Homeodomain Proteins Directly Regulate ATM Kinase Activity

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

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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.
Homeodomain Proteins Directly Regulate ATM Kinase Activity

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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.

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+ prostate cells are prone to formation of precancerous prostatic intraepithelial (PIN) lesions that increase in severity with aging of the animal (Asatiani et al., 2003; Biberich 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 homeodomain 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-Pouler 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 homeodomain 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 His-MBP 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 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 overexpressing 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 nucleus. 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 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 effect 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 pretreatment 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 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.

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 communoprecipitated 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 camptothecin (CPT) for 30 min and analyzed as in (C).

(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 containing 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.
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 4-OHT 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 (Giggin 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 blastocoel 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 5). 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 overexpression 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 homeodomain 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 thesame factors to change the effects of CDX2 on ATM activity.

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 (Vermezoic 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 pN01 plasmid cut with Affil 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_{2}O_{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 7.7, 70 mM sodium chloride, 5 mM magnesium chloride, 1 mM ATP, 0.35 mM DTT, and 50 μg/mL BSA [New England Biosciences]) for 60 min at 37°C in a volume of 10 μL. Phosphorylation of Ser15 of the GST-p53 peptide was detected via immunoblotting using a phosphospecific antibody (Calbiochem; PC461) as described previously (Lee and Paul, 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 μL 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-cholamidopropyldimethylammonio]-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 2.5× 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 uM 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 x g for 2 min. Cells were lysed with cell lysis buffer, and lysates were clarified at 10,000 x 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; GTX70222), 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 680-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-TAATCTCTCTGTTTATAGCTCTGACCT) annealed to its complementary DNA strand TP5922. Protein/DNA mixtures were separated on an 8% acrylamide native PAGE gel run in 0.5x 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.

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.

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AUTHOR CONTRIBUTIONS
T.E.J. performed all experiments with the exception of ATOM monomerization assays, which were performed by J.-H.L., and confocal imaging of cells, which was performed by L.R.M. and 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 BioBricks 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.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

Homeodomain Proteins Directly Regulate
ATM Kinase Activity

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Figure S1, related to Figure 1: SDS-PAGE analysis of recombinant proteins and multiple sequence alignment of homeodomain proteins. (A) Recombinant full-length homeodomain proteins with HisMBP epitope tag on the N-terminus and Flag epitope on the C-terminus were separated by SDS-PAGE and stained with Coomassie blue. (B) Recombinant C-terminal truncation homeodomain proteins with HisMBP epitope tag on the N-terminus were separated by SDS-PAGE and stained with Coomassie Blue. (C) Amino acid sequences of NKX3.1, NKX2.2, NKX2.1, TTF1, CDX2, and HOXB7 were aligned by the T-COFFEE Multiple Sequence Alignment Server and visualized with BoxShade program. Residues rendered in black are the consensus residue, and residues rendered in gray are chemically similar to the consensus residue.
Figure S2, related to Figures 1 and 2: Heterologous DNA-binding proteins do not affect ATM kinase activity. (A) Kinase assays were performed as in Fig. 1A in the main text with 12.5 nM, 25 nM, 50 nM, or 250 nM purified Lac Repressor. (B) ATM kinase assays were performed as in Fig. 2D in the main text with 6.25, 12.5 nM, 25 nM, or 50 nM purified Lac Repressor protein.
Figure S3, related to Figure 2. The ATM kinase assay was performed with purified recombinant ATM, 4 nM MRN, 70 pM DNA, and GST-p53 substrate with 12.5, 25, or 50 nM of full length homeodomain proteins or the homeodomain only of NKX2.2 as indicated. Reactions were separated by denaturing SDS-PAGE and phosphorylation of Ser15 in a GST-p53 substrate was detected with a phospho-specific antibody. Quantification of replicates (n >4) of ATM kinase assays are shown. Error bars indicate the SEM. * denotes p < 0.05 of Student’s two tailed T test in comparison to basal levels.
Figure S4, related to Figure 5: Intact homeodomains are important for effects on ATM. (A) ATM kinase reactions were performed in vitro with purified, recombinant ATM, 32 nM MRN, 70 pM DNA, and GST-p53 substrate with 12.5, 25, or 50 nM of Δ151-160 ΔCTD NKX3.1 or 50 nM ΔCTD 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 reactions were performed under oxidative conditions in vitro as in Fig. 1A with ATM, GST-p53 substrate, and 12, 25, or 50 nM CDX2 R214N ΔCTD protein, as indicated. "+++" indicates 50 nM CDX2 R214N ΔCTD. 70 pM DNA was added in lane 5. Phosphorylation was detected as in (A).
Figure S5, related to Figure 5: Model of the effect of homeodomain proteins on ATM kinase function. In the presence of a DSB, homeodomain proteins compete with MRN for binding to ATM and reduce the amount of active ATM. Simultaneously, homeodomain proteins stabilize the noncovalent dimer and prevent monomerization by the MRN complex. With activation via reactive oxygen species, stabilization of the ATM dimer by homeodomain proteins may result in a more rapid transition to an active covalent dimer. Alternatively, homeodomain binding to ATM results in a hyperactive conformation of the ATM covalent dimer.
Supplemental Experimental Procedures

Plasmid Construction

Invitrogen™ Gateway™ entry vectors were obtained for each homeodomain protein (DNASU). The LR Clonase II Enzyme Mix (ThermoFisher) was used to insert each gene into destination vectors, pDEST-HisMBP (a gift from David Waugh; Addgene plasmid #11085) or pDEST-Flag (a gift from Kyle Miller). For mammalian cell culture, homeobox genes in pDEST-Flag were moved into a pcDNA™5/FRT/TO (ThermoFisher) vector using restriction digest and re-ligation (NKX3.1 pTP3330, CDX2 pTP3257). For protein expression in E. coli, homeobox genes in pDEST-HisMBP were mutated to have an AflII restriction enzyme at the C-terminus site via QuikChange and a Flag epitope sequence was inserted via linker cloning (NKX3.1 pTP2762, CDX2 pTP2932, NKX2.2 pTP2799, HOXB7 pTP2821, TTF1 pTP2814, NKX2.5 pTP2813). C-terminal truncations of homeodomain proteins were made by introducing three stop codons with QuikChange (Agilent Technologies) after the homeodomain coding regions of each gene or inverse PCR was used to remove the C-terminus (NKX3.1(S185X) pTP3110, CDX2(K248X) pTP3118, NKX2.2(A186X) pTP3121, HOXB7(K197X) pTP3117, TTF1/NKX2.1(Q249X) pTP3601, NKX2.5(Q196X) pTP3122). Inverse PCR with the Q5® Site-Directed Mutagenesis Kit (New England Biolabs) was used to delete the N-terminus of NKX2.2 ΔC to generate homeodomain only, pTP3599. CDX2 point mutations were made using QuikChange into the CDX2 C-terminal truncation construct (E218Q pTP4034, H196W pTP4035, T212I pTP4036, Y222A pTP4037, H206N pTP4038, R214N pTP4039, T222A pTP4040, L199D pTP4041).

Wild-type Flag-ATM was a gift from M. Kastan, and HA-ATM was a gift from R. Abraham. Mre11, Rad50, Nbs1, and GST-p53 (1-102) peptide expression constructs were previously described (Bhaskara et al., 2007; Paull and Gellert, 1998, 1999). The plasmid encoding AID-ER-AsiSI was made by moving a cassette containing AID-ER-AsiSI (a gift from Gaelle Legube) into a derivative of lentiCRISPRv2 (a gift from Feng Zhang; Addgene plasmid # 52961) to make pTP4006. A pcDNA5-CDX2-Flag-mCherry (pTP4254) construct was the product of Gibson Assembly kit (New England Biosciences, E2611L) from two PCR products. The mCherry sequence was amplified from the pTP2750 plasmid with TP6384/6385 primers, and TP6387/6398 primers were used to amplify pTP3257. This served to insert the mCherry sequence C-terminal to the CDX2 coding region to create pTP4254. All constructs and mutations were confirmed by DNA sequencing. Details of plasmid construction available upon request.

Protein Expression and Purification

Expression plasmids were transformed into the BL21 expression strain of E. coli and grown in liquid culture under selection and induced with IPTG to a volume of 4 L. Bacterial cultures were spun at 4,000 xg for 10 minutes, washed with phosphate buffered saline, and spun at 4,000 xg for 10 minutes. Pellets were frozen in liquid nitrogen and stored at -80 °C. Pellets were thawed, resuspended, and lysed in 40 ml of buffer A (25 mM Tris-HCl pH 8.0, 10% glycerol, 1 mM dithiothreitol (DTT)) containing 1 M NaCl, 10 mM EDTA, 0.25% Tween® 20 (Sigma), 75 mg lysozyme, and 1 mM PMSF. The cell lysate was sonicated and ultracentrifuged at 125,000 xg rpm for 1 hour at 4 °C. The supernatant was incubated with amylose resin at 4 °C. Beads were washed and protein eluted with 10 mM maltose in Buffer A containing 100 mM NaCl and 0.25% Tween® 20. The resulting elution was incubated with Flag M2 magnetic beads (Sigma,
M8823-1ML) at 4 °C. Beads were washed with Buffer A containing 100 mM NaCl and eluted with 0.1 mg/ml 3x Flag peptide (Sigma) in Buffer A. C-terminal truncation proteins and homeodomain only mutants were purified using a similar protocol. However, rather than using magnetic flag beads, the amylose elution was bound to a HiTrap SP HP column (GE Healthcare, 17-1151-01). The column was washed with Buffer A, and proteins were eluted using a salt gradient.

GST-p53 (1-102) peptide was expressed in E. coli and purified as described previously (Lee and Paull, 2004). Mre11, Rad50, and Nbs1 expression constructs were used to make baculovirus that was co-expressed in S21 insect cells as described previously (Bhaskara et al., 2007). From these cells, MRN complex was purified following previous protocols (Bhaskara et al., 2007). Recombinant Flag-ATM expression construct was transfected into 40 to 60 150 mm dishes of human 293T cells (ATCC) using calcium phosphate transfection and purified as described previously (Lee and Paull, 2006). Bio-Flag-ATM was co-expressed with pCMV-myc-nuc-eGFP-BirA (a gift from Mauro Modesti) in 293T cells and purified using the same protocol. Bio-Flag-ATM/HA-ATM dimeric preparation was expressed in Sf21 insect cells and purified over anti-Flag and anti-HA resin sequentially as described previously (Lee and Paull, 2004). All purified proteins were run on SDS- PAGE gels with standards and stained with Coomassie blue. Quantification of proteins was performed using the Odyssey imaging system (LiCor).

**Mammalian Cell Culture:**

HCT116 Flp-In™ T-REx™ cells (gift, Daniel Durocher) were grown in DMEM (Invitrogen, 11995073) with 10% Tet System Approved FBS (Clontech, 631367) with 100 µg/ml Zeocin™ (Invivogen) and 5 µg/ml blasticidin (Life Technologies). Cells were co-transfected with pOG44 and each of the homeodomain expression constructs in pcDNA5/FRT/TO and grown under selection according to manufacturer instructions. Human 293T cells were grown in DMEM (Invitrogen, 11995073) with 10% FBS (Gemini Bio-Products, 900-208).

The coimmunoprecipitation experiment was performed with a 150 mm dish of HCT116 Flp-In™ T-REx™ cells with inducible CDX2-Flag, NKX3.1-Flag, or pcDNA5 vector only were induced for 16 hours with 10 µg/ml doxycycline (Dox) for 16 hours before being treated for 1 hour with 10 µM camptothecin (CPT, Sigma C9911-100MG). Cells were washed with phosphate-buffered saline (PBS) and lysed with 1 ml of cell lysis buffer (8 M urea, 50 mM Tris, pH 8.0, 5 mM CaCl₂, 30 mM NaCl, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, protease inhibitors (Pierce, 88666) and phosphatase inhibitors (Pierce, 88667)). Lysates were sonicated for 20 s on ice then centrifuged at 16,100 xg for 10 min. Protein concentration was quantitated using the Bradford assay with Coomassie Plus Protein Assay Reagent (Pierce, 23236), and volumes adjusted to normalize total protein. 10 µl of ATM antibody (Santa Cruz, sc-135663) were incubated with the cleared lysate for 3 hours at 4 °C. 10 µl of protein magnetic A/G beads (Pierce, 88803) were then added and incubated for 1 hour at 4 °C. Beads were washed 3 times with cell lysis buffer then boiled for 10 min. in 2.5 X SDS loading buffer. Immunoprecipitated material was separated by denaturing SDS-PAGE and analyzed by Western blotting.

Mouse embryonic stem cells were grown in DMEM with high glucose (Gibco) supplemented with 20% FBS, 1 mM sodium pyruvate, 50 µg/mL penicillin/streptomycin, 200 µM L-glutamine, 100 µM non-essential amino acids (Gibco) and 0.7% β- mercaptoethanol with Leukemia Inhibitory Factor (LIF). To induce differentiation, cells were grown in media containing every reagent without LIF. Cells were grown on plates coated with 0.1% gelatin.
**Fluorescence Microscopy:**

U2OS Flp-In™ T-REx™ cells containing an eGFP-CtIP construct stably incorporated in the Flp-In site were seeded on microscopy dishes and grown to 50% confluency. Cells were transfected with CDX2-mCherry using Fugene HD (Fisher Scientific, E2311) and induced with doxycycline (4 μM) for 48 hours before treatment. Cells were treated with 1 μM camptothecin (CPT, Sigma C9911-100MG) for 2 hours and allowed to recover for 2 hours before imaging. Cells were imaged using a Zeiss LSM 710 confocal microscope controlled by Zen software. Images were analyzed using ImageJ.

**Supplemental References**

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