The p53–Mdm2 interaction and the E3 ligase activity of Mdm2/Mdm4 are conserved from lampreys to humans

Cynthia R. Coffill,1,9 Alison P. Lee,2,9 Jia Wei Siau,1 Sharon M. Chee,1 Thomas L. Joseph,3 Yaw Sing Tan,3 Arumugam Madhumalar,4 Boon-Hui Tay,2 Sydney Brenner,2,5 Chandra S. Verma,3,6,7 Farid J. Ghadessy,1 Byrappa Venkatesh,2,8 and David P. Lane1

1p53 Laboratory (p53Lab), Agency for Science, Technology, and Research (A*STAR), Singapore 138648; 2Institute of Molecular and Cellular Biology, A*STAR, Singapore 138673; 3Bioinformatics Institute, A*STAR, Singapore 138671; 4National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110067, India; 5Okinawa Institute of Science and Technology Graduate University, Onna-son, Okinawa 904-0495, Japan; 6School of Biological Sciences, Nanyang Technological University, Singapore 637551; 7Department of Biological Sciences, National University of Singapore, Singapore 117543; 8Department of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 119228

The extant jawless vertebrates, represented by lampreys and hagfish, are the oldest group of vertebrates and provide an interesting genomic evolutionary pivot point between invertebrates and jawed vertebrates. Through genome analysis of one of these jawless vertebrates, the Japanese lamprey (Lethenteron japonicum), we identified all three members of the important p53 transcription factor family—Tp53, Tp63, and Tp73—as well as the Mdm2 and Mdm4 genes. These genes and their products are significant cellular regulators in human cancer, and further examination of their roles in this most distant vertebrate relative sheds light on their origin and coevolution. Their important role in response to DNA damage has been highlighted by the discovery of multiple copies of the Tp53 gene in elephants. Expression of lamprey p53, Mdm2, and Mdm4 proteins in mammalian cells reveals that the p53–Mdm2 interaction and the Mdm2/Mdm4 E3 ligase activity existed in the common ancestor of vertebrates and have been conserved for >500 million years of vertebrate evolution. Lamprey Mdm2 degrades human p53 with great efficiency, but this interaction is not blocked by currently available small molecule inhibitors of the human HDM2 protein, suggesting utility of lamprey Mdm2 in the study of the human p53 signaling pathway.

[Keywords: p53; Mdm2; Mdm4; evolution; lamprey]

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More than 50% of all human cancers contain mutations in the p53 tumor suppressor protein [Petitjean et al. 2007]. From among the three family members p53, p63, and p73, the founding member p53 is known to be a highly inducible transcriptional regulator that can activate or repress the activity of a large number of genes, resulting in diverse cellular effects, including cell cycle arrest, apoptosis, DNA repair, or metabolism [Vousden and Lu 2002; Reinhardt and Schumacher 2012; Hock and Vousden 2014; Khoo et al. 2014; Meek 2015]. The p53 key negative regulator Mdm2, which has a potent ubiquitin E3 ligase activity, acts in a heteromeric complex with its partner, Mdm4, to target p53 for degradation. The p53 response is activated in part through increased protein levels and decreased degradation when the p53/Mdm2/4 complex is disrupted; for example, in response to oncogene activation, nuclear stress, and DNA damage [Vousden and Lu 2002; Reinhardt and Schumacher 2012; Hock and Vousden 2014; Khoo et al. 2014; Meek 2015]. The significant role of p53 in response to DNA damage is highlighted by the recent discovery of multiple functional copies of the Tp53 gene in elephants [Abegglen et al. 2015]. The Tp63 gene also shows a response to DNA damage, acting as a major regulator of the response in female germ cells [Suh et al. 2006; Gonfloni et al. 2009; Levine et al. 2011]. The Mdm component of the p53 pathway has been conserved in evolution [Lu et al. 2009], as it is found in all jawed vertebrates and some invertebrate species. Some of the invertebrate Mdm homologs identified can be found in urochordates (e.g., sea squirt, Ciona intestinalis),

9These authors contributed equally to this work.

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cephalochordates (Florida lancelet, Branchiostoma floridae), molluscs (owl limpet, Lottia gigantean); bay mussel, Mytilus trossulus), hemichordates (acorn worm, Saccoglossus kowalevskii), arthropoda (deer tick, Ixodes scapularis), and placozoa (Trichoplax adhaerens) (Momand et al. 2011). However, the Caenorhabditis elegans and Drosophila melanogaster genomes lack an Mdm gene, but, notably, their Tp53-like gene activity is still induced by DNA damage [Brodsky et al. 2004]. The p53 family shares a common ancestor, and most conservation is seen in the DNA-binding domain. Tp53/Tp63/Tp73-like genes have been found in all animal species but are absent in plants and yeasts [Lu et al. 2009]. In addition, multiple isoforms of the p53 family members are produced by use of alternate initiation codons and by alternate splicing. Understanding more about this vital human tumor suppressor pathway is of high importance, as recent studies of p53 mutations in mice have shown that some p53 mutants lack substantial transcriptional activity yet retain some tumor suppressor functions [Brady et al. 2011; Li et al. 2012]. Indeed, mice that lack what were thought to be vital p53 downstream response genes—p21, Puma, and Noxa—are as tumor-resistant as wild-type mice, yet p53-null mice show an extraordinary propensity to develop cancer, suggesting that other activities of the p53 system are required for tumor suppression [Valente et al. 2013]. Biochemical studies of the evolutionary conservation of function of p53 family members and their regulators may help to identify the most conserved and essential functions of these complex and highly regulated proteins.

The extant vertebrates are divided into two major groups—the jawed vertebrates (gnathostomes) and the jawless vertebrates (cyclostomes)—that diverged ~500 million years ago. While the jawed vertebrates include cartilaginous fish, ray-finned fish, lobe-finned fish, amphibians, reptiles, birds, and mammals, the cyclostomes are represented by only two lineages, the lampreys and hagfish, which constitute a monophyletic group. The p53 pathway members have been investigated extensively in representative jawed vertebrates, including a cartilaginous fish, the elephant shark [Lane et al. 2011], and thus it has been inferred that genes for all three Tp53 family members as well as Mdm2 and Mdm4 were present in the common ancestor of jawed vertebrates. However, it is unclear whether jawless vertebrate genomes code for all of the components of the jawed vertebrate p53 pathway and whether the p53 pathway proteins in jawless vertebrates are functionally equivalent to their counterparts in humans and other jawed vertebrates.

Jawless vertebrates are regarded as “primitive” vertebrates because of their relatively simple morphological and physiological traits compared with jawed vertebrates. For example, jawless vertebrates possess a single median dorsal nostril, in contrast to ventral nostrils in jawed vertebrates, and lack mineralized tissues, articulate jaws, paired appendages, the pancreas, and the spleen [Osorio and Retaux 2008; Shimeld and Donoghue 2012]. The adaptive immune system of jawless vertebrates comprises variable lymphocyte receptors (VLRs) assemled from leucine-rich repeat modules (LRRs), in contrast to T-cell and B-cell receptors of jawed vertebrates that are generated from the immunoglobulin superfamily [Kasahara and Sutoh 2014]. A unique feature of lampreys (and probably hagfish) is the programmed loss of ~20% of germline DNA from somatic cells during early stages of embryonic development such that only the germline cells contain 100% of the genome [Smith et al. 2009]. This programmed genome rearrangement has been proposed to serve as a biological strategy to restrict pluripotency functions to the germline [Smith et al. 2012].

To date, draft genome sequences of two lamprey species, the sea lamprey (Petromyzon marinus) (genome size 2.4 Gb) [Smith et al. 2013] and the Japanese lamprey (Lethenteron japonicum) (genome size 1.6 Gb) [Mehta et al. 2013], have been generated. While the sea lamprey genome sequence was generated using DNA from a somatic tissue, the Japanese lamprey genome was sequenced using DNA from the testis. In this study, we mined the genome assembly of the Japanese lamprey for members of the p53 pathway and obtained their complete coding sequences by a combination of RT–PCR and S’/3’ RACE. To determine the extent of conservation of p53 pathway function during the evolution of vertebrates, we examined the biochemical function of the lamprey p53 (Lj-p53) and Mdm2 (Lj-Mdm2) proteins and their interactions with human p53 pathway proteins (Hs-p53 and HDM2). Lj-Mdm2 was remarkably active in degrading Hs-p53 when tested in a cellular background derived from mice lacking endogenous p53 and Mdm2, indicating that the p53-specific E3 ligase activity of Mdm2 already existed in the common ancestor of vertebrates.

Results

Lj-p53 family members

We searched the Japanese lamprey genome assembly [Japanese Lamprey Genome Project, http://jlampreygenome.imcb.a-star.edu.sg] for members of the p53 family using human and elephant shark p53, p63 and p73 protein sequences as the query [see the Materials and Methods]. Our searches identified a complete gene on scaffold 216 with high similarity to Tp53. The protein encoded by this gene lacks a SAM domain, thereby providing further evidence that it is likely the p53 ortholog of lamprey. The Lj-p53 protein shows an overall identity of 38% with the Hs-p53 protein. However, the identity is higher in the DNA-binding domain [60%] and the oligomerization domain [39%] (Table 1; Supplemental Fig. S1). By performing 5’ RACE using different sets of primers, we were able to identify four isoforms (Lj-p53, Lj-A27p53, Lj-A30p53, and Lj-A108p53) of the lamprey Tp53 gene [Fig. 1; Supplemental Fig. S2]. The Lj-A27p53 and Lj-A30p53 isoforms are produced by the use of alternate methionine residues as initiation codons, creating N’-terminal deletions of 27 and 30 amino acids, respectively. Such ΔN isoforms are a common feature of the p53 family members found in jawed vertebrates. They are functionally highly significant, as their orthologs studied from other organisms have impaired transcription activation...
function and can therefore act as repressors rather than activators of gene expression (Marcel et al. 2011). They also lack the critical N'-terminal Mdm-binding peptide, making them more resistant to Mdm-mediated proteasomal degradation. This is likely the case for the lamprey ΔN' isoforms, as the Mdm-binding peptide occurs at amino acids F15–W19 of Lj-p53. The third isoform found in this analysis is more complex, as it is generated by alternate splicing to produce a novel N'-terminal segment of 11 amino acids that is then fused in-frame to the main p53 sequence at amino acid 108. This truncated protein can be considered similar in general architecture to ΔNp53 and ΔNp73, with a 33% similarity; however, its overall similarity for p73 for these two species was higher, at 57% (Table 1). Lj-p63 possesses an N'-terminal transactivation domain (TAD) with a 33% similarity; however, Lj-p73, which lacks this domain, is more similar to Hs-Np73. The DNA-binding domain identities of Lj-p63 and Lj-p73 were 78% and 89%, respectively, to their human counterparts and were found to be significantly higher than that between Hs-p53 and Lj-p53 (64%). The SAM domain in Lj-p53 and Lj-p73 were 78% and 89%, respectively, to their human counterparts and were found to be significantly higher than that between Hs-p53 and Lj-p53 (64%). The SAM domain in Lj-p53 and Lj-p73 were 78% and 89%, respectively, to their human counterparts and were found to be significantly higher than that between Hs-p53 and Lj-p53 (64%).

## Table 1. Amino acid similarity between human and lamprey protein sequences

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<tr>
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<th>p53</th>
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<td>44%</td>
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<td>Oligomerization</td>
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<td>Ring finger</td>
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<td>—</td>
<td>56</td>
<td>60</td>
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* The N'-terminal (TAD1) is absent in Lj-p73.
* The zinc finger and acidic domains in Lj-Mdm4 are significantly truncated.

(-) Absent domains.

confirmed by RT-PCR. The proteins encoded by both genes contain a SAM domain, indicating that they are related to Tp63 and Tp73 genes of jawed vertebrates. In order to verify their identity, we carried out phylogenetic analysis (maximum likelihood) of all three lamprey proteins as well as p53, p63, and p73 proteins from selected jawed vertebrates. However, the phylogenetic analysis was unable to establish the identity of the lamprey proteins, as they were found to form independent branches with low bootstrap supports between the gnathostome p53 and p63 clades (Fig. 2), presumably due to the GC bias of lamprey genes [see Mehta et al. 2013]. We then compared the synteny of genes at the lamprey gene loci with the synteny of genes at human and coelacanth Tp53, Tp63, and Tp73 gene loci (Supplemental Fig. S3). However, the synteny was also not informative, as the genes flanking the lamprey genes were not conserved in either human or coelacanth loci. We then used detailed protein alignments to designate the gene on scaffold_21 as Lj-Tp63 gene and the gene on scaffold_926 as Lj-Tp73 gene.

The three members of the Lj-Tp53 gene family exhibit very similar exon–intron organizations with highly conserved intron positions and phases (Supplemental Fig. S4), which supports the hypothesis that they are the result of duplications of an ancestral gene. The protein sequence alignment for Lj-p63 [Supplemental Fig. S5A,B] and Lj-p73 [Supplemental Fig. S6A,B] with human and other jawed vertebrate proteins revealed the overall amino acid similarity between Hs-p63 and Lj-p63 to be 44%, while the overall similarity for p73 for these two species was higher, at 57% (Table 1). Lj-p63 possesses an N'-terminal transactivation domain (TAD) with a 33% similarity; however, Lj-p73, which lacks this domain, is more similar to Hs-Np73. The DNA-binding domain identities of Lj-p63 and Lj-p73 were 78% and 89%, respectively, to their human counterparts and were found to be significantly higher than that between Hs-p53 and Lj-p53 (60%). The SAM domains were found to have 61% and 69% identities between human and Lj-p63 and Lj-p73, respectively. For each domain analyzed, Lj-p73 consistently displayed the closest similarity to the human protein, followed by Lj-p63 and then Lj-p53.
RACE analysis identified three isoforms of the Lj-Tp63 gene, whereas no isoforms were identified for the Lj-Tp73 gene (Supplemental Fig. S7). All three isoforms of Lj-p63 have the same start site, which is 39 amino acids after the initiation of the longest Hs-p63 isoform, Hs-TAp63α (Supplemental Fig. S8A–C). The lamprey genome therefore does not appear to have an isoform equivalent to the well-described and important Hs-ΔNp63α, which is missing the first 94 amino acids that constitute a functional TAD. However, both zebrafish p63 and frog p63 (Supplemental Fig. S5A) have a translation start site similar to that of the Hs-ΔNp63 isoforms. Lj-p63_A is 774 amino acids and Lj-p63_B is 761 amino acids, but both terminate eight amino acids following the final amino acid of Hs-TAp63α based on the alignment of the full proteins. Lj-p63_C is 650 amino acids long and terminates at amino acid 613 of Hs-TAp63α but is longer than Hs-TAp63β or Hs-TAp63γ, which are missing 125 and 193 amino acids, respectively, at the C′ terminus of Hs-TAp63α. Therefore, in comparison with one another, the Lj-p63 isoforms lack amino acids in the central regions of the protein rather than at the N′-terminal or C′-terminal ends as found in human and other vertebrates.

Lj-Mdm2 and Lj-Mdm4 genes

Searches of the Japanese lamprey genome assembly identified several exons of an Mdm2 gene on scaffold_29. The coding sequence of this gene was completed by RT–PCR and 5′ RACE. The Lj-Mdm2 gene encodes a protein with 603 amino acids and includes all of the key functional domains such as the p53-binding domain, acidic region, zinc finger, and C′-terminal RING domain. Overall, it is 32% identical to human HDM2, with the RING finger domain exhibiting a higher identity of 56% (Table 1; Supplemental Fig. S9A,B). Consistent with other C′-terminal RING domain-containing proteins [Dolezelova et al. 2012], there are exactly 13 amino acids following the final cysteine residue, and, as with HDM2 [Fang et al. 2000], there are seven critical cysteine residues that are conserved for ubiquitin ligation activity and p53 degradation (Supplemental Fig. S10).

The Japanese lamprey genome also contains an Mdm4 gene [spread over two short scaffolds, 2349 and 14666] that encodes a 280-amino-acid protein. The full-length coding sequence of the Lj-Mdm4 was confirmed by RT–PCR. Alignment of this sequence with Mdm4 from a number of jawed vertebrate species shows that ~200 amino acids that are present in the middle of jawed vertebrate proteins are missing in the Lj-Mdm4 in two regions (Supplemental Fig. S11A,B). In the human protein, an acidic domain is found from amino acid 215 to amino acid 255, and a zinc finger is located from amino acid 290 to amino acid 332, but these regions are either partially or completely absent in Lj-Mdm4 in addition to ~50 amino acids located before the acidic domain. A caspase-3 cleavage site and a portion downstream from the zinc finger are also missing in Lj-Mdm4. Overall, the amino acid sequence similarity between Lj-Mdm4 and human HDM4 is 31%, with the highest identity in the RING domain at 60% [Table 1]. As with Lj-Mdm2, there are exactly 13 amino acids following the final cysteine residue in the RING domain. However, in contrast to jawed vertebrate Mdm4 proteins, which have six cysteine residues, Lj-Mdm4 contains seven—the same number found in Mdm2 [Momand et al. 2011]. This additional cysteine residue corresponds to C449 of human HDM2 but is absent in HDM4 and has been found to be important for p53 degradation and ubiquitination [Fang et al. 2000].
The synteny of genes flanking Lj-Mdm2 and Lj-Mdm4 and their orthologs in human and coelacanth are compared in Supplemental Figure S12. As with lamprey Tp53/Tp63/Tp73 loci, none of the genes flanking Lj-Mdm2 and Lj-Mdm4 is conserved in human and coelacanth loci. Interestingly, of the invertebrate genomes sequenced thus far and of those containing an Mdm gene, only one gene has been identified per organism, and this typically encodes a protein with six cysteine residues in its RING domain [Momand et al. 2011]. How many cysteine residues were present in the Mdm gene of the common vertebrate ancestor, whether this gene is closer to jawed vertebrates Mdm2 or Mdm4 genes, and whether the Mdm proteins of invertebrates are functional in terms of p53 degradation are all questions worth exploring.

Conservation of the p53–Mdm2 interaction in lamprey

In jawed vertebrates, p53 protein levels are highly regulated, and the main negative regulator is Mdm2. In order to determine whether the p53 pathway function is conserved in lamprey, we first investigated the ability of Lj-Mdm2 to bind to Lj-p53 and Hs-p53. Detailed sequence analysis of the p53-binding domain within Lj-Mdm2 (Fig. 3A) and the N’-terminal Mdm2-interacting region of p53 (Fig. 3B) showed that Lj-p53 contains the key F19 and W23 motifs [human protein numbering], which figure prominently in the crystal structure of the complex between a peptide derived from the TAD of p53 and the N’-terminal region of Mdm2 [Kussie et al. 1996]. Residue 26 is leucine in Hs-p53 and is one of three key residues required for binding to HDM2 [Hs-p53 F19, W23, and L26] (Bottger et al. 1997). In Lj-p53, this leucine is replaced by glycine (Figs. 3B, 4B), which incurs a complete loss of side chain interactions within the p53-binding cleft of HDM2. In vitro translation of Lj-p53, Lj-Mdm2, Hs-p53, and HDM2 in an Escherichia coli-based system showed that Lj-p53 is bound by both Lj-Mdm2 and HDM2. Furthermore, Lj-Mdm2 displayed binding to both Lj-p53 and Hs-p53 [Fig. 3C]. This was clearly visible despite the lower level of expression of the lamprey proteins in this in vitro system. Molecular models show that, structurally, both p53 peptides can easily be accommodated by the binding site in the N’-terminal domain of HDM2 (Fig. 4A) and Lj-Mdm2 (Fig. 4B). However, fluorescent polarization (FP) analysis showed that an Lj-p53 peptide was unable to displace an Hs-p53-based peptide [carboxylfluorescein [FAM]-labeled p53-binding peptide 12.1 [FAM-12.1]] from the first 125 amino acids of HDM2 (HDM2Nterm) (Fig. 4C; Supplemental Tables S2, S3). The ability of the two proteins to interact may therefore depend more on other sites of interaction between Lj-Mdm2 and Lj-p53. In Hs-p53, the leucine at position 26 is also critical in the interaction with HDM2 [Kussie et al. 1996], so the corresponding glycine residue in Lj-p53 was mutated to generate the peptide Lj-p5312–25(G22L). By FP analysis, it was demonstrated that this peptide was able to displace FAM-12.1 from HDM2Nterm but with a lower affinity compared with wild-type Hs-p5316–29 [Supplemental Table S2].

In order to understand the mechanistic basis for this differential binding of the p53 peptides to HDM2Nterm, we carried out modeling studies using extensive molecular dynamics simulations. We investigated the propensities of the peptides to partition themselves from an aqueous environment to the hydrophobic environment on the surface of HDM2Nterm. Simulations of the complex state [peptide bound to HDM2Nterm] suggest that, unsurprisingly, residue number 26 (Leu in Hs-p53; Gly in Lj-p53) is critical in the interaction between Lj-Mdm2 and Lj-p53. In Hs-p53, the leucine affords the largest discrimination between the human and lamprey peptides’ interactions with HDM2Nterm [Supplemental Table S1; Supplemental Fig. S13]. However, the overall affinity as predicted by the modeling suggests that the Lj-p5312–25(G22L) peptide has a higher affinity for HDM2Nterm than does Hs-p5316–29 [Supplemental Table S1]. To understand this discordance with respect to the above experimental observations, we examined the
In addition, the residues T18 and S20 in Hs-p53 are replaced by D14 and D16, respectively in lampreys. In humans, kinase-modulated phosphorylation of T18 or S20 (or replacement by phosphomimetic Asp/Glu) leads to an attenuation of the p53–HDM2 interaction and a concomitant stabilization of p53 (Meek 1999; Schon et al. 2002). The underlying mechanism engages an acidic patch on the surface of HDM2 [Lee et al. 2007]. The residues corresponding to T18 and S20 of Lj-p53 are both aspartic acids and would further contribute to the loss of affinity for HDM2Nterm. Thus, it is clear that the differences in the conformational landscapes of the Hs-p53 and Lj-p53 peptides and the physicochemical character of the HDM2 surface together modulate the differential binding of these peptides to HDM2Nterm.

To further explore the affinity of Lj-p53 with HDM2, we generated a full-length Lj-p53(G22L) expression construct to test its ability to bind to full-length HDM2 in an in vitro system. Figure 4D shows that, as predicted, the mutation Lj-p53(G22L) enhanced the ability of Lj-p53 to bind to HDM2. Conversely, this mutation displayed reduced ability to bind Lj-Mdm2 [Supplemental Fig. S15A]. These studies reflect on the interesting topic of the coevolution of proteins that must interact with each other for regulated function.

Conservation of the DNA binding of Lj-p53

The DNA-binding domain of Lj-p53 shows 60% homology with Hs-p53 [Table 1], and Figure 5A shows the alignment of the p53 DNA-binding domain from seven vertebrate species. The sequence-specific DNA-binding function of Lj-p53 could be demonstrated in an in vitro DNA-binding assay using in vitro synthesized proteins. Lj-p53 was able to bind to an optimized p53 consensus DNA-binding element, “ConA” [Fig. 5B; Goh et al. 2010], in this immunoprecipitation- and PCR-based assay but was only able to bind weakly to the Hs-p21 and Hs-PUMA p53-binding sites, reflecting a DNA-binding specificity subtly different from that of the human protein. This is consistent with Lj-p53 retaining certain critical contact residues, including K120, S241, R248, A276, C277, and R280 [Hs-p53 protein numbering], deduced from the X-ray structure analysis of the Hs-p53 and DNA interaction sites [Fig. 5A; Cho et al. 1994]. Lj-p53 was not able to induce a transcription response in a cell-based assay using a reporter gene system comprising the ConA response element [data not shown]. The lack of transcriptional activity could be due to the inability of Lj-p53 to recruit appropriate cotranscription factors in a human cell line, especially given the low sequence homology between the N-terminal domains (Table 1; Supplemental Fig. S1). In addition, a comparison of the sequences of the tetramerization domains of Hsp53 [EYFTLQIRGRGFERMFRELNEALEKDAQAG] and Ljp53 [ELFLIPVRGREYELLHILKESLMKOLVPQ] [39% of residues are identical and shown in bold, and 58% of residues are similar in character and shown underlined] suggests that the packing of the tetramers in humans is
mediated by larger side chains, thus suggesting that the human tetramer is likely more stable and hence may contribute to more effective transcription.

Conservation of E3 ligase activity in Lj-Mdm2

An analysis of the functional properties of the Lj-Mdm2 and Lj-Mdm4 proteins exploited key features of the known properties of the human homolog. HDM2 acts to promote the degradation of p53 through its action as an E3 ligase. Mutational studies have determined that the addition of amino acids to the C' terminus but not the N' terminus of HDM2 inhibits its enzymatic function, as do point mutations in the RING finger domain (Dolezelova et al. 2012). Figure 6A shows an alignment of the RING finger domain and the C' terminal sequence of a selection of vertebrate Mdm2 proteins. All proteins show a high degree of conservation, with all seven cysteine residues completely conserved. Mutation of cysteine residue 464 to an alanine residue inhibits the human protein (Fang et al. 2000), so the equivalent point mutation was made in the lamprey protein. The lamprey and all other sequenced Mdm2s have exactly 13 amino acids C' terminal to the last cysteine residue in the RING finger. Addition of amino acids at this C' terminus also inactivates the HDM2 protein (Dolezelova et al. 2012), so an HA tag was added to Lj-Mdm2 at this position as well as at the N' terminus of the protein in an alternative construct. The N'-terminally or C'-terminally tagged Lj-Mdm2 proteins were then tested for function by transfecting them along with Lj-p53 into double-knockout cells, which were fibroblasts derived from mice in which the endogenous Trp53 and Mdm2 genes had been deleted (de Rozieres et al. 2000). The results are remarkably clear (Fig. 6B). The N'-terminally HA-tagged Lj-Mdm2 protein was able to reduce Lj-p53 levels (Fig. 6B, lane 3), and this was partially blocked (Fig. 6B, lane 4) by the proteasome inhibitor MG132. In contrast, the C'-terminally tagged Lj-Mdm2 protein was unable to reduce the levels of Lj-p53 Fig. 6B, (Fig. 6B, lane 5). These results demonstrate that Lj-p53 is a target for E3 ligases and that the Lj-Mdm2 protein was able to function effectively within a mammalian cell, indicating the functional conservation of the ubiquitin–proteasome pathway within vertebrates. This implies specifically that the E2 ubiquitin complex protein of mouse cells was able to interact with the Lj-Mdm2 protein. Further evidence of this conservation comes from the study of the C464A mutation [human protein numbering] in Lj-Mdm2 [Fig. 6B, lane 7]. This point mutation acts just as has been reported for the human and mouse proteins (Fang et al. 2000). It abolishes the E3 ligase activity of Lj-Mdm2 [Fig. 6B, cf. lanes 7 and 3].

Western blot analysis of the function of Lj-Mdm4 [Supplemental Fig. S15B] showed that expression levels of both Lj-p53 and Lj-Mdm2 were decreased in the presence of very low levels of Lj-Mdm4 [we were unable to detect expression of the myc-tagged Lj-Mdm4 construct in the cell
line used. The ability of Lj-Mdm4 to degrade Lj-p53 [in the absence of Lj-Mdm2] [Supplemental Fig. S15B, cf. lanes 9 and 1] can be noted. This function is possibly due to the extra cysteine in the RING domain of Lj-Mdm4 (see above), but further mutational studies are needed to confirm this.

The disruption of the p53 Mdm2/Mdm4 interaction can activate the p53 response in the absence of DNA damage and, as such, is being actively pursued as a drug target. Currently, two classes of molecules are in clinical trial (Khoo et al. 2014). The first class, represented by the small molecule “Nutlin,” consists of conventional low-molecular-weight drugs, while the second class consists of p53-binding peptides that have been stabilized by cross-linking agents, represented here by the stapled peptide “Staplin.” It was of great interest to test these molecules for their binding ability in the lamprey system. Again, using in vitro translated proteins, it could be shown that, as expected, both Nutlin and Staplin could block the binding of in vitro translated proteins, it could be shown that, as expected, both Nutlin and Staplin could block the binding of in vitro translated proteins, it could be shown that, as expected, both Nutlin and Staplin could block the binding of in vitro translated proteins, it could be shown that, as expected, both Nutlin and Staplin could block the binding of in vitro translated proteins.

Discussion

The evolutionary conservation of the p53 pathway is remarkable and poses an intellectual challenge, as organisms in which the gene is deleted or lost are completely viable and fertile (Lozano 2010). One attractive explanation of this apparent dilemma is the concept of robustness,
where p53, as a highly inducible transcription factor, acts to ensure the genetic stability required for the long-term competitive success of a species (Lane 1992). Cyclostomes, comprising jawless vertebrates such as lampreys and hagfish, are the sister group of the living jawed vertebrates (gnathostomes) and thus are an important group for studying vertebrate evolution. Here we show that homologs of all members of the p53 family as well as the negative regulators Mdm2 and Mdm4 are present in the Japanese lamprey. Functional studies showed that sequence conservation was reflected in both biochemical activity and the ability of Lj-Mdm2 protein to function as a very effective E3 ligase in mouse cells. The coevolution of proteins that interact with and regulate each other is of great interest, and here the p53–Mdm2 interaction is a very clear example. Detailed biochemical and molecular dynamics simulations act to reinforce our understanding of this key molecular interaction, and the study of evolutionary variants of interacting partners that are targets of clinically relevant drugs is of marked interest. Thus, our finding that Staplin but not Nutlin can block the interaction of Lj-p53 or Hs-p53 with Lj-Mdm2Nterm is helpful in the design of more potent and specific inhibitors of HDM2 and may provide a useful genetic tool for the study of p53 regulation. Further elucidation of Lj-Mdm2 function and its response to these and other types of inhibitors in cellular systems may help in the dissection of critical post-translational modifications that regulate the p53 pathway, offering insights into the basis of tumor suppression by the human TP53 gene, given its high mutation rate in cancer.

Materials and methods

Identification of Tp53 family members and Mdm2 and Mdm4 genes in the lamprey genome

The Japanese lamprey genome assembly at the Japanese Lamprey Genome Project [http://lampreygenome.imcb.a-star.edu.sg] was searched for members of the Tp53 family and Mdm2 and Mdm4 genes by TBlastN using human and elephant shark p53, p63, p73, Mdm2, and Mdm4 protein sequences as queries. The genomic regions showing similarity to human or elephant shark protein sequences were searched against the NCBI nonredundant protein database using Blastx to confirm the identity of the gene. Any exons missing in the genome assembly were identified by sequencing RT–PCR or 5′/3′ RACE products. 5′RACE-ready single-strand cDNA was used as a template for RT–PCR. Total RNA was extracted from the gills, kidney, muscle, and intestine using Trizol reagent (GIBCO-BRL) according to manufacturer’s protocol. Single-strand cDNA was prepared from total RNA using Trizol reagent (GIBCO-BRL) according to manufacturer’s protocol. Single-strand cDNA was prepared from total RNA using Trizol reagent (GIBCO-BRL) according to manufacturer’s protocol. Single-strand cDNA was prepared from total RNA using Trizol reagent (GIBCO-BRL) according to manufacturer’s protocol. Single-strand cDNA was prepared from total RNA using Trizol reagent (GIBCO-BRL) according to manufacturer’s protocol. Single-strand cDNA was prepared from total RNA using Trizol reagent (GIBCO-BRL) according to manufacturer’s protocol.
have been deposited in GenBank (Lj-p53, KT960978; Lj-p63, KT960979; Lj-p73, KT960980; Lj-Mdm2, KT960981; and Lj-Mdm4, KT960982).

Phylogenetic analysis of Lj-p53, Lj-p63, and Lj-p73 sequences

The full-length protein sequences were aligned using ClustalW (default parameters) in MEGA6. Gaps in the alignment were removed completely, leaving a total of 252 positions in the final alignment. A maximum likelihood (JTT+G substitution model) bootstrap consensus tree was inferred from 100 replicates. The consensus tree is shown in Figure 2. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. The accession numbers of protein sequences that were used in phylogenetic analysis are Chick_p53 P53_CHICK, Chick_p63 F1N8Z7_CHICK, Chick_p73 XP_417545.3, Ciona_p53a NP_001122370.1, Ciona_p53b NP_001077306.1, Eshark_p53 AEW49898.1, Eshark_p63 AEW49891.1, Eshark_p73 AEW49690.1, Frog_p53 F7A9U0_X-ENTR, Frog_p63 F6ZCN7_XENTR, Frog_p73 F6T710_XENTR, Human_p53 P53_HUMAN, Human_p63 P63_HUMAN, Human_p73 HUMAN, Lancelet_p53 XP_002598770.1, Lancelet_p63like XP_002613954.1, Mouse_p53 P53_MOUSE, Mouse_p63 M63_MOUSE, Mouse_p73 P73_MOUSE, Zebrfish_p53 P53_DANRE, Zebrfish_p63 A7YYJ7_DANRE, and Zebrfish_p73 B05S76_DANRE.

Plasmids

For details about plasmids, see Supplemental Table S4.

Cell culture, immunoprecipitation, and Western blot analysis

Mouse embryonic fibroblast p53/Mdm2 double-knockout cells (a kind gift from Guillemira Lozano) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% (v/v) foetal calf serum (FCS) and 1% (v/v) penicillin/streptomycin. To ensure a high transfection efficiency to reliably measure p53 protein levels, double-knockout cells were cotransfected with various combinations of Hs-p53, Lj-p53, Lj-Mdm2, Lj-Mdm4, and vector plasmids using Nucleofector II (Lonza) according to the manufacturer’s instructions for mouse embryonic fibroblasts with MEF 2 Nucleofector solution and program A-023. In all cases, the total amount of plasmid DNA transfected per well was equalized by addition of parental vector. Four hours prior to harvesting, cells were treated with proteasome inhibitor MG132 (Selleckchem). Double-knockout cells were harvested 24 h after transfection and lysed with NP40 buffer (50 mM Tris-HCl at pH 7.4–8.0, 150 mM NaCl, 1% NP-40) supplemented with protease inhibitors. Following protein concentration determination by BCA protein assay (Thermo Scientific Pierce), equal protein amounts of sample were added to SDS-PAGE loading buffer and incubated for 5 min at 95°C. The proteins were then separated by SDS-PAGE and transferred to nitrocellulose. Immunoblottting was carried out with the relevant antibodies.

Immunoprecipitation and Western blot analysis

Protein G beads (Invitrogen) were incubated with 1 µg of anti-HA per 10 µL of beads for 1 h in PBST–3% BSA and subsequently washed twice in PBST–0.1% BSA. In vitro translation-expressed HDM2 or Lj-Mdm2 was incubated with the beads on a rotator for 30 min. The proteins were then separated by SDS-PAGE and transferred to nitrocellulose. Immunoblottting was carried out with the relevant antibodies.

DNA-binding assay

Protein G beads were incubated with 1 µg of anti-HA antibody per 5 µL of beads for 1 h in PBST–3% BSA and subsequently washed three times in PBST–0.1% BSA. In vitro translated Hs-p53 or Lj-p53 was incubated with the beads on a rotator for 30 min. PUMA, p21, 2ConA DNA template, or negative control DNA (20 ng) was added to the mixture and incubated for 1 h. The negative control DNA was added as a measurement of nonspecific binding by p53. The beads were finally washed three times in PBST–0.1% BSA and three times with PBS, and bound DNA was eluted by resuspension in 20 µL of nuclease-free water and incubated for 5 min at 95°C. Real-time PCR quantification of the eluates was performed using primers petF3 and WpetR1 at 250 nM each with SsoAdvanced Universal SYBR Green super mix (Bio-Rad Laboratories) and quantified via a CFX96 real-time system (Bio-Rad Laboratories). Data were interpreted as fold differences (calculated based on cycle threshold differences) over nonspecific DNA-binding control (neutral DNA).

Fluorescence anisotropy

Fluorescence anisotropy titrations of purified wild-type HDM2Nterm (both 1–125 and 6–125) with 50 nM FAM-12.1 (FAM-RFMDYWEGL-NH2) were performed as previously described (Brown et al. 2013; Chee et al. 2014) to first determine the dissociation constants for the peptide–protein interaction. Subsequent determination of apparent Ks of Nutlin, human, and lamprey peptides was performed by competitive fluorescence anisotropy in which titrations of the peptides were carried out with a constant concentration of HDM2 and FAM-labeled peptide (Brown et al. 2013; Chee et al. 2014). Readings were carried out using the Envision multilabel reader (PerkinElmer). All experiments were carried out in PBS (2.7 mM KCl, 137 mM NaCl, 10 mM Na2HPO4, 2 mM KH2PO4 at pH 7.4), 3% DMSO, and 0.1% Tween-20 buffer. All titrations were carried out in replicates (n = 2–6). Curve fitting was carried out using Prism 4.0 (GraphPad).

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References


The p53–Mdm2 interaction and the E3 ligase activity of Mdm2/Mdm4 are conserved from lampreys to humans

Cynthia R. Coffill, Alison P. Lee, Jia Wei Siau, et al.

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