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An autonomous chromatin/DNA-PK mechanism for localized DNA damage signaling in mammalian cells

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ABSTRACT

Rapid phosphorylation of histone variant H2AX proximal to DNA breaks is an initiating event and a hallmark of eukaryotic DNA damage responses. Three mammalian kinases are known to phosphorylate H2AX in response to DNA damage. However, the mechanism(s) for damage-localized phosphorylation remains incompletely understood. The DNA-dependent protein kinase (DNA-PK) is the most abundant H2AX-modifying kinases and uniquely activated by binding DNA termini. Here, we have developed a novel approach to examine enzyme activity and substrate properties by executing biochemical assays on intact cellular structures. We apply this approach to examine the mechanisms of localized protein modification in chromatin within fixed cells. DNA-PK retains substrate specificity and independently generates break-localized γH2AX foci in chromatin. *In situ* DNA-PK activity recapitulates localization and intensity of *in vivo* H2AX phosphorylation and requires no active cellular processes. Nuclease treatments or addition of exogenous DNA resulted in genome-wide H2AX phosphorylation, showing that DNA termini dictated the locality of H2AX phosphorylation *in situ*. DNA-PK also reconstituted focal phosphorylation of structural maintenance of chromatin protein 1, but not activating transcription factor 2. Allosteric regulation of DNA-PK by DNA termini protruding from chromatin constitutes an autonomous mechanism for break-localized protein phosphorylation that generates sub-nuclear foci. We discuss generalized implications of this mechanism in localizing mammalian DNA damage responses.

INTRODUCTION

The DNA within the human genome is highly ordered into structures that compact, organize and impart controls onto the DNA within each nucleus. The nucleosome is the basic unit of chromatin and contains two copies of each core histone, H2A, H2B, H3 and H4 (1). The histone variant H2AX comprises between 2 and 25% of the histone H2A complement in mammalian cells (2). H2AX has an extended carboxyl-terminal 'tail' with one additional serine (Ser139) that is phosphorylated on histones proximal to double-strand breaks (DSBs) within seconds of DNA damage (3). In the minutes to days following DNA damage, many proteins localize to sites of damage to coordinate chromatin remodeling, DNA repair, cell cycle control and ultimately determine cellular fates [reviewed in (4)]. Many damage responsive proteins such as MDC1, 53BP1, BRCA1, ataxia telangiectasia mutated (ATM), ataxia telangiectasia/Rad3 related (ATR), activating transcription factor 2 (ATF2), structural maintenance of chromatin protein 1 (SMC1) and the MRN complex (Mre11/Rad50/Nbs1) localize to phospho-H2AX (γH2AX) sites; however, the precise functional roles, mechanisms of localization, localized phosphorylation and spatiotemporal coordination of these many proteins remain areas of intensive research.

Within minutes of DNA damage, the diffuse nuclear staining of the DNA damage responsive (DDR) protein 53BP1 transitions to a nearly complete focal co-localization with γH2AX (5). Within 24 h following moderate DNA damage, most if not all of the DNA breaks are repaired, and the 53BP1/γH2AX foci are resolved (6,7). However, 53BP1 and γH2AX can remain in sub-nuclear focal staining patterns for days or even weeks following severe damage—presumptively until repair is completed (8). Importantly, 53BP1/γH2AX foci also occur in cells that have not been exogenously damaged. In these cases, critically shortened telomeres are at the center of the 53BP1/γH2AX foci and the

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source of the activated DNA damage responses (9,10). Persistent DDR foci are thought to be sites of unrepaired DSBs that signal cell cycle arrest, regardless of whether they arise from exogenous DNA damage, oncogene activation or critically shortened telomeres (11,12). Consequently, γH2AX and 53BP1 sub-nuclear foci have become widely used as surrogate markers for DNA DSBs, cellular senescence and powerful tools in elucidating cellular and molecular responses to DNA damage and critically shortened telomeres (12–15).

The family of phosphatidylinositol 3′-kinase-like kinases (PIKKs) is constitute serine/threonine protein kinases at the center of mammalian DNA damage responses (16). This family of enzymes includes ATM and ATR kinases, and the DNA-dependent protein kinase (DNA-PK). These kinases are largely responsible for initiating and maintaining DNA damage signals and all phosphorylate H2AX at DNA DSBs (17–19). Curiously, DNA-PK is by far the most abundant PIKK in human cells, at significantly lower levels in rodent cells, and entirely absent from nematodes, flies and yeast (20,21). In contrast, ATM is present at low levels in all eukaryotic cells and a vital sensor of DNA damage with severe cellular and organismal effects when inactivated (22–24). ATM, ATR and DNA-PK are all activated by DNA DSBs in cells, but only DNA-PK binds and is allosterically activated by DNA termini and has direct functions in DNA DSB repair and immunogenesis (25–27). The importance of ATM in DNA-damage sensing, signaling and repair is unequivocally established, and its functions appear to be similar in all eukaryotes from yeast to humans. In contrast, the functions of DNA-PK that are critical to vertebrate cell function but entirely dispensable in most eukaryotic species remain unclear. Regardless, DNA-PK is a key DDR kinase in human cells that phosphorylates H2AX in chromatin near DNA DSBs. Here, we investigate the mechanism of DNA-break localized phosphorylation of H2AX and other proteins by DNA-PK in the context of human chromatin.

MATERIALS AND METHODS

Cells and cell culture conditions

The cells used throughout this study were normal human diploid neonatal foreskin fibroblasts (HCA2) kindly supplied by Dr J. Campisi. Cells were grown in Dulbecco's Modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, and 100 U/ml of penicillin/streptomycin in humidified 10% CO_2 at 37°C. Cells were irradiated using a Pantak® x-ray generator (PANTAK Ltd) operating at 320 kV/10 mA with 0.5-mm copper filtration.

Enzymes, inhibitors and antibodies

Lambda phosphatase, DNAse I, Micrococcal nuclease and *RsaI* were obtained from New England BioLabs; recombinant protein phosphatase 1 isoform-α was from Calbiochem; and recombinant Akt1 was purchased from Invitrogen. DNA-PKcs was purified from human HeLa cells, and Ku70/80 from recombinant baculoviral infected Sf9 insect cells as described previously (28,29). Phosphatase inhibitors Microcystin and NaF were purchased from Sigma-Aldrich. Anti-53BP1 antibodies were procured from Bethyl Laboratories, anti-γH2AX and H2AX were from Upstate (Millipore), anti-phospho GSK3β was from Cell Signaling, anti-phospho SMC1 (Ser957) was from Millipore and anti-ATF2 (Ser490/498) from PhosphoSolutions. Secondary antibodies were donkey anti-mouse Alexa Fluor® 488 and anti-rabbit Alexa Fluor® 594 from Molecular Probes (Invitrogen).

In situ kinase assays

Cells were seeded at a density of 5×10^4 per well in 4-well chamber slides and exposed to X-rays or mock irradiated 24 h following seeding. Cells were fixed with fresh 4% paraformaldehyde for 10 min and permeabilized with phosphate buffered saline (PBS) containing 0.5% Triton X-100 by a 10-min incubation at room temperature. Cells were then incubated overnight at 4°C in blocking buffer (PBS, 1% bovine serum albumin, 4% normal donkey serum, Jackson ImmunoResearch). Fixation conditions were critical for preserving genomic structures, and over-fixation markedly reduced DNA-PK reconstitution activity. All *in situ* assays were carried out at room temperature (~22–25°C) with gentle rocking in '*in situ* reaction buffer' (ISB), 50 mM Hepes-NaOH pH 7.5, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM NaCl, 25 mM KCl and freshly added 1 mM dithiothreitol. All buffers were made with precautions to retain DNAse-free conditions (see Figure 2C). Cellular substrates were dephosphorylated by 1-h incubation at room temperature with a combination of 1600 U/ml of Lambda and 2 U/ml of PP1 phosphatases in ISB. Phosphatases were inhibited and removed by two serial washes with PBS supplemented with 20 mM NaF and 750 nM Microcystin. For DNA-PK assays, cells were pre-incubated for 5 min in ISB with 1.06 pmoles of DNA-PKcs and 2.0 pmoles of Ku70/80. The reactions were started by the addition of 5 mM adenosine triphosphate (ATP) and incubated for 90 min. Cells were washed with PBS followed by standard immunofluorescence staining protocols. For Akt1 assays, cells were incubated with 2.25 pmoles of recombinant human Akt1 kinase in 25 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 0.5 mM Na_3VO_4, 0.01% Triton X-100 and 5 mM ATP, followed by three washes with PBS. Treatments with DNAse I (10 U/ml), Micrococcal nuclease (10 000 Kunitz Units/ml) and *RsaI* (10 000 U/ml) enzymes were done before the DNA-PK assays by incubating the cells with 1 µl of the respective nucleases at room temperature for 30 min in the manufacturers reaction buffer. For oligonucleotide assays, DNA-PK was mixed on ice with the noted amounts of double stranded 67 bp oligonucleotide and assays executed as mentioned earlier.

Immunofluorescence

Cells were probed overnight at 4°C with primary antibodies in blocking buffer and then washed three times, 10 min each, with PBS. Cells were incubated with fluorochrome-conjugated secondary antibodies in blocking buffer for 30 min at room temperature and

washed three times with PBS, with the last wash containing the DNA counterstain 4′, 6-diamidino-2-phenylindole (DAPI) at 0.1 μg/ml. Slides were mounted in Vectashield®. Images were acquired with identical settings with an Olympus BX60 fluorescence microscope with Spotfire 3.2.4 software (Diagnostics Instruments) and processed with Photoshop CS2 (Adobe) and ImageJ (NIH) Software packages.

RESULTS

The redundancy and complexity of DNA damage responses in living cells and the difficulty of isolating intact chromatin structures have limited our ability to understand the detailed mechanisms of break-localized chromatin modification. We have developed a novel approach to investigate mechanisms of protein modification at DNA damage sites on intact chromatin. Biochemical reactions were carried out on fixed chromatin with purified enzymes, and reaction products were visualized using standard immunofluorescence microscopy and antibodies recognizing reaction products (i.e. γH2AX) (Figure 1A). *In situ* substrates were proteins that remained inside of permeabilized and fixed human cells in their native context (i.e. H2AX within chromatin) (Figure 1A).

Biochemical reactions on histones in fixed chromatin (*In Situ* biochemistry)

Covalent cross-links between proximal molecules are effective means to preserve cellular structures; however, such chemical treatments can render substrates and antibody epitopes unrecognizable by their partner proteins owing to steric interference. To test the feasibility of executing biochemistry on histone H2AX and other proteins fixed on chromatin, we incubated cells with a mixture of commercially available serine/threonine phosphatases (λ and rabbit muscle PP1 phosphatases). If active, this treatment should remove phosphate groups from all accessible serine and threonine residues in the cells, including H2AX-Ser139 modified by ATM, ATR and DNA-PK. Phosphatase activity would be evident as a loss of immunofluorescent γH2AX signal in treated cells relative to controls. Because 53BP1 and γH2AX subnuclear localization is practically coincident, cells were simultaneously probed with antibodies recognizing 53BP1 to mark damage sites independent of phosphorylation status (30) (Figure 1A). As expected, control cells showed almost complete co-localization of γH2AX and 53BP1 signal before phosphatase treatment (Figure 1B, merged). In phosphatase-treated cells, we observe a nearly complete loss of γH2AX signal after treatments, indicating robust phosphatase activity on H2AX-S139 *in situ* (Figure 1B, right). Residual γH2AX signal was detectable, but only marginally above background, and was ∼100-fold less intense than controls (Figure 1B, bottom 3D plots). In contrast, and as expected, the phosphorylation-independent recognition of 53BP1 by its antibody was not perturbed by phosphatase treatments (Figure 1B). Given that cellular proteins are cross-linked in fixed cells, and 53BP1 binds histones H4 and/or H3 (30,31), it is not unexpected that these foci are insensitive to phosphatase treatments. These data show that the S139 of γH2AX is accessible to exogenous enzymes and is not sterically blocked by chromatin structures, cross-linking or the presence of proteins such as MDC1, which directly binds γH2AX (6,32).

DNA-PK autonomously generates γH2AX foci on damaged chromatin

Phosphatase activity on γH2AX in fixed chromatin suggested that executing biochemical reactions on these molecules might be possible. Therefore, we next evaluated kinase activity on H2AX by incubating purified kinases with fixed cells that were pretreated with phosphatases. Dephosphorylation of cellular proteins by these treatments rendered H2AX-S139, and presumably all other phosphorylated serines and threonines, suitable kinase substrates. Before kinase reactions, the phosphatases were inactivated and removed by serial washes with buffer containing phosphatase inhibitors. The resulting dephosphorylated fixed cells were used as substrates for all subsequent *in situ* kinase reactions.

ATM, ATR and DNA-PK are active H2AX kinases in mammalian cells (17–19), but only DNA-PK is a highly abundant and readily purified soluble enzyme in human cells, a condition that is mimicked by our biochemical assays (33). We therefore investigated DNA-PK kinase activity on H2AX within fixed chromatin. Purified human DNA-PK was added to dephosphorylated cells using established *in vitro* kinase reaction conditions (28). DNA-PK reaction products were detected using antibodies recognizing γH2AX, and DNA damage sites were visualized by co-staining with 53BP1 antibodies. (Figure 1C). *In situ* DNA-PK activity was evident as striking increase in the γH2AX signal relative to the phosphatase-treated cells, while 53BP1 staining was not changed (Figure 1C). Surprisingly, *in situ* DNA-PK kinase activity was almost entirely restricted to sites marked by 53BP1—the sites in chromatin where H2AX had been previously phosphorylated *in vivo* (Figure 1C, compare merged images). To assess the extent of *in situ* H2AX phosphorylation by DNA-PK relative to *in vivo* kinases, we visualized immunofluorescent signal intensities in the third dimension (Figure 1D). In addition to reconstituting localization, *in situ* DNA-PK reactions reconstituted signal intensities comparable with that seen from *in vivo* kinase activities with the co-localized 53BP1 showing no changes (Figure 1D and E). To ensure that the observed kinase activity was inherent to DNA-PK and not from a chromatin-bound kinase such as ATM, we carried out reactions lacking DNA-PK or the DNA-binding subunit (Ku70/80) (Figure 1F). These incomplete reactions containing Mg-ATP were indistinguishable from phosphatase-treated cells and showed no kinase activity (Figure 1F). These data show that DNA-PK holoenzyme is required and sufficient for the observed *in situ* phosphorylation of H2AX. Remarkably, the addition of only purified active DNA-PK to fixed cells faithfully recapitulated *in vivo* H2AX phosphorylation patterns

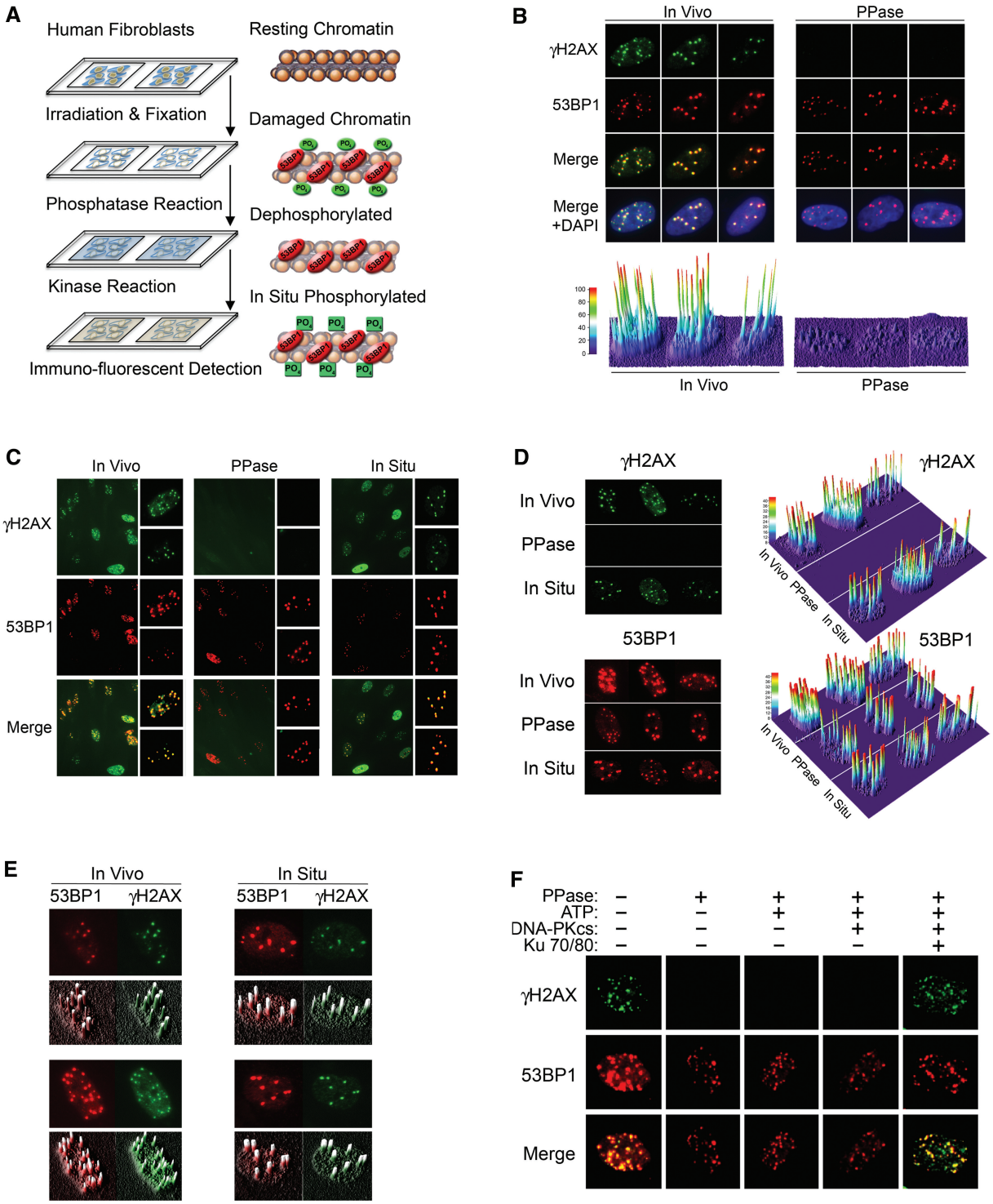

Figure 1. H2AX serine 139 is biochemically reactive in fixed chromatin. (**A**) Schematic representation of the *in situ* biochemical assay on human cells showing cell treatments (left), idealized diagram of DNA damage markers γH2AX and 53BP1 on chromatin through the assay process (right). (**B**) Primary human fibroblasts (HCA2) were fixed 48 h after X-ray treatments (10 Gy) and either untreated (*in vivo*) or treated with phosphatases (PPase) and probed for γH2AX and 53BP1 using immunofluorescent techniques; (bottom) 3D plot of γH2AX intensities aforementioned (scale is in arbitrary units). (**C**) *In situ* DNA-PK kinase assay showing X-ray-exposed controls ('*In Vivo*'), phosphatase (PPase), and DNA-PK kinase reacted ('*In Situ*') cells probed with γH2AX and 53BP1 antibodies as indicated. (**D**) Representative cells from *in situ* DNA-PK assays showing relative intensities of γH2AX and 53BP1 immunofluorescent signals. (**E**) Flat and 3D images of γH2AX and 53BP1 signals from control and *in situ* kinase cells are shown to reveal co-localization. (**F**) *In situ* kinase reactions lacking noted components showing γH2AX and 53BP1 signals.

and intensities independent of any active cellular processes (Figure 1C–F).

Localized H2AX context does not account for focal DNA-PK Activity

The reconstitution of γH2AX foci by exogenously added DNA-PK could be due to a number of factors including a localized exposure or modification of H2AX specifically at DNA-damage sites or local recruitment of DNA-PK by proteins fixed at damage sites. Any cellular process that established a localized H2AX 'status' or context making H2AX a DNA-PK substrate only proximal to breaks would account for the aforementioned observations. To begin testing these possibilities, we investigated whether break-localized exposure of H2AX-tails (Ser139) within damaged chromatin could account for localized DNA-PK activity. Human fibroblasts were irradiated and fixed at various time points up to 5 days after irradiation, then probed with antibodies recognizing unphosphorylated H2AX-tails. In contrast to the focal staining patterns of γH2AX, we observed pan-nuclear staining for H2AX-tails in both irradiated and control cells (Figure 2A). H2AX-tail staining completely lacked the focal pattern seen with γH2AX in damaged cells and did not notably change over time (Figure 2A). Although H2AX-tail staining showed small local variations in intensity, there was no apparent correlation between these slight variations and damage sites. Moreover, in contrast to γH2AX, the average signal intensity of H2AX-tail per nucleus did not change significantly under any condition tested (Figure 2B). These data are most consistent with the H2AX-S139 residue modified by DNA-PK being exposed throughout chromatin and not uniquely exposed at damage sites. Moreover, the exposure of H2AX-tails across the genome does not significantly change within 5 days following radiation-induced DNA damage. Therefore, changes in H2AX-S139 exposure at DNA damage sites does not appear to be responsible for localized DNA-PK kinase activity *in situ*.

Breaks in chromatin autonomously activate H2AX phosphorylation by DNA-PK

The preceding data show that H2AX-tails were exposed throughout chromatin in our assays but do not indicate that exposed H2AX-tails are suitable DNA-PK substrates. It remains possible that other histone modifications or proteins localized to breaks before fixation are responsible for the observed *in situ* localized DNA-PK activity. Alternatively, it is possible that *in situ* reconstitution of γH2AX foci by DNA-PK result from local allosteric activation of DNA-PK via binding to DNA termini (34–36). We reasoned that if local kinase activation by DNA DSBs were responsible for γH2AX foci reconstitution, then introduction of new DSBs into the genome should alter γH2AX *in situ* phosphorylation patterns. Conversely, if cellular processes such as protein modification or localization were responsible for local DNA-PK activity, then introduction of breaks into chromatin in fixed cells should not alter γH2AX patterns in fixed cells. To test these ideas, we incubated dephosphorylated fixed cells with the sequence-independent micrococcal nuclease and DNAse I as well as the restriction enzyme *RsaI*. Nuclease treatments before kinase reactions caused striking increases and complete delocalization of DNA-PK kinase activity on chromatin without altering 53BP1 patterns (Figure 2C). These distinct changes in γH2AX patterns could only be due to physical changes in chromatin, as no biological activity was occurring at the time of kinase reactions. Quantification of *in situ* γH2AX signal intensities in nuclease-treated cells reveals a rough correlation between γH2AX signal and the expected number of DSBs for various nucleases (Figure 2D). The robust kinase activity clearly shows that DNA-PK activity on H2AX is not limited by enzyme concentration, local H2AX context or *in situ* reaction conditions and that other factor(s) localize DNA-PK activity. Pan-nuclear H2AX phosphorylation after nuclease treatments is consistent with allosteric activation of DNA-PK by DNA ends being responsible for the location of γH2AX phosphorylation *in situ*. These data are consistent with an autonomous recognition of DNA breaks in chromatin by DNA-PK that activated the kinase to phosphorylate proximal H2AX molecules.

Activation of DNA-PK by DNA ends regulates γH2AX phosphorylation *in situ*

The aforementioned nuclease treatments significantly stimulated DNA-PK activity resulting in pan-nuclear H2AX phosphorylation. However, in addition to introducing numerous DNA breaks, these treatments may have also altered chromatin structures (Figure 2C and D). To discern whether the DNA termini or the disruption of chromatin were responsible for pan-nuclear phosphorylation of H2AX, DNA-PK kinase reactions were carried out with the addition of double-stranded DNA oligonucleotides. We reasoned that addition of excess soluble DNA termini would activate DNA-PK kinase but not perturb the chromatin structures or H2AX status. Thus, a reconstitution of γH2AX foci in these reactions would indicate that substrate and/or substrate context dictated spatially restricted H2AX phosphorylation by DNA-PK. Conversely, indiscriminant phosphorylation of H2AX would indicate that H2AX-tails are exposed and suitable DNA-PK substrates throughout chromatin; as a corollary, the DNA-PK activity observed in our assays was owing to localized allosteric activation of DNA-PK kinase by DNA ends.

Like the nuclease treatments described earlier in the text, reactions containing a molar excess of oligonucleotide DNA showed extremely high levels of pan-nuclear H2AX phosphorylation (Figure 2E, top left). Exponentially decreasing amounts of oligonucleotides were tested for DNA-PK activation and γH2AX staining patterns. At all concentrations tested, DNA-PK activity was observed throughout the nucleus as pan-nuclear rather than focal staining (Figure 2E). Quantification of the immunofluorescent signal intensity

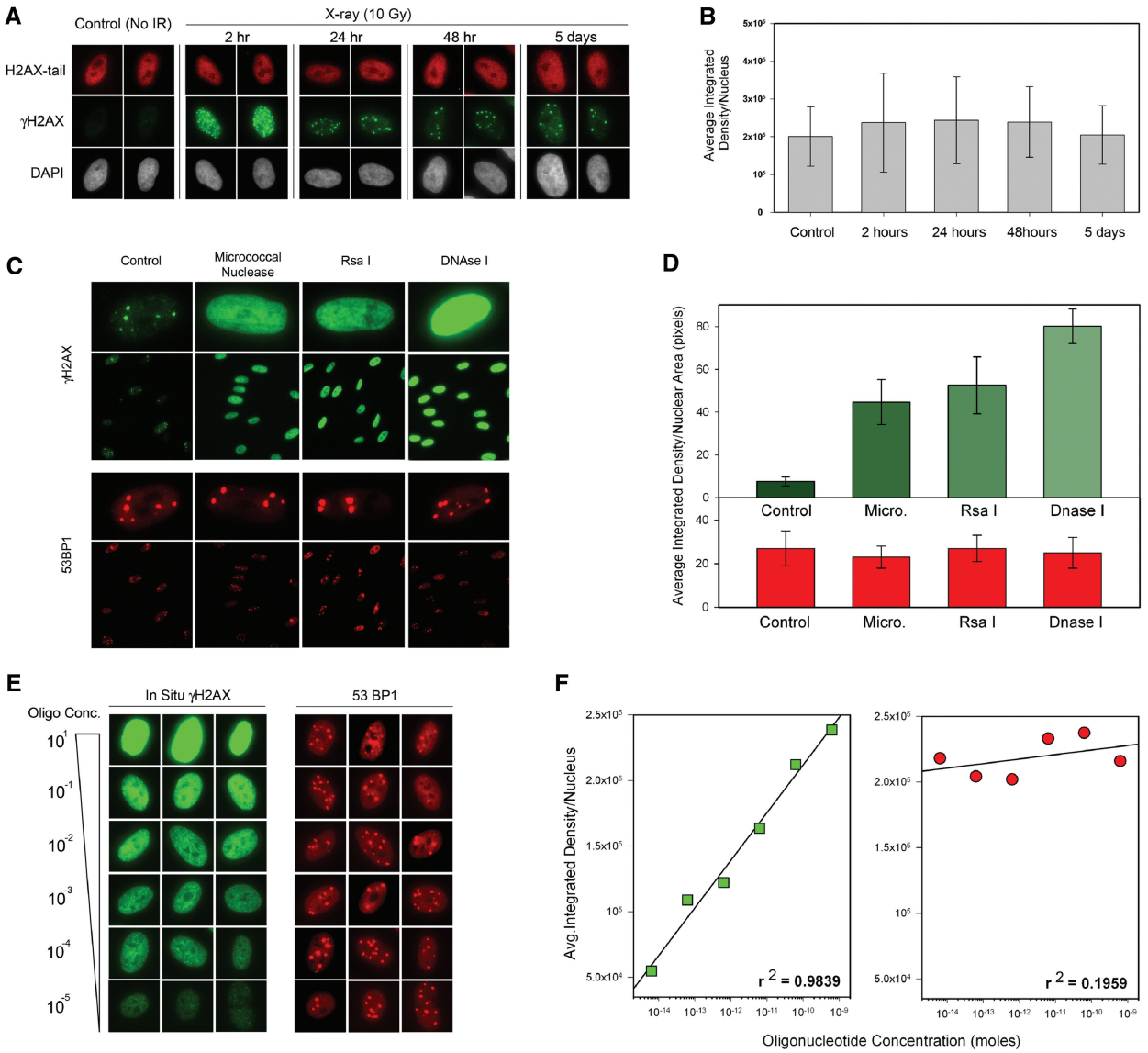

Figure 2. DNA-PK activity is spatially constrained by DNA termini. (**A**) Human fibroblasts were fixed 48 h after mock irradiation (No IR) or at different times following X-ray exposure (10 Gy), then probed with antibodies recognizing either the unphosphorylated H2AX-tail (red) or γH2AX (green). (**B**) Immunofluorescent H2AX-tail signals were measured from random fields of cells, and average integrated density/nucleus quantified and plotted ($n > 25$). (**C**) *In situ* DNA-PK kinase assays were carried out on dephosphorylated cells pre-treated with buffer (control) micrococcal nuclease, restriction enzyme *RsaI* or deoxyribonuclease I (DNAse I) as indicated, cells were probed for 53BP1 (red) and γH2AX (green) as noted. (**D**) γH2AX and 53BP1 immunofluorescent signals were quantified following *in situ* DNA-PK kinase reactions and noted treatments. The average integrated intensity/area (pixels) of nuclei ($n > 20$) from random fields was plotted. (**E**) Dephosphorylated fixed human fibroblasts were reacted with DNA-PK (1.0 pmol) in the presence of exponentially decreasing amounts (10^1 = 625 pmoles) of a 67 bp double-stranded oligonucleotide, then stained for γH2AX (green) and 53BP1 (red). (**F**) γH2AX and 53BP1 immunofluorescent signals from panels in E were quantified as the average integrated density/nuclei and plotted against the oligonucleotide concentration.

per nucleus revealed a linear relationship between the oligonucleotide concentration and γH2AX signal, whereas 53BP1 signal showed no correlation (Figure 2F). These data show that (i) H2AX-tails are exposed and suitable DNA-PK substrates throughout chromatin; (ii) modification of H2AX by DNA-PK is independent of break-localized proteins or any local modifications of H2AX or chromatin environments; and (iii) allosteric activation of DNA-PK by DNA termini is sufficient for H2AX phosphorylation in chromatin, and no active cellular processes are required.

Kinase substrate specificity is retained *in situ*

Enzymatic activities, including kinases, can be unnaturally promiscuous when under *in vitro* biochemical reaction conditions. To test enzyme specificity in our *in situ* reactions, we carried out assays with a second related but distinct enzyme/substrate set. Human Akt1/PKB (hereafter Akt1) is a member of the AGC enzyme family of serine-threonine protein kinases that phosphorylates numerous substrates involved in cell proliferation and survival including the mitochondrial glycogen synthase kinase β [GSK-3β, reviewed in (37)]. Akt1 acts

downstream of DNA-PK in the DNA damage response, and one isoform of Akt1 localizes and interacts with DNA-PK at DNA breaks (38). In addition, DNA-PK is required for damage-induced phosphorylation of Akt1 at a specific residue (39,40). For this context, it is important to note that the GSK-3β substrate is fixed in the mitochondria, whereas H2AX is fixed in the nucleus in our assays, but all cellular substrates are exposed to the added purified kinases.

To assess the general applicability of *in situ* kinase assays and evaluate *in situ* enzyme specificity, reactions with Akt1 and its cognate substrate GSK-3β were carried out on fixed cells. Before *in situ* kinase reactions, phosphates on serines and threonines were removed by treatment with phosphatases as aforementioned. Like γH2AX, staining of *in vivo* phosphorylated GSK-3β was practically abolished by phosphatase treatments, and as expected, dephosphorylated GSK-3β was not phosphorylated by exogenously added DNA-PK (Figure 3A). However, when cells were reacted with purified human Akt1 and probed with antibodies that recognize phospho-GSK-3β, *in situ* Akt1 kinase activity was plainly evident as reconstituted perinuclear and cytoplasmic staining of phospho−GSK-3β (Figure 3B). To test for substrate cross-reactivity, these same reactions were probed with antibodies recognizing γH2AX. These data

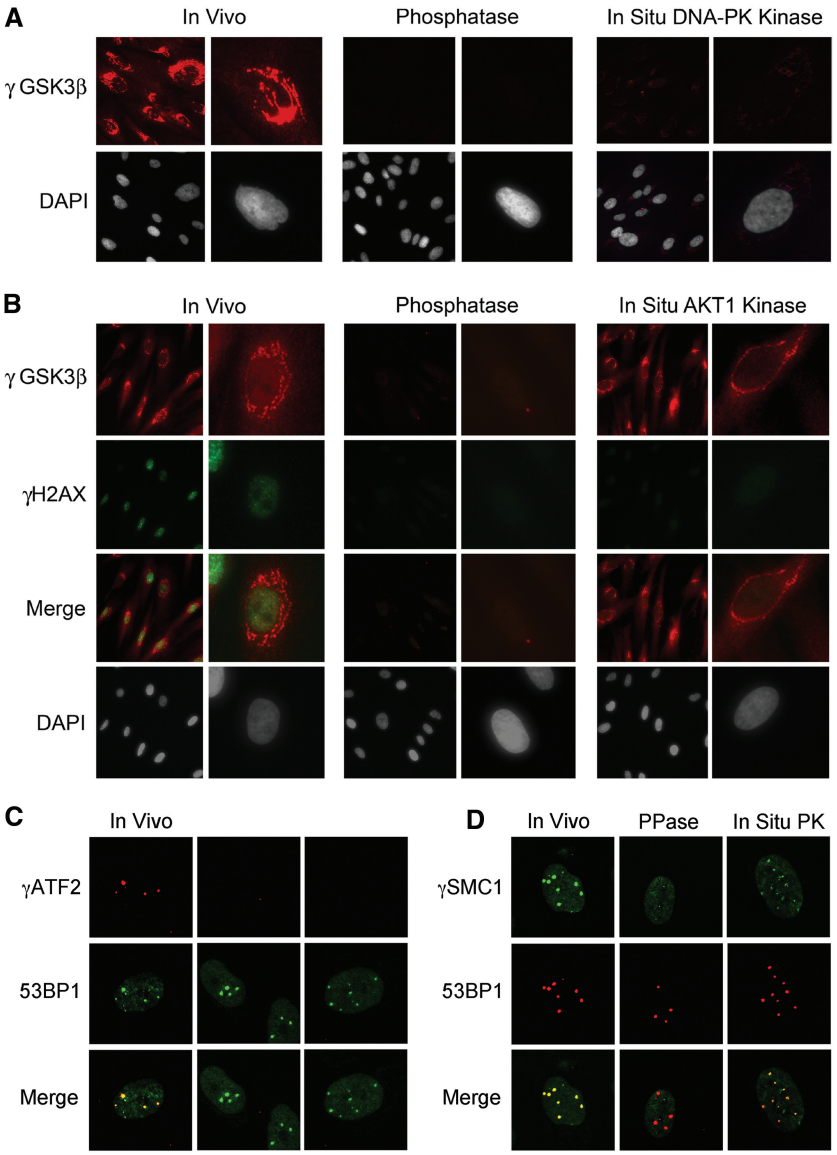

Figure 3. DNA-PK and Akt1 retain substrate specificity *in situ*. Fixed primary human fibroblasts either untreated (left panels), subjected to phosphatase treatment (middle panels) or dephosphorlated and reacted with kinases (right panels). Dephosporylated cells were treated with active human (**A**) DNA-PK or (**B**) recombinant human Akt1 and probed for phospho-GSK3β (γGSK3β red), γH2AX (green) and counterstained for DNA with DAPI (gray scale) as indicated. Standard *in situ* kinase reactions were carried out on fixed and dephosphorylated fibroblast cells and probed for (**C**) phospho-ATF2 (γATF2, red) and 53BP1 (green), or (**D**) phospho-SMC1 (γSMC1, green) and 53BP1 (red). The two-channel overlays are shown to evaluate localization to DNA damage sites in both cases (merge).

show that like DNA-PK, Akt1 retained substrate-specific kinase activity, and no γH2AX signal reconstitution was observed (Figure 3B). Consistent with known specificities of these kinases, the patterns of Akt1 and DNA-PK kinase activities were spatially and constitutionally distinct and showed no overlap *in situ*. These experiments establish that *in situ* biochemical kinase reactions can be carried out with various enzymes/substrates, and that these enzymes retain substrate specificity.

ATF2 is not phosphorylated by DNA-PK at DNA damage sites

Unambiguously determining PIKK on substrate specificity *in vivo* can prove difficult, in part owing to the significant substrate overlap between ATM, ATR and DNA-PK and the fact that similar stimuli activate these kinases (i.e. DNA damage). In addition, there are little data available that describe how these three kinases influence the others' activities. Further confounding these issues are single-site phospho-antibodies and the complexities of isolating a single PIKK kinase activity in living cells. Such experiments typically involve some combination of genetic defects, siRNA depletion and chemical inhibitor treatments to block the other PIKK kinases (41–43). Here, we exploit the novel ability of our *in situ* assays to directly probe an individual PIKK activity (DNA-PK) on substrates in the context of chromatin. These assays directly address the biochemical capabilities of DNA-PK on three dimensionally organized chromatin substrates and the associated DNA repair proteins in the context of DNA damage foci, an approach not possible before this work.

The ATF2 is a dual function transcriptional activator and DNA damage response protein (44). Like the other proteins discussed here, ATF2 localizes to sites of DNA damage and is phosphorylated at damage foci within the nucleus (45). ATM is required for ATF2 phosphorylation *in vivo*, and these modifications are important for function in DNA damage responses, cell cycle arrest and radiation resistance (45–47). To assess whether DNA-PK recognized and phosphorylated ATF2 at sites of damage, we carried out *in situ* DNA-PK assays and probed for phospho-ATF2 (Figure 3C). We find that like GSK-3β, DNA-PK does not phosphorylate ATF2 at sites of DNA damage, in sharp contrast to the robust DNA-PK activity on its native γH2AX substrate. These data show that substrate proximity to DNA breaks and active DNA-PK are not sufficient for phosphorylation at serines and threonines and that DNA-PK retains substrate specificity.

SMC1 is rcognized and phosphorylated by DNA-PK on chromatin

To further investigate DNA-PK substrate specificity with these assays, we chose to evaluate DNA-PK activity on SMC1. SMC1 is another protein that is phosphorylated in response to DNA damage proximal to DNA breaks in cells (48,49). ATM and ATR phosphorylate SMC1 on serine 966 and 957 in response to DNA damage, and these modifications are reported to be largely independent of DNA-PK (41). Furthermore, phosphorylation of SMC1 by ATM is the basis for assays that detect ATM deficiencies in humans (50,51). However, another study reports that viral infections prompted SMC1 phosphorylation that is DNA-PK-dependent and largely ATM/ATR independent (42). In addition, other studies indicate that SMC1 is linked to DNA-PK physically via a novel accessory protein that regulates both ATM and DNA-PK kinase activities (52,53). Despite these conflicting results, phosphorylation of SMC1 at S957 and S966 in response to DNA damage is generally ascribed to ATM and ATR kinase activity, and DNA-PK is usually not considered a relevant SMC1 kinase [reviewed in (54)].

To directly address these seemingly contradictory issues, we carried out foci reconstitution assays with DNA-PK and probed for SMC1 phosphorylation. In contrast to the results with ATF2 and GSK-3β, SMC1 was recognized and phosphorylated by DNA-PK at sites of damage on human chromatin (Figure 3D). Although *in situ* assays cannot directly address the full complexity of *in vivo* substrate/kinase interactions, these assays are useful indicators of biochemical capability, specificity and selectivity of kinase activity on contextually relevant substrates. Taken together, these results indicate that DNA-PK retains substrate selectivity *in situ*, and that SMC1 but not ATF2 is a DNA-PK substrate at sites of DNA damage in human chromatin.

DNA termini at γH2AX foci are altered by cellular processes over time

We next applied our *in situ* biochemical approach to investigate the nature of DNA termini at DNA damage foci at various times after DNA damage. Although the temporal phenomenon of fewer and larger γH2AX/53BP1 foci being observed over time is well-established (8,55,56), the existence of DNA DSBs at persistent γH2AX foci has been inferred but not demonstrated (57). Because *in situ* DNA-PK activity requires DNA termini, we used DNA-PK activity to probe fixed chromatin for DNA termini at persistent γH2AX foci. As described earlier in the text, DNA-PK faithfully reconstituted γH2AX foci in fixed cells at early times after irradiation (Figures 1 and 4A). In contrast, however, visual inspection reveals that both the intensity and frequency γH2AX foci reconstitution are markedly reduced or absent in cells after ~72 h following damage (Figure 4A and B). Quantification of γH2AX and 53BP1 foci size over time shows the clear increase in foci size over time as previously reported (8,55,56) (Figure 4C). In contrast to 53BP1 and the *in vivo* phosphorylated H2AX, the size of reconstituted γH2AX foci that are detectable at late time points was markedly smaller (Figure 4A–C). These data suggest that the temporal changes in γH2AX foci size and number are accompanied by a change in the nature and/or context of DNA termini within these foci. Notably, the loss or diminishment of DNA-PK activity at late foci suggests that *bona fide* DNA termini may not be present within persisting DNA damage foci. Alternatively, if breaks are present, the

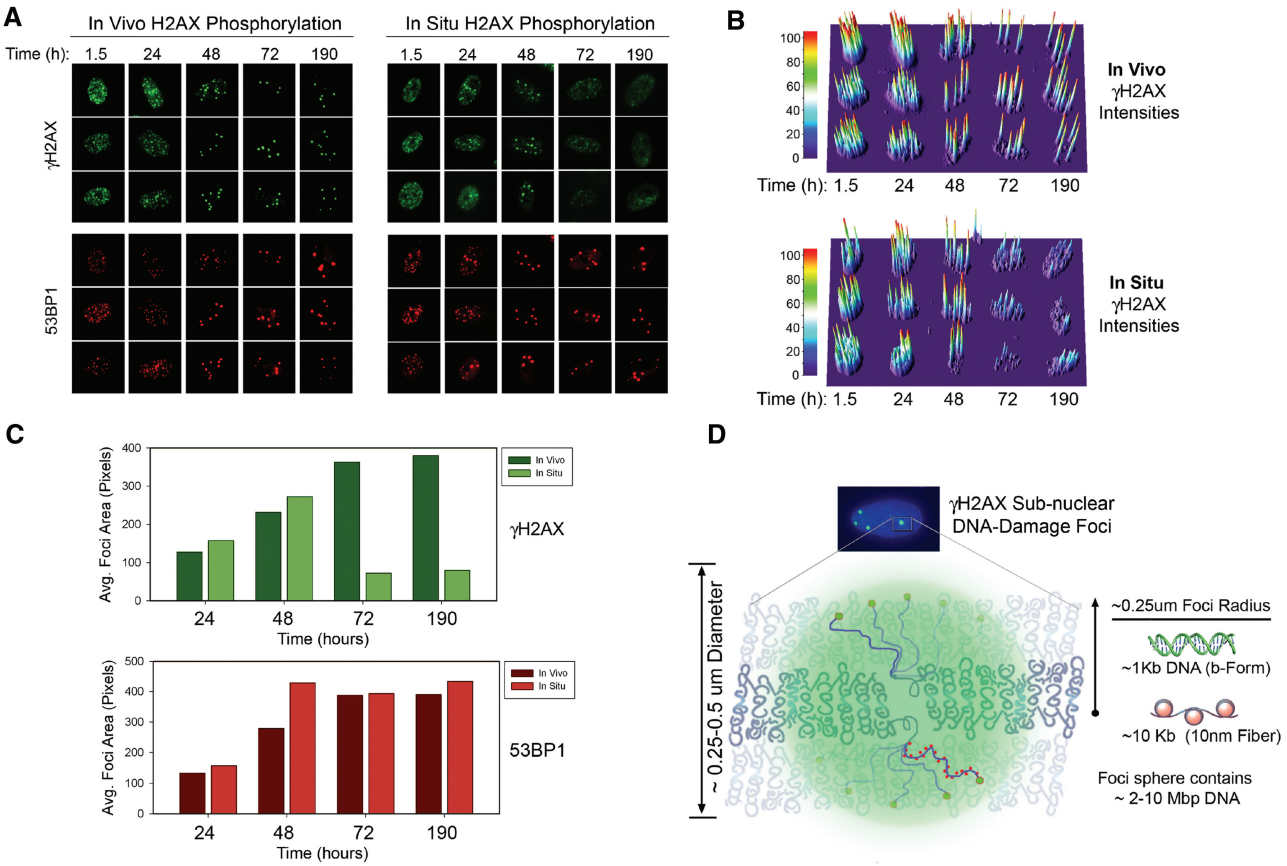

Figure 4. Persistent DNA damage foci fail to activate the DNA-PK. (**A**) HCA2 cells were fixed at noted time after X-irradiation (10 Gy) and subjected to *in situ* DNA-PK kinase assays with reaction products visualized with γH2AX (green) or 53BP1 (red) immunofluorescent staining. (**B**) Images captured under identical conditions of experiments in panel 'A' were plotted in three dimensions to reveal the relative *in vivo* and *in situ* γH2AX signal intensities. (**C**) The area of all of the discrete foci in panel 'A' was quantified, and the average foci area in pixels was plotted as a function of time. (**D**) Mechanistic model for localized H2AX phosphorylation by DNA-PK on chromatin at DNA DSBs. A single locally unwound DNA break is illustrated with translational motion depicted as fading lines within the sphere of DNA-PK kinase activity (green shading). DNA-PK is shown bound to the mobile DNA termini (green circle). Other damage-responsive molecules are represented binding epitopes exposed on chromatin unwinding (red circles, lower strand only). Dimensions of B-form DNA and 10-nm fiber required to span foci radius of 0.25 μM and approximate DNA content of foci volume are indicated (DNA content calculation assumes even DNA distribution within an average human nucleus).

termini are in a form that largely fails to activate DNA-PK kinase activity, possibly owing to changes in the nature of the terminus and/or chromatin/heterochromatin states as recently reported for slowly repaired DNA breaks (58).

DISCUSSION

The mechanism(s) for the spatial restriction of histone H2AX phosphorylation and the existence of *bona fide* DSBs at persistent γH2AX foci have remained largely unresolved questions (57). The relative roles of the three PIKKs in modifying substrates *in vivo* is not directly addressed here; however, *in situ* assays are a novel means to directly probe the mechanisms and biochemical capabilities of PIKKs in a cellular context. Here, we have applied this *in situ* biochemical approach to investigate modification of H2AX and other substrates by DNA-PK in chromatin, free of intracellular redundancy for PIKK kinases (15). Surprisingly, DNA-PK autonomously phosphorylated H2AX and SMC1 on fixed chromatin specifically reconstituting foci at sites that were phosphorylated *in vivo* (i.e. sites marked by 53BP1). Introduction of DNA breaks within chromatin or inclusion of exogenous DNA resulted in genome-wide H2AX modification (Figure 2). These *in situ* results indicate that H2AX tails were exposed and suitable DNA-PK substrates throughout chromatin and closely mimic *in vivo* observations with hyper-activated ATR kinase (59). Phosphorylation of H2AX by DNA-PK was neither dependent on the nature of H2AX at break sites nor did it require the localization of other proteins or any active cellular process. In addition, proximity of PIKK substrates to active DNA-PK was not sufficient for phosphorylation, as SMC1 but not ATF2 was modified by DNA-PK at damage sites in chromatin. Collectively, these data reveal an autonomous mechanism by which structural aspects of damaged chromatin and allosteric regulation of DNA-PK can account for localized modification of proteins that are visualized as sub-nuclear foci.

In considering such autonomous activity, we suggest a mechanistic model where DNA DSBs uniquely permit

rotational and translational movements in chromatin DNA that cause an immediate local unwinding of chromatin structure at DNA DSBs (Figure 4D). The nature and extent of this unwinding would be inherent to higher order chromatin organization and structures. The liberated termini would then freely diffuse in a volume dictated by the length of the 'unwound' region and its tethering to the larger intact chromatin structure. DNA-PK activity would be limited to H2AX and other proteins/substrates within the volume that the tethered DNA ends could reach. The movement of these DNA termini is by definition proximal to the break and the resulting spatial restriction of DNA-PK activity would result in break-localized protein modification visualized as sub-nuclear foci (Figure 4D).

Only DNA-PK is directly activated by DNA termini (25,60), but break-localized γH2AX foci occur in organisms wholly lacking DNA-PK and in mammalian cells deficient for DNA-PK (18,61). Therefore, mechanisms independent of DNA-PK activity and DNA-termini must be functional in localized H2AX phosphorylation in invertebrates and mammals. Considering these facts, we envision a more generalized DNA-damage signaling model. A break in the DNA 'thread' releases torsional and translational constraints resulting in a limited unraveling of chromatin. This conformational change in chromatin exposes sequestered allosteric activators and interfaces, including DNA termini. Break-responsive proteins like DNA-PK immediately recognize, bind and/or become activated by association with cognate-binding interfaces and again are spatially restrained by the limited motion of the tethered DNA strands and the extent of unwinding (Figure 4D). Alternatively, ATM and ATR may function by entirely different mechanisms, and this model may only be relevant to DNA-PK. Regardless, such a mechanism based on chromatin structure would facilitate many parallel, simultaneous and autonomous responses; each queuing on different molecular interfaces, but all immediately, locally and independently respond to DNA breaks.

Much of what is known about mammalian DNA damage responses and relevant proteins is entirely consistent with this proposed mechanism. First, damage foci are formed in cells lacking DNA-PK, ATM, Mre11/Rad50/NBS1 or H2AX itself (6,62–64), indicating independent parallel responses rather than a linear biochemical pathway. Microscopic observations reveal 'unwinding' as decondensed 10-nm fibers at repair foci, which may be limited by periodic chromatin anchoring points occurring 5–100 kb apart (65–69). Consistent with established dimensions, ~10 kb of 10-nm fiber would facilitate a sphere of kinase activity corresponding to a typical γH2AX foci size of 0.25–0.5 um^2, with a corresponding volume containing ~1–2 Mbp of DNA (55). Furthermore, modification of H2AX based on spatial proximity rather than linear expansion would account for the discontinuities observed in γH2AX along the megabases proximal to DNA breaks (70,71). Considering the steric limitations of a DNA terminus, the thousands of individual proteins visualized at 'damage-induced foci' are more likely distributed along regions of decondensed chromatin and associated with interfaces other than the terminus itself (Figure 4D). In fact, binding to chromatin interfaces other than the DNA termini are established for both the 53BP1 and MDC1 proteins that bind methylated histone H4 and γH2AX, respectively (30,72). Likewise, the activation of ATM in the absence of DNA termini/breaks and recruitment of proteins to damage sites via protein–protein interactions are entirely consistent with our proposed mechanism (73,74).

Apart from the generalized implications of this mechanism for other kinases, our data show that DNA-PK can autonomously recognize DNA termini in chromatin and become activated to phosphorylate H2AX. The reconstitution of γH2AX foci in fixed cells indicates that break-localized DNA-PK activity is independent of active cellular processes and inherent to DNA-PK and chromatin. We propose that these observations reflect a novel mechanism for break-localized chromatin modification based entirely on the biophysical properties of DNA-PK and chromatin (Figure 4D). Given the autonomy of this activity, the abundance of DNA-PK, and its affinity for DNA termini ($K_d \approx 10^{-10}$/M) (25), it is likely that allosteric activation of DNA-PK at break sites accounts for localized DNA-PK-mediated phosphorylation of proteins in living cells. An additional implication of this model is that DNA-PK bound to a single terminus could actively phosphorylate any number of proximal substrates in cis to initiate DNA-damage signaling, whereas the two termini would have to meet in space to facilitate DNA-PK auto-phosphorylation in trans. Such auto-phosphorylation of DNA-PK facilitates Artemis nuclease activity and brings about a conformational change in DNA-PK important for continued repair by the XRCC4-DNA Ligase IV complex (27,75,76). As a corollary to this line of reasoning, chromatin conformations that limit the meeting of the termini in space would disfavor repair. Although speculative, the residence of persisting DNA breaks in regions of heterochromatin (77) and the increased size of γH2AX foci at later time points (8,55,56) could be linked phenomena that can be rationalized by our proposed mechanistic model.

Unlike the universal importance of ATM in recognition and signaling of eukaryotic DNA damage, DNA-PK functions are unique to vertebrates. Exactly which aspects of vertebrate DNA repair processes have selected for the addition of DNA-PK remains unclear. These functions may simply be added layers of genome surveillance and repair to safeguard mammalian genomes for decades as opposed to days or months. Alternatively, the added DNA-PK functions may go hand-in-hand with the evolution of more complex chromatin organization that together ensures genetic integrity for decades. In any case, the formation of damage-induced sub-nuclear foci in living cells is independent of ATM, DNA-PK, H2AX or any known single gene product. This parallel redundancy in mammalian DNA repair processes has posed challenges to understanding specific mechanisms of DNA damage responses using traditional methods. Here, we have developed *in situ* biochemical assays to isolate the mechanism of DNA-PK kinase activity on chromatin substrates. We find that break-localized modification of H2AX in chromatin by DNA-PK relies on

allosteric activation by DNA-termini but is otherwise biochemically autonomous. The encoding of DNA damage signaling into higher order chromatin structure could be an elegant means to facilitate immediacy, redundancy, locality and autonomy for the many complex mammalian DNA repair processes. The ability to consider structural and spatial aspects of biochemistry in a cellular context may allow for novel insights into many cellular processes. Further *in situ* biochemical investigations may prove powerful additions to genetic and molecular techniques in deciphering the spatial and structural components of the complex cellular mechanisms of DNA repair and other cellular processes.

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