ID3 regulates the MDC1-mediated DNA damage response in order to maintain genome stability

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MDC1 plays a critical role in the DNA damage response (DDR) by interacting directly with several factors including γ-H2AX. However, the mechanism by which MDC1 is recruited to damaged sites remains elusive. Here, we show that MDC1 interacts with a helix-loop-helix (HLH)-containing protein called inhibitor of DNA-binding 3 (ID3). In response to double-strand breaks (DSBs) in the genome, ATM phosphorylates ID3 at serine 65 within the HLH motif, and this modification allows a direct interaction with MDC1. Moreover, depletion of ID3 results in impaired formation of ionizing radiation (IR)-induced MDC1 foci, suppression of γ-H2AX-bound MDC1, impaired DSB repair, cellular hypersensitivity to IR, and genomic instability. Disruption of the MDC1-ID3 interaction prevents accumulation of MDC1 at sites of DSBs and suppresses DSB repair. Thus, our study uncovers an ID3-dependent mechanism of recruitment of MDC1 to DNA damage sites and suggests that the ID3–MDC1 interaction is crucial for DDR.
The integrity of genomic DNA is challenged by genotoxic insults that originate from either normal cellular metabolism or external sources. To ensure proper maintenance of genomic integrity, eukaryotes have evolved a DNA damage response (DDR) system that senses damage and transduces this information within the cell in order to orchestrate DNA repair, cell-cycle checkpoints, chromatin remodeling and apoptosis. The functional importance of DDR in maintaining genomic integrity is highlighted by the fact that it is conserved among eukaryotes. Mutations that disrupt the activity of DDR components contribute directly to tumorigenesis; therefore, it is important to understand these complex mechanisms at the molecular level to further our understanding of cancer progression and treatment.

DNA double-strand breaks (DSBs), which are generated through ionizing radiation (IR) and through various DNA-damaging chemicals, are the most dangerous DNA lesions, because if they are not efficiently and accurately repaired, they can result in mutations, genomic rearrangements, and cell death, which can lead to cancer. The ability of cells to detect and properly repair DSBs is therefore essential for maintaining genome stability and preventing cancer. Central to the DSB checkpoint response is ATM protein kinase, which, when activated by DSBs, initiates a signaling cascade that starts with phosphorylation of the histone variant H2AX (γ-H2AX) at DSB sites, and is followed by recruitment of upstream factors including MDC1. MDC1 functions as an assembly platform to help localize and maintain signaling and repair factors at and around DSB sites. In this role, MDC1 amplifies DNA damage signals by binding to phosphorylated H2AX and subsequently binding and retaining additional DDR factors at sites of DNA damage. The accumulation of these DDR factors at DSB sites is generally believed to facilitate DNA damage repair and checkpoint control. Thus, MDC1 has been recognized as the “master regulator” that modulates a specific chromatin microenvironment required to maintain genomic stability.

MDC1-knockout (KO) mice show chromosomal instability, defects in DSB repair, radiosensitivity, and cancer predisposition. Furthermore, downregulation of MDC1 is associated with multiple cellular phenotypes including hypersensitivity of cells to DSBs, improper activation of the G2/M and intra-S checkpoints, aberrant activation of DNA damage-induced apoptosis, and inefficient phosphorylation of DDR regulatory proteins. It has been suggested that, in addition to its central role in the DDR, MDC1 directly mediates HR, and non-homologous end joining (NHEJ), activation of the decatenation checkpoint, regulation of the DNA replication checkpoint, mitosis, and spindle assembly checkpoint.

Clearly, MDC1 is quickly recruited to DNA damage sites, allowing multiple protein–protein interactions that are crucial for proper DDR processes. However, the precise mechanisms by which MDC1 is recruited to protect cells from the deleterious effects of DNA damage are not fully understood. The current study was initiated with the goal of better understanding how MDC1 is recruited to DNA damages sites and how the role of MDC1 in DDR is regulated in response to DNA damage. Since a tandem BRCA1 C-terminal (tBRCT) domain of MDC1 is essential for recruitment of MDC1 to DNA damage sites, we screen for tBRCT domain of MDC1-associated proteins and identify a helix-loop-helix (HLH) domain-containing protein called inhibitor of DNA-binding 3 (ID3), which we propose interacts directly with MDC1 and is a key factor in the interaction of MDC1 with γ-H2AX, recruiting MDC1 to DSB sites and regulating DDR function of MDC1.

Results

MDC1 interacts with ID3. Although the role of mammalian MDC1 in the DDR is well documented, its regulation and underlying mechanism of action are only partially understood. In order to better characterize the regulatory network relevant to MDC1 and to gain further insight into the molecular mechanism of action of MDC1 in the DDR, a yeast two-hybrid screen was performed using a HeLa cDNA plasmid library with the C-terminal fragment (amino acid 1882–2082) of human MDC1 as the bait. Out of the 2.6 × 10^7 transformants that were screened, 43 independent positive clones were isolated. When cDNA from each of the positive clones was sequenced, one was identified as 53BP1, a protein known to associate with the tandem BRCA1-C terminus (tBRCT) domain of MDC1. The other clones, which had no previously identified association with MDC1, encoded ID3 (NM_002167), PIA1 (NM_016166), UBE2I (NM_194259), KPNA2 (NM_0022266), ZNF114 (NM_153680), KIFC1 (NM_002263), CASP8AP2 (NM_001137667), C2orf44 (NM_025203), SRSF11 (NM_01190987), TINP1 (NM_014886), GPRC5C (NM_022036), and WWCI (NM_015238). Among these, DNA-binding protein inhibitor ID3 was particularly notable because this HLH-containing protein has been shown to activate a DNA repair process, and, correspondingly, when ID3 is inactivated, excess DNA damage accumulates.

To verify that an interaction between MDC1 and ID3 occurs in human cells, we used co-immunoprecipitation assays followed by western blotting to assess protein–protein interactions. As shown in Fig. 1a, b, endogenous MDC1 and ID3 co-immunoprecipitated reciprocally and, although the association occurred in non-irradiated cells, it was enhanced in response to DNA damage. To examine this further, we expressed HA-tagged MDC1 and GFP-tagged ID3 ectopically in HEK293T cells and attempted to immunoprecipitate MDC1 using anti-HA antibody. As shown in Fig. 1c, GFP-ID3 was observed in the anti-HA immunoprecipitate. Reciprocally, HA-MDC1 was immunoprecipitated together with GFP-ID3 using anti-GFP antibody (Fig. 1d) and these interactions were more pronounced following exposure to IR. This protein–protein interaction did not require the presence of DNA, as neither ethidium bromide nor DNase affected the co-immunoprecipitation (Supplementary Fig. 1a). Furthermore, ID3 appears to be the only member of the ID family of proteins with which MDC1 interacts, because we were unable to detect any interaction of MDC1 with ID1 or ID2 (Supplementary Fig. 1b).

To confirm that the tBRCT domain of MDC1 is the location at which ID3 binds, we examined the interaction between these two proteins using a series of internal deletion mutations of MDC1 (Supplementary Fig. 2a). We found that when expressed in HEK293T cells, all MDC1 mutants co-immunoprecipitated with GFP-ID3 except for a mutant lacking tBRCT domain (amino acids 1893–2082) (Supplementary Fig. 2b), indicating that this domain is indeed required for binding to ID3. To further analyze direct physical associations between ID3 and MDC1, a GST fusion to the tBRCT domain of MDC1 was made and used in a pull-down assay with IR-treated HeLa cell lysates. Immunoblotting of the pull-down samples revealed that the tBRCT domain of MDC1 interacts with ID3 (Fig. 1e); this polypeptide also pulled down the exogenously expressed GFP-tagged ID3 (Fig. 1f), confirming that ID3 directly binds to this region of MDC1 in vitro.

ATM-dependent and MDC1-dependent phosphorylation of ID3. To examine the potential role of ID3 in regulating DDR, we asked whether IR exposure induced phosphorylation of ID3. ATM/ATR-like kinases convey signals upstream of the DDR pathway by preferentially phosphorylating substrate proteins on either a serine or threonine residue that is typically followed by a glutamine residue in the so-called serine–glutamine (SQ) or threonine–glutamine (TQ) motif. We identified two...
putative ATM/ATR phosphorylation motifs in the ID3 amino acid sequence, threonine 62 (Thr62) and serine 65 (Ser65), both located in the conserved HLH domain (Fig. 2a). A sequence alignment of the human ID family of proteins, including ID1, ID2, and ID3, revealed that ID3 is the only member that possesses TQ (including Thr62) and SQ (including Ser65) motifs in the Helix2 domain, consistent with our earlier observation that ID3 is the only of the three family members relevant to this pathway. To test whether DNA damage induces phosphorylation of ID3, we immunoprecipitated ID3 from HeLa cells and examined phosphorylation levels before and after cells had been exposed to IR. Using antibodies that bind to phosphorylated serine or threonine, we showed that in IR-induced cells, serine was phosphorylated, but threonine was not (Fig. 2b). Western blot analysis, using lysates prepared from both control and irradiated cells and polyclonal rabbit antipSer65–ID3 antibody, confirmed that IR treatment enhanced the phosphorylation of endogenous ID3 at Ser65 (Fig. 2c). Recognition of pSer65–ID3 by this antibody was blocked by the presence of phosphorylated peptides but not by non-phosphorylated peptides (Supplementary Fig. 3a), and the recognition was inhibited by ID3 siRNA (Supplementary Fig. 3b), therefore used KU55933, an inhibitor of ATM kinase, and DNA-dependent protein kinase (DNA-PK) are largely responsible for initiating phosphorylation of ID3. ATM kinase and DNA-dependent protein kinase (DNA-PK) are largely responsible for initiating phosphorylation of ID3. We compared normal and MDC1-depleted HeLa cells and observed that IR-induced ID3 phosphorylation was attenuated when MDC1 was expressed at lower levels (Fig. 2e). Together, these results suggest that ATM and MDC1 act together in mediating phosphorylation of ID3 Ser65 in response to DNA damage in vivo.

Recruitment of phosphorylated ID3 to sites of DNA damage.

The finding that ID3 is phosphorylated at residue Ser65 after exposure to IR suggests that this form of ID3 may localize to site of DNA damage. To identify the cellular location of phosphorylated ID3, we used immunofluorescence staining with anti-pSer65–ID3 antibody. As shown in Fig. 3a, in IR-treated HeLa cells, foci of pSer65–ID3 were detected, and these foci co-localized with γ-H2AX, an intracellular protein that associates with damaged DNA. The staining of pSer65–ID3 was specific, because the number of foci decreased in the presence of a competing phosphorylated peptide but not in the presence of a non-phosphorylated peptide (Supplementary Fig. 3c). Because both ATM and MDC1 affect the phosphorylation of ID3, we looked for a role for either of these proteins on pSer65–ID3 foci formation. In the presence of the ATM inhibitor KU55933, a few IR-induced pSer65–ID3 foci were observed, whereas in the presence of NU7026, the DNA-PK inhibitor that had no effect in a

**Fig. 1** Protein–protein interactions between MDC1 and ID3. a HeLa cells, with or without exposure to IR, were collected after 3 h, and whole-cell lysates were subjected to immunoprecipitation using an anti-MDC1 antibody followed by western blotting using the antibodies indicated to the right of the blot. b HeLa cells were prepared as in a, and lysates were subjected to immunoprecipitation using an anti-ID3 antibody followed by western blotting using the antibodies indicated to the right of the blot. c HA-tagged MDC1 and GFP-tagged ID3 were co-transfected into HEK293T cells and exposed to IR. After 3 h, whole-cell lysates were subjected to immunoprecipitation using an anti-HA antibody followed by western blotting using the antibodies indicated to the right of the blot. d HEK293T cells were prepared as in c, and lysates were subjected to immunoprecipitation using an anti-GFP antibody followed by western blotting using the antibodies indicated to the right of the blot. e, f A GST-tagged fragment of MDC1 or a GST bead alone was incubated with total cell lysates from HeLa cells exposed to IR (e) or from GFP-ID3 transfected HEK293T cells exposed to IR (f). GST pull-downs were immunoblotted with antibodies as indicated. Uncropped blots of this figure accompanied by the location of molecular weight markers are shown in Supplementary Fig. 14.
previous experiment, the number of foci was comparable to the control, as expected (Fig. 3b). Likewise, in MDC1-depleted cells exposed to IR, accumulation of pSer65–ID3 at DSBs was strongly reduced (Fig. 3c, d), supporting the prediction that both MDC1 and ATM are required for efficient localization of ID3 under these conditions.

**ID3 is required for the recruitment of MDC1 to DSBs.** The above results, pointing to a biochemical interaction between ID3 and MDC1, prompted the prediction that ID3 is required for recruitment of MDC1 to DSBs. To test this hypothesis, we exposed control and ID3 knockdown HeLa and U2OS cells (Fig. 4a) to IR, and used immunofluorescence staining to detect MDC1 at various time points. As shown in Fig. 4b, c, in control siRNA-transfected cells, MDC1 foci formed rapidly in response to IR treatment and the percentage of cells containing a notable number of foci increased steadily for up to 1 h. In contrast, cells transfected with ID3-targeted siRNA had significantly fewer MDC1 foci and the percentage of cells with foci remained low throughout the entire time course. We also observed that HeLa cells with a stable knockdown of ID3, created using two different shRNAs, had dramatically less recruitment of MDC1 to DNA damage sites (Supplementary Fig. 4a, b). Similar results were obtained when DNA damage was induced in HeLa cells using the radiomimetic neocarzinostatin (Supplementary Fig. 4c). Introduction of shRNA-resistant ID3 into cells depleted of endogenous ID3, whereas γ-H2AX, a protein that acts upstream in the DDR pathway, still formed foci (Fig. 4f, g).

To further confirm the critical role for ID3 in recruitment of MDC1 to nuclear foci after DNA damage, we generated ID3–KO human U2OS cells using the CRISPR/Cas9 genome-editing system (Supplementary Fig. 5a–c). Noticeably, IR-induced MDC1 foci were significantly reduced in ID3 KO cells than in control cells (Supplementary Fig. 6a). Moreover, ID3 KO impaired the recruitment of NBS1, BRCA1, 53BP1, RNF8, and RNF168, but not γ-H2AX, at DSBs after IR exposure (Supplementary Fig. 6b, c). Together, these data suggest that ID3 is required for localization of MDC1 at DSBs thereby facilitating recruitment of downstream factors.

**pSer65–ID3 is essential for IR-induced MDC1 foci formation.** Phosphorylation of SQ or TQ motifs leads to altered interactions between proteins in the DNA repair complex and between other checkpoint proteins. Furthermore, proteins with BRCT domains, like MDC1, have been shown to recognize phospho-

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Ser/Thr motifs. Therefore, we predicted that phosphorylation of Ser65 within the HLH domain of ID3 is important for modulating the interactions between MDC1 and ID3. To test this, we generated GFP-tagged constructs that included the following versions of ID3: the HLH domain (amino acids 25–81), a mutant lacking the carboxyl terminus (ΔC; deletion of residues 82–119), and a mutant lacking the amino terminus (ΔN; deletion of residues 1–24) (Fig. 5a). ID3 from each of these constructs was co-expressed with full-length HA-tagged MDC1 in HEK293T cells. As shown in Fig. 5b, when the HLH domain was present, ID3 interacted with MDC1, but if the HLH domain was deleted, the interaction was impaired.

We then generated a point mutation within the HLH domain of ID3, replacing Ser65 with alanine (S65A). As a negative control, we exchanged Thr62 to alanine (T62A). As shown in Fig. 5c, the presence of the S65A mutation impaired interactions with MDC1, but the T62A mutation did not have a notable affect, suggesting that phosphorylation of ID3 at Ser65 is required for its interaction with MDC1. We next mutated Ser65 to aspartic acid (S65E), which mimics the phosphorylated state. This mutant interaction with MDC1. We next mutated Ser65 to aspartic acid (S65E), which mimics the phosphorylated state. This mutant interaction with MDC1 was abolished after treatment with phosphatase, supporting the conclusion that the interaction between ID3 and MDC1 is required for MDC1 to co-localize with sites of DNA damage.

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The BRCT domain of MDC1 is required for the formation of foci at sites of DNA damage. Because ID3 interacts with this same domain, and because depletion of ID3 decreased the formation of MDC1 foci, we hypothesized that a direct interaction between ID3 and MDC1 is required for MDC1 to co-localize with sites of DNA damage. Therefore, we predicted that phosphorylation of Ser65 within the HLH domain of ID3 is important for modulating the interactions between MDC1 and ID3. To test this, we generated GFP-tagged constructs that included the following versions of ID3: the HLH domain (amino acids 25–81), a mutant lacking the carboxyl terminus (ΔC; deletion of residues 82–119), and a mutant lacking the amino terminus (ΔN; deletion of residues 1–24) (Fig. 5a). ID3 from each of these constructs was co-expressed with full-length HA-tagged MDC1 in HEK293T cells. As shown in Fig. 5b, when the HLH domain was present, ID3 interacted with MDC1, but if the HLH domain was deleted, the interaction was impaired.

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be recruited to sites of DNA damage. We tested this using HeLa cells that had been transfected with ID3 shRNA in order to deplete the endogenous pool of protein, and then reconstituted with either an shRNA-resistant HLH domain (HLH ID3) or an HLH deletion mutant (ID3-ΔHLH) (Fig. 5e). The level of recruitment of HA-MDC1 to DSB sites in each cell type was then measured. As shown in Fig. 5f, there was significantly more recruitment of HA–MDC1 to DSBs in cells expressing HLH ID3 when compared to cells expressing ID3-ΔHLH, indicating that the HLH domain of ID3 is indeed involved in recruitment of...
MDC1. Moreover, immunofluorescence analysis suggested that when ID3 knockdown cells are reconstituted with either wild-type HLH domain of ID3 or the T62A HLH mutant, recruitment of MDC1 to DSBs occurred normally. But when reconstituted with the S65A HLH mutant, recruitment was significantly decreased (Fig. 5g,h). On the other hand, reconstitution of ID3 knockdown cells with the S65E HLH mutant, which mimics the phosphorylated state, restored MDC1 recruitment to normal levels. These results point to a requirement for a phospho-dependent interaction between MDC1 and ID3 in order for proper localization of MDC1 to sites of DNA damage.

**ID3 controls the interaction between γ-H2AX and MDC1.** The domains of MDC1 identified in this study as important for interactions with ID3 (Fig. 1) overlap with those previously identified as important for interactions with γ-H2AX. Therefore, we explored whether the lack of ID3 or H2AX would affect interactions between MDC1 and γ-H2AX. We found that in ID3 knockdown cells, but not H2AX knockdown cells, the association between MDC1 and γ-H2AX in response to IR irradiation was abolished (Fig. 6a,b), indicating that the interaction between MDC1 and γ-H2AX occurs downstream of the ID3–MDC1 interaction.

To more closely examine the structural differences in MDC1 when interacting with either ID3 or ID3 and γ-H2AX together, we used molecular dynamic (MD) simulations to compare the interactions. We observed that the most stable conformation of the MDC1–tBRCT domain, as derived from normal MD simulations, is similar to that determined through X-ray diffraction data, and the salt bridge network including Arg1933, Glu2063, and Thr2067 involved in the binding of γ-H2AX is also in agreement (Supplementary Fig. 8a). Interestingly, the distal C-terminal region of the tBRCT domain of MDC1 contains two positively charged lysine residues at positions 2071 (K2071) and 2075 (K2075) that are fully flexible, indicating that they may be relevant for binding to phospho-ID3. To explore this further, K2071 and K2075 were sequentially replaced with the neutral amino acid methionine in order to create both single-point and double-point mutants. The K2071M mutant bound to ID3-phosphorylated HLH (pHLH) equally as well as wild-type MDC1 (Fig. 6c). On the other hand, interactions between the K2076M mutant and ID3–pHLH were significantly decreased. Intriguingly, when both lysine residues were mutated, interactions were almost completely abolished. These results suggest a prominent role for K2075 in interactions with phospho-ID3, and perhaps a supportive role for K2071.

We then performed a docking simulation to more closely analyze the interactions between the tBRCT domain of MDC1 and the pHLH domain of ID3. Our simulation indicated the formation of a new hydrophobic core including Val67 of ID3 as a ligand and Phe1979, Ala2078, and Phe2079 of tBRCT as a receptor. The Cβ–methyl group of Thr2067 in tBRCT was located close to this cooperative hydrophobic core (Supplementary Fig. 8b). Thus, Arg1933 in the tBRCT domain, which is known to be involved in binding to the tail of γ-H2AX, was much more flexible when in a complex with pHLH than it is in tBRCT alone. Consequently, it is likely that the weakness in the salt bridge of the tBRCT–pHLH complex may allow for better capture of the γ-H2AX pentapeptide. Of note, the tBRCT–γ-H2AX–pHLH trimer contains a substantial salt bridge network with aggregation of hydrophobic side chains, including Phe1979, Ala2078, and Phe2079 of tBRCT and Val67 of pHLH (Fig. 6d), indicating that the stability of the salt bridge increased when γ-H2AX docked with the tBRCT–pHLH complex. Together, these results suggest a molecular mechanism by which the interaction of MDC1–tBRCT with ID3–pHLH increases binding affinity between MDC1–tBRCT and γ-H2AX.

**ID3 promotes DSB repair through its interaction with MDC1.** To define a possible role for ID3 in DDR, we first investigated whether cells lacking ID3 would be more sensitive to DNA damage. Cell viability, as measured by the clonogenic survival assay, was measured for HeLa cells with and without depletion of ID3 through introduction of each of two different shRNAs. Under normal conditions, the ID3 knockdown cells were equivalent to wild-type cells. However, the cells depleted in ID3 had a significant decrease in survival in response to IR (Fig. 7a). Moreover, the survival fractions of colonies following IR were much lower in ID3 KO cells than WT cells (Supplementary Fig. 6d).

We next investigated whether ID3 affects DSBs repair by measuring γ-H2AX staining and comet tail moments. We found that knockdown of ID3 in HeLa cells had significantly more residual DSBs than control cells, as evidenced by the increase in signal intensity of γ-H2AX staining (Fig. 7b) and by the increase in comet tail moments (Fig. 7c). Notably, depletion of both ID3 and MDC1 did not further increase comet tail length from what was observed with ID3 depletion alone (Fig. 7d,e), indicating that ID3 and MDC1 act in the same pathway. We also confirmed that the impaired DSB repair was due to an insufficient amount of ID3, because ID3 knockdown cells could be rescued through the introduction of shRNA-resistant ID3 (Fig. 7f,g). These results suggest that ID3 promotes DSB repair.

Because we observed a role for ID3 in DSB repair, we predicted that it was possible for the interaction between ID3 and MDC1 might also be to function in DNA repair. To test this, we stably transfected HeLa cells with ID3 shRNA in order to deplete endogenous ID3, reconstituted these cells with shRNA-resistant ID3–HLH or ID3–ΔHLH (Fig. 7h), and then measured the amount of DSB repair after IR exposure using the neutral comet assay. As shown in Fig. 7i, comet tails for cells depleted in ID3 or expressing ID3–ΔHLH were significantly longer than for those expressing ID3–HLH, suggesting that efficient DNA repair required the presence of the HLH domain. Further, we found that reconstitution of ID3-depleted cells with the S65E HLH mutant, but not the S65A HLH mutant, restored DSB repair...
Fig. 5 An interaction between ID3 and MDC1 is required for the recruitment of MDC1 at DSBs. a A schematic representation of wild-type ID3 (WT) and deletion constructs is shown. Domain boundaries, including the HLH region (25–81), are indicated using numbering for the human ID3 protein sequence. The relative affinity of MDC1 for the ID3 deletion mutants is indicated on the right side of the figure. b The HLH domain of ID3 is required for binding to MDC1. HEK293T cells were co-transfected with HA-tagged MDC1 and GFP-tagged deletion mutants of ID3, as indicated. After 48 h, cells were exposed to IR, and 3 h later, cell lysates were subjected to immunoprecipitation (IP) and immunoblotting (IB) using the indicated antibodies. * indicated heavy-chain. c HEK293T cells were co-transfected with HA-MDC1 and one of three different versions of GFP-ID3: WT, T62A, or S65A. Cell lysates were subjected to IP using an anti-HA antibody and IB using antibodies indicated to the right of the image. d HEK293T cells were co-transfected with HA-MDC1 and one of three different versions of GFP-ID3: WT, S65A, or S65E. Cell lysates were subjected to IP using an anti-HA antibody and IB using antibodies indicated to the right of the image. e ID3 shRNA + HA-MDC1. HEK293T cells were co-transfected with HA-MDC1 and GFP-tagged deletion mutants of ID3, as indicated. After 48 h, cells were exposed to IR, and 3 h later, cell lysates were subjected to immunoprecipitation (IP) and immunoblotting (IB) using the indicated antibodies. * indicated heavy-chain.

(Fig. 7j, k), suggesting that phosphorylated S65 of ID3 is necessary. These findings established that the characteristics of ID3 that are important for interactions with MDC1 are also necessary to promote DSB repair.

Another intracellular role for MDC1 is to regulate intra-S-phase and G2/M cell-cycle checkpoints in response to DNA damage. Thus, we looked for a role for ID3 in these cell-cycle checkpoints as well and found that an ID3 knockdown did not noticeably affect the G2/M checkpoints in response to IR (Supplementary Fig. 9). ID3 is involved in the transcriptional repression of p27, a key regulator of cell-cycle progression, and thus induced the G1/S transition. Indeed, HeLa cells...
transfected with ID3 siRNA were less proliferative compared to control siRNA-transfected cells (Supplementary Fig. 10). This may be explained by the fact that IR-induced G2/M checkpoints were not affected in ID3-deficient cells.

**ID3 promotes DSB repair and genomic stability.** DSBs can be repaired through one of two pathways, HR or NHEJ. Because we had established a role for ID3 in promoting DNA repair, the next step was to look more closely at specific pathways using well-established reporter assays for both HR and NHEJ35, 36. We found that ID3 depletion led to decreased HR repair (Fig. 8a, b). Knockdown of ID3 did not cause an additional reduction of HR (Supplementary Fig. 11a) and NHEJ repair (Supplementary Fig. 11b) in MDC1-depleted cells, suggesting that ID3 regulates HR and NHEJ repair through MDC1.

Next, we measured the number of metaphase spreads containing chromosomal breaks that result from exposure to IR. As shown in Fig. 8c, cells in which ID3 had been depleted had an increased number of chromosomal breaks following IR treatment when compared to control cells. We then performed array-comparative genome hybridization (array CGH)37, comparing the profiles of ID3-depleted and wild-type human fibroblast GM00637 and human embryonic lung fibroblasts MRC-5 cells. We found that chromosomal abnormalities were detected as chromosomal gains (dots over +0.5) and losses (dots below −0.5) that were widely distributed throughout the entire genome of ID3-depleted cells GM00637 (Fig. 8f and Supplementary Fig. 12) and MRC-5 cells (Supplementary Fig. 13), indicating that the DSB repair deficiency observed when ID3 is depleted results in a failure to maintain chromosome integrity.

**Discussion**

ID3 belongs to a family of proteins that consists of four members, ID1–ID4, which are ubiquitously expressed in many different tissues38. Most of the ID proteins contain a basic DNA recognition region that allows binding to either an E-box (CANNAG) or an N-box (CACNAG) in a promoter region; however, ID3 is lacking this region and instead, functions through dimerization with other transcriptional regulators39. Consequently, ID3 inhibits the activity of several transcription factors by directly binding and preventing their association with regulatory sequences in target genes39–41. ID3 plays an important role in controlling cell cycle, proliferation, differentiation, and apoptosis. ID3 has been also implicated as relevant to the pathology of vascular and metabolic disease42–44. These biological effects are mainly attributed to the aforementioned inhibition of transcription factors, specifically basic-HLH (bHLH) transcription factors. To our knowledge, the data presented here demonstrates for the first time that ID3 binds directly to proteins that are not transcription factors, and exerts its effects in a transcription-independent manner. We showed that ID3 directly binds to MDC1 and this interaction requires a tBRCT domain in MDC1 as well as an HLH domain in ID3. The BRCT domain of MDC1 is fairly conserved between human and murine proteins and it binds selectively to peptides containing a phosphorylated serine residue. Importantly,
**Fig. 7** ID3-depleted cells are sensitive to IR and are defective in DSB repair. a HeLa cells with a stable ID3 knockdown were exposed to the indicated doses of IR and assessed for colony forming ability (n = 3). The cell viability of untreated cells is defined as 100%. b, γ-H2AX foci (b) and comet moments (c) at indicated time points after exposure to IR in both control and ID3-depleted HeLa cells. Representative images (upper panel) and quantification (lower panel) of γ-H2AX foci are shown (n = 3). c Comet moments of complemented HeLa cells are shown as in (a). d Comet moments of complemented HeLa cells are shown as in (e). ID3-depleted cells were reconstituted with either HLH ID3 or an HLH deletion mutant (ΔHLH) of ID3 (n = 3). e Western blot of GFP-tagged wild-type (WT) ID3 or GFP alone (mock construct) expressed in ID3 knockdown HeLa cells. f Western blot of the indicated GFP-ID3 constructs reconstituted into ID3 knockdown HeLa cells is shown. g, h Western blot of GFP-ID3 Mock WT or GFP alone (mock construct) expressed in ID3 knockdown HeLa cells is shown. i Western blot of the indicated GFP-ID3 constructs reconstituted into ID3 knockdown HeLa cells is shown. j Western blot of the indicated GFP-ID3 constructs reconstituted into ID3 knockdown HeLa cells is shown. k Similar to i but with the indicated point mutants in the ID3 HLH domain expressed in ID3 knockdown HeLa cells (n = 3). Scale bars: 40 μm (b) and 200 μm (c, e, g, i, k). Uncropped blots of this Figure are shown in Supplementary Fig. 14. Data are presented as means ± s.d. P values are based on Student’s two-tailed t-test: **P < 0.01, ns, not significant.
a single phosphorylation of Ser65 in the HLH of ID3, is both necessary and sufficient for binding to MDC1. In addition, positively charged amino acid lysine at position 2075, which is fully flexible at the distal C-terminal region of tBRCT domain, plays an important role in association of MDC1-tBRCT with ID3-pHLH. ID1, ID2, and ID3 have a high degree of sequence similarity and have widely overlapping biological functions in many tissues. However, ID3 is the only one of these three proteins that contains Ser65 motifs in the HLH domain, which we propose is the reason why ID1 and ID2 did not interact with MDC1. This suggests that ID3 may have a unique role in DNA repair that is distinct from the other ID proteins.

The direct and highly specific interaction between ID3 and MDC1 suggests that they are functionally linked. Consistent with this idea, we have shown that ID3 becomes phosphorylated at Ser65 in response to IR, and that the localization of ID3 to DSBs in response to DNA damage resembles that of MDC1. Moreover, our findings suggest that functional ID3 is required for MDC1 to localize appropriately after IR treatment. When we compared the effect of various mutant ID3 proteins, we showed that a phosphorylated Ser65 in the HLH domain was required for proper localization of MDC1 to damaged DNA sites. Furthermore, we demonstrated that when cellular levels of ID3 were low, there were fewer DSB repair events, HR and NHEJ activities were suppressed, and cells are more sensitive to IR. Notably, ID3-deficient cells transfected with either wild-type ID3 or the HLH domain of ID3, but not ΔHLH ID3 or S65A ID3, fully rescued both IR-induced MDC1 foci and repair of DSBs. Thus, we conclude that ID3 contributes to the ability of MDC1 to regulate DNA damage repair by directly interacting with MDC1.

Our data also highlight the necessary role that ID3 plays in allowing binding between MDC1 and γ-H2AX, early DNA damage sensor protein. MDC1 mainly acts as an adapter protein that recruits DDR proteins to sites of DNA damage. After DNA damage, ATM is recruited to DSBs where it phosphorylates H2AX, and then γ-H2AX serves as a docking platform for MDC1, which binds to γ-H2AX via its tBRCT domain. Abrogation of the MDC1-γ-H2AX interaction disrupts IR-induced...
MDC1 foci formation and renders cells radiosensitive. In this study, we observed the interaction between MDC1 and γ-H2AX was abolished by depletion of ID3. Our MD simulations suggest that interaction of MDC1–tBRCT with ID3–pH2AX increases flexibility of Arg1933 of MDC1–tBRCT and therefore may give a better chance for catch the γ-H2AX. ID3 does not have obvious sequence homology with known DDR protein domains or relevant enzymatic active sites. Instead, ID3 may act as a scaffold for the MDC1–γ-H2AX complex by maintaining the interactions, stability, and DSβ targeting of these proteins. Consequently, depletion of ID3 diminishes both the protein–protein interactions and the DSβ-targeted activities of MDC1.

ID proteins are frequently overexpressed in many cancer cells, and disease severity and poor prognosis is associated with a high level of these proteins. As a result, ID proteins are now considered important targets for potential anti-cancer drugs as a way to counteract tumor progression. However, a KO of ID3 resulted in the accumulation of DNA damage in colon cancer initiating cells, and conversely, ectopic expression of ID3 activated DNA repair processes in pancreatic β cells, thereby supporting a role for ID3 in maintaining genomic stability. Our studies revealed that ID3 depletion led to significant increases in DNA damage accumulation and chromosomal aberrations. The development of genomic chromosomal abnormalities, which is a hallmark of cancer, in ID3-deficient non-transformed cells again supports the role of ID3 in maintaining chromosome stability. The association of ID3 with MDC1 and the effect on DSB repair promotion of chromosomal abnormalities and might subsequently contribute to tumorigenesis and cancer progression. This hypothesis is supported by a recent finding that ID3-KO mice develop T-cell lymphomas.

In summary, the interactions between ID3 and the DNA damage mediator protein MDC1 suggest that ID3 participates in the DNA damage signaling pathway to maintain genomic stability. The model depicted in Fig. 8g provides an integrated view of how MDC1 recruitment is linked to the upstream DNA damage signaling processes, and how ID3 may function at sites of DSBs. Thus, our data establish a mechanistic basis for the ID3 signaling processes, and how ID3 may function at sites of DSBs.

Plasmid constructs and transfection. The plasmids encoding wild-type MDC1 and various truncated MDC1 (Δ55–124, Δ20–40, Δ421–624, Δ525–1129, Δ1130–1661, Δ1893–2082) were obtained from Zhenhai Lou. For GST pull-down assay, the BRCT fragment of MDC1 was produced by PCR using wild-type MDC1 (pcDNA HA-MDC1) as template and then subcloned in the EcoRI/XhoI site of pGEX-4T-1 vector. To generate the full-length, N-terminal, C-terminal, HLH, ΔHLH constructs of ID3, each region of ID3 was amplified from human HeLa cDNA, and the PCR products were cloned into pEGFP-N3 vector. Point mutations of ID3 and tBRCT were generated by site-directed mutagenesis (Invitrogen). To construct the expression vector encoding mRNA-resistant GFP-tagged ID3, we performed mutagenesis using GEN-EART® Site-Directed Mutagenesis System (Invitrogen). The shRNA targeting MDC1 (5′-GGGACGACGCGGTACGAGG-3′), was designed by automated oligonucleotide design (NeoBioscience). The shRNA or pSilencer 2.1-U6-neo control shRNA or pSilencer 2.1-U6-neo ID3 shRNA using lipofectamine 2000 (Invitrogen) and selected in medium containing 500 μg/ml neomycin for 2–3 weeks. After selection, stably ID3 knockdown clones were confirmed by western blot analysis of ID3.

Immunoprecipitation assay and western blot analysis. Cells were lysed in ice-cold RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1mM phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin). Equal amounts of cell or tissue extracts were separated by 6%–12% SDS-PAGE followed by electrophoresis onto a polyvinylidene fluoride (PVDF) membrane (PALL life sciences). The membranes were blocked for 1 h with TBS-1 (50 mM NaCl and 0.1% Tween-20) containing 5% non-fat milk and then incubated with indicated primary antibodies overnight at 4 °C. The blots were washed four times for 15 min with TBS-1 and then incubated for 1 h with peroxidase-conjugated secondary antibodies (1:1000, Jackson ImmunoResearch, West Grove, PA). The blots were washed four more times with TBS-1 and developed using an enhanced chemiluminescence detection system (ECL; Millipore, Billerica, MA).

Methods

Cell culture and treatment. The human cervix carcinoma HeLa cells, human osteosarcoma U2OS cells and human embryonic kidney HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Lonza), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen). The human fibroblast GM00637 and human embryonic lung fibroblasts MRC-5 cells were cultured in Earle’s MEM containing 10% FBS, penicillin, and streptomycin. Cells were maintained in a humidified incubator with an atmosphere of 5% CO₂ at 37 °C. All cell lines were from the American Type Culture Collection (ATCC). 100 μg of DNA DSBs, exponentially growing cells were irradiated at 2 or 10 Gy from 137Cs source (GammaCell 3000 Irradiator, Best Theratronics) and allowed to recover at 37 °C incubator for various times. Inhibitors of ATM (Ku55933) and DNA-PKcs (NU7426) were from TOCRIS bioscience.

Antibodies. Polyclonal MDC1 antibody (R2) was raised in rabbit against GST fusion protein containing BRCT domain of MDC1 (residues 1882–2089 aa). Antibody against S65 phosphorylated ID3 was generated to phospho-peptide of tBRCT construct (1882–2089 aa). Antibodies against 5′-GGGACGACGCGGTACGAGG-3′ and 5′-GGGACGACGCGGTACGAGG-3′ were annealed and inserted into pSilencer2.1-U6-neo vector (Ambion). Cells were transfected with pSilencer2.1-U6-neo control shRNA or pSilencer 2.1-U6-neo ID3 shRNA using lipofectamine 2000 (Invitrogen) and selected in medium containing 500 μg/ml neomycin for 2–3 weeks. After selection, stably ID3 knockdown clones were confirmed by western blot analysis of ID3.
sample buffer, boiled for 5 min, and analyzed by western blotting using the appropriate antibodies (Supplementary Table 1).

**Immunofluorescence microscopy.** To visualize DNA damage foci, cells cultured on coverslips coated with L-lysine were irradiated at 2 Gy and allowed to recover at 37 °C for adequate times. Cells were washed twice with PBS and fixed with 4% paraformaldehyde for 10 min and ice-cold 96% methanol for 5 min, followed by permeabilization with 0.3% Triton X-100 for 10 min at room temperature. After permeabilization, coverslips were washed three times with PBS and then blocked with 5% BSA in PBS for 1 h. Cells were single or double immunostained with primary antibodies against the indicated proteins (Supplementary Table 1) overnight at 4 °C. Cells were washed with PBS and then stained with appropriate Alexa Fluor 488- (green, Molecular Probe), Alexa Fluor 594- (red, Molecular Probe) conjugated secondary antibodies (Supplementary Table 1). After washing, the coverslips were mounted onto slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Fluorescence images were taken using a confocal microscope (Zeiss LSM 510 Meta; Carl Zeiss) and analyzed with Zeiss microscope image software ZEN (Carl Zeiss). The foci number per cells was counted at least 100 cells. The error bars represent the standard error from three independent experiments.

**GST pull-down assay.** Bacterially expressed fusion proteins containing GST and the BRCT domains of MDC1 (residues 1889-2070) or GST alone immunoprecipitated onto Glutathion Sepharose 4B beads (GE Healthcare) and incubated with lysates prepared from HeLa cells or cells transiently transfected with GFP-ID3 expression vector for 3 h at 4 °C. Cells were lysed in TEN100 buffer (20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethane-sulfonfyl fluoride, 10 μg/ml pepstatin, and 10 μg/ml leupeptin). After a 2-h phosphatase treatment was required, TEN100 lysates were incubated with 400 units of λ- phosphatase (New England BioLabs) at 30 °C for 30 min. The GST beads were washed five times with NTEN buffer (0.5% NP-40, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 300 mM NaCl), and bound proteins were separated by SDS-PAGE and analyzed by western blotting using the appropriate antibodies.

**Chromosomal cell survival assay.** After treatment with irradiation, 5 × 10^4 cells were immediately seeded on 60 mm dish in triplicate and grown for 2–3 weeks at 37 °C to allow colonies to form. Colonies were stained with 2% methylene blue/50% ethanol and were counted. The fraction of surviving cells was calculated as the ratio for the plating efficiency of treated cells over untreated cells. Cell survival results are reported as the mean ± s.d. for three independent experiments.

**Single-cell gel-electrophoresis (Comet assay).** DSB repair was visualized by neutral single-cell agarose-gel electrophoresis. Briefly, individual cells were collected (≈ 10^5 cells per pellet), mixed with low-melting agarose, and layered over agarose-coated glass slides. The slides were maintained in the dark for all of the remaining steps. Slides were submerged in lysis solution (Cat.#4250-050-01, TREVIGEN® Instructions, Gaithersburg, MD, USA) for 1 h and incubated for 30 min in neutral electrophoresis solution (100 mM Tris, 300 mM Sodium Acetate at pH 9.0). After incubation slides were electrophoresed (−30 min at 1 V cm⁻¹ tank length), and then gently immerse slide in DNA Precipitation Solution (1.5 M NH₄Ac) for 30 min at room temperature. After air-dried, comet slide stained with SYBR green. Average comet tail moment was scored for 40–50 cells per slide using a computerized image analysis system (Komet 5.5; Andor Technology, South Windsor, CT, USA).

**HR assay.** To measure the HR repair, stable cell lines expressing DR-GFP vectors were generated by transfection using lipofectamine 2000. Clones were selected in the medium containing 500 μg ml⁻¹ neomycin for 2 weeks and screened for significant induction of GFP-positive cells following infection with I-SceI expressing adenovirus. U2OS-DR-GFP cells were transfected with control or ID3 siRNA using lipofectamine RNAiMAX, and then infected with I-SceI-carrying adenovirus at an estimated MOI of 10. After 72 h, GFP-positive cells were identified by fluorescence-activated cell sorting (FACS) and scored as the mean ± s.d. value from three independent experiments.

**NHEJ assay.** The NHEJ assay was measured in HeLa EJ5-GFP cells, using methods previously described. EJ5-GFP contains a promoter that is separated from a GFP coding region by puromycin resistance gene, which is flanked by two I-SceI sites that are in the same orientation. When the I-SceI-induced DSBR is repaired by NHEJ in HeLa EJ5-GFP cells, the puromycin gene is removed, and the promoter is rejoined to the rest of the GFP expression cassette, leading GFP expression. HeLa EJ5-GFP cells were kindly provided by Dr. Ke at the University of South Florida. Similar to the above HR assay, HeLa EJ5-GFP cells were transfected with control or ID3 siRNA using lipofectamine RNAiMAX, and then infected with I-SceI-expressing adenovirus at an estimated MOI of 10. After 3 days, the percentage of GFP-positive cells which had repaired the DSBRs generated by I-SceI was determined by flow cytometry. For each analysis, 10,000 cells were processed and each experiment was repeated three times.

**Chromosomal aberration analysis.** For chromosomal aberration analysis, indicated transfected-U2OS cells were treated with 1 Gy of γ-ray for 24 h. To arrest cells in metaphase, 300 ng ml⁻¹ colcemid (Sigma) was added 4 h before cell collection. Colcemid depolymerizes microtubules and inhibits the formation of mitotic spindle. Cells were collected in 15 ml tubes, gently resuspended in 40% of culture media for 10 min at 37 °C, and then fixed in equivalent volume of a freshly prepared fixative solution (3:1 mixture of methanol/acetic acids, Carnoy’s solution). After removal of supernatant, pellets were resuspended in fixative solution, dropped onto a cleaned glass slide and air-dried overnight. The slide was mounted in Vectashield with DAPI (Vector Laboratories). Metaphase images were captured using confocal microscope (Zeiss LSM 510 Meta; Carl Zeiss) and analyzed with Zeiss microscope image software ZEN (Carl Zeiss).

**CGH array and data analysis.** Human fibroblast GM00637 cells were stably transfected with control or ID3 shRNA, and genomic DNA was isolated using AccuPrep® Genomic DNA Extraction kit (Bioneer) according to the manufacturer’s instructions. Array CGH analysis was performed using the NimbleGen Human CGH 12 × 135 K whole-genome tiling v3.1 Array (Agilent Technologies). Human genomic DNA (1 μg) from ID3-depleted cells and reference DNA samples from control cells were independently labeled with fluorescent dyes (Cy3/Cy5), cohybridized at 65 °C for 24 h, and then subjected to the array. The hybridized array was scanned using NimbleGen’s MS200 scanner (NimbleGen systems Inc.) with 2 μm resolution. Log2-ratio values of the probe signal intensities were calculated and plotted with appropriate and reference signal intensities using Roche NimbleGen’s NimbleScan v2.5 software. Data are displayed and analyzed in Roche NimbleGen SignalMap software and CGH-explorer v2.55.

**G2/M checkpoint assay.** HeLa cells were transiently transfected with control or ID3 siRNA. After 48 h of transfection, cells were exposed to IR for 3 h and then placed in 100 ng ml⁻¹ nocodazole-containing media for 3 h, and the cells were collected and washed with PBS and then fixed with 1% formaldehde for 10 min at 37 °C. The cells were chilled on ice for 1 min and then permeabilized with 90% methanol at −20 °C overnight. The fixed cells were washed with PBS and blocked with incubation buffer (0.5% BSA in PBS) for 10 min. The cells were stained with anti-phospho-histone H3(S10)-Alexa Fluor 647-conjugated antibody (Cell Signal-ing Technology, Invitrogen, San Diego, CA). Staining of cell nuclei was performed in incubation buffer 6 h in darkness at room temperature, and the cells were then washed and resuspended in PBS containing 50 g ml⁻¹ propidium iodide. At least 10,000 cells were analyzed by fluorescence-activated cell sorting (FACSort, Becton Dickinson, San Jose, CA). The acquired data were analyzed using the CellQuest Pro software (Becton Dickinson).

**BrdU incorporation assay.** To determine the replication rate, control and ID3-depleted HeLa cells were seeded in a 48-well plate. After 48 h, 10 μl of BrdU solution (10 μg/ml) was added to the cells, and the culture was incubated for 2 h at 37 °C. After fixation of the cells, immune complexes were formed using peroxidase-coupled BrdU-antibodies. Colored products were measured in a microplate reader at 405 nm with a reference wavelength at ~490 nm. The relative DNA synthesis was calculated as the absorbance of ID3-depleted cells from the absorbance of control cells. The data are presented as the mean ± s.d. from triplicate experiments.

**Generation of U2OS-ID3-knockout cells.** The ID3 CRISPR (Guide sequence insert: 5'-CGAGGCGGCTTGCTCCTGGCTG3') constructs targeting exom 1 of the human ID3 gene were designed using the Cas9-Designer (http://www.rgenome.net/cas-designer). SpCas9 expression plasmids (500 ng) and sgRNA plasmids (500 ng) were transfected to 2 × 10^5 U2OS cells using 4D-nucleofector (Lonza) SE kit and program CM-104 in 20 ml Nucleofector Strips. Genomic DNA was isolated with the Nucleospin Tissue Kit (MACHEREY-NAGEL) 72 h post-transfection. Target sites were amplified with adapter primers using Phusion polymerase (New England Biolabs). The resulting deep sequencing libraries were subjected to paired-end sequencing with the MiSeq kit (Illumina). The MiSeq, paired-end reads were analyzed with adapter primers using Phusion polymerase (New England Biolabs). The MDCl–BRCT domain of MDC1/NFBD1–γ-H2AX complex (residues 1891–2083) is de

**Model system preparation.** The three-dimensional (3D) structure of both ID3 protein (PDB code: 2LEH) as ligand and MDC1/NFBD1–γ-H2AX complex (PDB code: 2AZW) as receptor are revealed by X-ray experiments. The MDCl–BRCT domain of MDC1/NFBD1–γ-H2AX complex (residues 1891–2083) is defined to bind the γ-H2AX tail (residues 138–142) in the phosphate-peptide recognition. For
the docking simulation, we need the isolated three systems; MDC1
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Statistical analysis
to explain the interaction with the receptor and the ligand.
newly special-binding site of the MDC1 BRCT domain is residues 2063
plex is built to restraint the distance between Lys2075 of the receptor and pSer65 of
conformation upon ligand binding. To predict more reliable protein-ligand com-
explore the vast conformational space of ligands in a short time but the major
condition between the specific-binding site and the ligand. To predict more reliable protein-ligand com-
plexes, MD simulation can deal with both ligand and protein in a complex
conformation in the atomic level, the MDC1
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Lys2075 of the receptor and pSer65 of ID3. The restraint distance is about 4.0 Å and the restraint force is about
100 KJ mol
Statistical analysis
Data in all experiments are represented as mean ± s.d. Two-tailed Student's t-test was conducted using GraphPad Prism software (GraphPad software, Inc.) and Excel (Microsoft). P values < 0.01 were considered statistically significant (**).

Data availability
The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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Author contributions
J.-H.L., S.-J.P. and H.J.Y. designed the experiments; J.-H.L., S.-J.P., M.-J.K., S.M.J. and S.-Y.J. performed the experiments; G.H. and I.-Y.C.: provided help for the experiments; C.K. and E.K. performed MD simulations; J.Y. and S.B. generated ID3 KO U2OS cells; J.-H.L., S.-J.P. and H.J.Y. analyzed data; J.-H.L., S.-J.P., E.K., S.B. and H.J.Y. wrote the manuscript.

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