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.

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; 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, 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).
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 (Yousden 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 GM16138, 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-SYSY 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 GM16138 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 GM16138 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 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.

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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.
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.

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

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**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).
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

**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.

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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, MDMP E3 ligase (Sen-gupta 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...
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% CO₂. 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).

**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% CO₂. DNA transfection was carried out using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) or X-tremeGENE 9 DNA (Roche, Indianapolis, IN) with ∼70% transfection efficiency.

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**p53 stability assays**

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

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