Brief Communication *Featured Article*

Histone H2A mono-ubiquitination and cellular transformation are inversely related in N-nitrosodiethylamine-induced hepatocellular carcinoma

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Abstract

Aberrant changes in histone post-translational modifications are encountered frequently in diseases like cancer. Although histone H3 post-translational modifications have been extensively studied in context of diseases, the functionally important histone H2A PTM H2A119ub (H2Aub) has not gained much attention. In this study, we report that H2Aub markedly decreases in hepatocellular carcinoma. Usp21, a H2A deubiquitinase, is probably responsible for decrease in H2Aub. In addition, the H2Aub levels showed an inverse correlation with H3S10 phosphorylation (H3S10p) and the proliferative state of the cells. Downregulation of H2Aub is also associated with increased expression of growth factor gene lipocalin 2. Interestingly, we show that treatment of cells with histone deacetylase inhibitor trichostatin A results in increase of H2Aub and decrease in H3S10p. Our work for the first time suggests the in vivo association of H3S10p, H4ac, and H2A119ub with cellular transformation.

Keywords: Histone post-translational modifications, deubiquitination, lipocalin 2, histone deacetylase inhibitor, cancer, epigenetics

***Experimental Biology and Medicine* 2016; 241: 1739–1744. DOI: 10.1177/1535370216649262**

Introduction

Histones, which organize chromatin, often undergo a variety of post-translational modifications (PTMs).[1] These modifications function either by disrupting chromatin contacts or by affecting the recruitment of various proteins to the chromatin and thereby, regulating transcription.[1] Histone H2A mono-ubiquitination at lysine 119 (H2Aub) is one such modification identified for polycomb-targeted gene silencing; H2Aub by PRC1 type complexes creates a binding site for Jarid2–Aebp2–containing PRC2 and promotes H3K27 trimethylation on H2Aub containing nucleosomes.[2] H2Aub is thus an integral component of a positive feedback loop establishing H3K27me3 mark and maintaining repressive state of the chromatin.[3,4]

Histone H2A was the first protein identified to be ubiquitinated and is one of the most abundant ubiquitinated protein in the nucleus.[4] Despite its abundance, apart from the study in which mono-ubiquitinated histone H2A was markedly down-regulated in prostate cancer,[5] this functionally important mark has not been investigated in other cancers. In addition, the drop in H2Aub is a pre-requisite for increase in H3S10p during S to G2/M transition in vitro.[6] Interestingly, H3S10p itself is a mark that is indispensable for cellular transformation.[7] Whether an antagonistic effect of these modifications on each other may exist in vivo has not been reported.

Using a hepatocellular carcinoma (HCC) model of rat, we show that the level of H2Aub and H4ac markedly decreases in tumor and is inversely correlated with gain in H3S10p. The enzyme Usp21 probably brings about this loss in ubiquitination. Upon treatment with HDAC inhibitor, the scenario is reversed with increase in H2Aub, H4ac, and loss of H3S10p, again pointing towards a possible correlation amongst these marks. Our results indicate that the drop in H2Aub might be functionally important in cancer progression and emphasizes the need of studying this modification and the cross talk with other modifications in more number of cancers.

Materials and methods

Animal experimentation

All the experiments were performed on male Sprague-Dawley rats (spp. *Rattus norvegicus*) after approval from

the Institutional Animal Ethics Committee, ACTREC. Protocol used to induce HCC is as previously described.[8] For liver tissue transplantation, small piece of ~3 mm^2 size liver and tumor tissue collected from the donor mice were washed in RPMI medium. Small skin incision was made at the flank region of NOD-SCID mice, and liver/tumor tissue was implanted aseptically under the subcutis. For histology analysis, liver tissues were excised, washed with ice-cold saline and either fixed in formalin for hematoxylin and eosin (H&E) staining or snap-frozen in liquid N_2. Further, IHC for proliferating cell nuclear antigen (PCNA) (Santacruz SC-96) and CycinD1 (SC-450) was done using VECTASTAIN® ABC kit (Vector Lab, P6200).

Transmission electron microscopy

Liver tissues were fixed with 3% glutaraldehyde and post-fixed with 1% osmium tetraoxide. Grids were contrasted by use of alcoholic uranyl acetate for 1 min and lead citrate for 30 s. The grids were then observed under a Carl Zeiss LIBRA120 EFTEM.

Histone extraction, acetic acid urea triton (AUT)-PAGE and Western blotting

Histones were extracted and purified as earlier.[9] AUT PAGE was done as described previously.[8] Gels were documented as image files and proteins spots were quantitated by ImageJ software (v1.42q, National Institutes of Health). For western, histones were resolved on SDS-PAGE, transferred, and probed with respective antibodies from Millipore or Abcam or CST (H2Aub, D27C4; H3S10p, M-06-570; H3S28p, ab-5169; Pan-acetyl, ab-61257; H3, M-05-499; H4, 07-108) according to the manufacturer's instructions (Millipore/Abcam).

Mass spectrometry

Histone spots of interest were subjected to matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (Bruker Daltonics, Bremen, Germany; Ultraflex II) as mentioned else were.[8]

Cell lines and HDAC inhibitors

Rat liver cell lines CL44 (preneoplastic) and CL38 (neoplastic) were used in the study. The cell lines were cultured in DMEM (invitrogen) media with 10% FBS and were maintained at 37°C with 5% CO_2 and 100 mg/ml streptomycin. Histone deacetylase inhibitor (HDACi), Trichostatin A (TSA: Sigma, T8552) was dissolved in absolute ethanol to prepare stock solution and cells were treated with different concentrations of TSA.

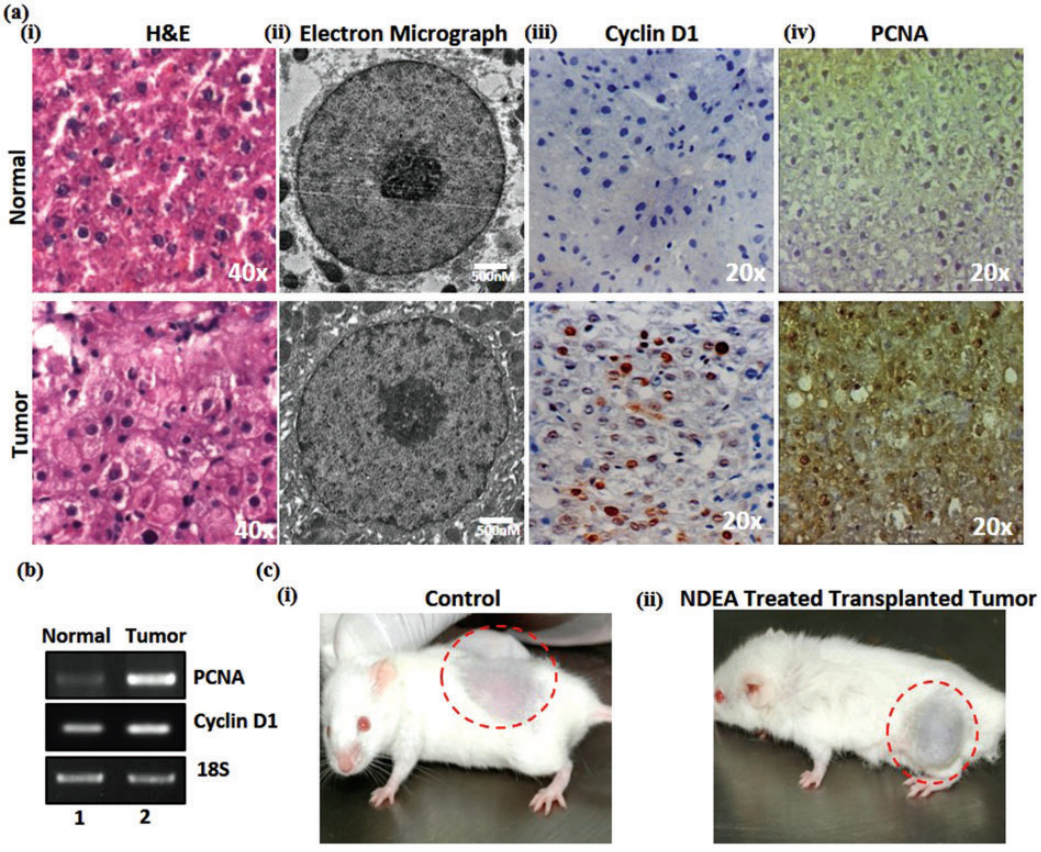

Figure 1 NDEA-mediated liver cell transformation. (a) (i) Comparative H&E, (ii) Electron micrographs, (iii) IHC of Cyclin D1 and (iv) PCNA of normal and four-month NDEA-treated liver tissues suggesting development of HCC. (b) (i) Semi-quantitative RT-PCR of Cyclin D1 and PCNA of the same tissues. (c) Implanting liver of (i) normal and (ii) NDEA-treated to NOD-SCID mice confirms cellular transformation in four months NDEA-treated rats.
NDEA: N-nitrosodiethylamine; H and E: hematoxylin and eosin; HCC: hepatocellular carcinoma; IHC: immuno-histochemistry. (A color version of this figure is available in the online journal.)

RT-PCR and real-time PCR

Total RNA (5 μg) prepared using TRIzol (invitrogen, 5596-026) was subjected to cDNA synthesis using random primers (Fermentas, K1632) as per the manufacturer's instructions. cDNA was subjected to PCR (NEB, M0271L) or real-time (SYBER green, Agilent Tech. 600882) analysis for the analysis of respective genes – **PCNA** (F: TCACAAAAG CCACTCCACTG, R: CATCTCAGAAGCGATCGTCA), **CyclinD1** (F:CGTGGCCACCTGGATGCTAGAG, R:TGCA GCAACTCCTCGGGGCGGAT), **Usp21** (F: ACTTCTCTCC GGCGTCTT, R:TGTGTGGTGAGCCATCTT), **RING1** (F: CCAAGCGGTCCCTACGGCC, R:CCTCGATACTGGAGC TCA), LCN2 (F:GGACCGAACGGTTCCAGG, R:CCCTGA CGAGGATGGAAG), **18SrRNA** (F:CGCGGTTCTATTTTGT TGGT, R:AGTCGGCATCGTTTATGGTC).

Cell proliferation assay

Proliferation was quantified by the ability of viable cells to reduce tetrazolium salt 3-(4,5-dimethylthiazol-2-y)-2, 5-diphenyl tetrasodium bromide (MTT) to a colored formazan product. Cell proliferation was then expressed as the percentage of absorbance (of formazan crystals) obtained in control cultures.

Results

Nitrosodiethylamine is a potent inducer of HCC in Sprague-Dawley rats

Nitrosodiethylamine (NDEA) administration for four months led to the development of HCC as confirmed by H&E staining, with cancer cells exhibiting evident atypia and large nuclei (Figure 1(a, i)). In addition, electron microscopy revealed several indentations giving rise to a more irregular shaped nucleus (Figure 1(a, ii)). This was further corroborated by high Cyclin D1 (Figure 1(a, iii)) and PCNA (Figure 1(a, iv)) expression in tumor sections by immunohistochemistry analysis and also their marked upregulation at the transcript level (Figure 1(b)). Also, 3 mm^2 liver tissue was excised from normal and NDEA administered animals (with tumor) and implanted into NOD-SCID-mice. Two weeks post-implantation tumors developed only in the mice with tumor tissue (Figure 1(c)). These results demonstrate the stable transformation of liver cells by administration of NDEA.

H2A ubiquitination markedly decreases in HCC

Histones were isolated from tissues and were resolved on AUT-PAGE (Figure 2(a)). A prominent decrease in Spot X

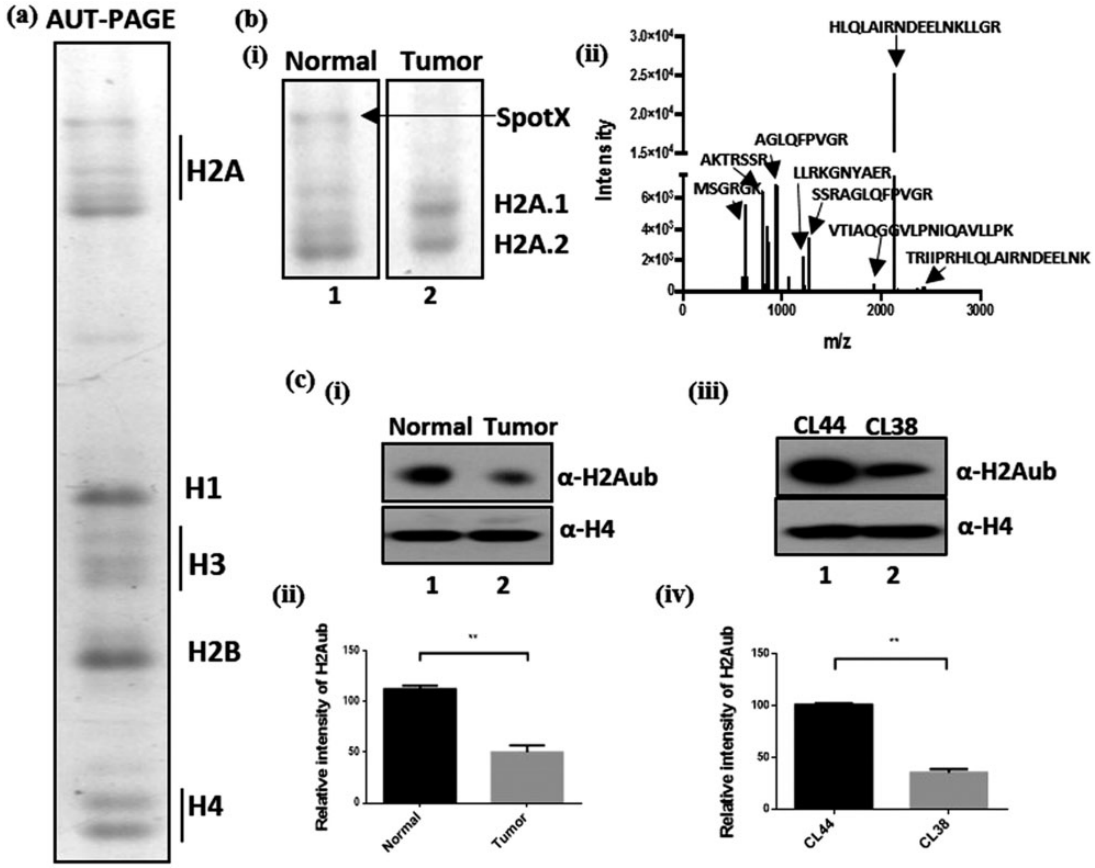

Figure 2 H2Aub levels in normal and tumor tissues. (a) Single dimensional AUT-PAGE profile of purified histones from normal tissues. Region marked spans the mobility of respective histones. (b) (i) Comparative AUT profile of normal and tumor tissues revealed differential levels of Spot X in enlarged H2A region. (ii) Mass spectrometry confirmed the identity of spot X to be as H2A. (c) Western blotting with antiH2Aub antibody revealed (i) decrease levels in HCC compared to normal, and also (iii) reduce levels of H2Aub in CL38 (Neoplastic) compared to CL44 (preneoplastic) cell lines. H4 was used as a loading control. (ii) & (iv) Densitometry analysis indicated decrease H2Aub in tumor and in CL38, respectively. Each histogram represents mean densitometry + SEM (*$p = 0.05$, **$p = 0.01$).
AUT-PAGE: acetic acid urea triton polyacrylamide gel electrophoresis.

was observed in tumor sample (Figure 2(b, i)). Increase in H2A.1 and decrease in H2A.2 were also observed in tumor which we have previously reported (Figure 2(b, i)).[8] MS–MS analysis of Spot X suggested that the band corresponds to H2A (Figure 2(b, ii)). Upshift of the band of H2A suggested the occurrence of mono-ubiquitination. This was further validated by subjecting the region of the AUT-PAGE to Western blotting with anti-H2Aub (Figure 2(c, i,ii)). We included liver cell lines derived from the same model system for our studies, CL44 (pre-neoplastic), and CL38 (neoplastic). Similar changes in H2Aub levels were observed on comparison of histones isolated from both the cell lines (Figure 2(b, iii,iv)). Our results suggest that overall there is decrease in the level of H2Aub in NDEA-induced liver cancer model.

Decrease in H2Aub is probably due to Usp21 and associates with upregulation of lipocalin 2

We looked for the levels of Usp21 as it was demonstrated to bring about H2A119 deubiquitination during liver regeneration.[10] Both in tumor tissue and neoplastic cell line CL38, a marked increase in the expression level of Usp21 was observed by quantitative (Figure 3(a, i)) and semi-quantitative PCR (Figure 3(a, ii)). We did not find any change in the expression level of the major H2A ubiquitinase RING1 (Figure 3(a, iii)), suggesting that the deubiquitination observed might be primarily owing to upregulation of the deubiquitinase.

H2Aub has been previously shown to localize to the promoter of genes like lipocalin 2 and repress their expression.[10] Hence, we checked the expression level of lipocalin 2 gene LCN2. A marked increase in the expression of LCN2 was observed in the CL38 cells and tumor tissue compared to their normal counterparts (by quantitative (Figure 3(b, i)) and semi-quantitative PCR (Figure 3(b, ii,iii)).

Increased cell proliferation may contribute to loss of H2Aub in tumor

Ubiquitination of H2A is dynamic, as suggested by the observations that global levels of H2Aub vary during the cell cycle.[6] Drop in the level of H2Aub has been shown to be a prerequisite for increase in the mitotic mark H3S10p during S to G2/M transition of cells.[6] Along with H3S10p increase, H3/H4 hypoacetylation is also seen during mitosis.[11] Indeed, when we looked into levels of H3S10p and H4ac in both tissues and cell lines (Figure 3(c, i,ii)), we observed a marked increase of H3S10p as opposed to decrease in H2Aub and H4ac. We further investigated the

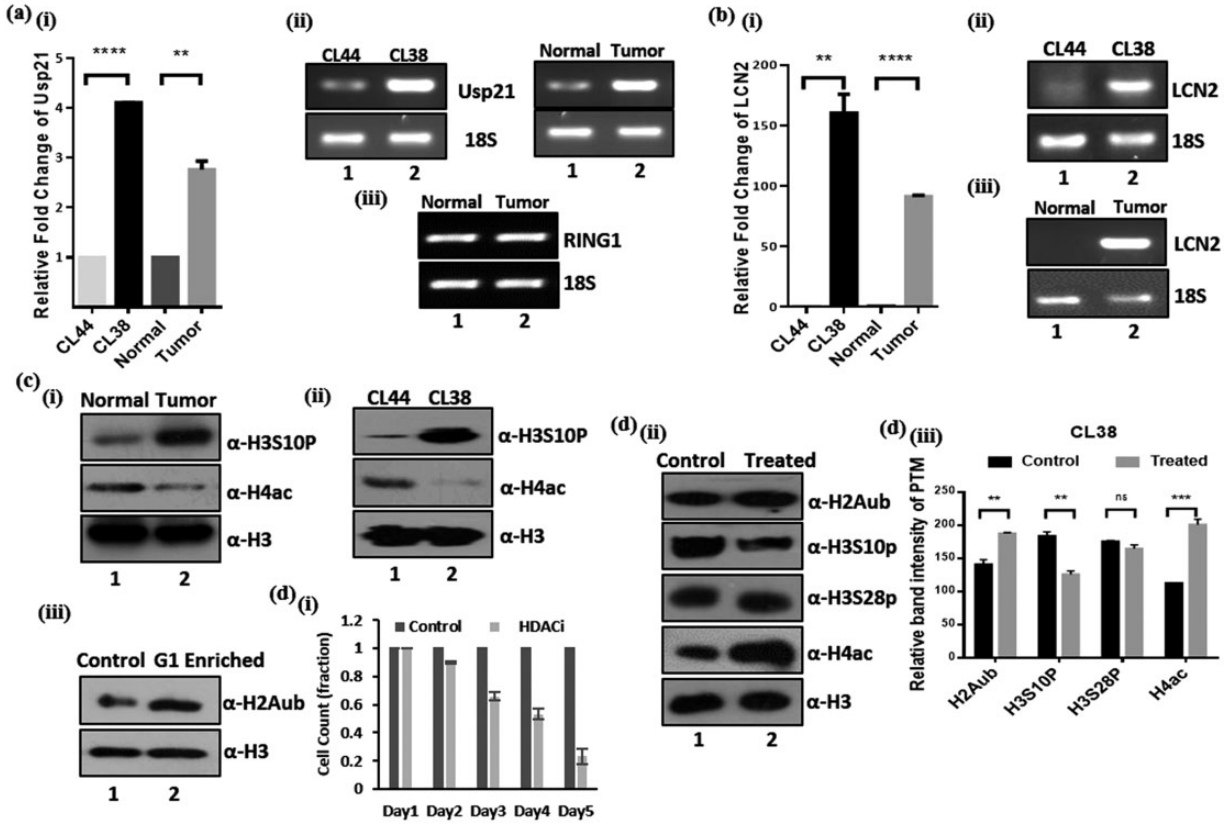

Figure 3 Decreased H2Aub associates with upregulation of lipocalin 2 expression. (a) Levels of Usp21, a H2A deubiquitinase enzyme in both cell lines (CL44 and CL38) and tissues (normal and HCC). (i) Real-time PCR and (ii) semi-RT-PCR. (iii) Semi-RT-PCR of H2A ubiquitinase, RING1 levels in tissues. 18 S rRNA was used as a loading control. (b) Level of lipocalin 2 (LCN2) in cell lines and tissues (i) Real-time PCR, (ii) semi-RT-PCR in both cell lines, and (iii) semi-RT-PCR in normal and HCC. (c) Relative protein levels of H3S10p and H4ac in (i) normal and HCC, (ii) CL38 and CL44 cell lines, and (iii) H2Aub in control and G1 enriched cells. (d) HDACi, TSA treatment of CL38 cell line (i) time-dependent decrease in cell proliferation as observed by MTT assay, (ii) histone PTM profile with indicated antibodies, and (iii) densitometry analysis of the western data.
HDACi: histone deacetylase inhibitor; TSA: trichostatin A.

possible relation between the levels of H2Aub and cell proliferation. In CL38, increased percentage of cells in S and G2/M population was observed as compared to CL44 (Figure S1(a, i,ii)). Consistent with this, H2Aub was low in CL38 cells and in tumor (Figure 2(c, i,ii)). Next, we enriched CL38 cells in G1-phase of the cell cycle by serum starvation (Figure S1(b)). An increase is H2Aub was found in G1-enriched cells (Figure 3(c, iii)) strengthening the inverse relationship between H2Aub and cell proliferation.

As we have seen decrease in H4ac in tumor, we wanted to investigate the effect of increase in H4ac on H3S10p and H2Aub. As histone deacetylase inhibitors (HDACi) are known to decrease cell proliferation,[12] we treated CL44 and CL38 cells with HDACi Trichostatin A. We observed decrease in proliferation of both CL44 (data not shown) and CL38 cells (Figure 3(d, i)) by MTT assay. Histones were isolated 48 h post drug-treatment and the levels of histone modifications were observed by immuno-blotting (Figure 3(d, ii)). The activity of the drug was confirmed by increase in the histone H4ac levels (Figure 3(d, ii,iii)). An increase in H2Aub was observed with a decrease in the level of H3S10p and H3S28p (another mitotic mark).

Discussion

Reduction in H2Aub has been reported in human pancreatic cancer[5] but otherwise this mark is poorly studied in cancers. In this study, we found marked decrease in H2Aub and increase in expression of gene lipocalin 2 in NDEA-HCC model. Interestingly, lipocalin 2 has been proposed to increase migration and metastasis of breast cancer cells.[13] We found the deubiquitinase Usp21 to be profoundly upregulated in HCC and no alteration in the level of ubiquitinase RING1. However, role of other ubiquitinases and deubiquitinases cannot be ignored. Along with H2Aub decrease, we have also observed a decrease in H4ac and increase in H3S10p. Our data thus suggest for the first time an inverse correlation between the global levels of cellular transformation mark H3S10p and H2Aub in vivo.

Upon treatment with HDACi, we found an increase in the level of H2Aub, H4ac, and drop in H3S10p. Previous studies have shown a decrease in H2Aub upon treatment with HDACi, valproic acid.[14,15] These differences in observation can be attributed to the use of different types of HDACi in the study. TSA is a class I and II specific inhibitor, without any specificity towards either of the enzymes.

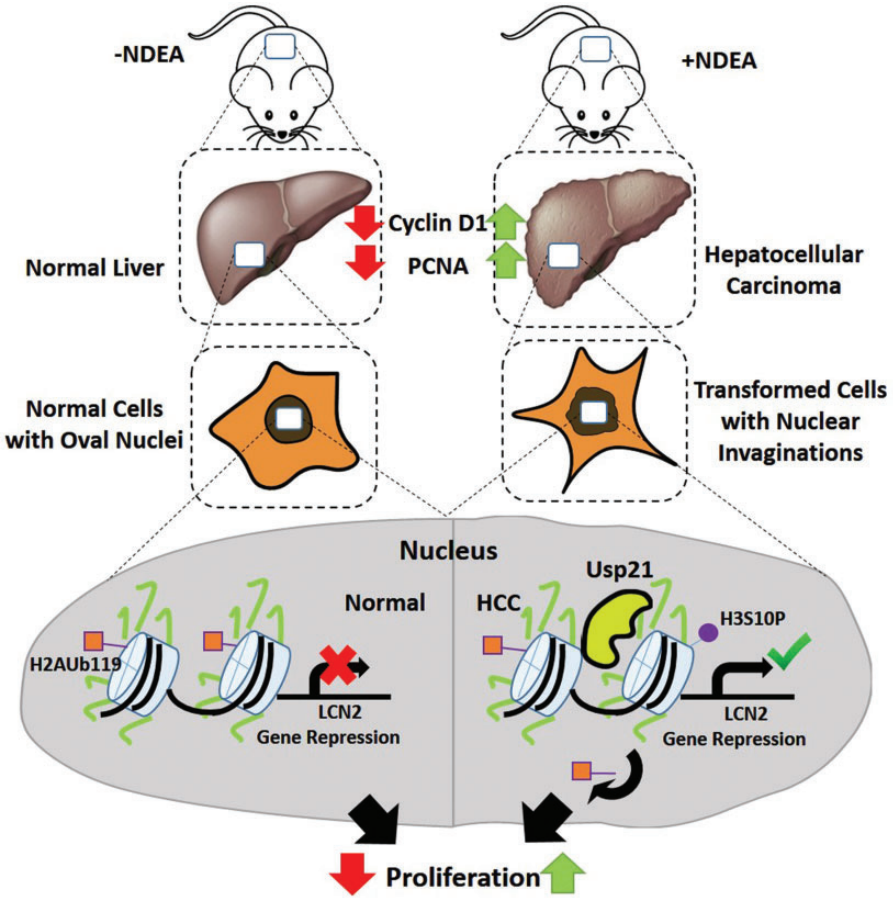

Figure 4 Proposed model for decrease in H2Aub and its association with other histone marks in HCC. NDEA treatment for four months led to stable cellular transformation with gross morphological changes in the nuclear shape compared to normal liver and increase in expression of cell proliferation markers (PCNA). At the molecular level, global decrease in H2Aub and increase in H3S10p levels was seen with simultaneous increase in lipocalin 2 (LCN2) expression, thus favoring cellular transformation of normal liver to HCC. (A color version of this figure is available in the online journal.)

On the other hand, VPA used in previous report has been shown to have selectivity towards HDAC2 amongst other HDACs.[16] Further, whether this increase in H2Aub seen upon TSA treatment is solely because of change in cell cycle profile of cells and H3S10p or whether there is involvement of cross-talk between the histone acetylation mark and H2Aub would be an interesting prospect to investigate.

In summary, we here show that NDEA administration leads to stable transformation of liver to HCC. This is associated with changes in shape and size of the nucleus along with increase in cell proliferation markers. At the molecular level, there is a marked decrease in the level of H2Aub in HCC. The loss of H2Aub also appears to be partly due to increase cellular proliferation and treatment with HDACi increases H2Aub in cells. Usp21 like in case of liver regeneration probably mediates this loss. This leads to upregulation of lipocalin 2, a growth factor whose role has been implicated in cancer progression (Figure 4). Future work should focus on understanding the functional implication of histone marks and whether the loss of H2Aub is a common hallmark of cancers.

Authors' contribution: SB majorly performed experiments. DR contributed in culture maintenance. AI carried out animal implantation. BK carried out histology and IHC experiments. SB and DR contributed in manuscript writing SG planned the experiments and wrote the manuscript.

ACKNOWLEDGEMENTS

The authors thank Dr. H. M. Rabes (University of Munich, Germany) for providing the CL44 and CL38 cell lines, ACTREC support for Gupta Lab and are grateful to all members of Gupta Lab, ACTREC for valuable discussion. S. B and D. R are supported by CSIR fellowship.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Bannister AJ, Kouzarides T. Regulation of chromatin by histone modifications. *Cell Res* 2011;**21**:381–95
2. Kalb R, Latwiel S, Baymaz HI, Jansen PWTC, Müller CW, Vermeulen M, Müller J. Histone H2A monoubiquitination promotes histone H3 methylation in polycomb repression. *Nat Struct Mol Biol* 2014;**21**:569–71
3. Bonner WM, West MH, Stedman JD. Two-dimensional gel analysis of histones in acid extracts of nuclei, cells, and tissues. *Eur J Biochem* 1980;**109**:17–23
4. Matsui S-I, Seon BK, Sandberg AA. Disappearance of a structural chromatin protein A24 in mitosis: implications for molecular basis of chromatin condensation. *Proceedings of the National Academy of Sciences* 1979;**76**:6386–6390
5. Glinskii AB, Yang Z, Li X, Hoffman RM. Essential role for activation of the polycomb group (PcG) protein chromatin silencing pathway in metastatic prostate cancer. *Cell Cycle* 2014;**5**:1886–1901
6. Joo H-Y, Zhai L, Yang C, Nie S, Erdjument-Bromage H, Tempst P, Chang C, Wang H. Regulation of cell cycle progression and gene expression by H2A deubiquitination. *Nature* 2007;**449**:1068–72
7. Choi HS, Choi BY, Cho YY, Mizuno H, Kang BS, Bode AM, Dong Z. Phosphorylation of histone H3 at serine 10 is indispensable for neoplastic cell transformation. *Cancer Res* 2005;**65**:5818–5827
8. Khare SP, Sharma A, Deodhar KK, Gupta S. Overexpression of histone variant H2A.1 and cellular transformation are related in N-nitrosodiethylamine-induced sequential hepatocarcinogenesis. *Exp Biol Med (Maywood)* 2011;**236**:30–5
9. Bonenfant D. Characterization of histone H2A and H2B variants and their post-translational modifications by mass spectrometry. *Mol Cell Proteomics* 2005;**5**:541–52
10. Nakagawa T, Kajitani T, Togo S, Masuko N, Ohdan H, Hishikawa Y, Koji T, Matsuyama T, Ikura T, Muramatsu M, Ito T. Deubiquitylation of histone H2A activates transcriptional initiation via trans-histone cross-talk with H3K4 di- and trimethylation. *Genes Dev* 2008;**22**:37–49
11. Li Y, Kao GD, Garcia BA, Shabanowitz J, Hunt DF, Qin J, Phelan C, Lazar MA. A novel histone deacetylase pathway regulates mitosis by modulating aurora B kinase activity. *Genes Dev* 2006;**20**:2566–79
12. Sakajiri S, Kumagai T, Kawamata N, Saitoh T, Said JW, Koeffler HP. Histone deacetylase inhibitors profoundly decrease proliferation of human lymphoid cancer cell lines. *Exp Hematol* 2005;**33**:53–61
13. Yang J, Bielenberg DR, Rodig SJ, Doiron R, Clifton MC, Kung AL, Strong RK, Zurakowski D, Moses MA. Lipocalin 2 promotes breast cancer progression. *Proc Natl Acad Sci U S A* 2009;**106**:3913–8
14. Krämer OH, Zhu P, Ostendorff HP, Golebiewski M, Tiefenbach J, Peters MA, Brill B, Groner B, Bach I, Heinzel T, Göttlicher M. The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2. *EMBO J* 2003;**22**:3411–20
15. Bommi PV, Dimri M, Sahasrabuddhe AA, Khandekar J, Dimri GP. The polycomb group protein BMI1 is a transcriptional target of HDAC inhibitors. *Cell Cycle* 2010;**9**:2663–73
16. Göttlicher M, Minucci S, Zhu P, Krämer OH, Schimpf A, Giavara S, Sleeman JP, Lo Coco F, Nervi C, Pelicci PG, Heinzel T. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J* 2002;**20**:6969–78

(Received February 3, 2016, Accepted April 19, 2016)