PP1α, PP1β and Wip-1 regulate H4S47 phosphorylation and deposition of histone H3 variant H3.3

Hui Zhang, Zhiquan Wang and Zhiguo Zhang*

Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905, USA

Received March 16, 2013; Revised May 19, 2013; Accepted June 10, 2013

ABSTRACT

Phosphorylation of histone H4 serine 47 (H4S47ph) is catalyzed by Pak2, a member of the p21-activated serine/threonine protein kinase (Pak) family and regulates the deposition of histone variant H3.3. However, the phosphatase(s) involved in the regulation of H4S47ph levels was unknown. Here, we show that three phosphatases (PP1α, PP1β and Wip1) regulate H4S47ph levels and H3.3 deposition. Depletion of each of the three phosphatases results in increased H4S47ph levels. Moreover, PP1α, PP1β and Wip1 bind H3-H4 in vitro and in vivo, whereas only PP1α and PP1β, but not Wip1, interact with Pak2 in vivo. These results suggest that PP1α, PP1β and Wip1 regulate the levels of H4S47ph through directly acting on H4S47ph, with PP1α and PP1β also likely regulating the activity of Pak2. Finally, depletion of PP1α, PP1β and Wip1 leads to increased H3.3 occupancy at candidate genes tested, elevated H3.3 deposition and enhanced association of H3.3 with its chaperones HIRA and Daxx. These results reveal a novel role of three phosphatases in chromatin dynamics in mammalian cells.

INTRODUCTION

Chromatin, an organized complex of DNA, RNA and proteins, encodes epigenetic information and maintains genome integrity. The basic repeating unit of chromatin is the nucleosome, which consists of 147 bp of DNA wrapped around a histone octamer comprising one histone (H3-H4)2 tetramer and two histone H2A-H2B dimers. In addition to canonical histones, histone variants also play an important role in marking chromatin into distinct functional domains and regulating diverse cellular processes including chromosome segregation and gene expression (1–3). In mammalian cells, there are two major histone H3 variants, CenH3 (CENPA) and H3.3. Compared with canonical histone H3.1 [H3.1 and H3.2, differing by only one amino acid (4–6), we refer H3.1 as the canonical H3 throughout the text for simplicity of discussion], CenH3 and H3.3, while adopting a similar structural fold as H3.1, have distinct functions. For instance, CenH3 is specifically localized at centromere and is important for the establishment and maintenance of a functional kinetochore (1). H3.3, although differing from H3.1 by only five amino acid residues, has unique functions that cannot be substituted by H3.1 (6–8). Early studies indicated that H3.3 was enriched at gene bodies of actively transcribed genes, and the levels of H3.3 at gene bodies positively correlated to gene expression (9–11). Recently, H3.3 has also been found at the promoters of both active and inactive genes in HeLa and embryonic stem cells (12,13). In addition, H3.3 has been detected at pericentriole heterochromatin (14,15). Thus, H3.3 likely impacts both active chromatin and heterochromatin. Supporting this idea, the levels of H3.3 have been found to play an important role in maintenance of epigenetic memory of actively transcribed states during nuclear transfer, and mutations at H3.3 compromise formation of heterochromatin during mouse development (15). More recently, it has been shown that H3FA3, one of two genes that encode H3.3, is frequently mutated in pediatric high grade brain tumors and H3.3 mutations are proposed to drive tumor formation (16,17). Thus, it is likely that H3.3 has diverse functions, and alterations of which will lead to human diseases.

The diverse functions of H3.3 are likely related to how H3.3 is deposited onto DNA to form nucleosomes. HIRA, which forms a complex with two other subunits, Cabin and UBN1, is an H3.3-H4 chaperone and aids in the assembly of H3.3-H4 into nucleosomes in a replication-independent manner (18,19). Recently, several groups have reported that the Death domain containing protein (Daxx), which forms a complex with the chromatin remodeling protein ATRX, is another H3.3 chaperone (13,20,21). H3.3 localization at genic regions depends on HIRA, whereas the H3.3 localization at telomeres depends on model protein ATRX, is another H3.3 chaperone and aids in the assembly of H3.3-H4 into nucleosomes in a replication-independent manner (18,19). Recently, several groups have reported that the Death domain containing protein (Daxx), which forms a complex with the chromatin remodeling protein ATRX, is another H3.3 chaperone (13,20,21). H3.3 localization at genic regions depends on HIRA, whereas the H3.3 localization at telomeres depends on...
on ATRX in mouse embryonic stem cells (13). Thus, different H3.3 chaperones likely regulate the deposition and localization of H3.3 at distinct genomic regions.

Mutational studies indicate that the three H3.3 specific residues play an important role in specifying H3.3 deposition (9,13). Indeed, the structure of H3.3-H4 and Daxx reveals that two H3.3 unique residues (Ala 87 and glycine 90) are important for the recognition of H3.3 by Daxx (22,23). In addition to these H3 residues, the structure of Daxx-H3.3-H4 complex reveals that Daxx makes extensive contact with histone H4. Similarly, Asf1 also interacts with both H3 and H4 as revealed by the structure of Asf1-H3-H4 complex (24). These findings indicate that histone H4 also mediate the interaction between H3.3-H4 and their chaperones, raising the possibility that modifications on histone H4 can impact the interactions between histone and H3-H4 binding proteins. Indeed, acetylation of H4K5, K12 by Hat1 in human cells enhances the interactions of importin 4 with H3.1, but not H3.3 (25). In addition, phosphorylation of histone H4S47 increases the interaction between H3.3-H4 and HIRA and consequently regulates H3.3 deposition (26). These results indicate that the levels of H4S47ph must be regulated to impact H3.3 deposition in cells.

H4S47 phosphorylation is catalyzed by Pak2, a member of the p21-activated serine/threonine protein kinase (Pak) family that consists of Pak1-Pak6 (27,28). Pak family members participate in a variety of cellular processes and have also been implicated in several cancers (28–30). However, the phosphatases that are involved in regulation and have also been implicated in several cancers (28–30).

**MATERIALS AND METHODS**

**Cell culture, transfection and infection**

HeLa and OVCAR5 cells were grown in Dulbecco’s modified Eagle’s medium (GIBCO) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. HCT116 cells were grown in RPMI 1640 (GIBCO) with 10% fetal bovine serum and 1% penicillin/streptomycin. Stable cell-lines (including those expressing e-H3.1, e-H3.3, each tagged with both the Flag and HA epitopes) were grown in the presence of 1 μg/ml Puromycin. Cells were incubated at 37°C with 5% CO₂. Transient transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Lentiviruses based on shRNA was packaged using 293T cells and infected into targeting cells following a procedures as described.

**Chromatin immunoprecipitation assay and real-time PCR**

Chromatin immunoprecipitation (ChIP) assays were performed as described (26). Briefly, for each ChIP assay, 2 × 10⁶ Cells were cross-linked with 1% (v/v) formaldehyde for 10 min at room temperature and quenched by addition of glycine to a final concentration of 125 mM. Cells were washed with 1× Phosphate buffered saline (PBS) (1 mM phenylmethylsulfonyl fluoride (PMSF)) and then resuspended in 1 ml lysis buffer [50 mM HEPES (pH 7.5), 1% TritonX-100, 140 mM NaCl, 1 mM EDTA, 0.1% (w/v) sodium deoxycholate and protease inhibitors]. The cell lysis was sonicated in a Bioruptor (Diagenode) to achieve a mean DNA fragment size of 0.5–1 kb base pairs. After clarification by centrifugation, the supernatants were incubated with 20 μl of anti-Flag agarose (M2 beads, Sigma) overnight at 4°C. The beads were washed extensively, and DNA-protein complex cross-link was reversed by boiling for 10 min in the presence of 10% chexol. The proteins were digested by Proteinase K at 55°C for 30 min. The beads were then centrifuged, and the supernatants containing DNA were collected. The immunoprecipitated DNA was analyzed using a real-time PCR machine with iQTM SYBRgreen PCR mastermix (Bio-Rad).

**Immunoprecipitation and western blot analysis**

The 293T cells were lysed using the lysis buffer containing 50 mM HEPES–KOH (pH 7.4), 100 mM NaCl, 1% NP40, 10% glycerol, 1 mM EDTA, 1 mM dithiotreitol (DTT) and protease and phosphatases inhibitors (1 mM PMSF, 1 mM Benzamindine, 0.1 mM NaVO₃, 10 mM NaF). After clarification by centrifugation, the supernatants were incubated with 25 μl of M2 (anti-Flag) beads at 4°C for overnight. The beads were washed using washing buffer [50 mM HEPES–KOH (pH 7.4), 100 mM NaCl, 0.01% NP40, 10% glycerol, 1 mM EDTA and proteinase inhibitors] for 5 min × four times. Proteins were dissolved in 1× SDS sample buffer [50 mM Tris (pH 6.8), 100 mM DTT, 2% SDS, 10% glycerol and 0.005% bromophenol blue] and then loaded onto SDS–PAGE gel. The gels were transferred onto nitrocellulose membranes (Biorad). The membranes were blocked in Tris-buffered saline containing 5% (w/v) skimmed milk powder and then were probed with primary antibodies against HIRA (Millipore), Flag (Sigma), p60 (Abcam), Daxx (Millipore), Asf1 as indicated. For the Flag-H3.1/H3.3 immunoprecipitation with depletion of the phosphatases, 293T cells were lysed using the lysis buffer [50 mM HEPES–KOH (pH 7.4), 200 mM NaCl, 0.5% NP40, 10% glycerol, 1 mM EDTA, 1 mM DTT and proteinase and phosphatases inhibitors 1 mM PMSF, 1 mM Benzamindine, 0.1 mM NaVO₃, 10 mM NaF] and denounced by 30 passages. After clarification by centrifugation, the lysates were incubated with 30 μl of M2 (anti-Flag) beads at 4°C for overnight. The beads were washed using washing buffer [50 mM
HEPES–KOH (pH 7.4), 100 mM NaCl, 0.01% NP40, 10% glycerol, 1 mM EDTA and protease inhibitors] for 5 min × six times. Then, the Flag-H3.1/H3.3 and the co-purified proteins were eluted with 2 mg/ml Flag peptide. The eluted proteins were precipitated using TCA and dissolved with 1 × SDS sample buffer and detected by western blot.

**Histone H3.3-SNAP labeling**

The SNAP staining was performed as described previously (31). Briefly, 10 μM SNAP block reagent was added to the medium at 37 °C for 30 min to quench the SNAP activity. Then, cells were washed with medium three times and incubated in the medium for another 30 min. After chasing for 8 h, 2 μM TMR was added to the medium for 15 min at 37 °C. Cells were then pre-extracted with TritonX100 and fixed in paraformaldehyde. A fluorescence microscope (100×) was used to record the SNAP staining and Image J was used to quantify the SNAP fluorescence intensity. For each experiment, >200 cells were counted. For the chromatin fraction assay, after the SNAP staining, the cells were collected by trypsin and washed with PBS. Cells were extracted with CSK buffer on ice for 5 min followed by high speed centrifugation. The chromatin pellet was washed with PBS again and boiled in 1 × SDS sample buffer. Proteins were separated by SDS–PAGE and SNAP-tagged proteins were detected by Typhoon 7900, and the total proteins were visualized by IRDye® Blue Protein Stain and used for loading controls.

**RESULTS**

**Phosphatases PP1α, PP1β and Wip-1 regulate the levels of H4S47 phosphorylation**

We have shown previously that Pak2 is the primary kinase that phosphorylates H4S47 in mammalian cells (26). To identify a phosphatase(s) responsible for the regulation of H4S47ph level, we purchased all available shRNAs targeting seven different catalytic subunits of Ser/Thr protein phosphatases from Sigma and tested how depletion of each affected H4S47 levels in HeLa cells. We found that cells treated with at least two shRNAs targeting PP1α, PP1β or Wip-1 resulted in increased H4S47 phosphorylation (Supplementary Figure S1). To confirm this result, PP1α, PP1β and Wip-1 were depleted from two other cell lines, HCT116 and OVCAR5. In both cell lines, depletion of each of the three phosphatases resulted in a dramatic increase in H4S47ph (Figure 1A and B). In addition, depletion of PP1α did not affect the expression of PP1β or Wip-1 (Figure 1C). Similar results were observed for depletion of PP1β and Wip1 (Figure 1D and E). Together, these results suggest that PP1α, PP1β or Wip-1 likely regulate H4S47ph independently.

**PP1α, PP1β and Wip-1 interact with H3-H4 in vivo and in vitro**

PP1α and PP1β are two members of PP1 family Ser/Thr phosphatases including PP1γ. Wip-1 is a member of PP2C family phosphatases (32,33). To understand how depletion of PP1α, PP1β or Wip1 affects the levels of H4S47 phosphorylation, we first determined whether each phosphatase interacted with H3-H4. Transiently expressed and Flag-tagged PP1α, PP1β, PP1γ or Wip1 was immunoprecipitated from 293T cells, and co-precipitated proteins were analyzed by western blot. H3 and H4 were detected in the precipitates of PP1α, PP1β and Wip-1, but not those of PP1γ (Figure 2A). Canonical histone H3.1 and histone H3 variant H3.3 differ by 5 amino acids (Figure 2B). In a reciprocal immunoprecipitation experiments, we tested whether each phosphatase co-purified with H3.1 and H3.3. PP1α, PP1β and Wip-1, but not PP1γ, were co-purified with histone e-H3.1 or e-H3.3 (Figure 2C). These results are consistent with idea that PP1α, PP1β and Wip-1, but not PP1γ, are H4S47ph phosphatases. To test this idea further, we asked whether each phosphatase purified from 293T cells interacted with recombinant H3.1-H4 or H3.3-H4 tetramers in vitro. As shown in Figure 2D–F, PP1α, PP1β and Wip-1 interacted with H3.1-H4 or H3.3-H4 in vitro. In addition, although PP1α and Wip-1 bound similar amounts of H3.1-H4 and H3.3-H4, we consistently observed that PP1β bound more H3.1-H4 than H3.3-H4 in vitro and in vivo (compare lanes 5–6 in Figure 2C and lanes 3–4 to lanes 5–6 in Figure 2E). These results demonstrate that phosphatases PP1α, PP1β and Wip-1 interact with H3-H4 complex in vivo and in vitro and suggest that PP1α, PP1β and Wip-1 may regulate the levels of H4S47ph by directly removing H4S47 phosphorylation.

**PP1α and PP1β regulate Pak2 phosphorylation**

The H4S47 kinase, Pak2, contains an auto-inhibitory domain at its N-terminus that binds the catalytic domain and inhibits catalytic activity of Pak2 (27). Therefore, Pak2 must be activated to phosphorylate its substrates including H4S47. It is known that phosphorylation of Pak2 T402 or S141 in vitro and in vivo (compare lanes 5–6 in Figure 2C and lanes 3–4 to lanes 5–6 in Figure 2E). These results demonstrate that phosphatases PP1α, PP1β and Wip-1 interact with H3-H4 complex in vivo and in vitro and suggest that PP1α, PP1β and Wip-1 may regulate the levels of H4S47ph by directly removing H4S47 phosphorylation.

**Depletion of PP1α, PP1β and Wip-1 affects H3.3 occupancy**

We have shown previously that depletion of Pak2 reduces the H3.3 occupancy (26). Therefore, we tested whether...
depletion of PP1α, PP1β or Wip-1 has any effect on H3.3 occupancy using a ChIP assay. As reported previously, e-H3.3 was enriched at the genome regions flanking the transcription start site (TSS) of TP53TG1, OSTF1 and the transcription terminal site (TTS) of TM4SF1, whereas e-H3.1 is enriched in the regions flanking TSS of TRIM42 and TTS of CSRP1 (Figure 4). Importantly, depletion of phosphatases PP1α, PP1β or Wip-1 led to a significant increase in the H3.3 occupancy at three H3.3-enriched genes (TM4SF1, TP53TG1 and OSTF1) tested. Interestingly, depletion of each of the phosphatases also led to the increased H3.3 occupancy at CSRP3, but not TRIM42, even though both are H3.1-enriched genes (Figure 4A). These results are consistent with the idea that PP1α, PP1β and Wip-1 regulate H4S47ph levels, which in turn promotes H3.3 deposition.

Depletion of PP1α, PP1β or Wip-1 did not impact the H3.1 occupancy at the two H3.1 candidate genes tested significantly (Figure 4B). As depletion of Pak2 results in increased e-H3.1 occupancy at these candidate genes (26), one would expect that depletion of H4S47ph phosphatase will have the opposite effect on e-H3.1 occupancy as that of Pak2 depletion. One possible explanation for the apparent discrepancy is that the effect of depleting
PP1α, PP1β or Wip-1 on H3.1 occupancy is marginal owing to the presence of two other H4S47ph phosphatases in cells. Nonetheless, our results indicate that PP1α, PP1β and Wip1 can regulate H3.3 occupancy at these candidate genes, revealing a novel function of these phosphatases in chromatin dynamics.

Deposition of newly synthesized H3.3 increases in cells with PP1α and PP1β depletion

To understand how depletion of PP1α, PP1β or Wip-1 affects H3.3 occupancy at chromatin, we established H3.3-SNAP stable cell line as described (31). A SNAP tag is a mutant form of O^6^-guanine nucleotide alkyltransferase that covalently reacts with benzylguanine. To monitor the deposition of newly synthesized H3.3, we performed the ‘quench-chase’ experiment as outlined in Figure 5A. Briefly, after depletion of PP1α, PP1β or Wip1 (Figure 5B), old H3.3-SNAP reacted with benzyl-guanine derivative and would not give rise to fluorescence. After washing away the blocker for 30 min, newly synthesized H3.3 (after 8h) was labeled with TMR and detected with fluorescence. As shown in Figure 5C and D, compared with non-targeting controls, depletion of PP1α or PP1β resulted in a significant increase in H3.3 intensity compared with control cells (NT). In addition, using an independent chromatin fraction assay, the effect of depletion of PP1α and PP1β on H3.3 deposition was also...
observed (Figure 5E and F). Interestingly, although deple-
tion of Wip-1 also resulted in an increase in H3.3 depos-
tion using both fluorescence and chromatin fraction
assays, the effect of Wip-1 depletion on H3.3 intensity
using the fluorescence-based assay from three independent
experiments was not statistically significant (P = 0.1)
(Figure 5D), which could be due to the mild increase in
H3.3 intensity in Wip-1-depleted cells. Together, these
results indicate that PP1α, PP1β and Wip-1 regulate
H3.3 deposition.

Depletion of PP1α, PP1β and Wip-1 results in increased
interaction between H3.3-H4 and its chaperones, Daxx
and HIRA

To understand how PP1α, PP1β and Wip-1 impact depos-
tion of H3.3, we performed in vivo immunoprecipitation
to determine whether depletion of each phosphatase
affects the association of H3.3-H4 with its chaperons,
HIRA and Daxx. As shown in Figure 6, depletion of phosphatases PP1α, β and Wip-1 resulted in increased asso-
ciation of H3.3 with HIRA and Daxx, two H3.3 chap-
erones. However, we did not observe consistent changes in
the association of H3.3 with other histone chaperones
including Asf1a after depletion of each phosphatase.
Furthermore, we did not observe consistent changes in
the association of H3.1 with H3.1 chaperones CAF-1 or
Asf1a and Asf1b (data not shown). Together, these results
suggest that that PP1α, PP1β and Wip1 affect H3.3 occu-
pancy through their impact on the association of H3.3-H4
with H3.3 chaperones.

DISCUSSION

H4S47ph is catalyzed by the Pak2 kinase. Here, we have
presented the following lines of evidence supporting the
idea that three phosphatases, PP1α, PP1β and Wip1,
regulate the level of H4S47ph. First, we show that deple-
tion of PP1α, PP1β and Wip-1, but not the catalytic
subunits of four other phosphatases, results in increased
H4S47ph levels in three different cell lines tested. Second,
we show that PP1α, PP1β and Wip-1 interact with H3-H4
in vitro and in vivo, suggesting that PP1α, PP1β and Wip-1
are directly involved in removing H4S47ph. Third, we
show that PP1α and PP1β, but not Wip1, interact with
Pak2, and depletion of PP1α and PP1β, but not Wip-1,
results in increased phosphorylation of Pak2T402 and
S141. Phosphorylation of these two residues is known to
be involved in Pak2 activation (27). Therefore, the
observed increase in H4S47ph levels in PP1α and PP1β
depleted cells is also likely due, in part, to the activation
of Pak2 in these cells. These results indicate that PP1α,
PP1β and Wip-1 regulate H4S47ph levels by acting as the
H4S47ph phosphatases, with PP1α and PP1β also
regulating the Pak2 kinase activity.

Although depletion of PP1α, PP1β or Wip1 results in
increased levels of H4S47ph, depletion of each does not
affect the expression of the other two phosphatases. These
results suggest that PP1α, PP1β and Wip1 regulate H4S47ph
levels independently. PP1α and PP1β are the
catalytic subunits of the PP1 phosphatase family phos-
phatases that consist of three distinct catalytic subunits,
PP1α, PP1β and PP1γ. PP1 family phosphatases
dephosphorylate many cellular proteins and achieve sub-
strate specificity through PIPs (protein phosphatase

Figure 5. Depletion of PP1α, PP1β and Wip-1 result in increased deposition of newly synthesized H3.3. (A) A schematic diagram outlining H3.3-
SNAP labeling procedure. HeLa cells stably expressing H3.3-SNAP were infected with viruses targeting PP1α, PP1β or Wip-1. H3.3-SNAP was
blocked with SNAP blocking reagent 72 h after infection. Eight-hour after removal of the blocking reagent, cells were either fixed for detection of
newly synthesized H3.3-SNAP, which reacted with TMR, using a fluorescence microscopy (C–D) or extracted for preparation of chromatin as
described in experimental procedures to detect new H3.3-SNAP using SDS–PAGE analysis (E–F). (B) PP1α, PP1β and Wip-1 were efficiently
knockdown as examined by real-time RT-PCR. The experiment was performed as described in Figure 1C. (C and D). Depletion of PP1α, PP1β and Wip-1
results in increased H3.3 fluorescence intensity. (C) The representative images. (D) The average and standard deviation of fluorescence
intensity from three independent experiments. Image J was used to quantify fluorescence intensity of at least 200 cells from each experiment. (E and
F) Deposition of new H3.3 was monitored by a chromatin fractionation assay. Chromatin fractions were prepared and new H3.3-SNAP was detected
using a Typhoon FLA 7000, and total proteins were detected by IRDye® Blue Protein Stain. The relative SNAP intensity over total protein was
reported as the average and standard deviation of three independent experiments. P-values were shown in D and F.
interacting proteins). These PIPs helps bridge the PP1 to a specific substrate (32). Therefore, it is possible that PP1α and PP1β use different PIPs to interact with H3-H4 or Pak2, which in turn regulates the levels of H4S47ph.

Using three independent different assays, we show that depletion of PP1α, PP1β and Wip-1 results in increased H3.3 deposition and occupancy. First, the H3.3 occupancy at H3.3-enriched genes as detected by ChIP assay increases after depletion of PP1α, PP1β or Wip1. Second, deposition of new H3.3 as measured by fluorescence intensity of H3.3-SNAP increases after depletion of PP1α, PP1β and Wip1. Finally, chromatin bound new H3.3-SNAP as detected by the chromatin fractionation assay increases in cells with depletion of PP1α, PP1β or Wip1. These results demonstrate a novel role of PP1α, PP1β or Wip1 in regulating H3.3 deposition/exchange.

Wip-1 plays an important role in DNA damage response by dephosphorylating activated ATM and Chk2 kinases (34). Wip-1 is also likely involved in aging process. For instance, the expression of Wip-1 is reduced during the aging process, and this reduction leads to increased expression of p16INK4a (a cell cycle inhibitor and a senescence marker in aging cells), through activation of p38MAKP kinase, which phosphorylates BMI1 (a component of PRC1 complex represses transcription of p16INK4a) and initiates the release of BMI1 from chromatin (35). In addition, overexpression of Wip-1 compromises RAS-induced senescence (36) in fibroblast cells. It is known that histone chaperone Asf1a and H3.3 chaperone HIRA are required for oncogenic RAS-induced senescence, possibly through their role in mediating H3.3 deposition (37). Because Wip-1 depletion results in increased H3.3 deposition, we suggest that the role of Wip-1 in RAS-induced senescence and/or in aging cells is at least partially due to its ability to regulate H3.3 deposition described here. Future studies are needed to test this idea.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS
The authors thank Dr Genevieve Almouzni for her advice on establishment of H3.3-SNAP technology before publication of her studies. They also thank Drs Almouzni and Ray-Gallet for their critical comments on the manuscript. They thank Dr Sherry L. Winter and Dr Irene L. Andrulis from University of Toronto for kindly providing PP1α, PP1β and PP1γ expressing plasmids. They also appreciate Dr Albert J. Fornace from Georgetown University for giving us the Wip-1 expressing plasmid.

FUNDING
NIH [GM81838, CA157489 and P50 CA136393]. Zhiguo Zhang is a Scholar of Leukemia and Lymphoma Society. Funding for open access charge: Mayo Clinic and Epigenomics Programme, Center of Individualized medicine.
Conflict of interest statement. None declared.

REFERENCES

1. Talbert,P.B. and Henikoff,S. (2010) Histone variants – ancient wrap artists of the epigenome. *Nat. Rev. Mol. Cell. Biol.*, 11, 264–275.

2. Burgess,R.J. and Zhang,Z. (2013) Histone chaperones in nucleosome assembly and human disease. *Nat. Struct. Mol. Biol.*, 20, 14–22.

3. Ransom,M., Dennehey,B. and Tyler,J.K. (2010) Chaperoning histones during repair and replication. *Cell*, 140, 183–195.

4. Elsaesser,S.J., Goldberg,A.D. and Allis,C.D. (2010) New functions for an old variant: no substitute for histone H3.3. *Curr. Opin. Genet. Dev.*, 20, 1–8.

5. Szenker,E., Ray-Gallet,D. and Almouzni,G. (2011) The double face of the histone variant H3.3. *Cell Res.*, 21, 421–434.

6. Schwartz,B.E. and Ahmad,K. (2005) Transcriptional activation and histone variant H3.3 is specific to regions bordering centromeres. *Cell Res.*, 15, 15–22.

7. Sakai,A., Schwartz,B.E., Goldstein,S. and Ahmad,K. (2009) HIRA is critical for a nucleosome assembly and human disease. *Nature*, 419, 1253–1256.

8. Couldrey,C., Carlton,M.B., Nolan,P.M., Colledge,W.H. and Evans,M.J. (1999) A retroviral gene trap insertion into the histone 3.3A gene causes partial neonatal lethality, stunted growth, neuromuscular deficits and male sub-fertility in transgenic mice. *Hum. Mol. Genet.*, 8, 2489–2495.

9. Ahmad,K. and Henikoff,S. (2002) The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly. *Mol. Cell.*, 9, 1191–1200.

10. Schwartz,B.E. and Ahmad,K. (2005) Transcriptional activation triggers deposition and removal of the histone variant H3.3. *Genes Dev.*, 19, 804–814.

11. Mito,Y., Henikoff,J.G. and Henikoff,S. (2005) Genome-scale profiling of histone H3.3 replacement patterns. *Nat. Genet.*, 37, 1090–1097.

12. Jin,C., Zang,C., Wei,G., Cui,K., Peng,W., Zhao,K. and Felsenfeld,G. (2009) H3.3/H2A.Z double variant-containing nucleosomes mark ‘nucleosome-free regions’ of active promoters and other regulatory regions. *Nat. Genet.*, 41, 941–945.

13. Goldberg,A.D., Banaszyński,L.A., Noh,K.M., Lewis,P.W., Elsaesser,S.J., Studler,S., Dewell,S., Law,M., Guo,X., Li.X. et al. (2010) Distinct factors control histone variant H3.3 localization at specific genomic regions. *Cell*, 140, 678–691.

14. Hake,S.B., Garcia,B.A., Kauer,M., Baker,S.P., Shabanowitz,J., Hunt,D.F. and Allis,C.D. (2005) Serine 31 phosphorylation of histone variant H3.3 is specific to regions bordering centromeres in metaphase chromosomes. *Proc. Natl Acad. Sci. USA*, 102, 6344–6349.

15. Santenard,A., Ziegler-Birling,C., Koch,M., Tora,L., Bannister,A.J. and Torres-Padilla,M.E. (2010) Heterochromatin formation in the mouse embryo requires critical residues of the histone variant H3.3. *Nat. Cell Biol.*, 12, 853–862.

16. Wu,G., Broniscer,A., McEachron,T.A., Lu,C., Paugh,B.S., Beckfors,J., Qu,C., Ding,L., Hueter,R., Parker,M. et al. (2012) Somatic histone H3 alterations in pediatric diffuse intrinsic pontine gliomas and non-brainstem glioblastomas. *Nat. Genet.*, 44, 251–253.

17. Sturm,D., Witt,H., Hovestadt,V., Khuong-Quang,D.A., Jones,D.T., Konermann,C., Pfaff,E., Tonjes,M., Sil, M., Bendor,S. et al. (2012) Hotspot mutations in H3F3A and IDH1 define distinct epigenetic and biological subgroups of glioblastoma. *Cancer Cell.*, 22, 425–437.

18. Ray-Gallet,D., Quivy,J.P., Scamps,C., Martini,E.M., Lipinski,M. and Almouzni,G. (2002) HIRA is critical for a nucleosome assembly pathway independent of DNA synthesis. *Mol. Cell.*, 9, 1091–1100.

19. Tagami,H., Ray-Gallet,D., Almouzni,G. and Nakatani,Y. (2004) Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. *Cell*, 116, 51–61.

20. Campos,E.I. and Reinberg,D. (2010) New chaps in the histone chaperone arena. *Genes Dev.*, 24, 1334–1338.

21. Drane,P., Ouarrarhni,K., Depaux,A., Shuaib,M. and Hamiche,A. (2010) The death-associated protein DAXX is a novel histone chaperone involved in the replication-independent deposition of H3.3. *Genes Dev.*, 24, 1253–1265.

22. Elsaesser,S.J., Huang,H., Lewis,P.W., Chin,J.W., Allis,C.D. and Patel,D.J. (2012) DAXX envelops a histone H3.3-H4 dimer for H3.3-specific recognition. *Nature*, 491, 560–565.

23. Liu,C.P., Xiong,C., Wang,M., Yu,Z., Yang,N., Chen,P., Zhang,Z., Li,G. and Xu,R.M. (2012) Structure of the variant histone H3.3-H4 heterodimer in complex with its chaperone DAXX. *Nat. Struct. Mol. Biol.*, 19, 1287–1292.

24. English,C.M., Adkins,M.W., Carson,J.J., Churchill,M.E. and Tyler,J.K. (2006) Structural basis for the histone chaperone activity of Asfl. *Cell*, 127, 495–508.

25. Zhang,H., Han,J., Kang,B., Burgess,R. and Zhang,Z. (2012) Human histone acetyltransferase 1 protein preferentially acetylates H4 histone molecules in H3.4-H4 over H3.3-H4. *J. Biol. Chem.*, 287, 6573–6581.

26. Kang,B., Pu,M., Hu,G., Wen,W., Dong,Z., Zhao,K., Stillman,B. and Zhang,Z. (2011) Phosphorylation of H4 Ser 47 promotes HIRA-mediated nucleosome assembly. *Genes Dev.*, 25, 1359–1364.

27. Bokoch,G.M. (2003) Biology of the p21-activated kinases. *Annu. Rev. Biochem.*, 72, 743–781.

28. Sellis,M.A. and Charnoff,J. (1997) Emerging from the Pak: the p21-activated protein kinase family. *Trends Cell Biol.*, 7, 162–167.

29. Kumar,R. and Yadavamdi,R.K. (2002) Emerging functions of p21-activated kinases in human cancer cells. *J. Cell. Physiol.*, 193, 133–144.

30. Su,M.K., Wong,E.S., Chan,H.Y., Kong,D.S., Woo,N.W., Tam,K.F., Ngan,H.Y., Chan,Q.K., Chan,D.C., Chan,K.Y. et al. (2009) Differential expression and phosphorylation of Pak1 and Pak2 in ovarian cancer: effects on prognosis and cell invasion. *Int. J. Cancer.*, 127, 21–31.

31. Ray-Gallet,D., Woofle,A., Vassias,I., Pellentz,C., Lacoste,N., Puri,A., Schulz,D.C., Pechelintsev,N.A., Adams,P.D., Jansen,L.E. et al. (2011) Dynamics of histone H3 deposition in vivo reveal a nucleosome gap-filling mechanism for H3.3 to maintain chromatin integrity. *Mol. Cell.*, 44, 928–941.

32. Shi,Y. (2009) Serine/threonine phosphatases: mechanism through structure. *Cell*, 139, 468–484.

33. Bollen,M., Peti,W., Ragusa,M.J. and Beullens,M. (2010) The extended PP1 toolkit: designed to create specificity. *Trends Biochem. Sci.*, 35, 450–458.

34. Oliva-Trastoy,M., Berthona,V., Chevalier,A., Ducrot,C., Marsolier-Kergoat,M.C., Mann,C. and Leteurur,F. (2007) The Wip1 phosphatase (PPM1D) antagonizes activation of the Chk2 tumour suppressor kinase. *Oncogene*, 26, 1449–1458.

35. Le Guenennec,X. and Bulavin,D.V. (2009) Wip1 phosphatase at the crossroads of cancer and aging. *Trends Biochem. Sci.*, 35, 109–114.

36. Zhang,X., Kim,J., Ruthazer,R., McDevitt,M.A., Wazer,D.E., Paulson,K.E. and Yee,A.S. (2006) The HBPI transcriptional repressor participates in RAS-induced premature senescence. *Mol. Cell. Biol.*, 26, 8252–8266.

37. Zhang,R., Pousovoitov,M.V., Ye,X., Santos,H.A., Chen,W., Daganzo,S.M., Erzberger,J.P., Serebriiski,I.G., Canutescu,A.A., Dunbrack,R.L. et al. (2005) Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. *Dev. Cell.*, 8, 19–30.