Review

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The *MDM2* gene family

Abstract: MDM2 is an oncoprotein that blocks p53 tumor suppressor-mediated transcriptional transactivation, escorts p53 from the cell nucleus to the cytoplasm, and polyubiquitylates p53. Polyubiquitylated p53 is rapidly degraded in the cytoplasm by the 26S proteasome. MDM2 is abnormally upregulated in several types of cancers, especially those of mesenchymal origin. MDM4 is a homolog of MDM2 that also inhibits p53 by blocking p53-mediated transactivation. MDM4 is required for MDM2-mediated polyubiquitylated of p53 and is abnormally upregulated in several cancer types. *MDM2* and *MDM4* genes have been detected in all vertebrates to date and only a single gene homolog, named *MDM*, has been detected in some invertebrates. *MDM2*, *MDM4,* and *MDM* have similar gene structures, suggesting that *MDM2* and *MDM4* arose through a duplication event more than 440 million years ago. All members of this small *MDM2* gene family contain a single really interesting new gene (RING) domain (with the possible exception of lancelet MDM) which places them in the RING-domain superfamily. Similar to MDM2, the vast majority of proteins with RING domains are E3 ubiquitin ligases. Other RING domain E3 ubiquitin ligases that target p53 are COP1, Pirh2, and MSL2. In this report, we present evidence that COP1, Pirh2, and MSL2 evolved independently of MDM2 and MDM4. We also show, through structure homology models of invertebrate MDM RING domains, that MDM2 is more evolutionarily conserved than MDM4.

Keywords: evolution; oncogene; p53; RING; tumor suppressor; ubiquitylation.

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Introduction

The *MDM2* gene was discovered as one of three genes (*MDM1, MDM2,* and *MDM3*) within an amplicon cloned from the tumorigenic mouse cell line 3T3DM (1–3). The genes have different sequences and only *MDM2* was found to be amplified in human cancers. In humans, the *MDM2* gene (also known as *HDM2*) is located on chromosome 12q14.3-q15 and most frequently expresses a 491 amino acid residue protein. *MDM2* is amplified at an overall frequency of 7% in human cancers and at a higher frequency within soft tissue sarcomas, osteosarcomas, and esophageal carcinomas (4, 5). In some cancers with no apparent *MDM2* amplification, MDM2 transcript levels are elevated by increased gene expression (6–8).

MDM2 protein negatively regulates the p53 tumor suppressor protein (9). The p53 tumor suppressor responds to cell stress by transcriptionally activating several genes responsible for DNA repair, cell cycle arrest, anti-angiogenesis, apoptosis, and autophagy (10). The particular downstream pathway activated by p53 depends on many conditions, including the severity of the stress, the nature of the stressor, and the cell type. Regulation of p53 primarily takes place at the protein stability level within a regulatory network where p53 is polyubiquitylated by MDM2 and subsequently degraded by the 26S proteasome (11–13). A key component of this network is the p53/MDM2 feedback loop, where p53 turnover is regulated by MDM2 and expression of MDM2 is under the transcriptional control of p53 (14–16). p53 transcriptionally activates *MDM2* through a p53-responsive element located in the first intron, and in turn, MDM2 targets p53 for degradation. This negative feedback loop keeps p53 levels relatively low, unless stress is applied to the cell.

Detailed examination of this negative feedback loop is worthwhile, especially in light of current interest in the development of small molecules to inhibit MDM2 activities. In normal cells, p53 activates the expression of *MDM2*. Upon cell stress, MDM2 and p53 are phosphorylated (17–24) and bind to proteins that physically separate MDM2 from p53 (25–27). MDM2 inhibits p53 through three linked actions. First, MDM2 binds to the transactivation

domain of p53 that sterically blocks access of p53 to basal transcription factors. Second, the E3 ubiquitin ligase activity of MDM2 mediates monoubiquitylation of p53, promoting the relocation of the p53-MDM2 complex from the nucleus to the cytoplasm (28). Third, once in the cytoplasm, MDM2 polyubiquitylates p53, leading to its degradation by the 26S proteasome (29). This negative feedback loop is further regulated by critical proteins including MDM4, HAUSP (USP7), ARF, Pirh2, MSL2, and COP1 (30–34).

The second member of the *MDM2* gene family is *MDM4* (sometimes known as *MDMX*, *HDM4*, or *HDMX*), first identified when its protein product was discovered as a novel p53 binding protein by screening a mouse cDNA expression library with radiolabeled p53 protein (35). The *MDM4* gene is located on human chromosome 1q32 and encodes a 490 residue protein. The *MDM4* gene is amplified or the MDM4 protein is overexpressed in 10%–20% of diverse tumors including lung, colon, stomach, and breast cancers, as well as 65% of retinoblastomas (36, 37). Similar to MDM2, MDM4 inhibits the transactivation function of p53 by sterically blocking its access to basal transcription factors (35, 38). Currently, the development of molecules that block p53-MDM2/MDM4 interactions is considered a promising strategy to combat cancers that contain inactive wild-type p53. Although still in the development and testing stage, small molecules have been shown to induce p53 tumor suppressor activities in animal models (39–41). In the cell, MDM2 and MDM4 form a heterodimer that strengthens the efficacy of MDM2's inhibitory activities (29, 42).

Careful mouse genetic studies indicate that MDM4 contributes more to inhibition of p53-mediated transcriptional transactivation while MDM2 contributes more to degradation of p53 (43). In line with such studies, MDM4 lacks robust E3 ligase activity *in vitro*. Instead, MDM4 is an E4 protein in the ubiquitylation pathway. In general, E4 proteins are responsible for recognizing monoubiquitylated substrates and guiding the conjugation of multiple ubiquitin units onto single lysine residue targets within the protein substrate, a process known as polyubiquitylation. Only after polyubiquitylation is the protein substrate recognized by the 26S proteasome for degradation. MDM4 forms a complex with MDM2, monoubuiquitylated p53 and E2 protein to assist MDM2 polyubiquitylate p53 (44). The really interesting new gene (RING) domains within MDM2 are critical for its ubiquitylation activity and, in addition, RING domains within MDM2 and MDM4 form the heterodimerization interfaces of these two proteins.

MDM2 and *MDM4* are paralogs that form a small family called the *MDM2* gene family within the superfamily of RING domain-bearing proteins. An analysis of the evolutionary history of *MDM2* and *MDM4* indicates that the paralogs arose from a duplication event more than 440 million years ago, at approximately the same time that the *p53* gene underwent duplication events to form p63 and p73 (45, 46). Both *MDM2* and *MDM4* paralogs are detected in vertebrates, but only one gene family member is detected in invertebrates, named *MDM*. This review discusses the *MDM2* gene family from an evolutionary perspective.

The ubiquitylation pathway

To appreciate the evolutionary perspective of the *MDM2* gene family, a brief background of ubiquitin-mediated protein modification is necessary because the domain responsible for this modification, the RING domain, is strongly conserved in orthologs of this family. Ubiquitylation is the covalent modification of protein lysine residues by addition of the small regulatory protein molecule ubiquitin (47). This process requires three enzymes: an ATP-dependent ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin ligase (E3). Upon activation, ubiquitin is transferred from E1 to a catalytic cysteine on E2, forming a thioester-linked conjugate. The E2-ubiquitin conjugate engages E3 and, together, they transfer ubiquitin from E2 to the ε-amino group of a lysine side chain on the target protein (48). In many instances, RING domains within E3s are interaction sites for E2s, and the presence of RING domains is assumed to be indicative of E3 ubiquitin ligase function (49, 50). Another component of the ubiquitylation pathway is E4 (discussed previously), discovered much later than other components of the ubiquitin pathway (51).

E3 ubiquitin ligases fall into two classes, those that contain a RING domain (with a few containing a structurally and functionally similar U-box domain) and those with a homologous to E6-AP carboxy-terminus (HECT) domain. Protein target specificity within the ubiquitin cascade is provided by E3 ubiquitin ligase. RING domain-containing E3 ubiquitin ligases, in most cases, mediate the transfer of ubiquitin by recruiting E2 ubiquitin-conjugated enzymes to the acceptor lysine residue on the target protein and enhancing this transfer (52). RING domains are not covalently bound to ubiquitin. HECT domains, however, form covalent intermediates between ubiquitin

and a cysteine residue within E3 prior to ubiquitin transfer to the target protein.

RING domains within the MDM2 family

Human MDM2 and MDM4 proteins exhibit 31% amino acid residue identity and possess similar patterns of protein domain organization (46). Both contain an N-terminal p53 binding domain, central acidic and zinc-binding domains, and a C-terminal RING domain. The p53 binding domain and RING domain are particularly well conserved between the human MDM2 and MDM4 paralogs (50.9% and 52.4% amino acid residue identity respectively). Invertebrates frequently code for only one MDM family protein and at the moment there are seven identified invertebrate species that contain *MDM* gene: lancelet, owl limpet, bay mussel, acorn worm, sea squirt, deer tick, and placozoa. The invertebrate MDM and human MDM2 protein sequences share identities that range from 21% to 27%, whereas the invertebrate MDM and human MDM4 protein sequences share identities that range from 19% to 26%. With the exception of sea quirt MDM, invertebrate MDMs exhibit higher identity to human MDM2 than to human MDM4, indicating that in six out of seven instances, human MDM2 and invertebrate MDMs are slightly more related to each other than human MDM4 and invertebrate MDMs. This increased relatedness is largely due to relatively high identity between the RING domains of human MDM2 and invertebrate MDMs. Furthermore, within the vertebrates, the RING domains of MDM2 orthologs exhibit a high degree of sequence identity (≥79% identity) compared to that of the RING domains of the MDM4 orthologs (≥52% identity). Overall, the RING domain of MDM2 is well conserved amongst vertebrate MDM2 orthologs as well as amongst invertebrate MDMs protein sequences.

MDM2 and MDM4 can form homodimers or heterodimers through RING domain interactions (42). Within the cell, the majority of MDM2 and MDM4 molecules form heterocomplexes that create efficient E3 functions towards p53 (53). In a broader scope, RING domain proteins can function as E3 ubiquitin ligases in either the monomeric or dimeric state. The RING proteins Pirh2, c-Cbl, PML, and CNOT4 facilitate their E3 ligase function through their RING domains in their monomeric forms (54–57). Other RING proteins, such as the cIAPs, BRCA1-BARD, and Ring1b-Bmi1 require RING dimerization for E3 function (58–60).

Other RING domain E3 ligases that target p53

Since the discovery of MDM2 and MDM4, other RING domain-containing E3 ligases that target p53 have come to light. Constitutive photomorphogenic 1 (COP1), also known as RING finger and WD repeat domain 2 (RFW2), was initially identified in *Arabidopsis* where it plays a critical role in plant growth and development in response to light (34). COP1 is conserved in higher plants and vertebrates; it consists of an N-terminal RING finger domain, an internal coiled coil domain, and C-terminal WD40 repeats (61). Mammalian COP1 targets p53 for degradation independently of MDM2 and is necessary for p53 turnover in cultured normal and cancer cells. Analogous to *MDM2* and *MDM4*, *COP1* is a p53-inducible gene (it contains a p53-responsive element within the *COP1* promoter region) and is part of a negative feedback loop (34). *COP1* is overexpressed in 81% of breast cancers and 44% of ovarian adenocarcinomas. In cancers that retain wild-type p53, *COP1* overexpression is correlated with a striking decrease in steady state p53 protein levels and attenuation of the downstream p53 target gene *CDKN1A* (also known as *CIP1*, *WAF1, PIK1, SDI1*).

Another E3 that targets p53 is p53-induced RING-H2 domain protein (Pirh2), originally identified as an androgen receptor N-terminal-interacting protein (ARNIP). Pirh2 is also known as RING finger and CHY zinc finger domain-containing protein 1 (RCHY1) (62). Pirh2 consists of an N-terminal CHY zinc finger domain, central RING domain, and a C-terminal Zinc finger domain (56). The best-known function of Pirh2 is its role in the p53/Pirh2 negative feedback loop, independent of MDM2 and COP1, in which Pirh2 inhibits p53 activity and is under the transcriptional control of p53. A p53-responsive element is located in the third intron of the *pirh2* gene. Similar to MDM2 and COP1, Pirh2 negatively regulates p53 function through ubiquitin-mediated proteolysis. Pirh2 has been shown to target p53 for degradation under DNA damaging conditions when MDM2 dissociates from p53 and fails to target p53 for degradation (33). The interaction of p53 and Pirh2 employs a two-site binding mode, where the Pirh2 N-terminus interacts with the p53 DNA binding domain and the Pirh2 C-terminus binds to the p53 tetramerization domain with enhanced specificity for the active tetrameric form of p53 (63). Mouse models indicate that overexpression of Pirh2 promotes tumorigenicity (64) and that its unphosphorylated form is detected in tumor cells (65).

A third E3 ligase with a RING domain that targets p53 is MSL2 (30). MSL2's RING domain has the same cross brace

zinc domain motif as MDM2 and MDM4 (see next section), but has a different Zn-coordination scheme (C3HC4 vs. MDM2/MDM4's C2H2C4). MSL2 ubiquitylates p53 at Lys 351 and Lys 357 residues, distinct from lys residues ubiquitylated by MDM2. Modification by MSL2 appears to expose a nuclear export motif within p53 as well as release p53 from MDM2. Overexpression of MSL2 does not target p53 for destruction but, rather, causes p53 accumulation in the cytoplasm.

Evolutionary relationships of human RING proteins

A sequence alignment of the RING domains of 24 human RING-containing proteins is presented in Figure 1. Sequences of the RING proteins were obtained from the UniProt protein database and limited to the range beginning with the first zinc coordinating cysteine and ending with the residue following the last zinc coordinating residue. The proteins listed in Figure 1 act as E3 ligases with the exception of MDM4. In general, the RING domains range from 40 to 60 residues and coordinate two zinc atoms through a zinc finger cross brace motif--a zinc finger motif with the consensus sequence Cys-X_2-Cys-$X_{9\text{-}39}$-Cys-$X_{1\text{-}3}$-His-$X_{2\text{-}3}$-Cys-X_2-Cys-$X_{4\text{-}48}$-Cys-X_2-Cys (49). However, the RING domains of MDM2 and MDM4 possess a C2H2C4 zinc-binding scheme that is unique among RING finger family members (66). Unlike all other known RING domains, four amino acid residues, instead of two or three, separate the third and fourth zinc coordinating residues (underlined above). In the human MDM2 RING domain, residues C438, C441, C461, and C464 coordinate the zinc atom Zn1, while H452, H457, C475, and C478 coordinate Zn2 (67). In the human MDM4 RING domain, residues C437, C440, C460, and C463 coordinate Zn1 while H451, H456, C474, and C477 coordinate Zn2. The MDM2 RING (residues 438–479) and MDM4 RING (residues 437–478) domains are located near their respective C-termini.

Analysis of the gene structures of p53-targeting RING proteins and the gene structures of other RING proteins from humans suggests that the *MDM2* gene family consists of just *MDM2* and *MDM4*. The products of gene duplication often retain gene structures that include the total number of exons and the exon lengths. In addition, the particular exon that encodes the RING domain relative to other exons in the gene is also often conserved in closely related gene family members. We analyzed the human RING-containing proteins listed in Figure 1 for maintenance of these gene structure features. Table 1 lists the number of

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>RNF4       CPICMDGYSEIVQNGRLIVSTEC---GHVF-CSQ-CLRDSLKNA---NTCPTCR
>Ring1b     CPICLDML------KNTMTTKEC---LHRF-CAD-CIITALRSGN--KECPTCR
>TRAF6      CPICLMAL-------REAVQTPC---GHRF-CKA-CIIKSIRDAG--HKCPVDN
>BRCA1      CPICLELI-------KEPVSTKC---DHIF-CKF-CMLKLLNQKKGPSQCPLCK
>Rad18      CGICFEYF------NIAMIIPQC---SHNY-CSL-CIRKFLSYK---TQCPTCC
>TRAF2      CSACRNVL-------RRPFQAQC---GHRY-CSF-CLASILSSGP--QNCAACV
>cIAP1      CKVCMDKE-------VSVVFIPC---GHLVVCQE-CAPSL-------RKCPICR
>cIAP2      CKVCMDKE-------VSIVFIPC---GHLVVCKD-CAPSL-------RKCPICR
>XIAP       CKICMDRN-------IAIVFVPC---GHLVTCKQ-CAEAV-------DKCPMCY
>Rififylin  CKICMDSP-------IDCVLLEC---GHMVTCTK-CGKRM-------NECPICR
>MGRN1      CVVCLSDL-------RDTLILPC---RHLCLCTS-CADTLRYQA---NNCPICR
>RING157    CVVCLSDV-------RDTLILPC---RHLCLCNT-CADTLRYQA---NNCPICR
>MEX3B      CSVCFESE-------VIAALVPC---GHNLFCME-CANRICEKSE--PECPVCH
>MEX3C      CVICFENE-------VIAALVPC---GHNLFCME-CANKICEKRT--PSCPVCQ
>IDOL       CMVCCEEE-------INSTFCPC---GHTV-CCESCAAQL-------QSCPVCR
>c-Cbl      CKICAEND-------KDVKIEPC---GHLM-CTS-CLTSWQESEG--QGCPFCR
>RNF103     CVVCLENF----ENGCLLMGLPC---GHVF-HQN-CIVMWLAGGR--HCCPVCR
>mdm2       CVICQGRP--------KNGCIVHGKTGHLMACFT-CAKKLKKRN---KPCPVCR
>mdm4       CSLCEKRP--------RDGNIIHGRTGHLVTCFH-CARRLKKAG---ASCPICK
>COP1       CPICFDMI-------EEAYMTKC---GHSF-CYK-CIHQSLEDN---NRCPKCN
>Pirh2      CPICLEDI---HTSRVVAHVLPC---GHLL-HRT-CYEEMLKEG---YRCPLCM
>MSL2       CCVCGHLL----QDPIAPTNSTC---QHYV-CKT-CKGKKMMMK---PSCSWCK
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Figure 1 Alignment of the RING domain sequences of 22 human proteins.
Residues critical for coordinating Zn atoms are highlighted. Five proteins reported to interact and regulate ubiquitylation of p53 are bordered. Alignment created by the ClustalW2 software program. Eight of the sequences were obtained by performing a BLAST analysis of the human, rat, and frog genomes using the MDM2 RING sequence as the query. The BLAST output revealed that eight proteins are present in all three organisms: MGRN1, XIAP, cIAP1, RNF103, Rififylin, Ring 157, MEX3B, and MEX3C. Ten RING containing proteins were added: MDM4, BRCA1, c-Cbl, cIAP2, IDOL, Rad18, hRing1b, RNF4, TRAF2, and TRAF6 obtained from a recent review of RING proteins (80). The proteins Pirh2, COP1, MLS2 were added because they are RING proteins that ubiquitinate p53.

Table 1 Human RING genes: number of exons, RING coding exons, and length of RING-coding exons.

Gene	Number of exons	Exon(s) coding for RING domain	Length of RING-containing exon(s)
RNF4	8	7 and 8	199
RING1b	7	3 and 4	377
TRAF6	7	2 and 3	447
BRCA1	23	3 and 4	212
RAD18	13	2 and 3	144
TRAF2	12	3 and 4	267
cIAP1	9	9	194
cIAP2	9	9	194
XIAP	7	7	194
Rififylin	7	7	182
MGRN1	12	10	160
RNF157	19	10	160
MEX3B	2	2	1454
MEX3C	2	2	1226
IDOL	7	6 and 7	511
c-Cbl	16	8 and 9	336
RNF157	19	10	160
MDM2	11	11	192
MDM4	11	11	190
COP1	20	2 and 3	158
Pirh2	9	6, 7, 8 and 9	252
MSL2	2	1 and 2	5202

exons, the RING-domain coding exon(s) and the length of exon(s) in each RING-domain containing gene. Four groups of closely related RING genes are observed (color shaded): (i) *cIAP1*, *cIAP2, XIAP*; (ii) *MGRN1* and *RNF157*; (iii) *MEX3B* and *MEX3C*; (iv) *MDM2* and *MDM4*. Through gene structure analysis it appears that non-MDM2/MDM4 RING-domain proteins that target p53, COP1, Pirh2, and MSL2, are not closely related to MDM2/MDM4 nor to other RING-domain proteins in this cohort.

As mentioned previously the most conserved domain in MDM2 is the RING finger domain, which binds to E2 and is responsible for dimerization. Figure 2 shows the results of neighbor-joining cluster analysis of RING domains of human RING proteins (68). Relatively short length branches connect proteins that are highly related. Cluster analysis confirms and extends the groupings of RING family members created from analysis of gene structures. Consistent with gene structure data, cluster analysis suggests that other RING-domain carrying E3s that target p53, COP1, Pirh2, and MSL2 evolved independently from the MDM2 family. Furthermore, it appears that COP1 and MSL2 are more related to one another than to Pirh2 and, overall, these three p53-targeting proteins are more related to each other than to the MDM2 family.

Structure analysis of MDM family members

Now that we have established that the two members of the *MDM2* gene family are *MDM2* and *MDM4*, it is instructive to deduce how invertebrate *MDM*s are related to this family. Invertebrate *MDM*s have been found in seven organisms (46) and, by sequence comparison analysis,

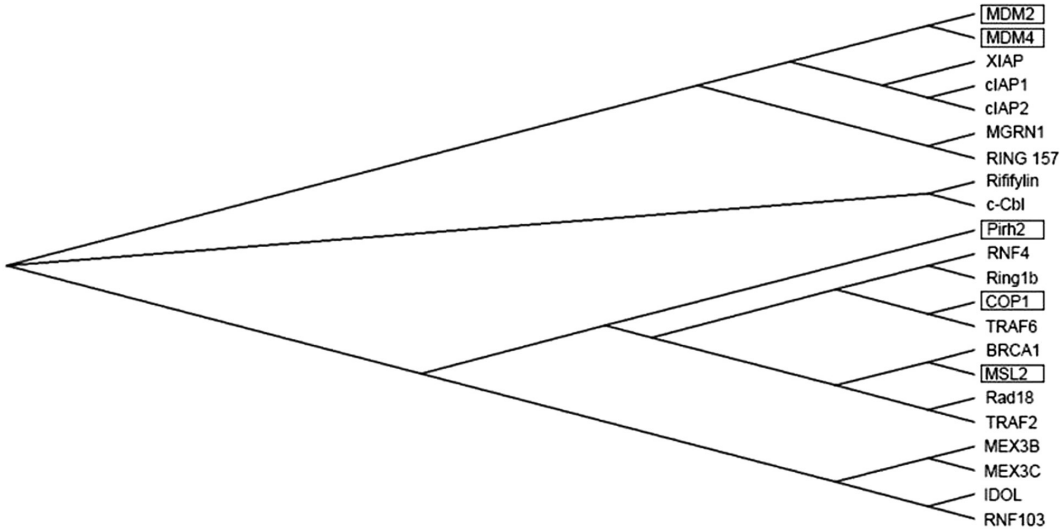

Figure 2 Cluster analysis of RING domains of 24 human proteins.
Bordered proteins ubiquitylate p53. All have E3 ligase activity with the exception of MDM4.

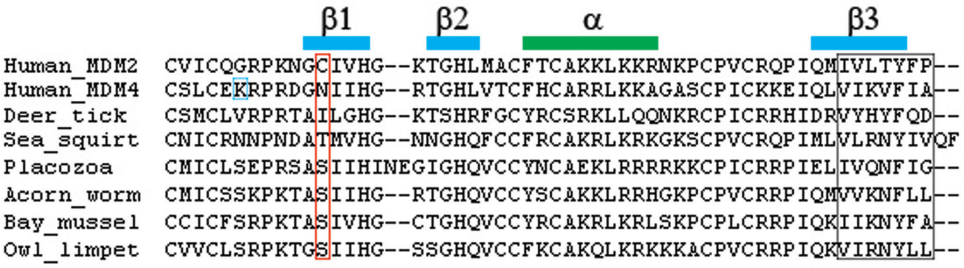

Figure 3 Alignment of human MDM2 with MDM RING domains and C-terminal residues.
Shown are the β-strand and α-helix regions in human MDM2 and MDM4. Blue rectangle shows site of MDM4 ubiquitylation by MDM2 in MDM2/MDM4 heterodimers. Red rectangle borders Cys449 position in human MDM2, which is critical for E3 ligase activity. Grey rectangle shows residues that are necessary for human MDM2 oligomerization aligned with human MDM4 and MDMs. Shown are the β-strand and α-helix regions in human MDM2 and MDM4. Lancelet RING domain could not be accurately aligned in this multiple sequence alignment. Sequences were aligned with Clustal Omega (81, 82).

the RING domains in six of the invertebrate MDM protein sequences exhibit greater percent identity to human MDM2 than to human MDM4, suggesting that the MDM2 RING may retain functions of invertebrate MDMs. An illustration of a potential conservation of invertebrate MDM2 function within MDM centers on E3 activity. Cys 449 in human MDM2 appears to be critical for E3 activity (69, 70), but not for maintenance of the RING structure (71). When Cys is replaced by Ser, MDM2 retains its E3 activity as assessed by *in vitro* p53 ubiquitylation experiments. But, when Cys is replaced by Ala, MDM2 loses its E3 activity. MDM4, which does not possess E3 activity, contains an Asn at position 449. Three of the six invertebrate MDMs with identifiable RING domains code for Ser in this position, suggesting that they also potentially possess E3 activity (red bordered residues in Figure 3). Other invertebrate MDMs code for Thr and Ile at this position. Interestingly, in cultured human cells an MDM2 with a Cys to Ser substitution does not support E3 activity (69), indicating that other components of the p53 ubiquitylation pathway in human cells require MDM2 to have a Cys at this position. The evidence suggests that Ser at this position can support E3 activity *in vitro*, suggesting that invertebrate MDMs are somewhat more similar to MDM2 than to MDM4. Experiments to test whether invertebrate MDMs actually possess E3 activity will clarify this issue.

A structure modeling experiment was conducted to assess whether RING domains of invertebrate MDMs are more structurally similar to human MDM2 RING or human MDM4 RING. Multiple sequence alignment of full-length sequences between human MDM2 and invertebrate MDMs was generated and the regions with the highest degree of conservation were used for modeling studies. This region consists of ten residues flanking the first zinc coordinating Cys through 13 residues flanking the last zinc coordinating Cys. The invertebrate MDM sequences corresponding to this conserved region of human MDM2 were submitted to the automated structure homology modeling software program Swiss Model to create structure models (72–74). All invertebrate MDMs produced a structure model with the exception of lancelet MDM because it lacked sufficient sequence similarity to potential structure templates available to Swiss Model. The template automatically selected by the software program to build the homology models was the RING-H2 finger domain (PDB# 2kiz) from the human Arkadia the RING-H2 protein. The six invertebrate RING models are shown in Figure 4. RING domains from X-ray crystallography structures of MDM2 and MDM4 are shown for comparison. The alpha helices in the MDM models and MDM2/MDM4 structures are maintained. MDM2 and MDM4 RING's contain distinct regions with antiparallel β-strands. In contrast, the invertebrate MDM structure models, with the exception of placozoa MDM, lack B-strands. Spatial comparisons were made between the maximum number of protein backbone atoms of the MDM RING models shared with those of the crystal structures of MDM2 and MDM4. Structure/model comparisons were conducted by calculating the root mean square deviations (RMSDs) (Table 2). The consistent lower RMSDs in MDM2/MDM comparisons for all invertebrate structures indicate that MDM2 is more structurally similar to MDM than is MDM4. Importantly, one invertebrate MDM RING domain (deer tick) has a slightly higher sequence identity to MDM4 than to MDM2; yet, the lower RMSD value suggests that deer tick MDM RING domain appears to be more structurally similar to MDM2. The model/structure comparison suggests that invertebrate MDMs are more structurally similar to MDM2 than to MDM4.

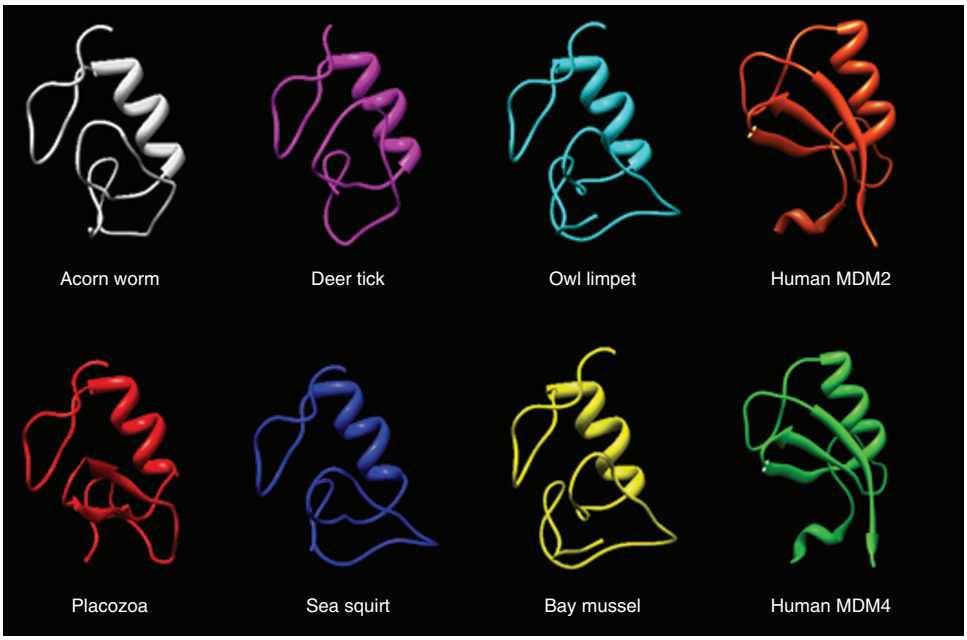

Figure 4 Models of RING domains of six invertebrate MDMs, X-ray structure of MDM2 RING, and X-ray structure of MDM4 RING.

Table 2 Comparison of MDM RING models to MDM2 and MDM4 RING structures (PDB#: 2vje).

	MDM2 RMSD (Å)	MDM4 RMSD (Å)	Number of atoms compared
Acorn worm	6.489	7.930	403
Sea squirt	6.518	6.818	383
Owl limpet	6.808	7.296	402
Placozoa	7.377	8.724	427
Bay mussel	7.441	8.493	410
Deer tick	8.090	8.131	384

Summary

Our analyses indicate that *MDM2* and *MDM4* constitute a two-gene family (*MDM2* gene family) that, in turn, belongs to the *RING* superfamily. Gene structure comparisons indicate that MDM2 and MDM4 are not closely related to other members of the *RING* superfamily. Cluster analysis of RING protein sequences further confirm that *MDM2* and *MDM4* evolved separately from other members of the superfamily. Other p53 targeting RING domain-containing genes, *COP1*, *Pirh2*, and *MSL2* are not closely related to the *MDM2* gene family. Furthermore, in invertebrates a single *MDM* gene is present. Invertebrate MDM RING protein sequence alignment and homology model structure comparisons to human MDM2 and human MDM4 suggests human MDM2 RING domain is more evolutionarily conserved than human MDM4.

Studies by Dehal and Boore (75) and others (76, 77) suggest that more than 440 million years ago two successive rounds of duplication (known as 2R) occurred in a common ancestor at the base of vertebrates. In accordance with this model starting from a single *MDM* gene, 2R would produce four paralogs of *MDM* genes. However, as four *MDM* paralogs are not detected in vertebrates one scenario to account for only two *MDM* paralogs in modern vertebrates is that a single paralog of *MDM* was deleted after the first round of duplication (after 1R). Another scenario is that two of the four paralogs were deleted after 2R. If the first scenario was correct, one would predict that lineages descended from an ancestor that emerged just after 2R would contain only two paralogs (i.e., *MDM2* and *MDM4*). Cartilaginous fish are thought to descend from an ancestor shortly after 2R and one species of cartilaginous fish (elephant shark, *Callorhinchus milii*) codes for *MDM2* and *MDM4* (78). At the time of this communication, genome sequencing of organisms that trace back to an evolutionary window between 1R and 2R, such as lamprey, has not been completed. It will be interesting to see what *MDM* genes exist in the lamprey genome. If only one *MDM* is present, it would lend support to the scenario where a deletion event occurred after 1R. If two *MDM*s are present, then the data would lend support to the scenario in which two *MDM* paralogs were deleted after 2R.

Our analysis suggests that MDM proteins do not have the capability of forming oligomers. X-ray crystal structure studies show that heterodimerization between human MDM2 and human MDM4 occurs when three β-strands from one monomer and three β-strands from a second monomer form a β-barrel (67). These β strands are labeled β1, β2 and β3 in Figure 3; β2 and β3 bracket an α-helix. According to our modeling studies, the α-helix is preserved in the invertebrate MDMs but the β-strands are not, with the exception of placozoa in which two small β strands form in the approximate locations of β1 and β2, but not β3.

Mutation analyses of human MDM2 show that the C-terminal five residues of MDM2 are critical for oligomerization and E3 activity and that oligomerization can be restored by replacing the C-terminal seven residues of MDM2 with the C-terminal seven residues of MDM4 (79). Figure 3 shows a sequence alignment of human MDM2, human MDM4, and six invertebrate MDMs from the RING domain to the carboxyl terminal ends of the sequences (with the residues aligned to C-terminal seven MDM2 residues bordered). As our modeling studies show that the MDMs do not form the three β-strands necessary to form a β-barrel, we suggest that MDMs act as monomers (analogous to other RING domain proteins with E3 activity such as Pirh2, c-Cbl, PML, and CNOT4) and ubiquitylate p53 without dimerization. Upon duplication and subsequent mutation during evolution, vertebrate MDM2 and MDM4 may have gained the capability of dimerization.

We speculate that dimerization would have posed difficulties for MDMs with E3 ligase activities unless there were mutations that led to MDM2- and MDM4-specific RING domains and C-terminal residues. As the dimerization property was acquired, a potential problem for the early evolving MDM could have arisen. Currently, dimeric MDM2 has been shown to auto-ubiquitylate, which leads to self-degradation (67). MDM2 self-degradation incapacitates its ability to properly regulate p53. Fortunately, within vertebrates MDM2 self-degradation is prevented by forming heterodimers with MDM4. Upon hetero-dimerization, MDM4 K442 is ubiquitylated by MDM2, thus protecting MDM2 from self-destruction, which allows MDM2 to survive and properly regulate p53. We note that invertebrate MDMs, that we suggest are monomeric, do not possess lysine at this position (see blue bordered residue in Figure 3). If the invertebrate MDMs were dimeric E3 ligases, they could potentially encounter auto-ubiquitination problems analogous to homodimeric MDM2. Thus, we suggest that dimerization property evolved after *MDM* gene duplication.

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