The RNA transport factor PHAX is required for proper histone H2AX expression and DNA damage response

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ABSTRACT

PHAX (phosphorylated adaptor for RNA export) promotes nuclear export of short transcripts of RNA polymerase II such as spliceosomal U snRNA precursors, as well as intranuclear transport of small nucleolar RNAs (snoRNAs). However, it remains unknown whether PHAX has other critical functions. Here we show that PHAX is required for efficient DNA damage response (DDR) via regulation of phosphorylated histone variant H2AX (γH2AX), a key factor for DDR. Knockdown of PHAX led to a significant reduction of H2AX mRNA levels, through inhibition of both transcription of the H2AX gene and nuclear export of H2AX mRNA, one of the shortest mRNAs in the cell. As a result, PHAX-knockdown cells become more sensitive to DNA damage due to a shortage of γH2AX. These results reveal a novel function of PHAX, which secures efficient DDR and hence genome stability.

Keywords: PHAX; histone mRNA; H2AX; DNA damage response

INTRODUCTION

DNA lesions, caused by environmental stresses such as DNA injuring chemicals, ultraviolet (UV) light, ionizing radiation, etc., rapidly induce DNA damage response (DDR) that involves DNA repair, cell cycle arrest, and apoptosis, leading to the maintenance of genome integrity and tissue homeostasis (Jackson and Bartek 2009). DNA lesions activate protein kinases such as ataxia telangiectasia mutated (ATM) kinase, Rad3-related (ATR) kinase, and DNA-dependent protein kinase, catalytic subunit (DNA-PKcs), which gather to the DNA damage sites and phosphorylate histone variant H2AX at Ser139 (Thiriet and Hayes 2005). The resultant phosphorylated H2AX (γH2AX) forms "DDR foci" on the injured chromatin sites, which function as platforms for recruiting other DDR effectors (Jackson and Bartek 2009). γH2AX-mediated formation of DDR foci is a mark of the early step of the DDR pathway and is critical for efficient DDR. The H2AX protein turns over rapidly in normal situations, but is stabilized by DNA damage (Atsumi et al. 2015).

To focus on the topic of this study, it is necessary to describe an apparently unrelated field, RNA export. We have previously shown that nuclear export of the spliceosomal U snRNA precursors requires two adaptor proteins, the cap-binding complex (CBC) and the phosphorylated adaptor for RNA export (PHAX) as well as CRM1, the export receptor for proteins containing a leucine-rich nuclear export signal and RanGTP (Ohno et al. 2000; Will and Luhrmann 2001). PHAX and CRM1 were also implicated in an intranuclear transport of small nucleolar RNAs (snoRNAs) (Boulon et al. 2004). We have subsequently shown that RNA polymerase II (RNAPII) transcripts are sorted according to their lengths (Ohno et al. 2002; Masuyama et al. 2004; McCloskey et al. 2012). The RNAPII transcripts shorter than 200–300 nt are funneled into the spliceosomal U snRNA export pathway involving PHAX and CRM1, while the transcripts longer than the threshold are funneled into the mRNA export pathway involving mRNA export factors, including the TREX components and the export receptor NXF1/TAP but excluding PHAX. Notably, mRNAs for histones including H2AX are the shortest mRNAs in the cells and their lengths are quite close to the above threshold. In addition, the majority of histone mRNAs are produced without splicing and polyadenylation as U snRNAs (Marzluff et al. 2008). Hence histone mRNAs are unique mRNAs resembling U snRNAs.

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Although it has been shown that nuclear export of histone mRNAs requires mRNA export factors (Erkmann et al. 2005), the requirement for U snRNA export factors such as PHAX has not been investigated.

In this study, we found that knockdown of PHAX led to a significant reduction in H2AX expression levels, through inhibitions of H2AX mRNA export as well as of H2AX promoter activity. In response to DNA damage, PHAX-knockdown cells failed to induce efficient DNA repair due to a shortage of γH2AX. These results reveal a novel function of PHAX, which secures efficient DDR and hence genome stability *via* regulation of histone H2AX expression.

RESULTS

PHAX knockdown leads to a reduction in tolerance to DNA damage

During our survey process for undocumented functions of PHAX, we examined cell viability upon stimulation with anti-cancer agents and UV. We found that PHAX is involved in the cell viability after stimulation causing DNA damage. In the experiments, U2OS cell was used because of its wild-type p53 expression. The viability of PHAX-knockdown (KD) U2OS cells was markedly reduced after UV irradiation as compared to that of the control cells (Fig. 1A, lower panels), whereas cell viability without UV irradiation was not significantly altered (Fig. 1A, upper panels). Similar results were obtained with the cells treated by anti-cancer drugs such as adriamycin (ADR, Fig. 1B) or camptothecin (CPT, Fig. 1C). Notably, the UV irradiation-mediated inductions of p53 and phosphorylated p53 were enhanced in PHAX-KD cells (Fig. 1D).

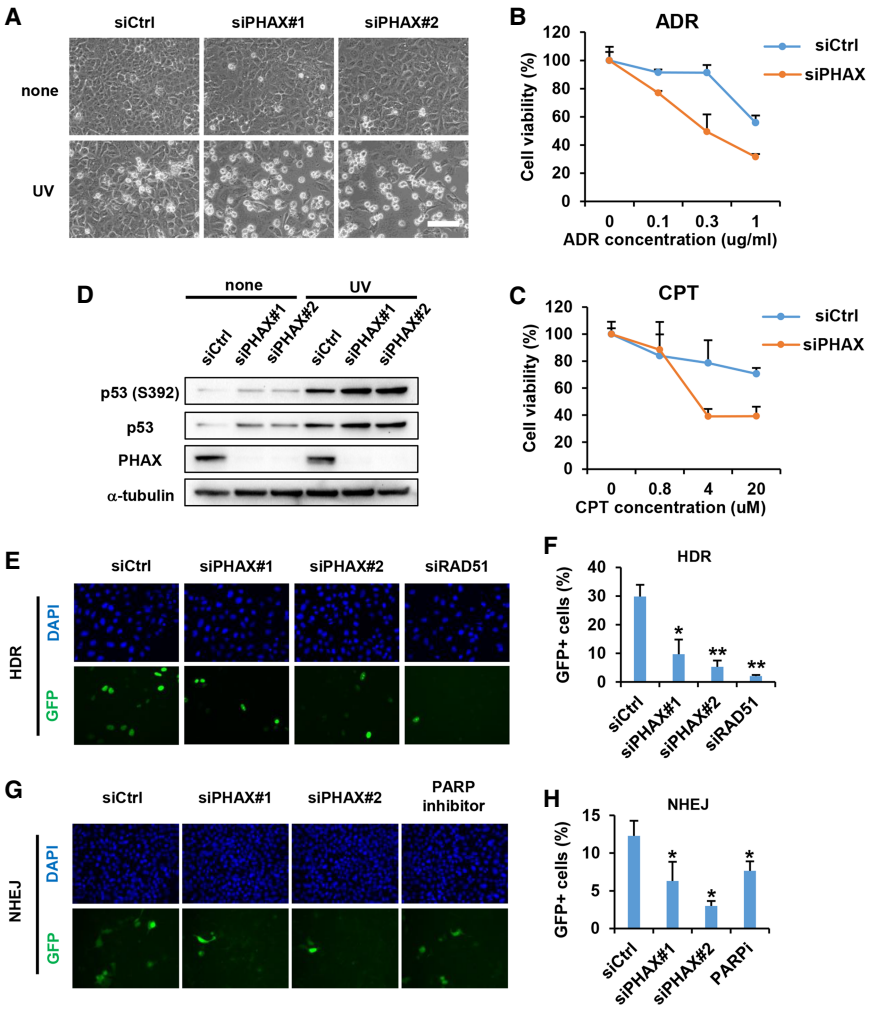

FIGURE 1. Inhibition of DNA repair by knockdown of PHAX. (*A*) U2OS cells were transfected with the indicated siRNAs, followed by UV-irradiation (20 J/m^2). After 6-h incubation, phase-contrast images were obtained. (*B*,*C*) U2OS cells were transfected with the indicated siRNAs, followed by ADR (*B*) or CPT (*C*) at the indicated concentrations. After 24-h incubation, cell viabilities were determined by alamarBlue assay. (*D*) U2OS cells were treated as in *A*. The indicated protein levels were determined by western blotting analysis. (*E–H*) U2OS cells were cotransfected with the indicated siRNAs and each reporter plasmid for DNA repair assay (pDRGFP for HDR [*E*,*F*] or pEJ2GFP for NHEJ [*G*,*H*]). Fluorescence microscopic images were obtained, and the numbers of GFP-positive cells were counted. As a positive control, the cells were treated with PARP inhibitor (PARPi) at 100 nM for 48 h. Data are the means ± S.D. ($n = 4$). (*) $P < 0.01$, (**) $P < 0.001$.

Since these results suggested that DDR may be disturbed in PHAX-KD cells, DNA repair efficiency in these cells was next examined. It is known that two major repairs, homologous-dependent repair (HDR) and nonhomologous end joining (NHEJ), are induced after double-strand DNA breaks (DSBs) (Shrivastav et al. 2008). PHAX-KD or control-KD cells were transfected with the reporter plasmids for each DNA repair assay (pDRGFP for HDR and pEJ2GFP for NHEJ, respectively), and the two DNA repair assays were performed (see Supplemental Fig. S1A for the HDR assay scheme). PHAX-KD cells showed a significant decrease in HDR efficiency as compared to the control cells (Fig. 1E,F; Supplemental Fig. S1B,C). This decrease was quite comparable to that of the positive controls, RAD51-KD cells (Fig. 1E,F) and H2AX-KD cells (Supplemental Fig. S1B,C; Shrivastav et al. 2008; Jackson and Bartek 2009). Efficiencies of NHEJ were also suppressed by over 50% in PHAX-KD cells (Fig. 1G,H). The effect of PHAX-KD was comparable to the positive control cells that had been treated by a PARP inhibitor, olaparib (Fig. 1G,H).

Note that there was no difference in transfection efficiency among the reporter plasmids under the experimental conditions (data not shown). Taken together, these results indicate that PHAX is required for efficient DSB repair.

PHAX knockdown leads to a reduction in H2AX expression

To clarify the cause of the inefficient DDR in PHAX-KD cells, we next examined the expression levels of several DDR factors. PHAX-KD promoted the phosphorylation levels of ATM and DNA-PKcs after ADR treatment, while γH2AX was not efficiently induced in PHAX-KD cells (Fig. 2A,B). Immunofluorescence cell staining analyses also demonstrated that the induction of γH2AX was inhibited in PHAX-KD cells (Fig. 2C). Similar results were obtained in UV-irradiated cells (Fig. 2D). When the PHAX-KD cells were transfected with a PHAX-expressing plasmid, the γH2AX expression level was largely restored (Fig. 2D), indicating that it is PHAX that promotes γH2AX expression.

To examine the cause of the low γH2AX expression, the total H2AX protein expression level was examined by using an antibody against H2AX, which recognizes both phosphorylated and unphosphorylated H2AX proteins. After treatment of the cells with UV or ADR, the expression levels of both total and phosphorylated H2AX proteins were lower in PHAX-KD cells as compared to those in the control cells, although their levels were constantly low without UV or ADR (Fig. 2E). These results demonstrated that PHAX-KD cells were not able to induce efficient H2AX protein expression and hence efficient DNA damage repair, because reduction of H2AX expression in fact leads to reduction in DNA repair efficiency (Supplemental Fig. S1B,C).

To examine why H2AX protein expression is low, we next measured mRNA levels of H2AX by quantitative reverse-transcription PCR (qRT-PCR) analysis. Regardless of the DNA damage induction, the steady state H2AX mRNA level was reduced by ~50% in PHAX-KD cells, as compared to control-KD cells (Fig. 3A). In addition, mRNA levels of various replication-dependent histones were also reduced by half in PHAX-KD cells (Fig. 3B). In contrast, expression of replication-dependent histone mRNAs, but not H2AX mRNA, was heavily depressed by the DNA damage induction probably due to a DNA replication arrest, consistent with a previous report (Dankert et al. 2016). Therefore it was not possible to determine the effect of PHAX-KD (data not shown). The reduction of H2A and H2AX mRNA levels was restored by a rescue of PHAX expression, at least partially (Fig. 3C, Supplemental Fig. S2A), indicating that it is PHAX that is critical for this phenomenon.

PHAX-KD had specific suppressive effects on histone gene expressions among the genes tested (Fig. 3D), although c-myc expression was enhanced for unknown reasons. Note that steady state U snRNA levels were not significantly altered by PHAX-KD despite PHAX's role as a U snRNA exporter (Fig. 3D; Supplemental Fig. S2B) likely because U snRNAs are very stable in the cell. This indicates that the reduction in histone mRNA levels in PHAX-KD cells was

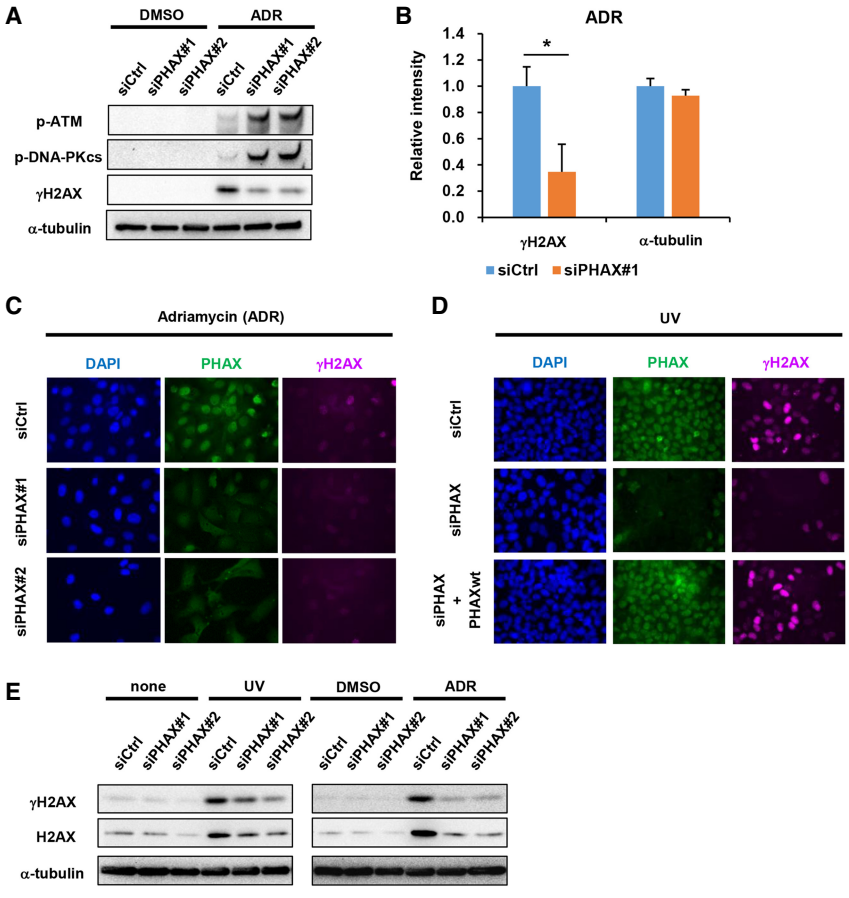

FIGURE 2. Suppression of H2AX expression by knockdown of PHAX. (*A*–*C*) U2OS cells were transfected with the indicated siRNAs, followed by treatment with ADR at 1 μg/mL. After 5-h incubation, the indicated protein levels were determined by western blotting analysis (*A*,*B*) and immunostaining analysis for PHAX and γH2AX (*C*). The intensities of protein bands were quantified (*B*). (*D*) U2OS cells were cotransfected with siPHAX and a PHAX-expressing plasmid, followed by UV-irradiation (20 J/m^2). After 5-h incubation, cells were fixed and immunostained for PHAX and γH2AX. (*E*) U2OS cells were treated as in *A* or *D*. The indicated protein levels were similarly determined.

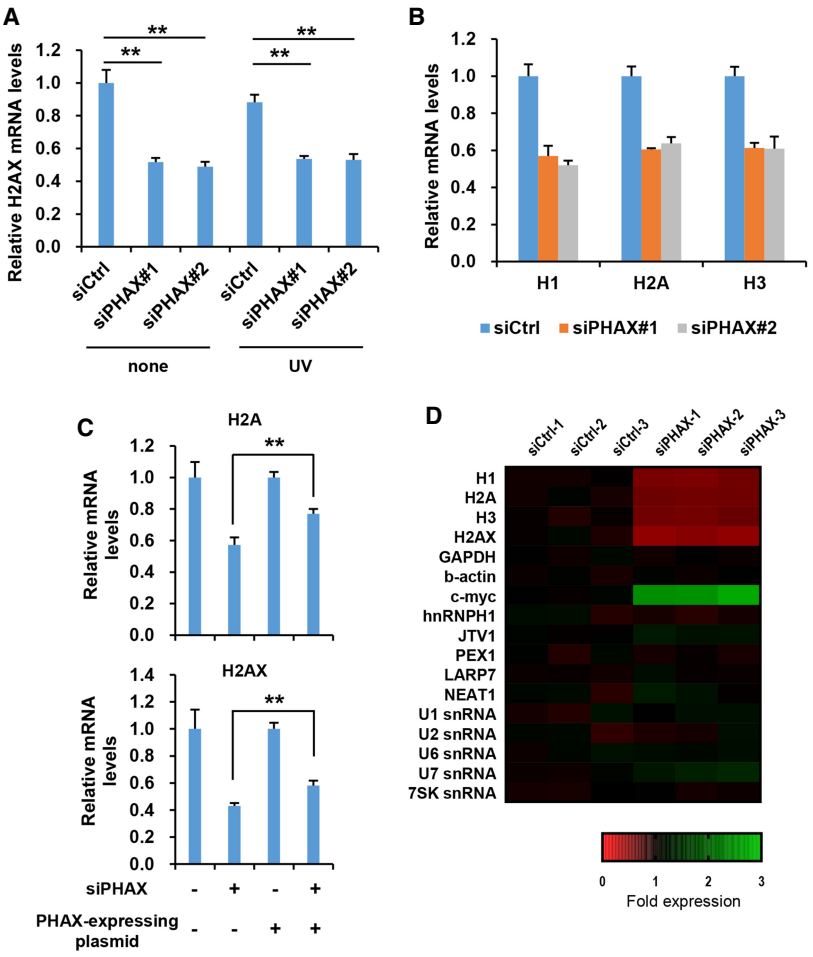

FIGURE 3. Reduction in H2AX mRNA levels by knockdown of PHAX. (*A*) U2OS cells were transfected with the indicated siRNAs, followed by UV-irradiation (20 J/m^2). After 6-h incubation, the indicated mRNA levels were determined by qRT-PCR analysis. (*B*) U2OS cells were transfected with the indicated siRNAs. After 48-h incubation, the indicated mRNA levels were similarly determined. (*C*) U2OS cells were cotransfected with siPHAX and a PHAX-expressing plasmid. After 48-h incubation, the indicated mRNA levels were similarly determined. (*D*) The indicated mRNA levels in siPHAX-transfected cells were determined by qRT-PCR analysis and were expressed in the form of a heat map. Data are the means ± S.D. (n = 3–4). (**) P < 0.001.

Since replication-dependent histone mRNAs, such as H1, H2A, H2B, H3, and H4, are transcribed during S phase (Marzluff et al. 2008), we also examined the cell cycle property of PHAX-KD cells. The KD of PHAX had no great effects on the cell cycle property, except that the cell population in G1 and S phase was only slightly increased and decreased, respectively (Supplemental Fig. S2E), indicating that the reduction in replication-dependent histone mRNA levels in PHAX-KD cells cannot be attributed to a disturbance of cell cycle. To further examine the effect of cell cycle property on histone gene expression, we synchronized the cell cycle in G1 phase by a double-thymidine block. Expression of replication-dependent histone mRNAs depends on cell cycle, while H2AX mRNA expression does not. Even in the condition of cell synchronization in G1 phase, H2AX mRNA expression was significantly decreased in PHAX-KD cells (Supplemental Fig. S2F), suggesting that PHAX regulates H2AX expression independently of cell cycle property.

We have so far shown that steady state mRNA levels of histones including H2AX are reduced by ~50% in PHAX-KD cells (Fig. 3A). It is worth pointing out that the protein levels of histones including H2AX are not significantly altered in normal situations without DNA damage induction (Fig. 2E, Supplemental Fig. S2G) but that the protein expression level of H2AX is heavily suppressed in DNA damaging conditions, probably due to the stabilization of H2AX protein in DNA damaging conditions (Fig. 2E).

not attributed to the interruption of U snRNA biogenesis and/or splicing.

PHAX accumulates in Cajal bodies (CBs), and the CBs are depleted when expression of PHAX is suppressed in cells (Lemm et al. 2006). In addition, UV treatment causes CB disassembly that is mediated by a proteasome activator PA28γ, and overexpression of PA28γ causes depletion of CBs even in the cells not treated by UV (Cioce et al. 2006). To examine whether CBs affect histone mRNA expression, we induced depletion of CBs in the cell by an overexpression of PA28γ (Cioce et al. 2006). In USOS cells overexpressing PA28γ, CBs were depleted (Supplemental Fig. S2C), but histone mRNA levels were not significantly altered (Supplemental Fig. S2D), suggesting that it was not CB depletion that affected histone mRNA levels.

PHAX knockdown leads to reductions in histone gene transcription

In order to examine whether PHAX is involved in histone mRNA expression at early steps such as transcription and RNA processing, histone mRNA levels during early steps were analyzed in PHAX-KD cells. RNA was pulse-labeled by 5-ethynyl uridine (5EU) for 1.5 h, and purified through an immunoprecipitation by an anti-5EU antibody. qRT-PCR analyses demonstrated that the nascent mRNA levels of H2A, H3, and H2AX but not GAPDH, were significantly decreased in PHAX-KD cells (Fig. 4A), suggesting that

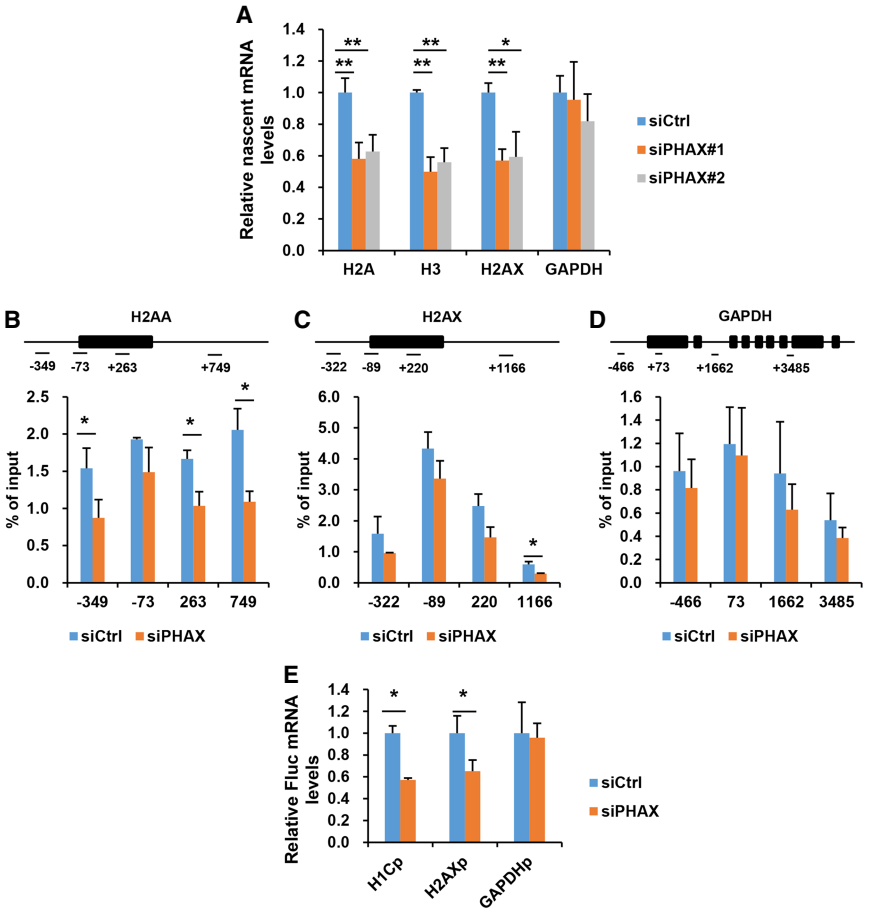

FIGURE 4. Suppression of histone mRNA transcription by knockdown of PHAX. (*A*) U2OS cells were transfected with the indicated siRNAs. After 48-h incubation, nascent RNAs were labeled by 5EU for 1.5 h. 5EU-labeled nascent mRNAs were determined by qRT-PCR analysis. (*B–D*) U2OS cells were transfected with siPHAX. After 48-h incubation, RNAPII-binding DNA levels on the indicated gene loci were determined by ChIP assay. (*E*) U2OS cells were cotransfected with siPHAX, an Rluc-expressing plasmid (pGL4-SV40p-Rluc), and the reporter plasmid carrying HIC, H2AX, or GAPDH promoter-driven Fluc expression cassette. After 48-h incubation, Fluc mRNA levels were determined by qRT-PCR analysis. Fluc mRNA levels were normalized by Rluc mRNA levels. Data are the means ± S.D. ($n = 3–4$). (*) $P < 0.05$, (**) $P < 0.01$.

histone mRNA transcription was partially inhibited by PHAX-KD. Chromatin Immunoprecipitation (ChIP) analyses revealed that RNAPII recruitments to H2AA and H2AX gene loci were reduced in PHAX-KD cells, as compared to those in control cells (Fig. 4B–D). Note that RNAPII recruitments to the promoter regions (H2AA: −349; H2AX: −322) were attenuated (Fig. 4B,C). This prompted us to examine the activity of histone gene promoters using reporter plasmids in which the promoter sequences from H1C and H2AX were fused to the coding sequence of the firefly luciferase (Fluc). The reporter assay confirmed that the promoter activities of H1C and H2AX were significantly decreased by PHAX-KD (Fig. 4E). These results indicate that PHAX regulates the transcription of the histone genes. The molecular mechanism for the transcription regulation is currently unknown. It is known that the transcription of replication-dependent histone mRNAs is driven by a transcriptional regulator NPAT (nuclear protein, ataxia-telangiectasia locus), which is recruited to histone gene clusters and forms a nuclear structure called the histone locus body (HLB) in the form of nuclear dots (Marzluff et al. 2008). The HLBs were detected in both control and PHAX-KD cells (Supplemental Fig. S3A), suggesting that PHAX-KD had no effects on the expression of NPAT and the formation of HLBs.

It was possible that PHAX KD might affect RNA stabilization. A previous report has demonstrated that PHAX and an exosome adaptor ZC3H18 are mutually exclusive during interaction with CBC-bound RNAs, and that in PHAX-KD cells, ZC3H18 is preferentially associated with CBC-bound RNAs, leading to destabilization of the RNAs (Giacometti et al. 2017). To examine the involvement of ZC3H18, ZC3H18 was knocked down simultaneously with PHAX. The expression of ZC3H18 proteins was successfully knocked down after transfection with siRNA against ZC3H18 (siZC3H18) (Supplemental Fig. S3B). Histone mRNA levels in PHAX/ZC3H18 double-KD (DKD) cells were not significantly altered as compared to those in PHAX-KD cells (Supplemental Fig. S3C), suggesting that ZC3H18 was not involved in the reduction in histone mRNA expression in PHAX-KD cells.

PHAX knockdown leads to a reduction of histone mRNA export

PHAX plays an important role in nuclear export of short transcripts of RNAPII including spliceosomal U snRNA precursors. Since histone mRNAs are the shortest mRNAs in the cell, we next examined whether PHAX is also involved in histone mRNA export. To this end, a tagged H2A- or H2AX-expressing plasmid was transfected into PHAX-KD or control-KD cells. As controls, U1ΔSm RNA-, or EGFP-expressing plasmid was also transfected. U1ΔSm RNA is a mutant U1 snRNA capable of export but not reimport (Hamm and Mattaj 1990). After incubation, the cells were harvested, fractionated into the cytoplasmic and the nuclear fractions, and the export efficiency (the cytoplasmic to nuclear ratio) of the RNAs transcribed from the transfected

plasmids as well as endogenous GAPDH mRNA were analyzed. The purity of each fraction was confirmed by analyzing endogenous NEAT1 RNA (mainly localized in the nucleus) and endogenous GAPDH mRNA levels (mainly localized in the cytoplasm) (Supplemental Fig. S4A). The export efficiency of H2A and H2AX mRNAs, as well as U1ΔSm RNA, but not EGFP and GAPDH mRNAs, was significantly decreased by PHAX-KD (Fig. 5A), while the export of all mRNAs but not of U1ΔSm RNA was severely reduced by NXF1/TAP-KD (Supplemental Fig. S4B,C; Erkmann et al. 2005), suggesting that efficient export of histone mRNAs requires PHAX on top of mRNA export machinery. Consistent with this is PHAX's association with various histone mRNAs as well as U1 snRNA but not GAPDH and β-actin mRNAs, as shown by immunoprecipitation analyses (Fig. 5B). To further examine whether PHAX is required for histone mRNA export in a different experimental system, a precursor of human H2A mRNA was microinjected into the nucleus of *Xenopus* oocytes (Fig. 5C). U6Δss RNA which has a small deletion in the single-stranded region was used as a marker for the efficacy of nuclear microinjection, because the U6Δss RNA is not imported into the nucleus if misinjected into the cytoplasm (Hamm and Mattaj 1990). The precursor H2A mRNA was processed to produce mature H2A mRNA after microinjection (Fig. 5D). Microinjection of the antibody against PHAX inhibited the export of processed H2A mRNA but not the control mRNA (Fig. 5D). These results confirm that PHAX regulates the export of histone mRNAs.

DISCUSSION

In this study, we have clarified a previously undocumented role of RNA transport factor PHAX. We have provided evidence showing that PHAX regulates histone H2AX expression and DDR. Since PHAX is required for efficient H2AX mRNA synthesis and nuclear export, PHAX-KD cell cannot efficiently induce γH2AX expression (Fig. 2) and hence DDR (Fig. 1) when DNA is damaged, due to a shortage of H2AX protein. Steady state mRNA levels of H2AX are modestly reduced (by ~50%) in PHAX-KD cells (Fig. 3A). This modest reduction of H2AX mRNA leads to a large reduction of the H2AX protein level, not in normal situations but in DNA damaging situations (Fig. 2). This is probably because H2AX protein rapidly turns over in the former situations but is highly stabilized in the latter (Atsumi et al. 2015). In addition, our data show that PHAX also regulates the production of mRNAs for replication-dependent histones similarly at the levels of both transcription (Fig. 4) and mRNA export (Fig. 5).

Although our results clearly indicate that PHAX regulates the transcription of histone genes quite specifically, the molecular mechanism for the transcription regulation is currently unknown. As shown in Figure 4B, the RNAPII recruitment to the promoter region is as high as that to the transcriptional start sites (TSSs), especially in the case of H2AA, although RNAPII usually stalls at TSS (Ji et al. 2013). This suggests that transcription of histone mRNAs might be progressed promptly without stalling of RNAP II at TSSs, and that the RNAPII recruitment to the promoter region might be a rate-limiting step. Recruitment of RNAPII to histone gene loci and their promoter activities were shown to be reduced in PHAX-KD cells (Fig.

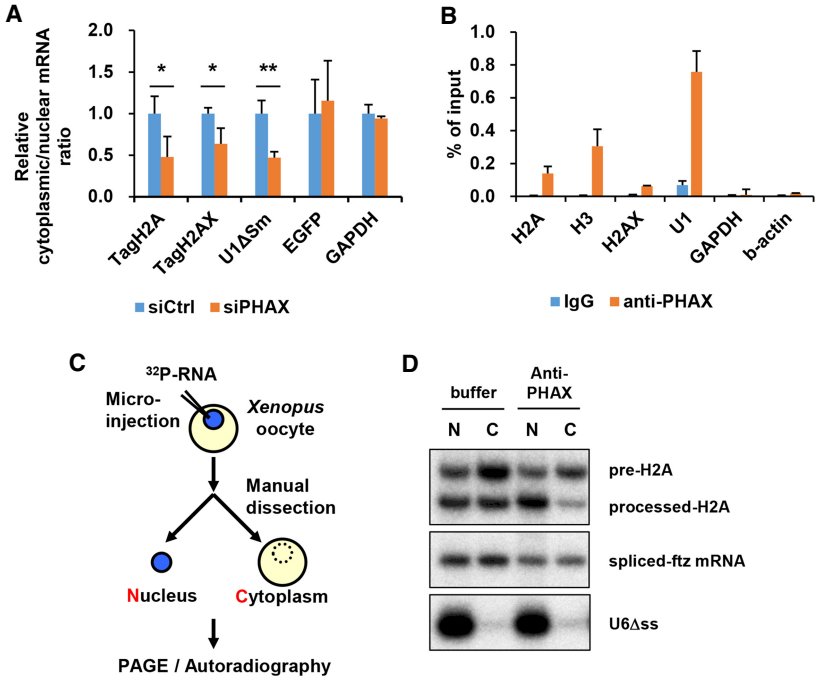

FIGURE 5. Reduction in histone mRNA export efficiencies by knockdown of PHAX. (*A*) U2OS cells were transfected with siPHAX, followed by transfection with plasmids expressing the indicated genes. After 3-h incubation, RNA was extracted from the nuclear and cytoplasmic fractions of the cells, and the indicated mRNA levels were determined by qRT-PCR analysis. (*B*) U2OS cells were lysed and immunoprecipitation was performed with an anti-PHAX antibody. PHAX-binding RNA levels were determined by qRT-PCR analysis. (*C*,*D*) A mixture of ^{32}P-labeled m7G-capped precursor H2A (pre-H2A), precursor ftz mRNA, and U6Δss was injected into the nucleus of Xenopus oocytes either alone or together with an anti-PHAX antibody. RNA was extracted from nuclear (N) and cytoplasmic (C) fractions 2 h after microinjection and analyzed by 8% denaturing PAGE followed by autoradiography. Pre-H2A was processed into mature H2A (shown as processed-H2A) and pre-ftz mRNA was spliced (spliced-ftz mRNA) in the nucleus. Data are the means ± S.D. ($n = 3$). (*) $P < 0.05$, (**) $P < 0.01$.

4), suggesting that PHAX could affect these steps. Furthermore, it is interesting that transcription of both replication-dependent histones and -independent histone H2AX is affected, since their transcription regulations are quite different. This suggests that PHAX may be involved in the basic transcription machinery common to all histone genes. PHAX is recruited to CBC together with an adaptor protein ARS2 that directly interact with FLASH (Kiriyama et al. 2009). FLASH is known to regulate histone gene transcription through a direct interaction with NPAT (Marzluff et al. 2008). Integrity of HLBs and their constituent NPAT are critical for the transcription of replication-dependent histones such as H1, H2A, H2B, H3, and H4 (Zhao et al. 2000; Marzluff et al. 2008). Nonetheless, we have at least shown that HLBs are not affected by PHAX-KD (Supplemental Fig. S3A). Further investigation of how PHAX is involved in the transcription of a variety of histone genes is necessary.

In U snRNA export, PHAX is recruited to CBC that has been bound to the cap structure and the recruited PHAX in turn recruits the export receptor CRM1 and RanGTP, leading to the RNA export to the cytoplasm (Ohno et al. 2000). In contrast, bulk mRNAs are bound by adaptor proteins including the TREX components and/or SR proteins and these adaptors in turn recruit the export receptor NXF1/TAP, leading to the bulk mRNA export to the cytoplasm (Kohler and Hurt 2007). Notably, the U snRNA export factors such as PHAX are usually excluded from the mRNA export complex (Masuyama et al. 2004). We have previously shown that the RNAPII transcripts shorter than 200–300 nt are funneled into U snRNA export pathway involving PHAX and CRM1, while the transcripts longer than the threshold are funneled into the mRNA export pathway involving mRNA export factors, including the TREX components and NXF1/TAP (McCloskey et al. 2012). Although histone mRNAs are the shortest mRNAs in the cells and their lengths are quite close to the above threshold, it has been already demonstrated that the histone mRNA export is carried out by NXF1/TAP-, but not CRM1-, mediated export pathway (Erkmann et al. 2005). Consistent with a previous study (Giacometti et al. 2017), our results demonstrated that PHAX is recruited to histone mRNAs unlike the bulk mRNAs (Fig. 5B) and that PHAX is required for the efficient nuclear export of histone mRNAs on top of the mRNA export factors (Fig. 5A,D). The effect of PHAX-KD in this process is relatively modest as compared to that of the NXF1/TAP-KD (Fig. 5A; Supplemental Fig. S4C). Therefore, it seems that PHAX plays only an optimizing role in the nuclear export of histone mRNAs. However, this modest decrease in mRNA export together with a modest decrease in gene transcription does lead to a large reduction in γH2AX expression in DNA damage situations. How PHAX plays a role in the mRNA export of histone mRNAs is currently unknown. Only what we know is that PHAX seems to be a part of the histone mRNA export complex (Fig. 5B) but that CRM1 seems not involved unlike in the case of U snRNA export (Erkmann et al. 2005). Further studies will be needed to clarify the mechanism underlying PHAX-mediated export of histone mRNAs.

Although there have already been several reports describing roles of RNA binding proteins in DDR (Dominguez-Sanchez et al. 2011; Adamson et al. 2012; Awwad et al. 2017), our unexpected results described here further reveal how the mechanism promoting the maintenance of genome integrity is multifarious and extensive.

MATERIALS AND METHODS

Cells, chemicals, and antibodies

U2OS cells (a human osteosarcoma cell line) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 µg/mL), and penicillin (100 U/mL). ADR (also known as doxorubicin), CPT, and PARPi (Olaparib) were purchased from FUJIFILM Wako Pure Chemical Corp., Sigma Aldrich Japan, and Selleckchem, respectively. The antibodies used in this study are described in Supplemental Table S1.

Plasmids

pDRGFP (plasmid 26475), pEJ2GFP (44025), pCBASceI (26477) were obtained from Addgene. Human PHAX cDNA containing FLAG-tag was amplified by PCR from a HeLa cDNA library and cloned into pcDNA3, resulting in pcDNA3-FLAG-PHAX. The sequences of the primers used in this study are described in Supplemental Table S2.

The reporter plasmids pGL4-H1Cp, -H2AXp, and -GAPDHp, which have sequences from the respective promoters upstream of the Fluc gene, were constructed as follows: The fragment containing each promoter was amplified by PCR using the corresponding primers and cloned into pGL4.10 (Promega). pGL4-SV40p-Rluc, a reporter plasmid carrying a SV40 promoter-driven renilla luciferase (Rluc) expression cassette, was purchased from Promega (sold as pGL4.73).

The fragment containing human H2A promoter and coding region was amplified by PCR from HeLa genomic DNA and cloned into pUC118, resulting in pUC118-hH2Ap-hH2A. To insert tag sequence (5′-TGATCGCGCTTCTCGTTG-3′), site-directed mutagenesis of pUC118-hH2Ap-hH2A was performed using appropriate primers, resulting in pUC118-hH2Ap-tag hH2A. A plasmid carrying human H2AX promoter and coding region, pUC118-hH2AXp-tag hH2AX, was similarly constructed. The sequences of the primers used in this study are described in Supplemental Table S2.

Transfection with siRNA

All siRNAs (Stealth siRNA of the 25-mer duplex) used in this study were obtained from Thermo Fisher Scientific. Cells were

transfected with the siRNAs using Lipofectamine2000 (Thermo Fisher Scientific) according to the manufacturer's instructions.

qRT-PCR analysis

Total RNA was isolated from cells using Sepasol-RNA I Super (Nacalai Tesque). cDNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA Synthesis Kit (Thermo Fisher Scientific). qRT-PCR analysis was performed using Fast SYBR Green Master Mix (Thermo Fisher Scientific) and StepOnePlus Real-Time PCR systems (Thermo Fisher Scientific). All data of qRT-PCR analysis were normalized by the data of GAPDH mRNA levels. The sequences of the primers used in this study are described in Supplemental Table S2.

Western blotting analysis

Western blotting assay was performed as previously described (Machitani et al. 2020). Briefly, whole-cell extracts were prepared and 10 µg of total protein per lane was loaded onto 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels. After electrophoresis under reducing conditions, bands of protein were transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore). After blocking with 5% skim milk prepared in TBS-T (Tween-20, 0.1%), the membrane was incubated with the primary antibodies, followed by incubation in the presence of horseradish peroxidase (HRP)-labeled anti-mouse or -rabbit IgG antibody (Jackson ImmunoResearch). The antibodies used in this study are described in Supplemental Table S1. The intensities of protein bands were quantified using Image J software.

Immunofluorescence cell staining

Immunofluorescence cell staining was performed as previously described (Izumi et al. 2014). Briefly, cells were fixed with 4% formaldehyde in PBS, permeabilized with 0.2% TritonX-100 in PBS, and blocked with 2% bovine serum albumin in PBS. The cells were incubated with the primary antibodies, followed by incubation in the presence of Alexa488-, Alexa568-, or Cy5-labeled secondary antibody (Thermo Fisher Scientific). The antibodies used in this study are described in Supplemental Table S1.

DNA repair assays

DNA repair assay was performed as previously described (Seluanov et al. 2010). Cells were cotransfected with pDRGFP (for HDR) or pEJ2GFP (for NHEJ) and pCBASceI using Lipofectamine2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. After 48- or 72-h incubation, fluorescence microscopic images were obtained, and the numbers of GFP-positive cells were counted.

Cell viability assay

For the evaluation of cell viability after UV treatment, cells were irradiated with UV (20 J/m^2). After 6-h incubation, phase-contrast photomicrographs of the cells were obtained.

For the evaluation of the cytotoxic activity of ADR and CPT, cells were treated with ADR or CPT at the indicated concentrations. After 24-h incubation, the cell viabilities were determined by staining with alamarBlue (Thermo Fisher Scientific) according to the manufacturer's instructions.

Determination of nascent RNA levels

U2OS cells were transfected with the indicated siRNAs. After 48-h incubation, nascent RNAs were labeled by 5EU at 0.5 mM for 1.5 h. 5EU-labeled RNAs were biotinylated and immunoprecipitated using Click-it Nascent RNA Capture Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The nascent RNA levels were determined by qRT-PCR analysis.

Chromatin immunoprecipitation (ChIP) assay

U2OS cells were transfected with siRNAs. After 48-h incubation, cells were treated with formaldehyde at a final concentration of 1% for crosslinking, and then genomic DNA was fragmented by sonication. The DNA fragment-protein complexes were immunoprecipitated using a mouse anti-RNA polymerase II CTD antibody (8WG16) (Abcam). The ChIP assay kit was purchased from Merck Millipore. The precipitated DNA copy numbers were determined by quantitative PCR analysis using the primers shown in Supplemental Table S2.

RNA immunoprecipitation experiments

RNA immunoprecipitation experiments were performed as previously described (McCloskey et al. 2012). Briefly, for formaldehyde crosslinking, cells were treated with formaldehyde at a final concentration of 1%. The reaction was quenched by adding a final 0.15 M glycine. After cells were washed with ice-cold PBS, cell pellets were resuspended in RIPA buffer. Cell lysate was prepared by sonication with Bioruptor (CosmoBio). After centrifugation, PHAX-binding RNA was immunoprecipitated using a mouse anti-PHAX antibody from the lysate. The precipitated RNA copy numbers were determined by qRT-PCR analysis.

Nuclear and cytoplasmic mRNA fractionation

U2OS cells were transfected with siPHAX, followed by transfection with tagged-H2A, -H2AX, -U1ΔSm (Izumi et al. 2014), or EGFP-expressing plasmids (pUC118-hH2Ap-taghH2A, -hH2AXp-taghH2AX, hU1p-hU1ΔSm, or pEGFP-C1 [Clontech], respectively). After 3-h incubation, the nuclear and cytoplasmic fractions of the cells were prepared using NE-PE Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. RNA was isolated from each fraction as described above, and the indicated mRNA levels were determined by qRT-PCR analysis.

Microinjection into Xenopus oocytes

For the human H2A plasmid, the human H2A fragment was amplified by PCR from a HeLa cDNA library and cloned into pBluescript

KS (+), resulting in pBS-hH2A. pBS-hH2A was linearized by *Not*I, followed by transcription by T7 RNA polymerase. ^{32}P-labeled U6Δss RNA and precursor ftz mRNA were prepared as described previously (Ohno et al. 2000). Microinjection of the ^{32}P-labeled RNAs into Xenopus oocytes was performed as previously described (Ohno et al. 2000). Nuclear and cytoplasmic RNA was recovered at 2 h after microinjection.

Statistical analysis

Statistical significance was determined using Student's *t*-test. Data are presented as the means ± S.D.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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