

Wip1 contributes to cell homeostasis maintained by the steady-state level of Wtp53

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Wip1, a human protein Ser/Thr phosphatase also called PPM1D, stands for wild-type p53 induced phosphatase 1. Emerging evidences indicate that Wip1 can act as an oncogene largely by turning off DNA damage checkpoint responses. Here we report an unrecognized role of Wip1 in normally growing cells. Wip1 can be induced by wild-type p53 under not only stressed but also non-stressed conditions. It can trigger G₂/M arrest in wild-type p53 containing cells, which was attributed to the decreased Cdc2 kinase activity resulting at least partly from a high level of inhibitory tyrosine phosphorylation on Cdc2 protein at Tyr-15. Furthermore, we also found that Wip1 not only causes G₂/M arrest but also decreases cell death triggered by microtubule assembly inhibitor in mouse fibroblasts when wild-type p53 function was restored. These results indicate that Wip1 can provide ample time for wild-type p53-containing cells to prepare entry into mitosis and avoid encountering mitotic catastrophe. Therefore, Wip1 may play important roles in cell/tissue homeostasis maintained by wild-type p53 under normal conditions, enhancing our understanding of how p53 makes cell-fate decisions.

Introduction

Nearly all aspects of cell life are regulated by reversible protein phosphorylation. Some examples include metabolic processes, gene regulation, cell cycle control, DNA damage responses, the organization of the cytoskeleton and cell adhesion.¹⁻⁸ There are no replacements for protein serine/threonine phosphatases, which play a crucial role in cell life. Protein phosphatase 2C is one of four major types of protein serine/threonine phosphatases [PP1, 2A, 2B and 2C] in eukaryotic cells. They in general have 11 motifs conserved within the subfamily,⁹ and these motifs could be identified in sequences of numerous other proteins, which are called PP2C-related proteins. It has been known that the PP2C family and its related proteins are essential for regulating cellular stress responses in eukaryotes.^{10,11}

The p53 is known to play crucial roles in cellular stress responses.^{12,13} p53 guardian function has been implicated in various aspects of cellular life besides stress conditions.¹⁴⁻¹⁶ The function of p53 is largely mediated through its transactivation activity to activate its target genes and fulfill its function.¹⁷⁻²³ Such as waf1/p21 is a cyclin-dependent kinase inhibitor that is capable of inhibiting cdk's activities and plays a major role in p53-dependent G₁ cell cycle checkpoint.²⁴⁻²⁶ Puma, Noxa, and others play important roles in mediating p53-dependent apoptotic responses.²⁷ G₁ and G₂ checkpoints are major genomic surveillance mechanisms,

which exist at G₁/S and G₂/M transitions respectively.^{28,29} Unlike p53's role in G₁ checkpoint responses, largely relying on transactivating p21/waf1,²⁴ a role for p53 in G₂ cell cycle arrest is less well defined. Cells that do not contain wtp53 are deficient for G₁ checkpoint response, but can undergo a G₂ arrest.³⁰⁻³² However, elevated steady-state level of p53 by ectopic expression is sufficient to induce cell cycle arrest at both G₁/S and G₂/M transitions by inducing p21/waf1, 14-3-3 δ , and/or Gadd45.³³⁻³⁹ Taken together, these results suggest that the role for p53 surveillance in G₂ phase of cell cycle is much more complicated and much less well defined compared with its role in controlling the progression of cells in G₁ phase of cell cycle.

Wip1 was identified from the screen of p53 targeted genes in response to ionizing radiation (IR) and has close similarity to a number of known type2C phosphatases.⁴⁰ The initial information that Wip1 expression in response to ionizing radiation (IR) is in a wtp53 dependent manner and ectopic expression of Wip1 can suppress colony formation, suggested that Wip1 might be involved in growth inhibitory pathways activated in response to DNA damage to contribute to p53 tumor suppressor activity.⁴⁰ However, the studies reported in the following decade indicate that Wip1 most likely functions as an oncogene. Analysis of primary human breast tumors provided the first hint for the role of Wip1 as an oncoprotein; Wip1 gene, also called PPM1D gene, is amplified and Wip1/PPM1D mRNA is overexpressed in 15%

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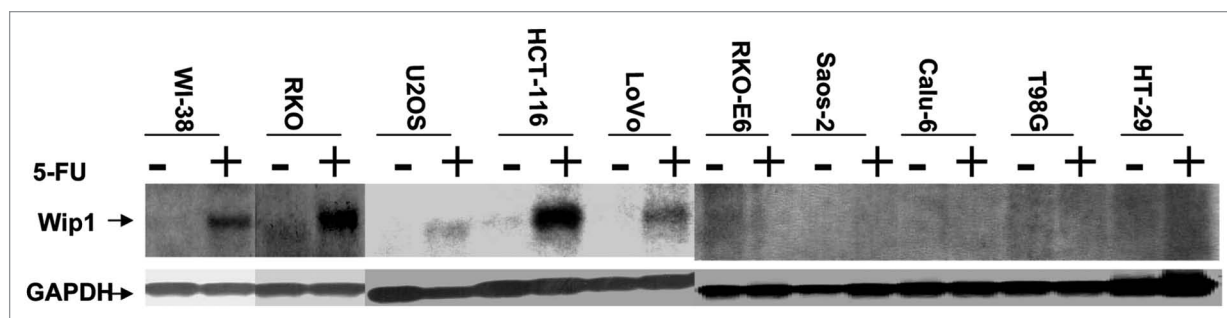


Figure 1. The expression of Wip1 depends on wtp53 status. Total RNA isolated from WI-38, RKO, U2OS, HCT-116 and Lovo, which all carry wtp53, and from RKO-E6, Saos-2, Calu-6, T98G and HT29, which all harbor mtp53 without (-) or with (+) the treatment of 5-FU respectively was hybridized to the ³²P-labeled Wip1 cDNA or GAPDH cDNA fragment. Wip1 mRNA level is substantially elevated only in cell lines having wtp53 following 5-FU treatment (+). (GAPDH used as RNA loading control). P21/waf1 mRNA expression was detected in the same batch of RNA samples used. The pattern of P21/waf1 mRNA expression is similar to the one of wip1 mRNA expression (data not shown), confirming the activity of wtp53.

of breast cancers.^{41,42} Substantial experimental evidence indicates that Wip1 can act as a negative regulator of several tumor suppressors through removing phosphate from these proteins, including ATM, CHK2, p38^{MAPK}, p53, INK4A and ARF.⁴³⁻⁴⁸ Modulation of these key checkpoint proteins by Wip1 ultimately can attenuate p53 tumor suppressor responses.^{43,45,46,49} Here we report that Wip1, working in concert with the steady-state level of functional p53, provides ample time for cells to be ready for mitosis, which is at least partly mediated through increased inhibitory phosphorylation level on Cdc2. This study advances our understanding of how wtp53 maintains cells at G₂/M boundary and how Wip1 functions as a target to contribute to p53 surveillance function as a cell-fate decision maker.

Results

Upregulation of Wip1 by a higher level of p53 expression in both stress and non-stressed cells. Wip1 was identified in cells exposed to ionizing radiation (IR) in a wtp53-dependent manner.⁴⁰ To define how specifically Wip1 induction relies on p53 status upon other stresses, DNA damage agent 5-FU (Fluorouracil) was used, which is often used clinically as an anti-cancer drug.⁵⁰ Human fibroblast WI-38 cell line and several tumor cell lines were examined for Wip1 expression following exposure to 5-FU. Four of them (Lovo, HCT116, RKO, U2OS) carry a wt p53 gene as does WI-38. The rest of them (HT29, Calu-6, Saos-2, T98G and RKO-E6) harbor mutations or functional defects of p53. Wip1 mRNA expression was determined by RNA gel blot analysis of RNAs from all cell lines with or without exposure to 5-FU. As shown in **Figure 1**, Wip1 expression was clearly detectable in 5-FU treated cells carrying wtp53, but not in untreated corresponding cells or in cells with deficient p53. This indicates that the induction of Wip1 in response to 5-FU treatment is dependent on the existence of wtp53. Taken together, wtp53 status appears to be essential for induction of Wip1 in stressed cells, consistent with findings that wip1 is a target of p53,^{40,48} which may partly mediate p53 function.

The induction of Wip1 in response to DNA damage triggered by IR or 5-FU demonstrates the coexistence between the

increased expression of Wip1 and wtp53 in stressed cells, suggesting their functions may partly depend on each other. To further reveal Wip1 expression relies on wtp53 status, we asked whether an increased expression of Wip1 depends on wtp53 is also true in non-stressed cells. GM47.23 cell line,⁵¹ a derivative of T98G that carries a hormone-inducible wild-type p53 transgene was used. RNA gel blot analysis of RNA from induced (wild-type p53⁺) and non-induced (endogenous mutant p53⁻) GM47.23 cells were performed. We found that Wip1 mRNA was detectable only in cells induced to express wtp53 (**Fig. 2A**). Wtp53 function was confirmed by the expression of p21, which was clearly detected only in Dex-induced GM cells, but not in non-Dex added GM cells or in GM parental cell line—T98G cells with or without Dex addition (**Fig. 2B**). These results demonstrate that wtp53 alone can upregulate the Wip1 expression, suggesting that a steady-state level of p53 also can regulate Wip1 expression, which is not solely depending on stress-activated wtp53.

Wip1 induction of G₂/M arrest. Past 30 y studies demonstrate that p53 is a critical checkpoint protein upon a variety of genotoxic stresses to guard genomic stability and suppress tumor development.^{13,52-54} However, emerging studies indicate that p53 also plays essential roles in cells that do not receive clear genotoxic stresses. But this aspect of p53 function remains much under-investigated compared with its function triggered by genotoxic stresses. Above study indicated that a higher steady-state level of wtp53 is also responsible for a higher level of Wip1 expression. We therefore anticipated that Wip1 may play important roles in mediating p53 function in cells growing under normal conditions. To this end, we decided to investigate how a changed magnitude of Wip1 expression affects non-stress cell behavior, in hope of providing new insights into roles of p53 in cells growing under normal conditions. U2OS-15 and TWIF stable cells, derived from parental cells carrying wtp53 or non-functional p53 respectively, were established, in which Wip1 transgene can be conditionally expressed upon the addition of a conditional inducer, Dex (**Fig. 3A and B**). The cell cycle analysis was subsequently performed, and Wip1 was found to be capable of inducing G₂/M arrest in U2-15 cells (**Fig. 3C**) but not in TWIF cells (**Fig. 3D**), suggesting Wip1 can provide enough time for wtp53-containing cells to

prepare entry into mitosis, and thus mediate the function of p53 in G₂/M check under normal growth conditions.

Wip1 induction of G₂/M arrest was also found in mouse cells with “normal” genetic background. Tumor cell lines may differ in ways other than only a p53 status. Therefore, it needs to be further validated that Wip1 induction of G₂/M arrest found in tumor cells shows a wtp53 dependent manner. We next tried to use two known genetic background cell lines—mouse fibroblasts with p53^{-/-} or p53^{+/+} derived from p53 knockout mice and normal mice respectively, as parental cell lines to establish two stable cell lines that can conditionally express Wip1 transgene. As shown in Figure 4A, the derivative cells of mouse fibroblasts with null p53 (named MF-41) were established and capable of conditionally expressing Wip1 transgene, but not those carrying wtp53, because the mouse fibroblasts with p53^{+/+} only survived a few passages. To directly study the relationship between wtp53 and the function of Wip1 seemed to be a problem when using mouse fibroblasts with wtp53, consistent with the concept that wtp53 can lead to cell arrest or/and apoptosis. Tsp53Val135 is murine p53 class II mutant, which holds wild-type (wt) conformation at 32°C and turns into mutant (mt) conformation at 37°C. It has been suggested as a tool to analyze whether, and how cells were growth-arrested or driven into apoptosis by wtp53.^{34,55,56} We thereby put tsp53 into MF41 stable cells, in which both Wip1 and wtp53 can be conditionally expressed (Fig. 4B). The clone 23 cells, derived from MF41, were established to conditionally express p53 protein at a level of about two-fold of endogenous p53 (Fig. 4B). Also, clone 23 cells at 37°C are basically like its parental cells MF41 wherein p53 is non-functional and Wip1 can be conditionally expressed; but at 32°C, the function of Wip1 can be studied on the basis of functional p53. Cell cycle studies were subsequently performed to define the relationship between wtp53 and Wip1 induction of G₂/M arrest in these cells carrying known genetic background. As usual, cells should be synchronized first for cell cycle analysis. Mouse fibroblasts, however, are too rigid to be synchronized when using conventional starvation method. The exponentially growing cells were thus tentatively used to prepare for flow cytometry. The cell cycle analysis (Fig. 4C) on asynchronized clone 23 cells (wip1⁺, tsp53⁺) at 32°C shows G₂ arrest under the condition of the expression of wip1, but, not at 37°C, indicating Wip1 induction of G₂/M arrest in normal cells also depends on wtp53.

Wip1 not only induces G₂/M arrest but also decreases cellular apoptotic response. Although we found consistent results between mouse fibroblasts carrying normal genetic background and human tumor cells with genomic instability, we did not stop to find a way of synchronization for these mouse cells used. Nishii et al. reported that microtubule formation inhibitor Nocodazole can be used to synchronize cells at early M phase reversibly with variable levels of apoptosis among cell types. Indeed, these mouse cells showed a relatively better G₂-rich population with a considerable percentage of cells undergoing apoptosis when treated with Nocodazole (Fig. 5A). Next, Both MF41 and Clone 23 cells were synchronized with Nocodazole and prepared for cell cycle study. We found that, at the 9-h point, the induction of Wip1 did not show any effect on cell population distribution in

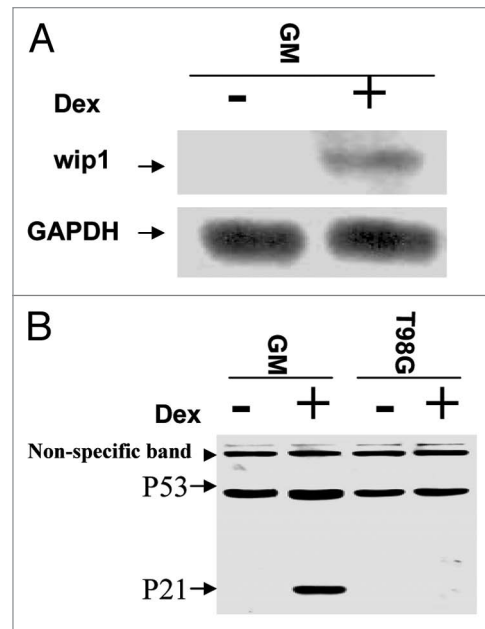


Figure 2. The Wip1 is induced by an elevated steady-state level of wtp53. (A) Autoradiogram of RNA gel blots using either Wip1 cDNA or GAPDH as probe. Lanes: marked (-), GM47.23 cells without Dex (control, no induction of wtp53); Marked (+), GM47.23 cells with Dex (for the induction of wtp53). (GAPDH used as a RNA loading control). The Wip1 mRNA can be detected in GM cells with the wtp53 induction. (B) Western blots analysis of p53 and p21 proteins in both GM and its parental cell line T98G cells. The p21 is only detected in GM cells with the addition of Dex (+), indicating the activity of wtp53.

comparison with non-induction of wip1 at both 32°C and 37°C; however, at the 12-h point, Wip1 induction induced a significant increase in G₂ cell percentage and a decreased percentage of sub-G₁ at 32°C, but, not at 37°C (Fig. 5B). These results indicate that Wip1 can induce G₂/M arrest and decrease cell death response when functional p53 is present (32°C). The corresponding controls were provided to make certain that Wip1 effect did not result from the temperature switch that may misinterpret the dependence on wtp53. As seen in Figure 5B, induction of Wip1 in MF41 cells has no effect on cell cycle progression either at 32°C or 37°C. Furthermore, western blotting analysis indicates that Wip1 protein expression responds to inducer Dex to indicate that the conditional expression system remains to be intact; Figure 5C, once again, suggests that the effect of Wip1 observed in the systems used is wtp53-dependent, and not a non-specific effect.

Wip1 induced G₂/M arrest is at least attributed to the decreased Cdc2 kinase activity. Induction of G₂/M arrest by a higher level of Wip1 expression was found in both tumor and normal cells carrying wtp53, indicating Wip1 must play substantial roles in regulating G₂/M cell phase transition of wtp53-containing cells. The transition from G₂ to M phase is controlled by Cdc2 kinase activity, and G₂ arrest is associated tightly with an inhibition of Cdc2 activity. Therefore, Cdc2 kinase activity was examined in U2-15 and MF-tstp53 cells (Clone 23 cells) with functional p53 (Fig. 6A and B) and non-functional p53

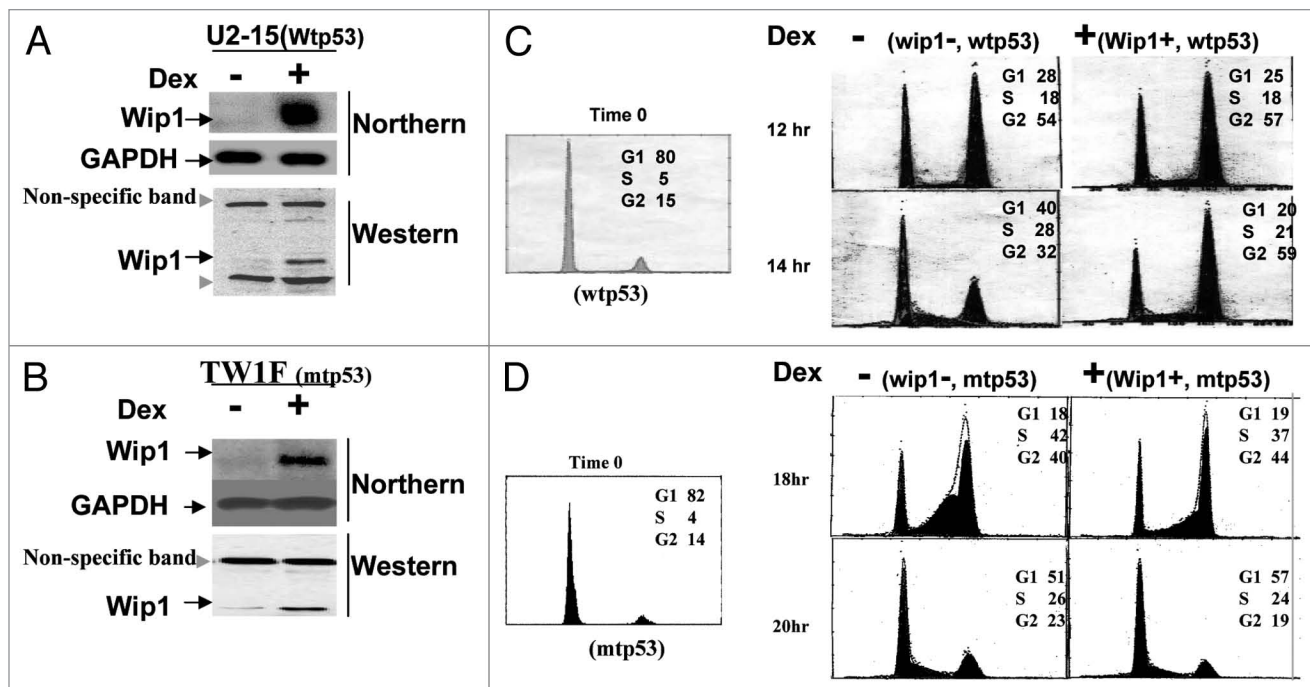


Figure 3. The Wip1 induces G_2/M arrest in cells carrying wtp53 but not in cells harbouring mtp53. (A and B) Establishment of conditional Wip1 expression cells. RNA gel blots were prepared using total RNA isolated from un-induced (-Dex) and induced (+Dex) clone U2-15 (A) and TW1F cells (B) derived from U2OS (wtp53) and T98G (mtp53) cell lines respectively, detected by ^{32}P -labeled Wip1 or GAPDH cDNA fragment. The Wip1 mRNA is increased substantially in cells with the addition of Dex. (GAPDH is loading control). The corresponding levels of Wip1 protein expression were also detected. (C) Wip1 induction of G_2/M arrest in U2-15 cells. Synchronized U2-15 cells (left part) were released and incubated with (+) or without (-) Dex. Cells were collected at the time of 8 h in 2 h interval respectively after starvation synchronization (left part). The fitting curves from Flow Cytometric Analysis showed (right part) are cell cycle profiles for cells tested at 12 and 14 h points. At 14 h Wip1 induction, U2-15 cells could not move from G_2/M into G_1 , as they did in the situation of non-induction of Wip1. (D) Induction of Wip1 has no effect on TW1F cells. Synchronized TW1F cells (left part) were released and incubated with (+) or without (-) Dex. Cells were collected at the time of 14 h in 2 h interval respectively. The fitting curves are cell cycle profiles of 18 h and 20 h points (right part). The transition from G_2/M peak to G_1 peak is similarly shown in both Wip1 induced (+) and non-induced (-) TW1F cells. (pMSG-PP2C stable cells were established, and used to conduct a similar cell cycle analysis; but induction of PP2C has no clear effect on cell cycle progression (data not shown), indicating G_2/M arrest triggered by Wip1 is not a general effect of PP2C phosphatase family).

(Fig. 6C). Cdc2/cyclinB1 complex immunoprecipitated from the soluble fraction of cells by using monoclonal antibody anti-Cdc2, was mixed with γ - ^{32}P -ATP and bovine histone H1 to perform a standard H1 kinase assay.⁵⁸ We found that the induction of Wip1 can decrease the activity of Cdc2/cyclinB1 in functional p53-containing cells (Fig. 6A and B), not in cells carrying non-functional p53 (Fig. 6C) in comparison with the non-Wip1 induced cells, which is not a result of a different amount of Cdc2 protein used in the assay. Therefore, consistent with cell cycle analysis on tumor and mouse cells, the reduction in Cdc2 kinase activity was also found in tumor cells and mouse fibroblasts carrying normal genetic background, which appears to be a plausible cause of G_2/M arrest induced by Wip1 (Fig. 6).

Increased level of tyrosine phosphorylation on Cdc2 may partially contribute to the decreased Cdc2 kinase activity. Cdc2 kinase activity basically can be affected by expression levels of Cdc2 itself or/and its partner cyclin B. To this point, we decided to examine expression levels of both proteins in these interested cells with or without overexpressed Wip1. Protein lysates, prepared from the same batch of U2-15 cells used for FACS and kinase assay,⁵⁸ were used for protein gel blot analysis on the levels of cdc2 and cyclin B protein expression. As shown in Figure 7A,

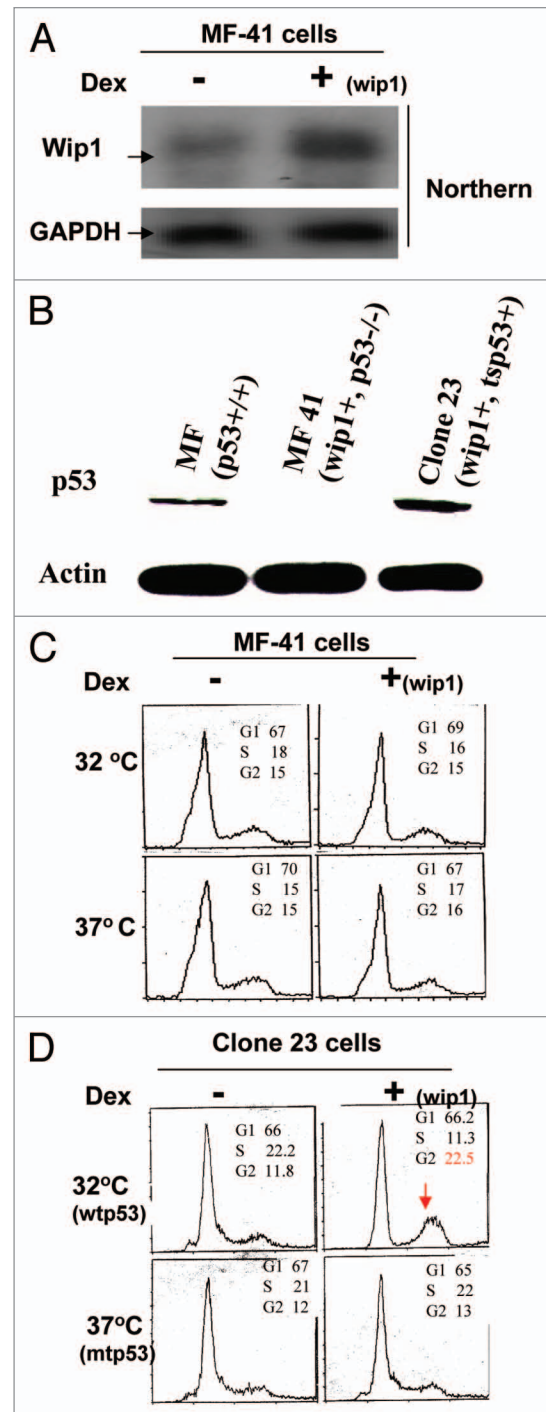
no significant difference in the levels of these proteins is detected when Wip1 is induced compared with un-induced control cells. Therefore, both levels of cdc2 protein and cyclin B1 protein did not appear to be a cause of decreased Cdc2 kinase activity. At G_2/M transition, Cdc2 kinase activity is not only determined by the levels of Cdc2 and cyclin B proteins but also by many post-translational modifications on Cdc2 protein, including an inhibitory tyrosine-15 phosphorylation level on Cdc2 protein.⁵⁹ The relative level of tyrosine-15 phosphorylation on Cdc2 protein was thereby examined by immunoprecipitation using an anti Cdc2 monoclonal antibody and western blotting of the immunoprecipitated Cdc2 by using a monoclonal antibody specifically for phosphorylated tyrosine-15 on Cdc2. The result showed that the level of tyrosine-15 phosphorylation was significantly increased on Cdc2 in Wip1 induced cells (Fig. 7B). Again, mouse clone 23 cells were correspondently treated and prepared for cyclin B and Cdc2 western blotting and detection of tyrosine phosphorylation level on Cdc2 protein, and the results were consistent with what shown in Figure 7B (data not shown). Collectively, Wip1 can promote an inhibitory level of tyrosine phosphorylation on Cdc2, which is able to interfere with Cdc2 kinase activity conferring a G_2/M arrest.

Figure 4. Wip1 induction induces G_2/M arrest in un-synchronized mouse cells carrying functional p53. (A) The Wip1 mRNA expression in mouse fibroblast ($p53^{-/-}$) derivative cells (MF-41). RNA gel blot was prepared using total RNA isolated from the induced (+) and un-induced (-) No.41 clone, which is named MF41, and detected by ^{32}P -Wip1 cDNA fragment. The Wip1 signal was increased in lane marked (+). (B) Western blot was prepared using 50 μ g/lane of cell soluble protein from mouse fibroblast (MF) ($p53^{+/+}$), MF 41 ($wip1^+$, $p53^{-/-}$) and clone 23 derived from MF41 ($tsp53^-$; $wip1^+$), detected with primary antibodies against p53 and actin respectively. The p53 protein signal is shown in MEF and clone 23, but not in MF41 cells. (The actin signal used as loading control). (C and D) Asynchronized MF-41 and clone 23 cells were collected after O/N induction (+) or non-induction (-) of Wip1 at both 32°C (wtp53) and 37°C (mtp53). The cell cycle profiles presented in DNA content histogram show that the Wip1 induction can clearly increase cell population in G_2 phase when functional p53 is present (at 32°C). (Red arrow head marks a higher G_{2M} of cells compared with other cells tested).

Discussion

Since the discovery of p53 over 30 y ago, it is now recognized that all cell fate pathways of live cells are virtually under the control of p53. Such extensive involvement indicates that p53 protein is acting as a major traffic controller in the cell signaling network.^{12,27,60} But still how p53 acts at a cross road of live or death cell signaling pathway is difficult to answer. Our finding demonstrates Wip1 is involved in such a pivotal, complicated task of p53. As shown in **Figures 1 and 2**, both stressed-activated p53 and elevated steady-state level of p53 can induce Wip1, indicating p53 function is attributed at least partly to Wip1 under both stressed and non-stressed conditions. A large body of evidences indicates p53 exerts pivotal roles in guarding genomic stability upon many stress conditions.^{12,54,61} However, it is less well understood for its role in protecting cells under non-stress conditions. We revealed that, in non-stressed cells, Wip1 can induce G_2/M arrest and decrease basal level cell death in wtp53-containing cells, suggesting Wip1 plays important roles in p53 maintaining cell/tissue homeostasis, consistent with the recent findings that p53 can maintain oocyte genomic stability via its target LIF (Leukemia inhibitory factor).^{14,62} Furthermore, our study revealed the mechanisms by which Wip1 induces G_2/M arrest. We found a decreased Cdc2 kinase activity, leading to a G_2/M arrest, is at least attributed to an elevated level of inhibitory tyrosine phosphorylation on Cdc2. At the moment, we remain unknown of how Wip1 caused elevated levels of tyrosine phosphorylation on Cdc2 protein. Further studies are needed on revealing more in-depth molecular controls by which Wip1 induces G_2/M arrest. Nonetheless, our study suggests that Wip1 can provide necessary time needed for wtp53-containing cells to have a regular cell cycle progression from G_2 to M; otherwise, cells will be rushed into M phase of cell cycle and commit suicide owing to a limited preparation time at G_2 . Therefore, Wip1 plays an important role in maintaining cells carrying wtp53 particularly at G_2/M cell cycle transition; and cells harbor mutated p53, or a low, null level of Wip1, would not have ample of time at G_2 phase of cell cycle, and thereby encounter a mitotic catastrophe.

Accumulated studies indicate that Wip1 can inactivate ATM, CHK2, p38^{MAPK}, MDM2 and p53 itself upon a variety of genotoxic stresses.^{43,45,46} Therefore Wip1 appears to act as an oncogene,



and promotes tumor formation. We believe that Wip1 plays an important role in p53 guarding the steady-state levels of cellular responses. This feature of Wip1 can be turned into either good or bad in a cell context dependent manner. As we discussed above, when Wip1 is not around under normal condition, wtp53 containing cells may lose a proper time needed before entry into mitosis, which was in agreement with our antisense study in U2OS cells showing a little faster G_2/M transition (data not shown). Understandably, these normal cells with proper cellular checkpoint responses would die when rushed into M phases. On the other hand, if cells are undergoing neoplastic transformation,

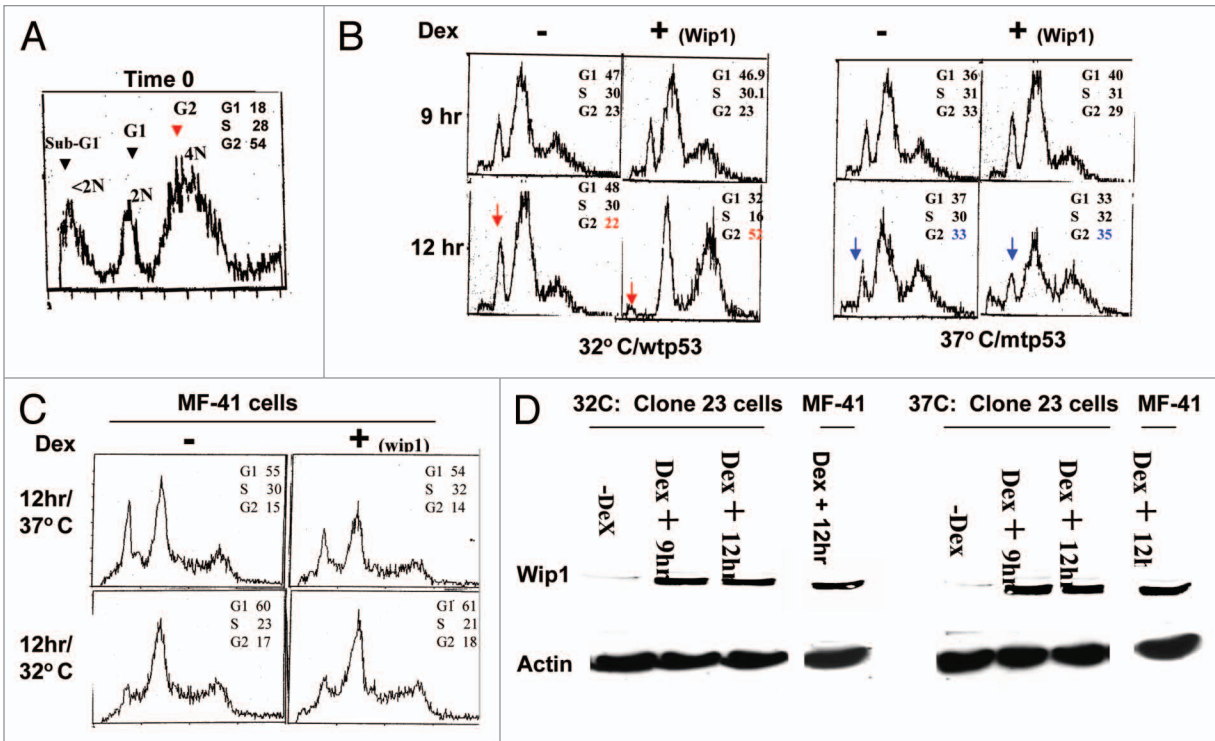


Figure 5. Wip1 induces G_2/M arrest but also decreases cellular apoptosis in Mouse fibroblasts. (A) Mouse fibroblasts were treated with Nocodazole (0.1 $\mu\text{g}/\text{ml}$) for 40 h. The cell cycle profile of these treated cells shows a relatively nice synchronization at G_2 population. (B) Cell cycle analysis on Nocodazole-synchronized clone 23 cells shows a better G_2 arrest triggered by Wip1 in cells carrying functional p53. The cell cycle profiles presented here in DNA content histogram are those of 9 h and 12 h at 32°C (left part) and 37°C (right part). Wip1 induction (+) can increase cell population in G_2 phase and decrease sub- G_1 peak (arrow pointed) at time of 12 h at 32°C, but not at 37°C. (C) Wip1 induction has no effect on synchronized MF-41 cells. (D) Wip1 protein expression in both MF41 cells and clone 23 cells upon the Dex addition. Left part is for cells at 32°C and right part is for cells at 37°C, and both show the correct levels of Wip1 protein expression in cells used for cell cycle analysis, indicating the conditional system remains intact.

the loss of Wip1 may favor the tumor suppression, as a result of cell rushing into M phase of cell cycle and encountering the death check suggested from our study (Fig. 5A), thereby Wip1 appears to act as an oncogene. This is also in agreement with an earlier reported study that silencing Wip1 suppressed tumor formation.⁴⁵ We believe tumor suppression activity of Wip1-loss is at least partly attributed to a shorter- G_2 preparation, which renders cells to commit a suicide. When Wip1 is overexpressed again in cells with a neoplastic context, the decreased apoptotic potential caused by a high level of Wip1 (Fig. 5B) would promote tumor formation. Amplification of wip1 gene functioning in breast tumorigenesis might be a scenario we discussed here, under which Wip1 helps tumors cells escaped from the elimination presumably enforced by a mitotic checkpoint. Therefore our study provides cellular behaviors influenced by Wip1, a part of which is consistent with the initial finding regarding the function of Wip1 at the cellular level, capable of inhibiting colony formation in tumor cells tested. We revealed that, in tumor cells, Wip1 can induce G_2/M arrest (Figs. 3C and 4C), which apparently contributes to colony formation; however, a decreased apoptotic response resulting from Wip1 (Fig. 4C), probably unable to show clearly owing to a low level of basal apoptotic response in some tumor cells, contributes little to promoting the colony growth. Cancer cells in general harbor a compromised apoptotic response

that may lead to a disguised phenotype when study gene functions in tumor cells. Indeed, when we reexamined Wip1 function in cells with normal genetic background, it turned that Wip1 not only causes G_2/M arrest but also decreases substantial apoptotic activity, which is consistent with the concept that Wip1 acts as an oncogene, thinking that apoptotic potential is a major determinant for tumor suppression. Here, the function of Wip1 we found is important for p53 guiding tissue homeostasis, because normal cells or tissues need to have ample of time to prepare entry into mitosis rather than rushing into M phase to be eliminated by surveillance mechanism. Collectively, Wip1 may perform a further check function for normal cells before entry into mitosis; otherwise cells would die in mitosis. This study reveals unrecognized roles of Wip1 in p53 guarding the steady-state levels of cellular responses, which can be easily extended and aligned with its known effects on cells carrying genetic instability, thereby providing further understanding of how p53 orchestrates cellular fate decisions to die or to live, and how these functions of p53 can be swung around by its targets in a cell context dependent manner.

Materials and Methods

Cell lines and cell culture. The following eight cell lines were obtained from the American Type Culture Collection (ATCC):

Saos-2, U-20S, T98G, HT-29, Lovo, HCT116, Calo-6 and WI-38. The RKO and RKO-E6 were generously provided by Dr. Vogelstein at the Johns Hopkins University. All the above cell lines were maintained routinely in MEM medium supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 µg/ml). Mouse fibroblasts with p53^{+/+} and p53^{-/-} were obtained from the laboratory of Dr. Lawrence A. Donehower. These cell lines were maintained routinely in DMEM/F-12 medium supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 µg/ml). All cell lines were incubated in 37°C incubator with 5% CO₂ ambient as described in reference 51. For a specific culture condition, the incubation temperature is 32°C, which will be specified in the experiment. For trypsin treatment, cells were washed in PBS-CMF (calcium and magnesium free) twice, followed by appropriate incubation time with 0.25% (m/v) trypsin at RT. Trypsin activity was quenched by addition of complete medium. GM47.23 cells are derivative cells of T98G that can conditionally express wtp53 upon the addition of Dexamethasone (Dex).⁵¹

Plasmids and sub-cloning. pMSG (purchased from Pharmacia) is an eukaryotic expression plasmid containing the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter and the gene encoding Eco-gpt selectable marker driven by the SV40 early promoter.^{63,64} Wip1 cDNA was inserted at EcoRI site of MCS of pMSG, and under the control of MMTV promoter. The plasmid pMSG-PP2C was a kind gift from Dr. Appella (NCI). pBabe-tsp53V135 was a kind gift from Dr. Skorski (Temple University).

RNA gel blotting analysis and the probe preparation. Total RNA isolation and RNA gel blotting were performed as described in references 23 and 51. Briefly, 15 µg of total RNA from each cell line was electrophoresed through a 1.0% agarose gel. The RNA was transferred to a nylon membrane, and UV-crosslinked. The membrane was pre-hybridized and hybridized with 10⁶ cpm/ml of radiolabeled cDNA fragments, and subsequently washed. The RNA-cDNA hybrids were exposed to X-ray films. To normalize the loading of RNA, the blot was stripped in the boiling water and rehybridized with radiolabeled cDNA fragment of GAPDH. 2.4 kb wip1 cDNA fragment and GAPDH fragment were labeled with a random hexamer primed synthesis kit (Promega).

Transfection and the stable clone(s) selection. The whole procedures were performed as described in reference 51. Briefly, 10 µg of pMSG-wip1 plasmid was transfected into a 100 mm dish of U2OS cells, T98G cells, Mouse fibroblasts (p53^{-/-}) or (p53^{+/+}), or 10 µg of pBabe-tsp53V135 was transfected into a 100 mm dish of MF41 cells. 48–72 h later, cells were reseeded in gpt⁺ medium⁵¹ for the selection of cells containing pMSG-wip1 or 2 µg/ml puromycin was added for selection of pBabe-tsp53V135. After 3–5 weeks of selection, each individual clone was isolated and expanded until further verified by RNA gel blot analysis and western blot analysis.

Western blot analysis and immunoprecipitation. As performed essentially in,^{64,65} primary antibodies targeting Cdc2, cyclinB1, phosphotyrosine-15 of CDC2, Wip1, actin, p53 or p21 (Santa Cruz) all were used at a ratio of 1:1,000 dilution, and the 2nd antibodies—horseradish peroxidase conjugated

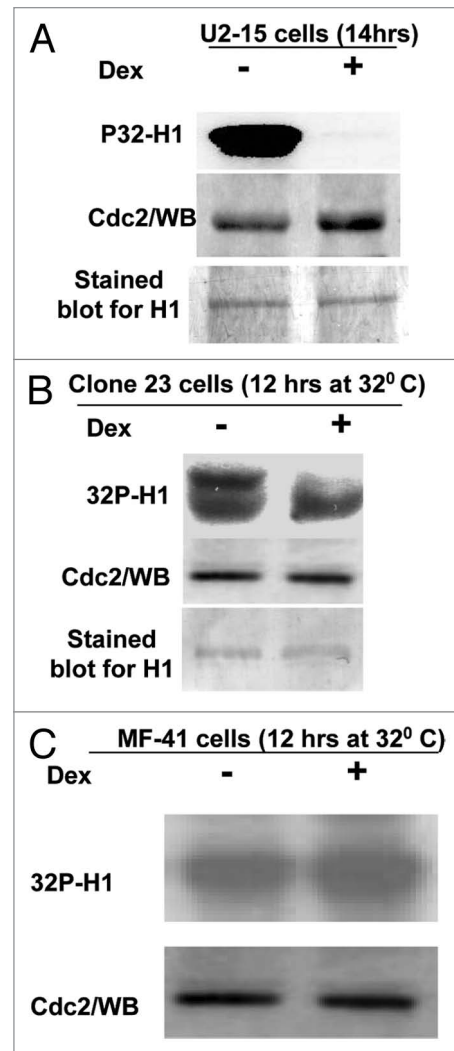


Figure 6. Induction of Wip1 inhibits Cdc2 kinase activity. (A) Wip1 modulates the kinase activity of Cdc2/cyclinB. Immune complexes from the total soluble protein of 12 h un-induced (Lane⁻) and induced (lane⁺) U2-15 cells using anti-cdc2 monoclonal antibodies were mixed with histone H1 and γ -³²P-ATP at 30°C for 30 min. Autoradiogram (top part) shows much less phosphorylated histone H1 in Lane⁺ than in Lane⁻. [Wb (middle part) for the control of cdc2 protein used in the assay and Coomassie blue-stained blot (bottom part) for H1 loading control]. (B and C) Cdc2 kinase activity is decreased upon Wip1 induction in mouse cells when functional p53 is restored. Similar to the experiment conducted in (A), the corresponding mouse cells used for cell cycle analysis showing a nice G₂/M arrest were used for preparing cell lysates, and subsequently Cdc2 IP and kinase assay were performed. The decreased Cdc2 kinase activity was found when Wip1 was induced in clone 23 cells (B) but not in MF-41 cells (C) at 32°C.

anti-rabbit or mouse IgG (Amersham) were used at a ratio of 1:10,000 dilution. The membrane was stained with Coomassie blue for the verification of the sample loading if actin signal was not detected. For IP, 1 µg of anti Cdc2 monoclonal Ab agarose conjugate (Santa Cruz) was added to 500 µg of cell lysates, and the mixture was rotated for binding at 4°C for 3 h or overnight. The pellet was washed two times with lysis buffer, another two times with kinase reaction buffer (if the precipitates needs for

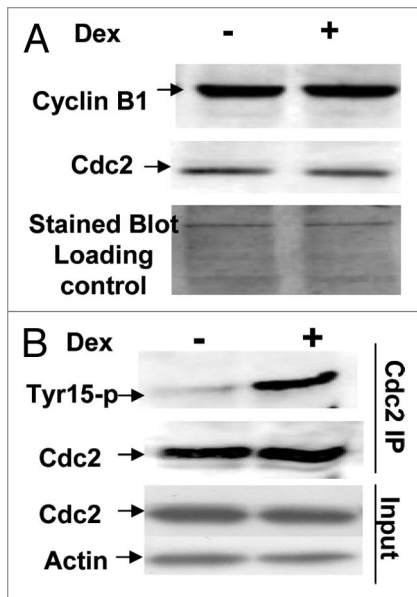


Figure 7. Induction of Wip1 increases the level of inhibitory tyrosine phosphorylation on Cdc2 protein. (A) The levels of nuclear cyclin B and cdc2 proteins are the same between the Wip1 induced and uninduced cells at time of 12 h. The western blot prepared from the nuclear fraction of Wip1 induced (+) and uninduced (-) U2-15 cells at time of 12 h, was blotted by cyclinB and cdc2 antibodies, respectively. There is no detectable difference in both protein levels of Cdc2 and cyclin B between lane (-) and lane (+). Cytoplasmic levels of both proteins were also the same between Wip1 induced and uninduced cells (data not shown) (the stained blot for the control of protein loading). (B) The level of inhibitory tyrosine phosphorylation on Cdc2 is increased when wip1 is induced. The immunoprecipitate from the total soluble protein of uninduced (lane-) and induced (lane+) U2-15 cells at time of 12 h using anti Cdc2 antibody was detected by anti-phosphotyrosine-15 monoclonal antibody. There is an increased level of inhibitory phosphotyrosine on Cdc2 protein in Lane (+). The same blot shown in the top part of (B) was stripped and detected using anti-Cdc2 antibody. There is no detectable difference in Cdc2 protein level between lane (-) and lane (+) (the middle part of B). The IP input control is provided (the bottom part of B).

kinase assay). If not, it was washed four times with lysis buffer, collecting agarose beads each time by centrifuging (CFG) at 2.5 k rpm for 5 min at 4°C.

Cell synchronization, flow cytometry and cell cycle analysis.

To synchronize the cells in G₀, exponentially proliferating cells at 80% confluence were starved in 0.1% serum medium for 72 h. To synchronize the cells in G₂, exponentially proliferating cells at 80% confluence were grown in the medium with 0.1 µg/ml Nocodazole medium for 40 h. 10⁶ cells per time point were trypsinized, and further prepared for flow cytometry.

Cdc2 kinase assay. The detail procedures were performed as described in reference 66. Briefly, the immunoprecipitates equilibrated with kinase buffer were mixed with 10 µl of kinase buffer

containing 50 µM ATP, 1.25 µCi [γ -³²P] ATP, and 1 µg of histone H1 (Roche). The mixture then was incubated at 30°C for 30 min, and reactions were terminated by the addition of SDS-sample buffer. The samples were analyzed by SDS-PAGE, followed by drying gel and autoradiography.

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