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Dephosphorylation of the C-terminal Tyrosyl Residue of the DNA Damage-related Histone H2A.X Is Mediated by the Protein Phosphatase Eyes Absent*

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In mammalian cells, the DNA damage-related histone H2A variant H2A.X is characterized by a C-terminal tyrosyl residue, Tyr-142, which is phosphorylated by an atypical kinase, WSTF. The phosphorylation status of Tyr-142 in H2A.X has been shown to be an important regulator of the DNA damage response by controlling the formation of γH2A.X foci, which are platforms for recruiting molecules involved in DNA damage repair and signaling. In this work, we present evidence to support the identification of the Eyes Absent (EYA) phosphatases, protein-tyrosine phosphatases of the haloacid dehalogenase superfamily, as being responsible for dephosphorylating the C-terminal tyrosyl residue of histone H2A.X. We demonstrate that EYA2 and EYA3 displayed specificity for Tyr-142 of H2A.X in assays *in vitro*. Suppression of *eya3* by RNA interference resulted in elevated basal phosphorylation and inhibited DNA damage-induced dephosphorylation of Tyr-142 of H2A.X *in vivo*. This study provides the first indication of a physiological substrate for the EYA phosphatases and suggests a novel role for these enzymes in regulation of the DNA damage response.

Unlike kinases, which are derived from a common ancestor, the opposing phosphatases have evolved in separate, structurally distinct families. In fact, a variety of catalytic mechanisms have been harnessed to facilitate protein dephosphorylation. The protein-Ser/Thr phosphatases, such as the PPP and PPM families, mediate dephosphorylation by using two metal ions at the active site, activating a water molecule for nucleophilic attack on the substrate phosphate in a single-step reaction (1). The family of Cys-dependent protein-tyrosine phosphatases (PTPs)[4] utilizes a two-step catalytic mechanism involving an essential nucleophilic cysteinyl residue, which forms a Cys-phosphate intermediate (2). More recently, a third family of Asp-dependent phosphatases belonging to the haloacid dehalogenase (HAD) superfamily (3) has been gaining prominence.

Various HAD phosphatases have been linked to fundamental aspects of control of cell function. One of the best characterized is FCP1, which is an important regulator of transcription through dephosphorylation of the C-terminal domain of RNA polymerase II (4). Chronophin has been shown to dephosphorylate Ser-3 in cofilin and thereby function as a regulator of the actin cytoskeleton (5). More recently, a HAD phosphatase known as Dullard has been shown to dephosphorylate the phosphatidic acid phosphatase lipin, functioning in a phosphatase cascade that regulates nuclear membrane biogenesis (6). In these cases, the HADs are functioning as protein-Ser/Thr phosphatases. However, there is now also an example of a member of the HAD superfamily with the capacity to dephosphorylate phosphotyrosyl residues in substrates.

Eyes Absent (EYA) was identified initially as a component of a network of transcription regulators, the retinal determination gene network that is responsible for eye development in *Drosophila*. It is now known to be involved in tissue and organ development in many organisms (7). There are four EYA proteins in mammals (designated EYA1–4) that are defined by a C-terminal domain of ~270 residues termed the EYA domain (ED) (7). This domain is required for interaction with the homeodomain protein Sine oculis (SO) in *Drosophila* (known as the SIX proteins in vertebrates) and the transcriptional regulator Dachshund (DACH). EYA and SIX function as a transcription factor complex, in which SIX mediates DNA binding, and EYA uses its N-terminal segment to function in transactivation. Considerable progress has been made in characterizing the function of EYA as a transcription factor (7). The demonstration of altered expression of the SIX-EYA-DACH network has also illustrated a potential role in cancer (8, 9). However, a major breakthrough came with the observation that the ED of EYA contains signature sequences from the HAD superfamily.

EYA is the first example of a transcription factor with intrinsic phosphatase activity (10–12). Characterization with phosphopeptides indicated specificity for phosphotyrosyl residues (10, 11), although one group suggested that EYA could function as a dual-specificity phosphatase (12). The phosphatase activity of EYA is important for switching SIX1-DACH function from transcriptional repression to activation, and inactivating mutations also impair EYA function in eye development (10–12). Furthermore, mutations in *eya1* that are associated with branchio-oto-renal syndrome have been shown to result in loss of

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The atomic coordinates and structure factors (code 3GEB) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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[4] The abbreviations used are: PTP, protein-tyrosine phosphatase; HAD, haloacid dehalogenase; ED, EYA domain; RNAi, RNA interference; siRNA, small interfering RNA; SH2, Src homology 2; DSBs, double-strand breaks.

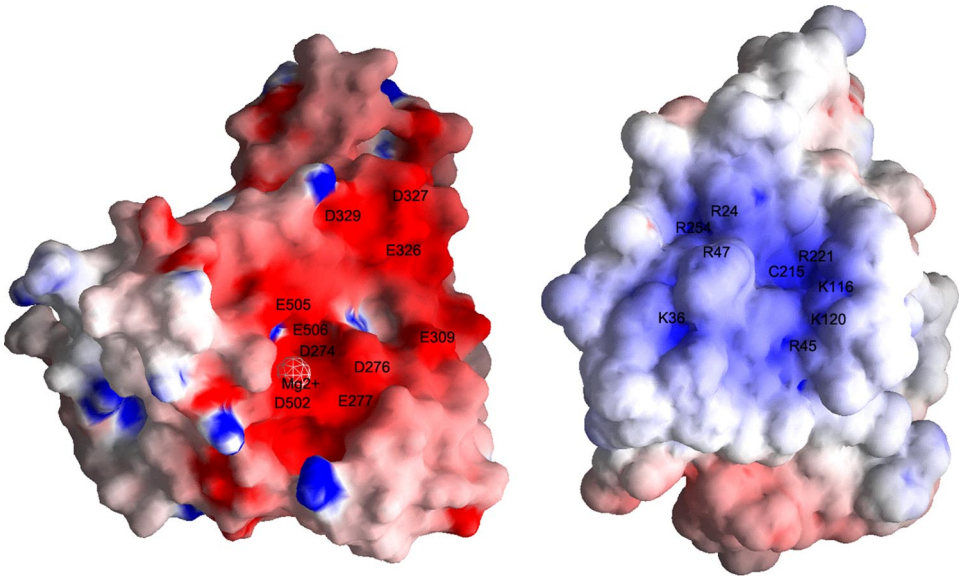

FIGURE 1. **Surface electrostatic potential of the ED of EYA2.** The surface electrostatic potentials of the ED of EYA2 (*left*; Protein Data Bank code 3GEB) and PTP1B (*right*; code 2HNP) are compared. Surface electrostatic potential was calculated with contours from −10 (*red*) to +10 (*blue*) kTe^{-1} (where *k* is the Boltzmann constant, *T* is temperature, and e is electron). The negatively charged residues near the active site are labeled in the EYA2 ED, whereas the positively charged residues are labeled in PTP1B. A bound magnesium ion in the ED of EYA2 is shown as a *meshed ball*, and the essential catalytic Cys residue (Cys-215) is labeled to indicate the catalytic core of PTP1B.

phosphatase activity (13). Nevertheless, the identity of downstream physiological substrates of EYA (cytosolic and nuclear) remains a major unresolved issue.

In this study, we report that a striking feature of the ED of EYA2, as revealed by the crystal structure, is the clustering of acidic residues around the active site to present a negatively charged surface. This suggests that substrates of the enzyme may have basic properties. Recently, phosphorylation of the C-terminal tyrosyl residue of the DNA damage-related histone H2A.X has been revealed as a new element in the control of the DNA damage response (14). Considering the known functions of EYA in the nucleus and the fact that histones are recognized as highly basic proteins, we sought to determine whether EYA could dephosphorylate histone H2A.X as a substrate. We present data from *in vitro* assays demonstrating the specificity of EYA for Tyr-142 in H2A.X. Furthermore, we show that suppression of EYA by RNA interference (RNAi) results in enhanced basal phosphorylation and inhibition of DNA damage-induced dephosphorylation of the C-terminal tyrosyl residue of histone H2A.X in cells. These data provide the first indications of a physiological substrate of EYA and suggest that the phosphatase may be an important component of the response to DNA damage.

MATERIALS AND METHODS

Protein Purification and Phosphatase Assays—Recombinant human EYA2 (residues 268–538) and EYA3 (residues 255–536) were produced in *Escherichia coli* strain BL21(DE3) as N-terminally His-tagged proteins and purified by nickel-nitrilotriacetic acid affinity chromatography. The sequence of the C-terminal H2A.X peptide was CPSGGKKATQASQEY. Phosphate release was measured using the malachite green detection assay.

RNAi—The small interfering RNA (siRNA) against *eya3* was a pool of three target-specific 19–25-nucleotide siRNAs (Santa Cruz Biotechnology). As a control, nonspecific non-targeting fluorescein-conjugated siRNAs were used. Cells were transfected 24 h after plating (5×10^3 cells/well in 6-well plates or 1×10^6 cells in a 10-cm culture dish) with up to 30 nM siRNA pool.

Reverse Transcription-PCR—Total RNA was phenol-extracted, precipitated, and reverse-transcribed to synthesize total cDNA. The resulting cDNA was then used as a template for PCR amplification using specific primers for EYA3 (5′-ATGGAAGAAGAGCAAGATTTACCAGAGC-3′ and 5′-GAGAAAATCAAGCTCTAAAGCCTGGTG-3′) and the loading control glyceraldehyde-3-phosphate dehydrogenase.

RESULTS

Distinct Surface Charge Properties of the ED of Human EYA2—We determined the structure of the ED of EYA2 at 2.4 Å resolution by the multiple isomorphous replacement method using two heavy atom derivatives. Details of the structural analysis will be described elsewhere.[5] The domain has a typical α/β-hydrolase fold and possesses the three conserved sequence motifs and the bound magnesium ion that are common to the active site of members of the HAD family (3), suggesting that EYA shares a common mechanism with other HADs. A striking feature is that with five aspartate and five glutamate residues exposed around the active site, the surface is predominantly negatively charged (Fig. 1). These residues show 100% sequence conservation among EYA homologs, suggesting the possibility that these phosphatases may recognize basic substrates. A comparison of the surface charge properties of the EYA2 ED with those of PTP1B, the prototype for the family of classical Cys-based PTPs, revealed a striking difference. Unlike the EYA2 ED, the surface of PTP1B, like that of most of the non-transmembrane Cys-based PTPs (15), is mostly positively charged close to the active site, suggesting differences in substrate recognition by these enzymes and EYA. In light of the highly basic nature of histones and the recent discovery that phosphorylation of the C-terminal tyrosyl residue of histone H2A.X (Tyr-142 in human and mouse H2A.X) may be of regulatory importance (14), we decided to test first a matched series of C-terminal H2A.X peptides as substrates.

EYA3 Displays Metal-dependent PTP Activity for the C-terminal Tyrosyl Residue of Histone H2A.X in Vitro—Synthetic peptides with a sequence derived from the C terminus of histone H2A.X were generated in monophosphorylated (Ser-139 or Tyr-142) and dual-phosphorylated (Ser-139 and Tyr-142) forms and tested as substrates of recombinant EYA2 (Fig. 2*A*)

[5] S.-K. Jung, *et al.*, manuscript in preparation.

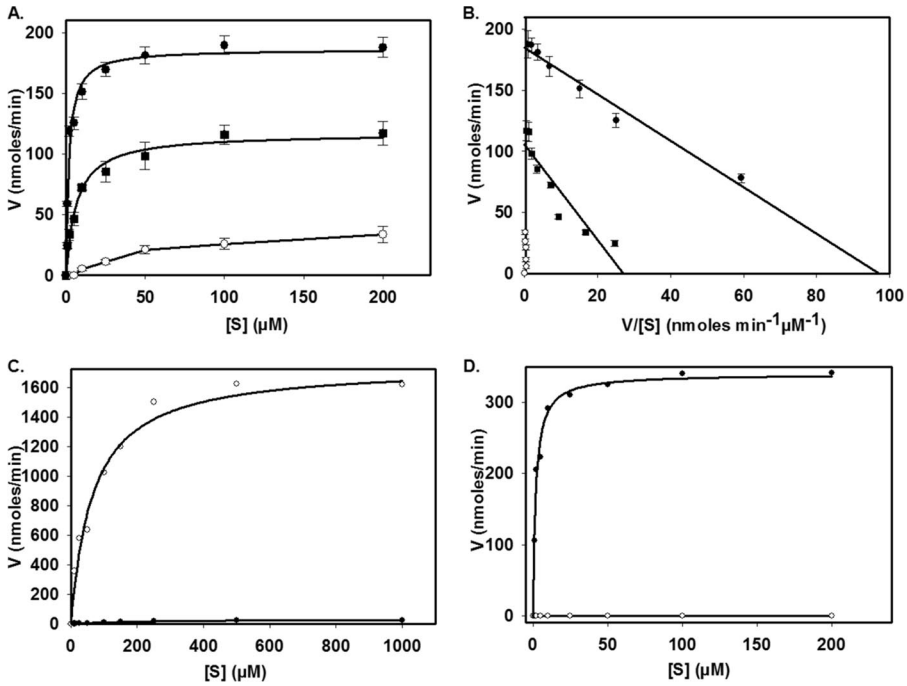

FIGURE 2. **Substrate specificity of EYA2 and EYA3.** *A* and *B*, the dephosphorylation of monophosphorylated H2A.X phospho-Tyr-142 (●), monophosphorylated H2A.X phospho-Ser-139 (○), and bisphosphorylated H2A.X phospho-Ser-139/phospho-Tyr-142 (■) peptides is shown. A plot of rate *versus* substrate concentration for dephosphorylation by EYA2 is shown in *A*, and an Eadie-Hofstee plot for EYA3 is shown in *B*. Data are the means ± S.D. from three experiments. *C*, the activity of EYA3 (●) was compared with that of PTP1B (○) against the peptide substrate pYEEY. *D*, the activity of EYA3 (●) was compared with that of PTP1B (○) against the monophosphorylated H2A.X phospho-Tyr-142 peptide. The enzymes were used at 10 nM.

and EYA3 (Fig. 2*B*). Both enzymes displayed classic Michaelis-Menten kinetics and dephosphorylated phospho-Tyr-142 with similar kinetic constants (EYA2, K_m = 1.9 μM and k_{cat} = 5.1 s^{-1}; EYA3, K_m = 1.8 μM and k_{cat} = 5.2 s^{-1}). In both cases, there was a dramatic decrease in the efficiency of dephosphorylation of phospho-Ser-139 (EYA2, K_m = 80 μM and k_{cat} = 0.9 s^{-1}; EYA3, K_m = 72 μM and k_{cat} = 0.9 s^{-1}). Interestingly, as illustrated for EYA3 (Fig. 2*B*), when the dual-phosphorylated form (phospho-Ser-139/phospho-Tyr-142) of the peptide was tested, an inhibitory effect was observed on both K_m and k_{cat} compared with the monophospho-Tyr-142 peptide (EYA2, K_m = 3.8 μM and k_{cat} = 2.9 s^{-1}; EYA3, K_m = 3.5 μM and k_{cat} = 3.2 s^{-1}). This indicates not only a striking preference of EYA2 and EYA3 for the dephosphorylation of phospho-Tyr-142 but also raises the possibility that the dephosphorylation of the C-terminal tyrosyl residue may be influenced by phosphorylation of the neighboring residue Ser-139 in a "cross-talk" pathway.

Specificity of the Effect of EYA3 on Histone H2A.X Phosphorylation Sites in Vitro—To investigate the substrate specificity of EYA3, we tested its activity against other phosphotyrosyl synthetic peptides. The monophosphorylated synthetic peptide pYEEY is derived from a well characterized SH2 domain-binding motif from the RNA-binding protein SAM68 (16). This peptide was an efficient substrate for PTP1B, with k_{cat} = 30 s^{-1}. In contrast, EYA3 showed no measurable activity against the pYEEY peptide (Fig. 2*C*). Similarly, another monophosphorylated synthetic peptide with the sequence pYEEI, which is a ligand for the Fyn SH2 domain (17), was readily dephosphorylated by PTP1B (k_{cat} = 29 s^{-1}); however, there was no measurable phosphatase activity when the peptide was incubated with EYA3. Similar results were obtained with EYA2. In the converse experiment, PTP1B was tested against the monophosphorylated synthetic peptide (phospho-Tyr-142) derived from histone H2A.X (Fig. 2*D*). Although PTP1B has been shown to dephosphorylate a wide variety of synthetic peptides *in vitro* (18), it displayed no measurable activity against the H2A.X phospho-Tyr-142 peptide. Overall, these data suggest that EYA2 and EYA3 have the potential to exhibit selectivity in the dephosphorylation of phospho-Tyr-142 of histone H2AX.

Suppression of EYA3 Expression by RNAi Enhances the Phosphorylation of Tyr-142 in Histone H2A.X in U2OS Cells—In light of the specificity for the H2A.X phospho-Tyr-142 peptide *in vitro*, we tested the effects of suppressing EYA expression in cells by RNAi. We transfected U2OS cells with siRNA pools (up to 30 nM) against *eya3*. Analysis by reverse transcription-PCR indicated that transfection of the siRNA pools reduced mRNA expression by ~80% for *eya3* (Fig. 3*A*). Under these conditions, we tested the phosphorylation status of Tyr-142 of histone H2A.X using a phospho-specific antibody in immunoblots of a total histone preparation (14). Interestingly, following suppression of EYA3, the level of H2A.X Tyr-142 phosphorylation was elevated relative to the control, consistent with a link between EYA3 and dephosphorylation of the C terminus of histone H2A.X (Fig. 3*B*). Furthermore, we tested the effects of DNA damage treatment on the phosphorylation status of H2A.X in the presence and absence of EYA3. In control cells, as expected, we observed that DNA damage induced dephosphorylation of Tyr-142 in H2A.X. In contrast, following suppression of *eya3*, this dephosphorylation was abrogated (Fig. 3*C*). Finally, treatment with the *eya3* siRNA pools, but not the control siRNA, induced the majority of the cell population to round up and lift off the plate, consistent with cell death.

DISCUSSION

Double-strand breaks (DSBs) are an extremely deleterious form of DNA damage that can lead to losses of large pieces of DNA during cell division. Incorrect repair may generate chromosomal rearrangements and mutations with the potential for cell death and carcinogenesis. Therefore, tight control over the repair process is required. The reversible phosphorylation of histone H2A.X is an important component of the cellular response to DSBs, natural or imposed, in what is beginning to be described as an "epigenetic landscape" for DNA damage and repair (19, 20).

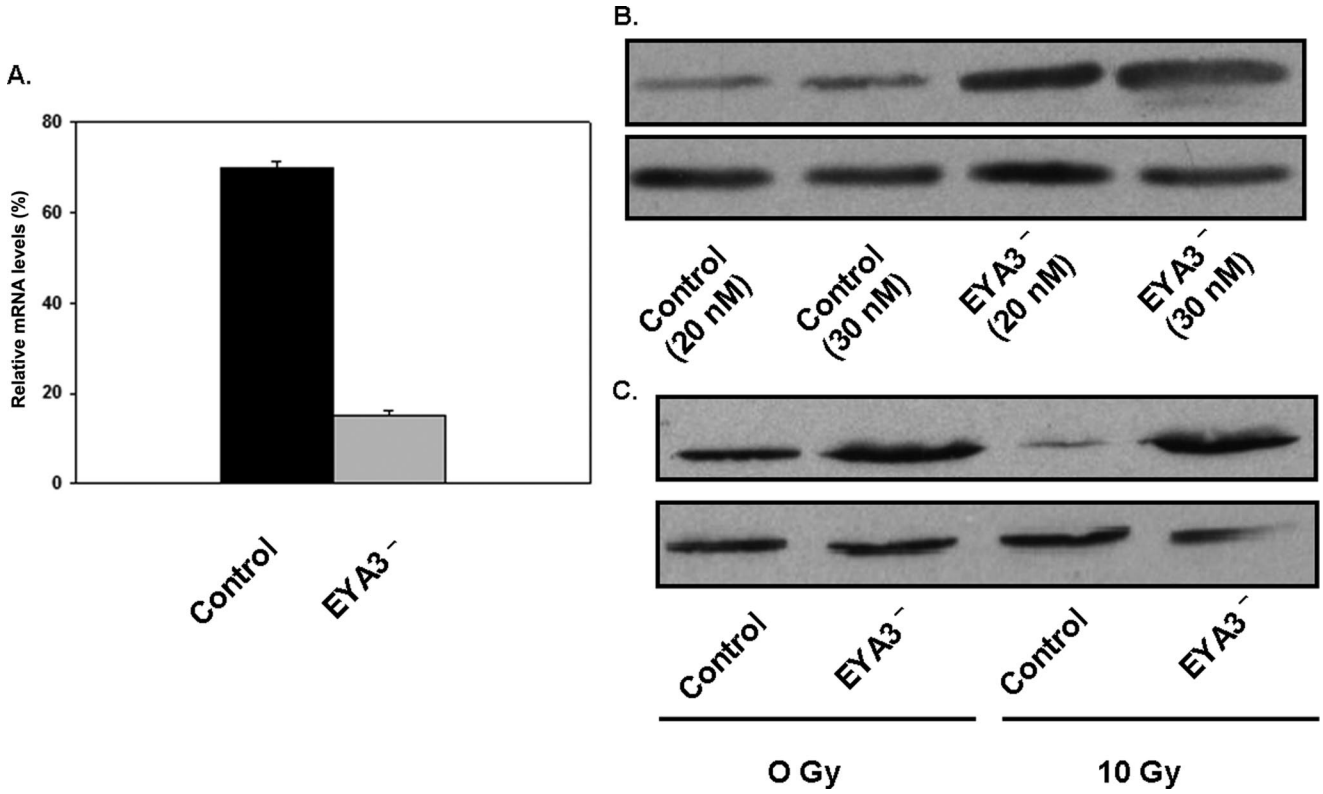

FIGURE 3. **Effects of suppression of *eya3* by RNAi on the phosphorylation of Tyr-142 in histone H2A.X.** *A*, expression of *eya3* was determined by reverse transcription-PCR of total RNA obtained from cultured U2OS cells transfected with 30 nM siRNA. The control cells were transfected with a fluorescently tagged siRNA pool. Glyceraldehyde-3-phosphate dehydrogenase mRNA expression was used as an internal standard. *B*, U2OS cells were transfected with *eya3* siRNA (up to 30 nM). Histones were isolated by acid extraction (27), resolved by SDS-PAGE, and subjected to immunoblotting with anti-phospho-Tyr-142 H2A.X antibody (*upper panel*) or anti-H2A.X antibody (*lower panel*) to assess the phosphorylation status of Tyr-142. *C*, U2OS cells were irradiated with 10 grays (*Gy*) of ionizing radiation in the presence or absence of 30 nM *eya3* siRNA. Histones were extracted before or 4 h post-irradiation and blotted with anti-phospho-Tyr-142 H2A.X antibody (*upper panel*) or anti-H2A.X antibody (*lower panel*) to assess the effect on DNA damage-induced dephosphorylation of H2A.X.

A histone octamer, which is composed of two molecules each of histones H2A, H2B, H3, and H4, forms the core of the nucleosome, the basic unit of chromatin. The variant H2A.X comprises ~10% of the H2A pool in mammalian cells and contains a unique C-terminal segment with sites of regulatory phosphorylation. Within minutes of genotoxic stress, such as ionizing radiation, histone H2A.X becomes phosphorylated at Ser-139 in its C-terminal segment by members of the PIKK family of Ser/Thr kinases, including ATM. Phosphorylated H2A.X decorates a broad region of ~1 megabase flanking the DNA break, which serves as a platform for recruiting molecules involved in damage repair and signaling. These regions are referred to as γH2A.X foci (19). There is evidence to suggest that this Ser/Thr phosphorylation of H2A.X is reversible and that the histone is dephosphorylated after repair is complete (21–23), emphasizing the importance of reversible Ser phosphorylation in the recovery from DNA damage. However, recently a new tier of control over the response to DNA damage has been identified, which involves reversible tyrosine phosphorylation of histone H2A.X (14).

In mammalian cells, histone H2A.X possesses a tyrosyl residue at its C terminus (Tyr-142), which is constitutively phosphorylated under normal growth conditions (14). Following DNA damage, such as in response to ionizing radiation, this residue becomes dephosphorylated, while γH2A.X, the Ser-139-phosphorylated form, appears. Phosphorylation of Tyr-142 is catalyzed by WSTF, a BAZ/WAL chromatin-remodeling factor that has now been shown to possess intrinsic protein kinase activity. WSTF together with SNF2H (an ISWI ATPase) constitutes the WICH complex (WSTF-ISWI ATPase-dependent chromatin-remodeling complex), which binds to histone H2A.X under normal growth conditions and has been implicated in DNA replication. Suppression of WSTF expression by RNAi led to a decrease in the phosphorylation of Tyr-142 in H2A.X, resulting in a rapid decline in the level of γH2A.X after ionizing radiation, unlike in control cells, in which the levels were sustained for hours. The recruitment of proteins critical for formation of γH2A.X, such as MDC1 and ATM, was defective, and the normal progression from small to large γH2A.X foci was not observed in the absence of WSTF. Phosphorylation of Ser-139 and formation of γH2A.X foci were also reduced in cells expressing H2A.X in which Tyr-142 was mutated to Phe or Leu, residues that cannot be phosphorylated, suggesting that the phosphorylation/dephosphorylation of these sites may be coordinated in a poorly understood "cross-talk" pathway. Clearly, the identity of the phosphatase(s) that dephosphorylates Tyr-142 is a critical component of this novel aspect of the DNA damage response, as it would be part of the enzyme system responsible for governing the steady-state balance of this regulatory phosphorylation mark.

Our data indicate that EYA3 may function as a PTP in the dephosphorylation of Tyr-142 in histone H2A.X. Whether the

other EYA family members (EYA1, EYA2, and EYA4) can also serve this function remains to be established. Our data reveal specificity in the effects of EYA3 on H2A.X. The k_{cat} for dephosphorylation of the monophosphorylated H2A.X phospho-Tyr-142 synthetic peptide was ~600-fold faster than that for the general phosphatase substrate *p*-nitrophenyl phosphate. In addition, whereas there was a robust activity against the H2A.X phospho-Tyr-142 peptide, EYA2 and EYA3 did not dephosphorylate other phospho-Tyr peptides modeled on sequences that are known to engage signaling molecules. In contrast, a classical Cys-dependent PTP, PTP1B, readily dephosphorylated these SH2 domain-containing peptide ligands but was inactive against the H2A.X phospho-Tyr-142 peptide. It will be interesting to explore further whether the C-terminal location of the phosphorylation site favors recognition by EYA. Our data also provide further support for specificity of the EYA phosphatases for tyrosyl residues in proteins. In contrast to the phospho-Tyr-142 peptide, EYA displayed substantially lower activity for the same peptide that was phosphorylated at the residue equivalent to Ser-139 of H2A.X. Furthermore, both affinity and turnover were impaired by the presence of phosphate at the seryl residue in the dual-phosphorylated peptide. This raises the possibility that dual phosphorylation of these sites in histone H2A.X may impair the ability of EYA to dephosphorylate the C-terminal tyrosyl residue, perhaps due to repulsive charge interactions between the second phosphorylation site and the acidic surface surrounding the active center of the enzyme.

We noted that suppression of *eya3* led to increased basal phosphorylation and abrogated DNA damage-induced dephosphorylation of Tyr-142 in histone H2A.X, consistent with a role for EYA3 as a physiological H2A.X phosphatase. We observed that suppression of *eya3* by RNAi was accompanied by a pronounced increase in cell death, which has also been reported to accompany suppression of EYA family members in other systems (24, 25). Most often, γ-phosphorylation of H2A.X at Ser-139 is equated with "sensing" DSBs, leading to DNA repair in the context of a chromatin template. It is interesting to note, however, that histone H2A.X has not only been shown to function as a "caregiver" of the genome in DNA repair but has also been implicated in the control of apoptosis in a poorly understood response (26). Thus, it is possible that the interaction of EYA and H2A.X may play a critical role in directing a cell's decision either to undergo apoptosis or the process of DNA repair. Dephosphorylation of Tyr-142 is important for the formation of γH2A.X foci and the protein-protein interactions that support the process of DNA repair (14). It will be important to examine how the activity of EYA is regulated and how the phosphorylation status of Tyr-142 influences the protein-protein interactions at the C terminus of H2A.X that may direct a choice between death and DNA repair. It has been shown that the gene for H2A.X maps to a region of the genome that is frequently mutated or deleted in a variety of cancers, with suggestions of a role as a tumor suppressor (26). It would be anticipated that further characterization of the functional interaction between EYA and H2A.X will yield new insights into links between DNA damage, repair, apoptosis, and carcinogenesis.

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