Characterization of the Ubiquitin/Nedd8 E3 Ligase Activity of the Mdm2/MdmX complex

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Abbreviations

°C	Degree celcius	mRNA	messenger-RNA
ADP	Adenosine Di-Phosphate	mu	murine
AMP	Adenosine Mono-Phosphate	n	nano
ATP	Adenosine Tri-Phosphate	N	Normal
bp	base pairs	ng	nanogram
cDNA	complementary DNA	nm	nanometer
ddH_2O	double-distilled water	mA	miliAmpere
DMSO	Dimethylsulphoxide	mM	miliMolar
DNA	Deoxyribonucleic acid	nt	nucleotide
DTT	Dithiothreitol	OD	Optical Density
E1	Ubiquitin-activating enzyme	ONPG	Orthonitrophenyl-β-
E2	Ubiquitin-conjugating enzyme		D- galactosidase
E3	Ubiquitin-protein ligase	PBS	Phosphate buffer
EDTA	Ethylenediamine tetraacetic acid		saline
FBS	Fetal bovine serum	PCR	Polymerase chain
HA	Haemagglutinin		reaction
hr	hour	RNA	Ribonucleic acid
hrs	hours	rpm	revolution per minute
i.e.	id est or that is	s	second
IgG	Immunoglobulin G	TAE	Tris-acetated-EDTA
IP	immunoprecipitation	Ub	ubiquitin
kDa	Kilodalton	V	Volt
LiAc	Lithium Acetate	wt	wild-type
M	Molar	µ	micro
min	minute	µl	microlitre
ml	mililitre	µg	microgram

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1 Introduction

Protein degradation is commonly accepted to be involved in the regulation of many fundamental processes within cells. Three decades ago, lysosomes were known to degrade most of the extracellular proteins and some intracellular proteins in the presence of certain stimuli in an unspecific manner. However, there were indications that at least some intracellular proteins can also be degraded by lysosomal free cell lysates in a controlled manner and that this type of degradation was ATP-dependent. During those days, it was hard for scientists to believe that the catabolism (i.e. degradation) of a protein should need energy (in the form of ATP), since hydrolysis of a peptide bond per se is an exergonic process. The existence of a non-lysosomal degradation system was further evident by the biochemical fractionation and *in vitro* reconstitution of a degradation machinery that was able to degrade several abnormal/misfolded proteins independent of lysosomes[1]. Subsequently, the components of this degradation machinery were identified and termed collectively as the ubiquitin-proteasome system.

1.1 The ubiquitin-proteasome system

In the ubiquitin-proteasome system (UPS), ubiquitin, a 76 amino acid long polypeptide, is first covalently attached to another protein, which is followed by the recognition and degradation of the ubiquitin attached protein by a protein complex having inherent protease activity, termed "proteasome"[2; 3; 4]. Modification by ubiquitin (ubiquitylation or ubiquitination) was the first example of a protein attached to another protein by a covalent bond. Since then, hundreds of proteins involved in a broad array of basic cellular processes have been unravelled to be modified by ubiquitin and degraded by the proteasome. Cell cycle[5], DNA repair[6; 7], endocytosis of cell surface receptors[8], differentiation and development are only few examples of basic cellular processes requiring a functional UPS for their proper activity. Considering the vast array of proteins modified by ubiquitin, it is not surprising that deregulation of the UPS has been implicated in the pathogenesis of many diseases[9; 10; 11].

1.1.1 The ubiquitin conjugation pathway

Ubiquitination to a substrate is achieved by a cascade of enzymatic reactions involving at least three different enzymes, E1, E2, and E3 (Fig 1)[12]. The reaction starts with the sequential binding of Mg^{2+}-ATP and ubiquitin to the ubiquitin activating enzyme (E1) to form an adenylate ubiquitin intermediate from which ubiquitin is transferred to the catalytic cysteine of E1 via the formation of a thioester bond. Hence, one E1 molecule binds two ubiquitin

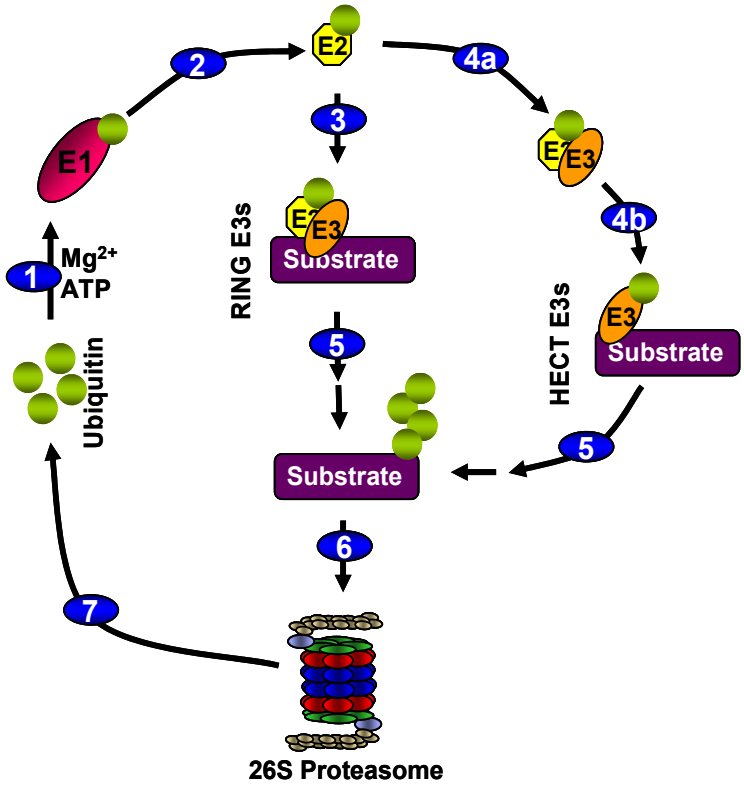

Fig 1. The ubiquitin proteasome pathway.

In the ubiquitin proteasome pathway, ubiquitin molecule is first activated by the ubiquitin activating enzyme (E1) in the presence of Mg2+ and ATP via formation of a high energy thioester bond (1). The activated ubiquitin molecule is then transferred to the ubiquitin conjugating enzyme (E2) by formation of a second high energy thioester bond with ubiquitin (2). Finally, the ubiquitin molecule is either transferred directly to the substrates by the RING-type E3 ligase (3) or transferred to the substrates via formation of a third high energy thioester bond with the catalytic cysteine of the HECT domain (4a) by the HECT-type E3 ligases (4b). Repetition of steps 1, 2, and 3/4 for several times finally attaches a polymer of ubiquitin on the substrates (5). The ubiquitinated substrates are then recognised and degraded by the 26S proteasome (6) and the ubiquitin molecules are recycled (7).

molecules- one in adenylate form and the other in thioester bonded form. Formation of a high energy thioester bond with the carboxyl group of the C-terminal glycine of ubiquitin (G76) activates the ubiquitin molecule. Ubiquitin loaded E1 interacts with the next enzyme of the cascade, an ubiquitin conjugating enzyme (E2), and catalyses the transfer of thioester bonded ubiquitin to the catalytic cysteine of E2. The thioester bonded ubiquitin-E2 complex next binds to an ubiquitin protein ligase (E3) and, in conjunction with E3 catalyses the formation of an isopeptide bond between the C-terminal carboxyl group of ubiquitin and the ε-NH_2 group of a lysine (K) residue of the substrate protein. The specificity of ubiquitin conjugation to the substrate is mainly governed by E3 in the ubiquitination pathway. The E3s recognize their cognate substrates by the presence of a structural motif in the substrate commonly known as ubiquitination signal[13].

Depending on their mode of action, E3 enzymes can be divided into two major classes- (i) HECT E3s, which form an ubiquitin thioester complex via the catalytic cysteine of the E3 before the final transfer of ubiquitin to the lysine of the substrate, and (ii) RING finger and RING finger like E3s, which do not form a thioester complex with ubiquitin but act as adaptor proteins to bring their cognate E2s in close proximity of substrate protein for ubiquitination.

In many cases, a polymer of ubiquitin (polyubiquitin chain) is attached onto lysine residue(s) of the substrate protein by the sequential repetition of the cascade followed by recognition and degradation by the proteasome. However, degradation by the proteasome is not always the fate of ubiquitinated proteins. In fact, the fate of the ubiquitinated proteins is decided by the number and the topology of ubiquitins attached to the protein[14]. Attachment of one molecule of ubiquitin to one lysine of the substrate (mono-ubiquitination) or several lysine of the substrate (multiple mono-ubiquitination) usually serves signalling functions. Moreover, different type of polyubiquitin chains can also serve different functions. For example, a polyubiquitin chain formed by forming an isopeptide bond between K48 of one ubiquitin molecule and G76 of another ubiquitin molecule (K48-linked polyubiquitin chain) serves mainly as a signal for proteasomal degradation[15]. In contrast, K63-linked polyubiquitin chains predominantly serve non-proteolytic functions, e.g. ribosomal function[6] and in post replicational DNA repair[6; 7]. The different types of ubiquitin chains have different topology, which presumably can be recognized by different sets of regulatory proteins leading to the proper fate of the substrate.

1.1.1.1 HECT E3s

The founding member of the HECT (homologous to E6-AP C terminus) E3 family was discovered in studies characterising the degradation of the tumour suppressor protein p53 by the oncogenic E6 protein of human papillomaviruses (HPVs). Later studies revealed that the HPVs E6 protein forms a complex with a cellular protein, termed E6-AP (E6-associated protein), and this complex functions as an E3 ligase to target p53 for ubiquitination and proteasomal degradation[16]. The C-terminal ~350 amino acids of E6-AP, termed HECT domain, showed homology to several proteins in databases[17] and contains a conserved catalytic cysteine required for the E3 activity of the E6/E6-AP complex. Full-length E6-AP presumably binds to substrates via binding sites in its N-terminal region and, contains E6-binding site and the catalytic HECT domain at the C terminus. E6 or E6-AP alone cannot bind p53, however, when E6 binds at the E6-binding site of E6-AP, then the E6/E6-AP complex efficiently binds to p53 and targets it for ubiquitination[18]. Furthermore, E6-AP has

been implicated in E6-independent binding and ubiquitination of several cellular proteins (e.g. the Src family tyrosine kinase Blk[19], the replication licensing factor Mcm7[20], estrogen and progesterone receptors[21]). However, the physiological significance of E6-AP-induced degradation of these proteins is not clear. In addition, the fact that mutations in the E6-AP gene (*UBE3A*) cause Angelman syndrome[22; 23], which is characterized by severe neurological abnormalities, predicts an important role of E6-AP in brain developement. Several other HECT E3s are known to perform important functions. Human Nedd4 (Rsp5 in yeast) is a well studied HECT E3 ligase, which is implicated in ubiquitination and degradation of Rpb1[24], the large subunit of RNA polymerase II, upon DNA damage. Nedd4 also ubiquitinates the kidney epithelial Na^+ channel (ENaC) for endocytosis[25]. Itch is another HECT E3 ligase, which targets a number of proteins including p73 (a member of the p53 family) for proteasomal degradation[26].

The HECT domain of HECT E3s facilitates the transfer of activated ubiquitin from cognate E2s to their catalytic cysteine followed by transfer of ubiquitin onto the lysine residue of the substrate via isopeptide bond formation. However, the mechanistic details of this catalytic process remain elusive. Crystal structure of the UbcH7/E6-AP HECT complex[27] predicts a conformational change in the complex after binding to the substrate to facilitate final transfer of ubiquitin to the substrate lysine residues. However, a detailed understanding of the molecular events underlying ubiquitin transfer to the substrate by HECT E3 ligases still awaits further research.

1.1.1.2 RING and RING finger like E3s

RING (really interesting new gene) finger domains are characterized by the presence of eight histidine and cysteine residues with a typical spacing in order to form a cross brace like structure (see Fig 2), which is stabilised by the presence of two zinc ions. The widely accepted consensus sequence for forming a RING domain is C-X_2-C-$X_{(9-39)}$-C-X-H -$X_{(2-3)}$-C/H-X_2-C-$X_{(4-48)}$-C-X_2-C[28]. Depending on the presence of C or H residues at the 5th position, the RING domains have been classified into two groups- C3HC4 (also known as HC-type RING), and C3H2C3 (also known as H2-type RING).

RING E3s were first discovered as integral part of several multi-subunit protein complexes- SCF (Skp1-cullin-F-box), APC (anaphase promoting complex) and CBC (cullin-elongin B and C) complexes. SCF complexes were initially identified as complexes consisting of at least three proteins Skp1, Cullin1 (Cdc53 in yeast), and a so called F-box protein. Later, it was shown that a RING finger protein ROC1 (or Rbx1) is also a part of the complex and it is essential for the E3 activity of the complex. Skp1, cullin1 and ROC1 are common to all SCF

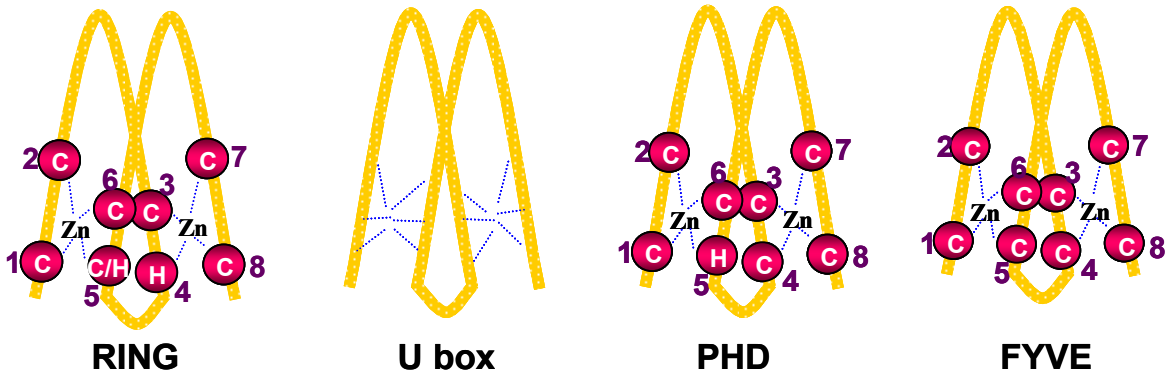

Fig 2. Schematic representation of RING finger and RING finger related motifs.

A RING finger domain is stabilized by the presence of eight cysteine (C) or histidine (H) residues, which form a cross brace like structure in the presence of zinc ion. RING finger domains are usually classified into CH or H2-type RING fingers depending on whether the 5th conserved residue is cysteine or histidine. In contrast, a U box is stabilised by the formation of several salt bridges and hydrogen bonds with the conserved charged and polar residues. PHD and FYVE finger domains are very similar to the RING finger domain. The PHD finger domain contains cysteine as 4th conserved residue and an invariant tryptophan before the seventh residue, whereas the FYVE finger domain uses only cysteine residues to stabilise its structure.

complexes. The F-box protein, which mediates the substrate specificity of SCF complex, is the variable component of the complex. Depending on the presence of different F-box protein, the SCF complexes are designated accordingly (for example, SCF^{Cdc4}, $SCF^{\beta\text{-}TrCP}$). The different SCF complexes perform different functions. For example, the $SCF^{\beta\text{-}TrCP}$ complex targets phosphorylated IκBα[29] and β-catenin[30] for proteasomal degradation. Another RING E3 complex is the anaphase promoting complex (APC), which is best known for its function in timely degradation of mitotic substrates[31]. The APC complex contains the RING finger protein Apc11 as its core. The CBC complex is composed of substrate binding components (for example, Von Hippel-Lindau tumour suppressor protein, pVHL), the adaptors Elongins B and C, the regulatory protein cullin2, and the RING finger protein ROC1.

The most recently discovered RING E3s have their substrate binding site and the catalytic RING domain on the same polypeptide and have therefore been termed single-subunit RING E3 ligases. Mdm2, c-Cbl, Parkin, BRCA1 are a few examples of single-subunit RING E3s. Mdm2 is well known for its ability to target p53 for proteasomal degradation in normal cells[32; 33]; c-Cbl targets activated receptor protein tyrosine kinases for ubiquitination and endocytosis[34]; and, mutations in the E3 activity of Parkin and BRCA1 have been implicated in Parkinson's disease[11] and familial breast carcinoma, respectively.

The mechanism underlying the substrate ubiquitination by RING finger E3s is different than HECT E3s. Unlike HECT E3s, RING E3s facilitate the direct transfer of ubiquitin from E2 to

the substrate lysine without forming an E3-Ub thioester intermediate. Hence, RING E3s function as a scaffold to bring the active site of E2 in close proximity to the lysine of the substrate. Structural data of RING E3s indicate that at least in some cases, RING fingers form dimers and the dimer formation is required for E3 activity. The dimer can be further stabilised by the presence of additional interacting residues adjacent to the N- and C terminus of the RING, as has been shown for the hetero-dimers of BRCA1/BARD1[35] and Ring1b/Bmi1[36], respectively. However, the structural details as to how RING E3s bring together E2s and their respective substrate proteins are not yet clear. One recent report indicates that binding of E2 to RING E3s causes allosteric changes in the E2, which may aid in bringing the catalytic cysteine of the E2 in close proximity to the substrate lysine residue for facilitating the catalysis of isopeptide bond formation[37].

Although U box domains do not have conserved cysteine and histidine residues, the structure of the U box domain is very similar to the structure of the RING domain[38]. The U box domain is stabilised by the presence of several salt bridges and hydrogen bonds involving conserved charged and polar residues. Another RING finger like domain is the PHD (plant homeodomain) finger, which also has consensus cysteine and histidine residues. However, PHD finger contains a cysteine rather than histidine residue at the fourth zinc coordination position and a conserved tryptophan before the seventh zinc coordination residue. Finally, another yet poorly defined RING related structure known is the FYVE finger. The characteristic feature of the FYVE finger is the inclusion of the sequence (R/K)(R/K)HHCR encompassing the third zinc coordination residue. Another defining feature of FYVE finger is that it uses only cysteine residues to coordinate zinc atoms. Although U box and PHD domains have been implicated to have E3 ligase activity, no protein containing a FYVE finger has been shown so far to have E3 ligase function.

1.1.2 The Proteasome

The 26S proteasome is a large multi-subunit and multi-catalytic protease found in all eukaryotes and, unlike other proteases, requires the polyubiquitination of substrate proteins prior to degradation into small peptides[2; 39]. Given the importance of the proteasome in degradation of a vast array of proteins, it is not surprising that mutations in the proteasome subunits are lethal for cells. Both nucleus and cytoplasm have been shown to be the site of degradation of polyubiquitinated proteins by the proteasome. The 26S proteasome consists of three sub-complexes, a catalytic 20S core particle and two identical 19S regulatory lid particles each present on the two extremities of the 20S core particle. The 20S core particle is a barrel shaped structure consisting of four stacked ring like structures, two outer identical α

rings and two inner identical β rings. Each α and β ring is composed of seven distinct subunits. The catalytic activity of the 20S core particle is localised to some of the β subunits. Several functions have been attributed to the 19S lid particle. One function is to recognise ubiquitinated proteins. The ATPase function of the 19S lid particle has been implicated in creating a gate in the α ring of the 20S core and also in unfolding of the ubiquitinated protein such that it can be inserted into the proteolytic chamber[40].

1.2 p53 and its family members

The tumour suppressor protein p53 is the most extensively studied protein in cancer biology. In 1979, p53 was identified as a binding partner of the SV40 large T antigen[41]. Subsequent studies revealed that p53 is a sequence specific DNA binding protein and acts as a transcriptional modulator and that the p53 gene is mutated in around 50% of all known human cancers[42; 43]. p53 is a short-lived protein that is very tightly maintained at threshold low levels within cells[44]. This low level of p53 is essential for the proper functioning of cells, as a decrease below the threshold level is oncogenic and increased levels cause cell cycle arrest or apoptosis or senescence. The low level of p53 is maintained by several RING finger E3 ligases including Mdm2[32; 33], PirH2[45], Cop1[5], Mule[46], and Topors[47]. However, Mdm2 appears to be the main regulator of p53 levels in normal cells by targeting p53 for ubiquitination and degradation. In addition, Mdm2 also inhibits the transactivation activity of p53 by directly binding to the p53 transactivation domain and thus inhibiting the binding of transcriptional coactivators (for example, p300 and CBP)[48].

Recently, two 53-related proteins, p73[49] and p63[50] (also known as p51, KET, p53CP, NBP, p40, p73L), have been identified. Structurally, p63 and p73 are more similar to each other than to p53. However, the three main functional domains of p53, the N-terminal transactivation domain (TA), the central DNA binding domain (DBD), and the C-terminal oligomerization domain (OD), are conserved in both p63 and p73 proteins (Fig 3). In addition, both p63 and p73 have C-terminal extensions. In contrast to p53, both the p63 and p73 gene produce multiple alternative splice variants with the respective proteins differing only in the length of C-terminal extension. At least 6 splice variants of p73 (α, β, γ, δ, ε, ζ) and 3 splice variants for p63 (α, β, γ) have been reported[51]. In addition, each splice variant can be produced in two forms (full-length and ΔN) by using two different promoters at the 5'-end of both p73 and p63 gene, which further adds to the complexity in the expression of p63 and p73. The ΔN form lacks the N-terminal transactivation domain and functions as a dominant-negative for the full-length protein, possibly by competing with the full-length protein for DNA binding[52].

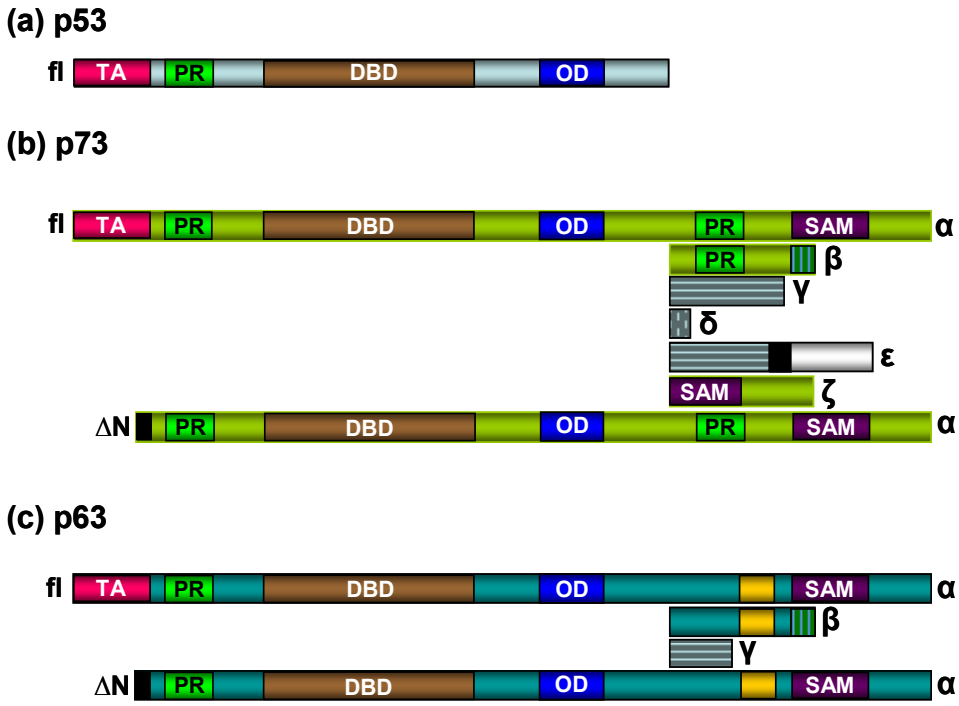

Fig 3. Schematic representation of p53 and its family members.
The p53 family contains three proteins- p53, p73 and p63. All the domains of p53 - transactivation (TA), proline rich (PR), DNA binding (DBD), and oligomerization (OD), are conserved in each of the three members of the p53 family. Unlike p53, several splice variants of p73 and p63 are expressed within cells, which mainly differ at their C terminus following the oligomerization domain. p73 has at least six (α, β, γ, δ, ε, and ζ), whereas p63 has three (α, β, and γ) splice variants. The α splice variant is always the full-length protein. In addition, each splice variant of p73 and p63 can be expressed with two different promoters. Promoter 1 produces the full-length (fl) protein, while promoter 2 produces an N-terminal truncated protein (ΔN) which lacks the transactivation domain.

Several functional properties of p63 and p73 are similar to p53 including homo-oligomer formation, DNA-binding, activation of the transcription of some of the p53-responsive genes, and induction of apoptosis[50; 53; 54]. However, unlike p53, both p63 and p73 are rarely mutated in cancers and in fact they are overexpressed in several cancers. Thus, p63 and p73 are unlikely to be classical tumour suppressor proteins. In addition, organ specific severe developmental abnormalities have been observed with p63 or p73 knockout mice, indicating tissue specific expression of p63 and p73 during development, whereas p53 is ubiquitously expressed in all tissues. p73 knockout mice show severe abnormalities mostly affecting brain functions, suggesting a role of p73 in the development of nervous system. Although p63 is very similar to p73, p63 knockout mice show very different characteristics. Unlike p73 knockout mice, which are viable, p63 null mice die shortly after birth due to significant skin, limb and craniofacial defects, suggesting a role of p63 in ectodermal development.

Similar to p53, Mdm2 also binds to p73 and inhibits its transcriptional activity. However, Mdm2 does not ubiquitinate and degrade p73[55]. In fact, p73 stability is regulated by Itch, a Nedd4 like HECT E3 ligase. Itch has been shown to bind to p73 and promotes its

proteasomal degradation[26]. In addition, p73 stability is also dependent on an intact Nedd8 (an ubiquitin like protein) attachment pathway[56]. The interaction of Mdm2 with p63 is still controversially discussed.

1.3 Mdm2

The *mdm2* (murine double minute 2) gene was initially identified as one of three genes (*mdm1, mdm2, and mdm3*) amplified in a spontaneously transformed BALB/c mouse cell line (NIH 3T3-DM)[57]. These three genes were located on short acentromeric extrachromosomal bodies (known as double minutes) and were present in >50 copies. Later, it was shown that overexpression of the *mdm2* gene product (Mdm2) was responsible for the transformed phenotype of the 3T3-DM cell line[58]. In 1992, Mdm2 was shown to inhibit the transactivation activity of p53 by directly binding to the transactivation domain of p53[59]. This showed that overexpression of Mdm2 was yet another mechanism to inactivate p53 and hence transform a cell. At that time, one third of human sarcomas were observed to overexpress Mdm2[57], which further strengthened the notion that Mdm2 is an oncoprotein. In 1997, it was observed that Mdm2 not only inhibits the transactivation activity of p53, but it also controls the levels of p53 by ubiquitination and proteasomal degradation[32; 33].

The importance of the *mdm2* gene for cell viability became further evident with the generation of Mdm2 knockout mice and Mdm2 hypomorphic allele containing mice, expressing ~30% of total Mdm2 level. Mdm2 knockout mice are embryonic lethal and die at 3.5dpc (days post coitum). Importantly, Mdm2 knockout mice are viable only in p53 null background[60; 61]. The mice with an Mdm2 hypomorphic allele have decreased body weight, defects in haematopoiesis and are more radiosensitive than normal mice[62]. These phenotypes are p53-dependent and further indicate that Mdm2 is the main regulator of p53 also in adult mice.

1.3.1 Mdm2 gene structure and protein domains

The *mdm2* gene is composed of 2 known promoters and 12 exons (Fig 4a). The first promoter (P1) is a constitutive promoter and the second promoter (P2) is p53-dependent due to the presence of a p53-responsive element. These promoters produce two proteins- a full-length Mdm2 termed p90 and an N-terminal deleted Mdm2 termed p76[63]. The truncated p76-Mdm2 cannot bind p53 and functions as a dominant negative by binding to p90-Mdm2[63; 64]. In addition, several splice variants of Mdm2 are known to be expressed in many human and mouse tumours, with Mdm2-A and Mdm2-B mRNAs representing the major splice variants observed in many human tumours[65]. Mdm2-A and Mdm2-B mRNAs have deletions of exons

4-9 and exons 4-11, respectively, and the respective proteins lack the p53-binding domain and hence have been suggested to function as dominant negative interactors for p90-Mdm2.

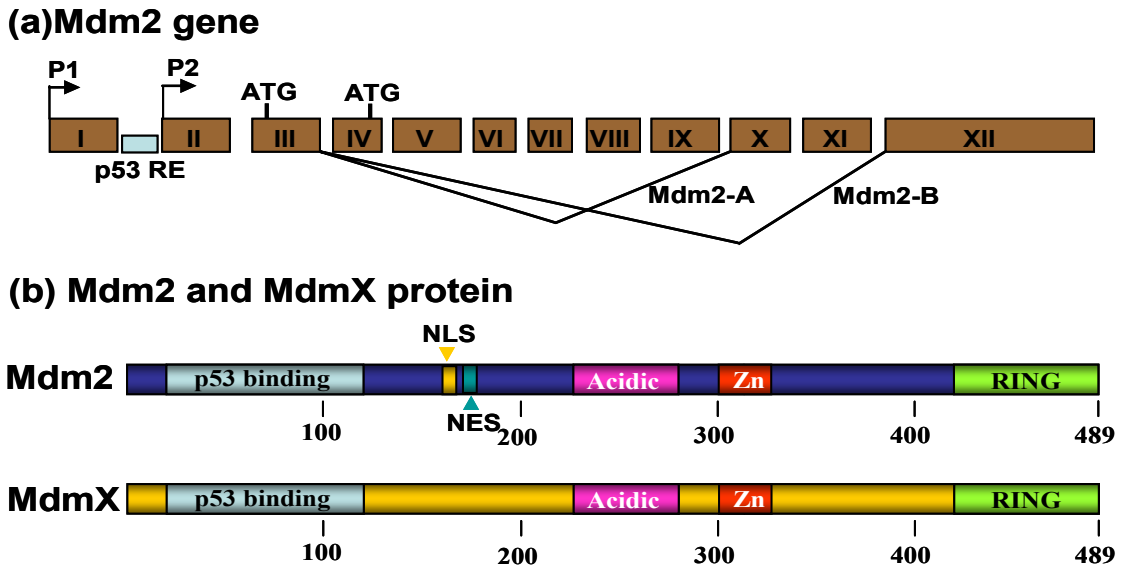

Fig 4. Mdm2 gene structure and the protein domains.

(a) Mdm2 gene is composed of 12 exons and 2 promoters- P1 and P2. P1 is the constitutive promoter, whereas the P2 promoter is controlled by the p53 protein due to the presence of p53 responsive elements (p53 RE). Both exons I and II are present in the 5' untranslated region. Exon III contains the first start codon which produces the full-length Mdm2 protein (p90), whereas exon IV contains the second start codon which is transcribed and translated to a N-terminal truncated Mdm2 protein lacking the p53 binding domain (p76). The splice variants Mdm2-A and Mdm2-B are produced by the deletion of exons IV to IX and IV to XI, respectively.

(b) A schematic representation of Mdm2 and MdmX polypeptides that illustrates the conserved N-terminal p53 binding domain, the central acidic domain followed by the zinc finger domain (Zn), and the C-terminal RING finger domain.

(c) Multiple alignment of the RING finger domains of Mdm2 and MdmX from different species. Most of the residues in the RING finger domain are conserved across all species. The conserved cysteine and histidine residues are shaded in red. Walker A motif sequences are shaded in yellow. The C-terminal 7 amino acid residues are shaded in green and the proposed dimerisation residues have been indicated by blue non-filled rectangles. Numbering of the amino acid residues is based on mouse Mdm2.

The full-length p90-Mdm2 protein is a 489 amino acid long polypeptide consisting of several domains (Fig 4b). At the very N terminus of Mdm2, the p53 interaction domain is present within residues 29-128. This domain of Mdm2 binds to the transactivation domain of p53. Hence, even if Mdm2 does not degrade p53, it interferes with the ability of p53 to interact with the transcriptional machinery. Downstream of the p53 binding domain, a nuclear localisation signal (NLS) and a nuclear export signal (NES) are present within residues 181-185 and 191-199, respectively. These two motifs are responsible for Mdm2 to shuttle in and out of the nucleus and provide another means to control p53 level in the nucleus[66]. The central acidic domain (amino acid 222-272) is responsible for the interaction with a number of proteins including p300/CBP and ribosomal proteins L5, L11 and L23. Following the acidic domain, a well conserved structure is present between residues 300-332, identified as zinc finger. Recently, solution structure of this domain has been reported[67]. However, the physiological function of this domain is still unknown. At the C terminus, a RING finger domain is present within residues 438-478. Within the RING domain of Mdm2, a nucleolar localisation signal and Walker A motif have been reported.

1.3.2 Structural requirements for p53 ubiquitination and degradation

In addition to the N-terminal Mdm2 binding domain, the oligomerization domain and the C-terminal regulatory domain of p53 are essential for Mdm2-mediated p53 ubiquitination and degradation[68]. A possible explanation for the requirement of the C-terminal regulatory domain could be the presence of six lysine residues at the very C terminus of p53, which could act as acceptors for ubiquitin. A p53 mutant, in which all the C-terminal six lysine residues were mutated to arginine (K370/372/373/381/382/386R; 6KR mutant), was reported to be impaired in Mdm2-mediated degradation[69], supporting the importance of these lysine residues for ubiquitination. However, a recent report indicates that a 6KR p53 mutant is still ubiquitinated *in vivo*[70]. Mapping of the lysine residues ubiquitinated in the 6KR p53 mutant revealed several lysine residues in the DNA binding domain of p53. Furthermore, mutations of these lysine residues impair the ability of Mdm2 to ubiquitinate and degrade p53. This indicates that the six lysine residues at the C terminus of p53 are not required for ubiquitination. Thus, the exact role of the C-terminal regulatory sequence of p53 in its degradation is still unclear. The oligomerization domain of p53 is necessary for Mdm2 binding and Mdm2-mediated p53 degradation[68 71].

Along with the N-terminal p53 binding domain and the RING finger domain, the acidic domain of Mdm2 has been found to be necessary for p53 ubiquitination and degradation as an acidic domain (residues 222-272) deletion mutant of Mdm2 is deficient in p53 degradation

activity[72]. Several possibilities have been suggested for the role of the acidic domain in p53 degradation. One possibility could be that Mdm2 directly interacts with the proteasome thereby facilitating p53 degradation[73]. Another possibility could be that the acidic domain is required for p300/CBP binding, which has been implicated in formation of polyubiquitin chain on p53. A recent report suggests that Mdm2 can only mono-ubiquitinate p53 and p300/CBP is required for polyubiquitin chain formation on mono-ubiquitinated p53[74]. Furthermore, the acidic domain of Mdm2 has been recently reported to be the another site for p53 interaction and has been suggested in stabilising the interaction of Mdm2 with p53 during p53 degradation[75]. However, the exact role of the acidic domain in p53 degradation requires further study.

1.3.3 The p53-Mdm2 feedback loop

It is well established that Mdm2 ubiquitinates and degrades p53. On the other hand, p53 binds to the p53-responsive elements within the P2 promoter of the *mdm2* gene and induces the expression of Mdm2[76]. This forms a negative feedback loop, known as the p53-Mdm2 loop, where p53 controls its own level in the cell by regulating the expression of its antagonist Mdm2. Furthermore, Mdm2 not only ubiquitinates p53 but it can also ubiquitinate itself[77] such that low levels of both p53 and Mdm2 are maintained in cells. Several post-translational modifications and the interaction with several regulator proteins have been reported to affect the p53-Mdm2 loop, both under non-stressed and stressed conditions of cells.

1.3.3.1 The p53-Mdm2 loop under non-stressed conditions

In the presence of growth factors and survival signals, Akt (also known as protein kinase B) dependent phosphorylation of S166 and S186 of Mdm2 has been observed[78]. These amino acid residues lie within close proximity to the nuclear localisation signal and the nuclear export signal of Mdm2 and evidence indicates that phosphorylation of S166 and S186 promotes nuclear localisation of Mdm2 which promotes p53 degradation[79]. However, the mechanism of Akt-induced nuclear localisation of Mdm2 is still unclear. Moreover, several phosphorylated serine residues within the acidic domain of Mdm2 (S240, 242, 246, 253, 256, 260, and 262) have been recently observed[80]. Mutation of these residues, individually or in combination, impairs the ability of Mdm2 to degrade p53. However, these Mdm2 mutants still ubiquitinate p53, suggesting that phosphorylation of these residues may be required for p300/CBP binding to the acidic domain of Mdm2. The identity of the protein kinase(s) required to modify these residues in cells is not yet known.

The interaction with several regulatory proteins has been also implicated in regulating the p53-Mdm2 loop- Gankyrin, YY1, Daxx, HAUSP, and MdmX possibly the most important ones. Gankyrin, originally identified as a protein involved in phosphorylation and degradation of the tumour suppressor protein pRB, has recently been reported to be involved in p53 degradation as well[81]. It appears that Gankyrin enhances Mdm2-mediated p53 degradation by enhancing the interaction of p53 with Mdm2. YY1 (Yin Yang 1) protein has also been reported to enhance p53-Mdm2 binding and p53 degradation[82], though, only in response to genotoxic stress. Both Daxx (death domain associated protein) and HAUSP (a ubiquitin specific protease, also known as USP7) have been shown to increase the Mdm2 stability by facilitating the deubiquitination of Mdm2, thereby enhancing p53 degradation. Interestingly, Daxx-mediated recruitment of HAUSP has been recently reported to be the mechanism of action of stabilisation of Mdm2 by Daxx[83]. MdmX, a protein structurally related to Mdm2, has been reported to have both inhibiting and enhancing effects on Mdm2-mediated p53 degradation. However, the mechanism of action of MdmX is unclear (for more details see below).

1.3.3.2 The p53-Mdm2 loop upon stress

In case of genotoxic stress (e.g. DNA damage), the negative effect of Mdm2 on p53 has been observed to be inhibited in order to activate p53. The activated p53 induces the expression of its target genes, which causes either cell cycle arrest or apoptosis depending on the intensity of DNA damage.

Several mechanisms have been implicated to overcome the negative effect of Mdm2 on p53 upon DNA damage. One major mechanism is to inhibit the complex formation between p53 and Mdm2. Several residues within the N-terminal interaction domain of both p53 and Mdm2 have been observed to be phosphorylated in response to a variety of DNA damaging agents including UV and ionizing radiation. DNA-PK (DNA-activated protein kinase) has been reported to phosphorylate S17 of Mdm2. Further studies indicate that indeed phosphorylation of S17 blocks the p53-Mdm2 interaction *in vitro*[84]. An NMR study of the N-terminal domain of Mdm2[85] revealed that residues 25-109 of Mdm2 form a hydrophobic cleft which accommodates the N-terminal region of p53. Residues 16-24 of Mdm2 form a “flexible lid”, which stabilises the N-terminal structure of Mdm2. Interestingly, S17 of Mdm2 has been observed close to T18 and S20 of human p53[85], both of which have been implicated in DNA damage-induced phosphorylation of p53 and in weakening the p53-Mdm2 interaction. However, a p53 mutant, in which all the N-terminal phosphorylation sites were substituted by non-phosphorylatable residues, has been observed to be stabilised by certain DNA-damaging

agents[44; 86]. This implies that phosphorylation-mediated regulation of the p53-Mdm2 loop is dependent on cell context and cell type.

Some additional post-translational modifications of Mdm2 have been observed upon DNA damage. Phosphorylation of S395 by the ATM (ataxia telangiectasia-mutated) kinase has been reported to inhibit p53 degradation and p53 nuclear export[87]. Since p53 nuclear export was originally assumed to be essential in order to efficiently degrade p53[88; 89], it was assumed that ATM dependent phosphorylation of S395 of human Mdm2 inhibits p53 degradation by inhibiting the nuclear export of p53. However, recent studies showed that Mdm2-dependent ubiquitination and degradation of p53 occurs both in the nucleus and cytoplasm[90]. Thus, it is unclear as to how S395 phosphorylation attenuates the ability of Mdm2 to degrade p53. Similarly, the protein tyrosine kinase c-Abl, has been shown to phosphorylate Mdm2 at Y394 and to be required for accumulation of p53 after DNA-damage[91]. Since Y394 phosphorylation in Mdm2 has also been reported to block ubiquitination and nuclear export of p53, the phosphorylation of both S395 and Y394 of Mdm2 may be required for accumulation of p53 after DNA damage. Furthermore, acetylation of several lysine residues in the RING finger domain of Mdm2 has been observed recently and has been shown to inhibit the p53 ubiquitination and degradation activity of Mdm2[92]. Also, several lysine residues at the very C terminus of p53 have been implicated to be acetylated by p300/CBP, which leads to increased transactivation activity of p53.

Dephosphorylation of Mdm2 has also been observed upon DNA damage. Cyclin G1, one of the first p53 responsive genes identified, together with its interacting partner protein PP2A (phosphatase 2A subunit) has been reported to interact with Mdm2 causing dephosphorylation of S166 and T216 [93]. Notably, phosphorylation of S166 and T216 has been implicated in destabilising p53 in normal cells and, hence, dephosphorylation of these residues provides a possible mechanism to stabilise p53 after DNA damage. Moreover, several serine residues in the acidic domain (S240, 242, 260, and 262) of Mdm2 have been observed to be rapidly dephosphorylated in response to ionising radiation[80]. However, the mechanism of dephosphorylation of the acidic domain residues in Mdm2 remains unclear.

Finally, another mode of regulation upon DNA damage is the inhibition of the p53-Mdm2 interaction by the tumour suppressor protein ARF ($p14^{ARF}$ in human, $p19^{ARF}$ in mouse), expressed as alternative reading frame from *INK4a* locus. Normally, $p14^{ARF}$ localises to the nucleolus by forming a tight complex with the nucleolar protein nucleophosmin B23. However, upon DNA-damage $p14^{ARF}$ redistributes into the nucleoplasm and binds to Mdm2 leading to translocation and hence sequestering of Mdm2 into the nucleolus[94]. This may cause p53 to remain in the nucleoplasm of the cell to perform its function.

1.3.4 p53 independent functions of Mdm2

The notion that Mdm2 has p53 independent functions has become evident with the observation that some tumours have both high levels of Mdm2 and mutations in the p53 gene. Subsequent studies revealed that Mdm2 binds and affects the function of a number of proteins including pRB, SP1, E2F1/DP1, $p21^{WAF1/CIP1}$, and histones H2A and H2B.

The interaction of the tumour suppressor pRB with Mdm2 has been reported to target pRB for proteasomal degradation by both ubiquitin-dependent[95] and ubiquitin-independent[96] mechanism. Furthermore, targeting of pRB by Mdm2 is independent of p53 since pRB-mediated cell cycle arrest at the G1 phase is enhanced by overexpression of an E3 inactive Mdm2 mutant in a p53 negative cell line[97]. Mdm2 has also been shown to interact and inhibit the activity of the transcriptional activator SP1[98]. Mdm2 binding to SP1 inhibits its DNA binding ability. However, the presence of pRB disrupts the Mdm2-SP1 interaction by competition, which leads to SP1 reactivation.

The E2F1 transcription factor interacts with DP1 and the hetero-dimer E2F1/DP1 activates transcription of several genes required for S-phase transition of the cell cycle. Mdm2 has been reported to interact with E2F1/DP1 and stimulate E2F1/DP1-dependent transcription[99]. In addition, Mdm2 has been implicated in blocking the apoptotic activity of E2F1[100].

$p21^{WAF1/CIP1}$ is a cyclin-dependent kinase (CDK) inhibitor, which induces cell cycle arrest at the G1 phase in a p53-dependent manner. The interaction of Mdm2 with $p21^{WAF1/CIP1}$ has been reported to target $p21^{WAF1/CIP1}$ to the proteasome in a ubiquitin-independent fashion[101]. The inhibitory effect of Mdm2 on $p21^{WAF1/CIP1}$ is also observed in p53 (-/-) and p53 (-/-)/pRB (-/-) human cell lines. Later on, it has been reported that Mdm2 directly delivers $p21^{WAF1/CIP1}$ to the proteasome by directly binding to the proteasome[102]. Finally, Mdm2 can mono-ubiquitinate histones H2A and H2B, which causes transcriptional repression[103].

1.4 MdmX

MdmX (also known as Mdm4) was originally identified in a cDNA screen as a p53 interacting partner that shows significant structural similarity to Mdm2[104]. Subsequently, MdmX was observed to interact with itself and with Mdm2 in the yeast two-hybrid interaction assay[105]. Interestingly, hetero-oligomers of Mdm2/MdmX appear to be more stable than Mdm2 or MdmX homo-oligomers[105]. The *mdmx* gene was mapped to chromosome 1q32 and has been observed to be overexpressed in a subset of gliomas having no mutations in p53 and Mdm2, suggesting an oncogenic role of MdmX[106]. Similar to Mdm2, MdmX interacts with p53 and inhibits the transactivation activity of p53. However, MdmX has no appreciable E3 ligase activity and, thus, does not ubiquitinate and degrade p53. The

role of MdmX in the regulation of p53 function was confirmed by the generation of MdmX null mice[107]. MdmX null mice, like Mdm2 null mice, are embryonic lethal and die 7.5-11.5 dpc, mainly due to overall growth deficiency and massive apoptosis in the neuroepithelium. The role of p53 in embryonic lethality of MdmX null mice is evident from the fact that MdmX null mice are viable in a p53 null background[108]. Furthermore, several p53 target genes including p21, Bax, and Apaf-1 are overexpressed in homozygous *mdmx* null mice. These experiments further suggest that Mdm2 cannot replace the p53 regulatory functions of MdmX, at least in the embryo and that both Mdm2 and MdmX are essential negative regulators of p53.

The MdmX polypeptide is structurally very similar to the Mdm2 polypeptide (Fig 4b). The three main domains of Mdm2, i.e. the p53-binding, the Zn-finger, and the RING finger domains, are conserved in MdmX and have significant similarity at the amino acid level. However, the nuclear localisation signal (NLS) as well as the nuclear export signal (NES) of Mdm2 are missing in MdmX. Co-expression of Mdm2 has been observed to localise MdmX into the nucleus and, hence, MdmX depends on Mdm2 for its nuclear localisation. Although MdmX has no appreciable E3 activity, the amino acid sequence of the RING finger domain of MdmX is very similar to Mdm2 and also forms a C2H2C4 type RING structure (Fig 4c). Importantly, it is the RING finger domain, by which MdmX interacts with Mdm2 to form hetero-oligomeric complexes.

MdmX was reported to be an inhibitor of Mdm2-mediated p53 degradation, presumably due to its ability to interact with Mdm2[109; 110; 111]. Thus, it was assumed that MdmX inhibits the ability of Mdm2 to interact with E2s by forming hetero-dimer with Mdm2 thereby inhibiting the ability of Mdm2 to ubiquitinate and degrade p53. However, the p53-dependent embryonic lethality of MdmX null mice does not support this hypothesis. Several studies showed that MdmX stabilises Mdm2 in several cell lines when overexpressed in higher amounts (MdmX:Mdm2>2:1). Hence, one possibility could be that Mdm2 alone is a very unstable protein in cell and that the presence of MdmX increases the half-life of Mdm2 so assisting Mdm2 to ubiquitinate and degrade p53. However, *in vitro* reconstituted p53 ubiquitination assays using bacterially expressed Mdm2 and MdmX showed that MdmX is able to enhance the intrinsic E3 activity of Mdm2 to ubiquitinate p53[112]. The observed enhancement of Mdm2-mediated ubiquitination of p53 by MdmX was further supported by RNA interference-mediated knockdown of either MdmX or Mdm2 within cells [112] showing that knockdown of MdmX by siRNA increased cellular p53. The mechanism of enhancement of Mdm2-mediated p53 ubiquitination was suggested to be the result of a stronger binding of the cognate E2, UbcH5, to the Mdm2/MdmX complex compared to Mdm2 alone. However, the

exact mechanism of action of MdmX in Mdm2-mediated p53 ubiquitination and degradation remained elusive.

Considering an essential role of MdmX in Mdm2-mediated p53 ubiquitination and degradation, one can postulate that, upon DNA damage, it is important to inactivate MdmX in order to activate p53. Indeed, MdmX has been observed to be degraded after DNA damage in cells in an Mdm2-dependent manner suggesting a function of p53-dependent induced expression of Mdm2 in the cells after DNA damage[113]. Furthermore, site specific phosphorylation of MdmX on S367 by Chk1/2 after DNA damage stimulates the ability of MdmX to interact with the 14-3-3 sigma protein, a cell cycle inhibitor, which further leads to enhancement of Mdm2-mediated degradation of MdmX in cells[114]. Moreover, caspase-3-mediated cleavage of MdmX has also been observed following DNA damage[115].

In contrast to Mdm2, almost nothing is known about potential interacting partners of MdmX. MdmX appears to interact with p73 and has been shown to inhibit the transactivation activity of p73 and enhance the stability[116]. Similar to Mdm2, conflicting results have been reported about the potential of interaction of MdmX with p63. One study showed that MdmX interacts with p63 and inhibits its function[117], while another study did not detect any interaction between p63 and MdmX[118]. Contrasting data were also reported for the interaction of $p14^{ARF}$ with MdmX. One group demonstrated that, like Mdm2, $p14^{ARF}$ interacts with MdmX and sequesters MdmX into the nucleolus[119]. In contrast, another group did not detect any interaction of p14ARF with MdmX[120]. However, similar to Mdm2, HAUSP has been reported to interact with and stabilise MdmX via its deubiquitination activity[121].

1.5 The Nedd8 conjugation pathway

After the discovery of the ubiquitin conjugation pathway, a number of ubiquitin like (ubls) proteins that can also be covalently linked to other proteins have been identified including Nedd8, Sumo-1/2/3, ISG15, and FAT10. Among these ubls, Nedd8 (also known as Rub1) is the closest kin of ubiquitin, as Nedd8 is ~57% identical and ~76% similar to ubiquitin at the amino acid level. However, to perform their distinct functions, ubiquitin and Nedd8 have to be attached to their correct substrates. The selectivity is achieved by similar mechanisms but distinct enzymes in the conjugation cascades of ubiquitin and Nedd8. The Nedd8 E1 is a hetero-dimer of APPBP1 and UBA3 corresponding to the N-terminal and C-terminal part of the single polypeptide of the ubiquitin E1, respectively. Given the striking similarity between ubiquitin and Nedd8 and ubiquitin E1 and Nedd8 E1, it is not surprising that Nedd8 can be activated by ubiquitin E1, although with an efficiency 100 times less than the efficiency of the activation of Nedd8 by Nedd8 E1[122]. The difference in the efficiency between proper and

cross activation by E1s is explained by crystal structure data of human APPBP1/UBA3 in complex with Nedd8[123]. Nedd8 contains an alanine residue at position 72 whereas ubiquitin has an arginine at the respective position. The Nedd8 E1 UBA3 subunit, which contacts Nedd8, possesses a conserved arginine at position 190, whereas ubiquitin E1 contains a glutamine (Q608) at the homologous position. A72 of Nedd8 has been shown to interact with R190 of the UBA3 subunit. Thus, the presence of R72 in ubiquitin causes repulsion from the R190 of UBA3 subunit of Nedd8 E1 resulting in a low efficiency of cross activation of ubiquitin by Nedd8 E1.

The crystal structure of Nedd8 E1 in complex with its cognate E2, Ubc12, further adds to the understanding of the selectivity of the respective conjugation pathways [124]. Ubc12 contains an N-terminal extension and a central E2 like domain. Both, the N-terminal extension and the central domain of Ubc12 form a unique bipartite contact with the hetero-dimer of Nedd8 E1 and this bipartite contact is essential for transfer of activated Nedd8 from E1 to Ubc12.

In contrast to the ubiquitin conjugation pathway, which has one E1, several E2s, and many E3s, the Nedd8 conjugation pathway has one E1, only one E2, and yet undefined number of E3s. A few RING finger family E3s including ROC1, c-Cbl, and Mdm2 have been reported to catalyse the attachment of Nedd8 to specific substrates. However, it is not yet clear if, similar to ubiquitin, Nedd8 can form chains, although free ubiquitin and Nedd8 dimers have been observed in the presence of E2-25K (an E2 enzyme) *in vitro*[122]. Furthermore, attachment of Nedd8 to ubiquitin occurs 5 times faster than the transfer of ubiquitin to ubiquitin, whereas the transfer of either Nedd8 or ubiquitin to Nedd8 is 50 times slower than the transfer of either of the molecules to ubiquitin[122]. These experiments indicate that Nedd8 is efficiently attached to ubiquitin, but has poor acceptor function. Moreover, *in vitro* synthesized K-48 linked chimeric chains, which contain three ubiquitin and one distal Nedd8 molecule (3Ub-Nedd8), are better substrates for the proteasome than K-48 linked tetra ubiquitin chains. However, the physiological relevance of these findings remains unclear. Recently, an interacting partner of Nedd8, NUB1 (Nedd8 ultimate buster 1), has been shown to target Nedd8 conjugated substrates to the proteasome[125]. Moreover, the Jab1/Csn5 subunit of COP9 signalosome has also been implicated in cleaving Nedd8 from substrate proteins[78].

The first substrate identified to be modified with Nedd8 was Cul1 (cullin1), an integral part of the SCF complex. Initially, the modification of Cul1 with Nedd8 was implicated in localisation of SCF complexes to the centrosome and regulation of the centrosome duplication cycle[126]. Later on, it was shown that Nedd8 attachment to Cul1 boosts the ubiquitin E3 ligase activity of the SCF complex by enhancing the interaction of the cognate ubiquitin E2 with the SCF complex and dissociation of an inhibitor of the SCF complex,

CAND1[127]. Further studies have shown that several members of the cullin family including Cul1, 2, 3, 4A, 4B, and 5 can be neddylated. Finally, Mdm2 has recently been shown to function as a Nedd8 ligase to modify p53 and p73 by Nedd8 and that modification inhibits the transactivation activity of p53[128] and p73[56].

2 Aim of the study

The RING finger proteins Mdm2 and MdmX share significant structural similarity with each other and are major antagonists of the tumor suppressor protein p53. Mdm2 is a member of the RING finger family of E3 ligases and targets p53 for ubiquitination and degradation by the 26S proteasome system. Unlike Mdm2, MdmX does not have appreciable E3 ligase activity and, hence, does not induce p53 degradation. Initial reports indicated that MdmX suppresses the E3 ligase activity of Mdm2 by forming hetero-oligomers with Mdm2. However, genetic studies reported that both Mdm2 and MdmX null mice are only viable in a p53 null background indicating that both Mdm2 and MdmX are important negative regulators of p53 and that they cannot replace each other's function, at least in developing embryos. Consistent with the genetic data, at least two independent groups observed that MdmX is enhancing the E3 ligase activity of Mdm2[112; 129]. One group reported that MdmX increases the E3 activity of Mdm2 to ubiquitinate itself[129], while the other group showed that MdmX enhances the E3 activity of Mdm2 to ubiquitinate p53 and itself[112]. In order to further understand the role of MdmX in p53 regulation, we aimed to characterize the E3 activity of the Mdm2/MdmX complex with a particular emphasis on the identification of the mechanism underlying MdmX-mediated enhancement of the E3 ligase activity of Mdm2.

3 Materials and Methods

3.1 Materials

3.1.1 Solutions and Media

	Composition
Laemmli running buffer (10X)	250 mM Tris-HCl pH 8.4, 2 M Glycine, 1% SDS
Laemmli stop buffer (2X)	62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% Glycerin, 100 mM DTT, 0.001 % Bromophenol blue (store at -20°C)
Stacking gel buffer	0.5M Tris pH-6.8, 0.4% SDS
Separating gel buffer	1.5M Tris pH-8.8, 0.4% SDS
DNA stop buffer (10X)	60% Saccharose, 0.25M EDTA, pinch of Bromophenolblue
Buffer Z (for β-gal assay)	100 mM NaH_2PO_4 pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-Mercaptoethanol (store at -20°C)
TAE-buffer (50X)	2M Tris-HCl,, 950 mM Acetic acid, 50mM EDTA
Transfer buffer	12.5mM Tris HCl, 100mM Glycine, pH 8.3
TNE-T (wash buffer for western blot)	10mM Tris-HCL pH 7.5, 2.5mM EDTA, 50mM NaCl, 0.1% Tween 20
TNN lysis buffer	0.1 M Tris-HCl pH 8.5, 0.1 M NaCl, 1% NP-40, 1mM pfefabloc, 1µg/mL A/L, 1mM DTT
Phosphate buffer saline PBS	137mM NaCl, 2.7mM KCl, 10.1mM NaH_2PO_4, 1.8mM KH_2PO_4, pH 7.4
Guanidinium lysis buffer	6M Guanidinium HCl, 100mM phosphate buffer pH8.0, 10mM Imidazol, 10mM Mercaptoethanol, 1mM pfefabloc, 1µg/mL A/L
Luria Broth (LB) medium	10g/L NaCl, 5g/L yeast extract, 10g/L Bacto-Trypton (pH-7.5)
Yeast complete medium (YPDA)- 1L	10g Yeast extract (Gibco BRL), 20g Bacto Peptone (Difco), 30ml Adenine (100mM) and 50ml Glucose (40%)
Yeast minimal medium (SC)- 1L	5g Yeast Nitrogen base (Gibco BRL), 15g Ammonium sulphate, 4g Amino acid mixture.

Yeast transformation solution A	1M Sorbitol, 10mM Bicine pH 8.35, 3% Ethyleneglycol, 5% DMSO.
Yeast transformation solution B	40% Polyethyleneglycol 1000, 200mM Bicine pH 8.35.
Yeast transformation solution C	150mM NaCl, 10mM Bicine pH 8.35.

3.1.2 Cell Strains and Cell lines

3.1.2.1 Bacterial Strains

-*E. coli DH5α*, Genotype: F^- ϕ80d lacZΔM15 Δ(lac) U169 deo R rec A1 hsdR17 ($r_k^- m_k^+$) supE44 λ^- thi-1 gyrA96 rel A1 (Gibco BRL)

-*E. coli BL 21* (DE3), Genotype: F^- *ompT hsdS*$_b$ ($r_b^- m_b^-$)*βgal dcm* (DE3) (Novagen)

3.1.2.2 Yeast Strains

KF1, Genotype: *MAT***a** *trp1-901 leu2-3,112 his3-200 gal4*Δ *gal80*Δ *LYS2*::*GAL1-HIS3 GAL2-ADE2 met2*::*GAL7-lacZ SPAL10-URA3*

3.1.2.3 Mammalian cell lines

H1299 Non small lung carcinoma

HEK293T Human embryonic kidney

3.1.3 Antibodies

	Characteristics	**Company**	**Dilution**
anti-p53 IgG, Ab6 (DO-1)	Monoclonal mouse	Calbiochem	1:1000
anti-HA IgG, HA.11	Monoclonal mouse	Hiss Diagnostics	1:1000
anti-Mdm2 IgG, SMP14	Monoclonal mouse	Santa Cruz Biotech	1:1000
anti-MdmX IgG, D19	Monoclonal goat	Santa Cruz Biotech	1:1000
anti-GAL4-BD IgG	Monoclonal rabbit	Santa Cruz Biotech	1:1000
anti-mouse IgG	Peroxidase conjugated goat	Dianova, Germany	1:5000
anti-rabbit IgG	Peroxidase conjugated goat	Dianova, Germany	1:5000
Anti-goat IgG	Peroxidase conjugated sheep	Sigma	1:50000
anti-mouse IgG	AlexaFluor 568 goat	Molecular Probes	1:1000

3.1.4 Expression Vectors

Vector	Use	Reference
pGEX-2TK-Spl	Bacterial expression of GST-fused proteins	Amersham Biosciences
pGBKT7	Yeast expression and two-hybrid analysis	Clontech
pGADT7	Yeast expression and two-hybrid analysis	Clontech
pPC86	Two-hybrid analysis	Invitrogen
pDB-leu	Yeast expression and two-hybrid analysis	Invitrogen
pcDNA3-HA	Mammalian expression, in vitro translation	D. Roth, MPI, Frankfurt
pSG 5.0	Mammalian expression, in vitro translation	Stratagene
pcDNA3.1(+)/(-)	Mammalian expression, in vitro translation	Invitrogen

3.1.5 cDNA library

T-cell Library

Fetal Brain Library

3.1.6 Primers

Name	Sequence	Description
RS1	CGCGGATCCGCATGTGCAATACCAACATG	5'-Hdm2, 1aa, BamH1
AH32	CCGCTCGAGTTAGGGGAAATAAGTTAGCAC	3'-Hdm2, 491aa+Stop, XhoI
RS10	CGCGAATTCATGTGCAATACCAACATGTCT	5'-Hdm2, 1aa, EcoRI
MS191	CGCGGATCCTTAGGGGAAATAAGTTAGC	3'-Hdm2, 491aa+stop, BamHI
RS13	TGTCCATGGCGCAACAGGACATCTTATG	5'-Hdm2 (K454A), NcoI
RS14	TGTCCATGGCAAATGCGGACATCTTATGGCC	5'-Hdm2 (T455C), NcoI
RS25	CGGCCTAAAAATAGCTGCATTGTTC	5'-Mdm2 (G446S)
RS26	GAACAATGCAGCTATTTTTAGGCCG	3'-Mdm2 (G446S)
RS40	ATTTGTCAAGGTCGACCTAAAAATGGTTGCATTGTCC ATAGCAAAACAGG	5'-Hdm2 (G453S), SalI
MS244	CGCGGATCCATGTGCAATACCAACATG	5'-Hdm2, 1aa, BamHI
RS89	CATGTGCAATACCAACATGTC	5'-Mdm2, 1aa, BamHI part
RS90	GCGCTCGAGTTAGTTGAAGTAAGTTAGCAC	3'-Mdm2, 489aa+stop, XhoI
RS93	GCGCTCGAGTTACATTTGAATTGGTTGTCTAC	3'-Hdm2, 484aa+stop, XhoI
RS99	GGGCGGCCTAAAAATGCATGCATTGTTCACGGC	5'-Mdm2 (G446A)
RS100	GCCGTGAACAATGCATGCATTTTTAGGCCGCCC	3'- Mdm2 (G446A)
RS101	GGCTGCATTGTGCACAGCAAGACTGGACAC	5'- Mdm2 (G451S)
RS102	GTGTCCAGTCTTGCTGTGCACAATGCAGCC	3'- Mdm2 (G451S)
RS103	GGCTGCATTGTTCACGGCGCCACTGGACACCTC	5'- Mdm2 (K452A)
RS104	GAGGTGTCCAGTGGCGCCGTGAACAATGCAGCC	3'- Mdm2 (K452A)
RS105	GCATTGTTCACGGCAAGTGTGGACACCTCATGTC	5'- Mdm2 (T453C)
RS106	GACATGAGGTGTCCACACTTGCCGTGAACAATGC	3'- Mdm2 (T453C)
RS107	GTTCACGGCAAGGCCGGCCACCTCATGTCATG	5'- Mdm2 (T453A)
RS108	CATGACATGAGGTGGCCGGCCTTGCCGTGAAC	3'- Mdm2 (T453A)
RS109	GTTCACGGCAAGTCTGGTCACCTCATGTCATG	5'- Mdm2 (T453S)
RS110	CATGACATGAGGTGACCAGACTTGCCGTGAAC	3'- Mdm2 (T453S)
RS120	GCAGACAGCCAATCCAAATGTAGCTGACCTGCTCAC	5'- Mdm2 (ΔC7)

	AAAAATAG	
RS121	CTATTTTTGTGAGCAGGTCAGCTACATTTGGATTGGC TGTCTGC	3’- Mdm2 (ΔC7)
RS122	CCTAAAAATGGCTGCGAGGTGCACGGCAAGACTGG	5’- Mdm2 (I448E)
RS123	CCAGTCTTGCCGTGCACCTCGCAGCCATTTTTAGG	3’- Mdm2 (I448E)
RS124	GGCAAGACTGGACACGAGATGTCATGTTTCACG	5’- Mdm2 (L456E)
RS125	CGTGAAACATGACATCTCGTGTCCAGTCTTGCC	3’- Mdm2 (L456E)
RS126	CAGCCAATCCAAATGGAGGTCCTAACTTACTTCAAC	5’- Mdm2 (I483E)
RS127	GTTGAAGTAAGTTAGGACCTCCATTTGGATTGGCTG	3’- Mdm2 (I483E)
RS128	CAATCCAAATGATTGTGGAGACCTACTTCAACTAGC TG	5’- Mdm2 (L485E)
RS129	CAGCTAGTTGAAGTAGGTCTCCACAATCATTTGGATT G	3’- Mdm2 (L485E)
RS133	GCGCTCGAGTTAGTTGAAGTAGGTCTCCAC	3’-Mdm2 (L485E), 489aa+stop, XhoI
RS134	GCGGAATTCATGTGCAATACCAACATGTC	5'-Mdm2, 1aa, EcoRI
RS2	GCGCCATGGCGATGACATCATTTTCCACC	5'-HdmX, 1aa, NcoI
RS3	GCGCCATGGCGATGCACTATTTAGGTCAG	5'-HdmX, 53aa, NcoI
RS4	GCGCCATGGCGATGCTAAGAAAGAATCTT	5'-HdmX, 101aa, NcoI
RS5	GCGCCATGGCGATGGAGGATTGCCAGAAT	5'-HdmX, 427aa, NcoI
LL14	CGCGGATCCTTATGCTATAAAAACCTTAATAAC	3'-HdmX, 490aa+Stop, BamHI
LL20	CGCGGATCCTTAGTTTTCTGTATCTGTTCTCTG	3'-HdmX, 426aa+Stop, BamHI
RS11	CGCGGATCCGCATGACATCATTTTCCACC	5'-HdmX, 1aa, BamHI
RS12		
RS22	CATTCTAGACAGGATGTGGGTACTGCC	5'-HdmX, 167aa, XbaI
RS94	GCGCTCGAGTTACAGCTGAATCTCTTTCTTGC	3'-HdmX, 483aa+stop, XhoI
RS140	GACGGGAACATTATTCACTCGAGGACGGGCCATCTT GTC	5’-HdmX (G452S)
RS141	GACAAGATGGCCCGTCCTCGAGTGAATAATGTTCCC GTC	3’-HdmX (G452S)
RS142	GGGAACATTATTCACGGCCGGGCGGGCCATCTTGTC	5’-HdmX (T454A)
RS143	GACAAGATGGCCCGCCCGGCCGTGAATAATGTTCCC	3’-HdmX (T454A)
RS144	CATGGAAGGACGGGACATGAAGTCACTTGTTTTCAC TGTGCC	5’-HdmX (L457A)
RS145	GGCACAGTGAAAACAAGTGACTTCATGTCCCGTCCT TCCATG	3’-HdmX (L457A)
RS146	GCAAGAAAGAGATTCAGCTCGAGATTAAGGTTTTTA TAGC	5’-HdmX (V484E)
RS147	GCTATAAAAACCTTAATCTCGAGCTGAATCTCTTTCT TGC	3’-HdmX (V484E)
RS148	GCAAGAAAGAGATTCAGCTCGTGATTGAGGTTTTTA TAGC	5’-HdmX (K486E)
RS149	GCTATAAAAACCTCAATCACGAGCTGAATCTCTTTCT TGC	3’-HdmX (K486E)
RS27	GCGCCATGGCGATGGCTCTGAAGAGAATC	5'-h-UbcH5b, 1aa, NcoI
RS28	CGCGGATCCTTACATCGCATACTTCTGAGT	3'-h-UbcH5b/a/c, 147aa+Stop, BamHI
RS51	GCGCCATGGCGATGGCGCTGAAGAGGATTC	5'-h-UbcH5a, 1aa, NcoI
RS74	GCGCCATGGCGATGGCGCTGAAACGGATT	5'-h-UbcH5c, 1aa, NcoI
RS111	CCCCTTTAAACCACCTAAGG	5'-UbcH5b, 62aa, DraI
RS112	GGGAATTCCATATGGACAAGAGTACTTTTG	3'-UbcH5b, 106aa, NdeI
RS114	GTGGTTTAAAGGGGTAATCTG	3’-UbcH5b, 62aa, DraI
RS115	CTCTTGTCCATATGTTCTCTG	5’-UbcH5b, 106aa, NdeI
RS29	GCGCCATGGCGATGGCTTCGAAACGGATC	5'-Ubc8, 1aa, NcoI
RS30	CGCGGATCCTTAGCCCATGGCATACTTCTG	3'-Ubc8, 148aa+Stop, BamHI

RS42	GCGGGATCCATGCTAATTAAAGTGAAGACG	5'-m/hNedd8, 1aa, BamHI
RS43	GGCAGAGATCTCTCGAGTTATCCTCCTCTAAGAGCC AACAC	3'-mNedd8, 76aa+stop, XhoI+BglII
RS60	GGCAGCTCGAGTTATGCTGCTCTAAGAGCCAACAC	3'-hNedd8, G75/76A, 76aa+stop, XhoI
RS61	GGCAGCTCGAGTTATGCTCCTCTAAGAGCCAACAC	3'-hNedd8, G76A, 76aa+stop, XhoI
RS68	GGCAGCTCGAGTGCTGCTCTAAGAGCCAACAC	3'-hNedd8, G75/76A, 76aa, XhoI
RS69	GGCAGCTCGAGTCCTCCTCTAAGAGCCAACAC	3'-hNedd8, 76aa, XhoI
RS116	GGCCTCGAGTTATCCTCCTCTAAGACGCAACACCAG	3'-hNedd8, A72R, 76aa+Stop, XhoI
RS119	GGCCTCGAGTTATCTCAGAGCCAACACCAG	3'-hNedd8, 74aa+Stop, XhoI
RS117	GGCCTCGAGTTACCCACCTCTGAGAGCGAGGACCAG	3'-hUb, R72A, 76aa+Stop, XhoI
Rs118	GGCCTCGAGTTATCTGAGACGGAGGACCAG	3'-hUb, 74aa+Stop, XhoI
HP5	GCGGGATCCATGCAGATCTTCGTGAAGAC	5'-hUb, 1aa, BamHI
RS52	GCGCCATGGCGATGATCAAGCTGTTCTCG	5'-hUBC12, 1aa, NcoI
RS53	GCGGAATTCTTATTTCAGGCAGCGCTC	3'-hUBC12, 183aa+stop, EcoRI
RS57	GCGGGAAGATCTTTATTTCAGGCAGCGCTC	3'-hUBC12, 183aa+stop, BglII
RS64	GCGGGATCCATGATCAAGCTGTTCTCG	5'-hUbc12, N1-26, 1aa, BamHI
RS65	GCGCTCGAGTTACTTCTTGCTGCTGCCC	3'-hUbc12, N1-26, 26aa+stop, XhoI
RS66	GCGCTCGAGATGATCAAGCTGTTCTCG	5'-hUbc12, 1aa, XhoI
RS67	GCGAGATCTTTACTTCTTGCTGCTGCCC	3'-hUbc12, 26aa+stop, BglII
RS72	GACCTCGAGGGCAACGTCGCCCTCAACATCCTCAG	5'-hUbc12, C111A, XhoI
RS73	GACCTCGAGGGCAACGTCTCCCTCAACATCCTCAG	5'-hUbc12, C111S, XhoI
RS80	GCGGGATCCATGGCCCAGTTCAATCTGCTG	5'-hp73, 61aa, BamHI
RS81	GCGGGATCCATGCTGTACGTCGGTGACCCCGCACGG CACCTCGCCACGGCCCAGTTCAA TCTGCTG	5'-hdNp73, 1aa, BamHI
RS82	GCGTCTAGATTAGTGGATCTCGGCCTCCG	3'-hp73, 636aa+stop, XbaI
RS41	AATTCTTAAAGAGGAGAAATTAACCATGGGATCGCA TCACCATCACCATCACG	5'-PSG vector, EcoRI&BamHI hangover
RS44	GATCCGTGATGGTGATGGTGATGCGATCCCATGGTT AATTTCTCCTCTTTAAG	3'-PSG vector, EcoRI&BamHI hangover
RS58	AATTCTTAAAGAGGAGAAATTAACCATGGGATACCC ATACGACGTCCCAGACTACGCGG	5'-PSG vector, HA-tag, EcoRI&BamHI hangover
RS59	GATCCCGCGTAGTCTGGGACGTCGTATGGGTATCCC ATGGTTAATTTCTCCTCTTTAAG	3'-PSG vector, HA-tag, EcoRI&BamHI hangover

3.1.7 Plasmids constructed during this study

No.	Common Name	Insert	Vector	Primers/Res. site
1705	X1, X2	fl-HdmX	pGBKT7	5'-RS2 (NcoI) 3'-LL14 (BamHI)
1706	21, 22	fl-Hdm2	pGADT7	5'-RS1 (BamHI) 3'-AH32 (XhoI)
1707	52-4, 52-5	ΔN52-HdmX	pGBKT7	5'-RS3 (NcoI) 3'-LL14 (BamHI)
1708	100-3, 100-4	ΔN100-HdmX	pGBKT7	5'-RS4 (NcoI) 3'-LL14 (BamHI)
1712	ΔRF2, ΔRF3	ΔRF-HdmX	pGBKT7	5'-RS2 (NcoI) 3'-LL20 (BamHI)
1713	RF3, RF4	RF-HdmX	pGBKT7	5'-RS5 (NcoI) 3'-LL14 (BamHI)
1714	G6	G448S-Hdm2	pcDNA 3.0	Subcloning

				(5'-BamHI, 3'-XhoI)
1715	G1, G5	G448S-Hdm2	pGEX-2TK-Spl	Subcloning (5'-BamHI, 3'-XhoI)
1716	ADX1, ADX2	fl-HdmX	pGADT7	Subcloning (5'-Blunt (NcoI), 3'-BamHI)
1718	BD21, BD24	fl-Hdm2	pGBKT7	5'-RS10 (EcoRI) 3'-MS191 (BamHI)
1724	XAD4	Δ225-288 HdmX	pGBKT7	5'-RS2 (NcoI) 3'-RS12 (AflII)
1725	XAD2	Δ174-290 HdmX	pGBKT7	5'-RS2 (NcoI) 3'-RS12 (AflII) (Accidently)
1728	PKA1, PKA2	K454A-Hdm2	pcDNA 3.0	5'-MS244 (BamHI) 3'-AH32 (XhoI) 5'-RS13 (NcoI) 3'-AH32 (XhoI)
1729	GKA4, GKA11	K454A-Hdm2	pGEX-2TK-Spl	5'-MS244 (BamHI) 3'-AH32 (XhoI) 5'-RS13 (NcoI) 3'-AH32 (XhoI)
1730	2GK7	G448S+K454A-Hdm2	pcDNA 3.0	Insert fallout from 2GK1 (BamHI+XhoI)
1731	2GK1, 2GK2	G448S+K454A-Hdm2	pGEX-2TK-Spl	5'-MS244 (BamHI) 3'-AH32 (XhoI) 5'-RS13 (NcoI) 3'-AH32 (XhoI)
1732	PTC1, PTC2	T455C-Hdm2	pcDNA 3.0	5'-MS244 (BamHI) 3'-AH32 (XhoI) 5'-RS14 (NcoI) 3'-AH32 (XhoI)
1733	GTC9, GTC10	T455C-Hdm2	pGEX-2TK-Spl	5'-MS244 (BamHI) 3'-AH32 (XhoI) 5'-RS14 (NcoI) 3'-AH32 (XhoI)
1734	2GT13	G448S+T455C-Hdm2	pcDNA 3.0	Insert fallout from 2GT2 (BamHI+XhoI)
1735	2GT2, 2GT3	G448S+T455C-Hdm2	pGEX-2TK-Spl	5'-MS244 (BamHI) 3'-AH32 (XhoI) 5'-RS14 (NcoI) 3'-AH32 (XhoI)
1742	D2, D3	Δ170-223aa-HdmX	pGADT7	5'-RS22 (XbaI) 3'-MS266 (XhoI)
1743	D34, D35	Δ170-223aa-HdmX	pGBKT7	5'-RS2 (NcoI) 3'-LL14 (BamHI)
1744	8BD2, 8BD6	fl-Ubc8	pGBKT7	5'-RS29 (NcoI) 3'-RS30 (BamHI)
1745	P11, P12	Δ170-223aa-HdmX	pcDNA 3.0	5'-MS265 (BamHI) 3'-MS266 (XhoI)
1746	G15, G16	Δ170-223aa-HdmX	pGEX-2TK-Spl	5'-MS265 (BamHI) 3'-MS266(XhoI)
1753	U51, U52	h-UbcH5b	pGBKT7	5'-RS27 (NcoI) 3'-RS28 (BamHI)
1756	N31, N32	Nedd8	pSG 5.0-Spl	5'-RS41 3'-RS44 (EcoRI & BamHI hangover)
1757	GGS6	G453S Hdm2	pGEX-2TK-Spl	Insert fallout from G1 by BamHI and SalI + 5'-RS40 (SalI)

				3'-AH32 (XhoI)
1761	LX28, LX31	Δ170-223aa-HdmX	pDBLeu	Insert fallout from D34 (5'NcoI, 3'NotI » blunt)
1767	BD31	Ubc12	pGBKT7	5'-RS52 (NcoI) 3'-RS53 (EcoRI)
1768	PGS1	G453S-Hdm2	pcDNA3.0	Insert fallout from GGS6 (BamHI+XhoI)
1777	BD45	UbcH5a	pGBKT7	5'-RS51 (NcoI) 3'-RS28 (BamHI)
1790	NN1	G75/76A Nedd8	pSG5.0-Spl	5'-RS42 (BamHI) 3'-RS60 (XhoI)
1791	NN7	G76A Nedd8	pSG5.0-Spl	5'-RS42 (BamHI) 3'-RS61 (XhoI)
1792	NHA13	HA-Nedd8	pSG5.0-Spl	5'-RS58 (EcoRI) 3'-RS59 (BamHI)
1793	PCN19	HA-Nedd8	pcDNA 3.0	Insert fallout from N31(BamHI+XhoI)
1794	N8, N9	N1-26 Ubc12	pSG5.0-Spl	5'-RS64 (BamHI) 3'-RS65 (XhoI)
1795	PN1, PN2	N1-26 Ubc12	pcDNA 3.0	5'-RS64 (BamHI) 3'-RS65 (XhoI)
1801	NNU10, NNU11	G75/76A Nedd8 -N1-26 Ubc12	pSG5.0-Spl (NSG7)	5'-RS66 (XhoI) 3'-RS67 (BglII)
1802	NNU5	Nedd8-N1-26 Ubc12	pSG5.0-Spl (NS3)	5'-RS66 (XhoI) 3'-RS67 (BglII)
1803	NCA1	C111A Ubc12	pSG5.0-Spl	5'-RS72 (XhoI) 3'-RS57 (BglII)
1804	NCS2	C111S Ubc12	pSG5.0-Spl	5'-RS73 (XhoI) 3'-RS57 (BglII)
1807	B5C5, B5C3	UbcH5c	pGBKT7	5'-RS74 (NcoI) 3'-RS28 (BamHI)
1808	H45, H46	ΔNp73	pcDNA3.1/HisC	5'-RS81 (BamHI) 3'-RS82 (XbaI)
1809	H39	ΔN61 p73	pcDNA3.1/HisC	5'-RS80 (BamHI) 3'-RS82 (XbaI)
1810	CG2, CG1	G446S Mdm2	pCoc	5'-RS25 (Quick-change) 3'-RS26 (Quick-change)
1813	P7H19, P7H20	ΔC7-Hdm2	pcDNA 3.0	5'-MS244 (BamHI) 3'-RS93 (XhoI)
1814	G7H21	ΔC7-Hdm2	pGEX-2TK-Spl	5'-MS244 (BamHI) 3'-RS93 (XhoI)
1815	P7X28	ΔC7-HdmX	pcDNA 3.0	5'-MS265 (BamHI) 3'-RS94 (XhoI)
1816	G7X33	ΔC7-HdmX	pGEX-2TK-Spl	5'-MS265 (BamHI) 3'-RS94 (XhoI)
1817	G11	G446S Mdm2	pGEX-2TK-Spl	5'-RS89 (BamHI overhang, blunt) 3'-RS90 (XhoI)
1818	PM1	wt-Mdm2	pcDNA 3.0	5'-RS89 (BamHI overhang, blunt) 3'-RS90 (XhoI)
1819	PG1S33	G446S Mdm2	pcDNA 3.0	5'-RS89 (BamHI overhang, blunt) 3'-RS90 (XhoI)
1820	CG11	G446A Mdm2	pCoc	5'-RS99 (Quick-change) 3'-RS100(Quick-change)
1821	CG21	G451S Mdm2	pCoc	5'-RS101(Quick-change)

				3'-RS102(Quick-change)
1822	CK31	K452A Mdm2	pCoc	5'-RS103(Quick-change) 3'-RS104(Quick-change)
1823	CT41	T453C Mdm2	pCoc	5'-RS105(Quick-change) 3'-RS106(Quick-change)
1824	CT51	T453A Mdm2	pCoc	5'-RS107(Quick-change) 3'-RS108(Quick-change)
1825	CT61	T453S Mdm2	pCoc	5'-RS109(Quick-change) 3'-RS110(Quick-change)
1830	5ab	UbcH5a/b chimera	pGBKT7	Insert fallout from BD45 (NcoI+DraI) 5'-RS111 (DraI) 3'-RS28 (BamHI)
1831	5ba	UbcH5b/a chimera	pGBKT7	Insert fallout from BD45 (NdeI+BamHI) 5'-RS27 (NcoI) 3'-RS112 (NdeI)
1833	PG1A4	G446A Mdm2	pcDNA 3.0	5'-RS89 (BamHI overhang, blunt) 3'-RS90 (XhoI)
1834	PG2S8	G451S Mdm2	pcDNA 3.0	5'-RS89 (BamHI overhang, blunt) 3'-RS90 (XhoI)
1835	PKA2	K452A Mdm2	pcDNA 3.0	5'-RS89 (BamHI overhang, blunt) 3'-RS90 (XhoI)
1836	PTC20	T453C Mdm2	pcDNA 3.0	5'-RS89 (BamHI overhang, blunt) 3'-RS90 (XhoI)
1837	PTA29	T453A Mdm2	pcDNA 3.0	5'-RS89 (BamHI overhang, blunt) 3'-RS90 (XhoI)
1838	PTS34	T453S Mdm2	pcDNA 3.0	5'-RS89 (BamHI overhang, blunt) 3'-RS90 (XhoI)
1839	G1A17	G446A Mdm2	pGEX-2TK-Spl	5'-RS89 (BamHI overhang, blunt) 3'-RS90 (XhoI)
1840	G2S27	G451S Mdm2	pGEX-2TK-Spl	5'-RS89 (BamHI overhang, blunt) 3'-RS90 (XhoI)
1841	TC38	T453C Mdm2	pGEX-2TK-Spl	5'-RS89 (BamHI overhang, blunt) 3'-RS90 (XhoI)
1842	TA49	T453A Mdm2	pGEX-2TK-Spl	5'-RS89 (BamHI overhang, blunt) 3'-RS90 (XhoI)
1843	TS70	T453S Mdm2	pGEX-2TK-Spl	5'-RS89 (BamHI overhang, blunt) 3'-RS90 (XhoI)
1844	KA75	K452A Mdm2	pGEX-2TK-Spl	5'-RS89 (BamHI overhang, blunt) 3'-RS90 (XhoI)
1845	PU74-3	Ub-74	pcDNA 3.0	5'-HP5 (BamHI) 3'-RS118 (XhoI)
1846	PURA7	R72A Ub	pcDNA 3.0	5'-HP5 (BamHI) 3'-RS117 (XhoI)
1847	PN74-14	Nedd8-74	pcDNA 3.0	5'-RS42 (BamHI) 3'-RS119 (XhoI)

1848	PNAR19	A72R Nedd8	pcDNA 3.0	5'-RS42 (BamHI) 3'-RS116 (XhoI)
1850	GU74-31	Ub-74	pGEX-2TK-Spl	Insert fallout from PU74-3 (BamHI, XhoI)
1851	GURA40	R72A Ub	pGEX-2TK-Spl	Insert fallout from PURA7 (BamHI, XhoI)
1852	GN74-43	Nedd8-74	pGEX-2TK-Spl	Insert fallout from PN74-14 (BamHI, XhoI)
1853	GNAR53	A72R Nedd8	pGEX-2TK-Spl	Insert fallout from PNAR19(BamHI, XhoI)
1855	NU74-62	Ub-74	pSG5.0-Spl	Insert fallout from PU74-3 (BamHI, XhoI)
1856	NURA67	R72A Ub	pSG5.0-Spl	Insert fallout from PURA7 (BamHI, XhoI)
1857	NN74-73	Nedd8-74	pSG5.0-Spl	Insert fallout from PN74-14 (BamHI, XhoI)
1858	NNAR79	A72R Nedd8	pSG5.0-Spl	Insert fallout from PNAR19(BamHI, XhoI)
1860	AD2C7-1	ΔC7-Hdm2	pGADT7	5'-RS1 (BamHI) 3'-RS93 (XhoI)
1861	ADXC7-1	ΔC7-HdmX	pGADT7	5'-RS11 (BamHI) 3'-RS94 (XhoI)
1862	CC7-11	ΔC7-Mdm2	pCoc	5'-RS120(Quick-change 3'-RS121(Quick-change
1863	CI1E21	I448E Mdm2	pCoc	5'-RS122(Quick-change 3'-RS123(Quick-change
1864	CL1E33	L456E Mdm2	pCoc	5'-RS124(Quick-change 3'-RS125(Quick-change
1865	CI2E43	I483E Mdm2	pCoc	5'-RS126(Quick-change 3'-RS127(Quick-change
1866	CL2E51	L485E Mdm2	pCoc	5'-RS128(Quick-change 3'-RS129(Quick-change
1867	ΔCM4	ΔC7-Mdm2	pGEX-2TK-Spl	5'-RS134 (EcoRI) 3'-RS93 (XhoI)
1868	GI1E1	I448E Mdm2	pGEX-2TK-Spl	5'-RS134 (EcoRI) 3'-RS90 (XhoI)
1869	GL1E15	L456E Mdm2	pGEX-2TK-Spl	5'-RS134 (EcoRI) 3'-RS90 (XhoI)
1870	GI2E34	I483E Mdm2	pGEX-2TK-Spl	5'-RS134 (EcoRI) 3'-RS90 (XhoI)
1871	GL2E24	L485E Mdm2	pGEX-2TK-Spl	5'-RS134 (EcoRI) 3'-RS90 (XhoI)
1872	PM2C7-51	ΔC7-Mdm2	pcDNA 3.0	Insert fallout from ΔCM4 (EcoRI, XhoI)
1873	PI1E7	I448E Mdm2	pcDNA 3.0	Insert fallout from GI1E1 (EcoRI, XhoI)
1874	PL1E13	L456E Mdm2	pcDNA 3.0	Insert fallout from GL1E15 (EcoRI, XhoI)
1875	PI2E20	I483E Mdm2	pcDNA 3.0	Insert fallout from GI2E34 (EcoRI, XhoI)
1876	PL2E25	L485E Mdm2	pcDNA 3.0	Insert fallout from GL2E24 (EcoRI, XhoI)
1877	ADΔC39	ΔC7-Mdm2	pGADT7	Insert fallout from ΔCM4 (EcoRI, XhoI)
1878	ADI1E61	I448E Mdm2	pGADT7	Insert fallout from GI1E1 (EcoRI, XhoI)
1879	ADL1E67	L456E Mdm2	pGADT7	Insert fallout from GL1E15

				(EcoRI, XhoI)
1880	ADI2E73	I483E Mdm2	pGADT7	Insert fallout from GI2E34 (EcoRI, XhoI)
1881	ADL2E79	L485E Mdm2	pGADT7	Insert fallout from GL2E24 (EcoRI, XhoI)
1882	ADM85	wt-Mdm2	pGADT7	5'-RS134 (EcoRI) 3'-RS90 (XhoI)
1883	ADG1S1	G446S Mdm2	pGADT7	5'-RS134 (EcoRI) 3'-RS90 (XhoI)
1884	ADG1A7	G446A Mdm2	pGADT7	5'-RS134 (EcoRI) 3'-RS90 (XhoI)
1885	ADG2S14	G451S Mdm2	pGADT7	5'-RS134 (EcoRI) 3'-RS90 (XhoI)
1886	ADTC25	T453C Mdm2	pGADT7	5'-RS134 (EcoRI) 3'-RS90 (XhoI)
1887	ADTA26	T453A Mdm2	pGADT7	5'-RS134 (EcoRI) 3'-RS90 (XhoI)
1888	BDΔC7	ΔC7-Mdm2	pGBKT7	Insert fallout from ADΔC39 (EcoRI, PstI)
1889	BDI1E43	I448E Mdm2	pGBKT7	Insert fallout from ADI1E61 (EcoRI, PstI)
1890	BDL1E49	L456E Mdm2	pGBKT7	Insert fallout from ADL1E67 (EcoRI, PstI)
1891	BDI2E55	I483E Mdm2	pGBKT7	Insert fallout from ADI2E73 (EcoRI, PstI)
1892	BDL2E61	L485E Mdm2	pGBKT7	Insert fallout from ADL2E79 (EcoRI, PstI)
1893	BDM37	wt-Mdm2	pGBKT7	Insert fallout from ADM85 (EcoRI, PstI)
1894	BDG1S	G446S Mdm2	pGBKT7	Insert fallout from ADG1S1 (EcoRI, PstI)
1895	BDG1A	G446A Mdm2	pGBKT7	Insert fallout from ADG1A7 (EcoRI, PstI)
1896	BDG2S	G451S Mdm2	pGBKT7	Insert fallout from ADG2S14 (EcoRI, PstI)
1897	BDTC	T453C Mdm2	pGBKT7	Insert fallout from ADTC25 (EcoRI, PstI)
1898	BDTA	T453A Mdm2	pGBKT7	Insert fallout from ADTA26 (EcoRI, PstI)
1899	GXG2S11	G452S HdmX	pGEX-2TK-Spl	5'-RS140(Quick-change) 3'-RS141(Quick-change)
1900	GXTA21	T454A HdmX	pGEX-2TK-Spl	5'-RS142(Quick-change) 3'-RS143(Quick-change)
1901	GXLE32	L457E HdmX	pGEX-2TK-Spl	5'-RS144(Quick-change) 3'-RS145(Quick-change)
1902	GXVE41	V484E HdmX	pGEX-2TK-Spl	5'-RS146(Quick-change) 3'-RS147(Quick-change)
1903	GXKE51	K486E HdmX	pGEX-2TK-Spl	5'-RS148(Quick-change) 3'-RS149(Quick-change)
1904	PXG2S61	G452S HdmX	pcDNA 3.0	5'-RS140(Quick-change) 3'-RS141(Quick-change)
1905	PXTA71	T454A HdmX	pcDNA 3.0	5'-RS142(Quick-change) 3'-RS143(Quick-change)
1906	PXLE82	L457E HdmX	pcDNA 3.0	5'-RS144(Quick-change) 3'-RS145(Quick-change)
1907	PXVE91	V484E HdmX	pcDNA 3.0	5'-RS146(Quick-change) 3'-RS147(Quick-change)

1908	PXKE102	K486E HdmX	pcDNA 3.0	5'-RS148(Quick-change) 3'-RS149(Quick-change)

3.1.8 Other plasmids used in this study

No.	Common Name	Insert	Vector	Reference
1	CMV-p53	P53	pRc-CMV	Martin Scheffner
2	GFP-R273H-p53	R273H-p53	pcDNA3.1(-) hygro	Martin Scheffner
3	pcHdm2	Hdm2	pcDNA3.1(-)	Martin Scheffner
4	pcHdmX	HdmX	pcDNA3.1(-)	Martin Scheffner
5	β-gal	β-gal	pRC-CMV	Martin Scheffner
6	p73α	p73α	pcDNA3.1/His C	Martin Scheffner
7	His-Ub	His-myc-ubiquitin	pcDNA3.1	Martin Scheffner
8	Herc2	Herc2 (Rcc1b domain)	pcDNA3-HA	Martin Scheffner
9	Rlim	Rlim	pcDNA3-HA	Martin Scheffner
10	Livinα	Livinα	pcDNA3-HA	Martin Scheffner
11	EAP30	EAP30	pcDNA3-HA	Martin Scheffner
12	Rho52	Rho52	pcDNA3.1	Martin Scheffner
13	hPC2	hPoly comb 2	pcDNA3-His	Martin Scheffner
14	E6-AP	E6-AP	pcDNA3-HA	Martin Scheffner
15	C820A E6-AP	C820A E6-AP	pcDNA3-HA	Martin Scheffner
16	PIASY	PIASY	pCI-Flag	Martin Scheffner
17	PIASXβ	PIASXβ	pCI-Flag	Martin Scheffner
18	PIAS1	PIAS1	pcDNA3-Flag	Martin Scheffner

3.2 Methods

3.2.1 PCR and Cloning

3.2.1.1 Polymerase Chain Reaction (PCR)

PCRs were set up using either Pfu-polymerase (Stratagene) or Triple-Mastermix (Eppendorf) according to manufacturer's instructions.

3.2.1.2 Site directed mutagenesis

Silent point mutations in Mdm2 and HdmX were generated using Pfu polymerase by the quick change site directed mutagenesis method (Stratagene). Complementary primers containing the required mutations were designed and used to generate nicked circular PCR products. The nicked circular PCR products were digested with *Dpn* I for 2 hours to digest the template DNA and 5 µl of the digested product was used to transform into *E. coli.*

3.2.1.3 Restriction digestion

The digestion reactions were set up in a volume of 50 µl. All restriction enzymes were obtained from either NEB or Invitrogen and the digestions were performed in the buffer

systems and temperature conditions according to the manufacturers' instructions. The digestions were set up for 2-4hrs.

3.2.1.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed according to the method described by Sambrook *et al.* (1989). Electrophoresis were typically performed with 1 % (w/v) agarose gels in 1x TAE buffer submerged in a horizontal electrophoresis tank containing 1x TAE buffer at 1-5 V/cm, except for the resolution of fragments less than 500 bp, 1.2-2% (w/v) agarose gels were used instead. The gels were analyzed under a UV Trans illuminator and photographed using a Gel documentation system (from Fujifilm).

3.2.1.5 Purification of DNA from Agarose gels

DNA fragments from restriction digestion or from PCR reactions were separated by agarose gel electrophoresis. The gel pieces containing the desired DNA fragments were carefully excised from ethidium bromide stained gels under a UV Trans illuminator. The DNA fragments were then purified from the excised gel pieces using the Qiagen gel extraction kit according to the manufacturer's instructions.

3.2.1.6 Ligation

Ligation reactions for the DNA fragments were performed in a volume of 30 µl at room temperature for 1 hour. For PCR cloning, the digested PCR products and the appropriate linearized plasmids were mixed in a molar ratio of 1:3. T4 DNA ligase (Gibco) were added according to the manufacturer's instructions. In addition, 1 µl of 0.1M DTT were also added into each reaction mix.

3.2.1.7 Transformation of DNA into *E. coli* by $CaCl_2$ method

Plasmid DNA (10 µl of a ligase reaction or ~100 ng of a purified plasmid) were mixed with 200 µl of $CaCl_2$-competent *E. coli* cells and incubated on ice for 30 minutes. The cells were then heat-shocked at 42ºC for 90 seconds and immediately transferred to ice for 5 minutes. 1 ml of pre-warmed (at 37ºC) SOC medium was added to the above transformation mix and incubated at 37ºC with shaking for 1 hour. Finally, the entire transformation mix or an appropriate dilution, was plated onto selection plates and the transformants were allowed to grow overnight at 37ºC.

3.2.1.8 Estimation of DNA concentration

Concentration of purified plasmid DNA was estimated using a UV spectrophotometer. (Biorad Smart spec Plus). Measurements were performed in quartz cuvettes using the standard OD_{260}1.0 = 50µg/ml DNA. The ratio $OD_{260}/OD_{280}$was determined to assess the purity of the sample.

3.2.2 Maintenance of bacterial cultures and mammalian cell lines

3.2.2.1 Bacterial cultivation and preparation of glycerol stocks

To maintain glycerol stocks of bacteria, each plasmid was transformed into *DH5α* cells and cultured overnight in LB medium containing the appropriate antibiotic at 37°C with shaking at 220 rpm. Glycerol stocks were prepared by mixing 500 µl of cooled sterile glycerol to 1ml of the overnight culture in a cryovial tube. The contents were then mixed and stored at -80 °C.

3.2.2.2 Maintenance of mammalian cell lines

All mammalian cell lines were incubated at 37°C under 95% humidity and 5% Carbon-di oxide. H1299 cells were cultured in RPMI-1640 (Sigma) medium supplemented with 10% (v/v) FBS. HEK293T cells were cultured in DMEM (Sigma) medium supplemented with 10% (v/v) FBS.

3.2.2.3 Freezing of cell lines in liquid nitrogen

Approximately 70% confluent plates of each cell line were trypsinized. The cells were then released from the plate with 4 ml medium and centrifuged at 1000 rpm for 1 minute at room temperature. The cell pellet was subsequently resuspended in 2 ml freezing medium (5.9 ml growth medium, 1.7 ml FBS, 1 ml DMSO) and aliquoted out into two cryovials and then transferred to a –80°C freezer. The cryovials were finally transferred to a liquid nitrogen tank the next day.

3.2.3 Transfection of mammalian cell lines

Cells were transiently transfected using either Lipofectamine 2000 (Invitrogen) or DOTAP (Roche). In transient transfections, transfection efficiency was normalized by the co-transfection of an expression construct that encodes for β-galactosidase (pRc/CMV-β gal, 200 ng).

3.2.3.1 Transfection using Lipofectamine 2000

Cells were transfected by lipofection using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In brief, cells were seeded onto a 6 cm or a 6-well plate a day before transfection to ensure 90% confluency on the day of transfection. For transfections into H1299 and HEK293T cells, the ratio of Lipofectamine 2000 to DNA was maintained at 1:1 (µl/µg).

3.2.3.2 Transfection using DOTAP

Cells were seeded onto a 6 cm plate 24 hours prior to transfection to ensure 50-70% confluency on the day of transfection. For each transfection in a 6 cm plate, 2 µg of total plasmid DNA was suspended in HBS and added to 14 µl of DOTAP (Roche). DNA-liposomal complexes were allowed to form for 10 min. Meanwhile, the medium from the plates were replaced with growth medium without FBS. The DNA-Liposomal complexes were then added to the cells and incubated in standard growth conditions. The medium was replaced with normal growth medium containing FBS after 4 hours of transfection.

3.2.4 Protein assays for mammalian cells

3.2.4.1 *In vivo* degradation assays for p53 and MdmX

Cells at 90% confluency in 6 cm plates were transfected using Lipofectamine 2000. After 20-24 hours of transfection, the cell monolayers were washed twice with cold PBS. The cells were then scraped from the plates into 100 µl of non-denaturing TNN buffer on ice for 30 minutes and centrifuged at 13,200 rpm for 30 minutes to remove cell debris. The supernatant was subsequently removed carefully from the cell pellet. Transfection efficiency was normalized by a β galactosidase assay and equilibrated amounts of the samples were boiled in laemmli buffer for 5 minutes at 95ºC and loaded onto SDS-PAGE gels.

3.2.4.2 *In vivo* ubiquitination and neddylation assays

Cells at 90% confluency in 6 cm plates were transfected using Lipofectamine 2000. After 20-24 hours transfection, the cells were lysed in 500 µl Gd-HCl buffer and 20 µl of protein A sepharose beads (equilibrated in Gd-HCl buffer) were added. The samples were incubated at 4ºC with rotation for 1 hour. After 1 hour, samples were centrifuged and 50µl of Ni-Agarose beads (equilibrated in Gd-HCl buffer) were added to the supernatant followed by incubation at 4ºC for 3-4 hours or overnight. The beads were then washed two times with Gd-HCl buffer followed by two more washes in a buffer containing one part of Gd-HCl buffer and four part of 50 mM Tris-Cl (pH 6.8) containing 20 mM imidazol. Finally, the samples were washed

two times with 50 mM Tris-Cl (pH 6.8) buffer containing 20 mM imidazol. The samples were boiled at 95 ºC for 5 minutes in 100 µl of laemmli buffer containing 200 mM imidazole and loaded onto SDS-PAGE gels.

3.2.4.3 β galactosidase assay

To a 96 well plate kept on ice, 120 µl of buffer Z was added per well. To each well, 10 µl of cell lysate and 5 µl of ONPG (Orthonitrophrnyl-β-D-galactopyranoside, concentration: 4mg/ml in 100mM NA_2HPO_4 pH 7.4) were added and mixed by a pipette. The plate was then incubated at 37°C till development of yellow color (approx 10-20 minutes) and the absorbance was measured at λ_{405} in an ELISA plate reader. All estimations were done in duplicates.

3.2.4.4 Bradford assay

Normalization of total protein amounts was performed using the Bio-rad Bradford reagent. To 5 µl of protein lysate, 795 µl of milli Q water was added. Then 200 µl of the reagent was added and after 5 min the absorbance was measured at λ_{595} using a Bio-Rad spectrophotometer 3000.

3.2.4.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was done according to the protocol of Laemmli (1970). 8-15% separating gels were used to analyse proteins based on their size. The loading amounts of proteins were normalized by either β-galactosidase analysis or Bradford assay. Normalized samples were boiled for 5 minutes at 95°C in Laemmli loading buffer prior to loading onto the gels. The gels were electrophoresed at constant current setting of 44 mA (for 1 gel) or 80mA (for 2 gels) for 200 minutes.

3.2.4.6 Western blotting

Protein samples were separated by SDS-PAGE and the gels were incubated in transfer buffer. Required sizes of PVDF membranes (Millipore) were cut and activated by their incubation in methanol for 10 seconds. The membranes were then incubated in transfer buffer. Western blotting was performed using a wet transfer apparatus (BIO-RAD) for 150 minutes at 40V. After transfer, the membranes were blocked with 5% (w/v) milk solution in TNE-T for 1 hour at room temperature. The membranes were then washed and incubated in primary antibody for 1 hour. The membranes were subsequently washed in TNE-T buffer (5 times, 10 minutes each) and incubated with secondary antibody conjugated with horse radish peroxidase for 1

hour. The membranes were washed once again for 1 hour with TNE-T buffer and they were developed using ECL (Enhanced Chemiluminescence) kit (Amersham) according to the manufacturer's instructions.

3.2.4.7 Immunoprecipitation (IP)

Cell lysates were prepared in TNN lysis buffer. The normalized amounts of cell lysates were precleared with 50 µl of protein A sepharose slurry (1:1 ratio of Protein A sepharose bead: TNN lysis buffer) by incubation at 4°C with rotation for 1 hour. Samples were then centrifuged and 1 µl of the antibody was added to the supernatant for incubation at 4°C with rotation for 1 hour. Then 50 µl of Protein A sepharose slurry was added and the samples were kept for further incubation at 4°C for 3-4 hours or overnight. The Protein A sepharose beads were washed four times with TNN lysis buffer and then 50 µl of 2X Laemmli loading buffer was added to the beads. The samples were boiled at 95°C for 5 minutes, centrifuged and the supernatants were analyzed by SDS-PAGE.

3.2.4.8 Immunoflorescence

Coverslips with diameter of 12 mm were sterilized by immersion in absolute ethanol for 15 minutes. They were then removed and allowed to dry. Single coverslips were aseptically placed into each well of a 24 well plate (Greiner) and cells were seeded according to required confluency. Cells were transfected using either Lipofectamine 2000 or DOTAP. After 20-24 hours post-transfection, the coverslips containing the transfected cells were carefully transferred to a new 24 well plate with the adherent cells facing upwards. The coverslips were washed twice with 500 µl of PBS containing 100 mM of MgCl2. Into each well, 500 µl of freshly prepared 4% Para-formaldehyde solution was added carefully and the plate was then kept at room temperature for 1 hour. The coverslips were washed twice with PBS and permeabilisation was performed with 500 µl of freshly prepared 0.1% Triton X-100 in PBS for 5 minutes on ice. After permeabilization, the coverslips were again washed twice with PBS. To each coverslip that contained fixed cells, 180 µl of primary antibody (in PBS containing 1% BSA, dilution 1:200) was added and incubation was done at room temperature for 1 hour. After 1 hour, the coverslips were washed 4 times with PBS to remove excess of unattached primary antibody. Excess PBS was also removed and the coverslips were incubated with 180 µl of secondary antibody for 1 hour in the dark (dilution 1:1000 in PBS containing 1% BSA). Following this incubation, the coverslips were once again washed 4 times with PBS. The coverslips were then mounted onto a glass slide using Gelvatol that

contained DAPI to visualize the nucleus. The coverslips were allowed to solidify and were subsequently observed by fluorescence microscopy.

3.2.5 *In vitro* biochemical assays

3.2.5.1 GST-tagged protein purification from bacteria

10 ml of LB media that contained ampicillin was inoculated with bacteria, which contained a plasmid that encoded the GST-fused protein. The culture was grown overnight at 37°C with shaking at 220 rpm. On the morning of the next day, a fresh culture was inoculated with a 1:10 dilution of the overnight grown culture and this culture was grown for 2 hours. To this culture, 0.1 ml of IPTG was added for the induction of protein expression for 4 hours at 37°C with shaking at 220 rpm. The culture was centrifuged and the cell pellet was resuspended in PBS containing 1% triton X-100. The cells were then lysed by sonication (duty cycle, 30-40%; output control, 3-4) for 1 minute using BRANSON sonifier 250 followed by centrifugation at 10,000g for 10 minutes at 4°C. To the supernatant, 200 µl of glutathione sepharose beads were added and incubated for 1 hour at 4°C with rotation. The beads were washed 5 times with PBS that contained 1% triton X-100. The GST-fused proteins were either used for the GST pull down assay (see below) or eluted by the addition of 100 µl (3 times) of 10 mM glutathione in 50 mM Tris-Cl (pH8.0) for use in the *in vitro* ubiquitination and neddylation assays.

3.2.5.2 Purification of Nedd8 E1 from H1299 cells

10 confluent 10 cm plates containing H1299 cells were scrapped and lysed in 500 µl of non-denaturing TNN buffer for 30 minutes. The cells were then centrifuged for 30 minutes at 13,200 rpm. During this time, a column was prepared that contained strong anion exchange sepharose beads (1 ml). The beads were washed with 15 bed volumes of $T_{25}N_{500}$ buffer. Next, the beads were equilibrated by washing with $T_{25}N_{50}$ (15 bed volumes). The cell lysates that were obtained after centrifugation of the lysed cells were loaded onto the column and it was allowed to flow through the column completely. The beads were washed once again with $T_{25}N_{50}$ (10 bed volumes) and Nedd8 E1 was eluted with $T_{25}N_{400}$ (5ml) that contained protease inhibitors and 1 mM DTT. The first 500 µl of the elutant was discarded and the next 1 ml fraction that contained Nedd8 E1 was collected and stored at -20°C.

3.2.5.3 *In vitro* ubiquitination assays

Proteins were S^{35}-radiolabelled using Rabbit Reticulocyte Lysate based kit (Promega) for *in vitro* transcription and translation as per supplier's instructions. 1 µl of the radiolabelled

protein was incubated with 50 ng of E1, 50 ng of E2, 20 µg of ubiquitin (Sigma), 1 mM DTT, 2 mM ATP, 4 mM $MgCl_2$ and Mdm2 or MdmX (50-200 ng) for 2 hours at 30°C. The reactions were stopped by the addition of Laemmli buffer and loaded onto SDS-PAGE gels.

3.2.5.4 *In vitro* neddylation assays

1 µl of the S^{35}-radiolabelled proteins was incubated with 50 ng of E1 (purified from H1299 cells or from bacteria), 50 ng of the bacterially expressed Ubc12, 20 µg of GST-Nedd8 purified from bacteria, 1 mM DTT, 2 mM ATP, 4 mM $MgCl_2$ and Mdm2 or MdmX (50-200 ng) for 2 hours at 30°C. The reactions were stopped by the addition of Laemmli buffer and loaded onto SDS-PAGE gel.

3.2.5.5 GST-pulldown assays

10 µl of the S^{35}-radiolabelled proteins were incubated with 20 µl of GST-tagged bacterially expressed proteins immobilised on glutathione-sepharose beads, 1 mM DTT, and 170 µl of TNN buffer for 3-4 hours at 4°C. The beads were washed 5 times with TNN buffer and then 50 µl of laemmli buffer was added. The samples were centrifuged briefly and the supernatants were loaded onto SDS-PAGE gels.

3.2.5.6 Fixation, amplification and drying of polyacrylamide gel containing radioactive proteins.

The polyacrylamide gel containing radioactive samples were fixed by submerging the gel in a solution containing 40% methanol and 10% acetic acid for 20 minutes. The signal of the radioactive proteins were amplified in NAMP100V (Amersham biosciences) for 20 minutes and then the gel was dried using a gel dryer from BIORAD. The dried gel was exposed to a Fugi imaging plate (Fugifilm) and analysed by a phospho-imager.

3.2.6 Yeast two-hybrid interaction system

3.2.6.1 Competent cell formation (low efficiency)

100 ml of fresh YPDA media was inoculated (0.2 OD) with overnight grown cultures of yeast cell (strain KF1) . Cells were grown at 30°C with shaking at 250 rpm till an OD of 0.8 was obtained. The cells were then pelleted down by centrifugation at 3000g for 5 minutes at room temperature. The pellet was washed with 50 ml of Yeast Transformation solution A and the supernatant was discarded. The pellet was then resuspended in 2 ml of Yeast transformation solution A. The competent cells were finally aliquoted (200 µl each) into 1.5 ml eppendorf tubes and stored at -80°C.

3.2.6.2 Transformation

200 µl of the competent cells were thoroughly mixed with 1-2 µg of DNA and then 1 ml of the Yeast transformation solution B was added. The mixture was vortexed and then incubated for 1 hour at 30°C. The transformed cells were washed with Yeast transformation solution C and then plated on their respective selection plates.

3.2.6.3 Reporter assay

After selection, the yeast cells were streaked on plates lacking the reporter markers (histidine, adenine, or uracil) and the growth of yeast cells were monitored for 4 days.

3.2.6.4 Expression and purification of proteins from yeast cells

10ml of YPDA media was inoculated with yeast cells stabily expressing the required proteins. The culture was grown for 5-6 hours until it reached an OD of 0.8. The cells were centrifuged at 3000g for 5 minutes and to the pellet 100 µl of TNN lysis buffer was added, which also contained protease inhibitors. To the above suspension, equal amounts of glass beads were also added and the cells were lysed by vortexing for 5 minutes (5 times, 1 minute each while keeping intermittently on ice for 1 minute). The liquid part of the mixture was separated from the glass beads by perforating the eppendorf tube and then it was centrifuged for 30 minutes at 13200 rpm. A Bradford assay was performed with the supernatant to quantify and normalise the amount of total protein extracted. 100µg of the total cell lysates were loaded on SDS-PAGE and probed by the indicated antibody.

3.2.7 Yeast two-hybrid screening

3.2.7.1 Competent cell formation (high efficiency)

100 ml of fresh YPDA media were inoculated (0.2 O.D.) with yeast cell (strain KF1) cultures that were grown overnight and contained a plasmid that encoded MdmX fused to a GAL4 DNA binding domain. Cells were grown at 30°C with shaking at 250 rpm till an OD of 0.8 was obtained. Cells were pelleted down by centrifugation at 3000 g for 5 minutes at room temperature. The pellet was washed with 50 ml of sterile ddH_2O. The pellet was then resuspended in 50 ml of 100 mM sterile LiAc and incubated at 30°C for 15 minutes. The cells were centrifuged and the pellet was used for high efficiency transformation.

3.2.7.2 High efficiency transformation

A transformation mix (3.6 ml i.e. 10X) containing 100 mM LiAc, 33% polyethylene glycol (PEG), 250 µg/ml single stranded salmon sperm DNA, 10 µg of library DNA (T cell or fetal

brain) was added to the competent cells prepared for high efficiency transformation. The mixture was thoroughly mixed and incubated at 30°C for 30 minutes. Heat shock treatment of the cells was performed by incubation of the cells for 20 minutes at 42°C and then centrifugation was done at 3000g for 5 minutes at room temperature. The pellet was resuspended in 10 ml of sterile ddH_2O and plated on solid media lacking leucine, tryptophan and adenine. The growth of the yeast cells was monitored for 7 days and the colonies were then streaked onto three different reporter plates that either lacked histidine, adenine, or uracil, respectively.

4 Results

Despite of having a RING finger domain very similar to Mdm2, MdmX does not work as an E3 ligase for p53. Nonetheless, MdmX is an important regulator of p53 protein stability in cells and overexpression of MdmX was reported to increase p53 stability[109; 110; 111]. To understand the role of MdmX in Mdm2-mediated p53 ubiquitination and degradation, Linares et al. reconstituted the ubiquitination reaction *in vitro* by using baculovirus expressed E1, bacterially expressed E2 and Mdm2. Interestingly, using the *in vitro* ubiquitination system, Linares et al. observed that MdmX enhances the E3 ligase activity of Mdm2 towards p53[112]. To obtain insight into the mechanism by which MdmX enhances the E3 ligase activity of Mdm2, we decided to perform a mutational analysis of the RING finger domains of Mdm2, its human ortholog Hdm2, and HdmX and to test the respective mutants - (i) in *in vitro* ubiquitination assay, (ii) for their ability to form homo- and hetero-oligomeric complexes, respectively, and (iii) to interact with members of the UbcH5 family.

4.1 Walker A motif in Mdm2 and MdmX

Mdm2 and MdmX are unique among RING E3 ligases in having a putative Walker A motif (see introduction). The consensus Walker A motif consists of (G/A)XXXXGK(T/S) and has a well defined role in binding nucleotides, in particular ATP and GTP. The Walker A motif in Mdm2 is represented by G(446)CIVHGKT and it has been reported to bind the adenine based nucleotides ATP, ADP and AMP[130]. Moreover, binding of ATP to the Walker A motif of Mdm2 sequesters Mdm2 into the nucleolus, independent of p14ARF. The Walker A motif in MdmX (GNIIHGKT) has not yet been studied. It should be noted, however, that HdmX has an imperfect Walker A motif (GNIIHGRT) and since replacement of K by R is known to significantly decrease the interaction with nucleotides, it can be assumed that the Walker A motif in HdmX has no role in nucleotides binding.

4.1.1 Several Walker A mutants of Mdm2 are impaired in their E3 ligase activity

The T residue in the Walker A motif of Mdm2 has been suggested to coordinate one of the zinc ions in the RING finger domain[131] suggesting that the ability to act as an E3 and the ability to bind ATP may affect each other in a positive or negative manner. In order to test whether the Walker A motif has any role for the E3 ligase activity of Mdm2, we mutated all the key residues in the Walker A motif of Mdm2 (G446S, G446A, G451S, K452A, T453C and T453A) and its human ortholog Hdm2 (G448S, G453S, K454A and T455C) by site

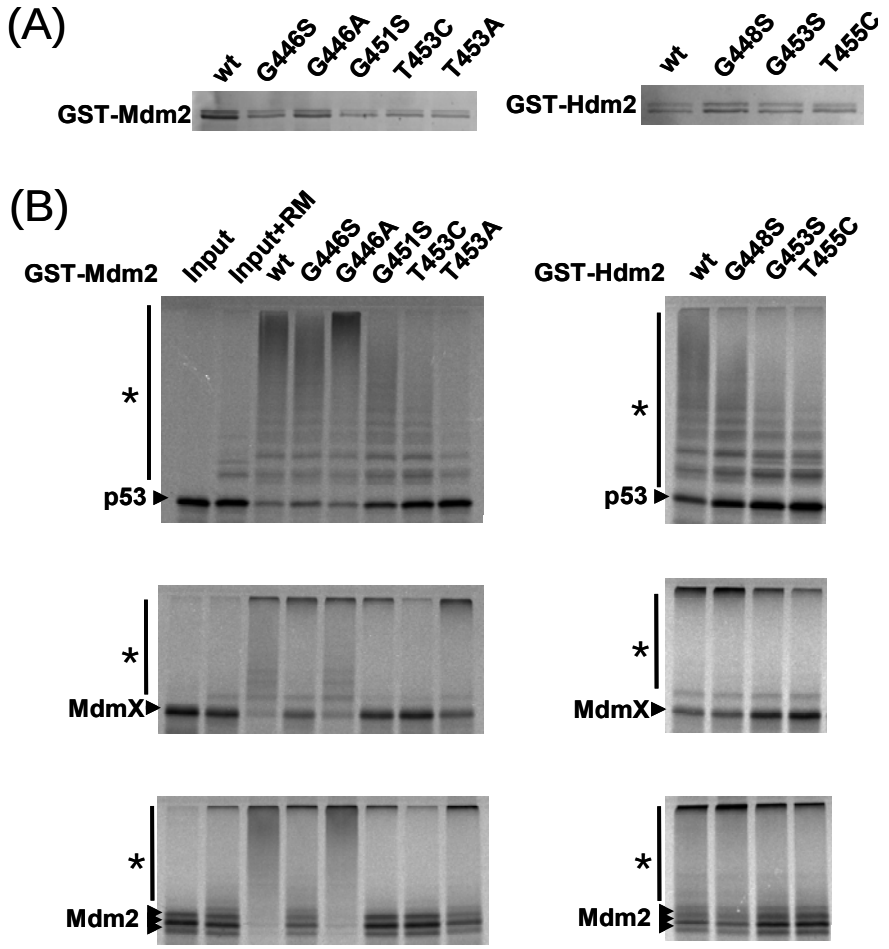

Fig 5. Mutations in the Walker A motif of Mdm2 impair its E3 activity.

(A) Indicated GST-tagged wild type (wt) or the Walker A mutants of Mdm2 and Hdm2 proteins have been expressed in *DH5α* and purified using glutathione immobilised on sepharose beads. The amounts of wild type or mutant Mdm2 and Hdm2 proteins were equilibrated using elution buffer.
(B) Similar amounts of bacterially expressed wild type or mutants of Mdm2 and Hdm2 were incubated with S^{35} labeled, *in vitro* translated p53 or MdmX or Mdm2 protein in a standard ubiquitination reaction for 2 hours at 30ºC. The reactions were stopped by adding similar amounts of 2X laemmli buffer followed by SDS-PAGE and fluorography. The non-modified and ubiquitinated forms of the indicated proteins are denoted by arrowhead(s) and asterisk, respectively.

directed mutagenesis. The respective mutants were expressed in bacteria as GST-fusion proteins and purified by affinity chromatography (see materials and methods 3.2.5.1). Equal amounts of each mutant (Fig 5A) were then used to test their E3 ligase activity towards p53 using S^{35} labelled, *in vitro* translated p53 as a substrate in a standard *in vitro* ubiquitination assay (see materials and methods 3.2.5.3).

Lysine to alanine mutants of Mdm2 (K452A Mdm2 and K454A Hdm2) were previously reported to have slightly increased the E3 ligase activity compared to wt-Mdm2[130]. We also observed a similar effect with the respective mutants of Mdm2 and Hdm2 (data not shown). Mutation of T453, the last residue of the Walker A motif, significantly affected the E3 ligase

activity of Mdm2 and Hdm2 (Fig 5B, upper panel), while mutation of G446 showed very little effect (G446S Mdm2, G448S Hdm2) or even increased the E3 activity (G446A Mdm2). Mutation of the second G residue in the Walker A motif (G451S Mdm2, G453S Hdm2) showed an intermediate effect.

Since Mdm2 does not only ubiquitinate p53, but also ubiquitinates MdmX and itself, the Walker A Mdm2 mutants were also tested for their ability to ubiquitinate wt-MdmX and wt-Mdm2. As shown in Fig 5B (middle and lower panels), the ability of the respective mutants to ubiquitinate wt-MdmX and wt-Mdm2 was affected in a similar manner as their ability to ubiquitinate p53. This indicates that the mutations affect an activity of Mdm2 that is generally required for Mdm2-mediated ubiquitination independent of the substrate used.

4.1.2 MdmX rescues the E3 ligase activity of the ligase defective Mdm2 Walker A mutants

Since MdmX has been reported to enhance the E3 activity of wt-Mdm2 towards p53[112], we tested the effect of MdmX on the E3 activity of the Walker A motif Mdm2 mutants. GST-tagged MdmX was expressed in *E. coli* and purified by affinity chromatography. GST-tagged MdmX was added together with the individual Walker A motif Mdm2 mutants, in a 1:1 ratio, to a standard *in vitro* ubiquitination reaction containing S^{35} labelled p53 as substrate. Interestingly, MdmX was able to rescue the E3 activity of all impaired Walker A motif Mdm2 mutants (Fig 6, upper panel). It is important to note that, in the absence of Mdm2, wt-MdmX did not ubiquitinate p53 indicating that the Walker A Mdm2 mutants together with wt-MdmX form an active E3 unit.

Using wt-MdmX as substrate in the *in vitro* ubiquitination assay, bacterially expressed MdmX was also able to rescue the activity, though not completely, of some of the significantly affected Walker A mutants (e.g. G451S and T453C of Mdm2 and the respective Hdm2 counterparts). The partial rescue of MdmX ubiquitination by GST-MdmX in the presence of the Mdm2 Walker A mutants could be possibly due to the interference of substrate (S^{35}-MdmX) and E3 enzyme (the Walker A Mdm2 mutants) interaction by GST-MdmX, as both S^{35}-MdmX and GST-MdmX interact with the RING finger domain of the Walker A Mdm2 mutants (see discussion).

The above mentioned *in vitro* ubiquitination assays were performed with human UbcH5b as an E2 enzyme (since a recent report suggested that Mdm2 uses UbcH5b and UbcH5c but not UbcH5a for p53 ubiquitination in cells[132]). In some experiments, p53 ubiquitination was studied in the presence of Ubc8 (an E2 from *A. thaliana* and homolog of human UbcH5 protein). The G448S and T455C Hdm2 mutants were tested for their ability to ubiquitinate

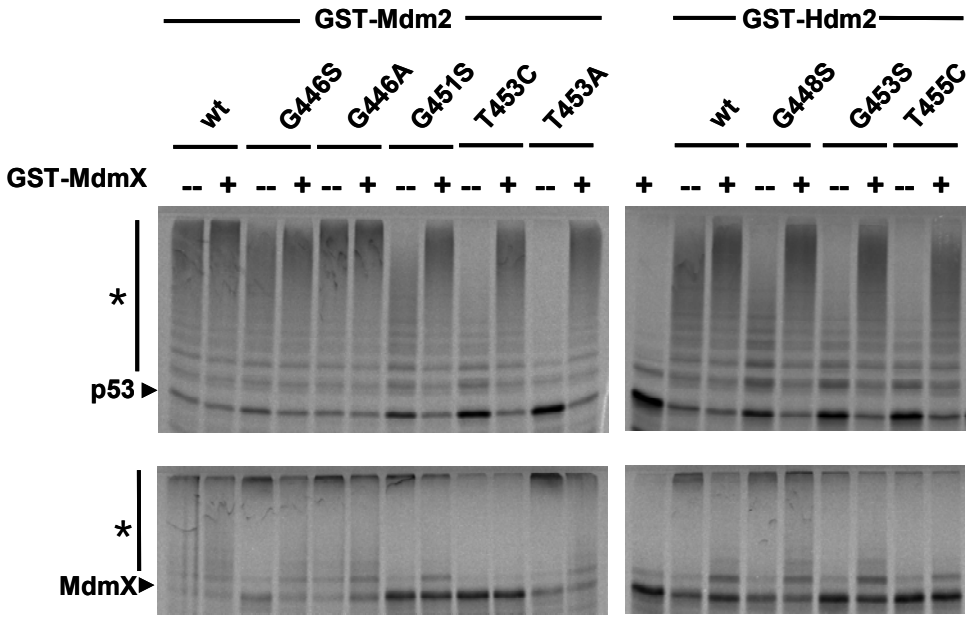

Fig 6. MdmX rescues the E3 activity of the ligase defective mutants of Mdm2.

The indicated GST-tagged Mdm2 and Hdm2 along with their mutants were purified from bacteria and each of them individually incubated in the absence or presence of equal amounts of bacterially expressed GST-tagged MdmX in an *in vitro* ubiquitination reaction containing *in vitro* translated p53 or MdmX. After 2 hours, the reactions were stopped and fluorography performed after SDS-PAGE. Arrowhead and asterisk denote the non-modified and ubiquitinated forms of the indicated proteins, respectively.

p53 using Ubc8 as E2. Interestingly, the G448S and T455C mutants were completely inactive in the presence of Ubc8 (compare Fig. 5B with Fig 7A). However, also under these conditions MdmX completely rescued the E3 ligase activity of these mutants.

In order to map the minimal domain/region of HdmX required to rescue the Walker A Mdm2 mutants, several deletion mutants of HdmX were constructed (a schematic diagram of the HdmX mutants is shown in Fig 7B). The ΔN101 HdmX mutant lacks the known p53-binding domain, ΔN300 HdmX lacks the p53-binding and the acidic domain, ΔN401 HdmX contains only the RING finger domain and ΔC62 HdmX lacks the RING finger domain. After expression and purification, the mutants were tested for their ability to rescue the E3 activity of the G448S Hdm2 mutant. All the mutants containing the RING finger domain of HdmX (ΔN101, ΔN300, and ΔN401) were able to rescue the E3 activity of the G448S Hdm2 mutant, whereas activity was not rescued by the ΔC62 HdmX mutant (Fig 7C). This indicates that the RING finger domain of HdmX is necessary and sufficient to rescue the E3 activity of the G448S Walker A Hdm2 mutant.

It was reported that Hdm2 uses K11, K48, and K63 of ubiquitin for polyubiquitin chain formation[112]. To test if mutation of the Walker A motif affects the usage of K of ubiquitin for

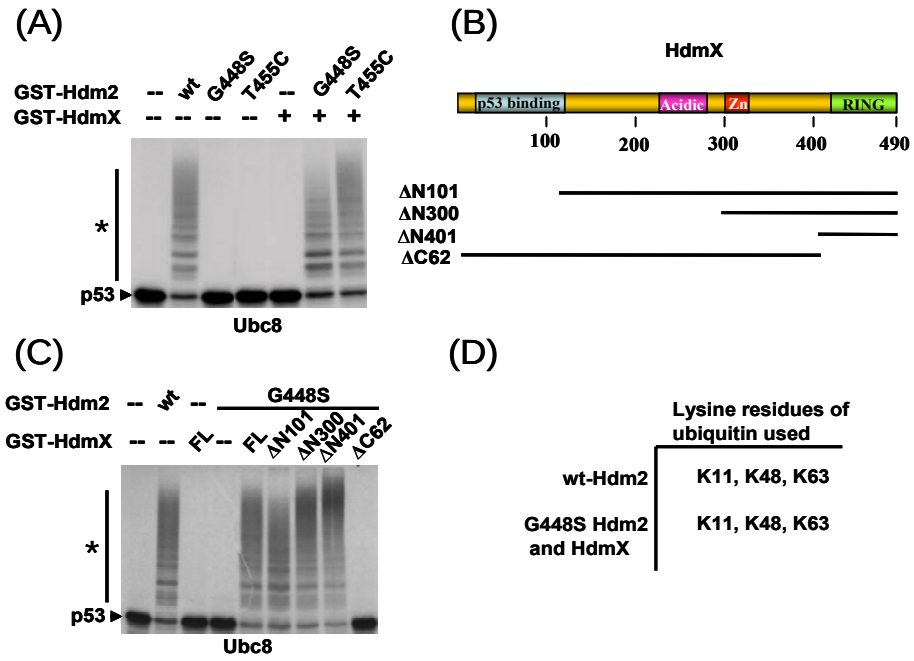

Fig 7. RING finger of HdmX is necessary and sufficient to rescue the E3 activity of the ligase defective Mdm2 mutants.

(A) Bacterially expressed wild type (wt) and mutants of Hdm2 were individually incubated with radiolabelled p53 in the absence or the presence of HdmX in a standard *in vitro* ubiquitination reaction. Notably, Ubc8 is used as E2 in the *in vitro* reaction instead of UbcH5b. The reactions were stopped and fluorography performed after SDS-PAGE. Arrowhead and asterisk denote the non-modified and ubiquitinated forms of p53, respectively.
(B) Schematic representation of full-length and several deletion mutants of HdmX that were constructed.
(C) The wild type (wt) and deletion mutants of HdmX were incubated with the G448S Hdm2 mutant in a standard *in vitro* ubiquitination reaction using Ubc8. The reactions were stopped and fluorography performed after SDS-PAGE. Arrowhead and asterisk denote the non-modified and ubiquitinated forms of p53, respectively.
(D) Mass spectroscopic analysis of the ubiquitinated forms of wt-Hdm2 and the G448S Hdm2/HdmX complex. The polyubiquitinated forms of wt-Hdm2 and the G448S/HdmX complex were excised from the gel and a MS-MS was performed to analyse the lysine residues of ubiquitin used to form polyubiquitin chain.

chain formation, we analysed the K residues of ubiquitin used in polyubiquitin chain formation by the G448S Hdm2 mutant/GST-HdmX complex compared to wt-Mdm2 by mass spectrometry. This revealed that the G448S Hdm2/GST-HdmX complex still uses K11, K48 and K63 of ubiquitin for polyubiquitin chain formation (see Fig 7D). However, whether these lysine residues were used to form three different polyubiquitin chains (i.e. K11 chains, K48 chains, and K63 chains) or they form mixed chains is not clear due to the limitation of mass-spectrometric techniques.

4.1.3 Mutation of the Walker A motif of HdmX does not impair its rescue activity

As indicated earlier, in contrast to MdmX, HdmX contains an imperfect Walker A motif consisting of G(447)NIIHGRT, which should not or only inefficiently bind to ATP. Since

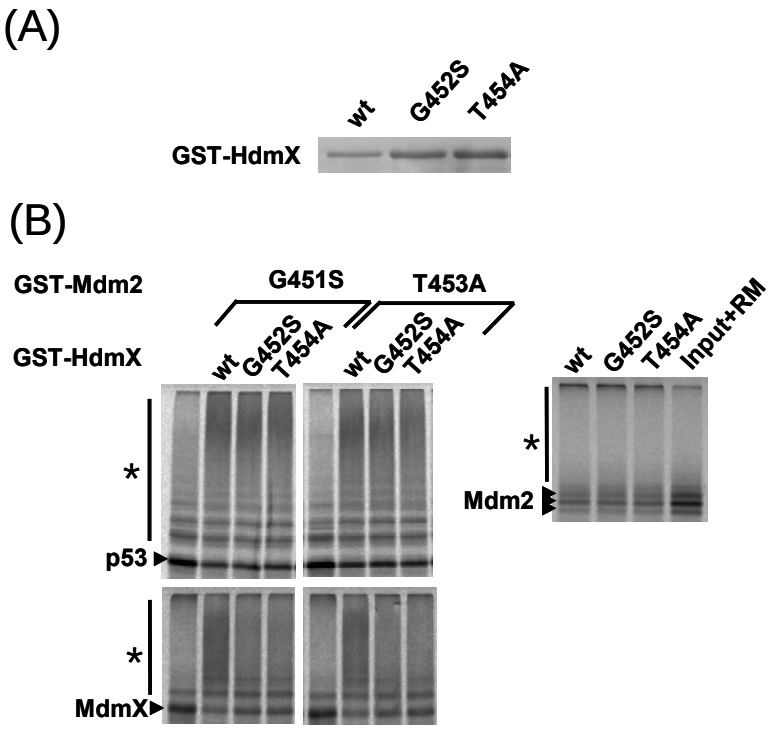

Fig 8. Effect of mutations of the Walker A motif residues of HdmX on its rescue activity.

(A) Indicated wild type (wt) or mutants of HdmX were expressed and purified from *E. coli*. The amounts of wild type and mutant proteins were equilibrated by adding elution buffer.
(B) HdmX or its mutants were incubated with Mdm2 Walker A mutants, as indicated, in an *in vitro* ubiquitination reaction in the presence of radiolabelled p53 or MdmX (left panel). In addition, HdmX or its mutants were also incubated with *in vitro* translated, radiolabelled Mdm2 in a normal ubiquitination reaction (right panel). The reactions were stopped and fluorography performed after SDS-PAGE to detect non-modified and ubiquitinated forms of proteins, which are indicated by arrowhead and asterisk, respectively.

MdmX was able to rescue the E3 activity of the Mdm2 Walker A mutants, we used HdmX to test the possibility that MdmX-mediated rescue is because of the presence of an intact Walker A motif in MdmX. As shown in Fig 8B left panel, this is not the case, since HdmX efficiently rescued the E3 activity of the G451S and T453A mutants of Mdm2. To substantiate this notion, two mutants of the HdmX Walker A motif, G452S and T454A, were constructed. Both HdmX mutants were able to rescue the E3 activity of the Mdm2 Walker A mutants tested for p53 ubiquitination (Fig 8B, upper left panel). This clearly shows that an intact Walker A motif is not required for HdmX to rescue the E3 activity of Mdm2 mutants. Similar to wt-HdmX, the HdmX Walker A mutants were also able to partially rescue the ubiquitination of HdmX by the Mdm2 Walker A mutants (Fig 8B, lower left panel). Furthermore, ubiquitination of wt-Mdm2 by HdmX is also not impaired by mutation of the HdmX Walker A motif (Fig 8B, right panel). Note that henceforth no distinction is made between MdmX and its human ortholog HdmX as similar results were obtained for both proteins.

4.1.4 Mdm2 Walker A mutants were highly ubiquitinated by wt-Mdm2

Some of the Walker A mutants of Mdm2 (i.e. G451S, T453C and T453A) were significantly impaired in their E3 activity. Since Mdm2 can also serve as its own substrate ("auto-ubiquitination"), we tested if the Walker A mutants of Mdm2 serve as substrates for wt-Mdm2 or wt-MdmX. The Walker A mutants of Mdm2 were *in vitro* translated and subsequently tested for ubiquitination in a standard *in vitro* ubiquitination assay. Interestingly, all three Walker A mutants (G451S, T453C and T453A) were highly ubiquitinated by wt-Mdm2 but not by the Mdm2 Walker A mutants and only inefficiently by wt-MdmX (Fig 9). This indicates that the Mdm2 Walker A mutants form an enzyme/substrate complex with wt-Mdm2 and that, at least in part, "auto-ubiquitination" of Mdm2 occurs in trans rather than in cis.

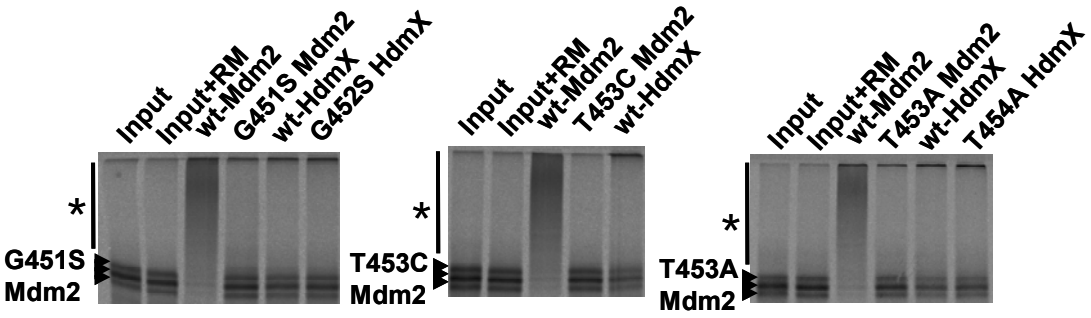

Fig 9. The Walker A Mdm2 mutants are highly ubiquitinated by wt-Mdm2.
Walker A mutants of Mdm2 were radiolabelled and incubated with wild type (wt) or mutants of Mdm2 and HdmX in an *in vitro* ubiquitination assay. The reactions were stopped and fluorography performed after SDS-PAGE to detect non-modified and ubiquitinated forms of proteins, which are indicated by arrowhead and asterisk, respectively.

4.1.5 MdmX rescues the E3 activity of Mdm2 Walker A mutants in H1299 cells

The Mdm2 Walker A mutants with significantly impaired E3 activity (i.e. G451S, T453C and T453A) were cloned into mammalian expression vectors to test their E3 activity in cotransfection assays within cells. H1299 (p53 null) cells were transfected with expression vectors encoding p53, His-tagged ubiquitin, and various mutants of Mdm2 in the absence or the presence of HA-tagged MdmX. After 20-24hr of transfection, cells were lysed in 6M Guanidinium hydrochloride (Gd-Hcl) buffer, followed by purification with Nickel conjugated beads followed by SDS-PAGE and western blotting with an antibody against p53 (hereafter termed "*in vivo* ubiquitination assay"). Consistent with the *in vitro* data, the Mdm2 Walker A mutants were severely affected in their ability to ubiquitinate p53 in H1299 cells.

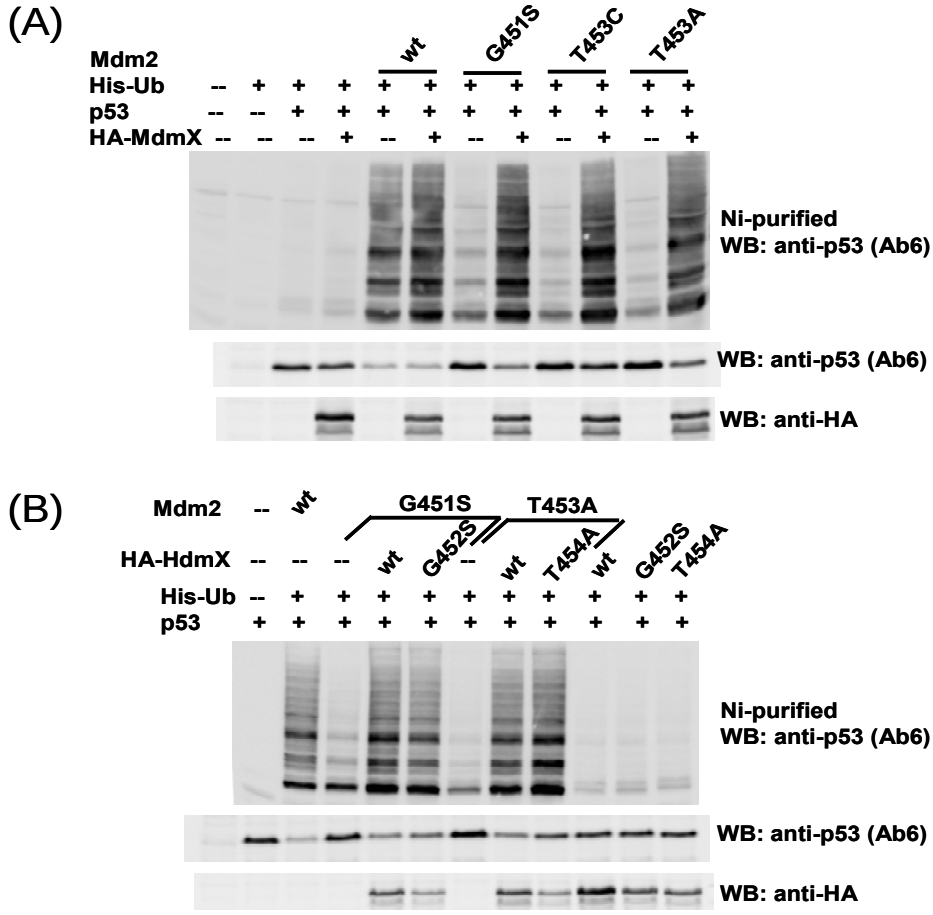

Fig 10. MdmX rescues the E3 activity of the ligase defective Mdm2 mutants in H1299 cells.

(A) Upper panel, the plasmids (Mdm2, 200 ng; HA-Mdmx, 600 ng; His-ubiquitin (His-Ub), 1 µg; and p53, 200 ng), as indicated, were co-transfected into H1299 cells. After 20-24 hours of transfection, cells were lysed in denaturing guanidinium hydrochloride buffer. His-Ub conjugated p53 were purified by Ni-beads followed by SDS-PAGE and western blot performed with a p53 antibody; Middle and lower panels, H1299 cells were transfected with plasmids as indicated in upper panel except β-gal was used in place of His-Ub and only 10 ng of p53 used for transfection. After 20-24 hours of transfection, cells were lysed in non-denaturing condition with TNN buffer followed by normalization of transfection efficiency by β-gal assay. The samples were analysed on a SDS-PAGE gel and western blot performed subsequently with indicated antibodies.
(B) H1299 cells were transfected with the indicated plasmids. The upper, middle and lower panels were treated as mentioned in (A).

Furthermore, coexpression of MdmX completely rescued the E3 activity of the Mdm2 Walker A mutants also in cells (Fig 10A, upper panel).

Mdm2 controls p53 levels within cells by ubiquitination and subsequent degradation of p53 by the 26S proteasome. As the Walker A mutants of Mdm2 were significantly affected in their ability to ubiquitinate p53 within cells, we next tested the ability of the Mdm2 Walker A mutants to degrade p53 in cotransfection assays as described above but in the absence of an ubiquitin expression construct (see materials and methods 3.2.4.1). 20-24hr post-transfection, cells were lysed in non-denaturing TNN buffer. Samples were normalized for transfection

efficiency using β-gal assay (see materials and methods 3.2.4.3) followed by protein separation by SDS-PAGE and western blotting with antibodies against p53 and HA. This showed that the Mdm2 Walker A mutants alone were not able to degrade p53. However, coexpression of MdmX restored p53 degradation (Fig 10A, middle and lower panels). MdmX coexpressed with either the G451S or T453A Mdm2 mutants degrades p53 like wt-Mdm2. However, MdmX coexpressed with the T453C Mdm2 mutant could only partially rescue the degradation of p53.

In the *in vitro* ubiquitination assay, the HdmX Walker A mutants were very similar to wt-MdmX in their ability to rescue the E3 activity of the Mdm2 Walker A mutants. Consistent with the *in vitro* data, the Walker A mutants of HdmX were also able to rescue the ability of the Walker A Mdm2 mutants to ubiquitinate and degrade p53 within cells (Fig 10B). This further confirms that an intact Walker A motif is not necessary for HdmX to rescue the E3 activity of the Mdm2 Walker A mutants.

Several reports suggest that MdmX stabilizes Mdm2[109; 110; 111], possibly by forming hetero-oligomers via their RING finger domains. Thus, one possibility could be that MdmX rescues the E3 ligase activity of the Mdm2 Walker A mutants by Mdm2 stabilisation. To test this possibility, Mdm2 Walker A mutants were expressed in HEK293T cells in the absence or the presence of MdmX (note that levels of ectopically expressed Mdm2 cannot be detected in H1299 cells). This revealed that the levels of the ectopically expressed Mdm2 Walker A mutants were not affected by coexpression of MdmX (Fig 11, lower panel). In order to show that MdmX is also able to rescue the activity of the Mdm2 Walker A mutants in HEK293T cells, we repeated the *in vivo* p53 ubiquitination assay in HEK293T cells. As in H1299 cells, the Mdm2 Walker A mutants were impaired in their ability to ubiquitinate p53 in HEK293T cells and MdmX rescued the E3 activity of the Mdm2 Walker A mutants (Fig 11, upper panel). However, it was not possible to show that MdmX rescues p53 degradation (data not shown), most likely due to the fact that HEK293T cells express adenovirus E1B and SV40 large T antigen, which are known to interfere with p53 degradation.

4.1.6 Mdm2 Walker A mutants oligomerize with wt-MdmX, wt-Mdm2, and themselves

Mdm2 is known to form homo-oligomeric complexes and this property has been suggested to be required for Mdm2 to exert its E3 activity[112; 133]. In order to get insight into the reason for the impaired E3 activity of the Mdm2 Walker A mutants, we tested the ability of the Mdm2 Walker A mutants to interact with p53 and themselves in GST-pulldown assays (Fig 12). *In vitro* translated, radiolabelled p53 was incubated with either GST bound glutathione

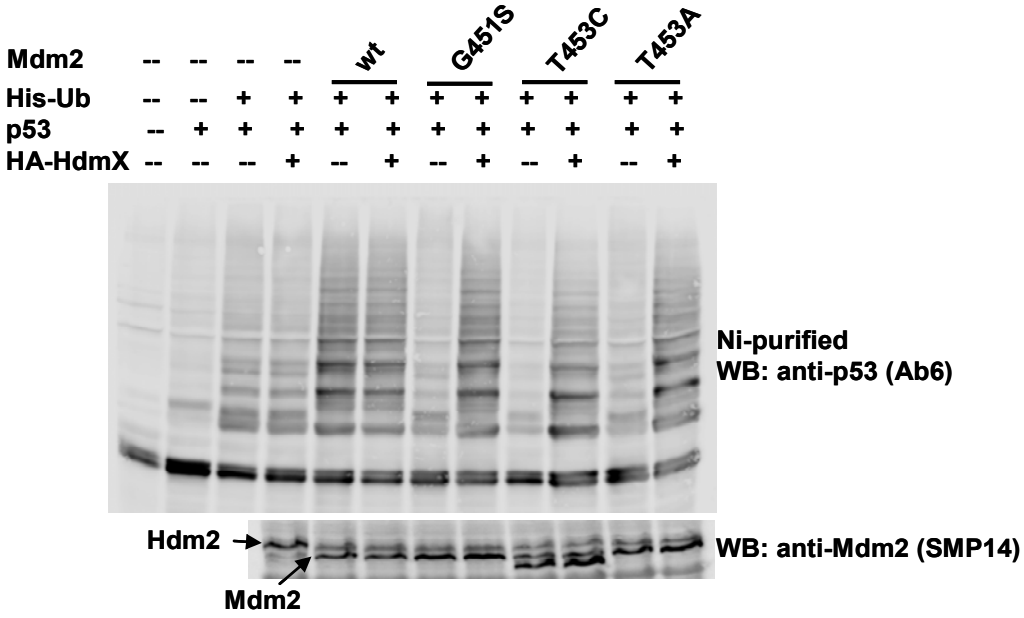

Fig 11. Effect of MdmX on the Walker A Mdm2 mutants in HEK293T cells.

Upper panel, HEK293T cells were transfected with the plasmids (Mdm2, 200 ng; HA-MdmX, 600 ng; His-ubiquitin (His-Ub), 1 μg; and p53, 200 ng), as indicated. 20-24 hours posttransfection, cells were lysed in denaturing buffer. His-Ub conjugated p53 were purified by Ni-beads followed by SDS-PAGE and western blot performed with a p53 antibody.
Lower panel, H1299 cells were transfected with plasmids as indicated in upper panel except β-gal was used in place of His-Ub and no p53 plasmid was transfected (Mdm2, 1 μg; HA-Mdmx, 3 μg; and β-gal, 200 ng). 20-24 hours posttransfection, cells were lysed in non-denaturing TNN buffer followed by normalization of transfection efficiency by β-gal assay. The samples were run on a SDS-PAGE gel and western blot performed subsequently with an Mdm2 antibody that detects both human and mouse Mdm2.

sepharose beads or GST-Mdm2 or GST-Mdm2 Walker A mutants for 3-4hr. Proteins were then separated by SDS-PAGE and p53 detected by fluorography. As expected (since the p53 binding site is located within the N-terminal 100 amino acid of Mdm2), none of the mutations in the Walker A motif of Mdm2 or Hdm2 interfered with p53 binding (Fig 12A, upper panel). Similarly, all the Mdm2 and Hdm2 Walker A mutants interacted with wt-MdmX or wt-Mdm2 with an efficiency similar to the interaction of wt-Mdm2 with wt-MdmX or wt-Mdm2 (Fig 12B, middle and lower panels). In addition, we also tested the interaction of the Mdm2 Walker A mutants with themselves or with the HdmX mutants. No significant differences were observed in the interaction efficiency (Fig 12B).

The oligomerization ability of the Mdm2 and Hdm2 Walker A mutants were also analysed by the yeast two-hybrid interaction assay. cDNA encoding wt-proteins or the mutants (as indicated in Fig 13A) were cloned into yeast two-hybrid vectors in fusion with the Gal4 transactivation domain (AD) or the Gal4 DNA binding domain (BD). Plasmids encoding the respective proteins were transformed into yeast cells (KF1) and tested for expression. All the proteins were expressed at similar levels in yeast cells (Fig 13A). To analyse the interaction

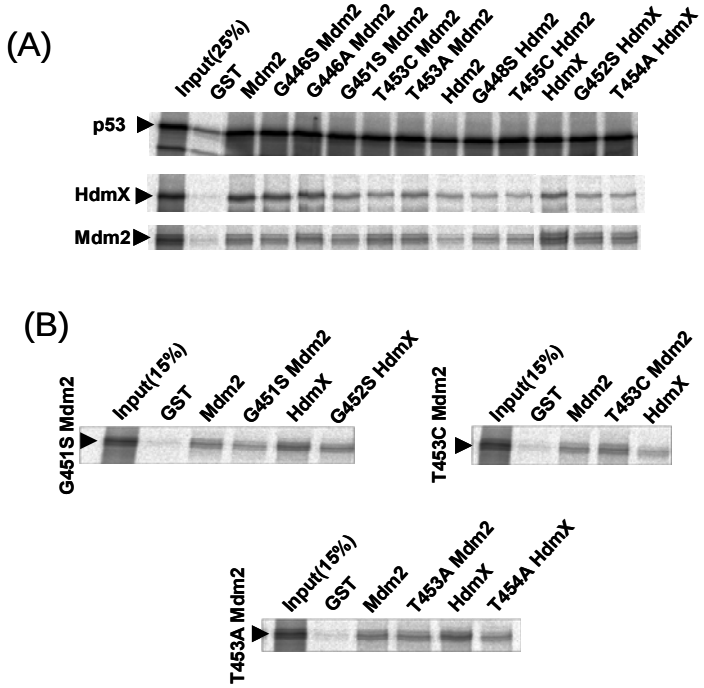

Fig 12. Interaction analysis of the Walker A Mdm2 mutants by GST-pulldown assays.

Arrowhead indicates S^{35} radiolabelled proteins that were incubated with either GST or GST-tagged proteins bound on glutathione sepharose beads, as indicated, for 4 hours at 4ºC. The beads were washed 5 times with TNN buffer containing 1mM DTT followed by SDS-PAGE and fluorography.

of the proteins, transformed yeast cells were streaked onto three different reporter plates lacking histidine, adenine or uracil. An interaction between expressed proteins will switch on the respective reporter genes in yeast cells and, the respective cells would be able to survive on media lacking histidine, adenine or uracil. The presence of these three different reporter genes enabled us to analyse the strength of the respective interactions, since a strong interaction is required to obtain growth on all plates, while an intermediate one will allow growth only on histidine and adenine deficient plates and a weak interaction will allow growth on only histidine deficient plates. All the Mdm2 Walker A mutants, except T453C, were able to interact with either wt-MdmX or wt-Mdm2 or themselves with an affinity similar to the interaction of wt-Mdm2 with itself or with wt-MdmX (Fig 13B, left panel). The T453C Mdm2 mutant was impaired in its homo-oligomer formation ability. However, the hetero-oligomer formation ability of the T453C Mdm2 mutant with wt-MdmX remained intact. Please note that Mdm2 interacted with Mdm2 weakly compared to Mdm2/MdmX interaction as the yeast cells expressing Mdm2 in fusion with the Gal4 DNA binding domain and the transactivation domain survived only on the reporter plates lacking histidine or adenine, respectively, compared to the yeast cells expressing Mdm2 and MdmX, which survived on all three reporter plates. This implies that hetero-oligomers of Mdm2 and MdmX

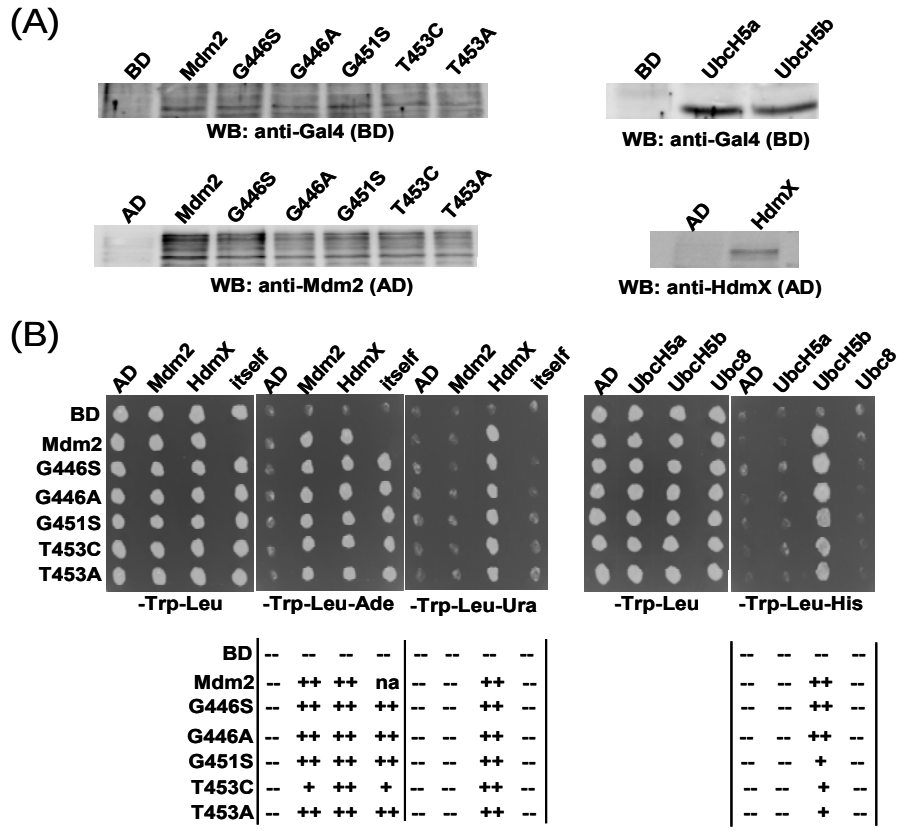

Fig 13. Interaction analysis of the Mdm2 Walker A mutants by the yeast two-hybrid system.

(A) The cDNA of Mdm2 and its mutants, MdmX, UbcH5a, UbcH5b, and *At* Ubc8 were cloned in fusion with the Gal4 activation domain (AD) and the Gal4 DNA binding domain (BD), as indicated, in the yeast two-hybrid vectors. The expression of the proteins in yeast was detected by western blot analysis.
(B) Upper panel, 1µg of each plasmid, as indicated, was transformed into yeast strain KF1 and selected on media lacking tryptophan (Trp) and leucin (Leu). After 3 days, the transformed yeast cells were streaked on reporter plates lacking either histidine (His), adenine (Ade), or uracil (Ura), respectively. Pictures were taken after 4 days of growth. Lower panel, the table indicates the growth of yeast cells after analysis of 10 different colonies for each transformation. --, no growth; ++, growth detected after 2days; and +, growth detected after 3-4days. na, not applicable.

interact more strongly compared to Mdm2 homo-oligomers. Taken together, the results obtained in GST-pulldown and yeast two-hybrid interaction assays suggest that mutations in the Walker A motif of Mdm2 do not significantly affect the oligomerization ability of Mdm2 except for the T453C Mdm2 mutant, which was impaired in forming homo-oligomers.

4.1.7 Interaction of Mdm2 Walker A mutants with E2s

As discussed in the introduction (1.1.1.2), the RING finger domains of some E3 ligases not only interact with themselves, but also interact with E2 enzymes. Hence, it is possible that mutation of the Walker A motif impairs the ability of Mdm2 to bind to its cognate E2s. To test this hypothesis, a GST-pulldown assay was performed using *in vitro* translated E2s

(UbcH5a, UbcH5b and *At* Ubc8) and GST-wt-Mdm2. However, an interaction of GST-wt-Mdm2 with UbcH5a, UbcH5b or *At* Ubc8 could not be detected, indicating that the interaction of Mdm2 with its E2s is rather labile (data not shown). Therefore, a yeast two-hybrid interaction assay was performed with the DNA binding domain of Gal4 fused to the E2s and the transactivation domain of Gal4 fused to wt-Mdm2 or wt-MdmX. UbcH5b weakly interacted with wt-Mdm2 and wt-MdmX as the respective yeast cells were only able to survive on histidine reporter plates. Interestingly, no interaction of Mdm2 and MdmX was detected with UbcH5a or *At* Ubc8, although the proteins were expressed at similar levels as UbcH5b (Fig 13A). It is important to note that yeast cells expressing UbcH5b and MdmX were growing more slowly on histidine reporter plates compared to yeast cells expressing UbcH5b and Mdm2, suggesting that the interaction of MdmX with UbcH5b is weaker than the interaction of UbcH5b and Mdm2. Finally, we analysed the interaction of the Mdm2 Walker A mutants with UbcH5b in the yeast two-hybrid assay. Interestingly, the G451S, T453C and T453A Mdm2 mutants interacted rather weakly with UbcH5b, as they were growing more slowly on histidine reporter plate compared to the cells expressing wt-Mdm2 and UbcH5b (Fig 13B, right panel). In contrast, the G446S and G446A mutants, which have wt-like E3 activity, interacted with UbcH5b with an efficiency similar to wt-Mdm2. To further analyse the strength of interaction of the Mdm2 Walker A mutants with UbcH5b, yeast cells were streaked on five different histidine reporter plates containing different concentrations (5, 10, 15, 20 and 25mM) of 3-AT (a drug which suppresses the histidine promoter in a dose dependent manner). All the yeast cells expressing the individual Mdm2 Walker A mutants along with UbcH5b were growing on all the 3-AT plates, but with slower growth kinetics than yeast cells expressing wt-Mdm2 and UbcH5b (data not shown).

As indicated above, UbcH5b and UbcH5c (not shown) but not UbcH5a or Ubc8 interacted with Mdm2 or MdmX in a detectable manner. However, Ubc8 can support Mdm2-mediated p53 ubiquitination in a standard *in vitro* ubiquitination assay. We therefore assume that there is a very weak interaction between Ubc8 and Mdm2, which cannot be detected under the condition of the yeast two-hybrid interaction assay. Multiple alignment of UbcH5a, UbcH5b, UbcH5c and *At* Ubc8 by ClustalW indicates that UbcH5b and UbcH5c are more similar to each other than to UbcH5a or *At* Ubc8. In order to investigate why UbcH5a did not interact with Mdm2 in yeast, we generated two chimeras of UbcH5a and UbcH5b (Fig 14B). In one chimera, the N-terminal 61 amino acid residues of UbcH5b were replaced by the respective region of UbcH5a (5ab chimera), while in the other chimera the C-terminal 41 amino acid residues of UbcH5b were replaced by the respective region from UbcH5a (5ba chimera). The interaction of these two chimeras, 5ab and 5ba, with wt-Mdm2 was analysed by yeast two-

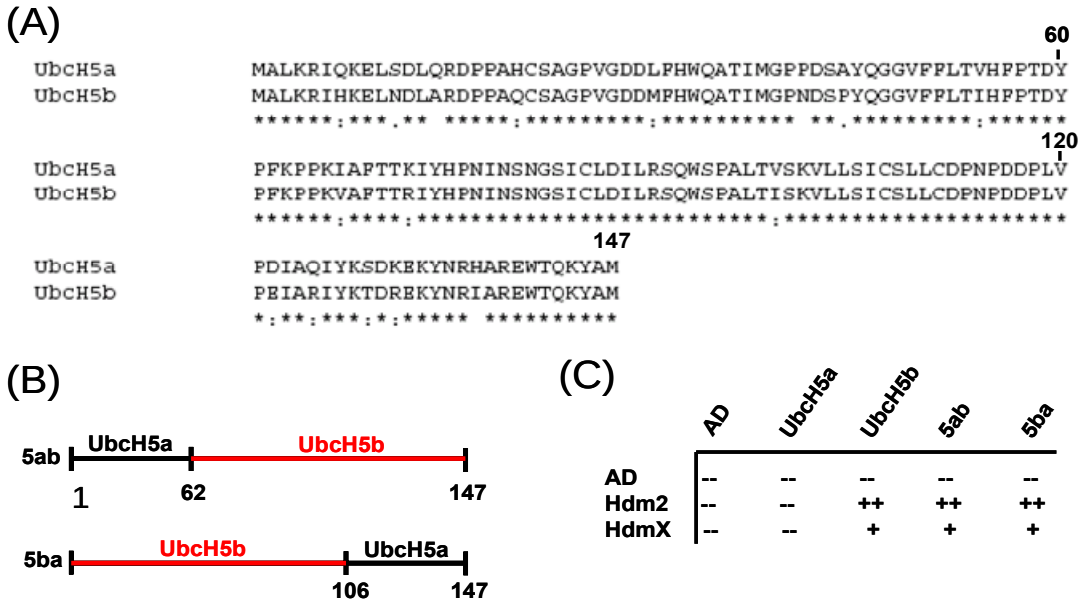

Fig 14. Interaction analysis of UbcH5a/UbcH5b chimeras with Mdm2 and MdmX.

(A) Alignment of UbcH5a with UbcH5b using ClustalW.
(B) A schematic representation of the two constructed chimeras of UbcH5a and UbcH5b.
(C) 1µg of each plasmid, as indicated, was transformed into yeast strain KF1 and selected on media lacking tryptophan (Trp) and leucin (Leu). After 3 days, the yeast cells were streaked on reporter plates lacking histidine (His). The table indicates growth of yeast cells after analysis of 10 different colonies for each transformation. --, no growth; ++, growth detected after 2days; and +, growth detected after 3-4days.

hybrid interaction assay (Fig 14C). Both chimeras interact with wt-Mdm2 with an affinity similar to the UbcH5b-Mdm2 interaction, suggesting that the difference between UbcH5b and UbcH5a may be located between amino acid residues 62-106 of UbcH5b (see discussion).

4.1.8 Interaction of RNA and nucleotides with the Mdm2 Walker A mutants

Mdm2 can bind RNA and it was previously reported that binding to polyG involves the Walker A motif[134]. Furthermore, according to Linares et al., polyG binds to the RING finger of Mdm2 and inhibits its E3 ligase activity by disrupting Mdm2 oligomerization[133]. p53 has also been reported to bind to RNA[135] and this association facilitates p53 to be soluble (A. Waniek, unpublished data). However, if MdmX also binds RNA is not known. We analysed the interaction of polyG with p53, MdmX, and Mdm2 by GST-pulldown assays. Interestingly, despite of having a conserved Walker A motif, MdmX was not able to interact with polyG (Fig 15, upper right panel). As reported, p53 and Mdm2 interacted with polyG (Fig 15, upper left and lower panels). Furthermore, we tested the interaction of the Hdm2 Walker A mutants with polyG. Surprisingly, all the Hdm2 Walker A mutants were able to

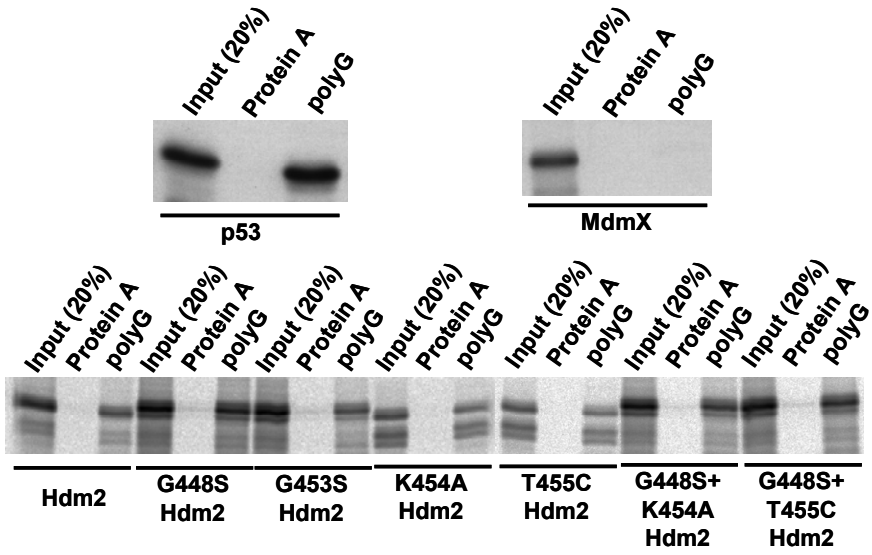

Fig 15. Interaction of polyG with the Walker A Hdm2 mutants.

p53, MdmX, Hdm2 and their mutant proteins were *in vitro* translated in the presence of S^{35} and incubated with sepharose beads containing either immobilised protein A or polyG RNA for 4 hours at 4ºC. Beads were then washed 5 times with TNN buffer containing 1mM DTT followed by SDS-PAGE and fluorography.

interact with polyG, even double mutations (G448S+K454A, G448S+T455C) of the Hdm2 Walker A motif did not abolish the interaction (Fig 15, lower panel). This suggests that the Walker A residues in Mdm2 are not responsible for polyG binding and that a different motif or surface of the RING finger domain of Mdm2 is responsible for polyG binding.

Not only RNA but also adenine based nucleotides (ATP, ADP, AMP) have been reported to bind to the Walker A motif of Hdm2[130]. To test whether the binding of nucleotides, like polyG RNA, has any effect on the oligomerization ability of Mdm2 and MdmX, GST-pulldown assays were performed in the presence or the absence of ATP. To this end, Mdm2 was *in vitro* translated and depleted of ATP by incubation with hexokinase and glucose (hexokinase converts glucose and ATP to glucose-6-phosphate and ADP). GST-Mdm2, GST-MdmX or GST-G448S Hdm2 mutant bound to glutathione sepharose beads were also depleted for ATP by incubating the beads with hexokinase and glucose. Then pulldown assays were performed in the absence of ATP (-ATP-ADP), the presence of ATP (+ATP-ADP), extra addition of ADP (-ATP+ADP) or the presence of ATP and extra ADP (+ATP+ADP). The results obtained (Fig 16) suggest that ATP does not detectibly affect the homo- and hetero-oligomerization abilities of Mdm2 and MdmX. However, it should be noted that ADP was always present in the reaction and, if ADP is able to replace ATP function, then it is hard to exclude a role of ATP in oligomerization of Mdm2 and MdmX.

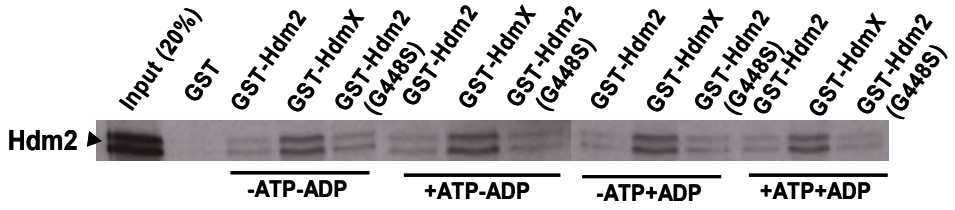

Fig 16. Effect of nucleotides on oligomer formation ability of Hdm2 and HdmX.
In vitro translated Hdm2 were incubated with sepharose beads containing either GST or GST-tagged proteins, as indicated, in the absence or the presence of ATP or ADP or both for 4 hours at 4ºC. Both *in vitro* translated protein and GST-protein bound sepharose beads were depleted of ATP by incubation with hexokinase and glucose. Beads were then washed with TNN buffer, followed by SDS-PAGE and fluorography.

4.2 Residues of Mdm2 and MdmX involved in oligomers formation

Structural data of the RING finger domain of several RING E3 ligases indicate that at least some RING finger domains of E3 ligases form homo- and hetero-dimers[35; 36; 136]. A recent report revealed that the U box protein Prp19 also forms a dimer via its U box, and the amino acid residues required for dimer formation have been identified[136]. Furthermore, this report also predicted amino acid residues of Mdm2 and MdmX involved in oligomeric complex formation. Importantly, the predicted residues (hereafter termed "dimerisation residues") are highly conserved throughout evolution in Mdm2 and MdmX (as shown with blue non-filled rectangles in Fig 4c). Two out of four predicted dimerisation residues are clustered at the very C terminus of Mdm2 and MdmX and are adjacent to the RING finger domains. As discussed before (see introduction 1.1.1.2), in some E3 ligases a role of such C-terminal extensions in stabilisation of the RING finger structure has been reported[35; 36]. Furthermore, oligomerization has been suggested to be required for the E3 activity of Mdm2[112; 133]. Hence, in order to test the importance of the predicted dimerisation residues and the C-terminal extension for the E3 activity and oligomer formation of Mdm2, we mutated the proposed dimerisation residues of Mdm2 and, in addition also deleted seven C-terminal amino acid residues of Mdm2 (these mutants are hereafter collectively termed "dimerisation mutants").

4.2.1 Dimerisation mutants have impaired E3 activity

GST-tagged Mdm2 dimerisation mutants were expressed in bacteria and purified by affinity chromatography. Equal amount of these dimerisation mutants (Fig 17A) were analysed for their ability to ubiquitinate radiolabelled p53 in a standard *in vitro* ubiquitination assay (Fig 17B, left panel). Deletion of the seven C-terminal amino acid residues (ΔC7) of either Mdm2 or Hdm2 completely abrogates the ability to ubiquitinate p53. Moreover, with the exception

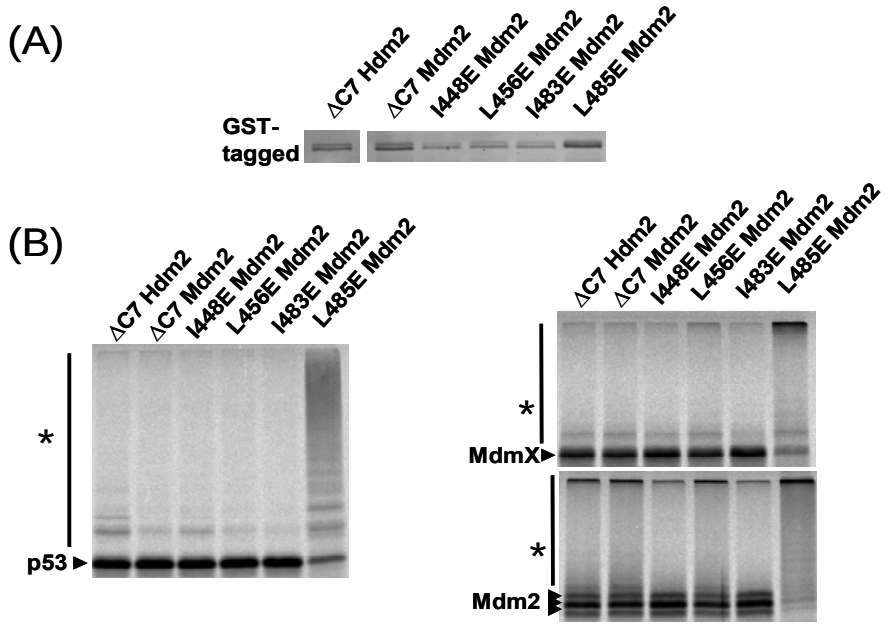

Fig 17. The E3 activity of the dimerisation mutants of Mdm2.

(A) GST-tagged dimerisation mutants of Mdm2 and Hdm2 were expressed in *DH5α* and purified using glutathione immobilised on sepharose beads. The amounts of the Mdm2 and Hdm2 mutant proteins were equilibrated using elution buffer.
(B) Similar amounts of bacterially expressed mutants of Mdm2 and Hdm2 were incubated with S^{35} labeled, *in vitro* translated p53 or MdmX or Mdm2 in a standard *in vitro* ubiquitination reaction for 2hr at 30ºC. The reactions were stopped by adding similar amounts of 2X laemmli buffer and separated on SDS-PAGE followed by fluorography. The non-modified and ubiquitinated forms of the indicated proteins are denoted by arrowheads and asterisk, respectively.

of the L485E Mdm2 mutant, all the other mutants were also impaired in ubiquitinating p53. The L485E mutant retained its E3 activity. Similarly, with the exception of the L485E mutant, all the Mdm2 mutants were also impaired in their activity to ubiquitinate MdmX or Mdm2 (Fig 17B).

4.2.2 Rescue of the E3 activity of the Mdm2 dimerisation mutants by MdmX

As MdmX was able to rescue the E3 activity of the Mdm2 Walker A mutants, we tested the ability of MdmX to rescue the E3 activity of the dimerisation mutants in standard *in vitro* ubiquitination assays. Interestingly, MdmX was not able to rescue the E3 activity of the ΔC7 Mdm2 and Hdm2 mutants. Similarly, the E3 activity of the I448E mutant was not rescued by MdmX. However, the activity of the L456E and I483E Mdm2 mutants was partially rescued by MdmX (Fig 18, upper panel). Furthermore, using MdmX as substrate, except the L485E Mdm2 mutant, none of the other dimerisation mutants were able to ubiquitinate MdmX even in the presence of bacterially expressed GST-MdmX (Fig 18, lower panel).

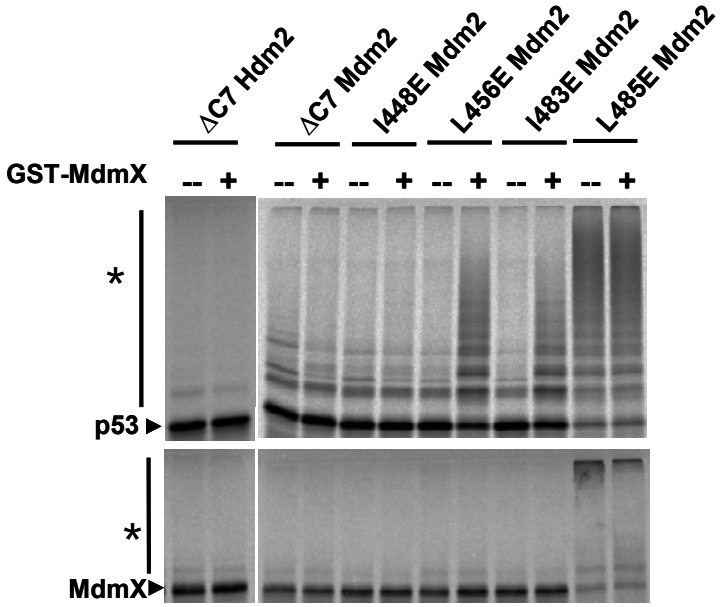

Fig 18. Effect of MdmX on E3 activity of the dimerisation mutants of Mdm2.
GST tagged Mdm2 and Hdm2 mutants were purified from bacteria and each of them individually incubated in the absence or presence of equivalent amounts of bacterially expressed GST-tagged MdmX with *in vitro* translated p53 or MdmX in an *in vitro* ubiquitination reaction. After 2 hours, reactions were loaded on SDS-PAGE and fluorography performed. The non-modified and ubiquitinated forms of indicated proteins are denoted by arrowheads and asterisk, respectively.

The L456E and I483E were the only two mutants whose p53 ubiquitination activity was partially rescued by MdmX *in vitro*. We incorporated similar mutations in HdmX, L457E and V484E, to test the importance of these residues for HdmX to restore the E3 activity of the L456E and I483E Mdm2 mutants. Additionally, a ΔC7 HdmX mutant was constructed. As shown in Fig 19B, unlike wt-HdmX, none of the HdmX mutants tested was able to rescue the E3 ligase activity of the L456E and I483E Mdm2 mutants, respectively. Moreover, the ΔC7 MdmX mutant was also defective in rescueing the E3 activity of the L456E and I483E Mdm2 mutants. Taken together, the data indicate that I448, L456, I483 and the C-terminal 7 amino acid residues of Mdm2 are required for E3 activity *in vitro*.

Next, the expression levels of the Mdm2 dimerisation mutants were determined in H1299 cells. All the Mdm2 mutants were expressed at readily detectable levels (Fig 20A). It is noteworthy that wt-Mdm2 expression was not detected under the same condition. Since it is known that wt-Mdm2 is very short lived in H1299 cells and since the dimerisation mutants lack E3 activity, a reasonable explanation for the observed difference in expression levels is that the dimerisation mutants of Mdm2 were not degraded like wt-Mdm2 and, hence, accumulated in transfected cells. This hypothesis is further supported by the observation that the L485E mutant, which acted like wt-Mdm2 in standard *in vitro* ubiquitination assay, was

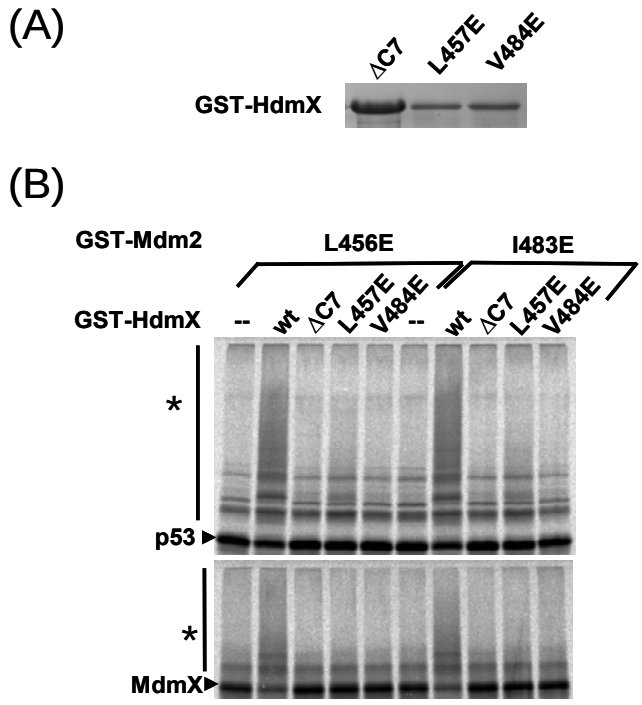

Fig 19. Effect of mutations of dimerisation residues of HdmX on its rescue activity.

(A) Mutants of HdmX, as indicated, were expressed and purified from *E. coli*. The amounts of mutant proteins were adjusted by adding elution buffer.
(B) HdmX or its mutants were incubated with the Mdm2 dimerisation mutants as indicated in an *in vitro* ubiquitination reaction in the presence of either radiolabelled p53 or MdmX. Fluorography was performed after SDS-PAGE to detect the non-modified and ubiquitinated forms of proteins, which are indicated by arrowhead and asterisk, respectively.

expressed at lower levels in cells compared to the other dimerisation mutants.

The dimerisation mutants of Mdm2 were tested for their ability to ubiquitinate p53 within cells by *in vivo* ubiquitination assays (see materials and methods 3.2.4.2). The ΔC7, I448E, L456E and I483E Mdm2 mutants were impaired in their ability to ubiquitinate p53. In contrast, the L485E Mdm2 mutant was only slightly impaired in ubiquitinating p53. Interestingly, co-expression of MdmX completely rescued the activity of the L485E Mdm2 mutant. The activity of the other Mdm2 dimerisation mutants was also partially rescued by co-expression of MdmX, but only the activity of the I483E Mdm2 mutant was rescued to a significant extent (Fig 20B, upper panel).

The dimerisation mutants of Mdm2 were further tested in p53 degradation assays in H1299 cells. Again, the ΔC7, I448E and L456E Mdm2 mutants were completely inactive in degrading p53 in the absence or the presence of co-expressed MdmX (Fig 20B, middle panel). The I483E Mdm2 mutant was partially able to degrade p53 but only in the presence of MdmX. The L485E Mdm2 mutant alone was impaired in degrading p53, however, in the presence of MdmX, the L485E mutant was degrading p53 like wt-Mdm2 (Fig 20C).

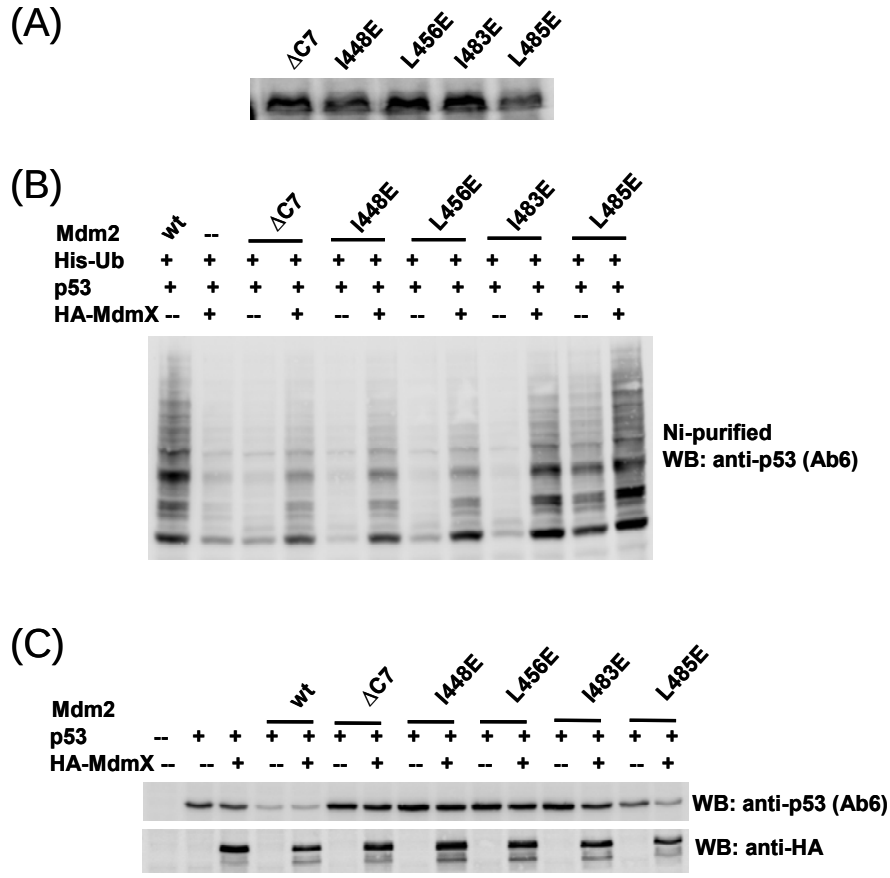

Fig 20. Effect of MdmX on the E3 activity of the dimerisation mutants of Mdm2 in H1299 cells.

(A) Expression of the dimerisation mutants of Mdm2 in H1299 cells. 1.5μg of the plasmids encoding the respective dimerisation mutants of Mdm2 were co-transfected into H1299 with 200 ng of β-gal plasmid. 20-24 hours posttransfection, cells were lysed in TNN buffer followed by normalisation of transfection efficiency by β-gal assay. The samples were run on a SDS-PAGE gel and western blot performed subsequently with an Mdm2 (SMP14) antibody.

(B) The plasmids (Mdm2, 200 ng; HA-Mdmx, 600 ng; His-ubiquitin (His-Ub), 1 μg; and p53, 200 ng), as indicated, were co-transfected into H1299 cells. 20-24 hours post-transfection, cells were lysed in denaturing guanidinium hydrochloride buffer. His-Ub conjugated p53 were purified by Ni-beads followed by SDS-PAGE and western blot performed with a p53 antibody.

(C) H1299 cells were transfected with plasmids as indicated with 200 ng of β-gal plasmid. 20-24 hours posttransfection, cells were lysed in non-denaturing TNN buffer followed by normalisation of transfection efficiency by β-gal assay. The samples were run on a SDS-PAGE gel and western blot performed subsequently with indicated antibodies.

Finally the HdmX mutants V484E and K486E, which are equivalent to the I483E and L485E Mdm2 mutants, respectively, were expressed from mammalian expression vector and tested for their ability to rescue the ability of the I483E and L456E Mdm2 mutants to ubiquitinate p53 in H1299 cells. Consistent with the *in vitro* data, only wt-MdmX, but not the V484E or K486E mutants, was able to rescue the p53 ubiquitination activity of the I483E and L485E Mdm2 mutants. Furthermore, in accordance to p53 ubiquitination, the HdmX mutants also failed to rescue the p53 degradation activity of the I483E and L485E Mdm2 mutants (Fig 21).

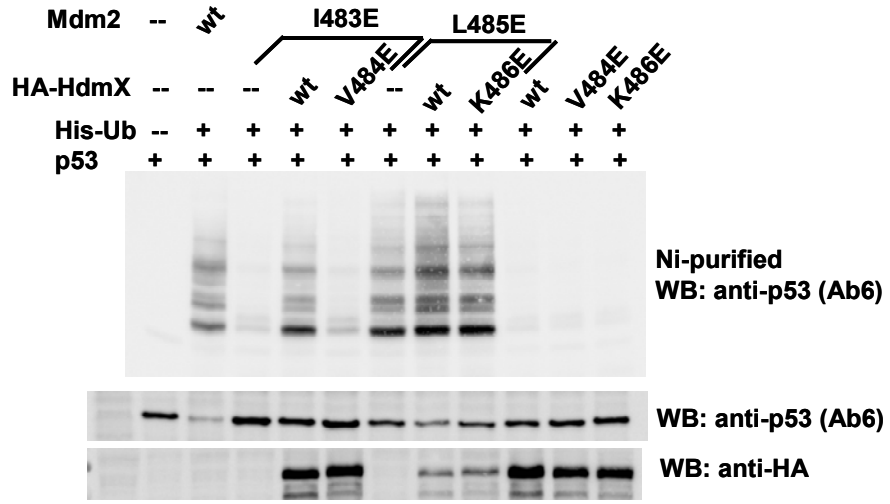

Fig 21. Mutations of the dimerisation residues in HdmX abrogate its rescue activity.
Upper panel, the plasmids (Mdm2, 200 ng; HA-Mdmx, 600 ng; His-ubiquitin (His-Ub), 1 µg; and p53, 200 ng), as indicated, were co-transfected into H1299 cells. 20-24 hours post-transfection, cells were lysed in denaturing guanidinium hydrochloride buffer. His-Ub conjugated p53 were purified by Ni-beads followed by SDS-PAGE and western blotting performed with a p53 antibody; Middle and lower panels, H1299 cells were transfected with plasmids as indicated in upper panel except β-gal was used in place of His-Ub and only10 ng of p53 used for transfection. After 20-24 hours of transfection, cells were lysed in non-denaturing TNN buffer followed by normalisation of transfection efficiency by β-gal assay. The samples were run on a SDS-PAGE gel and western blot performed subsequently with indicated antibodies.

4.2.3 Mdm2 dimerisation mutants are highly ubiquitinated by wt-Mdm2

The Walker A mutants of Mdm2, despite of having impaired E3 ligase activity, were highly ubiquitinated by wt-Mdm2 (see Fig. 9). Hence, we also tested the ability of the dimerisation mutants of Mdm2 to be ubiquitinated by wt-Mdm2. Unexpectedly, all the dimerisation mutants, which were completely abrogated in their E3 activity, were highly ubiquitinated by wt-Mdm2 (Fig 22A). This indicates that the dimerisation mutants are recognised as substrates by wt-Mdm2. We then tested whether the RING finger of Mdm2 is at all required for Mdm2 to serve as a substrate. To do so, ΔC62 Mdm2, which lacks the entire RING finger domain, was *in vitro* translated and tested for its ubiquitination by wt-Mdm2 in a standard *in vitro* ubiquitination assay. Surprisingly, ΔC62 Mdm2 was highly ubiquitinated by wt-Mdm2 (Fig 22B), indicating that the region of Mdm2 that is required as ubiquitination signal does not coincide with the RING finger domain. To test whether the RING finger domain of wt-Mdm2 is sufficient to ubiquitinate ΔC62 Mdm2, we tested the ability of two deletion mutants of Mdm2, ΔN401 and ΔN417, to ubiquitinate ΔC62 Mdm2. Once again, ΔC62 Mdm2 was highly ubiquitinated by the RING finger containing mutants of Mdm2 (Fig 22B), indicating that the RING finger domain cannot only interact with itself, but in addition with another yet unknown region in the Mdm2 polypeptide.

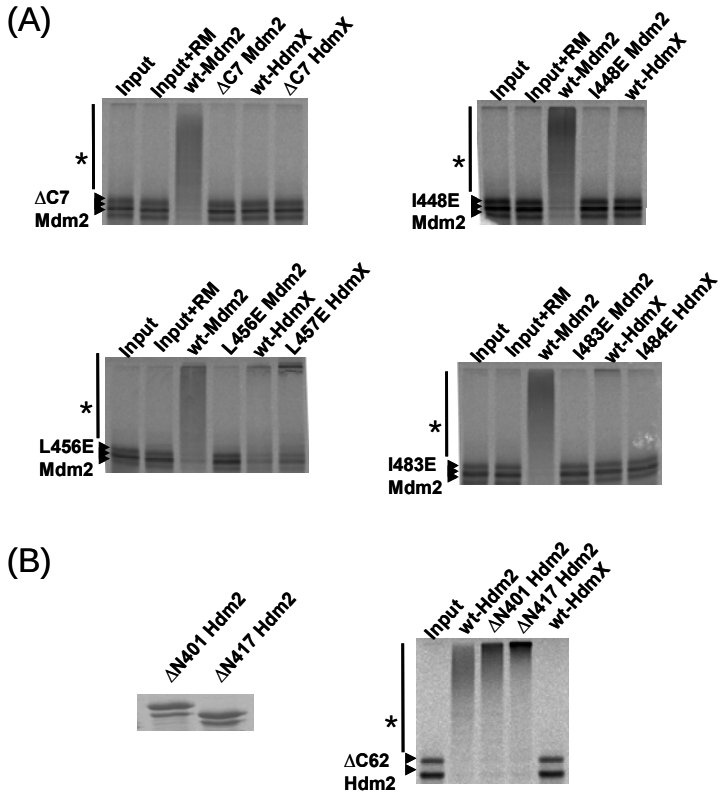

Fig 22. The RING domain is not required for Mdm2 to be ubiquitinated.
The dimerisation and RING finger deleted mutants of Mdm2 and Hdm2, respectively, were radiolabelled and incubated with wild type (wt) or mutant forms of Mdm2 and HdmX proteins in an *in vitro* ubiquitination assay. The non-modified and ubiquitinated forms of the indicated proteins were detected by radiography and are denoted by arrowheads and asterisk, respectively.

4.2.4 Oligomer formation and E2 binding ability of the Mdm2 dimerisation mutants

To characterise the binding properties, the Mdm2 and HdmX dimerisation mutants were first tested for their interaction with p53 in GST-pulldown assays. All the Mdm2 and HdmX dimerisation mutants were binding to p53 with an efficiency similar to wt-Mdm2 and wt-MdmX (Fig 23A, upper panel). Next, the Mdm2 and HdmX dimerisation mutants were analysed for interaction with wt-MdmX, wt-Mdm2, and the respective Mdm2 and HdmX mutants (Fig 23). Except the L485E mutant, all the dimerisation mutants of Mdm2 were significantly impaired in their ability to pulldown radiolabelled wt-MdmX, wt-Mdm2, and the respective Mdm2 and HdmX mutants. This provides a reasonable explanation for the finding that the L485E Mdm2 mutant, but not the other dimerisation mutants of Mdm2, has appreciable E3 activity.

The interaction abilities of the Mdm2 dimerisation mutants were also tested in the yeast two-hybrid system (see materials and methods 3.2.6). All the dimerisation mutants of Mdm2 were

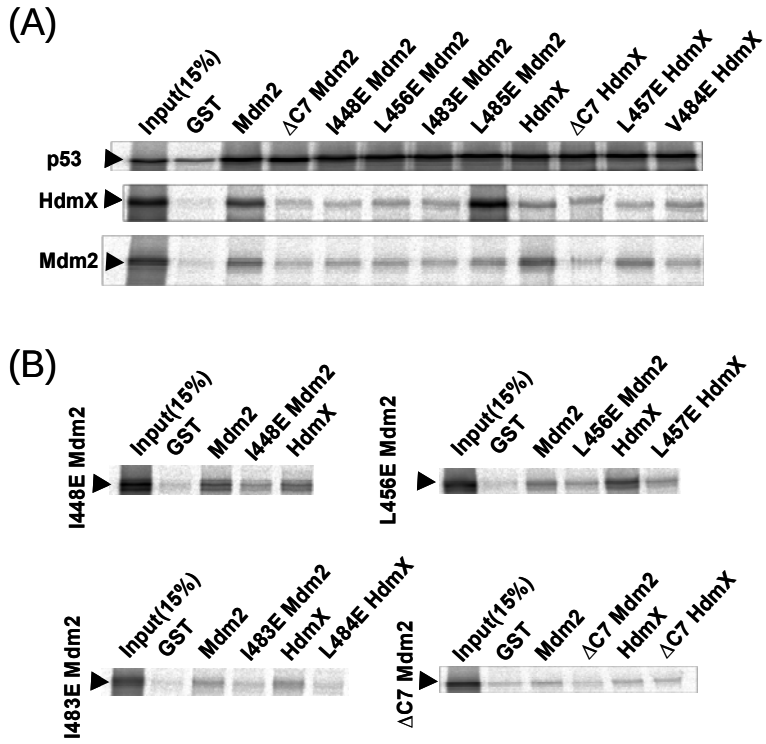

Fig 23. Interaction analysis of the dimerisation mutants of Mdm2 by GST-pulldown assay.

S^{35} radiolabelled proteins, denoted by arrowheads, were incubated with either GST or GST-tagged proteins bound on glutathione sepharose beads, as indicated, for 4 hours at 4ºC. The beads were washed 5 times with TNN buffer containing 1mM DTT followed by SDS-PAGE and radiography.

expressed as fusion proteins with the Gal4 DNA binding domain (BD) or the activation domain (AD) (Fig 24A) and tested for their ability to interact with wt-Mdm2, wt-MdmX, and themselves (Fig 24B, left panel). In accordance to the results obtained in GST-pulldown assays, with the exception of the L485E mutant, all the dimerisation mutants of Mdm2 were impaired for their ability to bind to wt-Mdm2 or themselves. The L485E Mdm2 mutant interacted with wt-Mdm2 with an affinity similar to wt-Mdm2/wt-Mdm2 interaction. The interaction of wt-MdmX with some of the Mdm2 dimerisation mutants was also affected. The L456E and L485E Mdm2 mutants were interacting with MdmX similar to wt-Mdm2/wt-MdmX interaction. Furthermore, the ability of the I483E Mdm2 mutant to interact with MdmX was less affected than the interaction of the ΔC7 and the I448E Mdm2 mutants with MdmX. The ΔC7 and I448E Mdm2 mutants were significantly affected in their ability to interact with MdmX.

We further tested the ability of the Mdm2 dimerisation mutants to interact with UbcH5b in the yeast two-hybrid interaction system (Fig 24B, right panel). Except the ΔC7 Mdm2 mutant, all the Mdm2 dimerisation mutants interacted with UbcH5b like wt-Mdm2. Since the

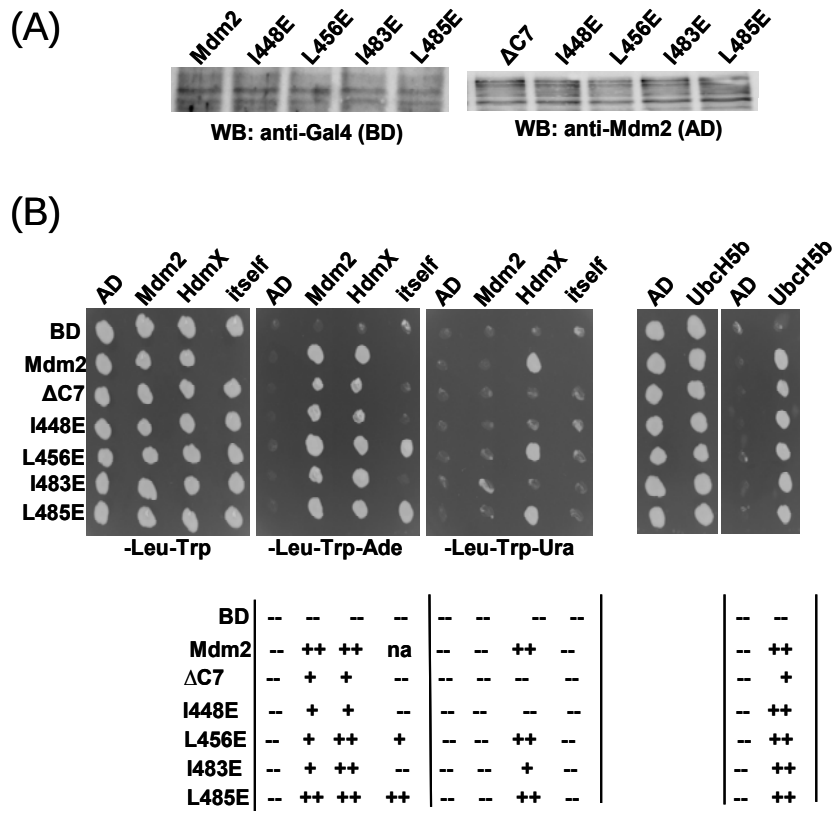

Fig 24. Interaction analysis of the dimerisation mutants of Mdm2 by the yeast two-hybrid system.

(A) The cDNA of Mdm2 and its mutants, MdmX, and UbcH5b were cloned in fusion with the Gal4 activation domain (AD) or its DNA binding domain (BD), as indicated, in the yeast two-hybrid vectors. The expression of the proteins in yeast was detected by western blot analysis.
(B) Upper panel, 1 µg of each plasmid, as indicated, was transformed into yeast strain KF1 and selected on media lacking tryptophan (Trp) and leucin (Leu). After 3 days, the transformed yeast cells were streaked on respective reporter plates, lacking either histidine (His), adenine (Ade), or uracil (Ura). Pictures were taken after 4 days of growth. Lower panel, the table indicates growth of yeast cells after analysis of 10 different colonies for each transformation. --, no growth; ++, growth detected after 2days; and +, growth detected after 3-4days. na, not applicable.

ΔC7 Mdm2 mutant was the only mutant among the Mdm2 dimerisation mutants that was somewhat impaired in interacting with UbcH5b, this suggests that oligomerization of Mdm2 plays no significant role in the interaction of the RING finger domain of Mdm2 with UbcH5b. The slightly impaired binding of the ΔC7 Mdm2 mutant to UbcH5b may be due to a slight alteration in the tertiary structure of the RING domain of the ΔC7 Mdm2 mutant.

4.2.5 Effect of Walker A and dimerisation mutants of Mdm2 on MdmX ubiquitination and degradation

Mdm2 not only ubiquitinates and degrades p53 but it can also ubiquitinate and degrade MdmX[137; 138]. The Walker A mutants and the dimerisation mutants of Mdm2 were tested for

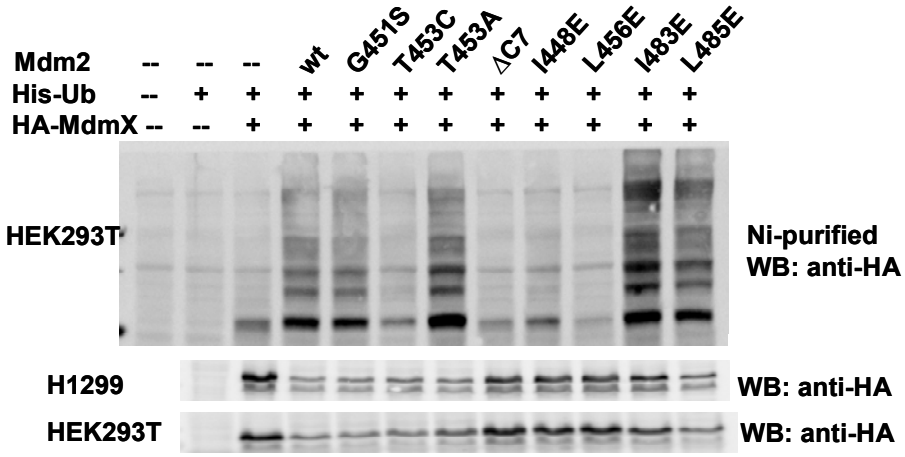

Fig 25. Effect of the Mdm2 mutants on HdmX ubiquitination and degradation.

Upper panel, the plasmids (Mdm2, 600 ng; HA-Mdmx, 600 ng; His-ubiquitin (His-Ub), 1 µg) were co-transfected into HEK293T cells. After 20-24 hours of transfection, cells were lysed in denaturing guanidinium hydrochloride buffer. His-Ub conjugated MdmX were purified by Ni-beads followed by SDS-PAGE and western blot performed with a HA antibody; Middle and lower panels, H1299 and HEK293T cells were transfected with plasmids as indicated in upper panel except β-gal was used in place of His-Ub. After 20-24 hours of transfection, cells were lysed in non-denaturing TNN buffer followed by normalisation of transfection efficiency by β-gal assay. The samples were run on a SDS-PAGE gel and western blot performed subsequently with a HA antibody.

their ability to ubiquitinate MdmX in HEK293T cells in an *in vivo* ubiquitination assay (Fig 25, upper panel). Like wt-Mdm2, the G451S and T453A Mdm2 mutants were able to ubiquitinate MdmX. However, no ubiquitinated MdmX was detected in the presence of the T453C Mdm2 mutant. Three Mdm2 dimerisation mutants, ΔC7, I448E, L456E, were completely abrogated in their ability to ubiquitinate MdmX. However, the other two Mdm2 dimerisation mutants (I483E and L485E) were active in ubiquitinating MdmX. Furthermore, we analysed the degradation of MdmX in H1299 and HEK293T cells (Fig 25, middle and lower panels). All three Walker A Mdm2 mutants tested were degrading MdmX both in H1299 and HEK293T cells with an efficiency similar to wt-Mdm2. Moreover, except L485E mutant, all the other Mdm2 dimerisation mutants were impaired in their ability to degrade MdmX in H1299 and HEK293T cells.

4.3 Neddylation

Recently, p53 and Mdm2 have been reported to be covalently modified with the ubiquitin like protein Nedd8[128]. In fact Mdm2 acts as Nedd8 E3 ligase for p53 and itself, although the physiological relevance of neddylation of Mdm2 is not known.

4.3.1 The *in vivo* neddylation assay

In order to test the ability of the various Mdm2 mutants to act as Nedd8 ligase, an *in vivo* neddylation assay similar to the *in vivo* ubiquitination assay was established using His-Nedd8. As shown in Fig. 27A, Mdm2-mediated neddylation of p53 could be readily observed in this system. We also tested the ability of MdmX to be modified by Nedd8. Indeed, MdmX was mono-neddylated in H1299 cells and, in the presence of ectopically expressed Mdm2, MdmX was modified with several moieties of Nedd8 (Fig 26A, left panel). To characterise the specificity of the *in vivo* neddylation assay, several RING E3 ligases (Rlim, Livin α and Rho52) available in our group were tested for their ability to be modified with Nedd8. Surprisingly, all the RING E3 ligases tested were modified with Nedd8 (Fig 26A, middle panel). Furthermore, several SUMO E3 ligases (i.e. human polycomb 2 (hPC2), PIASY, PIASXβ and PIAS1) were tested for their ability to act as Nedd8 substrates. Again, all these proteins were modified with Nedd8, mostly with one moiety of Nedd8 (Fig 26B, left panel). Moreover, E6-AP, a HECT E3 ligase, was also modified with Nedd8 in H1299 cells. The catalytically inactive E6-AP (C820A mutant) was modified to a lower extent than wt-E6-AP (Fig 26B, right panel), and this neddylation is possibly catalyzed by endogenous E6-AP, which may form a complex with the mutant[27]. Finally, two E6-AP interacting proteins, EAP 30 and Herc2 (Rcc1b domain), were analysed for their ability to be modified with Nedd8. Again, neddylation of both proteins was observed (Fig 26A, right and middle panels).

4.3.2 Mdm2 is the only Nedd8 E3 ligase for p53

Besides Mdm2, several other ubiquitin E3 ligases including Cop1 and Pirh2 have been reported to ubiquitinate p53 in normal cells[5; 45]. Moreover, E6-AP in complex with the HPV E6 protein also functions as an ubiquitin E3 ligase for p53 in HPV infected cells (see introduction 1.1.1.1). In order to determine whether, similar to Mdm2, the other ubiquitin E3 ligases for p53 are able to neddylate p53, *in vivo* p53 neddylation assays were performed with E6-AP in the presence of HPV 16E6 or 18E6. As shown in (Fig. 27A), p53 neddylation was not significantly enhanced by coexpression of E6-AP and the E6 proteins compared to p53 neddylation by Mdm2, suggesting that the E6/E6-AP complex does not act as Nedd8 E3 ligase for p53. It is important to note that, like Mdm2, E6/E6-AP complex was able to degrade p53 in cotransfection assays (data not shown). Furthermore, both Cop1 and Pirh2 were not able to modify p53 with Nedd8 (data not shown). However, it should be noted that in our *in vivo* ubiquitination assay, both Cop1 and Pirh2 were also not able to ubiquitinate p53.

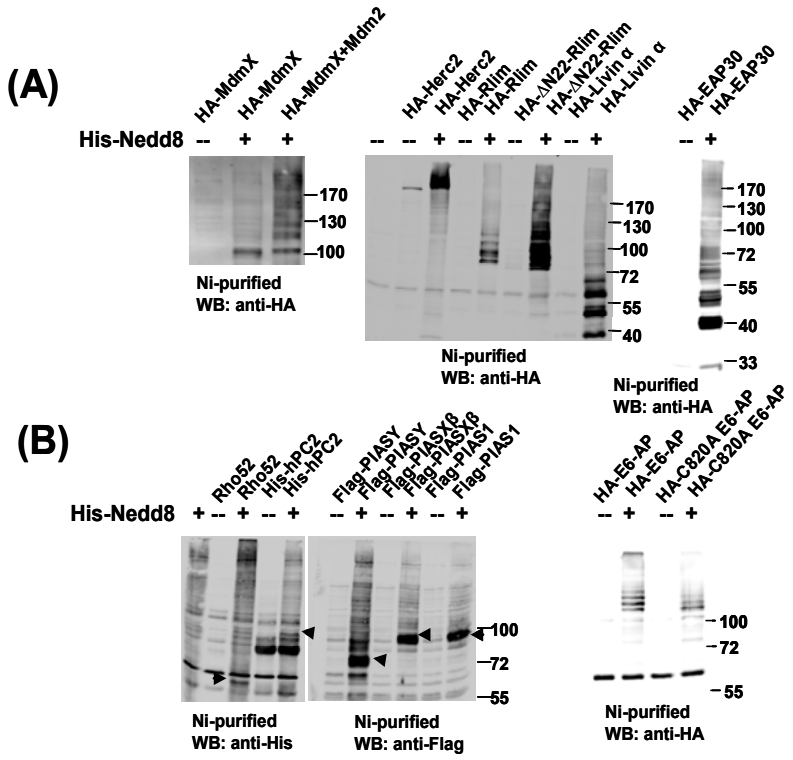

Fig 26. Neddylation of several diverse proteins.
1 µg of each plasmid, as indicated, was transfected into H1299 cells. 20-24 hours posttransfection, cells were lysed in denaturing guanidinium hydrochloride buffer. His-Nedd8 conjugated proteins were purified by Ni-beads followed by SDS-PAGE and western blot performed with indicated antibodies. Arrowheads in (B) indicate the putative Nedd8 modified forms.

4.3.3 Nedd8 is attached to its substrate by forming an isopeptide bond

Generally, ubiquitin and ubiquitin-like (ubl) proteins are attached to their substrates by forming an isopeptide bond with a lysine residue of the substrate and the C-terminal glycine residue of ubiquitin or ubl. The attachment of Nedd8 to so many substrates raised the question as to whether Nedd8 attachment is indeed via formation of an isopeptide bond. To test this possibility, we mutated the last glycine (G76A) or both glycines (G75/76A) at the C terminus of Nedd8 and these mutants were tested for their ability to modify p53 in the *in vivo* neddylation assay in H1299 cells. Unlike wt-Nedd8, none of the Nedd8 mutants were attached to p53 (data not shown), suggesting that p53 modification by Nedd8 is through formation of an isopeptide bond. Furthermore, we also expressed HA-Nedd8 from two different plasmids and tested the attachment of HA-Nedd8 to p53 by immuno-precipitation (IP) of the complex with an anti-HA antibody and blotting with p53 antibody. p53 was observed to be modified with HA-Nedd8 (Fig 27B). Taken together, the above experiments indicate that Nedd8 attachment to at least p53 is not an artefact of the methods or plasmids used in our assay system.

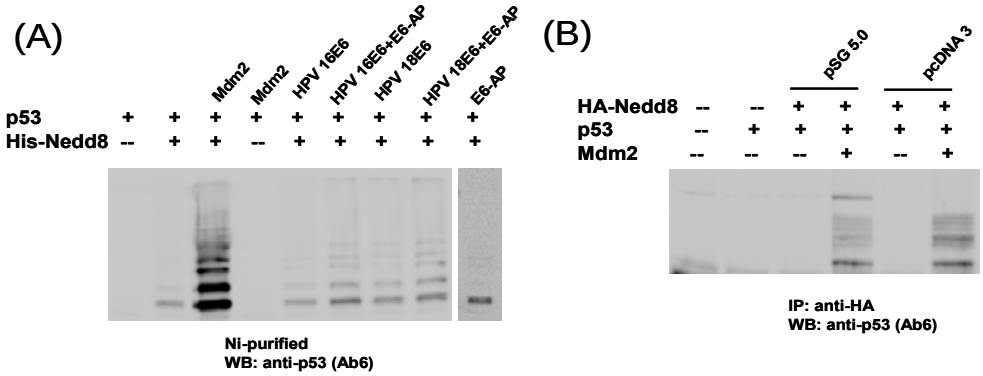

Fig 27. Only Mdm2 functions as E3 ligase for p53.
(A) The plasmids (Mdm2, 200 ng; p53, 200 ng; all others, 1 µg) were co-transfected into H1299 cells. After 20-24 hours of transfection, cells were lysed in denaturing guanidinium hydrochloride buffer. His-Nedd8 conjugated p53 were purified by Ni-beads followed by SDS-PAGE and western blot performed with a p53 antibody.
(B) H1299 cells were transfected with the indicated plasmids (Mdm2, 200 ng; p53, 200 ng; HA-Nedd8, 1 µg). HA-Nedd8 was expressed from two different plasmids, either pSG 5 (Stratagene) or pcDNA3 (Invitrogen). Cells were lysed in non-denaturing TNN buffer followed by treatment with protein A sepharose. The samples were then immuno-precipitated with 1 µg of HA antibody followed by SDS-PAGE and western blot performed with a p53 antibody.

4.3.4 Nedd8 attachment to p53 by Mdm2 Walker A and dimerisation mutants

Some of the Walker A mutants of Mdm2 were impaired in their ability to ubiquitinate and degrade p53. Here, we asked as to whether the Walker A mutants of Mdm2 were also impaired in their Nedd8 E3 activity and, if yes, whether MdmX can rescue the Nedd8 E3 activity of the Mdm2 Walker A mutants. As observed for p53 ubiquitination, the Walker A mutants of Mdm2 were impaired in neddylation of p53 and p53 neddylation was completely rescued by coexpression of MdmX (Fig 28, upper panel). Furthermore, the HdmX Walker A mutants were also tested for their ability to rescue the Nedd8 E3 activity of the Mdm2 Walker A mutants. Indeed, similar to the rescue of the ubiquitin E3 activity, the HdmX Walker A mutants were able to rescue the Nedd8 E3 activity of the Mdm2 Walker A mutants (Fig 28, lower panel).

The dimerisation mutants of Mdm2 were also tested for their ability to neddylate p53 in H1299 cells. Like their ubiquitin E3 activity, the Nedd8 E3 activity of all the dimerisation mutants, with the exception of L485E, was impaired. Furthermore, MdmX could only inefficiently rescue the Nedd8 E3 activity of the dimerisation mutants (Fig 29, upper panel). Taken together these results indicate that (i) MdmX functions as a positive cofactor for Mdm2-mediated neddylation of p53, (ii) Nedd8 E3 activity requires homo-oligomerization of Mdm2 or hetero-oligomerization with MdmX, and (iii) the ubiquitin E3 activity and the Nedd8 E3 activity of Mdm2 cannot be easily separated by mutation.

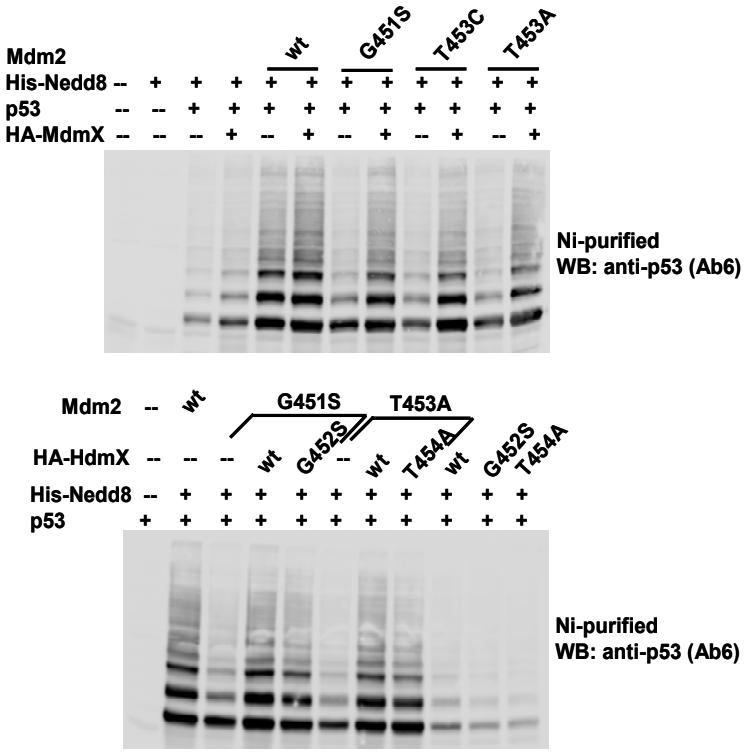

Fig 28. MdmX rescues the Nedd8 E3 activity of the ligase defective Mdm2 Walker A mutants.
The plasmids (Mdm2, 200 ng; p53, 200 ng; HA-MdmX, 600 ng; and His-Nedd8, 1 µg) were co-transfected into H1299 cells. After 20-24 hours of transfection, cells were lysed in denaturing guanidinium hydrochloride buffer. His-Nedd8 conjugated p53 were purified by Ni-beads followed by SDS-PAGE and western blot performed with a p53 antibody.

4.3.5 Effect of Nedd8 on p53 ubiquitination and vice versa

Since Mdm2 not only ubiquitinates p53 but also neddylates p53, we tested whether p53 neddylation affects p53 ubiquitination and *vice versa*. H1299 cells were transfected with plasmids encoding p53, Mdm2, and His-ubiquitin in the presence of increasing amounts of the HA-Nedd8 expression vectors. Cells were lysed with Gd-Hcl buffer followed by Ni-purification (see materials and methods) and p53 detected by SDS-PAGE followed by western blotting with p53 antibody. The results obtained indicate that Mdm2-mediated p53 ubiquitination is not affected by the presence of ectopically overexpressed HA-Nedd8. Similarly, p53 neddylation was also not affected by the presence of overexpressed HA-Ub (Fig 30). However, it became evident that neddylated forms of p53 accumulated much more efficiently than ubiquitinated forms of p53, in particular the forms corresponding to p53 molecules modified with one, two, or three moieties of Nedd8 and ubiquitin, respectively. The first band (labelled as 1 in Fig 30) represents p53 modified with one moiety of either His-Ub or His-Nedd8, depending on whether His-Ub or His-Nedd8 plasmids were used. The

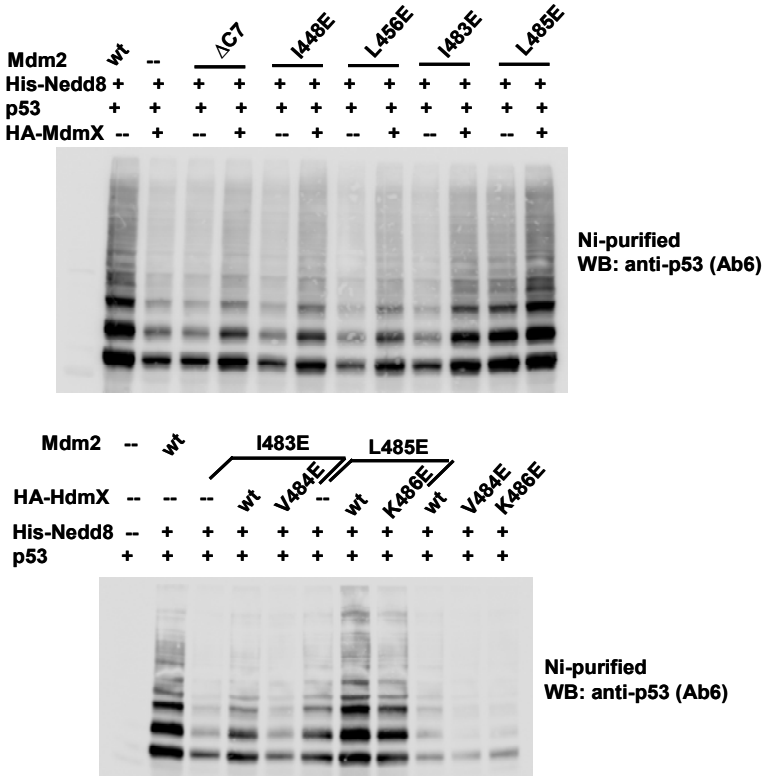

Fig 29. MdmX rescues the Nedd8 E3 activity of some of the dimerisation mutants of Mdm2.

The plasmids (Mdm2, 200 ng; p53, 200 ng; HA-MdmX, 600 ng; and His-Nedd8, 1 µg) were co-transfected in H1299 cells. After 20-24 hours of transfection, cells were lysed in denaturing guanidinium hydrochloride buffer. His-Nedd8 conjugated p53 were purified by Ni-beads followed by SDS-PAGE and western blot performed with a p53 antibody.

second and third bands (labelled as 2 and 3, respectively, in Fig. 30) could be p53 modified with different combinations of overexpressed and endogenous ubiquitin and Nedd8 molecules containing at least one molecule of His-Ub or His-Nedd8, depending on whether His-Ub or His-Nedd8 expression constructs were used.

From the above experiment, it was not clear whether one p53 polypeptide is modified with either ubiquitin or Nedd8 or whether both ubiquitin and Nedd8 can be attached to the same p53 molecule. To test this, H1299 cells were transfected with p53 and Mdm2 plasmids along with expression constructs for His-Ub and HA-Nedd8 or for His-Nedd8 and HA-Ub. In addition, the combinations of His-Ub/HA-Ub and His-Nedd8/HA-Nedd8 plasmids were also transfected along with the plasmids encoding p53 and Mdm2 to rule out the possibility that the tags used interfere with the ability of Ub and Nedd8 to be attached to the same molecule. Transfected cells were lysed in denaturing Gd-Hcl buffer followed by Ni-purification and upon SDS-PAGE, first blotted with anti-HA antibody. The membrane was then stripped and reblotted with the anti-p53 antibody. In the presence of His-Ub and HA-Ub, a smear was

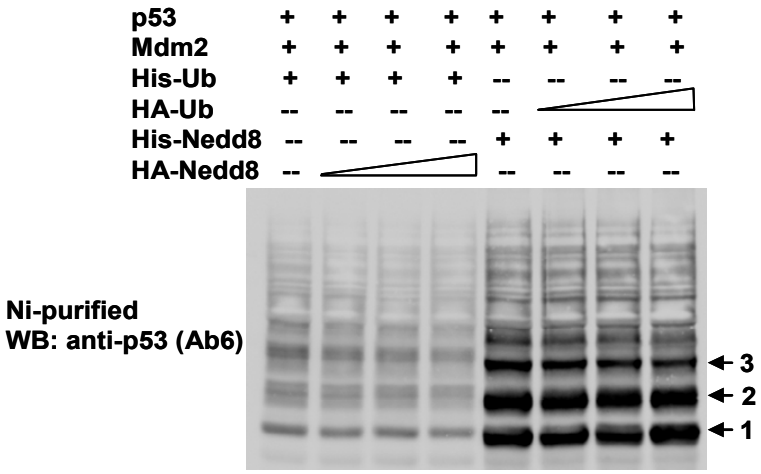

Fig 30. Ubiquitination of p53 is not affected by the presence of Nedd8, and vice versa.

The plasmids (Mdm2, 200 ng; p53, 200 ng; His/HA-ubiquitin (His/HA-Ub) and His/HA-Nedd8, 500 ng; or in increasing amounts 500 ng, 1 µg, and 2 µg) were co-transfected into H1299 cells. 20-24 hours posttransfection, cells were lysed in denaturing guanidinium hydrochloride buffer. His-Nedd8/Ub conjugated p53 were purified by Ni-beads followed by SDS-PAGE and western blot performed with a p53 antibody. The positions of p53 modified with one, two, or three moieties of Nedd8 are indicated by 1, 2, or 3, respectively.

observed in the blot probed with the HA antibody indicating that, in general, different tagged ubiquitins can be attached to the same molecule (Fig 31, left panel). Furthermore, in the presence of His-Ub and HA-Nedd8, two bands in the HA blot corresponded to the second and third modified p53 bands in the p53 blot (Fig 31, comparing left and right panels), suggesting that both ubiquitin and Nedd8 can be attached onto the same molecule of p53. Moreover, the presence of two corresponding bands in the HA and the p53 antibody blot in the presence of His-Nedd8 and HA-Nedd8 indicates that more than one molecule of Nedd8 can be attached to one molecule of p53. However, it remains unclear whether a Nedd8 chain can be attached on one lysine residue of p53 or whether several lysines of p53 are modified with mono-Nedd8.

Formation of ubiquitin chains is well documented. However, so far there has been no evidence for the existence of Nedd8 chains. It has been reported that *in vitro*, ubiquitin and Nedd8 can form an ubiquitin-Nedd8 dimer in the presence of E2-25K[122]. Thus, we were interested to determine if the lysine residues of Nedd8 can be used by ubiquitin or Nedd8 to form a chain on Nedd8 in H1299 cells. For this, the C-terminal two glycines of ubiquitin and Nedd8, respectively, were deleted so that these ubiquitin and Nedd8 mutants cannot be attached to other proteins but can serve as substrates. Different combinations of expression constructs of wt-Ub, wt-Nedd8, and their respective mutants were transfected in H1299 cells. 20-24hr posttransfection, the cells were lysed in Gd-Hcl buffer followed by Ni-purification and upon SDS-PAGE blotted with anti-HA antibody. In the presence of wild-type His-Ub

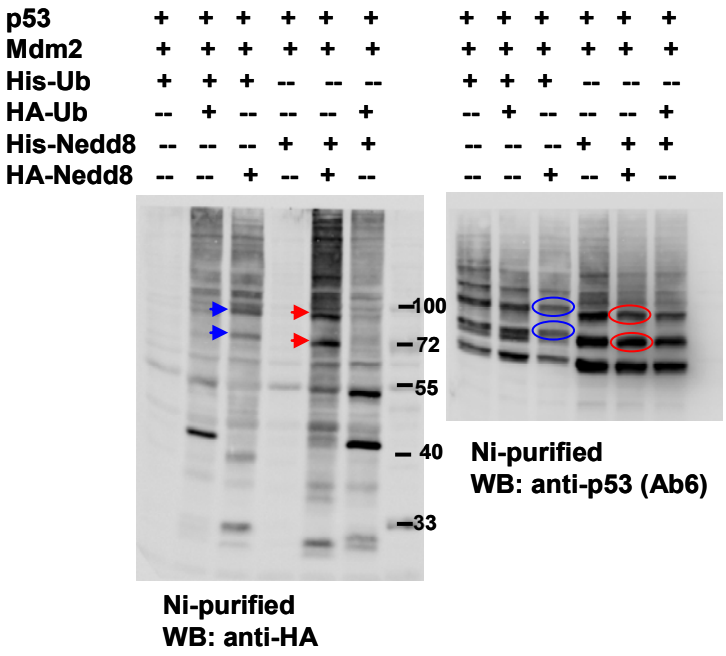

Fig 31. Ubiquitin and Nedd8 may attach on the same p53 molecule.

Left panel, the plasmids (Mdm2, 200 ng; p53, 200 ng; His/HA-ubiquitin (His/HA-Ub) and His/HA-Nedd8, 1 µg), as indicated, were co-transfected into H1299 cells. After 20-24 hours of transfection, cells were lysed in denaturing guanidinium hydrochloride buffer. His-Nedd8/Ub conjugated p53 were purified by Ni-beads followed by SDS-PAGE and western blot performed with a HA antibody; right panel, the blot was then stripped with 0.2M NaOH and reblotted with a p53 antibody. The arrowheads and circles of same colour indicate similar band by virtue of their size on SDS-PAGE.

and HA-Nedd8, a band running at around 25KDa was observed for ubiquitin and Nedd8 (labelled as dimer in Fig 32). However, this band disappeared in the presence of His-Ub and ΔG75/76 HA-Nedd8, indicating that ΔG75/76 HA-Nedd8 does not serve as substrate for ubiquitination. Interestingly, in the presence of ΔG75/76 His-Ub and wild type HA-Nedd8, the 25KDa band reappeared, which implies that ubiquitin serves as substrate for neddylation (see discussion).

Previous studies indicated that the amino acid residue at position 72 of ubiquitin (R) and Nedd8 (A) decides whether the respective protein is activated and attached via the ubiquitin or the Nedd8 conjugation pathways[122]. Replacement of A72 by R enables Nedd8 to be used by the ubiquitin pathway *in vitro*[122]. Thus, we mutated the 72nd residue of ubiquitin to A (R72A Ub) and of Nedd8 to R (A72R Nedd8) and tested their ability to be used by the ubiquitin system for *in vitro* ubiquitination of p53. Interestingly, mutation of R72A of ubiquitin significantly impaired the conjugation of this mutant to p53 and R72A ubiquitin mutant became very similar to Nedd8 in its efficiency to be conjugated to p53 in this *in vitro* system (Fig 33A). Nedd8 has been reported to be cross-activated by the ubiquitin pathway with an efficiency 100 times less than ubiquitin[122]. This explains why we observed a minute amount of p53 modified with Nedd8 or the R72A ubiquitin mutant under the conditions used.

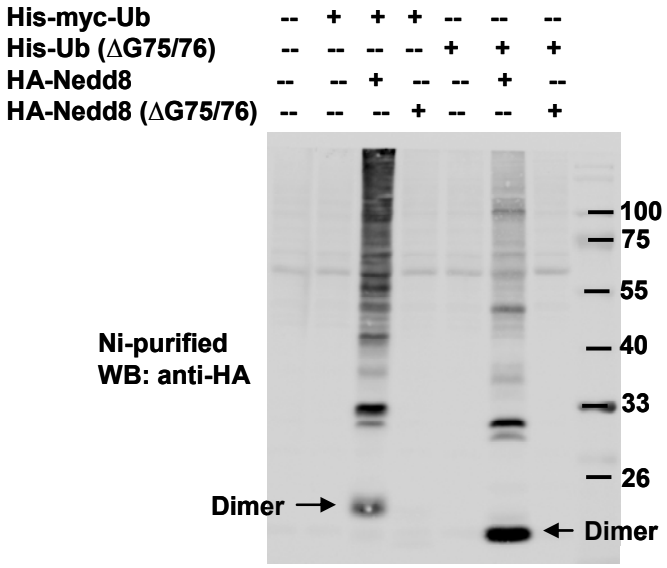

Fig 32. The lysine of Nedd8 cannot be used to form the dimer of ubiquitin and Nedd8.

The plasmids (His-ubiquitin (His-Ub) or its mutants and His-Nedd8 or its mutants, 1 μg), as indicated, were transfected into H1299 cells. After 20-24 hours of transfection, cells were lysed in denaturing guanidinium hydrochloride buffer. His-Nedd8 conjugated proteins were purified by Ni-beads followed by SDS-PAGE and western blot performed with a HA antibody.

Furthermore, the A72R Nedd8 mutant was attached to p53 with an efficiency similar to wt-ubiquitin. Thus, as reported, it is indeed feasible to activate Nedd8 via the ubiquitin conjugation pathway by replacing A72 by R in Nedd8. Next, we also tested the R72A ubiquitin and A72R Nedd8 mutants in our *in vivo* ubiquitination/neddylation assay. As shown before (see Fig. 30), neddylated forms of p53 accumulate in cells to much higher amounts than ubiquitinated form of p53. However, this phenotype was not reversed by the use of the ubiquitin R72A and Nedd8 A72R mutants (i.e. Nedd8 A72R modified p53 accumulated to higher amounts than ubiquitin R72A modified p53).

Finally, we tested the localisation of p53 in the presence of Nedd8 in H1299 cells. p53 is normally localised to the nucleus and Nedd8 localisation is also reported to be mostly nuclear. A plasmid encoding a GFP-tagged transactivation deficient mutant of p53 (R273H) was transfected along with the plasmid expressing HA-Nedd8 into H1299 cells. After 20-24hr of transfection, cells were fixed and immuno-stained with an anti-HA antibody. GFP and anti-HA fluorescence, which correspond to R273H p53 and Nedd8 expression, respectively, co-localised into discrete dots within the nucleus (data not shown). Neither GFP R273H p53 alone nor HA-Nedd8 alone, however, localised to these dots. The identity of these nuclear dots (e.g. PML bodies) remains unclear.

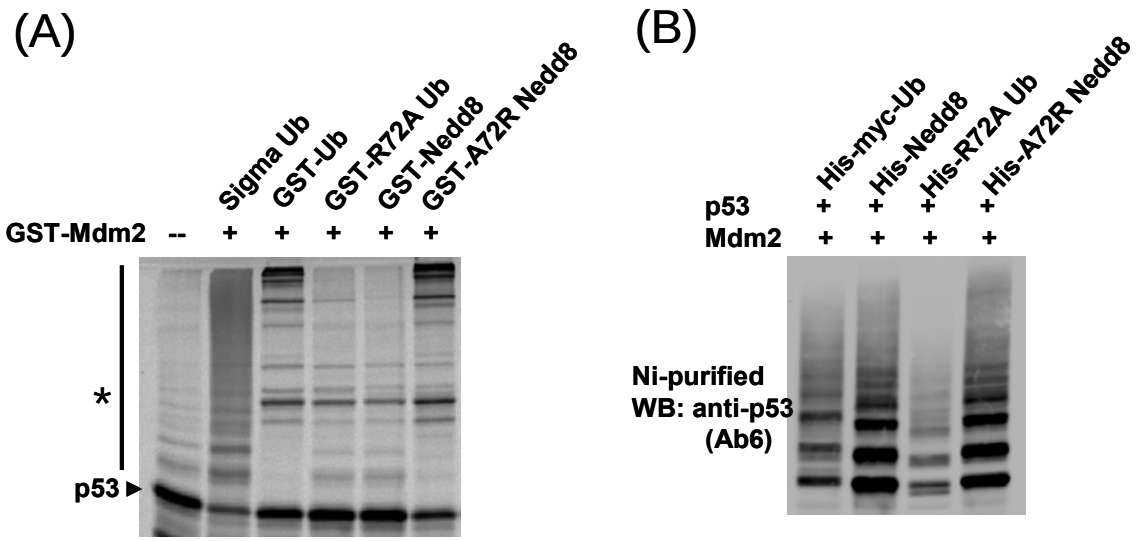

Fig 33. The A72R Nedd8 mutant is cross activated by the ubiquitination pathway, *in vitro*.

(A) Bacterially expressed GST-tagged ubiquitin (Ub), Nedd8 and their mutants were incubated with radiolabelled p53 in a standard *in vitro* ubiquitination assay. Untagged ubiquitin purchased from Sigma was also used in one of the reactions. Radiography was performed after SDS-PAGE to detect the non-modified and modified forms of p53, which are indicated by arrowhead and asterisk, respectively.

(B) The plasmids (His-ubiquitin (His-Ub) or its mutants and His-Nedd8 or its mutants, 1 μg) were transfected into H1299 cells. After 20-24 hours of transfection, cells were lysed in denaturing guanidinium hydrochloride buffer. p53 conjugated with His tagged proteins were purified by Ni-beads followed by SDS-PAGE and western blot performed with a HA antibody. The difference in the positions for wt-Ub modified p53 and the Ub mutant modified p53 on the blot was due to the fact that wt-Ub had a His-myc tag whereas its mutant had only the His tag.

4.3.6 MdmX Neddylation

As shown above, MdmX was modified with one moiety of Nedd8 in the absence of ectopically expressed Mdm2 in H1299 cells (see Fig. 26A, left panel). In the presence of overexpressed Mdm2, several slowly migrating forms of MdmX were observed, suggesting that one MdmX molecule can be modified with several Nedd8 moieties. In this part, we mapped the minimal region of MdmX required to be modified with Nedd8. Several deletion constructs of MdmX (ΔN 300, 1-375, RING finger only (RF)) were transfected into H1299 cells along with the His-Nedd8 expressing plasmid and determined which of the MdmX mutants can be modified with Nedd8. The ΔN 300 MdmX mutant, but not 1-375 MdmX and the RF mutant, was modified with Nedd8 (Fig 34, upper panel). This indicates that the RING finger of MdmX and the N-terminal region adjacent to the RING finger is necessary and sufficient for MdmX neddylation. Thus the N-terminal region (301-429) adjacent to the RING finger of MdmX may contain the lysine residue(s) that serves as acceptor for Nedd8. Furthermore, the minimal region/domain required in Mdm2 to enhance MdmX neddylation was also mapped. Several deletion mutants of Hdm2 (ΔN 300, ΔN 401 (RING finger only),

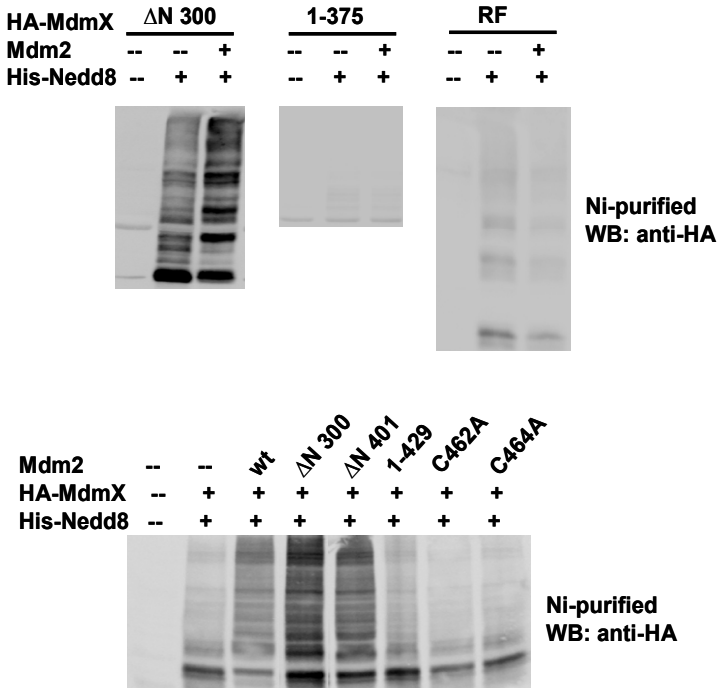

Fig 34. Mapping of the domain of MdmX and Mdm2 required for MdmX neddylation.

1 μg of the plasmids, as indicated were transfected into H1299 cells. After 20-24 hours post-transfection, cells were lysed in denaturing guanidinium hydrochloride buffer. His-Nedd8 conjugated MdmX or its mutants were purified by Ni-beads followed by SDS-PAGE and western blot performed with a HA antibody.

1-429 (missing RING finger), C464A and C462A Mdm2 (point mutants of RING finger)) were tested for their ability to enhance MdmX neddylation. The ΔN 300 and ΔN 401 Hdm2 mutants, which contain a functional RING finger domain, were able to enhance MdmX neddylation (Fig 34, lower panel). The other mutants of Mdm2, which lack the RING finger domain or contain a non-functional RING finger, were abrogated in their ability to enhance MdmX neddylation. This suggests that a functional RING finger domain in Mdm2 is necessary and sufficient for Mdm2 to enhance MdmX neddylation.

To obtain insight into the mechanism involved in MdmX neddylation, we attempted to set up an *in vitro* neddylation assay with bacterially purified Ubc12 (E2) and GST-Nedd8, and Nedd8 E1 purified from H1299 cells. Using these components, we observed that a rather small fraction of *in vitro* translated MdmX was modified with GST-Nedd8 in the presence of Mdm2 and this modification was dependent on the additional presence of E1 and Ubc12 (Fig 35A). Furthermore, in order to test the effect of neddylation on Mdm2-mediated p53 ubiquitination *in vitro*, we added the Nedd8 E1, E2 and GST-Nedd8 (termed "Nedd8 RM") to the *in vitro* p53 ubiquitination assay. However, due to the presence of high concentrations of salt in the Nedd8 RM (note that the Nedd8 E1 fraction contains 400mM of NaCl, which is required for its elution), we were not able to determine whether neddylation affects Mdm2-

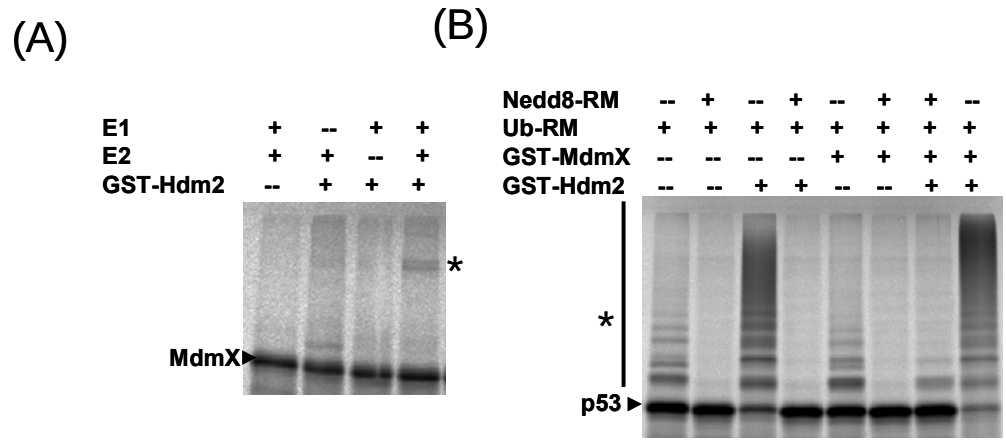

Fig 35. *In vitro* Neddylation of MdmX and its effect on p53 ubiquitination.

(A) Nedd8 E1 containing fraction of H1299 cells were incubated with bacterially expressed Ubc12 (E2) and radiolabelled MdmX in an *in vitro* neddylation reaction mix containing GST-Nedd8, MgCl2, ATP and DTT. After 2 hours, the samples were run on a SDS-PAGE gel and fluorography was performed subsequently. The non-modified and neddylated forms of HdmX are indicated by arrowhead and asterisk, respectively.
(B) The reaction mix (RM) and proteins were incubated with radiolabelled p53 in a standard *in vitro* ubiquitination assay. Nedd8 RM contains GST-Nedd8, Nedd8 E1 from H1299 cells and Ubc12. Ubiquitin (Ub) RM contains ubiquitin from Sigma, MgCl2, ATP and DTT as per standard ubiquitination reaction. After 2 hours, the samples were run on a SDS-PAGE gel and fluorography was performed subsequently. Non-modified and modified forms of the proteins are indicated by arrowhead and asterisk, respectively.

mediated p53 ubiquitination, as the respective salt concentrations completely inhibited the Mdm2-mediated p53 ubiquitination (Fig 35B). To circumvent this problem, bacterial expression constructs for Nedd8 E1 were obtained and Nedd8 E1 expressed and purified from bacteria. Using bacterially expressed Nedd8 E1, however, no neddylation of either MdmX or p53 was observed *in vitro*, for as yet unclear reasons.

The potential interaction of Ubc12 with Mdm2 and MdmX was analysed by GST-pulldown and yeast two-hybrid assays. Interaction of Ubc12 with MdmX or Mdm2 was not detected in either GST-pulldown or yeast two-hybrid assays (data not shown). Moreover, MdmX localisation in H1299 cells was monitored in the presence and the absence of Nedd8. MdmX mostly localised to the cytoplasm in the absence of ectopically expressed Nedd8. However, in the presence of overexpressed Nedd8, MdmX was mainly localised to a distinct region in the cytoplasm, which is very near to one of the two poles of the nucleus (data not shown). This type of localisation has been observed in the case of inclusion body formation[139] or the localisation of ubiquitinated forms of certain proteins[140].

4.3.7 p73 Neddylation

p73, a member of the p53 protein family (see introduction 1.2), has been reported to interact with Mdm2. However, the role of Mdm2 in p73 ubiquitination and degradation is controversial. Initial reports suggested that Mdm2 does not ubiquitinate and degrade p73[54; 55].

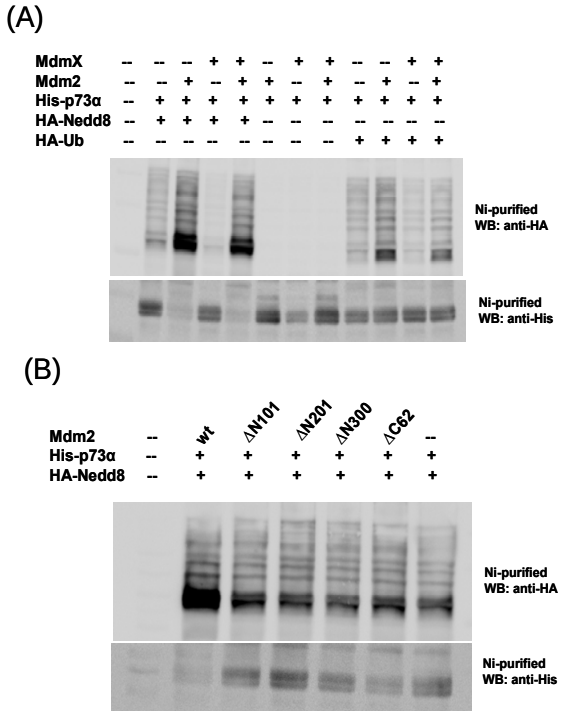

Fig 36. p73 neddylation.

1 µg of the plasmids were transfected into H1299 cells. After 20-24 hours of transfection, cells were lysed in denaturing guanidinium hydrochloride buffer. His-p73 was purified by Ni-beads followed by SDS-PAGE and western blot performed with indicated antibodies.

However, more recent results indicate that Mdm2 can also target p73 for ubiquitination. Here, we tested, whether p73 can be neddylated in H1299 cells and whether Mdm2 acts as Nedd8 E3 ligase for p73 neddylation. An expression constructs encoding His-tagged p73 was transfected into H1299 cells together with either HA-Nedd8 or HA-Ub expressing constructs and determined if p73 is neddylated and ubiquitinated, respectively (Fig 36A). Indeed, p73 was neddylated and p73 neddylation was significantly enhanced in the presence of ectopically expressed Mdm2. In contrast, Mdm2 was less active in modifying p73 with ubiquitin. Ectopically expressed MdmX did not modulate p73 ubiquitination or neddylation either alone or in the presence of overexpressed Mdm2.

Next, we mapped the minimal region/domain required of Mdm2 to enhance p73 neddylation. Several deletion constructs of Mdm2 (ΔN101, ΔN201, ΔN300 (all lacking the p53/p73 binding domain) and ΔC62 (lacking the RING finger domain of Mdm2)) were tested for their ability to enhance p73 neddylation in cells. All the mutants were defective in enhancing p73 neddylation (Fig 36B), indicating that a full-length Mdm2 containing both the p73 binding domain and the RING finger domain is required to enhance p73 neddylation.

4.3.8 Dominant negative mutants for Neddylation pathway

As mentioned above, the ubiquitination machinery can cross-activate Nedd8, though with an efficiency approx 100 times less than the efficiency to activate ubiquitin[122]. In the *in vivo* neddylation assay, almost all the proteins tested were modified with Nedd8, raising doubts as to whether overexpressed Nedd8 was indeed properly activated and conjugated by the neddylation pathway or cross-activated by ubiquitination pathway. To answer this question, we constructed several mutants of Ubc12 that should function in a dominant-negative manner for the neddylation pathway.

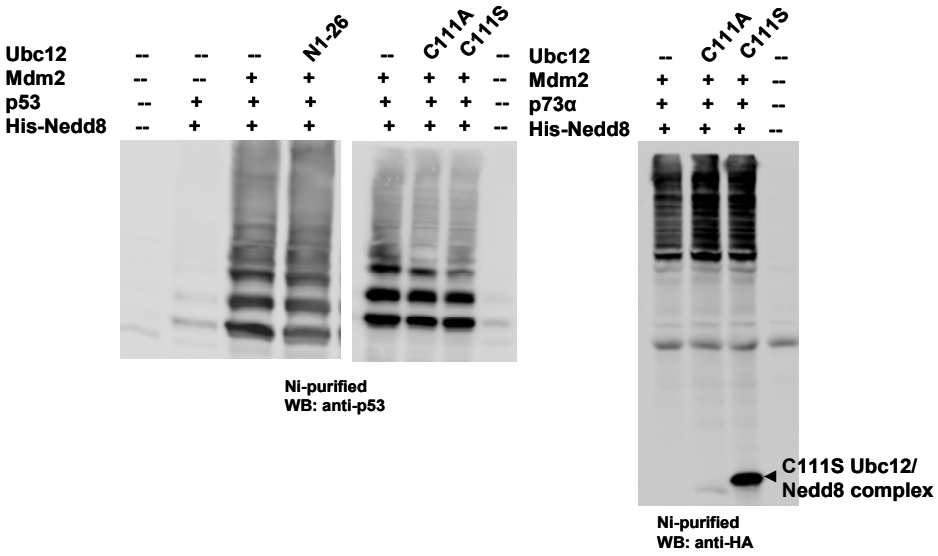

Fig 37. Dominant-negative mutants for the neddylation pathway.
Plasmids (p53, 200 ng; pCoc Mdm2, 200 ng; and rest of the plasmids, 1 µg), as indicated, were transfected into H1299 cells. After 20-24 hours of transfection, cells were lysed in denaturing guanidinium hydrochloride buffer. His-tagged proteins were purified by Ni-beads followed by SDS-PAGE and western blot performed with indicated antibodies. Note, Ubc12 is also His tagged, thus C111S Ubc12 mutant appears in western blot as a complex with HA-Nedd8.

Unlike the UbcH5 family members, Ubc12 has an N-terminal extension that is required for proper docking of Ubc12 to its cognate E1[124]. The interaction of Ubc12 with Nedd8 E1 is predicted to be unique as Ubc12 interacts with Nedd8 E1 via two domains- the N-terminal amino acids 1-26 and a C-terminal core E2 like domain. Both domains of Ubc12 are essential for proper transfer of activated Nedd8 from its cognate E1 to Ubc12. Furthermore, it has been reported that a chemically synthesised peptide corresponding to amino acid 1-26 of Ubc12 can efficiently block neddylation of Cul-1 *in vitro*, by blocking the proper docking of Ubc12 to its cognate E1[124]. Thus, we constructed a plasmid expressing a peptide consisting of amino acids 1-26 (N1-26) of Ubc12 and transfected it into cells to test its ability to interfere with p53 neddylation. The N1-26 Ubc12 coexpression construct was not able to interfere with p53

neddylation (Fig 37, left panel). However, the expression of peptide in cells could not be monitored due to the lack of a suitable antibody and due to the small size of the N1-26 Ubc12 peptide. Thus, in order to render the N1-26 peptide more stable and to ensure transport to the nucleus, we attached the peptide to wt-Nedd8 (Nedd8-N1-26) and to the G75/76A mutant of Nedd8 (Nedd8-G75/76A-N1-26). However, both constructs also failed to inhibit neddylation of p53 in H1299 cells (data not shown). In addition, we generated two coexpression constructs for Ubc12 mutants, in which the catalytic cysteine residue was mutated to either alanine (C111A) or serine (C111S). The C111S Ubc12 mutant has already been reported to function as a dominant-negative mutant of Nedd8 conjugation by virtue of forming a stable ester bonded complex with Nedd8 and thus, depleting Nedd8[141]. However, both Ubc12 mutants failed to significantly inhibit neddylation of either p53 or p73 in H1299 cells (Fig 37, middle and right panels), although the His-C111S Ubc12 mutant was observed to form a stable complex with HA-Nedd8, which migrates in SDS-PAGE at around 25KDa (Fig 37, right panel).

4.4 Screening of interacting partners of MdmX by the yeast two-hybrid system

In contrast to Mdm2, close to nothing is known about interaction partners of MdmX (besides p53). Thus, to obtain insight into the role of MdmX in cellular regulation, we aimed to use the yeast two-hybrid system to identify potential interacting partners of MdmX.

The foremost criterion of the yeast two-hybrid system is that the protein ("bait") fused with the Gal4 DNA binding domain does not act as transcriptional activator of the reporter genes used. To test whether full-length MdmX is able to switch on the reporter gene, an expression construct (BD-MdmX) encoding MdmX fused to the DNA-binding domain of the Gal4 transcription factor was transformed into KF1 yeast cells. This showed that BD-MdmX alone was able to switch on all the reporter genes tested. This implies that full-length MdmX has the ability to act as transcriptional transactivator in yeast cells. Hence, full-length MdmX cannot be used as bait in the yeast two-hybrid system. In order to identify the putative transactivation domain in MdmX, several deletion mutants of BD-MdmX were constructed (see Table 1) and their abilities to switch on the expression of the reporter genes were tested. Three of the constructs (RF, ΔAD, and ΔN-AD) were revealed as having no ability to switch on the reporter genes (Fig 38A). Thus the ΔN-AD MdmX mutant (having the smallest deletion) was selected for the yeast two-hybrid screen. However, before starting the actual two-hybrid screen, it was important to analyse whether ΔN-AD MdmX is functionally active. To do so, a yeast two-hybrid interaction assay was performed to test the ability of the ΔN-AD

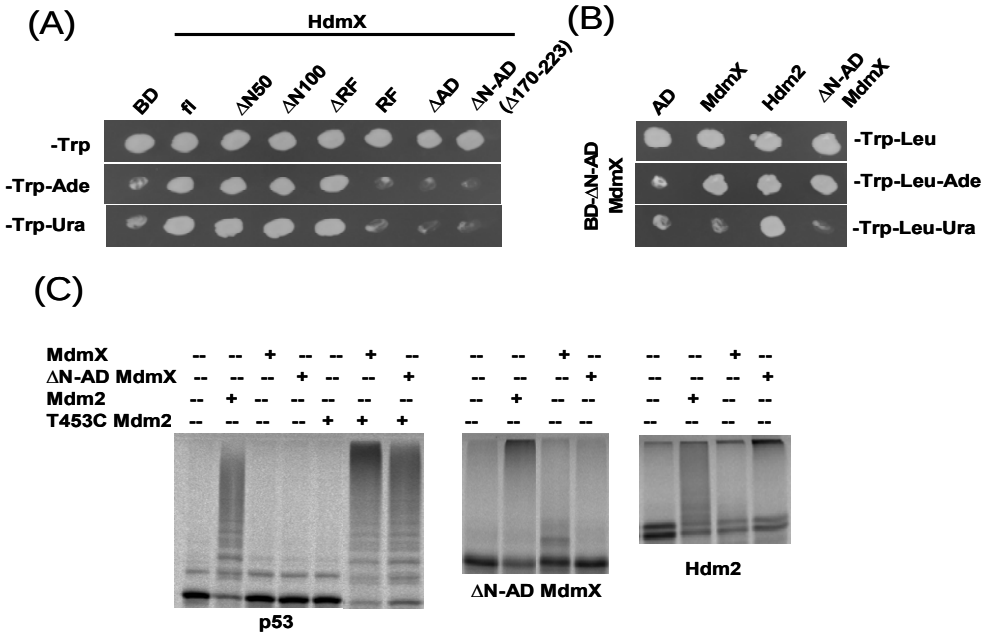

Fig 38. Characterization of MdmX to be used as bait in the yeast two-hybrid screen.

(A) Identification of the transactivation domain deficient MdmX. The cDNA of several deletion constructs of MdmX were cloned in fusion with the Gal4 DNA binding domain (BD), as indicated, in yeast two-hybrid vectors. The plasmids were transformed into yeast cells and after 3 days of selection on tryptophan (Trp) plate, the colonies were streaked on two reporter plates lacking either adenine (Ade) or uracil (Ura). Pictures were taken after 4 days of growth. RF, RING finger; ΔAD, deletion of 170-280 residues in MdmX.
(B) Interaction analysis of ΔN-AD MdmX. 1 μg of each plasmid, as indicated, was transformed into yeast strain KF1 and selected on media lacking tryptophan (Trp) and leucin (Leu). After 3 days, the yeast cells were streaked on reporter plates lacking either histidine (His), adenine (Ade), or uracil (Ura). Pictures were taken after 4 days of growth.
(C) Functional analysis of ΔN-AD MdmX. Bacterially expressed MdmX, ΔN-AD MdmX, Mdm2 and T453C Mdm2 were, individually, incubated with radiolabelled p53, ΔN-AD MdmX, or Mdm2 in a standard *in vitro* ubiquitination assay. The samples were run on a SDS-PAGE gel and fluorography was performed subsequently.

MdmX mutant to interact with wt-Mdm2 or wt-MdmX. The ΔN-AD MdmX mutant interacted with wt-Mdm2 or wt-MdmX with an efficiency similar to wt-Mdm2/wt-Mdm2 or wt-Mdm2/wt-MdmX interactions, respectively (Fig 38B). The p53 binding ability of the ΔN-AD MdmX mutant was analysed by GST-pulldown assay. Like wt-MdmX, the ΔN-AD MdmX mutant interacted with p53 (data not shown). Furthermore, the ability of the ΔN-AD MdmX mutant to rescue the E3 activity of the T453C Mdm2 mutant was analysed. The ΔN-AD MdmX mutant was able to rescue T453C-mediated *in vitro* ubiquitination of p53 (Fig 38C, left panel). Moreover, the ΔN-AD MdmX mutant was able to mediate ubiquitination of *in vitro* translated wt-Mdm2 and can also be ubiquitinated by wt-Mdm2 (Fig 38C, middle and right panels).

Table. 1. Yeast two-hybrid analysis of several deletion constructs of MdmX for their transactivation activity

MdmX constructs	Amino acid deleted	Transactivation activity	Interact with Mdm2	Interact with MdmX
Full-length	--	+	+	+
ΔN50	1-50	+	+	+
ΔN100	1-100	+	+	+
ΔRF	427-490	+	--	--
RF	1-426	--	+	+
ΔAD	170-280	--	+	+
ΔN-AD	170-223	--	+	+

Several deletion constructs of MdmX were transformed into yeast cells in order to perform the yeast two-hybrid assay. These mutants were analysed for their transactivation activity along with their ability to interact with Mdm2 and MdmX respectively. + and – denote yes and no, respectively.

The above results indicate that the ΔN-AD MdmX mutant can be used as bait to screen for interacting partners of MdmX. An expression plasmid encoding ΔN-AD MdmX (pDBleu-ΔN-AD) was transformed into yeast cells to obtain a yeast cell line stably expressing ΔN-AD MdmX. These yeast cells were further transformed with different amounts of library DNA (0.5, 1 and 2μg) to analyse the concentration of library DNA at which a maximum number of transformants can be obtained. Finally, two libraries (a T-cell library and a foetal brain library) were screened to identify potential interacting partners of ΔN-AD MdmX (about 20 million transformants each). 179 and 191 clones were obtained from the T-cell and the foetal brain library, respectively, growing on adenine reporter plates. However, due to time constraints, it was not possible to characterise the clones obtained within the frame of this thesis.

5 Discussion

5.1 Walker A motif Mdm2 mutants and p53

The results obtained in this study show that several Walker A motif mutants of Mdm2 (notably G451S, T453C and T453A) are significantly impaired in their E3 activity and that the defective ligase activity of the mutants is probably due to impaired homo-oligomer formation (in case of T453C) and/or weaker E2 binding (in case of G451S, T453C, and T453A). It has been previously suggested that the T453 residue (numbering of amino acid position is according to mouse Mdm2) is involved in coordinating one of the zinc ions. However, the recent determination of the NMR structure of the RING finger domain of human Mdm2 (429-491)[142], which was published during the preparation of this thesis, indicates that this is not the case (see Fig 4c, with zinc coordinating residues shaded in red). The NMR structure shows that the RING domain of Mdm2 folds into a tertiary structure (ββαβ topology) very similar to the structure of other RING domains and that in solution, the RING domain of Mdm2 exists as a symmetrical dimer (Fig 39). The T453 residue, which is located on the second β strand at the dimer interface, has been predicted to stabilize the dimer by forming a hydrogen bond with the side chain of H450 of the other subunit. Consistent with the NMR data, our yeast two-hybrid interaction assay also showed that mutation of T453 (i.e. T453C) impairs the homo-oligomer formation ability of Mdm2 (see Fig. 13b). Previously, it has been suggested that oligomerization is required for Mdm2 to function as E3 ligase[112; 133]. Hence, the impaired E3 activity of Mdm2 in case of the T453 mutation is probably explained by impaired homo-oligomer formation ability. The importance of T453 in hydrogen bond formation and dimer stabilisation is further supported by the wild-type E3 activity of a T453S Mdm2 mutant (data not shown) *in vitro*. In this case, the hydroxyl group of serine may be able to form the hydrogen bond in an appropriate manner and hence the T453S Mdm2 mutant retains its full E3 activity.

The G451S mutant was also impaired in its E3 activity and the NMR structure shows that G451 is located in the loop between the first and second β strand. Although this loop is also located at the dimer interface, G451 does not directly participate in dimer formation. Then, how does mutation of G451 affect the E3 activity of Mdm2? Our data show that, similar to the T453 mutation, the G451 mutation impairs the binding of Mdm2 to UbcH5b (see Fig. 13b). On the basis of our data, I propose that the mutation of G451 has a subtle influence on the dimer structure leading to a change in the structure at the E2 binding surface. The

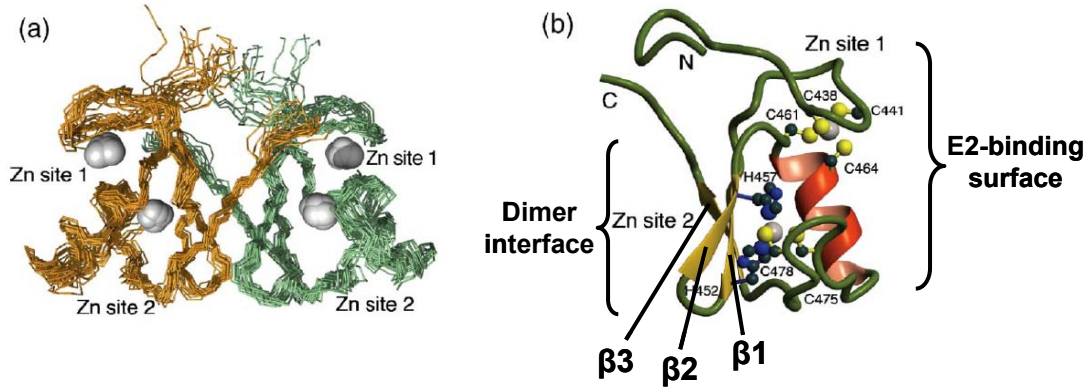

Fig 39. NMR structure of the RING domain of Mdm2.

(a) Solution structure of the dimer of the RING finger domain of human Mdm2, where two different subunits are shown in green and gold colour. The grey spheres represent the Zn ions.
(b) A ribbon diagram of the monomer of the RING finger domain of Mdm2. The monomer of Mdm2 folds into secondary structure with ββαβ topology. All three β strands forms the dimer interface while α helix lie at the opposite surface of the dimer interface. The side chains of the Zn-binding conserved residues are shown in balls. (Kostic et. al. J Mol Biol, 2006, 363(2); 433-50).

other Walker A motif mutants of Mdm2, G446S, G446A, and K452A, more or less retained their E3 ligase activity *in vitro*. Only the G446S Mdm2 mutant was slightly impaired in its E3 activity. A closer look at the location of G446 residue indicates that this residue is also located very near to the dimer interface, just prior to the start of the first β strand, and possibly also affects the dimer structure.

Our data further demonstrate that MdmX is able to completely rescue the E3 ligase activity of the ligase defective Walker A mutants of Mdm2 both *in vitro* and within cells (see Fig. 6 and 10a). A likely explanation for this finding is provided by the notion that the Walker A motif mutants of Mdm2 interact with MdmX with an efficiency similar to wt-Mdm2 as observed in the yeast two-hybrid interaction assay (Fig 13b). Thus, our data indicate that Mdm2/MdmX hetero-oligomers function as an E3 ligase for p53 within cells (which was previously disputed by other groups) and that Mdm2/MdmX hetero-oligomers are more robust than Mdm2 homo-oligomers. This type of enhancement of E3 ligase activity has also been observed in case of the BRCA1/BARD1 hetero-dimer, where BARD1 on its own has no appreciable E3 activity. However, when BARD1 interacts with BRCA1, the hetero-dimer is significantly more active than the BRCA1 homo-dimer[143]. The robust E3 activity of the BRCA1/BARD1 hetero-dimer has been suggested to be in part due to the ability of the BRCA1/BARD1 hetero-dimer to assemble non-K48 linked ubiquitin chains on itself[144], which stabilises BRCA1/BARD1 hetero-dimers. One of the Walker A Hdm2 mutant (G448S) was tested for its ability to form ubiquitin chains in the presence of MdmX. The mass spectrometry data show that, similar to wt-Mdm2 on its own, the G448S/MdmX complex

forms K11, K48 and K63 chains (Fig 7D). However, the actual proportion of respective chains synthesized by G448S/MdmX and by Mdm2 was not analysed due to technical constraints. Thus, it seems possible that the G448S/MdmX complex forms more K11 and K63 chains than Mdm2, which may predominantly form K48 chains. To resolve this issue, it will be important to perform quantitative mass spectrometry to quantify the amount of the respective chains synthesized in the presence of the G448S/MdmX complex and wt-Mdm2. Furthermore, it would also be informative to analyse the chain formation ability of the other Walker A Mdm2 mutants (e.g., G451S, T453C, and T453A) in the absence and in the presence of MdmX.

Since MdmX has been reported to stabilise Mdm2 within cells[109; 110; 111], we tested the stability of the Walker A motif mutants of Mdm2 in the presence of MdmX. We did not observe any stabilisation of the Mdm2 Walker A mutants in the presence of MdmX in HEK293T cells (Fig 11, lower panel). This indicates that the enhancement of the E3 ligase activity of the Walker A motif mutants of Mdm2 in the presence of MdmX is not due to the stabilisation of these mutants in cells.

Consistent with previous reports[105], we observed that the interaction between Mdm2 and MdmX was significantly stronger than the Mdm2/Mdm2 interaction, probably due to the presence of additional stabilising interactions in the hetero-oligomers compared to homo-oligomers. An NMR titration experiment revealed the saturation of hetero-dimer formation in the presence of the RING domains of Mdm2 and MdmX in a near 1:1 molar ratio[142]. The additional stabilising interactions in Mdm2/MdmX hetero-oligomers could be located within the respective RING domains or outside of the RING domains. Additional interactions located outside of the RING domain have been observed with other RING-RING interactions. For example, BRCA1 and BARD1 hetero-dimers, which involves the respective RING finger domains, are stabilised by the interaction of N-terminal extensions of BRCA1 and BARD1[35]. Similarly, Ring1b and Bmi1 hetero-dimers are also stabilised by interactions of the N-terminal extensions to the RING domains[36]. Hence, it is possible that MdmX and the Walker A motif mutants of Mdm2 interact more strongly due to the presence of additional non-RING interactions, which are missing in the case of Mdm2 RING homo-dimer formation. The solution structure of the Mdm2 RING dimer was determined with a RING finger domain bearing some amino acid residues N-terminal to the RING domain. No additional interactions between these N-terminal extensions or between the N-terminal extensions and the RING finger domain were observed[142]. However, the structure of the Mdm2/MdmX hetero-dimer was not determined, so that it is possible that the interaction of Mdm2 and MdmX involves regions in addition to the RING finger domain. Alternatively, it

is possible that MdmX interacts with Mdm2 with some additional interactions within the RING domain, which is supported by our observation that only the RING domain of MdmX is necessary and sufficient to rescue the activity of two of the Walker A mutants of Hdm2 *in vitro*.

On the basis of the data obtained, I propose a model where certain mutations in the Walker A motif of Mdm2 induce subtle changes in the structure of the RING dimer interface, which in turn leads to subtle changes in the structure of the E2 binding surface of Mdm2. These two structural changes synergistically impair the ability of Mdm2 to efficiently position the substrate and its cognate E2 for optimal transfer of activated ubiquitin from the E2 to the lysine of the substrate. However, in the presence of MdmX, more robust hetero-oligomers of MdmX and the Walker A motif mutants of Mdm2 are formed, which are more resistant to any change in the tertiary or/and quaternary structure compared to the respective homo-oligomers of the Walker A motif mutants of Mdm2. Hence, the hetero-oligomers of wt-MdmX and the Mdm2 Walker A mutants function like wt-Mdm2 in their E3 activity (see model in Fig 40).

Studies with Mdm2 and MdmX null mice propose overlapping but non-redundant functions for Mdm2 and MdmX in the regulation of p53. Mdm2 is proposed to inhibit p53- mediated apoptosis by negatively regulating p53 levels within cells[145], while MdmX inhibits p53-mediated cell cycle arrest by inhibiting the transactivation activity of p53[145]. Considering that no significant increase in p53 levels has been observed in MdmX null mice, how can we conclude that the ability of MdmX to enhance the E3 ligase activity of Mdm2 towards p53 is of physiological significance? I propose that the intracellular levels of Mdm2 are not sufficient to form Mdm2/Mdm2 homo-oligomeric complexes. However, due to the stronger interaction of Mdm2 with MdmX, an identical level of Mdm2 can form Mdm2/MdmX hetero-oligomeric complexes, which then can degrade p53 within cells. Furthermore, p53 is known to control *mdm2* gene expression[43]. Since MdmX inhibits the p53 transcriptional transactivation activity, knockdown of MdmX should result in enhanced expression of the Mdm2 protein. The elevated amounts of Mdm2 are then sufficient to allow the formation of Mdm2/Mdm2 homo-oligomeric complexes, which can efficiently degrade p53. Although our model is rather difficult to prove within cells, the data obtained here are consistent with it.

The hypothesis that intracellular levels of Mdm2 are not sufficient to control p53 levels is supported by a recent report, which indicates that MdmX null embryonic lethality can be completely rescued by 3-4fold higher expression of an Mdm2 transgene[146]. This indicates that in the absence of MdmX, the endogenous levels of Mdm2 are indeed not sufficient to control p53 levels, at least during embryonic development. However, it should be noted that

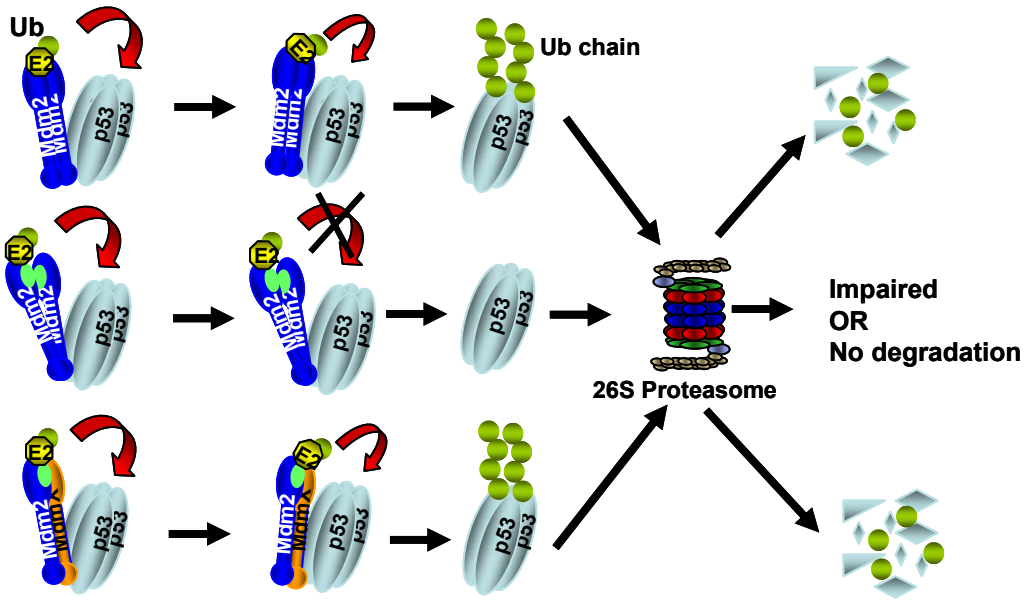

Fig 40. Mechanism of the rescue of the E3 activity of Mdm2 mutants by MdmX.

Normally, Mdm2 binds at its N-terminal (shown by blue ball like structure) to a tetramer of p53. Simultaneously, a dimer of RING finger domain (shown by blue elliptical shape structure) of Mdm2 is required to transfer ubiquitin from its cognate E2 to p53. The polyubiquitinated p53 is degraded by the 26S proteasome (upper panel). However, mutations (shown with green) in the RING finger domain of Mdm2 cause subtle changes in the tertiary structure of the RING domain, which leads to weakening of either E2 binding (in case of G451S, T453C, and T453A Mdm2 mutants) or/and dimer formation (in case of T453C, ΔC7, I448E, L456E, and I483E Mdm2 mutants), resulting in impaired or abrogated p53 ubiquitination and degradation (middle panel). Since MdmX binds much stronger to Mdm2, the presence of MdmX resists the subtle changes in tertiary structure of the RING domain of some of the mutants of Mdm2 and, hence, MdmX rescues the ability of the ligase defective Mdm2 mutants to ubiquitinate and degrade p53 within cells (lower panel).

several other molecules have also been reported to enhance the E3 activity of Mdm2 including Gankyrin, YY1, and Daxx[81; 82; 83]. Gankyrin and YY1 have been implicated in enhancing binding of Mdm2 to p53 with a subsequent increase in E3 activity of Mdm2. Daxx was reported to facilitate the binding of the ubiquitin specific protease HAUSP/USP7 to Mdm2 thereby stabilizing Mdm2 by preventing its auto-ubiquitination. However, these data are not contradictory to the proposed model in this thesis.

5.2 Walker A motif Mdm2 mutants and MdmX

The hetero-oligomers of MdmX with the Walker A mutants of Mdm2 (G451S, T453C, and T453A) were completely active in ubiquitinating p53 *in vitro* (see Fig. 6). However, these hetero-oligomers were impaired in their ability to ubiquitinate *in vitro* translated MdmX (Fig. 6). This apparent discrepancy may be explained by the fact that the p53 binding site is located at the N terminus of Mdm2 and MdmX, respectively, and does not involve the respective RING finger domains. However, the MdmX/Mdm2 interaction is mediated via the RING

finger domain. Hence, the *in vitro* translated S^{35}-MdmX has to compete with GST-MdmX for binding to the GST-Walker A Mdm2 mutants and, since GST-MdmX is present at much higher amounts than the S^{35}-labelled MdmX, complexes between GST-Mdm2 and S^{35}-labelled MdmX are not or only inefficiently formed. Consistent with this hypothesis, we observed that the hetero-oligomers of GST-Walker A mutants of Mdm2 and GST-MdmX, in a near 1:1 ratio, were efficiently ubiquitinated in *in vitro* ubiquitination assays (data not shown). Furthermore, similar to wt-Mdm2, the Walker A Mdm2 mutants can degrade MdmX both in H1299 and HEK293T cells (Fig. 25). However, we observed that the Walker A Mdm2 mutants were also impaired in their ability to ubiquitinate S^{35}-MdmX in the absence of GST-MdmX. A possible explanation for this observation could be that Mdm2 or Mdm2/MdmX are not active as dimer but as higher oligomeric structures and that for this oligomeric structures Mdm2 and MdmX have to be present in stoichiometric (i.e. 1:1) amounts. Indeed, size exclusion chromatography of bacterially expressed Mdm2 and MdmX indicates that both proteins can form higher oligomeric structures (data not shown).

5.3 Mdm2 and its interaction with UbcH5 family members

Using the yeast two-hybrid system, we were not able to show an interaction of Mdm2 with UbcH5a and Ubc8. However, we observed a weak binding of Mdm2 to UbcH5b and UbcH5c. Since Ubc8 supports Mdm2-mediated ubiquitination of p53 *in vitro*, I propose that there is a weak binding between Ubc8 and Mdm2, which cannot be detected in the yeast two-hybrid system. The hypothesis that, in comparison to UbcH5b/Mdm2 interaction, Ubc8 only weakly interacts with Mdm2 is supported by the observation that p53 ubiquitination by two Walker A mutants (G448S, T455C) is abrogated in the presence of Ubc8, while p53 ubiquitination by these mutants is impaired, but not abrogated, in the presence of UbcH5b. Since Ubc8 is more similar to UbcH5a than to UbcH5b or UbcH5c, two chimeras of UbcH5a and UbcH5b were constructed to map the region of UbcH5b responsible for the higher affinity to Mdm2 (Fig. 14). The observation that the two chimeras generated interact with Mdm2 with an efficiency similar to UbcH5b/Mdm2 interaction, indicates that swapping of either the N-terminal (in 5ab) or the C-terminal (in 5ba) domain of UbcH5b with UbcH5a does not impair the interaction ability of UbcH5b with Mdm2. Thus, it is possible that both the N terminus and the C terminus of UbcH5b together affect the efficiency of the interaction with Mdm2. Alternatively, It is possible that the central region in UbcH5b, though very much similar to UbcH5a except for 3 amino acids, is responsible for the stronger interaction. Indeed, the crystal structure of the c-Cbl/UbcH7 complex indicates that the residues located in the central region of UbcH7 are mainly responsible for its interaction with c-Cbl[27]. If this

is the case for UbcH5/Mdm2 interaction, then analysis of the interaction of two additional chimeras ("5aba" and "5bab"), which contain only the central region of UbcH5b and the other parts of UbcH5a in 5aba and *vice versa* in 5bab, with Mdm2 would be very informative. Furthermore, it would also be interesting to test the interaction of UbcH5 family members with Mdm2 using more sensitive methods, e.g. surface plasmon resonance or calorimetry.

5.4 Walker A motif of Mdm2 and its RNA binding ability

The Walker A motif of Mdm2 (GCIVHGKT) has been reported to bind adenine-based nucleotides (ATP, ADP and AMP)[130]. Generally, the residues between the two conserved glycines in the Walker A motif (GXXXXGKT/S) form a loop which surrounds the nucleotide and the highly conserved lysine residue binds to the phosphate of the nucleotide. The nucleotide binding property of Mdm2 has been challenged recently by the published structure of the RING domain of Hdm2, where the predicted loop forming residues (CIVH) are actually involved in the formation of the first β strand and are located at the dimer interface[142] (see Fig. 39). Moreover, a loop is formed right after the first β strand consisting of the glycine and lysine residues (GK) of the putative Walker A motif. The last threonine (T) of the Mdm2 Walker A motif folds into the second β strand. Based on the structure, it seems likely that the Walker A motif of Mdm2 does actually not bind nucleotides in a canonical manner. One possibility might be that nucleotides are binding to the Mdm2 RING domain via a region/residues other than the Walker A motif. Indeed, this assumption is supported by the observation that the tested Walker A Hdm2 mutants (including K454A) were still able to bind polyG RNA in GST-pulldown assays (see Fig. 15). Furthermore, since MdmX/HdmX does not interact with polyG RNA, it would be interesting to map the region/residues of Hdm2 required to interact with polyG by constructing chimeras of the RING finger domains of Hdm2 and HdmX.

5.5 The dimerisation mutants of Mdm2

Based on the structure of the U box of the Prp19 dimer, several hydrophobic residues have been proposed within (I448 and L456) or outside (I483 and L485, residues within the C-terminal extension) of the RING finger domain of Mdm2 to be involved in formation of homo-oligomers[136]. By mutational analysis of these residues, the evidence is provided that homo-oligomerization of Mdm2 is necessary for its E3 activity. The data show that several of the predicted dimerisation residues including the C-terminal 7 amino acid of Mdm2 are essential for the E3 activity of the Mdm2. Individual mutation of three (I448E, L456E and I483E) out of four predicted residues completely abrogated the E3 activity of Mdm2, both *in*

vitro and within cells. Mutation of the L485 residue did not affect the E3 activity of Mdm2. In support of the interaction studies performed in this thesis, the NMR structure of the Hdm2 RING dimer[142] indicates the importance of I448, L456, and I483 residues in oligomer formation. The I448 residue of Mdm2 is involved in the formation of the first β strand that forms the core of the hydrophobic dimer interface. Although no direct involvement of I448 has been observed in dimer formation of the Hdm2 RING domain, it is likely that replacing a hydrophobic residue (I448) by a negatively charged residue (glutamate) at the core of the hydrophobic interface disrupts the interaction. Furthermore, I448 is adjacent to the V449 residue, which is implicated in dimer formation. Hence, it is also possible that mutation of I448 residue interferes with the ability of V449 to interact with the other subunit. L456 and the region encompassing the C-terminal 7 amino acid residues have been observed to directly interact with the other subunit[142].

The GST-pulldown and yeast two-hybrid interaction data (Fig. 23 and 24B) further show that the proposed dimerisation residues are not only required for Mdm2 homo-oligomerization but also required for hetero-oligomerization with MdmX and that abrogation of the E3 ligase activity of several Mdm2 dimerisation mutants is due to their impaired ability to form homo- and hetero-oligomeric complexes, respectively. Moreover, those Mdm2 mutants (I483E, L485E) that are able to interact with MdmX are rescued for their E3 activity by MdmX. In addition, the ability of these mutants to interact with UbcH5b suggests that homo-oligomerization is not necessary for Mdm2 to bind to its cognate E2.

5.6 Mdm2 as substrate for ubiquitination

Several of the dimerisation mutants (e.g. I448E and ΔC7) and the Walker A mutants of Mdm2 have either no or impaired E3 activity. However, all these mutants are highly ubiquitinated by wt-Mdm2 (see Fig. 9 and 22). This indicates that E3 activity is not required for Mdm2 to serve as substrate for wt-Mdm2-mediated ubiquitination and that "auto-ubiquitination" of Mdm2 can, at least in part, occur in trans. This hypothesis is in agreement with the notion that E3 activity of Mdm2 requires homo-oligomerization or hetero-oligomerization with MdmX and that Mdm2 monomers do not function as E3 ligase. Furthermore, ubiquitination of the ΔC62 Mdm2 mutant (RING deleted) by either wt-Mdm2 or only the RING domain of Mdm2 shows that besides RING/RING interaction, there is an additional binding site for the RING finger domain located outside the RING domain (Fig. 41a). Furthermore, I propose that this other binding site of Mdm2 represents the ubiquitination signal for Mdm2 to ubiquitinate itself. If this is the case, then mapping and deletion (or point mutation) of the second binding site in Mdm2 should abrogate its ability to

be ubiquitinated by itself. However, whether Mdm2 auto-ubiquitination occurs also in cis is not clear yet (see model in Fig. 41b).

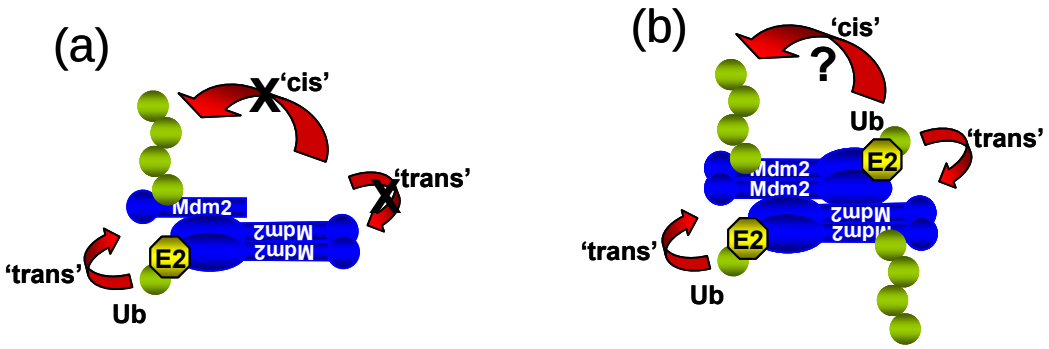

Fig 41. Mdm2 ubiquitinates itself in trans.
According to our observation, the RING finger domain of Mdm2 is not required for its ability to be ubiquitinated by itself, however, an intact RING domain of Mdm2 is necessary and sufficient to ubiquitinate RING-deleted Mdm2 molecule (a). It is only possible if the RING domain of Mdm2 interacts with another site additional to the RING domain and if Mdm2 follows a 'trans' (where a dimer of Mdm2 ubiquitinates another dimer) type of mechanism to ubiquitinate itself (b). However, it is still unclear that whether Mdm2 ubiquitinates itself following a 'cis' (where a dimer ubiquitinates itself) type of ubiquitination mechanism.

5.7 Neddylation

Most of the proteins tested were neddylated in the *in vivo* neddylation assay, except Cop1 (an ubiquitin E3 ligase for p53[5]). A common feature of all of the tested proteins is that they are somehow associated with pathways involved in conjugation of ubiquitin or ubl proteins. Recently, several proteins have been reported that can be ubiquitinated as well as neddylated including p53, Mdm2, EGFR, and p73[56; 128; 147]. Furthermore, also at least three E3 ligases (Mdm2, c-Cbl and ROC1) have been reported that can both ubiquitinate and neddylate the same substrate[127; 128; 147]. This indicates that it is possible that a given protein can be both ubiquitinated and neddylated, and that the ubiquitinated and neddylated forms of a given protein can perform two different functions. For example, ubiquitination of p53 leads to its degradation[128], whereas neddylation has been proposed to inhibit its transactivation activity[56]. The observation that Mdm2 can act as Nedd8 E3 ligase for p53 but not E6-AP (even in the presence of E6 proteins from oncogenic HPV16 and HPV18) suggests that HECT E3 ligases may not be able to accept Nedd8 from the E2 or that they cannot catalyse the final attachment of Nedd8 to substrate proteins. This assumption is supported by the fact that all the reported Nedd8 E3 ligases are RING-type E3 ligases. It would be interesting to test the ability of HECT E3 ligases to form thioester complexes with Nedd8.

Mdm2-mediated neddylation of p53 was reported two years ago[128]. However, it was not known if MdmX affects the Nedd8 E3 activity of Mdm2. Here, by studying the Nedd8 E3 ligase activity of several mutants of Mdm2, evidence is presented that Mdm2/MdmX hetero-oligomers not only function as ubiquitin E3 ligase but also as Nedd8 E3 ligase for p53 within cells (Fig. 28 and 29). This further supports the notion that MdmX is a positive cofactor for the E3 ligase activity of Mdm2. In case of the Walker A Mdm2 mutants, the impaired ubiquitin E3 activity of the mutants may be due to the weaker binding of UbcH5b. It is possible that the ubiquitin E2 (UbcH5) and Nedd8 E2 (Ubc12) bind to the same surface of Mdm2 and that the mutation in the Walker A motif also affects the binding of Ubc12 with Mdm2. However, in the yeast two-hybrid interaction assays, Ubc12 did not show any interaction with wt-Mdm2 or wt-MdmX (data not shown). This indicates that the interaction of Ubc12 with Mdm2 is too weak to be detected by yeast two-hybrid assay, which limits our ability to test the interaction of Ubc12 with the Walker A mutants of Mdm2. It would be interesting to test the interaction of Ubc12 and Mdm2 with more sensitive assays, e.g. surface plasmon resonance or calorimetry. However, these technologies require large amounts of purified protein, which is rather difficult to achieve with Mdm2 (since it is rather insoluble in bacteria). Another possibility could be that Ubc12 binds to Mdm2/MdmX hetero-oligomers rather than to Mdm2 homo-oligomers. To test this possibility, we are currently establishing a yeast three hybrid assay[148].

I attempted to address as to which process is more favourable in cells - p53 ubiquitination or p53 neddylation. Furthermore, since a recent report indicates that EGFR neddylation enhances the ability of the EGFR molecule to accept ubiquitin chains[147], another important question is whether neddylation of p53 affects p53 ubiquitination or *vice versa*. However, the data showed that ubiquitination or neddylation efficiency of p53 did not change in the presence of overexpressed Nedd8 or ubiquitin (see Fig. 30), respectively. This suggests that p53 ubiquitination does not affect neddylation of p53 and vice versa at least under the conditions used. However, we observed that Nedd8 modified p53 molecules are more readily detected in cells than ubiquitin modified p53 molecules (Fig. 30). Furthermore, the data suggest that one p53 molecule can be modified with both Ub and Nedd8 (Fig. 31) and, hence, p53 ubiquitination and neddylation can occur side by side on the same molecule of p53. In addition, the experiments indicate that free Nedd8 cannot serve as an ubiquitination substrate within cells, whereas free ubiquitin can be neddylated. This is consistent with a report, which indicates that the *in vitro* transfer of Ub or Nedd8 to Nedd8 by E2-25K is at least 50 times slower than the transfer of either molecule to Ub[122].

On the basis of our data, I propose a role for Nedd8 as a chain terminator in cells to hinder the ubiquitin conjugation pathway from forming very long chains and, at least under certain cellular conditions, this is probably advantageous for cells to maintain levels of free ubiquitin at a certain threshold level (Fig. 42). This model is supported by the notion that the kinetics of proteasomal degradation of a substrate conjugated to a tetraubiquitin K48-linked chain does not differ significantly from the one of a substrate conjugated to long chains. In addition, there is evidence that a substrate conjugated to a chain composed of three ubiquitin moieties and one distal Nedd8 moiety is a better substrate for proteasomal degradation than a substrate conjugated to a tetra ubiquitin chain[122]. Recently, NUB1 (Nedd8 ultimate buster-1) and NUB1L (NUB1 long, a splice variant of NUB1) have been shown to function as adaptors to target Nedd8 conjugated substrates to the proteasome[125], further suggesting that a chain composed of ubiquitin and Nedd8 can be targeted to the proteasome for degradation. However, it is not clear whether NUB1 and NUB1L indeed recognise substrates modified by mixed chains of ubiquitin and Nedd8. Taken together, the obtained data and published results favour the hypothesis of a chain termination role of Nedd8.

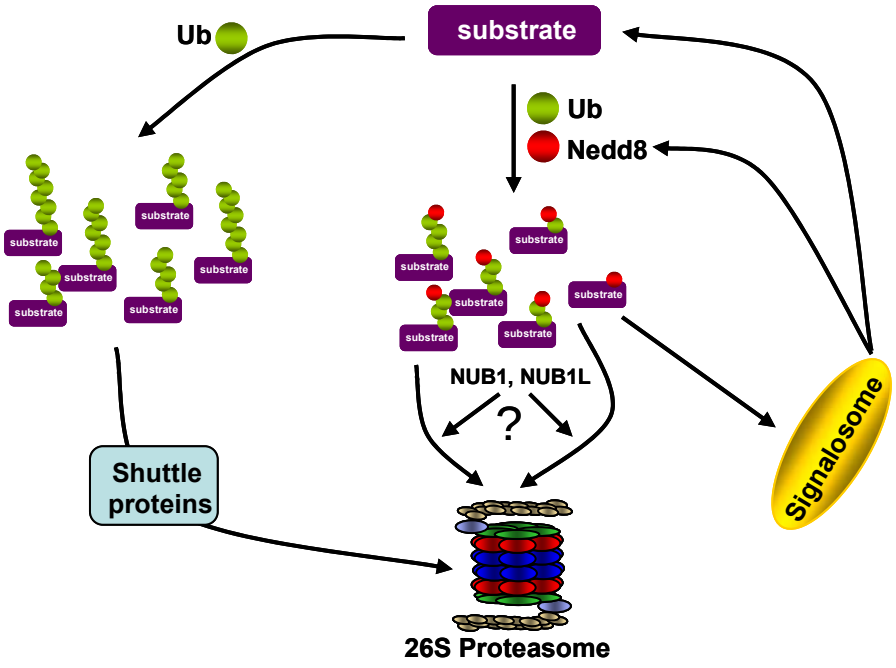

Fig 42. Model predicting the role of Nedd8 as a chain terminator.
Presence of Nedd8 shortens the formation of ubiquitin chains on the substrates and hinder the ubiquitin conjugation pathway from forming very long ubiquitin chains. The distal Nedd8 conjugated chains may be targeted to the proteasome by NUB1 and NUB1L, however, it is not clear whether NUB1 and NUB1L indeed recognise substrates modified by ubiquitin/Nedd8 mixed chains. The Nedd8 conjugated protein can also be cleaved by Signalosomes. This could result in maintaining a threshold level of ubiquitin within cells, if not in general, at least in certain conditions of cells.

The correct activation of ubiquitin and Nedd8 by their respective pathways is partly dependent on the presence of the residue at position 72 of ubiquitin and Nedd8 molecules. Ubiquitin contains arginine (R) at position 72 and Nedd8 contains alanine (A) at that position. Previous studies reported that switching the residue of Nedd8 at position 72 to the respective residue of ubiquitin enables Nedd8 to be activated by the ubiquitination machinery *in vitro*[122]. Similar to the reported data, our data show that A72R Nedd8 is conjugated to p53 in the presence of Mdm2 with an efficiency similar (or even slightly better) to wt-ubiquitin and that conjugation of A72R Nedd8 to p53 is dependent on the additional presence of UbcH5b (data not shown). However, due to the presence of sufficient amounts of ubiquitin E1 in the rabbit reticulocyte used in the *in vitro* ubiquitination assay, we were not able to test the ubiquitin E1 dependency of A72R Nedd8 conjugation to p53. Furthermore, similar to wt-Nedd8, the R72A ubiquitin mutant was not efficiently conjugated to p53. Interestingly, studies with these mutants in H1299 cells show that both wt-Nedd8 and A72R Nedd8 modified p53 accumulated in cells to higher levels than p53 modified with wt-ubiquitin and R72A ubiquitin (Fig. 33B). The accumulation of p53 modified with Nedd8 or the Nedd8 mutant in H1299 cells could be due to the inability of Nedd8 to accept ubiquitin or Nedd8 molecule for chain formation. This is supported by the data discussed above that A72R Nedd8 cannot be used to synthesize chains *in vitro*[122]. Please note that only mono-, di- and tri-Nedd8/ubiquitin modified p53 were accumulating within cells (Fig. 30) and not p53 molecules modified by four or more Nedd8/ubiquitin moieties, which may suggest that ≥ tetra-Nedd8/ubiquitin modified p53 is targeted to the proteasome for degradation. In contrast to Nedd8 and its A72R mutant, R72A ubiquitin may still form chains within cells and this targets p53 for degradation. It should be noted, however, that it is not clear whether, within cells, A72R Nedd8 is activated by the ubiquitination pathway or the neddylation pathway. If the A72R Nedd8 is activated by the ubiquitination pathway (as seen in the *in vitro* assay), then it is an intrinsic feature of the Nedd8 molecule that it cannot accept ubiquitin or Nedd8 molecule for chain formation. However, if A72R Nedd8 is activated by the neddylation pathway, then it may be the neddylation pathway which determines that Nedd8 cannot form chains. To solve this question, it would be important to establish an *in vitro* neddylation system to test how the neddylation pathway differentiates between wt-Nedd8 and A72R Nedd8. Furthermore, since A72R Nedd8 can be used in our *in vitro* ubiquitination assay, mass spectrometric analysis of A72R Nedd8 modified Mdm2 or MdmX would be very informative to determine whether A72R Nedd8 molecule can form chains on Mdm2 or MdmX.

Our data show that MdmX can be neddylated both *in vitro* and within cells and that Mdm2 functions as a Nedd8 E3 ligase for MdmX (Fig. 26A and 35A). Furthermore, mapping of the minimal domain required for MdmX to be neddylated by Mdm2 indicates that an MdmX mutant (ΔN300) containing the Zinc-finger region and the RING finger domain are necessary and sufficient for MdmX to be neddylated. Since the RING finger domain of MdmX alone cannot be neddylated, it is possible that the region spanning amino acids 301-429 contains the lysine (K) residue(s) required to form an isopeptide bond with Nedd8. Similarly, MdmX also requires amino acid residues in addition to the RING finger domain to be ubiquitinated by Mdm2 (unpublished data, Linares et al.).

The efforts to establish an efficient *in vitro* neddylation assay were not successful. Using a Nedd8 E1 containing fraction isolated from H1299 cells, we observed that very little amounts of MdmX were modified with Nedd8 (Fig. 35A). However, the E1 fraction purified from H1299 cells contained high concentration of salt. The presence of this high concentration of salt also completely inhibits Mdm2-mediated ubiquitination of p53, Mdm2, and MdmX. Furthermore, bacterially expressed Nedd8 E1 and E2 were not able to neddylate MdmX. One possibility could be that bacterially purified Nedd8 E1 or E2 or both were not active (i.e. they do not form thioester complexes with Nedd8). Hence, in the future, analysis of the thioester bond formation ability of Nedd8 E1 and E2 will be important. Furthermore, it is also possible that some component(s) of the Nedd8 attachment pathway are missing. Hence, performing the *in vitro* neddylation reaction in the presence of cell lysate and bacterially expressed E1 and E2 may be informative.

Our data showed that p73 is neddylated in H1299 cells and that p73 neddylation but not ubiquitination was significantly enhanced in the presence of ectopically expressed Mdm2 (Fig. 36). One recent report indicates that N-terminally deleted variants of p73 (ΔNp73; N-terminal 61 amino acids are replaced with 11 novel residues due to transcription start at the second promoter) are degrade faster upon DNA damage[149]. In order to test whether ΔNp73 (lacking the Mdm2 binding domain) can also be neddylated in H1299 cells, we constructed two mutants, ΔNp73 and ΔN61 p73 (missing the 11 novel residues), and tested their ability to be neddylated in H1299 cells. Both mutants were neddylated in H1299 cells and, as both the mutants lack the Mdm2 binding domain, Mdm2 was not able to enhance their neddylation (data not shown). Hence, it is likely that E3 ligases other than Mdm2 also mediate the neddylation of ΔNp73 and ΔN61 p73 within cells (or at least upon DNA damage).

In order to test whether ectopically expressed Nedd8 is activated and attached to p53 or p73 by the Nedd8 pathway or cross-activated by the ubiquitin pathway, we constructed several proposedly dominant-negative mutants of Ubc12. However, none of them was able to inhibit

neddylation of p53 or p73. The N-terminal 26 amino acid long peptide from Ubc12 was probably not stable in cells. Furthermore, our approach to tag the N1-26 Ubc12 peptide at the C terminus of Nedd8 also failed to inhibit neddylation of p53. The catalytic cysteine mutants of Ubc12, C111S and C111A, were generated considering that their expression will inhibit the activation of Nedd8 by titrating out Nedd8 by formation of stable Nedd8- C111S Ubc12 ester complexes[141]. Indeed, the C111S mutant was observed to form the ester bonded complex (Fig. 37). However, it failed to significantly inhibit p53 and p73 neddylation. Thus, higher amounts of the C111S Ubc12 mutant may be required to achieve any inhibition. Thus at present, we cannot conclude whether p53/p73 are neddylated by the Ubc12-dependent neddylation pathway or by another pathway. ASPP2 has been reported to inhibit the Nedd8 E1[150]. In addition, a temperature sensitive Nedd8 E1 containing Chinese hamster ovary (ts41 CHO) cell line has been shown to inhibit Nedd8 modification[151]. Thus, it would be interesting to use ASPP2 or the ts41 CHO cell line to solve this question.

5.8 Significance of the results obtained with respect to molecular approaches in the treatment of cancer

In the past 10 years, the p53-Mdm2 interaction has become a focus of cancer-related studies, due to the notion that activation of p53 in tumours expressing wt-p53 (approx 50% of human cancers) can be employed to eradication of these tumours. Most of the studies have been aiming at identifying molecules that can disrupt the p53-Mdm2 interaction and, thus, result in subsequent activation of p53 within cells. Indeed, Nutlin3a, an imidazoline based drug, is highly successful in disrupting the p53-Mdm2 interaction at a concentration of 2-10μM[152] and thus Nutlin3a is a promising drug for cancer therapy. However, more recently, it has been reported that the effect of Nutlin3a is compromised in tumours overexpressing MdmX and that Nutlin3a failed to disrupt the p53-MdmX interaction. Consequently, Nutlin3a also failed to suppress the growth of MdmX overexpressing cancer cell lines[153; 154]. In support of the latter finding, my studies indicate that, to treat cancers expressing wt-p53, it would be advisable to screen for drugs that not only inhibit the p53-Mdm2 interaction but also the p53-MdmX interaction, as p53-bound MdmX molecules can hetero-oligomerize with Mdm2 and may act as an efficient E3 ligase for p53 even in the presence of Nutlin3a.

Recently, attempts have been reported to screen for molecules that can inhibit the E3 activity of Mdm2[155]. Results obtained in this thesis revealed several residues in Mdm2, which can be targeted to not only inhibit Mdm2 homo-oligomer formation but also Mdm2/MdmX hetero-oligomer formation and hence abrogation of the E3 activity of the Mdm2. However, the strategy to abrogate Mdm2 E3 activity alone may not be successful in the treatment of

cancers, as E3-inactive Mdm2 and MdmX molecules can still interact with the N-terminal domain of p53 and thereby interfere with the transcriptional transactivation activity of p53. Hence, a strategy to inactivate the E3 activity of Mdm2/MdmX combined with disruption of the interaction of Mdm2 and MdmX with p53 would probably have the greatest potential to treat wt-p53 expressing cancers.

6 References

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7 Zusammenfasung

Die RING-Finger Proteine Mdm2 und MdmX zeigen große strukturelle Ähnlichkeiten zueinander und sind hauptsächliche Antagonisten des Tumorsupressorproteins p53. Mdm2 gehört zu der Familie der RING-Finger E3 Ligasen und ist verantwortlich für die Ubiqutitinierung von p53, was zu dessen Abbau durch das 26S Proteasom führt. Im Gegensatz zu Mdm2 besitzt MdmX keine nennenswerte E3 Ligase Aktivität und kann demzufolge alleine nicht den Abbau von p53 induzieren. Allerdings konnte in unserer Gruppe kürzlich gezeigt werden, dass MdmX die E3 Ligase Aktivität von Mdm2 *in vitro* verstärkt. Um einen genaueren Einblick in den Mechanismus zu erlangen, durch den MdmX die E3 Ligase Aktivität von Mdm2 erhöht, wurden Mutationsanalysen der RING-Finger Domänen von Mdm2 und MdmX durchgeführt. Wir konnten einige Aminosäurereste innerhalb der RING-Finger Domäne von Mdm2 identifizieren, die für dessen E3 Ligase Aktivität essentiell sind. Die Mutationen einiger dieser Reste beeinträchtigt die Fähigkeit von Mdm2, mit sich selbst oder mit MdmX zu interagieren, was darauf hindeutet, dass die Oligomerisierung für die E3 Ligase Aktivität von Mdm2 notwendig ist. Die Mutationen anderer Aminosäurereste beeinträchtigen nicht die Interaktion von Mdm2 mit sich selbst oder mit MdmX. Allerdings ist die Fähigkeit dieser Mutanten beeinträchtigt, mit dem Ubiquitin-konjugierenden Enzym UbcH5b zu interagieren. Interessanterweise kann MdmX die Aktivität der letzteren Mdm2 Mutanten wieder herstellen, sowohl *in vitro* als auch in der lebenden Zelle, was darauf hindeutet, dass Mdm2/MdmX Hetero-Oligomere in der Zelle eine Funktion als E3 Ligase für p53 ausüben können. Außerdem zeigen wir, dass Mdm2/MdmX Hetero-Oligomere nicht nur als E3 Ubiquitin Ligase für p53 fungieren, sondern auch die Modifikation von p53 mit dem Ubiquitin-ähnlichen Molekül Nedd8 katalysieren. Darüber hinaus sind sämtliche Mdm2 Mutanten, die in ihrer Ubiquitin Ligase Aktivität beeinträchtigt sind auch in ihrer Nedd8 Ligase Aktivität beeinträchtigt, wovon einige Mutanten in der Anwesenheit von MdmX wieder aktiv sind. Dies lässt vermuten, dass die molekularen Mechanismen der Mdm2-abhängigen Konjugation von Ubiquitin und Nedd8 an p53 zueinander sehr ähnlich sind. Zusätzlich konnte gezeigt werden, dass Ubiquitin und Nedd8 an das gleiche p53 Molekül angeheftet werden können, was darauf hinweist, dass Ubiquitin und Nedd8 in der Zelle möglicherweise gemischte Ketten bilden. Schließlich konnten wir durch den Einsatz verschiedener Ubiquitin- und Nedd8 Mutanten Hinweise erhalten, dass Nedd8, wenn es an Ubiquitin angeheftet wird, als Ketten-Terminator dienen könnte.

8 Abstract

The RING finger proteins Mdm2 and MdmX share significant structural similarity with each other and are major antagonists of the tumor suppressor protein p53. Mdm2 is a member of the RING finger family of E3 ligases and targets p53 for ubiquitination and degradation by the 26S proteasome system. Unlike Mdm2, MdmX does not have appreciable E3 ligase activity and, hence, does not induce p53 degradation on its own. However, our group previously reported that MdmX enhances the E3 ligase activity of Mdm2 *in vitro*. To obtain insight into the mechanism by which MdmX enhances the E3 ligase activity of Mdm2, a mutational analysis of the RING finger domains of Mdm2 and MdmX was performed. We identified several residues within the RING finger domain of Mdm2 that are essential for its E3 ligase activity. Mutation of some of these residues impairs the ability of Mdm2 to interact with itself or with MdmX indicating that oligomerization is required for Mdm2 to function as an E3 ligase. Mutation of other residues does not detectably interfere with the ability of Mdm2 to interact with itself or with MdmX. However, the ability of these mutants to interact with the ubiquitin conjugating enzyme UbcH5b is impaired. Interestingly, MdmX rescues the E3 ligase activity of the latter Mdm2 mutants, both in vitro and within cells indicating that Mdm2/MdmX hetero-oligomers can function as an E3 ligase for p53 within cells. Furthermore, we present evidence that Mdm2/MdmX hetero-oligomeric complexes not only function as ubiquitin E3 ligases for p53 but also facilitate modification of p53 with the ubiquitin-like molecule Nedd8. Moreover, all the Mdm2 mutants impaired for their ubiquitin ligase activity are also impaired for their Nedd8 ligase activity and some of them are rescued in their activity by MdmX. This suggests that Mdm2 employs similar molecular mechanisms to transfer both ubiquitin and Nedd8 to p53. In addition, we show that ubiquitin and Nedd8 can be attached to the same p53 molecule indicating that ubiquitin and Nedd8 may form mixed chains within cells. Finally, using several mutants of ubiquitin and Nedd8, we present evidence that Nedd8, when attached to ubiquitin, may function as chain terminator in cells.

9 Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie-abgesehen von unten angegebenen Teilpublikationen -noch nicht veröffentlicht worden ist, sowie dass ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Mats Paulsson betreut worden.

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10 Lebenslauf

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