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BAF53 is Critical for Focus Formation of γ-H2AX in Response to DNA Damage

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Abstracts: When DNA double-strand breaks (DSBs) were induced in mammalian cells, many DNA damage response proteins are accumulated at damage sites to form nuclear foci called IR-induced foci. Although the formation of foci has been shown to promote repair efficiency, the structural organization of chromatin in foci remains obscure. BAF53 is an actin-related protein which is required for maintenance of chromosome territory. In this study, we show that the formation of IR-induced foci by γ-H2AX and 53BP1 were reduced when BAF53 is depleted, while DSB- activated ATM pathway and the phosphorylation of H2AX remains intact after DNA damage in BAF53 knockdown cells. We also found that DSB repair efficiency was largely compromised in BAF53 knockdown cells. These results indicate that BAF53 is critical for formation of foci by γ-H2AX decorated chromatin at damage sites and the structural organization of chromatin in foci is an important factor to achieve the maximum efficiency of DNA repair.

Key words: BAF53, IR-induced foci, γ-H2AX, chromosomal subdomain

INTRODUCTION

DNA double-strand breaks (DSBs) induced by ionizing radiation and radiomimetic drugs lead to the formation of the IR-induced foci (IRIF; Rogakou et al., 1999). IRIF is formed by massive assembly of the DNA damage response (DDR) proteins at DNA damage sites. The phosphorylated H2AX (γ-H2AX) plays a central role in focus formation at sites of DNA damage. H2AX is a variant of H2A and is associated with DNA throughout whole genome (Rogakou et al., 1998). After induction of DNA double-strand breaks, H2AX is phosphorylated on serine 139. γ-H2AX provides docking sites for many DDR proteins, including the MRN complex (MRE11, RAD50, and NBS1), 53BP1, and BRCA1 (Paull et al., 2000: Bassing and Alt, 2004). Formation of IRIF has been proposed to restructure large segments of chromatin in order to allow the access of the repair factors to damage sites (van Attikum and Gasser, 2009) or to preserve the integrity of the epigenetic information written in these segments of chromatin (Koundrioukoff et al., 2004).

Expansion of γ-H2AX region results from the positive feedback loop interplayed by the MRN complex, ATM, MDC1, and γ-H2AX (Lou et al., 2006). The MRN complex recruits active ATM, and ATM phosphorylates H2AX. MDC1 then associates with γ-H2AX and recruits additional activated ATM to the sites. Additional docking of the MRN complex on γ-H2AX contributes to this positive feedback loop. Interestingly expansion of γ-H2AX is limited to megabase chromatin regions flanking the break and does not spread throughout the nucleus in mammals (Rogakou et al., 1999). However, barrier to the expansion of γ-H2AX is yet to be identified. In addition, how chromatin segments are structurally organized in IRIF is obscure.

It is conceptually simple and neat to propose that IRIF is formed based on the pre-existing higher-order chromatin structure of interphase chromatin. Interphase chromatin in mammals is indeed compartmentalized as stable chromosomal subdomains with size of several hundreds kilobase to megabase (Müller et al., 2004; Shopland et al., 2006). Although the organization of chromatin in chromosomal subdomain is controversial, most evidence available indicates that the chromosomal subdomain is a compact structure generated by clustering of chromatin loops or series of folding of the 30-nm chromatin fiber (Münkel et al., 1999; Belmont and Bruce, 1994). Therefore, if binding of the DDR proteins spreads throughout the chromosomal subdomain and its spreading is limited within the subdomain, it will be observed as microscopically discernible foci.

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Previously we showed that volume of chromosome territory is under regulation by BAF53 (Lee et al., 2007). When BAF53 is unavailable, volume of chromosome territory is expanded by 2 to 2.4 folds. Considering that chromosome territory consists of a collection of chromosomal subdomains and thus effective volume of chromosomal subdomain is a primary determinant of envelope volume of chromosome territory (Schardin et al., 1985; Kleckner et al., 2004), one likely cause of the expansion of chromosome territory is disintegration of chromosomal subdomain.

Taking advantage of this unique opportunity that integrity of chromosomal subdomain can be manipulated by BAF53, we examined in this study whether structure of chromosomal subdomain is prerequisite for the formation of IRIF. We found that formation of IRIF by γ-H2AX and 53BP1 was largely compromised by BAF53 knockdown although production of γ-H2AX was not reduced. We also found that DNA repair on DSBs became inefficient when BAF53 was depleted.

MATERIALS AND METHODS

Cell culture

NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO_2. siRNA interference was carried out as previously described (Lee et al., 2007).

Immunoblotting and immunofluorescent staining analysis

Immunoblotting and Immunofluorescent staining analyses were performed as previously described (Choi et al., 2001; Lim et al., 2008).

Antibodies

Immunofluorescence analysis was carried out using anti-γ-H2AX antibody (Upstate, #05-636) and anti-53BP1 antibody (Novus, #NB100-304). Immunoblotting analysis was carried out using anti-γ-H2AX antibody (Upstate, #05-636), anti-H2AX antibody (Bethyl Lab., #A300-083A), anti-phospho-Ser15 p53 antibody (Cell Signaling, #9284), anti-phospho-Thr68 Chk2 antibody (Cell Signaling, #2661). The BAF53 antibody is previously described (Lee et al., 2003).

Neutral Comet Assay

Neutral comet assay was performed using a kit (Trevigen; Gaithersburg, MD) according to the manufacturer's instructions. NIH3T3 cells (1×10^5 cells/mL) were mixed with low temperature melting agarose at a ratio of 1 to 10 volume (v/v) and spread on a slide glass. Cells were lysed with a lysis solution (2.5 M NaCl, 100 mM EDTA, pH 10, 10 mM Tris base, 1% sodium lauryl sarcosinate, 1% Triton X-100) at 4°C for 1 hr. After equilibrated in TBE solution (40 mM Tris/boric acid, 2 mM EDTA, pH 8.3), slides were electrophoresed at 1.0 V/cm for 10 min, and then stained for 25 min in SYBR Gold. For measuring of comet tail moments, more than 150 cells from each slide were counted using CASP (Comet Assay Software Project) program.

RESULTS

We generated DSBs by the treatment of 1.6 μM adryamycin for 2 hrs in NIH3T3 cells. After 30 min, phosphorylation of H2AX and formation of IRIF by γ-H2AX and 53BP1 were examined in the cells where expression of BAF53 was suppressed by siRNA interference. BAF53 knockdown alone did not result in phosphorylation of H2AX, suggesting BAF53 depletion did not cause any DNA damage (Fig. 1A). Next, we examined the effect of BAF53 knockdown on phosphorylation of H2AX induced by adriamycin, and found the similar level of γ-H2AX in BAF53 knockdown cells compared to non-treated control cells or control siRNA treated cells. This data indicate that BAF53 is not required for the phosphorylation of H2AX induced by DSBs.

Provided with that H2AX is normally phosphorylated in BAF53 knockdown cells, we next examined whether γ-H2AX is able to form microscopically discernible foci in BAF53 knockdown cells (Fig. 1B). Strikingly, these analyses showed that the number of γ-H2AX foci induced by adriamycin was drastically reduced in BAF53 knockdown cells compared to non-treated control cells or control siRNA treated cells. Similar impairment in IRIF formation of 53BP1 was observed in BAF53 knockdown cells exposed to adriamycin (Fig. 1C). 53BP1 is a protein to relocalize to damage sites and form IRIF. Taken together, there data suggest that lack of BAF53 interfere focus formation of γ-H2AX and 53BP1 although normal level of γ-H2AX is generated.

To further substantiate that DSB-activated ATM pathway remains intact in BAF53 knockdown cells, we examined two ATM-dependent phosphorylation events: phosphorylation of p53 on Ser15 (Banin et al., 1998) and phosphorylation of the Chk2 kinase on Thr68 (Matsuoka et al., 2000). Fig. 2 shows that lack of BAF53 had no significant effect on phosphorylation of p53 on Ser15 and phosphorylation of Chk2 on Thr68 induced by adriamycin.

Next, we used a single cell electrophoresis analysis (comet assay) for monitoring DNA repair efficiency in BAF53 knockdown cells (Fig. 3). Just after the treatment of adriamycin, approximately the same tail moments were observed in BAF53 knockdown cells compared to untreated control cells. On the other hand, the kinetic

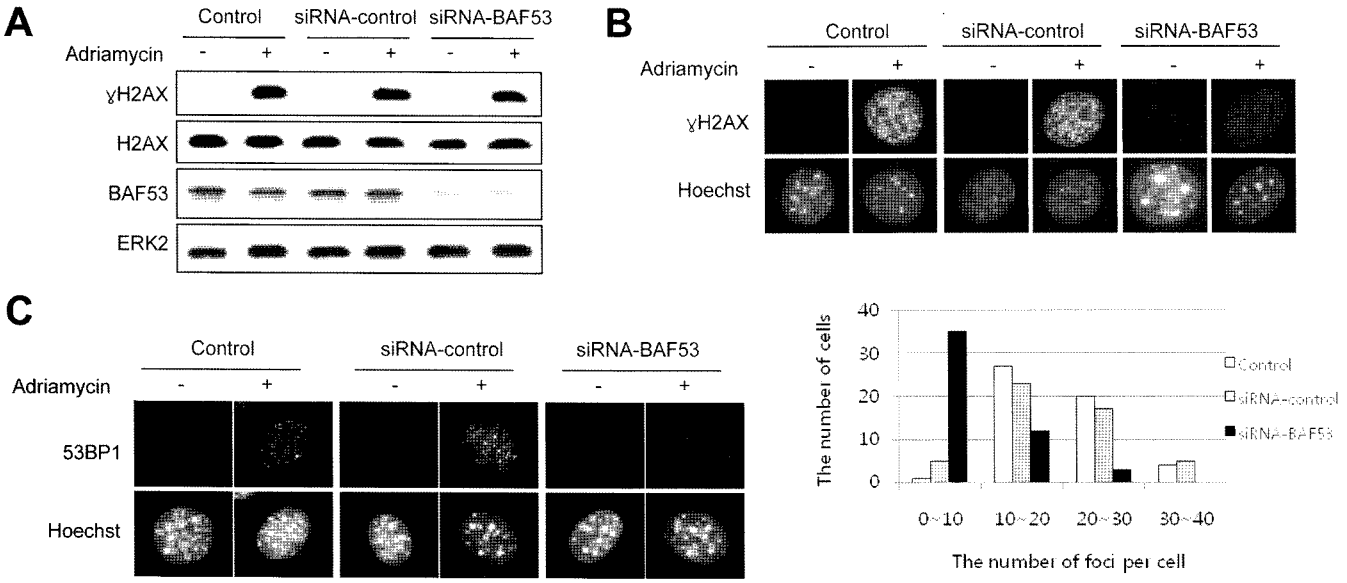

Fig. 1. BAF53 is required for focus formation. (A) NIH3T3 cells were transfected with either siRNA-control or siRNA-BAF53, and then after 48 hr the cells were treated with adriamycin (1.6 μM) for 2 hr. After recovery for 30 min, the extracts were prepared and subjected to immunoblotting analysis. (B) The cells treated as (A) were fixed and stained with anti-γH2AX antibody. Histogram shows the number of γH2AX foci per cell. (C) The cells treated as (A) were fixed and stained with anti-53BP1 antibody.

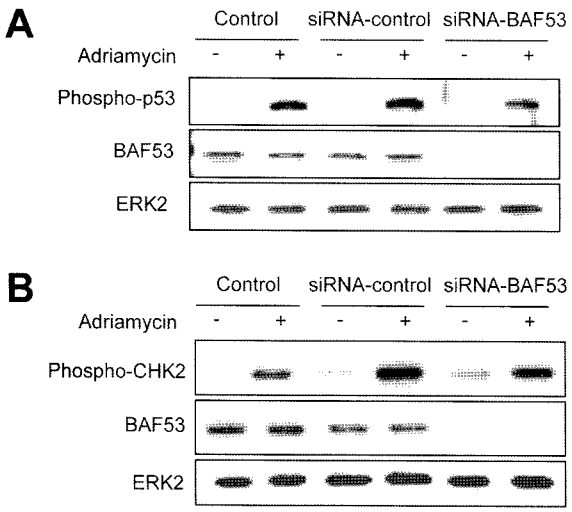

Fig. 2. DSB- activated ATM pathway remains intact in BAF53 knockdown cells. NIH3T3 cells were transfected with either siRNA-control or siRNA-BAF53, and then after 48 hr the cells were treated with adriamycin (1.6 μM) for 2 hr. After recovery for 30 min, the extracts were prepared and subjected to immunoblotting analysis using an antibody against phosphorylated Ser15 of p53 (A) or phosphorylated Thr68 of Chk2 (B).

profile of tail moment decrease was found to be much slower in BAF53 knockdown cells (Fig. 3B). This data indicate that BAF53 knockdown cells exhibited significantly lower repair efficiency than untreated control cells.

DISCUSSION

This study provides evidence that in BAF53 knockdown cells, γ-H2AX dose not form foci even though the normal level of γ-H2AX is produced after DNA damage. Failure in foci formation in BAF53 knockdown cells appears not to be limited to γ-H2AX. Relocalization of 53BP1 to IRIF was impaired in BAF53 knockdown cells. We also found that the formation of IRIF of Nbs1 and RPA was also compromised in BAF53-depleted HeLa cells (data not shown). An important implication of this finding is that the structural organization of chromatin in IRIF requires an assistance of machinery in which our data suggest BAF53 plays a pivotal role.

The structural framework of IRIF formation is obscure. Chromatin may be re-organized after DNA damage at damage sites in order to form IRIF. The chromatin modifying complexes containing BAF53 could play a role in this reorganization process (Sung et al., 2001). Considering the ability of BAF53 and β-actin in the mammalian SWI/SNF complex to mediate the formation of branching networks of actin filaments in vitro (Rando et al., 2002), BAF53-dependent actin filament networking within the γ-H2AX-containing chromatin segments may provide another way to bring the chromatin come closer. Previously it has been demonstrated that the Act3p/Arp4p, the yeast homolog of BAF53, interacts with core histones (Harata et al., 1999; Galarneau et al., 2000). It will be interesting to investigate whether γ-H2AX promotes the formation of BAF53-dependent actin filament networking around damage site.

While reorganization of chromatin after DNA damage can account for formation of foci, another possibility should be considered that BAF53 is required for the formation of chromosomal subdomain and IRIF is formed on the pre-

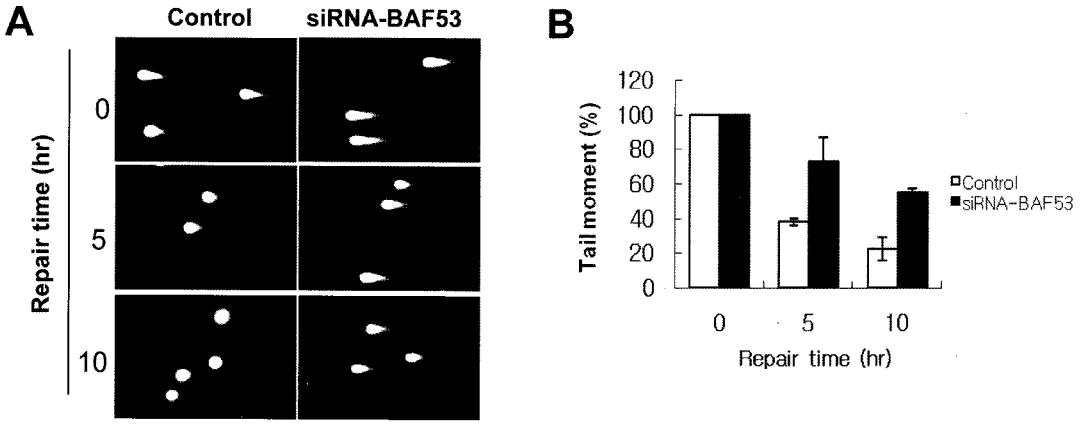

Fig. 3. Inability of BAF53 knockdown cells to achieve full efficiency of DSB repair. NIH3T3 cells were transfected with siRNA-BAF53, and then after 48 hr the cells were treated with adriamycin (1.6 μM) for 2 hr. After recovery for the indicated time, comet assay was performed. (A) The pictures showing representative comet images of the control and the BAF53 knockdown cells. (B) The comet tail moments were measured using CASP (Comet Assay Software Project) software. More than 150 cells were counted for each experimental point (mean±SD).

existing chromosomal subdomain. Several lines of evidence support that interphase chromatin consists of chromosomal subdomain (Müller et al., 2004; Shopland et al., 2006). The estimated size of chromosomal subdomain is several hundreds kilobase to megabase which is comparable to the estimated size of IRIF. Previously, we showed that BAF53 depletion leads to an expansion of chromosome territory which could be an indication of disintegration of chromosomal subdomains (Lee et al., 2007). According to this idea, when BAF53 is unavailable, chromosomal subdomains become disintegrated and IRIF cannot be formed even though phosphorylation of H2AX normally occurs at franking regions of damage site. Further studies are required to determine which of these possibilities is correct.

Previous studies on the physiological role of IRIF rely mostly on monitoring H2AX deficient cells (Celeste et al., 2002; Xie et al., 2004). However, this approach is not able to distinguish the contribution of structural organization as foci from other aspects of the roles of γ-H2AX. In this sense, this study is unique in that BAF53 interferes the formation of IRIF while the formation of γ-H2AX remains intact. Comet assay shows that DNA repair efficiency is largely reduced by BAF53 knockdown. This data clearly indicates that the formation of γ-H2AX itself cannot achieve the optimum condition for DNA repair and the bringing together of γ-H2AX-decorated chromatin segments is a key element that promote DNA repair events.

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