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ARTICLE *in* FEBS LETTERS · OCTOBER 2009

Impact Factor: 3.17 · DOI: 10.1016/j.febslet.2009.09.057 · Source: PubMed

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Polo-like kinase-1 phosphorylates MDM2 at Ser260 and stimulates MDM2-mediated p53 turnover

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ARTICLE INFO

Article history:
Received 14 August 2009
Revised 23 September 2009
Accepted 30 September 2009
Available online 13 October 2009

Edited by Varda Rotter

Keywords:
p53
Murne double-minute clone 2
Polo-like kinase-1
Phosphorylation

ABSTRACT

The E3 ubiqutin ligase, murne double-minute clone 2 (MDM2), promotes the degradation of p53 under normal homeostatic conditions. Several serine residues within the acidic domain of MDM2 are phosphorylated to maintain its activity but become hypo-phosphorylated following DNA damage, leading to inactivation of MDM2 and induction of p53. However, the signalling pathways that mediate these phosphorylation events are not fully understood. Here we show that the oncogenic and cell cycle-regulatory protein kinase, polo-like kinase-1 (PLK1), phosphorylates MDM2 at one of these residues, Ser260, and stimulates MDM2-mediated turnover of p53. These data are consistent with the idea that deregulation of PLK1 during tumourigenesis may help suppress p53 function.

Structured summary:
MINT-7266353: *MDM2* (uniprotkb:Q00987) *physically interacts* (MI:0915) with *PLK1* (uniprotkb:P53350) by *pull down* (MI:0096)
MINT-7266344, MINT-7266329: *MDM2* (uniprotkb:Q00987) *physically interacts* (MI:0915) with *PLK1* (uniprotkb:P53350) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-7266250: *PLK1* (uniprotkb:P53350) *phosphorylates* (MI:0217) *p53* (uniprotkb:P04637) by *protein kinase assay* (MI:0424)
MINT-7266241, MINT-7266318: *PLK1* (uniprotkb:P53350) *phosphorylates* (MI:0217) *MDM2* (uniprotkb:P23804) by *protein kinase assay* (MI:0424)
MINT-7266231, MINT-7266805, MINT-7266264, MINT-7266299: *PLK1* (uniprotkb:P53350) *phosphorylates* (MI:0217) *MDM2* (uniprotkb:Q00987) by *protein kinase assay* (MI:0424)

1. Introduction

The p53 tumour suppressor is a latent transcription factor that plays a pivotal role in preventing tumour development [1,2]. p53 is induced in response to a variety of cellular stresses, leading to cell cycle arrest at the G1/S and G2/M boundaries or the onset of apoptosis. p53 is also regulated by a number of important partner proteins including the E3 ubiquitin ligase, murne double-minute clone 2 (MDM2), which plays a critical role in the ubiquitylation and proteasome-dependent degradation of p53 [3].

Polo-like kinase-1 (PLK1) is essential for the maturation of centrosomes, entry into M phase, spindle formation and cytokinesis [4]. PLK1 is a critical target of the DNA damage-induced G2/M checkpoint and is down-regulated in an ATM/ATR-dependent fashion coincident with increases in p53 [5–7]. PLK1 is also thought to be oncogenic. For example, constitutively active mutants of PLK1 can override the G2/M checkpoint [6] and transform NIH3T3 cells [8]. Moreover, PLK1 is up-regulated in many human malignancies, consistent with a role in tumourigenesis, and is widely considered to be a potential therapeutic target [4].

In response to DNA damage, MDM2 undergoes rapid hypo-phosphorylation of several important regulatory phospho-serine residues within its acidic domain leading to loss of its ability to promote p53 degradation [9]. Consistent with this model, phosphorylation of the acidic domain can stimulate its interaction with a critical ubiquitylation signal in the core domain of p53 [10]. Although proteins kinases CK1, GSK3β and CK2 can modify several sites within the acidic domain [11–13], we still do not have a complete picture of the modifying enzymes that target this region. In the present study we show that PLK1 can interact with and phosphorylate MDM2 at Ser260, one of these important serine residues. We also show that PLK1 can stimulate the turnover of p53, and

Abbreviations: MDM2, murne double-minute clone 2; PLK1, polo-like kinase-1; MP, mini-protein; GST, glutathione S-transferase; TOPORS, topoisomerase I binding, arginine/serine-rich protein

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0014-5793/$36.00 © 2009 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2009.09.057

correspondingly inhibit its transcription function, in an MDM2-depenent manner.

2. Materials and methods

2.1. Cell lines, transfections and plasmids

HeLa (human cervical carcinoma-derived) cells and H1299 (human lung carcinoma-derived) cells were used in this study. Transfections were carried out using Oligofectamine reagent (Invitrogen Ltd.) as instructed by the manufacturer. Plasmids expressing p53 [14] or wild-type MDM2 [15] have been described previously.

2.2. Generation of tagged expression plasmids

The MYC-tagged human PLK1 construct was generated by PCR amplification of the MGC clone 2822226 (encoding human PLK1) using the following primers:

PLK1 Forward primer: ATGAGTGCTGCAGTGACTGCA
PLK1 Reverse primer: TTAGGAGGCCTTGAGACG

The PCR product was cloned downstream of a Myc 9E10 tag in the vector pSG5 (Stratagene). The inactive mutant PLK1 expression plasmid was constructed by introducing a K82M substitution into the wild-type PLK1 by site-directed mutagenesis as instructed by the manufacturer (Stratagene Inc.). For expression in *Escherichia coli*, the PCR product encoding PLK1 was cloned in the vector pGEX4T (GE Healthcare). Subsequent expression and purification of the glutathione S-transferase (GST)-PLK1 fusion protein was carried out as described elsewhere [16].

2.3. Antibodies and Western blot analysis

SDS–PAGE and western blotting was carried out using standard conditions. Nitrocellulose membranes were probed for the presence of PLK1 (antibodies PL2 and PL6, Zymed Laboratories Inc.) or for the MYC 9E10 tag, p53 (antibody DO1, Moravian Biotechnology Ltd.; or CM-1, from Sir D. Lane, Dundee), MDM2 (SMP14, D12, Santacruz Biotechnology Inc.; or 4B2, Moravian Biotechnology), EGFP (ab6556, Abcam Ltd.), or actin (20–33, Sigma–Aldrich). Appropriate secondary antibodies (HRP-conjugated) were from DAKO. Proteins were detected by enhanced chemiluminescence (Pierce Biotechnology Inc.).

2.4. Cell lysis and immunoprecipitation

These procedures were carried out as described elsewhere [17].

2.5. Luciferase reporter assays

H1299 cells were transfected with 50 ng/well of PG13 luciferase reporter plasmid (containing the synthetic PG13 p53-responsive element; [18]), 0.5 ng/well of SV-*Renilla* luciferase, 1 ng/well of p53 expression vector, 10 ng/well of MDM2 expression vector and 200 ng/well of PLK expression vector (all described above). Cell extracts were prepared and the luciferase activities were determined as described previously [18].

2.6. Protein kinase assays

Purified untagged MDM2 proteins were a gift from Dr. Mark Saville (University of Dundee). MDM2 kinase assays were performed in 60 mM HEPES–NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 1.2 mM DTT and 100 μM [γ-^{32}P]ATP (Amersham Pharmacia Biotech, at a specific activity of 12 Ci/mmol) in a volume of 20 μl. Assays were initiated by the addition of PLK1 protein kinase (generally 13 units [pmol/min] of activity) and incubated at 30 °C for 30 min. Reactions were terminated by adding SDS sample buffer and heating at 100 °C for 2 min. Phosphorylated proteins were detected following SDS–PAGE and autoradiography. Alternatively, a non-radioactive assay was used in which the ATP concentration was 100 μM and the phosphorylated proteins were detected by Western blotting using a phospho-serine 260 specific antibody (described below).

2.7. Generation and purification of Ser260 phospho-specific antibody

A rabbit polyclonal phospho-specific antibody was raised against the phosphopeptide: DSEDYS(phos)LSEEG (phospho-serine 260), coupled to keyhole limpet haemocyanin (Moravian Biotechnology Ltd.). Antibodies were affinity-purified on sepharose 4B resin to which the phosphorylated peptide had been covalently coupled and passed through a second column containing the unphosphorylated peptide to remove any antibodies that did not recognise the phosphorylated epitope.

2.8. GST-pulldown experiments

The GST-MDM2 fusion proteins used in this study have been described previously [19,20]. GST-pulldowns experiments were performed as described elsewhere [17].

3. Results

Analysis of the human MDM2 sequence revealed a PLK1 consensus phosphorylation site (E/D-X-S-Ψ-X-E: [21]) at Ser260 within the acidic domain of the protein (Fig. 1A). To investigate whether MDM2 is a substrate for PLK1, purified murine Mdm2 was incubated in the presence of [γ-^{32}P]ATP and in the presence or absence of purified recombinant PLK1. The data indicate that PLK1 can phosphorylate Mdm2 in vitro (Fig. 1B). Recombinant PLK1 was also able to phosphorylate human MDM2 (Fig. 1C); interestingly, p53, previously reported to be a substrate for PLK1 [5], performed only weakly as a PLK1 target in comparison with MDM2 (Fig. 1C). A previously characterised series of GST-(human) MDM2 fusion proteins comprising overlapping regions of MDM2 were also tested as substrates (these "mini-proteins" are termed MP1–MP4; see Fig. 1D; [20]). The data revealed that there are at least two sites of phosphorylation, located within amino acids 203–282 (major site) and 279–491 (minor site) (Fig. 1E). Following substitution of Ser260 (equivalent to ser258 in murine Mdm2) with an alanine residue in the MP3 GST-MDM2 fusion protein, phosphorylation by PLK1 in vitro was abolished, confirming that this residue is the PLK1 target (Fig. 2A). A Ser260(P) phospho-specific antibody was generated and purified: this antibody detected both human MDM2 and murine Mdm2 only after they had been incubated in the presence of ATP and active PLK1 in vitro (Fig. 2B). Notably detection of phosphorylated murine Mdm2 was considerably weaker than the human protein, possibly because there is a single amino acid difference between the human and murine proteins in the residues flanking the phosphorylation site (Fig. 1A). Several attempts to identify the minor phosphorylation site(s) between amino acids 279 and 491, using mass spectrometry followed by Edman degradation, failed to provide any rigorous identification, possibly owing to difficulties encountered in recovering sufficient phosphorylated material.

To determine whether PLK1 could phosphorylate MDM2 in a cellular context, wild-type or S260A mutant human MDM2 were

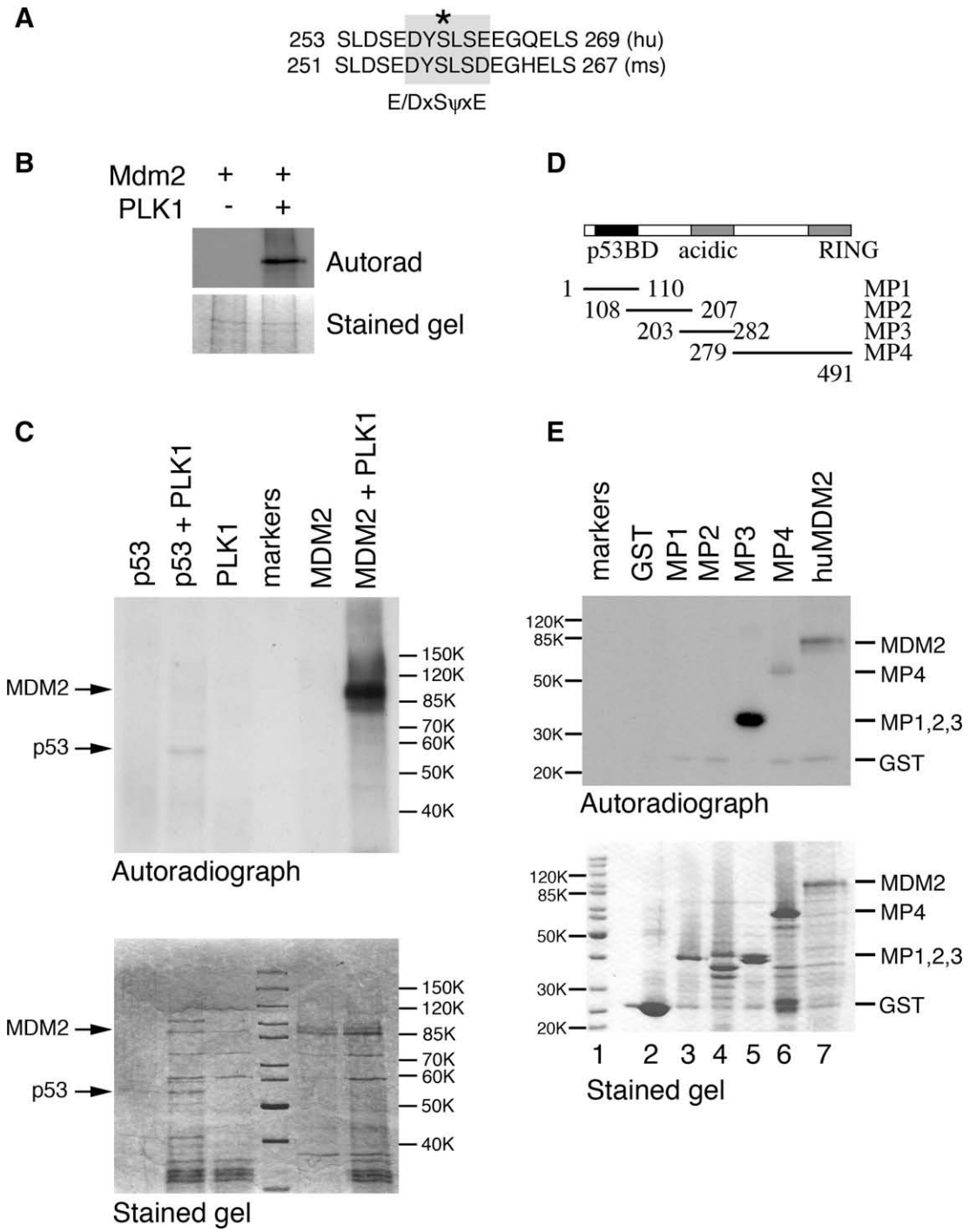

Fig. 1. PLK1 phosphorylates MDM2 in vitro. (A) Schematic of human MDM2 (upper) and murine Mdm2 (middle) amino acid sequences respectively, compared with a PLK1 consensus sequence (lower: [21]). The asterisk marks the phospho-acceptor residue. (B) Phosphorylation of murine Mdm2 by recombinant PLK1 in vitro. (C) Recombinant full length his-tagged human p53 or untagged full length human MDM2 were phosphorylated by recombinant PLK1 in vitro. (D) Schematic showing the regions of MDM2 present in a series of GST-MDM2 fusion "mini-proteins" MP1, -2, -3 and -4 used as substrates for protein kinase assays in panel (E) [19,20]. (E) GST alone or GST-MDM2 fusion proteins were phosphorylated in vitro by recombinant PLK1 in vitro. Full length untagged human MDM2 protein (lane 7) was also used as a substrate.

expressed transiently in H1299 cells in the presence or absence of active PLK1. MDM2 was immunoprecipitated from cell extracts and examined by western blotting using the Ser260P phospho-specific antibody. This antibody recognised wild-type MDM2 but not the S260A mutant (Fig. 2C). Strikingly, this signal was enhanced when wild-type PLK1, but not inactive mutant (K82M), was co-expressed, suggesting that PLK1 can phosphorylate Ser260 in cultured cells. Attempts to detect changes in endogenous MDM2 using the Ser260P phospho-specific antibody were inconclusive (data not shown). We suspect that, since this residue lies within a cluster of phosphorylation sites, the phosphorylation of neighbouring residues may diminish the interaction of the antibody with MDM2 thereby affecting the level of detectability.

To determine further whether MDM2 and PLK1 associate in cultured cells, endogenous proteins were examined by IP/western. Notably, endogenous PLK1 was observed to be present in

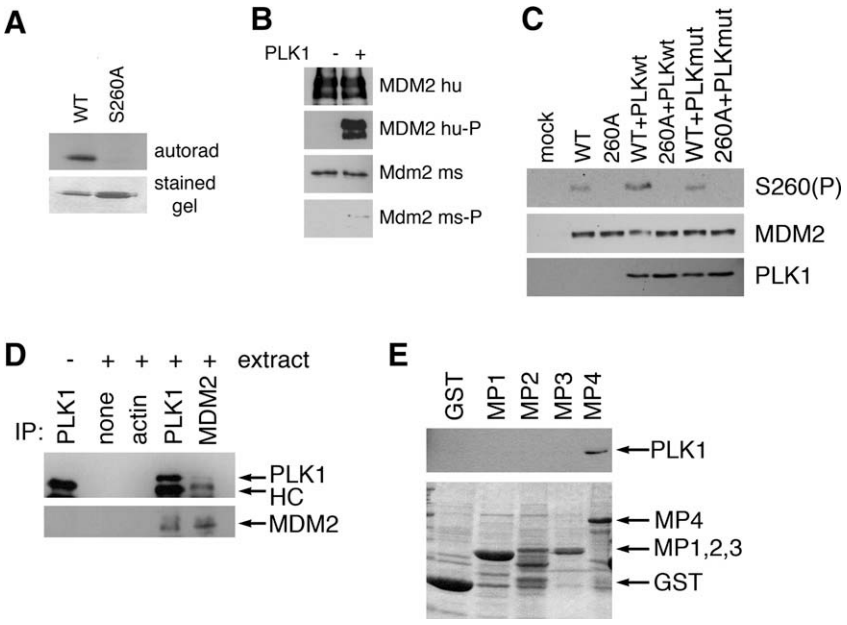

Fig. 2. PLK1 interacts with MDM2 in cultured cells and phosphorylates Ser260. (A) In vitro phosphorylation of wild-type or a S260A mutant of the MP3 mini-protein by recombinant PLK1. (B) Phosphorylation of human MDM2 (upper two panels) and murine Mdm2 (lower two panels) in vitro. The kinase assay conditions were as described above except that the ATP concentration was 100 μM and radiolabelled ATP was omitted. The phosphorylated MDM2/Mdm2 was detected using the rabbit polyclonal phospho-specific antibody raised against the phosphopeptide: DSEDYS(phos)LSEEG (human phospho-serine 260; see Section 2). (C) Transient transfection of H1299 cells with plasmids expressing wild-type or a S260A mutant of MDM2 together with wild-type or mutant (K82M) PLK1. After 48 h, MDM2 was immunoprecipitated from extracts using antibodies SMP14 and 4B2. S260 phosphorylation, MDM2 and PLK1 proteins were detected by western blotting. (D) Endogenous PLK1 (antibodies PL2 and PL6) or MDM2 (antibodies SMP14 and 4B2) proteins were immunoprecipitated from HeLa cells. The immunoprecipitates were analysed by western blotting. HC shows the position of the heavy chains of the antibody. Antibody alone (no extract) was run in the first lane to confirm the positions of the heavy chains. (E) HeLa cell lysates were incubated with GST or the GST-MDM2 mini-proteins (MP1–4 (Fig. 2B)) immobilised on glutathione-coupled sepharose 4B beads. The bound proteins were analysed by western blotting using anti-PLK1 antibodies (PL2 and PL6).

immunoprecipitates of MDM2 from HeLa cells (Fig. 2D, upper panel). Similarly, MDM2 was observed in immunoprecipitates for PLK1 (Fig. 2D, lower panel). GST-pulldown experiments, in which the MP1–MP4 mini-proteins described above were used to precipitate PLK1 from mammalian cell extracts, indicated that PLK1 can associate with the C-terminal portion of MDM2 (amino acids 279–491) (Fig. 2E). Polo-like kinases contain a "polo-box domain" which functions, in part, by interacting with a docking site containing tandem ser/thr residues that is distinct from the site of phosphorylation and provides additional specificity [22]. The C-terminal region of MDM2 contains several such tandem ser/thr residues that could act as contact points for the polo-box domain of PLK1 [22]. However, further analysis of the site(s) of interaction in vitro, using deletion derivatives of MP4, was inconclusive (data not shown).

To determine whether PLK1 could affect MDM2 function, p53-null H1299 cells were transfected with plasmids encoding p53 and/or MDM2 in the presence or absence of plasmids expressing active PLK1 or an inactive mutant of PLK1 (K82M substitution). Conditions were adjusted empirically such that expression of MDM2 reduced the level of p53 by about 50% (Fig. 3A). Under these conditions, co-expression of active PLK1 further decreased the levels of p53 (lanes 9–12). In contrast, the inactive PLK1 mutant appeared to rescue p53 from MDM2-mediated depletion (lanes 5–8). No effect on p53 levels was observed when PLK1 and p53 were expressed in the absence of MDM2 either in p53-null/MDM2-null MEFs (Fig. 3B) or in H1299 cells (data not shown). These data are consistent with the idea that PLK1 mediates its effect on p53 levels by regulating MDM2. To determine whether PLK1 could affect p53 function, p53-mediated transcription was examined (Fig. 3C). In the absence of p53, PLK1 had no detectable effect on expression of luciferase from the p53-responsive PG13 reporter plasmid. In contrast, PLK1 reduced p53-mediated stimulation of gene expression (Fig. 3C), consistent with previously published data [5]. Notably, inhibition of p53-dependent transcription by PLK1 was proportionately greater in the presence of MDM2 than in its absence (Fig. 3C). These data suggest that PLK1 may regulate p53 coordinately on at least two levels, one of which is MDM2-dependent.

4. Discussion

In the present study we show that: (i) PLK1 can phosphorylate MDM2 on Ser260 (Ser258 in murine Mdm2) both in vitro and in cultured cells (Figs. 1 and 2) and (ii) endogenous PLK1 and MDM2 proteins can interact in cultured cells; additionally, PLK1 interacts in vitro with the C-terminal domain of MDM2 which contains several tandem ser/thr resides that could act as contact points for the polo-box domain of PLK1 [22]. These data are consistent with the idea that PLK1 can interact with and phosphorylate MDM2 physiologically. We also show that PLK1 can influence the levels and activity of p53 in a manner that requires MDM2 (Fig. 3). Interestingly, recently published data indicate that PLK1 phosphorylates and activates TOPORS, one of several other p53-directed E3 ubiquitin- (and SUMO-) ligases, leading to increased p53 turnover [23]. Our data therefore fit with the idea that PLK1 can regulate p53 levels through parallel ubiquitylation pathways.

The acidic domain of MDM2 (Fig. 4) plays a critical functional role by providing a second point of contact between MDM2 and p53 (within the core region of p53) that permits efficient ubiquitylation and degradation of p53 [10,24,25]. The acidic domain is also a key target for numerous regulatory events that control p53 turnover. These include negative regulation by the ARF tumour suppressor [26] and by various ribosomal proteins ([27] and references therein). It is also modified by multiple phosphorylation events that normally maintain MDM2 activity [9]. Among these,

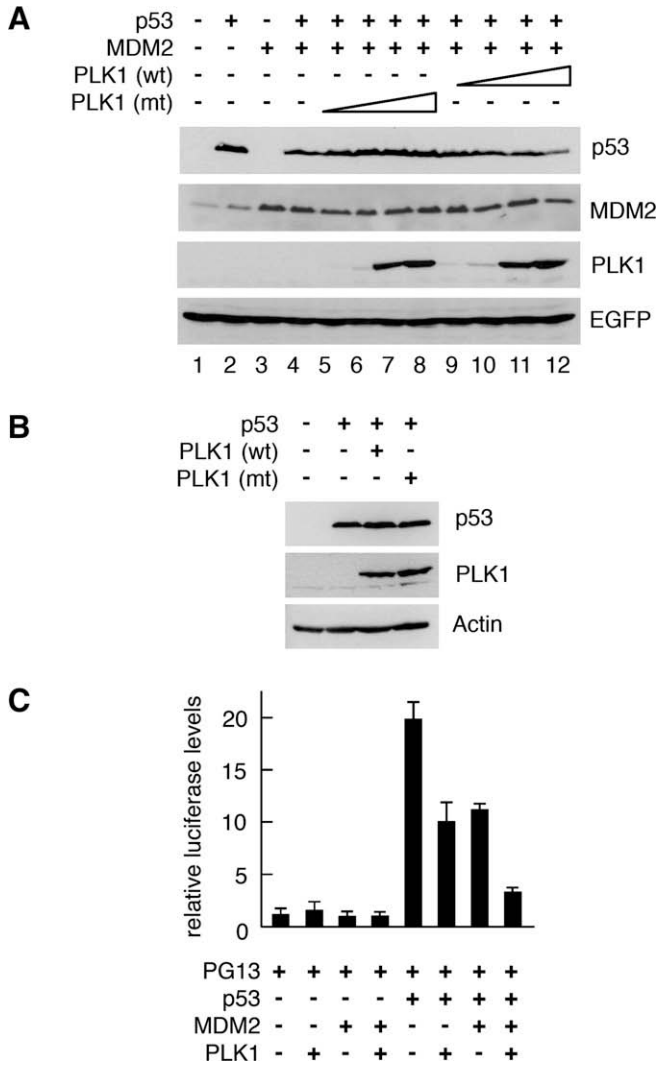

Fig. 3. PLK1 negatively regulates p53 protein levels and transcriptional activity. (A) Transient transfection of H1299 cells with plasmids expressing p53 [14], wild-type MDM2 [15] and increasing amounts of either wild-type or kinase-inactive (K82M) PLK1. The cells were lysed after 48 h and the proteins analysed by western blotting. (B) p53/MDM2 double knock-out (DKO) cells were transiently transfected with plasmids expressing p53 and either wild-type or inactive PLK1. Cells were harvested and analysed as described in panel (A). (C) p53 transcriptional activity was measured using the p53-responsive reporter plasmid PG13 (Section 2). Experiments were performed in triplicate and the data presented as the mean ± standard deviation. The level of p53 transcription in the presence of MDM2 is significantly reduced following the addition of PLK1 ($P = 0.05$).

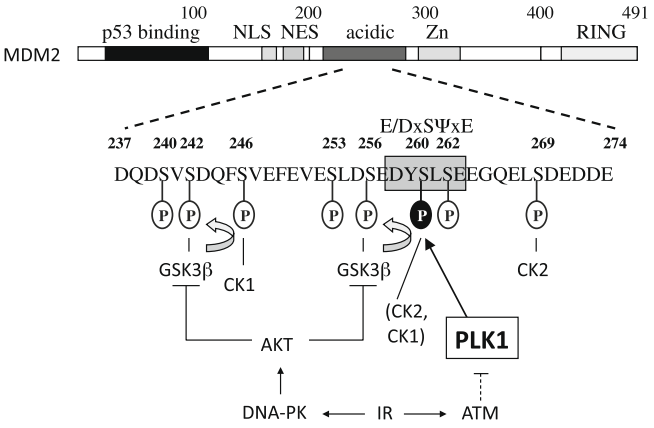

Fig. 4. Context of PLK1 phosphorylation site in MDM2. The schematic shows the salient features of MDM2 including the N-terminal p53 binding domain, the nuclear localisation (NLS) and export (NES) sequences, the acidic domain, the zinc finger (Zn) and the RING finger (RING). Part of the acidic domain is expanded to show the sequence containing the cluster of phosphorylation sites that stimulate MDM2 function. These undergo DNA damage-induce hypo-phosphorylation through a mechanism that is thought to involve DNA-PK/AKT-mediated inhibition of GSK3β, one of the key protein kinases that modify this region (discussed in the text). PLK1 phosphorylates Ser260 within this cluster of regulatory residues. This site can also be modified (weakly) by CK2 and possibly CK1 in vitro.

we showed previously that a S258/260A double mutant of murine Mdm2 (equivalent to residues 260/262 of human MDM2) had lost, in part, the ability to mediate p53 degradation [9,13]. These sites become hypo-phosphorylated in response to DNA strand breaks leading to the inhibition of MDM2-mediated p53 turnover and subsequent accumulation of active p53 [9,28] (Fig. 4). Recently published data has indicated that DNA damage-induced loss of phosphorylation of at least some of these sites can occur through a mechanism involving DNA-PK/AKT-mediated inactivation of GKS3β, one of the key protein kinases that modify this region [28] (Fig. 4). As with GSK3β, the rapid down-regulation of PLK1 in response to DNA damage [6,7] fits with the idea that hypo-phosphorylation may occur (at least in part) through inactivation of the protein kinases that modify this region (Fig. 4).

Several other protein kinases are now known to phosphorylate regulatory serine residues in the acidic domain [11–13] suggesting that this region may act as an integration point that monitors cellular homeostasis. As such, it may be sensitive to the action of various signalling molecules at any one time, each of which may be a contributor rather than a dominant effector. We suggest that PLK1 may play its part by contributing to maintenance of phosphorylation of this domain during normal cell cycle progression as cells are approaching mitosis. Regrettably, we have not been able to confirm this experimentally because siRNA-mediated knock-down of PLK1 arrests the cells in mitosis where they fail to express MDM2 (presumably because p53 cannot maintain its expression from the highly condensed chromatin) and undergo rapid p53-independent apoptosis (our unpublished data). Additionally, given that PLK1 is down-regulated as part of the G2/M checkpoint [5–7], we would also suggest that DNA damage-mediated inhibition of PLK1 might contribute to relieving suppression of the p53 pathway. This would also provide an additional, albeit indirect, layer of ATM-mediated regulation of the p53 pathway and would fit with the paradigm that ATM orchestrates events following DNA damage in a multifaceted and coordinated manner.

Acknowledgement

This study was supported by funding from Cancer Research UK.

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