A novel histone exchange factor, protein phosphatase 2Cγ, mediates the exchange and dephosphorylation of H2A–H2B

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In eukaryotic nuclei, DNA is wrapped around a protein octamer composed of the core histones H2A, H2B, H3, and H4, forming nucleosomes as the fundamental units of chromatin. The modification and deposition of specific histone variants play key roles in chromatin function. In this study, we established an in vitro system based on permeabilized cells that allows the assembly and exchange of histones in situ. H2A and H2B, each tagged with green fluorescent protein (GFP), are incorporated into euchromatin by exchange independently of DNA replication, and H3.1-GFP is assembled into replicated chromatin, as found in living cells. By purifying the cellular factors that assist in the incorporation of H2A–H2B, we identified protein phosphatase (PP) 2Cγ subtype (PP2Cγ/PPM1G) as a histone chaperone that binds to and dephosphorylates H2A–H2B. The disruption of PP2Cγ in chicken DT40 cells increased the sensitivity to caffeine, a reagent that disturbs DNA replication and damage checkpoints, suggesting the involvement of PP2Cγ-mediated histone dephosphorylation and exchange in damage response or checkpoint recovery in higher eukaryotes.

Introduction

In eukaryotic nuclei, DNA is wrapped around a protein octamer containing two copies of each of the core histones H2A, H2B, H3, and H4, forming nucleosomes, the fundamental units of chromatin (Luger et al., 1997). Nucleosomes are assembled with the assistance of chaperones or assembly complexes. During de novo nucleosome assembly, DNA is first wrapped around the H3–H4 tetramer before the addition of two H2A–H2B dimers (Loyola and Almouzni, 2004). Once assembled, these core histones are tightly bound to DNA, and the interactions must be loosened or remodeled to allow the access of molecular machineries (e.g., polymerases) to DNA. In living cells, the histone–DNA interaction and chromatin structure are expected to be continually altered during transcription, genome duplication, and damage recovery, and the remodeling of chromatin is often associated with histone exchange (Belotserkovskaya and Reinberg, 2004; Flaus and Owen-Hughes, 2004; Loyola and Almouzni, 2004; Henikoff and Ahmad, 2005; Kimura, 2005). On the other hand, nucleosome contexts on specific loci must be preserved to maintain epigenetic marks on histone tails (Turner, 2002). The modification of histones, including acetylation, methylation, and phosphorylation, plays essential roles in chromatin functions such as gene expression and chromosome segregation. Thus, fluidity and stability seem to be well balanced in nucleosomes in living cells.

Early studies of histone deposition and exchange in living cells used radiochemical labeling. The stable association of [3H]arginine-labeled H3–H4 with chromatin was demonstrated by cell fusion (Manser et al., 1980). In a series of studies analyzing the deposition of the newly synthesized radio-labeled histones into nucleosomes, it was found that linker histone...
H1 and H2A–H2B undergo exchanges independently of DNA replication and transcription (Louters and Chalkley, 1985; Jackson, 1990). Such different behaviors of different histone species are also seen in living cells using GFP-tagged proteins (Kimura, 2005). The linker histone H1 is rapidly exchanged within a few minutes, and core histones are more stably bound. Long-term observation and cell fusion experiments further revealed that a substantial fraction of H2B-GFP exchanges slowly in euchromatin, whereas most H3-GFP (which is the H3.1 variant and is referred to below as H3.1-GFP) and H4-GFP remain bound to chromatin. In addition to the slowly exchanging fraction of H2B-GFP, which exchanges independently of DNA replication and transcription, another rapidly exchanging fraction, probably coupled to transcription, has been observed (Kimura and Cook, 2001), which is in agreement with the dimer eviction observed during transcription (Kireeva et al., 2002; Belotserkovskaya and Reinberg, 2004).

The exchange and assembly of histones are regulated in a development- and differentiation-specific manner (Meshorer et al., 2006) by chaperones or assembly factors that are distinct for each histone variant. Histone H3 has several variants, which are deposited into specialized chromatin loci mediated differentially through the action of deposition complexes (Loyola and Almouzni, 2004; Tagami et al., 2004; Henikoff and Ahmad, 2005; Thiriet and Hayes, 2005). The modification pattern in the conserved tail is also distinctive in each variant (Hake et al., 2006). H3.3 has modifications associated with transcriptionally active chromatin, which is consistent with its localization on active genes; in contrast, replication-coupled H3.2 has mostly silencing modifications. Although the modification pattern of each histone is established after its assembly into nucleosomes (influenced by the surrounding chromatin state), some specific modifications are associated with nucleosome-free deposition forms. Such modifications are typically found in H4, whose deposition form is diacetylated throughout eukaryotes, and some acetylation is associated with newly synthesized H3 in human cells (Benson et al., 2006).

In addition to H3, variant-specific deposition and modification are found in H2A. Nucleosome assembly protein 1 (Nap1) and the related proteins are known as somatic H2A–H2B chaperones after Nap1's purification from HeLa cells on the basis of nucleosome assembly activity in vitro (Ishimi et al., 1984; Loyola and Almouzni, 2004; Henikoff and Ahmad, 2005). Although Nap1 is not essential for yeast viability, its disruption affects the expression level of ~10% of genes in clusters, suggesting a nucleosome maintenance function of Nap1 by depositing H2A–H2B (Ohkuni et al., 2003). Although Nap1 assists nucleosome assembly without ATP in vitro, complexes containing ATP-dependent chromatin remodeling activity have recently been shown to mediate the exchange of H2A–H2B dimers (Bruno et al., 2003; Kobor et al., 2004; Krogan et al., 2004; Mizuguchi et al., 2004). A complex containing yeast SWR1 (Swi2/Snf2-related ATPase 1) exchanges canonical H2A with H2AZ in nucleosome arrays, and SWR1 and H2AZ regulate an overlapping subset of genes. Another complex containing Tip60 (Tat-interacting protein 60) is involved in the exchange of phosphorylated H2Av (a Drosophila melanogaster histone H2A variant homologous to H2AX) with the unphosphorylated form at DNA lesions in Drosophila (Kusch et al., 2004). Thus, multiple mechanisms appear to exist to control the exchange of H2A variants at appropriate chromatin loci and in response to various stimuli, including DNA damages.

To understand the molecular mechanisms that regulate the assembly and exchange of histones in higher eukaryotes, we set
out to establish an in vitro system that mimics in vivo histone dynamics using permeabilized cells. When cells are treated with nonionic detergents such as Triton X-100 or saponin, cellular membranes are permeabilized and some proteins are extracted, but many nuclear functions remain active (Jackson and Cook, 1985; Pombo et al., 1999), and some nuclear structures can be manipulated by adding exogenous factors (Misteli and Spector, 1996; Maison et al., 2002). Therefore, we expected that exogenously added histones might be incorporated into chromatin in permeabilized cells by exchange or replication-coupled assembly. As expected, GFP-tagged histones were indeed incorporated into chromatin in permeabilized cells with the assistance of cellular factors. By purifying the factors assisting GFP-H2A–H2B incorporation, we identified the type 2C protein phosphatase (PP) 2Cγ/PPM1G (Travis and Welsh, 1997; Murray et al., 1999) in addition to the Nap1 family members. PP2Cγ directly bound to and dephosphorylated H2A–H2B, and its disruption in chicken DT40 cells caused hypersensitivity to checkpoint abrogation. Although PP2Cγ did not appear to be the major phosphatase for H2AX and H2B, dephosphorylation and exchange via PP2Cγ may function to allow full recovery from DNA damage.

Results

Histone assembly and exchange in permeabilized cells

As illustrated in Fig. 1 A, HeLa cells were permeabilized and incubated in cell extracts prepared from cells stably expressing GFP-tagged core histones whose expression levels were <10% of their endogenous counterparts (Kimura and Cook, 2001). After washing out the unincorporated materials, GFP-H2A localized to euchromatin, which was devoid of DAPI-dense heterochromatin (Fig. 1 B, inset), in most permeabilized cells (Fig. 1 B). In contrast, H3.1-GFP highlighted replicated chromatin, which was labeled with Cy3-dUTP. The euchromatic localization of GFP-H2A was confirmed by the overlapping signals with specific antibodies recognizing acetylated histone H3 (not depicted) and H4 (Fig. 1 C), which are associated with transcriptionally active chromatin (Turner, 2002). In contrast, GFP-H2A was excluded from inactive chromatin rich in K20-trimethylated H4 (Fig. 1 C; Turner, 2002). To confirm that GFP-H2A replaced the endogenous H2A in chromatin in permeabilized cells, GFP-containing mononucleosomes were prepared by immunoprecipitation using antibody directed against GFP, and the ratio of core histones was analyzed by SDS-PAGE and Coomassie staining (Fig. 1 D). The amount of H2A was roughly halved in GFP-H2A nucleosomes (Fig. 1 D), suggesting the incorporation of a dimer of GFP-H2A and H2B (GFP-H2A–H2B) into chromatin by exchange. This stoichiometry is unlikely to be created by the nonspecific aggregation of a GFP-H2A–containing histone octamer from the extract onto chromatin, as H2A–H2B and H3–H4 are present in different complexes in the chromatin-free fraction (see Fig. 5 B; Chang et al., 1997; Tagami et al., 2004).

We next used specific inhibitors to examine whether the incorporation of histones depends on transcription and/or DNA replication in permeabilized cells. Most H2A–H2B appeared to be exchanged independently of ongoing RNA polymerase II transcription and DNA replication, as the incorporation of GFP-H2A and H2B-GFP was still observed in the presence of α-amanitin and aphidicolin, respectively (Fig. 2 and not depicted). The incorporation of H3.1-GFP into chromatin was coupled to DNA replication, as the signal almost disappeared in the presence of aphidicolin (Fig. 2). These results are reminiscent of the different behaviors of H2A–H2B and H3.1–H4 observed in living mammalian cells (Louters and Chalkley, 1985; Jackson, 1990, Kimura and Cook, 2001; Benson et al., 2006).

Purification of the activity required for histone H2A–H2B incorporation from HeLa cell extract

To analyze whether GFP-H2A–H2B dimer alone can be incorporated into chromatin, permeabilized cells were incubated...
with GFP-H2A–H2B purified from HeLa cells expressing GFP-H2A–H2B (Fig. 3). Although GFP-H2A–H2B alone failed to be incorporated, its incorporation was restored when supplemented with HeLa cell extract (Fig. 3 C), suggesting the presence of soluble factors required for H2A–H2B exchange in the extract. By following the incorporation of GFP-H2A under a fluorescent microscope, we purified the activity required for H2A–H2B exchange using column chromatography (Fig. 4 A). The purest active fraction consisted of three major bands by SDS-PAGE (Fig. 4 B). Mass spectrometry analysis identified these polypeptides as PP2Cγ/PPM1G (Travis and Welsh, 1997; Murray et al., 1999), Nap1/Nap1L1 (Ishimi et al., 1984), and Nap2/Nap1L4 (Rodriguez et al., 1997). It was not surprising to find Nap1 and Nap2 in the active fractions, as they have been described as histone chaperones that assist nucleosome assembly in vitro (Ishimi et al., 1984; Rodriguez et al., 1997). In contrast, no link between PP2Cγ and histones was previously established, which prompted us to focus on the function of PP2Cγ in histone H2A–H2B exchange. The phosphatase might regulate the chaperone activity of Nap1/2 by altering their phosphorylation state. On the other hand, PP2Cγ might also mediate the H2A–H2B exchange as such because it has a unique acidic domain (Travis and Welsh, 1997) that could potentially interact with histone H2A–H2B.

Recombinant Nap1, Nap2, and PP2Cγ individually support H2A–H2B incorporation

To examine the relationship between H2A–H2B exchange activity and the individual proteins, we incubated permeabilized cells with the purified GFP-H2A–H2B and each recombinant protein fused to a histidine hexamer (His) tag expressed in and purified from *Escherichia coli* (Fig. 4, C and D). GFP-H2A was incorporated into chromatin in the presence of either His-Nap1, -Nap2, or -PP2Cγ (Fig. 4 D), and similar results were obtained when ATP was omitted from the system (Fig. S1, available at online).
Three recombinant proteins were mixed (Fig. 4 D). The incorporation of GFP-H2A–H2B were observed when these three proteins by gel filtration chromatography (Fig. 4 B) suggests their presence in a complex, only additive effects on the phosphorylation state of FLAG-PP2C could be substrates for the phosphatase. Therefore, we analyzed the phosphorylation state of FLAG-PP2C by immunoprecipitation using anti-FLAG agarose beads, substantial amounts of endogenous H2A and H2B were coprecipitated with FLAG-PP2Cγ but not with FLAG-ΔAcDo (Fig. 5 A). The interaction between basic proteins like histones and the acidic domain could occur through nonspecific binding as a result of the positive and negative charges. However, the immunoprecipitation experiments show specific binding of the phosphatase to H2A–H2B because only these two, but not the other histones (i.e., H1, H3, and H4), were coprecipitated even though all histone subtypes are positively charged. The complex formation between GFP-H2A–H2B and PP2Cγ in the cell extract (used in Fig. 1) was observed by immunoprecipitation using anti-GFP agarose beads (Fig. 5, B and C). The presence of PP2Cγ as well as Nap1 in the immunoprecipitates was confirmed by mass spectrometry (Fig. 5 B) and immunoblotting (Fig. 5 C). A two-hybrid cDNA library screen also yielded histone H2B as an interactor with PP2Cγ, and the interaction required the phosphatase’s acidic domain (unpublished data).

PP2Cγ dephosphorylates nucleosome-free histone H2A–H2B

The aforementioned results showing the physical interaction between PP2Cγ and H2A–H2B suggest that these histones could be substrates for the phosphatase. Therefore, we analyzed the phosphorylation state of FLAG-PP2Cγ–bound histones using acid-urea-Triton (AUT) gel electrophoresis and immunoblotting with specific antibodies directed against phosphorylated histones (Fig. 6 A). As expected, histones coprecipitated with the wild-type phosphatase were poorly recognized by antiphosphohistone antibodies. In contrast, histones bound to a phosphatase-inactive mutant (D496A) comprised detectable levels of phosphorylated molecules, including those related to DNA damage response and apoptosis such as Ser139-phosphorylated H2AX (called γ-H2AX; Rogakou et al., 1999) and Ser14-phosphorylated H2B (Cheung et al., 2003; Fernandez-Capetillo et al., 2004), although the overall migration pattern was similar to those bound to the wild-type phosphatase. As bulk nucleosomal histones were still phosphorylated in cells overexpressing the wild-type phosphatase (Fig. 6 A and by immunofluorescence; not depicted), only nucleosome-free H2A–H2B may be dephosphorylated by the phosphatase. Consistently, the purified His-PP2Cγ efficiently dephosphorylated nucleosome-free histones, including γ-H2AX in vitro (Fig. 6, C and D). As the D496A mutant still supported histone exchange in permeabilized cells (Fig. 4 D), the histone exchange and dephosphorylation do not appear to be coupled. These results suggest that a nucleosome-free H2A–H2B that binds to PP2Cγ may be dephosphorylated before its deposition into a nucleosome. Although we did not obtain positive data indicating the dephosphorylation of nucleosomal histones by PP2Cγ in overexpression and in vitro assays, it is also possible that additional cellular factors, which may be limited in the assays, stimulate the phosphatase activity or targeting toward the nucleosomal histones.

We next tested whether PP2Cγ has in vitro nucleosome assembly activity using a supercoiling assay (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200608001/DC1) in which the assembly of nucleosomes can be assessed by the formation of supercoils from relaxed circular DNA (Ishimi et al., 1984;
Coomassie-stained histones are shown. The 32P signals from all of the histones were analyzed by AUT gel electrophoresis. After transfecting FLAG-PP2Cγ (wild type [wt]) or FLAG-PP2Cγ (D496A) into 293T cells, PP2Cγ-bound and nucleosomal histones were separated in an AUT gel. Coomassie staining (left) and immunoblots with the indicated phospho-specific antibodies are shown. The positions of histone subtypes and their phosphorylated forms (arrows) are indicated. (B) Dephosphorylation of histones by PP2Cγ. 2 μg of 32P-labeled histones, which were phosphorylated by MSK1, were incubated with different amounts (lanes 2 and 5, 1 ng; lanes 3 and 6, 5 ng; lanes 4 and 7, 25 ng) of His-PP2Cγ [wt; lanes 2–4] or mutant (D496A; lanes 5–7) and separated by SDS-PAGE. The radioactivity and Coomassie-stained histones are shown. The 32P signals from all of the histones disappear by dephosphorylation with increasing amounts of His-PP2Cγ but not with His-PP2Cγ (D496A). (C) Dephosphorylation of H2AX by PP2Cγ. 1 μg histone H2A–H2B fraction prepared from 12 Gy-irradiated HeLa cells was incubated alone (lane 1) or with different amounts (lanes 2 and 5, 1 ng; lanes 3 and 6, 5 ng; lanes 4 and 7, 25 ng) of His-PP2Cγ [wt; lanes 2–4] or mutant (D496A; lanes 5–7) and separated by SDS-PAGE. The phosphorylation level of H2AX was analyzed by immunoblotting with anti-γ-H2AX. The Coomassie-stained gel (CBB) is shown as a loading control.

Rodriguez et al., 1997). Most of the plasmid DNA became supercoiled in the presence of Nap1, but only some supercoiled molecules accumulated even in the presence of high levels of PP2Cγ (Fig. S2), indicating that PP2Cγ has only weak de novo nucleosome assembly activity.

**Effect of PP2Cγ knockdown on H2A–H2B mobility in living HeLa cells**

To investigate whether PP2Cγ is involved in the regulation of H2A–H2B kinetics in living cells, we knocked down PP2Cγ in HeLa cells expressing histone-GFP using RNAi; 3 d after the transfection of a specific siRNA, the level of PP2Cγ decreased substantially to <5% of the normal level (Fig. 7, A and B). The mobility of H2A–H2B was analyzed by fluorescence recovery after photobleaching (Kimura and Cook, 2001). The recovery kinetics of both GFP-H2A and H2B-GFP decreased in cells transfected with PP2Cγ-specific siRNA compared with those with the control siRNA (Fig. 7, C and D), whereas the mobility of the linker histone H1c-GFP was unaffected (Fig. 7 E). These observations in living cells reflect the results from in vitro assays, suggesting that at least a part of H2A–H2B exchange is mediated by PP2Cγ as a histone chaperone in HeLa cells.

**PP2Cγ-deficient DT40 cells show hypersensitivity to caffeine**

To gain further insights into the biological function of PP2Cγ in vertebrate cells, we established PP2Cγ-deficient chicken DT40 cells by gene targeting (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200608001/DC1). As the deficient cells were generated by a simple knockout strategy to disrupt both alleles, PP2Cγ does not appear to be essential for cell growth. However, substantial growth defects were observed when DNA replication and damage checkpoints were abrogated by caffeine, which preferentially inhibits ataxia telangiectasia mutated– and ataxia telangiectasia and RAD3 related–dependent pathways, although its exact interfering points remain elusive (Kaufmann et al., 2003; Abraham, 2004). As shown in Fig. 8, PP2Cγ-deficient cells were more sensitive to caffeine compared with the wild type in a growth rate assay (Fig. 8 A) and in a colony formation assay (Fig. 8 B). In 2 mM caffeine, the wild-type cells continued to grow for 3 d, but PP2Cγ-deficient cells stopped growing at day 2. At a higher concentration (4 mM), the number of live cells (judged by the exclusion of trypan blue) became considerably lower after day 2 in PP2Cγ-deficient cells (Fig. 8 A). The colony formation assay revealed that the survival rate after 22 h of incubation in 4 mM caffeine was 35 ± 8 and 8.2 ± 0.3% in the wild-type and PP2Cγ-deficient cells, respectively (Fig. 8 B). As caffeine is known to sensitize cells to DNA double-strand breaks induced by ionizing radiation (Kaufmann et al., 2003; Abraham, 2004), we compared the sensitivity of these cells with γ-ray irradiation in the presence or absence of caffeine. Although PP2Cγ-deficient cells showed a similar radiation sensitivity to the wild type without caffeine, they became more sensitive when 1 mM caffeine was present in the colony-forming medium (Fig. 8 C).

These results indicate that PP2Cγ is not essential for DNA double-strand break repair but suggest its involvement in recovery from damage. As H2AX is known to be phosphorylated around damaged chromatin, its dephosphorylation is required for full recovery from the damage response (Chowdhury et al., 2005; Keogh et al., 2005). Even though PP2A is likely to be the major γ-H2AX phosphatase in higher eukaryotes (Chowdhury et al., 2005), PP2Cγ could be involved in a backup dephosphorylation and deposition pathway. To assess the role of PP2Cγ in γ-H2AX dephosphorylation, the phosphorylation level of H2AX (i.e., the signal detected with antibody directed against γ-H2AX) was compared between the wild-type and PP2Cγ-deficient cells in response to DNA damage combined with treatment with calyculin A, an inhibitor of PP1 and PP2A (Nazarov et al., 2003; Chowdhury et al., 2005). In both cells, γ-H2AX...
appeared at a similar level 2 h after irradiation (8 Gy) and disappeared by 8 h (Fig. 8 D, lanes 1–6); faint signals of apoptosis-associated H2B (S14) phosphorylation appeared by 8 h. When cells were incubated with calyculin A, γ-H2AX was accumulated by 8 h even in the wild-type cells, probably as a result of spontaneous or replication-associated damages, which is consistent with the involvement of PP2A in γ-H2AX dephosphorylation (Chowdhury et al., 2005). The levels of γ-H2AX and phospho-H2B (S14) were higher in PP2Cγ-deficient cells in the presence of calyculin A (Fig. 8 D, lanes 1–6 and 13–18) or not irradiated (lanes 7–12), and calyculin A was added (lanes 7–18). Cells were collected either immediately (0 h), 2, or 4 h after irradiation, and the levels of γ-H2AX and phosphorylated H2B (S14) were analyzed by immunoblotting. The Coomassie-stained gel (CBB) is shown as a loading control. KO, knockout.

Discussion

Identification of histone chaperones required for H2A–H2B incorporation into chromatin in permeabilized cells

To understand the biological function and molecular mechanisms of histone dynamics, we established a permeabilized cell-based assay for histone assembly and exchange. GFP-H2A and H2B-GFP were incorporated into euchromatin in permeabilized cells. This is consistent with the exchange of H2A–H2B in living cells, which can occur independently of DNA replication.
and transcription (Jackson, 1990; Kimura and Cook, 2001), preferentially in chromatin-containing acetylated H4 (Benson et al., 2006). H3.1-GFP assembled into replicated chromatin but contrasted to H2A–H2B, which is also reminiscent of the behavior in living cells (Kimura and Cook, 2001). By purifying the activity that assists GFP-H2A–H2B incorporation into chromatin in permeabilized cells, we identified three proteins—Nap1, Nap2, and PP2Cγ—in the purest fraction. Finding these Nap1-related proteins in our active fractions reassures us that the permeabilized cell-based assay has physiological relevance. The third protein we found was PP2Cγ, which harbors a unique acidic domain (Travis and Welsh, 1997) and was purified as a factor that stimulates spliceosome assembly in vitro (Murray et al., 1999).

Our analyses indicated that the phosphatase as such can assist the incorporation of H2A–H2B into chromatin in permeabilized cells and that it binds to and dephosphorylates histone H2A and H2B subtypes. Although the acidic domain of PP2Cγ could potentially mediate nonspecific electrostatic binding to basic proteins such as the histones, the fact that H2A–H2B was exclusively coimmunoprecipitated among all of the histones using FLAG-tagged phosphatase suggests that the interaction between PP2Cγ and H2A–H2B is specific. These histone chaperones do not require ATP for assisting H2A–H2B incorporation into chromatin in permeabilized cells as well as for in vitro nucleosome assembly with naked DNA. Because we followed the most active fractions that support GFP-H2A incorporation globally in euchromatin, other H2A–H2B exchange factors that are probably less abundant and act on more specific loci, including facilitating chromatin transcription (FACT; Belotserkovskaya and Reinberg, 2004) and ATP-dependent remodeling factors (Flaus and Owen-Hughes, 2004), were not found in the final preparation. Although Nap1/2 and PP2Cγ may mediate global H2A–H2B exchange independently of transcription and DNA replication, FACT may participate in transcription-coupled exchange. Future studies may reveal whether FACT supports H2A–H2B incorporation in a transcription-dependent manner in permeabilized cells.

A recent study revealed that ATP-dependent chromatin remodeling complexes can mediate histone exchange in addition to their remodeling function without the displacement of histone octamers (Flaus and Owen-Hughes, 2004). Therefore, it is also possible that the function of ATP-independent chaperones like Nap1/2 and PP2Cγ is solely to escort H2A–H2B and transfer the dimer to the ATP-dependent machineries, such as the yeast SWR1 complex that catalyzes the exchange between a canonical dimer and an H2AZ–H2B dimer (Mizuguchi et al., 2004). However, several lines of evidence suggest that the chaperones might also mediate H2A–H2B incorporation by themselves in addition to their escorting function. First, yeast Nap1 has the ability to exchange H2A–H2B in mononucleosomes in vitro (Park et al., 2005). Second, additional ATP is not required for H2A–H2B incorporation supported by Nap1/2 and PP2Cγ in permeabilized cells. Third, a substantial H2B-GFP recovery was still observed in living cells by FRAP even when the cellular ATP pool was depleted by treatment with sodium azide (unpublished data). Thus, although ATP-dependent factors might be required for the exchange of a dimer containing H2AZ at specific loci or during gene activation, ATP-independent chaperones may participate in the basal level of exchange of the major H2A and other variants. Alternatively, the major role of ATP-independent chaperones may be to deposit an H2A–H2B dimer into an incomplete nucleosome lacking a dimer, which can result from positive torsional stress (Jackson et al., 1994) or through ATP-driven eviction. This may account for the slow exchange rate of H2A–H2B in living cells despite the presence of a large pool of PP2Cγ (~10⁶ molecules/HeLa cell) diffusing almost freely in the nucleus (unpublished data).

**Involvement of PP2Cγ in DNA damage response**

To understand the biological function of PP2Cγ at the cellular level, we used chicken DT40 cells to create knockout cells by gene targeting. Although the deficient cells are viable, they show subtle growth retardation and a remarkable hypersensitivity to caffeine, which abrogates DNA replication and damage checkpoints. One possible mechanism to explain these phenomena is that the chaperone function together with the phosphatase activity plays a role in completing chromatin formation after DNA repair and/or replication by depositing dephosphorylated H2A–H2B molecules (Fig. 9). H2AX is phosphorylated around damaged chromatin (Rogakou et al., 1999), and its dephosphorylation is required for full recovery from damage responses. Also, H2AX molecules outside the damaged area are kept from undergoing phosphorylation for several hours. Although PP2A seems to play a major role in γ-H2AX dephosphorylation on chromatin (Chowdhury et al., 2005), we showed that PP2Cγ likewise mediates γ-H2AX and H2B dephosphorylation, as PP2Cγ-deficient cells showed a greater accumulation of γ-H2AX and phosphorylated H2B (S14) compared with wild-type cells when PP1 and PP2A were inhibited by calyculin A.

Although the eviction of γ-H2AX or phosphorylated H2B may be mediated by other proteins such as the *Drosophila* Tip60-containing complex (Kusch et al., 2004), PP2Cγ may passively deposit dephosphorylated H2A–H2B or H2AX–H2B...
to incomplete nucleosomes lacking one dimer. This view is consistent with the observed uncoupling of the chaperone function and phosphatase activity of PP2Cγ; histone dephosphorylation can occur at any time after the binding of PP2Cγ until deposition (Fig. 9). Most H2A–H2B that bound to PP2Cγ but away from nucleosomes was indeed dephosphorylated. The lack of PP2Cγ in the DT40 knockout cells may thus delay the recovery from damage. When checkpoints are functional, such a subtle repair defect would not be critical and might only cause a subtle delay in cell growth. However, when checkpoints are abrogated, more cells with damaged chromatin would enter into mitosis for catastrophe.

An alternative possibility is that the substrate specificity or phosphatase activity of PP2Cγ is regulated by binding to H2A–H2B (Fig. 9); the level of nucleosome-free H2A–H2B could be altered by damage or replication fork arrest. The type of H2A–H2B (Fig. 9); the level of nucleosome-free H2A–H2B is regulated by binding to checkpoint responses (Leroy et al., 2003; Lu et al., 2005), and the γ subtype in particular might take part in inactivating checkpoint responses by sensing the free H2A–H2B level in the nucleus. Finally, a link between chromatin-remodeling factors and alternative pre-mRNA splicing was recently reported (Batsche et al., 2006). Consistent with this observation, PP2Cγ was previously identified as a factor that stimulates pre-mRNA splicing in vitro (Murray et al., 1999), raising the interesting possibility that PP2Cγ coordinately regulates stress responses in mammalian cells at the level of chromatin and RNA splicing.

Concluding remarks

It is now widely acknowledged that histone modification is key for the regulation of chromatin functions. Recent studies further indicate that the deposition and exchange of appropriate histone variants to specific chromosome loci are also important for gene expression and genome integrity (Loyola and Almouzni, 2004; Henikoff and Ahmad, 2005). A connection between the histone modification and deposition has been shown typically in the case of histone H4; before replication-coupled assembly, the newly synthesized molecules are diacytethylated by HAT1 histone acetylase in the H3.1–H4 deposition complex (Chang et al., 1997; Tagami et al., 2004). Although diacytetyl is not a prerequisite for assembly (Ma et al., 1998), this modification contributes to the recovery from replication block-mediated DNA damage (Barman et al., 2006). Similarly, in the case of H2A–H2B and H2AX–H2B, the deposition of unphosphorylated forms mediated by PP2Cγ appears to play a role in DNA damage responses. Thus, controlling the incorporation of appropriately modified histones seems to be important for maintaining genome integrity. Future studies should reveal how individual ATP-independent chaperones and ATP-dependent remodeling complexes function in distinct exchange processes in different chromatin contexts. Although differences in histone exchange kinetics in vivo were shown decades ago (Manser et al., 1980; Louters and Chalkley, 1985), the biological significance of the exchange and the underlying molecular mechanisms are just emerging. The approach presented in this study may contribute to bridging the gap between live cell observations and biochemical analyses.

Materials and methods

Histone exchange and assembly in permeabilized cells

In typical experiments, Hela cells were plated in a 12-well plate containing 15-nm coverslips and were grown up to subconfluence. Cells were chilled on ice, washed twice in ice-cold physiological buffer (PB: 100 mM CH3COOK, 30 mM KCl, 10 mM Na2HPO4, 1 mM EDTA, 1 mM MgCl2, and 1 mM ATP; Jackson and Cook, 1983) containing 5% Ficoll (PB; pH 7.4; 1 ml per well; Nacalai Tesque), permeabilized in PB containing 0.1% Triton X-100 (1 ml; for 5 min on ice), and washed twice in 1 ml PB on ice. Cells were incubated for 1 h at 30°C in a reaction mixture containing cell extract [40%] or purified proteins supplemented with 100 μM each of NTP and dNTP (GE Healthcare), 0.4 μM Cy3-dUTP (PerkinElmer), and 800 μM MgCl2 in PB. For incubation, a coverslip was overlaid (cell side down) on a 100-μl drop of the reaction mixture on Parafilm covering a flat aluminum block in a water bath at 30°C. After washing twice in 1 ml PB for 5 min on ice in a 12-well plate, cells were fixed in 4% PFA (Electron Microscopy Sciences) in 250 mM Hepes-NaOH, pH 7.4 (Wako), for 20 min at room temperature, washed three times in 1 ml PBS, and DNA was counterstained with DAPI [12.5 ng/ml in PBS; 1 ml for 15 min; Nacalai Tesque]. After washing twice in 1 ml PBS, coverslips were mounted using Prolong Gold (Invitrogen). In some cases, ATP and the other nucleotides were omitted from PB and the reaction mixture.

For immunolabelling (Fig. 1 C), permeabilized cells were incubated in the reaction mixture containing 40% GFP-H2A extract and 2 μM Cy5-dUTP instead of Cy3-dUTP for 30 min at 30°C. After fixation, cells were treated with 1% Triton X-100 in PBS for 20 min, washed five times in PBS, and incubated in blocking buffer (0.2% gelatin, 1% BSA, and 0.05% Tween 20 in PBS, pH 8.0) for 30 min and then with rabbit polyclonal antibodies directed against hyperacetylated H4 (1:1,000; Upstate Biotechnology) or H4-trimethylated K20 (1:500; Abcam) in the same buffer for 3 h. Cells were washed in PBS containing 0.05% Tween 20 (PBST) three times for 10 min, incubated in Cy3-conjugated donkey anti-mouse IgG (1:500; Jackson Immunoresearch Laboratories) overnight at 4°C, and washed with PBST three times for 10 min before DAPI staining.

Fluorescence images were sequentially collected using a confocal microscope featuring 405-, 488-, 543-, and 633-nm laser lines with the optimized pinhole setting operated by the built-in software: either a microscope (LSM510 META; Carl Zeiss Microlmaging, Inc.) with a C-Apo 40× NA 1.2 objective lens (for Figs. 1 B and 7 B) or a microscope (FV-1000; Olympus) with a UPlanSApo 60× NA 1.35 lens (for Figs. 1 C and 2-4).

Image files were converted to tiff format using the operating software, merged, linearly contrast stretched (with the same setting in each set of experiments) using Photoshop version 7.01 (Adobe), and imported into Canvas 8 (Deneva) for assembly.

For chromatin immunoprecipitation, cells were centrifuged at 1,300 g for 10 min at 4°C each after sonication for buffer replacement. After the incubation and washing, nucleosomes were prepared, and GFP-containing nucleosomes were precipitated as described previously (Kanda et al., 1998; Kimura and Cook, 2001).

Preparation of cell extracts and protein purification

Hela cells and derivatives expressing H2B-GFP (Kanda et al., 1998) and H3.1-GFP were grown as described previously (Kimura and Cook, 2001), and lines expressing GFP-H2A and H1c-GFP were established by transfecting the expression vectors (Misteli et al., 2000; Perche et al., 2000). Cell extracts were prepared based on the study by Dignam et al. (1983) with modifications. The S100 extract was prepared using a 1.5 cell-packed volume of 10 mM CH3COOK, 3 mM KCl, 1 mM Na2HPO4, 1 mM MgCl2, 1 mM ATP, 1 mM EDTA, 10 mM Hepes-KOH, pH 7.4, and Complete protease inhibitor cocktail (EDTA-free; Roche) and dialyzed PB plus inhibitors (1.5 μg/ml leupeptin, 2.5 μg/ml aprotinin, and 1 μg/ml pepstatin A; Wako). The nuclear pellet was extracted using an equal volume of 20 mM Hepes-KOH, 0.6 M KCl, 0.2 mM EDTA, 25% glycerol, 1 mM DT, 1.5 mM MgCl2, and protease inhibitor cocktail (Roche) to yield the nuclear extract, which was also dialyzed against PB. Histone H2A–H2B and H3–H4 were separately purified from the nuclear pellet essentially according to Simon and Felsenfeld (1979), and the GFP-H2A–H2B fraction was separated from untagged H2A–H2B using gel filtration column chromatography (HiLoad Superdex 75; GE Healthcare).

To purify the activity assisting histone H2A–H2B incorporation in permeabilized cells, S100 extract was first fractionated through a histone H2A–H2B column, which was prepared by coupling 3 μg of the purified H2A–H2B to 1 ml NHydroxysuccinimide-ester-activated Sepharose (GE Healthcare) according to the manufacturer’s instructions. Approximately

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6 mg/ml Hela S100 extract was mixed with 5 M NaCl to yield a final salt concentration of 0.5 M before applying to the column (2 ml per run). After washing with five column volumes of PB containing 0.5 M NaCl, bound proteins were eluted with a linear gradient of NaCl (0.5–2 M in PB; 20 column volumes). Each fraction was concentrated, and the buffer was substituted to PB using Ultrafree 0.5 (Millipore) before use in the permeabilized cell assay with 10–20 μg/ml of purified GFP-H2A–H2B. The GFP signal was monitored using a cooled camera (Hamamatsu) and washing with another 5 M NaCl. The nucleoli was followed under a fluorescence microscope (Axiovert2; Carl Zeiss Microlmaging, Inc.). The active fractions eluted in ~1 M NaCl were further separated using a MonoQ 5/50 GL column (GE Healthcare) with a linear gradient of NaCl (0–2 M in PB; 20 column volumes). The most active fraction eluted in ∼0.6 M NaCl was concentrated and separated 10–20 μl ml fraction) on a Superose 12 (GE Healthcare). Proteins were identified by mass spectrometric analysis using a mass spectrometer (Ultraflex TOF/TOF; Bruker Daltonics) and by comparison between the determined molecular weights and theoretical peptide masses from the proteins registered in the National Center for Biotechnology Information.

Recombinant proteins and phosphatase assay
The cDNAs encoding human Nap1 (Nap1L1) and Nap2 (Nap1L4) were amplified by reverse transcription (Revertra Ace and oligo-dT18; TOYOBO) of HeLa RNA and PCR (high fidelity PCR master; Roche) using the following primers designed from GenBank/EMBL/DDB accession no. BT070023 (Nap1) and BC022090 (Nap2); Nap1 forward, TACCATAGGCGACATGTCACACACACACATG; Nap1 reverse, CACACACCCACCTATGGCTTACCTGGCTG; Nap2 forward, CCTCATATGCGCAGATCTGCTTTCATGGGGT; and Nap2 reverse, GACACACTACCTGTTGGAATCTTTCCGATCA.

The amplified products were purified (QIAGEN), digested with NdeI and HindIII, and ligated into pHITS1 (containing T7 promoter, D box, His sequence, and multicloning site; provided by H. Tabara, Kyoto University, Kyoto, Japan) digested with the same enzymes. The resulting plasmids (PP2Cγ and the mutants) were also constructed by inserting the PP2Cγ sequence (Murray et al., 1999) into pHIT51. Each plasmid was introduced into BL21-Gold (Stratagene), and expression was induced with 1 mM IPTG into cells expressing MSK1 (Upstate Biotechnology), mouse anti-phospho-H2AX Ser139 (1:1,000; Upstate Biotechnology), mouse anti-phospho-H2AX Ser139 (1:1,000; Upstate Biotechnology), and mouse anti-phospho-H2B Ser14 (clone 6C9; 1:20 hybridoma supernatant).

To produce anti-phospho-H2B Ser14, mice were immunized with a synthetic peptide KAAPKGGphospho-SKKAYKQGGC (Sigma Genosys) coupled to keyhole limpet hemocyanin (Kimura et al., 1994), and a hybridoma clone 6C9 was obtained by ELISA screening using the phosphorylated and nonphosphorylated peptides. As H2B Ser14 is phosphorylated during apoptosis (Cheung et al., 2003), the specificity was then checked by the specific appearance of positive signals in apoptosis-induced (etoposide treated) Hela cells by immunoblotting and immunofluorescence.

siRNA transfection and photobleaching
PP2Cγ-specific Stealth RNA (Invitrogen; nucleotide number 351-376 or 642-667 of GenBank/EMBL/DDB accession no. NM_177983) and the control RNA (Invitrogen; number 12935-300) were transfected using LipofectAMINE2000 (Invitrogen). Total cellular proteins were prepared 1–3 d after transfection, separated on 8% SDS-polyacrylamide gel, and immunoblotted (Kimura and Cook, 2001) with mouse monoclonal antibody directed against PP2Cγ (1:10,000; Murray et al., 1999) or α-tubulin (1:1,000; Oncogene Research Products) as a control. Cells grown on coverslips were transfected with Stealth RNA and fixed for immunofluorescence using the mouse anti-PP2Cγ (1:30,000) and Cy3-conjugated anti-mouse IgG (1:500; Jackson ImmunoResearch Laboratories).

For photobleaching studies, Hela cells expressing GFP-H2A, H2B-GFP, or H1c-GFP (Misteli et al., 2000) grown on glass-bottom dishes (MatTek) were transfected with Stealth RNA. 3 d later, the dish was set on an inverted microscope (LSM510 META; Carl Zeiss MicroImaging, Inc.) in an air chamber at 37°C, and the mobility was analyzed by photobleaching using the inverted microscope with a plan-Neofluor 40/NA 1.3 objective. For H2A and H2B, five confocal images were collected (512 × 512 pixels, zoom 3, maximum scan speed, pinhole 3.7 airy unit, IP505 emission filter, and 0.3% transmission of 458-nm Ar laser with 75% output power), one half of a nucleus was bleached using 100% transmission of 458 and 488 nm (eight iterations), and images were collected using the original settings every 5 min. For H1c, five images were collected (256 × 256 pixels, zoom 8, and scan speed 12), a 2 μm spot was bleached using 100% transmission of 458 and 488 nm (eight iterations), and images were collected using the original settings every 5 s (the graph in Fig. 7 E shows the points of every 10 s for ease of comparison). The fluorescence intensity of the bleached area was measured using MetaMorph software (Molecular Devices). After subtracting the background, the intensity was normalized to the initial intensity before bleaching.

DT40 cells
PP2Cγ-deficient DT40 cells were established using standard methods (Fig. S3; Fukagawa et al., 2004) and grown at 37°C. To measure the cell density, cells were mixed with trypan blue solution (Invitrogen), and the number of five cells excluding the dye was counted. To determine the sensitivity to caffeine and caffeine-induced irradiation, serially diluted cells were plated in methylcellulose plates with or without 1 mM caffeine (Sigma-Aldrich) and irradiated using a Gammacell 40 Exactor (Nordion). Colonies were counted 10–12 d after plating. For immunoblotting (Fig. 8 D), 4 × 105 cells/ml were irradiated and calyculin A (Sigma-Aldrich) was immediately added (final concentration of 10 ng/ml). A 1 ml aliquot was taken at each time point, and cells were collected (600 g for 2 min) and lysed in 100 μl of 2× SDS gel loading buffer.
Online supplemental material

Fig. S1 shows that ATP is not required for GFP-H2A incorporation into chromatin in permeabilized cells assisted by PP2Cγ or Nap1. Fig. S2 shows that PP2Cγ has weak de novo nucleosome assembly activity. Fig. S3 shows evidence for the generation of PP2Cγ knockout DT40 cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200608001/DC1.

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