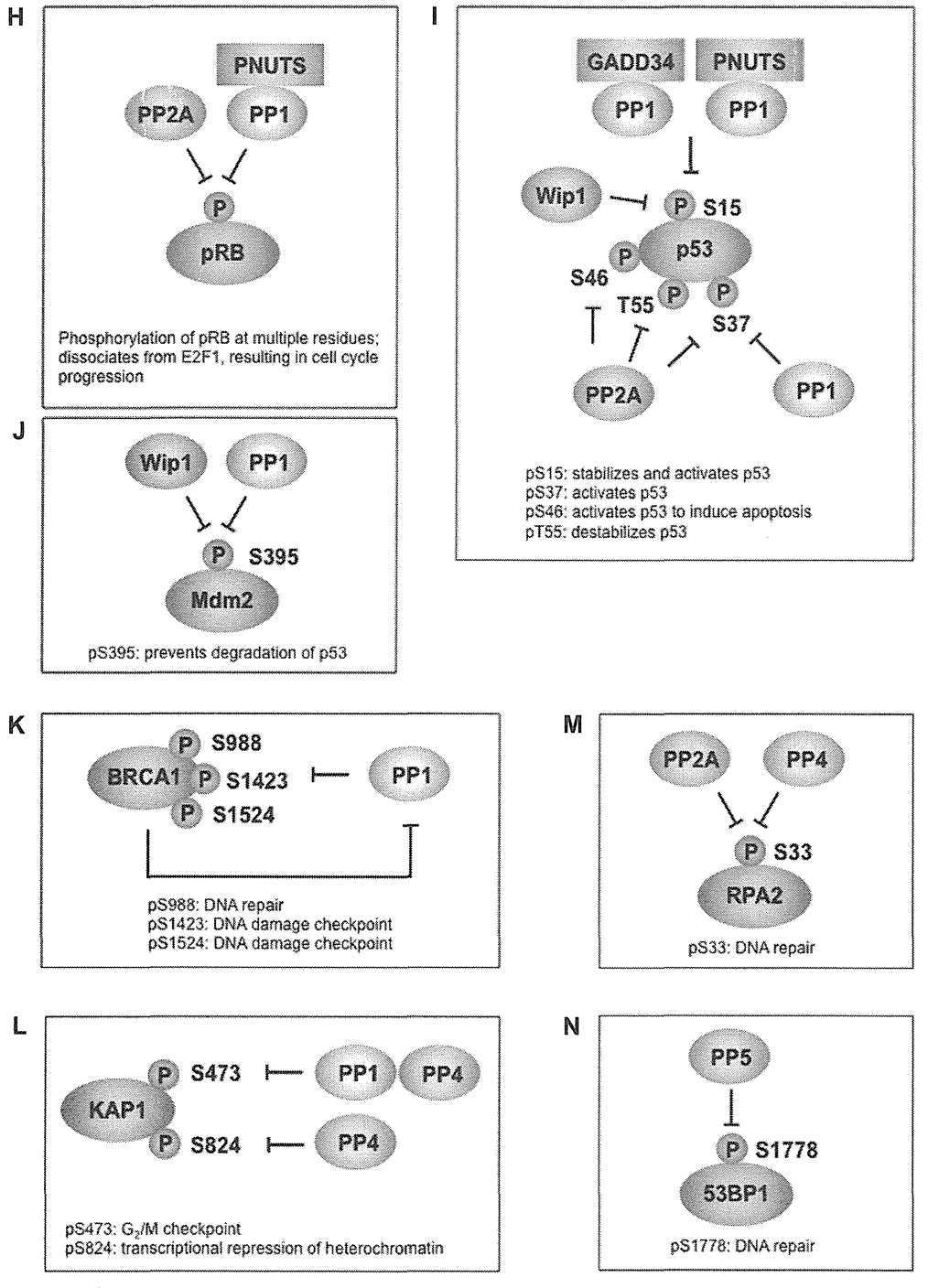

FIGURE 1 | autophosphorylation at T383 and T387. **(F)** γ-H2AX (the phosphorylated form of H2AX at S139) is required for recruitment of various DDR proteins to damaged sites and is dephosphorylated by PP2A, PP4, PP6, and Wip1. Rvb1/Tip60 is implicated in the removal of γ-H2AX. **(G)** PP1 dephosphorylates H3 at T11 following DNA damage, leading to transcriptional repression of cell cycle-regulated genes. **(H)** PP2A and PP1-PNUTS dephosphorylate pRb at multiple sites, leading to inhibition of E2F1 activity and cell cycle arrest. **(I)** p53 is dephosphorylated at S15 by PP1-GADD34, PP1-PNUTS, and Wip1, resulting in p53 inactivation. S37 dephosphorylation is also mediated by PP1 and PP2A. PP2A also dephosphorylates p53 at S46 and T55. **(J)** Wip1 and PP1 dephosphorylate Mdm2 at S395, which facilitates p53 degradation. **(K)** PP1 interacts with BRCA1 and dephosphorylates multiple sites of BRCA1. In addition to acting as a PP1 substrate, BRCA1 also plays a role in PP1 inhibition. **(L)** KAP-1 is dephosphorylated at S473 by PP1 and PP4, whereas S824 is dephosphorylated by PP4. **(M)** PP2A and PP4 are required for RPA2 dephosphorylation. **(N)** Phosphorylated 53BP1 is recruited to DNA damage sites to coordinate the localization of DDR factors and promote their activation. PP5 dephosphorylates 53BP1 at S1778, leading to 53BP1 release from DNA damage sites.

at S1981, is not induced in BAAT1-knockdown cells. Defects in ATM phosphorylation at S1981 observed in BAAT1-knockdown cells could be restored by OA treatment.

TRANSDUCER KINASES, Chk1/Chk2

PP2A

During the normal unperturbed cell cycle, Chk1 is phosphorylated on S317 and S345 by ATR, and in turn, phosphorylated Chk1 is antagonized by Chk1-regulated PP2A to maintain the status of Chk1 activity (**Figure 1D**). Thus, the activity of Chk1 is finely tuned in an ATR-Chk1-PP2A regulatory loop (Leung-Pineda et al., 2006).

PP2A was also reported to interact with Chk2 and regulates phosphorylation at T68 of Chk2 after DNA damage (Dozier et al., 2004; Liang et al., 2006; Freeman et al., 2010; **Figure 1E**). Studies have suggested that PP2A maintains Chk2 in an inactive state under normal conditions, while PP2A dissociates from Chk2 and permits the phosphorylation of Chk2 by ATM under DNA damage conditions. After completion of DNA repair, PP2A has a role in attenuating the DDR partly through dephosphorylation of Chk2.

Wip1

Wip1 binds Chk1 and dephosphorylates S345 and, to a lesser extent, S317, leading to inhibition of Chk1 activity (Lu et al., [illegible]; **Figure 1D**). Thus, Wip1 has a role in abrogating cell cycle checkpoints, in part through dephosphorylation of Chk1.

Wip1 also interacts with Chk2 and dephosphorylates Chk2 at T68 (Fujimoto et al., 2006; Oliva-Trastoy et al., 2007; **Figure 1E**). Knockdown of Wip1 leads to sustained phosphorylation of Chk2 at T68, promoting apoptosis in response to DNA damage. Consistent with this observation, overexpression of Wip1 antagonizes Chk2 activation. Thus, Wip1 is thought to play a negative role in DNA damage-induced apoptosis by dephosphorylation and inactivation of Chk2.

PP5

Upon UV irradiation, ATR-mediated phosphorylation of Chk1 at S345 is increased and maintained in PP5-depleted cells. After 24-h exposure to UV irradiation, this site is dephosphorylated to control levels, indicating that PP5 is not the only phosphatase mediating Chk1 at S345 (Amable et al., 2011). Importantly, PP5-knockout MEFs also exhibit prolonged and enhanced phosphorylation of Rad17, H2AX, and Chk1 at S317. However, contrary to this observation, one study has shown that knockdown of PP5 by antisense PP5 or ectopic expression of a catalytically inactive PP5 mutant leads to impairment of the ATR-mediated phosphorylation of Rad17 and Chk1 (Zhang et al., 2005). The precise functions of PP5 in the DDR remain to be determined.

PP1

The involvement of PP1 in checkpoint recovery is less well studied. However, a study in *Schizosaccharomyces pombe* demonstrated that dephosphorylation of Chk1 by the PP1 homolog Dis2 allows mitotic entry upon completion of DNA repair in G_2 phase (den Elzen and O'Connell, 2004; **Figure 1D**). However, in human cells, knockdown of PP1 does not change the phosphorylation status of Chk1 on S317, and PP1 does not dephosphorylate Chk1 directly (Leung-Pineda et al., 2006).

HISTONES AND HISTONE VARIANTS

H2AX-pS139 (γ-H2AX)

PP4 PP4 dephosphorylates γ-H2AX *in vitro*, and knockdown of PP4 shows persistent γ-H2AX without apparent deficiencies in DNA repair following IR, suggesting that PP4 has a direct role in the dephosphorylation of γ-H2AX (Nakada et al., 2008; **Figure 1F**). Indeed, PP4C knockdowned cells display a prolonged G2/M checkpoint arrest after IR. It is also reported that PP4 is required to repair DNA replication-mediated DNA damage and PP4 silenced cells are sensitive to DNA replication inhibitors (Chowdhury et al., 2008).

PP2A In response to DNA damage, PP2A forms foci and colocalizes with γ-H2AX and dephosphorylates γ-H2AX (Chowdhury et al., 2005; **Figure 1F**). However, since repair of damaged DNA is delayed in PP2A-depleted cells, the PP2A-dependent increase in γ-H2AX may be partly due to reduced repair (Chowdhury et al., 2005; Nakada et al., 2008).

PP6 Down-regulation of either PP6C or PP6R1 causes extensive γ-H2AX and persistent γ-H2AX foci formation following DNA damage, suggesting that PP6 plays a role in the dephosphorylation of γ-H2AX (Douglas et al., 2010; **Figure 1F**). It is important to note that knockdown of PP6 did not affect the phosphorylation of ATM at S1981, SMC1 at S957, or Chk2 at T68 (Douglas et al., 2010). In the context of cisplatin-induced DSBs, PP6 is required for homologous recombination; thus, persistent γ-H2AX in PP6-depleted cells can be explained by delayed DSB repair (Zhong et al., 2011).

Wip1 A recent study reported that Wip1 binds directly to H2AX and dephosphorylates it *in vitro* and *in vivo*, leading to reverse checkpoint signaling (Cha et al., 2010; **Figure 1F**). Moreover, ectopic expression of Wip1 reduces IR-induced γ-H2AX and foci formation for several DDR factors, leading to delayed DNA repair after IR. However, whether knockdown of Wip1 affects DNA repair efficiency remains unknown.

Histone H3

PP1 We have recently identified a novel function for Chk1 as a transcriptional regulator through phosphorylation of H3 at T11 (H3-pT11; Shimada and Nakanishi, 2008; Shimada et al., 2008). This phosphorylation appears to activate the GCN5 histone acetyltransferase complex, leading to H3K9 acetylation and transcription of critical cell cycle regulatory genes, such as *cdk1* and *cyclin B1*. Upon DNA damage, Chk1 rapidly dissociates from chromatin, H3T11 phosphorylation and H3K9 acetylation levels are reduced, and target genes are repressed. In addition to release of Chk1 from chromatin, we recently reported that activation of protein phosphatase 1 is involved in the reduction of H3-pT11 following DNA damage through suppression of T311 phosphorylation due to decreased Cdk1 activity (Shimada et al., 2010; **Figure 1G**).

THE EFFECTOR MOLECULES pRb, p53, AND Mdm2

PP1

Retinoblastoma tumor suppressor protein (pRb), which is negatively regulate cell cycle progression, can interact with all PP1

isoforms (Durfee et al., 1993; Vietri et al., 2006), and PP1 dephosphorylates and activates pRb at the mitosis-to-interphase transition (Alberts et al., 1993; Durfee et al., 1993; Ludlow et al., 1993; Nelson et al., 1997; **Figure 1H**). Importantly, recent data revealed that PP1 competes with Cdks for binding to pRb (Hirschi et al., 2010). PP1 regulatory factors have also been implicated in the regulation of pRb. One of the regulatory subunits, PNUTS dissociates from PP1 under hypoxia stress, leading to activation of PP1, dephosphorylation of pRb at T821, and inhibition of cell growth (Udho et al., 2002; Krucher et al., 2006). Importantly, depletion of PNUTS in cancer cells, but not in normal cells, induces apoptosis through the activation of PP1 and its subsequent regulation of pRb (Krucher et al., 2006; De Leon et al., 2008).

p53 is phosphorylated on pS15 upon DNA damage by ATM/ATR and contributes to stabilization and activation of p53 (Dumaz and Meek, 1999; Lu et al., 2005). Phosphorylation of p53 at S37, which is also transiently up-regulated upon DNA damage, is required for p53 transcriptional activity (Dohoney et al., 2004). PP1 dephosphorylates p53 at S15 and S37 *in vitro* and *in vivo*, reducing transcriptional activity and attenuating apoptosis (Li et al., 1998, 2006; **Figure 1I**). Growth arrest and DNA damage 34 (GADD34) is known to inhibit the binding of PP1 to p53 and prevent dephosphorylation of p53 at S15 (Li et al., 1998). In addition to GADD34, PNUTS also inhibits PP1-dependent dephosphorylation of p53 at S15 and plays a role in apoptosis via regulation of p53 (Lee et al., 2007b). Thus, the association of regulators such as GADD34 and PNUTS with PP1 is required PP1-mediated regulatory activity.

p53 is also regulated indirectly through Mdm2. DNA damage-induced phosphorylation of Mdm2 at S395 by ATM attenuates the ability of Mdm2 to promote nuclear export and degrade p53 (Maya et al., 2001). Once p53 is stabilized and activated, PP1 triggers the inactivation of the signaling cascade (**Figure 1J**). Dephosphorylation of Mdm2 inhibits it autoubiquitination, resulting in stabilization, which triggers degradation of p53 (Lu et al., 2007).

PP2A

PP2AC physically associates with pRb, p107, and p130 *in vivo* (Cicchillitti et al., 2003; Garriga et al., 2004) and mediates oxidative stress-induced dephosphorylation of these proteins (Cicchillitti et al., 2003; Magenta et al., 2008). pRb can also be dephosphorylated by PP2A after IR, which may trigger the recruitment of pRb to replication initiation sites, thereby suppressing abnormal replication (Avni et al., 2003; **Figure 1H**).

PP2A binds to p53 following IR and dephosphorylates multiple sites, S37, S46, and T55 to control p53 activity (Dohoney et al., 2004; Li et al., 2004; Mi et al., 2009a; **Figure 1I**). Under normal cell growth conditions, p53 is phosphorylated at T55 by TATA box binding protein-associated factor 1 (TAF1), resulting in Mdm2-mediated p53 degradation (Li et al., 2004). In response to DNA damage, two reactions trigger dephosphorylation of p53 at T55 and stabilization of p53. One is mediated by the dissociation of TAF1 from p53, while the other occurs through dephosphorylation of B56γ-containing PP2A complexes (Li et al., 2007). B56γ and PP2AC levels are increased upon DNA damage, contributing to PP2A-mediated dephosphorylation of p53 at T55 (Dohoney et al., 2004; Li et al., 2007).

Wip1

Wip1 can dephosphorylate p53 on S15 *in vitro* (Lu et al., 2005; **Figure 1I**). In addition, ectopic expression of Wip1 decreases p53 protein levels and S15 phosphorylation, whereas knockdown of Wip1 results in increased p53 protein levels and S15 phosphorylation. Thus, Wip1 mediates dephosphorylation of p53 at S15.

Wip1 is also known to target Mdm2 at S395, promoting the stability of Mdm2 and enhancing the interaction between Mdm2 and p53 (Maya et al., 2001; Lu et al., 2007; Yamaguchi et al., 2007; **Figure 1J**).

OTHERS

BRCA1

The breast cancer susceptibility gene BRCA1 plays multiple roles in the DDR, such as DNA repair and S and G_2/M checkpoint control (Huen et al., 2010). BRCA1 has a RING finger domain and two BRCA1 terminal domains (so-called BRCT domains) involved in associations with other proteins. DNA damage induces the phosphorylation of BRCA1 at multiple residues, such as S1524 and S1423 by ATM and ATR, respectively (Cortez et al., 1999; Tibbetts et al., 2000), and S988 by Chk2 (Lee et al., 2000). BRCA1 is rapidly localized to damage sites, which contain DNA repair proteins such as Rad51. The PP1α catalytic subunit interacts with BRCA1 and dephosphorylates the sites phosphorylated by ATM, ATR, and Chk2 (Liu et al., 2002; Hsu, 2007; **Figure 1K**). Mutational research of the PP1-binding motif in BRCA1 has revealed that the interaction between BRCA1 and PP1α is important for proper relocation of BRCA1 and Rad51 to DNA damage sites and consequently is important for the DNA repair function of BRCA1. In addition, BRCA1 inhibits PP1α activity, although the precise mechanism underlying this regulatory event remains to be determined (Liu et al., 2002).

KAP1

Phosphorylation of KAP-1 by ATM has been implicated in chromatin relaxation at sites of DSBs (Ziv et al., 2006; Goodarzi et al., 2008), a process that is necessary to permit the recruitment of DDR factors to the damaged DNA. Lee et al. (2010) extensively studied KAP-1 as a PP4 substrate and found that PP4 controls 2IR-mediated phosphorylation sites on KAP-1, i.e., ATM-dependent phosphorylation at S824, which is important for transcriptional repression of heterochromatin, and Chk2-dependent phosphorylation at S473, which is involved in the G_2/M DNA damage checkpoint (Lee et al., 2010; **Figure 1L**). Moreover, a recent study also revealed that PP1 mediates dephosphorylation of KAP1 at S473 and sumoylation of KAP-1 to counter the effect of ATM (Li et al., 2010; **Figure 1L**).

RPA2

RPA is a trimeric protein complex involved in DNA replication, DNA repair, and recombination. ATM, ATR, and DNA-PK phosphorylate one of the subunits, RPA2, and this phosphorylation event is important for the DNA repair function of the enzyme (Wang et al., 2001; Sakasai et al., 2006; Anantha et al., 2007). In addition to phosphorylation, timely dephosphorylation of RPA2 is required for the recruitment of the homologous recombination

factor Rad51 and RPA2 itself to damaged sites, which facilitates DNA repair (Lee et al., 2010). PP4 dephosphorylates the RPA2 subunit at multiple sites, of which S33 seems to be critical for the function of RPA2 after DNA damage, and PP4R2 mediates the DNA damage-dependent interaction of RPA2 and PP4C, the PP4 catalytic subunit (Lee et al., 2010; **Figure 1M**). It has also been reported that PP2A is involved in the dephosphorylation of RPA2 after hydroxyurea treatment (Feng et al., 2009; **Figure 1M**).

53BP1

53BP1 is phosphorylated and recruited to DNA damage sites and plays a role in the DDR, including the DNA damage checkpoint and DNA repair. PP5 has been shown to regulate the function of 53BP1 after DNA damage through dephosphorylation at S1778 and release of phospho-53BP1 foci following NCS treatment (Kang et al., 2009; **Figure 1N**).

CONCLUSION

Spatial and temporal phosphorylation/dephosphorylation events are critical for the cellular response to DNA damage. Although much work has focused on the regulation of kinases and phosphorylation events, recent reports has revealed the involvement of protein phosphatases in the DDR and have extensively documented the physiological roles of dephosphorylation. As discussed in this review, protein phosphatases have multiple functions in the activation and inactivation of the DDR through numerous dephosphorylation events. However, several questions remain to be investigated. First, it is not clear how the activity of each phosphatase is regulated to induce dynamic dephosphorylation events following DNA damage. In the case of PP1, DNA damage triggers dissociation of PP1 and its inhibitory subunits, resulting in the activation of PP1 (Tang et al., 2008). So far, nearly 700 PP1 interacting proteins (PIPs) have been isolated; the identification of specific PIPs for each isoform may also help us to understand the individual roles of these proteins. It is possible that each associated factor is modified by phosphorylation, thereby affecting its interaction with PP1 and altering PP1 activity. It is essential to analyze the precise mechanisms of activation for each phosphatase/substrate combination. Second, in some cases, distinct phosphatases are reported to control the same substrate sites. The purpose of such multi-phosphatase regulation is unclear. Moreover, it is not known whether specific phosphatases are directly involved in the dephosphorylation of target proteins because depletion of certain phosphatase causes multiple effects, including indirect effects on the phosphorylation of target proteins.

It can be speculated that multiple phosphatases regulate different populations of targets at specific regions, such as DNA damage sites and genes whose expression is repressed or activated. Future work is required to determine the precise spatial patterns of phosphorylation events at specific time points.

Importantly, protein phosphatases may be targets for cancer therapy. Mice lacking Wip1 are resistant to spontaneous and oncogene-induced tumors (Choi et al., 2002; Hirasawa et al., 2003; Saito-Ohara et al., 2003; Bulavin et al., 2004). In addition, double knockout of ATM and Wip1 in mice rescues several phenotypes observed in ATM-null mice, such as thymic lymphomas (Darlington et al., 2012), possibly due to enhanced DDRs caused by Wip1 depletion. Although inhibition of PP1 or PP2A has some effects on reduced tumor resistance to radiation or chemotherapy (Hamilton et al., 2009; Lu et al., 2009), it is difficult to apply protein phosphatase inhibitors for cancer therapy because they also affect many other cellular events *in vivo*. In contrast, targeting specific disruption of the interaction between substrates and protein phosphatases may be useful for cancer therapy. In fact, loss of Repo-Man has been reported to reduce anchorage-independent growth of tumor cells in soft agar (Peng et al., 2010). Thus, it is important for cancer therapy to understand the mechanisms underlying the functions and regulation of phosphatases in the DDR.

ACKNOWLEDGMENTS

This work was funded by Grants from the NEXT program. We thank Dr. Sharif for discussions on the manuscript and apologize for any failures to cite relevant articles due to space limitations.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 17 November 2012; paper pending published: 06 December 2012; accepted: 09 January 2013; published online: 31 January 2013.

Citation: Shimada M and Nakanishi M (2013) Response to DNA damage: why do we need to focus on protein phosphatases? Front. Oncol. 3:8. doi: 10.3389/fonc.2013.00008

This article was submitted to Frontiers in Radiation Oncology, a specialty of Frontiers in Oncology.

Biochemical and Biophysical Research Communications 432 (2013) 654–659

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Mitotic phosphorylation of MPP8 by cyclin-dependent kinases regulates chromatin dissociation

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ARTICLE INFO

Article history:
Received 23 January 2013
Available online 14 February 2013

Keywords:
MPP8
Cyclin B1
Cdk1
Chromatin

ABSTRACT

Repressive epigenetic modifications, DNA methylation at CpG sites and histone H3 lysine 9 (H3K9) methylation, are enriched in heterochromatin, which undergoes drastic changes in structure during mitosis. MPP8 (M phase phosphoprotein 8) has been proposed to regulate positive association between these two repressive modifications, but actual involvement of this protein in changes in the heterochromatin structure during mitosis remains elusive. We demonstrate here that MPP8 predominantly localized to, but dissociated from, chromatin during interphase and early mitosis, respectively. Chromatin dissociation from MPP8 appeared to correlate with the phosphorylation status of MPP8. Experiments using inhibitors of various mitotic kinases demonstrated that the chromatin dissociation of MPP8 during metaphase to anaphase was specifically regulated by cyclin B1-Cdk1. Indeed, cyclin B1-Cdk1 effectively phosphorylated MPP8 in vitro and on STA mutant of MPP8 (all possible sites phosphorylated by Cdk were substituted by alanine) failed to dissociate from chromatin during early mitosis. Taken together, our results indicate that the chromatin association of MPP8 is regulated by Cdk-dependent phosphorylation.

1. Introduction

Heterochromatin was originally defined as an unusually condensed chromatin region forming such structures as centromeres and G bands as seen in cytological studies [1]. Although heterochromatin is usually associated with transcriptional silencing [2], this structure also has other functional properties such as roles in DNA replication timing [3] and sister chromatid cohesion [4]. Methylation of histone H3 at lysine 9 as well as DNA methylation are the modifications that primarily characterize heterochromatin [5]. DNA methylation is known to associate with the methylated state of H3 lysine 9, providing clear in vivo evidence that it is strictly dependent on the presence of H3 lysine 9 methyltransferases in Neuropora [6]. Thus, impaired cooperation of DNA methylation with histone H3 methylation likely affects heterochromatin formation.

The heterochromatin of higher eukaryotes cannot be regarded as a static structure during the course of the cell cycle, but rather undergoes dynamic changes in its structure [7–9]. For example, HP1α (Heterochromatin protein 1), a heterochromatin organizer, recognizes H3 methylation at lysine 9 through its chromodomain, which is important for transporting it to heterochromatin regions [10]. Both H3 tri-methylation at lysine 9 and HP1 are thought to be essential for establishing and maintaining heterochromatin domains. In the G2 phase, HP1α is associated with heterochromatin, but it progressively dissociates from it at the G2-M boundary. This dissociation is dependent on Aurora B-mediated phosphorylation of histone H3 at Ser10 without changes in the level of H3 methylation at lysine 9 [7–10]. Although the exact function of this dissociation remains to be determined, null alleles of HP1 in Drosophila and *swi6* in fission yeast suggest their respective functions in proper mitotic chromosome segregation [11,12].

MPP8, originally identified as a novel M phase phosphoprotein by expression cloning, is composed of two functional domains, an amino-terminal chromodomain and a carboxy-terminal ankyrin domain [13]. Similar to HP1α, MPP8 predominantly localizes at the heterochromatin region during interphase [14] and has an important role in heterochromatin organization through regulation of the interplay between DNA methylation and histone H3 methylation [15]. The MPP8 chromodomain specifically binds to Dnmt3a methylated by G9a or GLP, and to self-methylated GLP.

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.02.027

Since the MPP8 chromodomain forms a dimer, dimeric MPP8 could form a Dnmt3a-MPP8-GLP/G9a complex, suggesting that this complex might cooperate in DNA methylation and H3 lysine 9 methylation in chromatin. Taken together with the essential function of MPP8 as a heterochromatin organizer and the observations regarding HP1, we predicted the dynamic distribution of MPP8 as well as HP1α during the onset of mitosis.

In this report, we demonstrated that MPP8, similar to HP1α, predominantly localized to but dissociated from chromatin during interphase and mitosis, respectively. Importantly, this chromatin release appeared to be regulated at least in part by Cdk-dependent phosphorylation.

2. Materials and methods

2.1. Cell culture and synchronization

HeLa cells and human diploid fibroblasts (MJ90) were grown in DMEM supplemented with 10% FBS. HCT116 cells were cultured in McCoy's 5a medium containing 10% FBS. MJ90 cells were synchronized at G0 by serum starvation for 3 days (in DMEM containing 0.5% FBS) and stimulated with DMEM containing 15% FBS. Cells were harvested at the indicated times after release and cell lysates were subjected to immunoblotting and FACS analysis. HeLa and HCT116 cells were synchronized at prometaphase by treating with nocodazole at a final concentration of 100 ng/ml for 12 h. Mitotically arrested cells were collected by a shake-off of the dishes. For Cdk1 inhibition, cells were treated with 9 μM RO-3306 for 30 min. For Aurora or Plk1 inhibition, cells were treated with 2 μM ZM 447439 for 4 h or with BI 2536 for 2 h, respectively.

2.2. Plasmid constructs and site-specific mutagenesis

The full-length cDNA of wild-type human MPP8 was obtained by RT-PCR and was ligated into eukaryotic expression vector pcDNA3.1 myc His. Point mutations of pcDNA3.1 hMPP8 myc His were generated by inverse PCR with a site-specific mutagenesis kit (Toyobo) using specific primers as follows;

TTGAGGCAGAGAGAAGAGAAAGCCCCAGATGATCTGAAAAAGAA AAAA,
TTTTTTCTTTTTCTTCTTTGGCGCAGTATCTTCTTTTGTCTCACTTTG,
GCCCCGAGAAAGGCTGAGG, CTTCTTTTTCCTCCTGCCTCTG,
GCGCCAAAGGGCCGGAG, TTGGGCAGATACAGGCATCAG,

2.3. Cell cycle analysis

Cells were harvested and fixed with 70% ethanol. They were then washed once with PBS, treated with RNase and stained with propodium iodide (PI). Flow cytometry was performed using a FACS CANTO2 flow cytometer (BD Biosciences).

2.4. Immunoblotting

Chromatin and nuclear soluble fractions, and a whole-cell lysate were prepared as previously described [16]. Antibodies used in this study were as follows; anti-MPP8 (16796-1-AP; Proteintech),

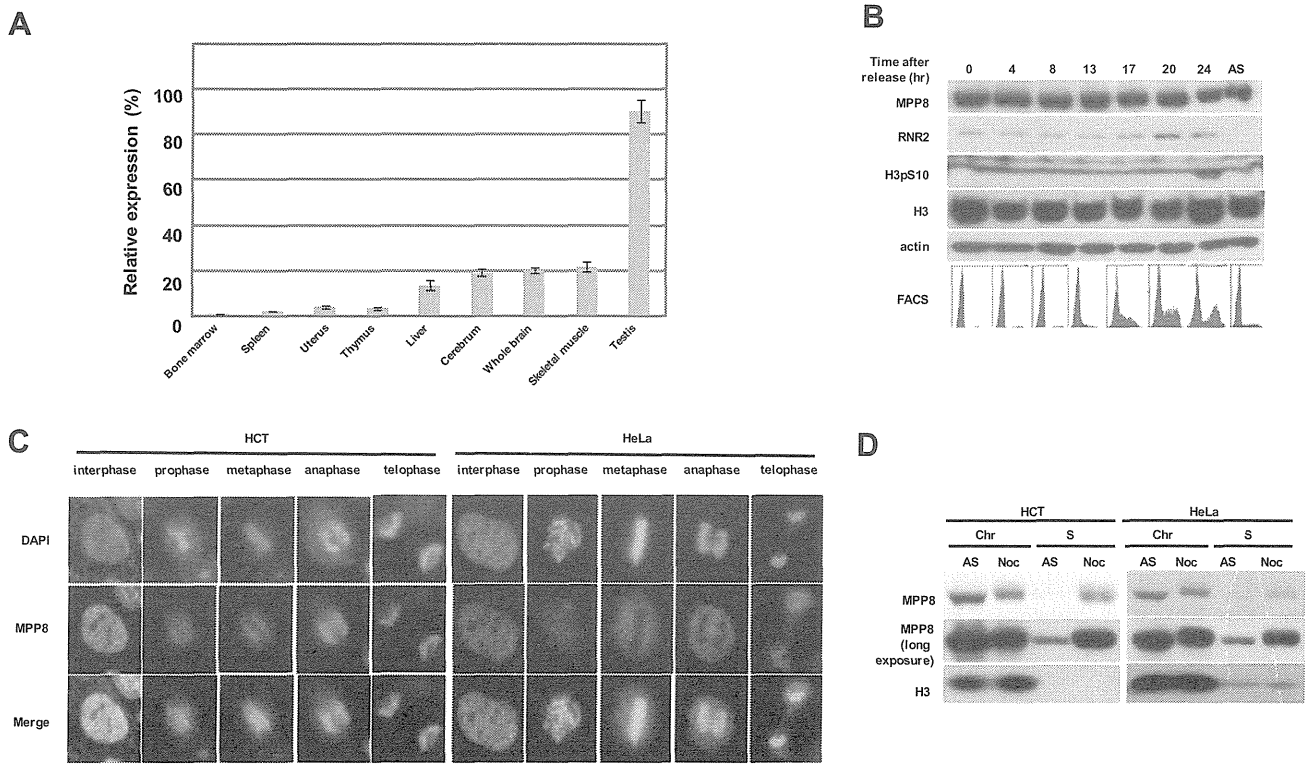

Fig. 1. Tissue-specific expression of MPP8 in mouse and subcellular localization of its protein during cell cycle progression. (A) MPP8 mRNA levels in several tissues of an ICR mouse were detected by quantitative real-time PCR (qRT-PCR). Samples were normalized against levels of beta actin. (B) The level of MPP8 did not vary throughout cell cycle progression. Serum starved MJ90 cells were stimulated by adding serum and were harvested at the indicated times. The cells were prepared for FACS analysis and whole cell lysates were prepared and subjected to immunoblotting with the indicated antibodies. (C) Immunohistochemical analysis of MPP8 in HCT116 cells and HeLa cells. Asynchronously growing HCT116 cells (left) and HeLa cells (right) were fixed and stained with anti-MPP8 antibodies and the nuclei were counterstained by DAPI. Representative images for cell cycle phases are shown. (D) Chromatin fractionation was performed using HCT116 cells (left) and HeLa cells (right) growing synchronously (AS) or synchronized at prometaphase by nocodazole (100 ng/ml, 12 h) (Noc). Chromatin (Chr) and soluble (S) fractions were analyzed by immunoblotting using the indicated antibodies.

anti-phospho-Ser10 histone H3 (06-570; Upstate), anti-phospho-Ser CDKs substrate (2324; Cell Signaling), anti-RNR2 (sc10846; Santa Cruz), anti-Cdk1 (sc54; Santa Cruz), anti-Cyclin B1 (sc245; Santa Cruz), anti-myc (sc40; Santa Cruz) and anti-beta-actin (ab2676-100; Abcam).

2.5. Immunohistochemical analysis

Cells on glass slides were fixed in 4% paraformaldehyde for 10 min at room temperature. Immunofluorescence analyses were performed with anti-MPP8 antibody and the nuclei were counterstained with DAPI as described previously [17].

2.6. Real time PCR

Total RNAs from specific tissues of ICR mice were purchased from UNITECH. Co., Ltd. Primers for the detection of MPP8 transcripts used in this study were as follows; MPP8 TTGGAAGCAGGAGCTTTTGT and TTGCAGTCAGCTCCACATTC, beta Actin AGAAAA TCTGGCACCACACC and AGAGGCGTACAGGGATAGCA.

2.7. Kinase assay

Recombinant-GST-fused MPP8 protein was bacterially expressed and purified with glutathione-sepharose beads (GE). The wild-type and a kinase-dead mutant of cyclin B1-Cdk1 kinase were immunopurified using Cyclin B1 antibodies from Sf9 cells infected with baculoviruses expressing Cyclin B1 and the wild type or kinase-dead mutant of Cdk1. In vitro kinase assays were performed as described [18].

3. Results and discussion

MPP8 is a mitotic phosphoprotein that has recently been reported to mediate interplay between de novo DNA methylation and histone H3K9 methylation [15]. Both DNA methylation and histone H3K9 methylation are involved in the heterochromatin structure, suggesting a function for MPP8 as a heterochromatin organizer. Given that HP1 proteins, a family of heterochromatin organizers, engaged in the dynamic behavior at the G2-M phase transition [19], MPP8 might also exert similar dynamic behavior during the course of the cell cycle and play a role in various cell-cycle-dependent events. To examine the cell-cycle-dependent function of MPP8, we first determined the tissue-specific expression of MPP8 using quantitative PCR. Levels of MPP8 transcripts were extremely varied in our murine model, showing the highest expression in testis (Fig. 1A). We then determined the expression of MPP8 during cell cycle progression. Using normal human fibroblasts synchronized by serum starvation, we found that the expression of MPP8 appeared constant during cell cycle progression although specific expression of RNR2 [20] and phosphorylation of histone H3 at serine 10 [21] were observed at S phase and M phase, respectively, confirming the synchronization of cell cycle progression (Fig. 1B).

We then determined the subcellular localization of MPP8 during M phase. Immunostaining of MPP8 showed a similar signal intensity throughout M phase, confirming its constant expression during the cell cycle. However, MPP8 dissociated from chromatin in early mitosis and re-associated in late mitosis, although its re-association occurred in anaphase in HCT116 cells and in telophase in HeLa cells (Fig. 1C). Consistent with this, cell fractionation

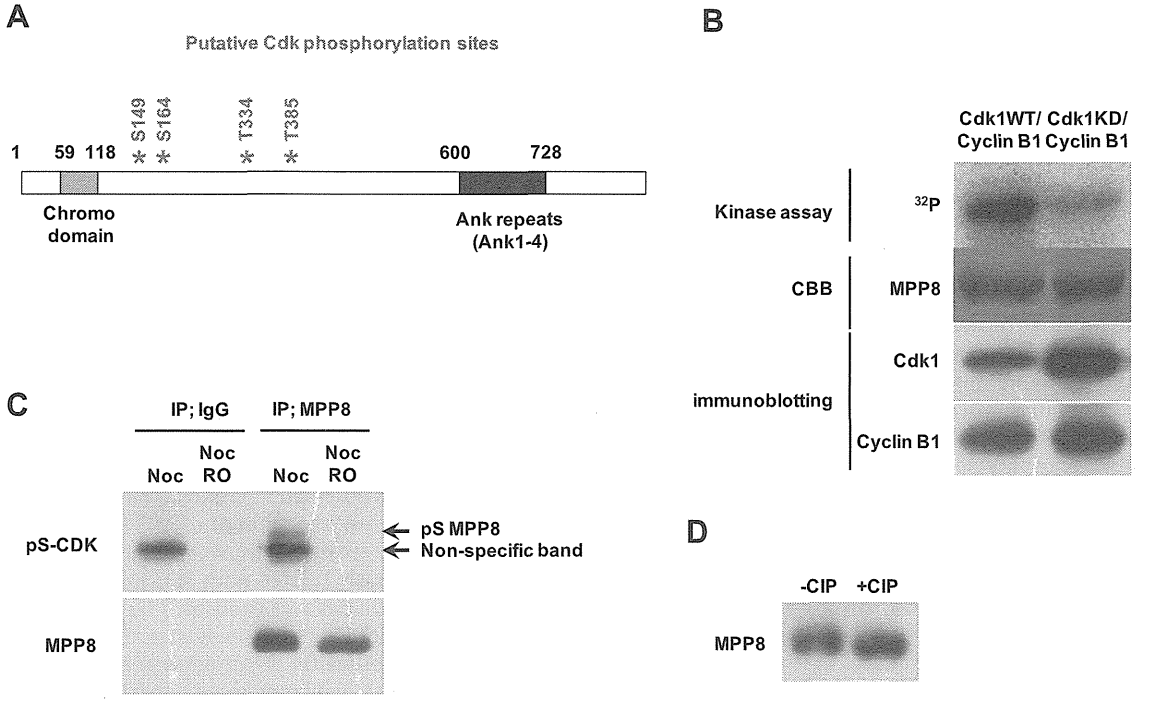

Fig. 2. MPP8 is phosphorylated by cyclin B1-Cdk1 in vitro and vivo (A) Schematic representation of Cdk1 phosphorylation sites on MPP. Putative phosphorylation sites are indicated by an asterisk *. (B) Immunopurified wild-type (WT) or kinase-deficient mutant (KD) of Cdk1 and Cyclin B1 kinase was used for in vitro kinase assays using purified recombinant GST-MPP8 as a substrate. Two top panels: products obtained after an in vitro kinase assay were separated by SDS PAGE and visualized by autoradiography (^{32}P) and staining with Coomassie brilliant blue (CBB). Bottom two panels: a reaction mixture without ^{32}P ATP was subjected to immunoblotting using the indicated antibodies. (C) Chromatin fractionation was performed using HCT116 cells treated with nocodazole (Noc) and the resultant chromatin fractions were solubilized and immunoprecipitated with anti-MPP8 antibodies or control IgG. The resultant immunoprecipitates were subjected to immunoblotting using anti-phospho-Ser CDKs substrate antibodies (pS-CDK) and anti-MPP8 antibodies. (D) Chromatin fractions from nocodazole-treated HCT116 cells were incubated with or without calf intestinal phosphatase (CIP) at 37 °C for 2 h. Immunoblotting was performed using anti-MPP8 antibodies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

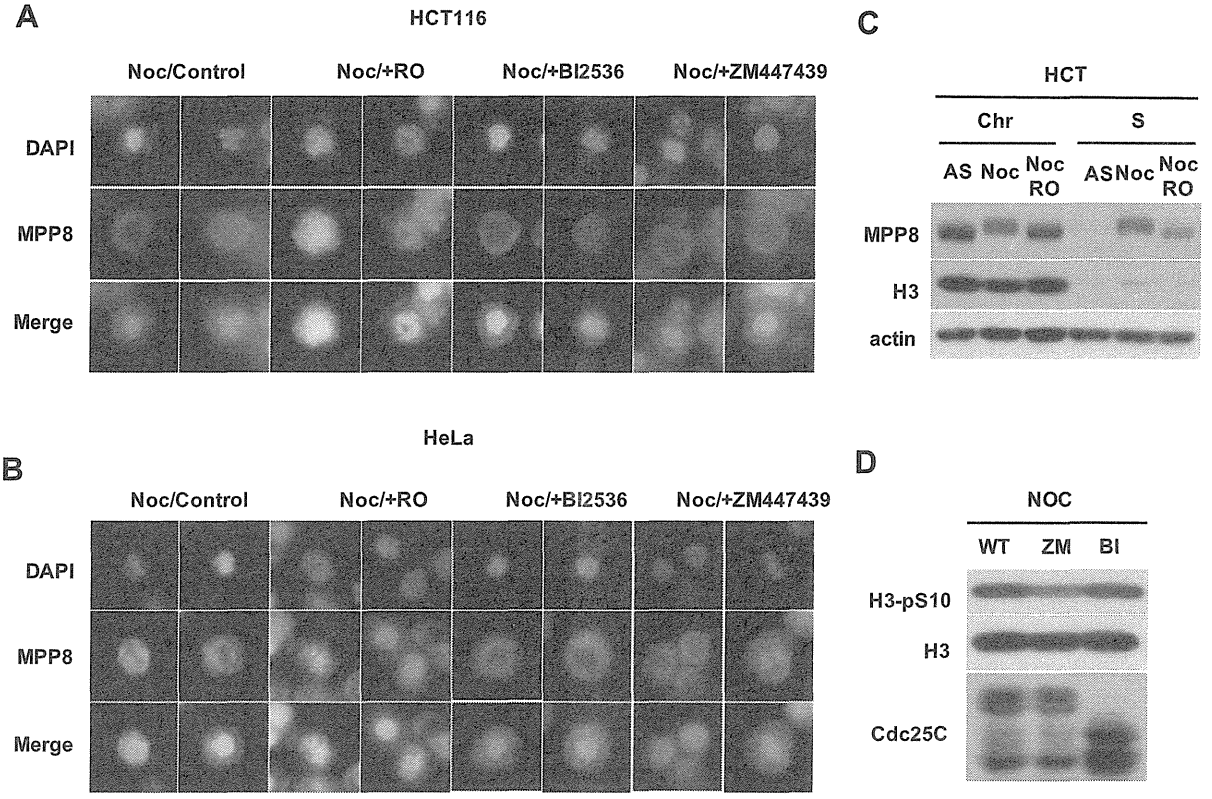

Fig. 3. Inhibition of Cdk1 specifically suppressed dissociation of MPP8 from chromatin during early mitosis. Asynchronously growing HCT116 cells (A) or HeLa cells (B) were treated with nocodazole for 12 h with or without 9 μM RO-3306, 4 μM BI2546 or 2 μM ZM447439 during the last 30 min, 2 h or 4 h, respectively. Mitotic cells were collected by mitotic shake off and spread on the cover glass for immunohistochemistry. (C) Chromatin fractionation was performed from HCT116 cells synchronized by nocodazole with (Noc RO) or without the Cdk1 inhibitor RO-3306 (Noc), or asynchronously growing HCT116 cells (AS). Chromatin (Chr) and soluble (S) fractions were subjected to immunoblotting using the indicated antibodies. (D) HCT116 cells synchronized by nocodazole with or without the 2 μM ZM447439 (ZM) and 4 μM BI2546 (BI) during the last, 2 h or 4 h, respectively. Mitotic cells were collected by mitotic shake-off, chromatin fractionation was performed, and immunoblotting was carried out using the indicated antibodies.

experiments revealed that a significant portion of MPP8 was detected in the soluble fraction collected from cells treated with nocodazole, but not from asynchronized cells (Fig. 1D). In addition, an upshift of the MPP8 band was obvious in cells treated with nocodazole but not in asynchronized cells, suggesting that MPP8 is likely to be phosphorylated during M phase. Taken together, the results indicated that MPP8 was specifically dissociated from chromatin during metaphase to anaphase.

Given that MPP8 is reported to be phosphorylated during M phase [13], we speculated that chromatin dissociation of MPP8 might be regulated by its mitotic phosphorylation. To clarify this point, we first examined whether MPP8 could be phosphorylated in vitro by cyclin B1-Cdk1, one of the major mitotic kinases. There are four putative phosphorylation sites targeted by Cdks in MPP8 (Fig. 2A). Immunopurified cyclin B-Cdk1 expressed in insect cells were incubated with GST-fused recombinant MPP8 produced in *Escherichia coli*. An in vitro kinase assay revealed that wild-type cyclin B1-Cdk1 effectively phosphorylated GST-MPP8, whereas the level of MPP8 phosphorylation was significantly compromised when GST-MPP8 was incubated with the kinase-dead mutant of cyclin B1-Cdk1 (Fig. 2B).

We then examined whether endogenous MPP8 was phosphorylated by Cdks during M phase. MPP8 was immunoprecipitated, using its specific antibodies, from cells treated with nocodazole or nocodazole and RO3306, a Cdk1-specific inhibitor, and then subjected to immunoblotting using anti-phospho-Ser CDKs substrate antibodies. These antibodies specifically recognized MPP8 protein in cells treated with nocodazole, but not with nocodazole and RO3306 (Fig. 2C). In addition, we also found a downshift of the MPP8 band in cells treated with nocodazole and RO3306. To further confirm whether the upshift of the MPP8 band in cells treated with nocodazole was due to phosphorylation, we treated immunopurified MPP8 from nocodazole-exposed cells with calf intestinal phosphatase. We found a clear downshift of the MPP8 band in the CIP-treated samples (Fig. 2D). Taken together, MPP8 was phosphorylated specifically during M phase by cyclin B1-Cdk1.

We then examined whether chromatin dissociation of MPP8 during metaphase and anaphase was regulated by its Cdk1-dependent phosphorylation. Immunostaining of MPP8 revealed that although dissociation of MPP8 was obvious in prometaphase HCT116 cells treated with nocodazole, this dissociation was strongly suppressed when cells were simultaneously treated with RO3306, but not with BI2536, a PLK specific inhibitor, or with ZM447439, an Aurora kinase inhibitor (Fig. 3A). A similar dissociation of MPP8 was observed in prometaphase in HeLa cells (Fig. 3B). Consistent with this, the amount of MPP8 in the soluble fraction from nocodazole-treated cells was reduced when HCT116 cells were simultaneously treated with RO3306 (Fig. 3C). Inhibition of Aurora and Plk kinases was confirmed by suppression of H3 pS10 and phospho-Cdc25C, respectively (Fig. 3D). Thus, these results suggested that chromatin dissociation of MPP8 during metaphase and anaphase was regulated by its phosphorylation by cyclin B1-Cdk1.

Finally, we examined whether the Cdk phosphorylation site mutant of MPP8 retained chromatin binding during early mitosis in order to further confirm the Cdk1-dependent dissociation of MPP8 from chromatin. We substituted serine or threonine residues in four putative phosphorylation sites of MPP8 by replacing them with alanine (STA mutant) and expressed the mutant in HeLa cells (Fig. 4A). Importantly, immunostaining of ectopically expressed

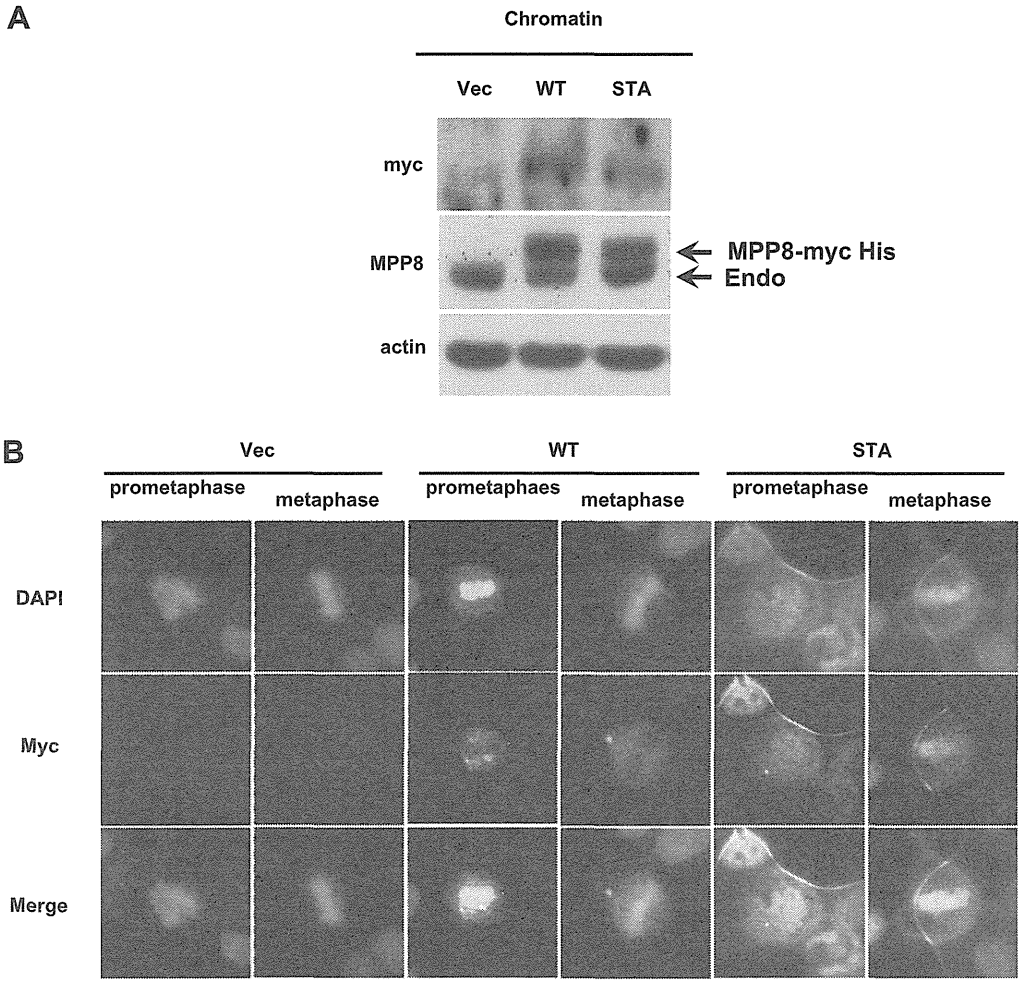

Fig. 4. MPP8 with mutations at CDK phosphorylation sites localized on metaphase chromosomes. (A) Asynchronously growing HeLa cells were transiently transfected with expression vectors expressing MPP8 WT, MPP8 STA (S149A, S164A, T334A and T385A) or empty vector. Three days after transfection, the cells were collected for chromatin fractionation and immunoblotting was performed using the indicated antibodies. (B) The transfected cells described in (A) were fixed and stained with anti-myc monoclonal antibody and the nuclei were counterstained with DAPI. Representative images during prometaphase to metaphase are shown.

MPP8 revealed that although wild-type MPP8 dissociated from chromatin during early mitosis, its STA mutant retained chromatin during this period (Fig. 4B). Thus, the results suggested that Cdk1-mediated phosphorylation of MPP8 regulated its chromatin dissociation during early mitosis.

In summary, our results clearly demonstrated that chromatin localization of MPP8 was regulated by cyclin B1-Cdk1-dependent phosphorylation during early mitosis. As to the physiological role of MPP8 dissociation from mitotic chromatin, removal of MPP8 might be important for enhancing the accessibility of factors essential for mediating proper chromosomal condensation and segregation during early mitosis. In this respect, a similar mitotic dissociation of HP1α from chromosomal arms was proposed to be essential for maintaining the proper structure of mitotic chromosomes [7–9], although dissociation of HP1α from mitotic chromatin was mainly regulated by Aurora B-mediated H3 phosphorylation at Ser 10. This dissociation might also include further modifications of H3-tail (H3 acetylation at lysine 14) [10,22]. In this regard, the binding of MPP8 chromodomain to methylated H3K9 is also reduced in the presence of H3pS10 [23]. Thus, chromatin dissociation of heterochromatin organizers during early mitosis may be commonly required for maintaining a proper heterochromatin structure during the course of the cell cycle regardless of the regulatory mechanisms.

Acknowledgment

We thank Ms. Yoshie Chiba and Ms. Hiromi Matsuo for technical assistance. This work was supported in part by the NEXT program awarded to M.S.

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PLOS ONE

Increased Protein Stability of CDKN1C Causes a Gain-of-Function Phenotype in Patients with IMAGe Syndrome

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Abstract

Mutations in the proliferating cell nuclear antigen (PCNA)-binding domain of the *CDKN1C* gene were recently identified in patients with IMAGe syndrome. However, loss of PCNA binding and suppression of CDKN1C monoubiquitination by IMAGe-associated mutations hardly explain the reduced-growth phenotype characteristic of IMAGe syndrome. We demonstrate here that IMAGe-associated mutations in the *CDKN1C* gene dramatically increased the protein stability. We identified a novel heterozygous mutation, c.815T>G (p.Ile272Ser), in the *CDKN1C* gene in three siblings manifesting clinical symptoms associated with IMAGe syndrome and their mother (unaffected carrier). PCNA binding to CDKN1C was disrupted in the case of p.Ile272Ser, and for two other IMAGe-associated mutations, p.Asp274Asn and p.Phe276Val. Intriguingly, the IMAGe-associated mutant CDKN1C proteins were fairly stable even in the presence of cycloheximide, whereas the wild-type protein was almost completely degraded via the proteasome pathway, as shown by the lack of degradation with addition of a proteasome inhibitor, MG132. These results thus suggested that the reduced-growth phenotype of IMAGe syndrome derives from CDKN1C gain-of-function due to IMAGe-associated mutations driving increased protein stability.

Citation: Hamajima N, Johmura Y, Suzuki S, Nakanishi M, Saitoh S (2013) Increased Protein Stability of CDKN1C Causes a Gain-of-Function Phenotype in Patients with IMAGe Syndrome. PLoS ONE 8(9): e75137. doi:10.1371/journal.pone.0075137

Editor: Bin He, Baylor College of Medicine, United States of America

Received May 5, 2013; **Accepted** August 9, 2013; **Published** September 30, 2013

Funding: The authors have no support or funding to report.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

IMAGe syndrome (OMIM 614732) was originally defined as an association of intrauterine growth restriction, metaphyseal dysplasia, adrenal hypoplasia congenita, and genital anomalies [1]. A number of familial and sporadic cases, which show clinical heterogeneity, have been reported [1–8]. The genetic cause of this syndrome has recently been shown to be mutations in the proliferating cell nuclear antigen (PCNA)-binding domain of the *CDKN1C* gene [9].

CDKN1C (p57Kip2), CDKN1A (p21Cip1), and CDKN1B (p27Kip1) belong to the Cip/Kip family of cyclin-dependent kinase (CDK) inhibitors (Figure 1A), which negatively regulate cell cycle progression by inhibiting G1 CDKs [10,11]. The *CDKN1C* gene is located at 11p11.5, which harbors a cluster of imprinted genes and is expressed only from the maternal allele. Mutations across the length of the *CDKN1C* gene have been identified in patients with Beckwith-Wiedemann syndrome (BWS), which is characterized by an over-growth phenotype and an association with certain cancers; loss-of-function of CDKN1C promotes cell proliferation giving rise to an over-growth phenotype [11,12]. In contrast, the clinical symptoms of patients with IMAGe syndrome strongly suggest that mutations in their *CDKN1C* gene are associated with gain-of-function of the CDKN1C protein, although disruption of PCNA binding and suppression of CDKN1C monoubiquitination do not directly correlate with the *CDKN1C* gain-of-function [9], and truncation mutants of CDKN1C lacking PCNA binding were also identified in BWS patients (Figure 1A) [11,12].

In the present study, we identified a novel maternally inherited mutation in the PCNA-binding domain of the *CDKN1C* gene in three siblings manifesting symptoms associated with IMAGe syndrome. Molecular investigations demonstrated that the IMAGe-associated mutations caused a dramatic increase in the stability of the CDKN1C proteins that probably results in a functional gain.

Subjects and Methods

Subjects

Three siblings, patient 1 (male, III-1 in Figure 2), 2 (female, III-2), and 3 (male, III-3), were born from non-consanguineous Japanese parents with normal adult heights (father (II-1), 182 cm; mother (II-2), 158 cm) and normal birth body weights and lengths. There is no other sibling in this family. The mother's parents (I-1, I-2) and younger sister (II-4) were born with a normal body weight and length and are of normal adult height. All individuals other than the siblings in this family manifest no clinical symptoms associated with IMAGe syndrome. The siblings and their parents were subjected to molecular genetic analysis.

Clinical profiles of the siblings are summarized (Table 1). In brief, they presented with severe intrauterine growth restriction, frontal bossing, and a flattened nasal bridge. The males presented with hypospadias and bilateral cryptorchidism. All three siblings had experienced recurrent episodes of acute adrenal insufficiency, and their adrenals could not be detected by image analysis; their adrenal function is currently well managed by hydrocortisone replacement therapy. No metaphyseal dysplasia was observed.

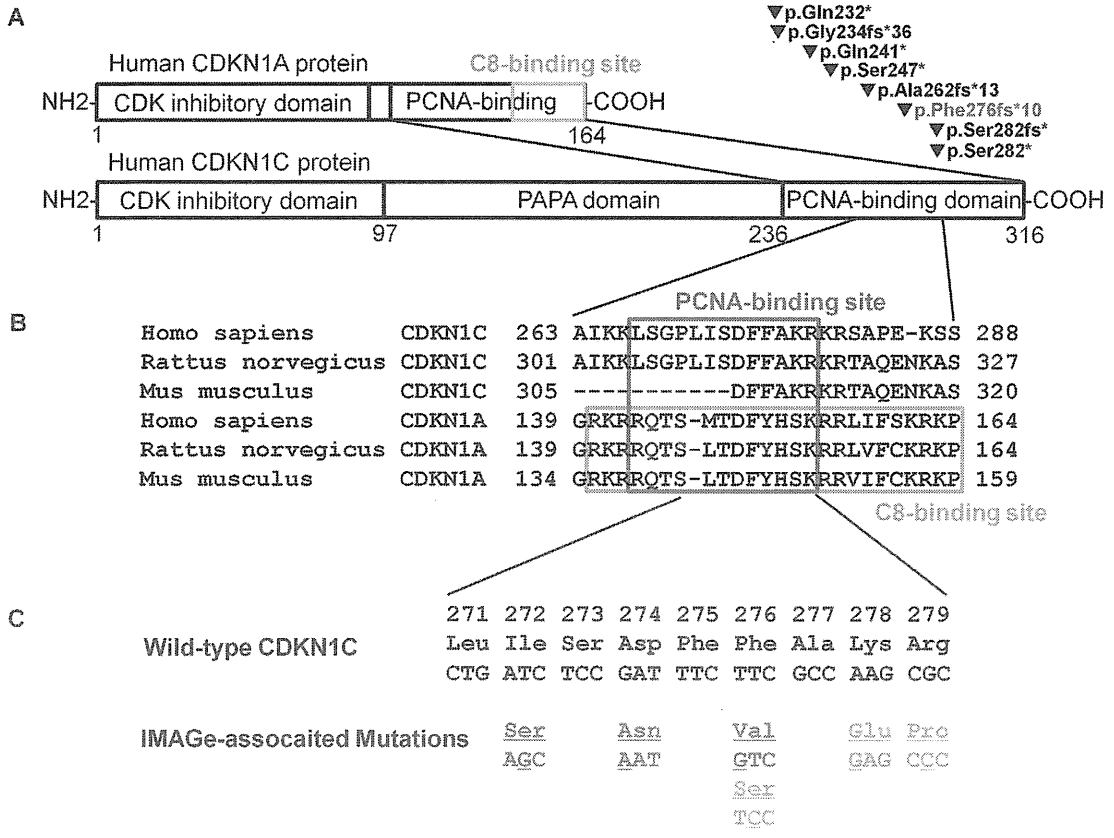

Figure 1. Structure of CDKN1C and CDKN1A proteins and IMAGe-associated mutations. (A) Schematic representation for the structures of human CDKN1C and CDKN1A proteins and for the BWS-associated truncation mutations in the PCNA-binding domain of CDKN1C. The green closed square represents the C8-binding site [13]. Numbers below the schemas represent the locations of amino acid residues. Filled inverted triangles denote the truncation mutants in the PCNA-binding domain of CDKN1C reported in patients with BWS [11,12]. The blue characters represent the mutation analyzed in this article (p.Phe276fs*10). (B) Alignment of amino acid sequences around the PCNA- and C8-binding sites in human, rat, and mouse CDKN1C and CDKN1A. The numbers above the set of sequences represent the amino acid residues. The blue closed square represents the PCNA-binding site [17] and the green closed square represents the C8-binding site [13]. Multiple sequence alignment was performed by using ClustalW (http://www.genome.jp/tools/clustalw/). Accession numbers of the amino acid sequences described here are as follows: NP_000067.1, Homo sapiens CDKN1C; NP_001028929.1, Rattus norvegicus CDKN1C; NP_001155096.1, Mus musculus CDKN1C; AAH13967, H. sapiens CDKN1A; AAI00621, R. norvegicus CDKN1A; and AAH02043, M. musculus CDKN1A. (C) Amino acid and nucleotide sequences of the PCNA-binding domain in the wild-type and IMAGE-associated mutant *CDKN1C* genes in human. Numbers on the top line represent amino residues of the CDKN1C protein based on accession number NM_000076.2. Red characters represent the mutation reported in this article (c.815T>G and p.Ile272Ser). Blue and green characters represent mutations described in the previous report [9]: blue characters represent mutations analyzed in this article (p.Asp274Asn and p.Phe276Val). Underlined characters represent substituted residues and nucleotides.
doi:10.1371/journal.pone.0075137.g001

They presented with severe growth failure without growth hormone deficiency, and are undergoing growth hormone replacement therapy. Their psychomotor development is normal, although a vocal tic was observed in patient 2. No abnormal ophthalmologic findings including eyeball size were identified.

We obtained written informed consent for molecular studies from the patients and the parents. The Institutional Review Board of Nagoya City West Medical Center and Nagoya City University Graduate School of Medical Sciences approved this research. We also obtained written consent to publish this article from the patients and the parents.

Exome Sequencing

Genomic DNA was isolated from the peripheral leukocytes of the siblings and parents by a standard procedure. Exome sequencing was performed with a SureSelect Human All Exon 44 Mb kit (Agilent, Santa Clara, CA) and high-throughput sequencing of pair-end reads was conducted with a HiSeq2000 system (Illumina, San Diego, CA). Data was analyzed by using a CLC Genomic Workbench 5.1 (CLC bio, Aarhus, Denmark) under the default settings.

Sanger Sequencing

Sanger sequencing was performed by using a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) with primer pairs 1–6 (Table 2).

Plasmid Constructs and Mutagenesis

Wild-type plasmid encoding human *CDKN1C* cDNA with 3×FLAG tag at the N-terminal was constructed by insertion of a DNA fragment from pBS-human *CDKN1C* plasmid (GenBank, U22398) digested at *Sma*I and *Hind*III sites, into pCMV-3Tag-1B vector (Stratagene, La Jolla, CA) digested at *Eco*RV and *Hind*III sites. Mutant plasmids were constructed by site-directed mutagenesis with a KOD Plus Mutagenesis Kit (TOYOBO, Osaka, Japan) and primer pairs M1–4 (Table 2). Each expression plasmid was purified by using a QIAGEN Plasmid Midi Kit (QIAGEN, Hilden, Germany).

Cell Culture and Transfection

HEK293T and HeLa cells were cultured in DMEM with 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were transiently transfected with expression plasmids by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for HEK293T cells and Nucleofector 2b (Lonza, Basel, Switzerland) for HeLa cells according to the manufacturer's instructions.

Flow Cytometry

At 48 h after transfections, HEK293T and HeLa cells were fixed in 70% ethanol, stained by propidium iodide, and then subjected to cell cycle analysis by FACSCanto II (BD, Franklin Lakes, NJ).

Western Blot Analysis

Cell lysates were prepared from HEK293T cells 48 h after transfection, and then immunoprecipitated with ANTI-FLAG M2 Agarose Affinity Gel (Sigma-Aldrich, St. Lois, MO). Western blot analysis was performed with input and immunoprecipitated (IP) samples by using primary antibodies against FLAG (Sigma Aldrich; F2555, 1:1000 dilution), CDKN1C (Cell Signaling Technology, Danvers, MA; 2557S, 1:1000 dilution), and PCNA (Abcam, Cambridge, UK; ab92729, 1:1000 dilution). To analyze protein stability, HEK293T cells were treated with 0.1 mg/ml cycloheximide and 0.01 mg/ml MG132 (Sigma Aldrich) for 48 h.

Results

Identification of a Novel Mutation in the *CDKN1C* Gene

We carried out exome sequencing of the three siblings and their parents to identify a disease-causing mutation in the siblings. Pair-end reads with an average length of 90 bp were aligned to the human reference genome sequence (GRCh37/hg19), and simple nucleotide variations (SNVs) and small insertions and deletions (Indels) were called. The single nucleotide polymorphism (SNP) database dbSNP build 135 served as a reference for registered SNPs, and non-synonymous SNVs were extracted. We identified 260 variations, including homozygous and compound heterozygous variations inherited in an autosomal recessive pattern and *de novo* heterozygous variations. During the bioinformatic analysis, we were notified of the report by Arboleda et al. [9] describing mutations in *CDKN1C* in patients with IMAGe syndrome, and subsequently, we identified a non-synonymous A to C SNV with low coverage at position chr11:2,905,905 in *CDKN1C* in the three siblings (Figure S1). This mutation was not included in the 260 candidate SNVs because it exhibited an autosomal dominant pattern of inheritance. Sanger sequencing confirmed the presence of this SNV representing c.815T>G and p.Ile272Ser (based on accession number NM_000076.2), in the PCNA-binding domain of the *CDKN1C* gene, in the three siblings and their mother (Figures S2A–C, S2E). No mutation was detected in other coding regions of the *CDKN1C* gene in the siblings. The mother was found to be an unaffected carrier, while the father was homozygous for the wild-type allele (Figure S2D).

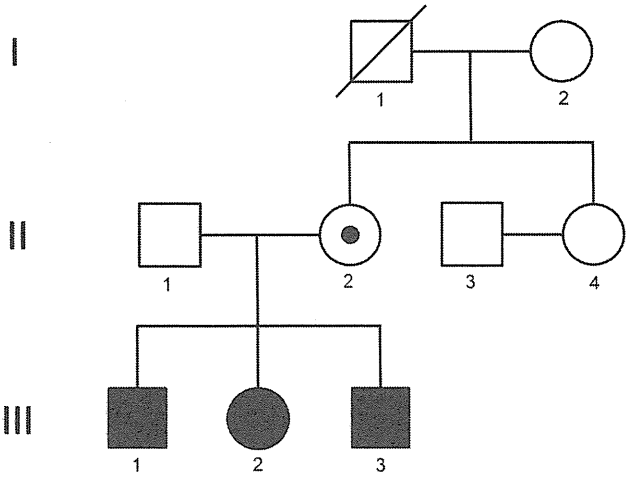

Figure 2. Pedigree of the family with IMAGe syndrome. Filled squares and circles represent the male and female patients, respectively. Closed squares and circles represent the male and female unaffected individuals, respectively. Small filled circles with large closed circles or squares represent unaffected carriers. Diagonal lines represent deceased individuals.
doi:10.1371/journal.pone.0075137.g002

IMAGe-associated Mutations in the *CDKN1C* Gene did not Compromise the Protein Product Activities to Arrest the Cell Cycle

We carried out a cell cycle analysis to investigate the effects of IMAGe-associated and BWS-associated mutations on cell cycle progression. HeLa and HEK293T cells were transfected with plasmids expressing wild-type and p.Ile272Ser mutant protein, as well as two known IMAGe-associated mutant proteins, p.Asp274Asn and p.Phe276Val [9], and a BWS-associated mutant protein, p.Phe276fs*10, caused by a frame-shift mutation in DNA encoding residues 276 to 285 to generate a nonsense codon at residue 286 [12]. Mock transfections were performed by using pCMV-3Tag-1B vector. FACS analysis to ascertain the proportion of cells in the various cell stages was performed 48 h later on fixed and stained cells (Figure 3). In both HeLa and HEK293T cells, the percentage of cells in the G1-phase was increased by the transfection of wild-type plasmid compared with mock transfection. Moreover, the percentages of G1-phase cells were increased further by transfection of IMAGe-associated mutant plasmids compared with wild-type plasmid, but were slightly decreased by transfection of the BWS-associated mutant plasmids. However, the differences observed did not reach significance.

Loss of PCNA Binding in the IMAGe-associated Mutations

We performed Western blot analyses to investigate the expression and PCNA-binding ability of wild-type CDKN1C protein, three IMAGe-associated mutant CDKN1C proteins, p.Ile272Ser, p.Asp274Asn and p.Phe276Val, and a BWS-associated mutant CDKN1C protein, p.Phe276fs*10. Cell lysates were prepared 48 h after transfection, and both input and IP samples were subjected to Western blot analysis by using primary antibodies against FLAG, CDKN1C, and PCNA (Figure 4). In both input and IP samples, anti-FLAG and anti-CDKN1C antibodies detected the IMAGe-associated mutant proteins, p.Ile272Ser, p.Asp274Asn, and p.Phe276Val, at the same molecular weight (~57 kDa) and abundance as the wild-type protein. In contrast, the BWS-associated mutant protein, p.Phe276fs*10, was detected by anti-FLAG antibody at a smaller molecular weight and markedly reduced expression level compared with the wild-type protein. Moreover, the p.Phe276fs*10 mutant protein was not detected by the anti-CDKN1C antibody in the input or IP samples, indicating that it had lost immunogenicity to the antibody. Anti-PCNA antibody detected endogenous PCNA binding to wild-type CDKN1C but not to the IMAGe-associated mutant proteins, p.Ile272Ser, p.Asp274Asn, and p.Phe276Val, nor to the BWS-associated mutant protein,

Table 1. Clinical profiles of three siblings with IMAGe syndrome.

	Patient 1 (male)	Patient 2 (female)	Patient 3 (male)
Present Age	15 years	12 years	10 years
Gestational Age	37 weeks 1 day	39 weeks 1 day	37 weeks 4 days
Body Weight at Birth	1374 g (−3.6 SD)	1772 g (−3.8 SD)	1808 g (−2.8 SD)
Body Height at Birth	38 cm (−4.5 SD)	41 cm (−4.4 SD)	41 cm (−3.2 SD)
Onset of Adrenal Insufficiency	4 months	1 month	8 days
Hydrocortisone Replacement Therapy	From 5 years	From 2 years	From 8 days
Growth Hormone Replacement Therapy	From 11 years with height 105.7 cm (−5.5 SD)	From 8 years with height 102.1 cm (−4.5 SD)	From 6 years with height 95.7 cm (−3.7 SD)
Present Height	128.4 cm (−5.4 SD)	130.2 cm (−3.4 SD)	121.6 cm (−2.3 SD)
Metaphyseal Dysplasia	Not observed from 5 years	Not observed from 3 years	Not observed from 1 year
Other Bone Disease	Thin proximal phalanges, Perthes disease at 13 years	Thin proximal phalanges	Thin proximal phalanges, Cervical supine anomaly
Genital Anomalies	Hypospadias, Cryptorchidism	Not observed	Hypospadias, Cryptorchidism
Puberty	Pubic hair at 11 years	Menarche at 12 years	Not observed

doi:10.1371/journal.pone.0075137.t001

p.Phe276fs*10, in IP samples. We repeated the same experiments by using HeLa cells, and we confirmed the expression of wild-type and mutant CDKN1C proteins and endogenous PCNA proteins (data not shown).

Increased Protein Stabilities in the IMAGe-associated Mutations

Given that a binding site for the C8 alpha-subunit of the 20S proteasome, defined in CDKN1A (Figure 1A–B), overlaps the PCNA-binding site [13], and that the motif is strongly conserved between CDKN1C and CDKN1A (Figure 1B), we analyzed the protein stability of wild-type and IMAGe-associated mutant CDKN1C proteins. HEK293T cells were transfected with wild-type and IMAGe-associated mutant plasmids (p.Ile272Ser, p.Asp274Asn, and p.Phe276Val), and the cells were treated with DMSO, cycloheximide, and MG132. Cell lysates were prepared after the treatment and analyzed by Western blotting (Figure 5). There were no differences in expression levels among wild-type and the three IMAGe-associated mutant proteins without treatment (Figure 5A) or with DMSO for 48 h (Figure 5B). In the presence of cycloheximide for 48 h, the expression levels of wild-type protein were markedly reduced, whereas IMAGe-associated mutant proteins remained the same, i.e., these proteins were remarkably stable (Figure 5C). The expression levels of wild-type proteins were also remarkably improved when treated with MG132 and cycloheximide (Figure 5E). The increased expression levels of IMAGe-associated mutant proteins were minimal when treated with MG132 alone (Figure 5D). These results indicated that the protein degradation of CDKN1C is mediated via a proteasome pathway and that the degradation is severely impaired by IMAGe-associated mutations.

Discussion

Here we describe a novel mutation in the PCNA-binding domain of the *CDKN1C* gene in three Japanese siblings who manifest most of the IMAGe-associated symptoms: i.e., intrauterine growth restriction, adrenal hypoplasia congenita, and genital anomalies in males. Metaphyseal dysplasia, which was originally defined as one of the symptoms of IMAGe syndrome [1], was not observed in the siblings, but other bone disorders were evident. We consider that metaphyseal dysplasia might not be an essential component of IMAGe syndrome, although CDKN1C might play

Table 2. Nucleotides sequences of Primer Pairs.

Primer Pair	Forward Primers	Reverse Primers
1	5′-CAGGAGCCTCTCGCTGAC-3′	5′-CTTTAATGCCACGGGAGGAG-3′
2	5′-GGCGACGTAAACAAAGCTGA-3′	5′-GGGCTCTTTGGGCTCTAAAC-3′
3	5′-CGTTCCACAGGCCAAGTGCG-3′	5′-GCTGGTGCGCACTAGTACTG-3′
4	5′-CGTCCCTCCGCAGCACATCC-3′	5′-CCTGCACCGTCTCGCGGTAG-3′
5	5′-TGGACCGAAGTGGACAGCGA-3′	5′-GGGGCCAGGACCGCGACC-3′
6	5′-CGGAGCAGCTGCCTAGTGTC-3′	5′-CTTTAATGCCACGGGAGGAGG-3′
M1	5′-GCTCCGATTTCTTCGCCAAGCGCAAG-3′	5′-TCAGAGGCCCGGACAGCTTCTTGATC-3′
M2	5′-AATTTCTTCGCCAAGCGCAAGAGATC-3′	5′-GGAGATCAGAGGCCCGGACAGCTTC-3′
M3	5′-GTCGCCAAGCGCAAGAGATCAGCGCC-3′	5′-GAAATCGGAGATCAGAGGCCCGGACAGC-3′
M4	5′-AGTCGCCAAGCGCAAGAGATCAGCGCC-3′	5′-GAAATCGGAGATCAGAGGCCCGGACAGC-3′

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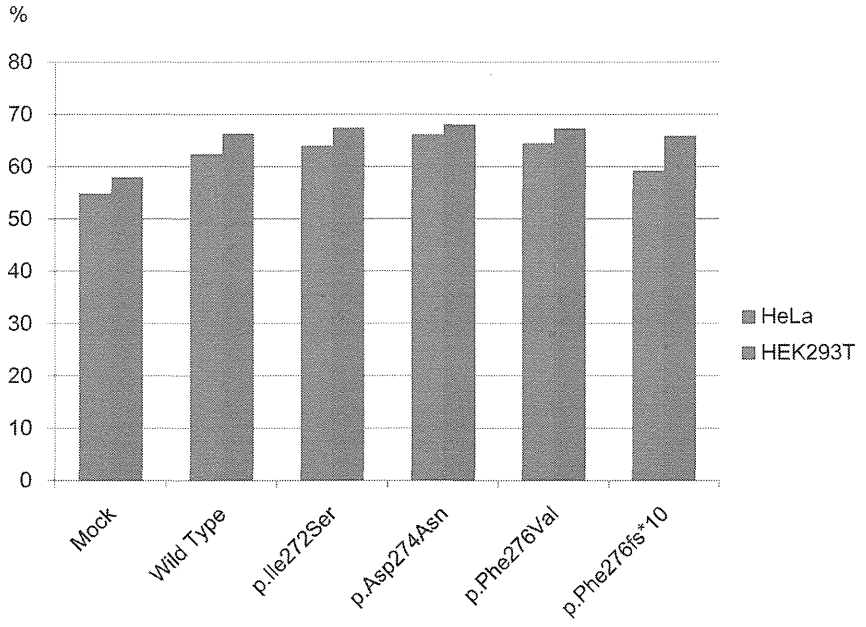

Figure 3. Cell cycle analysis. HeLa and HEK293T cells were transfected with plasmids expressing wild-type or one of four mutant CDKN1C proteins, p.Ile272Ser, p.Asp274Asn, p.Phe276Val, and p.Phe276fs*10. Mock transfections were performed by using pCMV-3Tag-1B vector alone. At 48 h after transfection, cells were fixed in 70% ethanol, stained by propidium iodide, and then subjected to cell cycle analysis by FACSCanto II. Percentages of the cells in G1 phase are presented by bar graphs: blue bars, HeLa cells; red bars, HEK293T cells.
doi:10.1371/journal.pone.0075137.g003

a role in bone disorders because *CDKN1C* knockout mice present with several bone anomalies [14–16]. Although over-expression of IMAGe-associated mutations in *Drosophila melanogaster* results in moderate to severe restriction in eyeball size [9], no ophthalmologic abnormalities were identified in the siblings.

The PCNA-binding motif was originally defined in CDKN1A [17], and is strongly conserved between CDKN1A and CDKN1C (Figure 1A–B). In CDKN1C, the disruption of PCNA binding partially reduces the ability of CDKN1C to suppress myc/RAS-mediated transformation [18]. In a previous study [9], p.Phe276Val and p.Lys278Glu resulted in a complete loss of PCNA binding, and p.Asp274Asn was identified in a sporadic case of IMAGe syndrome. Our findings that the amino acid changes of p.Ile272Ser, p.Asp274Asn, and p.Phe276Val resulted in the complete loss of PCNA binding indicate that Ile272 as well as Asp274 and Phe276 in humans are crucial for the ability of

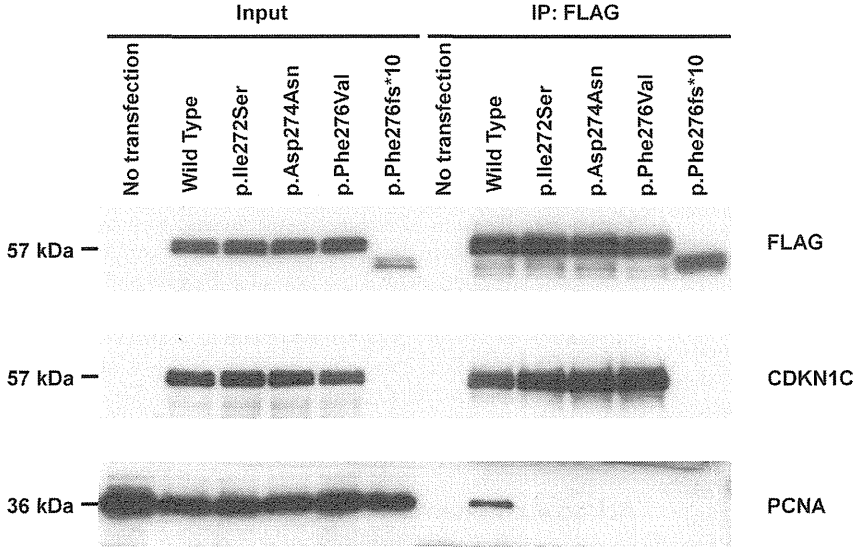

Figure 4. Western blot analysis. HEK293T cells were transiently transfected with plasmids expressing FLAG-tagged wild-type or one of four mutant CDKN1C proteins, p.Ile272Ser, p.Asp274Asn, p.Phe276Val, and pPhe276fs*10. Forty-eight hours after transfection, cell lysates were prepared and immunoprecipitated by anti-FLAG antibody. Both input and immunoprecipitated (IP) samples were subjected to Western blot analysis with antibodies against FLAG, CDKN1C, and PCNA.
doi:10.1371/journal.pone.0075137.g004

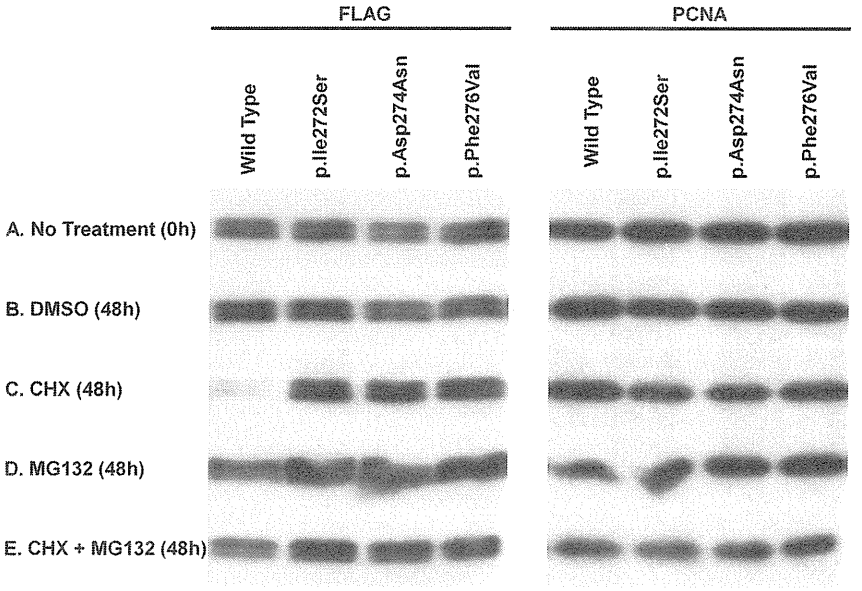

Figure 5. Protein stability assay. HEK293T cells were transiently transfected with plasmids expressing FLAG-tagged wild-type or one of three mutant CDKN1C proteins, p.Ile272Ser, p.Asp274Asn, and p.Phe276Val. At 48 h after transfection, cells were treated with DMSO, cycloheximide (CHX), or MG132 for 48 h. Cell lysates were prepared at 0 h (no treatment) or at 48 h (DMSO, CHX, MG132, CHX+MG132), and subjected to Western blot analysis using anti-FLAG antibody for detecting the CDKN1C proteins and anti-PCNA antibody as a loading control.
doi:10.1371/journal.pone.0075137.g005

CDKN1C to bind PCNA (Figure 1C). However, lack of PCNA binding in CDKN1C per se is not directly involved in the gain-of-function phenotype observed in IMAGe syndrome patients because CDKN1C truncation mutants lacking PCNA binding, i.e., p.Phe276fs*10 (Figure 4), were also identified in BWS patients (Figure 1A) [11,12], in whom the loss-of-function phenotype of CDKN1C was always observed.

The stability of Cip/Kip family CDK inhibitors is tightly regulated by ubiquitination and proteasome-mediated degradation in a manner dependent on the cell cycle stage [19,20]. For CDKN1C, two distinct degradation complexes mediated by polyubiquitination and proteasome-associated degradation have been identified: the Skp1/Cul1/F-box (SCF)-type E3 ubiquitin ligase complex (SCF^{Skp2} complex) [21] and the TGF beta1-activated, Smad-dependent transcription of the gene encoding F-box protein (FBL12) ubiquitin ligase complex (SCF^{FBL12} complex) [22]. A Thr310 mutation in the CDKN1C protein compromised the effect of Skp2 on the degradation of CDKN1C protein, suggesting that phosphorylation at this residue is required for SCF^{Skp2} complex-mediated ubiquitination [21]. The SCF^{FBL12}-mediated degradation of CDKN1C protein also requires its phosphorylation at Thr310 [22]. On the other hand, ubiquitination-independent degradation promoted by the C8 alpha-subunit of the 20S proteasome was identified in CDKN1A. Interestingly, the C8 interaction domain of CDKN1A completely overlaps the PCNA-binding site (Figure 1A–B) [13]. This degradation pathway is mediated by mouse double minute 2 (MDM2) and mouse double minute X (MDMX), both of which trigger the degradation of CDKN1A in G1 and early S phases [23,24]. In addition, the 14-3-3 tau protein plays a role in promoting MDM2-mediated CDKN1A degradation through binding to MDM2, CDKN1A, and the C8 subunit of the 20S proteasome [25].

It is not clear whether CDKN1C is degraded by the C8 alpha-subunit of the 20S proteasome; however, it is possible that IMAGe-associated mutations disrupt binding of these two molecules because IMAGe-associated mutations are located on the putative binding site of the C8 alpha-subunit of the 20S proteasome in CDKN1C.

In conclusion, our findings clearly demonstrated that IMAGe mutations in the *CDKN1C* gene significantly stabilized the protein products. Therefore, an increase in the CDKN1C protein level can easily lead to the typical gain-of-function phenotypes observed in IMAGe syndrome patients.

Supporting Information

Figure S1 Mapping of exome sequencing. Mapping results of pair-end reads around chr11:2,905,905 (GRCh37/hg19) in patient 1 (A), patient 2 (B), and patient 3 (C) by exome sequencing are presented. The vertical red lines denote the nucleotide position on chr11:2,905,905 (GRCh37/hg19). Genomic DNAs were isolated from peripheral leukocytes from three siblings and both parents by a standard procedure.
(TIF)

Figure S2 Results of Sanger sequencing. Results of Sanger sequencing to validate the A to C substitution at chr11:2,905,905 (GRCh37/hg19) in patient 1 (A), patient 2 (B), patient 3 (C), father (D), and mother (E) are presented. A heterozygous A to C substitution at chr11:2,905,905 representing c.815 T>G in the *CDKN1C* gene (p.Ile272Ser amino acid change) was identified in the three siblings (A, B, C) and their mother (E). The father (D) was found to be homozygous for the wild-type allele.
(TIF)

Acknowledgments

We are grateful to Dr. Chisato Yamada for valuable technical advice. We wish to thank all members in the laboratories of Department of Pediatrics and Neonatology, and Department of Cell Biology, Nagoya City University Graduate School of Medical Sciences, for helpful assistance.