A Novel Acetylation Cycle of Transcription Co-activator Yes-associated Protein That Is Downstream of Hippo Pathway Is Triggered in Response to S\textsubscript{N}2 Alkylation Agents*\textsuperscript{a,b}

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**Background:** YAP is a target molecule of the Hippo pathway.
**Results:** YAP acetylation and deacetylation were mediated by CBP/p300 acetyltransferase and SIRT1 deacetylase, respectively.
**Conclusion:** A YAP acetylation/deacetylation cycle is located downstream of the Hippo pathway.
**Significance:** The discovery that YAP undergoes an acetylation cycle advances our understanding of YAP functions.

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Yes-associated protein (YAP)\textsuperscript{2} is a transcriptional co-activator that acts downstream of the Hippo signaling pathway and regulates multiple cellular processes. Although cytoplasmic retention of YAP is known to be mediated by Hippo pathway-dependent phosphorylation, post-translational modifications that regulate YAP in the nucleus remain unclear. Here we report the discovery of a novel cycle of acetylation/deacetylation of nuclear YAP induced in response to \textsubscript{S}N\textsubscript{2} alkylation agents. We show that after treatment of cells with the \textsubscript{S}N\textsubscript{2} alkylation agent methyl methanesulfonate, YAP phosphorylation mediated by the Hippo pathway is markedly reduced, leading to nuclear translocation of YAP and its acetylation. This YAP acetylation occurs on specific and highly conserved C-terminal lysine residues and is mediated by the nuclear acetyltransferases CBP (CREB-binding protein) and p300. Conversely, the nuclear deacetylase SIRT1 is responsible for YAP deacetylation. Intriguingly, we found that YAP acetylation is induced specifically by \textsubscript{S}N\textsubscript{2} alkylating agents and not by other DNA-damaging stimuli. These results identify a novel YAP acetylation cycle that occurs in the nucleus downstream of the Hippo pathway. Intriguingly, our findings also indicate that YAP acetylation is involved in responses to a specific type of DNA damage.

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**This article contains supplemental Table S1 and Figs. S1–S3.**

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2 The abbreviations used are: YAP, yes-associated protein; hYAP, human YAP; meYAP, medaka; TEAD, TEA domain transcription factor; Lats, large tumor suppressor; MMS, methyl methanesulfonate; CBP, cAMP-response element-binding protein (CREB)-binding protein; p300, E1A-binding protein p300; HDAC, histone deacetylase; NAM, nicotinamide; SIRT, siruin; DMS, dimethyl sulfate; MNU, N,N-methylenebis(N,N-dimethylformamide); MNS, N,N-methylenebis(N,N-dimethylsulfonamide); WRN, Werner syndrome helicase; ABH, AlkB homolog; ASCC3, activating signal co-integrator 1 complex subunit 3; Ab, antibody; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.
YAP Acetylation Cycle Downstream of the Hippo Pathway

DNA alkylating agents (14, 15). Endogenous and environmental alkylating agents react with specific sites on the nucleic acid bases of DNA or RNA to generate methylated bases that are cytotoxic or mutagenic (16). The alkylating agents generating such bases are classified as either \( S_{N}2 \) or \( S_{N}1 \), depending on their reaction mechanism. The distribution of the methylated products on nucleic acid bases depends not only on the type of alkylating agent but also on whether the nucleic acid targeted is in a single-stranded or double-stranded state (16, 17). \( S_{N}2 \) alkylating agents, but not \( S_{N}1 \) agents, can act on single-stranded DNA and RNA to generate 1-methyladenine and 3-methylcytosine. However, the signal transduction events mediating cellular responses to damage caused by \( S_{N}2 \) alkylating agents are undefined. In this study we show that YAP is involved in specific cellular responses to \( S_{N}2 \) alkylating agents and provide the first evidence that YAP can be post-translationally modified via acetylation in the nucleus.

EXPERIMENTAL PROCEDURES

Plasmids—pCMV-FLAG-hYAP, pCMV-FLAG-TEAD4, pcDNA-VP16-hYAP, and pcS2-meYAP were constructed by inserting PCR-amplified DNA fragments into the indicated vectors. Sequences of PCR primers are listed in supplemental Table S1. The meYAP-13KR, meYAP-12KR/K, and hYAP-KR mutants were constructed by site-directed mutagenesis. Other plasmids used in this study have been described elsewhere (18).

Reagents—Methyl methanesulfonate (MMS), cisplatin, doxorubicin and \( N\)-methyl-\( N\)-nitrosourea (MNU) were purchased from Sigma. Camptothecin, \( N\)-methyl-\( N\)-nitro-\( N\)-nitrosooguanidine (MNNG), and dimethyl sulfate (DMS) were purchased from Wako Pure Chemical Industries.

Antibodies—Anti-YAP antibody (Ab) has been described previously (19). Polyclonal Abs recognizing Lys-494-acetylated hYAP or Lys-497-acetylated hYAP were generated in rabbits using synthetic peptides corresponding to acetylated human YAP (residues 489–499) (C-VLAATK(Ac)-LDKES) or acetylated human YAP (492–502) (C-ATKLDK(Ac)-ESFLT), respectively. For immunoizations, these peptides were conjugated via cysteine to maleimide-activated keyhole limpet hemocyanin. Anti-Ac-YAP (Lys-494) and Anti-Ac-YAP (Lys-497) polyclonal Abs were affinity-purified using the acetylated peptide and the SulfoLink Immobilization Kit for Peptides (Thermo Scientific). Anti-FLAG (M2) Ab was purchased from Sigma; anti-\( \beta\)-actin Ab, anti-CBP (C-20) Ab, and anti-p300 (N-15) Ab were from Santa Cruz; anti-pYAP (Ser-127) Ab and anti-pan-AcK Ab were from Cell Signaling; anti-SIRT1 Ab was from Millipore; anti-HA Ab was from Immunology Consultants Laboratory.

Cell Culture and Transfection—For routine culture, HeLa cells and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (normal medium). Transfection with FuGENE HD was performed according to the manufacturer’s instructions (Roche Applied Science).

Retroviral Vector Infection—To generate HeLa cells stably expressing hYAP-WT or hYAP-2KR, 293 retroviral packaging cells were transfected with empty retroviral vector or pCLNCX-hYAP constructs. At 48 h post-transfection, retroviral supernatant was supplemented with Polybrenne, passed through a 0.22-\( \mu \)m filter, and used to infect HeLa cells. At 36 h post-infection, cells were selected by adding G418 to the culture medium.

RNA Interference—HeLa cells were transfected with siRNA using DharmaFECT 1 (Dharmacon Inc.) according to the manufacturer’s instructions. siRNAs specific for CBP (siGENOME SMART pool human CREBBP, M-003477-02), p300 (siGENOME SMART pool human EP300, M-003486-04), or SIRT1 (siGENOME SMART pool human SIRT1, M-003540-01) and control siRNA (siGENOME Non-Targeting siRNA Pool #1, D-001206-13), were obtained from Dharmacon Inc.

MTS Assays—HeLa cells (2 \( \times \) 10\(^3\)) were plated in a 96-well dish in 100 \( \mu \)l of normal medium/well. For assessment of MMS sensitivity, cells seeded for 24 h were exposed for 1 h to medium containing an increasing concentration (0–3 mm) of MMS. The MMS-containing medium was then replaced with normal medium, and cell survival was assessed using the MTS assay (Promega) at 48 h after MMS exposure.

Immunoprecipitation—Immunoprecipitation and co-immunoprecipitation assays were performed as previously described (18) with some modifications. HeLa cells and HEK293T cells were seeded in 10-cm dishes and treated with DNA-damaging agents or transfected with the appropriate expression vectors as described in the figures. Cells were homogenized in immunoprecipitation buffer (150 mm NaCl, 5 mm EDTA, 15 mm MgCl\(_2\), 1% Nonidet P-40, 1 mm DTT, 0.5% deoxycholic acid, and 50 mm Tris-HCl, pH 8.0) or co-immunoprecipitation binding buffer (150 mm NaCl, 1 mm EDTA, 0.5% Nonidet P-40, 1 mm EGTA, 5% glycerol, and 20 mm Tris-HCl, pH 7.4) containing protease inhibitor mixture tablets. Homogenates were centrifuged for 10 min at 15,000 \( \times \) g. Total protein from the supernatant was incubated for 12 h at 4 °C with the appropriate Abs as described in the figures plus 20 \( \mu \)l of protein G-agarose beads (GE Healthcare). The beads were washed 3 times with immunoprecipitation buffer or co-immunoprecipitation wash buffer containing 1% Nonidet P-40 and boiled in SDS sample buffer. The supernatant was fractionated by SDS-PAGE and analyzed by standard immunoblotting.

Immunofluorescence—HeLa cells were cultured on a glass coverslip and fixed with 4% paraformaldehyde in PBS at room temperature. After treatment with 0.2% Triton X-100 in PBS, the cells were incubated with blocking solution (5% bovine serum albumin in TBS) before incubation with primary Abs for 1 h at room temperature. Cells were washed with PBS and incubated for 1 h with Alexa 488- or 546-conjugated secondary Abs. After PBS washes, coverslips were mounted and viewed on a Carl Zeiss confocal microscope equipped with LSM510 software.

Luciferase Reporter Assay—HEK293T cells or HeLa cells were plated in 12-well plates at 1.5 \( \times \) 10\(^5\) cells per well and incubated overnight. Cells were transfected the next day with 20 ng of firefly luciferase reporter plasmid (p5xUAS-c-fos-promoter-Luc or 8xGT-IIC-\( \delta \)S1LucII) and 5 ng of sea pansy luciferase reporter plasmid (pRL-CMV; from Promega) plus 40 ng of total expression plasmids (as indicated in the figures). The pcDNA vector was added as needed to make up the total
To investigate whether hYAP underwent a qualitative change in response to MMS treatment, we carried out a screen to identify post-translational modifications of hYAP. We used anti-YAP Ab to immunoprecipitate endogenous hYAP from lysates of MMS-treated HeLa cells and performed immunoblotting using several Abs that recognize specific post-translational modifications. As shown in Fig. 1D, we found that the endogenous hYAP present in MMS-treated HeLa cells could be detected by anti-pan-AcK Ab, which specifically recognizes acetylated lysine residues. Moreover, this hYAP acetylation was induced in a dose-dependent manner (Fig. 1E). These results clearly show that endogenous hYAP is acetylated in response to MMS treatment.

hYAP Acetylation Is Mediated by CBP/p300—The above data prompted us to identify the histone acetyltransferases responsible for hYAP acetylation. Because MMS treatment induced both the nuclear translocation and acetylation of hYAP, we focused on the abilities of CBP, p300, and CLOCK, which are histone acetyltransferases localized in the nucleus, to acetylate hYAP (18, 20). We found that co-expression in HEK293T cells of HA-tagged CBP or HA-p300 with FLAG-tagged hYAP significantly increased hYAP acetylation (Fig. 2A). In contrast, hYAP acetylation was not induced by CLOCK under conditions in which the well-established CLOCK target BMAL1b was acetylated (Fig. 2B). Thus, hYAP acetylation is induced specifically by the closely related histone acetyltransferases CBP and p300.

To determine whether hYAP physically interacts with CBP/p300, we first performed co-immunoprecipitation assays which revealed that HA-CBP co-immunoprecipitated with FLAG-hYAP but not with FLAG-TEAD4 (Fig. 2C). To confirm this result, we performed mammalian two-hybrid assays (21) in which hYAP fused to the VP16 transactivation domain (VP16-hYAP) was co-expressed with GAL4-CBP and not with VP16-BMAL1b fused to the GAL4 DNA binding domain. Only when VP16-hYAP was co-expressed with GAL4-CBP and not with GAL4-BMAL1b was luciferase reporter activity significantly increased (Fig. 2D). These results indicate that hYAP physically interacts with CBP.

To investigate the effect of MMS treatment on CBP/p300-induced hYAP acetylation, we treated HEK293T cells expressing HA-CBP or HA-p300 with MMS. Notably, acetylation of endogenous hYAP was synergistically enhanced by a combination of CBP/p300 expression and MMS treatment (Fig. 2E). These data imply that CBP/p300 serves as the histone acetyltransferase that acetylates hYAP in response to MMS treatment.

CBP-mediated Acetylation of YAP Targets Its C-terminal Lysine Residues—To identify the hYAP lysines targeted by CBP-mediated acetylation, we first compared the predicted amino acid sequences of human YAP, mouse YAP, and the YAP (meYAP) present in the small fish medaka. This analysis showed that there are 13 lysines shared by hYAP and meYAP (Fig. 3A and supplemental Fig. S1) and that meYAP contains no
additional lysines. This evolutionary conservation of critical lysine residues allowed us to utilize meYAP protein for the identification of common hYAP acetylation sites.

To determine precisely which YAP lysines are acetylated by CBP/p300, we generated a meYAP-13KR mutant in which all lysine residues were replaced by arginine residues. As expected, CBP-mediated acetylation of meYAP-13KR was completely abolished (Fig. 3B). We then restored individual lysines one at a time in meYAP-13KR to generate a series of meYAP-12KR/K mutants in which each retained a single, different lysine residue. Strikingly, restoration of Lys-430 or Lys-433 allowed resumption of CBP-mediated acetylation of meYAP-13KR (Fig. 3C). Consistent with this result, a meYAP-2KR mutant in which Lys-430 and Lys-433 were replaced with arginines exhibited dramatically reduced CBP-mediated acetylation compared with wild type (WT) meYAP (Fig. 3D). Finally, we determined whether hYAP is also acetylated at its conserved C-terminal lysines. Analogous to our results with meYAP, mutation of the C-terminal lysines 494 and 497 in FLAG-hYAP significantly reduced its CBP-mediated acetylation (Fig. 3E). Taken together, these data demonstrate that evolutionarily conserved lysine residues at the C terminus of YAP are targets for CBP-mediated acetylation.

CBP/p300 Mediates Lys-494 Acetylation of hYAP upon MMS Treatment—To investigate site-specific hYAP acetylation at the endogenous level, we prepared polyclonal Abs that specifically recognized the hYAP protein acetylated at Lys-494 or Lys-497. We first confirmed that these Abs detected exogenously expressed WT hYAP in the presence of HA–CBP expression but did not recognize mutant hYAP proteins bearing altered acetylation sites (data not shown). Next, we used these Abs to confirm that both Lys-494 and Lys-497 of endogenous hYAP are acetylated upon MMS treatment (Fig. 3F). Immunofluorescence analysis using anti-Ac-YAP (Lys-494) Ab showed that exogenously expressed FLAG-hYAP-WT, but not FLAG-hYAP-2KR, was acetylated after MMS treatment (Fig. 3G). Because the anti-AcYAP (Lys-497) Ab was unsuitable for immunofluorescence analysis, we utilized the anti-AcYAP (Lys-494) Ab for subsequent studies.
To investigate whether MMS-induced acetylation of hYAP Lys-494 is mediated by CBP/p300, we down-regulated CBP and/or p300 expression in HeLa cells using RNA interference (RNAi). Double knockdown of CBP and p300 completely reduced endogenous hYAP Lys-494 acetylation upon MMS treatment (Fig. 4D), indicating that MMS-induced acetylation of hYAP at Lys-494 can indeed be mediated by either CBP or p300. Because CBP and p300 are both nuclear proteins, we speculated that hYAP Lys-494 acetylation occurs in the nucleus. To determine the subcellular localization of endogenous Lys-494-acetylated hYAP, we performed an immunofluorescence analysis of HeLa cells transfected with HA-CBP. Endogenous Lys-494-acetylated hYAP was detected mainly in the nuclei of these cells (Fig. 4E). Taken together, these results indicate that the acetylation of hYAP Lys-494 that is induced by MMS is mediated by CBP and/or p300 and takes place in the nucleus.

SIRT1 Is Responsible for Deacetylation of hYAP Lys-494—Lysine acetylation is a reversible protein modification, and deacetylation of acetylated lysine is catalyzed by histone deacetylases (HDAC). HDACs are grouped into four classes according to phylogenetic analysis and sequence homology (22). To identify the HDACs responsible for deacetylation of hYAP Lys-494, we utilized class-specific HDAC inhibitors. When we treated HeLa cells with nicotinamide (NAM), an inhibitor of class III HDACs, MMS-induced acetylation of endogenous hYAP Lys-494 was markedly increased (Fig. 5A). In contrast, treatment with trichostatin A, an inhibitor of class I and II HDACs, had no effect on hYAP Lys-494 acetylation. Consistent with these results, CBP-mediated hYAP Lys-494 acetylation was also enhanced by NAM treatment but not by trichostatin A (Fig. 5B). Intriguingly, even in the absence of MMS, acetylation of endogenous hYAP Lys-494 was induced in a time-dependent manner after NAM treatment (Fig. 5C).
These results suggest that the acetylation-deacetylation cycle involving hYAP Lys-494 operates regardless of extrinsic DNA damage and that the deacetylation of this residue is catalyzed by a class III HDAC. The class III HDAC Sirtuin 1 (SIRT1) is reportedly transcriptionally up-regulated by MMS treatment and involved in responses to this agent (23). These observations prompted us to test whether SIRT1 might mediate hYAP Lys-494 deacetylation. Exogenous expression of SIRT1 in HeLa cells significantly decreased MMS-induced acetylation of hYAP Lys-494 (Fig. 5D). Consistent with this finding, knockdown of SIRT1 enhanced MMS-induced hYAP Lys-494 acetylation (Fig. 5E). Thus, SIRT1 is at least one of the class III HDACs responsible for deacetylating Lys-494-acetylated hYAP.

**SN2 Alkylating Agents Specifically Induce Both Acetylation and Ser-127 Dephosphorylation of hYAP**—YAP has previously been shown to be involved in cellular responses to a variety of DNA-damaging agents (10, 24, 25). Therefore, we investigated the effects of several DNA-damaging agents other than MMS on both hYAP Ser-127 phosphorylation and hYAP acetylation. Because cisplatin treatment induces nuclear translocation of hYAP (10) and H2O2 treatment activates the Hippo pathway (6, 19), we treated HeLa cells with cisplatin or H2O2 and examined hYAP Ser-127 phosphorylation and hYAP acetylation. Intriguingly, hYAP Ser-127 phosphorylation was down-regulated in a dose-dependent manner after cisplatin treatment but up-regulated in a dose-dependent manner upon H2O2 treatment (Fig. 6A). However, in contrast to MMS, neither cisplatin nor H2O2 induced the acetylation of endogenous hYAP (Fig. 6B). Nor was hYAP acetylation induced in HeLa cells by treatment with other DNA-damaging agents, including camptothecin or doxorubicin (supplemental Fig. S2). These data indicate that hYAP regulation by the Hippo pathway is differentially altered by different types of DNA damage and that hYAP acetylation may be a regulatory event specific to DNA alkylating agents such as MMS.

As mentioned above, alkylating agents are classified as being of either the S,N1 or S,N2 type depending on their reaction mechanism (16). Because MMS is an S,N2 alkylating agent, we first investigated whether another S,N2 agent could induce hYAP acetylation by treating HeLa cells with DMS. As expected, DMS treatment also resulted in a dose-dependent decrease in hYAP Ser-127 phosphorylation (Fig. 6C) and a dose-dependent increase in hYAP acetylation (Fig. 6D). We then treated HeLa cells with either of two S,N1 alkylating agents, MNNG or MNU. Phosphorylation of hYAP Ser-127 was reduced by MNNG treatment but not altered upon MNU exposure (Fig. 6E). Intriguingly, neither MNNG nor MNU could induce hYAP acetylation (Fig. 6F). These results indicate that S,N2 alkylating agents specifically induce two relevant events, 1) the release of hYAP from Hippo pathway-mediated cytoplasmic retention and 2) the up-regulation of CBP/p300-mediated hYAP acetylation in the nucleus.

**hYAP Acetylation Affects Cellular Responses to MMS Treatment**—YAP is known to be an important regulator of cell viability (2). To put our results in a biological context, we inves-
tigated whether hYAP acetylation influenced cellular responses to MMS treatment. We established HeLa cells stably expressing hYAP-WT or hYAP-2KR by retroviral vector infection and exposed them to increasing concentrations of MMS. Cell survival was assessed using a tetrazolium-based colorimetric viability assay (MTS). Interestingly, expression of hYAP-2KR significantly decreased cell survival compared with hYAP-WT expression (Fig. 7A), indicating that hYAP acetylation affects the sensitivity of HeLa cells to MMS treatment.

Next, we examined whether hYAP acetylation is important for its nuclear translocation after MMS exposure. As shown in supplemental Fig. S3, both the FLAG-hYAP-WT and FLAG-hYAP-2KR proteins accumulated in the nuclei of MMS-treated HeLa cells. Thus, the MMS-induced nuclear localization of hYAP is not affected by hYAP acetylation.

To investigate the physiological significance of hYAP acetylation in the nucleus, we co-transfected 293T cells with a synthetic YAP responsive luciferase reporter (8xGT-IIC/H925451LucII) (26) and FLAG-hYAP-WT or FLAG-hYAP-2KR. These cells were then exposed to MMS, and luciferase activity was measured. As shown in Fig. 7B, cells expressing FLAG-hYAP-2KR exhibited significantly higher luciferase activity than cells expressing FLAG-hYAP-WT, indicating that the transcriptional coactivator activity of hYAP is regulated by its acetylation. Taken together, these data suggest that cell fate decisions made in response to MMS-induced DNA damage may be governed by hYAP-dependent gene induction that is regulated by hYAP acetylation.

**DISCUSSION**

In this study we present evidence for a novel YAP acetylation cycle downstream of Hippo signaling. We have shown that MMS treatment causes a reduction in hYAP Ser-127 phosphorylation mediated by the Hippo pathway, promoting the nuclear translocation of this transcriptional co-activator. Through a screen for post-translational modifications of hYAP, we have demonstrated that hYAP is acetylated at the endogenous level upon MMS treatment. This MMS-induced acetylation of YAP is mediated by the nuclear acetyltransferases CBP and p300, occurs on conserved C-terminal lysine residues, and can be reversed by SIRT1. Importantly, this acetylation-deacetylation cycle of hYAP is triggered specifically in response to S,N2 alkylating agents and not by other types of DNA-damaging stimuli. A schematic model summarizing our findings appears in Fig. 8.
Our results have revealed that Lys-494 and Lys-497 of hYAP are targets for CBP-mediated acetylation. In previous reports, CBP and p300 were shown to directly acetylate a variety of proteins at a consensus motif (KX[K/R]XK) (27–30). Notably, hYAP contains this consensus motif (99KLDK497), and the hYAP acetylation sites identified in our experiments are consistent with the positions of two of the lysine residues present in this motif. Interestingly, although the acetylation consensus motif is conserved among vertebrate YAPs (human, mouse, and medaka), it is not found in either Yki (yorkei), the Drosophila homologue of YAP, or in TAZ (transcriptional coactivator with PDZ-binding motif), a vertebrate paralogue of YAP (4). Thus, tight regulation governs the state of hYAP acetylation induced in response to S2 alkylation agents.

A number of studies have reported that CBP/p300-mediated acetylation of several proteins is important for cellular responses to DNA damage. For example, the Werner syndrome helicase (WRN) helps to promote the survival of cells with damaged DNA. CBP-mediated acetylation of WRN triggered by DNA damage inhibits the ubiquitination-dependent degradation of this helicase and thus increases its stability (31). Similarly, DNA damage induces p300 to acetylate and stabilize Nijmegen breakage syndrome 1, which plays a crucial role in homologous recombination repair (32). The acetylation of proteins involved in DNA damage responses is often countered by SIRT1-mediated deacetylation (33), and acetylated WRN and Nijmegen breakage syndrome 1 are both substrates of SIRT1 (34, 35). The importance of such acetylation-deacetylation cycles has been highlighted by the significant increase in chromosomal aberrations identified in SIRT1-deficient cells (36). Our study has revealed that hYAP also undergoes reversible acetylation and deacetylation mediated by CBP/p300 and SIRT1, respectively. More importantly, our results indicate that acetylation of hYAP influences both its transcriptional co-activator activity and the sensitivity of hYAP-expressing cells to MMS-induced DNA damage.

Different genotoxic agents induce different types of DNA damage, and these lesions trigger lesion-specific DNA repair mechanisms. YAP has previously been shown to participate in responses to DNA-damaging agents such as cisplatin, adriamycin, ultraviolet radiation, and ionizing radiation (10, 24, 25), but the lesion-specific aspects of YAP regulation were not defined in these studies. In this report we have demonstrated that hYAP Ser-127 phosphorylation mediated by the Hippo pathway is differentially altered depending on the type of DNA-damaging agent encountered. Our data show that DNA-damaging stimuli can be classified into at least three groups based on their effects on the subcellular localization and post-translational modification of hYAP: Group I, Hippo pathway-mediated hYAP Ser-127 phosphorylation is up-regulated and hYAP remains in the cytoplasm; Group II, hYAP Ser-127 phosphorylation is down-regulated, and hYAP undergoes nuclear translocation but is not acetylated; Group III, hYAP Ser-127 phosphorylation is down-regulated, and hYAP undergoes both nuclear translocation and acetylation. In line with previous reports (6, 19), we have demonstrated that H2O2 treatment of HeLa cells results in up-regulated hYAP Ser-127 phosphorylation (Group I), whereas cisplatin treatment down-regulated Ser-127 phosphorylation but had no effect on acetylation (Group II). Treatment of HeLa cells with MNNG had a similar effect, placing this agent in Group II. The most interesting aspect of our work is the identification of the new Group III, represented solely by S2 alkylation agents, which both down-regulate Ser-127 hYAP phosphorylation and induce hYAP acetylation.

Our findings indicate that mere nuclear accumulation of hYAP is not enough to induce its acetylation, and another factor(s) must play an important role in this reaction. Along these lines, we demonstrated that CBP and p300 are key mediators of MMS-induced hYAP acetylation, implying that up-regulation of CBP/p300 activity may be the additional factor contributing to hYAP acetylation. Collectively, our data imply that the specific cellular response to DNA lesions caused by S2 alkylation agents involves the coupling of hYAP nuclear translocation induced by Ser-127 dephosphorylation with the activation of CBP/p300-mediated acetylation.

S2 alkylation agents, but not S1 agents, generate 1-methyladenine and 3-methylcytosine residues in single-stranded DNA and RNA. These cytotoxic lesions are known to be repaired by AlkB family dioxygenases (16). In mammalian cells, AlkB homolog 2 (ABH2) removes 1-methyladenine lesions in genomic DNA and thus is critical for cellular defense against MMS treatment (17). In addition, ABH3 collaborates with ASCC3 helicase to remove 3-methylcytosine lesions in certain tumor-derived cell lines (37). However, the mechanisms regulating ABH2 and ABH3 in response to S2 alkylation agents remain largely unknown. Intriguingly, 1-methyladenine accumulates in aging ABH2-deficient mice (17) and 3-methylcytosine accumulates in ABH3 or ASCC3 knockout cells (37), clearly showing that such cytotoxic methylation events occur endogenously. Relevant mechanisms regulating S2 alkylation agents demand further study.
vant in this context is the fact that S-adenosylmethionine, which is the methyl group donor in the majority of enzymatic methylation events, can act as an endogenous S_{2}2 alkylling agent and generates the same methylated adducts on DNA as MMS (38). We found that even in the absence of MMS exposure, acetylation of endogenous hYAP was observed after treatment with the class III HDAC inhibitor NAM. Thus, the hYAP acetylation cycle downstream of the Hippo pathway may occur in response to endogenous single-strand DNA/RNA alkyllation events induced by physiological S_{2}2 alkylling agents. It is, therefore, tempting to speculate that Hippo-YAP signaling may be involved in the intrinsic ALKB-mediated DNA repair pathway.

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