The Regulatory Potential of OTUD4 in DNA Damage Repair

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THE REGULATORY POTENTIAL OF OTUD4 IN DNA DAMAGE REPAIR

By

Abigail R. Lubin

A THESIS

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Master of Science

Coral Gables, Florida

May 2017
UNIVERSITY OF MIAMI

A thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science

THE REGULATORY POTENTIAL OF OTUD4 IN DNA DAMAGE REPAIR

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The choice of DNA repair pathway and the subsequent initiation of a select pathway in response to a DNA lesion are complex, and many questions remaining as to their regulation. We identified the deubiquitinase OTU deubiquitinase 4 (OTUD4) as a novel putative interactor of Xeroderma pigmentosum complementation group C protein (XPC), one of the crucial sensors of DNA damage in global genome nucleotide excision repair (GG-NER). The goal of this study was to establish a greater understanding of the role of OTUD4 in the DNA damage response. While the complexity of the structure and function of OTUD4 remain to be fully unraveled, our study found that knockout of OTUD4 led to altered XPC ubiquitination and, intriguingly, to a specific sensitivity to cisplatin. Further studies will allow more understanding of the role of OTUD4 in the DNA damage response.
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Chapter 1: Introduction

DNA damage results from a variety of sources, both endogenous and exogenous, and living organisms harbor various pathways to repair each specific type of damage. The DNA damage response in humans comprises a number of repair mechanisms, including nucleotide excision repair (NER), non-homologous end-joining (NHEJ), and the Fanconi anaemia (FANC) pathway. For a review of DNA repair mechanisms in humans and their links to disease, please see [1].

As reviewed in [2], NER repairs bulky adducts such as cyclopyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) induced by UV irradiation. Xeroderma pigmentosum, complementation group C (XPC), in complex with UV excision repair protein RAD23 homolog B (RAD23B) and centrin 2, serves as a primary damage sensor within global genome NER, the subpathway of NER responsible for detecting and repairing bulky adducts in DNA throughout the genome [3-6]. The crystallization of the Rad4-Rad23 complex (the yeast ortholog of the XPC-RAD23B complex) allowed for the determination of how XPC recognizes DNA damage [7]. When recognizing DNA lesions, the TGD (transglutaminase-homology domain) and BHD1 (beta-hairpin domain) domains of XPC bind to the dsDNA upstream of the damage while the BHD2 and BHD3 domains of XPC bind the undamaged DNA opposite to the lesion. The BHD2 and BHD3 domains encircle the DNA, and the BHD3 domain is inserted through the double helix, flipping out the two bases containing the lesion. Additionally, DNA damage-binding protein 2 (DDB2) aids XPC in the recognition of CPDs; the opening in the double helix created by the adduct is not large enough for the insertion of the BHD3 domain of XPC on its own, and DDB2 widens this gap, recruiting XPC to the lesion [8].
The indirect binding of the DNA lesion allows XPC to recognize a variety of lesions, a prescient characteristic as the number of lesions known to be recognized by XPC has increased dramatically. These lesions include stereoisomeric benzo[a]pyrenyl-DNA lesions and lesions typically repaired by base excision repair (BER) such as tetrahydrofuran mimicking an abasic lesion and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG). [9-12]. We recently reviewed the expanding role of XPC in damage recognition and further roles outside of DNA damage repair [13]. In addition to lesion binding, XPC can bind undamaged DNA, and the crystal structure of the Rad4-Rad23 complex bound to undamaged DNA “is essentially indistinguishable” compared to that of the Rad4-Rad23 complex bound to damaged DNA [14, 15]. Due to the relatively indiscriminate nature of XPC binding, a pressing question is how XPC distinguishes damaged from undamaged DNA. A kinetic gating mechanism has been proposed, suggesting the specificity for repair of damaged lesions arises from competition between DNA opening and site residence time [14]. XPC recognition is the first of many verification steps undertaken by NER to ensure repair of only damaged bases [16-19].

Because XPC is an important initiator of GG-NER, its regulation is essential and occurs through several methods of regulation. p53 transcriptionally regulates XPC, and XPC association with RAD23 has been suggested to promote XPC stabilization [20-22]. Spatial control of XPC exists through DNA-dependent mobility and nuclear-cytoplasmic shutting [23]. Importantly, XPC ubiquitination by ubiquitination two different E3 ligases, DDB2 and RNF111, has emerged as essential in regulating XPC [24, 25]. The DDB2 process has been more thoroughly studied and is known to ubiquitinate XPC after DNA damage, though the precise role of either in XPC ubiquitination has yet to be thoroughly
determined [24, 26]. One report suggests that XPC ubiquitination leads to its degradation, while other lines of evidence suggest that degradation does not occur following ubiquitination and instead leads to stronger DNA [27]. RNF111 ubiquitinates XPC following addition regulation via sumoylation [25, 28]. An interesting hypothesis has been postulated, proposing that differential ubiquitination (or sumoylation) controls the response of XPC to different damage types [29, 30].

OTUD4 is a homologously identified putative cysteine deubiquitinase. Based on homology with the other OTU deubiquitinases, the OTU domain of OTUD4 comprises amino acids 34 to 155 of the 1114 aa protein. The primary active site and catalytic cysteine are amino acids 39 to 45 and cysteine 45, respectfully. An in vitro system indicated that a truncated version of OTUD4 specifically cleaves Lys48-linked ubiquitin chains [31]. Recently, OTUD4 has been shown to affect alkylation damage repair [32]. There have been additional links of OTUD4 to ataxia and hypogonadotropic hypogonadism in humans as well as to dorsoventral patterning in zebrafish [33-35].
Chapter 2: Materials and methods

2.1 Yeast two hybrid screenings.

DDB2 or XPC were fused to the Gal4 DNA-binding domain (DNA-BD) within the Y2H Gold strain and mated with a library of prey proteins (SMART-based Normalized Yeast Two-Hybrid cDNA Library) expressed as fusions to the Gal4 activation domain within the Y187 strain. Association between the two domains leads to the transcription of four independent reporter genes (AUR1-C, ADE2, HIS3, and MEL1) which allow for screening of interactions. Putative interactors were isolated, and their DNA was sequenced. Result sequences were verified for quality and identified using NCBI BLAST.

2.2 Co-immunoprecipitation.

HCT116 cells were lysed, and the extracts were incubated overnight with no antibody, an XPC antibody, or an OTUD4 antibody. The extracts were then incubated for two hours with protein G beads before the beads were washed and isolated a Western blot analysis was performed.

2.3 CRISPR.

CRISPR guides were designed to target OTUD4: CTCTCGAAGATAGTGAATAC and TGAAGAATATTTAAAGCGTT. HCT116 cells were transfected with a construct expressing the bacterial Cas9 nuclease or a construct expressing both the bacterial Cas9 nuclease and a guide RNA targeting OTUD4. Individual colonies from the transfection with the construct expressing both the bacterial Cas9 nuclease and a guide RNA targeting OTUD4 were selected, allowed to repopulate, and screened for OTUD4 expression.
2.4 Plasmids.

The variable loop comprises amino acids 94 to 104, and the His loop comprises amino acids 143 to 148. To understand the importance of the domains of OTUD4, two truncated versions of the protein were generated with two mutated versions of the protein designed for future studies. The truncated proteins are OTUD4.part and OTUD4.short. OTUD4.part lacks the first 31 amino acids of the protein, and OTUD4.short lacks the first 65 amino acids of the protein including the catalytic cysteine residue. The mutated proteins are OTUD4.full.C45A and OTUD4.full.delC. OTUD4.full.C45A was designed with the catalytic cysteine converted into an alanine, and OTUD4.full.delC was designed without the Cys loop. All constructs are expressed within the p3xFlag vector, and OTUD4.full designates the full-length protein expressed within the p3x-Flag vector. Graphic depictions of the catalytic domain of OTUD4 and of the plasmids can be seen in Fig. 3.10.

2.5 Treatment with UV irradiation.

HCT116 and HCT116 OTUD4−/− cells were treated with UV irradiation (254 nm) at 20, 40, or 100 J/m² using a germicidal lamp or at 1, 2, or 4 J/m² in a cross linker.

2.6 Chemicals.

Cells were treated with H₂O₂, cisplatin, MG-132, and/or cycloheximide.

2.7 Western blot analysis and immunoprecipitation.

HCT116 cells and HCT116 OTUD4−/− cells were collected by scraping in 2X SDS buffer and boiled for 20 min. Lysates were separated by SDS–PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 3% milk in PBS for at room temperature and incubated at 4°C with primary antibodies against actin, GAPDH (5174, Cell Signaling Technology, Inc.), FLAG (F1804, Sigma-Aldrich Co. LLC.), IgG, MDM2
(sc-965, Santa Cruz Biotechnology, Inc.), OTUD4 (ab106971, Abcam), p53 (05-224, EMD Millipore Corporation), cleaved PARP (44698G, Invitrogen Corporation), PML (sc-966, Santa Cruz Biotechnology, Inc.), tubulin (GTX11304, GeneTex Inc.), or XPC (X1129, Santa Cruz Biotechnology, Inc.). Membranes were washed with PBS containing 0.05% Tween and incubated with anti-rabbit and anti-mouse secondary antibodies. The membranes were incubated with Pierce™ ECL Western Blotting Substrate (32109, Thermo Fisher Scientific Inc.) and images of the antibody complexes were captured with x-ray film.

2.8 Clonogenic assays.

10^3 HCT116 or HCT116 OTUD4−/− were seeded and incubated at 37°C and 5% CO₂. After 24 hours, the media was removed, the cells were washed, and media containing 0, 1, 2, or 4 μM cisplatin was introduced or cells were irradiated. Cells were incubated at 37°C and 5% CO₂. After 7 days, the surviving cells were washed, fixed with 4% paraformaldehyde, washed, and stained with 0.1% crystal violet. All residual crystal violet was removed, and the colonies and stain were allowed to dry overnight. Images of the plates were taken using a UVP GelDoc-It® Imager, and the colonies were then counted using OpenCFU [36]. Cell numbers were normalized, and a student’s t-test was performed.

2.9 HPRT gene mutation assay.

HCT116 and HCT116 OTUD4−/− cells were mock treated or treated with 2 μg/mL 6-thioguanine. Cells were allowed to grow for 7 or 11 days and, and surviving cells were fixed and stained with 0.1% crystal violet. Colonies were then counted using OpenCFU [36]. Representative plates were imaged using a UVP GelDoc-It® Imager.
2.10 **Nuclear-cytoplasmic fractionation.**

HCT116 and HCT116 *OTUD4*<sup>-/-</sup> cells were mock treated or treated with 40 J/m<sup>2</sup> UV irradiation. The cells were lysed, and the nuclear and cytosplasmic protein fractions were separated using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (78833, Thermo Fisher Scientific Inc.).

2.11 **RT-PCR.**

HCT116 cells were treated cisplatin or irradiated. Cells were incubated at 37°C and 5% CO<sub>2</sub> for a period of time, and then the cells were lysed, and the RNA was extracted. Reverse transcription was performed, and PCR amplification of the resulting cDNA was performed using oligonucleotide primers for OTUD4 and actin. The PCR products were run on an agarose gel, and images were obtained using a UVP GelDoc-It® Imager.

2.12 **Microscopy.**

HCT116 cells on coverslips were mock treated or treated with 20 J/m<sup>2</sup> global UV irradiation or with 100 J/m<sup>2</sup> local UV irradiation covered with micropore membrane. The cells were then incubated at 37°C and 5% CO<sub>2</sub> for the times indicated. The cells were then fixed and washed, and the DNA was denatured. Cells were then blocked, treated with primary and secondary antibodies, and washed. The secondary antibodies were conjugated to Alexa Fluor 568. Coverslips were then mounted in ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were visualized by a Zeiss Axiovert 200 M fluorescence microscope, courtesy of Dr. Ralf Landgraf.
Chapter 3: Results

3.1 Yeast Two-Hybrid screenings of DDB2 and XPC identify putative interactors of the two DNA damage sensing proteins, including OTUD4, an OTU deubiquitinase.

To identify putative interactors of the two essential DNA damage sensing proteins of global genome nucleotide excision repair, we performed two yeast two-hybrid screenings with DDB2 and XPC. The screenings identified 113 putative interactors of DDB2 (Table 3.1) and 49 putative interactors of XPC (Table 3.2, published in [37]).

The deubiquitinase OTUD4 was identified as a potential interactor of XPC in the yeast two-hybrid system [37]. To validate the XPC-OTUD4 interaction, we performed a co-immunoprecipitation (Co-IP) (Fig. 3.1). XPC was detected after pull-down of OTUD4 in HCT116 cells.

3.2 XPC is very stable and is recycled after UV irradiation.

To get a better understanding of XPC stability, we performed a Western blot analysis after treating cells with cycloheximide, cycloheximide and UV irradiation, or cycloheximide, UV irradiation, and MG-132 (Fig. 3.2). After treatment with cycloheximide, XPC protein levels decreased though some amount of the protein was detected even after 24 hours. After treatment with cycloheximide and UV irradiation, unmodified XPC protein levels decreased while modified XPC levels increased and then decreased. Intriguingly, as when the cells were not irradiated, some amount of the protein was detected even after 24 hours. After treatment with cycloheximide, UV irradiation, and MG-132, XPC protein levels similarly decreased though not to the same extent as in the absence of MG-132, modified XPC levels increased though not to the same extent as in the absence of MG-132, and, as when the cells were not irradiated and/or treated with MG-132, some amount of the protein was detected even after 24 hours.
Figure 3.1. Co-immunoprecipitation of XPC and OTUD4. A. HCT116 cells were lysed, and the extracts were incubated with no antibody, an XPC antibody, or an OTUD4 antibody. Associated proteins were collected by protein A/G beads and separated using SDS-PAGE. Protein associations were analyzed by immunoblotting with an XPC antibody. Originally published in [37]. B. HCT116 cells were lysed, and the extracts were incubated with an IgG antibody, an XPC antibody, or an OTUD4 antibody. Associated proteins were collected by protein A/G beads and separated using SDS-PAGE. Protein associations were analyzed by immunoblotting with XPC and OTUD4 antibodies.
Though some XPC is degraded in a UV-independent manner, the majority of the protein is recycled with a stability exceeding 24 hours. HCT116 cells were treated with 10 μg/mL cyclohexamide, UV (254 nm, 40 J/m²), or 10 μM MG-132 as indicated on top of the blots. Cells were incubated at 37°C and 5% CO₂, and after the time indicated on top of the blots, the cells were lysed, and proteins were separated using SDS-PAGE. Unmodified and modified protein levels were analyzed by immunoblotting with XPC and tubulin antibodies.

### 3.3 There is a differential response of OTUD4 and XPC to DNA damaging agents.

Having identified OTUD4 in association with XPC and as XPC has been implicated in a variety of DNA damage repair pathways, we then asked what was the response of OTUD4 and XPC levels (both unmodified and ubiquitinated) after treatment with various DNA-damaging agents (Fig. 3.3). HCT116 cells were treated with hydrogen peroxide, UV irradiation, or cisplatin, and Western blot analysis was performed using OTUD4, XPC, and tubulin antibodies. After treatment with hydrogen peroxide, unmodified XPC levels remained fairly constant and some faint hints of increased modified XPC levels were seen while OTUD4 levels visibly increased during the 7 hours after treatment. OTUD4 levels were even more drastically augmented after treatment with cisplatin, while both unmodified and modified XPC levels increased slightly in the 24 hours after treatment. Conversely, OTUD4 levels remained stagnant after treatment with UV irradiation while
unmodified levels of XPC decreased and then increased and modified levels increased and then decreased for three hours after treatment.

To get a comprehensive picture of OTUD4 and XPC localization before and after UV irradiation, we performed nuclear-cytoplasmic fractionation and confocal microscopy (Fig. 3.4). For nuclear-cytoplasmic fractionation, HCT116 cells were treated with UV irradiation, the nuclear and cytoplasmic fractions were separated, and Western blot analysis was performed using OTUD4, XPC, PARP, GAPDH, and p53 antibodies. Interestingly, Both OTUD4 and XPC translocate to some extent in response to UV irradiation. OTUD4 localizes to the cytoplasm in the absence and presence of damage. However, a distinct amount of OTUD4 translocates to the nucleus one hour after UV irradiation. Comparatively, unmodified and modified XPC localizes to the nucleus in the absence and presence of damage, while a small amount of both unmodified and modified XPC translocates to the cytoplasm one hour after UV irradiation. For confocal microscopy, HCT116 cells were irradiated, fixed on coverslips, and imaged after incubation with fluorescence-conjugated secondary antibodies and mounting in ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI). OTUD4 was seen in the nucleus one hour after treatment with 20 J/m² global irradiation or 1, 15, or 30 minutes after 100 J/m² local irradiation. As with the nuclear-cytoplasmic fractionation, microscopy indicated that OTUD4 localizes to the cytoplasm in the presence of damage. Further, OTUD4 did not colocalize with CPD foci.
Figure 3.3. OTUD4 and XPC are differentially induced by a variety of damaging agents. A. HCT116 cells were mock treated (NO H2O2) or treated with 0.4 mM H2O2. Cells were incubated at 37°C and 5% CO2, and after the time indicated on top of the blots, the cells were lysed, and proteins were separated using SDS-PAGE. Unmodified and modified protein levels were analyzed by immunoblotting with OTUD4, XPC, and tubulin antibodies. B. HCT116 cells were mock treated (NO UV) or irradiated with 40 J/m² UV (254 nm). Cells were incubated at 37°C and 5% CO2, and after the time indicated on top of the blots, the cells were lysed, and proteins were separated using SDS-PAGE. Unmodified and modified protein levels were analyzed by immunoblotting with OTUD4, XPC, and tubulin antibodies. C. HCT116 cells were mock treated (NO CIS) or treated with 50 μM cisplatin. After 1 hour, the media was removed, the cells were washed, and cisplatin-free media was introduced. Cells were incubated at 37°C and 5% CO2, and after the time indicated on top of the blots, the cells were lysed, and proteins were separated using SDS-PAGE. Unmodified and modified protein levels were analyzed by immunoblotting with OTUD4, XPC, and tubulin antibodies. D. HCT116 cells were mock treated (NO CIS) or treated with 10 or 80 μM cisplatin. After 1 hour, the media was removed, the cells were washed, and cisplatin-free media was introduced. Cells were incubated at 37°C and 5% CO2, and after 24 hours, the cells were lysed, and the RNA was extracted. Reverse transcription was performed, and PCR amplification of the resulting cDNA was performed using oligonucleotide primers for OTUD4 and actin. The PCR products were run on an agarose gel, and images were obtained using a UVP GelDoc-It® Imager. E. HCT116 cells were mock treated (NO UV) or irradiated with 40 J/m² UV (254 nm). Cells were incubated at 37°C and 5% CO2, and after the time indicated on top of the blots, the cells were lysed, and the RNA was extracted. Reverse transcription was performed, and PCR amplification of the resulting cDNA was performed using oligonucleotide primers for OTUD4 and actin. The PCR products were run on an agarose gel, and images were obtained using a UVP GelDoc-It® Imager.
Figure 3.4. Cellular location. A. Nuclear-cytoplasmic fractionation. OTUD4 localizes to the cytoplasm in the absence and presence of damage. However, a distinct amount of OTUD4 translocates to the nucleus after UV irradiation. OTUD4 is primarily located in the cytoplasm with translocation to the nucleus 1 hour after UV irradiation while XPC is primarily located in the nucleus with mild translocation of both modified and unmodified moieties to the nucleus 1 hour after UV irradiation. HCT116 cells were mock treated (NO UV) or irradiated with 40 J/m2 UV (254 nm). Cells were incubated at 37°C and 5% CO2, and after the time indicated on top of the blots, the cells were lysed, and the nuclear and cytoplasmic fractions were separated. Proteins were then separated using SDS-PAGE. Unmodified and modified protein levels were analyzed by immunoblotting with XPC, OTUD4, p53, PARP, and GAPDH antibodies. C = cytoplasm, N = nucleus.

B-D. Microscopy. OTUD4 is primarily located in the cytoplasm and does not translocate in response to UV irradiation. OTUD4 localizes to the nucleus and cytoplasm in the absence of damage. B. HCT116 cells on coverslips were irradiated with 20 J/m2 UV (254 nm) incubated at 37°C and 5% CO2. After 60 minutes, the cells were washed and fixed, and the DNA was denatured. Cells were then blocked and incubated with OTUD4 and CPD antibodies. Cells were then treated with a secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 568. Coverslips were mounted in ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were visualized by a Zeiss Axiovert 200 M fluorescence microscope.

C. HCT116 cells on coverslips were irradiated with 100 J/m2 UV (254 nm) using a micropore membrane and incubated at 37°C and 5% CO2. After the time indicated to the side of the images, the cells were washed and fixed, and the DNA was denatured. Cells were then blocked and incubated with OTUD4 and CPD antibodies. Cells were then treated with a secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 568. Coverslips were mounted in ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were visualized by a Zeiss Axiovert 200 M fluorescence microscope.

D. HCT116 or HCT116 OTUD4-/- cells on coverslips were irradiated with 100 J/m2 UV (254 nm) using a micropore membrane and incubated at 37°C and 5% CO2. After the time indicated to the side of the images, the cells were washed and fixed, and the DNA was denatured. Cells were then blocked and incubated with XPC and CPD antibodies. Cells were then treated with a secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 568. Coverslips were mounted in ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were visualized by a Zeiss Axiovert 200 M fluorescence microscope.
3.4 Decreased levels of OTUD4 lead to altered protein and ubiquitination levels of XPC in response to UV irradiation and cisplatin treatment.

Initial studies indicated that knockdown of OTUD4 led to increased XPC ubiquitination after UV irradiation [37]. To further examine the effects of OTUD4 knockdown on XPC ubiquitination after UV irradiation, we knocked down OTUD4 in HCT116 cells using shRNA targeting the 3’ UTR of the protein (Fig. 3.5). The cells were treated with UV irradiation, and Western blot analysis was performed using OTUD4, XPC, and tubulin antibodies. Compared to HCT116 cells treated with scrambled shRNA, cells with decreased levels of OTUD4 displayed augmented amounts of both unmodified and ubiquitinated XPC levels two hours after treatment with UV irradiation. Intriguingly, unmodified levels of XPC were higher in OTUD4 knockdown cells compared with control cells even before treatment with UV irradiation.

Figure 3.5. Knockdown of OTUD4 via the 3’ UTR leads to increased levels of XPC ubiquitination. HCT116 cells were transfected an shRNA construct targeting the 3’ UTR of OTUD4. Cells were then mock treated (NO UV) or irradiated with 40 J/m² UV (254 nm). Cells were incubated at 37°C and 5% CO₂, and after the time indicated on top of the blots, the cells were lysed, and the proteins separated using SDS-PAGE. Unmodified and modified protein levels were analyzed by immunoblotting with XPC, OTUD4, and tubulin antibodies.
An unfortunate disadvantage of using RNA knockdown to control protein levels is the inability to scrounge away all of the targeted protein. Therefore, we established a knockout cell line of OTUD4 using CRISPR to better analyze the role of the protein in DNA damage and in the cell. To examine the effects of OTUD4 knock out on XPC ubiquitination after UV irradiation, HCT116 OTUD4\(^{-/-}\) cells were treated with UV irradiation, and Western blot analysis was performed using OTUD4, XPC, and tubulin antibodies (Fig. 3.6). The effects of OTUD4 knockout were similar to those of OTUD4 knockdown. Compared to HCT116 cells, HCT116 OTUD4\(^{-/-}\) cells displayed augmented amounts of both unmodified and ubiquitinated XPC levels one to three hours after treatment with UV irradiation. Also as in OTUD4 knockdown cells, unmodified levels of XPC were higher in HCT116 OTUD4\(^{-/-}\) cells compared with HCT116 cells even before treatment with UV irradiation.

As seen in Fig. 3.3, XPC and OTUD4 each respond differently to UV irradiation and cisplatin treatment. To examine the effects of OTUD4 knock out on XPC ubiquitination after treatment with cisplatin and see whether they matched those after UV irradiation, HCT116 OTUD4\(^{-/-}\) cells were treated with cisplatin, and Western blot analysis was performed using OTUD4, XPC, and tubulin antibodies (Fig. 3.7). Similarly to after UV irradiation, HCT116 OTUD4\(^{-/-}\) cells displayed augmented amounts of both unmodified and ubiquitinated XPC levels compared to HCT116 cells 24 hours after treatment with various does of cisplatin. However, XPC levels showed an increase 24 hours after cisplatin treatment compared to the decrease and increase after UV irradiation treatment, and modified levels of XPC were significantly lower after cisplatin treatment compared to after UV irradiation. However, as in OTUD4 knockdown and knockout cells, unmodified levels
of XPC were higher in HCT116 OTUD4−/− cells compared with HCT116 cells even before treatment with cisplatin.

Figure 3.6. Knockout of OTUD4 was confirmed, and leads to increased protein levels and ubiquitination levels of XPC in response to UV irradiation. A. HCT116 cells were transfected with a construct expressing the bacterial Cas9 nuclease or a construct expressing both the bacterial Cas9 nuclease and a guide RNA targeting OTUD4. Individual colonies from the transfection with the construct expressing both the bacterial Cas9 nuclease and a guide RNA targeting OTUD4 were selected, allowed to repopulate, and screened for OTUD4 expression. HCT116 cells, cells transfected with the construct expressing the bacterial Cas9 nuclease (pspCas9), and cells grown from a colony lacking OTUD4 expression after transfection with the construct expressing both the bacterial Cas9 nuclease and a guide RNA targeting OTUD4 (CRISPR (clone K)) were lysed. Proteins were separated using SDS-PAGE, and protein levels were analyzed by immunoblotting with OTUD4 and tubulin antibodies. B, C. HCT116 and HCT116 OTUD4−/− cells were mock treated (NO UV) or irradiated with 40 J/m2 UV (254 nm). Cells were incubated at 37°C and 5% CO2. After the time indicated on top of the blots, the cells were lysed, and the proteins separated using SDS-PAGE. Unmodified and modified protein levels were analyzed by immunoblotting with XPC, OTUD4, and tubulin antibodies.
Figure 3.7. Knockout of OTUD4 leads to increased protein levels and ubiquitination levels of XPC in response to cisplatin treatment. HCT116 and HCT116 OTUD4−/− cells were mock treated (NO CIS) or treated with 50 μM cisplatin. After 1 hour, the media was removed, the cells were washed, and cisplatin-free media was introduced. Cells were incubated at 37°C and 5% CO₂, and after the time indicated on top of the blots, the cells were lysed, and proteins were separated using SDS-PAGE. Unmodified and modified protein levels were analyzed by immunoblotting with OTUD4, XPC, and tubulin antibodies.

3.5 Knockout of OTUD4 conveys sensitivity to certain DNA damaging agents.

Since there is a differential effect of OTUD4 knockout on XPC ubiquitination and stability after treatment with UV irradiation or cisplatin, we then asked if there was a similar differential effect of OTUD4 knockout on cell survival in HCT116 cells after treatment with DNA damaging agents. Using a clonogenic assay, we treated HCT116 and HCT116 OTUD4−/− cells with UV irradiation or cisplatin and measured colony formation after several days. Interestingly, while knockout of OTUD4 did not seem to alter cell survival after UV irradiation, knockout of OTUD4 rendered cells much more sensitive to cisplatin (Fig 8–9). Indeed, the calculated IC50 of the knockout cell line is half that of the control cell line (Fig. 3.9).
Figure 3.8. Knockout of OTUD4 does not alter the sensitivity of cells to UV irradiation. 10^5 HCT116 or HCT116 OTUD4^-/- were seeded and incubated at 37°C and 5% CO₂. After 24 hours, the media was removed, and the cells were washed. The cells were irradiated with 0, 5, 10, or 20 J/m² UV (254 nm), and media was refreshed. Cells were incubated at 37°C and 5% CO₂. After 3 days, the media was removed, the cells were washed, and media containing the same concentration of cisplatin was introduced. After an additional 3 days, the media was removed, and the surviving cells were washed, fixed with 4% paraformaldehyde, washed, and stained with 0.1% crystal violet. All residual crystal violet was removed, and the colonies and stain were allowed to dry overnight. Images of the plates were taken using a UVP GelDoc-It® Imager, and the colonies were then counted using OpenCFU [36].

3.6 OTUD4 structure and function are more complicated than at first glance.

To determine if the exogenously expressed full-length or truncated protein recover the phenotype of wild-type HCT116 cells, p3x-Flag-OTUD4.full, p3x-Flag-OTUD4.short, or p3x-Flag were expressed in HCT116 OTUD4^-/- cells. The cells were treated with UV irradiation, and Western blot analysis was performed using OTUD4, XPC, and tubulin antibodies. Not only did expressing p3x-Flag-OTUD4.full not recover the expected decreased ubiquitination of XPC after UV irradiation as compared to in HCT116 OTUD4^-/- cells expressing the vector, but the HCT116 OTUD4^-/- cells expressing p3x-Flag-OTUD4.short actually demonstrated an augmented level of XPC ubiquitination after UV irradiation as compared with that of HCT116 OTUD4^-/- cells expressing the vector or the exogenous full-length protein (Fig. 3.10).
Figure 3.9. Knockout of OTUD4 increases the sensitivity of cells to cisplatin treatment. A. Representative images of the clonogenic assay performed in B. B. $10^3 \text{HCT116 or HCT116 } OTUD4^{-/-}$ were seeded and incubated at 37°C and 5% CO$_2$. After 24 hours, the media was removed, the cells were washed, and media containing 0, 1, 2, or 4 μM cisplatin was introduced. Cells were incubated at 37°C and 5% CO$_2$. After 3 days, the media was removed, the cells were washed, and media containing the same concentration of cisplatin was introduced. After an additional 3 days, the media was removed, and the surviving cells were washed, fixed with 4% paraformaldehyde, washed, and stained with 0.1% crystal violet. All residual crystal violet was removed, and the colonies and stain were allowed to dry overnight. Images of the plates were taken using a UVP GelDoc-It® Imager, and the colonies were then counted using OpenCFU [36].
Figure 3.10. OTUD4 domains. A. OTU domain. B. OTUD4 mutants. C. Differential ubiquitination of XPC after reintroduction of OTUD4 variants. HCT116 OTUD4<sup>−/−</sup> cells were transfected with p3xFlag, p3xFlag-OTUD4.full, or p3xFlag-OTUD4.short. After 48 hours, cells were mock treated (NO UV) or irradiated with 40 J/m² UV (254 nm). Cells were incubated at 37°C and 5% CO₂. After the time indicated on top of the blots, the cells were lysed, and the proteins separated using SDS-PAGE. Unmodified and modified protein levels were analyzed by immunoblotting with XPC, OTUD4, and tubulin antibodies. D. Overexpression of mutant OTUD4. HCT116 cells were transfected with p3xFlag or p3xFlag-OTUD4.short. After 48 hours, cells were mock treated (NO UV) or irradiated with 40 J/m² UV (254 nm). Cells were incubated at 37°C and 5% CO₂. After the time indicated on top of the blots, the cells were lysed, and the proteins separated using SDS-PAGE. Unmodified and modified protein levels were analyzed by immunoblotting with FLAG, XPC, OTUD4, p53, and actin antibodies.
To understand the effects of OTUD4 in a cell, we asked whether OTUD4 knockout lead to an alteration in the mutation frequency at the HPRT locus. Using a clonogenic assay, we treated HCT116 and HCT116 OTUD4<sup>−/−</sup> cells with 6-thioguanine and measured colony formation after several days (Fig. 3.11). The mutation frequency of HCT116 cells was determined to be $1.38 \times 10^5$ mutations/cell at the HPRT locus and mutation frequency of HCT116 OTUD4<sup>−/−</sup> cells was determined to be $4.22 \times 10^6$ mutations/cell at the HPRT locus, indicating that knockout of OTUD4 leads to a decrease in the mutation frequency at the HPRT locus.

Figure 3.11. Knockout of OTUD4 leads to an increased number of mutations at the HPRT locus. $10^5$ HCT116 or HCT116 OTUD4<sup>−/−</sup> cells were seeded and incubated at 37°C and 5% CO<sub>2</sub> in media without 6-thioguanine. $10^6$ HCT116 or HCT116 OTUD4<sup>−/−</sup> cells were seeded and incubated at 37°C and 5% CO<sub>2</sub> in media containing 2 μg/mL 6-thioguanine. After 7 days for the cells in media without 6-thioguanine and after 11 days for the cells in media containing 2 μg/mL 6-thioguanine, the media was removed, and the surviving cells were washed, fixed with 4% paraformaldehyde, washed, and stained with 0.1% crystal violet. All residual crystal violet was removed, and the colonies and stain were allowed to dry overnight. Images of the plates were taken using a UVP GelDoc-It® Imager, and the colonies were then counted using OpenCFU [36].
Having established that the knockout of OTUD4 leads to increased sensitivity to cisplatin, we then asked whether OTUD4 was involved in cisplatin-induced apoptosis. HCT116 and HCT116 OTUD4<sup>−/−</sup> cells were treated with cisplatin, and Western blotting analysis was performed using OTUD4, XPC, PARP, p53, PML, MDM2, and tubulin antibodies to determine the influence of OTUD4 knockout on the levels of apoptosis-associated proteins 24 hours after treatment with varying doses of cisplatin (Fig. 3.12). Surprisingly, though p53 and MDM2 levels were not changed between HCT116 and HCT116 OTUD4<sup>−/−</sup> cells after cisplatin treatment, levels of cleaved PARP and certain isoforms of PML were increased in HCT116 OTUD4<sup>−/−</sup> cells compared to HCT116 cells. These data suggest that OTUD4 is involved in a p53-independent apoptotic pathway.

**Figure 3.12. Knockout of OTUD4 leads to altered levels of apoptosis-associated proteins in response to cisplatin treatment.** A. HCT116 and HCT116 OTUD4<sup>−/−</sup> cells were mock treated (NO CIS) or treated with 1, 2, or 4 μM cisplatin. Cells were incubated at 37°C and 5% CO2, and 24 hours, the cells were lysed, and proteins were separated using SDS-PAGE. Unmodified and modified protein levels were analyzed by immunoblotting with OTUD4, XPC, PARP, p53, and tubulin antibodies. B. HCT116 and HCT116 OTUD4<sup>−/−</sup> cells were mock treated (NO CIS) or treated with 1, 2, or 4 μM cisplatin. Cells were incubated at 37°C and 5% CO2, and 24 hours, the cells were lysed, and proteins were separated using SDS-PAGE. Unmodified and modified protein levels were analyzed by immunoblotting with OTUD4, PARP, p53, PML, MDM2, and tubulin antibodies.
Chapter 4: Discussion

4.1 OTUD4 and XPC involvement in DNA damage repair.

The differential induction of OTUD4 and XPC is intriguing in that there appears to be an inverse relationship between XPC modification and OTUD4 levels (Fig. 3.3). Both hydrogen peroxide and cisplatin induce an increase in OTUD4 levels, while ubiquitinated or modified XPC levels barely increase. Potentially, induction of OTUD4 could lead to increased deubiquitination of XPC after treatment with certain DNA damaging agents, therefore serving to control XPC modification and even response to DNA damage. Alternatively, UV irradiation does not induce an increase in OTUD4 levels but does induce a major increase in ubiquitination of XPC. While the focus of XPC involvement in DNA damage repair has classically focused on NER, there have been reports of XPC involvement in BER and ICL repair. Further inquiry into the exact role of XPC in these damage repair pathways and the identification of a characteristic that distinguishes this role in BER and ICL repair compared to NER could shed light on how ubiquitination regulates XPC and, further, how OTUD4 and other deubiquitinases help to regulate the protein.

The fractionation and microscopy data showing cytoplasmic localization of OTUD4 agree with previous reports of OTUD4 in the cytoplasm (Fig. 3.4). The discrepancy between the primary localization of OTUD4 in the cytoplasm and XPC in the nucleus poses an issue to the association of the two proteins. However, both proteins appear to translocate at the same time after UV irradiation, and the data do not rule out an indirect effect of OTUD4 on XPC. Further localization analyses using the HCT116 OTUD4<sup>−/−</sup> cell line could help delineate the role of OTUD4 in XPC translocation.
4.2 OTUD4 influence on XPC ubiquitination and stability.

The increase in unmodified and modified XPC levels after treatment with UV irradiation in cells with decreased amounts of OTUD4 or knocked out indicates that OTUD4 is involved in the deubiquitination and/or destabilization of XPC (Fig. 3.5–6). However, several questions remain. Is OTUD4 involved in simply the deubiquitination or destabilization of XPC and the other activity is an indirect effect? The higher unmodified levels of XPC in OTUD4 knockdown and HCT116 \( OTUD4^{-/-} \) cells compared with control cells even before treatment with UV irradiation indicates that OTUD4 affects XPC levels through altering XPC stability as opposed to through deubiquitination. So which protein is the target of OTUD4 deubiquitination whose degradation or altered function leads to decreased levels of unmodified and modified XPC? Furthermore, the exact function of the ubiquitination of XPC is not fully known. While reports suggest that ubiquitination aids in XPC binding to DNA, further understanding of XPC ubiquitination function could shed light on the role of OTUD4 and XPC deubiquitination. It has been postulated that differential ubiquitination could act as a regulator of the DNA damage response.

Though a certain amount of XPC does appear to degrade over the course of 24 hours, the half-life of the protein is very long, and degradation is not augmented in response to UV irradiation (Fig. 3.2). These data support previous reports of XPC not degrading after UV irradiation and subsequent ubiquitination. Though some XPC is degraded in a UV-independent manner, the majority of the protein is recycled with a stability exceeding 24 hours. Additionally, while we show the half-life of XPC to be long, XPC levels are known to drop after UV irradiation and then recover, though the reliance of this level fluctuation on degradation and/or recycling is as-of-yet unknown. Further understanding
of the regulation of XPC levels could help with understanding the role of OTUD4 and XPC stability.

Further transfections of p3x-Flag-OTUD4.full, p3x-Flag-OTUD4.short, and p3x-Flag in HCT116 $OTUD4^{-/-}$ cells analyzed against HCT116 cells would allow a direct comparison of the XPC ubiquitination levels of wild-type cells with cells expressing no, full-length, or truncated OTUD4 for a more thorough understanding of XPC ubiquitination levels after UV irradiation in cells expressing different forms of OTUD4. However, the fact that overexpression of full-length OTUD4 is not able to recover the wild-type XPC ubiquitination phenotype of HCT116 cells after UV irradiation and that the truncated form of OTUD4 lacking the catalytic site leads to higher levels of XPC ubiquitination after UV irradiation raises several questions (Fig. 3.10). Is the exogenous OTUD4 protein functional? Or does the endogenous protein get modified in some unknown yet essential way that prohibits the expression of functional protein without further knowledge? And more worryingly, is OTUD4 serving to deubiquitinate XPC or even serving as a deubiquitinase within the context of NER? Other deubiquitinases have been shown to serve as a non-enzymatic scaffold of chemical reactions. Could OTUD4 be such a protein scaffold? Or operating indirectly on XPC ubiquitination through an as-of-yet unknown mechanism?

4.3 Larger implications: OTUD4 involvement in cell survival and apoptosis.

The differential effect of OTUD4 knockout on XPC ubiquitination and stability after UV irradiation or cisplatin treatment speaks to the potential role of OTUD4 in regulating XPC throughout the different DNA repair pathways (Fig. 3.6–8). Further, knockout of OTUD4 does not alter the sensitivity of cells to UV irradiation, yet leads to an increase in the
sensitivity of cells to cisplatin treatment (Fig 8–9). The differential effect of OTUD4 knockout on cell survival after treatment with UV irradiation or cisplatin further supports this divergence. If XPC ubiquitination or stability play a role in determining XPC function or even pathway choice, the role of OTUD4 in this regulation could indicate a bigger significance for OTUD4 in DNA damage repair.

As the initial experiments indicated a similarity between hydrogen peroxide and cisplatin in their influence on XPC and OTUD4 levels, and OTUD4 showed a differential response to UV irradiation and cisplatin treatment including a drastic influence on the IC50 of cisplatin in HCT116 cells, a continuation of these studies involving an examination of the role of OTUD4 in hydrogen peroxide would be prudent (Fig. 3.3, 8–9). Should HCT116 OTUD4−/− cells demonstrate a similar or comparable phenotype in response to hydrogen peroxide as in response to cisplatin, the role of OTUD4 in DNA damage repair could be understood to a greater extent.

Though p53 and PARP are both involved in apoptotic pathways and typically demonstrate similar alterations in induction after treatment with damaging agents, there are apoptotic pathways which involve one or the other protein. OTUD4 appears to be involved in a p53-independent apoptotic pathway (Fig. 3.12). Further Western blot analysis using additional antibodies could be used to identify in which apoptotic pathway OTUD4 is involved.

### 4.4 Future studies.

Of particular concern is the differential response of OTUD4 to various DNA damaging agents and complexity of the OTUD4 response. Further confirmation of the results shown here as well as thorough examination of the exact molecular mechanism of this
deubiquitinase are warranted. However, OTUD4 has the potential to serve as an anti-apoptotic and mitogenic deubiquitinase susceptible to inhibition as a chemosensitization strategy.
## Table 3.1. Yeast Two Hybrid Results for DDB2, damage-specific DNA binding protein 2, 48kDa (*Homo sapiens*)

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