiquitination facilitates removal of XPC from the damage site [75]. Sequential polyubiquitination of sumoylated XPC by RING finger protein 111 (RNF111) also mediates release of XPC bound to damaged DNA, making way for downstream factors to further the NER process [65, 76, 77].

In addition, XPC interacts with other proteins to regulate its protein stability. For example, XPC-binding protein Rad23 (yeast homolog of HR23B) was shown to stabilize XPC [78, 79] (Figure 3). Inhibition of the proteasome pathway or overexpression of Rad23 increases the stability of Rad4 (yeast homolog of XPC) [80]. The authors further show that Rad23 significantly impacts NER capacity via two independent but simultaneous mechanisms [80, 81].

With p53-null cell lines, retinoblastoma protein RB was shown to increase the half-life/stability of XPC protein to enhance NER, through a direct interaction with XPC [82].

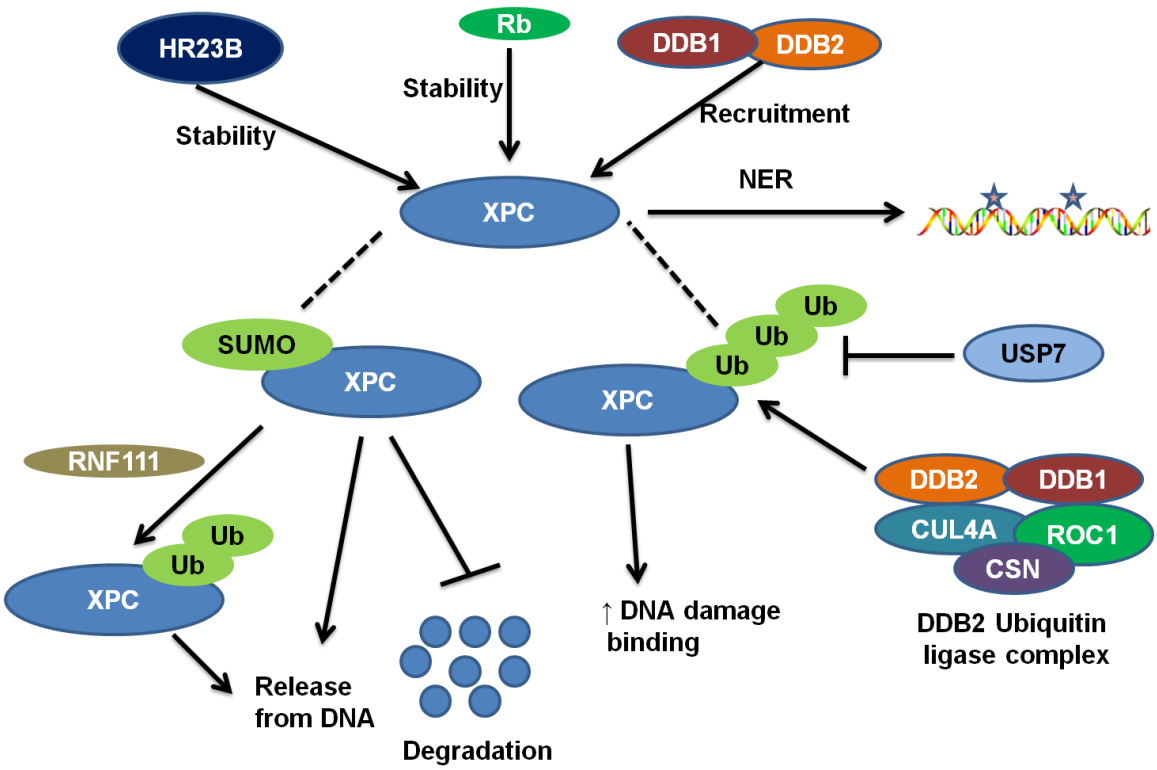

Figure 3: Post-translational regulation of XPC.

XPC regulation by immunosuppressants in organ transplant recipients

Organ transplant patients are at high risk of developing skin cancer [83]. These skin cancers have long been attributed to the immunosuppressive therapy post-transplant, and the level of immunosuppression affects the development of skin cancer [83, 84]. However, cyclosporin A (CsA), an immunosuppressant used for organ transplant recipients, promotes UVB-induced skin carcinogenesis in an immunosuppression-independent manner by (i) impairing DNA repair by suppressing XPC transcription , and (ii) impairing checkpoint and

DNA damage response by upregulating CypA [85]. Other reports have also shown that CsA inhibits NER in fibroblasts and lymphoblasts [86, 87]. In contrast to keratinocytes, in fibroblasts CsA inhibits NER by reducing XPA and XPG but not XPC [88]. In keratinocytes, the immunosuppressants tacrolimus and mycophenolate mofetil reduce UVB-caused DNA damage repair and apoptosis, and tacrolimus also impairs UVB-mediated checkpoint signaling, and thus may promote skin cancer in both an immunosuppression-dependent and -independent manner [89].

Regulation of XPE/DDB2

As an essential factor in DNA damage recognition, DDB2 is regulated at both transcriptional and post-translational levels. In response to UVC, DDB2 transcription is regulated by p53 in human cells, but not in mice [90, 91]. In addition, DDB2 activity is modulated by multiple pathways. UV irradiation was shown to cause constitutive photomorphogenesis 9 (COP9) signalosome (CSN) dissociation from the DDB2 complex [69, 92]. This in turn increased the ubiquitin-ligase activity of DDB2 [69, 93]. Upon being polyubiquitinated by the DDB complex itself, DDB2 signals for its degradation by proteasomes and loses its DNA binding activity [68]. Poly (ADP)-ribosylation (PARylation) of DDB2 was shown to inhibit DDB2 ubiquitination and degradation, to allow DDB2 more time to mediate chromatin modification [94, 95]. In addition to DDB2 complex-mediated chromatin regulation, DDB2 itself can regulate NER through chromatin remodeling by (i) Poly (ADP-ribose) polymerase 1 (PARP-1) mediated PARylation of chromatin, and (ii) recruiting chromatin remodeler Amplified in Liver Cancer 1 (ALC1) [94, 96-98].

Regulation of CSA and CSB

As an ubiquitin-ligase in TC-NER, CSA activity is decreased by UV irradiation *via* rapid association of COP9 signalosome (CSN) with CSA [69]. CSA was shown to ubiquitinate CSB to target it for degradation *via* the ubiquitin proteasome pathway, ultimately aiding the reinitiating of transcription after DNA repair [99]. UVSSA (UV-sensitive syndrome protein) was shown to recruit USP7 to deubiquitinate and stabilize CSB, thus opposing CSA-mediated ubiquitination and degradation of CSB [100, 101].

Regulation of RNA pol II

Following UV-induced DNA photoproduct formation at actively transcribed genes, stalled RNA pol II recruits CSA and CSB [102-105]. RNA pol II is then polyubiquitylated by CSA- and CSB-complex, which leads to the degradation of RNA pol II in the proteasomes to allow the recruitment of downstream NER factors [102-105]. Alternatively, RNA pol II degradation factor 1 (Def1) can mediate degradation of RNA pol II via ubiquitination independent of TC-NER [106]. In addition, the VHS (Vps-27, Hrs and STAM) domain of UVSSA was found to be essential for ubiquitination and dephosphorylation of RNA pol II and for efficient TC-NER, and this ubiquitination of RNA pol II does not target RNA pol II for degradation [107]. The authors also suggest that since UVSSA interacts with TFIIH, UVSSA may ubiquitinate RNA pol II by recruiting TFIIH, helping to remove stalled RNA pol II from the damage sites to allow the TC-NER factors access to the DNA damage [107].

XPD regulation

XPD functions at the merging point of the GG-NER and TC-NER pathways. XPD is a

part of the TFIIH complex, and along with XPB serves to unwind the recognized DNA damage via its helicase activity. In keratinocytes, immediately after UVB irradiation, XPD normally undergoes a small decrease, followed by its upregulation [58].

XPA regulation

XPA level and activity are regulated through various mechanisms under physiological and stress conditions. XPA level is increased by deficiency in toll-like receptor 4 (TLR4) [108]. In keratinocytes, αMSH/MC1R complex (α-Melanocyte-Stimulating Hormone/ Melanocortin 1 Receptor complex) enhances the GTPase activity of XPA-binding protein 1 (XAB1), which in turn induces nuclear translocation of XPA, thus regulating NER for UVB-induced DNA damage via a pigmentation-independent mechanism [109] (Figure 4). PKA-mediated ATR phosphorylation enhances ATR-XPA interaction and rapid recruitment of XPA to DNA damage sites, ultimately promoting NER and reducing UVB or UVC-caused mutations [110]. In addition, PARP-1 directly interacts with XPA to enhance NER [111]. Arsenic is known to inhibit NER, and zinc was shown to protect against the detrimental effects of arsenic on DNA damage [112-115]. In cell-based systems, XPA and PARP-1 (both zinc finger proteins) were shown to be molecular targets for arsenite [116-119]. Cadmium, copper, nickel and cobalt also inhibited XPA by decreasing binding of XPA to UVC-damaged DNA [120]. The mechanism of NER impairment by metals needs to be further investigated.

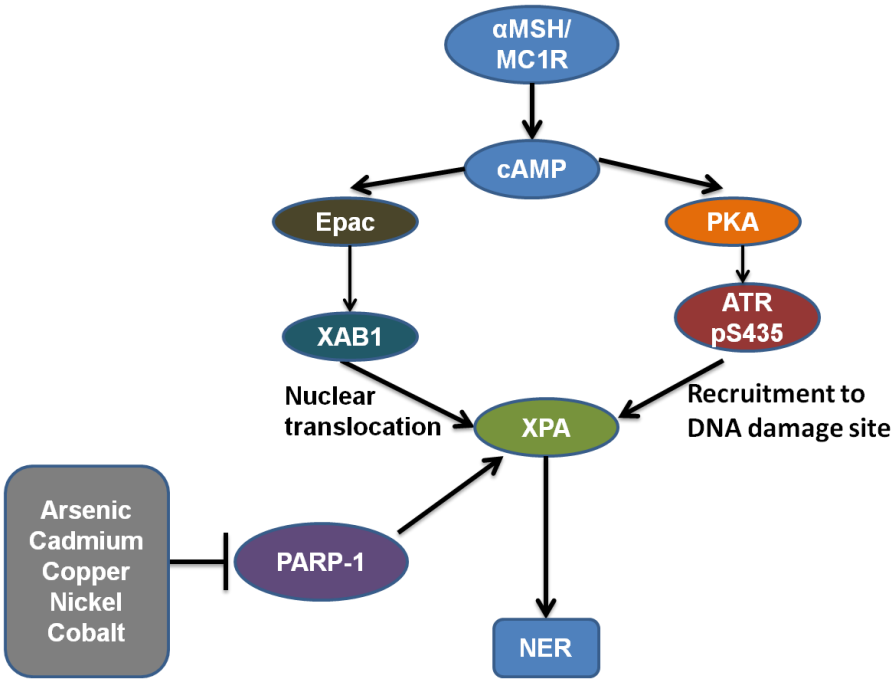

Figure 4: Molecular Regulation of XPA.

Regulation of RPA and PCNA

RPA binds to the unwound DNA damage site and to XPA. XPA and RPA together define the cleavage sites and strand specificity for the downstream nucleases. After excision of the damaged DNA by the nucleases, PCNA promotes filling of the gap by DNA synthesis. Transcription of RPA and PCNA may be regulated by E2F1 (E2F Transcription Factor 1) and E2F4, which were found to bind to the promoter region of RPA3 and PCNA genes [121]. E2F4 and p130 repressors, under growth arrest conditions, were also found to bind to the PCNA promoter region in a genome wide binding screen [60].

Regulation of XPG/ERCC5

XPG/ERCC5 participates in cleavage and excision of the damaged DNA lesion *via* its endonuclease activity. XPG level and activity are regulated by transcription and protein-protein

interaction, respectively. In H23 or H460 human lung adenocarcinoma cell lines, CCAAT/enhancer-binding protein gamma (CEBPG) was shown to increase ERCC5 transcription [122] (Figure 5). Human interferon β (IFN-β) treatment was shown to increase XPG mRNA levels in fibroblasts isolated from CSA and CSB patients with defects in TC-NER [123]. IFN-β-mediated upregulation of XPG could be a possible mechanism for IFN-β-mediated resistance to UVC-induced cell death, in a TC-NER-independent manner [124]. Other mechanisms may also have a role in the effect of IFN-β and remain to be determined.

Gadd45 (Growth Arrest And DNA-Damage-Inducible) has been shown to improve NER [125, 126]. Gadd45a directly interacts with XPG to cause active DNA demethylation [127]. Gadd45a-mediated DNA demethylation probably stimulates DNA repair via XPG and XPB, due to the association between DNA repair and DNA demethylation [127, 128]. The precise mechanism remains to be elucidated.

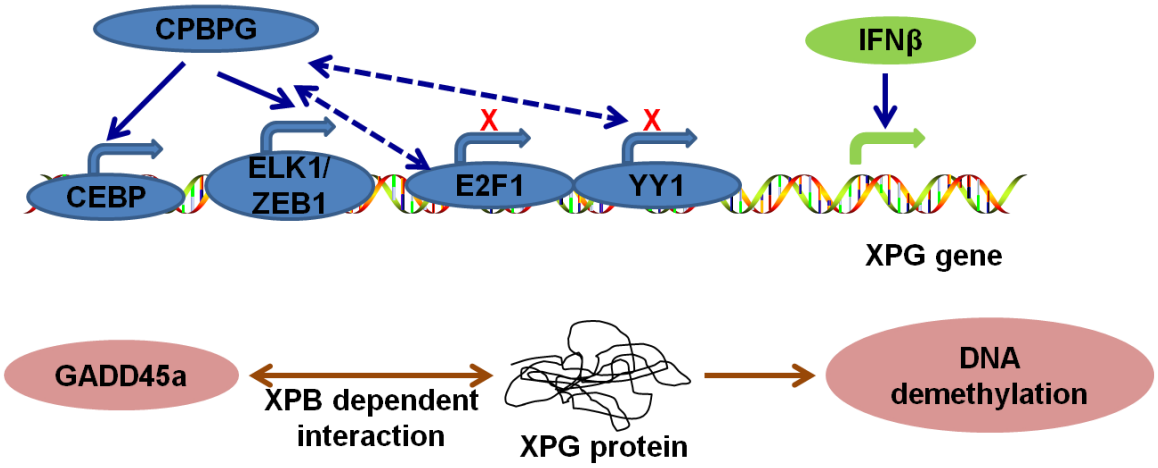

Figure 5: Molecular regulation and interactions of XPG.

ERCC1 regulation

XPF-ERCC1 dimer participates in cleavage and excision of the damaged DNA lesion *via*

its endonuclease activity. ERCC1 mutations contributing to NER disorders have rarely been found. A patient with a homozygous Exon 7 mutation in ERCC1 is reported to have CS symptoms, and a patient with ERCC1 deficiency had developmental failure and a mild defect in NER [129, 130]. Ercc1 knockout mice have similar critical developmental disorders and neonatal lethal characteristics [131]. Deletion of Smad4 (SMAD family member 4) decreases ERCC1 transcription to cause defective CPD repair via reduced Snail expression, leading to increased UVA and UVB-induced SCC in murine models with keratinocyte-specific Smad4 loss [132].

Other NER Regulatory Pathways

Melanin, MSH, and Melanocortin

In melanocytes with functional MC1R, total melanin and eumelanin contents (MC and EC) were found to be inversely proportional to CPD damage [133]. Additionally, melanocytes with loss of MC1R function have higher UVB-induced CPD damage and lesser repair of these lesions, independent of their total melanin and eumelanin content [133].

MSH and adrenocorticotropic hormone (ACTH) have been shown to activate MC1R, leading to the increased repair capacity of UVB-induced DNA photoproducts and decreased ROS generation [134-137]. Forskolin was also shown to have effects similar to those of αMSH on UV-induced repair of DNA photoproducts, due to forskolin-mediated activation of the cAMP pathway, a downstream pathway common to melanocortins [134, 138, 139]. Tetrapeptide and tripeptide analogs of melanocortin, containing a modified αMSH core with N capped groups and C terminal modifications respectively, also enhance CPD repair, by activating MC1R as MSH does [134, 140, 141].

Chromatin modification

Chromatin modification may be an important regulator of NER, since chromatin in an open conformation facilitates the binding of DNA repair factors to the damaged DNA [67, 102, 142, 143]. In cell extracts and reconstituted human excision nuclease systems with reconstituted nucleosomes, the NER rate was reduced to only 10% of that in naked DNA [144]. The repair kinetics for acetylaminofluorene-guanine (AAF-G) adduct is increased by the SWI/SNF complexes (SWItch/sucrose nonfermentable) through multiple mechanisms [145-155]. In addition, by increasing the access of NER factors to nucleosomal DNA, NER can be increased by ACF (Asymmetric Crying Facies), an ATP-utilizing chromatin assembly and remodeling factor [156-158].

The DDB2 complex also facilitates NER by carrying out ubiquitination of H2A, H3, and H4 histone proteins, indicating DDB2's role as a chromatin remodeler to allow the NER factors access to damaged DNA lesions [96-98]. Histone acetylation mediates chromatin unfolding even after lesion detection, which is important for efficient NER. Indeed, histone acetyltransferases, such as GCN5 (general control of amino-acid synthesis 5), have been shown to be involved in this process [159-162]. p53 was also shown to mediate whole genome relaxation to facilitate lesion detection and NER [162]. The UV-DDB complex also associates with GCN5 and p300, suggesting another probable mechanism of DDB-mediated chromatin regulation to facilitate NER [163-165].

E2F1 has been shown to facilitate NER by recruiting GCN5 at sites of UVC- or UVB-induced DNA photoproducts [161]. GCN5 mediates histone H3 Lysine 9 (H3K9) acetylation to allow increased access of NER factors to the damaged DNA [161]. The S29A mutation (S29 in mice, equivalent to human S31) in E2F1 hinders E2F1 recruitment and E2F1-mediated

recruitment of GCN5 and H3K9 acetylators at damage sites, reducing access of the NER factors XPC and XPA to the DNA damage lesions [166]. After removal of DNA damage, restarting of transcription is enhanced by rapid removal and exchange of H2A and H2B at UVC-induced DNA photoproducts, and by placement of H3.3 histone [167-169]. In yeast, the loss of H2A histone variant HTZ1 (H2A.Z) inhibits UVC-induced CPD damage removal [170]. HTZ1 promotes CPD repair by recruiting GCN5, leading to histone H3K9/K14 acetylation and increasing Rad14 (ortholog of XPA) binding to damaged DNA [170]. Through their histone acetylase activity, p300 and CREB Binding Protein (CBP) redundantly lead to DDB2 recruitment to CPD lesions in compacted chromatin regions, facilitating repair of UVC-induced CPD lesions [171]. p300 phosphorylation at S1834 is seminal for efficient CPD repair through facilitating DDB2 recruitment to the CPD lesion [171].

Conclusion and Perspectives

UV-induced DNA repair, or NER, essentially removes DNA damage by inevitable environmental factors like solar UVB radiation and air pollutants. NER is vital to maintaining genomic integrity to protect animals and humans from skin, lung and brain cancer as well as neurological and developmental disorders, and thus justifiably has multiple factors and signaling mechanisms for its regulation. Recent studies have demonstrated that UV-induced DNA repair is regulated at multiple levels including transcriptional modulation and post-translational modifications. Both extracellular cues and intracellular signaling regulate UV-induced DNA repair capacity. These regulations are achieved through modulating the availability or activity of individual repair factors, or modifying chromatin structure. Better understanding of the NER regulation may offer new opportunities to enhance the NER capacity and therefore improve our

ability to combat cancer initiation and progression, as both processes involve genetic mutations and/or genomic instability.

CHAPTER 3: MATERIALS AND METHODS

Human Skin Tumor Samples

All human specimens were studied after approval by The University of Chicago Institutional Review Board. Formalin-fixed, paraffin-embedded tissue blocks were obtained from the archives in the tissue bank of Section of Dermatology, Department of Medicine, University of Chicago. Non–sun-exposed nonlesional normal epidermis (Normal, n = 21), AK (AK, pre-malignant, n = 15), and SCC (SCC, malignant, n = 16) were used for immunohistochemical analysis.

Animal Treatments

All procedures were approved by the University of Chicago Institutional Animal Care and Use Committee. Female hairless SKH-1 mice (n = 9, 4-6 weeks old, Charles River Laboratories) were randomized and exposed to UVB (100 mJ/cm^2, dose without visible sunburn) dorsally or sham-irradiated, three times a week for 25 weeks. Mouse skin or tumors were fixed in formalin and used for immunohistochemical analysis.

Cell Culture

Human HaCaT keratinocytes (kindly provided by Prof. N. Fusenig of German Cancer Research Center (DKFZ), Heidelberg), human embryonic kidney cells HEK293T (ATCC), HEK293T USP11-knockout cells (ΔUSP11, kindly provided by Dr. Daniel Durocher of The Lunenfeld-Tanenbaum Research Institute, Canada), and XPC-deficient (XPC^{Null}) immortalized skin fibroblasts (GM15983, also known as XP4PA-SV-EB, obtained from Coriell Cell Repositories, Camden, New Jersey) were cultured in a monolayer in 95% air/5% CO_2 (vol/vol) at

37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Normal human epidermal keratinocytes (NHEK) cells (Clonetics, Lonza) were cultured in KGM Gold BulletKit medium (Clonetics, Lonza). Where indicated, cells were treated with the mTOR inhibitor rapamycin (25nM, LC Laboratories, R-5000), the casein kinase II inhibitor CX-4945 (5μM, Selleck Chemicals, S2248), the ATM kinase inhibitor KU-60019 (1μM, Selleck Chemicals, S1570), the PI3K inhibitor LY294002 (10μM, Promega, V1201), the DNA-PK inhibitor NU7441 (1μM, Selleck Chemicals, S2638), the JNK inhibitor TCS JNK 60 (10μM, Torcis, 3222), the p38 inhibitor SB203580 (10μM, Promega, V1161), the ERK inhibitor PD98059 (20μM, Promega, V1191), the ROCK1 inhibitor Y27632 (10μM, Torcis, 1254), or with the vehicle (DMSO, Sigma-Aldrich). The concentrations of the kinase inhibitors were chosen based on our previously used concentrations and from literature [172-181].

UVB Radiation

Cells were irradiated with UVB using UV Stratalinker 2400 with UVB bulbs (Stratagene) after washing twice with PBS as described previously [182, 183]. Control samples were treated similarly and sham irradiated. The Goldilux UV meter with a UVB detector (Oriel Instruments) was used to monitor the UVB dose weekly. There is no detectable UVC emission from our system.

Plasmids, transfection and site-directed mutagenesis

pRK5myc plasmids with wild-type (WT) and C275/283S mutant (csmt) USP11 were kindly provided by Dr. Ruey-Hwa Chen. The plasmids were transfected into HEK293T ΔUSP11

cells using X-tremeGENE 9 according to the manufacturer's instructions (Roche) as described previously [184]. Human XPC gene was sub-cloned from the pCMV6-XL5 vector (Origene) to a Gateway pENTR vector (Invitrogen). The resulting pENTR-XPC was used for recombination reaction with pLenti CMV Puro Dest destination vector (Addgene, 17452; deposited by Eric Campeau) to generate the pLenti-XPC lentiviral expression vector, according to the manufacturer's instructions (Invitrogen). The S61A, S94A, T169A, S397A, S399A, S883A, S884A, S892A and S892D point mutations of wild-type pLenti-XPC plasmid were generated by site-directed mutagenesis with the QuikChange XL kit according to the manufacturer's instructions (Stratagene, 200521). The following primers were used to generate the mutations: S61A sense 5'- TGAACCCCCAGGATGAGCGCAGCCTCTTTTCCTC-3' and S61A antisense 5'- GAGGAAAAGAGGCTGCGCTCATCCTGGGGGTTCA-3', S94A sense 5'- AAAGGATGAAGCCCTCGCCGATGGGGATGACCTC-3' and S94A antisense 5'- GAGGTCATCCCCATCGGCGAGGGCTTCATCCTTT-3', T169A sense 5'- CTGCTCTGGCGCTTCAATCTCTATCTCCACTGG-3' and T169A antisense 5'- CCAGTGGAGATAGAGATTGAAGCGCCAGAGCAG-3', S399A sense 5'- GCAAGCCCTCCTCCGCCGAGGAAGATGAGG-3' and S399A antisense 5'- CCTCATCTTCCTCGGCGGAGGAGGGCTTGC-3', S397A sense 5'- CGGAGCAAGCCCGCCTCCAGCGAGG-3' and S397A antisense 5'- CCTCGCTGGAGGCGGGCTTGCTCCG-3', S884A sense 5'- CCTCCTCTTCATCAGCAGAGAGTCCACCTCC-3' and S884A antisense 5'- GGAGGTGGACTCTCTGCTGATGAAGAGGAGG-3', S883A sense 5'- CCTCTTCATCAGAAGCGAGTCCACCTCCTGC-3' and S883A antisense 5'- GCAGGAGGTGGACTCGCTTCTGATGAAGAGG-3', S892A sense 5'-

GCTTCTGCTTGAGCGCTGGTCCCCTCC-3' and S892A antisense 5'-GGAGGGGACCAGCGCTCAAGCAGAAGC-3', S892D sense 5'-GCCGCTTCTGCTTGATCGCTGGTCCCCTCCTC-3' and S892D antisense 5'-GAGGAGGGGACCAGCGATCAAGCAGAAGCGGC-3'. All mutants were confirmed by sequencing.

siRNA transfection

siRNAs targeting human USP11, DDB1, CK2A1, or CK2A2 (*ON-TARGETplus SMARTpool*) and Control siRNA (*ON-TARGETplus Non-targeting siRNA)* were purchased from GE Healthcare Dharmacon Inc. Nucleofector (Amaxa, Gaithersburg, MD) was used to electroporate cells with siRNA as previously described [185-187].

Lentiviral Production and Infection

Human shUSP11 (USP11 MISSION shRNA TRCN0000011090, Sigma-Aldrich) and shCon constructs (obtained from Seungmin Hwang); or pLenti vector and pLenti-XPC (wild type or mutant S61A, S94A, T169A, S397A, S399A, S883A, S884A, S892A, S892D) constructs were co-transfected with pCMVdelta8.2 and pVSV-G plasmids into 293T cells as previously described using X-tremegene 9 (Roche Applied Science) to produce lentiviral particles [188]. The packaged lentivirus (in supernatants from transfected 293T cells at 24-48 hours) was used to infect HaCaT or XPC^{Null} cells along with polybrene (8 μg/mL, Sigma). Stable cell lines were selected using 1 μg/mL puromycin for 7 days.

Western Blotting

Western Blotting was performed as described previously using an SDS-PAGE electrophoresis system [189]. Briefly, cells were lysed in RIPA buffer (Pierce, Rockford, CA) supplemented with Protease and Phosphatase inhibitor cocktail (Thermo Scientific) and harvested. Equal amounts of protein were subjected to SDS-PAGE electrophoresis and electrophoretic transfer to nitrocellulose membranes. 5% nonfat milk in TBST (Thermo Fisher Scientific) was used to block membranes prior to probing with primary and secondary antibodies. The following antibodies were used: XPC (Sigma-Aldrich, X1129), USP11 (Bethyl Laboratories, Inc., A301-613A), CK2A2 (Bethyl Laboratories, Inc., A300-199A), GAPDH (Santa Cruz, sc-25778), Histone H3 (Santa Cruz, sc-8654), DDB1 (Santa Cruz, sc-137132), XPB (Santa Cruz, sc-293), CK2A1 (Santa Cruz, sc-365787), myc (Cell Signaling Technology, 2278S), XPA (Kamiya biomedical, MC-340), phosphorylated S94 XPC (AMS Biotechnology (Europe) Limited), and CK2B (Abcam, ab133576).

Determination of CPD damage in genomic DNA by immuno-slot-blot assay

Determination of CPD and 6-4PP using a slot blot assay was performed as previously described [183, 190, 191]. Briefly, DNA was extracted from cells collected at various times after UV exposure using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). The absorbance at 260 nm from a NanoDrop 1000 (NanoDrop products, Wilmington, DE) was used to determine the concentration of DNA. CPD monoantibody (TDM-2, Cosmo Bio Co., Koto-Ku, Tokyo, Japan) was used to quantify CPD in the DNA with slot blot (Bio-Rad). To determine repair kinetics, percentage (%) repair was calculated by measuring optical density at the specified times and

comparing it to that at time zero hours, since at zero hours 100% of the CPD damage was present after UVB prior to repair.

Chromatin fractionation

The chromatin-bound protein fraction was extracted from cells using the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Fisher Scientific #78840) according to the manufacturer's instructions. The resulting chromatin-bound protein fraction was analyzed by Western blotting.

Local UV irradiation and fluorescent labeling

The local UV irradiation procedure was carried out as previously described, with some modifications [192, 193]. Briefly, cells were UVC-irradiated (254 nm, 100 J/m^2) through an isopore polycarbonate filter with 5-μm diameter pores (Millipore Co., Bedford, MA). After incubation for indicated times, cells were fixed, permeabilized, and DNA was denatured with 2M HCl for 30 min at room temperature. Blocking was performed using 5% normal goat serum in PBS (Invitrogen, Carlsbad, California) for 30 min at room temperature, followed by incubation with primary and secondary antibodies for 30 min at 37°C. The samples were mounted in Prolong Gold Antifade Reagent with DAPI (Invitrogen, Carlsbad, California). The antibodies used were CPD (TDM-2, COSMO BIO Co.), XPC (Santa Cruz, sc30156), XPB (Santa Cruz, sc-293), Alexa Fluor 488 $F(ab')_2$ fragment goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG antibodies.

Immunoprecipitation

Immunoprecipitation was carried out as described previously using anti-USP11 (Bethyl Laboratories, Inc., Rabbit, A301-613A) antibody [184].

Immunohistochemical analysis

Immunohistochemical analysis for USP11 levels was carried out by using anti-USP11 antibody (Atlas Antibodies, Cat # HPA037536) by the Immunohistochemistry core facility at the University of Chicago. The protein levels were visualized with the diaminobenzidine (DAB) method (brown color) in mouse skin, and Vina Green™ Chromogen Kit (green color, to exclude the contribution of endogenous brown pigmentation) in human skin, respectively. USP11 levels in tissue sections were scored blindly by two independent investigators as strong (3), moderate (2), weak (1), or absent (0), as previously published [178, 194].

In vitro cell proliferation assay

Cell proliferation was analyzed using CellTiter 96 AQueous non-radioactive cell proliferation assay (MTS) (Promega) as described previously [195]. Briefly, XPC^{Null} cells expressing pLenti-vector, pLenti-XPC WT or mutant constructs S892A, S94A were seeded into 24 well tissue culture plates. 24, 48 and 72 hours later, cell viability was determined by MTS assay by incubation with MTS solution for 1 hour at $37^{o}C$, and subsequent measurement of absorbance with a TECAN Infinite M200 plate reader at 490 nm.

Determination of apoptosis

Apoptosis was determined by propidium iodide (PI) assay followed by flow cytometry, as

described previously [196]. Briefly, XPC^{Null} cells expressing pLenti-vector, pLenti-XPC WT and mutant construct S94A or S892A were exposed to UVB (30 mJ/cm^2) or sham irradiation and incubated for 24 hours. After incubation, floating apoptotic cells in the supernatant were combined with attached cells collected by trypsinization followed by neutralization. The cells were collected by centrifugation at 1,000 rpm for 5 min at 4^oC, washed with cold PBS, and fixed with cold 70% ethanol. Fixed cells were stained with propidium iodide (20 μg/ml, with 0.1 mg/ml RNase in PBS) and incubated for 30 min at room temperature. Propidium iodide staining for apoptosis was quantified by flow cytometry using BD FACSort (BD Biosciences).

Statistical analyses

Statistical analysis was performed with Prism 5 (GraphPad software, San Diego, CA, USA). Sample size calculations were performed with StatMate 2.00 (GraphPad). The number of mouse or human tissue used in the *in vivo* mouse model is determined based on 80% power, a two-sided test with a significance level of 0.05. Two independent investigators double blindly scored the normal and tumor sections as no staining (0), weak (1), moderate (2) or strong (3) for USP11. Data were shown as the mean of three independent experiments and analyzed by Student's *t*-test (two-tailed). Immunohistochemical analysis was analyzed by the Mann-Whitney *U* test (two-tailed). *p*-value <0.05 was considered statistically significant. Error bars were shown as standard errors of the mean (S.E.).

CHAPTER 4: REGULATION OF XPC DEUBIQUITINATION BY USP11 IN REPAIR OF UV-INDUCED DNA DAMAGE

Summary

Nucleotide excision repair (NER) is the most versatile DNA repair pathway for removing DNA damage caused by UV radiation and many environmental carcinogens. Although the core NER proteins have been identified and characterized, molecular regulation of NER remains poorly understood. Particularly, post-translational regulation of XPC is largely unknown. Here we show that ubiquitin-specific peptidase 11 (USP11) positively regulates NER by deubiquitinating xeroderma pigmentosum complementation group C (XPC) and promoting its retention at the DNA damage sites. In addition, UV irradiation induces both USP11 recruitment to the chromatin and USP11 interaction with XPC in an XPC-ubiquitination-dependent manner. Furthermore, we found that USP11 is down-regulated in chronically UV-exposed mouse skin and in skin tumors from mice and humans. Our findings indicate that USP11 plays an important role in maintaining NER capacity, and suggest that USP11 acts as a tumor suppressor *via* its role in DNA repair.

Introduction

Nucleotide excision repair (NER) is the most versatile DNA repair system for removing various forms of bulky DNA damage induced by environmental carcinogens, including solar ultraviolet B (UVB) radiation and air pollutants [13, 197, 198]. Defective GG-NER in humans leads to the Xeroderma pigmentosum (XP) syndrome, characterized by an increased risk of carcinogenesis in various organs including the skin, lungs and brain [16, 17, 26, 199]. The risk is especially increased significantly at a very young age for non-melanoma and melanoma skin

cancers, the most common cancer in the United States [1, 6, 27]. Of the xeroderma pigmentosum complementation group A-G (XPA-XPG) factors identified in the GG-NER process, XPC plays a vital role in the initial DNA damage recognition step in GG-NER [16, 17, 193, 199-201], and in preventing carcinogenesis in various organs, especially skin carcinogenesis [202-206].

XPC is regulated at various levels including genetic, transcriptional, post-translational, and by immunosuppression [207]. After UV exposure, XPC is polyubiquitinated by the UV-DDB E3 ligase complex, consisting of DDB1 (DNA damage-binding protein 1), DDB2, CUL4A (Cullin-family E3-ligase adaptor protein) and ROC1 (E3-ligase RING domain) [68, 69, 208-210]. Ubiquitination of XPC by the UV-DDB complex enhances XPC binding to the DNA damage site and is essential for its DNA damage recognition function in NER [68, 208, 210]. Subsequently XPC is sumoylated and then undergoes a second ubiquitination event by RING finger protein 111 (RNF111), which mediates XPC release from the damage site, and also promotes efficient NER [72, 77, 209-211]. Since ubiquitination of XPC does not promote XPC degradation, it must probably be deubiquitinated and recycled [68]. Even though the biochemical function of XPC in the NER process has been extensively studied, regulation of XPC by deubiquitination is largely unknown. Only recently, USP7 was identified as a deubiquitinase for XPC, which promoted the NER process [74]. Identifying novel regulators of XPC deubiquitination could provide more drug-susceptible targets than XPC to modulate XPC activity in the NER process and prevent skin cancer.

Ubiquitin specific peptidase 11 (USP11) is a member of the ubiquitin-specific proteases (USPs) family of deubiquitinase enzymes [212]. USP11 participates in various signaling pathways and biological processes such as TGFβ signaling, pro-inflammatory signaling, viral replication, and NF-κB signaling, by regulating deubiquitination and protein stability of various

targets such as TβRII, ALK5, LPA1, NP protein, and IκBα [213-217]. Additionally, USP11 has emerged to positively regulate DNA double-strand break (DSB) repair by regulating PALB2 deubiquitination, by modifying recruitment of RAD51 and 53BP1 to the DNA damage site dependent on its catalytic activity, and by interacting with BRCA2 independent of its catalytic activity [218-221]. Proteomic analysis by Havugimana and colleagues predicted that USP11 and XPC interact as part of a protein complex [222]. However, the regulatory and functional role of USP11 in NER is unknown.

The objective of this study was to determine the role of USP11 in the NER pathway. We further determined whether the mechanism of USP11 activity was *via* regulating deubiquitination of XPC, as well as USP11's role in skin cancer.

Results

USP11 promotes UVB-induced DNA damage repair

To determine whether USP11 affects repair of UVB-induced DNA damage, we measured the difference in UV-induced DNA damage repair between control and USP11-inhibited cells. We focused on the repair of cyclobutane pyrimidine dimers (CPD), since CPD is the main photoproduct of UV-induced DNA damage in humans, and unrepaired CPD damage leads to skin cancer [223]. In HaCaT cells, both siRNA- and shRNA-mediated USP11 knockdown significantly inhibited CPD repair (Figure 6A-6F). The experimental conditions and low dose for UV radiation (20 mJ/cm^2) were chosen to avoid significant changes in cell proliferation and apoptosis after UV exposure, which could affect DNA damage measurements (data not shown). Our results indicate that USP11 positively regulates repair of UV-induced CPD DNA damage, and suggests a tumor suppressive function of USP11 in skin cancer.

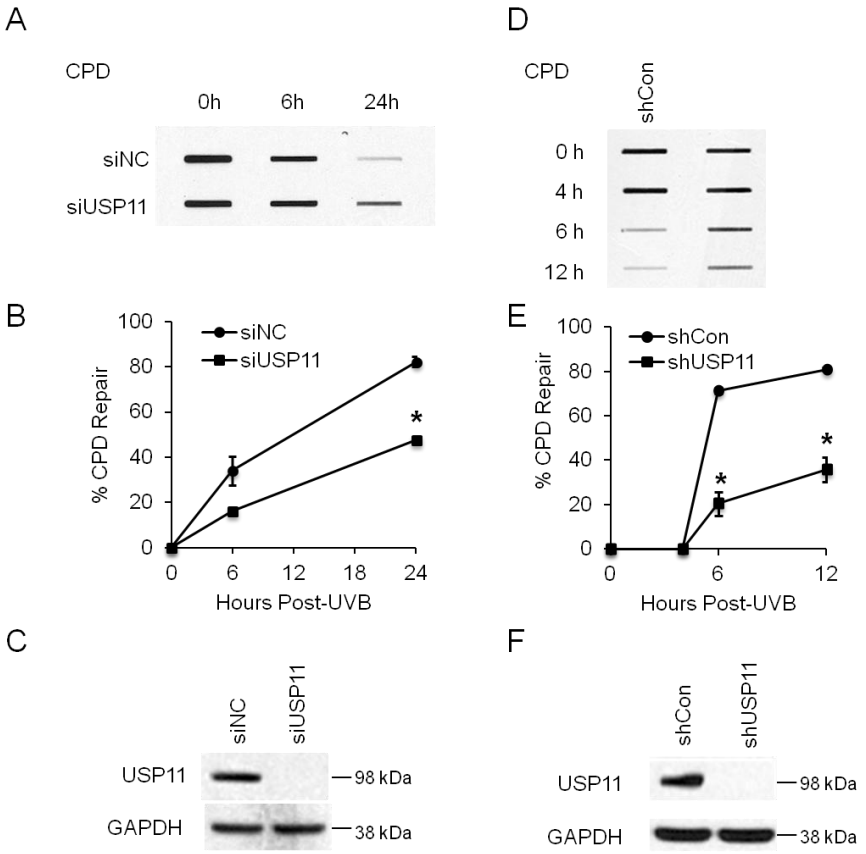

Figure 6: USP11 promotes UV-induced DNA damage repair.
(A, D) Slot blot analysis of the levels of CPD at indicated times post-UVB (20 mJ/cm^2) in HaCaT cells transfected with siRNA targeting USP11 (siUSP11) or non-targeting control siRNA (siNC) (A), and HaCaT cells stably infected with a lentiviral vector expressing control shRNA (shCon) or shRNA targeting USP11 (shUSP11) (D). (**B, E)** Quantification of percentage (%) of CPD repair (B) from (A), and (E) from (D). *, $P < 0.05$, compared with siNC and shCon groups respectively, Student's *t*-test. **(C, F)** Immunoblot analysis of USP11 and GAPDH in HaCaT cells transfected with siUSP11 or siNC (C), and stably infected with a lentiviral vector expressing shCon or shUSP11 (F). The results were obtained from three independent experiments.

USP11 deubiquitinates XPC at the chromatin following UVB damage

To elucidate the mechanism by which the deubiquitinase USP11 affects UVB-induced DNA damage repair, we determined whether USP11 regulates deubiquitination of XPC after UVB exposure, since ubiquitination of XPC is important for its efficient function in the NER process [68, 77]. Using biochemical and biological approaches, Sugasawa and colleagues have

demonstrated that UV induces XPC ubiquitination through the UV-DDB complex, and that XPC immunoblot analysis detects several bands for ubiquitinated XPC (migrated slower than non-ubiquitinated XPC), in addition to non-ubiquitinated XPC [68]. Thus we used this immunoblot method to detect XPC ubiquitination. In HaCaT keratinocytes, knockdown of USP11 by siRNA increased ubiquitinated XPC levels at 1.5 h post-UVB as compared to control siRNA, while it did not affect the levels of XPC ubiquitination at 0.5 h post-UVB (Figure 7A). This indicates that USP11 is important for XPC deubiquitination after UV exposure. Since at 6 h the XPC ubiquitination levels decreased to a similar level in both the siUSP11 and control groups, XPC was eventually deubiquitinated in the siUSP11 group, possibly by a mechanism independent of USP11. Previous studies have shown that XPC is degraded after UV damage [72]. With MG132 proteasome inhibitor treatment, the siUSP11 group did not show difference in XPC levels compared to control, indicating that USP11 does not affect degradation of XPC after UV damage. Similarly, after UV damage in 293T ΔUSP11 cells, USP11 deficiency increased XPC ubiquitination levels only at a later time point (2 h) after UV damage compared to WT cells, not at earlier time points (0.5 h and 1.5 h) (Figure 7B). Previous studies indicate that XPC is recruited to the DNA damage site and is ubiquitinated to promote its binding to the damage site [68]. To confirm that changes in XPC ubiquitination by USP11 occur at the chromatin, we examined changes in XPC ubiquitination by USP11 in the chromatin-bound XPC protein fraction. In the chromatin-bound protein fraction, USP11 knockdown increased ubiquitination of XPC at a later time point (60 min), but not earlier ones, as compared with control (shCon) cells (Figure 7C). Our findings indicate that USP11 mediates XPC deubiquitination at the chromatin following UVB damage.

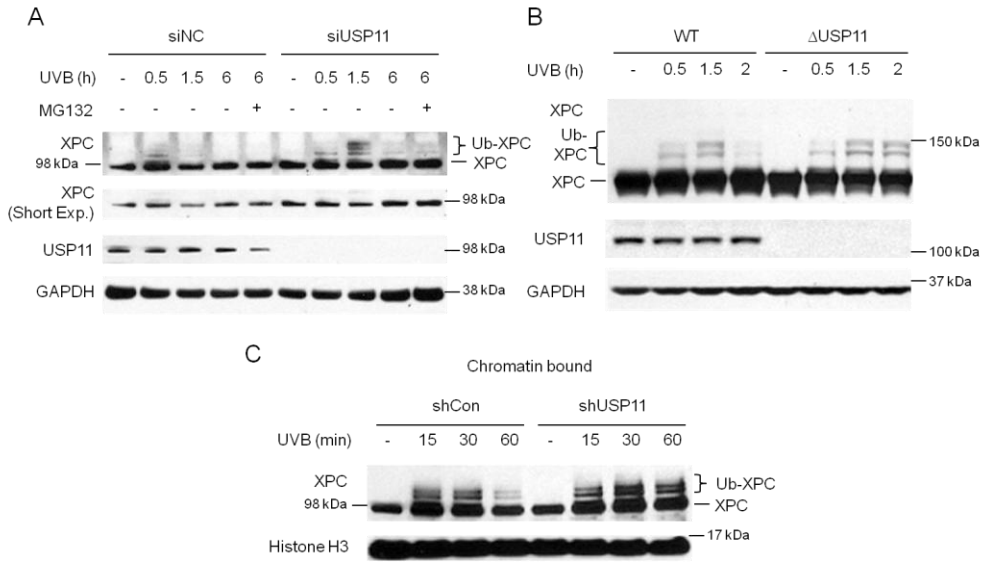

Figure 7: USP11 deubiquitinates XPC at the chromatin following UVB damage.
(A, B) Immunoblot analysis of XPC, USP11, and GAPDH in HaCaT cells transfected with siUSP11 or siNC and treated with or without MG132 (10 μM) 1 h prior to UVB exposure (20 mJ/cm^2) (A), 293T WT and ΔUSP11 cells (B) at the indicated times post-UVB (20 mJ/cm^2). **(C)** Immunoblot analysis of XPC and histone H3 using chromatin-bound protein fractions from HaCaT cells stably infected with a lentiviral vector expressing shCon or shUSP11 at the indicated times post-sham or -UVB (20 mJ/cm^2) irradiation. The results were obtained from three independent experiments.

Catalytic activity of USP11 is essential to regulation of XPC deubiquitination and NER after UVB exposure

To determine whether the deubiquitinase activity of USP11 is necessary for USP11-mediated deubiquitination of XPC, we assessed the difference in XPC ubiquitination levels after UV damage between wild-type USP11- and C275/283S mutant (csmt) USP11-added 293T cells with USP11 genetic deletion (ΔUSP11). The C275/283S mutant USP11 is a catalytically inactive mutant of USP11. Expression of wild-type USP11 decreased XPC ubiquitination levels, whereas expression of csmt USP11 had little effect (Figure 8A). This indicates that the catalytic activity of USP11 is essential for USP11-mediated deubiquitination of XPC after UVB. Additionally, csmt USP11 expression in 293T ΔUSP11 cells showed decreased CPD repair after UVB

irradiation as compared with WT USP11-expressing cells (Figure 8B and 8C), indicating that the catalytic activity of USP11 is vital for its effect on CPD repair. These results indicate that the catalytic activity of USP11 is necessary to mediate XPC deubiquitination and to promote UV-induced DNA damage repair.

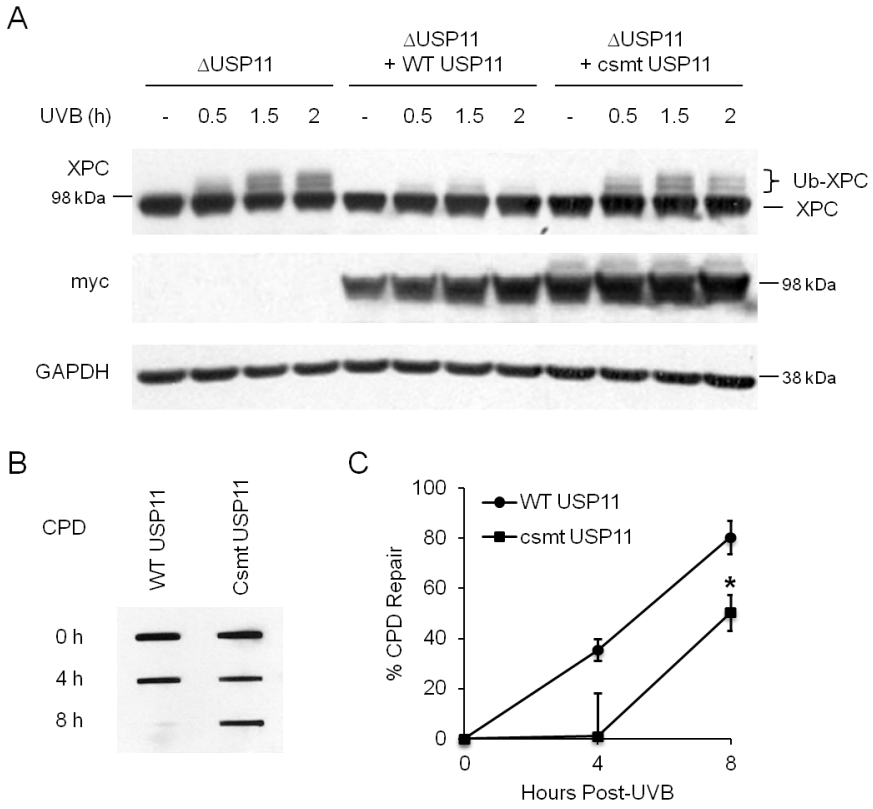

Figure 8: Catalytic activity of USP11 is essential to regulating XPC deubiquitination and NER after UVB exposure.

(A) Immunoblot analysis of XPC, myc, and GAPDH in 293T ΔUSP11 cells transfected with or without myc- tagged wild-type (WT) and catalytic mutant USP11 plasmids (csmt USP11) at the indicated times post-UVB (20 mJ/cm^2) or -sham irradiation. **(B)** Slot blot analysis of the CPD levels at indicated times after UVB (5 mJ/cm^2) in 293T ΔUSP11 cells transfected with WT USP11 or csmt inactive mutant USP11 plasmids. **(C)** Quantification of percentage (%) of CPD repair from (B). *, $P < 0.05$, compared with WT USP11 group, Student's *t*-test. The results were obtained from three independent experiments.

USP11 knockdown leads to premature dissociation of XPC from DNA damage sites by VCP/p97

Next we determined whether USP11 affected XPC localization to the DNA damage site. We used a local UV radiation method, in which cells were exposed to UV radiation through a micropore filter leading to the formation of sub-nuclear localized DNA damage foci, and we evaluated colocalization of XPC to the CPD DNA damage foci. In both shCon and shUSP11 HaCaT cells, similar amounts of XPC colocalized with CPD damage foci at 15 min post-UV irradiation (Figure 9A and 9B). However, at a later time point (30 min) after UV exposure, shUSP11 cells showed significantly reduced colocalization of XPC with CPD foci as compared with shCon cells. These results indicate that USP11 knockdown mediates premature dissociation of XPC from the DNA damage site, while it does not affect XPC recruitment to the damage site. To determine the mechanism by which USP11 affects XPC dissociation from the damage site, we asked whether VCP/p97 might play a role, since VCP/p97 had been found to mediate XPC removal from DNA damage sites [224]. We determined the difference in XPC localization to CPD damage foci between HaCaT shCon and shUSP11 cells with or without a potent and specific inhibitor for VCP, NMS-873 [225]. We found that NMS-873 pretreatment inhibited the effect of USP11 deficiency on premature dissociation of XPC from the DNA damage site (Figure 9C and 9D). These findings demonstrate that USP11 promotes proper retention of XPC at the DNA damage site by preventing VCP/p97-dependent XPC removal. To determine whether the effect of USP11 on XPC retention affects the downstream NER pathway, we assessed the impact of USP11 on recruitment of XPB to the damage site. shUSP11 HaCaT cells showed significantly reduced colocalization of XPB with CPD foci as compared with shCon cells at 30 min post-UV irradiation (Figure 9E and 9F). These results indicate that USP11 knockdown decreases recruitment of XPB to the DNA damage site.

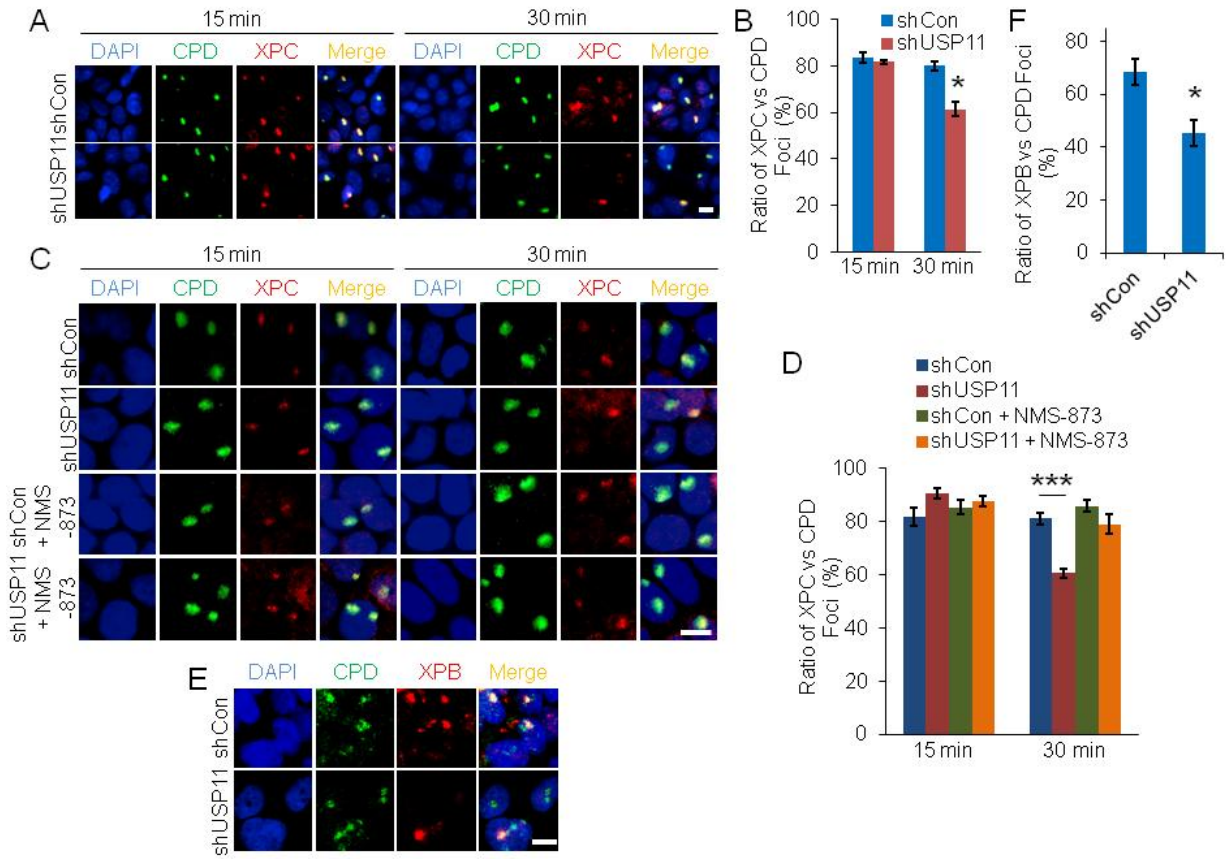

Figure 9: USP11 knockdown leads to premature dissociation of XPC from the DNA damage sites by VCP/p97.

(A, C) Immunofluorescence assay of the colocalization of XPC with sub nuclear CPD in HaCaT cells stably infected with a lentiviral vector expressing shCon or shUSP11 at 15 min, 30 min post-UV (10 mJ/cm^2) through a 5 μm micropore filter (A) or cells pretreated with NMS-873 (10μM) or vehicle for 1 hour prior to UV exposure (C). Scale bar, 10 μm. **(B, D)** The ratio of XPC to CPD foci (B) from (A), and (D) from (C) were calculated by analyzing 100 foci for merged fluorescent signals of XPC and CPD foci. *, $P < 0.05$, compared with shCon group, Student's *t*-test. **(E)** Immunofluorescence assay of the colocalization of XPB with sub nuclear CPD in HaCaT cells stably infected with a lentiviral vector expressing shCon or shUSP11 at 30 min post-UV (10 mJ/cm^2) through a 5 μm micropore filter. Scale bar, 10 μm. **(F)** The ratio of XPB to CPD foci from (E) was calculated by analyzing 100 foci for merged fluorescent signals of XPB and CPD foci. *, $P < 0.05$, compared with shCon group, Student's *t*-test. The results were obtained from three independent experiments.

UVB induces USP11 recruitment to the chromatin

To determine the regulation of USP11 by UV irradiation, we first examined the effect of UV on USP11 protein levels and stability. In HaCaT cells, USP11 levels did not change after UV exposure, nor did they increase with MG132 treatment, indicating that UV does not regulate USP11 levels or stability (Figure 10A). Similarly, USP11 levels did not change after UV exposure in NHEK cells, confirming that UV does not regulate USP11 protein levels (Figure 10B). Another mechanism by which UV could regulate USP11 is by affecting its localization. USP11 is mainly localized in the nucleus, and UVB irradiation had no effect on USP11 localization in the nucleus (Figure 10C). However, UV exposure increased USP11 protein levels in the chromatin-bound protein fraction, indicating that UV irradiation induces USP11 recruitment to the chromatin in parallel with XPC ubiquitination in response to UV-induced DNA damage (Figure 10D).

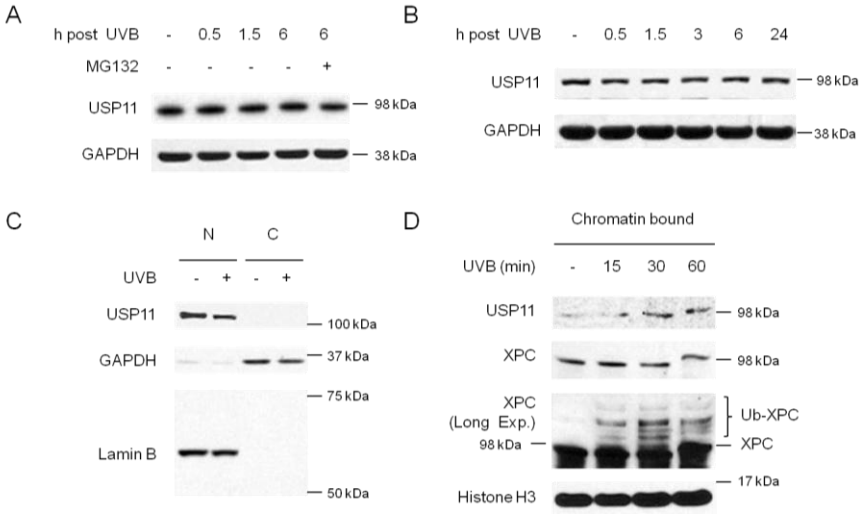

Figure 10: UVB induces USP11 recruitment to the chromatin.
(A, B) Immunoblot analysis of USP11 and GAPDH in HaCaT cells (A), and normal human epidermal keratinocyte (NHEK) cells (B) with or without MG132 treatment (10 μM) 1 h prior to UVB (20 mJ/cm^2). **(C)** Immunoblot analysis of USP11, GAPDH, and Lamin B using nuclear [N] and cytoplasmic [C] fractions from HaCaT cells with or without UVB (20 mJ/cm^2, 30 min). **(D)** Immunoblot analysis of USP11, Histone 3, and XPC using chromatin bound protein fractions from HaCaT cells over a time course post-UVB (20 mJ/cm^2). The results were obtained from three independent experiments.

UVB induces physical interaction of USP11 with XPC dependent on XPC ubiquitination levels

To determine whether UV regulates USP11 interaction with XPC, we performed co-IP for USP11 and immunoblotted for XPC. We found that USP11 and XPC indeed interacted, and that UV irradiation increased USP11-XPC interaction (Figure 11A). To determine whether UV-induced XPC ubiquitination regulates USP11 interaction with XPC, we determined the effect of DDB1 knockdown on USP11-XPC interaction, since DDB1 is a critical protein in the UV-DDB complex that mediates UV-induced XPC ubiquitination [226]. DDB1 knockdown reduced the UV-induced XPC ubiquitination levels and inhibited USP11-XPC interaction as compared with control cells (Figure 11B). These results demonstrate that XPC ubiquitination levels are critical for UV-induced interaction of USP11 with XPC. We also found that USP11 did not interact with other NER factors or chromatin factors (data not shown), suggesting that USP11 acts *via* its interaction with XPC in the NER process.

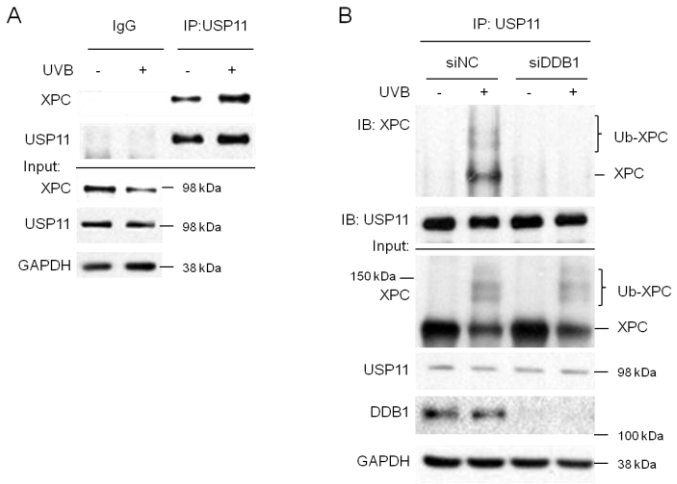

Figure 11: UVB induces USP11 interaction with XPC dependent on XPC ubiquitination levels.

(A) Immunoblot analysis of XPC, USP11, and GAPDH following immunoprecipitation using control species matched IgG and anti-USP11 antibody in HaCaT cells treated with or without UVB (20 mJ/cm^2, 1.5 h). **(B)** Immunoblot analysis of XPC, USP11, DDB1, and GAPDH in total cell lysates (input) or following immunoprecipitation using anti-USP11 antibody in HaCaT cells transfected with siRNA targeting DDB1 (siDDB1) or non-targeting control siRNA (siNC), and then treated with or without UVB (20 mJ/cm^2, 1 h). The results were obtained from three independent experiments.

USP11 is down-regulated in mouse skin with chronic UV exposure, and in human and mouse skin tumors

To determine the regulation of USP11 by UV exposure and in UV-induced skin cancer, we evaluated the protein levels of USP11 by immunohistochemical staining in skin tissue from sham-irradiated and chronic UVB-irradiated mice (n = 9). We found that USP11 levels were high (score 2 or 3) in all sham-irradiated skin tissue (9/9), in ~45% of the chronic UV-irradiated non-tumor tissue (4/9), and in none of the chronic UV-irradiated tumor tissue (0/9) (Figure 12A and 12B). The differences in USP11 levels among these tissues were found to be statistically significant by the Mann-Whitney U test (p = 0.0006 for sham versus chronic UV non-tumor tissue, $p < 0.0001$ for sham versus chronic UV tumor tissue, and p = 0.0023 for chronic UV tumor versus chronic UV non-tumor tissue). These results indicate that USP11 is down-regulated in UV-irradiated skin and skin tumors, and implicate USP11 as a tumor suppressor in skin cancer.

To determine the role of USP11 in human skin cancer, we evaluated the protein levels of USP11 by immunohistochemical staining in normal human skin tissue (Normal, n = 21), actinic keratosis (AK, pre-malignant, n = 15), and squamous cell carcinoma (SCC, malignant, n = 16). We found that USP11 levels were high (score 2 or 3) in ~90% of normal skin tissue (19/21), in ~46% of AK samples (7/15), and in none of the SCC tissues (0/16) (Figure 12C and 12D). The differences in USP11 levels among these tissues were found to be statistically significant by the Mann-Whitney U test ($p < 0.0001$ for Normal versus AK, $p < 0.0001$ for Normal versus SCC, and $p < 0.0001$ for SCC versus AK). These results indicate that USP11 is down-regulated in both AK and SCC as compared with normal skin, and suggest that USP11 acts as a tumor suppressor and that USP11 down-regulation is an early event in human skin cancer development.

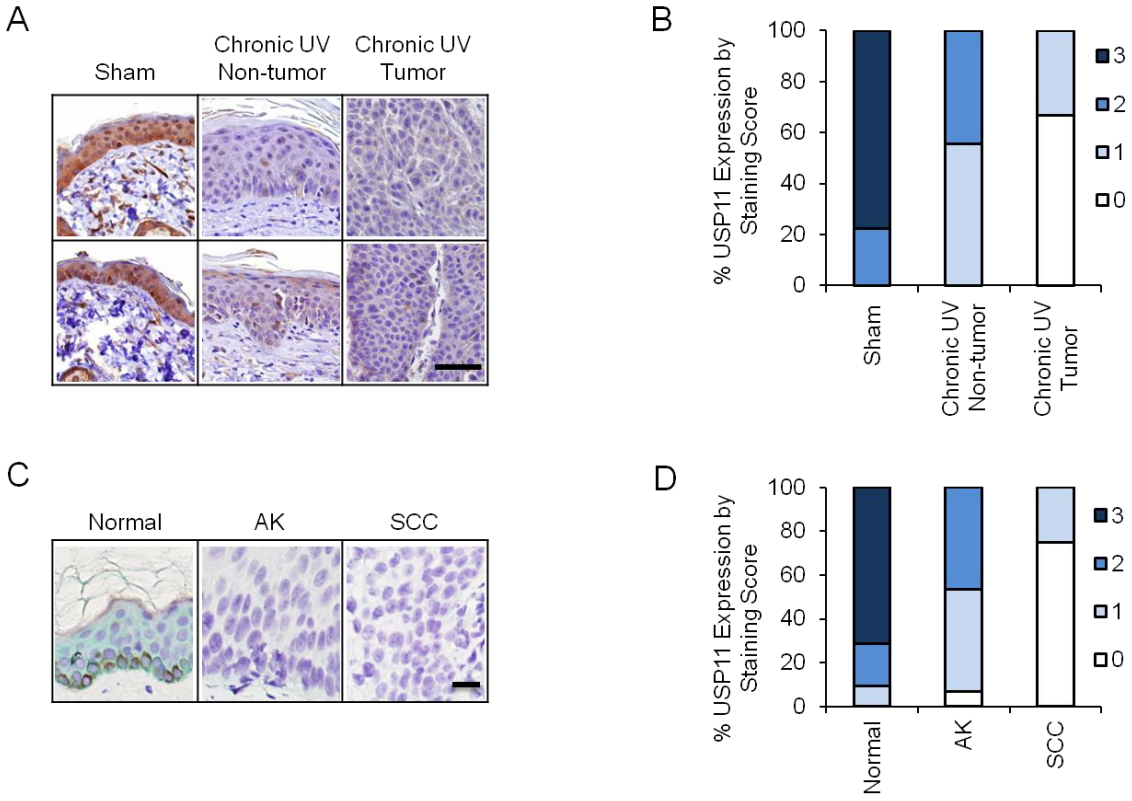

Figure 12: USP11 is down-regulated in mouse skin with chronic UV exposure, and in human and mouse skin tumors.

(A) Representative immunohistochemical analysis of USP11 protein levels (brown) in sham or chronic UVB-irradiated mouse skin with or without tumor. Scale bar, 50 μm. **(B)** Percentage of samples (in stacked column format) for each score of USP11 protein levels in chronic UVB-irradiated mouse skin (tumor and non-tumor) and sham-treated mouse skin (n = 9). **(C)** Representative immunohistochemical analysis of USP11 protein levels (green) in normal human skin (Normal), actinic keratosis (AK), and squamous cell carcinoma (SCC). Scale bar, 20 μm. **(D)** Percentage of samples (in stacked column format) for each score of USP11 protein levels in Normal (n = 21), AK (n = 15) and SCC (n = 16) human skin. The Mann–Whitney U test was used for statistical analysis (B, D).

Conclusion

In summary, we have identified USP11 as a novel post-translational regulator of the NER pathway (Figure 13). Upon UVB exposure, USP11 is recruited to the chromatin and binds to the ubiquitinated XPC. USP11 mediates XPC deubiquitination, thus preventing its premature removal from the damage site by VCP/p97, and promoting proper retention of XPC for its

efficient damage recognition function in NER. USP11 is down-regulated in mouse skin with chronic UVB irradiation and skin tumors from mice and humans. Our data indicate that USP11 is a positive regulator for NER, and suggest that USP11 acts as a tumor suppressor in UV-induced skin cancer.

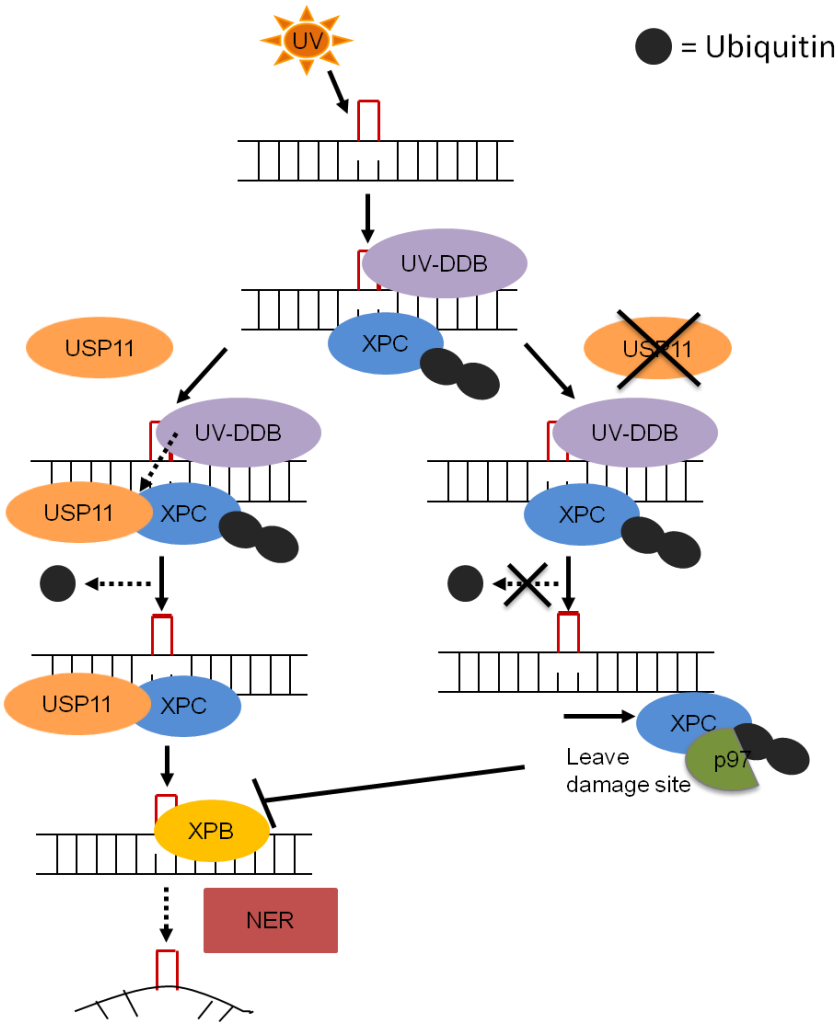

Figure 13: Schematic diagram of USP11 mediated regulation of XPC deubiquitination in nucleotide excision repair.

UVB induces USP11 recruitment to the chromatin and promotes the interaction of USP11 with ubiquitinated XPC. Then USP11 deubiquitinates XPC and promotes the proper association of XPC with the DNA damage site for positively regulating nucleotide excision repair.

CHAPTER 5: PHOSPHORYLATION OF XPC REGULATES ULTRAVIOLET-INDUCED DNA DAMAGE REPAIR

Summary

Nucleotide excision repair (NER) is the most versatile DNA repair system that removes bulky DNA damage induced by various endogenous and exogenous factors, including UV radiation. The function of NER factors, including xeroderma pigmentosum group C (XPC), can be regulated by post-translational modifications such as ubiquitination. However, the role of phosphorylation in XPC function remains unknown. Here, we show that phosphorylation of XPC acts as a novel post-translational regulatory mechanism of the NER pathway. We show that XPC is phosphorylated at serine 94. Moreover, after UVB irradiation, XPC phosphorylation regulates recruitment of ubiquitinated XPC and its downstream NER factors to the chromatin. In addition, upon evaluating the predicted kinases for XPC phosphorylation, we found that casein kinase II (CK2) promotes NER. Furthermore, CK2 kinase mediates XPC phosphorylation at serine 94, and also promotes recruitment of ubiquitinated XPC to the chromatin after UVB irradiation. Our findings have identified XPC phosphorylation as a novel mechanism for regulating NER following UV-induced DNA damage.

Introduction

XPC and other NER factors have been shown to be regulated by post-translational modifications [207]. XPC has been found to be ubiquitinated and sumoylated post-UV irradiation [68, 209, 227]. Ubiquitination of XPC regulates binding of XPC to the DNA damage site, and promotes the NER process [68, 209, 227]. XPC modification by SUMO-1 functions to increase the stability of XPC protein after UV exposure [209]. Another critical post-translational

modification that determines protein activity is phosphorylation. Modification of the phosphorylation state of XPC protein is likely to control its activity in NER. High throughput screening studies have identified various phosphorylation sites on XPC, namely serine (S) 61, 94, 397, 399, 883, 884, and 892 and threonine (T) 169 (Figure 14) [172, 228-230]. However, the function of XPC phosphorylation in NER has not yet been explored. Identifying phosphorylation as a novel regulator of XPC function and the kinase regulators of XPC phosphorylation could yield novel molecular targets to regulate NER and thus prevent skin cancer.

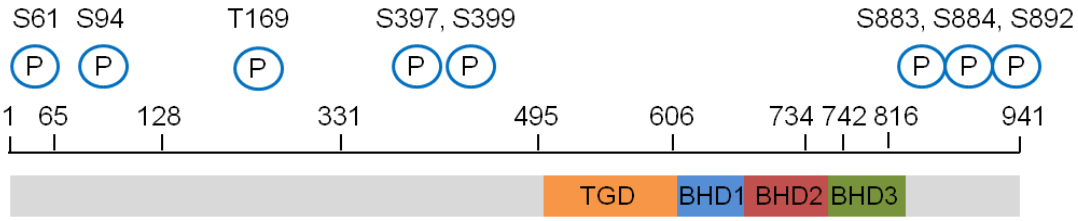

Figure 14: Schematic depicting potential phosphorylation sites on XPC protein.

One kinase that could possibly regulate XPC function is casein kinase II (CK2), a ubiquitous serine/threonine protein kinase [231, 232]. CK2 is a constitutively active kinase, which exists as a tetramer of two catalytic (α and/or α') and two regulatory (β) subunits [231, 232]. CK2 modulates various cellular processes such as the cell cycle, transcription, apoptosis and cell survival by phosphorylating numerous substrates [231, 232]. Multiple functions have associated CK2 with various diseases [231]. The positive impact of CK2 on cell survival and its upregulation in various cancers imply that CK2 plays an important role in promoting cancer [231]. CK2 kinase activity was also found to promote DNA double-strand break (DSB) repair by dissociating HP1-β from chromatin [233], by facilitating NBS1-MDC1 interaction [234, 235], and by promoting association of DNA-PKcs with DNA ends [236]. CK2 subunit alpha' (CK2A2) was found to interact with XPC by high-throughput affinity-purification mass spectrometry

analyses [237, 238]. However, the role of CK2 in regulating XPC phosphorylation and NER is unknown.

The objective of this study was to elucidate the role of XPC phosphorylation in the NER pathway, as well as to determine whether CK2 could be the upstream kinase regulating phosphorylation of XPC in NER.

Results

XPC phosphorylation at S61, T169, S397, S399, S883, and S884 does not affect UVB-induced DNA damage repair

To determine whether phosphorylation of XPC affects repair of UVB-induced DNA damage, we measured the difference in UV-induced DNA damage repair between wild-type (WT) XPC and dephosphomimetic mutant (Ser/Thr → Ala) XPC-expressing cells (Figure 15A). In XPC^{Null} cells, WT XPC expression significantly increased CPD repair compared to the vector control (Figure 15A-C). These results are consistent with the NER promoting function of XPC. Moreover, we only detected CPD in UVB treated and not in sham (no UV) controls, verifying the specificity of our CPD antibody (Figure 15D). Compared to WT XPC-expressing XPC^{Null} cells, S397A mutant XPC expression had no effect on CPD repair (Figure 15E), and neither did S399A, T169A, S883A, S884A or S61A XPC expression (Figure 15F-J).

Similar to CPD repair, in XPC^{Null} cells, WT XPC expression significantly increased 6-4PP repair compared to the vector control (Figure 16A-B). These results are consistent with the NER promoting function of XPC. The low dose of UV irradiation (20 mJ/cm^2) and culture conditions were selected to avoid significant effects on cell proliferation and apoptosis post-UV, which could potentially alter the DNA repair capacity (data not shown). Moreover, we only

detected 6-4PP in UVB treated and not in sham (no UV) controls, verifying the specificity of our 6-4PP antibody (Figure 16C). 6-4PP repair was also not affected by these six mutations compared to WT XPC (Figure 16D-H). Thus XPC phosphorylation at S61, T169, S397, S399, S883, and S884 does not affect UVB-induced DNA damage repair.

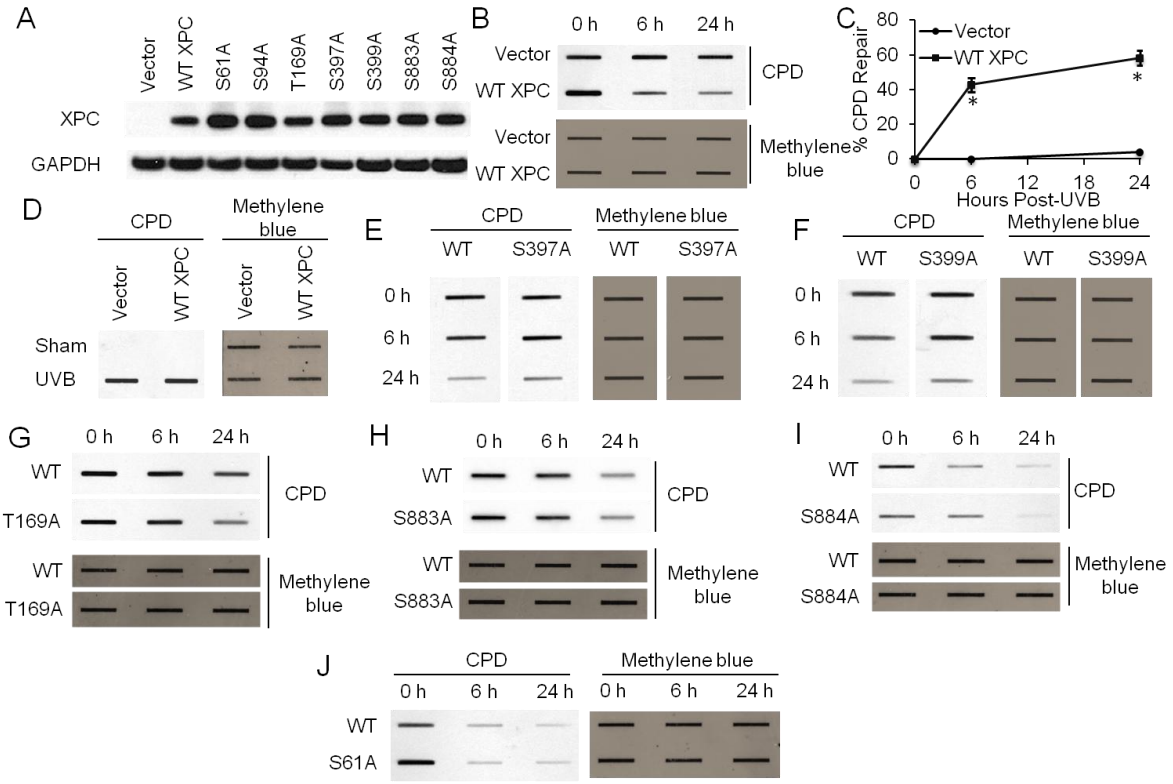

Figure 15. Role of XPC phosphorylation at S61, T169, S397, S399, S883, and S884 in CPD repair.

(A) Immunoblot analysis of XPC and GAPDH in XPC^{Null} cells expressing pLenti vector or pLenti-XPC (WT or Ser/Thr→ Ala mutant) constructs. **(B)** Slot blot analysis of the levels of CPD at the indicated times post-UVB (20 mJ/cm^2) in XPC^{Null} cells expressing pLenti vector or pLenti-WT XPC. Methylene blue staining was used for loading control. **(C)** Quantification of percentage (%) of CPD repair from (B). *, $P < 0.05$, compared with vector, Student's *t*-test. **(D)** Slot blot analysis of the levels of CPD in sham control and UVB (20 mJ/cm^2) irradiated XPC^{Null} cells expressing pLenti vector or pLenti-WT XPC. Methylene blue staining was used for loading control. **(E-J)** Slot blot analysis of the levels of CPD at the indicated times post-UVB (20 mJ/cm^2) in XPC^{Null} cells expressing pLenti-XPC WT and mutant constructs S397A (E), S399A (F), T169A (G), S883A (H), S884A (I), S61A (J). Methylene blue staining was used for loading control. The results were obtained from three independent experiments.

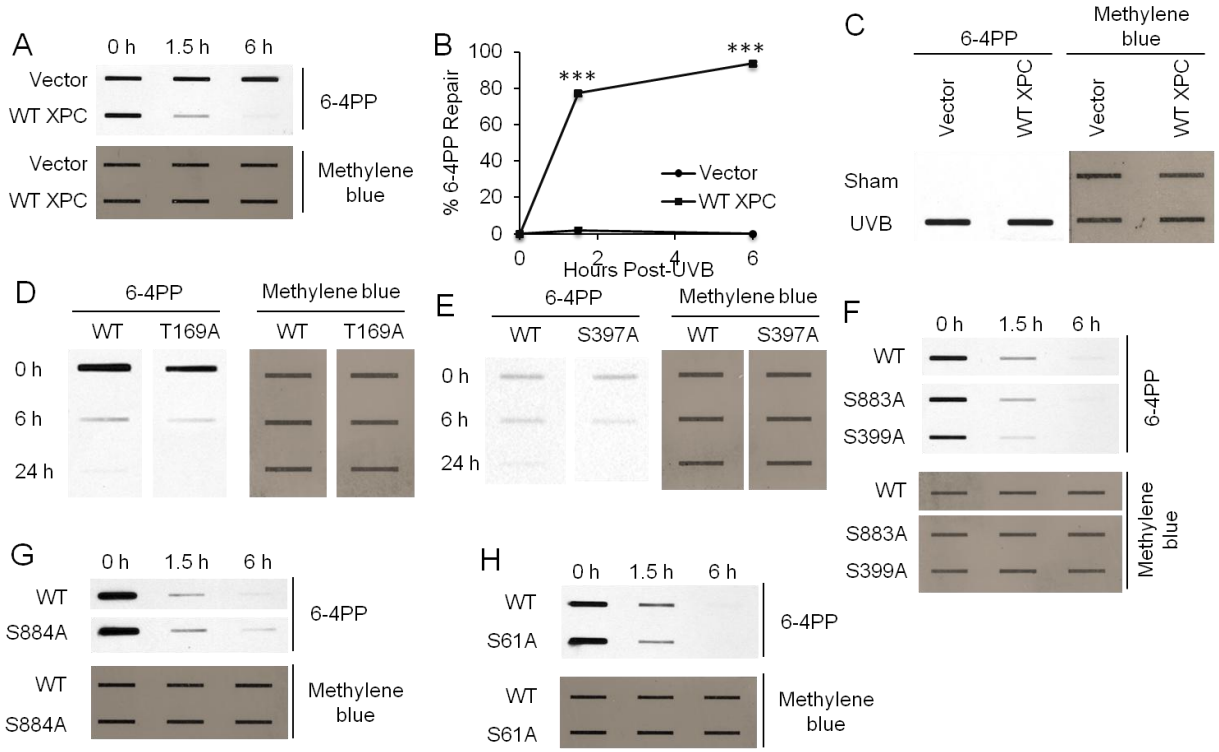

Figure 16. Role of XPC phosphorylation at S61, T169, S397, S399, S883, and S884 in 6-4PP repair.

(A) Slot blot analysis of the levels of 6-4PP at the indicated times post-UVB (20 mJ/cm^2) in XPC^{Null} cells expressing pLenti vector or pLenti-WT XPC. Methylene blue staining was used for loading control. **(B)** Quantification of percentage (%) of 6-4PP-repair from (A). ***, $P \leq 0.001$, compared with vector, Student's *t*-test. **(C)** Slot blot analysis of the levels of 6-4PP in sham control and UVB (20 mJ/cm^2) irradiated XPC^{Null} cells expressing pLenti vector or pLenti-WT XPC. Methylene blue staining was used for loading control. **(D-H)** Slot blot analysis of the levels of 6-4PP at the indicated times post-UVB (20 mJ/cm^2) in XPC^{Null} cells expressing pLenti-XPC WT and mutant constructs T169A (D), S397A (E), S883A or S399A (F), S884A (G), S61A (H). Methylene blue staining was used for loading control. The results were obtained from three independent experiments.

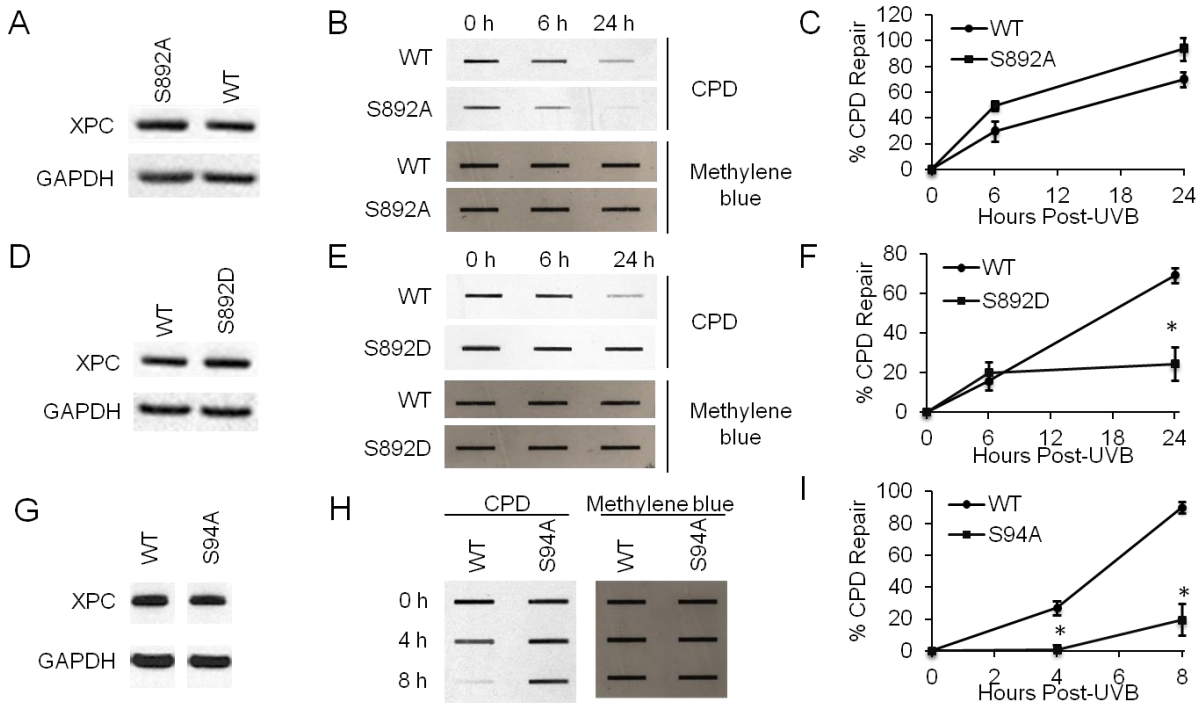

Figure 17. Role of XPC phosphorylation at S892 and S94 in CPD repair.
(A, D, G) Immunoblot analysis of XPC and GAPDH in XPC^{Null} cells expressing pLenti-XPC WT or mutant constructs S892A (A), S892D (D), S94A (G). **(B, E, H)** Slot blot analysis of the levels of CPD at the indicated times post-UVB (20 mJ/cm^2) in XPC^{Null} cells expressing pLenti-XPC WT or mutant constructs S892A (B), S892D (E), S94A (H). Methylene blue staining was used for loading control. **(C, F, I)** Quantification of percentage (%) of CPD repair (C) from (B), (F) from (E), and (I) from (H). *, $P < 0.05$, compared with WT, Student's *t*-test. The results were obtained from three independent experiments.

XPC phosphorylation at S892 and S94 regulate UVB-induced DNA damage repair

Next we determined the role of XPC phosphorylation at serine 892 (S892) and serine 94 (S94). In XPC^{Null} cells, S892A XPC expression did not have less CPD repair compared to WT XPC (Figure 17A-C), but S892D phosphomimetic XPC expression significantly decreased it (Figure 17D-F), as did S94A XPC (Figure 17G-I). Results were similar for 6-4PP repair: S892A XPC expression in XPC^{Null} cells did not have less repair compared to WT XPC (Figure 18A-B), while S892D XPC and S94A XPC significantly decreased it (Figure 18C-F). These results

indicate that XPC phosphorylation at S892 inhibits UVB-induced DNA damage repair, while phosphorylation at S94 promotes it.

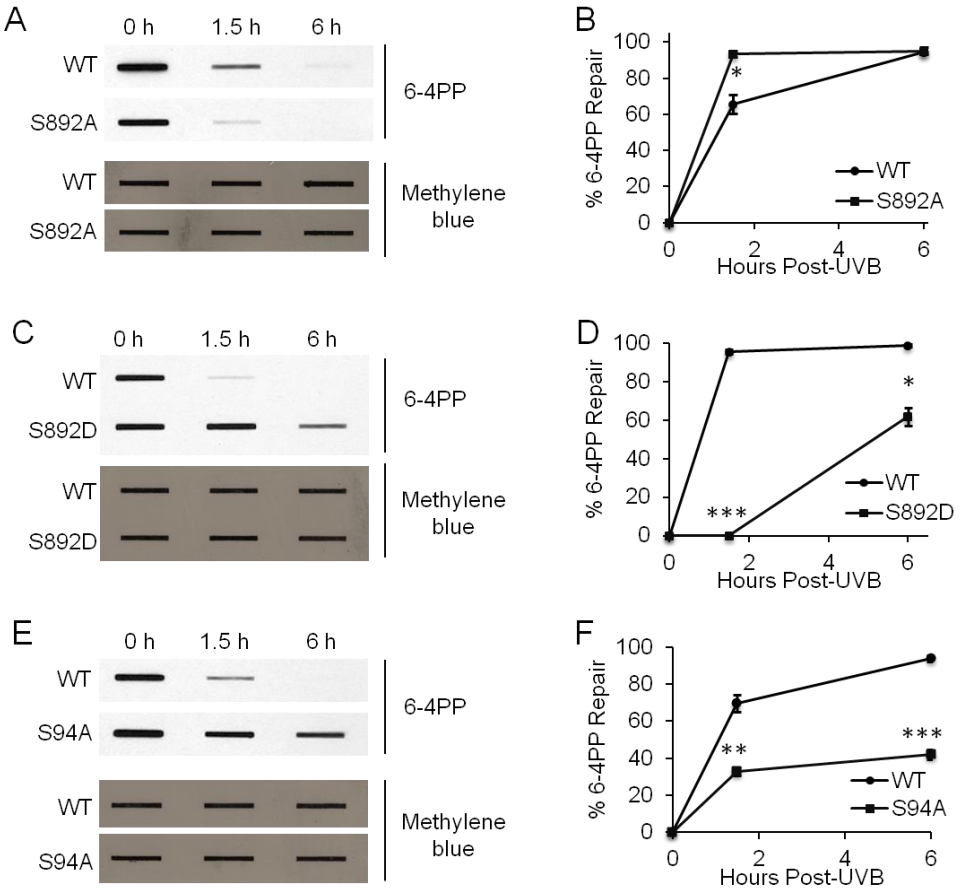

Figure 18. Role of XPC phosphorylation at S892 and S94 in 6-4PP repair.
(A, C, E) Slot blot analysis of the levels of 6-4PP at the indicated times post-UVB (20 mJ/cm^2) in XPC^{Null} cells expressing pLenti-XPC WT or mutant constructs S892A (A), S892D (C), S94A (E). Methylene blue staining was used for loading control. **(B, D, F)** Quantification of percentage (%) of 6-4PP repair (B) from (A), (D) from (C), and (F) from (E). *, $P < 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; compared with WT, Student's *t*-test. The results were obtained from three independent experiments.

XPC phosphorylation regulates recruitment of NER factors to the chromatin post-UVB irradiation

To determine the mechanism by which phosphorylation of XPC regulates NER, we first examined the effect of XPC phosphorylation on XPC levels. As compared with WT XPC, S94A

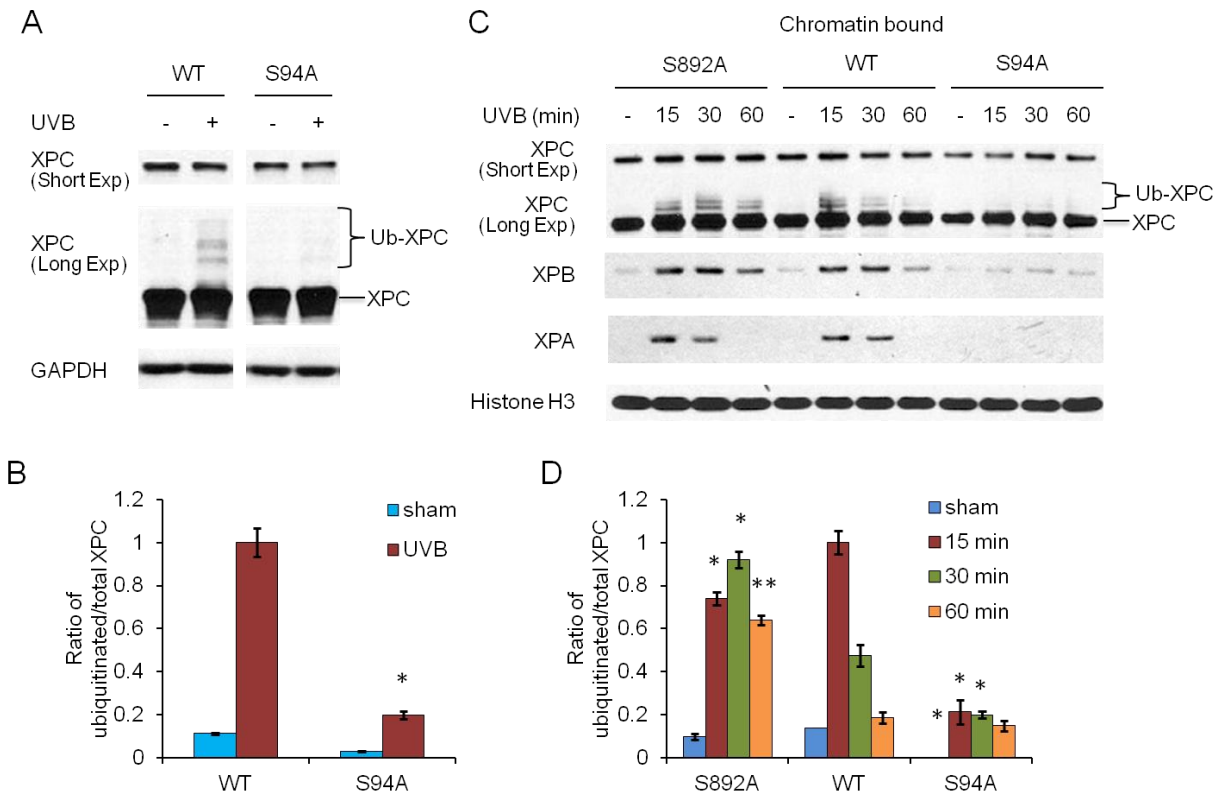

Figure 19. Role of XPC phosphorylation in recruitment of NER factors to the chromatin post-UVB irradiation.

(A) Immunoblot analysis of XPC and GAPDH 30 min after UVB exposure (20 mJ/cm^2) in XPC^{Null} cells expressing pLenti-XPC WT or mutant S94A. **(B)** Quantification of ratio of ubiquitinated/total XPC fraction from the Western blots in (A). *, $P < 0.05$, compared with WT, Student's *t*-test. **(C)** Immunoblot analysis of XPC, XPB, XPA, and Histone H3 using chromatin-bound protein fractions from XPC^{Null} cells expressing pLenti-XPC WT or mutant constructs S892A or S94A, at the indicated times post-UVB exposure (20 mJ/cm^2). **(D)** Quantification of ratio of ubiquitinated/total XPC fraction from the Western blots in (C). *, $P < 0.05$, **, $P \leq 0.01$; compared with WT, Student's *t*-test. The results were obtained from three independent experiments.

XPC showed similar levels of XPC prior to and after UVB exposure (Figure 19A). However, as compared with WT XPC, S94A XPC decreased ubiquitinated XPC levels after UV damage (Figure 19A-B). Since ubiquitination of XPC has been shown to regulate XPC binding to the DNA damage [68], we determined the effect of XPC phosphorylation on XPC binding to the damaged DNA. As compared with WT XPC-expressing XPC^{Null} cells, S892A XPC increased

ubiquitinated XPC levels bound to the chromatin at 30 and 60 min after UV damage (Figure 19C-D). However, at 15 min after UV exposure, S892A XPC decreased ubiquitinated XPC levels bound to the chromatin as compared with WT XPC-expressing XPC^{Null} cells, suggesting a delay and prolonging of the ubiquitinated XPC binding to chromatin with the S892A mutation. In contrast, compared to WT XPC, S94A XPC expression decreased ubiquitinated XPC levels bound to the chromatin at 15 and 30 min after UV damage (Figure 19C-D). The completion of transient upregulation of XPC ubiquitination at the longer time of 60 min after UV exposure may explain the little difference in chromatin bound ubiquitinated XPC levels between S94A and WT XPC at that time. Furthermore, as compared with WT XPC, S892A XPC had little effect on the XPB and XPA levels bound to the chromatin after UV exposure, while S94A XPC decreased them (Figure 19C). These findings demonstrate that XPC phosphorylation at S94 promotes ubiquitinated XPC recruitment and recruitment of the downstream NER factors to the chromatin after UV damage.

XPC phosphorylation does not affect either cell proliferation or UVB-induced apoptosis

Next we determined the cellular function of XPC phosphorylation at S94 and S892. Compared to WT XPC, S892A and S94A XPC had little effect on cell proliferation in XPC^{Null} cells (Figure 20A). S94A XPC had little effect on cell apoptosis compared to WT XPC post UVB-irradiation (Figure 20B). S892A XPC also had little effect on cell apoptosis compared to WT XPC (Figure 20C). These results indicate that XPC phosphorylation at S94 and S892 has no effect on cell proliferation and UVB-induced apoptosis.

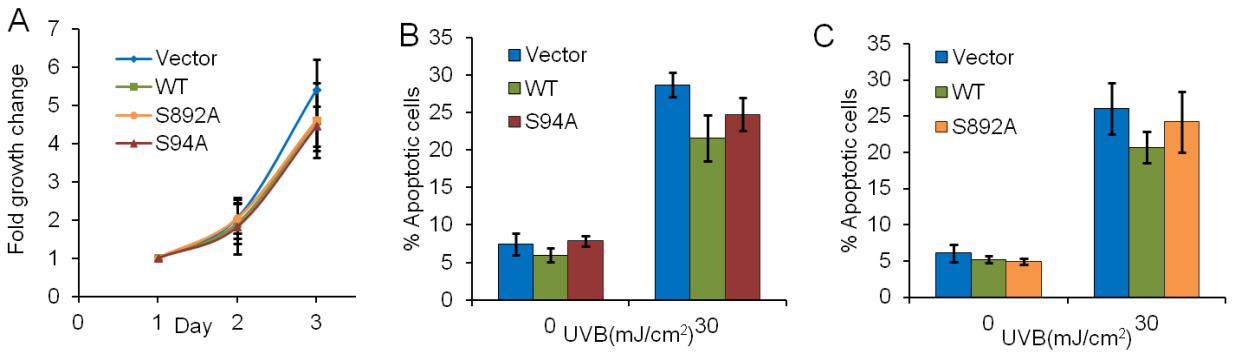

Figure 20. Role of XPC phosphorylation in cell proliferation and UVB-induced apoptosis. **(A)** MTS cell proliferation assay of XPC^{Null} cells expressing pLenti-vector, pLenti-XPC WT or mutant constructs S892A, S94A. **(B, C)** Propidium iodide assay followed by flow cytometric analysis of apoptosis at 24 h post-UVB (30 mJ/cm^2) or post-sham in XPC^{Null} cells expressing pLenti-vector, pLenti-XPC WT and mutant construct S94A (B), or S892A (C). The results were obtained from three independent experiments.

Inhibition of CK2 kinase decreases XPC phosphorylation at S94

To determine whether XPC is phosphorylated at S94 in cellular models, we utilized antibodies specific for XPC phosphorylation at S94. We found that XPC is phosphorylated at the S94 site in WT XPC-expressing XPC^{Null} cells under basal conditions as well as after UV exposure (Figure 21A). To determine the upstream kinase regulating XPC phosphorylation at S94, we used pharmacological small molecule inhibitors to screen the role of various candidate kinases in XPC phosphorylation. The kinases selected were either predicted to phosphorylate XPC or previously identified to play a role in UV response, including CK2, mTOR, ATM, PI3K, DNA-PK, JNK, p38, ERK, and ROCK1 [172, 239-244]. XPC phosphorylation at S94 was not affected by the ATM kinase inhibitor KU-60019, the PI3K inhibitor LY294002, the DNA-PK inhibitor NU7441, or the JNK inhibitor TCS JNK 60 (Figure 21B). Interestingly, XPC phosphorylation at S94 was increased following the treatment with the mTOR inhibitor rapamycin, the p38 inhibitor SB203580, the ERK inhibitor PD98059 or the ROCK1 inhibitor Y27632 (Figure 21B), which requires future investigation to elucidate the mechanism. However,

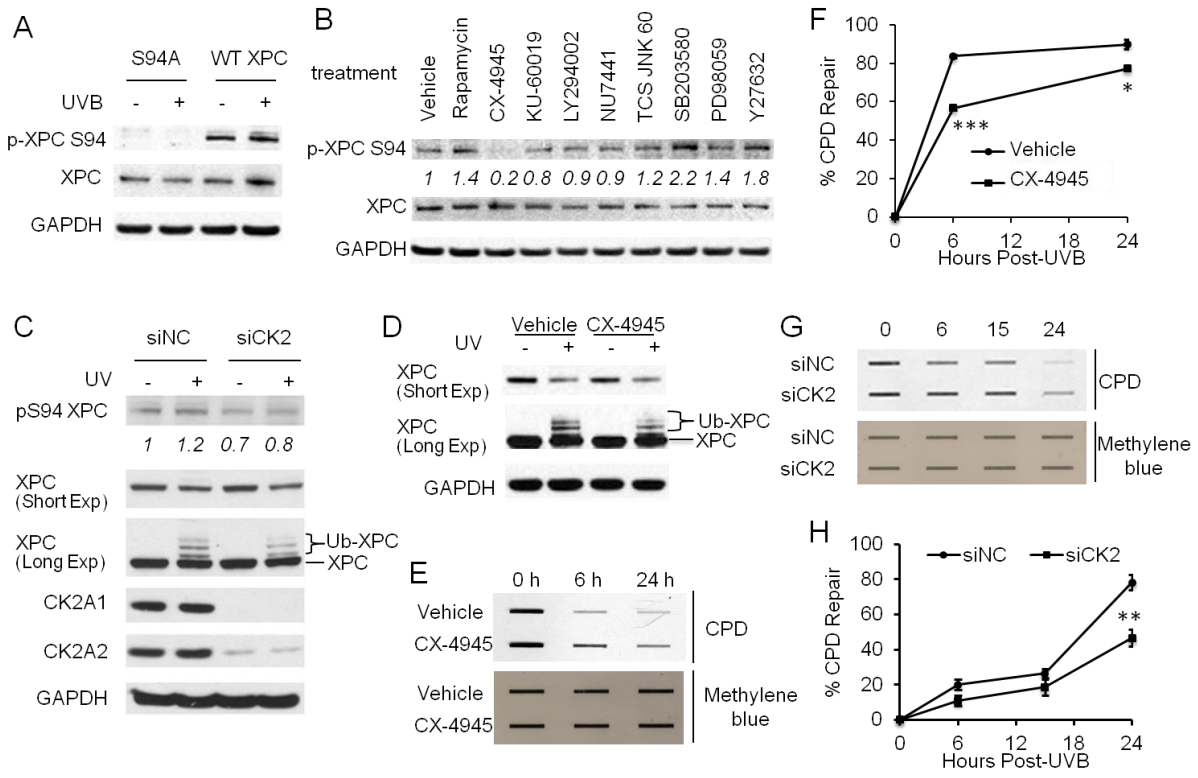

Figure 21. Role of CK2 kinase in XPC phosphorylation and CPD repair.

(A) Immunoblot analysis of XPC phosphorylated at S94 (p-XPC S94), total XPC, and GAPDH 30 min after UVB exposure (20 mJ/cm^2) in XPC^{Null} cells expressing pLenti-XPC WT or mutant S94A. **(B)** HaCaT cells were treated with vehicle, rapamycin (25 nM), CX-4945 (5 μM), KU-60019 (1 μM), LY294002 (10 μM), NU7441 (1 μM), TCS JNK 60 (10 μM), SB203580 (10 μM), PD98059 (20 μM), or Y27632 (10 μM) for 1 hour. The levels of XPC phosphorylated at S94 (p-XPC S94), total XPC and GAPDH were analyzed by immunoblot assay. **(C)** Immunoblot analysis of XPC phosphorylated at S94 (p-XPC S94), total XPC, CK2A1, CK2A2, and GAPDH 30 min after UVB exposure (20 mJ/cm^2) in HaCaT cells transfected with siRNA targeting CK2A1 and CK2A2 (siCK2) or non-targeting control siRNA (siNC). **(D)** HaCaT cells were pretreated with vehicle or CK2 inhibitor CX-4945 (5μM) for 1 h, exposed to UVB (20 mJ/cm^2), and incubated for 30 min. The levels of XPC and GAPDH were analyzed by immunoblot assay. **(E)** Slot blot analysis of the levels of CPD at the indicated times post-UVB (20 mJ/cm^2) in HaCaT cells pretreated with vehicle or CX-4945 (5 μM) for 1 h. Methylene blue staining was used for loading control. **(F)** Quantification of percentage (%) of CPD repair from (E). *, $P < 0.05$; ***, $P \leq 0.001$; compared with the vehicle group, Student's *t*-test. **(G)** Slot blot analysis of the levels of CPD at the indicated times post-UVB (20 mJ/cm^2) in HaCaT cells transfected with siRNA targeting CK2A1 and CK2A2 (siCK2) or non-targeting control siRNA (siNC). Methylene blue staining was used for loading control. **(H)** Quantification of percentage (%) of CPD repair from (G). **, $P < 0.01$; compared with the siNC group, Student's *t*-test. The results were obtained from three independent experiments.

treatment of HaCaT cells with CX-4945, a potent and specific inhibitor of CK2 [245], decreased levels of phosphorylated XPC at S94, while it did not affect the total XPC protein level (Figure 21B). Similarly, knockdown of CK2 kinase in HaCaT cells also decreased levels of phosphorylated XPC at S94 before and after UV exposure compared to negative control, while it did not affect the total XPC protein level (Figure 21C). These results suggest that CK2 kinase mediates XPC phosphorylation at S94 in keratinocytes.

Inhibition of CK2 kinase reduces NER capacity and CK2 recruitment is associated with ubiquitinated XPC binding to the chromatin after UVB exposure

Since XPC phosphorylation at S94 was found to affect XPC ubiquitination (Figure 19A-D) and CK2 is critical for S94 phosphorylation (Figure 21B-C), we assessed the effect of CK2 kinase on levels of ubiquitinated XPC. CX-4945 decreased the levels of ubiquitinated XPC post-UVB irradiation (Figure 21D). Knockdown of CK2 kinase using siRNA also decreased the levels of ubiquitinated XPC post-UVB irradiation (Figure 21C). To determine whether CK2 affects repair of UVB-induced DNA damage, we measured the difference in repair of UV-induced CPD between CX-4945 and vehicle treated HaCaT keratinocytes. CX-4945 significantly decreased CPD repair post-UVB irradiation (Figure 21E-F). Similarly, knockdown of CK2 kinase using siRNA significantly decreased CPD repair post-UVB irradiation (Figure 21G-H). Furthermore, although the total cellular levels of CK2A1, CK2A2, and CK2B were unaffected by UV exposure (Figure 22A), we found that CK2A2 and CK2B are recruited to the chromatin in HaCaT cells, and that UVB irradiation increased the levels of CK2A2 bound to the chromatin (Figure 22B). Additionally, CX-4945 treatment decreased the levels of ubiquitinated XPC bound to chromatin post-UVB irradiation, as well as inhibited UV-induced CK2A2 recruitment to the

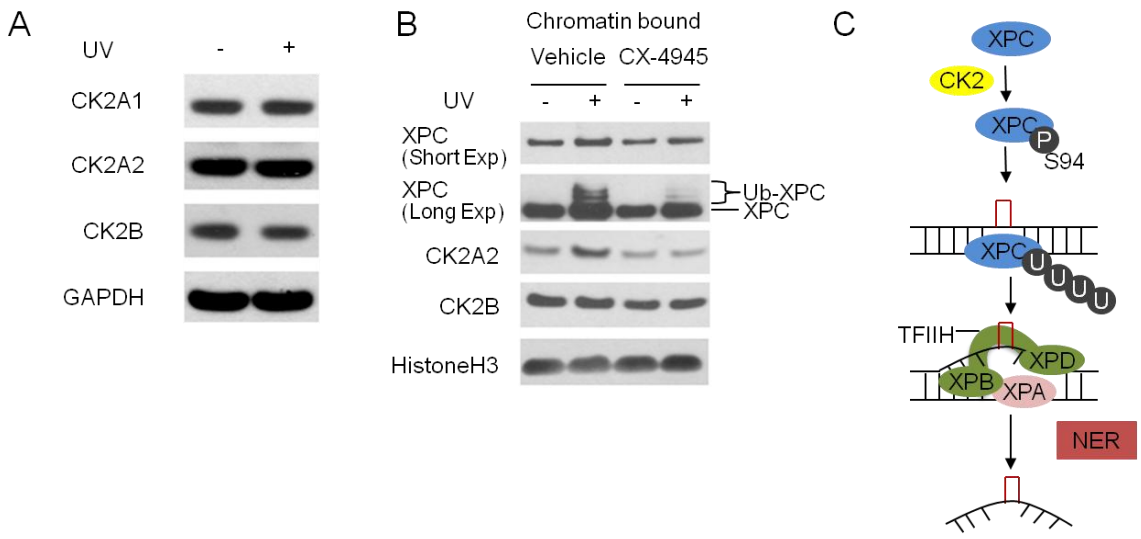

Figure 22. UVB regulation of CK2 levels and CK2 recruitment to the chromatin; and schematic of role of CK2 in XPC phosphorylation and NER.

(A) Immunoblot analysis of CK2A1, CK2A2, CK2B and GAPDH in HaCaT cells 30 min after UVB exposure (20 mJ/cm^2). **(B)** HaCaT cells were pretreated with vehicle or CK2 inhibitor CX-4945 (5μM) for 1 h, exposed to UVB (20 mJ/cm^2), and incubated for 30 min. The levels of XPC, CK2A2, CK2B and Histone H3 were analyzed by immunoblot assay in the chromatin-bound protein fractions. The results were obtained from three independent experiments. **(C)** Schematic diagram of the role of XPC phosphorylation in nucleotide excision repair. XPC is phosphorylated at S94 under basal conditions and after UV exposure through CK2 kinase. Further, XPC phosphorylation at S94 promotes binding of ubiquitinated XPC and downstream NER factors XPB and XPA to the damaged chromatin. This provides a molecular mechanism for S94 mediated XPC phosphorylation to promote its GG-NER repair capacity. Thus XPC phosphorylation is a previously unrecognized regulatory mechanism of XPC function in the GG-NER process.

chromatin (Figure 22B).Our findings demonstrate that inhibition of CK2 reduces UVB-induced DNA damage repair, and that CK2A2 recruitment to the chromatin following UVB damage coincides with ubiquitinated XPC binding to the chromatin, suggesting a critical role of CK2 in NER.

Conclusion

In summary, we found that XPC phosphorylation at S94 regulates the NER pathway (Figure 22C). XPC is phosphorylated at S94 under basal conditions and after UV exposure

through CK2 kinase. Further, XPC phosphorylation at S94 promotes binding of ubiquitinated XPC and downstream NER factors XPB and XPA to the damaged chromatin. This provides a molecular mechanism for S94 mediated XPC phosphorylation to promote its GG-NER repair capacity. Thus XPC phosphorylation is a novel and previously unrecognized regulatory mechanism of XPC function in the GG-NER process.

CHAPTER 6: DISCUSSION AND FUTURE DIRECTIONS

Regulation of XPC deubiquitination by USP11 in repair of UV-induced DNA damage

USP11 functions in various pathways and biological processes including TGFβ signaling, pro-inflammatory signaling, viral replication, and NF-κB signaling as well as DNA double-strand break repair [213-221]. However the role of USP11 in UV-induced DNA damage repair is unknown. We have identified a novel function of USP11 in UV damage repair. We found that USP11 positively regulates the NER process. At the molecular level, USP11 regulates deubiquitination of XPC and its retention at the DNA damage site following UV damage. Since CPD, and not 6-4PP, is responsible for UV-induced skin carcinogenesis, the positive regulation of CPD repair by USP11 suggests a tumor suppressor role of USP11 in skin cancer [223]. Furthermore, USP11 is down-regulated in mouse skin with chronic UVB irradiation and skin tumors from mice and humans. Our findings demonstrate a crucial role of USP11 in UV-induced DNA damage repair and suggest USP11 as a tumor suppressor in skin cancer. These insights into the mechanism of USP11 action on XPC deubiquitination and NER suggest that USP11 could be a promising target for treatment of skin cancer. Moreover, the mechanisms delineated here are also relevant to other NER-associated cancers, such as lung and brain cancers [199, 203, 205]

Deubiquitinase activity of USP11 in relation to other deubiquitinases for XPC

Ubiquitination of XPC has a significant impact on XPC's function in NER [68, 69, 208, 209]. Recently USP7 has been identified as a deubiquitinase for XPC, preventing XPC degradation and promoting the NER process [74]. Here we identify another deubiquitinase, USP11, which can deubiquitinate XPC at the chromatin after UV damage. Furthermore, we show that the catalytic activity of USP11 is essential to regulating XPC deubiquitination in the NER

process. Since the inhibition of USP11 and USP7 individually has been found to regulate XPC deubiquitination, USP7 and USP11 are non-redundant for regulating XPC deubiquitination. However, as USP7 and USP11 have been found to interact with the same Polycomb complex components [246], it is possible that USP7 and USP11 may interact to regulate NER. It is unclear yet whether they might act synergistically to regulate XPC deubiquitination in NER. Further studies are needed to elucidate whether USP7 and USP11 act synergistically, or which deubiquitinase plays a dominant role, to regulate XPC deubiquitination in NER. USP11 was found to preferentially act on K63 ubiquitin chain linkages [247]: it would be interesting to elucidate the ubiquitin linkage type and the upstream ubiquitin ligase for USP11-mediated deubiquitination of XPC, as well as the amino acid sites on XPC being deubiquitinated by USP11.

Regulation of USP11 and XPC interaction in UV response

Our results also demonstrated that XPC ubiquitination levels regulate UVB-induced USP11-XPC interaction. Reduction in XPC-ubiquitination levels by DDB1 knockdown abolished UVB-induced USP11-XPC interaction. This is further supported by the association of USP11's recruitment to the chromatin with the parallel XPC ubiquitination. We also found that USP11 did not interact with other NER factors and chromatin factors that could affect NER capacity after UV exposure, suggesting that XPC is the downstream effector of USP11 in the NER process. Although a recent study suggested that USP11 levels may decrease post-UVC insult (50 mJ/cm^2) [220], we did not find any change in USP11 levels after UVB damage (20 mJ/cm^2). This might be due to the difference in the cellular model systems, U2OS cells [220]

versus keratinocytes in our study, or the different types or dose of UV radiation used. Future investigation will elucidate the specific response to UV radiation in different cell types.

Mechanism of USP11 activity to affect XPC function in NER

We found that USP11 promotes the damage recognition function of XPC in NER, by preventing premature dissociation of XPC from the DNA damage site. In addition to efficient XPC recruitment to the DNA damage site [248], proper XPC retention at the damage site is critical for efficient NER. Conversely, when XPC dissociation is delayed beyond the optimum time, it hinders access and recruitment of the downstream NER factors to the damage site and decreases NER capacity, underscoring the importance of the appropriate duration for XPC retention at the damage site [77, 224]. We also found that USP11 inhibition decreases XPB recruitment to the DNA damage site. Our results suggest that USP11 inhibition mediated premature dissociation of XPC from the damage site compromises XPC function to recruit downstream NER factors like XPB to the DNA damage site. We found that USP11 prevents premature dissociation of XPC from the damage site through inhibiting XPC removal by the ubiquitin-selective segregase VCP/p97. Since VCP/p97 mediates removal of ubiquitinated XPC from the DNA damage site to impact genomic stability [224], it is likely that deubiquitination of XPC by USP11 prevents VCP/p97 interaction with ubiquitinated XPC and subsequent removal of XPC from the damage site.

Role of USP11 in skin cancer

The role of USP11 in cancers is complex. USP11 acts as a tumor suppressor in lung adenocarcinoma and brain tumors [249, 250], but has tumor promoting characteristics in colon

cancer, melanoma, pancreatic cancer, and cervical cancer [251-255]. However, the significance of USP11 in skin cancer is unknown. We found that human and mouse skin tumors associated with UV damage show down-regulation of USP11, suggesting that USP11 acts as a tumor suppressor in skin cancer. Additionally, pre-cancerous AK in human skin and UVB-irradiated non-tumor mouse skin showed a decrease in USP11 protein levels in the epidermis as compared with normal skin. Our findings suggest that USP11 functions as a tumor suppressor in the early stages of skin carcinogenesis associated with UV exposure. The function of USP11 in promoting the NER process further supports the tumor suppressive role of USP11 in skin cancer. It remains unknown how chronic UV radiation down-regulates USP11. It is possible that chronic UV exposure alters the microenvironment of the skin, which can lead to USP11 down-regulation. It is also possible that UV exposure causes inactivating mutations in USP11, leading to down-regulation of USP11 or its activity. Future investigation will be needed to elucidate how chronic UV down-regulates USP11 levels. Such insights into mechanisms of USP11 down-regulation by UV exposure and those of promoting USP11activity could lead to translational strategies for prevention of skin carcinogenesis. Moreover, previous studies have indicated that NER could contribute to therapeutic resistance in cancer, especially with agents like cisplatin [256]. Consequently, USP11 inhibitors like mitoxantrone, and more specific USP11 inhibitors developed in the future, have the potential for cancer therapy in skin cancer and other cancers with NER involvement [254, 257].

Phosphorylation of XPC regulates ultraviolet-induced DNA damage repair

Post-translational modification of proteins by phosphorylation plays an important role in various biological functions such as metabolism, cell proliferation, cell-cycle control, cell

survival, and inflammation [258]. Several NER factors, such as XPA, RPA, XPB and RNA Polymerase II, were shown to be phosphorylated [207, 259-264]. Phosphorylation sites for NER factor XPC have been recently identified [172, 228-230]. However, the role of XPC phosphorylation in NER is unknown. We found that XPC phosphorylation at S94 and S892 regulates the NER pathway. At the molecular level, phosphorylation of XPC regulates recruitment of ubiquitinated XPC and its downstream NER factors to the chromatin following UV damage. We also show that XPC is phosphorylated at S94 in cellular models, both under basal conditions and after UV irradiation. Additionally, we found that CK2 kinase may play an important role in XPC phosphorylation at S94 in cellular models. Our results have identified XPC phosphorylation as a novel post-translational regulatory mechanism for UV-induced DNA damage repair. Our data indicate that XPC phosphorylation regulates XPC function in NER. The mechanisms delineated here are also applicable to prevention of cancers in the skin, lungs and brain, since defects in XPC in humans cause increased risk of these cancers [27].

XPC phosphorylation at S94 and S892 affect UVB-induced DNA damage repair

XPC was shown to be regulated post-translationally by various modifications such as ubiquitination and sumoylation [68, 209, 227]. Here we identify another post-translational modification, phosphorylation, as a regulator of XPC function in NER. Specifically, we found that XPC phosphorylation at S94 promoted CPD and 6-4PP repair. Even though XPC dephosphomimetic mutant S892A had little effect on GG-NER capacity, the phosphomimetic mutant S892D inhibited CPD and 6-4PP repair, indicating that prolonged phosphorylation at S892 negatively impacts GG-NER. Future studies are needed to explain the divergent response of dephosphomimetic and phosphomimetic mutants for S892 on repair capacity. However, it is

possible that transient phosphorylation of XPC at S892 may not impact repair capacity greatly in a process whose basis is sequential events, whereas prolonged phosphorylation is detrimental to DNA repair. We show that phosphorylation-site mutants for S94 and S892 individually regulate NER. Future studies will determine at which of the two sites phosphorylation is dominant, or how the two collectively participate to regulate XPC activity in NER. Interestingly, results for the S892D mutant suggest better CPD repair than 6-4PP repair. Compared to previous findings that partial correction of XPC results in better CPD repair, our data suggests that the S892D mutant behaves similar to partial correction of XPC activity, probably such that the activity specific for 6-4PP repair is compromised upon phosphorylation at S892 [265]. Moreover, we found little effect of XPC phosphorylation at S94 and S892 on cell proliferation and UVB-induced apoptosis, suggesting that regulation of XPC activity in NER is the primary function of XPC phosphorylation in the UV response. We show that XPC phosphorylation at S94 and S892 affect the repair of CPD, the major UVB DNA damage products causing skin cancer [20]. Future studies are needed to establish their role in skin carcinogenesis.

Physiological relevance of XPC phosphorylation at S94 in cellular models

Our findings indicate that XPC is phosphorylated at S94, under basal conditions and after UV exposure. A prior study showing that wild-type p53-induced phosphatase 1 (WIP1) inhibits NER suggested that WIP1 can dephosphorylate XPC at S892 and XPA at S196 [266]. However, the study only showed WIP1-mediated XPC dephosphorylation at S892 *in vitro* [266], and future studies will confirm phosphorylation at S892 in cellular models. The study left unexplored the effect of XPC phosphorylation on NER, as well as the dependence of WIP1 activity on XPC dephosphorylation for its effect on NER [266]. To our knowledge, ours is the first study

evaluating the role of XPC phosphorylation on NER, and showing that XPC is phosphorylated in cellular models under physiological conditions. Although XPC phosphorylation at S94 after UV exposure supports its function in the NER process, its role under basal conditions is so far unknown. Future investigations will determine whether XPC phosphorylation observed under basal conditions contributes to NER-independent functions of XPC, such as cell metabolism and oxidative DNA damage [267-269]. Future studies would also be needed to elucidate the physiological conditions and NER-independent functions of XPC phosphorylation at S61, T169, S397, S399, S883 and S884, in the skin or other tissues.

Mechanism of function of XPC phosphorylation in the NER pathway

We found that XPC phosphorylation regulates recruitment of NER factors to the chromatin post UVB-irradiation. Recognition of DNA damage and subsequent recruitment of downstream NER factors is the main function of XPC in NER [270]. Ubiquitination of XPC mediates recruitment of XPC to DNA damage sites and is critical for its DNA damage recognition function in NER [68, 248]. We have shown that XPC phosphorylation at S94 led to increased amounts of ubiquitinated XPC bound to the chromatin, respectively, after UV damage. These findings suggest that phosphorylation regulates XPC activity of damage recognition through ubiquitinated XPC recruitment in NER. It is possible that S94 phosphorylation of XPC may modify XPC structure and activity, and thus XPC interaction with the UV-DDB ubiquitin ligase complex, which in turn increases ubiquitinated XPC levels bound to the chromatin. We have also shown that XPC phosphorylation S94 led to increased amounts of XPB and XPA bound to the chromatin after UV damage. In contrast, even though S892A XPC increased ubiquitinated XPC levels bound to the chromatin, it had little effect on XPB and XPA

recruitment to the damaged chromatin. This effect coincides with the little difference in GG-NER capacity for the S892A mutant. These findings suggest that XPC phosphorylation could also regulate XPC activity of downstream NER factor recruitment to the chromatin. XPC phosphorylation may affect recruitment of downstream NER factors *via* affecting recruitment of ubiquitinated XPC to the damage site. It is also possible that XPC phosphorylation may directly affect recruitment of downstream NER factors. For example, the S892 site of XPC belongs to the XPB binding region of the protein, and phosphorylation at this site might directly affect XPC binding to XPB. Future studies will elucidate the specific mechanism by which phosphorylation of XPC affects recruitment of ubiquitinated XPC and downstream NER factors to the chromatin.

Role of CK2 kinase in XPC phosphorylation and NER

Our findings identify CK2 as the potential kinase mediating XPC phosphorylation at S94 both before and after UV irradiation in cellular models. Our data demonstrate that CK2 positively regulates CPD repair and UVB-induced ubiquitinated XPC levels, similar to regulation by XPC phosphorylation at S94. CK2 was previously shown to mediate XPB phosphorylation at S751, which inhibited TFIIH activity in NER [262]. However, our data did not recapitulate the negative regulation of NER by CK2 [262]. This may be due to a difference in the model systems in those studies (HeLa and fibroblasts) [262] compared to our system (HaCaT keratinocytes). CK2 was also found to phosphorylate centrin 2 leading to decreased interaction of centrin 2 with XPC [271]. Findings from other studies that centrin 2 interaction with XPC enhanced NER [272] suggest that it is likely that CK2-mediated phosphorylation of centrin 2 would inhibit NER efficiency. Future studies would be needed to elucidate the specific outcome of CK2-mediated centrin 2 phosphorylation on NER efficiency.

Although we cannot exclude the possible inhibitory effect of CK2 on NER *via* phosphorylation of centrin 2, the effect of CK2-mediated direct phosphorylation of XPC on NER would probably dominate, since XPC activity is more crucial to NER than centrin 2 [273]. Furthermore, we found that CK2A2 and CK2B were recruited to the chromatin, however we were unable to detect CK2A1 in the chromatin bound fraction. Thus we can infer that CK2A1 may not be recruited to the chromatin, suggesting that interaction with XPC and XPC phosphorylation is specifically mediated by the CK2A2 catalytic subunit. It is also possible that the strength of CK2A1 interaction with the chromatin is weaker and hence could not be detected in this assay. We also show that UV irradiation promoted CK2A2 recruitment to the chromatin. Since the total levels of CK2 proteins are unchanged after UV exposure, our results suggest that the recruitment of CK2A2 to the chromatin mediates CK2 function in the DNA repair process. Since CK2B recruitment to the chromatin was not affected by UV exposure, it is possible that CK2B may be associated with non-specific binding to the chromatin. Additional CK2A2 may be recruited or incorporated into the complex superficially in response to UV exposure, possibly by interaction with XPC or other repair proteins.

We also found that inhibition of CK2 activity decreased ubiquitinated XPC bound to the chromatin after UV exposure, and that UVB-induced recruitment of CK2 coincided with the recruitment of ubiquitinated XPC to the damaged chromatin (Figure 22B). Since CK2 inhibition also prevented UVB-induced CK2A2 recruitment (Figure 22B), our data suggests that UVB-induced recruitment of CK2A2 to the damaged chromatin mediates CK2 activity of promoting XPC ubiquitination and binding to damaged DNA. This supports our conclusion that XPC phosphorylation at S94 regulates ubiquitinated XPC recruitment to the UV-damaged chromatin. We were unable to detect XPC phosphorylation at S94 in the chromatin-bound protein fraction,

possibly due to the low abundance of the S94 phosphorylation or the low sensitivity of the antibody. This data agrees with our mechanistic model that phosphorylation of XPC occurs upstream of XPC ubiquitination and binding to UV-damaged chromatin. In addition, DNA-dependent protein kinase (DNA-PK), ataxia-telangiectasia mutated (ATM), and CK2 kinases are predicted to phosphorylate XPC at S892 [239]. Future studies would be needed to explore which specific kinase phosphorylates XPC at S892 under physiological conditions. Future studies may also explore the application of CK2 activators for promoting UV-induced DNA damage repair, consequently leading to improved prevention of skin aging and cancer.

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