

Acetylated H4K16 by *MYST1* protects UROtsa cells from arsenic toxicity and is decreased following chronic arsenic exposure

William Jaime Jo^{a,1}, Xuefeng Ren^{b,1}, Feixia Chu^c, Maria Aleshin^b, Henri Wintz^a, Alma Burlingame^c, Martyn Thomas Smith^b, Chris Dillon Vulpe^{a,*}, Luoping Zhang^{b,*}

^a Department of Nutritional Sciences and Toxicology, University of California Berkeley, Berkeley, CA 94720, USA

^b Department of Environmental Health Sciences, School of Public Health, University of California Berkeley, Berkeley, CA 94720, USA

^c Department of Pharmaceutical Chemistry, University of California San Francisco, San Francisco, CA 94158, USA

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ABSTRACT

Arsenic, a human carcinogen that is associated with an increased risk of bladder cancer, is commonly found in drinking water. An important mechanism by which arsenic is thought to be carcinogenic is through the induction of epigenetic changes that lead to aberrant gene expression. Previously, we reported that the *SAS2* gene is required for optimal growth of yeast in the presence of arsenite (As^{III}). Yeast *Sas2p* is orthologous to human *MYST1*, a histone 4 lysine 16 (H4K16) acetyltransferase. Here, we show that H4K16 acetylation is necessary for the resistance of yeast to As^{III} through the modulation of chromatin state. We further explored the role of *MYST1* and H4K16 acetylation in arsenic toxicity and carcinogenesis in human bladder epithelial cells. The expression of *MYST1* was knocked down in UROtsa cells, a model of bladder epithelium that has been used to study arsenic-induced carcinogenesis. Silencing of *MYST1* reduced acetylation of H4K16 and induced sensitivity to As^{III} and to its more toxic metabolite monomethylarsonous acid (MMA^{III}) at doses relevant to high environmental human exposures. In addition, both As^{III} and MMA^{III} treatments decreased global H4K16 acetylation levels in a dose- and time-dependent manner. This indicates that acetylated H4K16 is required for resistance to arsenic and that a reduction in its levels as a consequence of arsenic exposure may contribute to toxicity in UROtsa cells. Based on these findings, we propose a novel role for the *MYST1* gene in human sensitivity to arsenic.

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Introduction

Bladder cancer is the most common urologic malignancy and the leading cause of cancer death in patients with urinary tract malignancies (Jemal et al., 2007). Chronic exposure to arsenic through drinking water is strongly linked to increased incidence and mortality of bladder cancer (Smith et al., 1998; Chu and Crawford-Brown, 2007; Marshall et al., 2007). For example, during 1958–1970, people who lived in region II of Chile were exposed to high concentrations of arsenic in water, and excess deaths from lung and bladder cancers predominated ten years after reduction of exposures (Marshall et al., 2007; Yuan et al., 2007).

Both DNA hypermethylation and altered histone acetylation have been observed in tumors from patients with bladder cancer (Chen et al., 2007; Brait et al., 2008), suggesting that aberrant epigenetic changes are associated with the development of this disease. There is increasing evidence indicating that epigenetic dysregulation plays an important role in the development of bladder cancer induced by arsenic (Marsit et al., 2006; Chai et al., 2007). Global DNA hypomethylation and focal DNA hypermethylation are both implicated in arsenic-induced malignant transformation *in vivo* and *in vitro* (Chen et al., 2001; Benbrahim-Tallaa et al., 2005; Chanda et al., 2006). Further, chronic exposure to arsenic alters DNA methylation and induces aberrant gene expression (Zhao et al., 1997; Xie et al., 2007). Moreover, arsenic induces aberrant acetylation of histone 3 (H3) at the *loci* of the proto oncogenes *c-jun* and *c-fos*, which in turn correlates with up-regulation of these genes (Li et al., 2003), and increases global acetylation of H3 at lysine 9 through inhibition of histone deacetylases (Ramirez et al., 2007). Therefore, arsenic also impairs the normal regulation of histone modifications, which may result in gene expression changes.

In a genome-wide, parallel phenotypic screen of yeast deletion mutants, we identified several genes associated with epigenetic changes as essential for optimal growth in the presence of arsenicals

* Corresponding authors. L. Zhang is to be contacted at School of Public Health, University of California, Berkeley, CA 94720, USA. Fax: +1 510 642 0427. C.D. Vulpe, 317 Morgan Hall, Department of Nutritional Sciences and Toxicology, University of California, Berkeley, CA 94720, USA. Fax: +1 510 642 0535.

E-mail addresses: williamjo@berkeley.edu (W.J. Jo), xfr@berkeley.edu (X. Ren), fcchu@cgl.ucsf.edu (F. Chu), mariaaleshin@berkeley.edu (M. Aleshin), wintz@berkeley.edu (H. Wintz), alb@cgl.ucsf.edu (A. Burlingame), martynts@berkeley.edu (M.T. Smith), vulpe@berkeley.edu (C.D. Vulpe), luoping@berkeley.edu (L. Zhang).

¹ These authors contributed equally to the work.

(Jo et al., 2009). These results suggest that epigenetic regulation is required in response to arsenic. One of the identified genes, *SAS2*, is required for the resistance of yeast to arsenite (As^{III}). This gene encodes the catalytic subunit of the heterotrimeric something about silencing (SAS) complex, which is responsible for the acetylation of histone 4 at lysine 16 (H4K16) (Shia et al., 2005). The SAS complex (Sas2p–Sas4p–Sas5p) is involved in transcriptional activation and silencing, chromatin-mediated boundary formation (Kimura et al., 2002; Suka et al., 2002; Shia et al., 2006a; Shogren-Knaak et al., 2006), and may play a role in DNA damage repair and maintenance of nuclear integrity (Lafon et al., 2007). The human ortholog of the yeast Sas2p, MYST1, is a histone acetyltransferase responsible for the acetylation of H4K16. Loss of *MYST1* decreases levels of acetylated H4K16 and the efficiency of double-strand break repair after induction of DNA damage with ionizing radiation (Taipale et al., 2005; Gupta et al., 2008). Loss of *MYST1* also leads to G₂/M cell cycle arrest, nuclear morphological defects, spontaneous chromosomal aberrations, and reduced transcription of certain genes (reviewed in Gupta et al., 2008). In addition, hypoacetylation of H4K16 is commonly found in human tumors and cell lines, and loss of acetylation at histone 4 (H4) appears to occur during malignant transformation (Fraga et al., 2005). Thus, current evidence suggests a connection between *MYST1*, H4K16 acetylation status, and human cancer (Lafon et al., 2007; Rea et al., 2007).

In the current study, UROtsa cells were used to explore the potential role of H4K16 acetylation by *MYST1* in the response to As^{III} and its more toxic metabolite, monomethylarsonous acid (MMA^{III}). Both arsenic forms are detected in urine and in exfoliated bladder epithelial cells collected from people exposed to arsenic (Hernandez-Zavala et al., 2008). Because individuals who excrete a higher proportion of ingested arsenic as methylated forms are at higher risk of bladder cancer (Steinmaus et al., 2006), it was considered of particular importance to evaluate the effects of MMA^{III} . The UROtsa cell line, originally isolated from a primary culture of normal human uroepithelium, has previously been used as a model for bladder epithelium and arsenic-induced bladder cancer (Sens et al., 2004; Bredfeldt et al., 2006; Eblin et al., 2008). As^{III} and MMA^{III} have previously been shown to induce malignant transformation in this model, partially through alterations in DNA methylation and histone acetylation (Jensen et al., 2008, 2009). The acetylation of H4K16 by *SAS2* and its modulation of arsenic toxicity were also studied in yeast in the current study. The results presented here show that acetylated H4K16, the status of which is determined by *MYST1* probably in conjunction with other determinants in humans, is necessary for protection against arsenicals and suggest that reduction in the levels of acetylated H4K16 in bladder epithelial cells could increase the risk of arsenic carcinogenesis.

Methods

Cultures of yeast strains and human UROtsa cells. Yeast strains were from the BY4743 (Invitrogen, Carlsbad, CA), and RMY200 and JTY102TU (generous gift from Prof. Michael Grunstein, University of California, Los Angeles, CA) backgrounds. Growth was conducted in rich media (yeast extract–peptone–dextrose, YPD) at 30 °C with shaking at 200 rpm. UROtsa cells (generously provided by Prof. Petia Simeonova, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention) were cultured at a starting cell density of 4–5 × 10⁴ cells/ml in RPMI 1640 (Mediatech, Inc, Manassas, VA) with L-glutamine, 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Omega Scientific, San Diego, CA), under standard human cell culturing conditions.

Arsenical exposures. Sodium arsenite (NaAsO_2 , As^{III}) was purchased from Sigma-Aldrich (St. Louis, MO). Monomethylarsine oxide (MMA^{III} O, MMA^{III}) was a generous gift of Prof. Miroslav Styblo (University of North Carolina, Chapel Hill, NC). MMA^{III} O hydrolyzes to

MMA^{III} in solution (Petrick et al., 2001). Stock solutions were prepared in sterile Milli-Q water, protected from light and stored at –80 °C until use. Yeast cells were treated with concentrations ranging from 0 to 300 µM of either As^{III} or MMA^{III} . UROtsa cells were treated, once they reached 80%–90% confluence in culture, with As^{III} at 1, 3, and 10 µM or with MMA^{III} at 0.3, 1, and 3 µM.

Yeast growth assay. Yeast strains were pre-grown in YPD media to mid-log phase, diluted in fresh media to an optical density at 595 nm (OD_{595}) of 0.0165, and inoculated into a 48-well microplate. Stock solutions of arsenicals were added to the desired final concentrations with at least three replicate wells per dose. Plates were incubated in a Tecan GENios spectrophotometer set to 30 °C, intermittent shaking and OD_{595} measurements at 15-minute intervals for a period of 24 h. Raw absorbance data were averaged for all replicates, background-corrected, and plotted as a function of time. The area under the curve (AUC) was calculated for the cultures in each well using Prism version 5.01 (GraphPad Software, Inc., La Jolla, CA), the treatments averaged, and expressed as a percentage of the control.

Retrovirus-mediated MYST1 gene knockdown. The *MYST1* RNAi design and sequences have been reported before (Taipale et al., 2005). With the modifications, the top strand cDNA sequence of the *MYST1* shRNA construct (sh-*MYST1*) was 5'-GATCCGAGACCATAAGATTACTGTTT-CAAGAGAACAGTAAATCTTATGGTCTTTTTTACCGGTG-3', and the bottom strand was 5'-AATTCACGCGTAAAAAAGACCATAAGATTACTGTTCTC-TTGAAACAGTAAATCTTATGGTCTCG-3'. The non-silencing shRNA control was modified from the non-target shRNA control (Sigma-Aldrich), and the control sequence used for the shRNA (sh-NSC) was 5'-GATCCGCAACAAGATGAAGAGCACCATTCAAGAGATTGGTG-CTTTCATCTTGTGCTTTTTTACCGGTG-3' for top strand, and 5'-AATTCACGCGTAAAAAAGCAACAAGATGAAGAGCACCAATCTTTGAA-TTGGTGCTTTCATCTTGTGCG-3' for bottom strand. These oligonucleotide pairs containing *Bam*HI and *Eco*RI overhangs were annealed and ligated to a linearized RNAi-Ready pSIREN-RetroQ-ZsGreen vector digested with *Bam*HI and *Eco*RI (BD Biosciences Clontech). The RNAi-Ready pSIREN-RetroQ-ZsGreen vector is a self-inactivating retroviral expression vector designed to express a small hairpin RNA using the human U6 promoter. The resultant constructs were amplified, purified, and sequenced. The UROtsa cells were transfected with Lipofectamine 2000 reagent following the manufacturer's instruction (Invitrogen). After incubation at 37 °C for 8 h, the supernatant fraction containing the retroviral vector was removed and replaced with normal growth medium. Cells grown for 48–72 h were assessed by fluorescence microscopy. The ZsGreen fluorescent marker yields a bright green fluorescence, permitting direct monitoring of the delivery efficiency. Finally, the cell populations were sorted by the DAKO-Cytomation MoFlo High Speed Sorter (Dako North America, Carpinteria, CA), and the green fluorescent cells were purified and collected for continuing culture. The green fluorescent cells were used for additional experimentation.

Cytotoxicity assay (MTT assay). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed to assess the effect of *MYST1* silencing on cell viability after arsenic treatment. Cells were cultured in 96-well plates in a volume of 100 µl of media per well at a density of 5 × 10⁴ cells/ml. Twenty-four hours after incubation with As^{III} or MMA^{III} (3 replicates/arsenical concentration), 10 µl of sterile MTT dye (Sigma-Aldrich; 5 mg/ml) was added to each well, and plates were incubated at 37 °C for 4 h. Then, the culture medium was removed and 200 µl of DMSO was added and thoroughly mixed in for 10 min. Spectrometric absorbance at 570 nm was measured in a microplate reader.

Human tissue array and real-time quantitative PCR assay. TaqMan-based real-time quantitative polymerase chain reaction (RT qPCR)

was performed to quantify *MYST1* expression in human normal tissues in the Human Rapid-Scan Plate (OriGene Technologies, Inc., Rockville, MD). The primers and probe used for amplification of *MYST1* and β -actin control were ordered from Applied Biosystems (Foster City, CA). For quantification of transcripts, relative gene expression was calculated using the $\Delta\Delta C_T$ method.

Mass spectrometry analysis of H4K16 acetylation. Human UROtsa cells were grown in SILAC RPMI 1640 medium containing L-lysine HCl (light) or L-lysine 2HCl ($U-^{13}C_6$, 98%; $U-^{15}N_2$, 98%, heavy) (Cambridge Isotope Laboratories Inc., Andover, MA), supplemented with 10% dialyzed FBS and antibiotics. Cells were cultured for five passages to ensure complete labeling of proteins before being exposed to As^{III} or MMA^{III} for 24 h or 7 days. Treatment and control cultures were mixed in a 1:1 ratio, and the core histone proteins were extracted and purified as described before (Shechter et al., 2007), separated by 4%–20% SDS-PAGE, and visualized with Coomassie staining. In-gel digestions on histone bands were performed utilizing the procedure described at <http://ms-facility.ucsf.edu/ingel.html>.

Mass spectrometric analysis was carried out as described previously (Chu et al., 2006). Briefly, the tryptic digest of core H4 was separated by a $75\text{-}\mu\text{m} \times 15\text{-cm}$ reverse-phase capillary column at a flow rate of 330 nl/min. The HPLC eluent was connected directly to the micro-ion electrospray source of a QSTAR XL mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). LC-MS data were acquired in an information-dependent acquisition mode, cycling between 1-s MS acquisition followed by 3-s low-energy CID data acquisition. The centroided peak lists of the CID spectra were searched against the National Center for Biotechnology Information (NCBI) protein database using Batch-Tag (Chalkley et

al., 2005). Protein N-terminus and lysine acetylation; lysine mono-, di-, and trimethylation; arginine mono- and dimethylation; phosphorylation; and lysine ubiquitination were considered as variable modifications. Quantitative calculation of identified peptides was carried out by the UCSF Search Compare program in ProteinProspector package, which performs peak list generation, SILAC- and extracted ion current-based quantitation, expectation value calculation, statistic analysis of identified peptide, data filtration, and presentation. SILAC ratio of each peptide was normalized against the average SILAC ratio of all identified peptides in each histone protein.

Immunoblot analysis. Total cell lysates were prepared from 5×10^6 cells using 300 μl of radioimmunoprecipitation assay lysis buffer. Nuclear extracts were collected from 1×10^7 cells using a nuclear extraction kit (Millipore, Billerica, MA) according to the manufacturer's protocol. Protein concentrations in cell lysates and nuclear extracts were determined by the DC assay (Bio-Rad Laboratories, Inc., Hercules, CA). Equal protein amounts were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted for *MYST1* (Novus Biologicals, Littleton, CO) and β -actin (Sigma-Aldrich) or acetyl-H4, acetyl-H4K16, and H4 pan (Millipore). Proteins were visualized using the enhanced chemiluminescence method (Amersham Biosciences, United Kingdom) as per the manufacturer's protocol. Film was exposed and developed using a Konica SRX-101 developer (Konica Minolta Medical Imaging USA, Wayne, NJ). Images were quantified using the ImageJ software (NIH, Bethesda, MD). Each measured protein was normalized to either of the loading controls β -actin or H4 pan.

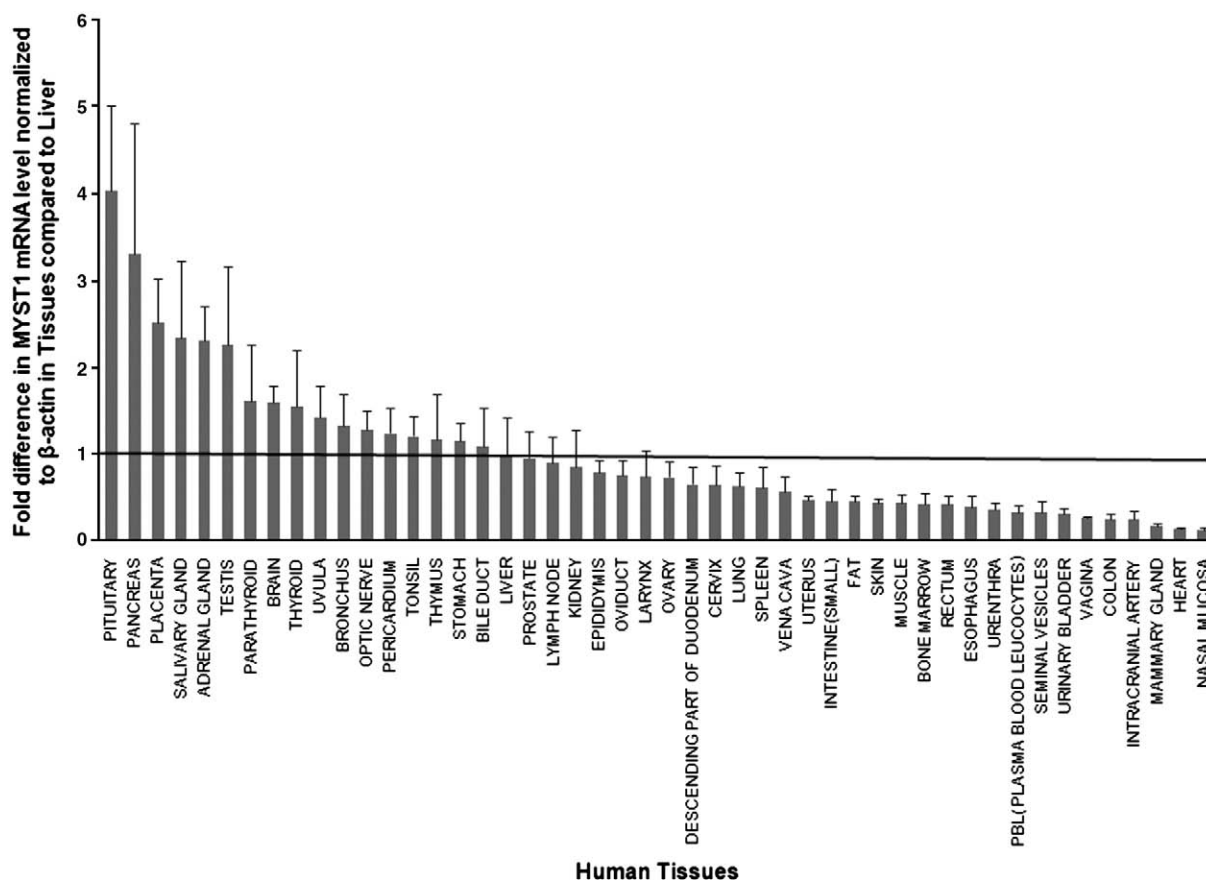


Fig. 1. Comparison of *MYST1* gene expression in human tissues. Expression of *MYST1* was quantified by RT qPCR in cDNA from a panel of 48 normal human tissues. Transcript levels of *MYST1* were determined using primers designed to amplify a defined region of the gene. *MYST1* mRNA was expressed at detectable levels in the majority of the tissues analyzed. Expression of *MYST1* in liver was selected as the reference for comparison with all other tissues.

Data analysis. Statistical analyses were performed using one-way analysis of variance followed by a post-hoc test if the group means were significantly different. Levels of significance were denoted in graphs as * p value < 0.05, ** p value < 0.01, and *** p value < 0.001.

Results

mRNA expression of *MYST1* in human tissues

MYST1 is a recently characterized gene, and a search of the expressed sequence tag (EST) database revealed sequences matching the cDNA of *MYST1* in many human tissues with varied expression levels. To experimentally measure and compare the mRNA expression of *MYST1* across tissues, real-time quantitative PCR analysis was performed in cDNA from a panel of 48 human tissues contained in a tissue array (Fig. 1). Using the liver as reference, it was found that *MYST1* is highly expressed in tissues such as the pituitary gland and placenta; moderately expressed in others including the stomach, prostate, kidney, lung, skin, and urinary bladder; and weakly expressed in the mammary gland, colon, and heart. The presence of detectable levels of *MYST1* mRNA in several normal human tissues indicates a role for this gene in regular cellular metabolism and supports studying its relation to arsenic sensitivity in humans. Long-term exposure to As^{III} has been associated with cancers in the lung, urinary bladder, and skin (Tchounwou et al., 2003) and, interestingly,

correlates with the lower *MYST1* expression levels observed in these tissues.

Deletion of yeast *SAS2* and silencing of human *MYST1* induces arsenic sensitivity

The growth phenotype of the *SAS*-deletion mutants was evaluated in the presence of either As^{III} or MMA^{III} (Fig. 2). Deletion strains and their isogenic counterpart BY4743 wild type were treated with equitoxic doses equivalent to the concentration that induced 20% growth inhibition (IC_{20}) and $2 \times IC_{20}$, which were 300 and 600 μM for As^{III} and 150 and 300 μM for MMA^{III} , respectively. In these treatments, *sas2* Δ , *sas4* Δ , and *sas5* Δ exhibited decreased growth in As^{III} relative to wild type, but not in MMA^{III} . These results showed a specific requirement of the *SAS* genes for optimal growth in As^{III} , despite the higher toxicity of MMA^{III} .

To investigate the role of *MYST1* in arsenic toxicity in mammals, the expression of *MYST1* was knocked down in UROtsa cells by approximately 75% compared to controls (Fig. 3A). Transfected UROtsa cells did not have an altered doubling time or appearance in culture. The UROtsa sh-*MYST1* (*MYST1* knockdown) and UROtsa sh-NSC cells (vector control) were then treated with either As^{III} or MMA^{III} at concentrations up to the IC_{50} for 24 h. The dose ranges used included doses equivalent to high environmental exposures encountered by humans. Arsenical treatments induced a dose-dependent decrease in viability in both cell lines. In the absence of

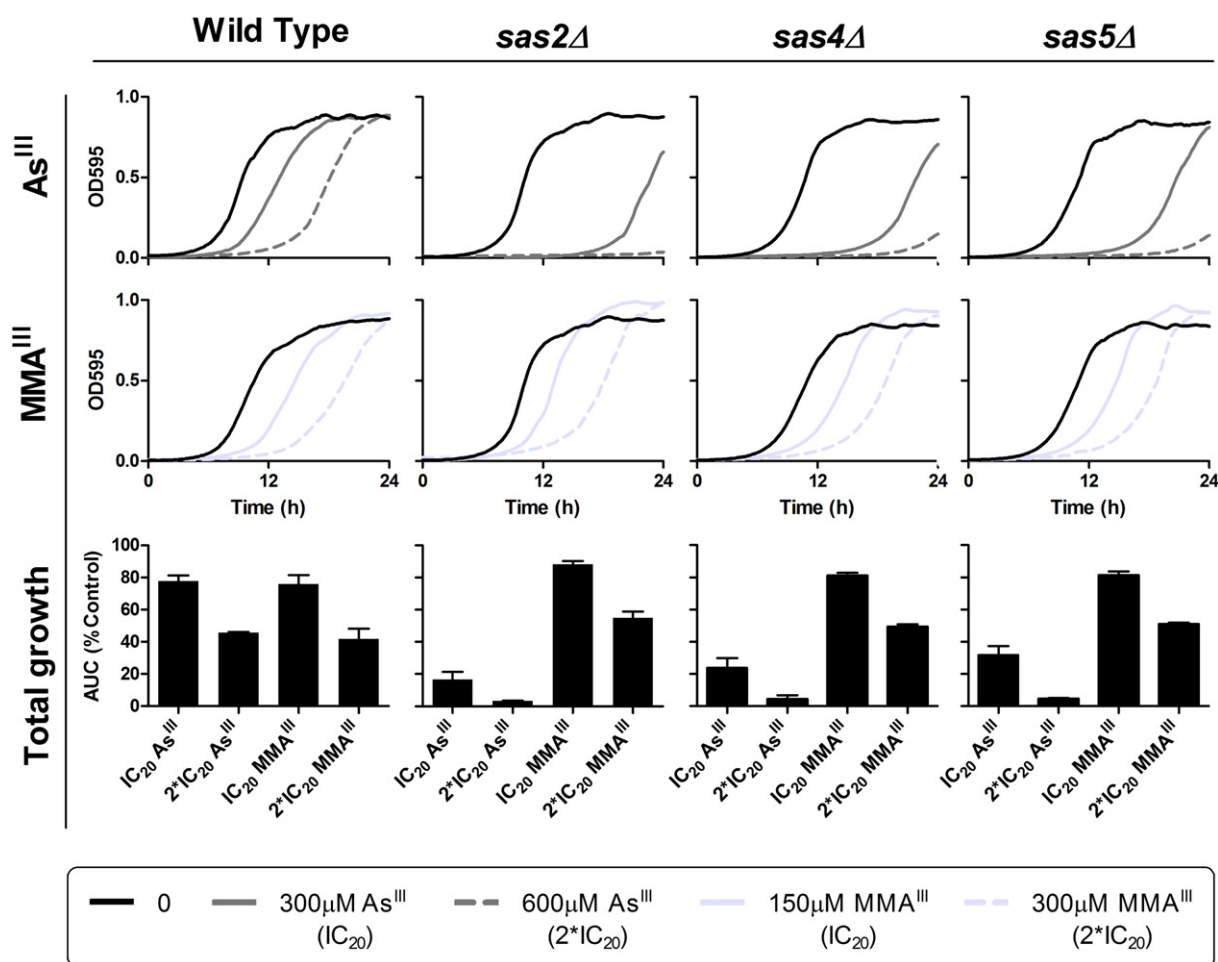


Fig. 2. Deletion of the *SAS* genes in yeast results in sensitivity to arsenite. The strains *sas2* Δ , *sas4* Δ , *sas5* Δ and their isogenic counterpart BY4743 wild type were treated with equitoxic doses equivalent to the IC_{20} and $2 \times IC_{20}$, which were 300 and 600 μM for As^{III} and 150 and 300 μM for MMA^{III} , respectively. Growth curves show the average optical density of the cultures at 595 nm (OD_{595}) for each treatment as a function of time for a period of 24 h. The bars represent the mean area under the curve (AUC) for three technical replicates with SE. At the doses tested, the *SAS* mutants displayed reduced growth in As^{III} relative to the wild type strain but not in MMA^{III} .

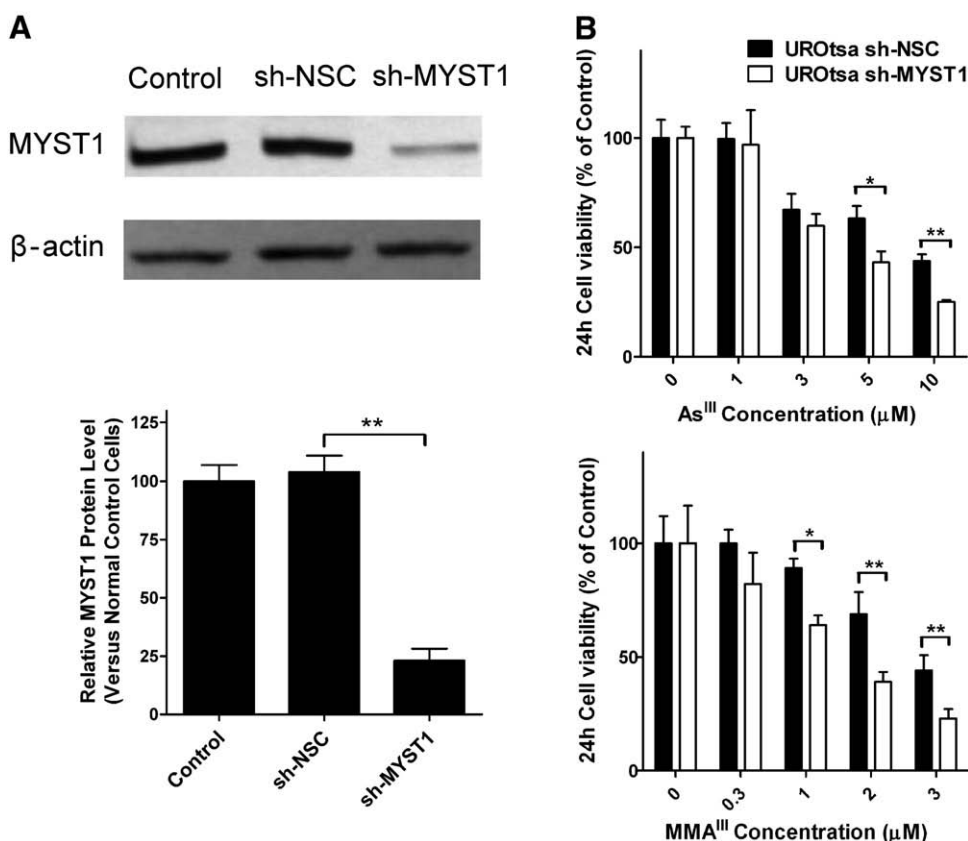


Fig. 3. Knockdown of *MYST1* in human UROtsa cells induces sensitivity to arsenic. (A) Western blot analysis of whole cell lysates with anti-MYST1 shows a reduction of approximately 75% in MYST1 protein levels, relative to vector controls, in human UROtsa cells after knockdown with shRNA constructs targeting *MYST1*. The nonspecific control (NSC) shRNA had no effect on the protein level of MYST1. β -Actin was used as the loading control. (B) UROtsa sh-NSC and UROtsa sh-MYST1 cells were treated with increasing concentrations of As^{III} and MMA^{III} for 24 h. Cell viability was evaluated with MTT, a dye that is reduced by viable cells, resulting in a color that can be quantified by spectrophotometry. Bars represent the average of three independent experiments with SD. Treatment with either As^{III} or MMA^{III} resulted in a dose-dependent decrease in viability between the two cell lines. At the IC₅₀, UROtsa sh-MYST1 cells displayed a reduction in viability of approximately 30% and 50% after As^{III} (10 μ M) and MMA^{III} (3 μ M) exposure, respectively, relative to control UROtsa sh-NSC cells. These results indicated that arsenicals were more cytotoxic in UROtsa sh-MYST1 cells. * p <0.05, ** p <0.01.

arsenic treatment, there was no effect of MYST1 knockdown on cell viability, relative to vector control. Unlike deletion of the SAS genes in yeast, silencing of *MYST1* led to increased sensitivity to both As^{III} and MMA^{III} (Fig. 3B). MMA^{III} was more potent than As^{III} in producing toxicity; at the IC₅₀, the viability of UROtsa sh-MYST1 cells was about 70% of the UROtsa sh-NSC cells post-As^{III} exposure (10 μ M), as opposed to only 60% after MMA^{III} treatment (3 μ M).

As^{III} resistance in yeast is related to acetylation of H4K16 and its modulation of chromatin state

The growth phenotype of a H4K16→R yeast mutant, containing a non-acetylable arginine residue at position 16 in place of lysine (Suka et al., 2002), was evaluated to further investigate the role of H4K16 acetylation in As^{III} resistance. This strain exhibited slow growth compared to the wild type and was sensitive to high doses of As^{III} (Fig. 4A). In addition, the growth of this mutant was evaluated in the presence of cytotoxic concentrations of As^{III}, cadmium, copper and zinc, and sodium chloride (Fig. 4B). Only treatment with As^{III} significantly decreased growth relative to wild type, therefore showing that H4K16 acetylation is specifically required for resistance to As^{III}.

In yeast, the histone deacetylase Sir2p antagonizes the histone acetyltransferase activity of Sas2p by removing acetyl groups from acetylated lysine residues (Landry et al., 2000). These two proteins normally establish a gradient of histone acetylation in yeast chromosomes that ranges from a hypoacetylated state in telomeric-proximal regions to an hyperacetylated state in telomeric-distal regions,

associated with heterochromatin and euchromatin, respectively (Kimura et al., 2002; Suka et al., 2002). Deletion of *SIR2* increases H4K16 acetylation. Deletion of *SAS2* reduces H4K16 acetylation and induces the spreading of heterochromatin from telomeric-proximal to telomeric-distal regions, which is reversed by further deletion of *SIR2* (Suka et al., 2002). In order to determine whether the alteration in heterochromatic regions influences the sensitivity of yeast to As^{III}, the growth phenotypes of *sas2Δ*, *sir2Δ*, and *sas2Δsir2Δ* were evaluated in its presence. Deletion of *SAS2*, as previously shown, resulted in sensitivity to As^{III}. Deletion of *SIR2* in the double knock-out strain was dominant over deletion of *SAS2* (Figs. 4C and D), and was in agreement with the proposed model for Sas2p and Sir2p function (Kimura et al., 2002; Suka et al., 2002). Both *sir2Δ* and *sir2Δsas2Δ* strains were insensitive to As^{III} in liquid media and resistant to As^{III} when grown on agar plates (Figs. 4C and D), indicating that H4K16 acetylation plays a role in As^{III} resistance. Silencing of genes located in telomeric-proximal regions is reduced in both of these mutant strains, suggesting that H4K16 acetylation in yeast influences the response to arsenic toxicity through its modulation of chromatin state. In an open chromatin conformation induced by acetylated H4K16, cells are resistant to As^{III}, whereas in a closed state induced by deacetylated H4K16, cells are more sensitive to arsenic toxicity.

Arsenic exposure and H4K16 acetylation in UROtsa cells

MYST1 is responsible for the acetylation of H4K16 in human cells (Taipale et al., 2005; Gupta et al., 2008), and silencing of this gene in UROtsa cells led to increased sensitivity to both As^{III} and MMA^{III} (Fig. 3B).

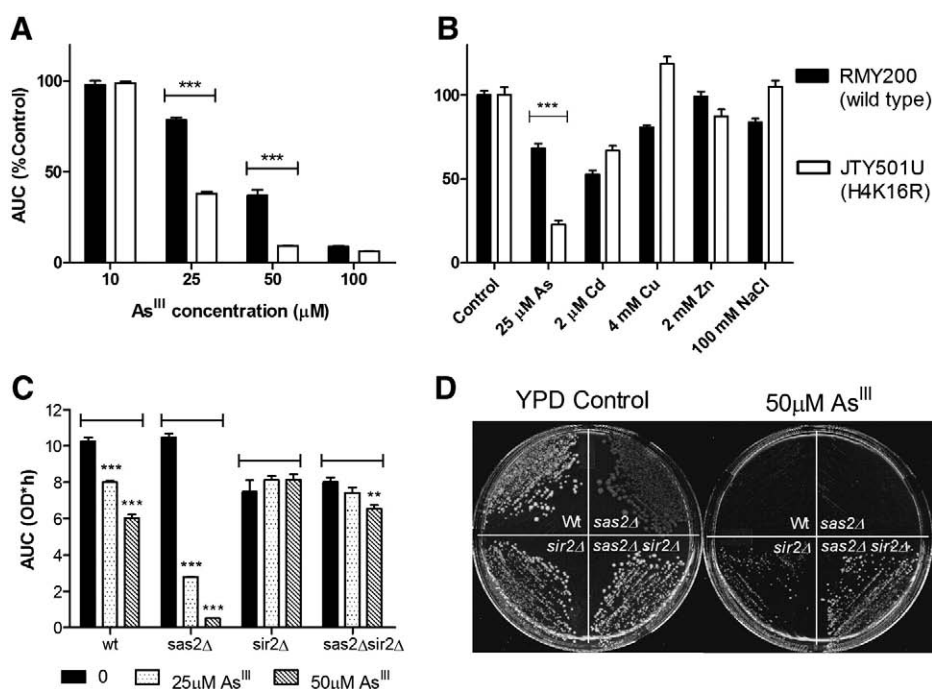


Fig. 4. Acetylation of H4K16 is required for yeast resistance to As^{III}. Mutant strains and their isogenic counterpart wild type (RMY200) were grown in rich media containing different As^{III} concentrations. The optical density of the cultures was measured at 595 nm for 24 h and used to calculate the area under the curve (AUC). The bars represent the mean AUC for three technical replicates with SE. ***p*<0.01, ****p*<0.001. (A) Decreased growth in rich media and sensitivity of H4K16→R mutant, containing a non-acetyltable arginine residue in place of the lysine, at As^{III} doses of 25 μM and higher relative to the wild type strain. (B) The presence in media of growth inhibitory concentrations of cadmium chloride, copper sulfate, zinc chloride, or sodium chloride does not decrease H4K16→R mutant growth relative to the wild type strain. (C) Deletion of the histone deacetylase gene *SIR2* induces resistance to As^{III} and is dominant over the As^{III}-sensitive phenotype that results from deletion of the histone acetyltransferase gene *SAS2*. Growth assays for wild type, *sas2Δ*, *sir2Δ*, and *sas2Δsir2Δ* strains in 0, 25, and 50 μM As^{III} show a dose-dependent decrease in growth for wild type and *sas2Δ* but no effect on *sir2Δ* and *sas2Δsir2Δ*. (D) Plate assays show resistance of *sir2Δ* and *sas2Δsir2Δ* to As^{III} compared to the wild type. Yeast strains were grown for 3 days on YPD agar in the presence or absence of As^{III}. The wild type and *sas2Δ* strains were unable to grow in medium with As^{III} but *sir2Δ* and *sas2Δsir2Δ* did.

Acetylation levels of H4 and H4K16 were compared between UROtsa sh-MYST1 and UROtsa sh-NSC cells and were slightly reduced in H4 and significantly reduced in H4K16 (Fig. 5A). The correlation between decreased H4K16 acetylation and decreased viability after As^{III} and MMA^{III} treatments provides evidence that this epigenetic modification is required for resistance against these two arsenicals in UROtsa cells.

Next, the ability of arsenic to alter H4K16 acetylation levels was determined. UROtsa cells were cultured in medium containing either heavy or light lysine to allow quantitative comparison through mass spectrometry of the epigenetic changes on histones. The analysis showed that arsenical treatment reduced levels of H4K16 acetylation in UROtsa cells. There was a dose- and time- dependent decrease in SILAC ratio (treatment/control) for the tetra-acetylated peptide corresponding to the N-terminus of histone H4, GKacGGKacGLG-KacGGAKacR, after As^{III} and MMA^{III} treatments (Table 1). Treatment with MMA^{III} for 24 h resulted in only a slight decrease in SILAC ratio at 0.3 and 1 μM, but a significant decrease at 3 μM (Fig. 5B). Interestingly, the SILAC ratios were significantly reduced for both 0.3 and 1 μM after 7 days of treatment and were comparable. Similarly, in the case of As^{III}, only the highest dose of 10 μM led to a significant decrease (about two-fold) in the SILAC ratio for acetylated H4K16 peptide after 24 h of treatment. Acetylation of H4K16 was significantly decreased after 1 and 3 μM As^{III} treatment when the UROtsa cells were treated for 7 days.

These findings were then confirmed by immunodetection of acetylated H4K16. Compared to the untreated control, the H4K16 acetylation levels were slightly but significantly reduced in histone extracts from UROtsa cells following 7 days of treatment with either 3 μM As^{III} or 1 μM MMA^{III} (Fig. 5C). These slight changes are not surprising, given the presence of relatively low levels of total H4 in the

nuclear extracts, which contained a mixture of histones. Further, acetylation may not occur at all H4K16 residues in the cell but is likely targeted to specific chromosomal locations. In addition, as acetylation of H4K16 is thought to play a role in normal epigenetic processes the relatively high basal level of H4K16 acetylation makes the detection of small differences between treatment and control challenging. There were no apparent differences in H4K16 acetylation after 24 h of exposure to As^{III} or MMA^{III} (data not shown).

In spite of the difference in the magnitude of changes observed between mass spectrometry and immunoblot analyses, the trend of decreased H4K16 acetylation after arsenical exposure was consistent between the two. Overall, the data show that both As^{III} and MMA^{III} decrease H4K16 acetylation in UROtsa cells.

Discussion

Millions of people worldwide are exposed to arsenic through consumption of contaminated drinking water. Although a direct correlation between exposure to this metalloid and increased cancer risk is well documented, the mechanisms involved in arsenic carcinogenesis are not fully understood. Because inorganic arsenic is metabolized to methylated trivalent species with greater genotoxic potential (Mass et al., 2001) and are all found in urine, it is not surprising that the bladder is a target of arsenic carcinogenesis.

Histone acetylation is commonly associated with transcriptionally active euchromatin. Acetylation of specific lysine residues can decrease the affinity of histones for DNA, making the DNA more accessible to transcription factors and components of the transcriptional machinery (Shia et al., 2006b). Arsenic is known to induce alterations in histone acetylation that are associated with aberrant gene expression. Studies aiming to characterize the effects of arsenic

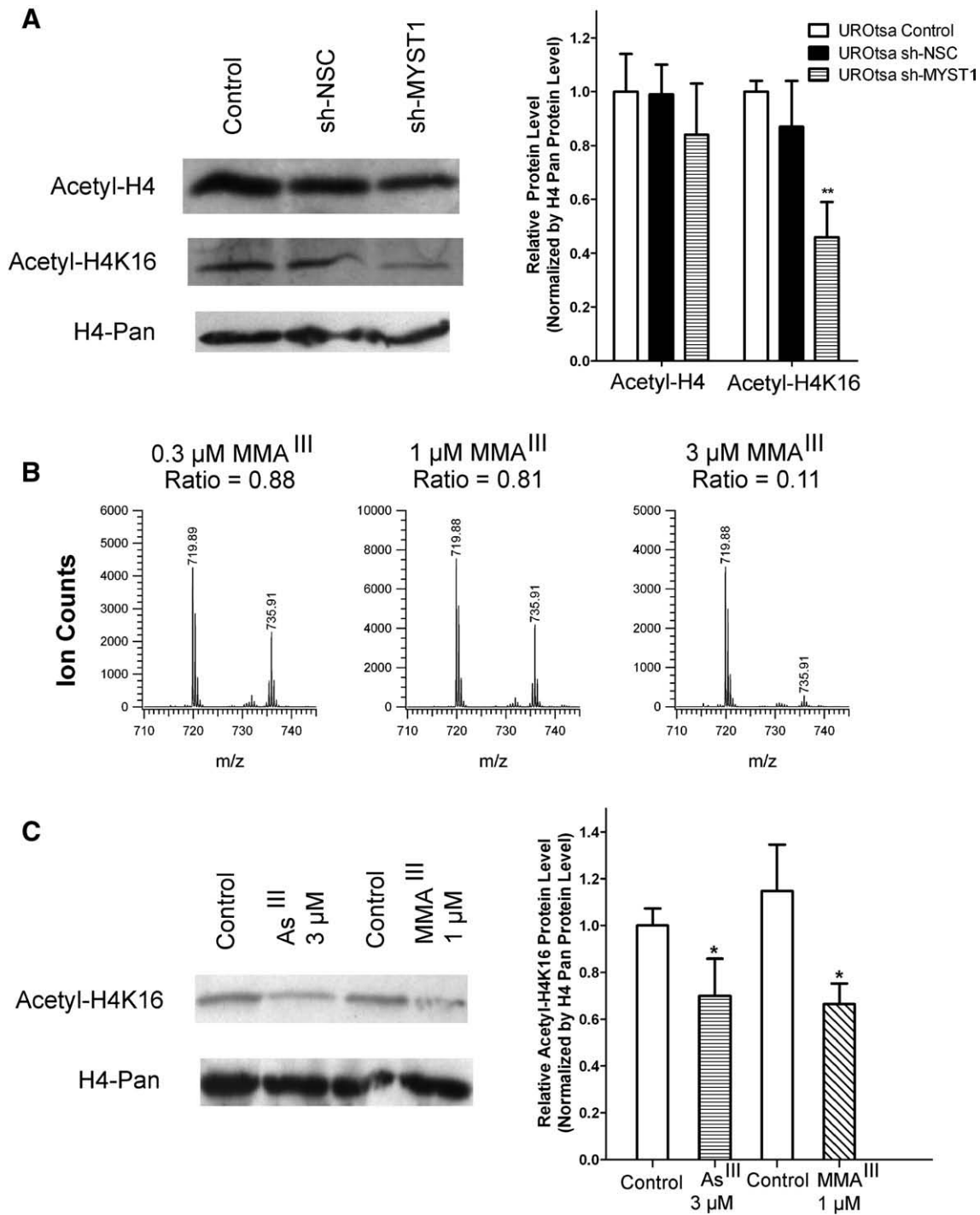


Fig. 5. Reduction of acetylated H4K16 levels in UROtsa cells after treatment with arsenicals. (A) Silencing of *MYST1* leads to a significant reduction in acetylated H4K16 levels. Nuclear extracts were run on SDS-PAGE gels, transferred onto nitrocellulose membranes, and probed with anti-acetyl H4 and anti-acetyl H4K16. Equal loading was checked by probing the membrane with anti-H4 pan. Silencing of *MYST1* in UROtsa cells (sh-*MYST1*) significantly reduce acetylated H4K16 relative to the vector (sh-NSC) and non-transfected controls. Densitometry analysis shows a significant decrease in acetylated H4K16 after knockdown of *MYST1* (** $p < 0.01$). (B) Mass spectrometry analysis of representative SILAC sample for MMA^{III}. UROtsa cell cultures were labeled with either heavy or light lysine and treated with arsenic or left untreated, respectively. Histones were extracted from culture mixtures at a ratio of 1:1 and analyzed by LC-MS. Treatment with 3 μM MMA^{III} for 24 h decreases the SILAC ratio (treatment/control) for the tetra-acetylated peptide GKacGGKacGLGKacGGAKacR labeled with light (m/z 719.88, control) or heavy (m/z 735.91, treatment) lysine. MMA^{III} treatments at the lower concentrations did not alter the SILAC ratio significantly. (C) Representative immunoblot images of nuclear extracts from arsenic-treated and control cultures comparing acetylated H4K16 levels. Nuclear extracts were run on SDS-PAGE gels, transferred onto nitrocellulose membranes, and probed with anti-acetyl H4 and anti-acetyl H4K16. Equal loading was checked by probing the membrane with anti-H4 pan. Acetylated H4K16 levels in UROtsa cells are reduced after treatments with 3 μM As^{III} and 1 μM MMA^{III} for 7 days. Densitometry analysis shows a significant decrease in acetylated H4K16 in cells treated with As^{III} and MMA^{III} after knockdown of *MYST1* (* $p < 0.05$).

on N-tail histone modifications and its relation to transcriptional changes have focused on the acetylation of H3 (Li et al., 2003; Ramirez et al., 2007). Moreover, the hyperacetylation of H3 at specific gene promoters in UROtsa cells transformed by chronic exposure to As^{III}

and MMA^{III} (Jensen et al., 2008) further link these changes to malignant transformation. Therefore, the induction of alterations in histone acetylation coupled with aberrant gene expression may play a role in arsenic carcinogenesis.

Table 1
SILAC ratios^a of acetylated H4K16 in UROtsa cells exposed to arsenic and controls.

Time ^b	As ^{III}			MMA ^{III}		
	1 μ M	3 μ M	10 μ M	0.3 μ M	1 μ M	3 μ M
1 day	0.86	0.78	0.53	0.88	0.81	0.11
7 days	0.48	0.38	N/A ^c	0.55	0.57	N/A ^c

^a SILAC ratio (treatment/control) for each experiment has been normalized to the mean value of unmodified peptides. UROtsa cells were labeled with heavy lysine before arsenic treatment and with light lysine for the untreated control.

^b Treatment period with arsenical.

^c N/A: not available. Samples could not be processed due to reduced cell number caused by cytotoxicity.

This study focused on the N-tail histone acetylation at a different lysine residue, H4K16. We demonstrate that H4K16 acetylation mediates arsenic resistance in human UROtsa cells. We showed this through silencing of the *MYST1* gene, which encodes the histone acetylase primarily responsible for H4K16 acetylation. Furthermore, we show that MMA^{III} and As^{III} decrease H4K16 acetylation in a dose- and time-dependent manner, suggesting that the functions mediated by acetylated H4K16 could be impaired after chronic exposure to arsenic. Hypoacetylated H4K16 appears early and accumulates during tumor development in a mouse model of multistage skin carcinogenesis, suggesting that loss of H4K16 acetylation occurs during malignant transformation and does not arise as a consequence of it (Fraga et al., 2005). Because H4K16 is commonly hypoacetylated in human tumors, loss of H4K16 acetylation may play a role in the pathogenesis of different human cancers. In bladder epithelial cells, a reduction in H4K16 acetylation caused by chronic arsenic exposure could be detrimental and contribute to bladder carcinogenesis. As a major determinant of H4K16 acetylation status, expression of *MYST1* may constitute a potential biomarker of carcinogenesis in tissues associated with As-induced cancer.

The homologous proteins primarily responsible for the acetylation of H4K16 in yeast and humans, Sas2p and MYST1, respectively, belong to the MYST (*MOZ*, *Ybf2/Sas3*, *Sas2*, and *TIP60*) family of histone acetyltransferases. Members of this family share a conserved MYST domain containing a DNA binding and a zinc finger motif. The putative role of the MYST family in human cancer has been reviewed recently (Avvakumov and Cote, 2007; Lafon et al., 2007). Knockdown of *MYST1* resulted in a considerable reduction in acetylated H4K16 levels, indicating that the bulk of H4K16 acetylation is catalyzed by the MYST1 protein and confirming previous findings in other cell lines (Taipale et al., 2005). These results provide evidence that the observed sensitivity of UROtsa cells to arsenic after *MYST1* knockdown is due to a decrease in acetylated H4K16.

The sensitivity to both As^{III} and MMA^{III} is indicative of common mechanisms by which these two arsenicals are toxic to humans and common biological processes in the cellular response to them. In spite of this similarity, MMA^{III} was more potent than As^{III} both at decreasing viability in *MYST1* knockdown cells and at reducing H4K16 acetylation in UROtsa cells. Compared to a previous study, UROtsa cells were more sensitive to As^{III} than in the work presented here, while sensitivity to MMA^{III} was comparable between the 2 (Drobna et al., 2005). While the exact reasons are unknown, possible contributions for the observed difference in sensitivity to As^{III} may be related to cell culture conditions, timing of the treatments during culture, and purity of the As^{III} used. Deletion of *SAS2* in yeast, on the other hand, increased sensitivity only to As^{III}. Yeast has different but overlapping detoxification mechanisms against MMA^{III} and As^{III}. For example, the arsenic resistance genes *ARR* are exclusively involved in detoxification of inorganic arsenic. Therefore, *SAS2* may be associated with an As^{III}-specific protection mechanism, while detoxification of the different arsenic species in humans may be mediated through a single pathway in which *MYST1* plays a common role.

Acetylation of H4K16 is a reversible, post-translational histone modification in eukaryotes that is associated with changes in gene expression. Thus, the requirement of acetylated H4K16 in the resistance to arsenic could be due to its ability to promote or maintain the expression of genes involved in response to arsenic stress. In yeast chromosomes, the H4K16 acetylation state defines heterochromatic and euchromatic regions and is associated with gene transcription (Kimura et al., 2002; Suka et al., 2002). Deletion of *SAS2* and mutation of lysine in H4K16 specifically induced sensitivity to arsenic and not to other chemical stressors. Interestingly, deletion of *SIR2*, which results in hyperacetylation of H4K16, induced resistance to As^{III} and further confirmed the importance of this epigenetic change. The H4K16 acetylation of chromosomal regions in yeast is associated with euchromatin. Therefore, H4K16 acetylation mediates arsenic resistance by promoting euchromatin formation in yeast chromosomes. These results show that *SAS2* is not the only determinant factor in arsenic resistance modulated by H4K16 acetylation status. Furthermore, the data suggest that any factor that can affect H4K16 acetylation has the potential to impact yeast sensitivity to arsenic.

Unlike traditional carcinogens that form metabolites covalently bound to DNA, As^{III} is not a potent mutagen but may act as a cocarcinogen by enhancing the mutagenicity of certain agents such as UV radiation (Danaee et al., 2004; Rossman et al., 2004). Decreased H4K16 acetylation as a consequence of environmental exposure to arsenic could render bladder epithelial cells more susceptible to genotoxic agents, such as chemicals found in cigarette smoke. Interestingly, smokers exposed to arsenic through drinking water are at a much higher risk of bladder cancer than exposed nonsmokers (Steinmaus et al., 2003).

There is limited knowledge of the risk factors that predispose individuals to the adverse health effects associated with long-term exposure to arsenic. Because knockdown of *MYST1* reduced acetylation of H4K16 and resulted in sensitivity to arsenic, it seems reasonable to consider a novel role for the *MYST1* gene in human sensitivity to arsenic in UROtsa cells. Furthermore, because *MYST1* is expressed in a variety of human tissues, the effects of arsenic on acetylated H4K16 levels and of decreased MYST protein expression on arsenic sensitivity need to be investigated in other cell types. Studies to determine potential mechanisms, other than *MYST1*, involved in the reduction of acetylated H4K16 levels following arsenic exposure are warranted. Our results suggest that genetic and/or environmental factors that can alter normal H4K16 acetylation could influence sensitivity to arsenic in humans. Importantly, the future identification of genes whose expression levels are epigenetically regulated by acetylated H4K16 in UROtsa cells will not only identify additional sensitivity biomarkers of arsenic exposure but also contribute to understanding the mechanisms of arsenic toxicity and carcinogenicity in the bladder.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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