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Interplay of YEATS2 and GCDH mediates histone crotonylation and drives EMT in head and neck cancer

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eLife Assessment

These **useful** findings assigned a novel functional implication of histone acylation, crotonylation. Although the mechanistic insights have been provided in great detail regarding the role of the YEATS2-GCDH axis in modulating EMT in HNC, the strength of evidence for the manuscript is **incomplete**. The patient cohort is very small, with just 10 patients; to establish a significant result the cohort size should be increased. Furthermore, the functional implication of p300 is also to be looked into.

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Abstract

The regulation of gene expression is an integral cellular process orchestrated by epigenetic marks like histone modifications. Perturbations in the activity or abundance of epigenetic factors can lead to tumorigenesis. Remarkably, several metabolites influence the epigenetic landscape in cells. Here, we investigated the interplay between a highly expressed epigenetic factor, YEATS2, and a metabolic enzyme, GCDH, in regulating epithelial-to-mesenchymal transition in head and neck cancer. We report that the histone reader YEATS2 is responsible for increased invasive potential in head and neck cancer in an SP1-dependent manner. YEATS2 functions by maintaining histone crotonylation, and its abrogation leads to a global decrease in the H3K27Cr mark. Mechanistically, we report that YEATS2 maintains high H3K27Cr levels at the promoter of the EMT-promoting gene *SPARC*. Further, we found that the addition of the H3K27Cr mark is also dependent on the crotonyl-CoA-producing enzyme GCDH. Overall, we describe a novel mechanism of interplay between epigenetics and metabolism in head and neck tumorigenesis, which results in the enhanced expression of EMT-related genes in a histone crotonylation-dependent manner.

Introduction

Head and neck cancer is an umbrella term associated with a number of cancers of the oral cavity and pharynx. Like other solid tumors of epithelial origin, head and neck cancer (HNC) involves a multi-step progression towards malignancy, of which transition from an epithelial to a mesenchymal phenotype is a pivotal event [1]. This epithelial-to-mesenchymal transition (EMT) involves dramatic changes like loss of cell polarity and acquisition of migratory and invasive properties in the tumor cells [2]. Underlying these remarkable external changes in tumor cells, are equally notable changes in gene expression and signalling pathways. Moreover, widespread alteration in the gene expression program of cancer cells undergoing EMT can be a result of either altered activity or the change in abundance of several epigenetic factors. Tumor-specific epigenomic changes thus allow HNC cells to tweak the expression levels of multiple genes at once, thereby leading to a drastic transformation in phenotype during metastasis. These large-scale changes are due to the rapid and global nature of epigenetic alterations [3].

Similar to epigenetic changes, metabolic rewiring is another important hallmark of cancer cells capable of carrying out vast tumor-promoting changes rapidly. Numerous reports in the past have documented instances of loss-of-function mutations or changes in the gene expression of enzymes involved in key metabolic processes such as glycolysis or citric acid cycle to promote oncogenesis [4]. However, the discovery of non-canonical functions of several enzymes and their atypical localization in the nucleus has intrigued cancer researchers of late. Metabolic enzymes are now believed to play a more direct role in influencing oncogene expression via crosstalk with the epigenome. One such phenomenon is the role of metabolites produced as a result of aberrant metabolism in acting as substrates for epigenetic factors. A ground breaking study in this regard by Wellen et al. reported the nuclear localization of a mitochondria-resident protein ACLY (adenosine triphosphate–citrate lyase). Nuclear pool of ACLY was found to affect histone acetylation directly by producing acetyl-coenzyme A (CoA) in colon carcinoma cells [5]. Moreover, another study reported a direct interaction involving α-ketoglutarate dehydrogenase (α-KGDH) and a histone acetyltransferase leading to the production of localized succinyl-CoA in nucleus, which in turn led to histone succinylation and enhanced expression of genes related to tumor cell proliferation [6]. M2 isoform of pyruvate kinase (PKM2) is another metabolic enzyme known to exhibit a non-canonical function by directly affecting gene transcription. We have recently reported the role of nuclear PKM2-HIF1α -p300 axis in maintaining high levels of H3K9Ac marks at the promoter of *PFKFB3*, which thereby leads to its increased expression in hypoxic breast cancer cells [7]. The reports stated herein, along with others [8], delineate the role of the nexus between aberrant metabolism and the epigenome in regulating various aspects of tumorigenesis in a wide variety of cancers. However, the significance of this partnership in regulating EMT, which is one of the most vital events in cancer, remains largely unexplored.

In this study, we have made an attempt to dissect the role of previously unknown link between epigenetic factors and metabolism in promoting EMT in head and neck cancer. To explore tumor-promoting epigenetic factors in HNC we analyzed large gene expression dataset and narrowed down our search to YEATS2. This gene was found to be overexpressed in HNC, had significant correlation with EMT, and also showed association with poor patient prognosis. YEATS2 (YEATS-domain containing protein 2) is a histone reader protein capable of recognizing epigenetic marks such as histone acetylation and crotonylation [9]. Through RNA-seq, it was revealed that silencing of YEATS2 led to a decrease in the expression of multiple EMT-associated genes. Further, the transcription factor SP1 was found to be responsible for YEATS2 expression in HNC. While exploring the relationship of YEATS2 with cancer cell metabolism, we found that YEATS2 is co-expressed with crotonyl-CoA producing GCDH (glutaryl-CoA dehydrogenase) in HNC, and the downregulation of either of these two genes led to a decrease in H3K27Cr levels in HNC cells. The H3K27Cr mark has been previously reported to activate the transcription of pro-tumorigenic *ETS1*

in colorectal cancer [10]. Corroborating with the previous finding, high H3K27Cr levels at the promoter region was associated with an increase in the expression of EMT-related *SPARC* gene. Subsequent investigation revealed that YEATS2 and GCDH worked synergistically in maintaining higher levels of H3K27Cr at the promoter of *SPARC*, thereby causing an increase in its expression. Finally, H3K27Cr ChIP-seq in YEATS2-abrogated cells uncovered the role of YEATS2 in maintaining global histone crotonylation on the promoter of a large number of genes, many of those genes being associated with EMT. Altogether, we have elucidated a novel link that unifies epigenetic regulation and metabolic rewiring in contributing toward the enhancement of EMT in head and neck cancer.

Results

YEATS2 is overexpressed in head and neck cancer

In order to study cancer-associated epigenetic factors, we analysed publicly available The Cancer Genome Atlas (TCGA) mRNA expression dataset. The analysis aimed at obtaining genes coding for epigenetic factors that are overexpressed in HNC, and are also associated significantly to overall patient survival (**Figure 1A**). The analysis resulted in a list of 10 epigenetic factors that are upregulated in HNC and whose expression levels are associated with poor overall patient survival (**Figure 1B**, **Table 1**). Out of these 10 epigenetic factors, *YEATS2*, *RUVBL1* and, *MRGBP* were not explored rigorously in HNC prior to our study. The expression of these three epigenetic factors was thus evaluated at mRNA level using reverse-transcription quantitative real-time PCR (RT-qPCR) in pairs of 8 head and neck normal and cancer tissues collected as a part of this study. We found that *YEATS2*, that encodes for a histone reader protein, was the only gene that was significantly upregulated in cancer as compared to normal tissues (**Figure 1C-D**, **Figure 1—figure supplement 1A-B**). *YEATS2* was also found to be upregulated in publicly available HNC microarray datasets GSE30784 and GSE9844 (**Figure 1E-F**). The association of YEATS2 with EMT was then established by significant positive correlation seen between its expression and the expression of genes included in an EMT gene signature in TCGA data (**Figure 1— figure supplement 1C**). On further investigation using immunoblotting, we found that the expression of YEATS2 protein was increased in a set of head and neck cancer vs. matched normal tissues (**Figure 1G**, **Figure 1—figure supplement 1D**).

In order to study the role of YEATS2 in the progression of HNC, we performed RNA-seq in YEATS2-silenced BICR10 cells. The shRNA-mediated silencing of YEATS2 led to the downregulation of 2,494 genes, whereas 2,073 genes were found to be upregulated (**Figure 1H**, **Supplementary file 1**). Overrepresentation analysis revealed that hallmark genesets from MSigDb (Molecular signature database) like OXIDATIVE PHOSPHORYLATION and EPITHELIAL TO MESENCHYMAL TRANSITION were some of the top pathways enriched among the downregulated genes (**Figure 1I**). On the other hand, pathways like TNFA SIGNALING VIA NFKB and G2M CHECKPOINT were enriched among the upregulated genes (**Figure 1— figure supplement 1E**). These set of results hint towards the role of YEATS2 in being one of the essential epigenetic factors responsible for HNC tumorigenesis, mediated via global control of EMT genes' expression.

YEATS2 is associated with increased invasion in head and neck cancer cells

Since YEATS2 was found to influence the expression of EMT-related genes globally in our RNA-seq data, we checked the expression of EMT markers in shControl vs. sh*YEATS2* BICR10 and SCC9 cells and it was found that Twist1, N-cadherin and, Vimentin showed a decrease (**Figure 2A**, **Figure 2—figure supplement 1A**). Also, the number of invading cells significantly reduced in invasion assay on YEATS2 downregulation, indicating that YEATS2 could be playing a role in maintenance of EMT phenotype in HNC cells (**Figure 2B**, **Figure 2—figure supplement 1B**). Conversely, the

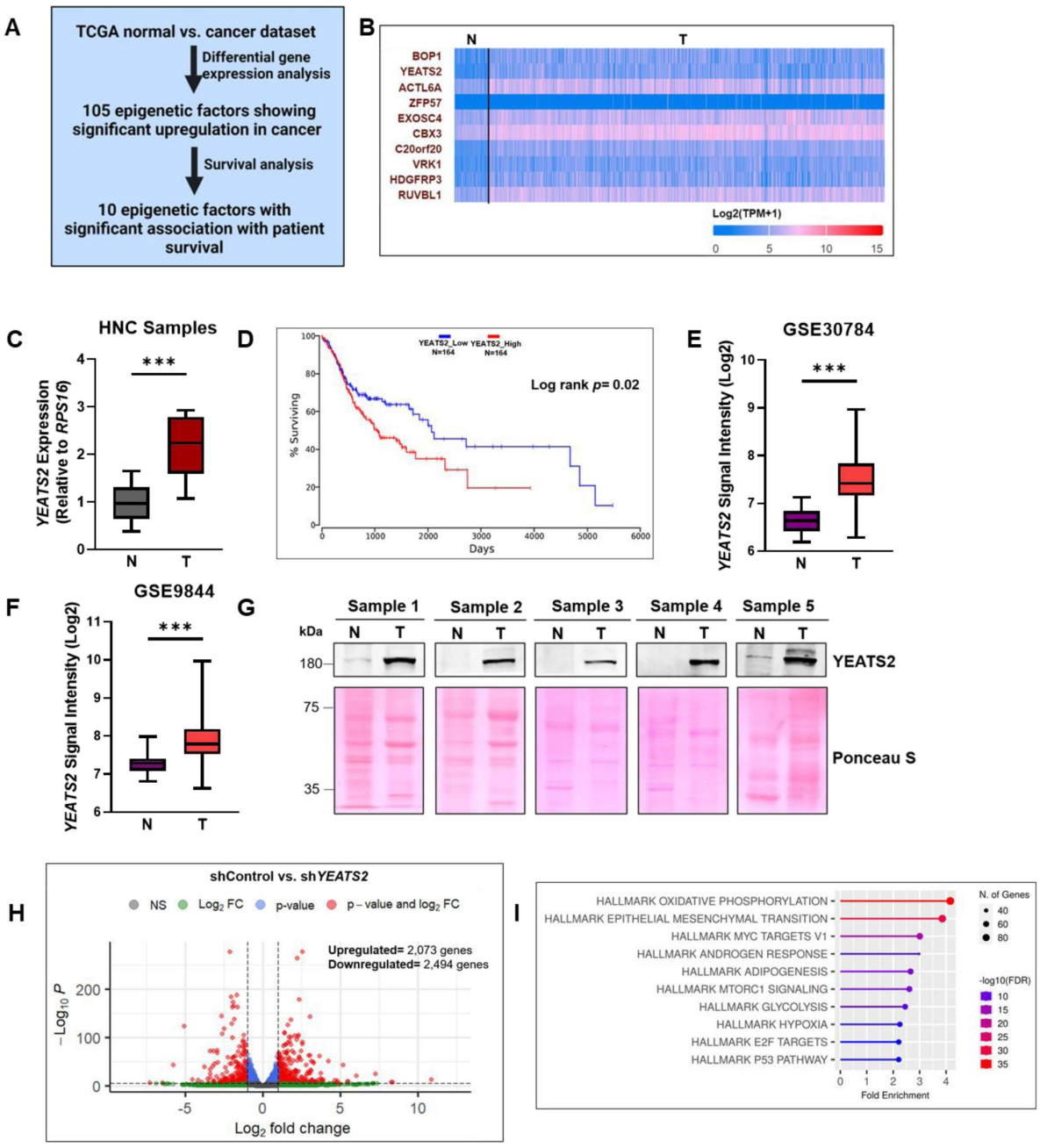


Figure 1.

YEATS2 is upregulated in head and neck cancer.

(A) Computational strategy used to find epigenetic factors with poor patient prognosis overexpressed in HNC. **(B)** Heatmap depicting the TCGA mRNA expression of top 10 epigenetic factors shortlisted using strategy mentioned in (A). **(C)** RT-qPCR result showing mRNA expression of *YEATS2* in HNC samples (n= 8). **(D)** Kaplan-Meier curve of *YEATS2* showing association with overall head and neck cancer patient survival. **(E-F)** Gene expression profile of *YEATS2* in publicly available HNC microarray datasets, GSE30784 (E) and GSE9844 (F). **(G)** Immunoblot showing protein levels of YEATS2 in HNC samples. **(H)** Volcano plot of differentially expressed genes in RNA-seq analysis of shControl vs. sh*YEATS2* in BICR10 cells. **(I)** Results of overrepresentation analysis of genes significantly downregulated in shControl vs. sh*YEATS2* RNA-seq data. Error bars, min to max; two-tailed t test, ∗∗∗$p < 0.001$.

Gene	logFC	FDR	Tumor	Normal	Delta
BOP1	1.182631	5.56E-14	1846.239	864.7273	2183.419
YEATS2	1.633844	1.72E-31	4568.299	1601.364	7463.89
ACTL6A	1.46826	1.29E-23	4672.807	1848.864	6860.898
ZFP57	1.389347	0.001025	45.83591	19.11364	63.68196
EXOSC4	1.012761	4.50E-11	1902.724	1026.273	1927.005
CBX3	1.206759	2.17E-31	8511.786	3997.455	10271.67
C20orf20/ MRGBP	1.560447	2.50E-43	1660.077	610.0455	2590.463
VRK1	1.132196	2.07E-19	1109.871	565.4318	1256.592
HDGFRP3	1.058652	5.38E-12	1729.21	879.2727	1830.632
RUVBL1	1.14195	1.71E-24	4335.722	2125.955	4951.178

Table 1.

Results for differential gene expression analysis for 10 shortlisted epigenetic factors.

expression of Twist1 was increased and there was significant upregulation in invasive capacity of cells when BICR10 and SCC9 cells were transfected with YEATS2-overexpression construct (**Figure 2C**, **Figure 2—figure supplement 1C-D**). Wound healing assay was performed to assess the cell migration property of HNC cells and it was found that YEATS2-silenced cells migrated slower than control cells (**Figure 2D**, **Figure 2—figure supplement 1E**). On the other hand, overexpressing YEATS2 in BICR10 and SCC9 cells resulted in faster wound healing as compared to control cells (**Figure 2E**, **Figure 2—figure supplement 1F**). A 3D invasion assay was also performed to check the ability of tumor spheres to invade a collagen matrix in YEATS2 knockdown and overexpressed conditions. It was observed that cells a reduced number of cells invaded the collagen matrix when YEATS2 was downregulated. The opposite pattern was observed in both the cell lines when YEATS2 was overexpressed (**Figure 2F-G**, **Figure 2—figure supplement 1G-H**). All of the above observations led us to the conclusion that YEATS2 is one of the important epigenetic factors responsible for conferring head and neck cancer cells with invasive properties.

SP1 regulates the expression of YEATS2 in head and neck cancer

In order to investigate the molecular mechanism behind the upregulated expression of YEATS2 in HNC, YEATS2-promoter fragments of variable lengths were cloned upstream of Firefly luciferase reporter gene in the pGL3-Basic vector plasmid. Luciferase assay was performed in two different HNC cell lines, BICR10 and SCC9. A significant decrease in the luciferase activity was observed for shorter promoter-deletion construct (Luc-311) compared to the full-length construct (Luc-508) (**Figure 3A**, **Figure 3—figure supplement 1A**). Transcription factor binding-site analysis was then performed with the sequence unique to Luc-508, the region potentially responsible for transcriptional regulation of YEATS2. This analysis revealed the presence of multiple consensus binding sites of SP1 and KLF5 transcription factors. Expression profiles of these transcription factors in the TCGA mRNA expression dataset for HNC showed that unlike KLF5, SP1 was significantly upregulated in tumor vs. normal patient data (**Figure 3—figure supplement 1B-C**). Furthermore, shRNA-mediated silencing of SP1 led to decreased expression of YEATS2 in the HNC cell lines (**Figure 3B-C**, **Figure 3—figure supplement 1D-E**). Further, on performing SP1-ChIP assay, we found significant enrichment of SP1 on *YEATS2* promoter when compared with control IP in both the cell lines (**Figure 3D**, **Figure 3—figure supplement 1F**). Next, we also performed luciferase assay using Luc-508 construct in SP1-knockdown condition, and found a decreased luciferase activity as compared to control (**Figure 3E**). To further validate the direct involvement of SP1 in the transcriptional regulation of YEATS2, 3 of the 4 SP1 consensus binding sites in the full-length YEATS2 Luc-508 were mutated using site-directed mutagenesis. As expected, after performing luciferase assay, nearly 50% decrease in luciferase activity was observed for the mutant promoter construct, compared to its wild-type counterpart (**Figure 3F**, **Figure 3—figure supplement 1G**). We then looked into the status of invasion in sh*SP1* cells and found that the EMT-marker Twist1 was reduced in these cells. However, the ectopic expression of YEATS2 was able to rescue Twist1 expression partially (**Figure 3G**). We then performed invasion assay to check the effect of YEATS2 overexpression in the background of SP1-knockdown, and found that there was a decrease in invasion in sh*SP1* cells, which was rescued by overexpression of YEATS2 (**Figure 3H**, **Figure 3—figure supplement 1H**). These results indicate that SP1 is directly responsible for YEATS2 upregulation in HNC.

YEATS2 and GCDH regulate histone crotonylation in HNC

After establishing the role of YEATS2 as one of the important epigenetic factors in maintaining EMT-phenotype in HNC cells, we wanted to delve into the mechanism of YEATS2-mediated regulation of gene expression. Being a histone reader with a varying magnitude of affinity for multiple histone modifications, the function of YEATS2 could be potentially influenced by the metabolic state of the cell since metabolic intermediates can act as substrates for histone modifying proteins. Therefore, in order to explore the influence of cellular metabolism on the dynamics of histone modifications maintained by YEATS2, we wanted to look into the metabolic

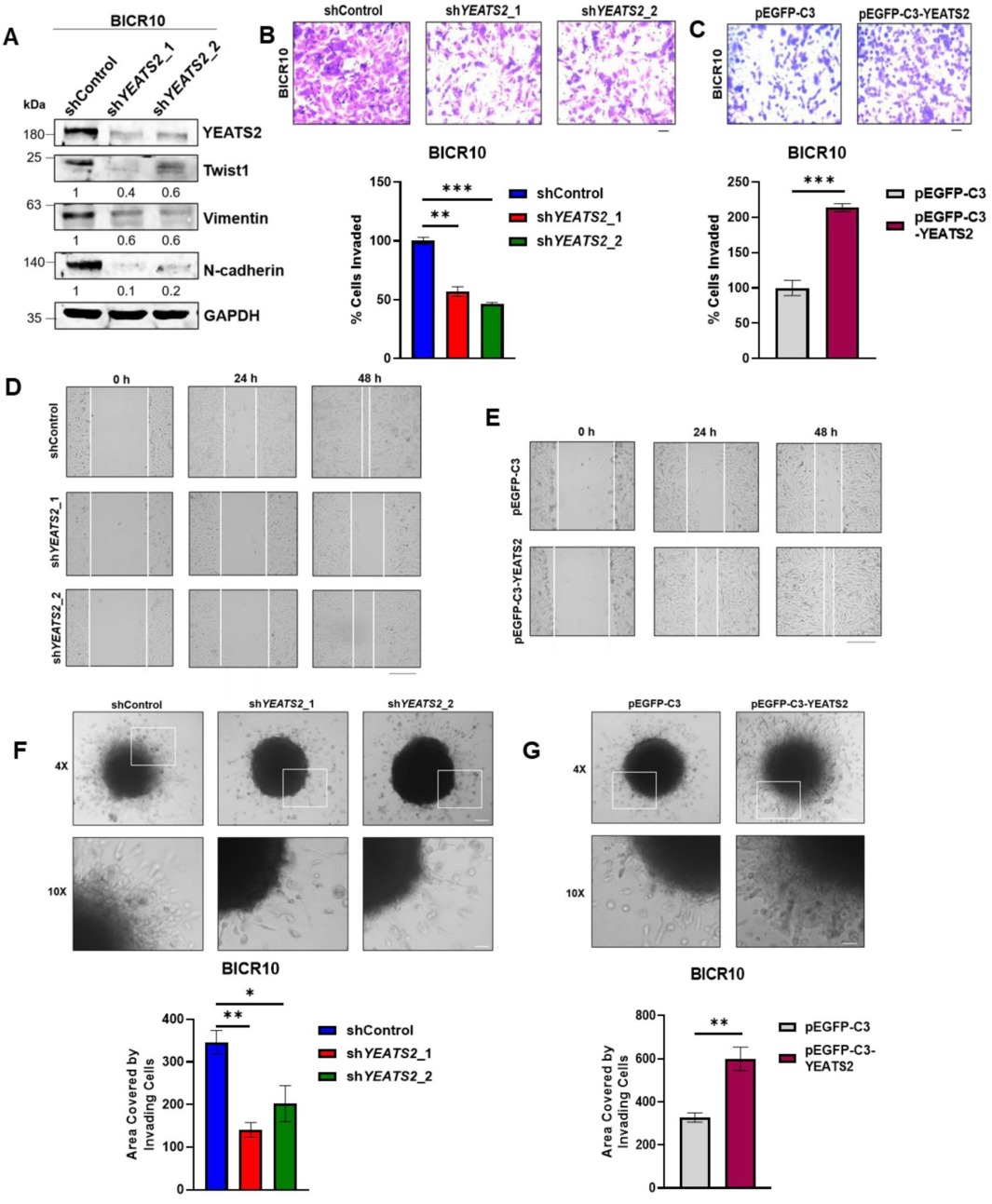


Figure 2.

YEATS2 drives EMT in head and neck cancer cells.

(A) Immunoblot showing the expression levels of various EMT factors upon YEATS2 knockdown in BICR10. **(B-C)** Results of invasion assay with quantification (below), after knockdown (B) or overexpression (C) of YEATS2 in BICR10 (Scale bar, 200 μm). **(D-E)** Wound healing assay performed after knockdown (D) or overexpression (E) of YEATS2 in BICR10 (Scale bar, 275 μm). **(F-G)** Results of 3D invasion assay showing change in invasive potential of BICR10 cells in collagen matrix after silencing (F) or overexpression (G) of YEATS2 (quantification shown below). (Scale bar: 4X, 200 μm; 10X, 50 μm) Error bars, mean ± SEM; two-tailed t test, $*p < 0.05$, $**p < 0.01$, n= 3 biological replicates.

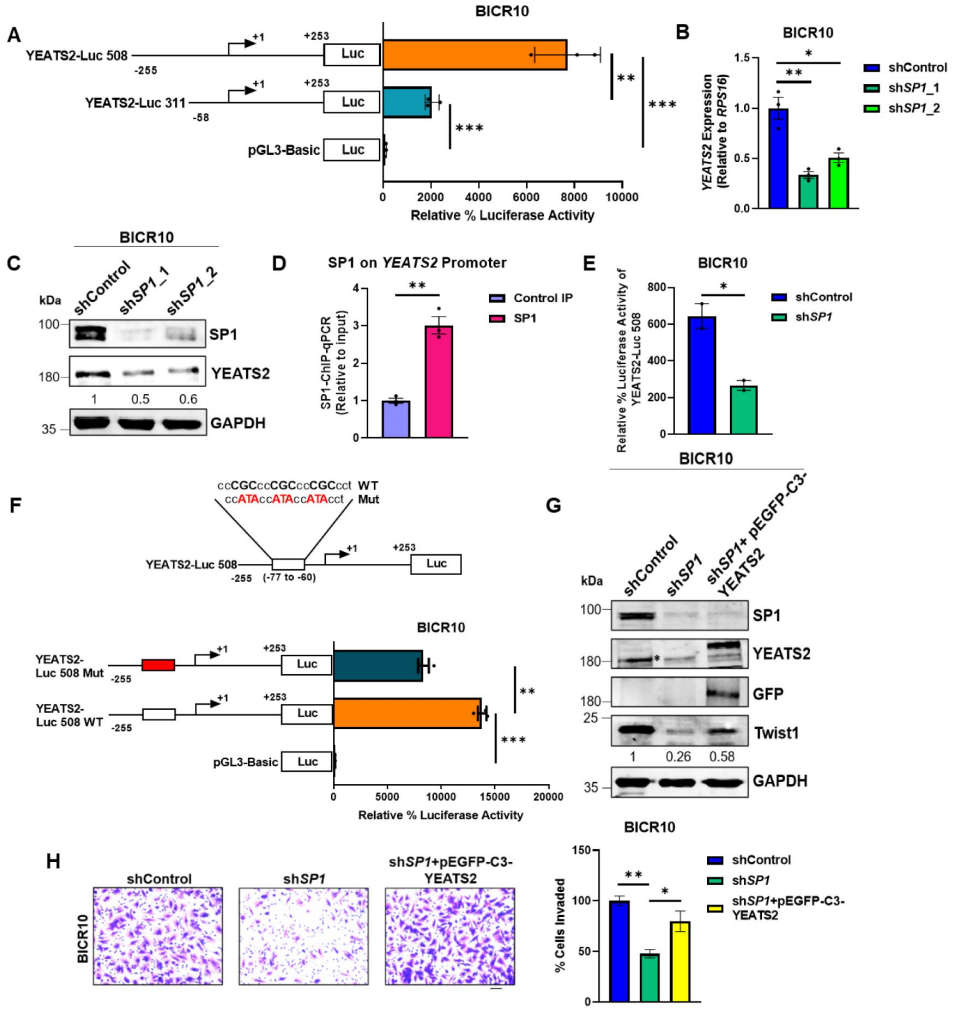


Figure 3.

Regulation of EMT by YEATS2 is SP1-dependent in HNC.

(A) Luciferase assay results showing the difference in relative luciferase activity of the two YEATS2 promoter deletion constructs in BICR10. **(B)** Plot showing decrease in mRNA expression of *YEATS2* on SP1-knockdown in BICR10 cells. **(C)** Immunoblot showing the reduced expression level of YEATS2 upon SP1-knockdown in BICR10. **(D)** Plot depicting SP1 binding on *YEATS2* promoter in SP1-ChIP assay in BICR10. **(E)** Plot showing difference in relative luciferase activity of YEATS2 Luc-508 in shControl vs. sh*SP1* BICR10 cells. **(F)** Schematic showing mutation of SP1-binding site sequence in YEATS2 Luc-508 construct (above), and relative luciferase activity of wild-type (WT) vs. mutant (Mut) YEATS2 Luc-508 in BICR10 (below). **(G)** Immunoblot depicting the decreased Twist1 levels on SP1 knockdown and its subsequent rescue of expression upon YEATS2 overexpression in BICR10 (* indicates YEATS2 band). **(H)** Invasion assay images (with quantification on right) showing decrease and rescue of the percentage of invaded cells in sh*SP1* BICR10 cells, and sh*SP1* cells with YEATS2 overexpression, respectively. Scale bar, 200 µm. Error bars, mean ± SEM; two-tailed t test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, n = 3 biological replicates.

enzymes that are co-expressed with YEATS2 in HNC. For this, we performed gene set enrichment analysis (GSEA) with a comprehensive list of essential metabolic pathways [11] and found out that KEGG_LYSINE_DEGRADATION was the top-most pathway associated with high *YEATS2* expression in TCGA samples (**Figure 4A**). The saccharopine pathway (canonical pathway for lysine degradation) results in conversion of lysine to 2 molecules of acetyl-CoA, with several intermediates including crotonyl-CoA (**Figure 4B**) [12]. Since it is known that YEATS2 has a higher affinity for binding crotonyl groups on histone (specifically on H3K27) than other histone substrates [9], we hypothesized that the crotonyl-CoA producing enzyme and part of lysine degradation pathway, glutaryl CoA dehydrogenase (GCDH) would have increased expression in HNC. We found that *GCDH* was upregulated and had an inverse pattern of expression with the downstream enzyme *ECHS1* in TCGA cancer vs. normal tissue dataset (Figure 4—figure supplement 1A-B). Further, we found that the mRNA expression of *GCDH* was significantly upregulated in head and neck cancer samples relative to their matched normal tissues (**Figure 4C**). Moreover, correlation analysis performed between the expression levels of GCDH and YEATS2 in TCGA showed significant Spearman correlation coefficient value of 0.32 (**Figure 4—figure supplement 1C**). We then proceeded to check the levels of histone crotonylation (H3K27Cr) in a set of protein samples obtained from HNC tissues and found that the abundance of this histone mark was higher in tumor as compared to normal (**Figure 4D**, **Figure 4—figure supplement 1D**). Since mitochondrial fraction of GCDH cannot influence histone crotonylation directly, we checked its subcellular localization in HNC tissues. Using immunohistochemistry, we detected the presence of nuclear GCDH in HNC tissues and observed that the regions that stained strongly for nuclear GCDH, also showed abundance in H3K27Cr (**Figure 4E**, **Figure 4—figure supplement 1E**). Conversely, the weakly stained regions for nuclear GCDH were less abundant in H3K27Cr levels. This established an association between the presence of H3K27Cr marks and the expression of GCDH in HNC. Further, on checking the levels of H3K27Cr on YEATS2- and GCDH-knockdown using immunoblotting and immunofluorescence, we found that the cells silenced for either of the two genes caused a decrease in H3K27Cr levels (**Figure 4F-I**, **Figure 4—figure supplement 1F-G**). On the other hand, an increase in H3K27Cr signals was observed when YEATS2 was overexpressed ectopically in BICR10 cells (**Figure 4—figure supplement 1H**). These results hint toward histone crotonylation in HNC cells being jointly regulated by the expression of both the epigenetic factor YEATS2, and the metabolic enzyme GCDH.

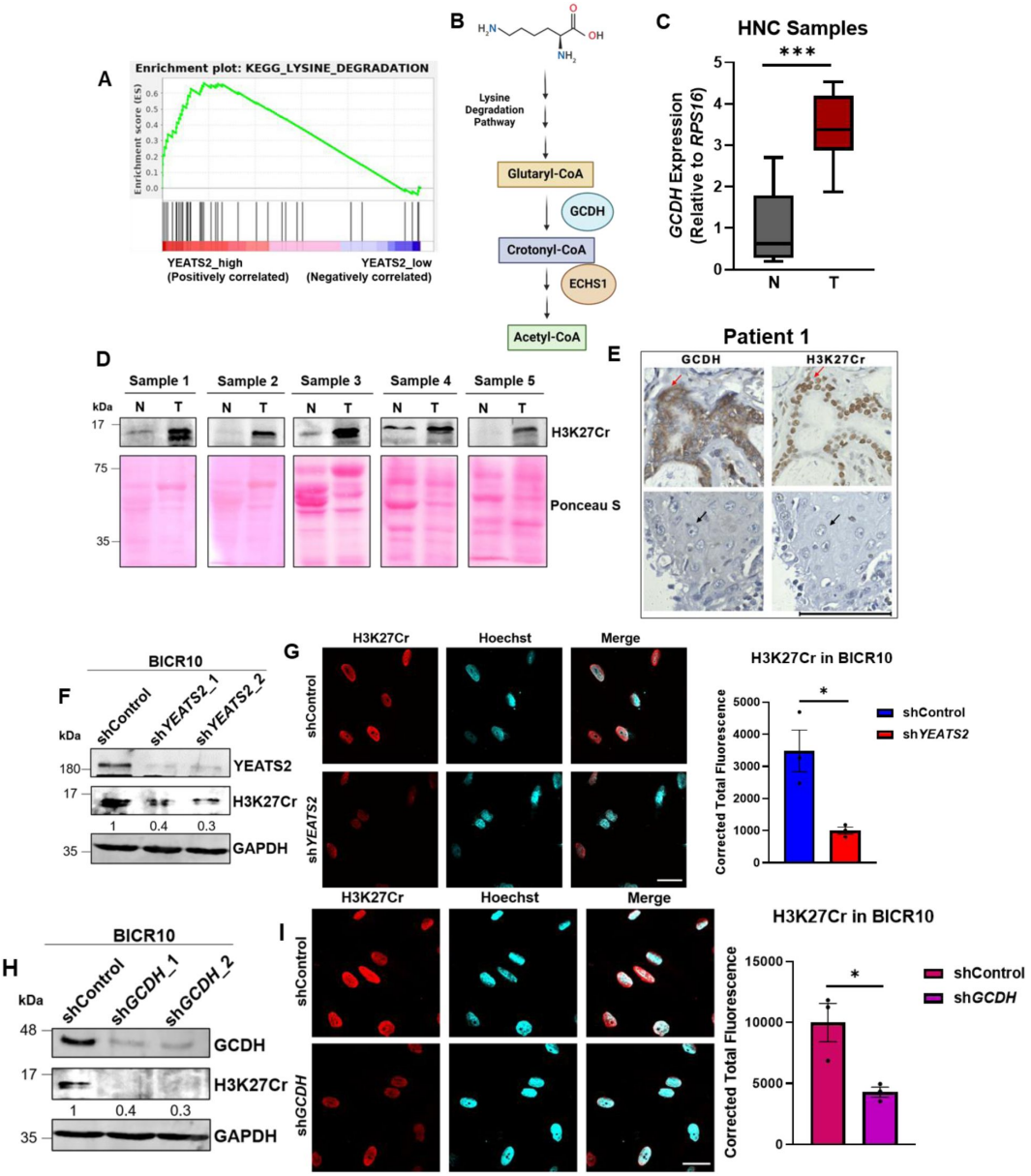
YEATS2 regulates expression of EMT-associated gene *SPARC*

In order to check the effect of YEATS2 on global gene expression, we performed YEATS2 ChIP-seq to obtain genome-wide binding sites of this protein (**Figure 5— figure supplement 1A, Supplementary file 2**). We then performed integration of those genes having YEATS2 ChIP-seq peaks on their promoters with the genes that were downregulated in sh*YEATS2* vs. shControl RNA-seq data, and the genes included in an EMT gene signature. We found that *SPARC* was the common gene present in all 3 sets (**Figure 5A-B**, **Figure 5—figure supplement 1B**). The mRNA expression level of *SPARC* in TCGA dataset was also found to be higher in tumor as compared to normal (**Figure 5—figure supplement 1C**). Therefore, we chose to investigate *SPARC* as a direct gene target of YEATS2 in mediating EMT in HNC. Secreted protein acidic and rich in cysteine (SPARC) is a secreted glycoprotein known to be a part of extracellular matrix (ECM). It interacts with various components of ECM and is known to play a role in processes like tissue remodeling and angiogenesis [13,14]. By performing YEATS2 ChIP-qPCR in YEATS2-downregulated cells, we found that the binding of YEATS2 on *SPARC* promoter decreased on its knockdown in BICR10 as well as SCC9 (**Figure 5C**). We then checked the expression of SPARC at the RNA level on YEATS2-downregulation. SPARC expression was decreased in YEATS2-knockdown conditions in both the cell lines (**Figure 5D**, **Figure 5—figure supplement 1E**). Since SPARC is a secretory protein, we investigated its level in conditioned media obtained from shControl and sh*YEATS2* BICR10 cells. We found a decrease in the secreted pool of SPARC on YEATS2-knockdown (**Figure 5E**). We also observed a decrease in the cellular levels of SPARC protein in SCC9 (**Figure 5—figure supplement 1F**). Since the binding of a histone reader protein to a gene promoter cannot solely

Figure 4.

YEATS2 and GCDH regulate histone crotonylation in HNC.

(A) GSEA plot showing enrichment of LYSINE_DEGRADATION_PATHWAY in TCGA samples stratified as YEATS2_high as compared to YEATS2_low samples. **(B)** Schematic depicting the canonical lysine degradation pathway highlighting the step that leads to crotonyl-CoA production via GCDH. **(C)** RT-qPCR result showing enhanced mRNA expression of *GCDH* in tumor vs. normal samples (n= 8). **(D)** Immunoblot showing enhanced levels of H3K27Cr in tumor vs. normal samples. **(E)** Representative IHC images showing the colocalization of nuclear GCDH with H3K27Cr (Scale bar, 100 μm). Cells with nuclear GCDH-H3K27Cr localization indicated by red arrows, whereas non-nuclear staining indicated by black arrows. **(F and H)** Immunoblot depicting the decrease in H3K27Cr levels on (F) YEATS2- and (H) GCDH-knockdown in BICR10 cells. **(G and I)** Immunoflorescence images depicting the decrease in H3K27Cr levels on (G) YEATS2- and (I) GCDH-knockdown in BICR10 cells (Scale bar, 5 μm). Error bars, mean ± SEM; two-tailed t test, $*p < 0.05$, $***p < 0.001$. n = 3 biological replicates.



lead to its increased expression, we explored the possibility of the involvement of a writer protein in collaborating with YEATS2 to maintain higher levels of SPARC in HNC. For this we looked at expression of the known writers of H3K27Cr mark [15] in TCGA dataset, and discovered that p300 was the only protein that had increased expression in tumor vs. normal HNC dataset (Figure 5—figure supplement 1G). On performing p300-ChIP assay, it was found that its binding was reduced in YEATS2-silenced BICR10 cells when compared to the shControl cells, indicating the potential role of p300 in leading to increased expression of SPARC in HNC cells (**Figure 5F**). Next, we wanted to check the effect of SPARC on invasion. It was observed that the decrease in invasion seen on YEATS2-downregulation was rescued by SPARC overexpression (**Figure 5G**). This indicates that SPARC is a direct target of YEATS2 and acts as a downstream effector of YEATS2 in maintaining invasive phenotype in HNC cells.

Maintenance of H3K27Cr marks on *SPARC* promoter is dependent on YEATS2

To investigate the mechanism of YEATS2-mediated gene regulation we checked the status of histone modifications, both acetylation and crotonylation at H3K27 residue. We performed ChIP-qPCR assay to check the abundance of both the marks at the *SPARC* promoter, we found that only H3K27Cr mark showed significant downregulation (**Figure 6A-B**, **Figure 6—figure supplement 1A-B**). This indicated that SPARC expression was controlled by a non-acetyl histone mark i.e., crotonylation. Since the substrate that is used in addition of crotonylation marks on histones is crotonyl-CoA, we supplemented the cells with its precursor molecule sodium crotonate [16], and saw that there was an increase in H3K27Cr levels on *SPARC* promoter (**Figure 6C**, **Figure 6—figure supplement 1C**). We also observed an increase in expression of SPARC at mRNA and protein levels in both the cell lines (**Figure 6D-E**, **Figure 6— figure supplement 1D-E**). We then checked the effect of crotonate on cancer cell invasiveness and observed that cells treated with crotonate invaded in greater numbers in Matrigel than untreated cells in both the HNC cell lines (**Figure 6F**, **Figure 6—figure supplement 1F**). Finally, we checked whether crotonate treatment can rescue the effect of YEATS2 downregulation on SPARC expression. We observed significant rescue neither in H3K27Cr levels on *SPARC* nor in SPARC protein expression, when YEATS2-downregulated cells treated with crotonate (**Figure 6G-H**). With these observations, we can conclude that H3K27Cr-mediated increase in SPARC expression is subject to the availability of YEATS2 in HNC cells.

GCDH expression is SP1-dependent and it regulates H3K27Cr-mediated SPARC expression with YEATS2 synergistically

As previously shown, both YEATS2 and GCDH regulate the levels of histone crotonylation in HNC. So, we hypothesized that the presence of GCDH could be essential for production of crotonyl-CoA, which could be added to histones by histone-modification machinery consisting of YEATS2 and crotonyltransferases. On performing ChIP assay with GCDH-downregulated cells, we found that H3K27Cr marks on *SPARC* promoter reduced relative to control cells (**Figure 7A**). Also, knocking down of GCDH led to decrease in SPARC at protein and mRNA levels (**Figure 7B-C**, **Figure 7—figure supplement 1A**). We then sought to explore the possibility of cooperativity between YEATS2 and GCDH in maintaining histone crotonylation. For this, we performed co-immunoprecipitation; however, we did not observe an interaction between GCDH and YEATS2 (**Figure 7—figure supplement 1B**), ruling out the possibility of the localized production of crotonyl-CoA at a specific gene locus [17]. We then checked whether both the genes are regulated by the same transcription factor i.e., SP1. TCGA gene expression dataset showed a significant correlation between the expression levels of SP1 and GCDH, hinting at the possibility of SP1 transcriptionally regulating GCDH in HNC (Figure 7—figure supplement 1C). Thus, we performed SP1-knockdown and found a decrease in GCDH levels in both HNC cell lines (**Figure 7D**, **Figure 7—figure supplement 1D**). On performing SP1-ChIP we found a significant

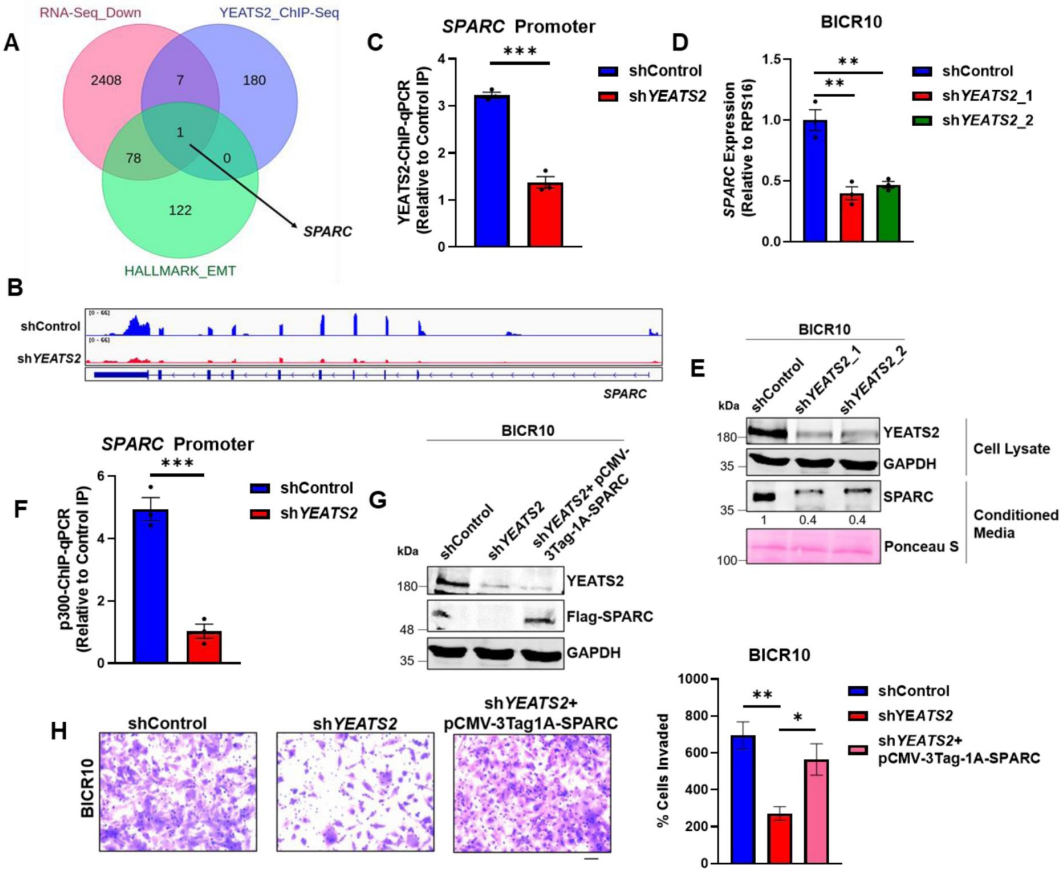


Figure 5.

YEATS2 regulates expression of EMT-related SPARC in HNC.

(A) Venn diagram showing *SPARC* gene obtained after integration of RNA-seq data (genes downregulated in shControl vs. sh*YEATS2*), YEATS2 ChIP-seq data and, HALLMARK_EPITHELIAL_TO_MESENCHYMAL gene signature. **(B)** Integrative genome viewer (IGV) plot showing decrease in SPARC expression in shControl vs. sh*YEATS2* RNA-seq data. **(C)** YEATS2-ChIP-qPCR results showing decreased binding of YEATS2 on *SPARC* promoter in sh*YEATS2* BICR10 cells. **(D)** qRT-PCR results showing decreased expression of *SPARC* on YEATS2-knockdown in BICR10. **(E)** Immunoblot showing decreased expression of SPARC in conditioned media derived from shControl and sh*YEATS2* BICR10 cells. **(F)** p300-ChIP-qPCR results showing decreased binding of YEATS2 on *SPARC* promoter in sh*YEATS2* BICR10 cells. **(G-H)** Immunoblot depicting the overexpression of Flag-tagged SPARC in sh*YEATS2* BICR10 cells (G), and Invasion assay images (with quantification on right) showing decrease and rescue of the percentage of invaded cells in shControl vs. sh*SP1* BICR10 cells, and sh*SP1* cells with YEATS2 overexpression, respectively. Scale bar, 200 µm. $**p < 0.01$, $***p < 0.001$, n = 3 biological replicates.

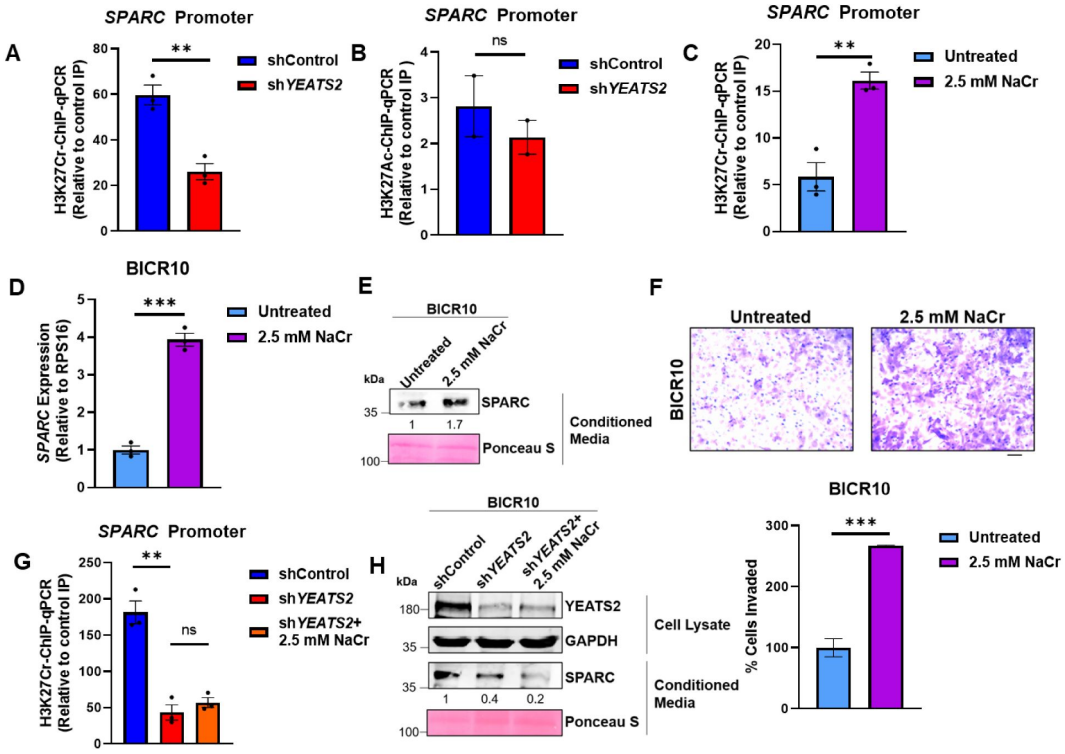


Figure 6.

Supplementation of sodium crotonate increases SPARC levels in YEATS2-dependent manner in HNC cells.

(A) H3K27Cr-ChIP-qPCR results showing decrease in H3K27Cr enrichment on *SPARC* promoter on YEATS2 knockdown. **(B)** H3K27Ac-ChIP-qPCR results showing non-significant change in H3K27Ac enrichment on *SPARC* promoter on YEATS2 knockdown. **(C)** H3K27Cr-ChIP-qPCR results showing increase in H3K27Cr enrichment on *SPARC* promoter on treating BICR10 cells with 2.5 mM sodium crotonate (NaCr). **(D-E)** RT-qPCR (D) and immunoblot (E) showing enhanced SPARC expression in untreated BICR10 cells vs. BICR10 cells treated with 2.5 mM NaCr. **(F)** Invasion assay images (with quantification below) showing increased invasion on treating BICR10 cells with 2.5 mM NaCr (Scale bar, 200 µm). **(G-H)** Immunoblot showing inability of cells to rescue SPARC expression on YEATS2-knockdown (G) and, H3K27Cr-ChIP-qPCR data depicting the lack of significant difference in H3K27Cr levels between sh*YEATS2* vs. sh*YEATS2*+ 2.5 mM NaCr cells (H). Error bars, mean ± SEM; two-tailed t test, ns-non-significant, $**p < 0.01$, $***p < 0.001$.

occupancy of SP1 on *GCDH* promoter in BICR10 and SCC9 (**Figure 7E**, **Figure 7—figure supplement 1E**). These results suggest that SP1 is responsible for high expression of both GCDH and YEATS2 in HNC. The canonical location of GCDH in lysine degradation pathway is mitochondria. However, having detected the presence of nuclear pool of GCDH in HNC tissues (**Figure 4E**, **Figure 4—figure supplement 1D**), we wanted to investigate the role of YEATS2 in affecting the subcellular localization of GCDH in HNC cells. We detected the presence of GCDH in nucleus in BICR10 using IF, and observed that the levels of nuclear GCDH diminished in sh*YEATS2* cells as compared to shControl cells (**Figure 7F**). This indicates that YEATS2 might be involved in regulating the transport of GCDH inside nucleus indirectly by an unknown mechanism. In order to further investigate this epigenome-metabolism interplay, we checked the role of YEATS2 and GCDH in regulating SPARC expression in the absence of SP1. The dual overexpression of YEATS2 and GCDH in SP1-silenced cells rescued the SPARC levels to a greater degree; whereas, YEATS2 and GCDH when overexpressed alone, could only partially rescue SPARC expression (**Figure 7G**). Hereby, we establish that one of the axes involved in regulation of EMT in HNC is dependent on the synergistic maintenance of H3K27Cr marks on *SPARC* gene by YEATS2 and GCDH.

YEATS2 regulates gene expression by maintaining H3K27Cr levels globally

We have previously shown that the expression of a large number of genes are affected by YEATS2 downregulation (**Figure 1G**). For establishing a direct relationship between YEATS2-mediated histone crotonylation and gene expression, we performed ChIP-sequencing to check global H3K27Cr levels in shControl and sh*YEATS2* BICR10 cells. We found that downregulation of YEATS2 led to a significant decrease in H3K27Cr marks at 1,583 locations in the genome (**Figure 8A**). Further, out of these locations, 1,166 were found to be present at the promoter regions of corresponding genes. As expected, overrepresentation analysis using these set of genes revealed EPITHELIAL TO MESENCHYMAL TRANSITION as one of the enriched hallmark genesets (**Figure 8B**). We then overlapped the genes with reduced H3K27Cr levels with genes that showed downregulation in shControl vs. sh*YEATS2* RNA-seq. A total of 147 genes were found to be common in both the sets (**Figure 8C**, **Supplementary file 3**). Among these 147 genes, some examples of EMT-associated genes were *ERG* and *CREB3L2* (**Figure 8D-E**). Thus, the role of YEATS2 in the H3K27Cr-mediated regulation of EMT-related genes was further substantiated with the genome-wide alteration in H3K27Cr observed on YEATS2 downregulation.

Discussion

In our study, we identified YEATS2 as an important epigenetic regulator that plays an oncogenic role in head and neck tumor progression. We have shown that the histone reader regulates expression of important EMT-related genes. The mechanism employed by YEATS2 for gene regulation was also investigated. The YEATS domain confers unique properties to this epigenetic factor, making it suitable for binding with modifications other than acetylation [18]. As stated earlier, YEATS2 has a binding affinity for a variety of chemical groups. Among all histone modifications screened, YEATS2 had the highest affinity for histone crotonylation, specifically at the histone H3 lysine 27 position [19]. There have been conflicting reports about the role of crotonylation at the H3K27 position. YEATS domain-containing GAS41 was found to recruit SIN3A-HDAC1 complex in a H3K27Cr-dependent manner at the promoters of tumor suppressor genes, thereby leading to their repression in colorectal cancer [20]. Conversely, another study in colorectal cancer showed that high H3K27Cr at the promoter of *ETS1* gene led to an increase in its expression and promoted metastasis[10]. In our findings, we have corroborated the latter study by showing the association of H3K27Cr with the activation of an EMT-associated gene.

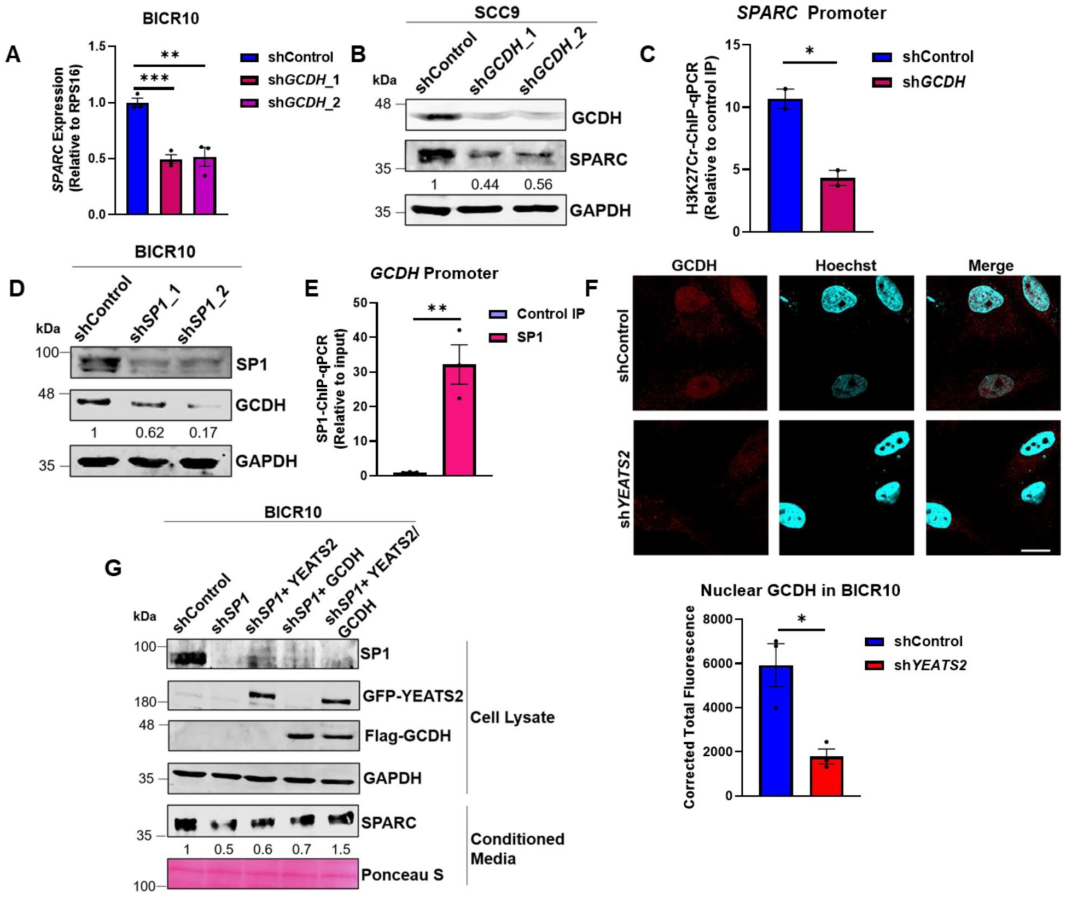


Figure 7.

GCDH expression is SP1-dependent and regulates H3K27Cr-mediated SPARC expression with YEATS2 synergistically.

(A) qRT-PCR results showing decrease in *SPARC* expression on GCDH-knockdown in BICR10 cells **(B)** Immunoblot showing the reduced expression of SPARC upon GCDH-knockdown in SCC9 cells. **(C)** Decrease in H3K27Cr levels on *SPARC* promoter in sh*GCDH* BICR10 cells. **(D)** Immunoblot showing the reduced expression of GCDH on SP1-knockdown in BICR10 cells. **(E)** Plot showing SP1 binding on *GCDH* promoter in SP1-ChIP assay in BICR10. **(F)** IF images showing reduced nuclear localization in sh*YEATS2* BICR10 cells. Scale bar, μm. **(G)** Immunoblot depicting decreased SPARC expression on SP1 knockdown and its subsequent rescue upon YEATS2 and GCDH overexpression in sh*SP1* BICR10 cells. Error bars, mean ± SEM; two-tailed t test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, n = 3 biological replicates.

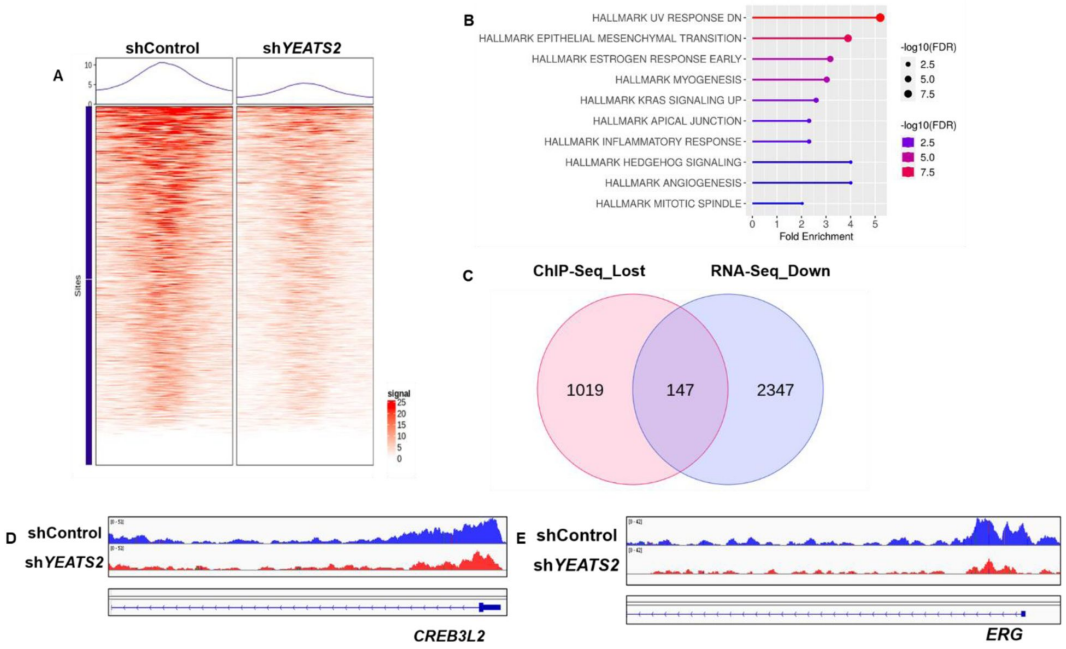


Figure 8.

YEATS2 regulates gene expression by maintaining H3K27Cr levels globally.

(A) Differential ChIP-seq profile of H3K27Cr in shControl vs. sh*YEATS2*. **(B)** Overrepresentation analysis showing pathways enriched among genes with decreased H3K27Cr enrichment in H3K27Cr ChIP-seq data. **(C)** Overlap of genes with reduced H3K27Cr on their promoter and downregulated genes in RNA-seq. **(D-E)** IGV plot showing representative examples of genes common in (C).

With the discovery of a plethora of novel histone modifications, researchers have been facing the challenge to understand the role of these marks in influencing gene expression. A group of such histone marks, known as the non-acetyl acylation marks, consist of a wide variety of chemical moieties which differ in structure and chemical properties [21,22]. Due to the differences amongst these histone marks, it is obvious to assume that the proteins interacting with such histone modifications would not be the same as those interacting with histone acetylation or methylation. YEATS domain is one of the domains that has affinity for non-acetyl histone acylation marks. There are 4 members in the family of YEATS-domain containing proteins, AF9, YEATS2, ENL and, GAS41 [18]. By using multiple high-throughput next generation sequencing techniques, we have strengthened the evidence for the role of YEATS2 in upregulating the expression of EMT-associated gene targets by maintaining a non-acetyl histone mark on their promoters. Global abrogation of pro-EMT genes was seen in RNA-seq data of YEATS2-silenced cells, which was underlaid by a simultaneous decrease seen in H3K27Cr levels, seen in H3K27Cr ChIP-seq, on the same set of downregulated genes. This established a direct relationship between tumorigenic gene expression and histone crotonylation in cancer. Furthermore, in order to thoroughly investigate this link, we have integrated different sets of NGS data and found that invasion-associated gene *SPARC* gene is a direct target of H3K27Cr-mediated regulation by YEATS2, and is possibly one of the many ways in which the EMT-promoting effect of YEATS2 is relayed downstream. As a part of the tumor microenvironment, SPARC mediates ECM-tumor cell communication. Several reports have suggested the role of SPARC in contributing towards a pro-invasive phenotype in cells multiple cancer types [23,24]. Presence of SPARC in tumor stroma was reported to be responsible for recruitment of immunosuppressive myeloid cells in high grade breast carcinomas [25]. In this study, we observed that the SPARC was capable of rescuing the decrease in invasive properties of YEATS2-silenced cells, making it the functional downstream effector of YEATS2.

Our study demonstrates the cooperation between an epigenetic factor and a metabolic enzyme working together to mediate EMT-promoting gene expression. This crosstalk has not been reported in head and neck cancer previously. Since epigenetic changes and aberrant metabolism both are major hallmarks of cancer, it is important to investigate the metabolism-epigenetics axis in order to yield new therapeutic targets [26,27]. Increasing evidences suggest that the state of chromatin in a cell is a reflection of the metabolic enzymes functioning inside the nucleus. Through gene set enrichment analysis, we found that there was a significant correlation between YEATS2 and the lysine degradation pathway in HNC. This led to the observation that one of the enzymes crucial for the production of crotonyl-CoA, GCDH is overexpressed in HNC. Previous reports have suggested that metabolic enzymes could affect epigenomic changes by functioning closely with epigenetic factors. Studies showing the presence of multi-subunit complexes consisting of a metabolite-producing enzyme, DNA-binding protein and, a histone writer are a testament to the fact that metabolism-epigenome collaboration is more direct than previously thought [28]. Although we did not observe direct association of GCDH and YEATS2, we did establish that the nuclear presence of GCDH causes an increase in histone crotonylation in HNC. As per the evidence provided by us, it could be proposed that the surge in GCDH in YEATS2-high cancer cells affects the chromatin by increasing the pool of crotonyl-CoA inside nucleus, which acts as a substrate for histone modifying enzymes. Since YEATS2 works by recognizing histone marks, maintenance of crotonylation at promoter regions could be dependent on sites with a constitutive basal level of H3K27Cr or other marks, as a histone reader cannot recognize a genomic locus devoid of pre-existing histone marks. The presence of such constitutive sites for histone crotonylation, which could act as an anchor for YEATS2 upon its upregulation in cancer cells, remains to be investigated. Moreover, we have shown that SP1 transcriptionally regulates both YEATS2 and GCDH. This hints towards a possibility of a direct axis initiated by an unknown upstream factor working in order to modify the chromatin in such a manner that it benefits the cancer cell by causing an increase in the tumorigenic properties of the cell. In conclusion, we have presented a sophisticated interplay between YEATS2 and GCDH, where the upregulation of both

factors contributes towards the enhancement of EMT in head and neck cancer by changes in the global EMT-favoring gene expression, mediated through promoter histone crotonylation (**Figure 9**).

Methods

Cell Culture

Human head and neck cancer cell lines BICR10 and SCC9 were obtained from European Collection of Authenticated Cell Cultures (ECACC). HEK293T cell line was obtained from American Type Cell Culture (ATCC). Cells were cultured in media recommended by ECACC and ATCC, supplemented with 10% FBS (FBS; Sigma, F7524), 100 units/mL of penicillin and streptomycin (Invitrogen, 15140122) and 2 mmol/L L-glutamine (Sigma, G7513). All cell lines were cultured in a humidified atmosphere at 37°C and 5% CO_2. Transfection of overexpression- and promoter-deletion-constructs was performed using polyethylenimine (PEI) (Polysciences, 23966). For crotonate supplementation, cells were treated with 2.5 mM sodium crotonate for 36 h before harvesting.

Head and neck cancer sample collection

The study was approved by the Institute Ethics Committee of the Indian Institute of Science Education and Research Bhopal, India. Head and neck cancer samples were collected after obtaining informed consent from patients undergoing surgery at Bansal Hospital, Bhopal, India. Details of patients are given in **Table 2**.

Bioinformatics Analyses

In order to study cancer associated epigenetic factors we analyzed The Cancer Genome Atlas (TCGA) mRNA expression dataset using TCGAbiolinks package [29] in R to obtain epigenetics-related genes that are significantly upregulated in head and neck cancer as compared to normal tissues. Survival analysis was then performed using an online tool OncoLnc (*http://www.oncolnc.org*). Results of this analysis are given in **Table 1**. To obtain the expression of individual genes in TCGA, online platforms like Gepia2 [30] and UALCAN [31]. Correlation analysis was done using Gepia2; EMT signature used was downloaded from MSigDb database [32]. GSEA was performed as described previously[33]. Stratification of TCGA gene expression data on the basis of YEATS2 expression was done and the enrichment of various metabolic pathways [11] in the YEATS2_high vs. YEATS2_low group was performed using GSEA software [34].

RNA Interference

SCC9 and BICR10 cells (2×10^5) were seeded in a 6-well plate. After 24 h of seeding, cells were infected with lentivirus containing small hairpin RNA (shRNA) (Sigma, Mission Human Genome shRNA Library) against *YEATS2*, *SP1* and *GCDH* or shControl plasmid with 8 µg/mL polybrene (Sigma, H9268) containing media. Selection was then done using puromycin at 1 µg/mL (Sigma, P9620) for 3 days before harvesting.

Molecular cloning

The *YEATS2* gene was cloned in pEGFP-C3, whereas *SPARC* and *GCDH* were cloned in pCMV-3Tag-1A overexpression plasmid (amplified using Phusion Polymerase, NEB, M0530S). *YEATS2* was cloned between SalI and SmaI sites, *SPARC* between BamHI and HindIII sites, and *GCDH* between HindIII and SalI sites. The construct was then confirmed using colony PCR and Sanger sequencing. Promoter deletion constructs of YEATS2 were cloned in pGL3-Basic vector between KpnI and HindIII RE sites. cDNA or genomic DNA from BICR10 cells was used as template in all PCR reactions.

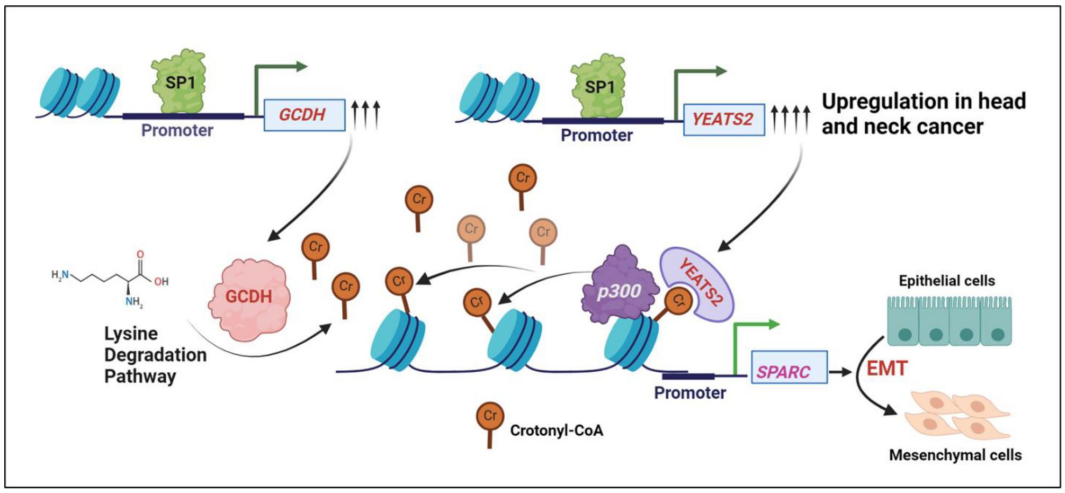


Figure 9.

Schematic showing YEATS- and GCDH-mediated regulation of EMT through SPARC upregulation in head and neck cancer.

Table 2.

Clinical characteristics of patients.

Patient No.	Age	Sex	Clinical Details	Histologic Grade
Patient 1	37	Male	Carcinoma Left lower alveolus	G1, well differentiated
Patient 2	60	Male	Carcinoma Left Buccal mucosa	G1, Well differentiated
Patient 3	41	Male	Carcinoma Left Buccal mucosa	G1, Well- differentiated
Patient 4	47	Male	Carcinoma Vallecula-Post CT/RT.	G1, Well differentiated
Patient 5	43	Male	Carcinoma Left buccal mucosa	G1, Well differentiated
Patient 6	33	Male	Carcinoma Left lower GBS	G2, Moderately differentiated
Patient 7	60	Male	Carcinoma Left lateral border of tongue	G2, Moderately differentiated
Patient 8	62	Male	Carcinoma Upper alveolus/palate	G1, Well differentiated
Patient 9	37	Male	Carcinoma Left lateral border of tongue	G1, Well differentiated
Patient 10	33	Male	Carcinoma Left lateral border of tongue with oropharyngeal involvement	G2, Moderately differentiated

Site directed mutagenesis

The site-directed mutant construct of the YEATS2 promoter-deletion construct was prepared by using a strategy previously described [35]. Oligonucleotides with mutations in the SP1-binding sites present from -71 bp to -60 bp upstream of the YEATS2 TSS (ccCGCccCGCccCGCcct to ccATAccATAccATAcct) were used. The wild-type YEATS2 Luc-508 promoter-deletion construct was used as a template. The primers used are given in **Table 3**. The mutation was confirmed by DNA Sanger sequencing.

Luciferase assay

BICR10 and SCC9 cells (2.5 ×10[5]) were seeded in 6 well plate. After 24 h, cells were co-transfected with either YEATS2-promoter deletion wild-type constructs or SP1-site mutated constructs and pRL-TK Renilla (Promega, E2231) luciferase plasmid. After 24 h of transfection cells were lysed using passive lysis buffer (1% Triton X-100, 25mM Tricine pH 7.8, 15mM potassium phosphate pH 7.8, 15mM magnesium sulphate, 4mM EGTA, 1mM DTT). The firefly/ Renilla luciferase activity was measured using a luminescence detecting plate reader (CYTATION 5, BioTeK).

Transwell invasion assay

BICR10 and SCC9 (2 × 10 [4]) cells resuspended in FBS-free media were added to the upper chamber of transwell setup over a Matrigel layer (Corning, 356230) and incubated for 24 h in a cell culture incubator. Lower chamber of the setup contained media supplemented with FBS. The non-migrated cells in the upper layer of Matrigel were removed, and cells migrated to the lower chamber of transwell were fixed in 4% formaldehyde and then stained with crystal violet (0.05% in 10% methanol in 1 × PBS). Five random fields were counted using an inverted microscope (Olympus CKX41).

Wound healing assay

BICR10 and SCC9 (1.2 x 10[5]) cells were seeded in a 12-well plate. 12 h after the cells had attached, a scratch was made in the center of each well and wound healing was monitored for 2 days. Three random regions per well were monitored for migration using EVOS FL Auto 2 Imaging System by Thermo Scientific.

3D invasion assay

BICR10 and SCC9 (1 x 10[4]) cells were seeded in 96 Well Round (U) Bottom Plate. 48 hours after cell seeding, spheres were transferred to a different flat bottom 96 well plate coated with collagen matrix (Rat Tail Collagen I, Corning 354236) and invasion was checked after 4 days. Images were taken using Olympus CKX41 microscope.

Immunoblotting

Cells were lysed using urea lysis buffer (8M urea, 2M thiourea, 2% CHAPS, 1% DTT) supplemented with 1× protease inhibitor cocktail (PIC; leupeptin 10–100 µM, pepstatin 1µM, EDTA 1–10 mM, AEBSF <1 mM) at 4°C for 30 min and spun at maximum speed (16,900 ×g) in a 4°C centrifuge for 1 hr. Equal amount of samples were loaded on denaturing polyacrylamide gel and transfer was done to activated PVDF membrane. After transfer, the blots were incubated with recommended dilutions of primary antibodies overnight at 4°C, followed by 40 min incubation with secondary antibody. The blots were scanned using Odyssey Membrane Scanning System. Antibodies used: YEATS2 (24717-1-AP), SP1 (9389S), SPARC (5420S), GCDH (HPA043252), N-Cad (ab19348), Vimentin (ab137321), Twist1 (46702S), GAPDH (5174S), GFP (T0006), Flag-tag (Novus, NBP1-06712SS), H3K27Cr (PTM Bio, PTM-545RM).

	Forward primer 5'-3'	Reverse primer 5'-3'
YEATS2 Luc-508	CGGGGTACCGATTAATAGGGAAGCTCATACACG	CCCAAGCTTAAACAATGCCCCGGAGAG
YEATS2 Luc-311	CGGGGTACCATCCCTGGGAGCTCCGCCCCGA	CCCAAGCTTAAACAATGCCCCGGAGAG
YEATS2 Luc-508 Mut	AGCCCGGACCAGCCCCGCCCATACCATACCATACCTCATCCCTGGGAGCTCCG	CGGAGCTCCCAGGGATGAGGTATGGTATGGTATGGGCGGGGCTGGTCCGGGCT
YEATS2 Overexpression	ATAAGAATGCGGCCGCATGTCTGGAATCAAGCGAACCATCA	ACGCGTCGACTCACTGGTCCTCATTCAATATTCC
SPARC Overexpression	CCCAAGCTTATGAGGGCCTGGATCTTCTTTC	CGCGGATCCTTAGATCACAAGATCCTTGTCG

Table 3.

Sequences of primers used in this study.

Table 3. (continued)

GCDH Overexpression	CCCAAGCTTATGGCCCTGAGAGGCGTCT	ACGCGTCGACTCACTTGCTGGCCGTGAACG
YEATS2 RT-qPCR	TGCACAACAGTCTGAAGGAATG	TGGCAGCCTTGCAGGTT
MRGBP RT-qPCR	GAAGAACTCCTCAGACTTGGG	CTTGGCAGCACTGGGACT
RUVBL1 RT-qPCR	CAGAAATCACAGACAAACTTCGAG	TGGATGCAAAGATGACGAT
SPARC RT-qPCR	GCAGCAATGACAACAAGACC	AAGGCCCGATGTAGTCCAG
RPS16 RT-qPCR	AAACGCGGCAATGGTCTCATCAAG	TGGAGATGGACTGACGGATAGCAT
SPARC Promoter ChIP-qPCR	AGATCAAGACACTTGGGCCT	GATGACCCAGAACAGCCTCT
YEATS2 Promoter ChIP-qPCR	GTCCCAGCCCGGACCAGCC	CCCCGCACGTCAGCAGCTG
GCDH Promoter ChIP-qPCR	CGGATTCTAGGAGGAACCAATG	CGAGGCTACAGTGCAACTGAC

Table 4.

Sequences of shRNA used in this study.

shRNA	**Target Sequence 5'-3'**
shYEATS2_1	CCGGCCAGTCAGAAATCTGTTCTATCTCGAGATAGAACAGATTTCTGACTGGTTTTTTG
shYEATS2_2	CCGGCGTCAGAGTTCAAGTTCATTTCTCGAGAAATGAACTTGAACTCTGACGTTTTTTG
shSP1_1	CCGGCCCAAGTTTATTTCTCTCTTACTCGAGTAAGAGAGAAATAAACTTGGGTTTTT
shSP1_2	CCGGGCAGCAACTTGCAGCAGAATTCTCGAGAATTCTGCTGCAAGTTGCTGCTTTTT
shGCDH_1	CCGGATGGGATTTCTGACGAGTATCCTCGAGGATACTCGTCAGAAATCCCATTTTTTG
shGCDH_2	CCGGGATGAGAGCAGACTCCATTTACTCGAGTAAATGGAGTCTGCTCTCATCTTTTTG

Immunoflorescence

Cells were plated at a seeding density of 2 × 10^5 on a coverslip in a 6-well plate. Next day, cells were washed thrice with ice-cold PBS followed by 4% formaldehyde fixation and permeabilization with 0.1% Triton X-100 for 15 min. Blocking was done for 1 h at RT using 2% BSA solution. Cells were then incubated with primary antibodies overnight at 4°C. Cells were washed thrice with PBS and incubated with Alexa-Flour 555 anti-rabbit IgG secondary antibody for 1 h at RT. The cells were then counterstained with Hoechst 33342 (Invitrogen, HI399) and mounted using fluoroshield (Sigma, F6182). Imaging was performed using Olympus FV3000 confocal laser scanning microscope with a 60× Plan Apo N objective (oil, 1.42 NA), and image analysis was done using Image J software.

Immunohistochemistry

Formalin-fixed, paraffin-embedded human head and neck cancer tissue sections were obtained from Bansal Hospital, Bhopal, India. Clinical characteristics of patients used in the study are presented in **Table 2**. As described previously [36], slides were kept at 65°C for 2 h on hot plate, deparaffinized and, rehydrated as described previously. Heat induced antigen retrieval was then performed using 10 mM sodium citrate buffer (pH 6 in microwave for 14 min). 1:10 dilution of 3% hydrogen peroxidase in methanol was used to quench endogenous peroxidase. Blocking was done with 3% BSA. Primary antibodies against H3K27Cr (1:50) and GCDH (1:200) were used. HRP/DAB-chromogenic based Super Sensitive Polymer-HRP Detection System kit (BioGenex, QD430-XAKE) was used as per manufactures' instructions for staining. All slides were counterstained with Harris' hematoxylin (Merck). The images were captured by Thermo Scientific Invitrogen EVOS FL Auto 2 Imaging System. Images were then processed in Adobe Photoshop Version 7.0.

Chromatin immunoprecipitation

ChIP assay was performed as described previously [37]. Briefly, cells were fixed with 1% formaldehyde and quenched by 0.125 M glycine followed by cell lysis. Further, cross-linked chromatin was sonicated to a chromatin fragment length of 100–400 bp approximately and chromatin was immunoprecipitated using YEATS2, H3K27Cr, H3K27Ac and SP1 antibody or control (immunoglobulin G) antibody overnight at 4°C followed by addition of Protein G Dynabeads (Invitrogen, 10004D). The obtained complex and input DNA were decrosslinked in TE buffer with 1% SDS and proteinase K (Invitrogen, 25530049) at 65°C for overnight. Eluted DNA was then purified using PCR purification kit (QIAGEN, 28106). The immunoprecipitated (IP) DNA and 5% input were analyzed by qPCR using locus specific primers (**Table 3**) and SYBR Green Master Mix (Promega, A6002). IP DNA values were normalized to input using the following formula: 2^ (Ct_input - Ct_IP). Resultant values were subsequently normalized to IgG control IP DNA values. The relative values are represented as mean ± SEM of triplicates.

RT-qPCR

Total was extracted using TRIzol (Invitrogen, 15596026) according to the manufacturer's instructions. cDNA was synthesized from 3 μg of total RNA by PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa, 6110A). Amplification reactions were performed on light cycler 480 II (Roche) using Go tag SYBR Green master mix (Promega, 75665). The gene expression was calculated using the following formula: 2^ (Ct_control - Ct_target) where RPS16 is taken as control. Sequence of each primer used is given in **Table 3**.

RNA-seq

RNA from shControl/ sh*YEATS2* BICR10 cells was extracted using TRIzol. QIAseq Stranded mRNA Lib Kit (Qiagen, 180441) kit was used to prepare libraries. Paired end sequencing (2×150 bp) of these libraries was performed on Novaseq 6000 platform. Fastq files obtained after sequencing

were aligned to GRCh38 human genome using STAR aligner [38]. Read counting for each sample was done using HTseq2 [39]. Differential expression analysis was then performed using DESeq2 [40]. Genes with significant change in expression are listed in Supplementary file 1.

ChIP-seq

Eluted DNA obtained after performing ChIP assay in BICR10 cells using YEATS2 antibody. TruSeq ChIP Library Preparation Kit (IP-202-1012) was used to prepare libraries. Paired end sequencing (2×100 bp) of these libraries was performed on Novaseq 6000 platform. Fastq files obtained after sequencing were aligned to GRCh38 human genome using STAR aligner. Peak calling was performed using MACS2 [41]. Genomic distribution of YEATS2 peaks was obtained using ChIPSeeker. Genes having YEATS2 peak in their promoter region are listed in Supplementary file 2. For H3K27Cr ChIP-seq, ChIP assay was performed using chromatin from shControl and sh*YEATS2* BICR10 cells. Libraries were made using NEBNext Ultra II DNA library preparation for Illumina Kit (NEBNext #E7645S) and paired end sequencing (2×150 bp) was performed on Novaseq 6000 platform. Fastq files were aligned to hg38 genome using Bowtie2 [42], followed by peak calling with MACS2 and differential binding analysis using DiffBind [43].

Statistical analysis

Data are presented as mean ± SEM unless otherwise stated. At least three independent biological replicates have been performed for each experiment. All statistical analyses were conducted using GraphPad Prism 8 software. Statistical tests used and specific *p* values are indicated in the figure legends.

Data availability

YEATS2 and H3K27cr ChIP-Seq data is deposited at GEO database (GSE275975). RNA sequencing data from shControl and shYEATS2 cells has been deposited at GEO database (GSE275977). This study does not report any original code.

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Additional information

Author contributions

S.S. and D.P. conceived and designed the study. D.P., R.J. and P.K. performed the experiments and analysed the results. D.P. wrote the manuscript with inputs from all other authors. S.S. supervised the experiments and manuscript preparation. S.S. acquired funding for the study.

Additional files

Figure supplements

Supplementary file 1. *Differentially expressed genes in shControl vs. shYEATS2 RNA-seq data (FDR-adjusted p < 0.05).*

Supplementary file 2. *List of genes having YEATS2 ChIP-seq peak in their promoters.*

Supplementary file 3. *List of genes common between ChIP-seq_Lost and RNA-seq_Down groups.*

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Reviewer #1 (Public review):

Summary:

This manuscript investigates a mechanism between the histone reader protein YEATS2 and the metabolic enzyme GCDH, particularly in regulating epithelial-to-mesenchymal transition (EMT) in head and neck cancer (HNC).

Strengths:

Great detailing of the mechanistic aspect of the above axis is the primary strength of the manuscript.

Weaknesses:

Several critical points require clarification, including the rationale behind EMT marker selection, the inclusion of metastasis data, the role of key metabolic enzymes like ECHS1, and the molecular mechanisms governing p300 and YEATS2 interactions.

Major Comments:

(1) The title, "Interplay of YEATS2 and GCDH mediates histone crotonylation and drives EMT in head and neck cancer," appears somewhat misleading, as it implies that YEATS2 directly drives histone crotonylation. However, YEATS2 functions as a reader of histone crotonylation rather than a writer or mediator of this modification. It cannot itself mediate the addition of crotonyl groups onto histones. Instead, the enzyme GCDH is the one responsible for generating crotonyl-CoA, which enables histone crotonylation. Therefore, while YEATS2 plays a role in recognizing crotonylation marks and may regulate gene expression through this mechanism, it does not directly catalyse or promote the crotonylation process.

(2) The study suggests a link between YEATS2 and metastasis due to its role in EMT, but the lack of clinical or pre-clinical evidence of metastasis is concerning. Only primary tumor (PT) data is shown, but if the hypothesis is that YEATS2 promotes metastasis via EMT, then evidence from metastatic samples or in vivo models should be included to solidify this claim.

(3) There seems to be some discrepancy in the invasion data with BICR10 control cells (Figure 2C). BICR10 control cells with mock plasmids, specifically shControl and pEGFP-C3 show an

unclear distinction between invasion capacities. Normally, we would expect the control cells to invade somewhat similarly, in terms of area covered, within the same time interval (24 hours here). But we clearly see more control cells invading when the invasion is done with KD and fewer control cells invading when the invasion is done with OE. Are these just plasmid-specific significant effects on normal cell invasion? This needs to be addressed.

(4) In Figure 3G, the Western blot shows an unclear band for YEATS2 in shSP1 cells with YEATS2 overexpression condition. The authors need to clearly identify which band corresponds to YEATS2 in this case.

(5) In ChIP assays with SP1, YEATS2 and p300 which promoter regions were selected for the respective genes? Please provide data for all the different promoter regions that must have been analysed, highlighting the region where enrichment/depletion was observed. Including data from negative control regions would improve the validity of the results.

(6) The authors establish a link between H3K27Cr marks and GCDH expression, and this is an already well-known pathway. A critical missing piece is the level of ECSH1 in patient samples. This will clearly delineate if the balance shifted towards crotonylation.

(7) The p300 ChIP data on the SPARC promoter is confusing. The authors report reduced p300 occupancy in YEATS2-silenced cells, on SPARC promoter. However, this is paradoxical, as p300 is a writer, a histone acetyltransferase (HAT). The absence of a reader (YEATS2) shouldn't affect the writer (p300) unless a complex relationship between p300 and YEATS2 is present. The role of p300 should be further clarified in this case. Additionally, transcriptional regulation of SPARC expression in YEATS2 silenced cells could be analysed via downstream events, like Pol-II recruitment. Assays such as Pol-II ChIP-qPCR could help explain this.

(8) The role of GCDH in producing crotonyl-CoA is already well-established in the literature. The authors' hypothesis that GCDH is essential for crotonyl-CoA production has been proven, and it's unclear why this is presented as a novel finding. It has been shown that YEATS2 KD leads to reduced H3K27cr, however, it remains unclear how the reader is affecting crotonylation levels. Are GCDH levels also reduced in the YEATS2 KD condition? Are YEATS2 levels regulating GCDH expression? One possible mechanism is YEATS2 occupancy on GCDH promoter and therefore reduced GCDH levels upon YEATS2 KD. This aspect is crucial to the study's proposed mechanism but is not addressed thoroughly.

(9) The authors should provide IHC analysis of YEATS2, SPARC alongside H3K27cr and GCDH staining in normal vs. tumor tissues from HNC patients.

https://doi.org/10.7554/eLife.103321.1.sa2

Reviewer #2 (Public review):

Summary:

The manuscript emphasises the increased invasive potential of histone reader YEATS2 in an SP1-dependent manner. They report that YEATS2 maintains high H3K27cr levels at the promoter of EMT-promoting gene SPARC. These findings assigned a novel functional implication of histone acylation, crotonylation.

Concerns:

(1) The patient cohort is very small with just 10 patients. To establish a significant result the cohort size should be increased.

(2) Figure 4D compares H3K27Cr levels in tumor and normal tissue samples. Figure 1G shows overexpression of YEATS2 in a tumor as compared to normal samples. The loading control is

missing in both. Loading control is essential to eliminate any disparity in protein concentration that is loaded.

(3) Figure 4D only mentions 5 patient samples checked for the increased levels of crotonylation and hence forms the basis of their hypothesis (increased crotonylation in a tumor as compared to normal). The sample size should be more and patient details should be mentioned.

(4) YEATS2 maintains H3K27Cr levels at the SPARC promoter. The p300 is reported to be hyper-activated (hyperautoacetylated) in oral cancer. Probably, the activated p300 causes hyper-crotonylation, and other protein factors cause the functional translation of this modification. The authors need to clarify this with a suitable experiment.

(5) I do not entirely agree with using GAPDH as a control in the western blot experiment since GAPDH has been reported to be overexpressed in oral cancer.

(6) The expression of EMT markers has been checked in shControl and shYEATS2 transfected cell lines (Figure 2A). However, their expression should first be checked directly in the patients' normal vs. tumor samples.

(7) In Figure 3G, knockdown of SP1 led to the reduced expression of YEATS2 controlled gene Twist1. Ectopic expression of YEATS2 was able to rescue Twist1 partially. In order to establish that SP1 directly regulates YEATS2, SP1 should also be re-introduced upon the knockdown background along with YEATS2 for complete rescue of Twist1 expression.

(8) In Figure 7G, the expression of EMT genes should also be checked upon rescue of SPARC expression.

https://doi.org/10.7554/eLife.103321.1.sa1

Author response:

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *This manuscript investigates a mechanism between the histone reader protein YEATS2 and the metabolic enzyme GCDH, particularly in regulating epithelial-to-mesenchymal transition (EMT) in head and neck cancer (HNC).*
>
> *Strengths:*
>
> *Great detailing of the mechanistic aspect of the above axis is the primary strength of the manuscript.*
>
> *Weaknesses:*
>
> *Several critical points require clarification, including the rationale behind EMT marker selection, the inclusion of metastasis data, the role of key metabolic enzymes like ECHS1, and the molecular mechanisms governing p300 and YEATS2 interactions.*

We would like to sincerely thank the reviewer for the detailed, in-depth, and positive response. We are committed to implementing constructive revisions to the manuscript to address the reviewer's concerns effectively.

> *(1) The title, "Interplay of YEATS2 and GCDH mediates histone crotonylation and drives EMT in head and neck cancer," appears somewhat misleading, as it implies that YEATS2 directly drives histone crotonylation. However, YEATS2 functions as a reader of histone crotonylation rather than a writer or mediator of this modification. It cannot itself mediate the addition of crotonyl groups onto histones. Instead, the enzyme GCDH is the one responsible for generating crotonyl-CoA, which enables histone crotonylation. Therefore, while YEATS2 plays a role in recognizing crotonylation marks and may regulate gene expression through this mechanism, it does not directly catalyse or promote the crotonylation process.*

We thank the reviewer for raising this concern. As stated by the reviewer, YEATS2 functions as a reader protein, capable of recognizing histone crotonylation marks and assisting in the addition of this mark to nearby histone residues, possibly by assisting the recruitment of the writer protein for crotonylation. Our data indicates the involvement of YEATS2 in the recruitment of writer protein p300 on the promoter of the *SPARC* gene, making YEATS2 a regulatory factor responsible for the addition of crotonyl marks in an indirect manner. Thus, we have decided to make changes in the title by replacing the word “mediates” with “regulates”. Therefore, the updated title can be read as: “Interplay of YEATS2 and GCDH regulates histone crotonylation and drives EMT in head and neck cancer”.

> *(2) The study suggests a link between YEATS2 and metastasis due to its role in EMT, but the lack of clinical or pre-clinical evidence of metastasis is concerning. Only primary tumor (PT) data is shown, but if the hypothesis is that YEATS2 promotes metastasis via EMT, then evidence from metastatic samples or in vivo models should be included to solidify this claim.*

We appreciate the reviewer’s suggestion. Here, we would like to state that the primary aim of this study was to delineate the molecular mechanisms behind the role of YEATS2 in maintaining histone crotonylation at the promoter of genes that favour EMT in head and neck cancer. We have dissected the importance of histone crotonylation in the regulation of gene expression in head and neck cancer in great detail, having investigated the upstream and downstream molecular players involved in this process that promote EMT. Moreover, with the help of multiple phenotypic assays, such as Matrigel invasion, wound healing, and 3D invasion assays, we have shown the functional importance of YEATS2 in promoting EMT in head and neck cancer cells. Since EMT is known to be a prerequisite process for cancer cells undergoing metastasis(1), the evidence of YEATS2 being associated with EMT demonstrates a potential correlation of YEATS2 with metastasis. However, as part of the revision, we will use publicly available patient data to investigate the direct association of YEATS2 with metastasis by checking the expression of YEATS2 between different grades of head and neck cancer, as an increase in tumor grade is often correlated with the incidence of metastasis(2).

> *(3) There seems to be some discrepancy in the invasion data with BICR10 control cells (Figure 2C). BICR10 control cells with mock plasmids, specifically shControl and pEGFP-C3 show an unclear distinction between invasion capacities. Normally, we would expect the control cells to invade somewhat similarly, in terms of area covered, within the same time interval (24 hours here). But we clearly see more control cells invading when the invasion is done with KD and fewer control cells invading when the invasion is done with OE. Are these just plasmid-specific significant effects on normal cell invasion? This needs to be addressed.*

We appreciate the reviewer for the thorough evaluation of the manuscript. The figure panels in question, Figure 2B and 2C, represent two different experiments performed independently,

the invasion assay performed after knockdown and overexpression of YEATS2, respectively. We would like to clarify that both panels represent results that are distinct and independent of each other and that the method used to knockdown or overexpress YEATS2 is also different. As stated in the Materials and Methods section, the knockdown is performed using lentivirus-mediated transfection (transduction) of cells, on the other hand, the overexpression is done using standard method of transfection by directly mixing transfection reagent and the respective plasmids, prior to the addition of this mix to the cells. The difference in the experimental conditions in these two experiments might have attributed to the differences seen in the controls as observed previously(3). Hence, we would like to state that the results of figure panels Figure 2B and Figure 2C should be evaluated independently of each other.

> *(4) In Figure 3G, the Western blot shows an unclear band for YEATS2 in shSP1 cells with YEATS2 overexpression condition. The authors need to clearly identify which band corresponds to YEATS2 in this case.*

The two bands seen in the shSP1+pEGFP-C3-YEATS2 condition correspond to the endogenous YEATS2 band (lower band, indicated by * in the shControl lane) and YEATS2-GFP band (upper band, corresponding to overexpressed YEATS2-GFP fusion protein, which has a higher molecular weight). To avoid confusion, the endogenous band will be highlighted (marked by *) in the lane representing the shSP1+pEGFP-C3-YEATS2 condition in the revised version of the manuscript.

> *(5) In ChIP assays with SP1, YEATS2 and p300 which promoter regions were selected for the respective genes? Please provide data for all the different promoter regions that must have been analysed, highlighting the region where enrichment/depletion was observed. Including data from negative control regions would improve the validity of the results.*

Throughout our study, we have performed ChIP-qPCR assays to check the binding of SP1 on *YEATS2* and *GCDH* promoter, and to check YEATS2 and p300 binding on *SPARC* promoter. Using transcription factor binding prediction tools and luciferase assays, we selected multiple sites on the *YEATS2* and *GCDH* promoter to check for SP1 binding. The results corresponding to the site that showed significant enrichment were provided in the manuscript. The region of *SPARC* promoter in YEATS2 and p300 ChIP assay was selected on the basis of YEATS2 enrichment found in the YEATS2 ChIP-seq data. We will provide data for all the promoter regions investigated (including negative controls) in the revised version of the manuscript.

> *(6) The authors establish a link between H3K27Cr marks and GCDH expression, and this is an already well-known pathway. A critical missing piece is the level of ECSH1 in patient samples. This will clearly delineate if the balance shifted towards crotonylation.*

We thank the reviewer for their valuable suggestion. To support our claim, we had checked the expression of GCDH and ECHS1 in TCGA HNC RNA-seq data (provided in Figure 4—figure supplement 1A and B) and found that GCDH showed increase while ECHS1 showed decrease in tumor as compared to normal samples. We hypothesized that higher GCDH expression and decreased ECHS1 expression might lead to an increase in the levels of crotonylation in HNC. To further substantiate our claim, we will check the abundance of ECHS1 in HNC patient samples as part of the revision.

> *(7) The p300 ChIP data on the SPARC promoter is confusing. The authors report reduced p300 occupancy in YEATS2-silenced cells, on SPARC promoter. However, this is paradoxical, as p300 is a writer, a histone acetyltransferase (HAT). The absence of a reader (YEATS2) shouldn't affect the writer (p300) unless a complex relationship between*

> *p300 and YEATS2 is present. The role of p300 should be further clarified in this case. Additionally, transcriptional regulation of SPARC expression in YEATS2 silenced cells could be analysed via downstream events, like Pol-II recruitment. Assays such as Pol-II ChIP-qPCR could help explain this.*

Using RNA-seq and ChIP-seq analyses, we have shown that YEATS2 affects the expression of several genes by regulating the level of histone crotonylation at gene promoters globally. The histone writer p300 is a promiscuous acyltransferase protein that has been shown to be involved in the addition of several non-acetyl marks on histone residues, including crotonylation(4). Our data provides evidence for the dependency of the writer p300 on YEATS2 in mediating histone crotonylation, as YEATS2 downregulation led to decreased occupancy of p300 on the *SPARC* promoter (Figure 5F). However, the exact mechanism of cooperativity between YEATS2 and p300 in maintaining histone crotonylation remains to be investigated. To address the reviewer's concern, we will perform various experiments to delineate the molecular mechanism pertaining to the association of YEATS2 with p300 in regulating histone crotonylation. Following are the experiments that will be performed:

(a) Co-immunoprecipitation experiments to check the physical interaction between YEATS2 and p300.

(b) We will check H3K27cr levels on the *SPARC* promoter and SPARC expression in p300-depleted HNC cells.

(c) Rescue experiments to check if the decrease in p300 occupancy on the *SPARC* promoter can be compensated by overexpressing YEATS2.

(d) As suggested by the reviewer, Pol-II ChIP-qPCR at the promoter of *SPARC* will be performed in YEATS2-silenced cells to explain the mode of transcriptional regulation of SPARC expression by YEATS2.

> *(8) The role of GCDH in producing crotonyl-CoA is already well-established in the literature. The authors' hypothesis that GCDH is essential for crotonyl-CoA production has been proven, and it's unclear why this is presented as a novel finding. It has been shown that YEATS2 KD leads to reduced H3K27cr, however, it remains unclear how the reader is affecting crotonylation levels. Are GCDH levels also reduced in the YEATS2 KD condition? Are YEATS2 levels regulating GCDH expression? One possible mechanism is YEATS2 occupancy on GCDH promoter and therefore reduced GCDH levels upon YEATS2 KD. This aspect is crucial to the study's proposed mechanism but is not addressed thoroughly.*

The source for histone crotonylation, crotonyl-CoA, can be produced by several enzymes in the cell, such as ACSS2, GCDH, ACOX3, etc(5). Since metabolic intermediates produced during several cellular pathways in the cell can act as substrates for epigenetic factors, we wanted to investigate if such an epigenetic-metabolism crosstalk existed in the context of YEATS2. As described in the manuscript, we performed GSEA using publicly available TCGA RNA-seq data and found that patients with higher YEATS2 expression also showed a high correlation with expression levels of genes involved in the lysine degradation pathway, including GCDH. Since the preferential binding of YEATS2 with H3K27cr and the role of GCDH in producing crotonyl-CoA was known(6,7), we hypothesized that higher H3K27cr in HNC could be a result of both YEATS2 and GCDH. We found that the presence of GCDH in the nucleus of HNC cells is correlated to higher H3K27cr abundance, which could be a result of excess levels of crotonyl-CoA produced via GCDH. We also found a correlation between H3K27cr levels and YEATS2 expression, which could arise due to YEATS2-mediated preferential maintenance of crotonylation. This states that although being a reader protein, YEATS2 is affecting the promoter H3K27cr levels, possibly by helping in the recruitment of p300 (as shown in Figure

5F). Thus, YEATS2 and GCDH are both responsible for the regulation of histone crotonylation-mediated gene expression in HNC.

We did not find any evidence of YEATS2 regulating the expression of GCDH in HNC cells. However, we found that YEATS2 downregulation reduced the nuclear pool of GCDH in head and neck cancer cells (Figure 7F). This suggests that YEATS2 not only regulates histone crotonylation by affecting promoter H3K27cr levels (with p300), but also by affecting the nuclear localization of crotonyl-CoA producing GCDH. Also, we observed that the expression of YEATS2 and GCDH are regulated by the same transcription factor SP1 in HNC. We found that the transcription factor SP1 binds to the promoter of both genes, and its downregulation led to a decrease in their expression (Figure 3 and Figure 7).

We would like to state that the relationship between YEATS2 and the nuclear localization of GCDH, as well as the underlying molecular mechanism, remains unexplored and presents an open question for future investigation.

> *(9) The authors should provide IHC analysis of YEATS2, SPARC alongside H3K27cr and GCDH staining in normal vs. tumor tissues from HNC patients.*

We thank the reviewer for their suggestion. We are consulting our clinical collaborators to assess the feasibility of including this IHC analysis in our revision and will make every effort to incorporate it.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *The manuscript emphasises the increased invasive potential of histone reader YEATS2 in an SP1-dependent manner. They report that YEATS2 maintains high H3K27cr levels at the promoter of EMT-promoting gene SPARC. These findings assigned a novel functional implication of histone acylation, crotonylation.*

We thank the reviewer for the constructive comments. We are committed to making beneficial changes to the manuscript in order to alleviate the reviewer's concerns.

> *Concerns:*
>
> *(1) The patient cohort is very small with just 10 patients. To establish a significant result the cohort size should be increased.*

We thank the reviewer for this suggestion. We will increase the number of patient samples to assess the levels of YEATS2 and H3K27cr in normal vs. tumor samples.

> *(2) Figure 4D compares H3K27Cr levels in tumor and normal tissue samples. Figure 1G shows overexpression of YEATS2 in a tumor as compared to normal samples. The loading control is missing in both. Loading control is essential to eliminate any disparity in protein concentration that is loaded.*

In Figures 1G and 4D, we have used Ponceau S staining as a control for equal loading. Ponceau S staining is frequently used as an alternative for housekeeping genes like GAPDH as a control for protein loading(8). It avoids the potential for variability in housekeeping gene expression. However, it may be less quantitative than using housekeeping proteins. To address the reviewer's concern, we will probe with an antibody against a house keeping gene as a loading control in the revised figures, provided its expression remains stable across the conditions tested.

> *(3) Figure 4D only mentions 5 patient samples checked for the increased levels of crotonylation and hence forms the basis of their hypothesis (increased crotonylation in a tumor as compared to normal). The sample size should be more and patient details should be mentioned.*

A total of 9 samples were checked for H3K27cr levels (5 of them are included in Figure 4D and rest included in Figure 4—figure supplement 1D). However, as a part of the revision, we will check the H3K27cr levels in more patient samples.

> *(4) YEATS2 maintains H3K27Cr levels at the SPARC promoter. The p300 is reported to be hyper-activated (hyperautoacetylated) in oral cancer. Probably, the activated p300 causes hyper-crotonylation, and other protein factors cause the functional translation of this modification. The authors need to clarify this with a suitable experiment.*

In our study, we have shown that p300 is dependent on YEATS2 for its recruitment on the SPARC promoter. As a part of the revision, we propose the following experiments to further substantiate the role of p300 in YEATS2-mediated gene regulation:

(a) Co-immunoprecipitation experiments to check the physical interaction between YEATS2 and p300.

(b) We will check H3K27cr levels on the *SPARC* promoter and SPARC expression in p300-depleted HNC cells.

(c) Rescue experiments to check if the decrease in p300 occupancy on the *SPARC* promoter can be compensated by overexpressing YEATS2.

(d) Pol-II ChIP-qPCR at the promoter of *SPARC* will be performed in YEATS2-silenced cells to explain the mode of transcriptional regulation of SPARC expression by YEATS2.

> *(5) I do not entirely agree with using GAPDH as a control in the western blot experiment since GAPDH has been reported to be overexpressed in oral cancer.*

We would like to clarify that GAPDH was not used as a loading control for protein expression comparisons between normal and tumor samples. GAPDH was used as a loading control only in experiments using head and neck cancer cell lines where shRNA-mediated knockdown or overexpression was employed. These manipulations specifically target the genes of interest and are not expected to alter GAPDH expression, making it a suitable loading control in these instances.

> *(6) The expression of EMT markers has been checked in shControl and shYEATS2 transfected cell lines (Figure 2A). However, their expression should first be checked directly in the patients' normal vs. tumor samples.*

We thank the reviewer for the suggestion. To address this, we will check the expression of EMT markers alongside YEATS2 expression in normal vs. tumor samples.

> *(7) In Figure 3G, knockdown of SP1 led to the reduced expression of YEATS2 controlled gene Twist1. Ectopic expression of YEATS2 was able to rescue Twist1 partially. In order to establish that SP1 directly regulates YEATS2, SP1 should also be re-introduced upon the knockdown background along with YEATS2 for complete rescue of Twist1 expression.*

To address the reviewer's concern regarding the partial rescue of Twist1 in SP1 depleted-YEATS2 overexpressed cells, we will perform the experiment as suggested by the reviewer. In

brief, we will overexpress both SP1 and YEATS2 in SP1-depleted cells and then assess the expression of Twist1.

> *(8) In Figure 7G, the expression of EMT genes should also be checked upon rescue of SPARC expression.*

We thank the reviewer for the suggestion. We will check the expression of EMT markers on YEATS2/ GCDH rescue and update Figure 7G in the revised version of the manuscript.

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