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Research article

Pilot RNAi screening using mammalian cell-based system identifies novel putative silencing factors including Kat5/Tip60

Anuroop Venkateswaran Venkatasubramani 1,2, Katy McLaughlin 1, Giovanny Rodriguez Blanco 1, Vladimir Larionov 3, and Alexander Kagansky 1,*

1 Institute of Genetics and Molecular Medicine, MRC Human Genetics Unit, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, United Kingdom
2 Department of Biosciences, P.O. Box 65 (Viikkinkaarri 1), 00014, University of Helsinki, Finland
3 Center for Cancer Research, National Cancer Institute, Building 37, Room 5032A, Bethesda, MD 20892, USA

* Correspondence: Email: alexander.kagansky@ed.ac.uk; Tel: +44-131-651-1080; Fax: +44-131-651-8800.

Abstract: Epigenetic silencing is the reversible inactivation of a gene transcription which occurs as a result of changes in the structure of the chromatin that can be successfully inherited from parent to daughter cells. It involves non-genetic mutations within the genome, as well as post-transcriptional and post-translational mechanisms. Existence of these mechanisms at various levels warrants their role in development and disease and thus it is crucial to study different factors and mechanisms of silencing. The aim of our study was to establish a method for rapid screening for the loss of epigenetic silencing in mammalian cells, to identify factor(s) involved in epigenetic silencing, and to get insights into their mechanism of action. For this purpose, we used RNAi screening approach using shRNAs that targeted our genes of interest. We employed a modified mouse cell line which contained a GFP transgene under the control of CMV promoter which has been silenced by epigenetic modifications. Our screening identified several proteins as epigenetic silencing regulators including Kat5/Tip60, an acetyltransferase of MYST family of proteins. To characterize its function, we performed preliminary experiments using microscopy and Western blot analysis of histone marks. We observed changes in H4 acetylation levels in Kat5/Tip60 knockdown cells. Our study thus serves as a pilot for a genome-wide silencing screening using mammalian cells, and provides preliminary results suggesting that Kat5 can be considered as a silencing factor, which, we propose, could function by maintaining H4 acetylation patterns.
Keywords: RNAi screening; acetyltransferase; Kat5/Tip60; silencing; epigenetics; chromatin; histone; acetylation

Abbreviations

SEM = Standard error of the mean; RNAi = RNA interference; shRNAs = short-hairpin RNAs; GFP = Green Fluorescent Protein; FACS = Fluorescent Activated Cell Sorting; MOI = Multiplicity of Infection; TSA = Trichostatin-A; scrRNA = TagRFP-shRNA; CRISPR = Clustered, regularly interspaced, short palindromic repeats

1. Introduction

Epigenetics refers to heritable changes in gene expression that can be observed irrespective of the changes in the DNA sequence [1]. Such processes can have varied outcomes depending on the structural organization of the chromatin. An “open chromatin”, technically termed euchromatin, would assist the expression of a gene while heterochromatin (“closed chromatin”), would result in the loss of expression [2]. Recent efforts allowed to model gene expression, taking into account multiple factors, including nucleosome dynamics, and binding of activators and repressors [3]. Loss of gene expression, referred to as epigenetic gene silencing, is one of the commonly observed phenomenon in disease mechanisms [4]. It can be affected by non-coding RNAs and nuclear remodelling factors, in addition to modification of cytosine and histone tails [5], eventually resulting in loss of gene transcription or inhibition of translation [6].

Research in epigenetics has been on a wide variety of mechanisms and regulatory functions. For instance, particular modifications or silencing marks, such as the abundance of H2A ubiquitylation or H3 lysine 27 (H3K27) trimethylation have been observed in regions of double stranded breaks. One of the most commonly studied roles of epigenetics in this aspect is the DNA repair mechanisms. Implications of interplay between chromatin modifying factors and repair mechanisms have been well studied especially in the case of DOT1L, HIRA and FACT [7]. Furthermore, the role of epigenetic factors in pluripotency and development have also been increasingly researched. The major pluripotency factors (Oct4, Sox2 and Nanog [OSN]) regulate the expression of genes which aids the transcription of Polycomb and MLL complex components. In turn, these complexes repress transcription factors in a lineage-specific manner. Such co-operation between the repressive complexes and OSN factors are significant for the maintenance of pluripotent state [8]. In addition, non-coding RNAs such as micro-RNAs (miRNAs) and long non-coding RNAs (lncRNAs) have been a major focus of interest with respect to developmental mechanisms. With advancements in RNA-sequencing methodologies and CRISPR system, a number of such non-coding RNAs are being identified and characterized [9].

Nonetheless, the ubiquitous nature of epigenetics have broadened its focus and research aims to disease mechanisms and drug resistance. Of the many diseases that can be associated with epigenetics, the most common and widely studied have been cancers. Cancers can arise due to the activation of an oncogene or by repression of tumour-suppressors, such as CDKN2A hypermethylation, which leads to lung cancer [10]. As mentioned above, different mechanisms such as DNA and histone modifications and RNAi (RNA-interference) are potential regulators of gene
expression and are thus involved at different stages of carcinogenesis [11]. Apart from cancers, there are number of other diseases from cardiovascular to autoimmune disorders which occur due to various epigenetic events. HOTAIR, a well-known non-coding RNA, which usually plays a role in repression of HOX genes by recruiting Polycomb complex, has been observed to be involved in aortic calcification due to its role in stress response and as a repressor of calcification genes [12]. Similarly, global decrease in histone acetylation at H3 and H4 of CD4+ T cells, have been associated with systemic lupus erythematosus (SLE), an autoimmune disorder [13]. Additionally, epigenetics would be able to provide a comprehensive understanding of personalized medicine due to its role in drug resistance. Cetuximab, a drug that is administered for colon cancer, is ineffective for patients with mutated BRAF and KRAS genes. However, some patients with wild-type copy of those genes, also do not show any response to the drug. SIRT2, a histone deacetylase, has been identified as a marker for drug resistance in such cases. SIRT2 acetylates MEK, a downstream component of KRAS pathway, which inhibits its phosphorylation activity. Loss of SIRT2 would thus result in MEK phosphorylation of ERK, another component of KRAS pathway, resulting in activation of transcription and in turn conferring drug resistance [14].

![Figure 1. Hypothesis of the research study: The modified cell-line contains an epigenetically silenced CMV promoter (marked in red) which controls a GFP reporter (marked in white). Administration of shRNA to knockdown a factor of interest, would result in the de-repression of the promoter (marked in white), if that particular gene is involved in epigenetic mechanism and thus should express GFP (marked in green). While when a non-targeted shRNA is used, no expression of GFP should be observed.](image)

Furthermore, current research have complicated the epigenetic landscape by hypothesizing the role of lipids in gene regulation [15] and the observation of factors involved in both repression and activation. For instance, H3.3, a variant of histone H3, has generally been associated with
transcriptional activation [16]. However, recent studies [17] suggested that H3.3 could act as a negative regulator of gene expression. In support of this, another study [18] observed that H3.3 is being deposited in the heterochromatic regions by ATRX and gets enriched with H3K9me3, thus potentially posing a negative regulatory role. Such complex nature of epigenetics and its involvement in myriads of processes, substantiates the comprehensive elucidation of its factors and their mechanisms. The aim of our study was to identify factor(s) involved in epigenetic silencing and to possibly get insights into their molecular function. We used mouse cells, termed C127, which contained a GFP transgene, downstream of a CMV promoter that has been epigenetically silenced. Short-hairpin RNAs (shRNAs) were used for knocking-down targets of interest by RNAi mechanism. We hypothesized that knockdown of targets involved in silencing should result in de-repression of promoter and hence the expression of GFP (Figure 1). Our screen identified seven hits out of which, KAT5/Tip60 was particularly interesting due to its role as an acetyltransferase [19]. Based on earlier reports and our preliminary experiments, we were able to suggest that KAT5/Tip60 positively regulates silencing by maintaining the patterns of histone H4 acetylation.

2. Materials and Methods

2.1. Cell lines and cell culture

Genetically modified mouse mammary adenocarcinoma, referred to as C127 was used for screening and analysis (kindly provided by Dr. Elisabeth Martinez). These cells contained a GFP reporter transgene under the control of CMV promoter that has been silenced. De-repression of CMV promoter and hence expression of GFP was observed after treatment with known modulators such as Trichostatin-A (TSA). The integration of GFP was a single insertion at Chromosome 4 (Dr. Elisabeth Martinez, personal communication). The methodology for the production of this cell-line has been explained in [20].

The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies) containing 1% v/v Penicillin/Streptomycin with 10% Fetal Calf Serum (FCS). The GFP leakage was maintained to be less than 5% and was checked during each experiment.

2.2. Lentiviral particles

Mission® shRNA lentiviral particles were purchased from Sigma-Aldrich. The targets of interest for screening were chosen based on previous implications, role in cancer, homologs and presence of putative domains that can bind to different modifications. Table 1 gives the list of genes that were chosen for screening while their corresponding shRNA sequences are given in Supplementary S1 and plasmid map in Supplementary S2.

2.3. Fluorescence-activated cell sorting (FACS)

GFP readout was analyzed by FACS. Cells were collected by trypsinization and re-suspended in PBS and analyzed using BD LSRSorter™ and BD FACSDiva™ software (BD Biosciences).
Table 1. List of genes that were chosen for the screening.

<table>
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<th>Targets of interest</th>
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<td>Wdhd1</td>
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<td>Brd2</td>
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<td>Parp2</td>
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<td>Chd4</td>
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2.4. RNAi screening

Multiplicity of infection (MOI), puromycin selection (Gibco® Life technologies) and polybrene (Millipore, CAT#TR-1003-G) concentration were assessed according to manufacturer’s protocol. Cells at a concentration of 20,000 cells/well (counted using Coulter counter) were seeded onto a 96-well flat bottom plate (Griener CELLSTAR®) and transducted according to manufacturer’s instructions. 48 hours after puromycin treatment, they were analysed by FACS. Tag-RFP shRNA (scrRNA in further contexts) was used as negative control and Trichostatin-A (TSA) at 4μM for 24 hours was used as positive GFP control. Once the candidate hits were identified, cell lines with stable knockdowns were obtained following manufacturer’s instructions.

2.5. RNA extraction and Quantitative real-time PCR (qRT-PCR)

RNA was extracted using TRIzol® reagent (Invitrogen) and its concentration measured using Qubit® 2.0 Fluorometer. RNA was treated with DNA-free™ kit to eliminate DNAse contamination (Ambion® Life Technologies). cDNA was synthesized using Promega Reverse Transcription following manufacturer’s protocol.

For qRT-PCR, LightCycler® 480 SYBR Green I Master (Roche) was used with LightCycler® 480 Instrument II (Roche). Primer sequences and the program followed are given in Supplementary S3.

2.6. Immunofluorescence (IF) staining and analysis

Cells cultured on a coverslip were fixed with 1% Paraformaldehyde (PFA) for 10min at room temperature (RT) followed by permeabilization with 0.2% Triton X-100 for 10min at RT and blocked using 5% donkey serum in PBS for 15min in a humidified chamber. This was followed by primary and secondary antibody incubation for 1hr each with PBS washes between them. H3K9me3 (a gift from Hiroshi Kimura) mouse monoclonal antibody at 1:100 dilution with PBS was used as the primary antibody while Cy5 (Invitrogen) at 1:500 dilution with PBS was used as secondary antibody.
Vectashield® (Vector laboratories) was used as mounting medium and DAPI was used to visualize the nuclei.

Images were taken using Zeiss Axioplan 2 microscope at 100x (Zeiss Plan-APOCHROMAT 100x/1.4 oil DIC) using iVision software and Zeiss observer Z1 microscope at 10x (Zeiss FLUAR 10x/0.50) using MetaMorph v7.8.8.0 (Molecular Devices). CellProfiler v2.1.1 [21] was used for analysing the images. The intensities were calculated at 10x magnification by taking the average at 5 different positions with a minimum of 200 cells each.

2.7. Extraction of histones

Cells were cultured on a 147mm dish and scraped in PBS when they reach 95–100% confluency. Histones were extracted using acid extraction method followed in [22].

2.8. Western blot

10μg of histones were run on 10–20% Novex® Tricine gel and transferred on to a 0.20μm PVDF membrane (GE Healthcare Life Sciences) using Trans-Blot® SD Semi-Dry Transfer cell (Bio-Rad) following manufacturer’s protocols (Buffer composition: Supplementary S4).

The following monoclonal histone antibodies (kind gift from Hiroshi Kimura) were used at 1:1000 dilution: i) H3K9me3 (CMA318); ii) H4K5Ac (200Nb 4A7); iii) H4K8Ac (200Nd 53B2); iv) H4K12Ac (200Nf 50B3); and v) H4K16Ac (200f 27F10). Histone H3 antibody (ab1791, Abcam) was used as a loading control. The secondary antibody was Goat anti-mouse HRP conjugate (Thermo scientific). NuGlo ECL substrate (Alpha Diagnostics International) was used followed by detection using LI-COR Odyssey® Fc system. Densitometric analysis was performed using Image Studio™ software v3.1 (LI-COR Biosciences).

2.9. Statistical tests

All experiments were done in triplicates, except Western blot which was done in duplicate. Screening data was log transformed and one-tailed t-test was performed to identify the significant hits. A 50% quartile cut-off for number and percentage of GFP cells was also calculated on the screening data. One-tailed Student’s t-test was performed for qPCR and Western blot data. All statistical calculations and graphs were obtained using GraphPad PRISM® v6.

3. Results

3.1. KAT5, a candidate hit from the screening

RNAi screen was set up and optimization was performed (Materials and Methods) to score the GFP de-repression 96 hours post-transduction. Initial experiments were carried out with scrRNA to determine the optimum conditions for transduction (Supplementary S5). FACS data for optimization are given in Supplementary S6. The best condition chosen was MOI of 2.0 with polybrene concentration of 8 μg/ml and serum-free media with puromycin concentration of 2 μg/ml, where a transduction efficiency of 65% was achieved. Once the conditions for transduction were optimized,
RNAi screening was performed with selected factors given in Table 1. The screening was performed in triplicates and the percentage of GFP expressing cells was assessed by FACS. In order to be considered a candidate hit, three conditions were followed: i) Number and percentage of GFP cells should pass the median quartile cut-off; ii) Statistical significance based on one-tailed t-test; iii) At least two shRNAs per gene should pass the above conditions. As can be observed from Figure 2, 7 genes were obtained as candidate hits from the screening: Chd4, G9a, H2afy, Kat5, Prdm16, Tal1 and Tdrd3. Details of candidates and the respective p-values are given in Supplementary S7 while graph of GFP percentages of other shRNAs from screening are given in the Supplementary S8.

Some of the genes such as Chd4, G9a, H2afy and Prdm16 have well described roles in epigenetic silencing. However, Kat5 was particularly interesting because of its role as an acetyltransferase. FACS data of GFP from Kat5 knockdown cells from screening is given in Supplementary S9. In addition to Kat5 knockdown cell line, cell lines with Dnmt1, Hdac1 and Suv39h1 knockdowns were made to compare the GFP de-repression. Strikingly, Figure 3, shows that Kat5 knockdown cells have higher percentage of GFP+ cells than the knockdowns of known silencing factors. We then checked for the efficiency of Kat5 knockdown using qPCR and observed a 50% knockdown at the mRNA level (Figure 4a). Furthermore, we were also able detect more GFP expression in Kat5 knockdown cells at mRNA level as compared to negative control (Figure 4b).

![Figure 2](image_url)

Figure 2. Graph showing percentage of GFP expressing cells for candidate hits obtained from RNAi screening following the above mentioned conditions. Error bars and asterisks indicate the SEM and significance level based on one-tailed t-test respectively. Screening was done in 3 independent biological replicates. [* − p ≤ 0.05; ** − p ≤ 0.01] (GFP control—TSA treated; Negative control—TagRFP shRNA)
Figure 3. Graph showing percentage of GFP expressing cells for Kat5, Dnmt1, Hdac1 and Suv39h1 knockdown cell lines. Error bars and asterisks indicate the SEM and significance level based on one-tailed t-test respectively. Samples were averaged over triplicates [∗p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001] (GFP control—TSA treated; Negative control—TagRFP shRNA).

Figure 4. Partial knockdown of Kat5 leads to alleviation of GFP silencing. a) qPCR data showing the fold change for Kat5 expression; b) qPCR data showing the fold change for GFP expression. Error bars and asterisks indicate the SEM and significance level based on one-tailed t-test respectively. 4 replicates were used in both cases and expression level was normalized to that of β-actin [∗p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001]
3.2. Distribution of H3K9me3 was unaltered in Kat5 knockdown cells

We then wanted to characterize the role of Kat5 in silencing. We first asked if the distribution of chromocenters or the classic hallmark of silencing, H3K9me3, could be altered. We performed microscopy at 100x magnification to visualize the distribution of H3K9me3, and the DAPI stained chromocenters. We observed that the loss of Kat5 did not have an apparent effect on the distribution of H3K9me3 (Figure 5a). In addition, we imaged the cells at 10x magnification and calculated the intensity of H3K9me3. Indeed, differences were not statistically significant (Figure 5b) further confirming that the loss of silencing does not occur from reduction of H3K9me3 levels and apparent heterochromatin structure. Furthermore, this observation was validated by Western blot which indicated that the levels of H3K9me3 remain unchanged by Kat5 knockdown (Figure 6a and b).

3.3. Kat5 knockdown cells showed reduced levels of histone H4 acetylation

Earlier reports in yeast suggested that acetylation patterns of specific residues in H4 were important for maintenance of silencing at heterochromatin and telomeres [23,24]. Since Kat5 can acetylate 4 lysine residues in H4 tail, namely, H4K5, -K8, -K12 and -K16 [19], we hypothesized that loss of Kat5 can modify the patterns of H4 acetylation and hence could disrupt the silencing at the CMV promoter thereby resulting in GFP expression. We extracted histones from Kat5 knockdown and scrRNA cell lines and performed Western blot for 5 different modifications: i) H3K9me3, ii) H4K5ac; iii) H4K8ac; iv) H4K12ac and v) H4K16ac, with total histone H3 as loading control. We were able to validate that H3K9me3 was unaltered supporting the results from microscopy. Additionally, we found that acetylation levels in Kat5 knockdown cells were lower than those in scrRNA-transduced cells (Figure 6a). Densitometric analysis was also performed by normalizing the intensities to that of H3 (Figure 6b) and the reduction in H4K16ac was observed to be significant, which provides a credible outcome to our hypothesis mentioned earlier. Based on these results, we could suggest that Kat5 might regulate silencing by maintaining the patterns of H4 acetylation.

4. Discussion

Our use of a mouse cell based system with a silenced GFP transgene provided a convenient model of epigenetically silenced locus in a mammalian system similar to that of the adenine or uracil marker in yeast or position-effect variegation in fruit flies. As a result, our study identified seven factors, Prdm16, Kat5, Tal1, Tdrd3, G9a, Chd4 and H2afy some of which have already been characterized for their roles in epigenetic silencing, which indeed validates our screening. Our study also identified two novel factors, TDRD3 and KAT5, whose role in silencing have been implicated before but reports on their mechanisms of action have not been well described. TDRD3 is a co-transcriptional activator which acts as an effector molecule in recognizing histone arginine methylation marks deposited by CARM and PRMT1 [25]. The only implication of its role in silencing is an indirect mechanism through its involvement in piRNA biogenesis, although the mechanism is not yet known [26]. We were particularly interested in studying the role of KAT5, specifically because it is an acetyltransferase. KAT5 is a histone lysine (K) acetyltransferase belonging to the MYST family that acetylates H4 at -K5, -K8, -K12, -K16; H3 at K14 and H2A at -K4 [19]. It is one of the components of human NuA4 complex [27] and has nucleosome binding
domain which aids in the formation of complexes involved in acetylation at nucleosomes [28]. In addition, it has been well studied for its role in DNA damage response through its interaction with various kinases. Most importantly, mutation of KAT5 has been linked to various cancers [28].

![Figure 5](image)

**Figure 5.** Overall heterochromatin structure and H3K9 trimethylation is not affected by Kat5 knockdown. a) H3K9me3 staining and DAPI used for visualization of chromocenter distribution. Knockdown and Negative control: Cells with and without Kat5 knockdown; b) Box-plot of H3K9me3 intensities between the TagRFP shRNA (negative control) and Kat5 knockdown cells (ns—not significant). Images were taken at 10x magnification at 5 different positions each having at least 200 cells and analysed using CellProfiler v2.1.1. Whiskers indicate the maximum and minimum quartiles.
Figure 6. Analysis of global levels of histone modifications in Kat5 knockdown. a) Western blot of different modifications at H4 and H3K9me3. Total H3 was used as a loading control. The intensities were calculated using ImageStudio™ v3.1; b) Densitometric analysis of Western blot with average intensity values from duplicates. Intensities were calculated using ImageStudio™ v3.1 and normalized to the intensities of total histone H3. Error bars and asterisks indicate the SEM and significance level based on one-tailed t-test respectively. [* \( p \leq 0.05; ** \( p \leq 0.01; \) ns—not significant].

Acetyltransferases, in general, have been associated with gene expression and transcriptional activation. However, our study identified Kat5 as a putative silencing factor. This supports earlier reports which suggest that Kat5 may be associated with HDAC7 and KLF4 by forming a repressive complex in regulating the promoter of HDC [29]. In addition, involvement of Kat5 in silencing the expression of viral genes by repressing EIA promoter through H4 acetylation and down regulation of...
Notch-IC via Kat5’s HAT activity have also been implicated. However, they have suggested that such acetylation of histones might be involved in recruitment of a repressive complex rather than a more direct outlook [30,31].

Reports suggested that Kat5 can bind to H3K9me3 [32]. We thus hypothesized that the level of H3K9me3 might be altered due to knockdown of Kat5, through which it might regulate silencing. However, we were able to observe no change in the distribution of H3K9me3 or any changes in the chromocenters between Kat5 knockdown and scrRNA cells. This was also confirmed by Western blot to assess the global levels of H3K9me3.

Studies by Braunstein et al., 1996, on *Saccharomyces cerevisiae* suggested that acetylation of H4 is very important for the maintenance of heterochromatin structure. They hypothesized that specific patterns of histone acetylation, especially at H4K5 and H4K12, play a significant role in silencing at heterochromatin. Besides, H4K12 acetylation is observed at higher level as compared to acetylations at other residues. The same pattern of acetylation was found at the centric heterochromatin of *Drosophila melanogaster*. Indeed, such patterns of acetylation were observed only at the transcriptionally silenced locus as compared to the active regions [23]. In addition, a study showed that loss of Hat1, also called as Kat1, in *Schizosaccharomyces pombe* resulted in the loss of silencing at Telomeres. Hat1 is member of MYST family of acetyltransferases, similar to Kat5, and is involved in acetylation of H4K12 and H4K5 [24]. Since Kat5 can acetylate 4 residues at histone H4, we hypothesized that Kat5 might be able to regulate silencing on a more direct context by maintaining the patterns of histone acetylation. Based on our results, where a reduction in the levels of acetylation was observed, it might be possible that Kat5 has a more direct role in silencing. This observation supports the results obtained by Gupta et al., where they observed that the H4 acetylation activity of Kat5 was important for silencing at EIA promoter locus [30]. Furthermore, the reduction at H4K16 was statistically significant. This is an important observation because, in the context of mammals, acetylation at H4K16 is abundant and plays an important role in transcription. It negatively regulates the formation of 30nm fiber in chromatin and circumvents the mobilization of nucleosomes by impeding the function of ACF enzyme and ATP-dependent chromatin remodelling (ADCR) activity [33] which explains its role as an activating mark. Studies also indicate that normal levels of acetylation at H4K16 in male *D. melanogaster* was linked to highly expressed X chromosome. Such evidences might indicate the reason for unacetylated lysine 16 in yeast in promoting silencing [34]. Further, [23], suggested that the role played by H4K12ac in heterochromatin maintenance in *S. cerevisiae* could be played by H4K16ac in the context of mammals and vice versa. The presence of a dedicated H4K16ac factor in mammals, MOF [35], adds credibility to the above hypothesis. Such patterns of histone acetylation might also exist as a synthetic lethality between H4K16 and H4K12 [23].

5. Conclusion

Our study using a modified mouse cell line hosting an epigenetically silenced GFP reporter provided interesting results, although they were preliminary. Our aim was to find novel silencing factors and try to understand their possible molecular function. We were able to point out few factors which might possess a role in silencing, using a system which was previously used in yeasts with tremendous success leading to functional identification of hundreds of novel chromatin-associated factors and other proteins. Indeed, this methodology can now be extended to a genome-wide
approach through RNAi or the recently developed CRISPR system. Our screening suggested Kat5 as a silencing protein with an insight into its putative molecular mechanism. However, further experiments such as RNA and ChIP sequencing will have to be performed in order to confirm its role at a global level and to identify the involvement of other factors, if any. Additionally, mutational studies of specific residues of Kat5 should prove the catalytic function of Kat5 in epigenetic silencing. Further analysis of the proteins implicated in silencing through this screening should reveal novel mechanism of action in chromatin, if any and studying them in a more comprehensive manner in the context of cancer would provide new perspectives to their potential as drug targets.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions Statements

AK and VL conceived the study; AK and GRB supervised the study; AVV, KM, and AK designed the experiments; AVV performed the experiments; AK, AVV and GRB analysed the data, AVV and AK wrote the manuscript; AK, GRB, VL, and KM made manuscript revisions.

References


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