DNA sequence features in the establishing of H3K27ac [version 1; referees: 2 approved]

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Abstract
The presence of H3K27me3 has been demonstrated to correlate with the CpG content. In this work, we tested whether H3K27ac has similar sequence preferences. We performed a translocation of DNA sequences with various properties into a beta globin locus to control for the local chromatin environment. We demonstrate that H3K27ac is not linked to CpG content of the sequence, while extremely high GC-content may contribute to the establishment of this mark.

Keywords
histone modification, H3K27ac, GC-content, CpG dinucleotides

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Introduction
Histone modification is a key mechanism of epigenetic regulation. Histone modification varies between cells in some genomic locations but not others. This observation raises the question to what extent histone modifications depend on the underlying nucleotide sequences. It has been reported that the attraction of PRC2 complex and consequent H3K27me3 is correlated with the local density of CpG dinucleotides. More complex sequence patterns, such as transcription factor (TF) binding sites (TFBS), also affect the presence of histone modifications; SUZ12, a member of the PRC2 complex, binds DNA in a sequence-specific manner; NRCF and ZBTB33 recruit histone deacetylase. The ENCODE project demonstrated a specific histone modification pattern around binding sites of many TFs.

Since the same lysine residue cannot be both methylated and acetylated, the presence of H3K27ac is negatively correlated with the presence of H3K27me3. Although there are several pieces of evidence showing that H3K27me3 is established at least partially in a sequence-specific manner, it is unclear if H3K27ac — an antagonistic activator to a repressive H3K27me3 — shares any sequence specific patterns. A computational approach predicted some TF to be linked to H3K27ac, yet the results were dependent on the background set. In this work, we perform a direct experiment to test whether a specific genomic sequence is capable of recovering H3K27ac.

Methods
Cas9 target selection and plasmid design
We selected a site for Cas9 in the intergenic region of human beta globin locus using services CCTop (hg38) and Off-Spotter (hg38). The chosen targeting sequence was CTTGTCCCTGCAGGGTATTA. Then we designed targeting oligonucleotides (listed in Table 2) for pSpCas9(BB)-2A-Puro (pX-459 plasmid, a gift from E.P.). Oligonucleotides Bgl1Cas_F and Bgl1Cas_R were diluted to 10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, heated and ligated into a plasmid with modified multiple cloning site containing ordered sites for BclI and XhoI.

Selection plasmid design
Selection cassette design (HSV thymidine kinase, 2A peptide, G418R in one frame) was performed with Ugene. HSVtk and NeoR sequences were PCR amplified with Phusion polymerase (Thermo Scientific) from pHSVTk-Neo (a gift from E.P.) with primers HSVtk_F, HSVtk_R, and G418_F, G418_R, correspondingly. T2A peptide coding sequence, corresponding to amino acid sequence GSGEGRGSLTCCGDDNPGP, was synthesized by hybridization of oligonucleotides T2A(+) and T2A(-) in annealing buffer. Hybridized duplex was treated with T4 PNK (Thermo Scientific) and gel purified. NeoR fragment was digested with XbaI (Thermo Scientific), T2A fragment was digested with NheI (Thermo Scientific), then these fragments were ligated and the fragment of expected size was gel purified. This T2A-NeoR fragment was digested with XhoI (Thermo Scientific), fragments were ligated and the fragment of predicted length was gel purified again. This HSVtk-T2A-NeoR fragment was double digested with EcoRI (Thermo Scientific) and BshTI (Thermo Scientific) and ligated with pX459-b1 double digested with EcoRI and BshTI. This step gave the plasmid with full length selection cassette called pHSVtk-T2A-G418R. Then plasmid pHSVtk-T2A-G148R was double digested with XbaI and Acc65I (Thermo Scientific), gel purified and ligated with hybridized AdaptUp(+) -AdaptUp(-) duplex. A successful insert was verified by digestion of newly introduced restriction sites: Sall and BamHI. This plasmid was called pAdaptUp-HSVtk-T2A-G148R. This plasmid was digested with NotI (Thermo Scientific), treated with FastAP (Thermo Scientific), gel purified and ligated with AdaptDown(+) -AdaptDown(-) duplex to introduce 2xBpiI site that generate half-sites for BclI and XhoI after cleavage. Resulting plasmid was transformed into E. coli Top-10 cells and called pAdaptUp-HSVtk-T2A-G418R-AdaptDown. To obtain LoxP-flanked sequences, we used a pBK-CMV-derived plasmid with modified multiple cloning site containing ordered sites for BclI, NheI, and XhoI. This plasmid was transformed into E. coliJM110 to eradicade Dam methylation, double digested with BclI and NheI, dephosphorylated, gel purified and ligated with LoxP(+) -LoxP(-) duplex, treated with T4 PNK. This plasmid was transformed into E. coli Top10 cells and called pLOXp. To obtain homology regions that flank Cas9 cleavage site we PCR amplified them from Caki1 gDNA, using primer pairs Bgl1Up_F and Bgl1Up_R for an upstream fragment and Bgl1Down_F and Bgl1Down_R for a downstream fragment. These PCR fragments were double digested with NheI and XhoI and ligated with pLoxP, double digested by the same sites and dephosphorylated. Ligation products were transformed into E. coli JM110, and purified plasmids were digested with BclI and XhoI to yield upstream and downstream fragments of DNA bearing LoxP site on its end. Upstream fragment was ligated with pAdaptUp-HSVtk-T2A-G148R-AdaptDown double digested with Sall and BamHI to give pUp1L-HSVtk-2a-G418R-AdaptDown. Downstream fragment was ligated with pUp1L-HSVtk-2a-G418R-AdaptDown treated with BpiI and dephosphorylated. This step brought us pUp1L-HSVtk-2a-G418R-L1Down.

Stably transfected cell line generation
We co-transfected Caki1 cells with pX459-b1 and pUp1L-HSVtk-2a-G418R-L1Down using Lipofectamine 3000 (Thermo Scientific) in 2 cm² wells following manufacturers instructions. After one week of Puromycin (3 µg/ml) selection, cells were split into 96-well plates and selected with 1 mg/ml G418 for two weeks. Cells from successfully growing clones were split into two equal aliquots, one for growth and another for genomic DNA isolation. Clones were checked for presence of insert with primer
pair BGL1pcr_F - BGL1pcr_R that surround the insertion site. One clone (called Caki1-GcvS-G418R) with homozygous insertion was selected for further work.

Construction of recombination target
Plasmid pBK-CMV was digested with SacI and HpaI, blunted with T4 polymerase, self-ligated, transformed into E. coli Top10 and called pBKCMVdHpaSacI. LoxP(+) oligo was PNK treated and annealed with LoxP(-) to form a duplex. Bait(+) and Bait(-) oligos were annealed and ligated with hemi-phosphorylated LoxP duplex. Ligation products were resolved on 3% agarose gel, the longest fragment was excised and purified with QiAquick gel extraction kit, treated with T4 PNK and ligated to NheI treated and dephosphorylated pBKCMVdHpaSacI. Ligation product was transformed into E. coli Top10 and called p1L-bait-L1. Ten sequences chosen to be insertion targets were PCR amplified from genomic DNA of Caki1 cells with primer pairs 01_Hi_Ac_F...10_No_Ac_GC53_R. Amplicons were gel purified, diluted to a concentration of 100nM, treated with T4 PNK and ligated with a plasmid p1L-bait-L1 treated with Ecl136II and dephosphorylated. Then library of ligation products was transformed into E. coli Top10, plasmid library was purified using plasmid mini kit (Evrogen, cat. no. BC021) and co-transfected with pBS598 EF1alpha-EGFPcre (Addgene) to Caki1-GcvS-G418R cells for Cre-mediated recombination exchange of the insertion. After 3-day growth Ganciclovir (2µM) was added to eliminate cells that did not undergo recombination. After selection for 10–14 days survived cells were grown to subconfluent monolayer in 10cm dishes and then ChIP on H3K27Ac was performed.

ChIP
ChIP was performed according to Abcam X-chip protocol with the following modification: we increased the number of washes in High Salt buffer from one to three times. Sonication was performed in PCR tubes (SSIbio, cat. no. 3245-00) on ice using Sonics vibra cell VCX 130 with an eight-element probe (cat. no. 630-0602). Sonication setup was: 10s pulse, 20s pause, 75% power, total sonication time is 30min. We used 2µg of Anti-H3K27Ac antibody (Abcam, ab4729) per ChIP. Control ChIP was performed in the same conditions with Caki1 cells.

Nested PCR
After ChIP 1ng of obtained DNA fragments was PCR amplified with primer pair BaitF - BaitR. At this step we amplified all DNA sequences inserted between this primer pair. Then 10ng of PCR amplicons from the first step was used as a template for a PCR with primer pairs 01_Hi_Ac_FUBP1_F...10_No_Ac_GC53_R, each pair in an individual reaction tube.

Results and discussion
None of the GC- and CpG rich promoter regions, that were acetylated in their original genomic loci (rows 1–3 in Table 1 and lanes 1–3 in Figure 1) recovered H3K27ac after relocation to a foreign genomic context in the beta globin locus, suggesting that H3K27ac may not depend directly on such features. An alternative explanation is that some of the CpG dinucleotides became methylated in a foreign genomic environment. Surprisingly, two extremely GC-rich but CpG poor (and, therefore, unmethylated) sequences (rows 5, 6 in Table 1 and lanes 5, 7 in Figure 1) gained H3K27ac in the foreign environment, while in their native environment (in their original genomic location) they had no H3K27ac. Sequences 5 and 6 are located far from promoters of known genes. Sequence 5 contains a lowly expressed CAGE cluster, representing a weak alternative promoter of the KMT2D gene (ZENBU). Therefore, we conclude that the gain of H3K27ac in these regions is unlikely to be explained by transcriptional activity. The lack of H3K27ac in sequence 6 in the native location may be due to the presence of the antagonistic mark H3K27me3, which is lost after translocation to the foreign

| Sample number | Genome location, hg38 | Gene name | Length, bp | GC, % | CpGs | H3K27Ac, native location | H3K27ac, foreign location | H3K27Me3, native location |
|---------------|-----------------------|-----------|------------|-------|------|------------------------|--------------------------|-------------------------|
| 1             | chr1:62435851-62436130 | USP1      | 280        | 59.6  | 15   | yes                    | no                       | no                      |
| 2             | chr1:77979434-77979693 | FUBP1     | 260        | 58    | 27   | yes                    | yes                      | no                      |
| 3             | chr:86913854-86914119 | HS2ST1    | 266        | 56.4  | 16   | yes                    | no                       | no                      |
| 4             | chr:63473349-63473556 | -         | 208        | 65    | 1    | no                     | yes                      | yes                     |
| 5             | chr:49033153-49033412 | -         | 260        | 66.6  | 1    | no                     | yes                      | no                      |
| 6             | chr:81127213-81127450 | -         | 238        | 67.7  | 2    | yes                    | yes                      | yes                     |
| 7             | chr:227381301-227381590| -         | 290        | 26.6  | 1    | no                     | no                       | no                      |
| 8             | chr:108505575-108505854 | -         | 280        | 37.5  | 0    | no                     | no                       | no                      |
| 9             | chr:50816997-50817284 | -         | 288        | 51.7  | 0    | yes (only GM12878)     | no                       | yes (in some cells)     |
| 10            | chr:X:48988617-48988801 | -         | 185        | 52    | 1    | no                     | no                       | no                      |
| Name            | Sequence                                                                 |
|-----------------|--------------------------------------------------------------------------|
| Bgl1Cas_F       | CACCGCTTGTCCCTGCAGGGGTATTA                                               |
| Bgl1Cas_R       | AAACTAATACCCGTAGGGGACAGACAG                                             |
| HSVtk_F         | AATTACCCTGGTATGGCTCTGTACACCCCTGC                                        |
| HSVtk_R         | AATTTGTCACGCTTATGCTCCCTGACGTCACCAGGTAGTCAGACGAG                         |
| T2A(+)          | AAAGCTCGAGGGCAGCTGGGAGGGGAGGGGAGGAGGCTGAACTATGCGGTG                        |
| T2A(-)          | ATTCGCTAGCTGGCAGGTTCAGATTCCTCCGTAGGATAGTCAGACGAG                         |
| G418R_F         | ATTATCTAGAATGAAAGATGGAGATTGC                                              |
| G418R_R         | TAATGAAATTCTCAGAGAACACTCTCGAAGAGG                                       |
| AdaptUp(+)      | CTAGCAGTCTCGACTTAAAGGATCC                                              |
| AdaptUp(-)      | GTACATGGATCTTTAGCAGCT                                                   |
| AdaptDown(+)    | GGCCTGCGATCTGCTTCTAAGACAGGAGCAGT                                        |
| AdaptDown(-)    | GGCCGCTGAGCTTCTTTAGGAGACAGGAGCAGT                                       |
| LoxP(+)         | CTAGCATAACTTCGTATAATGTGCTATAGCCAAGAGTTAT                                  |
| LoxP(-)         | GATCAAATATCTCAGATGAGGACATTGAGAAGATG                                       |
| Bgl1Up_F        | TCCACTCGAGACCTGGAAACCCATGTCG                                            |
| Bgl1Up_R        | GAATGCTAGCTCTCGTTACACTCTAGCTCAC                                             |
| Bgl1Down_F      | CATCGCTAGCCAGCCAGCATACCCAGGCAATAAG                                      |
| Bgl1_Down_R     | TGCTCTCGAGAAATGGACAGCAGCCAC                                            |
| BGL1pcr_F       | TGCTGACGATACCATCTACCC                                                 |
| BGL1pcr_R       | GTGAAATACGACCTGAGCCT                                                     |
| Bait(+)         | GATCACACTCCTCGAAGGATGAGAGAG                                             |
| Bait(-)         | GATCACCAGAAGGTGCTCTAGGAGAGAGAGG                                         |
| Bait-seq_F      | CACACTCCTCTCGAAGGATGAGAGG                                             |
| Bait-seq_R      | CCACACTCCTCTCGAAGGATGAGAGG                                             |
| 01_Hi_Ac_FUBP1_F | CCGCTATGGGCTATACCTCAG                                                  |
| 01_Hi_Ac_FUBP1_R | ATTAAACCCACCCCTCAGG                                                  |
| 02_Hi_Ac_USP1_F | GGCTGAAACGTATCCCTGCTAAG                                              |
| 02_Hi_Ac_USP1_R | GCAGATGCTGTAGAGGAGGAG                                                  |
| 03_Hi_Ac_HS2ST1_F | GGGGAGAAGAGGAGAGGATCAACC                             |
| 03_Hi_Ac_HS2ST1_R | TGGGAAATGGTGATGCGAT                                               |
| 04_No_Ac_GC66_F | GCCAGTAAGGACCACCTCAGGAC                                              |
| 04_No_Ac_GC66_R | GCTAATACGTCGACGCCC                                                   |
| 05_No_Ac_GC67_F | CTGCTGCCACAGATGAGGCCG                                               |
| 05_No_Ac_GC67_R | ACAAAACAGCTCGTGAGGCCG                                               |
| 06_No_Ac_GC68_F | CCCCATGCGAACAAAGCAAG                                                    |
| 06_No_Ac_GC68_R | CCCACCACCTCCAGAGGAG                                                   |
| 07_No_Ac_GC28_F | CTGCGAGAGGATGGGTAGGGCAAG                                              |
| 07_No_Ac_GC28_R | GGCTGAGAGGATGGGTAGGGCAAG                                              |
| 08_No_Ac_GC39_F | AGGTAAGTACAACCATCCACCTCCAC                                             |
| 08_No_Ac_GC39_R | AAATCCCTTTATCACCACCCACC                                               |
| 09_No_Ac_GC51_F | TGAGGTAGTACCTCGTGAGGGGAGG                                              |
| 09_No_Ac_GC51_R | ACAACCCCAAGTCTGACCA                                                   |
| 10_No_Ac_GC53_F | TTCAGACACACTCATCTCCAC                                                   |
| 10_No_Ac_GC53_R | GGTTAAGGATGGTCTGAGATTCC                                                |
environment. Yet, for sequence 5, H3K27me3 is absent in all cell types reported in ENCODE. We conclude that the establishment of H3K27ac is not dependent on a CpG content of the sequence per se, as opposed to its antagonistic mark H3K27me3, while it might depend on an extremely high GC-content.

**Data availability**

Dataset 1: Gel image of amplification of target sequences from native genomic loci compared with amplification from beta-globin locus with or without ChIP on H3K27ac. DOI, 10.5256/f1000research.13441.d192774

**Competing interests**

No competing interests were disclosed

**Grant information**

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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The study is focused on question of relation of primary genomic sequences and H3K27 acetylation preferences. Original method, based on the translocation of the different CG% and CpG -based sequences to the same genomic locus, followed by H3K27ac analysis was employed. The results suggest that that the context is more important than GC or CpG properties of the genomic sequence itself. The results of certain interest for the wide pool of researcher working with epigenetic aspects. At the same time, I believe that involvement of more techniques, such as Chip-Seq would be suggested to confirm the results. Also, it is not clear from the text how many replicates were taken and how general is the conclusion, if, for example, different genomic loci (or different genome, for example extremely GC or AT-rich one) will be used.

Is the work clearly and accurately presented and does it cite the current literature? Yes

Is the study design appropriate and is the work technically sound? Yes

Are sufficient details of methods and analysis provided to allow replication by others? Yes

If applicable, is the statistical analysis and its interpretation appropriate? Yes

Are all the source data underlying the results available to ensure full reproducibility? Partly

Are the conclusions drawn adequately supported by the results? Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
Vasily N Aushev

Department of Environmental Medicine and Public Health, Icahn School of Medicine at Mount Sinai, New York City, NY, USA

This study aimed to test whether H3K27 acetylation depends on the properties of the target sequence itself, or rather defined by the surrounding genomic context. To assess this question, authors translocated sequences with different CG% and CpG content, to the same beta globin locus, and then tested their H3K27ac in the new location.

As three tested highCG-highCpG sequences lost their H3K27ac in the new location, authors conclude that the context is more important than GC or CpG properties of the sequence itself. At the same time, two other sequences actually acquire H3K27ac in the same new location, and only one of those can be possibly explained by the antagonistic H3K27me3 mark.

Authors describe their methods with all the details which should be sufficient for reproducibility of their main results. I had, however, some minor technical questions regarding their plasmids construction procedure. For example: 1) From the sequence of T2A oligos they provide, peptide sequence seems to be PSPLPSPCCLTCDVEENPGP, not GSGGRGSSLTCGDVEENPGP. The latter one would be present in the original pX459 plasmid, but not in the constructed one. 2) To my understanding, annealing of T2A(+) and T2A(-) oligos will produce a hybrid with only 18bp double-stranded overlap - for further digestion with NheI, its single-stranded ends need to be built up with some polymerase; I don't see this step in the Methods. 3) I don't see XhoI site in the T2A-NeoR fragment, and don't see any other potential site compatible with SalI of HSVtk fragment. In addition, it is recommended that all the source plasmids used should be properly referred - either by their original publication, or by the sequence reference in any available repository (Genbank, Addgene, etc); referring as "pHSVTK-Neo (a gift from E.P.)" is not sufficient if the plasmid's sequence cannot be explicitly found by that name. Finally, authors should double-check their text for typos in the names of all constructs and genes (for ex., GAPGH), be sure to use all the titles exactly as they are originally defined (for ex., PX459 instead of pX-459, etc). All these issues, however, are minor and do not distort the main content of the paper.

From the technical point of view, the only question I had is about the final readout for H3K27ac: authors perform nested PCR followed by visualization of the PCR products on the gel. I am wondering this method was chosen and not qPCR (as more quantitative) or ChIP-seq (as more unbiased).

One of my main concerns is about the reproducibility of the findings: authors do not report any biological or technical replicates which makes the results potentially questionable.

Talking about the interpretation of the results, I am not sure how strong can be the conclusion based on only 10 tested sequences, and even more so, only one genomic context. Even with this limited number of tests, some results cannot be easily explained (for example, acquired H3K27ac for sample #5), so in future it would be necessary to expand the panel of both sequences and contexts.

Besides above-mentioned questions (hopefully authors can address at least some of them in the revised version), I think the paper is clearly of scientific interest and adds important piece of knowledge to its field.
Is the work clearly and accurately presented and does it cite the current literature? Yes

Is the study design appropriate and is the work technically sound? Yes

Are sufficient details of methods and analysis provided to allow replication by others? Yes

If applicable, is the statistical analysis and its interpretation appropriate? Not applicable

Are all the source data underlying the results available to ensure full reproducibility? Yes

Are the conclusions drawn adequately supported by the results? Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: Cancer biomarkers, oncogenesis, signal transduction, miRNA regulation

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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