Published in final edited form as:
J Biol Chem. 2008 June 27; 283(26): 18012–18023. doi:10.1074/jbc.M709695200.

Elevated Levels of Oncogenic Protein Kinase Pim-1 Induce the p53 Pathway in Cultured Cells and Correlate with Increased Mdm2 in Mantle Cell Lymphoma*,S

Carol Hogan[‡], **Caroline Hutchison**[§], **Lynnette Marcar**[‡], **Diane Milne**[‡,¶], **Mark Saville**[‖], **John Goodlad**[§], **Neil Kernohan**[§], and **David Meek**[‡,1]

[‡]Biomedical Research Centre, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, United Kingdom

[§]Division of Pathology and Neuroscience, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, United Kingdom

[¶]Maternal and Child Health Sciences, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, United Kingdom

[‖]Division of Surgery and Oncology, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, United Kingdom

Abstract

Mutation of the *p53* gene is a common event during tumor pathogenesis. Other mechanisms, such as *mdm2* amplification, provide alternative routes through which dysfunction of the p53 pathway is promoted. Here, we address the hypothesis that elevated expression of *pim* oncogenes might suppress p53 by regulating Mdm2. At a physiological level, we show that endogenous Pim-1 and Pim-2 interact with endogenous Mdm2. Additionally, the Pim kinases phosphorylate Mdm2 *in vitro* and in cultured cells at Ser^{166} and Ser^{186}, two previously identified targets of other signaling pathways, including Akt. Surprisingly, at high levels of Pim expression, as would occur in tumors, active, but not inactive, Pim-1 or Pim-2 blocks the degradation of both p53 and Mdm2 in a manner that is independent of Mdm2 phosphorylation, leading to increased p53 levels and, proportionately, p53-dependent transactivation. Additionally, Pim-1 induces endogenous ARF, p53, Mdm2, and p21 in primary murine embryo fibroblasts and stimulates senescence-associated β-galactosidase levels, consistent with the induction of senescence. Immunohistochemical analysis of a cohort of 33 human mantle cell lymphomas shows that elevated expression of Pim-1 occurs in 42% of cases, with elevated Pim-2 occurring in 9% of cases, all of which also express Pim-1. Notably, elevated Pim-1 correlates with elevated Mdm2 in MCL with a *p* value of 0.003. Taken together, our data are consistent with the idea that Pim normally interacts with the p53 pathway but, when expressed at pathological levels, behaves as a classic dominant oncogene that stimulates a protective response through induction of the p53 pathway.

The p53 tumor suppressor is a potent transcription factor that promotes the arrest or elimination of hyperproliferative or genotoxically damaged cells (reviewed in Refs. 1 and

*This work was supported by Cancer Research UK, the Association for International Cancer Research, and the Biomedical Research Centre, Dundee.

S The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1-6.

[1]To whom correspondence should be addressed: Biomedical Research Centre, Ninewells Hospital, Level 5, Dundee DD1 9SY, United Kingdom. Fax: 44-1382-669993; E-mail: d.w.meek@dundee.ac.uk..

Europe PMC Funders Author Manuscripts

2). Under normal circumstances, Mdm2, an E3[2] ubiquitin ligase, mediates ubiquitylation and proteasome-dependent degradation of p53 (3). Cellular stresses that induce p53 target the p53-Mdm2 interaction with the effect of attenuating p53 degradation (4). *mdm2* is overexpressed or amplified in a range of human tumors that retain wild type p53 with the outcome that p53 levels are thought to be suppressed (3).

Mdm2 plays a pivotal role in integrating signals coming into the p53 pathway. For example, the Mdm2 inhibitor, ARF, is induced by hyperproliferative signals, leading to inhibition of p53 and Mdm2 degradation (5-9) and, consequently, the elimination of cells with tumorigenic potential (10). Mdm2 is also regulated by a series of phosphorylation and dephosphorylation events, mainly, but not exclusively, in response to DNA damage (reviewed in Ref. 11). Among these, serine residues 166 and 186 were identified as targets of the Akt protein kinase and shown to be modified in established cell lines in response to serum and to individual survival factors (12-18). Interaction with and phosphorylation by Akt was variously reported to lead to nuclear localization of Mdm2, increased ubiquitylation of p53, increased degradation of p53, decreased association with ARF, and increased interaction with p300 (12-18), events consistent with lowering p53 levels and increasing the threshold required to initiate arrest or apoptosis. Recently, however, this paradigm has been challenged in a mouse model for prostate cancer, where loss of PTEN, which promotes constitutive activation of Akt, leads to induction of the p53 pathway and, consequently, cellular senescence (19).

The Pim proteins (Pim-1, Pim-2, and Pim-3 (also known as KID-1)) are a family of short lived protein serine/threonine kinases that are expressed at low levels physiologically, mainly in hematopoietic cells, and are transiently induced in response to a host of cytokines (reviewed in Refs. 20 and 21). Mice lacking expression of all three Pim kinases develop normally but are smaller than their wild type counterparts and have impaired responses to certain hematopoietic growth factors (22). Pim and Akt kinases have overlapping but independent regulatory roles in hematopoietic cells (23) and share a growing number of common substrates. Among these, Mdm2 was shown to be phosphorylated by Pim-1 *in vitro* (24). However, neither the site(s) of phosphorylation nor the effects of Pim-1 on Mdm2 function were established.

From a pathological perspective, elevated expression of Pim kinases has been implicated in tumor development and particularly in lymphomagenesis (assessed in Refs. 21, 25, and 26). For example, the *Pim-1* and *Pim-2* genes were originally identified as common proviral integration sites in Moloney murine leukemia virus-induced lymphomas (25). Expression of *Pim-1* or *Pim-2*, driven by the immunoglobulin heavy chain enhancer, predisposes

[2]The abbreviations used are:

E3	ubiquitin-protein isopeptide ligase
ARF	alternative reading frame
GST	glutathione S-transferase
MEF	mouse embryo fibroblast
DKO	double knock-out
SA-β-galactosidase	senescence-associated β-galactosidase
RSK	ribosomal S6 kinase

transgenic mice to T cell lymphomas and cooperates potently with Myc in the development of B cell leukemias *in utero* in transgenic mice (27, 28). Pim kinases are significantly overexpressed in various human cancers, including prostate cancer (29), lymphoma (30), leukemia (31), multiple myeloma (32), pancreatic cancer (33), and colon cancer (34), suggesting a contributory role in human pathogenesis.

The percentage of human hematological malignancies showing p53 mutation is low (15% as compared with 50% in tumors of the esophagus, ovary, and colorectum (International Agency for Research on Cancer data base)), suggesting that mechanisms other than p53 mutation (such as ARF loss or *mdm2* amplification) are likely to be significant in promoting dysfunction of the p53 pathway in these tumors. Pim-1 can associate with Mdm2 (24), suggesting the possibility that elevated expression of oncogenes such as *pim* might contribute to suppressing the p53 response by regulating Mdm2 function. In order to explore this hypothesis, we investigated the ability of the Pim kinases to interact with Mdm2 *in vitro* and in cultured cells. Surprisingly, we find that rather than suppressing the p53 response, Pim behaves as a classic dominant oncogene that leads to the induction of ARF, p53, p21, and senescence-associated (SA)-β-galactosidase, markers consistent with the onset of cellular senescence. Additionally, we find a striking correlation between elevated levels of Pim-1 and Mdm2 in human mantle cell lymphoma and, consistent with this observation, find that Pim-1 and Mdm2 can stabilize each other in cultured cells. These data raise the possibility that deregulated expression of Pim may prompt a protective p53 response but may also contribute to deregulated Mdm2 expression.

EXPERIMENTAL PROCEDURES

Plasmids

Plasmids expressing GST-Mdm2 fusion proteins have been described elsewhere (35, 36). Mdm2 in pCHDM1B and His_6-tagged ubiquitin in pMT 107-ubiquitin were generously provided by Dr. Dimitris Xirodimas (University of Dundee). Luciferase reporter plasmids PG13-luc and SV-*Renilla* luciferase were generously provided by Dr. Jean-Christophe Bourdon (University of Dundee). A pcDNA3 derivative expressing CD20 was obtained from Dr. Mark Saville (University of Dundee). A pcDNA3 derivative expressing p14ARF was provided by Dr. Sonia Lain (University of Dundee).

Construction of Expression Plasmids for the Pim Kinases

Plasmids encoding full-length human Pim-1, Pim-2, and Pim-3 (Mammalian Gene Collection clones) were obtained from Geneservice Ltd. UK and were amplified by PCR and cloned into pGEX-4T-1 to allow high level expression of GST-tagged Pim-1, Pim-2, and Pim-3 kinases in *Escherichia coli* and subsequent rapid purification of glutathione-Sepharose beads. For mammalian cell expression, Pim-1 and Pim-2 cDNAs were cloned into pSG9M, a derivative of the pSG5 vector (Stratagene) containing a 5′ Myc 9E10 epitope tag.

Site-directed Mutagenesis

Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) following the protocol recommended by the manufacturer. The mutant plasmids were sequenced to verify the presence of the appropriate mutation(s) and to ensure the absence of any fortuitous undesired mutations.

Cell Culture

H1299 (p53-null lung carcinoma-derived), U2OS (wild type p53-positive osteocarcinoma-derived), and p53-null/Mdm2-null murine embryo fibroblasts (DKO-MEFs) were cultured

in a humidified 5% CO_2 atmosphere at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen). Nalm-6 cells (wild type p53-positive human pre-B cell line) were cultured in RPMI 1640 medium (Invitrogen). The Dulbecco's modified Eagle's medium and RPMI 1640 medium were supplemented with 10% (v/v) fetal bovine serum (Biosera), 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin (all from Invitrogen). Early passage MEFs from Pim-1,-2,-3 triple knock-out mice (22) and MEFs from wild type littermates were kindly provided by Dr. A. Berns (Netherlands Cancer Institute) and were grown in Dulbecco's modified Eagle's medium supplemented with 15% (v/v) fetal bovine serum (Biosera) and 2 mM L-glutamine. Primary mouse embryonic fibroblasts (MEFs), prepared from C57BL/6 mice by Dr. Larry Higgins (University of Dundee) were maintained in gelatin-coated flasks and cultured in Iscove's modified Dulbecco's medium supplemented with 10% (v/v) fetal bovine serum, 10 ng/ml human recombinant epidermal growth factor, and 1% insulin/transferrin/selenium (all from Invitrogen).

Transfection of Established Cell Lines

H1299 cells were transfected with plasmids using the calcium phosphate method (37). DKO-MEFs were transfected using FuGENE 6 transfection reagent (Roche Applied Science), according to the manufacturer's instructions.

Transfection of Primary MEFs and CD20 Enrichment

Primary MEFs were seeded at 8.5×10^5 cells/6-cm plate and transfected 24 h later using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Cells were transfected with 2.5 μg of plasmid expressing CD20 together with 5.5 μg of plasmid expressing Pim-1 kinase or the empty expression vector (pSG9M). Transfected cells were enriched for CD20 expression 48 h post-transfection using Magnetic Dynabeads panmouse IgG as instructed by the manufacturer (Invitrogen). Selected cells were washed twice with phosphate-buffered saline before lysis for Western blotting.

Immunoprecipitation

Cells were lysed at 4 °C in Igepal lysis buffer (10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 150 mM NaCl, 1% (v/v) Igepal) 50 mM NaF, 1 mM sodium orthovanadate, 1 mM benzamidine, and a protease inhibitor mixture (Calbiochem)) and incubated for 30 min on ice. Lysates were cleared by centrifugation at 12,000 × *g* at 4 °C for 20 min. Equal amounts of protein were added to 40 μl of Protein G-Sepharose beads (prepared as a 50% suspension in lysis buffer) and 1 μg of antibody and rocked at 4 °C for 4 h. The beads were then washed three times in lysis buffer and denatured in SDS-PAGE sample buffer.

Antibodies and Western Blot Analysis

SDS-PAGE and Western blotting was carried out using standard conditions. Nitrocellulose membranes were probed for the presence of p53 (antibody DO1 (Moravian Biotechnology) or CM-5 (38)), Mdm2 (SMP14 or D12 (Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)) or 4B2 (Moravian Biotechnology)), the Myc 9E10 epitope (9E10; Cancer Research UK), p21 (sc-397; Santa Cruz Biotechnology), Pim-1 (12H8; Santa Cruz Biotechnology), Pim-2 (1D12; Santa Cruz Biotechnology), ARF (polyclonal antibody from Dr S. Lain (University of Dundee)), or actin (20-33; Sigma). Western analysis using phosphospecific antibodies was carried out as described elsewhere (12). Two phosphospecific antibodies, previously described (12), were used to detect Mdm2 phosphorylated at serine 166 and 186, respectively. Secondary antibodies used were horseradish peroxidase-conjugated rabbit anti-mouse (DakoCytomation), goat anti-rabbit (DakoCytomation), or donkey anti-goat (sc-2020; Santa Cruz Biotechnology). Proteins were detected by enhanced chemiluminescence according to the manufacturer's instructions (Pierce).

GST Pull-down Experiments

GST-Mdm2 fusion proteins (35, 36) (or GST alone as control) were bound to glutathione-Sepharose beads and subsequently incubated with extracts of H1299 cells that had been transfected with 10 μg of Pim-1 expression vector. After extensively washing the beads, immobilized proteins were eluted in 2× SDS sample buffer and detected by Western blotting.

In Vivo Ubiquitylation Assay

The ubiquitylation assay relies on the tranfection of cells with a plasmid encoding His-tagged ubiquitin followed by the capture and purification of ubiquitylated proteins from cell extracts using nickel-agarose beads. This assay has been described in significant detail elsewhere (39). The purified ubiquitylated proteins were analyzed by Western blotting.

Luciferase Reporter Assays

H1299 and p53/Mdm2-double knock-out (DKO) MEF cells (3×10^4/well) were seeded onto a 24-well plate and transfected in triplicate using Fugene. Luciferase reporter plasmids PG13-luc (50 ng (40)) and SV-*Renilla* luciferase (0.5 ng) were transfected along with plasmids expressing p53 (1 ng (41)), Mdm2 (20 ng (42)), Pim-1 kinase (50-200 ng), and empty expression vector (pSG9M or pcDNA3), as appropriate. Transfected cells were harvested 24 h post-transfection by adding 100 μl of passive lysis buffer (Promega) per well. 20 μl of protein extract was analyzed in a luminometer using the Dual Luciferase reporter assay kit (Promega). Variations in transfection efficiencies were corrected by determining the *Renilla* luciferase activity of the sample.

In Vitro Protein Kinase Assays

Mdm2 kinase assays were carried out using GST-Mdm2 fusion proteins (35, 36) immobilized on glutathione-Sepharose beads or purified recombinant Mdm2 as substrate. Kinase assays were carried out in 20 μl of kinase buffer (10 mM $MgCl_2$ 50 mM Tris/HCl, pH 7.5) containing 20 μM [γ-^{32}P]ATP (specific activity 12 Ci/mmol), and either recombinant Pim-1 (30 ng), Pim-2 (30 ng), or Pim-3 (30 ng). Reactions were incubated at 30 °C for 30 min (2 h for kinase reactions for *in vitro* ubiquitination assays) and then terminated in 2× SDS sample buffer. Phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography.

Immunohistochemistry

Standard immunohistochemistry was carried out using the ChemMate system (DakoCytomation) according to the manufacturer's instructions. After blocking, tissue sections were exposed to the primary antibody (Pim-1, goat polyclonal C-20; Pim-2, mouse monoclonal 1D12; Mdm2, mouse monoclonal antibody 2A9) for either1hor over-night in a humidified chamber at 4 °C. Immunohistochemical analysis was conducted using a biotin-streptavidin immunoperoxidase method (ABC Elite; Vector Laboratories) and diaminobenzidine in 0.03% hydrogen peroxide as a chromogen agent (Dako) with the Autostainer and the peroxidase/diaminobenzidine ChemMate detection kit with the TechMate system. Copper sulfate was applied to intensify the signal. Following staining, the slides were dehydrated through ascending grades of alcohol before being cleared in Histoclear and finally mounted, using an automated mounter (Leica CV 5000) with DPX mountant. Negative controls were prepared by replacing the primary antibody with antibody diluent alone (biotin) and buffer for Autostainer (antibody diluent for TechMate). Cell blocks containing H1299 cells transfected with plasmids encoding either Pim-1 or Pim-2 were used as positive controls for staining, and a mock-transfected pellet acted as a further negative control (see below).

To ensure that the specificity of antibodies against Pim-1 and Pim-2 was retained for immunohistochemical staining of sections of formalin-fixed paraffin-embedded archival clinical material, H1299 cells were transfected with plasmids expressing either human Pim-1 or Pim-2 (as a Myc (9E10) fusion protein) or empty vector. The cells were then harvested, and, following a brief wash in phosphate-buffered saline, they were resuspended in human plasma before the addition of bovine thrombin to form a fibrin clot. The clot was then fixed in neutral buffered formalin for 24-48 h and then processed to form a paraffin wax block in the same way as diagnostic tissue samples. Sections from these cell pellets were then stained to detect Pim-1, Pim-2, and Myc with a standard immunohistochemical technique following microwave antigen retrieval in citric acid buffer, pH 6.0.

RESULTS

Pim Kinases Phosphorylate Mdm2 at Serine Residues 166 and 186

To determine whether Mdm2 is a substrate for members of the Pim kinase family, purified recombinant Mdm2 or a series of glutathione *S*-transferase fusion proteins comprising overlapping regions of Mdm2 were incubated in the presence of [γ-^{32}P]ATP together with recombinant Pim-1, Pim-2, or Pim-3. The GST-Mdm2 fusion proteins (see *schematic* in Fig. 3) are designated as follows with Mdm2 amino acid numbers given in parentheses: MP1-(1-110), MP2-(108-207), MP3-(203-282), and MP4-(279-491) (35, 36). Pim-1 phosphorylated full-length Mdm2 and the MP2 miniprotein but did not phosphorylate GST alone or any of the other GST-Mdm2 fusion proteins (Fig. 1*A*). These data indicate that the phosphorylation site(s) is located within amino acids 110-207 of Mdm2.

Pim-1 preferentially phosphorylates serine or threonine residues preceded by a cluster of arginine and/or lysine residues ((K/R)(K/R)R(K/R)L(S/T)*X*) (43). There are two potential Pim-1 phosphorylation sites within the region encompassing amino acids 110-207 of Mdm2: serines 166 and 186, respectively. To determine whether either of these residues was phosphorylated by Pim-1 *in vitro*, Pim-1 kinase assays were performed using GST-Mdm2 fusion proteins in which serine to alanine amino acid substitutions had been introduced at positions 166 and 186. The data indicate that the S186A mutant was only a very poor substrate for Pim-1 (Fig. 1*B*). Substitution of Ser^{166} by alanine appeared to have little effect on phosphorylation of Mdm2 by Pim-1, but a S166A/S186A double mutant showed no detectable phosphorylation by Pim-1, indicating that some phosphorylation of Ser^{166} was occurring. Alanine substitution of nearby serine residues 157 or 188, which are targets of other protein kinases (such as MAPKAPK-2 and Akt, respectively (12, 15, 18)) did not alter the ability of Pim-1 to phosphorylate Mdm2, underscoring the specificity of the reaction. Similar data were obtained when GST-full-length wild type and -phosphorylation site mutant Mdm2 proteins were used as substrates (data not shown). Two-dimensional tryptic phosphopeptide mapping of the full-length proteins revealed a number of phosphopeptides consistent with phosphorylation of serines 166 and 186, respectively, based on our previous identification of these phosphopeptides (12) (Fig. 1*C*). Phosphorylation of Ser^{166} and Ser^{186} *in vitro* could also be detected using previously reported anti-Ser(P)^{166}- and anti-Ser(P)^{186} phosphospecific antibodies (Fig. 1*D*) (12).

Further *in vitro* analyses were carried out in which Pim-1 was substituted with Pim-2 or Pim-3. Both of these protein kinases were also found to phosphorylate Mdm2 at serines 166 and 186 (data not shown). Interestingly, these phosphorylation sites have been identified as targets of several other signaling pathways, including those mediated by Akt, MK2, DAP kinase, pp90^{RSK}, and MSK-1 (12-18, 35, 44, 45) (see also supplemental Fig. 1), raising the strong possibility that these enzymes may regulate Mdm2 by convergent signaling routes (Fig. 1*E*).

Pim-1 Interacts with and Phosphorylates Mdm2 in Cultured Cells

To determine whether Mdm2 and Pim-1 could interact in cultured cells, H1299 cells (a human lung carcinoma-derived p53-null cell line) were transfected with plasmids expressing Mdm2 together with wild type active Pim-1 or an inactive mutant of Pim-1 (K67M; supplemental Fig. 2). Immunoprecipitation of Mdm2 followed by Western analysis indicated that significant levels of Pim-1 associated with Mdm2 irrespective of whether the protein kinase was active or inactive (Fig. 2*A*). In a reciprocal analysis, Mdm2 was found to be present in Pim-1 immunoprecipitates (Fig. 2*A*). Similar data were obtained when Pim-2 was co-expressed with Mdm2 (data not shown). (Association with Pim-3 was not examined due to the lack of a Pim-3-specific antibody.) Using the anti-Ser(P)^{166} and anti-Ser(P)^{186} phosphospecific antibodies, immunoprecipitated Mdm2 was observed to be phosphorylated at Ser^{166} (possibly by endogenous Akt (12-18)), but not detectably at Ser^{186}, inthe absence of co-transfected Pim-1 (Fig. 2*B*). When active Pim-1 was co-expressed with the Mdm2, phosphorylation of Ser^{186} was clearly evident and was accompanied by a stimulation of Ser^{166} phosphorylation. The inactive mutant of Pim-1 was not able to promote significant phosphorylation of these residues. These data indicate that Pim-1 can phosphorylate Mdm2 in a cultured cell background. Once again, similar data were obtained when Mdm2 was co-expressed with Pim-2 (data not shown).

To determine whether endogenous Mdm2 and Pim proteins could interact, co-immunoprecipitation experiments were conducted using extracts of various cell lines. Mdm2 was observed to co-immunoprecipitate with Pim-1 from U2OS cells (Fig. 2*C*) and LnCAP cells (data not shown). Notably, the Mdm2 present in the immunoprecipitate had a slightly slower mobility, consistent with the possibility that this was a phosphorylated form of the molecule. In reciprocal immunoprecipitations, Pim-1 was observed to be present in the Mdm2 immunoprecipitates (Fig. 2*C*). It was also possible to detect co-immunoprecipitation of Mdm2 with Pim-2 in NALM-6 cell extracts (Fig. 2*D*); once again, the co-immunoprecipitating Mdm2 had a slightly slower mobility. When NALM-6 cells were treated with the phosphatidylinositol 3-kinase inhibitor, LY294002, Ser^{166} phosphorylation was significantly reduced, but there was little change in the degree of Ser^{186} phosphorylation (Fig. 2*E*). Although these data indicate that Ser^{186} can be phosphorylated independently of the Akt protein kinase, we cannot attribute this directly to Pim-2 in the current absence of a specific Pim inhibitor.

Pim-1 Requires at Least Two Distinct Elements within Amino Acids 1-207 of Mdm2 for Extended Interaction with Mdm2

To explore further the interaction between Mdm2 and Pim-1, GST pull-down experiments were carried out in which the ability of a series of GST-Mdm2 fusion proteins to capture Pim-1 expressed in mammalian cell lysates was measured. The data indicate that although Pim-1 can effectively phosphorylate the MP2 miniprotein (GST linked to amino acids 108-207), it only associates with this protein very weakly (or transiently) in the pull-downs (Fig. 3; see the long exposure). Similarly, there is a weak interaction with MP4 but no detectable association with MP1 or MP3. Interestingly, however, a GST fusion protein encompassing Mdm2 amino acids 1-207 (MP10) bound Pim-1 even more tightly than the GST-full-length Mdm2, strongly suggesting that two distinct elements, one from within amino acids 1-110 and the other within amino acids 108-207, cooperate in mediating interaction with Pim-1. Significantly, since MP9 (which encompasses Mdm2 amino acids 1-178) shows only extremely weak binding, one of the binding elements is likely to involve the Pim-1 consensus sequence (amino acids 181-186).

Pim-1 Increases Mdm2 Levels and p53 Ubiquitylation but Does Not Promote p53 Turnover

Phosphorylation of serines 166 and/or 186 in Mdm2 (in the context of Akt-mediated signaling) has been suggested by several groups to mediate a reduction in p53 levels and an increase in the threshold required to initiate arrest or apoptosis (13, 14, 16). To determine whether Pim-1-mediated phosphorylation of Mdm2 function can influence the p53 response, H1299 cells were transfected with plasmids encoding wild type p53, Mdm2, and increasing amounts of Pim-1 or Pim-2 (under conditions that gave p53 levels closely matching endogenous p53 levels in U2OS cells. We have used this assay previously to show that TAFII250, an activator of Mdm2, can stimulate Mdm2-mediated ubiquitylation and turnover of p53 (see Ref. 42) (Fig. 3). Surprisingly, although Mdm2 alone was able to reduce the levels of p53, increasing concentrations of either Pim-1 or Pim-2 did not significantly suppress p53 levels any further (Fig. 4*A*). In contrast, as the Pim levels increased, the levels of p53 actually increased slightly over and above that seen with Mdm2 alone. Notably, both Pim-1 and Pim-2 stimulated the levels of Mdm2 with a corresponding increase in the presence of higher molecular weight bands in the p53 blot, presumed to be ubiquitylated p53. To confirm this modification and to determine whether phosphorylation had altered the intrinsic ability of Mdm2 to mediate transfer of ubiquitin to p53, the ability of Pim to influence Mdm2-dependent ubiquitylation of p53 in cultured cells and *in vitro* was examined directly. Wild type Pim-1, but not an inactive mutant, stimulated the ability of Mdm2 to ubiquitylate p53 in H1299 cells (Fig. 4*B*). Notably, however, this was accompanied by a proportionate increase in Mdm2 levels. Interestingly, stimulation of p53 ubiquitylation by an S166A/S186A double mutant of Mdm2, which cannot be phosphorylated by Pim, was equally affected by the presence of the wild type Pim. These data suggested that the increased ubiquitylation resulted from the increased Mdm2 levels but ruled out, however, the possibility that this was mediated by the phosphorylation of Mdm2 *per se*. Consistent with this idea, direct phosphorylation of Mdm2 by Pim-1 did not affect its ability to ubiquitylate p53 *in vitro* (Fig. 4*C*). Very similar data were obtained when Pim-2 was used in place of Pim-1 (data not shown).

Additionally, it was noted that, in the absence of ectopically expressed Mdm2, Pim-1 could cause a slight increase in the levels of p53 (compare *lanes 1* and *2* in Fig. 4*B*). To determine whether this might have been influenced by the low level of endogenous Mdm2 present in H1299 cells, the levels of p53 were measured in the presence and absence of Pim-1 in p53-null/Mdm2-null MEFs (double knock-out or DKO cells). Pim-1 did not significantly affect p53 levels in this background (Fig. 4*D*), consistent with the idea that the increased p53 in the H1299 was related to the presence of endogenous Mdm2. When increasing amounts of wild type, but not inactive mutant, Pim-1 were expressed in the H1299 cells, there was a corresponding 2-fold increase in p53 transcriptional activity (Fig. 4*E, i* and *ii*, respectively). Notably, the increase was p53-dependent (Fig. 4*E, iii*), and no significant increase was seen when the measurement was carried out in the DKO cells (lacking endogenous Mdm2; Fig. 4*E, iv*). Taken together, these data suggest that elevated expression of Pim-1 or Pim-2 blocks the degradation of Mdm2 and p53. The elevated p53, although containing a proportionately larger amount of ubiquitylated p53, is transcriptionally active. The increase in ubiquitylated p53 observed could reflect an accumulation of a proportion of molecules that are marked simply for degradation. Alternatively, other mechanisms could be at play (*e.g.* the tagging of transcriptionally active molecules for subsequent degradation as suggested by Collins and Tansey (46)). The appearance of ubiquitylated species of p53 is a natural occurrence following induction; for example, phosphorylation of key sites in Mdm2 leads to an inhibition of p53 degradation but does not affect p53 ubiquitylation (47).

Mdm2 and Pim-1 Stabilize Each Other in Cultured Cells

The observation that increased Mdm2 levels occurred in a manner proportionate to the increase in Pim levels suggested that Pim might affect Mdm2 stability. To address this issue, H1299 cells were transfected with plasmids expressing wild type or phosphorylation site mutant (S166A/S186A) Mdm2 together with plasmids expressing active or inactive Pim-1. The turnover of the expressed proteins was then measured by cycloheximide chase. The data (Fig. 5) indicate that active Pim-1 can increase the stability of both wild type Mdm2 (Fig. 5, *A* and *B*) and the S166A/S186A mutant (Fig. 5, *C* and *D*). The Pim-1-KD mutant retained a weak ability to increase the Mdm2 half-life; (also evident in Fig. 4*B*, *Mdm2 panel*). Given that the K67M mutant is completely inactive (supplemental Fig. 2), this is not due to any residual protein kinase activity and may reflect a degree of protection provided by the protein-protein interaction (Fig. 2*A*). (Of additional interest, Pim-1 is stabilized in the presence of both the wild type and S166A/S186A mutant of Mdm2 (Fig. 4, *E* and *F*), but the mechanism of this effect is not clear at present.) In summary, therefore, the data suggest that elevated Pim kinase activity leads to Mdm2 stabilization in a manner that does not require its ability to directly phosphorylate Mdm2.

Phosphorylation by Pim Increases the Ability of Mdm2 to Interact with Its Inhibitor, ARF

One possible explanation for the observation that Pim-1 prevents degradation of p53 and Mdm2 is that Pim-1, which has established oncogenicity, may behave in the manner of many deregulated oncogene products by inducing the p53 pathway through a mechanism(s) involving the ARF tumor suppressor. ARF is a potent inhibitor of Mdm2 with a complex mode of action that can block the degradation of both p53 and Mdm2 (3, 7, 48). To address the possibility of ARF involvement, the influence of Pim-1 on the Mdm2-ARF interaction was examined. H1299 cells were transfected with plasmids expressing wild type or S166A/S186A Mdm2, wild type or inactive Pim-1, and ARF. Co-immunoprecipitation experiments (Fig. 6*A*) revealed that ARF associated with both the wild type Mdm2 and the S166A/S186A mutant in the presence or absence of active or inactive Pim-1. Interestingly, however, there was a notable stimulation of this interaction only when wild type Mdm2 was co-expressed with active Pim-1. These data suggest that Pim-1 can up-regulate Mdm2-ARF association in a manner that requires phosphorylation of Mdm2 by Pim-1. To explore this interaction further, the effect of Pim-1 on Mdm2-ARF association was examined *in vitro*. U2OS cells (which do not express ARF) were transfected with plasmids expressing Mdm2 in the presence or absence of active Pim-1, and lysates were prepared from these cells 36 h post-transfection. In separate plates, U2OS cells were transfected with a plasmid expressing ARF, and, as before, lysates were made 36 h post-transfection. The lysates containing the ARF were then mixed with equal volumes of the lysates from the Mdm2 alone or Mdm2 plus Pim-1 transfections. Mdm2 was then immunoprecipitated, and the amount of co-immunoprecipitating ARF was determined by Western blotting. The data (Fig. 6*B*) indicate that Mdm2 that had been co-expressed with Pim-1 was able to bind significantly more ARF than the Mdm2 that had not been co-expressed with the kinase. These data support the idea that phosphorylation by Pim-1 stimulates the ability of Mdm2 to interact with ARF. Nevertheless, this is a minor effect, and, although increased association with available ARF may contribute subtly to blocking p53/Mdm2 degradation, the finding that Mdm2 stabilization can occur independently of its phosphorylation by Pim-1 strongly suggests that an additional and more powerful mechanism is at play.

Oncogene expression can also lead to increased nucleolar localization of ARF as part of its mechanism of action. To determine whether Pim-1 stimulates the migration of ARF to the nucleolus, H1299 cells were transfected with plasmids expressing wild type Pim-1, the inactive mutant Pim-1, or empty vector as control and examined by immunofluorescence microscopy. This approach, however, was inconclusive, since (*a*) a high proportion of the

cells (>60%) already displayed a significant level of ARF in the nucleolus, and (*b*) significant levels of nucleolar ARF could not be observed in a small proportion (5-10%) of Pim-1-expressing cells (supplemental Fig. 3).

Pim-1 Induces ARF and the p53 Pathway and Stimulates Senescence-associated β-Galactosidase Levels in Primary Murine Embryo Fibroblasts

If ARF (which is expressed in the H1299 cells) plays a key role in the elevation of p53 and Mdm2 levels by Pim, expression of Pim in cells lacking ARF should not alter the levels of p53 and Mdm2. To test this idea, Pim-1 was expressed stably in clones of U2OS cells (which do not express ARF). A typical example of such a Pim-1-expressing clone in comparison with cells in which only the vector is present is shown in Fig. 7*A*. The data in this figure indicate that elevated Pim-1 had no effect on the (endogenous) levels of p53 or Mdm2.

Most, if not all, established cell lines have lost the ability to induce p53 through the ARF pathway. Thus, demonstrating endogenous p53-mediated events in primary cells or animal models can provide a critical route toward validating hypotheses. In order to determine whether Pim-1 could indeed induce the endogenous p53 pathway, primary MEFs were transfected with a plasmid expressing wild type Pim-1 or, as control, the empty vector. Due to the low transfection efficiency obtained with primary cells, the cells were co-transfected with a plasmid expressing the CD20 surface marker and were enriched, 48 h after transfection, by capture on magnetic beads coated with anti-CD20 antibody. Analysis of the cell extracts showed that ARF and p53 were elevated in the cells expressing the Pim-1 protein kinase (Fig. 7*B*). Consistent with induction of ARF and p53, elevated levels of p21 and Mdm2 were also observed. A typical response of these cells to the DNA damage-inducing agent, cis-platin, is also shown for comparison. In this latter case, there were no detectable changes in the levels of ARF.

Oncogene-induced activation of the p53 pathway in fibroblasts can lead to the induction of cellular senescence. To determine whether overexpression of Pim-1 in primary MEFs induced characteristics consistent with the onset of senescence, the cells were stained, 72 h post-transfection, for SA-β-galactosidase. Although there was a detectable background of cells expressing SA-β-galactosidase, wild type, but not the K67M mutant, Pim-1 gave rise to a small but significant elevation in the level of this senescence marker (Fig. 7*C*). As a positive control, wild type p53 expression showed a similar but slightly greater increase in the number of SA-β-galactosidase-staining cells. (Typical micrographs of the staining are shown in Fig. 7*D*.) These results are consistent with the idea that Pim-1 can behave as a dominant oncogene that is capable of activating the p53 pathway, leading to oncogene-induced senescence. Although our data strongly suggest that this is likely to occur through the induction of ARF, we cannot rule out the possibility that other mechanisms may contribute, especially given that protein kinases, such as Pim, have a great many different substrates.

Pim Kinases Are Expressed in Mantle Cell Lymphoma and Correlate with Elevated Levels of Mdm2

Based on the results of the biochemical studies described above, Pim kinases can interact with Mdm2 and modify its level of expression and activity. In order to determine whether there was evidence that this interaction might also occur *in vivo* in human tumor tissue, a relevant clinical tumor model was sought. Gene expression studies have indicated that the levels of Pim-1 mRNA are elevated in mantle cell lymphoma (49, 50), a form of small B cell non-Hodgkin's lymphoma characterized by specific morphological appearances, immunohistochemical profile, and karyotypic abnormalities (51). A series of 33 cases of

mantle cell lymphoma was therefore identified for immunohistochemical analysis. To ensure that the specificity of the Pim-1 and Pim-2 antibodies was retained for immunohistochemical analysis of sections of formalin-fixed paraffin-embedded archival clinical material, H1299 cells transiently expressing Myc(9E10)-tagged human Pim-1 or Pim-2 were resuspended in a fibrin clot and stained under appropriate conditions. The results demonstrated that the commercial antibodies could detect Pim-1 and Pim-2 in such material and that antibody specificity had been preserved (supplemental Fig. 4).

To investigate the relationship between Pim and Mdm2 in mantle cell lymphoma, the tissue sections were examined by immunohistochemistry. The results (Table 1) indicate that in 42% of cases (14 cases), expression of Pim-1 was detectable in the tumor cells, with three cases additionally showing positive staining for Pim-2; no case was positive for Pim-2 alone. (Typical micrographs of the staining are shown in Fig. 8.) Staining was cytoplasmic, finely granular with a suggestion of accentuation around the nuclear membrane. Notably, Mdm2 staining was significantly stronger ($p = 0.003$) in those cases in which Pim kinase expression was detected by immunohistochemistry (Fig. 8 and Table 1). This observation mirrors our findings showing increased Mdm2 stability in cultured cells upon Pim-1 overexpression (Fig. 5) and implies that Pim-1 expression under pathological conditions may result in elevated levels of Mdm2.

DISCUSSION

The initial purpose of the present study was to question whether Pim-mediated phosphorylation of Mdm2 might be a mechanism by which the p53 pathway could be suppressed in a manner that does not select for mutation of the *p53* gene. This potential mechanism was suggested by the finding that the Akt signaling pathway, which has significant overlap with Pim signaling (23), can phosphorylate Mdm2 in cultured cells and stimulate its ability to degrade p53 (12-18). The current study revealed that each of the Pim kinases phosphorylates two residues in Mdm2, serines 166 and 186, both *in vitro* and in cultured cells (Figs. 1 and 2). Significantly, these resides had previously been identified by a number of groups as targets of several signaling pathways including, principally, the Akt pathway (12-18, 35, 44, 45). Notably, inhibition of the phosphatidylinositol 3-kinase/Akt pathway by the inhibitor, LY294002, failed to inhibit Ser^{186} phosphorylation, consistent with phosphorylation of this residue by a protein kinase(s) that is independent of the phosphatidylinositol 3-kinase pathway. (LY294002 can also directly inhibit Pim-1, but we find that high levels (greater than 50 μ_M) are needed for significant inhibition (data not shown).) Regrettably, at the time of writing, there are no specific inhibitors of the Pim kinases available with which to pharmacologically assess endogenous Pim-mediated phosphorylation of Mdm2. Nevertheless, our demonstration that Mdm2 can co-immunoprecipitate with Pim-1 or Pim-2 (depending on the availability of either kinase in a given cell line) favors the idea that there is a physiological interaction between these proteins. This interaction is likely to involve the basic residues immediately N-terminal to the Ser^{186} phosphorylation site together with an additional contact(s) within the first 110 amino acids (Fig. 3). Alternatively, it is possible that the N-terminal domain can disengage an inhibitory region within amino acids 108-207 that masks the availability of the Pim recognition determinants. Our identification of the Ser^{166} and Ser^{186} phosphorylation sites also supports the idea that Mdm2 may be regulated by convergent signaling at these residues by various pathways, including those mediated by Akt, MAPKAP kinase-2, DAP kinase, Pim, and possibly $pp90^{RSK}$ and MSK-1 (12-18, 35, 44, 45) (Fig. 1*E* and supplemental Fig. 1). Such regulation may occur within a given cell and integrate Mdm2 regulation by two or more pathways. Alternatively, the prevailing pathway phosphorylating these residues may be a function of cell type.

Our findings suggest that the influence of active Pim kinase on the p53 pathway appears to operate at two levels. On one level, Pim phosphorylates Mdm2 at two sites, Ser^{166} and Ser^{186}, which have previously been reported by others to be phosphorylated through several independent pathways, including those mediated by Akt, MK2, DAP kinase, $pp90^{RSK}$, and MSK-1 (12-18, 35) (see also supplemental Fig. 1). The functional relevance of these phosphorylation sites has remained uncertain, and various effects have been reported, including nuclear localization of Mdm2, increased ubiquitylation of p53, increased degradation of p53, decreased association with ARF, and/or increased interaction with p300 (12-18). The emerging model based on these *in vitro* studies proposed that phosphorylation activates Mdm2 and promotes its nuclear entry, leading to increased p53 ubiquitylation and turnover. In contrast, we find that Mdm2 is predominantly nuclear even when it cannot be phosphorylated at Ser^{166} and/or Ser^{186}, suggesting that phosphorylation is not required for nuclear entry of Mdm2 (supplemental Fig. 5, *A* and *B*). This is not an artifact of fixation, because a mutant Mdm2 lacking the NLS clearly shows significant cytoplasmic staining. It is possible that association of Mdm2 with Pim (in our experiments) or Akt (in experiments of other researchers) may have a bearing on subcellular location over and above the phosphorylation mediated by these enzymes. Our observation of a significant level of both Pim-1 and Mdm2 in the cytoplasm of mantle cell lymphoma sections would fit with this idea or would suggest that additional, as yet undetermined, factors might additionally affect localization (Fig. 8). It is also possible that differences in expression levels or status of the cultured cells used could influence these observations. We also find that Pim-1 can stimulate the Mdm2/ARF interaction in transfection experiments in cultured cells (Fig. 6). This increased binding of Mdm2 to ARF is subtle, however, but may permit fine tuning of an escalation mechanism that increases the readiness of the p53 pathway following proliferative signals that engage Pim (or other proliferative pathways). To examine this further, we compared the levels of p53 or Mdm2 in wild type MEFs with MEFs in which all three Pim kinases had been knocked out (22) (supplemental Fig. 6). Regrettably, this comparison did not reveal any significant differences. This is not surprising, however, given that (*a*) in all of the cell lines/MEFs we have used, the levels of endogenous Pim proteins are extremely low or undetectable, making their contributions under normal cell culture conditions difficult to assess, and (*b*) the sites in Mdm2 phosphorylated by Pim are targeted by potentially several different signaling pathways/enzymes (Fig. 1*E*), making it very difficult to completely eliminate signaling to this part of the Mdm2 molecule by knock-out or knockdown. Given the significant degree of overlap between the Akt and Pim pathways (23) (and possibly other pathways focusing on this region), the physiological significance of these modifications may ultimately have to be resolved by the generation of an S166A/S186A knock-in mouse. Such a mouse would additionally provide an appropriate means of assessing the physiological role of these modifications without the need for transfection and ectopic expression of mutant cDNAs and could provide early passage cells with an intact p53 pathway for *in vitro* analysis. An additional point worth considering is that we know very little about the region of Mdm2 that is targeted by Pim, Akt, and these other pathways. This region is clearly important for nucleocytoplasmic shuttling, and the evidence to date suggests strongly that it is a focal point for multisignal integration. The possibility remains, therefore, that this region plays a critical, as yet undefined, role in Mdm2 biology (perhaps pertaining to other functions in Mdm2 that are independent of p53). Thus, the binding of ARF may be *a* function that is regulated by phosphorylation but not *the* function.

On a second and significantly more potent level, Pim appears to stimulate the induction of ARF in early passage MEFs, leading to increased p53 and Mdm2 levels (Fig. 7). This is accompanied by increased p21 levels and the appearance of senescence-associated β-galactosidase. This is consistent with the idea that high level expression of Pim, as can occur during tumor development, stimulates a protective p53 response, potentially driving cells toward senescence. Notably, this induction of the p53 pathway is absent in U2OS cells,

which lack ARF expression, but can be reconstituted in H1299 cells, which express ARF but lack endogenous p53, upon ectopic expression of p53, Mdm2, and Pim. Although this attractive hypothesis remains to be tested in an *in vivo* context (*e.g.* in mice expressing elevated expression of Pim-1 or Pim-2) it is entirely consistent with a recent study describing a mouse model for prostate cancer in which the absence of PTEN, which promotes constitutive activation of Akt, leads not to the suppression but rather to the activation of the p53 pathway and, consequently, cellular senescence (19).

Our observation that active Pim-1 and Mdm2 appear to stabilize each other is worth noting (Fig. 5). As suggested above, stabilization of Mdm2 may occur simply through the induction of ARF, which is known to block the degradation of both p53 and Mdm2 itself (7, 52). Alternatively, given that Mdm2 and Pim-1 interact stably both *in vitro* (Fig. 3) and in cultured cells (Fig. 2), it is possible that their association could provide protection against degradation and may have physiological (Fig. 2, *C* and *D*) or indeed pathological significance (see below). The finding that inactive Pim-1 retains some ability to stabilize Mdm2 (Fig. 5) would certainly lend support to this idea. Additionally, we note that others have independently observed stabilization of Mdm2 by Pim-1 (24) and indeed by Akt (15, 18).

The finding that 42% of the tissue samples in our mantle cell lymphoma cohort show elevated Pim-1 protein levels is, in itself, striking (Table 1 and Fig. 8). These data support previous observations of elevated Pim-1 mRNA in mantle cell lymphoma (49, 50) and suggest that Pim-1 may be a contributory factor in the development of this disease. Notably, far fewer of the tumors showed elevated Pim-2 levels, and these were observed only where there was already noticeable Pim-1 expression. One interpretation of these data is that there is a selective preference for Pim-1 that favors mantle cell lymphoma tumorigenesis. Alternatively, the mechanisms that lead to elevated expression of Pim-1 may be more susceptible to activation during the tumor development process than those for Pim-2. Equally striking is the finding of a correlation between elevated Pim-1 and Mdm2 (Table 1). This raises the possibility that, as reflected in our cell culture analyses (Fig. 5), Pim-1 may stabilize Mdm2 (or *vice versa*), possibly (as our model predicts) through induction of the p53 pathway. Alternatively, the Pim-1·Mdm2 complex may have an as yet undetermined specific biochemical function that plays a role in the development of the disease. These are important issues that should be addressed in future experiments.

A model describing the findings of the present study is shown in Fig. 9 and can be summarized as follows. 1) Mdm2 can associate with p53 and promote p53 ubiquitylation, export to the cytoplasm, and degradation by the proteasome under normal unstressed conditions. 2) Pim-1 can associate with Mdm2 under normal conditions of cell growth. Phosphorylation of Mdm2 under these circumstances does not appear to affect localization of Mdm2 but may have other, as yet unknown, effects or indeed play a more subtle role, such as fine tuning ARF binding in a manner that would contribute to putting the cell in a greater state of readiness to activate the p53 pathway, if required, during proliferation. Phosphorylation by Pim may be integrated with converging signals from other pathways, depending on the type of cell and its growth/survival status. 3) Pim-1 can also elevate ARF levels independently of Mdm2 phosphorylation, leading to inhibition of p53 degradation (and possibly of Mdm2). This mechanism would underlie a protective induction of the p53 pathway during tumorigenesis. 4) Mdm2 and Pim-1 can form a tight complex that may help protect both Mdm2 and Pim-1 from degradation. Additionally, this complex could play an as yet undefined role in the pathogenesis of mantle cell lymphoma. It may be possible to test these ideas more directly in the future using a mouse model for Pim-1-initiated lymphomagenesis, such as that described by Berns and co-workers (53).

Europe PMC Funders Author Manuscripts

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are very grateful to Dr. Larry Higgins (University of Dundee) for the preparation and provision of primary murine embryo fibroblasts, to Dr. Anton Berns (Netherlands Cancer Institute) for providing Pim-1, -2, -3 triple knock-out primary murine embryo fibroblasts, to the Tayside Tissue Bank (Ninewells Hospital, Dundee) for provision of human lymphoma samples and for immunohistochemistry, and to Dr. Jean-Christophe Bourdon (University of Dundee) for assistance with luciferase assays.

REFERENCES

1. Horn HF, Vousden KH. Oncogene. 2007; 26:1306–1316. [PubMed: 17322916]
2. Toledo F, Wahl GM. Nat. Rev. Cancer. 2006; 6:909–923. [PubMed: 17128209]
3. Michael D, Oren M. Semin. Cancer Biol. 2003; 13:49–58. [PubMed: 12507556]
4. Ashcroft M, Taya Y, Vousden KH. Mol. Cell Biol. 2000; 20:3224–3233. [PubMed: 10757806]
5. de Stanchina E, McCurrach ME, Zindy F, Shieh S-Y, Ferbeyre G, Samuelson AV, Prives C, Roussel MF, Sherr CJ, Lowe SW. Genes Dev. 1998; 12:2434–2442. [PubMed: 9694807]
6. Kamijo T, Zindy F, Roussel MF, Quelle DE, Downing JR, Ashmun RA, Grosveld G, Sherr CJ. Cell. 1997; 91:649–659. [PubMed: 9393858]
7. Stott FJ, Bates S, James MC, McConnell BB, Starborg M, Brookes S, Palmero I, Ryan K, Hara E, Vousden KH, Peters G. EMBO J. 1998; 17:5001–5014. [PubMed: 9724636]
8. Zhang Y, Xiong Y, Yarbrough WG. Cell. 1998; 92:725–734. [PubMed: 9529249]
9. Zindy F, Eischen CM, Randle DH, Kamijo T, Cleveland JL, Sherr CJ, Roussel MF. Genes Dev. 1998; 12:2424–2433. [PubMed: 9694806]
10. Christophorou MA, Ringshausen I, Finch AJ, Swigart LB, Evan GI. Nature. 2006; 443:214–217. [PubMed: 16957739]
11. Meek DW, Knippschild U. Mol. Cancer Res. 2003; 1:1017–1026. [PubMed: 14707285]
12. Milne D, Kampanis P, Nicol S, Dias S, Campbell DG, Fuller-Pace F, Meek D. FEBS Lett. 2004; 577:270–276. [PubMed: 15527798]
13. Zhou BP, Liao Y, Xia W, Zou Y, Spohn B, Hung MC. Nat. Cell Biol. 2001; 3:973–982. [PubMed: 11715018]
14. Mayo LD, Donner DB. Proc. Natl. Acad. Sci. U. S. A. 2001; 98:11598–11603. [PubMed: 11504915]
15. Ashcroft M, Ludwig RL, Woods DB, Copeland TD, Weber HO, MacRae EJ, Vousden KH. Oncogene. 2002; 21:1955–1962. [PubMed: 11960368]
16. Ogawara Y, Kishishita S, Obata T, Isazawa Y, Suzuki T, Tanaka K, Masuyama N, Gotoh Y. J. Biol. Chem. 2002; 277:21843–21850. [PubMed: 11923280]
17. Gottlieb TM, Leal JF, Seger R, Taya Y, Oren M. Oncogene. 2002; 21:1299–1303. [PubMed: 11850850]
18. Feng J, Tamaskovic R, Yang Z, Brazil DP, Merlo A, Hess D, Hemmings BA. J. Biol. Chem. 2004; 279:35510–35517. [PubMed: 15169778]
19. Chen Z, Trotman LC, Shaffer D, Lin HK, Dotan ZA, Niki M, Koutcher JA, Scher HI, Ludwig T, Gerald W, Cordon-Cardo C, Pandolfi PP. Nature. 2005; 436:725–730. [PubMed: 16079851]
20. Wang Z, Bhattacharya N, Weaver M, Petersen K, Meyer M, Gapter L, Magnuson NS. J. Vet. Sci. 2001; 2:167–179. [PubMed: 12441685]
21. White E. Genes Dev. 2003; 17:1813–1816. [PubMed: 12897050]
22. Mikkers H, Nawijn M, Allen J, Brouwers C, Verhoeven E, Jonkers J, Berns A. Mol. Cell Biol. 2004; 24:6104–6115. [PubMed: 15199164]
23. Hammerman PS, Fox CJ, Birnbaum MJ, Thompson CB. Blood. 2005; 105:4477–4483. [PubMed: 15705789]

24. Ionov Y, Le X, Tunquist BJ, Sweetenham J, Sachs T, Ryder J, Johnson T, Lilly MB, Kraft AS. Anticancer Res. 2003; 23:167–178. [PubMed: 12680209]
25. Allen JD, Berns A. Semin. Cancer Biol. 1996; 7:299–306. [PubMed: 9110407]
26. Fox CJ, Hammerman PS, Cinalli RM, Master SR, Chodosh LA, Thompson CB. Genes Dev. 2003; 17:1841–1854. [PubMed: 12869584]
27. Allen JD, Verhoeven E, Domen M, van der Valk M, Berns A. Oncogene. 1997; 15:1133–1141. [PubMed: 9294606]
28. Verbeek S, van Lohuizen M, van der Valk M, Domen J, Kraal G, Berns A. Mol. Cell Biol. 1991; 11:1176–1179. [PubMed: 1990273]
29. Dhanasekaran SM, Barrette TR, Ghosh D, Shah R, Varambally S, Kurachi K, Pienta KJ, Rubin MA, Chinnaiyan AM. Nature. 2001; 412:822–826. [PubMed: 11518967]
30. Yoshida S, Kaneita Y, Aoki Y, Seto M, Mori S, Moriyama M. Oncogene. 1999; 18:7994–7999. [PubMed: 10637510]
31. Amson R, Sigaux F, Przedborski S, Flandrin G, Givol D, Telerman A. Proc. Natl. Acad. Sci. U. S. A. 1989; 86:8857–8861. [PubMed: 2682662]
32. Claudio JO, Masih-Khan E, Tang H, Goncalves J, Voralia M, Li ZH, Nadeem V, Cukerman E, Francisco-Pabalan O, Liew CC, Woodgett JR, Stewart AK. Blood. 2002; 100:2175–2186. [PubMed: 12200383]
33. Li YY, Popivanova BK, Nagai Y, Ishikura H, Fujii C, Mukaida N. Cancer Res. 2006; 66:6741–6747. [PubMed: 16818649]
34. Popivanova BK, Li YY, Zheng H, Omura K, Fujii C, Tsuneyama K, Mukaida N. Cancer Sci. 2007; 98:321–328. [PubMed: 17270021]
35. Burch L, Scott M, Pohler E, Meek D, Hupp T. J. Mol. Biol. 2004; 337:115–128. [PubMed: 15001356]
36. Kurki S, Peltonen K, Kiviharju T, Latonen L, Ojala P, Meek D, Laiho M. Cancer Cell. 2004; 5:465–475. [PubMed: 15144954]
37. Webster GA, Perkins ND. Mol. Cell Biol. 1999; 19:3485–3495. [PubMed: 10207072]
38. Midgley CA, Owens B, Briscoe CV, Thomas DB, Lane DP, Hall PA. J. Cell Sci. 1995; 108:1843–1848. [PubMed: 7657708]
39. Xirodimas D, Saville MK, Edling C, Lane DP, Lain S. Oncogene. 2001; 20:4972–4983. [PubMed: 11526482]
40. Hupp TR, Meek DW, Midgley CA, Lane DP. Cell. 1992; 71:875–886. [PubMed: 1423635]
41. Dumaz N, Meek DW. EMBO J. 1999; 18:7002–7010. [PubMed: 10601022]
42. Allende-Vega N, Saville MK, Meek DW. Oncogene. 2007; 26:4234–4242. [PubMed: 17237821]
43. Palaty CK, Clark-Lewis I, Leung D, Pelech SL. Biochem. Cell Biol. 1997; 75:153–162. [PubMed: 9250363]
44. Jackson MW, Patt LE, LaRusch GA, Donner DB, Stark GR, Mayo LD. J. Biol. Chem. 2006; 281:16814–16820. [PubMed: 16621805]
45. Weber HO, Ludwig RL, Morrison D, Kotlyarov A, Gaestel M, Vousden KH. Oncogene. 2005; 24:1965–1972. [PubMed: 15688025]
46. Collins GA, Tansey WP. Curr. Opin. Genet. Dev. 2006; 16:197–202. [PubMed: 16503126]
47. Blattner C, Hay TJ, Meek DW, Lane DP. Mol. Cell Biol. 2002; 22:6170–6182. [PubMed: 12167711]
48. Sherr CJ. Nat. Rev. Cancer. 2006; 6:663–673. [PubMed: 16915296]
49. de Vos S, Krug U, Hofmann WK, Pinkus GS, Swerdlow SH, Wachsman W, Grogan TM, Said JW, Koeffler HP. Diagn. Mol. Pathol. 2003; 12:35–43. [PubMed: 12605034]
50. Zhu Y, Hollmen J, Raty R, Aalto Y, Nagy B, Elonen E, Kere J, Mannila H, Franssila K, Knuutila S. Br. J. Haematol. 2002; 119:905–915. [PubMed: 12472567]
51. Swerdlow SH, Williams ME. Hum. Pathol. 2002; 33:7–20. [PubMed: 11823969]
52. Llanos S, Clark PA, Rowe J, Peters G. Nat. Cell Biol. 2001; 3:445–452. [PubMed: 11331871]
53. van Lohuizen M, Verbeek S, Krimpenfort P, Domen J, Saris C, Radaszkiewicz T, Berns A. Cell. 1989; 56:673–682. [PubMed: 2537153]

54. Xirodimas DP, Stephen CW, Lane DP. Exp. Cell Res. 2001; 270:66–77. [PubMed: 11597128]

Europe PMC Funders Author Manuscripts

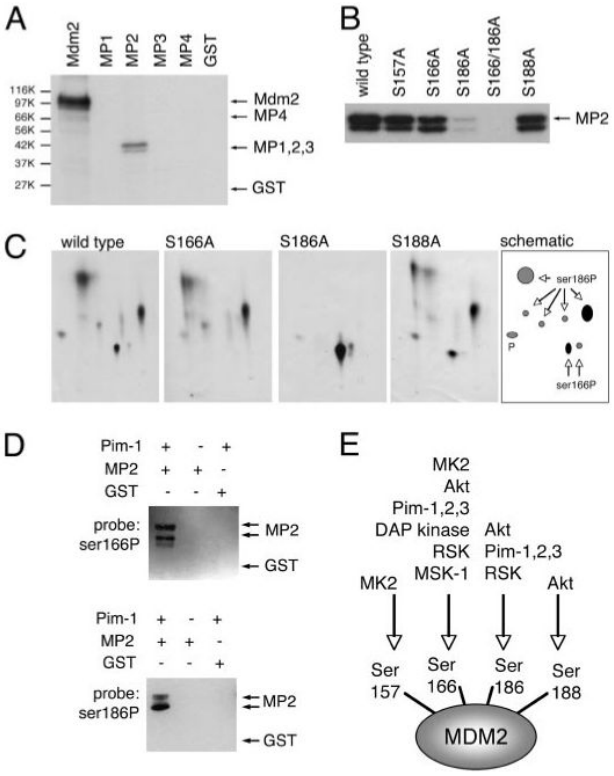

FIGURE 1. Pim-1 phosphorylates Ser^{166} and Ser^{186} in Mdm2 *in vitro*

A, phosphorylation of full-length human Mdm2 and GST fusion miniproteins, MP1–MP4, by recombinant GST-Pim-1. ^{32}P-Labeled phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. *B*, phosphorylation of the MP2 miniprotein (GST-linked through its C terminus to amino acids 108-207 of Mdm2) or a series of Ser to Ala substitution mutants of MP2 by Pim-1. *C*, tryptic phosphopeptide mapping of full-length GST-tagged Mdm2 and GST-tagged Mdm2 in which either Ser^{166}, Ser^{186}, or Ser^{188} is substituted by alanine. The *schematic map* shows identities of the phosphopeptides. *P*, free phosphate. *D*, Western blot analysis of *in vitro*-phosphorylated MP2 miniprotein using the Ser(P)^{166} and Ser(P)^{186} phosphospecific antibodies. *E*, schematic showing the Ser^{157}, Ser^{166}, Ser^{186}, and Ser^{188} phosphorylation sites and the signaling enzymes reported to modify these residues in Mdm2. Ser^{157} is modified by MK2; Ser^{166} is modified by Akt, Pim-1, Pim-2, Pim3, DAP kinase, RSK, MSK-1, and MK2; Ser^{186} is modified by Akt, Pim-1, Pim-2, Pim-3, and RSK; and Ser^{188} is modified by Akt (12-18, 35, 44, 45). Although RSK has been reported to regulate Mdm2 (44), our data identify Ser^{166} and Ser^{186} as potential phosphorylation sites for this kinase (supplemental Fig. 1).

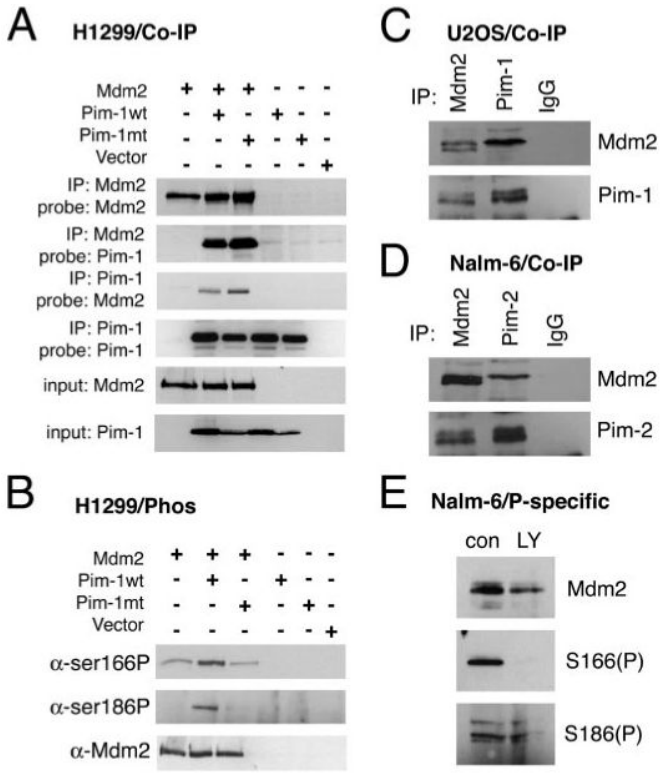

FIGURE 2. Pim-1 associates with and phosphorylates Mdm2 in cultured cells

A and *B*, H1299 cells were transfected with vector alone or a plasmid expressing wild type Mdm2, together with plasmids expressing wild type Pim-1 or an inactive mutant of Pim-1. Immunoprecipitation and Western analysis was performed as indicated in the *panel. C* and *D*, co-immunoprecipitation analysis was carried out, as indicated in the *panels*, on extracts of U2OS (from a total of 10 × 10-cm plates) and NALM-6 cells (from 5 × 15-cm plates), respectively, that had been pretreated with 20 μ_M MG132 for 4 h. *E*, Western analysis was performed on extracts of NALM-6 cells that had been pretreated with 20 μ_M MG132 for 4 h. Additionally, one plate of cells was pretreated with 20 μ_M LY294002.

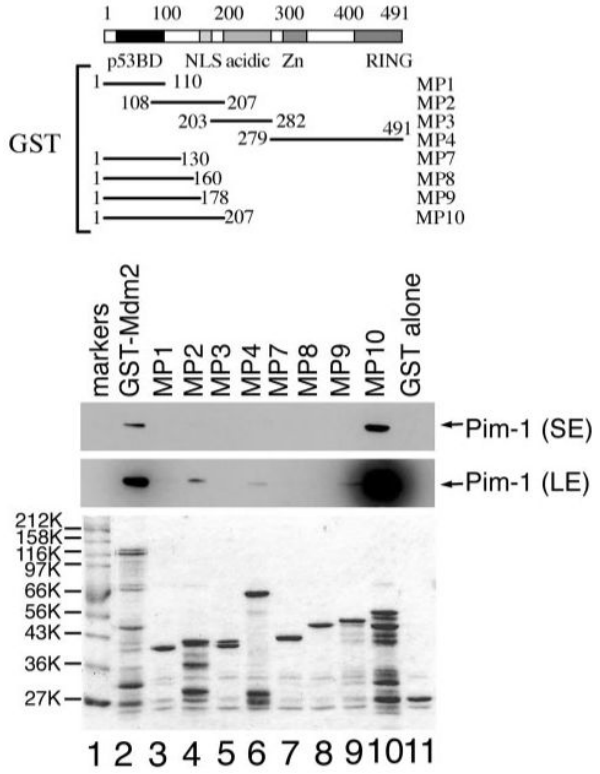

FIGURE 3. Pim-1 interacts with two regions in the N terminus of Mdm2

GST pull-down experiments were carried out using a series of GST-Mdm2 fusion proteins linked through the C terminus of GST to overlapping regions of Mdm2 (MP1–MP10). The regions of Mdm2 encompassed within these proteins are shown in the *upper panel*. The ability of Pim-1 to associate with these miniproteins in pull-down assays is indicated in the *upper middle panel* (short exposure (*SE*)). A long exposure (*LE*) of this autoradiograph is shown in the *lower middle panel*. The *bottom panel* shows a Coomassie-stained gel of the purified miniproteins used in the assay.

Europe PMC Funders Author Manuscripts

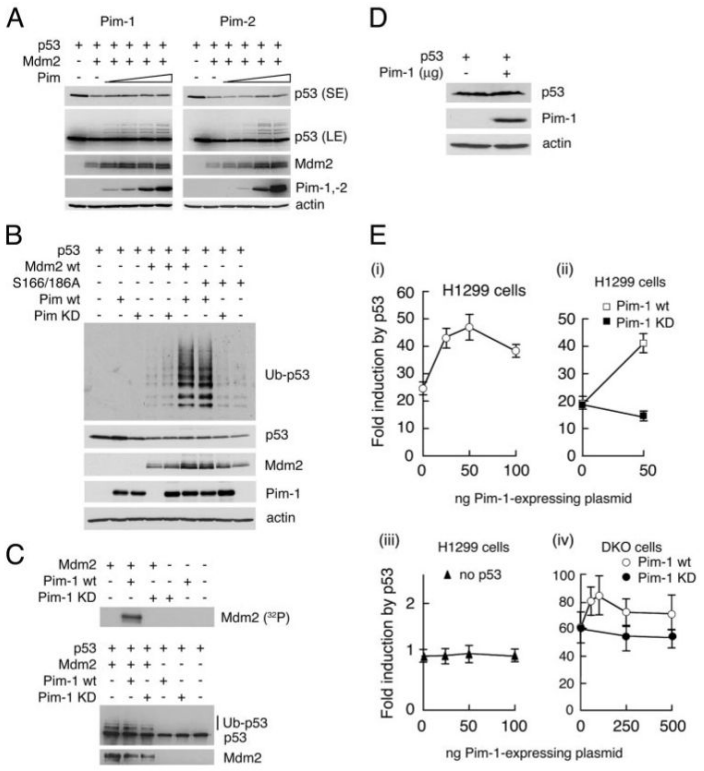

FIGURE 4. Expression of Pim-1 or Pim-2 increased Mdm2 levels and p53 ubiquitylation

A, H1299 cells were transfected with plasmids encoding wild type p53 (20 ng), Mdm2 (5 μg), and increasing amounts of Pim-1 or Pim-2 (up to 5 μg). The cells were harvested after 36 h, and the extracts were resolved by SDS-PAGE. p53, Mdm2, Pim-1, Pim-2, and actin were detected by Western blotting. *B*, H1299 cells were transfected as in *A* together with a plasmid expressing His_6-tagged ubiquitin. After 36 h, the cells were lysed in guanidinium hydrochloride, and the ubiquitylated proteins were isolated on Ni^{2+}-agarose beads as described (54). Ubiquitylated p53 was detected by Western blotting (*upper panel*). Alternatively, the cells were lysed in SDS-PAGE sample buffer and analyzed by Western blotting to detect p53, Mdm2, Pim-1, and actin (*lower panels*). *C*, Mdm2-mediated ubiquitylation of p53 *in vitro*. Mdm2 was incubated in the presence of [γ-^{32}P]ATP and wild type Pim-1 or, as control, in the presence of the Pim-1 inactive mutant (Pim-1 KD) to achieve stoichiometric phosphorylation. The phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography (*upper panel*). In the *lower panel*, phosphorylated Mdm2 (prepared in the presence of active Pim-1) and unphosphorylated Mdm2 (either not phosphorylated or prepared in the presence of inactive Pim-1) were compared for their ability to ubiquitylate p53 *in vitro* as described (54). *D*, DKO cells (*i.e.* $p53^{-/-}$, $Mdm2^{-/-}$ MEFs) were transfected with plasmids expressing p53 (10 ng) and Pim-1 (1 μg). The levels of p53, Pim-1, and actin were measured by Western blotting. *E*, H1299 or DKO cells were transfected with the p53-responsive luciferase plasmid PG13-luc together with the control luciferase plasmid SV-*Renilla*. The cells were additionally transfected with plasmids expressing p53 (1 ng) together with increasing amounts of plasmids expressing active or inactive Pim-1, as indicated. Following cell lysis, luciferase activities were determined as described under “Experimental Procedures.”

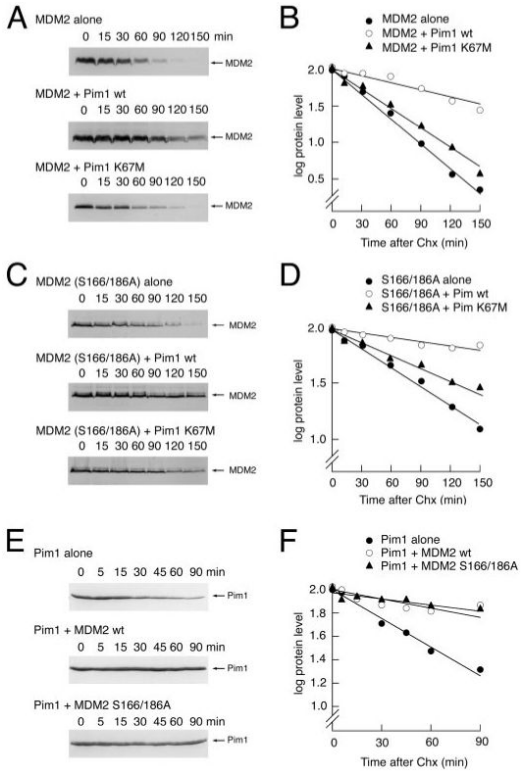

FIGURE 5. Co-expression of Mdm2 and Pim-1 leads to stabilization of both proteins

A, H1299 cells were transfected with 4 μg of Mdm2 expression plasmid together with 4 μg of wild type or kinase-dead Pim-1 expression plasmids or, as control, empty vector. 36 h post-transfection, the cells were treated with cycloheximide (*CHX*;10 μg/ml) and harvested at the indicated time points. Extracts were analyzed by Western blotting using the SMP-14 and 4B2 anti-Mdm2 antibodies. The signals obtained for Mdm2 were quantitated by densitometry (*B*). *C*, the experiment was carried out as described in *A* except that a plasmid expressing the S166A/S186A mutant of Mdm2 was substituted for wild type Mdm2. The signals obtained for S166A/S186A mutant Mdm2 were quantitated by densitometry (*D*). *E*, H1299 cells were transfected with 4 μg of Pim-1 expression plasmid either alone or with 4 μg of wild type or S166A/S186A mutant Mdm2 expression plasmids. The amount of promoter was balanced with empty expression vector. 36 h post-transfection, the cells were treated with CHX (10 μg/ml) and harvested at the indicated time points. Extracts were analyzed by Western blotting using the 12H8 anti-Pim-1 antibody. The signals obtained for Pim-1 were quantitated by densitometry (*F*).

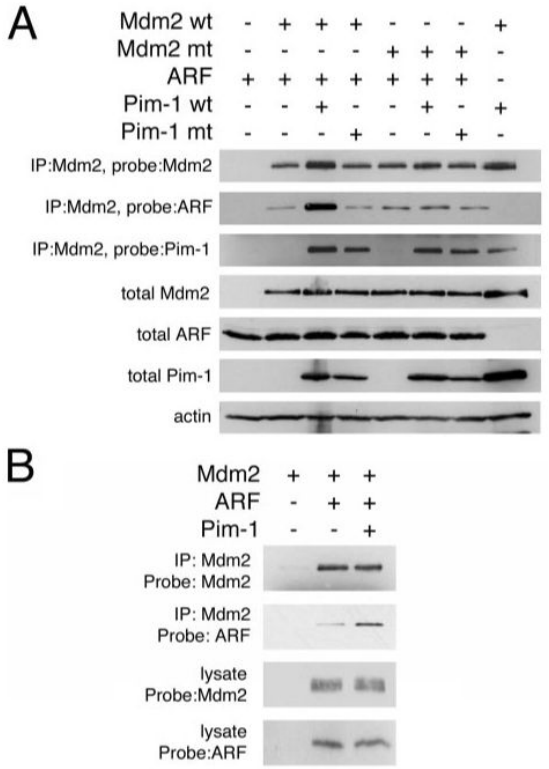

FIGURE 6. Pim-1 promotes the interaction between Mdm2 and ARF in a manner dependent on Pim-1 kinase activity

A, H1299 cells were transfected as indicated with 4 μg of ARF, 4 μg of wild type or S166A/S186A mutant Mdm2, and 4 μg of wild type or kinase-dead Pim-1 expression plasmids and the required amount of empty expression vector to balance promoter levels. MG132 (20 μM) was added to the cells 6 h before lysis. Cell lysates were immunoprecipitated with anti-Mdm2 antibodies (SMP-14/4B2). Immunoprecipitates were analyzed by Western blotting with anti-Mdm2 antibodies (SMP-14/4B2), anti-ARF antibody, and anti-Pim-1 antibody (12H8), as indicated. Total Mdm2, ARF, Pim-1, and actin were analyzed by Western blotting with anti-Mdm2 antibodies (SMP-14/4B2), an anti-ARF polyclonal antibody, anti-Pim-1 antibody (12H8), and anti-actin antibody (20-33). *B*, U2OS cells were transfected with either 2.5 μg of Mdm2 expression plasmid together with 2.5 μg of Pim-1 expression plasmid or, as control, empty vector. 36 h post-transfection, cells were lysed, and extracts were made. U2OS cells were also transfected, separately, with 5 μg of ARF expression plasmid, and again the cells were lysed 36 h post-transfection, and extracts were prepared. Equal volumes of lysate containing ARF were mixed with the lysates from cells transfected with plasmids expressing Mdm2 alone or Mdm2 and Pim-1. Mdm2 was then immunoprecipitated with anti-Mdm2 antibodies (SMP-14/4B2), and the amount of ARF co-immunoprecipitating was measured by Western blotting with an anti-ARF polyclonal antibody. Total Mdm2 and ARF in the extracts were analyzed by Western blotting with anti-Mdm2 antibodies (SMP-14/4B2) and anti-ARF antibody.

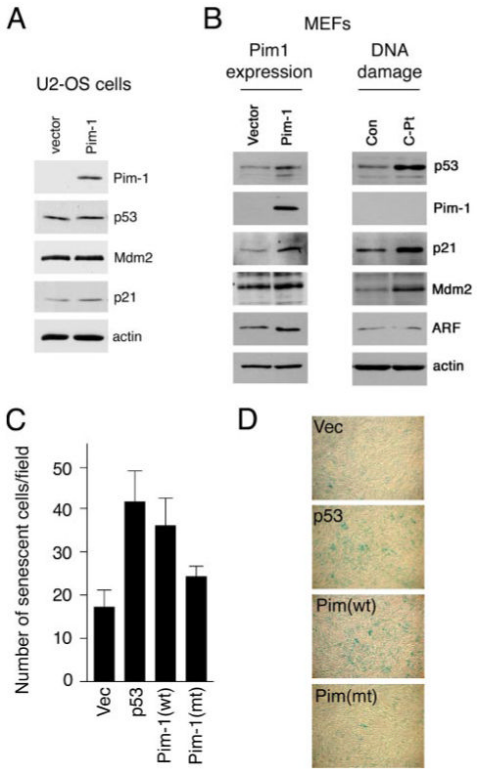

FIGURE 7. Pim-1 induces the p53 pathway and stimulates senescence-associated β-galactosidase levels in primary mouse embryo flibroblasts

A, U2OS cells were transfected with plasmids expressing wild type Pim-1 or empty vector (as control) together with plasmid pSV2neo (conferring neomycin resistance). G418-resistant clones were selected and screened for Pim-1 expression. Pim-1-expressing cells, or cells that had received vector, were grown to 70-80% confluence and harvested for analysis by Western blotting with anti-Pim-1 antibody (12H8), anti-p53 antibody (DO-1), anti-Mdm2 antibodies (SMP14 and 4B2), anti-p21 antibody (C-19), and anti-actin antibody (20-33). *B*, primary MEFs were transfected (Fugene) with 2.5 μg of a plasmid encoding the CD20 cell surface marker and either 5.5 μg of Pim-1 expression vector, as control, or empty vector. Cells were harvested 48 h post-transfection, and the transfected population was enriched by capture on magnetic beads coated with anti-CD20 antibody. Cell lysates were analyzed by Western blotting (*left panels*) with anti-Pim-1 antibody (12H8), anti-p53 antibody (DO-1), anti-Mdm2 antibodies (SMP14 and 4B2), anti-p21 antibody (C-19), anti-ARF antibody, and anti-actin antibody (20-33). Untransfected MEFs were also treated with the DNA damage-inducing drug, cis-platin (20 μ_M for 6 h). Cell lysates were analyzed by Western blotting (*right panels*) in the same manner as the Pim-1-expressing cells. *C*, primary MEFs were transfected (triplicate plates with Lipofectamine 2000) with 8 μg/plate of plasmid encoding wild type Pim-1, K67M mutant Pim-1, p53 (as positive control), or empty vector (as negative control). 72 h post-transfection, the cells were stained for SA-β-galactosidase as an indicator of cellular senescence (Senescence Detection Kit; Calbiochem). Blue cells were counted in each of 20 randomly selected fields per plate. The *bars* represent the average number of blue cells per field with the S.D. values shown as *error bars*. *D*, typical images of early passage MEFs transfected as indicated and stained for SA-β-galactosidase.

Europe PMC Funders Author Manuscripts

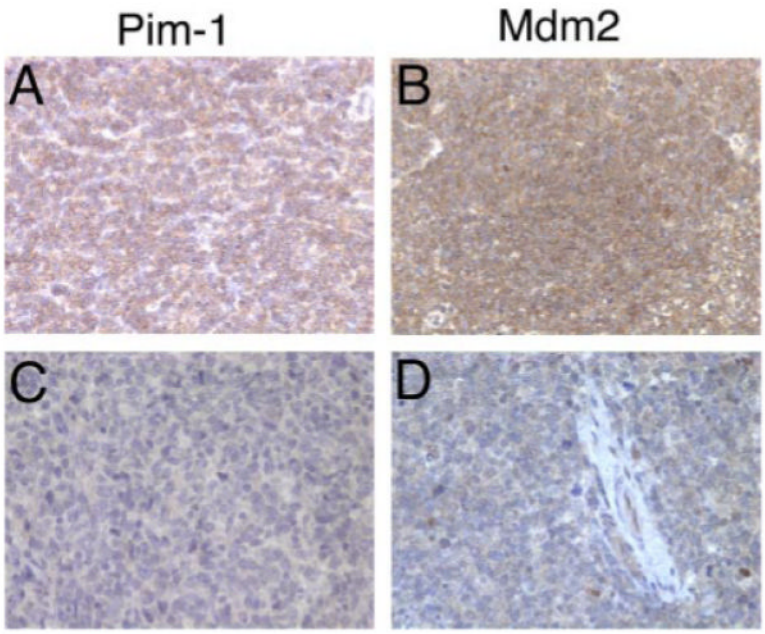

FIGURE 8. Elevated levels of Pim-1 and Mdm2 are present in tissue sections of human mantle cell lymphomas

Mantle cell lymphoma sections were stained for Pim-1 (*A* and *C*) and Mdm2 (*B* and *D*). Pim-1 was detected using the goat polyclonal anti-Pim-1 C-20, and Mdm2 was detected with the monoclonal antibody 2A9. Cases in which Pim-1 kinase expression can be detected in lymphoma cells by immunohistochemistry show stronger staining for Mdm2 (*A* and *B*) than those cases in which staining for Pim-1 kinase is not apparent (*C* and *D*).

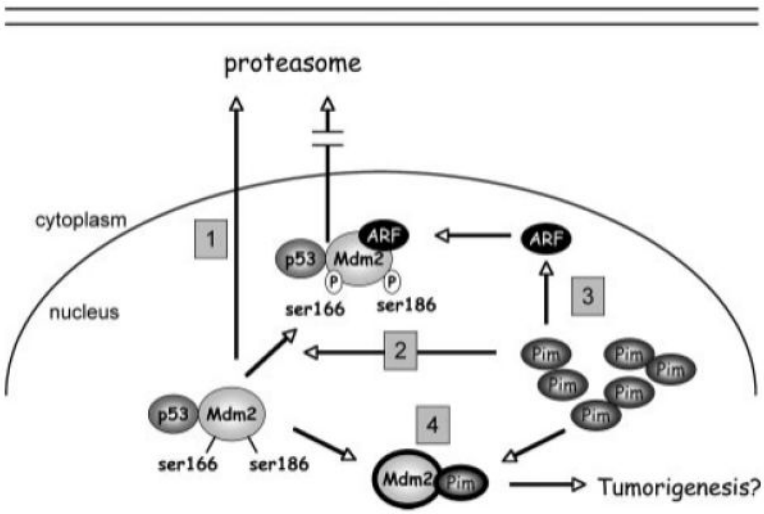

FIGURE 9. Model depicting the relationship between Pim-1 and the p53 pathway
See "Discussion" for a discussion of this model.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

TABLE 1

Staining by immunohistochemistry of Mdm2, Pim-1, and Pim-2 in a cohort of 33 samples of tissue from human mantle cell lymphoma

Sections of human mantle cell lymphoma were stained for Pim-1, Pim-2, and Mdm2 as described under "Experimental Procedures." It should be noted that 1) all cases positive for Pim-2 were also positive for Pim-1 (*i.e.* no case was Pim-1-negative and Pim-2-positive) and 2) using Fisher's Mdm2 in the tumor samples correlated with a p value of 0.003.

Protein	Positive staining	Negative (or weak (w)) staining
Pim-1	14 (42%)	19 (58%)
Pim-2	3 (9%)	30 (91%)
Mdm2	9 (27%)	24 (73%) (w)