J Biosci (2020)45:19
DOI: 10.1007/s12038-019-9986-z

Cellular environment controls the dynamics of histone H3 lysine 56 acetylation in response to DNA damage in mammalian cells

RAGHAVENDRA VADLA[1,2,†], NIRUPAMA CHATTERJEE[1,†] and DEVYANI HALDAR[1]*

[1]*Centre for DNA Fingerprinting and Diagnostics, Uppal, Hyderabad 500 039, India*

[2]*Graduate Studies, Manipal University, Manipal, India*

**Corresponding author (Email, devyani@cdfd.org.in)*

[†]*These authors contributed equally to this work.*

Epigenetic changes play a crucial role in sensing signals and responding to fluctuations in the extracellular environment. How the cellular micro-environment affects DNA damage response signalling in chromatin context is not extensively studied. Histone acetylation is dynamic and very sensitive to changes in the extracellular environment. Existing literature on H3 lysine 56 acetylation (H3K56ac) levels upon DNA damage in mammals presents a conflicting picture. The occurrence of both increased and decreased H3K56ac upon DNA damage in our experiments led us to investigate the role of the micro-environment on H3K56ac. Here, we show that the global levels of H3K56ac increase as cells grow from low density to high density while SIRT1 and SIRT6 expression decrease. Additionally, rising lactic acid levels increase H3K56ac. Our results show that cell density and accumulation of metabolites affect dynamics of H3K56ac in response to DNA damage. Upon DNA damage, H3K56ac increases in low density cells with low initial acetylation, while acetylation decreases in high cell density cells. These results highlight that H3K56ac levels upon DNA damage are dependent on the metabolites in the extracellular milieu which impact chromatin structure by regulating chromatin modifying enzymes. Accumulation of lactic acid at high cell density reflects conditions similar to the tumour micro-environment. As H3K56ac increases in tumours, lactic acid and low pH might alter H3K56ac in tumours, leading to deregulated gene expression, contributing to tumour progression.

Keywords. Chromatin; histone acetylation; lactic acid; micro-environment; sirtuin

Abbreviations: H3K56ac, Histone H3 lysine 56 acetylation; DDR, DNA damage response; HDAC, Histone deacetylase; CM, Conditioned medium; SIRT1, Sirtuin 1; SIRT6, Sirtuin 6

1. Introduction

Cells sense environmental cues such as soluble factors, genotoxic agents, nutrients, oxygen, pH, chemical nature of growth surfaces, etc., and respond by activating various signalling pathways (Ruprecht *et al.* 2017; Xiao *et al.* 2017). Signalling from extracellular molecules such as growth factors, nutrients, cytokines, hormones and neurotransmitters convey specific information via receptors on the surface of target cells, activating intracellular signalling cascades and inducing specific cellular responses. Most organisms transition between catabolic and anabolic states according to the availability of nutrients by adapting specific mechanisms to survive and grow in the altered environment. Dynamic regulation of chromatin is fundamental to the adaptive mechanisms eukaryotic cells employ to change cellular phenotype in response to fluctuating environmental conditions. The epigenome is a sensor and it responds to alterations in the extracellular environment, and yet very little is known about how this information is transmitted to the epigenetic regulatory machinery. Environmental regulation of the

This article is part of the Topical Collection: Chromatin Biology and Epigenetics.

Published online: 20 January 2020

epigenome severely affects health and disease, but how the chromatin regulatory machinery responds to such effects is not understood (Lu and Thompson 2012).

Epigenetic modifications are the interface between cellular metabolism and DNA metabolism. Histone acetylation is a crucial player in transcription, DNA replication and DNA damage response. Histone acetylation is dynamically controlled by histone acetyl transferases and histone deacetylases (HDACs) and the activity of these enzymes, in turn, are affected by concentration of metabolites in the cell. Histone acetylation is affected by the levels of acetate in yeast and that of glucose and lactate in mammals. Glucose is a source of acetyl CoA required for acetylation in mammals, while lactate has been shown to be a weak inhibitor of histone deacetylases; thus, the presence of lactate in medium is shown to increase acetylation (Wellen *et al.* 2009; Latham *et al.* 2012).

Histone H3K56ac is a core domain acetylation mark which is actively involved in transcription, DNA replication and DNA damage response. The molecular function of this acetylation mark in DNA damage response is not well understood. On DNA damage, acetylation at this residue is known to increase in case of yeast (Masumoto *et al.* 2005; Haldar and Kamakaka 2008); however, in mammalian cells, conflicting results on H3K56ac level has been reported. There are studies showing both increase as well as decrease in H3K56ac upon DNA damage in mammalian cells (Vempati *et al.* 2010; Das *et al.* 2009; Tjeertes *et al.* 2009). Deacetylation of H3K56ac upon DNA damage is required for successful completion of DNA repair by NHEJ pathway (Toiber *et al.* 2013; Tjeertes *et al.* 2009). However, it is not known whether one of these responses is more often observed or both the responses may occur based on cellular micro-environment, experimental setup, etc. Further, the conditions that lead to the increase and decrease in acetylation at H3K56 and their significance is also not clear. Since, we have observed both increased and decreased H3K56ac upon DNA damage in our experiments, depending on cell confluency, we have investigated the role of cellular micro-environment on H3K56ac. In this context, here we check whether H3K56acetylation is affected by cellular micro-environment (cell growth conditions in mammalian cell culture) and, if so, could this altered initial epigenetic state be the cause for the differential H3K56 acetylation levels observed earlier upon DNA damage.

In the present study, we report that the dynamics of H3K56ac upon DNA damage are dependent on the initial acetylation status of the cell, which in turn is dependent on the level of metabolites in the extracellular environment. Thus, in low cell density when metabolites are less, cells are likely to be in hypo-acetylated state and show an increase in H3K56ac upon DNA damage, whereas the reverse is true in cells growing in high density. High H3K56ac levels can be achieved by increasing lactic acid levels. These observations also explain the conflicting results reported previously by different laboratories. Based on the cellular environment, both the earlier reported responses could be obtained. Here, we have identified conditions which result in increase and decrease in H3K56 acetylation in response to DNA damage in cell culture. We have also found that SIRT6 deacetylates H3K56ac when treated with DNA damaging agents at high cell density. Collectively, this study reports that H3K56 acetylation levels were low in sparse culture and increased gradually as the culture became confluent. This increase in acetylation coincided with increase in glycolytic activity and decrease in sirtuin 1 (SIRT1) and SIRT6 expression. Increased glycolysis and secretion of lactate lead to hyper-acetylation at H3K56 in high density conditions and the initial level of acetylation at this site during DNA damage determine the rates of acetylation post damage. These observations provide a link between metabolites, deacetylases and increase in H3K56ac.

2. Materials and methods

2.1 *Cell culture and treatment*

All cell lines used in the experiments viz. HeLa, A549, U2OS and HADF, HEK293T, NIH3T3 were cultured in DMEM supplemented with 10% fetal bovine serum 2.5 mM glutamine, 100 mg/ml of penicillin and 100 mg/ml of streptomycin at 37°C in 5% CO2 incubator. For experiments done in low glucose medium DMEM containing 1 g/L glucose was used.

For inducing DNA damage, cells were treated with 2 mM MMS, 10 mM hydroxyurea and 5 μM bleomycin for 2, 12 and 1 h respectively. All reagents were purchased from Sigma-Aldirch unless mentioned otherwise.

2.2 *Growth curve*

293T cells (2×10^5 cells) were seeded in each P100 dishes. Each plate was harvested for every 24 h for 10 days. Cell number was counted by haemocytometer method before harvesting. Growth curve was plotted by number of days on X-axis and cell number on

Y-axis. Histones were isolated from harvested cells before performing Western blot.

2.3 *Preparation of conditioned medium*

HeLa cells (1×10^6) were seeded in p100 dishes in complete DMEM and grown till plates are over confluent. Medium was collected (conditioned medium) from overgrown cells plate and added to cells in P100 dishes, which are at low density (less confluent) and incubated for 24 h before harvesting. Histones were isolated and Western blot was performed.

2.4 *Lactic acid treatment*

HeLa cells (3×10^6) were seeded in P100 dishes. 24 h post seeding cells were treated with different concentrations of lactic acid for 24 h. Histones were isolated post treatment before Western blot.

2.5 *Whole cell lysate preparation*

All cell lines were lysed using NETN buffer (20 mM Tris-HCl, pH8, 100 mM NaCl, 1 mM EDTA, 0.5% (v/v) Nonidet P-40) containing protease and phosphatase inhibitors. After harvesting, cells were washed with 1× PBS and centrifuged to collect cell pellet. Cell pellet was resuspended in NETN lysis buffer (freshly added with PIC) and incubated on ice for 10 min. After incubation, samples were sonicated in water bath biorupter for 3 cycles (10 s on, 10 s off). Protein amount was estimated by Bradford method. The whole cell lysates were boiled for 5 min in 4× SDS loading dye and resolved by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blotting to detect specific proteins. The intensity of protein bands was quantified using ImageJ software (NIH, USA).

2.6 *Acid extraction of histones*

Histones were extracted as described by Shechter *et al.* (2007). Approximately 1×10^7 cells were re-suspended in 1 ml hypotonic lysis buffer (10 mM Tris–Cl pH 8.0, 1 mM KCl, 1.5 mM MgCl2 and 1 mM DTT) and incubated for 30 min on rotator at 4°C. The nuclei was pelleted by spinning in centrifuge at 10,000*g* for 10 min at 4°C. After discarding the supernatant, the pellet (nuclei) was re-suspended in 400 ml 0.4 N H_2SO_4 followed by incubation at 4°C on rotator for overnight. The nuclear debris was removed by spinning the samples at 16,000*g* for 10 min at 4°C and the supernatant was transferred into a fresh tube.132 µl of TCA (TriChloroAceticacid) was added drop by drop and tube was inverted several times to mix the solution (final concentration of TCA is 33%) followed by incubation on ice for 30 min. histones were pelleted by centrifugation at 16,000*g* for 10 min at 4°C and supernatant was discarded. The histone pellet was washed with ice-cold acetone and spinning in microcentrifuge at 16,000*g*, 5 min at 4°C. The supernatant was removed and histone pellet was air dried for 20 min at room temperature The histone pellet was dissolved in appropriate volume of nuclease free water.

Western blot: Western blot was performed by using histones obtained by acid extraction method or from whole cell lysates obtained by lysis in NETN buffer. 20 µg of histones or 50 µg of whole cell lysate were loaded on 15% gels and run on a constant voltage of 100 V till the dye front reached the end of the gel. The proteins were transferred on PVDF membrane electrophoretically at 4°C for 2 h and blocked by immersing in 5% fat free milk solution for 30 minutes at room temperature. Blocking was followed by overnight incubation in primary antibody at 4°C (H3K56c1:1000, Abcam, Ab76307). After three washes in PBST the blot was exposed to secondary antibody (HRP conjugated anti-rabbit IgG,1:10,000 BIORAD) for 1 h at room temperature. After three washes in PBST to remove the unbound antibodies the blot was developed using Chemidoc (protein Simple).

Immunofluorescence: For immunofluorescence, cells were seeded on coverslips. The cells were fixed in 3.4% formaldehyde for 15 minutes at room temperature. After fixing, the cells were permeabilized by 0.1% Triton X100 for 15 min at room temperature followed by blocking using 2% BSA for 30 min at room temperature. After each step the cells were washed in PBS thrice for 5 min. Blocking was followed by incubation in primary antibody (1:200 for H3K56ac Abcam 76307,) for 2 h at room temperature. The cells were given three washes of PBS of 10 minutes each and incubated in secondary antibody Rabbit or mouse IgG conjugated to Alexa 488 or Alexa 647 (1:5000, Invitrogen, USA) for 1 h at room temperature. After three final washes in PBS to remove the unbound antibody, DAPI (Diaminophenyl indiaole, Sigma Aldrich) was added for 5 min to stain the nucleus and washed off. The cells were finally mounted on slides using mounting medium containing anti-fade agent.

2.7 *siRNA transfections*

Small interfering RNA (siRNA) against SIRT6 was obtained from Ambion (Invitrogen, USA). All siRNA transfections were performed using Lipofectamine™ 2000 transfection reagent (Invitrogen, USA) according to manufacturer instructions. HeLa cells (1×10^{5}) HeLa were seeded in 12-well plates and grown overnight in medium without antibiotics. In two separate tubes, Lipofectamine 2000 reagent was diluted in DMEM and siRNA was diluted in DMEM and incubated for 5 min at room temperature (RT). After 5 min lipofectamine and siRNA complexes were mixed and incubated at RT for 20 min. The Lipofectamine and siRNA complex was then added to each well containing cells and incomplete DMEM. After 6 h medium was replaced with complete DMEM and incubated for 48–72 h.

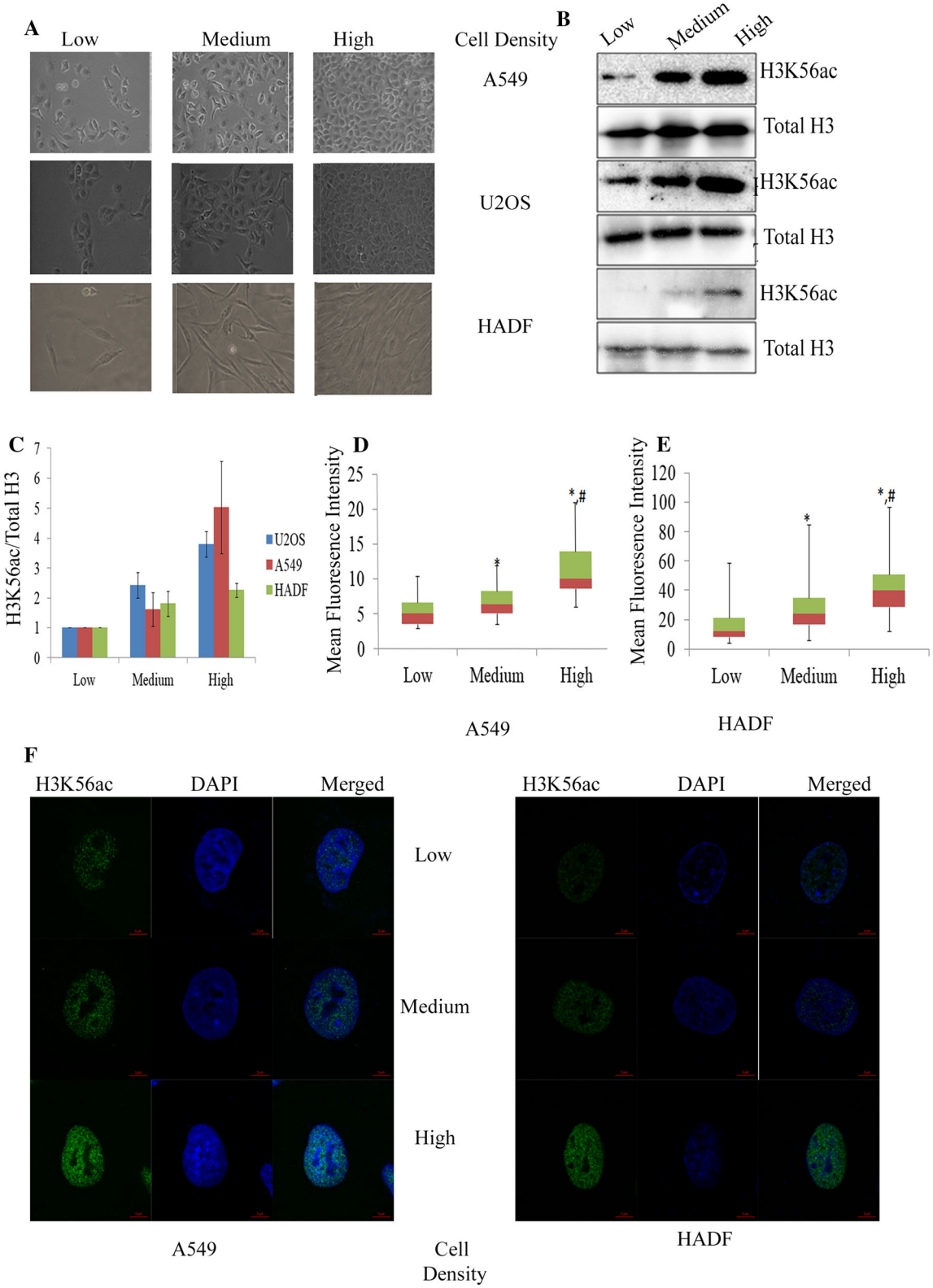
3. Results

3.1 *H3K56ac levels increase from low to high cell density*

In fluctuating environmental conditions, including altered nutrient availability and various forms of stress, cells adapt by dynamic regulation of chromatin, yet how this information is transmitted to the epigenetic regulatory machinery is unclear. Histone acetylation is dynamically controlled by histone acetyl transferases (HATs) and histone deacetylases (HDACs), which in turn are sensitive to levels of metabolites like glucose, lactate, NAD+, etc.,making histone acetylation very sensitive to external environment (Sebastián *et al.* 2012; Latham *et al.* 2012). Earlier, we have reported that H3K56 acetylation increases in response to DNA damage (Vempati *et al.* 2010). However, conflicting reports of decrease in H3K56 acetylation level on DNA damage and both the observations in different experiments in our laboratory prompted us to investigate whether both the reported responses occur in mammalian cells, or if decrease in acetylation is more prevalent. Environmental factors such as changes in pH, cell density and any other type of change in cell culture conditions affects cellular processes (Fabrizio and Wei 2011) Therefore, we hypothesized that the observed differential levels of H3K56ac in response to DNA damage might be due to differences in cell density, cell culture conditions or treatment time periods in these studies. We started this work by investigating whether H3K56 acetylation level changes as cell grow in cell culture. In order to study the effect of change in external micro-environment on histone H3K56ac under cell culture conditions, we devised a model of low and high density cell culture. To determine the level of acetylation of histone H3K56, we seeded cells at very low density (1×10^{6} cells in a 100 mm dish) and allowed the culture to grow and harvested cells at three cell densities, i.e. lower than 30%, 70% and 100% confluent cultures (figure 1A), prepared whole cell extracts and checked the H3K56 acetylation level by Western blot using anti- H3K56Ac antibody (figure 1B and C). The experiment was done in two cancerous cell lines A549 and U2OS, and one noncancerous line HADF using Western blotting (compare with figure 1). The level of H3K56 acetylation in one noncancer cell line HADF and one cancerous cell line A549 was also checked by Immunofluorescence using anti-H3K56ac antibody (figure 1D). We observed a significant increase in the levels of H3K56 from low density to high density culture in all the cell lines tested ($p<0.05$).

3.2 *Histone acetylation increases as mammalian cells in culture grow from log to stationary phase: H3K56ac is more responsive to external environment than H4K16ac*

The growth curve of cells in culture is made up of three phases: the latent phase also called lag phase before growth begins, the exponential growth phase also called log phase in which cell numbers increase rapidly, and the final or stationary phase in which the rapid increase in cell numbers gradually slows. To further confirm our above observation, we have performed growth curve experiment with 293T cells to determine the level of H3K56ac during various phases of growth of cells in culture. Cells were seeded at 2×10^{5} cells in 100 mm dishes in DMEM and grown for 10 days. Cells were harvested every 24 h and cell number was estimated by counting cell number using haemocytometer. Cell number has exponentially increased during log phase and remained constant during stationary phase (figure 2A). Growth curve showed three phases of growth lag, log and stationary phase. Histones were isolated and levels of H3K56ac and H4K16ac were analysed by Western blot. Increase in acetylation was observed in both the residues, but the increase was more prominent in the case of H3K56ac (figure 2B). Next, we checked whether the increase in acetylation is dependent on glucose by growing cells in low glucose (1 g/l), since glucose and generation of acetyl coA is


A
Low
Medium
High
Cell Density
B
Low
Medium
High
A549
H3K56ac
Total H3
U2OS
H3K56ac
Total H3
HADF
H3K56ac
Total H3
C
H3K56ac/Total H3
U2OS
A549
HADF
Low
Medium
High
D
Mean Fluoresence Intensity
Low
Medium
High
A549
E
Mean Fluoresence Intensity
Low
Medium
High
HADF
F
H3K56ac
DAPI
Merged
Low
Medium
High
A549
Cell Density
HADF

◀**Figure 1.** H3K56ac acetylation levels increase with cell growth/ proliferation. (**A**) Cancerous cell lines A549, U2OS and noncancerous human adult dermal fibroblast (HADF) cell line were seeded in low cell density 1×10^6 cells/10 cm dish and allowed to grow till fully confluent. Cells were collected at low (20–30% confluent); medium (70% confluent) and high (100% confluent). (**B**) Western blot showing the levels of H3K56ac and total H3 proteins in low, medium and high cell densities. (**C**) Graph representing the intensity of H3K56ac normalized to the intensity of total H3 for each experiment. Fold change was calculated by considering the intensity of low density culture as 1. * and # indicate significant difference with low density and medium intensity respectively ($p<0.05$, one-way ANOVA) Graph represents the mean±SEM for three independent experiments. (**D**) Box and whisker plot showing the mean fluorescence in 100 cells from low, medium and high density of cell lines A549 (* and # represent significant difference from low and medium density respectively ($p<0.05$, Student's *t*-test). (**E**) Box and whisker plot showing the mean fluorescence in 100 cells from low, medium and high density HADF cells in culture (* and # represent significant difference from low and medium density respectively ($p<0.05$, Student's *t*-test). (**F**) H3K56ac levels as measured by immunofluorescence in HADF and A549 cells in three different cell densities (Low, medium and High). Experiment was repeated three times. One representative image is shown.

pre-requisite for acetylation in mammals. Our observed decrease in H3K56ac under low glucose medium implying that indeed glucose is essential for histone acetylation. Together our results indicate that H3K56ac levels increases from low to high density cells in culture and H3K56ac is more sensitive to changes in the cellular micro-environment than H4K16ac.

3.3 *Accumulation of metabolites causes increase in acetylation of histone H3K56 via regulation of sirtuins*

It has been reported that human tumour cells when grown in cultures for high densities resulted in medium acidification caused in part by lactate accumulation, which mirrors the accumulation of ethanol and some acetic acid, and the acidification of the medium in *S. cerevisiae* (Fabrizio and Wei 2011). Conditioned medium induces chronological senescence in cancer cells (Leontieva and Blagosklonny 2011). We next examined whether accumulation of metabolites in the external medium that influences H3K56ac. Therefore, we examined the effect of conditioned medium (CM) on H3K56ac. To study whether addition of CM to less confluent cells induces increase in H3K56 acetylation, conditioned media that was collected from the dense culture of 293T cells and was added to cells plated at low density and H3K56ac was checked by Western blot. The H3K56ac levels were increased in low density cells treated with conditioned medium (figure 3A), suggesting that conditioning of medium due to accumulation of factors or depletion of nutrients during growth of cells in culture affects H3K56ac. Our observations show that indeed the change in external environment (fresh to conditioned medium) was enough to increase H3K56ac and the levels were similar to a high density culture with exhausted medium. It has been reported that lactic acid accumulated during culturing of cells in the medium increases histone H4 acetylation by acting as a HDAC inhibitor (Latham *et al.* 2012) and since lactate is one of the metabolites that accumulates in exhausted medium, we checked its effect on H3K56ac by adding different concentration of lactic acid in low density HeLa cells. H3K56ac levels were directly proportional to the lactic acid levels as revealed by our experiments (figure 3B). Since lactate is known as a mild inhibitor of HDACs, we next went on to check whether the level of human sirtuin1 (SIRT1) and sirtuin6 (SIRT6) change in low and high density culture as H3K56ac is deacetylated by these enzymes. SIRT1 and SIRT6 levels come down under high density conditions (figure 3C). Next, as H3K56ac is a major substrate of SIR6 deacetylase, we examined the levels of SIRT6 protein in cells grown to high confluency in unchanged medium and fresh medium. SIRT6 levels has increased in cell grown in fresh medium (figure 3D), suggesting that low levels of H3K56ac in high confluency might be due to increased SIRT6 levels. This observed increased level of SIRT6 in fresh medium under the high density cell culture conditions indicated that H3K56ac levels in high density medium is due to increased metabolites like lactate and coincides with decrease in SIRT1 and SIRT6.

3.4 *Initial acetylation levels determines the dynamics of histone H3 lysine 56 acetylation in response to DNA damage*

The level of H3K56ac changes in response to DNA damage. It also co-localizes with DNA damage marker γ-H2AX (Das *et al.* 2009; Vempati *et al.* 2010). However, conflicting results from various groups have been reported with respect to the changes in levels of this acetylation upon DNA damage. Increase in H3K56ac on DNA damage was observed by three groups (Das *et al.* 2009; Vempati *et al.* 2010; Yuan

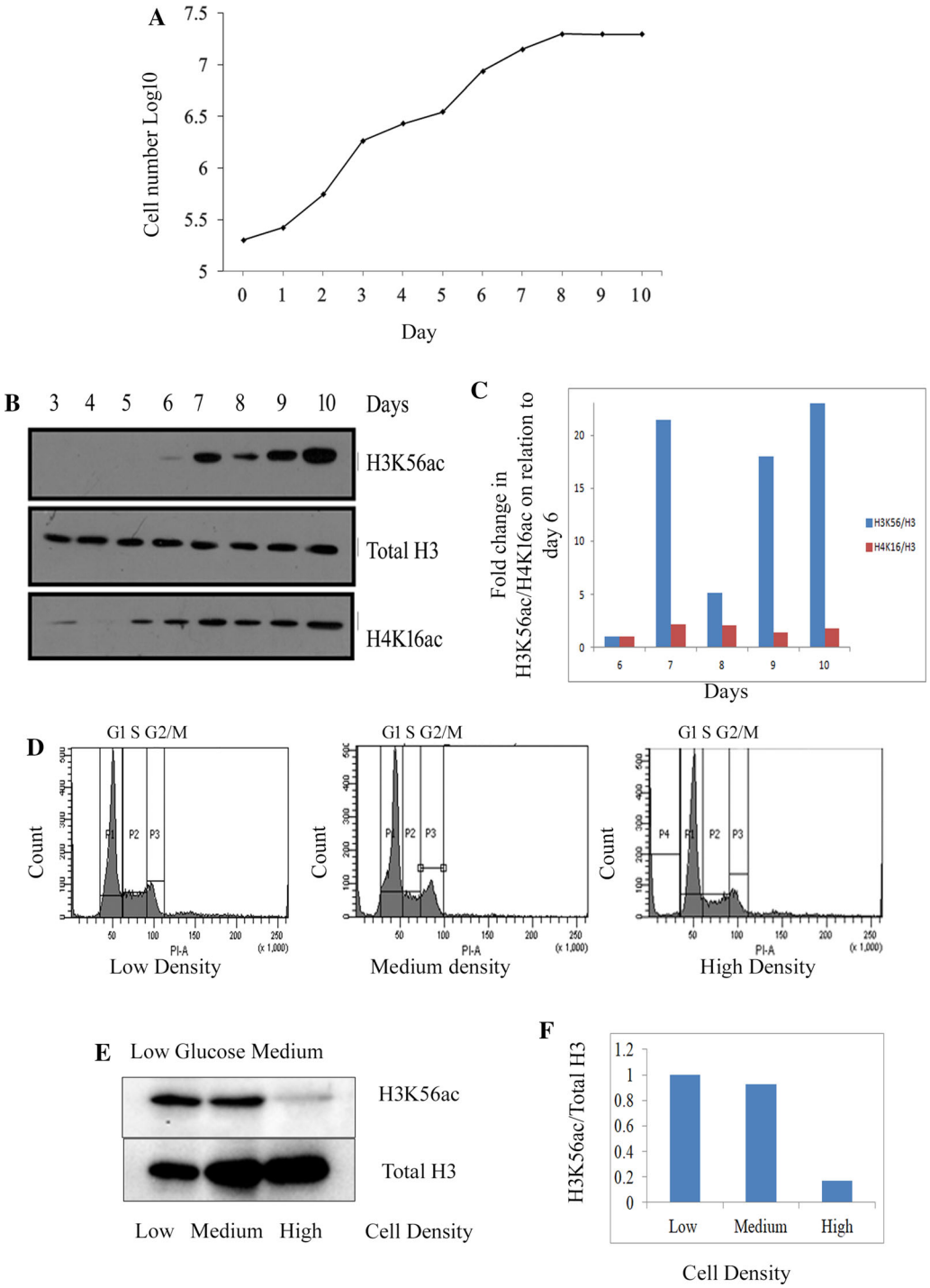


Figure 2. Levels of H3K56ac increase as cell density increases and H3K56ac is more responsive to external environment than H4K16ac. (**A**) 293T cells (2×10^5) were seeded in 100 mm dishes in DMEM. Cells were harvested every day and cells number was determined by counting using haemocytometer. The growth curve was generated by plotting cell number on the Y-axis and number of days on the X-axis. (**B**) Histones were isolated and levels of H3K56ac, H4K16ac and total H3 was analysed by Western blot from third day after seeding. (**C**) Quantification of fold change in H3K56ac and H4K16ac as compared to 6th day after seeding (note the dip in H3K56ac on 8th day is due to addition of fresh medium). (**D**) Distribution of cells in different phases of cell cycle at 2nd, 4th and 8th day (Low, medium and high density) after seeding shows decline in S phase population but does not show any significant cell death. (**E**) Level of H3K56ac in HeLa cells cultured in low glucose medium. Cells were seeded in low density and allow to grow in low glucose medium till 72 h. Cells were collected at three different densities (low 20–30% confluent) (medium 60–70% confluent) and (high 90–100% confluent). (**F**) Quantification of fold change in H3K56ac normalized to total H3 in relation to the low density cells.

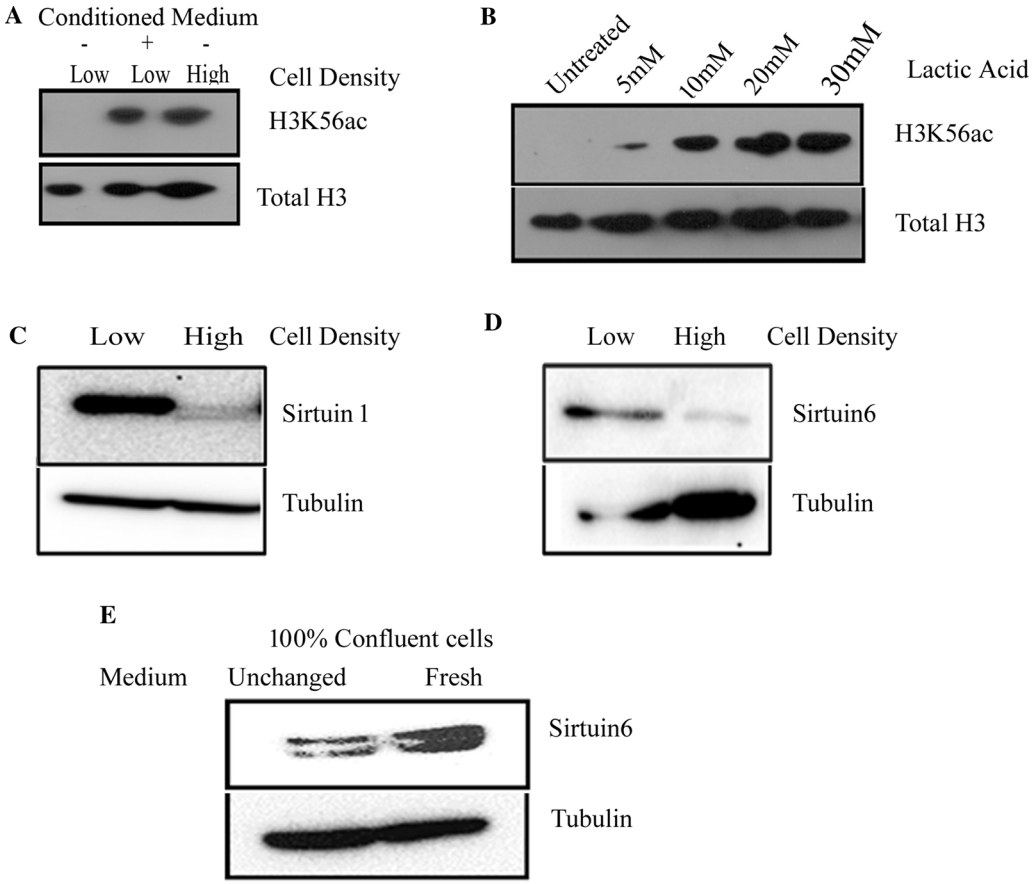


Figure 3. Accumulation of metabolites cause increase in H3K56ac via regulation of sirtuins. (**A**) H3K56ac levels were checked in Hela cells in low density under fresh and conditioned medium and in high density with conditioned medium. (**B**) H3K56ac and Total H3 were checked in Hela cells treated with different concentrations of lactic acid. (**C**) Protein level of sirtuin 1 in HeLa cells under low and high density conditions. Tubulin is used as a loading control. (**D**) Protein level of sirtuin6 in HeLa cells under low and high density conditions. Tubulin is used as a loading control. (**E**) SIRT6 levels in HeLa cells in high density culture (100% confluent) with unchanged medium and fresh medium.

et al. 2009) and it was shown to decrease in experiments carried out by other groups (Tjeertes *et al.* 2009).

The contradictory results reported earlier and our observation that H3K56ac varies with cell density, prompted us to hypothesise that dynamics of H3K56 acetylation upon DNA damage is dependent on cell density and initial acetylation levels. To test this hypothesis, we induced DNA damage under two different cell density conditions: (a) in very low cell density in fresh medium, where the initial acetylation levels were observed to be low and (b) in high cell density where the initial acetylation levels are generally high. Initially, we treated HADF and HeLa cells seeded at low and high density with various DNA damaging agents like methyl methane sulphonate (MMS), hydroxyurea (HU) and bleomycin and checked the levels of H3K56ac by Western blot. Our results indicated that in the low cell density cells, which had low levels of H3K56ac initially, acetylation increased in response to DNA damage. On the contrary, in medium or high cell density cells, which had higher initial acetylation, H3K56ac levels decreased upon DNA damage (figure 4A and B). To further confirm the observed difference in H3K56ac levels in response to DNA damage, we also performed immunofluorescence experiments in HeLa and HADF cells on treatment with DNA damaging agents at low and high density and observed the same differential H3K56ac dynamics by Western blot (figure 4C, D, E; the data for HeLa cells has not been shown).

3.5 *Sirtuins deacetylate H3K56 acetylation in response to DNA damage*

The histone deacetylases, SIRT1 and SIRT6 which deacetylates H3K56ac are involved in double strand

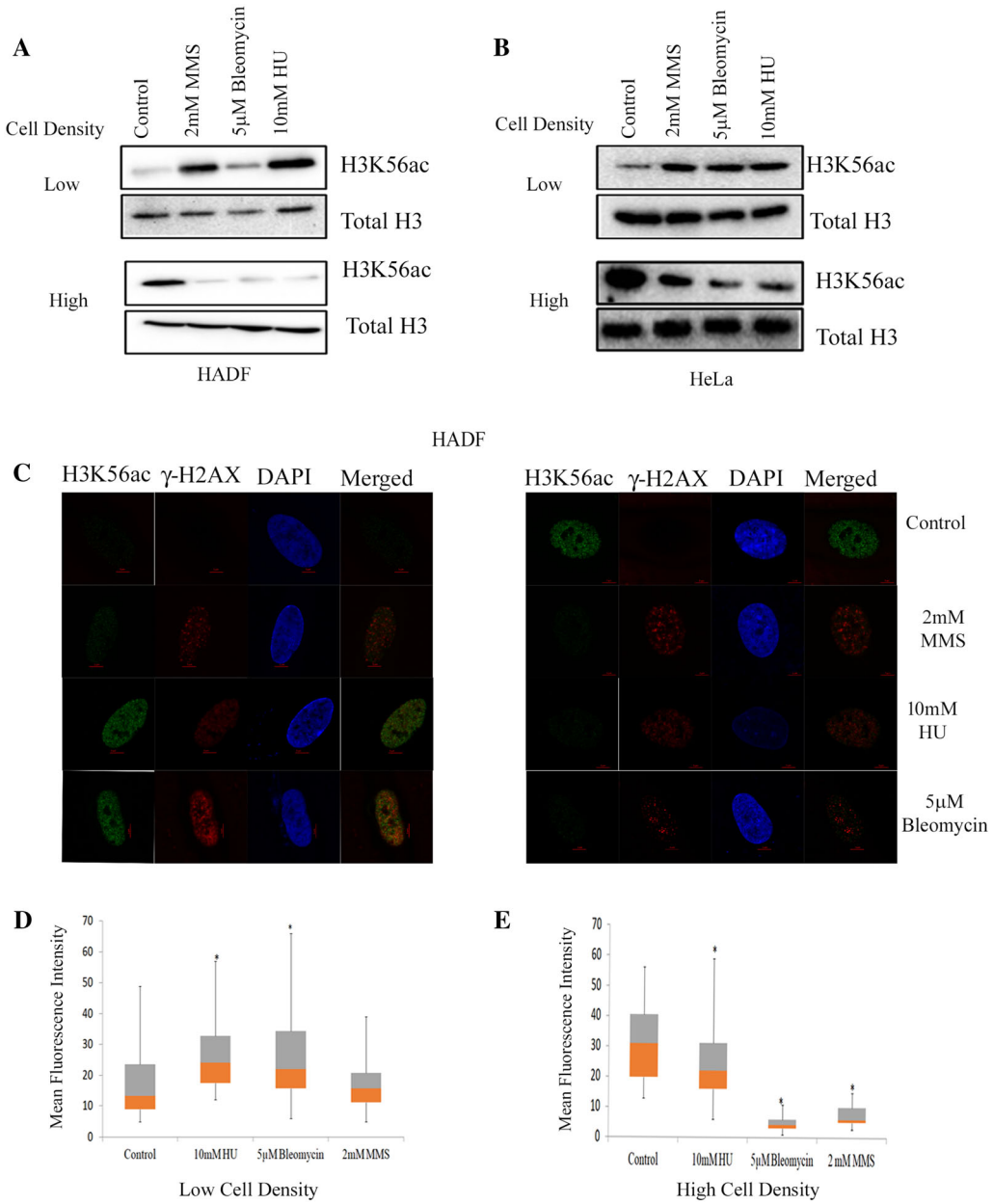


Figure 4. Initial levels of H3K56ac determine its dynamics upon DNA damage. (**A**) H3K56ac and total H3 levels in primary human fibroblast HADF seeded at low and high cell density (2×10^5 cells/10 cm dish) and high cell density (5×10^5 cells/10 cm dish) treated with 5 μM bleomycin, 2 mM MMS and 10 mM HU. (**B**) H3K56ac and total H3 levels in cervical cancer line HeLa seeded at low and high cell density (1×10^6 cells/10 cm dish) and high cell density (1×10^6 cells/10 cm dish) treated with 5 μM bleomycin, 2 mM MMS and 10 mM HU. (**C**) H3K56ac levels by immunofluorescence in HADF cells seeded at low and high cell density (control and cells treated with 2 mM MMS, 5 μM Bleomycin and 10 mM HU). γ-H2AX staining was done to verify DNA damage. (**D** and **E**) Box and whisker plot representing the Mean Fluorescence Intensity of H3K56ac in control and different treatment groups of HADF in low and high density [50–100 cells were counted for each experimental group in three independent experiments, box and whisker plot are representative of one experiment (*shows significant difference with control ($p<0.05$)].

break repair by both homologous repair and nonhomologous end joining pathways. SIRT1 regulates homologous recombination (HR) by deacetylating Nijmegen breakage syndrome 1 (NBS1) which results in recruitment of (MRE11)–RAD50–NBS1 (MRN) complex to the site of the DSB (Yuan *et al.* 2007). It

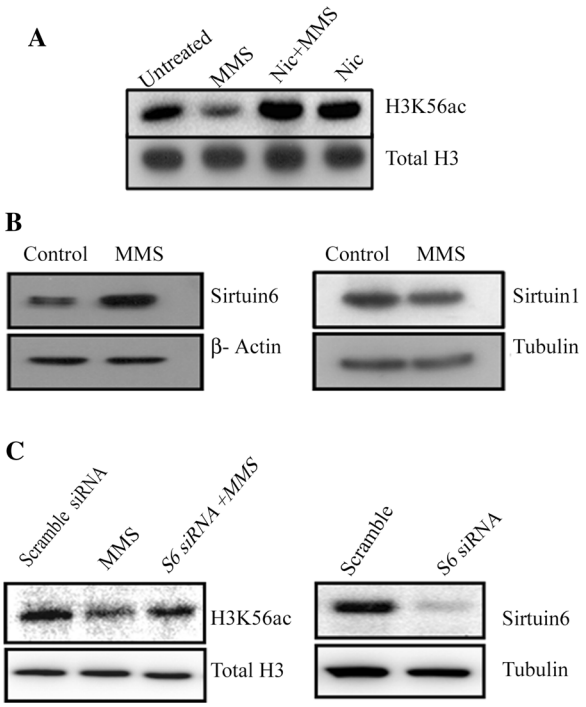


Figure 5. Sirtuins deacetylate H3K56 on DNA damage. (**A**) HeLa cells were seeded at high cell density and treated with MMS or pre-treated with nicotinamide (Nic) for overnight and then treated with MMS for 2 h. Whole cell lysates were analysed for H3K56ac by Western blot. (**B**) Levels of SIRT6 increases on MMS treatment. HeLa cells were seeded at high density and treated with MMS for 2 h. Whole cell lysates were analysed for SIRT6 and SIRT1 levels by Western blot. (**C**) SIRT6 deacetylates H3K56 on MMS treatment. HeLa cells were transfected with scramble or SIRT6 siRNA for 48 h and then treated with MMS for 2 h. Whole cell lysates were analysed for indicated proteins by Western blot. The Western blot data of SIRT6 in SIRT6 siRNA-transfected cells obtained with the same lysates as shown for K56ac level on the left.

has been reported that SIRT6 deacetylates H3K56ac around the DSB and recruits the chromatin remodeller SNF2H. To examine whether sirtuins deacetylate H3K56ac on DNA damage at high cell density, we have pre-treated HeLa cells at high density with nicotinamide which is a sirtuin inhibitor and then treated with MMS for 2 h. Deacetylation of H3K56 on MMS treatment was rescued in cells pre-treated with nicotinamide (figure 5A), suggesting that sirtuins deacetylate H3K56ac on DNA damage. Next, we examined whether SIRT1 and SIRT2 levels were affected on MMS treatment by treating HeLa cells with MMS for 2 h. We observed increased SIRT6 expression on MMS treatment, whereas SIRT1 levels were unchanged (figure 5B). It has been reported that SIRT6 deacetylates H3K56 near sites of DSB (Toiber *et al.* 2013). We knocked down SIRT6 by siRNA in HeLa cells and treated with MMS and monitored H3K56ac levels. Our results show that deacetylation of H3K56 was rescued in SIRT6 depleted cells on MMS treatment (figure 5C), indicating that SIRT6 deacetylates H3K56 acetylation in response to DNA damage.

4. Discussion

Here, we described the effect of metabolic factors that accumulate in cellular micro-environment during growth of cells in culture, on the levels of H3K56ac upon DNA damage. When transformed cells grow in culture, the exponential increase in cell number is accompanied by release of metabolites such as lactic acid, causing acidification of the culture medium. Continuous culturing of cells under overgrown conditions and lactate accumulation cause chronological senescence (CS), which is defined as loss of replicative viability in stationary culture (Leontieva and Blagosklonny 2011). Furthermore, CS can be prevented by neutralization of acidity. Increased cell density in cell culture affect the regulation of YAP by Hippo signalling resulting in deregulation of miRNA biogenesis (Mori *et al.* 2014; Gregory 2014). The phosphorylated H2AX (γ-H2AX) protein is stabilised at high confluency due to intercellular contact by activating γ-catenin pathway, resulting in activated DNA damage response (Kang *et al.* 2012). These studies suggest that during normal culture conditions multiple factors affect the cellular signalling pathways. We have found that H3K56ac induction at high confluency is not due to increased intercellular contact (our unpublished results). When cell culture medium was replenished and H3K56ac was examined at high density, we observed H3K56ac was maintained at low levels even at high density, suggesting that intercellular contact has no effect on H3K56ac. Further, H3K56ac was induced in low density cells when treated with conditioned medium, suggesting that depletion of nutrients or accumulation of metabolic factors in high density culture medium alter H3K56ac levels. Cancer cells in culture produce lactic acid as a product of glycolytic metabolism which results in acidification of the micro-environment. It has been shown that lactic acid acts a weak HDAC inhibitor resulting in increased H4 acetylation at high densities in culture (Latham *et al.* 2012). In that context, treating cells with lactate induced H3K56ac. As H3K56ac is majorly regulated by sirtuins, whether lactate also inhibits NAD+ dependent sirtuins has to be studied. Our results

showed downregulation of sirtuin 1 (SIRT1) and sirtuin 6 (SIRT6) expression in high density cultures, thus indicating that external metabolites like lactic acid which are known deacetylase inhibitors reduce the expression of deacetylases SIRT1 and SIRT6 resulting in increased H3K56ac. Additionally, compared to another histone acetylation mark H4K16ac, H3K56ac is more sensitive to conditioned medium. Lowered pH in conditioned medium could also contribute to increased acetylation. Although studies that link acidic conditions to higher H3K56ac are lacking, it has been shown that mTORC1 mediated by TSC1/2 is downregulated in low pH condition (Balgi *et al.* 2011). Rapamycin treatment delayed acidification of conditioned medium by reducing lactate production (Leontieva and Blagosklonny 2011). In a previous study from our lab, we have shown that H3K56c levels are controlled by mTORC2-dependent signalling (Vadla and Haldar 2018). It is well known that the pH of the tumor microenvironment is acidic (Zhang *et al.* 2010). Increase in levels of H3K56ac has been reported in various tumors including gliomas (Das *et al.* 2009). In this context, it would be interesting to investigate the crosstalk between pathways that lowered pH and mTORC2 regulation of H3K56ac in tumor initiation and progression (McBrian *et al.* 2013).

The role of H3K56ac in the DNA Damage Response (DDR) in yeast is well understood and it is known to play a role in recombination based repair (Wurtele *et al.* 2012). However, in case of mammals, the exact role of H3K56ac is not well understood. Different studies reported conflicting responses of H3K56ac on DNA damage. While some report increased acetylation on damage (Das *et al.* 2009; Vempati *et al.* 2010), others observed decrease in acetylation on DNA damage (Tjeertes *et al.* 2009; Yuan *et al.* 2009). In this present work, we report cell growth conditions under which both increase as well as decrease in H3K56ac upon DNA damage could occur. At low density, the acetylation increased upon DNA damage, while it decreased in the high density culture. It is noteworthy, that the initial acetylation is low in sparse culture as compared to the confluent, indicating that the initial H3K56acytelation level decides the dynamics of H3K56ac post DNA damage. Further, we observed that DNA damage is associated with increased SIRT6. SIRT6 is known to deaceylate H3K56ac on DNA damage (Toiber *et al.* 2013). Therefore, we went on to investigate the dynamics of H3K56ac on DNA damage in real time by inducing damage by laser irradiation (our unpublished data). Our observation was similar to previous study that reported deacetylation of H3K56ac deacetylates DNA damage. Yet, our study differs from the previous one in the respect that we have determined the levels of H3K56ac at very early time points (<30 s) in cells grown to two different cell densities. Interestingly, we observed that H3K56ac persists for a longer time i.e. around 2 min in low cell density, while in high cell density the deacetylation was observed as early as 20 s (our unpublished data). Since, initial acetylation is low in the sparse culture, we propose that H3K56ac mark plays an early role in DNA damage and certain threshold level of H3K56ac is required to initiate the DNA damage response signalling.

As acetylation is known to increase chromatin opening by loosening the interaction between histone and DNA as well as by recruiting bromodomain containing chromatin remodelers (Wellen *et al.* 2009), now we trying to understand the role of in H3K56ac in chromatin changes on DNA damage. On treatment with bleomycin under the two different cell densities, we observed chromatin opening in low density and chromatin compaction in high density. It is possible that our results indicate that increase in acetylation at H3K56 relates to chromatin opening, while the opposite is true for high density. Recent studies using photoactivable GFP have shown that chromatin opening after damage is for a very short period of 1.5 min (Burgess *et al.* 2014), which probably indicates that threshold level of acetylation may be required for chromatin relaxation in low density cells which may be followed by deacetylation of chromatin. Further studies are needed to establish this early role of H3K56ac in DDR signalling in mammals. Collectively, the results presented here suggest that H3K56ac is very sensitive to levels of metabolite in extracellular milieu and it plays a role in the initial steps of DNA damage response signalling possibly by altering chromatin structure.

Acknowledgements

The research was funded by grant from Centre for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad, India, which is funded by Department of Biotechnology (DBT), India. NC was supported by CDFD and RV supported by CSIR-Research Fellowships and CDFD Core Funding. Sincere thanks to members of the Laboratory of Chromatin Biology and Epigenetics, CDFD.

References

Balgi AD, Diering GH, Donohue E, Lam KK, Fonseca BD, Zimmerman C, Numata M and Roberge M 2011 Regulation of mTORC1 signaling by pH. *PLoS One* **6** e21549

Burgess R, Burman B, Kruhlak MJ, and Misteli T 2014 Activation of DNA damage response signaling by condensed chromatin. *Cell Rep.* **9** 1703–1717

Das C, Lucia MS, Hansen KC and Tyler JK 2009 CBP/p300-mediated acetylation of histone H3 on lysine. *Nature* **459** 113–117

Fabrizio P and Wei M 2011 Conserved role of medium acidification in chronological senescence of yeast and mammalian cells. *Aging* **3** 1127–1129

Gregory RI 2014 Hippo signaling regulates microprocessor and links cell-densitydependent histone H3 on lysine. *Nature* **459** 113–117

Haldar D and Kamakaka RT 2008 Schizosaccharomyces pombe Hst4 functions in DNA damage response by regulating histone H3 K56 acetylation. *Eukat. Cell* **7** 800–813

Kang MA, So EY and Ouchi T 2012 Deregulation of DNA damage response pathway by intercellular contact. *J. Biol. Chem.* **287** 16246–16255

Latham T, Mackay L, Sproul D, Karim M, Culley J, Harrison DJ , Hayward L, Langridge-Smith P and Gilber Nick Ramsahoy BH 2012 Lactate, a product of glycolytic metabolism, inhibits histone deacetylase activity and promotes changes in gene expression. *Nucleic Acids Res.* **40** 4794–4803

Leontieva OV and Blagosklonny MV 2011 Yeast-like chronological senescence in mammalian cells: phenomenon, mechanism and pharmacological suppression. *Aging* **3** 1078–1091

Lu C and Thompson CB 2012 Metabolic regulation of epigenetics. *Cell Metab.* **16** 9–17

Masumoto H, Hawke D, Kobayashi R and Verreault A 2005 A role for cell-cycle-regulated histone H3 lysine 56 acetylation in the DNA damage response. *Nature* **436** 294–298

McBrian MA, Behbahan IS, Ferrari R, Su T, Huang TW, Li K, Hong CS, Christofk HR, Vogelauer M, Seligson DB and Kurdistani SK 2013 Histone acetylationregulates intracellular pH. *Mol. Cell* **493** 10–21

Mori M, Triboulet R, Mohseni M, Schlegelmilch K, Shrestha K, Camargo FD and Gregory RI 2014 Hippo signaling regulates microprocessor and links cell-densitydependent miRNA biogenesis to cancer. *Cell* **156** 893–906

Ruprecht V, Monzo P, Ravasio A, Z, Makhija E, Strale OP, Gauthier N and Shivashankar GV 2017 How cells respond to environmental cues – insights from bio-functionalized substrates. *J. Cell Sci.* **130** 51–61

Sebastián C, Zwaans BM, Silberman DM, Gymrek M, Goren A, Zhong L and Mostoslavsky R 2012 The histone deacetylase SIRT6 is a tumor suppressor that controls cancer metabolism. *Cell* **151** 1185–1199

Shechter D, Dormann HL, Allis CD and Hake SB 2007 Extraction, purification and analysis of histones. *Nat. Protoc*. **2** 1445–1457

Tjeertes JV, Miller KM and Jackson SP 2009 Screen for DNA-damage-responsive histone modifications identifies H3K9Ac and H3K56Ac in human cells. *EMBO J.* **28** 1878–1889

Toiber D, Erdel F, Bouazoune K, Silberman DM, Zhong L, Mulligan P, Sebastian C, Cosentino C, *et al.* 2013 SIRT6 recruits SNF2H to DNA break sites, preventing genomic instability through chromatin remodelling. *Mol. Cell* **51** 454–468

Vadla R and Haldar D 2018 Mammalian target of rapamycin complex 2 (mTORC2) controls glycolytic gene expression by regulating histone H3 lysine 56 acetylation. *Cell Cycle* 17 110–123

Vempati RK, Jayani RS, Notani D, Sengupta A, Galande S and Haldar D 2010 p300-mediated acetylation of histone H3 lysine 56 functions in DNA damage response in mammals. *J. Biol. Chem*. 285 28553–64

Wellen KE, Hatzivassiliou G, Sachdeva UM, Bui TV, Cross JR and Thompson C B 2009 ATP-citrate lyase links cellular metabolism to histone acetylation. *Science* **324** 1076–1080

Wurtele H, Kaiser GS, Bacal J, St-Hilaire E, Lee EH, Tsao S, Dorn J and Maddox P 2012 Histone H3 lysine 56 acetylation and the response to DNA replication fork damage. *Mol. Cell Biol*. **32** 154–172

Xiao J, Jin R, and Wagner D 2017 Developmental transitions: integrating environmental cues with hormonal signalling in the chromatin landscape in plants. *Genome Biol.* **18** 88

Yuan J, Pu M, Zhang Z and Lou Z 2009 Histone H3-K56 acetylation is important for genomic stability in mammals. *Cell Cycle* **8** 1747–1753

Yuan Z, Zhang X, Sengupta N, Lane WS and Seto E 2007 SIRT1 regulates the function of the Nijmegen breakage syndrome protein. *Mol. Cell* **27** 149–162

Zhang X, Lin Y and Gillies RJ 2010 Tumor pH and its measurement. *J. Nucl. Med*. **51** 1167–1170