The ataxia-telangiectasia mutated (ATM) protein is a key signaling molecule that modulates the DNA damage response. However, the exact mechanism by which ATM regulates DNA damage repair has not yet been elucidated. Here, we report that ATM regulates DNA damage repair by phosphorylating lysine-specific demethylase 2A (KDM2A), a histone demethylase that acts at sites of H3K36 dimethylation. ATM interacts with KDM2A, and their interaction significantly increases in response to DNA double-stranded, but not single-stranded, breaks. ATM specifically phosphorylates KDM2A at threonine 632 (T632) following DNA damage, as demonstrated by a mutagenesis assay and mass spectrometric analysis. Although KDM2A phosphorylation does not alter its own demethylase activity, T632 phosphorylation of KDM2A largely abrogates its chromatin-binding capacity, and H3K36 dimethylation near DNA damage sites is significantly increased. Consequently, enriched H3K36 dimethylation serves as a platform to recruit the MRE11 complex to DNA damage sites by directly interacting with the BRCT2 domain of NBS1, which results in efficient DNA damage repair and enhanced cell survival. Collectively, our study reveals a novel mechanism for ATM in connecting histone modifications with the DNA damage response.

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INTRODUCTION

Ataxia-telangiectasia mutated (ATM) is a Ser/Thr protein kinase that has a crucial role in the cellular response to double-stranded breaks (DSBs). Following DSB induction, a large heterogeneous group of proteins forms several complexes at DSB sites. Protein kinase activation, including ATM, ATR (ATM- and Rad3-related) or DNA-PK (DNA-dependent serine/threonine protein kinase) then occurs to regulate cellular responses to DNA damage. ATM has been reported to primarily influence the DNA damage response by phosphorylating and/or recruiting several effector proteins, such as S3BP1 (p53 binding-protein 1), BRCA1 (breast cancer gene 1) or Abi tyrosine kinase, at DNA damage sites to promote efficient DNA repair or induce other cellular processes. In addition, ATM can modulate histone modifications to regulate the DNA damage response. For example, upon DSB induction, ATM rapidly phosphorylates the histone H2A variant H2AX to form γH2AX foci in the megabase chromatin domains flanking the sites of DNA damage, which is considered to be a classical marker for DSB sites. It has also been reported that histone H1 is markedly, but transiently, dephosphorylated in an ATM-dependent manner following low doses of ionizing radiation. In addition, histone acetylation is linked to the ATM-modulated DNA damage response, as ATM regulates chromatin organization by associating with the histone deacetylase HDAC1 and, subsequently, enhancing its deacetylase activity to regulate chromosomal integrity. Moreover, ATM was recently shown to modulate histone methylation. For example, ATM phosphorylates H4K20 methyltransferase multiple myeloma SET domain in response to ionizing radiation, which is important for H4K20 methylation, S3BP1 recruitment and the DNA damage response at damage sites. It is becoming increasingly clear that H3K36 methylation is an important modification for DNA damage repair. For example, H3K36me3 is required for the recruitment of the mismatch recognition protein hMutSα (hMSH2-hMSH6) onto chromatin and for the regulation of DNA mismatch repair. In addition, the SETD2-dependent histone H3K36me3 is required for homologous recombination repair and genome stability. However, the primary immediate methylation change after DSB is H3K36me2, as H3K36me2 increases at the DNA damage sites soon after γ-radiation, defining a histone methylation event that enhances DNA damage repair.

H3K36me2 is mainly catalyzed by nuclear receptor-binding SET domain-containing protein 1 (NSD1), Metnase, and is primarily demethylated by lysine-specific demethylase 2A (KDM2A, also known as FBXL11 or JHDM1A), which contains the well-known chromatin-binding domains PHD-type zinc-finger, CxxC-type zinc-finger and an enzymatic jmjdomain. The demethylation activity of KDM2A is associated with the transcriptional repression of ribosomal RNA, satellite repeats or DUSP3. In addition, KDM2A demethylates a non-histone protein, the p65 subunit of nuclear factor-xB at K218 and K221, to inhibit nuclear factor-xB activity. Furthermore, KDM2A overexpression reduces the accumulation of H3K36me2 at the DSB site and therefore inhibits DSB repair. Although the importance of H3K36 dimethylation in the DNA damage response is recognized, how the H3K36...
modification enzymes, such as KDM2A, are themselves regulated is completely unknown.

Based on the critical role of both ATM and H3K36 methylation in modulating the DNA damage response, we hypothesized that ATM may interact with H3K36 methylation to coordinate this response. In this study, we present evidence that ATM interacts with and phosphorylates KDM2A at threonine 632 (T632) in response to DSBs, which impairs the chromatin-binding capacity of KDM2A. Consequently, H3K36 dimethylation at the DNA damage sites is increased and facilitates the recruitment of the MRE11 complex near the DNA damage sites to aid DNA damage repair through a direct interaction with the BRCT2 domain of NBS1. Overall, this study is the first to demonstrate that KDM2A is a novel ATM substrate and KDM2A phosphorylation is critical to the DNA damage response.

RESULTS

ATM and KDM2A interact in vivo

As both ATM and H3K36 methylation are related to DSB, it was expected that ATM regulates H3K36-related histone methyltransferases or demethylases. To investigate whether ATM and H3K36 methyltransferases or demethylases interacted, a co-immunoprecipitation (co-IP) assay was performed in HEK293T (transformed human embryonic kidney) cells with overexpressed Flag-tagged ATM (Flag-ATM). As shown in Figure 1a, Flag-ATM did not interact with the two H3K36 demethylases JHDM1B and JMJD2B or the H3K36 methyltransferase NSD1. Interestingly, ATM did clearly interact with endogenous KDM2A, which is an H3K36me1/2 demethylase. To confirm this result, HEK293T cells were co-transfected with yellow fluorescent protein (YFP)-tagged ATM (YFP-ATM) and Flag-KDM2A, and the extracts were subjected to a co-IP assay. As shown in Figure 1b, YFP-ATM and Flag-KDM2A interact and phosphorylates KDM2A at threonine 632 (T632) in response to DSBs, which impairs the chromatin-binding capacity of KDM2A. Consequently, H3K36 dimethylation at the DNA damage sites is increased and facilitates the recruitment of the MRE11 complex near the DNA damage sites to aid DNA damage repair through a direct interaction with the BRCT2 domain of NBS1. Overall, this study is the first to demonstrate that KDM2A is a novel ATM substrate and KDM2A phosphorylation is critical to the DNA damage response.

Figure 1. Interaction between ATM and KDM2A. (a) Whole-cell lysates of HEK293T cells transfected with Flag-ATM were precipitated with anti-Flag antibody, and probed with the indicated antibodies. (b) HEK293T cells were transfected with YFP-ATM and Flag-KDM2A. At 48 h after transfection, the whole-cell lysates were precipitated with anti-GFP antibody, and probed with anti-GFP or anti-Flag antibody. (c) Whole-cell lysates of HEK293 cells were precipitated with anti-ATM or anti-KDM2A antibody, and the interactive components were then analyzed by immunoblotting. (d) HEK293 cells were treated with 5-aza-CdR at 5 μM for 48 h, adriamycin at 1 μM for 12 h, etoposide at 20 μM for 4 h, ultraviolet radiation (UV) for 5 min. The cell extracts were then precipitated with anti-ATM antibody and probed with anti-KDM2A antibody. The relative intensity of the interaction between ATM and KDM2A was quantified and is shown as a histogram. The ‘NC’ indicates negative control without any treatment. Student’s t-test (two-tailed): 5-aza-CdR versus NC, P < 0.01; adriamycin versus NC, P < 0.05; etoposide versus NC, P < 0.01; UV versus NC, P > 0.05. (e) HEK293 cells were treated with etoposide at concentration of 5 or 20 μM for 4 h. A co-IP assay was performed as described in d. Data are shown as means ± s.d.; n = 3. Student’s t-test (two-tailed): 5 μM versus 0 μM, P < 0.05; 20 μM versus 0 μM, P < 0.01.
clearly interacted. In addition, endogenous ATM and KDM2A interacted with each other as well, as demonstrated by co-IP in HEK293 and HCT116 cells (Figure 1c, Supplementary Figure 1a).

To investigate whether the interaction between ATM and KDM2A has a biological function within the cell, HEK293 cells were treated with different DNA damage reagents, such as the DSB inducers, adriamycin,22 etoposide23 or 5-aza-2′-deoxycytidine (5-aza-CdR),24 and the DNA single-stranded break inducer ultraviolet radiation.25 As shown in Figure 1d, the interaction between endogenous ATM and KDM2A markedly increased after treatment with adriamycin, etoposide or 5-aza-CdR, but not following ultraviolet radiation. This finding suggests that the interaction between ATM and KDM2A may be specifically associated with DSBs. In addition, the interaction between ATM and KDM2A was dose dependent when treated with etoposide (Figure 1e). However, neither the interaction between KDM2A and ATR nor the interaction between KDM2A and PARP1 was detected (Supplementary Figures 2a and c), which further suggests that the ATM–KDM2A interaction is a specific response to DSBs.

KDM2A is a novel ATM substrate that responds to DNA DSBs. As ATM interacted with KDM2A, it is hypothesized that KDM2A is a novel ATM substrate. In general, ATM kinase has a high substrate specificity, as it recognizes the Ser-Gln (SQ) or Thr-Gln (TQ) motif and phosphorylates Ser/Thr in these motifs.26,27 To investigate whether KDM2A is phosphorylated by ATM, we used a specific antibody recognizing the phosphorylated Ser/Thr ATM/ATR substrate antibody28 that recognizes the particular phosphorylation of S/TQ motifs (p-S/TQ) in ATM/ATR substrates to detect S/TQ phosphorylation in KDM2A. As shown in Figures 2a and b, KDM2A phosphorylation significantly increased after etoposide treatment. This effect was abolished by λ phosphatase (λ-PPase) treatment, indicating that KDM2A is phosphorylated at its serine or threonine in the S/TQ motifs in response to DNA damage. Consistent with the previous result (Supplementary Figure 2c), we did not detect any PARylation of KDM2A following etoposide treatment (Supplementary Figure 2d). To further identify which of the four S/TQ motifs (S11Q, T115Q, S180Q and T632Q) was phosphorylated within KDM2A, in vitro phosphorylation assays were performed with endogenous activated (S198I-phosphorylated) ATM29,30 and two glutathione S-transferase (GST)-fused truncation mutants of KDM2A (1-560aa/560-680aa). The catalyzed KDM2A fragments were then subjected to mass spectrometry analysis, which indicated that T632, but not S11, T115 or S180, was phosphorylated by ATM (Supplementary Figure 3). In addition, cells expressing mutated KDM2A (T632A) (T632 to alanine) exhibited reduced levels of S/TQ phosphorylation compared with cells expressing wild-type KDM2A (Figure 2c). Furthermore, an in vitro phosphorylation assay using 32P-labeled ATP revealed that the KDM2A (T632A) mutant blocked ortho-32P incorporation into KDM2A (Figure 2d). These findings suggest that T632 is a bona fide predominant site in KDM2A for DSB-inducible phosphorylation.

To analyze the role of T632-phosphorylated KDM2A in responding to DNA damage, a rabbit polyclonal antibody was responding to DNA damage (Figure 2h). In addition, no pT632 was detected in the ATM-mutated A-T cells,12 but pT632 level was induced in A-T cells overexpressing ATM in response to DNA damage (Figure 2i). These data further support the idea that ATM is the kinase that phosphorylates KDM2A at T632 after DSBs. KDM2A phosphorylation at Thr632 abrogates its chromatin-binding capacity

Subsequently, to detect whether T632 phosphorylation of KDM2A affects its demethylase activity, an in vitro demethylation assay was performed. It is unlikely that KDM2A phosphorylation at T632 is related to its demethylase activity because wild-type KDM2A, the unphosphorylated KDM2A (T632A) (Supplementary Figure 4), and the phosphomimetic KDM2A (T632E) (T632 to glutamate) mutant had similar in vitro demethylation efficiencies (Supplementary Figure 5). As the PHD zinc-finger domains of nuclear proteins are able to bind histone lysines33 and because T632 is located within KDM2A’s PHD domain (Figure 3a), we examined whether T632 phosphorylation affected KDM2A’s ability to bind chromatin. Interestingly, KDM2A levels were markedly decreased in the chromatin fraction and, accordingly, an increase in KDM2A appeared in the soluble fraction following treatment in HEK293 (Figure 3b) or HCT116 cells (Supplementary Figure 1b). However, KDM2A pT632 was not detected in the chromatin fraction and was only enriched in the soluble fraction of HEK293 (Figure 3b) or HCT116 cells (Supplementary Figure 1b). In addition, HEK293 cells were separately transfected with different KDM2A constructs, including wild-type KDM2A and T632A or T632E mutants (Supplementary Figure 6). The enrichment of exogenous KDM2A was detected in the chromatin fraction. As shown in Figure 3c, the strongest binding capacity of KDM2A to chromatin was detected in the cell extracts with the overexpressed T632A mutant, whereas a very weak chromatin-binding capacity was observed in cell extracts with the overexpressed phospho-mimetic T632E mutant. Moreover, the interaction of the T632A mutant and histone H3 increased, and the T632E mutant failed to interact with H3, as assayed by co-IP (Figure 3d). To further investigate the influence of KDM2A phosphorylation on localization of H3K36me2 and KDM2A, an immunofluorescence assay was performed. As shown in Figures 3e and f, most of the HEK293 cells that overexpressed the T632E mutant exhibited obvious H3K36me2 staining, but H3K36me2 did not fully colocalize with the overexpressed T632E mutant. However, H3K36me2 staining was not detected in most of the cells overexpressing wild-type KDM2A or the T632A mutant (Figures 3e and f). Together, these data demonstrate that the phosphorylation of KDM2A at T632 abrogates its chromatin-binding capacity but does not affect its demethylase activity.

An ATM-KDM2A signaling pathway is required for H3K36me2 increase in response to DNA damage. As T632 phosphorylation of KDM2A decreased its chromatin-binding capacity, we hypothesized that H3K36me2 would increase in response to DNA damage. This prediction is consistent with the evidence that H3K36me2 was induced at DSBs, but H3K36me3 induction was absent following ionizing radiation and I-Scel- or AsiI-induced DNA double-strand break induction.8,10,12,13 Immunoblotting assays were performed to detect H3K36 methylation changes in HEK293 cells after etoposide treatment. H3K36 dimethylation significantly increased at the early stage of DNA damage response, which is consistent with a previous study,13 and H3K36 monomethylation correspondingly decreased in a time- and dose-dependent manner (Figure 4a, Supplementary Figures 7a and b). However, similar changes at other typical lysine sites were not observed (Supplementary Figure 7c). In addition, these changes of H3K36 dimethylation were further confirmed in HEK293 cells treated with adriamycin, etoposide or 5-aza-CdR. By contrast, ultraviolet radiation did not significantly affect H3K36 dimethylation (Figure 4b). Similar changes of H3K36 dimethylation were also observed in HCT116 and HeLa cells in response to etoposide treatment (Supplementary Figure 1c), suggesting that the increase in H3K36me2 is a universal response to DSBs.
We next determined whether the signaling pathway of ATM-KDM2A is required to induce H3K36me2. As shown in Figure 4c, H3K36me2 did not increase in ATM-mutated A-T cells but significantly increased in A-T cells that overexpressed ATM in response to etoposide treatment. In addition, the H3K36me2 increase was significantly alleviated in HEK293 cells treated with the ATM-specific inhibitor KU55933 (Figure 4d). However, ATR knockdown did not affect H3K36me2 levels after etoposide treatment (Supplementary Figure 2b). Moreover, H3K36me2 significantly decreased in HEK293 cells that overexpressed wild-type KDM2A.

Figure 2. Induction of KDM2A phosphorylation by ATM at Thr632 in response to DNA damage. (a) HEK293T cells transfected with Flag-KDM2A were treated with 40 μM etoposide for 2 h. The cell extracts were precitated with anti-Flag antibody, and subjected to immunoblotting. (b) Flag-KDM2A immunoprecipitates from untreated or etoposide-treated HEK293T cells in the absence or presence of lambda phosphatase (λ. PPase) were subjected to immunoblotting. (c) HEK293T cells transfected with wild-type Flag-KDM2A, S11A, or T632A mutant were treated with etoposide and an immunoprecipitation assay was performed as described in a. (d) KDM2A fragments were incubated with pATMATM and [γ-32P] ATP, and subsequently separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), stained by Coomassie brilliant blue (CBB) or exposed by autoradiography. The arrow indicates phosphorylation of KDM2A. # Represents specific protein bands. The ‘NC’ here indicates negative control without KDM2A fragment. (e) HEK293 cells were treated with 5-aza-CdR at 5 μM for 48 h, adriamycin at 1 μM for 12 h, etoposide at 40 μM for 2 h, ultraviolet radiation (UV) for 5 min. The cell extracts were then subjected to immunoblotting. The ‘NC’ indicates negative control without any treatment. (f) The cell extracts of HEK293 cells treated with etoposide for 2 h at the indicated doses were subjected to immunoblotting. (g) Immunoblotting of pT632 after treating HEK293 cells with etoposide at 20 μM for the indicated time periods. (h) ATM inhibitor KU55933 was introduced into HEK293 cells in the absence or presence of etoposide at 40 μM for 2 h, and pT632 was detected by immunoblotting. (i) A-T cells transfected with pcDNA or Flag-ATM separately were treated with etoposide at 40 μM for 2 h and subjected to immunoblotting.
but not in cells that overexpressed the T632E mutant (Figure 4e). This finding indicates that KDM2A phosphorylation is responsible for the increase in H3K36me2. Collectively, these findings suggest that the ATM-KDM2A signaling pathway is essential for the increase of H3K36me2 in response to DNA damage.

H3K36me2 interacts with the MRE11 complex by directly binding to NBS1. As H3K36me2 increase and recruitment of the MRE11 complex onto chromatin occurs during an early stage of the DNA damage response, it is possible that H3K36me2 and the MRE11 complex are involved in the repair process. This interaction may play a role in the efficient repair of DNA damage in response to genotoxic stress.

Figure 3. Decreased binding capacity of KDM2A to chromatin by phosphorylation of KDM2A at Thr632. (a) Schematic representation of the KDM2A protein. KDM2A contains a PHD zinc finger, a CxxC-type zinc finger, and a Jmjd domain. Thr632 is located in the PHD zinc finger. (b) HEK293 cells were treated with etoposide at 40 μM for 2 h, and were fractionated into soluble fraction and chromatin fraction. Immunoblotting of KDM2A was performed. Equal amount of KDM2A was loaded, and immunoblotted with anti-pT632 antibody. (c) HEK293 cells were transfected with wild-type KDM2A, the T632A or T632E mutant. At 48 h after transfection, the chromatin fractions were separated and subjected to immunoblotting. Data are also shown as means ± s.d.; n = 3. Student’s t-test (two-tailed): T632A versus WT, P < 0.01; T632E versus WT, P < 0.01; T632A versus T632E, P < 0.01. (d) Whole-cell lysates of HEK293T cells transfected with wild-type KDM2A, the T632A and T632E mutant were precipitated with anti-Flag antibody, and then the interactive components were analyzed by immunoblotting. (e) HEK293 cells were transfected as described in c. The cells were fixed and subjected to confocal fluorescent microscopy with Flag and H3K36me2 antibodies. The merge channel was the combination of the two colors. The arrows indicate representative positively KDM2A-overexpressed cells, and the symbols ‘#’ indicate the normal untransfected cells. (f) The quantification of H3K36me2-positive cells from the experiment for which results are shown in e. H3K36me2-positive cells refer to the cells with the comparable H3K36me2 staining to that of the normal untransfected cells inside. The percentage of H3K36me2-positive cells for each condition was calculated from a minimum of 100 cells. Data are shown as means ± s.d. from three independent experiments.
complex interact when responding to DNA damage. To test this possibility, HEK293T cells were transfected with Flag-tagged MRE11, RAD50 or NBS1, and the cell extracts were subjected to co-IP assays. As shown in Figures 5a and c, all exogenous members of the MRE11 complex interacted with H3K36me2, and this interaction significantly increased in response to etoposide treatment. In addition, an endogenous reciprocal co-IP also indicated an increased interaction between H3K36me2 and the MRE11 complex members after DSB induction (Figure 5d). However, the mRNA and protein levels of the MRE11 complex components were not obviously changed after etoposide treatment (Supplementary Figure 8). To determine whether the interaction between H3K36me2 and the MRE11 complex is direct, in vitro GST pull-down assays were performed using GST-fused MRE11, RAD50 and NBS1 proteins and biotin-tagged H3 peptides. It was identified that H3K36me2 interacted with NBS1 directly, but not with MRE11 or Rad50. NBS1 did not interact with other H3 peptides (Figures 5e and f). Furthermore, the interaction between H3K36me2 and NBS1 was dependent on peptide concentration (Figure 5g).

To further determine which functional domain of NBS1 is required for the interaction between H3K36me2 and NBS1, a peptide pull-down assay was then performed, using deleted mutants of NBS1 and biotin-tagged H3K36me2. NBS1 contains a FHA (forkhead-associated) domain and a BRCT (BRCA1 C-terminus) domain in its N terminus, a BRCT domain in its central region, and a MRE11-head-associated) domain and a BRCT (BRCA1 C-terminus) domain in its C-terminus (328-460). To determine which region is required for the interaction between NBS1 and H3K36me2, in vitro GST pull-down assays were performed using GST-NBS1(218-327) and GST-NBS1(328-460) proteins, as well as biotin-tagged H3K36me2 peptide. As shown in Figure 6b, NBS1(218-327), but not NBS1(328-460), interacted with H3K36me2, indicating that the BRCT2 domain of NBS1 is required for the interaction between H3K36me2 and NBS1. In addition, two deleted BRCT2 mutants were constructed, and peptide pull-down assays using the two mutants were performed. As shown in Figure 6c, NBS1-BRCT2(d218-247) did not interact with H3K36me2, indicating that the N-terminal of the BRCT2 domain, which contains an α helix (a1) and a β sheet (B1),37 is critical for the interaction between H3K36me2 and the BRCT2 domain of NBS1. Together, these results suggest that H3K36me2 interacts with NBS1 directly through the BRCT2 domain of NBS1.

Enriched H3K36me2 facilitates MRE11 complex recruitment to DNA damage sites

Next, to determine whether H3K36me2 is required for the recruitment of the MRE11 complex to damaged DNA, HEK293 cells were transfected with pcDNA, wild-type KDM2A or KDM2A-T632E mutant. An immunofluorescence assay was then performed to observe MRE11 foci formation. As shown in Figures 7a and b, MRE11

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Figure 4. Increased H3K36me2 in response to DNA DSB and the role of ATM-KDM2A signaling pathway in H3K36me2 increase. (a) HEK293 cells were treated with etoposide at 40 μM for the indicated time periods and subjected to histone acid extraction assay. Immunoblotting was performed with the indicated antibodies. (b) HEK293 cells were treated with 5-aza-CdR at 5 μM for 48 h, adriamycin at 1 μM for 12 h, etoposide at 40 μM for 2 h, and ultraviolet radiation (UV) for 5 min. The acid-extracted histones were subjected to immunoblotting with H3K36me2 antibody. The 'NC' here indicates negative control without any treatment. (c) A-T cells were transfected with pcDNA or Flag-ATM separately and treated with etoposide at 40 μM for 2 h. The acid-extracted histones were subjected to immunoblotting. (d) The ATM-specific inhibitor KU55933 was introduced into HEK293 cells treated with etoposide at 40 μM for 2 h, and H3K36me2 was detected by immunoblotting. (e) HEK293 cells were transfected with pcDNA, wild-type KDM2A or the T632E mutant separately, and treated with etoposide at 40 μM for 2 h. The whole-cell lysates and acid-extracted histones were subjected to immunoblotting.
foci formation was significantly inhibited in cells overexpressing wild-type KDM2A. However, MRE11 foci formation in cells that overexpressed the phospho-mimetic T632E mutant was similar to that of cells that overexpressed nonspecific pcDNA. A U2OS DRGFP (direct repeat green fluorescent protein) cell line and an endonuclease I-SceI were introduced to induce a targeted single DNA damage site to further determine the role of KDM2A phosphorylation in MRE11 complex recruitment. As shown in Figures 7c and d, an increase in H3K36me2 and recruitment of the MRE11 complex to the DNA near the I-SceI site were observed upon I-SceI transfection. The relative enrichment of the MRE11 complex significantly decreased in U2OS cells that overexpressed wild-type KDM2A, but not in cells that overexpressed the T632E mutant. However, KDM2A knockdown did not significantly affect MRE11 recruitment in response to DNA damage (Supplementary Figure 9a).

We also confirmed that the KDM2A phosphorylation that is associated with the recruitment of the MRE11 complex is ATM dependent. As shown in Figures 7e and f, the enrichment of H3K36me2 on chromatin increased in A-T cells that overexpressed ATM in response to DNA damage, but not in A-T cells that overexpressed pcDNA. Although the enrichment of MRE11 on chromatin was upregulated in both types of A-T cells in response to etoposide treatment, the MRE11 enrichment on chromatin was higher in A-T cells that overexpressed ATM than in A-T cells that overexpressed pcDNA (P < 0.05). Consistently, MRE11 recruitment in response to DNA damage also decreased after treatment with the ATM-specific inhibitor KU55933 (Supplementary Figure 9b). These data clearly demonstrate that ATM-mediated KDM2A phosphorylation is essential for MRE11 complex recruitment to DNA damage sites.

KDM2A phosphorylation is required for efficient DNA damage repair and enhanced cell survival

It has been clearly demonstrated that the MRE11 complex has a crucial role in DNA damage repair. A comet assay was performed to directly measure the repair efficiency of...
etoposide-induced DNA damage. By comparing the length and the area of DNA tails, more serious DNA damage was retained in HEK293 cells that overexpressed wild-type KDM2A compared with cells that overexpressed pcDNA or the phospho-mimetic T632E mutant (Figures 8a and b). In addition, the γH2AX level (a marker of DNA damage) was detected during DNA damage recovery. As shown in Figures 8c and e, the γH2AX level and the number of γH2AX foci in HEK293 cells that overexpressed wild-type KDM2A were much higher at later time points than that in cells that overexpressed pcDNA or the T632E mutant. Finally, a colony formation assay was performed to quantify cell survival rate. Cells that overexpressed wild-type KDM2A exhibited a decreased survival rate in response to DNA damage, whereas cells that overexpressed the KDM2A (T632E) mutant did not experience a similar effect (Figure 8f). These results suggest that KDM2A overexpression inhibits DNA damage repair, which is consistent with a previous study and that ATM-mediated KDM2A phosphorylation is critical for DNA damage repair and cell survival.

**DISCUSSION**

In this study, KDM2A was identified as a novel substrate of ATM. DSB enhanced the interaction between ATM and KDM2A, which induced ATM-mediated phosphorylation of KDM2A at T632. The specific KDM2A T632 phosphorylation compromised its chromatin-binding capacity and thus alleviated its ability to demethylate H3K36me2. Consequently, H3K36 dimethylation level, especially at DNA damage sites, significantly increased. High levels of H3K36me2 recruited the MRE11 complex to the DNA damage sites via a direct interaction with NBS1 and thus contributed to the ability of cells to repair DSBs.

It has recently been reported that histone methylation has a critical role in the DNA damage response. For example, the DSB repair component 53BP1 and its fission yeast homolog Crb2 are recruited to the sites of DNA damage by the methylated histone H3 lysine 79 (H3K79) or methylated histone H4 lysine 20 (H4K20) [8,42-43]. Previous studies have also demonstrated that H3K36 dimethylation/trimethylation mediates the DNA damage response.
response. H3K36me2 level is increased in response to DNA DSBs, whereas H3K36me3 level does not change. The increase in H3K36me2 level is associated with the early response to DNA DSBs and promotes the recruitment of Ku70 or NBS1 to DSB sites.13 However, H3K36me3 constitutively recruits LEDGF (lens epithelium-derived growth factor p75) to chromatin. Following DNA DSBs, LEDGF recruits CtIP to promote end resection and then facilitates RPA and RAD51 recruitment.10 Therefore, both H3K36me2 and H3K36me3 are critical for DNA DSB, but they act in different stages of the DNA damage response. Our study contributes evidence that H3K36 dimethylation is critical in the DNA damage repair. Distinct from other reports,6–13 our study elucidated a mechanism by which the ATM-KDM2A signaling pathway modulated H3K36me2 changes, MRE11 complex recruitment and DNA damage repair.

The PHD-type zinc-finger domain, one of the main domains of KDM2A, has been demonstrated to bind to histone lysines. For example, the PHD fingers of inhibitor of growth 2 and phosphatase and tensin homolog deleted on chromosome 10 of Jumonji A-T-rich interactive domain 1C can bind to tri-methylated histone H3K4 (H3K4me3),44,45 whereas the PHD finger of Junonji A-T-rich interactive domain 1C can bind to tri-methylated histone H3K9 (H3K9me3).46 By contrast, the LSD1
Figure 8. Regulation of DNA repair by KDM2A phosphorylation. (a) HEK293 cells were transfected with pcDNA, wild-type Flag-KDM2A or the T632E mutant, and treated with etoposide at 40 μM for 2 h. Soon after 2-h etoposide treatment, cells were washed free of drug and then incubated for 18 h. Cells were collected for comet assays. A representative image of cells under each condition is presented. (b) Quantification of the tail moments from the experiment for which results are shown in a. The tail moment for each condition was calculated from a minimum of 100 cells. Data are shown as means ± s.d. (n ≥ 100). Student’s t-test (two-tailed): pcDNA versus WT, P < 0.001, pcDNA versus T632E, P > 0.05, WT versus T632E, P < 0.001. (c) HEK293 cells were transfected with the indicated expression constructs and treated with etoposide as described in a. Cells were washed free of drug, incubated for the indicated time periods and whole-cell lysates were subjected to immunoblotting. (d) HEK293 cells were transfected with the indicated expression constructs and treated with etoposide as described in a. Cells were washed free of drug, incubated for the indicated time periods and fixed for microscopic imaging of γH2AX after etoposide treatment. (e) Quantification of γH2AX-positive cells from the experiment for which results are shown in d. γH2AX-positive cells refer to cells with ≥ 10 γH2AX foci inside. The percentage of γH2AX-positive cells for each condition was calculated from a minimum of 100 cells. Data are shown as means ± s.d. from three independent experiments. (f) HeLa cells stably expressing pcDNA, wild-type Flag-KDM2A or the T632E mutant were treated with etoposide at 20 or 40 μM for 2 h and washed free of drug. Then cells were trypsinized and equally plated into six-well plates. Ten days later, the cell colonies were stained and counted. Data are shown as means ± s.d. from three independent experiments. Student’s t-test (two-tailed): 20 μM, pcDNA versus WT, P < 0.05, pcDNA versus T632E, P > 0.05, WT versus T632E, P < 0.01; 40 μM, pcDNA versus WT, P < 0.001, pcDNA versus T632E, P > 0.05, WT versus T632E, P < 0.05.
complex component BHC80 can bind to unmethylated H3K4 via its PHD finger to stabilize the complex at its targeted promoters to repress transcription. It has not yet been reported that the PHD domain of KDM2A binds to methylated histones, and KDM2A is recruited to CpG islands by CxxC-type zinc finger rather than by PHD zinc finger, however, the PHD domain of KDM2A may still affect its chromatin-binding ability. In this study, T632 of KDM2A was located just within the PHD domain, and the phosphorylation of KDM2A at T632 affected its chromatin-binding capacity, which further strengthens the importance of PHD zinc-finger domains in the chromatin-binding ability. However, Thr632 is located at the N-terminal of the PHD domain, which is near to the CxxC-type zinc finger, and it is possible that phosphorylation of KDM2A at Thr632 may have affected its chromatin-binding ability by influencing the CxxC-type zinc finger. It remains unknown how phosphorylation of Thr632 influences the chromatin-binding ability of KDM2A, and KDM2A phosphorylation may exert allosteric effects that influence its chromatin-binding activity.

Histone modification enzymes are often regulated in response to DNA damage. Modification of the enzymes themselves is associated with their enzymatic activity. For example, in our previous study, the histone methyltransferase SET7/9 interacted with and methylated histone methyltransferase SUV39H1 in response to DNA damage, resulting in decreased SUV39H1 methyltransferase activity and genome instability. However, it is unlikely that the modifications of all histone methyltransferases/demethylases are related to their enzymatic activity. For example, it has been recently reported that PARP1-mediated PARylation of histone demethylase KDM4D is essential for the recruitment of KDM4D to damaged DNA but does not influence demethylase activity. In addition, histone demethylase KDM4A is ubiquitinated by RNF8 and RNF168 and then degraded to promote S3BP1 accumulation at damaged sites in response to DNA damage. Moreover, phosphorylation of the yeast KDM4 ortholog Rph1 occurs upon ultraviolet irradiation and induces the release of Rph1 from chromatin. Similarly, this study reports that ATM-mediated KDM2A phosphorylation at T632 did not affect its demethylase activity but significantly decreased its chromatin-binding capacity in response to DNA damage.

The MRE1 complex participates in DNA damage repair and coordinates the response to broken chromosomes. However, the mechanism of MRE1 complex recruitment to DSBs is still unclear. For example, the MRE1 complex may recognize DSBs and directly target the damaged sites. Alternatively, a previous study has shown that PARP1 accumulates at DSB sites and is essential for the rapid accumulation of MRE1 and NBS1 at DNA damage sites through its direct interaction with MRE1. However, NBS1 appears to be much more critical to the recruitment of the MRE1 complex in response to DNA damage. MDC1 and yH2AX have been shown to promote MRE1 complex recruitment after DNA damage by directly binding to the FHA/BRCT domains of NBS1. In addition, Rad17 is required for the early recruitment of the MRE1 complex to DSB sites through direct interaction with the FHA domain of NBS1. In our study, we identified that H3K36me2 is also required for MRE1 complex recruitment to DSB sites by interacting with NBS1, such that H3K36me2 binds directly to the BRCT2 domain of NBS1, but not the FHA or BRCT1 domains. This novel finding explains a mechanism of how the MRE1 complex binds to damaged DNA. Consistent with our study, H3K36me2 was shown to interact with NBS1 upon ionizing radiation by co-IP, which further confirms the recruitment of the MRE1 complex by H3K36me2. Although the structural basis of this H3K36me2-NBS1 interaction is not yet clear, our study further supports the essential role of the MRE1 complex in the DNA damage response.

It has been described that KDM2A is frequently overexpressed in non-small cell lung cancer tumors and gastric cancers and can promote the growth and motility of cancer cells. This study is the first to report that KDM2A phosphorylation is critical for DNA damage response and cell survival. Therefore, KDM2A may be a new therapeutic target for cancer treatment. This study provides a useful strategy with which to design anticancer therapies and, eventually, cure cancer by manipulating histone methylation and demethylation at specific sites upon DNA damage-related chemical therapy.

MATERIALS AND METHODS

Chromatin immunoprecipitation assay

Cells were fixed with formaldehyde and lysed with lysis buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 1% sodium dodecyl sulfate). After sonication, the supernatant was collected by centrifugation and precleared in diluted lysis buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100) with protein G or A sepharose and salmon DNA. The precleared samples were incubated with the indicated antibody and protein G or A sepharose. The beads were washed and heated at 65°C to reverse the cross-link. DNA was purified and real-time PCR was performed with primers as follows: 5′-TCTCTTCAAGGAGCAGGCAAAC3′ (sense) and 5′-TTATGAGTTTACTCCA GCTTGGCG-3′ (antisense); genomic control: 5′-CTCTGCAAAAGCCTTACAA-3′ (sense) and 5′-TTACTGTGACACTGC-3′ (antisense).

Peptide pull-down assay

Biotin-tagged H3 peptides were immobilized on streptavidin beads in phosphate-buffered saline with rotation at 4°C overnight. Then the beads were washed with phosphate-buffered saline and incubated with cell lysates. After three washes with washing buffer (20 mM Tris-HCl pH 7.5, 1 mM EDTA, 500 mM NaCl), the beads were subjected to Coomassie brilliant blue staining and immunoblotting.

Histone acid extraction assay

Cells were lysed in hypotonic lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM KCl, 1.5 mM MgCl2 and 2 mM DTT, protease inhibitors), then pelleted the intact nuclei by spinning. Discard supernatant, re-suspend nuclei in 0.4 N H2SO4 and incubate for at least 30 min. Spin samples and transfer the supernatant containing histones. Add trichloroacetic acid and incubate on ice for 30 min. Collect histone pellet by spinning, wash with acetone and dissolve in ddH2O.

Comet assay

Comet assay has been described before. Briefly, cells were mixed gently with pre-melted low-temperature-melting agarose at a volume ratio of 1 to 1 (v/v) and spread on glass slides. The slides were then submerged in precooled lysis buffer at 4°C for 90 min. After rinsing, the slides were electrophoresed at 1.0 V/cm for 20 min, and then stained with propidium iodide. Fluorescence images for at least 100 nuclei were captured using an Olympus FV1000-IX81 Confocal Microscope (Tokyo, Japan). The images were analyzed by CometScore Version 1.5 software (TriTek Corp., Sumerduck, VA, USA) for tail moment.

Colony formation assay

Cells were plated and exposed to 40 μM etoposide for 1 h. Etoposide was removed by three rinses using phosphate-buffered saline. Cells were trypsinized and plated into six-well plates in an equal number. After 10 days, methanol fixation and staining with methylene blue was undertaken to identify visible colonies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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