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# A site-directed mutagenesis study of the MdmX RING domain

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ABSTRACT

Mdm2 and MdmX are important negative regulators of the tumor suppressor p53. Structurally homologous Mdm2 and MdmX inhibit p53 by directly blocking p53 transcriptional activation. Mdm2 also modifies and targets p53 for 26S proteasome dependent protein degradation through E3 ligase activity mediated by its C-terminal RING domain. However, MdmX lacks intrinsic E3 ligase activity and fails to catalyze ubiquitination of p53 despite containing a conserved RING domain. Thus, a comparative structural analysis between the Mdm2 and MdmX RING domains offers a unique way to elucidate the distinct functions of the two proteins in ubiquitination. We performed site-directed mutagenesis of the MdmX RING domain and found that the substitution of the residue N448 for cysteine and the substitution of the residue K478 for arginine granted MdmX RING domain ubiquitination activity. The structural analysis of the Mdm2 and MdmX RING domains revealed that the residue C449 of Mdm2 (structurally homologous to MdmX RING N448) located at the Mdm2 RING dimer interface is critical for the stability of the RING dimer structure, while the residue R479 (structurally homologous to MdmX RING K478) plays a role in recruiting and activating the ubiquitin E2 conjugating enzyme. This study provides new insight into the molecular mechanism of Mdm2 RING domain mediated ubiquitination.


## 1. Introduction

Mdm2 (Murine Double Minute 2), the key negative regulator and primary E3 ligase for the tumor suppressor protein p53, is strongly associated with cancer development and progression. The *MDM2* gene is amplified or overexpressed in more than 10% of all cancers, 40–60% of human osteogenic sarcomas and about 30% of soft tissue sarcomas [1–3]. Overexpression of Mdm2 is more frequently detected in low-grade and advance-stage hematological malignancies where p53 is commonly inactivated through negative regulation rather than gene mutation [4]. Mdm2 functions as a master negative regulator of the tumor suppressor p53. Acting as a transcription factor, the p53 protein responds to genomic or oncogenic stress signals to activate a wide range of genes that cause tumor suppression by cell cycle arrest, apoptosis and senescence. The interaction between Mdm2 and p53 directly inhibits p53 transcription activity and blocks p53 dependent activation of downstream effectors [5]. More importantly, Mdm2 is the primary E3 ligase for p53, which modifies p53 by a process known as ubiquitination and targets p53 for the 26S-proteasome mediated protein degradation [6]. The critical role of Mdm2 in p53 regulation was clearly demonstrated by the p53 dependent embryonic lethality of Mdm2 knockout mice, which can be rescued by deletion of the p53 gene [7,8]. Thus, aberrant regulation of Mdm2 is a key factor in the inactivation of p53 in cancer cells and represents an important therapeutic target to restore p53 tumor suppressive activity in many forms of malignancy. In contrast to the rich structural knowledge on the interaction between p53 and Mdm2, little information is available about the active site for Mdm2 E3 ligase activity, which hinders the development of Mdm2 inhibitors targeting its E3 ligase activity. Ubiquitination activity of Mdm2 is attributed to its C-terminal RING domain, which performs a direct transfer of ubiquitin from a ubiquitin E2 conjugating enzyme onto its substrate proteins. Several research studies have made attempts to characterize the mechanism of RING domain mediated ubiquitin transfer; however, mechanistic details still remain poorly understood [9–11].

MdmX, Murine Double Minute X (also referred to as Mdm4), is another important negative regulator of p53. It is homologous to Mdm2 in both domain arrangement and inhibition of p53 transcriptional activity [12,13]. Similar to Mdm2, *MDMX* gene knockout in mice caused p53 dependent embryonic lethality, and the phenotype was rescued with a *TP53* gene knock-out [14]. Mdm2 and MdmX form a dimer via their C-terminal RING domains, which

*Abbreviations:* Mdm, Murine Double Minute; RING, Really Interesting New Gene.

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leads to Mdm2 stabilization and more efficient inhibition of p53 [15]. Although the MdmX RING domain shares 54% sequence identity with the Mdm2 RING domain and is structurally indistinguishable, it does not possess intrinsic E3 ligase activity [15–17]. However, the molecular basis for the lack of E3 ligase activity of the MdmX RING domain is still unknown.

Previously, our group and others have mapped the active site of the Mdm2 RING domain by mutating specific residues and testing whether these mutations resulted in a loss of the RING domain function [9–11,18]. Through this approach, a unique surface area was identified which appeared to contribute to Mdm2 E3 ligase activity [9]. It was formed by the basic residues surrounding the E2 binding site and the residues at the C-terminus of the RING domain responsible for mediating Mdm2 dimerization. This approach provided a quick road map for understanding the mechanism of Mdm2 mediated ubiquitination. However, it did not pinpoint the exact role of each residue, or reveal whether the mutation affected Mdm2 structurally or catalytically. Here we conduct a site-directed mutagenesis study on the RING domain of MdmX to further delineate the mechanism of the Mdm2 RING domain-mediated ubiquitination and identify the residues critical for this process. A comparative structural analysis revealing these subtle differences between the Mdm2 and MdmX RING domains will offer critical information on the active site of the RING domain that makes Mdm2 active, but MdmX inactive.

## 2. Materials and methods

### 2.1. Molecular cloning and site-directed mutagenesis

MdmX RING (416–491) was cloned from the human MdmX (1–491) into pET-15b (Novagene) and pGEX-2TK (GE) expression vectors. Mutagenesis of the MdmX RING domain was carried out using QuikChange® Site-Directed Mutagenesis Kit.

### 2.2. Protein expression and purification

All proteins were expressed in *Escherichia coli* BL21 DE3 Codon Plus cells (Stratagene) using 1 mM IPTG for 16 h at 16 °C. 150 μM $ZnCl_2$ was added to the growing bacterial cultures. $His_6$-tagged and GST-tagged wild-type and mutated proteins were purified using standard nickel and GST affinity chromatography procedures, respectively. All protein samples were stored at −80 °C until use.

### 2.3. In vitro ubiquitination assays

A single ubiquitination reaction contained 0.1 μg of E1, 0.2 μg of UbE2D2, and 5 μg of ubiquitin. 0.5 μg of either Mdm2 RING or MdmX RING ($His_6$-tagged RING domains) was used as E3. For MdmX ubiquitination, 0.5 μg of GST-tagged MdmX full-length was added into the ubiquitination reaction. The reactions were performed in 20 μl in the buffer containing 50 mM Tris pH 7.6, 5 mM $MgCl_2$, 2 mM DTT, 0.1× protease inhibitor, and 2 mM ATP. Following a 90-minute incubation at 30 °C, 5× SDS–PAGE sample buffer was added to stop the reactions. Polyubiquitination was visualized by immunoblotting using a ubiquitin specific monoclonal antibody (Covance). Ubiquitination of the GST-tagged MdmX was visualized using a monoclonal anti-GST antibody (GE Healthcare).

### 2.4. Protein structural analysis

Solution structure of the Mdm2 RING homodimer (PDB 2HDP) and crystal structure of the Mdm2/MdmX RING heterodimer (PDB 2VJE) were used to analyze the Mdm2 or MdmX RING domain [10,19]. The computer model of the E2–E3 complex was generated using the crystal structure of the cIAP2–UbcH5B complex (PDB 3EB6) and the crystal structure of the Mdm2/MdmX RING heterodimer (PDB 2VJE) [10,20]. The Mdm2 RING and MdmX RING subunits were aligned with the cIAP2 RING domain. PyMol was used for structural analysis and for generating figures.

## 3. Results

### 3.1. Sequence analysis of the Mdm2 and MdmX RING domains

It is hypothesized that amino acid differences between the RING domains of Mdm2 and MdmX may be responsible for their functional differences. To test this hypothesis, a sequence and structural analysis of the Mdm2 and MdmX RING domains was performed to compare their catalytic sites. As shown by the sequence alignment, the Mdm2 and MdmX RING domains are highly conserved throughout evolution (Fig. 1A). There are 30 conserved residues forming the core structure of the RING domain composed of three β-strands and one α-helix. Among these conserved residues are six cysteines and two histidines, which are important for the coordination of two zinc ions and essential for proper folding of the RING domain [21]. There are 34 different residues between Mdm2 and MdmX that are distributed throughout the RING domain structure with the majority located in the loop region (Fig. 1A, non-shaded residues within sequence alignment). The fact that these residues were not conserved between Mdm2 and MdmX indicates the possibility of these residues being responsible for E3 ligase activity of the Mdm2 RING domain. In order to understand the role of these residues in the Mdm2-mediated ubiquitination, a set of residues of the MdmX RING domain were chosen to be mutated into the corresponding Mdm2 RING residues. These residues include MdmX S438 and E441 located in close proximity to the first zinc coordination site; T459, H462, and K478, located at the putative E2 enzyme interaction surface, and N448 and R453 within the dimerization interface of the RING domain dimer (Fig. 1B). Site-directed mutagenesis of these MdmX RING domain residues was performed, and the mutated MdmX RING domains were tested for their E3 ligase activity.

### 3.2. E3 ligase activity of the MdmX RING domain mutants

In this study, three single MdmX RING domain mutants (N448C, T459A, and K478R) and four double mutants (S438V/E441Q, N448C/K478R, R453K/K478R, and T459A/H462T) were generated. The mutant MdmX RING domains were purified as $His_6$-tagged proteins and tested for their E3 ligase activity using *in vitro* ubiquitination assays.

We tested the ability of MdmX RING domain mutants to perform autoubiquitination, which is a covalent attachment of ubiquitin molecules onto the E3 ligase itself (wild-type and mutated MdmX RING domains) (Fig. 2A). In this assay, the wild-type Mdm2 RING domain was used as the positive control and the wild-type MdmX RING domain as the negative control. Each reaction included E1, E2 (UbE2D2/UbcH5B), $His_6$-tagged E3 (Mdm2 RING, MdmX RING, or MdmX RING mutant), ubiquitin, and ATP. The efficiency of the E3 ligase in each reaction was evaluated based on the amount of the polyubiquitin chain produced, which was detected by immunoblotting using an antibody against ubiquitin. As expected, Mdm2 RING domain demonstrated strong autoubiquitination activity. MdmX T549A mutant did not show any autoubiquitination activity. MdmX K478R, T459A/H462T, S438V/E441Q, and R453K/K478R mutants gained weak ubiquitination activity. Interestingly, a point mutation N448C and a double

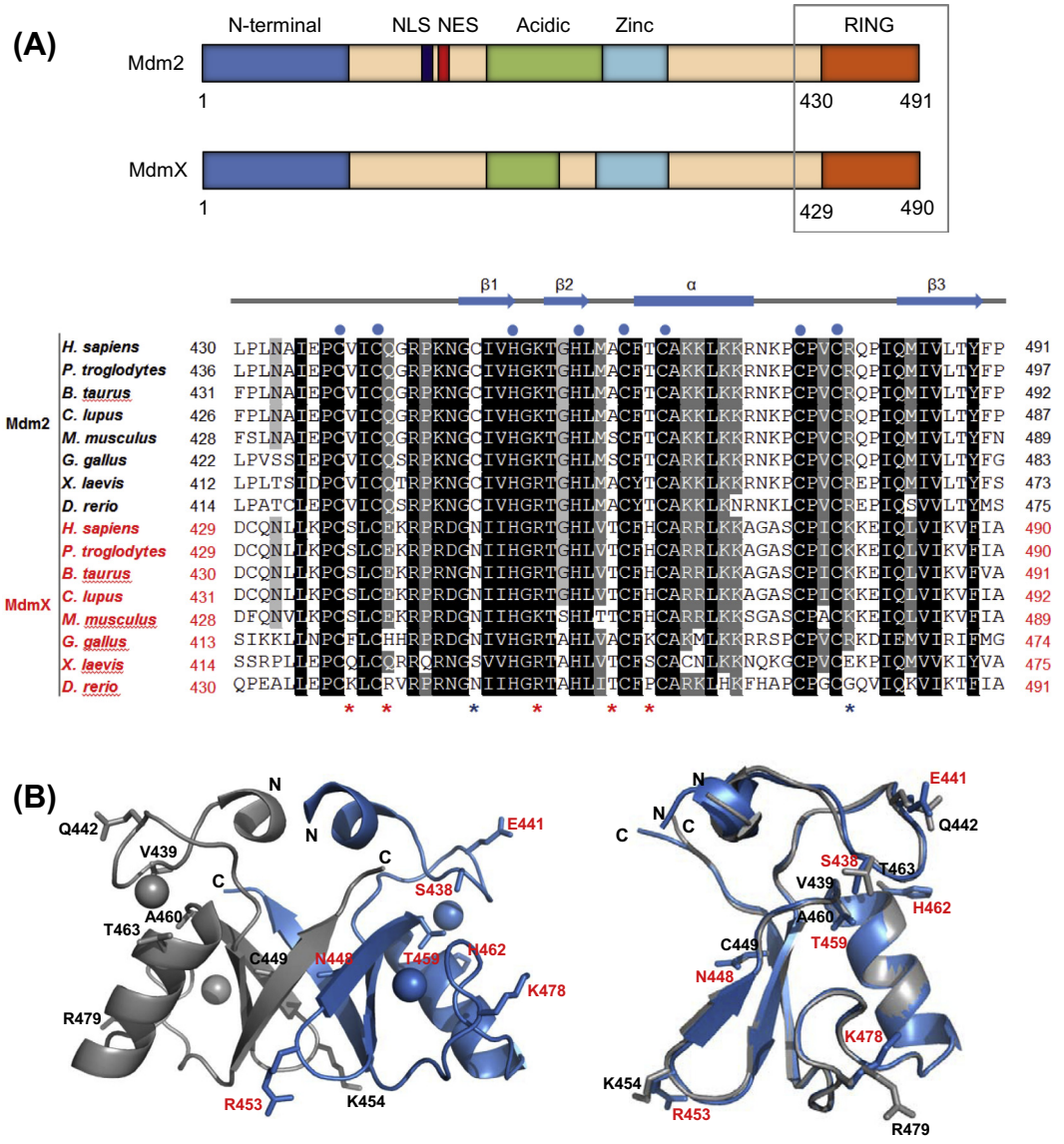


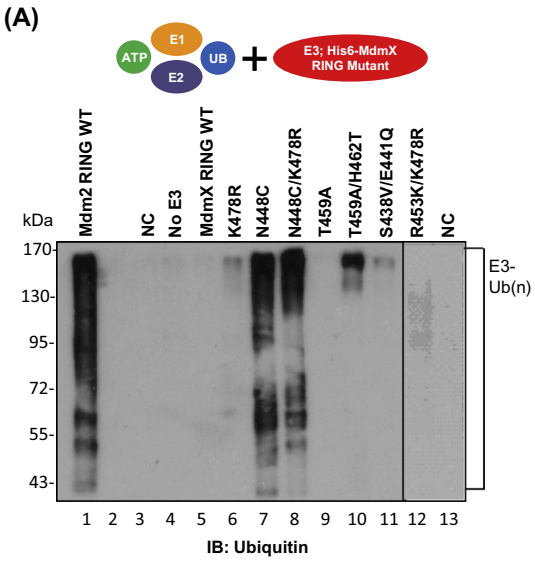

**Fig. 1.** Sequence analysis of the Mdm2 and MdmX RING domains. (A) Top panel, Domain arrangement of Mdm2 and MdmX. Bottom panel, Secondary structure and sequence alignment of the Mdm2 and MdmX RING domains from different species. Conserved and less conserved residues between Mdm2 and MdmX RING domains are highlighted in black and gray, respectively. The zinc coordinating cysteine and histidine residues are indicated by blue circles. The residues that were mutated within the dimerization and E2 binding regions are shown by blue and red stars, respectively. (B) Left panel, Ribbon diagram of the crystal structure of the Mdm2/MdmX RING domain heterodimer (PDB 2VJE). Right panel, Structural superposition of the Mdm2 and MdmX RING domain monomers. The Mdm2 RING domain is colored in gray and MdmX RING in blue. The Mdm2 and MdmX RING domain residues under investigation are shown in sticks and labeled in black and red, respectively.

mutation N448C/K478R granted the MdmX RING domain the ability to ubiquitinate itself similarly to the Mdm2 RING domain. This suggested that the respective residues Mdm2 C449 and R479 play important roles in the Mdm2-mediated ubiquitination (Fig. 2B).

Following autoubiquitination, the MdmX RING domain mutants were tested for the ability to perform substrate ubiquitination (Fig. 2B–D). As MdmX is one of the ubiquitination substrates of Mdm2, the wild-type full-length GST-tagged MdmX was used as a substrate in an independent *in vitro* ubiquitination experiment. Ubiquitinated full-length MdmX was detected by immunoblotting using an anti-GST tag antibody (Fig. 2B and C). As expected, the Mdm2 RING domain showed strong ability to ubiquitinate the full-length MdmX, whereas the MdmX RING domain showed no ubiquitination activity. In the experiment using the MdmX domain mutants as the E3 ligase, only the double substitution N448C/K478R was able to ubiquitinate the full-length MdmX similarly to wild-type Mdm2 activity. MdmX N448C and K478R showed weak activity in ubiquitination of the GST-tagged full-length MdmX. In addition, the total polyubiquitination was detected using a ubiquitin specific antibody (Fig. 2B and D). Consistent with the results from the previous autoubiquitination assay (Fig. 2A), MdmX K478R showed weak E3 ligase activity, while MdmX N448C and N448C/K478R demonstrated strong activity in catalyzing polyubiquitination (Fig. 2D). Thus, the Mdm2 RING domain residues C449 and R479 were found to be important for ubiquitination of the full-length MdmX. Interestingly, MdmX N448C could not ubiquitinate the substrate full-length MdmX similarly to wild-type Mdm2 despite its ability to catalyze polyubiquitination.

### 3.3. Structural analysis of the MdmX RING domain mutants

Since the mutants MdmX N448C and K478R were able to gain E3 ligase activity similarly to levels seen in wild-type Mdm2, structural analysis of the Mdm2/MdmX RING domain was carried out to




(B)

| E3 ligase | Auto-ubiquitination activity | Substrate ubiquitination activity |
|---|---|---|
| Mdm2 RING WT | ++++ | ++++ |
| MdmX RING WT | --- | --- |
| K478R | + | + |
| N448C | +++ | + |
| N448C/K478R | +++ | +++ |
| T459A | --- | --- |
| T459A/H462T | ++ | --- |
| S438V/E441Q | + | --- |
| R453K/K478R | + | ND |

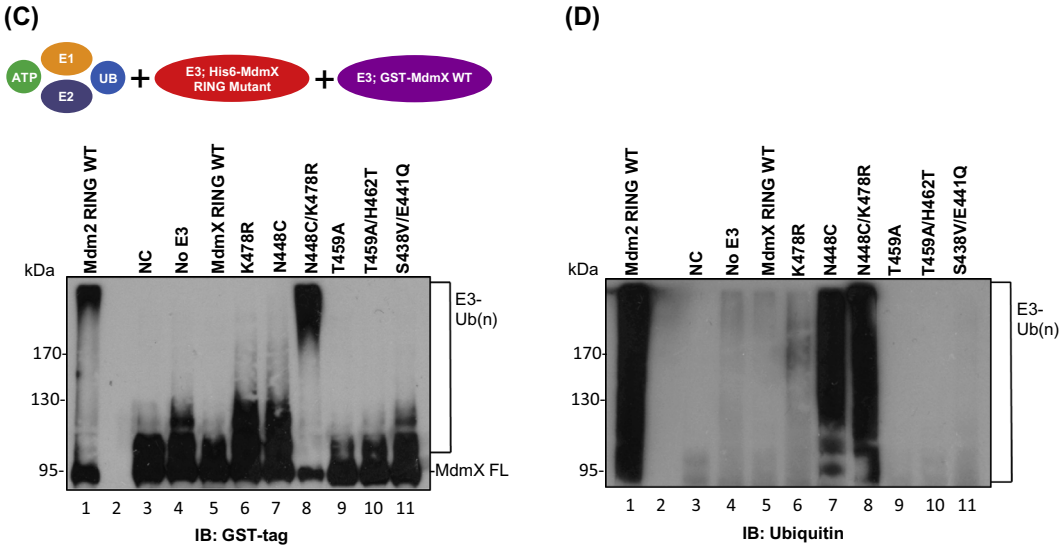



**Fig. 2.** Ubiquitination activity of the MdmX RING domain mutants. (A) Autoubiquitination activity of the Mdm RING domains was investigated with an *in vitro* ubiquitination assay containing E1, E2, ubiquitin, and ATP using the MdmX RING domain mutants as an E3 ligase. Ubiquitination activity was detected using a ubiquitin specific antibody. (B) E3 ligase activity exhibited by the wild-type Mdm2 and MdmX RING domains, as well as of the mutated MdmX RING domains is estimated based on the intensity of the polyubiquitination signal and is summarized in the table. (C and D) Ubiquitination of the GST-tagged MdmX by the MdmX RING domain mutants. (C) Ubiquitination of the GST-MdmX was detected using an antibody against GST. (D) Total ubiquitination was detected using a ubiquitin specific antibody.

understand the roles of these two residues in the Mdm2 RING domain mediated ubiquitination.

In the Mdm2/MdmX dimer structure, Mdm2 C449 and MdmX N448 are located at central β-barrel with the side chains protruding into the central space from the opposite sides. When overlaying the Mdm2 RING and MdmX RING domain monomers, Mdm2 C449 and MdmX N448 reside at the same position of the β1-sheet (Figs. 1C and 3A). Since both residues are buried in the hydrophobic core upon dimerization of the Mdm2 and MdmX RING domains, they are not likely to make direct contact with an E2 enzyme. However, two observations were made for MdmX N448 emphasizing the important role of this residue in ubiquitination. (1) MdmX N448 possesses a polar side chain, which creates a different intra-molecular interaction network in comparison to the structurally homologous Mdm2 C449. Specifically, in the hetero-dimer structure of Mdm2/MdmX RING domain, the δ-NH2 of MdmX N448 forms a hydrogen bond with the γ-OH of Mdm2 T488, which stabilizes the central β-barrel of the dimer (Fig. 3B). However, in the case of MdmX dimer, the polar side chain of two N448 residues in the middle of the hydrophobic core would be expected to destabilize the dimer structure due to decreased hydrophobicity of the core. (2) MdmX N448 could affect the hydrogen bonding network of the neighboring residues. The side chain of asparagine is known to actively participate in hydrogen bonding with the backbone of the neighboring residues [22]. In the hetero-dimer structure of the Mdm2/MdmX RING domain, δ-carboxyl oxygen of MdmX N448 resides within hydrogen bonding distance (3 Å) of the backbone amide group of MdmX I449 suggesting that N448 can potentially affect the hydrogen bonding network and local dynamics of the neighboring MdmX H456 and I449. H456 is critical for zinc coordination, whereas I449 forms part of the hydrophobic surface essential for recruitment of the ubiquitin-conjugating enzyme E2 [9]. The presence of MdmX N448 may have a negative effect on stability of the RING structure as well as an allosteric effect on the E2 binding. In contrast, the side chain of Mdm2 C449 displays more hydrophobicity and is not expected to interfere with the local hydrogen bonding network.

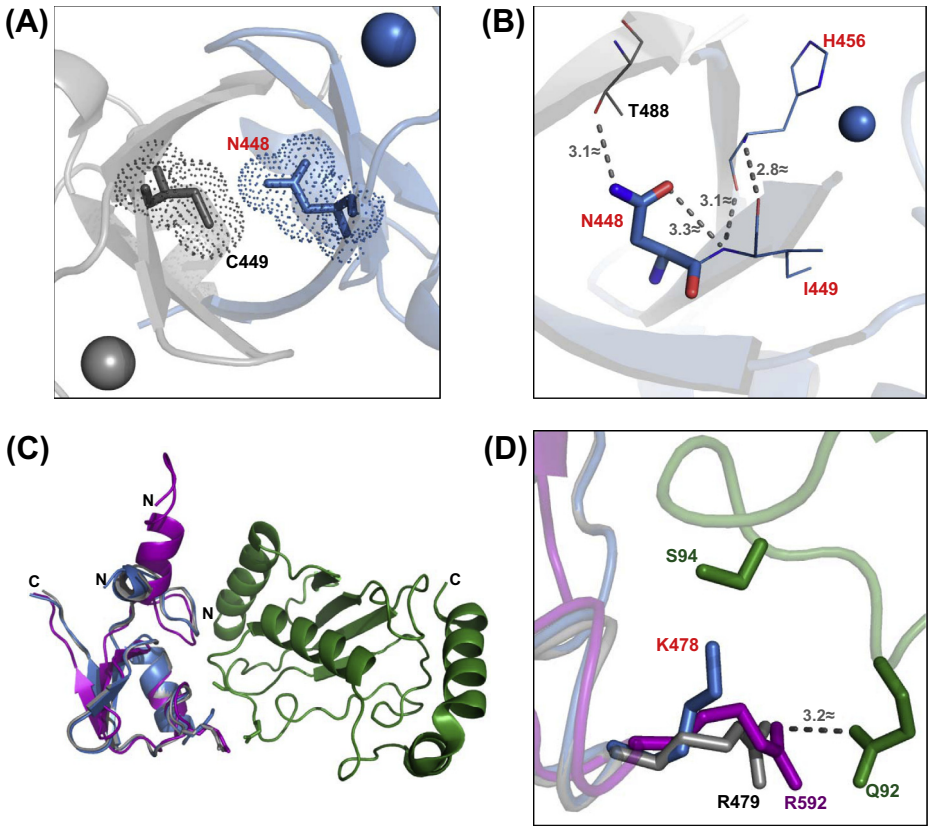



**Fig. 3.** Structural analysis of the Mdm2 and MdmX RING domains. (A) The dimerization region of the Mdm2/MdmX RING domain heterodimer (Mdm2: gray; MdmX: blue). Mdm2 C449 and MdmX N448 are shown in sticks and labeled in black and red, respectively. (B) The hydrogen bonding network around the MdmX N448. Mdm2 T488 is labeled in black. MdmX residues are labeled in red. (C) The structural model of the Mdm2/MdmX RING domain:E2 complex based on the crystal structure of the cIAP2 RING domain:E2 (PDB 3EB6, PDB 2VJE). The Mdm2 RING is colored in gray, MdmX RING in blue, cIAP2 RING in purple, E2/UbcH5B in green. (D) The molecular interactions between the RING domains and E2. The Mdm2 R479 residue is labeled in black, MdmX K478 in red, cIAP2 R592 in purple, and E2/UbcH5B S94 and Q92 in green. A polar contact between cIAP2 R592 and E2/UbcH5B Q92 is shown with dashed lines.

In addition, we investigated the role of Mdm2 R479 and its counterpart K478 in MdmX. Mdm2 R479 and MdmX K478 reside close to the second zinc ion and at the edge of the hydrophobic surface required for E2 binding. The side chains of the two residues point away from the structure and are solvent-exposed. Since the RING structure of Mdm2 is highly homologous to cIAP2, the complex structure of cIAP2 RING domain and UbE2D2 (PDB 3EB6) was used to model E2 binding for the Mdm2 RING domain. When the RING domains of Mdm2 and MdmX were superimposed with cIAP2 RING domain, Mdm2 R479 and MdmX K478 were found to reside at the same position as cIAP2 R592. cIAP2 R592 forms a hydrogen bond with UbE2D2 Q92, a critical residue within the catalytic cleft of the E2. The interaction is not only important for the E2-RING domain binding, but also essential for allosteric activation of ubiquitin transfer from the catalytic cysteine of the E2 to a substrate lysine residue [23]. While the side chain of Mdm2 R479 overlaps with cIAP2 R592 pointing towards UbE2D2 Q92, the side chain of MdmX K478 moves away from the position, suggesting Mdm2 R479 is more favorable in recruiting and activating the E2, which is consistent with the data that MdmX K478R is more active in catalyzing ubiquitination.

## 4. Discussion

In this study, structural and functional analyses of the Mdm2 and MdmX RING domains were performed to further explore and characterize the E3 ligase active site. Specifically, a number of residues within the Mdm2/MdmX dimerization surface and the E2 binding surface of the Mdm2 RING domain were selected and introduced into the MdmX RING domain at the corresponding sites. Since MdmX lacks intrinsic E3 ligase activity, the introduced Mdm2 residues that granted the MdmX RING domain ubiquitination activity were considered critical for Mdm2 RING domain mediated E3 ligase activity.

Function of Mdm2 as a ubiquitin ligase depends on: (1) dimerization via the RING domain, and (2) recruitment of a ubiquitin E2 conjugating enzyme. In this study, two Mdm2 residues C448 and K454 at the dimerization interface were introduced into the MdmX RING domain at the residues N449 and R453, respectively. The mutant N449C at the Mdm2/MdmX dimerization interface results in MdmX RING E3 ligase activity. Based on the structural analysis of the Mdm2/MdmX RING domain complex, N449 may affect the local hydrogen-bonding network and negatively influence RING dimerization and E2 binding [9,11,18]. The role of Mdm2 K454 and MdmX R453 was studied in the double mutant MdmX R453K/K478R. Even though MdmX K478R single mutant acquired E3 ligase activity as discussed below, MdmX R453K/K478R mutant showed very little effect, suggesting that K454 does not contribute to E3 ligase activity of the Mdm2 RING domain. This is consistent with the previous studies that reported that mutation of Mdm2 K454 did not affect the ubiquitination function of Mdm2 [18,24].

We also introduced the Mdm2 residues V439, Q442, A460, T463 and R479 into MdmX at the residues S438, E441, T459, H462 and K478, respectively. These residues are within or close to the E2 binding site. K478R, T459A/H462T, and S438V/E441Q mutations

granted MdmX RING weak autoubiquitination activity. This implies that hydrophobicity and charge distribution in the E2 binding site play an important role in determining E3 ligase activity. Mdm2 RING R479 has been mutated in previous studies, and its substitution abolished E3 ligase activity of Mdm2 [9,10]. Structural analysis of the Mdm2/MdmX RING domain indicates that Mdm2 R479 plays a critical role in both E2 binding and activation of ubiquitin transfer from E2 to a substrate. Interestingly, only MdmX N448C/K478R was able to perform autoubiquitination and ubiquitination of MdmX full length, further implying the importance of these two residues in the function of the Mdm2 RING domain.

This study aimed to identify the residues in the Mdm2 RING domain essential for its E3 ligase activity. A number of residues located within the Mdm2/MdmX dimerization interface and the E2 binding site were determined to be essential for ubiquitination activity. These results confirmed the significance of maintaining integrity of the hydrophobic core of the Mdm2 RING domain and the critical role of the E2 binding region for its E3 ligase activity. This study provides further insight into the mechanism of the Mdm2 RING domain mediated ubiquitination and a structural basis for future rational design of Mdm2 inhibitors targeting its E3 ligase activity.

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