XPC promotes MDM2-mediated degradation of the p53 tumor suppressor

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ABSTRACT **Although ubiquitin receptor Rad23 has been implicated in bringing ubiquitylated p53 to the proteasome, how Rad23 recognizes p53 remains unclear. We demonstrate that XPC, a Rad23-binding protein, regulates p53 turnover. p53 protein in XPC-deficient cells remains ubiquitylated, but its association with the proteasome is drastically reduced, indicating that XPC regulates a postubiquitylation event. Furthermore, we found that XPC participates in the MDM2-mediated p53 degradation pathway via direct interaction with MDM2. XPC W690S pathogenic mutant is specifically defective for MDM2 binding and p53 degradation. p53 is known to become stabilized following UV irradiation but can be rendered unstable by XPC overexpression, underscoring a critical role of XPC in p53 regulation. Elucidation of the proteolytic role of XPC in cancer cells will help to unravel the detailed mechanisms underlying the coordination of DNA repair and proteolysis.**

Monitoring Editor
Thomas Sommer
Max Delbrück Center for Molecular Medicine

Received: Jun 3, 2013
Revised: Nov 7, 2013
Accepted: Nov 12, 2013

INTRODUCTION

One early major discovery that propelled the ubiquitin (Ub)/proteasome system to the forefront in biological research is its tight control on the cellular concentration of p53 tumor suppressor, a master regulator of cell survival and death (Vousden and Prives, 2009; Wade *et al.*, 2010; Brooks and Gu, 2011). Over the years, many laboratories have elucidated the mechanisms of Ub ligase–mediated p53 ubiquitylation under various conditions. One pivotal regulatory mechanism is MDM2-mediated p53 degradation. Specifically, the MDM2 Ub ligase E3 binds to p53 and then covalently attaches Ub onto p53 with the help of E1 and E2 enzymes. Thus far, the studies on the MDM2-p53 circuit concentrated on the mechanisms underlying the MDM2-p53 interaction and MDM2-catalyzed Ub conjugation onto p53 (Wade *et al.*, 2010; Brooks and Gu, 2011). How the Ub-tagged p53 is then transferred to the proteasome remains unclear.

Rad23 can directly bind Ub and the proteasome and has been shown to act as an adaptor that shuttles the ubiquitylated proteins to the proteasome (Finley, 2009). Rad23 is required for the degradation of a subset of substrates, including p53 (Glockzin *et al.*, 2003; Dantuma *et al.*, 2009). Because multiple Rad23-like adaptor molecules (e.g., Dsk2, Ddi1) exist and have distinct substrate specificity in vivo, an important unresolved issue is how Rad23 recognizes specific targets (Finley, 2009; Liu and Walters, 2010). One possible helper of Rad23 is its binding protein XPC, a key factor for a conserved DNA repair pathway termed nucleotide excision repair (NER; Friedberg *et al.*, 2006; Sugasawa, 2008). Mutations in XPC can cause xeroderma pigmentosum (XP), a genetic disease that often leads to skin cancer, and are also associated with increased risks for Hodgkin's disease and other malignancies, including cancer of the lung, bladder, prostate, and pancreas (Hollander *et al.*, 2005; Friedberg *et al.*, 2006; Hirata *et al.*, 2007; El-Zein *et al.*, 2009). Not all the phenotypes associated with XPC patients can be easily explained by DNA repair defects, suggesting that XPC has an important non-NER activity. Consistent with this conjecture, the Lys939Gln allele of XPC is associated with a high risk of cancers, yet wild-type NER activity (Khan *et al.*, 2000; Friedberg *et al.*, 2006; Hirata *et al.*, 2007; El-Zein *et al.*, 2009).

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E13-05-0293) on November 20, 2013.

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Abbreviations used: FBS, fetal bovine serum; GFP, green fluorescent protein; GST, glutathione *S*-transferase; NER, nucleotide excision repair; RNAi, RNA interference; Ub, ubiquitin; XP, xeroderma pigmentosum.

The proteolytic involvement of Rad23 prompted us to examine whether XPC and its yeast counterpart Rad4 are involved in proteolysis. We demonstrated that yeast Rad4 also participates in the Rad23-mediated degradation pathway, as the function of Rad23 requires an intact Rad4-binding XPCB motif and these two proteins share common substrates (Li *et al.*, 2010). However, no physiological XPC degradation substrate is known in mammalian cells. In this paper, we demonstrate that XPC is specifically involved in the Ub ligase MDM2-mediated p53 degradation pathway. Moreover, pathogenic XPC mutation disrupts its MDM2 binding and impairs p53 degradation, suggesting that XPC may work with MDM2 to bring p53 to the proteasome. p53 is a central regulator of the DNA damage response that triggers an array of cellular events to restore genomic integrity, including transcription induction, cell cycle arrest, and apoptosis (Wade *et al.*, 2010; Brooks and Gu, 2011). p53 regulates XPC transcription (Adimoolam and Ford, 2002; Sengupta and Harris, 2005), and our results for XPC-mediated p53 degradation suggest a negative-feedback loop reminiscent of the feedback control of p53 by MDM2 (Vousden and Prives, 2009; Brooks and Gu, 2011). In response to DNA damage, p53 boosts transcription of XPC to meet the demand of DNA repair, which also plants the seed for its own demise, facilitating postdamage recovery. Interestingly, upon UV irradiation, p53 becomes stabilized in normal cells but is further degraded in cells expressing higher levels of XPC. Our results reveal a novel function of XPC in proteolysis and set the stage to further delineate the functions of XPC in proteolysis and the interplay between protein degradation and DNA repair.

RESULTS

p53 degradation is compromised in XPC-deficient cells

No physiological substrate of XPC is known. Because human Rad23 was shown to regulate p53 (Glockzin *et al.*, 2003), we examined whether XPC is involved in p53 turnover. We used the XPC-normal human skin cell line AG13145 and XPC-deficient cell line GM11638, which contains little, if any, XPC protein due to an initiation codon mutation (Khan *et al.*, 2009; Supplemental Figure S1A). A plasmid expressing Myc-tagged p53 was transfected into cells from both cell lines. p53 was efficiently degraded in normal skin cells, but markedly stabilized in XPC-deficient cells (Figure 1A), suggesting that p53 turnover is regulated by XPC. Though the experiments were done in the absence of exogenous DNA damage, one possibility is that compromised p53 degradation in XPC-deficient cells could be due to impaired NER activity. Hence, we assessed p53 stability in cells with deficient XPA (Figure S1B), an NER factor downstream of XPC. Interestingly, XPA deficiency did not attenuate p53 turnover (Figure 1B), suggesting that the specificity of XPC for p53 degradation may be unrelated to its role in NER.

To further confirm the involvement of XPC in p53 turnover, we generated an XPC knockdown in AG13145 cells (Figure 1C, left panel). We found that p53 degradation is impaired in XPC knockdown cells (Figure 1C), supporting a positive role of XPC in p53 turnover. We also assessed whether endogenous p53 is regulated by XPC. We generated an XPC knockdown in the SH-SY5Y neuroblastoma cell line (Isaacs *et al.*, 2001), which allows easy detection of p53 under nonstress conditions (Figure 1D). XPC knockdown led to compromised degradation of endogenous p53 (Figures 1D and S1C), suggesting that XPC is involved in endogenous p53 turnover as well. In contrast, XPA knockdown did not significantly alter degradation of endogenous p53 (Figures 1E and S1D).

Furthermore, we found that p53 coimmunoprecipitates XPC (Figure 2A), supporting the intimate involvement of XPC in p53 regulation. To determine the specific step in which XPC is involved, we evaluated whether p53 ubiquitylation was affected in XPC-deficient cells. p53 was efficiently multi-ubiquitylated in XPC-normal AG13145 cells (Figure 2B). Interestingly, more ubiquitylated p53 was accumulated in XPC-deficient GM11638 cells (Figure 2B), suggesting that XPC functions after the p53 ubiquitylation step, consistent with the proposed role of Rad4 in proteolysis. Interestingly, p53 coimmunoprecipitates endogenous S10a (a proteasome subunit) in XPC-normal AG13145 cells, but the p53-S10a association is markedly disrupted in XPC-deficient GM11638 cells (Figure 2C). The expression of wild-type XPC in the XPC-deficient cells restored the p53-S10a association (Figure 2C, lane 4). These results support the conjecture that XPC facilitates the transfer of substrates to the proteasome (see Figure 6E later in this article).

XPC functions in the MDM2-mediated p53 degradation pathway

Proteasomal substrates are selected for degradation by specific E3 Ub-protein ligases that mark the substrates with Ub molecules. Multiple E3s are known to facilitate p53 ubiquitylation under various conditions (Wade *et al.*, 2010; Brooks and Gu, 2011). To determine which specific E3 may work in concert with XPC, we evaluated whether XPC could interact with two major E3s (i.e., human MDM2 and Pirh2) involved in p53 degradation (Wade *et al.*, 2010; Brooks and Gu, 2011). We transiently transfected green fluorescent protein (GFP)-tagged XPC and Myc-tagged MDM2 or Pirh2. Interestingly, XPC associated with MDM2 specifically (Figure 3A) and directly (see Figure 6C later in this article), but did not bind Pirh2 (Figure 3B), potentially implicating XPC in the MDM2 pathway. Furthermore, overexpression of human MDM2 reduced p53 levels significantly in XPC-normal cells but not in XPC-deficient cells (Figure 3C), suggesting that XPC and MDM2 act in the same pathway for p53 degradation. In contrast, Pirh2 overexpression reduced p53 levels in both XPC-normal and XPC-deficient cells (Figure 3D), indicating that Pirh2 and XPC function independently in p53 turnover. We also determined whether UV exposure affects the MDM2-XPC interaction. UV damage led to attenuated association between MDM2 and XPC in vivo (Figure S2), likely due to reduced MDM2 levels (Figure S2), which is consistent with previous studies (Perry, 2004). Together the data support the involvement of XPC specifically in the MDM2-mediated p53 degradation pathway.

XPC expression stimulates p53 degradation following UV irradiation

To determine whether XPC is a rate-limiting component for p53 turnover, we assessed the effect of XPC overexpression on p53 degradation with or without UV damage. We cotransfected a plasmid bearing Myc-p53 with a plasmid expressing XPC or an empty vector into XPC wild-type AG13145 cells. Under normal conditions, XPC overexpression did not stimulate p53 turnover (Figure 4A), suggesting sufficient XPC present under nonstress conditions. On UV irradiation, p53 was stabilized, as previously reported (Wade *et al.*, 2010; Brooks and Gu, 2011). However, XPC overexpression enhanced p53 degradation following DNA damage (Figure 4B), suggesting that XPC is a rate-limiting factor for p53 turnover in the presence, but not the absence, of UV-induced DNA damage. We also used the SH-SY5Y neuroblastoma cell line, in which endogenous p53 can be detected (Figure 4, C and D). Similarly, we found that XPC overexpression promoted endogenous p53 turnover following UV exposure (Figure 4D). The parsimonious interpretation is that, upon DNA damage, most if not all XPC proteins are normally mobilized to search for DNA lesions and facilitate subsequent DNA repair; excess XPC would then promote p53 degradation.

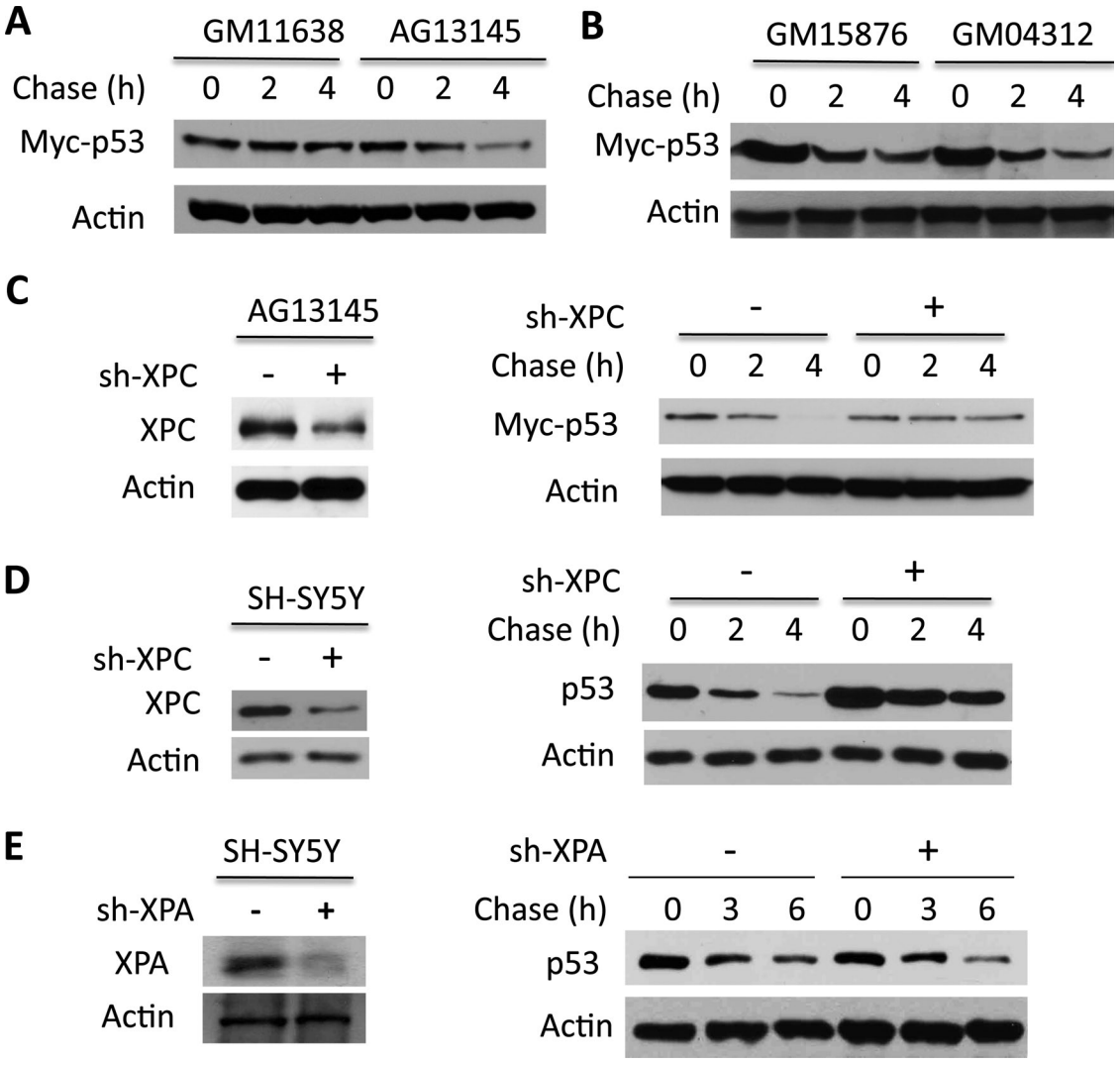

FIGURE 1: XPC is involved in p53 turnover. (A) p53 degradation is impaired in XPC-deficient cells. Myc-tagged p53 was transfected into XPC-normal primary AG13145 cells and XPC-deficient GM11638 cells. Protein synthesis was stopped by cycloheximide to start the chase. Samples were collected at indicated time points and analyzed by immunoblotting with anti-Myc antibody. For ensuring equal loading, actin levels in the extracts were determined by Western blotting in all of the subsequent expression shutoff experiments (bottom panel). (B) XPA deficiency does not affect p53 turnover. Myc-p53 stability in XPA-deficient GM04312 and XPA-complemented GM15876 cells was performed as described above. (C) Effect of XPC knockdown on p53 degradation in primary skin cells. XPC-normal skin fibroblast AG13145 cells were transfected with or without the XPC RNAi plasmid. Cell lysates were subjected to immunoblotting to analyze the levels of XPC and actin (loading control; left panel). XPC-normal or knockdown cells were split into six-well dishes and subjected to a cycloheximide chase to assess the degradation kinetics of Myc-p53 (right panel). (D) Endogenous p53 is stabilized in XPC knockdown SH-SY5Y cells. XPC knockdown in SH-SY5Y cells was carried out as above (left panel). The degradation of endogenous p53 was performed as in (A), except that anti-p53 antibody was used to probe endogenous p53 in SH-SY5Y cells. (E) XPA knockdown does not impair p53 degradation in SH-SY5Y cells. The degradation of endogenous p53 in SH-SY5Y wild-type or XPA knockdown cells was performed as in (D).

Mutations in XPC impair p53 turnover

Defects in XPC constitute one of the most common forms of XP in patients (Hanawalt, 2003; Friedberg *et al.*, 2006). Among the pathogenic mutations of XPC known to be associated with inherited XP, most of them are stop-codon mutations that lead to premature termination of XPC. To determine whether XPC mutants could maintain efficient proteolysis, we investigated whether the expression of two XPC alleles bearing single point mutations (i.e., W690S, K939Q) would restore p53 degradation in XPC-deficient cell lines. W690S was identified in one XP patient and conferred compromised DNA repair (Hanawalt, 2003; Friedberg *et al.*, 2006). Despite normal NER activity, the K939Q allele has been linked to increased cancer susceptibility (Khan *et al.*, 2000; Friedberg *et al.*, 2006; Hirata *et al.*, 2007; El-Zein *et al.*, 2009). Plasmids bearing XPC variants were cotransfected with a plasmid expressing Myc-p53 into XPC-deficient GM11638 cells. Interestingly, the expression of wild type but not XPC variants in the XPC-deficient cells restored efficient degradation of p53 (Figure 5, A and B), suggesting that XPC mutations may affect the turnover of substrates (e.g., p53) and thereby contribute to some disease phenotypes.

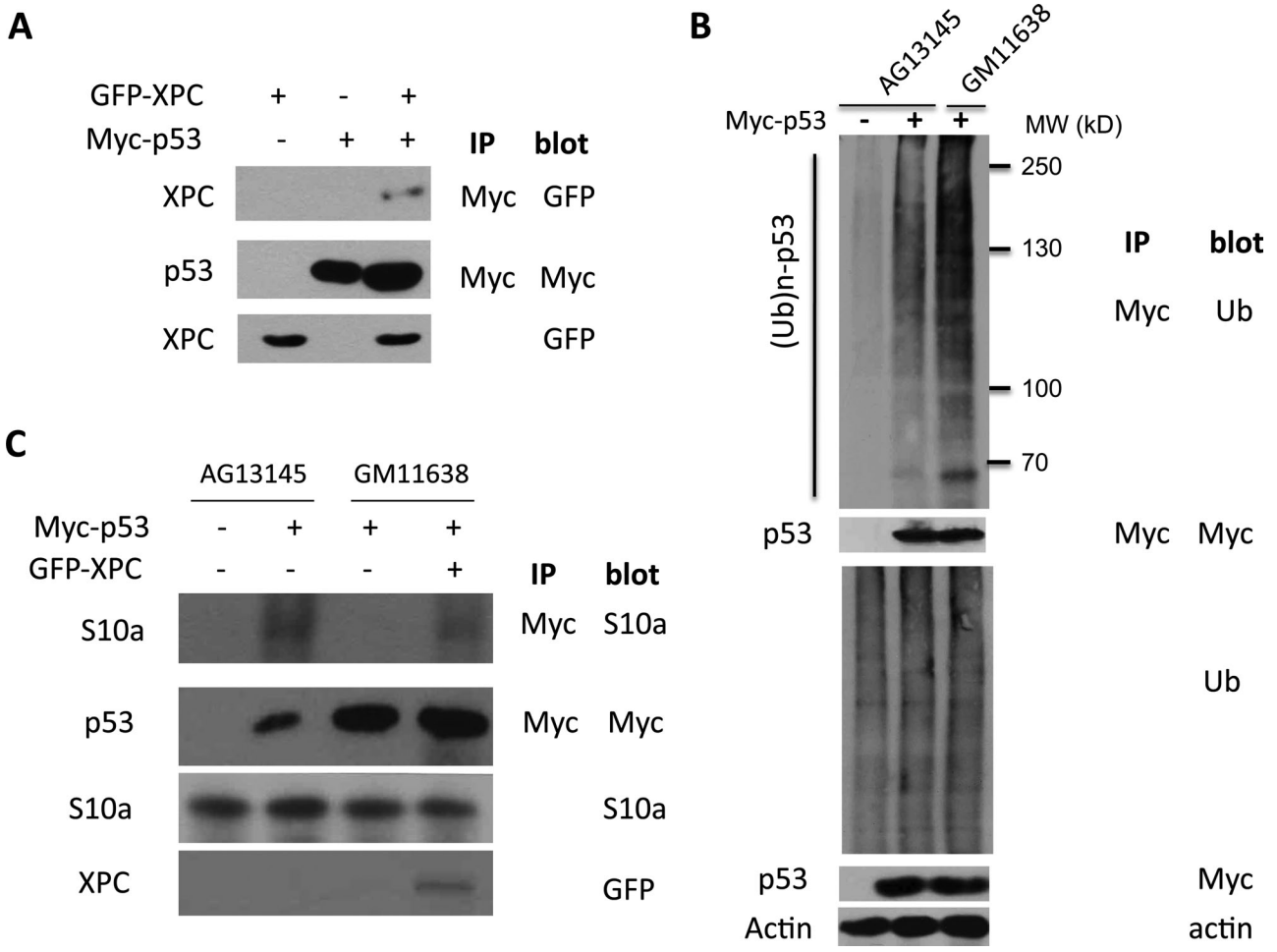

FIGURE 2: XPC is required for the interaction between p53 and the proteasome. (A) XPC immunoprecipitates p53. Plasmids expressing GFP-tagged XPC and/or Myc-tagged p53 were cotransfected into AG13145 cells. At 48 h posttransfection, cell extracts were immunoprecipitated (IP) with anti-Myc antibody, resolved by SDS–PAGE, and immunoblotted with anti-GFP antibody (top panel). The antibodies for immunoprecipitation (IP) and immunoblot (blot) are shown to the right of the panels. Levels of XPC and p53 in the extracts were also determined (bottom panels). (B) p53 remains ubiquitylated in XPC-deficient cells. XPC-normal or XPC-deficient cells were transfected with a plasmid bearing Myc-p53 or an empty vector. Cell lysates were immunoprecipitated with anti-Myc antibody and subsequently analyzed with anti-Ub antibody for ubiquitylated p53 (top panel), as previously described (Okuda-Shimizu and Hendershot, 2007). Levels of Myc-p53, Ub conjugates, and actin in cell extracts were determined by Western blotting with relevant antibodies (bottom panels). (C) The p53-proteasome association is compromised in XPC-deficient cells. Plasmid expressing Myc-p53 was introduced into XPC-normal cells (AG13145) and XPC-deficient cells (GM11638). In lane 4, the plasmid expressing GFP-XPC was also transfected into XPC-deficient cells. Immunoprecipitations were done as described in (A).

We also evaluated whether overexpression of XPC mutants could enhance p53 turnover in response to DNA damage. Although wild-type XPC stimulated p53 degradation following UV irradiation, overexpression of XPC variants did not promote p53 turnover under such conditions (Figure 5, C and D), demonstrating the association of proteolysis defects with XPC mutations.

What might be the molecular event(s) altered by XPC mutations? We assessed the associations between the XPC allele and its two partners, human Rad23 and MDM2, in vivo and in vitro. Interestingly, the W690S mutation disrupted its association with endogenous MDM2, but retained normal Rad23 association in mammalian cells (Figure 6, A and B). K939Q mutation also disrupted the MDM2-XPC interaction (Figure S3). We set up further in vitro binding assays with purified proteins to ascertain the effects of the W690S mutation. Wild-type and the W690S allele of Flag-XPC, glutathione *S*-transferase (GST)-MDM2, and His6-Rad23 were expressed in insect cells (Fong *et al.*, 2011) or *Escherichia coli* and purified separately (Figure S4A). We found that wild-type XPC can directly bind MDM2 (Figure 6C). Moreover, XPC W690S mutant is specifically defective for MDM2 binding (Figure 6C) but competent for Rad23 interaction (Figure 6D), suggesting that the MDM2-XPC interaction may be critical for p53 degradation (Figure 6E).

To determine the region of MDM2 responsible for XPC binding, we purified a series of MDM2 deletion mutants in the form of GST fusion proteins from bacteria, as previously described (Dai *et al.*, 2004; Figure S4B). Neither the N-terminal fragment (aa 1–301) nor the C-terminal fragment (aa 291–491) alone, encompassing the p53-binding domain or RING finger motif (Figure S4B), exhibited efficient XPC binding (Figure S4C), suggesting that intact MDM2 may be required for its association with XPC.

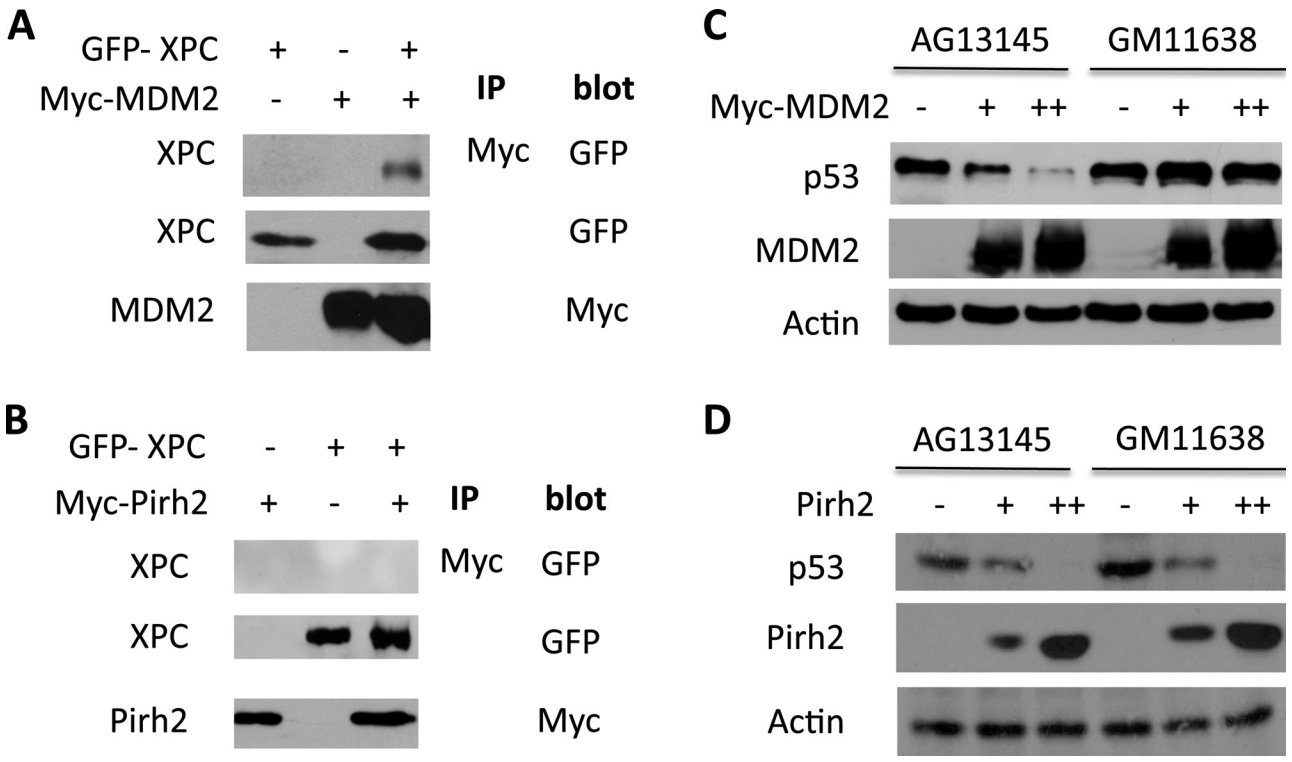

FIGURE 3: The functional relationship between XPC and MDM2. (A and B) XPC associates with human MDM2, but not Pirh2. Plasmids bearing GFP-XPC and Myc-tagged MDM2 or Pirh2 were transiently transfected into HEK293 cells. At 48 h posttransfection, cell extracts were subjected to immunoprecipitation with anti-Myc antibody; this was followed by immunoblotting with anti-GFP antibody (top panel). Ten percent inputs were analyzed by immunoblotting to determine the expression of GFP-XPC, MDM2, and Pirh2 (bottom panels). (C) Human MDM2-promoted p53 degradation requires XPC. XPC-normal and XPC-deficient cells were transfected with Myc-tagged p53 and increasing amounts of MDM2. Levels of p53, MDM2, and actin in cell extracts were determined by immunoblotting with the various antibodies indicated. (D) Pirh2-facilitated p53 degradation is independent of XPC. The experiments were performed as described in (C), except Pirh2 E3 was used.

DISCUSSION

How a substrate is delivered to the proteasome remains poorly understood (Finley, 2009; Liu and Walters, 2010). Although Rad23 has been shown to be a key regulator of the substrate delivery process, it requires other proteins to assist in facilitating specific substrate degradation, as other UBA/UBL proteins exist and regulate distinct substrates (Dantuma *et al.*, 2009; Finley, 2009; Liu and Walters, 2010). We previously demonstrated that yeast Rad4 is involved in Ub-mediated proteolysis as well (Li *et al.*, 2010). The interaction between Rad23 and Rad4 is critical for their proteolytic function, because the Rad23 mutant defective in Rad4-binding exhibits impaired substrate degradation (Li *et al.*, 2010). In this paper, we extend the proteolytic function of Rad4 to its human homologue, XPC. Like Rad23, XPC appears to be involved in a postubiquitylation step of protein degradation, as substrates are stabilized but efficiently ubiquitylated in XPC-deficient cells (Figure 2B).

Given Rad23's direct interactions with the proteasome and Ub conjugates, its function is easy to envision, although the underlying mechanism for its action in vivo remains elusive (Dantuma *et al.*, 2009; Finley, 2009). A central issue lies in how Rad23 differentiates and recognizes specific targets in vivo. As Rad23 is involved in a subset of Ub-mediated substrate degradation, Ub-binding activity alone is unlikely to be sufficient for sorting and selecting various substrates in vivo. Although Rad23 was shown to regulate p53 turnover and bind ubiquitylated p53 via its UBA domain (Glockzin *et al.*, 2003), it was unclear whether Rad23 recognizes ubiquitylated p53 on its own or with some help. XPC acts after p53 ubiquitylation (Figure 2B) and appears to be a key link between p53-the proteasome (Figure 2C) and p53-Rad23 (unpublished data). As a binding partner of Rad23, how might XPC help Rad23 in the degradation of p53? We found that XPC directly binds to the MDM2 Ub ligase, leading to a model that the XPC-MDM2 interaction helps Rad23 gain access to p53 (Figure 6E). We propose that XPC brings Rad23 close to p53 via MDM2 binding, which in turn promotes the recognition of ubiquitylated p53 by Rad23. Mutations that disrupt specific protein–protein interaction involved would be key to establish the MDM2-XPC-Rad23 connection in escorting p53 to the proteasome.

In DNA repair, XPC works with Rad23 to probe for UV-induced helical distortions along the DNA, a damage recognition step that is pivotal for DNA repair. The phenotypes associated with XPC mutations were attributed previously to defects in DNA repair (Friedberg *et al.*, 2006). However, not all XPC disease phenotypes (e.g., autism, hypoglycinemia) could be easily explained by their DNA repair deficiencies (Khan *et al.*, 1998, 2009; Friedberg *et al.*, 2006). Supporting a non-NER activity for XPC, studies have revealed that the Lys939Gln allele is associated with a high risk of cancers, yet has wild-type NER activity (Khan *et al.*, 2000; Friedberg *et al.*, 2006; Hirata *et al.*, 2007; El-Zein *et al.*, 2009). The observed association of impaired p53 degradation with XPC mutations raises an interesting possibility that compromised proteolysis may contribute to XP pathogenesis or

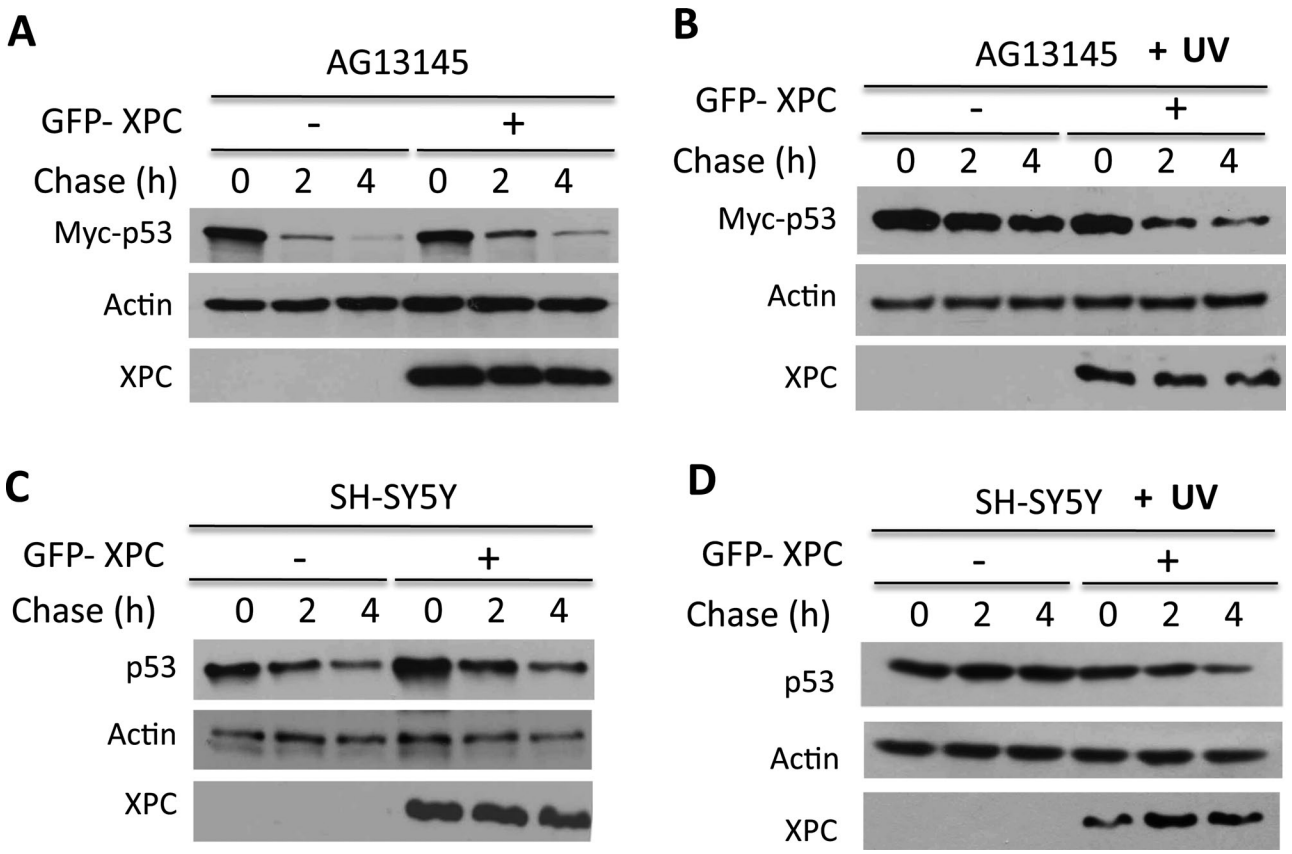

FIGURE 4: Effects of XPC overexpression on p53 degradation in the absence or presence of DNA damage. (A and B) XPC-normal AG13145 cells were transfected with Myc-tagged p53 and GFP-XPC or a control vector. The experiments were done without (A) or with (B) treatment of 25 J/m^2 UV light, and were subsequently processed as described in Figure 1A to evaluate Myc-p53 degradation kinetics. (C and D) The experiments were carried out as above, except that SH-SY5Y cells were used to detect the effects of XPC overexpression on the degradation of endogenous p53.

cause other unexplored phenotypes in XPC patients. XPC is likely to have more proteolytic substrates in addition to p53. It will be crucial to identify other XPC substrates and uncover the biological significance of these degradation events in healthy cells as well as during disease progression.

Our data also establish XPC as a novel regulator of p53 degradation. The p53 tumor suppressor, frequently found mutated in human cancers, plays a central role in mammalian DNA damage response (Vousden and Prives, 2009; Wade *et al.*, 2010). On UV irradiation, p53 is activated, which in turn elicits a number of downstream events, including transcriptional responses, cell cycle arrest, and induction of a DNA repair program to maintain genome stability (Vousden and Prives, 2009; Wade *et al.*, 2010). In response to UV-induced DNA damage, p53 plays diverse roles in NER that include promoting histone modification to allow damage recognition, targeting XPB to the lesion, and stimulating nuclear import of XPA. Interestingly, p53 uses its transcriptional activation activity to regulate UV-inducible expression of XPC (Adimoolam and Ford, 2002), suggesting a negative-feedback loop reminiscent of the relationship between p53 and its transcription target, MDM2 E3 ligase (Sengupta and Harris, 2005; Brooks and Gu, 2011). It is important to note, however, that, unlike MDM2, XPC directly participates in the DNA repair reaction as a DNA damage recognition factor. It is tempting to speculate that, in response to DNA damage, XPC is up-regulated in a p53-dependent manner to meet the demands of NER but is later freed from its repair-related role(s), functioning then to turn down p53 levels and reset cells back to their nondamaged state. In this scenario, the functional relationship between p53 and XPC facilitates the coupling of DNA repair and the damage recovery process.

Our data implicate XPC in the MDM2-facilitated p53 degradation pathway, a key regulatory module that normally keeps p53 at low level (Wade *et al.*, 2010). On DNA damage, the hold of MDM2 E3 on p53 is disrupted, leading to p53 stabilization and activation. Drugs that modulate the p53-MDM2 interaction have been explored as a therapeutic avenue against cancer. Identification of XPC as a key p53 regulator presents another means to manipulate p53 levels and activity that may have therapeutic benefits.

The participation of XPC in proteolysis adds another link between the Ub system and NER (Ulrich, 2012). Besides Rad23 and XPC, three NER factors (i.e., DDB2, TFIIH, CSA) are also components of the Ub system; specifically, these three proteins are Ub-protein ligases that target substrates for ubiquitylation. Among ~20 NER factors identified, several proteins (e.g., CSB, XPV) have been shown to be ubiquitylated and degraded. Although we have much to learn about the coupling between the Ub system and NER, their functional intertwining is evident (Ulrich, 2012). Ub is emerging as a key player in both the stress response and poststress recovery that require rapid cellular programming and reprogramming. Undoubtedly, further dissection of the biological function of

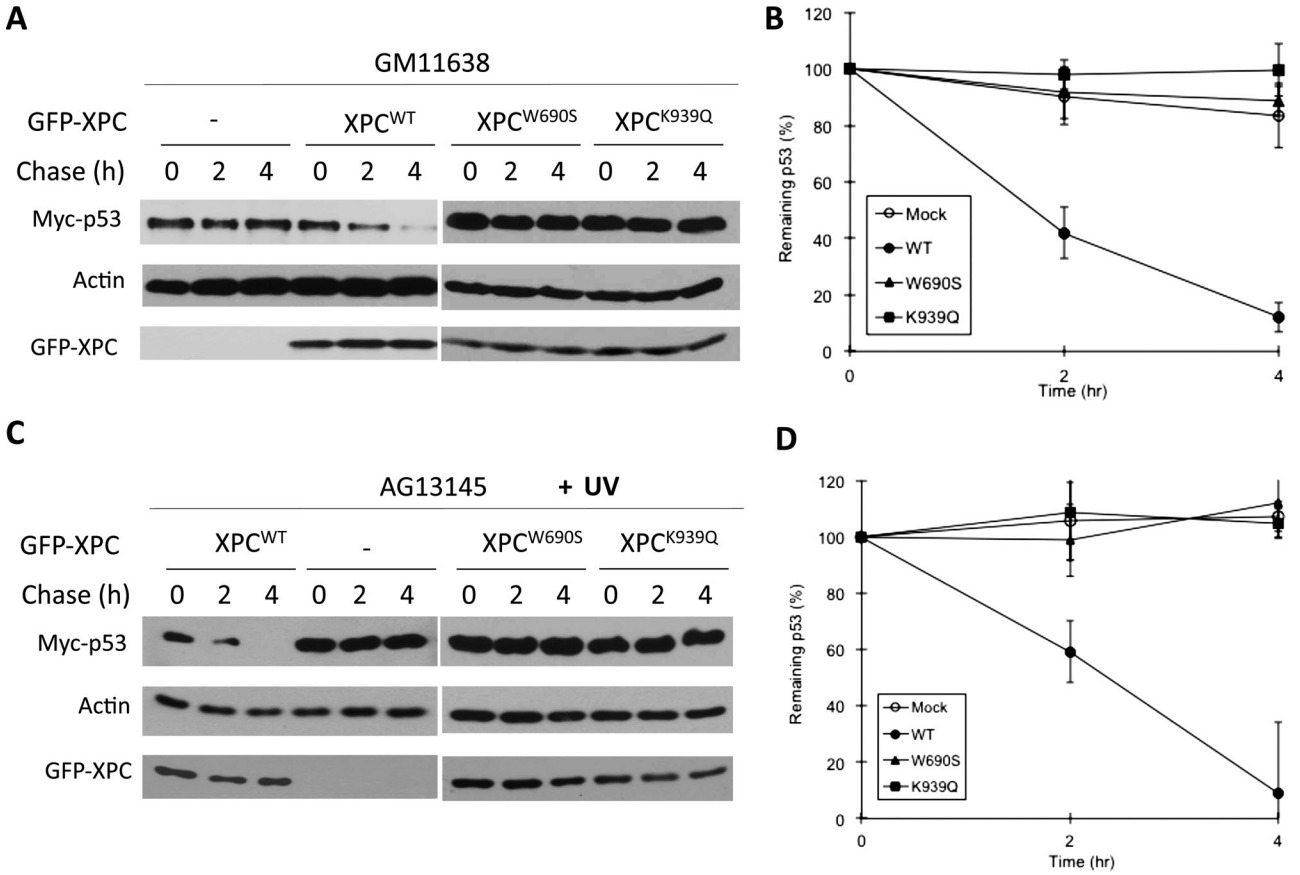

FIGURE 5: The differential effects of XPC alleles on p53 degradation. (A) Wild-type but not mutant XPC renders p53 unstable in XPC-deficient cells. XPC-deficient GM11638 cells were transfected with plasmids bearing wild-type or mutant XPC alleles (i.e., W690S, K939Q) or an empty vector. The half-life of Myc-p53 under each condition was determined as described in Figure 1A. (B) Quantitation of the data in (A) for Myc-p53. (C) Effects of overexpression of XPC derivatives on p53 turnover following UV irradiation in AG13145 cells. The experiments were performed as described in Figure 4B, except that XPC mutant alleles are included. (D) Quantitation of the data in (C).

XPC will help to unravel the intimate roles of Ub in proteolysis and NER.

The XPC-Rad23 pathway may not be the only substrate delivery route for p53. MDM2 was previously shown to bind the proteasome and further facilitate the association between p53 and the proteasome (Kulikov *et al.*, 2010), raising the possibility that MDM2 promotes not only p53 ubiquitylation but also its transfer to the proteasome. These two pathways are not necessarily mutually exclusive of each other. p53 is known to be regulated by more than 10 different Ub ligases under various conditions (Vousden and Prives, 2009; Wade *et al.*, 2010). MDM2 can promote p53 ubiquitylation in several different ways, on its own or with the help of other proteins (e.g., UBE4B, p300; Brooks and Gu, 2011), and may also be involved in multiple pathways of transferring p53 to the proteasome under different conditions. Further analysis is required to determine whether dysregulation of the events after p53 ubiquitylation is involved in p53-related cancers.

MATERIAL AND METHODS

Cell lines and plasmids

XPC wild-type primary cell line AG13145 and XPC-deficient cell line GM11638 and XPA-deficient and XPA-complemented cells were purchased from the Coriell Institute for Medical Research (Camden, NJ) and cultured using DMEM containing 15% fetal bovine serum (FBS). p53 gene is not mutated in GM11638 cells. SH-SY5Y cells were cultured in DMEM containing 10% FBS. All media reagents were purchased from Cellgro (Manassas, VA). Cells were incubated at 37°C and maintained with 5% CO_2. DNA transfection was carried out using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) or X-tremeGENE 9 DNA (Roche, Indianapolis, IN) with ~70% transfection efficiency.

The plasmids expressing GFP-tagged XPC wild-type or mutant alleles (e.g., W690S) were kind gifts from H. Naegeli. The plasmid bearing Flag-tagged XPC for baculovirus expression or His6-tagged Rad23 (i.e., hHR23b) for *E. coli* expression were obtained from R. Tjian and K. Sugasawa. The plasmids expressing GST-MDM2 derivatives were obtained from Hua Lu. The K939Q mutation was introduced to GFP-XPC by site-directed mutagenesis. The plasmids expressing human MDM2, p53, or Pirh2 have been described previously (Yan *et al.*, 2010).

p53 stability assays

For p53 stability experiments, identically transfected cells were treated with 100 μg/ml cycloheximide at ~48 h posttransfection.

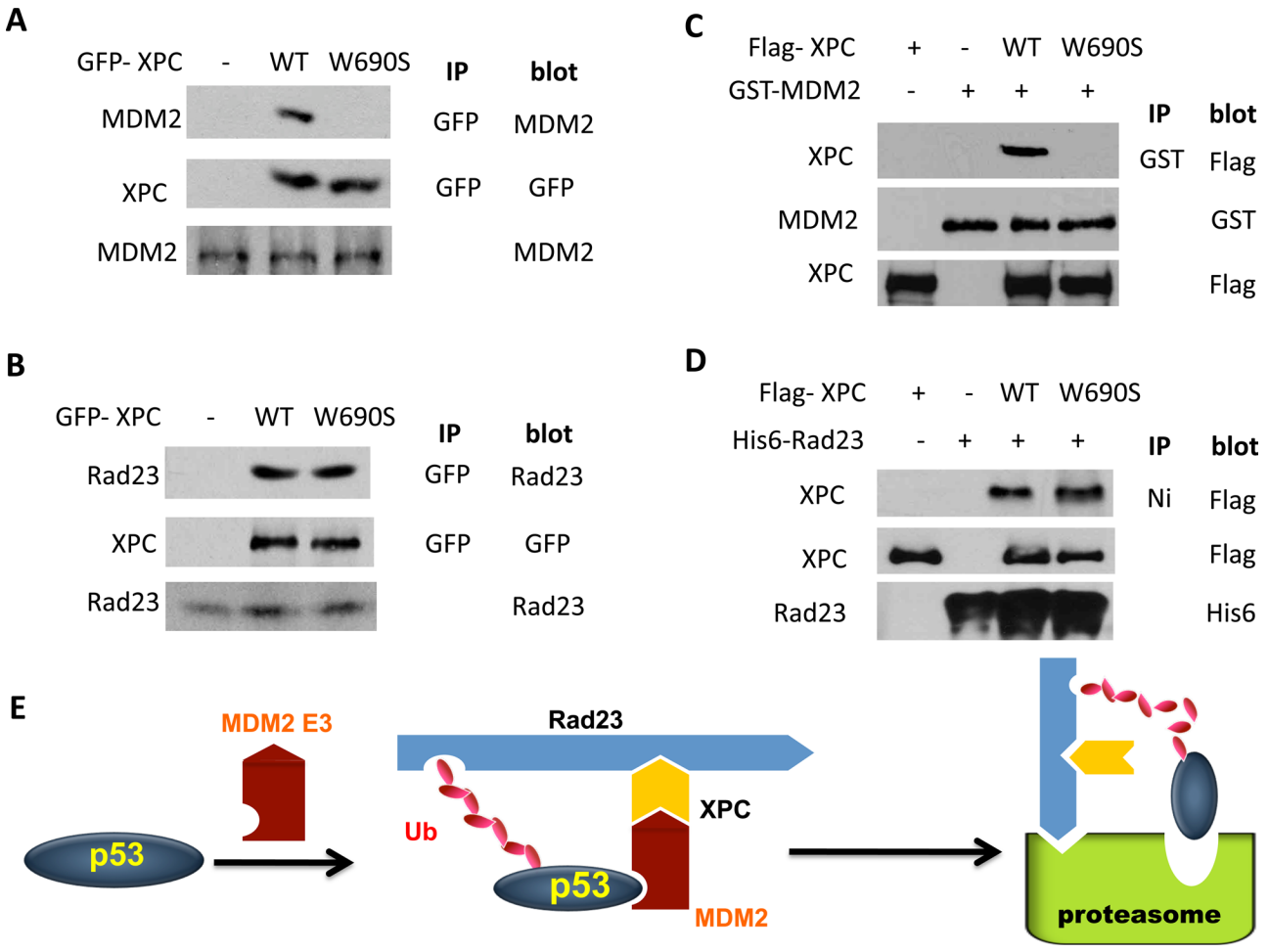

FIGURE 6: W690S mutation affects the binding of XPC to MDM2 but not human Rad23 in mammalian cells and in vitro. (A) W690S mutation disrupts XPC's association with endogenous MDM2. AG13145 cells were transiently transfected with a vector plasmid or a plasmid bearing GFP-XPC wild-type or W690S mutant allele. The binding experiments were carried out using beads coated with anti-GFP antibody. Endogenous MDM2 associated with GFP-XPC was detected by an anti-MDM2 antibody (top panel). Expressions of GFP-XPC and MDM2 in these samples are shown in the bottom panels. (B) W690S allele maintains the XPC-Rad23 interaction in AG13145 cells. Immunoprecipitations were done as described in (A), except that an anti-Rad23 antibody (H00005887-B02P; Novus Biologicals, Littleton, CO) was used to detect endogenous human Rad23. (C and D) Coimmunoprecipitation analysis of in vitro interactions between XPC and MDM2 (C) or Rad23 (D) purified Flag-XPC (2 μg) and GST-MDM2 (2 μg; Novus Biologicals) or His6-Rad23 were mixed in various combinations, as indicated, in the presence of glutathione Sepharose or Ni-NTA resin. Associated XPC was detected by anti-Flag antibody (top panels). Input proteins are shown in the bottom panels. (E) A model for XPC-mediated p53 degradation. XPC forms a stable complex with Rad23, which contains two separate domains that can directly bind Ub and the proteasome. In p53 degradation, MDM2 E3 recognizes and tags p53 with Ub chains (red dots). The MDM2-XPC binding then recruits the XPC-Rad23 complex to the site of p53 ubiquitylation, which in turn facilitates the p53 recognition by Rad23. The Rad23-proteasome interaction brings ubiquitylated cargo to the proteasome for destruction.

Cells were harvested at indicated time points (Figures 1, 4, and 5) and lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.4) supplemented with protease inhibitors. Ectopic p53 or endogenous p53 proteins were analyzed by anti-Myc or anti-p53 (DO-7; Abcam, Cambridge, MA) antibody, respectively. To ensure equal loading, we used the stable protein actin as the loading control. For induction of genomic stress, cells were UV irradiated at 25 J/m^2. After a 6-h recovery, p53 stability assays were performed as described above.

RNA interference assays

For XPC knockdown, a targeting sequence (5′-TTTCTGAGGAGAG-GACCTA-3′) synthesized by Invitrogen was ligated to the pcDNA6.2-GW/miR vector (Invitrogen, Carlsbad, CA). Transfection of RNA interference (RNAi) plasmids was carried out using Lipofectamine RNAiMAX (Life Technologies). At 72 h after transfection, cells were harvested and subjected to p53 stability assays. Rabbit polyclonal anti-XPC antibody was purchased from Sigma-Aldrich (St. Louis, MO). XPA was similarly knocked down using small interfering RNA (sc-36853; Santa Cruz Biotechnology, Santa Cruz, CA) and detected by anti-XPA antibody (Santa Cruz).

In vivo ubiquitylation detection

Cells plated in 100-mm plates were transfected with the plasmid expressing Myc-tagged p53 or the empty vector. Cells were then harvested at 48 h after transfection from each plate and lysed in SDS

lysis buffer (50 mM Tris-HCl, pH8.0, 0.6% SDS) as previously described (Okuda-Shimizu and Hendershot, 2007); SDS lysis buffer preserves covalent ubiquitylation but disrupts protein–protein interaction. The extracts were incubated with Sepharose beads coated with anti-Myc antibody for 4 h. The bound proteins were analyzed by immunoblotting with anti-Ub antibody (Enzo Life Sciences, Farmingdale, NY).

Coimmunoprecipitation assay

For the coimmunoprecipitation assay between XPC and MDM2, AG13145 cells were cotransfected with pGFP-XPC and pCMV-Myc-MDM2 plasmids. Cell extracts were prepared with lysis buffer (5 mM EDTA, 50 mM Tris-HCl, PH 7.5, 150 mM NaCl, 0.5% NP-40) followed by immunoprecipitation with beads coated with the specific antibodies indicated (Figures 2, 3, and 6), resolved by SDS–PAGE and immunoblotting, separately, with anti-GFP (Sigma-Aldrich) and anti-Myc (Covance, Princeton, NJ). Other coimmunoprecipitations were carried out similarly, and various antibodies (anti-MDM2, Sigma-Aldrich; anti-S10a, Enzo; and anti-Rad23 [i.e., hHR23b], Novus Biologicals) were used for detecting relevant proteins.

ACKNOWLEDGMENTS

We are grateful to K. Sugasawa, H. Naegeli, R. Tjian, and H. Lu for reagents. We thank Z. Yuan and P. Zhou for advice and the members of Rao laboratory for discussion. H.R. is supported by grants from the National Institutes of Health (GM 078085, P30 CA054174), the Welch Foundation (AQ-1747), the U.S. Department of Defense (W911NF-11-10466), and a Fudan University Key Laboratory Visiting Scholarship. K.H. is supported by grants from the National Science and Technology Key Program of China (2009ZX09301-011) and the National Basic Science Research Program of China (2010CB912602).

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