MDM2 interacts with MDMX through their RING finger domains

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Abstract The N-terminus of MDM2 proto-oncoprotein interacts with p53 and down modulates p53 activity by inhibiting transcriptional activity and promoting p53 degradation. MDMX is structurally related to MDM2 and also binds to p53. However, the function of MDMX has not been clarified yet. We found that MDM2 hetero-oligomerized with MDMX through their C-terminal RING finger domains. Yeast two-hybrid analysis revealed that the hetero-oligomerization between MDMX and MDM2 was more stable than the homo-oligomerization of each protein. MDM2 has been shown to be degraded by the ubiquitin-proteasome pathway, while MDMX was a stable protein. Interaction of MDMX with MDM2 through the C-terminal RING finger domains resulted in inhibiting degradation of MDM2. These data indicate that MDMX functions as a regulator of MDM2.

Key words: MDM2; MDMX; p53; RING finger

1. Introduction

The p53 tumor suppresser gene product plays an important role in the prevention of malignancies. The function of this protein is lost in many cancers and sarcomas. p53 exhibits DNA binding activity and functions as a transcription factor. It induces the expression of a number of genes including p21/WAF/Cip1, Bax, cyclin G, GADD45, and MDM2, which are involved in cell cycle regulation, DNA repair and apoptosis [1–3]. Among them, MDM2 has been suggested as being a negative feedback regulator of p53 [4].

The MDM2 oncogene was first cloned as an amplified gene on a murine double-minute chromosome in the 3T3DM cell line [5]. Overexpression of MDM2 increased the tumorigenic potential of immortalized NIH3T3 cells, demonstrating that the MDM2 protein does possess oncogenic activity [6]. The MDM2 gene was found to be amplified in a significant proportion of human sarcoma as well as in a variety of other tumors [7].

The gene encodes the 489 amino acid polypeptide that contains a p53 binding motif at the N-terminus, an acidic region and two putative metal binding motifs (zinc finger motif and RING finger motif) [8]. The expression of MDM2 increases at least 1 h after the induction of p53 and has been thought to suppress the activity of p53 by masking the N-terminal transactivation domain of p53 [9,10]. Furthermore, recent reports indicate that MDM2 accelerates p53 degradation and $p19^{ARF}$ has been shown to regulate this process [11–14]. MDM2 C-terminal regions are suggested to function as ubiquitin ligase E3 for p53 [15,16], and MDM2 itself is degraded by the ubiquitin-proteasome pathway [17].

MDMX, which is structurally similar to MDM2, was cloned as a p53-binding protein [18,19]. Like MDM2, MDMX interacts with the N-terminal region of p53 and partially inhibits p53 transcriptional activity. Importantly, expression of MDMX seems to be regulated differently from that of MDM2 [18]. The level of MDMX mRNA is unchanged after UV irradiation, in contrast to MDM2 mRNA. However, the physiological function of MDMX is unknown.

In the present study, we screened MDM2 C-terminus-interacting proteins by using a yeast two-hybrid system. We found that the C-terminal region of MDM2 containing the RING finger motif bound to the comparable region of MDMX. This interaction is dependent on the presence of the RING finger domain and hetero-oligomerization between MDM2 and MDMX was more stable than homo-oligomerization of each protein. This study is the first demonstration of hetero-oligomeric interaction between the two-related RING finger domains. Coexpression of MDMX inhibited degradation of MDM2, suggesting that MDMX modulates ubiquitin-proteasome dependent proteolysis of MDM2 by interacting with the C-terminal region.

2. Materials and methods

2.1. Yeast two-hybrid assay

The cDNAs encoding the N-terminal half (1–294) and the C-terminal half (294–491) of human MDM2 were cloned into a pBTM116 vector in frame with the LexA DNA-binding domain to yield pBTM-MDM2 vectors. These vectors were transfected into yeast strain L40 as described [20]. The human thymus cDNA library in the pGAD10 vector (Clontech) was introduced into the L40 transformant bearing pBTM-JH1 by the LiCl method. Yeast expressing interacting proteins was identified by growth on media lacking leucine, tryptophan and histidine, and confirmed by assaying for β-galactosidase activity [20].

2.2. Binding assay between MDM2 and MDMX in vitro and in vivo

Full length MDM2 cDNA was fused with N-terminal Myc epitope tag sequence and subcloned into a mammalian expression vector pCAGGS [20]. Full length MDMX cDNA was subcloned into pcDNA3 with N-terminal Myc-epitope tag [20]. C-terminal truncated MDMX (MDMXΔC) was obtained by deleting the *Eco*RI/*Xba*I fragment from pcDNA3-MDMX. The resulting cDNA lacked a C-terminal 96 amino acid containing the RING finger domain. GST-MDM2 or GST-MDMX RING finger domain constructs were obtained by the subcloning of appropriate PCR fragments into pGEX-4T vectors (Pharmacia).

For in vitro binding assay, GST-RING finger domains were produced in NM522 bacteria and purified on GSH-Sepharose in the presence of 1 mM $ZnCl_2$. MDM2 and MDMX or MDMXΔC were transiently expressed in 293 cells by the calcium phosphate precipitation method. After 2 days of transfection, cells were lysed in 1 ml of a lysis buffer (20 mM HEPES buffer pH 7.3, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride and 1% aprotinin), then centrifuged. Cell extracts were incubated with 20 μl (50%

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v/v) of GST-RING finger fusion protein bound to GSH-Sepharose, then washed extensively with phosphate buffered saline containing 0.5% NP-40, and analyzed by immunoblotting with anti-anti-Myc (9E10) antibody as described [20].

For in vivo binding, MDM2 was co-expressed with MDMX or MDMXΔC in 293 cells, then immunoprecipitated with monoclonal anti-MDM2 antibody (Santa Cruz Biotechnology). After 2 h incubation on ice, 20 μl protein G-Sepharose was added to lysate and incubated for a further 2 h. The beads were washed three times, then subjected to immunoblotting with anti-Myc antibody.

2.3. Degradation assay

MDM2 was co-expressed with MDMX or ΔC-MDMX in 293 cells. After 30 h transfection, 30 μg/ml cycloheximide with or without 10 μM lactacystin [17] was added to the culture medium and cells were further cultured for 0, 3, 6, or 12 h. After lysis, cell extracts were subjected to 7% SDS-PAGE and immunoblotting with anti-Myc.

3. Results

3.1. Identification of MDMX as a MDM2-binding protein

To isolate MDM2-interacting protein, we screened several yeast two hybrid libraries using MDM2 the N-terminal (1–294) or C-terminal half (294–491) as baits (Fig. 1a). From thymus cDNA library, we isolated six independent positive clones of various lengths of MDMX genes by screening with the C-terminal half but not with the N-terminal half. One of the clones contained full length MDMX cDNA (Fig. 1a; CT-2) and the shortest one contained only 58 amino acids including C-terminal RING finger domain (codon 432–490) (Fig. 1a; CT-34). Therefore, the C-terminal half of MDM2 that contains the Zinc finger motif and the RING finger motif interacts with the RING finger domain of MDMX.

We compared the strength of MDM2/MDMX interaction by using yeast two-hybrid assay (Fig. 1b). The yeast strain transfected with pBTM-MDMX (a bait containing the LexA-binding domain BD) and pGAD-MDMX (a prey containing Gal4-activation domain AD) did not grow without histidine (His), indicating that MDMX did not form homo-oligomer (Fig. 1b, A). On the other hand, the yeast strain transfected with pBTM-MDM2 and pGAD-MDM2 grew very slowly, suggesting that the two MDM2 molecules interact weakly (Fig. 1b, C). However, MDMX and MDM2 interacted more strongly (Fig. 1b, B and D). These data indicate that MDMX and MDM2 preferentially form hetero-oligomer. Similar results were obtained by quantitative β-galactosidase assay (data not shown).

To identify the region of MDM2 that interacts with the RING finger domain of MDMX, several C-terminal regions of MDM2 were examined for interaction with the MDMX RING finger domain using yeast two-hybrids (Fig. 2a). As shown in Fig. 2b, the MDMX RING finger domain, but not the zinc finger domain, did interact with the RING finger domain of MDM2 (Fig. 2b, C, G). The MDMX RING finger domain did not interact with the zinc finger domain or the internal domain of MDM2 (B,D). The MDMX Zinc finger domain did not interact with any regions of MDM2 (E–H). These data indicate that MDM2 and MDMX forms hetero-oligomer through their RING finger domains.

3.2. Binding of MDMX with MDM2 in vitro and in vivo

To confirm the interaction between MDM2 and MDMX in vitro and in vivo, we subcloned a Myc-epitope tagged version of the full length MDM2 (Myc-MDM2) and full length MDMX (Myc-MDMX) into mammalian expression vectors

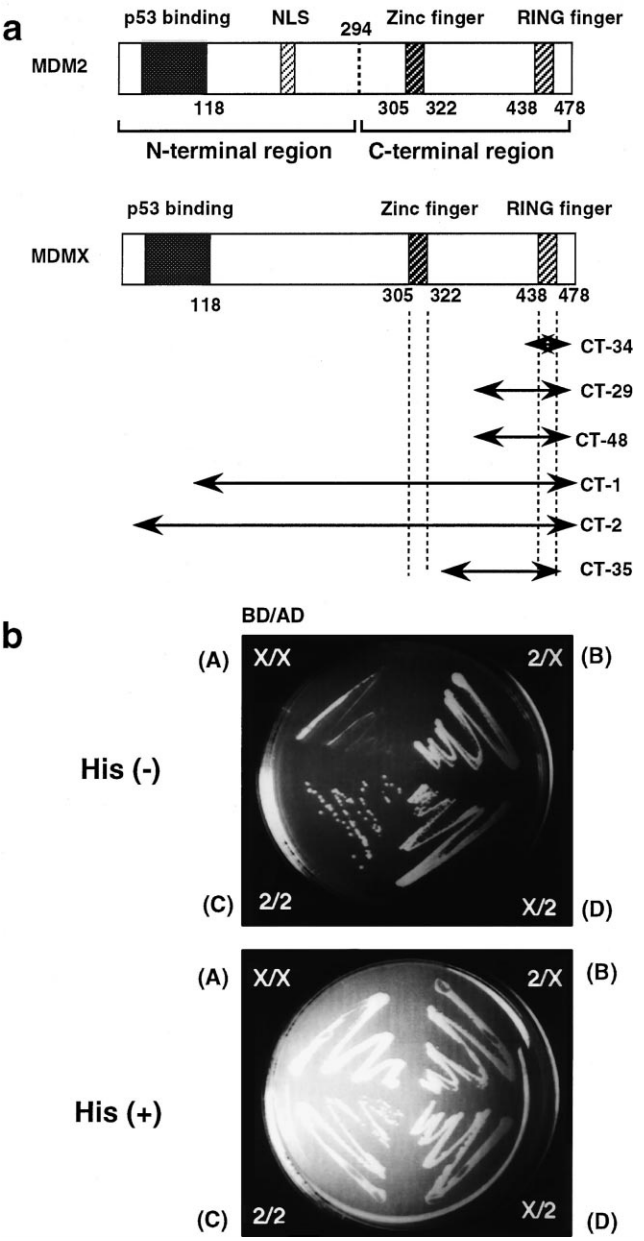

Fig. 1. MDM2 binds to the C-terminal region of MDMX. a: Structure of MDM2 used as bait and MDMX cDNA clones obtained by yeast two hybrid screening from a human thymus cDNA library. Positions (codon numbers) for p53 binding region, nuclear localization signal (NLS), zinc finger and RING finger are shown. CT-34, CT-29, CT-48, CT-1, CT-2 and CT-35 are clones obtained by screening using MDM2 C-terminal region as bait. b: Growth of L40 strain transfected with MDM2 and/or MDMX cDNAs either in the presence (His^+) or absence (His^-) of histidine. BD/AD indicates the combination of binding domain (bait) and activation domain (prey). Yeast L40 was transfected with pBTM-MDMX (full length) and pGAD-MDMX (A), pBTM-MDM2 (C-terminal half region) and pGAD-MDMX (B), pBTM-MDM2 and pGAD-MDM2 (C) or pBTM-MDMX and pGAD-MDM2 (D).

and transiently transfected into 293 cells. The MDM2 RING finger domain, MDM2 zinc finger domain or MDMX RING finger domain were fused to GST and purified from bacteria. These GST-fusion proteins immobilized on GSH-Sepharose were incubated with cell extracts containing Myc-MDM2 or Myc-MDMX (Fig. 3a). The MDM2-RING

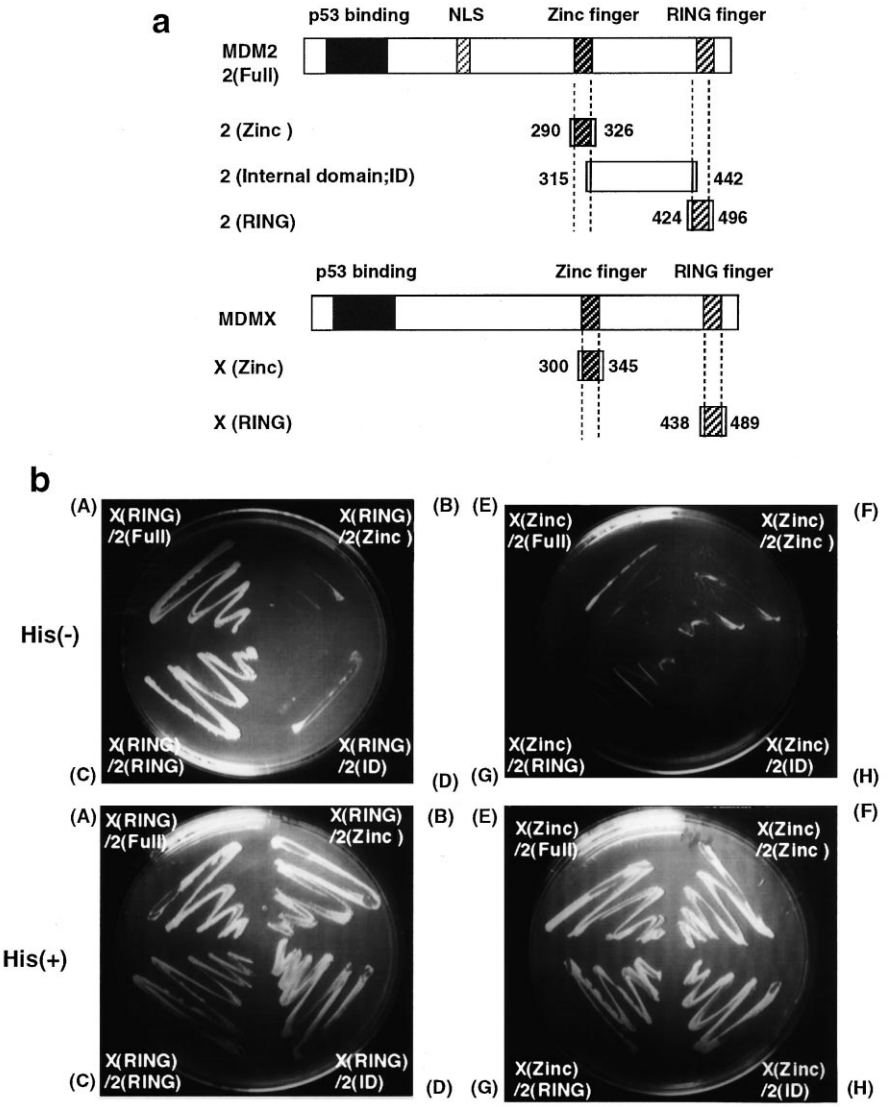

Fig. 2. RING finger domain interacts with MDMX RING finger domain. a: cDNA fragments corresponding to the zinc finger domain (Zinc), internal domain (ID), and the RING finger domain (RING) of MDM2 and MDMX were subcloned in pBTM (bait) or pGAD (prey). The numbers indicate positions for subcloned regions. b: Growth of yeast L40 strains expressing indicated combinations of MDM2 and MDMX regions cloned in pBTM/pGAD as in Fig. 1b, in the presence (His^+) or absence (His^-) of histidine.

finger domain (GST-2RING) but not the MDM2 zinc finger domain (GST-2Zinc) could capture Myc-MDM2 and Myc-MDMX (lanes 5,6). The MDMX RING finger domain (GST-XRING) bound strongly to Myc-MDM2 (lane 12), and weakly to Myc-MDMX (lane 11). Unlike the result of the yeast two-hybrid assay, we detected relatively strong homo-oligomeric interaction between GST-MDM2 RING and Myc-MDM2 in vitro (lane 6). This may be due to the very high concentration of GST-RING finger domain in vitro. MDMX lacking C-terminal RING finger domain (MDMXΔC) did not bind to RING finger domains of MDM2 and MDMX (lanes 4,10).

Next we examined the hetero-oligomer formation of MDM2 and MDMX in vivo. MDM2 was co-expressed with either full length MDMX or MDMXΔC in 293 cells, and immunoprecipitated with an anti-MDM2 monoclonal antibody. As shown in Fig. 3b, Myc-MDMX was co-immunoprecipitated with anti-MDM2 (lane 11), while Myc-MDMXΔC was not co-precipitated (lane 12). The intensity of the MDMX band co-precipitated with MDM2 was almost equal to that of the MDM2 band, suggesting that the interaction between MDM2 and MDMX is quite strong and stable. These data indicate that MDMX interacts with MDM2 through its C-terminal RING finger domain in vivo as well as in vitro.

3.3. *Effect of co-expression of MDMX on MDM2 stability*

Since the MDM2 C-terminal region has been shown to associate with ubiquitin-ligase activity, we examined ubiquitin-proteasome dependent degradation of MDM2 in the presence or absence of MDMX. Stability of the proteins was examined by immunoblotting after adding cycloheximide to block new protein synthesis. As shown in Fig. 4a, Myc-MDM2 was degraded with a half-life about 3–6 h. Degradation was almost completely abrogated by the proteasome inhibitor, lactacystin (Fig. 4b), confirming that the degradation of MDM2 is dependent on proteasome [17]. The appearance of the multiple higher molecular bands of MDM2 in the presence of proteasome inhibitor suggests the ubiquitination of

the molecule [17]. In contrast, MDMX was a very stable protein. The protein level of MDMX was not much changed after 12 h of cycloheximide treatment (Fig. 4c). When full length MDMX was co-expressed in MDM2, degradation of MDM2 was markedly suppressed (Fig. 4d). In contrast, Myc-MDMXΔC, which lacked a RING finger domain and did not interact with MDM2, could not affect the half-life of MDM2 (Fig. 4e). These data indicate that MDMX stabilizes MDM2 by interacting with the MDM2 RING finger domain.

4. Discussion

In this report, we demonstrated that MDMX hetero-oligomerizes with MDM2 through their RING finger domains. The RING finger motif is a subgroup of the zinc finger motif, which was originally found to be involved in protein-DNA interaction. However, more and more examples suggest that the RING finger domain is involved in protein-protein interactions rather than protein-DNA interactions. For example, the N-terminal RING finger domain of BRCA-1, the hereditary breast and ovarian cancer tumor suppresser gene, interacts with BAP-1, a novel ubiquitin hydrolase that enhances BRCA-1-mediated cell growth suppression [21]. The BRCA-1 RING finger domain also binds to BARD-1, which also contains a RING finger motif in the N-terminus. Like MDM2 and MDMX, BRCA-1 and BARD-1 may hetero-oligomerize through their RING finger domains [22]. BARD-1 contains a BRCT (BRCA1 C-terminus) domain similar to that of BRCA-1, suggesting a role of BARD-1 in cell cycle regulation. Although the meaning of hetero-oligomer formation between BARD-1 and BRCA-1 has not been clarified, specific hetero-oligomeric interaction among two-related RING finger domains may be a common mechanism of hetero-oligomerization of the two related cell cycle regulator proteins.

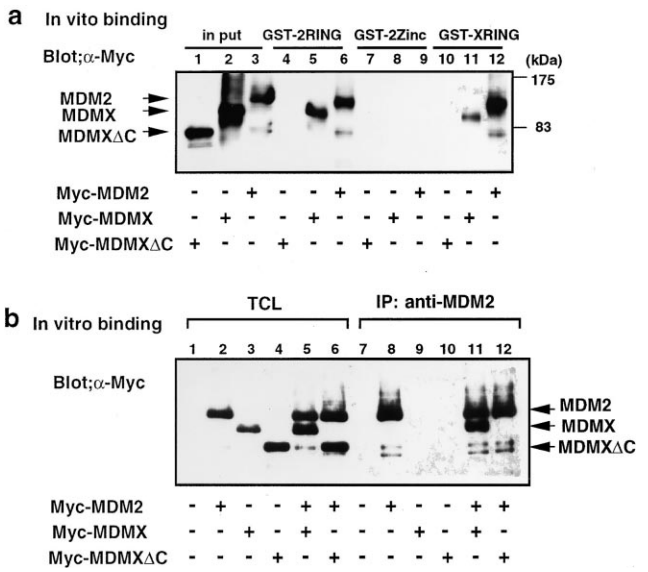

Fig. 3. MDM2 and MDMX interact through their RING finger domains in vitro (a) and in vivo (b). a: GST was fused with MDM2 RING finger domain (GST-2RING), MDM2 zinc finger domain (GST-2Zinc) and MDMX RING finger domain (GST-XRING) as shown in Fig. 2a. Immobilized GST-fusion proteins on GSH-Sepharose were incubated with 293 cell extracts containing full length Myc-MDM2, Myc-MDMX or C-terminal truncated MDMX (Myc-MDMXΔC). Bound proteins were analyzed by immunoblotting with anti-Myc antibody. b: 293 cells were transfected with indicated combination of plasmids carrying Myc-MDM2, Myc-MDMX or Myc-MDMXΔC. After 30 h, total cell lysates (TCL) or immunoprecipitates with anti-MDM2 monoclonal antibody (IP: anti-MDM2) were subjected to SDS-PAGE, followed by immunoblotting with anti-Myc antibody. Positions for MDM2, MDMX and MDMXΔC are shown.

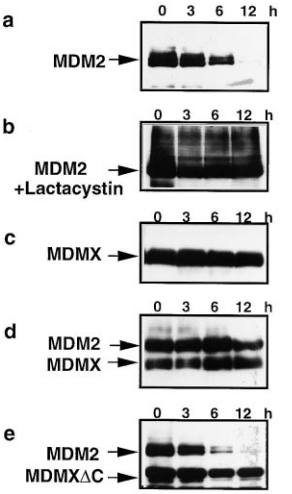

Fig. 4. The effect of MDMX co-expression on MDM2 stability. 293 cells were transfected with plasmids carrying full length Myc-MDM2 alone (a,b) or Myc-MDMX alone (c), or Myc-MDM2 with full length MDMX (d) or with Myc-MDMXqC (e). After 30 h transfection, 30 μg/ml cycloheximide was added into the culture medium (a–e). 10 μM lactacystin was also included in the culture medium with cycloheximide in b. After indicated periods of incubation, cells were lysed, then immunoblotting with anti-Myc antibody was performed.

MDM2 has been shown to promote degradation of p53. The C-terminal RING finger region of MDM2 has been suggested as playing a role in the ubiquitin-proteasome dependent degradation of p53 and MDM2 itself [23]. Honda et al. demonstrated that MDM2 functions as a ubiquitin ligase E3 for P53 in vitro [15]. However, the molecular mechanism of ubiquitination of MDM2 and p53 in vivo has not been clarified yet. Interestingly, Zhang et al. reported that $p19^{ARF}$ binds to the C-terminal region of MDM2 and promotes degradation of MDM2, resulting in the stabilization of p53 [13]. Therefore, MDMX seems to have a completely opposite effect on MDM2 degradation and probably on p53 stability. In addition, MDMX contains p53 binding motif at the N-terminus, which can also potentially inactivate p53 [18,19]. We found that MDMX synergistically inhibits transactivation activity of p53 in cooperation with MDM2 (data not shown). These data suggest that MDMX positively regulates MDM2 function for p53 inactivation. It is of interest whether MDMX possesses oncogenic activity like MDM2, which we are currently examining. Alternatively the MDM2-MDMX complex may play a role in regulation of the cell cycle or apoptosis, which is independent of p53, since MDM2 has been shown to possess both p53-dependent and independent functions [24,25].

MDM2 has been shown to be cleaved by CPP32-like protease during apoptosis [26,27]. This cleavage region is in the internal region between the zinc finger motif and the RING finger motif, thus the cleaved product of MDM2 cannot interact with MDMX. The meaning of this cleavage during apoptosis is not clear at present. However, MDMX may be involved in the regulation of MDM2 function during apopto-

sis. MDMX is highly expressed in the thymus. Therefore, MDMX-MDM2 interaction may be involved in T cell development by regulating p53-dependent or independent apoptosis.

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