Regulation of the antioncogenic Chk2 kinase by the oncogenic Wip1 phosphatase

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Abstract

The antioncogenic Chk2 kinase plays a crucial role in DNA damage-induced cell-cycle checkpoint regulation. Here we show that Chk2 associates with the oncogenic protein Wip1 (wild-type p53-inducible phosphatase 1) (PPM1D), a p53-inducible protein phosphatase. Phosphorylation of Chk2 at threonine68 (Thr68), a critical event for Chk2 activation, which is normally induced by DNA damage or overexpression of Chk2, is inhibited by expression of wild-type (WT), but not a phosphatase-deficient mutant (D314A) of Wip1 in cultured cells. Furthermore, an *in vitro* phosphatase assay revealed that Wip1 (WT), but not Wip1 (D314A), dephosphorylates Thr68 on phosphorylated Chk2 *in vitro*, resulting in the inhibition of Chk2 kinase activity toward glutathione S-transferase-Cdc25C. Moreover, inhibition of Wip1 expression by RNA interference results in abnormally sustained Thr68 phosphorylation of Chk2 and increased susceptibility of cells in response to DNA damage, indicating that Wip1 acts as a negative regulator of Chk2 in response to DNA damage.

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Abbreviations: ATM, ataxia-telangiectasia mutated; FCS, foetal calf serum; PMSF, phenylmethyl sulphonyl fluoride; PP2C, type 2C protein phosphatases; Wip1, wild-type p53-inducible phosphatase 1

Introduction

The Chk2 tumour suppressor protein is an evolutionarily conserved nuclear protein kinase that plays a crucial role in the response to DNA damage and helps to maintain genomic stability by regulating cell cycle checkpoints, DNA repair, and apoptosis.^{1–8} Upon DNA damage, Chk2 is activated by phosphorylation of threonine68 (Thr68) by ATM (ataxia-telangiectasia mutated).^{9–13} Activated Chk2 then phosphorylates its downstream effectors, including the tumour suppressors p53, BRCA1, PML, E2F-1, and Cdc25 phosphatases,^{14–19} thereby regulating cellular responses following DNA damage. Although the molecular basis of Chk2 kinase activation and roles of Chk2 in checkpoint activation are rather well understood, it remains largely unknown as to how activated Chk2 is inhibited to release from checkpoint arrest or to prevent cells from undergoing Chk2-mediated apoptosis.

In yeast cells, it has been recently reported that protein phosphatases play essential roles in inactivating cell-cycle checkpoint arrest induced by DNA damage. In *Schizosaccharomyces pombe*, the type I protein phosphatase Dis2 abrogates Chk1 phosphorylation and activation by dephosphorylating the phosphorylated-Ser345 in Chk1, thereby allowing mitotic entry following repair of damaged DNA.²⁰ On the other hand, in *Saccharomyces cerevisiae*, the type 2C protein phosphatases (PP2C), Ptc2 and Ptc3, bind to Rad53, the *S. cerevisiae* orthologue of Chk2, and inactivate Rad53 presumably by dephosphorylating Rad53, leading to checkpoint inactivation upon a DNA double-strand break.²¹ Thus, it can be assumed that protein phosphatases may mediate the termination of DNA damage-induced cell-cycle checkpoint arrest to restart cell cycle by dephosphorylating and inactivating checkpoint kinases. Despite the high degree of conservation between cell-cycle checkpoint in yeast and mammals, it remains unknown whether or not protein phosphatases are involved in the termination of DNA damage-induced cell-cycle checkpoint arrest in mammals.

In mammals, the PP2C family of protein phosphatases consists of at least seven distinct isoforms, and have been implicated in stress response signalling.^{22–24} Among members of the PP2C family, in particular, Wip1 (wild-type p53-inducible phosphatase 1) (PPM1D) possesses unique biological characteristics. Wip1 is induced by DNA damage in a p53-dependent manner, and inhibits ultraviolet (UV)-irradiation-induced p38 activation by dephosphorylating Thr180 in p38, thereby inhibiting the function of p53.^{24,25} It has been reported that the *Wip1* (*PPM1D*) gene is amplified or overexpressed in various human cancers, including breast

cancers,^{26–29} and that overexpression of Wip1 (PPM1D) complements the oncogenes *Ras*, *Myc*, and *Neu1* for transformation of wild-type mouse embryonic fibroblasts (MEFs).²⁶ More recently, it has been shown that Wip1 associates with the nuclear isoform of uracil DNA glycosylase, UNG2, and that Wip1 suppresses DNA damage-induced base excision repair (BER) activity by dephosphorylating and inactivating UNG2.³⁰

In this study, we show that Wip1 interacts with Chk2 both physically and functionally in the nuclei. Wip1 dephosphorylates Thr68 in activated Chk2 both *in vitro* and *in vivo*, and inactivates Chk2 kinase activity toward Cdc25C. Furthermore, inhibition of Wip1 expression by RNA interference (RNAi) with *Wip1* siRNA or *p53* siRNA results in abnormally sustained Thr68 phosphorylation and kinase activation of Chk2 in response to DNA damage. We further show that RNAi-mediated inhibition of Wip1 expression results in enhanced apoptosis following DNA damage, and that ectopic expression of Wip1 suppresses Chk2-mediated apoptosis. Collectively, our *in vitro* and *in vivo* evidence suggests that Wip1 acts as a negative regulator of Chk2 during DNA damage responses, and that Wip1 plays an essential role in terminating DNA damage-induced cell-cycle checkpoint activation or in preventing cells from undergoing Chk2-mediated apoptosis in response to DNA damage.

Results and Discussion

Association of Chk2 with Wip1 in the nuclei

To better understand the roles and regulation of Chk2 during the DNA damage responses, we performed yeast two-hybrid screening using human Chk2 as a bait to identify potential associating molecule(s). From this screen, we identified Wip1, a p53-inducible oncogenic nuclear protein serine (S)/threonine (T) phosphatase^{24–28} (data not shown). To test whether Chk2 can associate with Wip1 *in vitro*, we performed pull-down analyses using glutathione *S*-transferase (GST)-Chk2 (wild-type (WT)) or GST-Wip1 (WT) purified from *Escherichia coli*. As shown in Figure 1a, Flag-tagged Wip1 (WT) expressed in 293T cells associated specifically with GST-Chk2 (WT), but not with GST. Conversely, haemagglutinin (HA)-tagged Chk2 (WT) expressed in 293T cells associated specifically with GST-Wip1 (WT), but not with GST (Figure 1b). These results indicate that Chk2 associates with Wip1 *in vitro*. Since it has been reported previously that both Chk2 and Wip1 are localized in the nuclei,^{7,17,25,31} their intracellular distribution was examined by immunofluorescence in 293T cells coexpressing HA-tagged GST-Chk2 (GST-HA-Chk2) and Flag-tagged Wip1 (Flag-Wip1) (see Materials and Methods). GST-HA-Chk2 and Flag-Wip1 colocalized primarily in the nuclei (see Supplementary Information). To determine whether Chk2 can associate with Wip1 *in vivo*, Flag-Wip1 (WT) was transiently expressed in 293T cells, either alone or with GST-HA-Chk2 (WT). Cells were harvested, and protein association was evaluated by co-precipitation with glutathione-Sepharose, followed by anti-Flag-immunoblotting. As shown in Figure 1c, in cells expressing both GST-HA-Chk2 and Flag-Wip1, glutathione-

Sepharose precipitation specifically co-precipitated Wip1, indicating that Chk2 associates with Wip1 in 293T cells.

We next examined whether endogenous Chk2 and Wip1 can interact in intact cells. To this end, we generated polyclonal anti-Chk2 and anti-Wip1 antibodies raised against human Chk2 peptides (amino acids (a.a.) 523–543) and GST-human Wip1 (a.a. 1–458), respectively (see Materials and Methods), and evaluated specificities for their antigen recognition. As shown in Figure 1d (left panel), immunoblotting with anti-Chk2 antibody clearly detected HA-Chk2 (upper band) and endogenous Chk2 (lower band) in whole-cell lysates and HA-Chk2 in anti-HA immunoprecipitates from 293T cells expressing HA-Chk2, and preadsorption of anti-Chk2 antibody with GST-Chk2 prior to immunoblotting resulted in a failure of the detection of HA-Chk2, confirming the specificity of anti-Chk2 antibody. Similarly, immunoblotting with anti-Wip1 antibody specifically detected Flag-Wip1 (Figure 1d, right panel). Specificities of anti-Wip1 and anti-Chk2 antibodies were further confirmed by an RNAi experiment where the bands detected by anti-Wip1 and anti-Chk2 antibodies were diminished in cells treated with *Wip1* siRNAs and *Chk2* siRNAs, respectively (Figure 4, data not shown). We examined the association of endogenous Chk2 and Wip1 in MCF7 cells that express Wip1 at a higher level. As shown in Figure 1e, endogenous Chk2 and Wip1 associated in MCF7 cells.

Effect of Wip1 on Thr68 phosphorylation of Chk2 induced by DNA damage or overexpression of Chk2

It was shown that ectopic overexpression of Chk2 in 293T cells results in its electrophoretic mobility shift (Figure 2a),^{13,32} indicating auto- or transphosphorylation. In fact, electrophoretic mobility shift induced by overexpression of Chk2 was abrogated following treatment of cell extracts with CIP or BAP (data not shown). To examine the functional significance of the association between Wip1 and Chk2, HA-Chk2 (WT) or HA-Chk2 (DK) was expressed in 293T cells along with either Flag-Wip1 (WT) or Flag-Wip1 (D314A). Chk2 electrophoretic mobilities were monitored by anti-HA-immunoblotting of whole-cell lysates. Overexpression of Chk2 (WT), but not Chk2 (DK), resulted in decreased electrophoretic mobility (Figure 2a), indicating that Chk2 kinase activity is required for this electrophoretic mobility shift. It had been shown previously that phosphorylation of Chk2 on Thr68 is required for full activation of Chk2 via auto- (*trans*- or *cis*)phosphorylation.^{9–13,31–34} We therefore investigated the role of Thr68-phosphorylation in the electrophoretic mobility shift of Chk2. We found that a Chk2 mutant, the alanine68 mutant (HA-Chk2 (T68A)), did not undergo the mobility shift when overexpressed (data not shown), indicating that Thr68 is required. Intriguingly, this mobility shift was abrogated by coexpression of Wip1 (WT), but not Wip1 (D314A) (Figure 2a), suggesting that Wip1 phosphatase activity can counteract Chk2 phosphorylation either directly or indirectly.

It has been shown previously that γ -irradiation induces Thr68-phosphorylation and modification (activation) of Chk2.^{9–13} Hence, we examined Thr68-phosphorylation and

Figure 1 Association of Chk2 kinase with Wip1 phosphatase both *in vitro* and *in vivo*. **(a)** Whole-cell lysates (WCLs) from 293T cells expressing Flag-Wip1 were subjected to a pull-down assay (see Materials and Methods). Amounts of Flag-Wip1 bound to GST-Chk2- or GST-conjugated glutathione-Sepharose were determined by anti-Flag-immunoblotting. Upper panel, anti-Flag-immunoblotting of pull-down samples or WCLs; lower panel, CBBR staining shows amounts of the respective fusion proteins applied to the pull-down assay. **(b)** WCLs from 293T cells expressing HA-Chk2 were subjected to pull-down assay. Amounts of HA-Chk2 bound to GST-Wip1- or GST-conjugated glutathione-Sepharose were determined by anti-HA-immunoblotting. Upper panel, anti-HA-immunoblotting of pull-down samples or WCLs; lower panel, CBBR staining shows amounts of the respective fusion proteins applied to the pull-down assay. **(c)** WCLs from 293T cells, expressing Flag-Wip1 (WT) in the presence or absence of GST-HA-Chk2 (WT), were subjected to affinity precipitation with glutathione-Sepharose, followed by anti-Flag- and anti-HA-immunoblotting, respectively. Upper and middle panels, anti-Flag- and anti-HA-immunoblotting of glutathione-Sepharose-precipitated proteins, respectively; lower panel, anti-Flag-immunoblotting of WCLs. The results shown are representative of three independent experiments. **(d)** HA-Chk2 (left) and Flag-Wip1 (right) were expressed transiently in 293T cells. Anti-HA or anti-Flag immunoprecipitates or WCLs from the respective transfectants were immunoblotted with anti-Chk2 (left) or anti-Wip1 (right) antibodies, respectively. The specificity of the respective antibodies was assessed by preadsorption with corresponding GST fusion proteins as indicated. Expressions of HA-Chk2 and Flag-Wip1 were verified by anti-HA- and anti-Flag-immunoblotting, respectively. The arrow and asterisk indicate the positions of HA-Chk2 and endogenous Chk2, respectively. **(e)** WCLs or immunoprecipitates with anti-Chk2 or control (preimmune) antibodies prepared from MCF7 cells were subjected to anti-Wip1 (upper) or anti-Chk2 (lower) immunoblotting.

mobility shift of HA-Chk2 (WT) coexpressed with Flag-Wip1 (WT) or Flag-Wip1 (D314A) in 293T cells, before or after γ -irradiation, by immunoblotting with antiphospho-Chk2 (Thr68). As shown in Figure 2b (upper panel), ectopic overexpression of HA-Chk2 *per se* induced some Thr68-

autophosphorylation, but this phosphorylation, and that of the endogenous Chk2, was enhanced following γ -irradiation, in cells not coexpressing Flag-Wip1 (WT). Antiphospho-Chk2 (Thr68) antibody failed to detect HA-Chk2 (T68A) expressed in 293T cells, demonstrating that this antibody specifically

recognizes phosphorylated-Thr68 in Chk2 (data not shown). However, Thr68-phosphorylation and electrophoretic mobility shift of HA-Chk2 (WT) were inhibited in cells coexpressing Wip1 (WT), but not Wip1 (D314A) (Figure 2b), indicating that the phosphatase activity of Wip1 is required for the inhibition of Chk2 Thr68-phosphorylation and mobility shift induced by overexpression of Chk2 and/or γ -irradiation, although it was unclear whether Wip1 acted directly on Chk2. Since Wip1 belongs to the PP2C family of protein phosphatases, we examined whether or not other members of the PP2C family can exhibit similar effects. Coexpression of human PP2C α 2, mouse PP2C β , PP2C ϵ , or PP2C ζ fails to inhibit Thr68-

phosphorylation and electrophoretic mobility shift of HA-Chk2 (WT) (Figure 2b, data not shown), indicating that the observed effects are at least selective to Wip1 among members of the PP2C family.

Effect of Wip1 on other phosphorylation sites in Chk2 induced by DNA damage or overexpression of Chk2

We next examined the effect of Wip1 (WT) on phosphorylation of several S and T residues other than Thr68 of Chk2, induced upon ectopic overexpression of Chk2 and/or γ -irradiation, by immunoblotting with a series of phospho-Chk2 antibodies. As shown in Figure 2c, ectopic overexpression of HA-Chk2 *per se* induced weak phosphorylation of Ser19, Ser33/35, and Thr432 and strong (or apparent) phosphorylation of Thr387 and Ser516 of Chk2, and these phosphorylations were further enhanced following γ -irradiation. Phosphorylation of Ser19, Ser33/35, Thr387, and Thr432 of HA-Chk2 induced by its overexpression and/or γ -irradiation was also inhibited in cells coexpressing Wip1 (WT), but not Wip1 (D314A) (Figure 2c). The result presented in Figure 2b suggests that dephosphorylation of phosphorylated-Thr68 in Chk2 by Wip1 may inhibit subsequent phosphorylation of Chk2 at several S/T residues, or that Wip1 may dephosphorylate these phosphorylated residues in Chk2 (see Figure 3a). We also examined the effect of Wip1 (WT) on endogenous Chk2, and found that expression of Wip1 (WT), but not Wip1 (D314A), in 293T cells also inhibited Thr68-phosphorylation and mobility shift of the endogenous Chk2 (Figure 2d).

Dephosphorylation of Ser19, Ser33/35, Thr68, and Thr432, but not Thr387 and Ser516 in Chk2 by Wip1 *in vitro*

We next addressed the question of whether or not Wip1 could dephosphorylate Thr68 or Thr387 in Chk2. To this end, GST-Wip1 (WT) and GST-Wip1 (D314A) were expressed in *E. coli*

Figure 2 Inhibition of Chk2 Thr68-phosphorylation and electrophoretic mobility shift by Wip1 (WT), but not Wip1 (D314A), before and after γ -irradiation. (a) Wild-type (WT) or dead-kinase mutant (DK) of HA-Chk2 proteins were expressed transiently in 293T cells along with either WT or a phosphatase-deficient mutant (D314A) of Flag-Wip1 proteins. WCLs from the respective transfectants were subjected to anti-HA- (upper) or anti-Flag- (lower) immunoblotting, respectively. (b) HA-Chk2 (WT) proteins were expressed transiently in 293T cells along with either Flag-Wip1 (WT), Flag-Wip1 (D314A), or Flag-PP2C α 2. Before or after γ -irradiation (10 Gy), WCLs were subjected to antiphospho-Chk2 (Thr68)-immunoblotting. The arrow and asterisk indicate the positions of HA-Chk2 and of endogenous Chk2, respectively. Upper, middle, and lower panels, antiphospho-Chk2 (Thr68)-, anti-HA-, and anti-Flag-immunoblotting of WCLs, respectively. Flag-PP2C α 2 expressed in 293T cells is catalytically active, since it can dephosphorylate phosphorylated-p38 (data not shown). (c) HA-Chk2 (WT) proteins were expressed transiently in 293T cells along with either Flag-Wip1 (WT) or Flag-Wip1 (D314A) proteins. Before or after γ -irradiation (10 Gy), WCLs were subjected to antiphospho-Chk2 (Ser19, Ser33/35, Thr68, Thr387, Thr432, or Ser516)- or anti-HA-immunoblotting as indicated. WCLs were also subjected to anti-Flag-immunoblotting. (d) Flag-Wip1 (WT) or Flag-Wip1 (D314A) was expressed transiently in 293T cells. Before or after γ -irradiation (10 Gy), WCLs from the respective transfectants were subjected to antiphospho-Chk2 (Thr68)-immunoblotting. Upper, middle, and lower panels, antiphospho-Chk2 (Thr68)-, anti-Chk2-, and anti-Flag-immunoblotting of WCLs, respectively

and purified using glutathione-Sepharose beads. Purified GST-Wip1 (WT), but not GST-Wip1 (D314A), exhibited phosphatase activity toward *p*-nitrophenyl phosphate (*p*NPP) (data not shown), confirming that each functions as expected with respect to phosphatase activity. In these assays, it was found that the *p*NPP phosphatase activity of purified GST-Wip1 (WT) is Mg^{2+} dependent (data not shown), consistent with previous reports that Wip1 displays characteristics of the PP2C family of protein phosphatases.²⁵ These purified GST fusion proteins were subjected to an *in vitro* phosphatase assay using Thr68- and Thr387-phosphorylated HA-Chk2 as a substrate (see Materials and Methods). GST-Wip1 (WT), but not GST-Wip1 (D314A), dephosphorylated Thr68 in Chk2 *in vitro* (Figure 3a), and this activity was time dependent and Mg^{2+} dependent (data not shown), characteristic of Wip1. On the other hand, GST-Wip1 (WT) failed to dephosphorylate Thr387 in Chk2 *in vitro* (Figure 3a). These results are consistent with the idea that Wip1 can dephosphorylate the phosphorylated-Thr68 in Chk2 induced by γ -irradiation or ectopic overexpression of Chk2, and that Thr68-phosphorylation of Chk2 is a prerequisite event for (*trans*)-phosphorylating Thr387 (and possibly Thr383) in Chk2 (see Figure 2c).^{31–34} We also examined whether Wip1 could dephosphorylate Ser19, Ser33/35, Thr432, and Ser516 in Chk2. As shown in Figure 3a, GST-Wip1 (WT), but not GST-Wip1 (D314A), dephosphorylated-Ser19, -Ser33/35, -Thr432, but not Ser516 in Chk2 *in vitro*. The results of our *in vitro* phosphatase assays (Figure 3a) (data not shown),²⁴ are summarized in Table 1. As shown, Wip1 appears to dephosphorylate preferably a phosphorylated S or T followed by a glutamine (Q) *in vitro*.

Inhibition of Chk2 kinase activity by Wip1-mediated dephosphorylation of Thr68 in Chk2

It can be assumed that dephosphorylation of the phosphorylated-Thr68 in Chk2 by Wip1 may result in the inhibition of Chk2 kinase activity. To investigate this hypothesis, HA-Chk2 activated by ectopic overexpression and/or γ -irradiation was

Figure 3 Dephosphorylation of phosphorylated-Thr68 in Chk2 and inhibition of Chk2 kinase activity by Wip1 *in vitro*. **(a)** Phosphorylated-HA-Chk2 on Ser19, Ser33/35, Thr68, Thr387, Thr432, or Ser516 was prepared from γ -irradiated 293T cells overexpressing HA-Chk2 (WT), followed by anti-HA immunoprecipitation. Reaction was initiated by the addition of an equal amount (1 μ g) of the respective GST fusion proteins, and an *in vitro* phosphatase assay was performed (see Materials and Methods). Samples prepared after phosphatase reaction were immunoblotted with antiphospho-Chk2 (Ser19, Ser33/35, Thr68, Thr387, Thr432, or Ser516) or anti-HA antibodies, respectively. **(b)** Phosphorylated-HA-Chk2 on Thr68 was immunoprecipitated and treated with purified GST-Wip1 (WT) or GST-Wip1 (D314A) *in vitro*. Subsequently, two-third of each sample was subjected to an *in vitro* kinase (IVK) assay using GST-Cdc25C as a substrate and the rest was immunoblotted with either antiphospho-Chk2 (Thr68) or anti-HA antibodies. A histogram shows quantification of Chk2 kinase activities toward GST-Cdc25C. Data are expressed as the means $\pm$ S.D. in triplicate assays from a representative experiment of three. The arrows indicate the positions of GST-Cdc25C. **(c)** 293T cells expressing HA-Chk2 along with either Flag-Wip1 (WT) or Flag-Wip1 (D314A) were subjected to γ -irradiation. Anti-HA-immunoprecipitates of WCLs from the respective transfectants were subjected to an IVK assay using GST-Cdc25C as a substrate. WCLs were subjected to antiphospho-Chk2 (Thr68)-, anti-HA-, or anti-Flag-immunoblotting, as indicated. A histogram shows quantification of Chk2 kinase activity toward GST-Cdc25C. Data are expressed as the mean $\pm$ S.D. in triplicate assays from a representative experiment of three. The arrows indicate the positions of GST-Cdc25C

Table 1 Selectivity of dephosphorylation of phosphorylated serine and/or threonine residue by Wip1 *in vitro*

													Dephosphorylation by Wip1 <i>in vitro</i>
Chk2	S19	G	S	S	A	C	S ^a	Q	P	H	G	S	+
	S33/S35	G	S	S	S ^a	<u>Q</u>	S ^a	<u>Q</u>	G	I	S	S	+
	T68	L	E	T	V	S	T ^a	<u>Q</u>	E	L	Y	S	+
	T383	T	S	L	M	R	T ^a	<u>L</u>	C	G	T	P	ND
	T387	R	T	L	C	G	T ^a	P	T	Y	L	A	—
	T432	F	S	E	H	R	T ^a	<u>Q</u>	V	S	L	K	+
Chk1	S516	V	L	A	Q	P	S ^a	<u>T</u>	S	R	K	R	—
	S317	L	K	Y	S	S	S ^a	<u>Q</u>	P	E	P	R	+ ^{b,c}
	S345	Q	G	I	S	F	S ^a	<u>Q</u>	P	T	C	P	+ ^{b,c}
Cdc25C	S216	L	Y	R	S	P	S ^a	<u>M</u>	P	E	N	L	— ^{b,d}
p53	S15	V	E	P	P	L	S ^a	<u>Q</u>	E	T	F	S	+ ^{b,c}
	S20	S	Q	E	T	F	S ^a	<u>D</u>	L	W	K	L	— ^c
	S33	E	N	N	V	L	S ^a	P	L	P	S	Q	— ^e
	S46	D	D	L	M	L	S ^a	P	D	D	I	E	— ^{c,e}
p38	T180	T	D	D	E	M	T ^a	<u>G</u>	Y	V	A	T	+ ^e
UNG2	T6	M	I	G	Q	K	T ^a	<u>L</u>	Y	S	F	F	+ ^f

^aPhosphorylated serine or threonine residues; ND = not determined. ^bAs reported by Lu *et al.* (2005). ^cSee Supplementary Information. ^dN Onishi *et al.*, unpublished data. ^eAs reported by Takekawa *et al.* (2000). ^fAs reported by Lu *et al.* (2004). Glutamine residues following phosphorylated serine or threonine residues were underlined.

immunoprecipitated with anti-HA antibody, treated with purified GST-Wip1 (WT) or GST-Wip1 (D314A), and was subjected to an *in vitro* kinase assay using GST-Cdc25C (a.a. 200–256) as a substrate (see Materials and Methods). However, it is possible that GST-Wip1, associated with HA-Chk2, may exhibit phosphatase activities toward phosphorylated-GST-Cdc25C by HA-Chk2 in this assay. It was found that GST-Wip1 (WT) failed to dephosphorylate the phosphorylated-GST-Cdc25C by HA-Chk2 *in vitro* (data not shown), indicating that phosphorylated-GST-Cdc25C is not a substrate of Wip1 at least *in vitro*. Considering the finding that Wip1 dephosphorylates the phosphorylated-Ser33/35 and -Thr68, but not phosphorylated-Thr387 in Chk2 *in vitro* (Figure 3a), the result also suggests the substrate specificity of Wip1. Interestingly, incubation of immunoprecipitated HA-Chk2 (activated by its ectopic overexpression and/or γ -irradiation) with GST-Wip1 (WT), but not GST-Wip1 (D314A), resulted in a drastic inhibition of Chk2 kinase activity toward GST-Cdc25C *in vitro* (Figure 3b). Furthermore, coexpression of Flag-Wip1 (WT), but not Flag-Wip1 (D314A) in 293T cells, resulted in a drastic inhibition of Chk2 kinase activity *in vitro* (Figure 3c), confirming that Wip1 inhibits Chk2 kinase activity by dephosphorylating the phosphorylated-Thr68 in Chk2.

Abnormally sustained Thr68 phosphorylation and kinase activity of Chk2 by inhibition of Wip1 expression

Considering our results indicating that Wip1 dephosphorylates and inactivates Chk2, it is likely that Wip1 acts as a negative regulator of Chk2 during DNA damage responses. To test such a possibility, we performed an RNAi experiment with Wip1 siRNAs or control siRNAs and monitored the effects of inhibition of Wip1 expression on the extent of Thr68-phosphorylation and kinase activity of Chk2 following γ -irradiation. In this experiment, p53-proficient MCF7 and

HCT116 cells were utilized (see Materials and Methods). At 3 days after transfection, expression levels of Wip1 protein in cells transfected with Wip1 siRNAs decreased drastically compared to cells transfected with control siRNAs, as assayed by immunoblot analysis (Figure 4a, data not shown). In cells transfected with control siRNAs, Thr68-phosphorylation and kinase activity of Chk2 increased following γ -irradiation and decreased rapidly thereafter, while increased Thr68-phosphorylation and kinase activity of Chk2 were abnormally sustained in cells transfected with Wip1 siRNAs following γ -irradiation (Figure 4a, data not shown). A sustained electrophoretic mobility shift of Chk2 was also observed in cells transfected with Wip1 siRNAs following γ -irradiation compared to cells transfected with control siRNAs. These results support the notion that Wip1 induced by DNA damage acts as a negative regulator of Chk2 in the DNA damage response.

Since Wip1 was identified as 'wild-type p53-inducible phosphatase 1',²⁵ we next examined the effect of inhibition of p53 expression by p53 siRNA on the extent of γ -irradiation-induced expression of Wip1 and Thr68-phosphorylation of Chk2 (see Materials and Methods). As shown in Figure 4b, in MCF7 cells transfected with control siRNAs, upon γ -irradiation apparent induction of p53 proteins (reached maximal levels within 2 h) followed by induction of Wip1 proteins (reached maximal levels between 2 and 4 h) were observed, while transfection of MCF7 cells with p53 siRNAs resulted in inhibition of induction of both p53 and Wip1 proteins following γ -irradiation. Consistent with suppressed expression of Wip1 proteins in MCF7 cells transfected with p53 siRNAs following γ -irradiation, increased Thr68-phosphorylation of Chk2 was abnormally sustained in cells transfected with p53 siRNAs following γ -irradiation compared to cells transfected with control siRNAs (Figure 4b). Thus, the result indicates that p53 contributes to dephosphorylation of the phosphorylated-Thr68 in Chk2 at later points (4–8 h) of DNA damage-induced responses by regulating the induction of Wip1 proteins following γ -irradiation.

Figure 4 Sustained Thr68 phosphorylation and kinase activity of Chk2 by *Wip1* siRNAs-mediated inhibition of *Wip1* expression in response to DNA damage. (a) MCF7 cells were transfected with either *Wip1* siRNAs or control siRNAs (see Materials and Methods). At 3 days after transfection, cells were exposed to γ -irradiation. At the indicated time points, the respective WCLs were subjected to immunoblotting with antiphospho-Chk2 (Thr68) and anti-Chk2 as indicated. At the indicated time points (0, 4, and 8 h), anti-Chk2 immunoprecipitates of the respective WCLs were subjected to an IVK (see Materials and Methods). A histogram shows quantification of Chk2 kinase activity toward GST-Cdc25C. Data are expressed as the means $\pm$ S.D. of relative kinase activities (relative to the level in nonirradiated cells with control siRNA) in three independent experiments. (b) MCF7 cells were transfected with either *p53* siRNAs or control siRNAs. At 4 days after transfection, cells were exposed to 10 Gy γ -irradiation. At the indicated time points after γ -irradiation, WCLs from the respective transfectants were subjected to immunoblotting with anti-p53, anti-Wip1, antiphospho-Chk2 (Thr68), or anti-Chk2 antibodies, and the intensity of the respective bands was quantified. Histograms show quantification of expression levels of p53 and Wip1 proteins, respectively (upper, middle panels). Relative levels of Thr68-phosphorylation of Chk2 (lower panel) were determined by normalization with levels of Chk2 proteins in the respective samples. Data are expressed as the mean $\pm$ S.D. (relative to the levels in nonirradiated cells with control siRNA) in three independent experiments. Representative results (at 4 h after γ -irradiation) of the immunoblots are also indicated

Antagonistic effect of Wip1 on Chk2-dependent apoptosis

To understand the role of Wip1 in regulating cellular function, we monitored the effect of inhibition of Wip1 expression on the extent of γ -irradiation-induced apoptosis by TUNEL assays

(see Materials and Methods). As shown in Figure 5a, MCF7 cells transfected with *Wip1* siRNAs, but not control siRNAs, exhibited remarkably enhanced apoptosis following γ -irradiation, indicating that Wip1 negatively regulates γ -irradiation-induced apoptosis by dephosphorylating and inactivating Chk2. Similar results were obtained when p53-proficient

HCT116 cells, transfected with *control* siRNAs or *Wip1* siRNAs, were subjected to TUNEL assays following γ -irradiation (data not shown). Furthermore, γ -irradiation-induced apoptosis was inhibited by *Chk2* siRNAs (data not

shown). Moreover, it was found that ectopic expression of HA-Chk2 (WT) by itself in MCF7 cells enhanced γ -irradiation-induced apoptosis, but was inhibited by coexpression of Flag-Wip1 (WT), but not Flag-Wip1 (D314A), indicating that Wip1 antagonizes Chk2-mediated apoptosis in a phosphatase activity-dependent manner (Figure 5b). It has been reported that phosphorylation of Ser516 in Chk2 is required for radiation-induced apoptosis.³⁵ Thus, the inhibition of Chk2-mediated apoptosis by Wip1 following γ -irradiation can be explained by our observation that ectopic coexpression of Wip1 inhibits phosphorylation of Ser516 in Chk2 (Figure 2c). Taken together, these results indicate that enhanced apoptosis induced by *Wip1* siRNA is dependent both on γ -irradiation and on Chk2, and that Wip1 acts as a negative regulator during γ -irradiation-induced apoptosis. In this respect, it is of interest to note that fibroblasts from *Wip1*-null embryos exhibit a decreased proliferation rate.³⁶ It has been reported that Wip1 contributes to suppression of UV-irradiation-induced apoptosis by dephosphorylating and inactivating p38.²⁴ However, γ -irradiation-induced apoptosis of MCF7 cells transfected with *Wip1* siRNAs was unaffected in the absence or presence of the specific inhibitor of p38, SB203580 (data not shown).

Chk2 mutations have been found in patients having a variant form of the familial multicancer Li-Fraumeni syndrome, and Chk2 kinase is considered to be a tumour suppressor.^{7,8,37} In contrast, Wip1 phosphatase is considered to be an oncogenic protein, on the basis of the findings that the gene encoding Wip1 (PPM1D) is amplified in human breast cancers,^{26–28} and that disruption of the *Wip1* gene activates p53 and p16^{Ink4a}–p19^{Arf} pathway via p38 MAPK signalling, resulting in the suppression of *in vitro* transformation of MEFs and of *in vivo* tumorigenesis by oncogenes.^{38,39} It has also been reported that following DNA damage ATM phosphorylates Chk2 at Thr68, an event critical for the activation of Chk2.^{9–13} Here we show that Wip1 dephosphorylates Thr68 in Chk2 both *in vivo* and *in vitro* (Figures 2 and 3), and may act as a negative regulator of Chk2 (Figures 4b and 5). It has been reported that Ptc2 and Ptc3, two members of the PP2C family, bind to Rad53 and inactivate Rad53-dependent pathways in *S. cerevisiae*.²¹ Considering our results, it is likely that Ptc2 and Ptc3 inactivate Rad53 by dephosphorylating it. Furthermore, we provide evidence suggesting that Wip1 may

Figure 5 Antagonistic function of Wip1 on Chk2-dependent apoptosis. (a) MCF7 cells were transfected with either *Wip1* siRNAs or *control* siRNAs. At 3 days after transfection, cells were nonirradiated or exposed to 20 Gy γ -irradiation, and cultured for 36 h. Cellular apoptosis was then examined by TUNEL assays (see Materials and Methods). A histogram shows the fraction of apoptotic cells determined by dividing the number of TUNEL-positive cells by the total number of cells counted. About 1000 cells from three to four randomly chosen fields were counted. Decreased expression levels of Wip1 in cells treated with *Wip1* siRNAs were confirmed by anti-Wip1-immunoblotting (data not shown). (b) GST-HA-Chk2 (WT), GST-HA-Chk2 (DK), Flag-Wip1 (WT), or Flag-Wip1 (D314A) in combination were expressed transiently in MCF7 cells as indicated. After transfection, cells were exposed to 20 Gy γ -irradiation, and cultured for 36 h. Cellular apoptosis was examined by TUNEL assays (see Materials and Methods). A histogram shows the fraction of apoptotic cells determined by dividing the number of TUNEL-positive cells by the total number of anti-HA-positive cells. About 1000 cells from three to four randomly chosen fields were counted. Expression of GST-HA-Chk2 and Flag-Wip1 proteins was assessed by immunostaining with anti-HA and anti-Flag (data not shown) antibodies

negatively regulate γ -irradiation-induced apoptosis by dephosphorylating and inactivating Chk2 (Figures 4 and 5). However, at present we cannot rule out the possibility that Wip1 may dephosphorylate and inactivate ATM or other proteins, thereby inhibiting Chk2 function. Interestingly, it has been recently reported that Wip1 dephosphorylates the nuclear form of uracil DNA glycosylase (UNG2) and Chk1, thereby suppressing BER and intra-S and G2/M checkpoint regulation, respectively.^{30,40} In the case of UNG2 and p38, phosphorylation sites on these molecules, which can be targets for Wip1-mediated dephosphorylation, reveal consensus sequences 'phospho-T-X-phospho-Tyr (Y) (or phospho-T-X-Y, X: optional a.a.)'.⁴¹ In this respect, it is important to note that our *in vitro* evidence as well as previous report⁴⁰ indicate the presence of additional target sequences for Wip1, which are 'phospho-S-Q' or 'phospho-T-Q', well-known fingerprints of ATM kinase (see Table 1). Future study to identify a substrate(s) for Wip1 in addition to phosphorylated-Chk2, -Chk1, -p38,²⁴ and -UNG2³⁰ may contribute to our understanding of the roles of Wip1 during DNA damage-induced cellular responses.

Materials and Methods

Plasmid constructions

The human Chk2 coding region was amplified by PCR using human placenta cDNA as a template, and subcloned into the mammalian expression vector pcDNA3 (Invitrogen). The mutant Chk2 (T68A) was generated by replacing Thr68 with Ala using the Transformer TM Site-Directed mutagenesis kit (Clontech). The kinase-dead mutant of Chk2 (DK) replaces Lys249 with Arg and was created by PCR. A PCR-based procedure was employed to attach an HA tag at the C-terminal end of Chk2; to generate the expression vectors, pcDNA-*Chk2* (WT)-HA, pcDNA-*Chk2* (T68A)-HA, pcDNA-*Chk2* (DK)-HA, and pEBG-*Chk2* (encoding GST-HA-*Chk2*) were constructed to attach GST and HA tags at the N- and C-terminal ends of Chk2, respectively. pGEX-3X-*Chk2*, encoding GST-*Chk2* fusion proteins, was constructed to attach GST tag at the N-terminal end of Chk2; pGEX-3X-*Cdc25C*, encoding GST-*Cdc25C* (a.a. 200–256 of human *Cdc25C*), was constructed by subcloning of the PCR amplified *Cdc25C* cDNA fragment into the pGEX-3X to generate GST-*Cdc25C*. pCMV-*Wip1* (WT), and pCMV-*Wip1* (D314A), encoding the wild-type and phosphatase inactive mutant of Wip1, respectively, were constructed as described previously.²⁴ A PCR-based procedure was employed to attach a FLAG-tag sequence at the C-terminal end of Wip1 to generate pcDNA-*Wip1* (WT)-FLAG and pcDNA-*Wip1* (D314A)-FLAG. pGEX-5X-*Wip1* (WT) and pGEX-5X-*Wip1* (D314A), encoding GST-*Wip1* (WT) and GST-*Wip1* (D314A), respectively, were constructed as described previously.²⁴ pcDNA-*PP2C α 2* (WT)-FLAG, encoding the wild-type Flag-*PP2C α 2*, was constructed as described previously.²³ pcDNA-*Chk1*-myc, encoding the wild-type Chk1 of mouse origin, was a gift from Noboru Motoyama (National Institute for Longevity Sciences, Japan).

Antibodies, cells, and DNA transfection

The following antibodies were used: mouse monoclonal antibody (MoAb) M2 (Eastman Kodak) recognizes the Flag peptide sequence (DYKDDDDK). Mouse MoAb 12CA5 (Boehringer Mannheim) and rat MoAb 3F10 (Roche) recognize the peptide sequence (YPYDVPDYA) derived from the human influenza HA protein. Mouse MoAb 9E10 (Santa

Cruz) recognize the peptide sequence (EQKLISEEDL) derived from human c-myc protein. Rabbit polyclonal anti-Chk2 antibody was raised against peptides corresponding to a.a. 523–543 of human Chk2. Rabbit polyclonal anti-Wip1 antibody was raised against GST-Wip1 (a.a. 1–458). Mouse monoclonal anti-Wip1 (WC 10) antibody was from Trevigen. Mouse monoclonal anti-p53 (DO-1) antibody was from Santa Cruz. Rabbit polyclonal anti-phospho-Chk1 (Ser317 and Ser345) antibodies, anti-phospho-Chk2 (Ser19, Ser33/35, Thr68, Thr387, Thr432, and Ser516) antibodies, and anti-phospho-p53 (Ser15, Ser20, and Ser46) antibodies were purchased from Cell Signaling. Alexa Fluor 546 (goat anti-mouse IgG, red) and Alexa Fluor 488 (goat anti-rat IgG, green) were purchased from Molecular Probes. SB203580, a specific inhibitor of p38 MAP kinase (p38 α , p38 β , and p38 δ), was from Promega. HEK293T (293T), MCF7 cells were maintained in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% (v/v) foetal calf serum (FCS). HCT116 cells were maintained in McCoy's 5a medium (GibcoBRL) supplemented with 10% (v/v) FCS. It should be noted that 293T cells bear p53 inactivated by adenovirus E1B and SV40 T-antigen, while MCF7 and HCT116 cells are p53-proficient breast adenocarcinoma and colorectal carcinoma cells, respectively. Transient cDNA transfection into cells was performed using the calcium phosphate method³ or Lipofectamine Reagent, PLUS Reagent, and Lipofectamine 2000 Reagent (GibcoBRL) according to the manufacturer's instructions.

Preparation of cell lysates and co-(immuno) precipitation analysis

Transfected cells were solubilized with lysis buffer (50 mM Tris-HCl (pH 7.4), 0.5% (v/v) NP-40, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethyl sulphonyl fluoride (PMSF), 10 μ g/ml aprotinin), and cell lysates were prepared by centrifugation at 12 000 $\times$ g for 15 min at 4°C. Subsequently, the cell lysates were subjected to pull-down assay/immunoblot analysis or co-(immuno) precipitation/immunoblot analysis. For co-(immuno) precipitation analysis, the cell lysates were precleared with protein A-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4°C. The precleared supernatants were then co-(immuno) precipitated with glutathione-Sepharose beads (Amersham Pharmacia Biotech) or with anti-HA antibody conjugated to protein A-Sepharose beads for 2 h at 4°C. The resultant precipitates were washed five times with lysis buffer, and eluted with Laemmli sample buffer.

Expression and purification of GST fusion proteins

The plasmids encoding the GST fusion proteins, GST-*Chk2* (WT), GST-*Wip1* (WT), GST-*Wip1* (D314A), and GST-*Cdc25C* (a.a. 200–256), were constructed using the pGEX plasmids (Amersham Pharmacia Biotech). GST fusion proteins expressed in *E. coli* DH5 α were extracted with phosphate-buffered saline (PBS) containing 1% (v/v) Triton X-100, 1 mM EDTA, 1 mM PMSF, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin, and were isolated with glutathione-Sepharose beads. To further purify GST-*Wip1* (WT), GST-*Wip1* (D314A), and GST-*Cdc25C* for phosphatase and kinase assays, fusion proteins were eluted from beads by incubating with 25 mM glutathione (reduced), followed by dialysis prior to use.

Pull-down assay and immunoblot analysis

The cell lysates were precleared with glutathione-Sepharose for 1 h at 4°C. The precleared supernatant was then incubated with the respective GST fusion proteins conjugated with glutathione-Sepharose beads for 1 h

at 4°C, washed five times with lysis buffer, and eluted with Laemmli sample buffer. Precipitates either from the pull-down assay or whole-cell lysates were separated by SDS-PAGE (10% PAG), and transferred onto PVDF membrane filters (Immobilon P, Millipore). The membranes were immunoblotted with the respective antibodies, and bound antibodies were visualized with HRP-conjugated antibodies against mouse or rabbit IgGs (Bio-Rad) using the chemiluminescence reagent (Renaissance, NEN). Quantification of the immunoblots was performed by densitometric scanning of the film using an Image Analysis system with NIH Image 1.62 software.

In vitro phosphatase assay and kinase assays

To prepare phosphorylated-HA-Chk2 (WT), 293T cells were transfected with the expression vector encoding HA-Chk2 (WT). For *in vitro* kinase assay, 24 h after transfection, cells were exposed to 10 Gy γ -irradiation, and harvested 1 h later. Phosphorylated-HA-Chk2 (WT) was immunoprecipitated with anti-HA antibody, washed five times with lysis buffer, and resuspended in 50 μ l phosphatase buffer (50 mM Tris-HCl (pH 7.5), 30 mM MgCl₂, 1 mg/ml bovine serum albumin, 0.05% 2-mercaptoethanol). The *in vitro* phosphatase reaction was initiated by addition of purified GST-Wip1 (WT) or GST-Wip1 (D314A), and allowed to incubate for 1 h at 30°C. Samples were separated by SDS-PAGE (9% PAG), and transferred onto PVDF membrane filters. Immunoblot analysis was performed with anti-phospho-Chk2 (Ser19, Ser33/35, Thr68, Thr387, Thr432, or Ser516) antibody to monitor the extent of Ser19, Ser33/35, Thr68, Thr387, Thr432, or Ser516-phosphorylation in Chk2 in the respective samples. The amounts of phosphorylated HA-tagged Chk2 (WT) were assessed by anti-HA immunoblot analysis. Kinase activities of Chk2 after treatment with bacterial expressed GST-Wip1 (WT) or GST-Wip1 (D314A) *in vitro* were determined as follows: samples treated with either GST-Wip1 (WT) or GST-Wip1 (D314A) were washed once with kinase buffer (10 mM HEPES-NaOH (pH 7.5), 5 mM MgCl₂, 2 mM DTT), resuspended in 30 μ l of the kinase buffer containing 2 μ g GST-Cdc25C (a.a. 200–256), and 15 μ Ci of [γ -³²P]ATP (5000 Ci/mmol; Amersham), and incubated for 30 min at 30°C. The reactions were terminated by the addition of Laemmli sample buffer, separated by SDS-PAGE (12% PAG), and were subjected to autoradiography. Kinase activities of Chk2 in cells coexpressing Flag-Wip1 (WT) or Flag-Wip1 (D314A) or in cells transfected with Wip1 siRNAs or control siRNAs (see below) were also determined similarly using GST-Cdc25C (a.a. 200–256) as a substrate following anti-HA or anti-Chk2 immunoprecipitation, respectively.

siRNA

The siRNA duplexes were 21 bp including a 2-base deoxynucleotide overhang synthesized by Dharmacon Research. The sequence of the Wip1 siRNA oligo was UUGGCCUUGUGCCUACUAA. The sequence of the p53 siRNA oligo was GCAAUGGAUGAUUGAUGC. The control siRNA oligo used (Scramble II Duplex) was GCGCGCUUUGUAG GAUUCG. Cells were transfected with siRNA duplexes using GeneSilencer (Gene Therapy Systems) following the manufacturer's instructions.

TUNEL assays

Cells were transfected with the Wip1 siRNAs or control siRNAs or GST-HA-Chk2 or Flag-Wip1 as described above, subjected to γ -irradiation (20 Gy), and cultured for 36 h. By using the MEBSTAIN Apoptosis Kit II (MBL), TUNEL assays were performed following the protocol recommended by the manufacturer. In brief, cells were washed with PBS, fixed

with 1% (w/v) formaldehyde in PBS for 15 min at 4°C, and ethanol/acetic acid solution (2:1) for 5 min at –20°C. DNA fragmentation was nick end-labelled with biotinylated dUTP, mediated by TdT for 1 h at 37°C, and subsequently stained with FITC-conjugated avidin. The nuclei were stained with DAPI or Hoechst. Samples were visualized using an inverted confocal microscope (Zeiss).

Immunofluorescence

Cells grown on coverslips coated by rat-tail collagen were fixed in 4% paraformaldehyde/PBS for 15 min at room temperature and then permeabilized with PBS containing 0.1% Triton X-100 for 15 min at room temperature. After blocking in PBS with 10% FCS for 30 min, cells were incubated with primary antibodies, anti-FLAG MoAb (M2, 1:500), and/or anti-HA monoclonal Ab (3F10, 1:200), in PBS/10% FCS for 30 min at room temperature. Cells were washed twice with PBS and then incubated with secondary antibodies, Alexa Fluor 546 (goat anti-mouse IgG, 1:500) and/or Alexa Fluor 488 (goat anti-rat IgG, 1:200), in PBS/10% FCS at room temperature for 30 min. After two washes in PBS, the cells were mounted with Pristine Mount (Research Genetics) and analysed with an inverted confocal microscope (Zeiss).

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