Phosphorylation of $H2A.X^{\text{Tyr39}}$ positively regulates DNA damage response and is linked to cancer progression

Yan Liu$^{1,2}$, Yue-Hong Long$^1$, Shu-Qing Wang$^3$, Yu-Feng Li$^2$ and Jing-Hua Zhang$^2$

1 College of Life Science, North China University of Science and Technology, Tangshan, China
2 Cancer Institute, Affiliated Tangshan People’s Hospital of North China University of Science and Technology, Tangshan, China
3 Hospital of North China University of Science and Technology, Tangshan, China

Keywords
DSBs; EYA2; H2A.X; phosphorylation

Introduction
Histone H2A.X$^{S139\text{ph}}$ ($\gamma$-H2A.X; H2A.X phosphorylated on the 139th serine residue) is an early marker of DNA double-strand breaks (DSBs) [1] and guides the recruitment of the molecules required for DNA repair and chromosome remodeling at regions of DSBs. Incorrect repair of DSBs leads to instability of the whole genome and to cancers [2,3]. Therefore, knowing whether $\gamma$-H2A.X can reflect the status of genotoxic effects and cancer is of value to researchers and clinicians [1,4,5]. In current opinion, a high level of $\gamma$-H2A.X is a common characteristic of greater genome instability in premalignant lesions and cancer cells [1,6,7]. Currently, researchers are attempting to induce DSBs and prevent efficient DNA repair in cancer cells to promote apoptosis and cure cancers [4,8].

$\text{Tyr142}$ of H2A.X can be stably phosphorylated (H2A.X$^{Y142\text{ph}}$) under normal circumstances by a newly identified kinase, Williams–Beuren syndrome

Abbreviations
53BP1, p53 binding protein 1; ATM, ataxia telangiectasia mutated; BRCA1, breast cancer protein 1; DDR, DNA damage response; DR-GFP, direct-repeat green fluorescent protein; DSB, DNA double-strand break; EYA2, eyes absent 2; HA, hemagglutinin; IF, immunofluorescence; IP, immunoprecipitation; IR, ionizing radiation; MDC1, mediator of DNA damage checkpoint protein 1; NBS1, Nijmegen breakage syndrome 1; RNF168, ring finger protein 168; RNF8, ring finger protein 8; siRNA, small interfering RNA; TNM, tumor node metastasis; WSTF, Williams–Beuren syndrome transcription factor.
transcription factor (WSTF) [9]. Persistent high levels of Tyr142 phosphorylation potentiate DNA damage-induced apoptotic responses while inhibiting repair. This function is based on recruitment of apoptosis-related factors to DSB sites and nearby regions, and the prevention of simultaneous recruitment of repair factors. Promotion of sensitivity to chemotherapeutic drugs and apoptotic responses in cancer cells can be achieved by the cooperation of phosphorylation at Tyr142 and Ser139 [9,10]. The modification status of H2A.X may be used to evaluate the efficiency of certain treatments and prognoses [8,11,12]. The cooperation of phosphorylation at Ser139 and Tyr142 is important for DSB repair processes and cancer treatment [9,10]. Results of H2A.X studies have revealed a substantial role in human diseases and sophisticated H2A.X modulation patterns. Moreover, a recent study has indicated that phosphorylation of H2A.X Tyr142 is more complicated than anticipated. For example, WSTF is not a necessary kinase for H2A.X Tyr142 phosphorylation during sperm development [13]. This phenomenon reveals the complexity and diversity of the modifications and functions of H2A.X.

In addition to Tyr142, there are another three tyrosine residues on H2A.X, Tyr39, Tyr50 and Tyr57. According to a study of human embryonic kidney cells, Tyr142 is the only phosphorylated Tyr residue in mammalian embryonic kidney cells [14]. Considering the complexity of the modifications of H2A.X and its great significance in the diagnosis and therapy of cancers, we expanded the range of analyses and identified a novel phosphorylation site of H2A.X, Tyr39, in a variety of cancer cells. Eyes absent 2 (EYA2) was confirmed as a phosphatase of Tyr39 in H2A.X, and low expression of EYA2 protein in tumor cells maintains a certain level of Tyr39 phosphorylation. An increase in the level of Tyr39 phosphorylation may be a prerequisite for the formation and maintenance of γ-H2A.X, which influences cell survival after ionizing radiation (IR) treatment. Interestingly, EYA2 protein and H2A.X Tyr39ph correlate with tumor formation and differentiation, and patient survival rates.

Results

H2A.X Tyr39 is a target of phosphorylation in cancer cells

We transiently transfected a hemagglutinin (HA)-tagged H2A.X-expressing plasmid with Tyr mutated to Phe, mimicking an unphosphorylated Tyr, into the colon cancer cell line HCT15 [14,15]. The whole Tyr phosphorylation status was analyzed by western blotting after immunoprecipitation (IP) with an antibody against the HA tag. The T39F mutation dramatically decreased the tyrosine phosphorylation level of H2A.X. We considered that Tyr39 may be the major Tyr phosphorylation site in HCT15 cells (Fig. 1A). No obvious phosphorylation signal was detected for Tyr142, which has been reported as a unique phosphorylated Tyr residue in mammalian embryonic kidney cells [14]. For confirmation, an antibody that specifically recognized H2A.X with Tyr39 phosphorylation (anti-Y39ph) was prepared and applied to screen multiple cancer cell lines. H2A.X Tyr39ph was identified in
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various types of cancer cells including colon (HCT15, SNUC4, WIDR, SW620 and SW480), pancreas (SU.86.86, Hs766T, SW1990, PANC-1, MIAPaCa-2 and HPAF-II), liver (HHCC, HepG2, Hep3b and SMMC7721) and breast cancers (MDA-MB-231, MCF7 and T47D) as well as osteosarcoma (U2OS, HOS and SW1353), and the level of H2A.XY39ph was positively correlated with H2A.XS139ph (P < 0.01, Fig. 1B). The HCT15 cell line and pancreas cancer cell line SU.86.86, in which phosphorylation of Tyr142 was detected (Fig. 1C), were chosen for further investigation.

Tyr39 and the next residue, Ala40, are conserved in human, mouse and Xenopus laevis, and the residues on both flanks are the same in human and mouse, but not in X. laevis (Fig. 1D). H2A.XY39ph increased by approximately 3.5-fold at 10 min after IR treatment, and the high level of phosphorylation was maintained for about 6 h (Fig. 2A). Of note, the increase in the level of H2A.XY39ph occurred before Ser139 phosphorylation, which was upregulated at 20 min after IR exposure. These results indicated that Tyr39 of H2A.X was phosphorylated in normal cells without IR exposure, and H2A.XY39ph increased after IR treatment and may be correlated with H2A.XS139ph.

To further confirm the relationship between the phosphorylation of Tyr39 and Ser139 in H2A.X after IR treatment, an HA-tagged H2A.XY39F mutation plasmid was transfected into the cells. Following IR treatment, no H2A.XT139ph signal was detected on the mutant H2A.X protein (Fig. 2B). This result suggests that the phosphorylation on Tyr39 could be a precondition for γ-H2A.X, a DNA damage response (DDR) factor, in HCT15 cells. To complement our western blotting data with another technique, immunofluorescence (IF) was used to detect H2A.X phosphorylation on sites of laser microirradiation. H2A.XY39ph accumulation occurred within 5 min of DNA damage induction. By contrast, H2A.XS139ph accumulation occurred nearly 10 min after treatment of laser microirradiation (Fig. 2C). Next, we used the estrogen receptor-I Ppol system to induce DSBs in Hs766T cells [14]. Chromatin immunoprecipitation (ChIP) analysis revealed that H2AXY39ph and H2AXS139ph were present at different time points flanking the I-Ppol cut site (Fig. 2D).

In SU.86.86 cells, we wanted to observe the status of H2A.XS139ph with down-regulation of WSTF via a specific small interfering RNA (siRNA), because the decrease in H2A.XY142ph is necessary for the extension of γ-H2A.X after IR treatment. Down-regulation of WSTF and H2A.XY142ph did not affect the status of H2A.XS139ph for both wild-type and mutated H2A.X protein (Fig. 2E). Without IR treatment, the proliferation capability of H2A.XY39F/− SU.86.86 cells was negatively regulated after expression of the H2A.XY39F mutant (Fig. 2F). These results indicated that phosphorylation of Tyr39 may be involved in the regulation of cell proliferation, because the cell number was not significantly affected by the expression of wild-type H2A.X.

EYA2 dephosphorylates H2A.X Tyr39

Because we could not identify the kinases of Tyr39 in H2A.X (data not shown), we investigated potential phosphatase molecules. First, we examined EYA family members that have been reported to associate with H2A.X [14]. The results showed a significant increase in H2A.XY39ph following siRNA knockdown of EYA2 (Fig. 3A). Furthermore, H2A.X with Tyr39 phosphorylation was extracted from SU.86.86 cells to react with purified Flag-tagged EYA2 to confirm that H2A.XY39ph was dependent on the tyrosine phosphatase EYA2. As shown in Fig. 3B, the EYA2 D209N [16] mutant, which lacked Tyr phosphatase activity, failed to remove the phosphate group on Tyr39. Next, IF experiments were performed to confirm the western blot results. As shown in Fig. 3C, exogenous EYA2 expression induced a decrease in H2A.X phosphorylation levels before or after laser-microirradiation.

Next, we examined the association between EYA2 and H2A.X before and after IR exposure. An association was observed before IR treatment, but it was weakened following IR treatment. We also detected an increase of phosphorylation on Tyr39 and Ser139 in H2A.X (Fig. 3D). We presumed that the up-regulation of Tyr39 phosphorylation could be a consequence of dissociation of EYA2 from H2A.X following IR treatment, which promoted Ser139 phosphorylation. Therefore, we investigated the relationship of EYA2 and DNA damage repair. First, a neutral comet assay [17] was used to test the extent of DNA damage in the cells. Cell samples were collected at various time points from wild-type and EYA2−/− cells following 5 Gy IR treatment (Fig. 4A). The lack of EYA2 increased the capability of the cells to repair damaged DNA compared with wild-type cells. However, the expression of H2A.XY39F attenuated this increase.

Next, we analyzed the role of EYA2 in homology-directed repair using a gene conversion lentivirus assay [18]. As shown in Fig. 4B, the repair capability of cells transfected with EYA2 siRNA was enhanced compared with that of cells transfected with control siRNA. However, the enhancement was reversed by transfection of the H2A.XY39F-expressing plasmid. These data
indicated that knockdown of EYA2 strengthened the capability of cells to repair damaged DNA, and this function was dependent on H2A.X Tyr39.

We next investigated whether EYA2 was involved in the regulation of tumor cell proliferation, because we had found that phosphorylation of H2A.X Tyr39 regulated cell proliferation. Hence, we knocked down EYA2 in HCT15 colon cancer cells by siRNA, and observed enhancement of cell proliferation (Fig. 4C). This result was consistent with a previous study of pancreatic carcinoma [19]. However, the reported correlation in lung cancer cells [20] and breast cancer cells

**Fig. 2.** H2A.X Tyr39ph is involved in the regulation of DDR. (A) Histone proteins were extracted from HCT15 cells after 2 Gy IR exposure. Western blot analysis was performed with the indicated antibodies. Western bands were measured using densitometry. The experiment was repeated five times. Data are shown as the mean ± standard deviation (SD). (B) HCT15 cells were treated with 5 Gy IR at 48 h after transfection with HA-tagged wild-type or mutated H2A.X-expressing plasmids. Cells were collected at various times points, followed by IP assays with an antibody against the HA tag and western blotting with the indicated antibodies. (C) In Hs766T cells, DNA damage was generated by laser microirradiation followed by IF (5, 10 min after damage) with the indicated antibodies. (D) Hs766T cells were transfected with ER-I-PpoI-expressing vector, and after 48 h the cells were cultured with 4-hydroxytamoxifen (4-OHT). Finally, the cells were analyzed by ChIP using specific antibodies and primers around 3.9 kb downstream of the I-Ppo cut site. Values represent the mean ± SD of percentage input from quintuple samples. (E) Combinations of HA-tagged wild-type H2A.X or Y39F mutant plasmid with WSTF-specific siRNA or control siRNA were transfected into SU.86.86 cells, and the cells were treated with 10 Gy IR 48 h after transfection. H2A.X protein was precipitated using an HA tag antibody at 6 h after IR exposure, and western blot analysis was performed to detect phosphorylation. (F) Cell counting kit (CCK)-8 assays to assess cell proliferation were performed following transfection. The experiment was repeated five times. Data are shown as the mean ± SD.
was in contrast to our results. Furthermore, we observed a decrease in the enhancement of proliferation after expression of the H2A.X Y39F mutant (Fig. 4C). This result revealed the involvement of EYA2 in the regulation of cell proliferation.

We drew preliminary conclusions based on the above results. EYA2 is the phosphatase of H2A.X Tyr39, and phosphorylation of this site is necessary for the formation of c-H2A.X. EYA2 is involved in the regulation of DNA damage repair and cell proliferation, which is dependent on H2A.X Tyr39. Next, we elucidated the mechanisms involved in these processes.

**EYA2 promotes cell survival and proliferation**

It has been reported that γ-H2A.X determines the recruitment of DNA repair factors or apoptosis factors at or around DSB regions [22]. We showed that phosphorylation of H2A.X Tyr39 promoted the formation and maintenance of γ-H2A.X. Therefore, we investigated whether EYA2 and H2A.X Y39ph regulate tumor cell proliferation by regulating apoptosis and DNA damage repair.

**Mediator of DNA damage checkpoint protein 1 (MDC1)** [23,24] is a critical protein for the recruitment of ataxia telangiectasia mutated (ATM) [25], the major kinase of Ser139, which is also needed for the expansion of H2AX phosphorylation following DNA damage [2]. The recruitment of MDC1 was tested in the absence of phosphorylation on Tyr39 in H2A.X of H2AX−/− HCT15 cells that had been transfected with wild-type or Y39F mutant H2A.X-expressing plasmids. The results revealed substantial recruitment of ATM and MDC1 on wild-type H2A.X. On the Y39F mutant H2A.X, because of the absence of Tyr39
Fig. 4. EYA2 and H2A.X regulate cell survival and proliferation. (A) Samples of wild-type or EYA2−/− HCT15 cells were collected at various time points following IR exposure. Unrepaired DNA was quantified as a comet tail moment using COMETSCORE software. At least 60 cells were analyzed in each sample. The experiment was repeated five times. Data are shown as the mean ± SD. Differences between data at each time point were analyzed by Student’s t test; *P < 0.05, **P < 0.01. (B) A homology-directed repair (HR) experiment was performed with U2OS cells with integration of DR-GFP [18]. Cells were transfected with adenovirus expressing I-SceI, which leads to DSBs in DR-GFP reporter. The percentage of GFP-positive cells in each sample was normalized from the number of GFP-positive cells in the control siRNA-treated population. The experiment was repeated five times. Data are shown as the mean ± SD. The significance was analyzed by Student’s t test; **P < 0.01. The efficiency of siRNA was determined by western blotting. (C) Cell counting kit (CCK)-8 assays to assess cell proliferation were performed following transfection of siRNA and the plasmid. The experiment was repeated three times. Data are shown as the mean ± SD. (D) Wild-type or Y39F mutant H2A.X-expressing plasmids were transfected into H2AX−/− HCT15 cells. At 5 h after 5 Gy IR exposure, H2A.X was immunoprecipitated using a specific antibody, followed by western blot analysis. (E) MDC1–NBS1 interaction. (F) The recruitment of RNF8–RNF168–BRCA1 complex, and the recruitment of 53BP1 were analyzed through co-IP as described in (D). (G) Cells were treated with 5 Gy IR after transfection of control or EYA2-specific siRNAs. H2A.X was coimmunoprecipitated with associated factors at 1 h after IR treatment. Western blotting was applied to analyze the protein factors.
Phosphorylation, ATM and MDC1 recruitment was down-regulated (Fig. 4D). Moreover, the MDC1–Nijmegen breakage syndrome 1 (NBS1) complex, whose retention on the DSB regions facilitates survival, was tested (Fig. 4E). The interaction between MDC1 and NBS1 was not obviously affected by H2A.X\textsuperscript{Y39F} mutation, but the retention was down-regulated (Fig. 4D,F).

p53 binding protein 1 (53BP1) could activate ATM after DSBs [26]. The ring finger protein 8 (RNF8)–ring finger protein 168 (RNF168)–breast cancer protein 1 (BRCA1) complex is essential to the regulation of ubiquitination-mediated DDR [27]. Next, whether the recruitment of the RNF8–RNF168–BRCA1 complex and the recruitment of 53BP1 are dependent on H2A.X Tyr39 phosphorylation was investigated (Fig. 4F). The results confirmed that the status of H2A.X\textsuperscript{Y39ph} affects DDR through regulating the recruitment of DDR pathway factors.

Furthermore, in SU.86.86 cells, we determined whether the EYA2 knockdown-induced increase of Tyr39 phosphorylation could drive the association of ATM and MDC1 with H2A.X. Western blotting was performed with immunopurified endogenous H2A.X. As expected, H2A.X-associated ATM and MDC1 were obviously increased following knockdown of EYA2 (Fig. 4G).

For further confirmation, confocal based IF experiments were performed to check the kinetics of the recruitment process and thereby whether retention of the DDR proteins is hampered due to lack of this specific phosphorylation. The results revealed that EYA2 overexpression induced a quicker dissociation of DDR proteins compared with that in control groups (Fig 5A).

All these results support the conclusion that EYA2-targeted phosphorylation of H2A.X Tyr39 is vital for the formation of γ-H2A.X and recruitment of DNA damage repair factors after DNA damage. Next, we knocked down EYA2 in HCT15 cells with specific siRNA. Compared with the control group, cells showed a low level of apoptosis with the decrease of EYA2 protein, whereas the apoptosis response was partially recovered by transfection of the H2A.X\textsuperscript{Y39F} plasmid (Fig. 5B). These results indicated that EYA2-targeted phosphorylation of H2A.X Tyr39 was involved in the promotion of cell survival and contributed to a stable cell number. The lack of EYA2 may increase the rate of DNA damage; as shown in Fig 4G, the level of γ-H2A.X was increased following the knock-down of EYA2 after IR treatment. But the rate of repair was indeed increased in \textit{EYA2}\textsuperscript{−/−} cells (Fig 4A) and the cells showed a lower level of...
apoptosis with the decrease of EYA2 protein compared with the control group (Fig 5B). The cells may be more capable of repairing DNA damage following the decrease of EYA2 through different pathways.

**H2A.X\(^{Y39ph}\) correlates with cancer progression**

It has been reported that EYA2 protein levels are lower in colon cancer cells [28] and pancreatic cancer cells [19] than in normal cells. These reports indicate that EYA2 may be a tumor suppressor gene. To further identify the expression status of EYA2 and H2A.X\(^{Y39ph}\) in human cancers, we collected fresh tumor and paired adjacent tissues from patients with colon, pancreas, liver, prostate, breast, lung, cervical, gastric, skin and kidney cancers. Proteins were extracted from the tissues and analyzed by western blotting. We found that EYA2 levels were lower in cancer tissues than in matched adjacent tissues except in breast and lung cancers. Levels of H2A.X\(^{Y39ph}\) were higher in cancers than in adjacent tissues with the highest in colon cancer (Fig. 6A).

We chose colon cancer for further analyses. A total of 130 samples were collected, and immunohistochemistry (IHC) was used to analyze the samples. In cancer tissues, the phosphorylation level of H2A.X Tyr39 negatively correlated with the levels of EYA2 protein and positively correlated with Ki-67, a marker for cancer cell proliferation (Fig. 6B,C). This result agreed with that obtained in cell lines. In addition, a higher H2A.X Tyr39 phosphorylation level appeared to correlate with poorer differentiation, because phosphorylation of H2A.X Tyr39 gradually increased with the histological grade (Fig. 6D).

Furthermore, clinical-pathological data were collected for correlation analysis with phosphorylation of H2A.X Tyr39. The results showed that phosphorylation of H2A.X Tyr39 significantly and positively correlated with the histological grade, tumor size and advanced tumor node metastasis (TNM) stage \((P < 0.01, \text{Table 1})\). According to the follow-up data (Fig. 6E), patients were at risk of a low survival rate with a higher level of H2A.X\(^{Y39}\) phosphorylation; a significant negative correlation was shown between them \((P < 0.01)\).

**Discussion**

Activation of oncogenes and/or inactivation of tumor suppressor genes through genetic and/or epigenetic mechanisms confers on cancer cells the anomalous capability of proliferation by aberrantly activating self-renewal pathways. This phenomenon might be the consequence of dedifferentiation during tumorigenic transformation of somatic cells, inhibition of cancer cell differentiation, or neoplastic transformation of cancer stem cells [29,30].

To interpret the results of our proliferation experiments, we presumed that cancer cell proliferation and self-renewal mechanisms, which share many common signal pathways and factors [30,31], control tumor cell differentiation and growth. Although the detailed mechanisms require further investigation, collectively our results indicated that down-regulation of EYA2 and up-regulation of H2A.X\(^{Tyr39}\) phosphorylation promoted cancer cell proliferation.

The similarity between the proliferation and self-renewal capabilities of poorly differentiated cancer cells and normal stem cells results in difficulty in preserving normal stem cells and tissue repair when killing cancer cells during clinical treatment. This effect contributes to the toxicity of cancer therapy [30]. We were interested in the status of H2A.X Tyr39 in normal stem cells with the expectation that phosphorylation of H2A.X Tyr39 could be a useful diacritical point for cancer therapy. Moreover, phosphorylation of H2A.X Tyr39 is a prerequisite for the formation of γ-H2A.X. Therefore, the functions of phosphorylated H2A.X Tyr39 warrant more extensive and thorough exploration in both normal and cancer cells.

An interesting result that we obtained in breast and lung cancers was the increase in H2A.X\(^{Y39}\) phosphorylation without EYA2 down-regulation (Fig. 4B). It has been reported that EYA2 is aberrantly upregulated in lung adenocarcinoma cells and breast cancer cells through different mechanisms [20,21,32], and the increasing level of EYA2 spurs tumor cell growth by regulating the proliferation and migration capacities of the cells. These findings indicate that different enzymes or mechanisms function in these two kinds of cancer cells and in the complex functions and modifications of H2A.X. Modification of H2A.X in different tissue types and developmental stages is worthy of further investigation.

**Materials and methods**

**Ethics**

All studies performed with human cancer specimen and mice were approved by the Ethics Committee and Animal Care Committee of North China University of Science and Technology, and informed consent was obtained from all patients.
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Table 1. Correlation of H2A.XY39ph and clinicopathological parameters in breast cancer. Detection was by immunohistochemistry and quantification was with ImageJ software.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Case no.</th>
<th>H2A.XY39ph</th>
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<td>LN metastasis</td>
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<tr>
<td>Positive</td>
<td>91</td>
<td>19.05 ± 1.39**</td>
</tr>
<tr>
<td>Negative</td>
<td>39</td>
<td>10.42 ± 1.37</td>
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<td>Tumor size</td>
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<td>&gt; 3 cm</td>
<td>71</td>
<td>19.95 ± 1.51**</td>
</tr>
<tr>
<td>≤ 3 cm</td>
<td>59</td>
<td>9.95 ± 1.35</td>
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<tr>
<td>Histological grade (differentiation)</td>
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<tr>
<td>III (poor)</td>
<td>51</td>
<td>19.50 ± 1.39**</td>
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<td>I–II (well + moderate)</td>
<td>29 + 50</td>
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<tr>
<td>III + IV</td>
<td>35 + 33</td>
<td>19.95 ± 1.33**</td>
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<tr>
<td>I + II</td>
<td>23 + 39</td>
<td>9.05 ± 1.51</td>
</tr>
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<td>&gt; 60 years</td>
<td>77</td>
<td>20.89 ± 1.31**</td>
</tr>
<tr>
<td>≤ 60 years</td>
<td>53</td>
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Values are means ± SD. Data were analyzed by independent-samples t test; **P < 0.01.

Cell culture and patient samples

HCT15 and SU.86.86 cells were obtained from the Cell Culture Center of Peking Union Medical College and were cultured as recommended. U2OS cells, EYA2+/ HCT15 cells, H2A.X−/− HCT15 cells and H2A.X−/− SU.86.86 cells were gifts from Professor Li-Ren Ma (North China University of Science and Technology). Cell line authentication and validation by short tandem repeats was performed on 9 July 2015. All cell lines were routinely screened for the presence of mycoplasma (Mycoplasma Detection Kit, Roche Diagnostics, Beijing, China).

The patient samples of matched adjacent normal tissues and cancer tissues were provided by Central Lab of Cancer Institute, Tangshan People’s Hospital (Tangshan, China). Samples from patients with complete information for clinicopathological characteristics were selected.

Antibodies

The antibodies against γ-H2AX (cat. no. 9718), phosphotyrosine (cat. no.9411), MDC1 (05-1572) and H2AX (cat. no.7631) were purchased from Cell Signaling Technology (Shanghai, China). The antibodies against β-actin (cat. no.TA09) and Flag-tag (cat. no.TA08) were purchased from Zhongshan Golden Bridge Biotechnology Co. (Beijing, China). The antibody against EYA2 (sc-100325) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Plasmid construction and siRNA

The coding regions of human H2A.X were amplified from HeLa cDNA by polymerase chain reaction (PCR). The PCR products were subcloned into Flag-tag vectors and sequenced. The H2A.X mutant was constructed using the TaKaRa MutanBEST Kit (cat. no. R401), as recommend by the manufacturer. siRNAs were purchased from GenePharma (Shanghai, China).

Immunohistochemical analysis

Tumor sample was scored by an HSCORE method that combines the values of immunoreaction intensity and the percentage of tumor cell staining [33]. Immunohistochemistry was performed with a 2-step plus-Poly-HRP Anti-Mouse/Rabbit IgG Detection System (PV-9000, Zhongshan-Jinqiao, Beijing, China) as recommended. The chi-squared test was applied to analyze the correlations between discrete variables. Statistical significance was determined with two-sided log-rank analysis.

Immunoprecipitation and western blot

IP assays and western blot were performed as described previously [34]. Briefly, the cell extracts were incubated with a magnetic beads antibody complex for 5 h at 4 °C. Then the complex was washed and the immunoprecipitated proteins were analyzed by SDS/PAGE, transferred onto PVDF membranes and detected using each antibody.

Neutral comet assays

The single cell gel electrophoresis assay was conducted as described previously and the comet tail moment was determined with the comet data analysis software ( Comet Assay IV, Perceptive Instruments, UK).

Fig. 6. Colon cancer progression correlates with EYA2 and H2A.XY39ph phosphorylation. (A) Western blot analysis of EYA2 and H2A.XY39ph in paired fresh tissues of several kinds of human tumors. Ten paired samples were included for each type of cancer. Scores were determined by analyzing the protein bands of western blots against β-actin or total H2A.X. Data are shown as the mean ± SD. (B) The expression were determined by evaluating the extent and intensity of immunopositivity as described in Materials and methods. The ratio between H2A.XY39ph and Ki-67 was plotted and a positive correlation was significant (P < 0.001). H2A.XY39ph negatively correlates with the level of EYA2 (P < 0.005). (C) Representative human tumor and adjacent normal section analyses with specific antibodies. (D) The number of I–III grade cases are 29, 50 and 51. The scores were determined by evaluating the extent and intensity of immunopositivity. Values are means ± SEM. P-values were determined by the two-tailed unpaired t test; **P < 0.01. (E) The 5-year survival rate was determined by the Kaplan–Meier survival curves illustrate the relationship between an increased level of H2A.XY39ph and shorter overall survival. The survival rate of patients is shown on the y axis, and the x axis indicates the survival in months.
analyzed by COMETSORE software [17]. Briefly, cells were arrested at G1 by treatment with thymidine for 24 h. The cells were treated with 5 Gy IR and cultured in an incubator for 0–6 h. The slides were immersed in neutral lysis solution for 6 h. The supernatant was discarded and the pellet of cells resuspended by pipetting with low melting point agarose following cell centrifugation. Electrophoresis was performed at 18 V for 30 min (0.5 V cm−1). Then, the slides were stained with 12 μg mL−1 propidium iodide for 15 min and analyzed with a fluorescence microscope.

Homologous recombination assays
U2OS cells (105; direct-repeat green fluorescent protein (DR-GFP)) were plated and cultured for 20 h before transfection with EY42-specific siRNAs or control siRNAs. Twenty-four hours later, cells were transfected with a recombination-defective adenovirus expressing I-SceI to induce DNA DSBs in the DR-GFP reporter regions harboring the endonuclease recognition site, which produces GFP-expressing cells after repair through homologous recombination. The results of homologous recombination repair were analyzed using flow cytometry to detect cells expressing GFP. Quantification was by examination of at least 20 000 events per condition. The dividing line between cells with or without GFP was set to the background level for the cells expressing GFP in the internal control. This gate was used for the I-SceI-positive cells to evaluate homology-directed repair results [35,36].

Cell proliferation assay
The cell proliferation assay was performed with the Cell Proliferation and Cytotoxicity Assay Kit (Cell Counting Kit-8 (CCK-8), C0037), from Beyotime Institute of Biotechnology (Shanghai, China). Briefly, 100 μL of HCT15 cells suspension (5 × 103 cells per well) was dispensed in a 96-well plate. The plate was pre-incubated for 24 h in a cell culture incubator. Then, transfection experiments with siRNAs were performed with Lipofectamine 2000 (Invitrogen, Shanghai, China). The cells were incubated for different lengths of time as indicated. CCK-8 solution (10 μL) was added to each well and the plate incubated for 2 h. Finally, absorbance at 450 nm was measured.

Acknowledgements
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Conflict of interest
The authors declare that they have no conflict of interest.

Author contributions
YL, WSQ and JHZ had the idea for the experiment and supervised the project. YL, YHL, SQW and YFL, carried out the experiment. YL, SQW and YHL wrote the manuscript.

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