Homeodomain Proteins Directly Regulate ATM Kinase Activity

Tanya E. Johnson1,2, Ji-Hoon Lee1,2, Logan R. Myler1,2, Yi Zhou1, Trenell J. Mosley3, Soo-Hyun Yang4, The Bio-Bricks for Molecular Machines FRI Stream4, Nadima Uprety1, Jonghwan Kim1, and Tanya T. Paull1,2,5,*

1Department of Molecular Biosciences, The University of Texas at Austin, Austin, TX 78712, USA
2Howard Hughes Medical Institute, The University of Texas at Austin, Austin, TX 78712, USA
3Department of Human Genetics, Emory University School of Medicine, Atlanta, GA 30322, USA
4College of Natural Sciences, The University of Texas at Austin, Austin, TX 78712, USA
5Lead Contact

SUMMARY

Ataxia-telangiectasia mutated (ATM) is a serine/threonine kinase that coordinates the response to DNA double-strand breaks and oxidative stress. NKX3.1, a prostate-specific transcription factor, was recently shown to directly stimulate ATM kinase activity through its highly conserved homeodomain. Here, we show that other members of the homeodomain family can also regulate ATM kinase activity. We found that six representative homeodomain proteins (NKX3.1, NKX2.2, TTF1, NKX2.5, HOXB7, and CDX2) physically and functionally interact with ATM and with the Mre11-Rad50-Nbs1 (MRN) complex that activates ATM in combination with DNA double-strand breaks. The binding between homeodomain proteins and ATM stimulates oxidation-induced ATM activation In Vitro but inhibits ATM kinase activity in the presence of MRN and DNA and in human cells. These findings suggest that many tissue-specific homeodomain proteins may regulate ATM activity during development and differentiation and that this is a unique mechanism for the control of the DNA damage response.

Graphical Abstract

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

AUTHOR CONTRIBUTIONS

T.E.J. performed all experiments with the exception of ATM monomerization assays, which were performed by J.-H.L., and confocal imaging of cells, which was performed by L.R.M. Y.Z. established the protocol for the ATM/MRN kinase reactions. T.J.M. expressed and purified several different C-terminal deletion CDX2 mutants. Point mutations and initial expression and purification of CDX2 mutants were performed by the Freshmen Research Initiative Bio-Bricks cohort as part of their undergraduate research projects under the supervision of S.-H.Y. N.U. and J.K. provided reagents and helped with the ESC culture. T.T.P. directed the project. T.E.J. and T.T.P. co-wrote the paper.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.06.089.

DECLARATION OF INTERESTS

The authors declare no competing interests.
In Brief

Johnson et al. demonstrate that several members of the homeobox transcription factor family bind directly to the ATM protein kinase and to the MRN complex, inhibiting activation of ATM by DNA double-strand breaks. These results suggest that homeobox proteins may act as tissue-specific regulators of the DNA damage response.

INTRODUCTION

Ataxia-telangiectasia mutated (ATM) is a serine/threonine kinase encoded by the ATM gene that is critical for coordinating the response to DNA double-strand breaks (DSBs) and regulating oxidative stress in cells (Paull, 2015). Loss of functional ATM protein results in ataxia-telangiectasia, a pleiotropic disorder characterized by progressive neurodegeneration, predisposition to cancer, immunodeficiency, and sensitivity to DNA-damaging agents (Anheim et al., 2012; Lavin, 2008; Perlman et al., 2012; Shiloh and Ziv, 2013). As the main signal transducer after double-strand breaks, ATM phosphorylates hundreds of downstream targets that coordinate DNA repair, cell cycle checkpoint activation, and apoptosis (Shiloh and Ziv, 2013).

In unstressed cells, ATM is sequestered as an inactive dimer (Bakkenist and Kastan, 2003). Upon DNA damage, ATM is recruited to the sites of DNA double-strand breaks within seconds by the Mre11-Rad50-Nbs1 (MRN) complex through multiple points of interaction where it undergoes a transition to an active, autophosphorylated monomer (Lee and Paull, 2004, 2005; Uziel et al., 2003). In oxidative stress conditions, ATM transitions from an inactive noncovalent dimer to an active covalent dimer with multiple disulfide cross-links that is capable of phosphorylating targets in an MRN-independent manner (Barzilai et al., 2002; Ditch and Paull, 2012; Guo et al., 2010a).

Homeobox genes encode a family of transcription factors that were first documented in Drosophila for their importance in tissue-specific gene expression during development and in adult tissues (Biggin and McGinnis, 1997; Gehring and Hiromi, 1986; McGinnis and Krumlauf, 1992). Expression of homeodomain proteins is often deregulated in cancer, and
many of these proteins are thought to be tumor suppressors or oncogenes (Bhatia-Gaur et al., 1999; Kim and Nirenberg, 1989; Wang et al., 2009). NKX3.1 is a homeodomain-containing transcription factor that is important for the development of the embryonic prostate and regulating prostate-specific genes in adult tissues (He et al., 1997; Tan et al., 2012). NKX3.1 is located on chromosome 8p21, a region that often undergoes a loss of heterozygosity (LOH) in prostate cancers (Asch-Kendrick et al., 2014; Chuang et al., 2007). NKX3.1−/− mice are prone to formation of precancerous prostatic intraepithelial (PIN) lesions that increase in severity with aging of the animal (Asatiani et al., 2003; Bieberich et al., 1996; Eide et al., 2013; Yang et al., 2004). Recently, new roles for NKX3.1 have emerged in DNA repair signaling. NKX3.1 co-localizes at DNA damage foci and can physically associate with many DNA repair factors in prostate cells (Bowen et al., 2007; Yang et al., 2014). Multiple studies have shown that NKX3.1 causes attenuation of DNA damage signaling events in prostate cells after DNA damage treatments via modulation of ATM kinase activity (Bowen et al., 2007, 2013). Binding of NKX3.1 to ATM stimulates kinase activity in In Vitro kinase assays with MRN/DNA or hydrogen peroxide (Bowen et al., 2013). The highly conserved homeodomain was required for this effect, raising the intriguing possibility that other homeodomain proteins may affect ATM signaling in other tissue types.

In this study, we further investigate the effects of NKX3.1 on ATM and compare this to several other representative members of the homeobox transcription factor family. The effects of these proteins on ATM activation In Vitro depend on the type of activation used: DNA double-strand breaks or oxidative stress, where the transcription factors are generally stimulatory of oxidative activation of ATM but inhibit activation by the MRN complex. Analysis of the mechanism of this effect using NKX3.1 and the gastrointestinal-specific factor CDX2 shows that both proteins bind to ATM directly and exhibit a strong interaction with the MRN complex. Taken together, these results suggest that homeodomain proteins may serve as tissue-specific regulators of ATM kinase activity.

RESULTS

Several reports in the literature have described effects of home-odomain proteins on the DNA damage response, often with direct association with proteins that function in DNA double-strand break repair (Bowen and Gelmann, 2010; Bowen et al., 2013; Chiba et al., 2012; Renouf et al., 2012; Rubin et al., 2007; Schild-Poulter et al., 2001; Soret et al., 2016; Yang et al., 2014; Zhang et al., 2014). Since the effects of NKX3.1 on ATM were reported to be through the homeodomain itself, we hypothesized that other homeodomain transcription factors may also affect ATM activity. To test this idea, five additional homeodo-main proteins (NKX2.5, CDX2, TTF1, HOXB7, and NKX2.2) were chosen based on homology to NKX3.1 as well as documented involvement in cancer or DNA repair. The full-length homeodomain proteins were expressed and purified as fusion proteins with a HisMBP tag on the N terminus and a Flag affinity tag on the C terminus (Figure S1).

Homeodomain Proteins Stimulate ATM in the Oxidative Pathway

ATM can be directly activated by oxidative conditions through the transition from an inactive noncovalent dimer to an active, covalently linked dimer (Guo et al., 2010b).
Because NKX3.1 was previously shown to activate ATM \textit{In Vitro} under oxidizing conditions (Bowen et al., 2013), we hypothesized that other homeodomain transcription factors might also show this activity. To test this idea, we added purified, full-length homeodomain proteins to recombinant ATM in the presence of hydrogen peroxide, which activates ATM to a similar extent as the addition of MRN and DNA (Guo et al., 2010b). We found that all of the homeodomain proteins stimulated ATM phosphorylation of the substrate, a GST-p53(1–102) fusion protein, in this reaction in a dose-dependent manner (Figure 1A; quantitation of multiple experiments shown in Figure 1B). The sole exception to this was CDX2, which had no effect.

Homeodomain-containing transcription factors have unique N- and C-terminal extensions flanking the highly conserved DNA-binding motif (Figure 1C) (Gehring and Hiromi, 1986). Sequence alignment of each of the homeodomain proteins revealed that the least conserved region between CDX2 and the rest of the proteins is the C terminus due to a proline/glutamine-rich patch on CDX2 absent in the other homeodomain proteins (Figure S1). To test the role of this C-terminal extension, we truncated CDX2 after the homeodomain and found that this promoted stimulation of ATM kinase by CDX2, suggesting that the unusual C-terminal domain blocks the stimulatory effect of the N-terminal domain and central homeodomain of CDX2 (Figures 1C and 1D). We also truncated the rest of the homeodomain proteins immediately after the homeodomain (Figure 1C) and tested their activity in the ATM kinase assay with hydrogen peroxide (Figure 1D). These results also showed that all of the C-terminal truncated proteins as well as a homeodomain by itself (here shown with the homeodomain of NKX2.2 125–185 aa) could stimulate ATM through this pathway. In contrast, the MBP domain purified alone did not affect ATM oxidative signaling (Figure 1D); neither did the addition of an unrelated DNA-binding protein, Lac Repressor (Figure S2).

**Oxidative Signaling via ATM Is Not Regulated by Homeodomain Proteins in HCT116 Cells**

To investigate whether homeodomain proteins affect signaling of ATM kinase after oxidative stress in cells, we utilized an HCT116 human colon adenocarcinoma cell line containing a FLP recombination site adjacent to a CMV promoter under control of two copies of the tet operator in the genome (Flp-In T-Rex) (O’Gorman et al., 1991; Panier et al., 2012). Constructs containing NKX3.1 with a Flag tag on the C terminus or empty vector were integrated into these cells, allowing for induction of protein expression upon addition of doxycycline. The cells were treated with hydrogen peroxide, and downstream ATM targets were probed. In U2OS cells after treatment with low levels of hydrogen peroxide, ATM signaling can be observed at Thr68 of Chk2 but not Ser824 of Kap1, consistent with oxidative activation of ATM in the absence of DNA double-strand breaks (Guo et al., 2010a). However, in the case of HCT116 cells, treatment with hydrogen peroxide resulted in phosphorylation of Kap1, indicative of reactive oxygen species (ROS)-induced DNA damage (Figure 1E). Under these conditions, we found that overexpression of NKX3.1 caused an inhibition of ATM activity. In order to specifically examine ROS-activated ATM, we tested menadione, a reagent that induces intracellular ROS via redox cycling that has been shown to induce oxidative signaling of ATM (Schroeder et al., 2013; Yang et al., 2016). In HCT116 cells transiently over-expressing Flag-ATM, NKX3.1 expression was induced
with doxycycline and cells were treated with varying doses of menadione. Under these conditions, we observed ATM activation by menadione as evidenced by Chk2 (Thr68) phosphorylation in the absence of Kap1 modification, but there was no statistically significant difference in ATM signaling with NKX3.1 overexpression, suggesting that NKX3.1 does not influence ATM in the oxidative stress pathway, at least not in these cells (Figure 1F).

The expression of homeodomain proteins activates ATM under conditions of oxidative stress In Vitro yet shows no stimulatory effect in cells. We hypothesized that, due to the presence of abundant DNA in the nucleus, homeodomain proteins may be tethered to DNA and unable to stimulate ATM in the nucleoplasm. To test this idea, we titrated DNA into the ATM kinase assay In Vitro with hydrogen peroxide and NKX3.1. The addition of DNA to this reaction had no effect on ATM activation with hydrogen peroxide alone but removed the stimulatory effect of NKX3.1 on ATM at higher DNA concentrations (Figure 1G). This result suggests that the presence of DNA is an important functional regulator of the interactions between homeodomain proteins and ATM and could explain the lack of observed stimulation by NKX3.1 in the presence of oxidative stress in HCT116 cells.

Homeodomain Proteins Inhibit ATM Kinase Activity in the Presence of MRN and DNA

Recombinant, purified ATM can be stimulated over 100-fold In Vitro by the addition of MRN complex and DNA (Lee and Paull, 2005). This recapitulates the activation of ATM that occurs during canonical DNA signaling in which ATM is recruited to the site of the DNA double-strand break by the MRN complex (Shiloh and Ziv, 2013). MRN promotes monomerization of ATM and increases the affinity of ATM for its substrates (Lee and Paull, 2004, 2005).

We previously found that full-length NKX3.1 stimulates ATM kinase activity In Vitro, with the homeodomain itself sufficient for this effect, and that NKX3.1 binds to purified ATM directly (Bowen et al., 2013). The ATM kinase reactions showing a stimulatory effect of NKX3.1 we performed previously contained a minimal level of MRN necessary for ATM activation (4 nM), and low levels of MRN were necessary to see this effect (Figure 2A). We also observed a stimulatory effect of all the homeodomain domain proteins we tested, with most showing approximately 2-fold stimulation (Figure S3). However, with higher levels of MRN (32 nM) and preincubation of MRN with DNA and ATP, we observe that the addition of NKX3.1 to the reaction resulted in inhibition of ATM (Figure 2A).

Since the presence of DNA has a strong effect on the role of NKX3.1 in the oxidative reaction (Figure 1), we performed the ATM kinase assay with varying concentrations of linearized plasmid DNA in the reaction. However, this did not alleviate the ability of NKX3.1 to inhibit ATM (Figure 2B); in fact, the inhibition became more pronounced with higher levels of DNA in the reaction. This result also shows that it is unlikely that homeodomain proteins sequester double-strand break ends and thus prevent MRN binding.

We also considered the possibility that homeodomain proteins compete with the substrate in ATM kinase assays since NKX3.1 is known to be phosphorylated by ATM (Bowen et al., 2013). To test this, we examined the effect of full-length NKX3.1 on ATM stimulation by
MRN and DNA under a wide range of p53 concentrations, with the idea that much higher levels of substrate may eliminate the effect of the transcription factor. Instead, we observed that the inhibitory effect of NKX3.1 on ATM was independent of the concentration of substrate (Figure 2C).

To determine whether ATM inhibition is a general feature of homeodomain proteins, all of the full-length proteins we purified were tested In Vitro with ATM in the presence of MRN and DNA (Figure 2D). We found that all of the homeodomain proteins exhibited an inhibitory effect on ATM in the presence of high levels of MRN, with the most extreme effects observed with NKX3.1, NKX2.5, and NKX2.2 (quantitation of multiple assays shown in Figure 2E). In contrast, an unrelated DNA-binding protein, Lac Repressor, had no effect on ATM kinase activity in this assay (Figure S2).

We also considered the possibility that the C-terminal extensions present in the homeodomain proteins affect the degree to which it alters ATM activity. To address this, we tested the C-terminal truncation mutant of CDX2 and compared it to full-length CDX2 and to His-MBP purified as a control in ATM activation by MRN and DNA, similar to our comparisons with the oxidative reaction in Figure 1. These assays showed that C-terminal truncation of CDX2 does not affect its inhibitory effect on ATM in the presence of MRN and DNA (Figure 2F). Addition of His-MBP alone had no effect on ATM kinase activity, while a truncation containing only the homeodomain of NKX2.2 showed partial inhibition, showing that the homeodomain alone is sufficient to inhibit ATM activity. Taken together, these results suggest that homeodomain proteins as a group show the ability to inhibit ATM kinase activity in the presence of high levels of MRN and that the homeodomain itself is important for this effect.

**CDX2 Disrupts the Functional Interaction between ATM and MRN and Prevents ATM Monomerization**

The MRN complex has been shown to augment ATM kinase activity In Vitro by monomerization of the ATM dimer and increasing affinity of ATM for substrates (Lee and Paull, 2004, 2005). We reasoned that effects of homeodomain proteins on this reaction could be through binding to MRN or ATM and that they may affect monomerization and/or substrate recruitment. Recombinant NKX3.1 was previously shown to interact with ATM protein in In Vitro pull-down assays (Bowen et al., 2013); here, we used CDX2 as a comparison because it has well-documented roles in embryonic differentiation as well as in cancer (Gross et al., 2008; Mallo et al., 1998). In order to determine whether CDX2 similarly binds ATM, we purified recombinant biotinylated ATM protein and tested binding to a C-terminal truncation of CDX2 in a pull-down assay. We found that CDX2ΔCTD interacts strongly with ATM (Figure 3A). We also investigated whether direct interactions occur between CDX2 and MRN since other homeodomain proteins have been shown to interact with numerous other DNA repair proteins (Bowen et al., 2013; Renouf et al., 2012; Rubin et al., 2007; Schild-Poulter et al., 2001; Yang et al., 2014). To test this, we used biotinylated MRN complex, which was incubated with purified CDX2ΔCTD before isolation on streptavidin-coated magnetic beads. This result also shows direct binding of CDX2ΔCTD to the MRN complex (Figure 3B).
One simple explanation for the ability of CDX2 to inhibit ATM kinase activity may be that it reduces ATM binding affinity to the substrate. However, when bio-ATM was incubated with CDX2 and the binding of GST-p53 substrate was monitored in the absence of MRN, we found that the presence of CDX2 had no effect on ATM affinity for the substrate (Figure 3C). We then investigated whether CDX2 influences ATM/MRN interactions. Biotinylated ATM and MRN complex were incubated together for 15 min before addition of CDX2 protein. ATM was then isolated with magnetic streptavidin beads, and the levels of MRN and CDX2 bound to ATM were assessed by western blot. These results show that increasing amounts of CDX2 in the binding reaction lead to a concurrent decrease in the amount of MRN bound, suggesting that CDX2 disrupts the ATM/MRN interaction (Figure 3D). When this experiment was repeated in the presence of GST-p53 substrate, it was apparent that this disruption of ATM/MRN association results in a loss of substrate binding in the presence of CDX2 (Figure 3E).

Since our results with the ATM kinase assay suggest that many homeodomain-containing proteins have the ability to affect ATM activation, we also validated these results with NKX3.1. Similar to CDX2, we found that the N terminus of NKX3.1 containing the homeodomain and N-terminal extension (NKX3.1ΔCTD) is capable of binding directly to MRN (Figure 3F). In a combined ATM/MRN binding assay, we also observe that addition of NKX3.1ΔCTD to ATM blocks binding between ATM and MRN (Figure 3G).

Another key function of the MRN complex is to trigger the monomerization of the inactive ATM dimer, resulting in an active, autophosphorylated monomer (Bakkenist and Kastan, 2003; Lee and Paull, 2005). In order to assess the effect of CDX2 protein on ATM dimer dissociation, recombinant ATM was expressed and purified as a dimer as previously published (Lee and Paull, 2006). Briefly, BioFlag-ATM and HA-ATM were co-expressed in 293T cells and purified using sequential anti-Flag and anti-HA affinity chromatography, resulting in the purification of the intact dimer. To quantify ATM dimer dissociation, the BioFlag-ATM/HA-ATM dimer was bound to magnetic streptavidin beads, incubated with the MRN complex, and the presence of HA-tagged ATM was quantified in the supernatant by western blotting. Consistent with previous results, the incubation of dimeric ATM with MRN caused HA-ATM to dissociate into the supernatant (Figure 3H). Upon addition of CDX2, there was reduced dissociation of HA-ATM from the BioFlag-ATM/HA-ATM dimer (quantification as indicated, Figure 3H). This suggests that CDX2 may also serve to stabilize the ATM dimer either directly or through disruption of the ATM/MRN interaction.

**Homeodomain Proteins Inhibit ATM Signaling in Colon Cancer Cells after DNA Damage Treatment**

NKX3.1 has previously been shown to accumulate at sites of DNA damage in prostate cells (Bowen and Gelmann, 2010; Bowen et al., 2013; Erbaykent-Tepedelen et al., 2014; Zhang et al., 2016). Since our *In Vitro* experiments in this study focused primarily on CDX2, we examined the localization of an mCherry-tagged form of CDX2 in U2OS cells expressing GFP-tagged CtIP, a well-characterized DNA damage response factor that is known to form foci at sites of DNA double-strand breaks (Sartori et al., 2007). We observed co-localization...
of CDX2 with CtIP in cells even without DNA damage but found that exposure of the cells to camptothecin (CPT) resulted in a higher level of colocalization (Figure 4A).

As an additional test for interaction between CDX2 and ATM, we used the HCT116 Flp-In T-Rex cells described above (O’Gorman et al., 1991). Constructs containing CDX2 with a Flag tag on the C terminus or empty vector were integrated into these cells, allowing for induction of protein expression upon addition of doxycycline. HCT116 cells normally have undetectable levels of endogenous CDX2 as measured by western blot, so there should not be any basal effects of CDX2, although other homeodomain proteins are present in these cells at low levels (Boisvert et al., 2010). CDX2 expression was induced for 16 hr with doxycycline, and then treated with CPT for an hour before isolating endogenous ATM protein with anti-ATM antibody bound to magnetic beads. Western blots of the immunoprecipitated material showed that CDX2 is present in a complex with ATM after CPT treatment (Figure 4B). Experiments in parallel with HCT116 cells expressing NKX3.1 also confirmed the interaction reported previously between NKX3.1 and ATM (Bowen and Gelmann, 2010).

To determine the effect of CDX2 on ATM signaling, we treated with the DNA-damaging agents etoposide or CPT, and the phosphorylation of downstream ATM targets Kap1 (Ser824) and Chk2 (Thr68) was monitored by western blot as a readout of ATM activity. We found that phosphorylation of ATM targets is attenuated when CDX2 is overexpressed after treatment with either etoposide or CPT (Figures 4C and 4D). Overexpression of NKX3.1 in HCT116 cells also reduced the phosphorylation of ATM targets after CPT treatment (Figure 4E; quantitation of multiple NKX3.1 and CDX2 experiments shown in Figure 4F). To confirm that these phosphorylation events were indeed due to ATM kinase activity and not another closely related kinase of the phosphatidylinositol 3-kinase-related kinase (PIKK) family, chemical inhibitors were used to inhibit ATM or DNA-PKcs, respectively, before treatment with CPT (Figure 4G). Upon pre-treatment with the ATM inhibitor KU55933, there was a large reduction in phosphorylation of Kap1 and Chk2, whereas this was not seen with the DNA-PKcs inhibitor NU7441 (Figure 4G). This result reinforces the conclusion that the effect of homeodomain proteins on DNA damage signaling is through ATM kinase activity.

**CDX2 Expression Does Not Improve DNA Repair**

Other groups have documented reduced DNA damage-related phosphorylation events after CPT treatment in the presence of overexpressed NKX3.1 in prostate cells (Bowen and Gelmann, 2010; Erbaykent-Tepedelen et al., 2014). However, the reduction in phosphorylation of ATM targets such as the histone variant H2AX was explained in these studies as enhanced DNA repair rather than reduced signaling. To address this question directly, we expressed the restriction enzyme AsiSI fused to the estrogen receptor hormone-binding domain with an auxin-inducible degron (Aymard et al., 2014), which was stably integrated into HCT116 Flp-In T-REx cells. Upon addition of 4-hydroxytamoxifen (4OHT), AsiSI is transported into the nucleus and generates double-strand breaks, which can be stopped by the addition of auxin (Agger et al., 2005; Littlewood et al., 1995). Cells were induced for expression of CDX2-Flag for 16 hr, and then treated with 4OHT for 4 hr before...
the media was changed and supplemented with auxin to induce degradation of AsiSI and recovery from the DNA damage. After 10–14 days of recovery, cells with overexpression of CDX2 had fewer colonies compared to uninduced cells and those containing vector only, suggesting that CDX2 reduces rather than increases the efficiency of DNA repair (Figure 4H). This effect was only observed with 4OHT addition, showing that the lower survival with CDX2 expression was dependent on AsiSI translocation.

**CDX2 Mutants Abrogate the Effect of CDX2 on ATM Activity *In Vitro* and in Human Cells**

Because the homeodomain alone was sufficient to inhibit ATM kinase activity in the MRN/DNA-mediated pathway, we hypothesized that surface residues in this domain are responsible for the effect on ATM. Examining the recent crystal structure of CDX2 in contact with DNA (Yin et al., 2017), we identified surface-exposed residues as potential ATM binding interfaces (Figure 5A). For instance, R214 extends into solution from the third helix of the homeodomain (shown in purple in Figure 5A). Point mutations were made in this residue in the HisMBP-CDX2 C-terminal deletion construct; the mutant protein was purified and tested in the ATM kinase assay. The R214N protein was completely inactive in blocking ATM activity (Figure 5B; quantification in Figure 5C). However, when the mutant was tested for binding to a Cy3-labeled oligonucleotide containing a CDX2 consensus binding site using a gel mobility shift assay, we found that it also lacked DNA binding ability (Figure 5D). This mutant also lacked the ability to bind to ATM and MRN, and, upon binding, the MRN complex remained associated with biotinylated ATM (Figure 5E). Since C-terminal truncated CDX2 affects ATM in the oxidative assay (Figure 1), we also tested the effect of the R214N mutant in that context and found that the mutation blocks inhibition of ATM (Figure S4), consistent with our observation that this mutant lacks the ability to bind to ATM.

We also tested seven other mutants with single-amino acid changes in surface residues and found that, in general, those with reduced ability to bind DNA were also unable to inhibit ATM in the MRN/DNA-mediated kinase assay to similar levels as wild-type (Figure 5F). These results suggest that the highly conserved DNA-binding homeodomain is responsible for both DNA and ATM binding. We also expressed a mutant containing a deletion of several residues in the homeodomain that was reported to be deficient in ATM binding (Bowen et al., 2013) and found that this mutant is also deficient in repression of ATM activation through MRN and DNA (Figure S4), consistent with our results showing the importance of the homeodomain itself in promoting ATM interactions.

To test whether the CDX2-R214N mutant is deficient in ATM inhibition in cells, we utilized the FlpIn T-Rex system in HCT116 cells to induce overexpression of the mutant protein. The presence of CDX2-R214N had no effect on ATM signaling after CPT treatment in these cells in comparison to the inhibitory effect of the wild-type protein (Figures 5G and 5H), consistent with the conclusion that this mutation abrogates ATM binding.

**ATM Signaling Is Reduced in Embryonic Stem Cells Undergoing Differentiation**

Homeodomain-containing proteins are often induced in a tissue-specific manner during development, and high levels of these proteins can be transiently present in differentiating...
cells (Biggin and McGinnis, 1997). An example of this is in embryonic stem cells (ESCs) during early mammalian development, where the CDX2 homeodomain protein is upregulated in cells that go on to form the trophectoderm, an epithelial cell layer surrounding the blastocoele and the inner cell mass (Sasaki, 2010). Little is known about the DNA damage response during these types of transitions mediated by homeodomain proteins, so we examined ATM-dependent phosphorylation events in mouse ESCs maintained in a pluripotent state by leukemia inhibitory factor (LIF), in culture. LIF withdrawal triggers differentiation of ESCs toward all three germ layers as well as trophectoderm lineage—a transition that occurs with rapid upregulation of CDX2 (Sasaki, 2010). In this system, we observed that phosphorylation of Kap1 in response to DNA damage caused by CPT is severely reduced in differentiated cells that have high levels of CDX2, a result consistent with our observation of CDX2 inhibition of ATM activation in the DNA damage response (Figure 5I). The phosphorylation of Kap1 in this system is, in fact, attributable to ATM and not DNA-PK, as determined by sensitivity to the ATM inhibitor KU55933 (Figure 5J). Thus, ATM activity is markedly reduced at a developmental transition coincident with CDX2 upregulation and differentiation in mouse ESCs.

DISCUSSION

There are several reports in the literature documenting effects of homeodomain-containing proteins on the DNA damage response or on DNA repair-related proteins (Bowen and Gelmann, 2010; Bowen et al., 2013; Chiba et al., 2012; Renouf et al., 2012; Rubin et al., 2007; Schild-Poulter et al., 2001; Soret et al., 2016; Zhang et al., 2014), which prompted us to examine the overall relationship between these proteins and ATM activation. Bowen and Gelmann (2010) showed that NKX3.1 colocalizes with autophosphorylated ATM at sites of DNA damage in PC-3 prostate cancer cells and that γH2AX foci, as well as phospho-Ser1981 ATM foci at sites of laser-induced damage, were visually reduced with NKX3.1 depletion, leading the authors to conclude that NKX3.1 in this situation was activating ATM. Our contribution to the subsequent study showed that, under conditions of low MRN concentration In Vitro, purified NKX3.1 stimulates ATM in concert with DNA breaks (Bowen et al., 2013), consistent with this interpretation.

Here, we find that NKX3.1, as well as other homeodomain-containing proteins, shows multiple points of interaction with the DNA damage response: direct binding to ATM, to the MRN complex, and to the DNA itself that stimulates ATM through the canonical pathway. We find that NKX3.1 can promote stimulation of ATM In Vitro, although this effect is strongly dependent on the level of MRN present. With high levels of MRN in the reaction, we observe an inhibitory effect of NKX3.1 as well as other homeodomain transcription factors including CDX2, NKX2.2, NKX2.5, TTF1, and HOXB7. The mechanistic basis for this inhibition appears to be the homeodomain-dependent reduction of MRN-ATM association, which coincides with reduced ATM monomerization and reduced recruitment of substrate (see model in Figure S5). Consistent with this result, we also observe an overall decrease in ATM signaling with CDX2 or NKX3.1 over-expression in response to DNA damage in HCT116 cells. The dependence on MRN levels that we measure In Vitro, however, suggests the possibility that there may be different responses in cells depending on the expression level of MRN components as well as the levels of homeodomain proteins.
Interestingly, levels of Rad50 were shown to be much lower in PC-3 prostate cancer cells compared to other cancer cell lines (Kavitha et al., 2010), perhaps explaining previous observations that NKX3.1 has an overall stimulatory effect on ATM in this cell line.

We speculate that the stimulatory effect of homeodomain proteins on ATM under conditions of low MRN may be related to the tendency of MR complexes, particularly the human complex, to self-associate In Vitro (de Jager et al., 2004). Human MR that we produce in insect cells is present in monomeric, dimeric, and multimeric forms, with the dimeric forms consisting of either globular or hook-linked assemblies (Lee et al., 2013). Currently, we do not know which of these forms activates ATM, although it is clear that association of the globular domains of Rad50 with ATP binding is essential. If the multimeric interactions are in fact non-productive and are alleviated by homeodomain proteins binding to MRN, then it is conceivable that the homeodo-main proteins could have a positive effect on ATM activation under circumstances of limiting MRN. With higher levels of MRN, there should be enough of the productive complexes present that the positive effect of homeodomains is outweighed by the negative effect of homeodomains binding to ATM itself and blocking association between ATM and MRN (Figure 3).

Other reports have linked homeodomain proteins to DNA repair; specifically, CDX2 was shown to bind to the Ku70/80 heterodimer and to inhibit DNA-PKcs (Renouf et al., 2012), while HOXB7 was also shown separately to bind to the same factors and to increase survival of mammary cells to DNA damage (Rubin et al., 2007). We have not explored the functional relationships with non-homologous end joining (NHEJ) factors in this study, but it is possible that structural similarities between ATM and DNA-PKcs may underlie the observations that multiple different homeodomain-containing proteins associate with members of the PIKK family. We did investigate whether overexpression of homeodomain proteins in HCT-116 cells alters the efficiency of DNA damage survival, showing that CDX2 induction reduces colony formation after global double-strand break formation. These results are generally consistent with the role of ATM in promoting DNA repair (Blackford and Jackson, 2017) but do not preclude effects on other PIKK family kinases.

Interestingly, we observed that residues on the surface of CDX2 involved in binding of DNA were also important for ATM interaction and inhibition. DNA was not required for CDX2 inhibition of ATM or binding to ATM or MRN, however, and treatment of the homeodomain protein with benzonase did not change the effects of CDX2 on ATM activity In Vitro (data not shown). The evidence points most directly toward a molecular mimicry such that the homeodomain has affinity toward both DNA and the ATM protein. This phenomenon has occurred in other proteins, for instance, the single-stranded DNA binding complex RPA, which is regulated by many protein factors through one or more of its DNA-binding interfaces (Sugitani and Chazin, 2015).

ATM can also be activated directly through disulfide bond formation between ATM subunits (Guo et al., 2010b), and here we observe a striking increase in activity induced by homeodomain proteins In Vitro. Our finding that CDX2 blocks monomerization of ATM by MRN is consistent with the hypothesis that it is actually preserving ATM dimer conformation and thus increasing activation via oxidative stress. However, we observed that...
the presence of DNA masks this stimulatory role of CDX2 In Vitro, consistent with the idea that the DNA-binding surface of the homeodomain is responsible for the interactions with ATM. We also did not observe stimulation of ATM under oxidative conditions in HCT116 cells; nevertheless, it is still conceivable that a stimulatory role may be seen in other cells or with other homeodomain proteins.

It is interesting that the homeodomain family of proteins appears to have a general capability of modulating ATM activity since these proteins are often upregulated at transition points during development that can involve periods of rapid proliferation, during which it may be preferable to attenuate the DNA damage response in order to reduce the probability of cell cycle arrest. Although ATM-deficient mammals do develop with normal tissue differentiation, studies in zebrafish have shown that ATM is activated during early embryogenesis in the absence of exogenous damage (Verduzco et al., 2012). A balance between cell cycle progression and cell cycle arrest is likely necessary for proper transit through developmental programs to avoid propagation of DNA damage yet also to avoid terminal arrest, an idea supported by experiments in mice, Drosophila, and worms (Cao et al., 2006; Song et al., 2004; Verduzco et al., 2012). In C. elegans, tissues in the soma have a markedly lower DNA damage response compared to the germline, a phenomenon mostly related to regulation of gene expression in this organism, but again suggesting that attenuation of the DNA damage response in differentiating tissues is an evolutionarily conserved phenomenon (Vermezovic et al., 2012). We also observed this pattern of ATM activity attenuating in differentiating mouse ESCs, a transition dependent on upregulation of CDX2 (Sasaki, 2010).

In summary, we have demonstrated that several homeodomain proteins representative of this large family of transcription factors have effects on ATM activity that stem from direct interaction with ATM and with the MRN complex. Thus, these diverse DNA-binding proteins have the potential to alter DNA damage signaling in tissue-specific ways correlating with their developmental programs. The effects of homeodomain proteins may also have important consequences in cancer since upregulation or misexpression of these transcription factors is observed in some tumor types (Haria and Naora, 2013). Further work is clearly necessary to decipher the relationship between these factors and ATM activation in these different biological settings.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**

See Supplemental Experimental Procedures.

**Protein Expression and Purification**

See Supplemental Experimental Procedures.

**In Vitro ATM Kinase Assays**

*In Vitro* ATM kinase assays with MRN and DNA were performed with 1 nM ATM, 16 nM MRN (except in Figure 2 as noted), 200 nM GST-p53 (1–102), and 2 ng of pNO1 plasmid
cut with AflIII restriction enzyme. In Vitro oxidative ATM kinase assays were performed with 2 nM ATM, 150 nM GST-p53 (1–102) substrate, and 0.008% hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) as described previously (Guo et al., 2010b). ATM kinase assays were performed with 0.8–250 nM purified homeodomain protein, as indicated in figure legends. Kinase assays were performed in kinase buffer (25 mM 3-(N-morpholino)propanesulfonic acid [MOPS], pH 7, 70 mM sodium chloride, 5 mM magnesium chloride, 1 mM ATP, 0.35 mM DTT, and 50 mg/mL BSA [New England Biosciences]) for 60 min at 37°C in a volume of 10 nL. Phosphorylation of Ser15 of the GST-p53 peptide was detected via immunoblotting using a phosphospecific antibody (Calbiochem; PC461) as described previously (Lee and Paull, 2004).

**Immunoprecipitation and Immunoblotting**

Bio-Flag-ATM was incubated with varying amounts of MRN, GST-p53 (1–102) peptide, or homeodomain protein, as indicated in figure legends. Proteins were incubated at room temperature for 30 min. Then, 2.5 mL of Dynabeads kilobaseBINDER magnetic streptavidin beads (Invitrogen) were pre-washed with Buffer A containing 100 mM NaCl and 1 mg/mL BSA and incubated with the protein mixture for 15 min at 4°C in the presence of 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma). Biotinylated protein and associated factors were isolated using a magnet and were washed three times with Buffer A containing 100 mM NaCl and 0.1% CHAPS. Beads were resuspended in 2.53 SDS loading buffer and boiled for 10 min before loading onto a 9% denaturing SDS-PAGE gel. Proteins were transferred for 3 hr at 400 mA and analyzed by western blotting as indicated in figure legends.

**DNA Damage, Oxidative Stress Treatment, and Western Blotting**

HCT116 Flp-In T-REx cells were treated with 10 μg/mL doxycycline (Dox) for 16 hr to induce the expression of homeodomain proteins. KU-55933 (EMD Millipore) and NU-7441 (R&D Pharmaceuticals; 3712) were used to treat cells for 1 hr for ATM or DNA-PK inhibition, respectively. DNA-damaging agents and hydrogen peroxide treatments were used to treat cells as indicated in figure legends. After treatment, cells were scraped into PBS and centrifuged at 800 × g for 2 min. Cells were lysed with cell lysis buffer, and lysates were clarified at 10,000 × g for 10 min. The protein concentrations of the lysates were quantified using Bradford assay, and protein levels were normalized for all samples. Primary antibodies used were anti-ATM (Santa Cruz), anti-phospho-Ser1981-ATM (Abcam), anti-Kap1 (Abcam; ab22553), anti-phospho-Ser824-Kap1 (Bethyl Laboratories; A300–767A), anti-Nbs1 (Genetex; GTX70224), anti-Rad50 (Genetex; GTX70228), anti-Mre11 (Genetex; GTX70212), anti-MBP (Rockland; 200–401–385), anti-Flag (Sigma; A1205), anti-β-actin (Cell Signaling; 4970), anti-Chk2 (Genetex; GTX70295), anti-phospho-Thr68-Chk2 (Cell Signaling; 2661S), anti-phospho-Ser15-p53 (Abcam; ab1431), and anti-phospho-Ser15-p53 (mouse) (Assay Biotech; A7180). Alexa Fluor 68-anti-rabbit and IRDye 800 anti-mouse were the secondary antibodies used. Membranes were scanned and quantified using the Odyssey system (Li-Cor).

**Fluorescence Microscopy**

See Supplemental Experimental Procedures.
**DNA Binding Assays**

CDX2 proteins were incubated for 30 min at room temperature with 6 nM oligonucleotide containing the CDX2 consensus binding site composed of TP5921 (Cy3-TAATCTCTCTGTATAGCTCTGACCT) annealed to its complementary DNA strand TP5922. Protein/DNA mixtures were separated on an 8% acrylamide native PAGE gel run in 0.53 TBE for 90 min at 80 V and visualized with a Bio-Rad Gel Doc XR system.

**Statistical Analyses**

Data are expressed as mean values ± SEM or ± SD as indicated in figure legends. Data were compared using unpaired Student’s t test with p values less than 0.05 considered to be statistically significant.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**ACKNOWLEDGMENTS**

We thank members of the Paull laboratory for helpful suggestions and to Edward Gelmann for initial ideas and reagents. Lac Repressor was a gift from Ilya Finkelstein. Homeobox genes were obtained from DNASU Plasmid Repository. L.R.M. is supported by NIH Grant F99CA212452.

**REFERENCES**


Highlights

- Homeobox transcription factors block ATM signaling through the MRN complex
- NKX3.1 and CDX2 bind directly to ATM as well as to MRN in the absence of DNA
- NKX3.1 and CDX2 prevent monomerization and activation of ATM by MRN
- CDX2 localizes to DNA damage sites, and overexpression reduces DNA damage survival
Figure 1. Homeodomain Proteins Stimulate ATM Kinase Activity In Vitro in Oxidizing Conditions but Not in Human Cells

(A) The ATM kinase assay was performed with purified recombinant ATM incubated with GST-p53 substrate and 0.75, 1.5, 3.125, 6.25, 12.5, 25, or 50 nM of each homeodomain protein and hydrogen peroxide, as indicated. Reactions in lanes 3 and 4 contained 50 nM homeodomain protein. Reactions were separated by denaturing SDS-PAGE, and phosphorylation of Ser15 in a GST-p53 substrate was detected with a phospho-specific antibody.

(B) Quantification of replicates (n > 4) of ATM kinase assays shown in (A).

(C) Diagrams of homeodomain proteins with C-terminal and N-terminal truncations.

(D) Quantitation of ATM kinase assays performed as in (A) with 50 nM of C-terminally truncated homeodomain proteins, the homeodomain only of NKX2.2, or purified HisMBP only in the presence of 0.008% hydrogen peroxide with three replicates.

(E) HCT116 Flp-In T-Rex cells with NKX3.1-Flag were induced with 10 μM doxycycline for 16 hr before treatment with hydrogen peroxide for the indicated time points.

(F) HCT116 Flp-In T-Rex cells were transiently transfected with an ATM expression construct. After 48 hr, cells were induced to express NKX3.1-Flag with 10 μM doxycycline...
for 16 hr before treatment with menadione for 30 min at the indicated doses. Cells were harvested and analyzed for substrate phosphorylation as indicated.

(G) ATM kinase assays were performed In Vitro in triplicate with purified recombinant ATM, 50 nM NKX3.1, 0.008% hydrogen peroxide, and 175, 350, or 700 pM of DNA. For all assays, error bars indicate SEM. The asterisk (*) denotes p < 0.05 of Student’s two-tailed t test in comparison to basal levels.
Figure 2. Homeodomain Proteins Inhibit MRN and DNA-Mediated Stimulation of ATM Kinase Activity *In Vitro*

(A) ATM kinase reactions were performed *In Vitro* with purified, recombinant ATM, 4 nM (‘+’) or 32 nM (‘++++’) MRN, DNA, and GST-p53 substrate with 12.5, 25, or 50 nM of full-length NKX3.1. Reactions were separated by denaturing SDS-PAGE, and phosphorylation of Ser15 in a GST-p53 substrate was detected with a phospho-specific antibody.

(B) ATM kinase assays as in (A) with 50 nM NKX3.1, 16 nM MRN, and 1, 5, 10 or 20 ng (175, 350, or 700 pM) of linearized plasmid DNA.

(C) ATM kinase assays as in (A) but with 50 nM NKX3.1, 16 nM MRN, and 50, 100, 200, or 400 nM GST-p53 peptide as indicated.

(D) ATM kinase reactions with ATM, 16 nM MRN, DNA, and GST-p53 substrate and 12.5, 25, or 50 nM of each homeodomain protein as indicated.

(E) Quantification of triplicates of ATM kinase assays shown in (D).

(F) 50 nM CDX2 full-length, C-terminal truncation of CDX2, or the homeodomain only of NKX2.2 with HisMBP epitope in ATM kinase assays as in (D) in the presence of MRN and DNA. Error bars indicate the SEM. The asterisk (*) denotes p < 0.05 of Student’s two-tailed t test in comparison to basal levels.
Figure 3. CDX2 Disrupts Functional ATM/MRN Interactions and Prevents ATM Monomerization

(A) Purified Bio-Flag ATM (30 nM) was incubated with 10, 50, or 250 nM HisMBP-CDX2ΔCTD protein and magnetic streptavidin-coated beads. Bound protein was isolated, separated by denaturing SDS-PAGE, and analyzed by western blotting with anti-MBP antibody.

(B) Purified Bio-MRN (25 nM) was incubated with 10, 50, or 250 nM HisMBP-CDX2ΔCTD and magnetic streptavidin-coated beads. Bound protein was analyzed as in (A) with anti-MBP antibody.

(C) Purified Bio-Flag ATM (30 nM) was incubated with 50 or 250 nM of HisMBP-CDX2ΔCTD protein and 100 nM GST-p53 substrate with magnetic streptavidin-coated beads as indicated. Bound protein was analyzed as in (A) with anti-MBP and anti-GST antibodies as indicated.

(D) Purified Bio-Flag-ATM (30 nM) was incubated with recombinant MRN complex (25 nM) and HisMBP-CDX2ΔCTD protein (10, 50, or 250 nM) with magnetic streptavidin-coated beads. Bound protein was analyzed as in (A) with anti-MBP and anti-MRN antibodies as indicated.

(E) Purified Bio-Flag-ATM (30 nM) was incubated as in (D) in the presence of GST-p53 substrate protein (100 nM). Bound protein was analyzed as in (A) with anti-MBP, anti-MRN, and anti-ATM antibodies as indicated.
(F) Purified Bio-MRN (25 nM) was incubated with 10, 50, or 250 nM HisMBP-NKX3.1DCTD and magnetic streptavidin-coated beads. Bound protein was analyzed as in (A) with anti-MBP, Rad50, and Nbs1 antibodies.

(G) Purified Bio-Flag-ATM (30 nM) was incubated with recombinant MRN complex (25 nM) and HisMBP-CDX2 DCTD protein (10, 50, and 250 nM) with magnetic streptavidin-coated beads. ‘+++’ indicates 250 nM HisMBP-NKX3.1ΔCTD. Bound protein was analyzed as in (A) with anti-MBP and anti-MRN antibodies as indicated.

(H) Purified Bio-Flag-ATM/HA-ATM dimeric complex was incubated with magnetic streptavidin-coated beads. After washing, purified MRN complex was added in the presence or absence of CDX2 ΔCTD protein (10, 50, or 250 nM). After incubation and isolation of biotinylated ATM, the supernatant containing monomeric ATM was separated by denaturing SDS-PAGE and analyzed by western blotting with anti-HA, anti-Rad50, and anti-MBP antibodies as indicated.
Figure 4. CDX2 Attenuates ATM Signaling in Colon Cancer Cells

(A) Representative images (top) of U2OS cells containing nuclear CDX2-mCherry and eGFP-CtIP foci in the presence or absence of CPT treatment. Quantification (bottom) of total cells showing greater than five co-localized CDX2 and CtIP foci in the nucleus in the absence or presence of CPT. Three independent replicates with N > 50 cells for each condition were analyzed.

(B) Endogenous ATM in HCT116 Flp-In cells containing inducible Flag-CDX2, Flag-NKX3.1, or pcDNA5 vector only was isolated by immunoprecipitation with anti-ATM antibody and coimmunoprecipitated proteins were analyzed by western blotting with anti-Flag antibody.

(C) HCT116 Flp-In T-Rex cells were induced for CDX2-Flag expression for 16 hr with 10 μM doxycycline, and then treated with 2.5, 5, or 10 μM of etoposide (ETP) for 30 min. Total
cell lysates were prepared and analyzed for phosphorylation of ATM-dependent targets as indicated.

(D) HCT116 Flp-In T-Rex cells were induced for CDX2-Flag expression for 16 hr with 10 μM doxycycline, and then treated with 2.5, 5, or 10 μM of camptothecin (CPT) for 30 min and analyzed as in (C).

(E) HCT116 Flp-In T-Rex cells with inducible CDX2-Flag, NKX3.1-Flag, or pcDNA5 were incubated for 16 hr with 10 μM doxycycline, and then treated with 10 μM CPT for 30 min and analyzed as in (C).

(F) Quantification of normalized pKap1/Kap1 ratio with triplicate biological replicates as in (E).

(G) HCT116 Flp-In T-Rex cells were induced for CDX2-Flag expression for 16 hr with 10 μM doxycycline, pre-treated with PIKK inhibitors (10 μM NU7441 DNA-PK inhibitor or 100 μM KU 55933 ATM inhibitor) for 1 hr; media was changed; and cells were then treated for 30 min with 10 μM CPT.

(H) HCT116 Flp-In T-Rex cells containing inducible CDX2 or vector only were infected with lentivirus containing the AID-ER-AsiSI construct and stably selected with puromycin. Cells were induced for CDX2 expression for 16 hr with 10 μM doxycycline before treatment with 4OHT to induce translocation of AsiSI in the nucleus for 4 hr. Media was changed and supplemented with auxin to degrade remaining AsiSI, and colonies formed after 12 days were counted. Cells were plated in triplicate. For all graphs in this figure, error bars represent SD. The asterisk (*) denotes p < 0.05 of Student’s two-tailed t test in comparison to untreated cells.
Figure 5. Mutation of the CDX2 Homeodomain Blocks Inhibitory Effect on ATM Kinase Signaling

(A) Crystal structure of CDX2 bound to methylated DNA PDB: 5LTY (Yin et al., 2017).

(B) ATM kinase assay as in Figure 2D with wild-type CDX2ΔCTD and mutant CDX2ΔCTD R214N proteins (12.5, 25, and 50 nM, respectively). Reactions were separated by denaturing SDS-PAGE, and phosphorylation of Ser15 in a GST-p53 substrate was detected with a phospho-specific antibody; total substrate was detected with anti-GST antibody.

(C) Quantification of 3 ATM kinase assays as shown in (B). Error bars indicate SD. The asterisk (*) denotes p < 0.05 of Student’s two-tailed t test in comparison to basal levels.

(D) Gel mobility shift assays with wild-type and R214N CDX2ΔCTD proteins (16, 23, 35, 53, 79, 119, 178, 267, and 400 nM, respectively) on a double-stranded Cy3-labeled DNA duplex containing a CDX2 consensus site.

Cell Rep. Author manuscript; available in PMC 2018 September 07.
(E) Purified BioFlag-ATM was incubated with MRN complex and increasing amounts of CDX2ΔCTD wild-type or CDX2ΔCTD R214N protein, and then isolated with magnetic streptavidin-coated beads. Bound proteins were isolated, separated by denaturing SDS-PAGE, and analyzed by western blotting with antibodies directed against ATM, MRN components, or MBP.

(F) Table summarizing the effect of 50 nM of each CDX2 mutant on ATM in MRN/DNA kinase assay as in (B) and apparent K_D of each mutant binding to Cy3-labeled oligonucleotide containing CDX2 binding consensus sequence as in (D).

(G) HCT116 Flp-In T-Rex cells with CDX2-Flag or CDX2 R214N-Flag were induced with 10 μM doxycycline for 16 hr before treatment with 5 μM CPT for 30 min.

(H) Quantification of normalized phospho-Kap1 signal from triplicate experiments as in (G). Cells were plated in triplicate. Error bars represent SD. The asterisk (*) denotes p < 0.05 of Student’s two-tailed t test in comparison to cells without doxycycline.

(I) Schematic depicting differentiation of mouse embryonic stem cells upon deprivation of LIF factor (top). Mouse embryonic stem cells (mESCs) grown in either LIF+ or LIF− media to induce differentiation were treated with increasing CPT doses for 30 min before harvesting, SDS-PAGE, and western blotting (bottom).

(J) mESCs in LIF+ media were treated with ATM inhibitor (KU55933, 100 μM) or DNA-PK inhibitor (NU 7441, 10 mM) for 1 hr before 30 min of damage treatment with 1 μM CPT before harvesting, SDS-PAGE, and western blotting.