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Award Number:
W81XWH-09-1-0297

TITLE:
Identification of Estrogen Receptor Beta Binding Sites in the
Human Genomes

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REPORT DATE:
April 2010

TYPE OF REPORT:
Annual summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

X Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE					Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-4-2010		2. REPORT TYPE ANNUAL SUMMARY			3. DATES COVERED (From - To) 1 April 2009 - 31 March 2010	
4. TITLE AND SUBTITLE Identification of Estrogen Receptor Beta Binding Sites in the Human Genomes					5a. CONTRACT NUMBER W81XWH-09-1-0297	
					5b. GRANT NUMBER	
					5c. PROGRAM ELEMENT NUMBER	
					5d. PROJECT NUMBER	
6. AUTHOR(S) Thien Le Email: lethien@uchicago.edu					5e. TASK NUMBER	
					5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) UNIVERSITY OF CHICAGO, THE 5801 S ELLIS AVE CHICAGO IL 60637-5418					8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. ARMY MEDICAL RESEARCH AND MATERIEL COMMAND FORT DETRICK, MARYLAND 21702-5012					10. SPONSOR/MONITOR'S ACRONYM(S)	
					11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited						
13. SUPPLEMENTARY NOTES						
14. ABSTRACT With the recent discovery of ERbeta, the role of estrogens in the prostate becomes more complex and interesting. We hypothesize that ERbeta plays an essential role in the development and progression of prostate cancer. The major goal of this study is to use ChIP-seq to map ERbeta genome-wide binding sites and identify its target genes. We have successfully generated three cell lines stably expressing ERbeta as a FLAG-tagged fusion protein which are used to study the genomic functions of this steroid receptor. So far, the mapping of ERbeta was optimized in MCF7-C4-12-ERbeta. This provided the optimal conditions for our next step, which is mapping ERbeta binding sites in a prostate cancer cell line stably expressing this protein. Within the human genome, where ERbeta binds to and how it functions there will provide a better understanding of ERbeta genomic role in the normal and cancerous prostate. Such finding will undoubtedly provide considerable contribution to our knowledge of the complex interactions between androgen receptor, estrogen receptors, and possibly other proteins involved in the development of the prostate.						
15. SUBJECT TERMS Genome-wide mapping; estrogen receptor beta						
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT		18. NUMBER OF PAGES	
a. REPORT	b. ABSTRACT	c. THIS PAGE			19a. NAME OF RESPONSIBLE PERSON USAMRMC	
U	U	U	UU		19b. TELEPHONE NUMBER (Include area code)	
					17	

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INTRODUCTION

Beside being the major target of androgens, the prostate is also influenced by estrogens. In the adult male, 17-beta-estradiol (E2), classically considered the female sex hormone, is mainly produced by adipose tissue, adrenal glands, testicles and the prostate¹. On the cellular and molecular level, E2 mainly exerts its effect via the two estrogen receptors, ERalpha and ERbeta. ERalpha, as a marker for breast cancer, has been heavily studied, leading to a myriad of improvement in breast cancer treatment and prevention². On the other hand, the functions of ERbeta, the recently-discovered subtype, remain largely elusive if not controversial. Interestingly, ERbeta knock-out mice were reported to develop prostatic hyperplasia at late age³. Moreover, different lines of studies have suggested that ERbeta played an important role in anti-proliferation⁴, immunoprotection⁵, and detoxification in the prostate⁶. Therefore, ERbeta strikes as an important component in prostatic normal development as well as tumorigenesis. Because ERbeta regulates gene expression mainly at the transcription level, the proposed study is designed to map ERbeta genome-wide binding sites in a cell-based model for prostate cancer using ChIP-seq technology. In conjunction with detecting active promoters, marked by H3K4me3⁷, our results will provide a better knowledge of ERbeta target genes as well as how ERbeta regulates their expressions on the global scale. Ultimately, this will give rise to a better model to study this nuclear receptor.

BODY

Aim 1 – To identify genome-wide ERbeta binding sites

The lack of a reliable antibody for ERbeta has led to many controversies surrounding the presence and functions of this steroid⁸. Due to the same reason, detecting ERbeta binding sites, via chromatin immunoprecipitation (ChIP) assays, has also been delayed. Therefore, we propose to generate cancer cell lines stably expressing ERbeta as a FLAG-tagged fusion protein (FLAG-ERbeta). Since specific and reliable antibodies raised against the FLAG are broadly available, the fusion protein will be efficiently immunoprecipitated in ChIP conditions.

In order to map ERbeta genome-wide binding sites in a non-biased manner, we choose ChIP followed by massive parallel sequencing (ChIP-seq) as our main approach. This is because, in the last couple years, this technology has been widely proven to produce reliable data with higher resolution compared to the conventional ChIP-chip assay (ChIP followed by tiling microarray).

Also, in order to study ERbeta genomic functions without the interference of ERalpha, we wish to introduce the FLAG-ERbeta into two well-characterized prostate cancer cell lines LNCaP, PC3 (in which ERalpha is undetectable), and a breast cancer cell line MCF7-C4-12 (a derivative of the MCF7 cell line which does not express ERalpha).

Generate cell lines stably expressing FLAG-tagged ERbeta:

The DNA construct of the fusion protein was subcloned into a lentiviral vector. This vector carried a green fluorescent protein (GFP), which would served as a selective marker in the later step. Although ERbeta and GFP were expressed independently (due to two different promoters), they were integrated into the cell genome as a cassette. Therefore the expression of GFP in the cells could validate the integration of the FLAG-ERbeta into the cell genome. Following viral infections, cells were passaged a few times to assure stable expression, then sorted based on their GFP status. The sorting step was done twice to select for cells that had high level of DNA-to-genome integration. A representative data of the sorting step is presented in figure 1.

Due to a variety of reasons (low viral infection efficiency, low cell growth rates, etc.), we were not able to generate the prostate cancer cells stably expressing FLAG-ERbeta at first. However, the DNA construct of the fusion protein appeared to be integrated into the genome the MCF7-C4-12 cells at a much higher rate. The efficiency of viral infection could be ranked highest in MCF7-C4-12 cells, low in PC3 cells, and very low in LNCaP cells. Moreover, the MCF7-C4-12 cells grew at a very fast pace, which allowed generating material for the ChIP assay much more easily. Therefore, we used the MCF7-C4-12 cells stably expressing FLAG-ERbeta (C4-12-ERbeta) to optimize the condition for the ChIP-seq assay, while developing the prostate cancer cells stably expressing FLAG-ERbeta.

Mapping ERbeta genomic binding sites

C4-12-ERbeta cells were serum-starved for 24 hours before treated for 1 hour with 10nM estradiol (E2). Cells treated with the same volume of vehicle (ethanol) were used as control. The cells were crosslinked and proceeded through ChIP assay, in which anti-FLAG M2 antibody (Sigma-Aldrich) was used to pull down the FLAG-ERbeta. The immunoprecipitated DNA libraries were prepared according to the Illumina ChIP-seq library prep kit. Samples were then submitted to high throughput sequencing with Solexa technology at the IGSB High-throughput Genome Analysis Core (Argonne National Laboratory, IL).

As we proceeded through the ChIP-seq assay with the C4-12-ERbeta cells, our data confirmed the high efficiency of the optimized ChIP-seq conditions. We were also able to identify more than 5000 binding sites ERbeta binding sites in this breast cancer cell model at low stringency (5% FDR) and more than 3000 binding sites at high stringency (1% FDR). The global distribution of ERbeta binding sites at high stringency is presented in figure 2. Moreover, our motif analysis identified binding motifs of ERs, known ERbeta interactors as well as those of novel interactors (figure 2).

Recently, we have been successful in generating a PC3-ERbeta as well as an LNCaP-ERbeta, both of which stably expressed the FLAG-ERbeta as intended (figure 1 B and C). Novel ERbeta binding sites identified in the ChIP-seq assay using C4-12-ERbeta were also found enriched in PC3-ERbeta cells (Figure 3). However, the enrichment levels appeared to be lower than those seen in C4-12-ERbeta. This difference could be due to the epigenetic differences between cell types.

In progress

We are still in the process of validating the ChIP condition in the recently generated LNCaP-ERbeta cells. Because the LNCaP cell line is considered a better model for prostate cancer, we will proceed to ChIP-seq to map ERbeta genome-wide binding sites with the LNCaP-ERbeta cells. The experimental conditions as well as analysis, which were optimized using the C4-12-ERbeta cells, will be applied on these LNCaP-ERbeta cells. Our results will lead to a global view of ERbeta transcription activity in prostate cancer cells. Furthermore, analyzing ERbeta binding sites in breast cancer cells and prostate cancer cells will allow a comprehensive understanding on functions of this steroid receptor in different epigenomic contexts.

Aim 2: To identify genes directly regulated by ERbeta

In order to evaluate the functionalities of ERbeta binding sites, we first proposed to use gene expression microarray to identify genes regulated by ERbeta at different time points. However, using this approach, it would be difficult to link ERbeta binding events and its direct target genes. On the other hand, since we submitted the proposal, emerging evidence had showed that trimethylation of lysine 4 on histone 3 (H3K4me3) was a reliable marker for active promoters⁷, hence actively transcribed genes. Therefore, we now propose mapping this histone modification in conjunction with mapping ERbeta binding sites. Consequently,

actively transcribed genes will be detected at the same time of capturing ERbeta binding events.

Because of the favored characteristics of the C4-12-ERbeta cells (stated above), these cells were also used to optimize mapping H3K4me3 at 1 hour of E2 treatment (which was the same time point of capturing ERbeta binding events). This was also done with ChIP-seq, in which an antibody specifically against this histone modification (Millipore) was used. The binding profile of H3K4me3 around gene transcription start sites in our assay appeared to be bimodal, which was similar to reported binding profiles of H3K4me3 at active promoters⁷ (Figure 4). Therefore, we have successfully identified genes being actively transcribed at the same time of ERbeta binding events in this breast cancer cell model.

In progress

The experimental design and conditions, which were optimized using C4-12-ERbeta, will be applied on LNCaP-ERbeta to map H3K4me3 at 1 hour of E2 treatment. The combined analysis of ERbeta binding events and the concurrent transcribed genes will lead to the comprehensive understanding of ERbeta functionality in the genome.

Aim 3 - Confirmation of ERbeta target genes focusing on prostatic angiogenesis

Not initiated yet.

KEY RESEARCH ACCOMPLISHMENTS

- We have successfully generated three cell lines stably expressing FLAG-tagged ERbeta:
 - Prostate cancer cells: PC3-ERbeta and LNCaP-ERbeta
 - Breast cancer cells: MCF7-C4-12-ERbeta
- We have optimized the condition for mapping ERbeta genomic binding sites using MCF7-C4-12-ERbeta
 - We have validated novel ERbeta binding sites in MCF7-C4-12-ERbeta and PC3-ERbeta cells

REPORTABLE OUTCOMES

We have generated three cell lines stably expressing FLAG-ERbeta, which can be used in different assays to analyze ERbeta functions:

- o MCF7-C4-12-ERbeta
- o PC3-ERbeta
- o LNCaP-ERbeta

CONCLUSION

In summary, we have generated three different cell lines stably expressing ERbeta as a FLAG-tagged fusion protein. We have successfully used the breast cancer cells C4-12-ERbeta to optimize mapping ERbeta genomic binding sites as well as to identify novel binding regions of this steroid receptor in these cells. The combination of this finding with mapping H3K4me3, a marker for active promoters, has allowed us to comprehensively investigate ERbeta functions on a global scale. We will apply these optimized conditions to investigate ERbeta genomic functions in our recently generated LNCaP-ERbeta cells. The overall results will undoubtedly lead to a better understanding of ERbeta functions in different epigenomic contexts. Consequently, this will shed more lights on the role of ERbeta in the normal development and tumorigenesis in the breast and prostate.

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SUPPORTING DATA

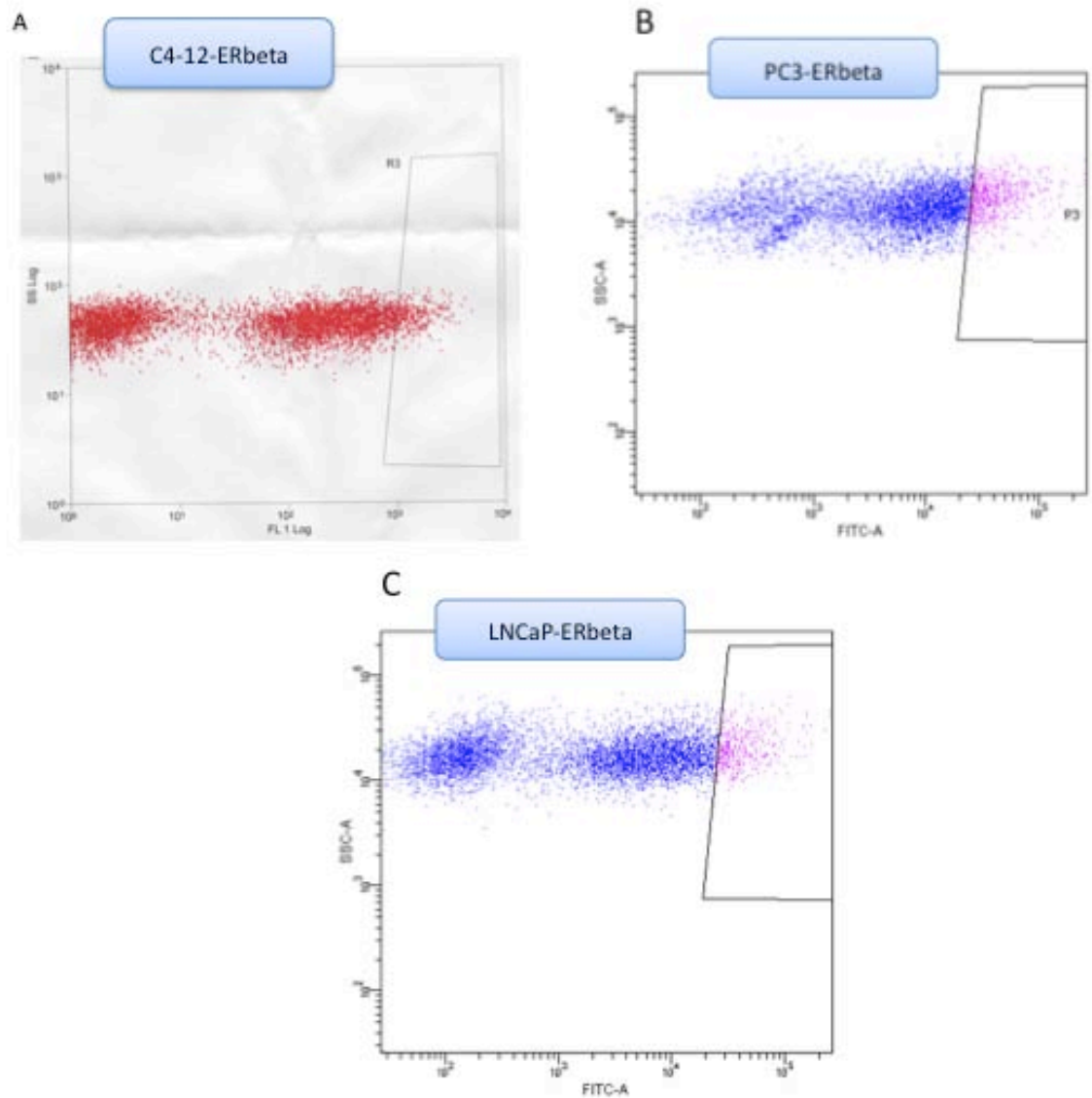
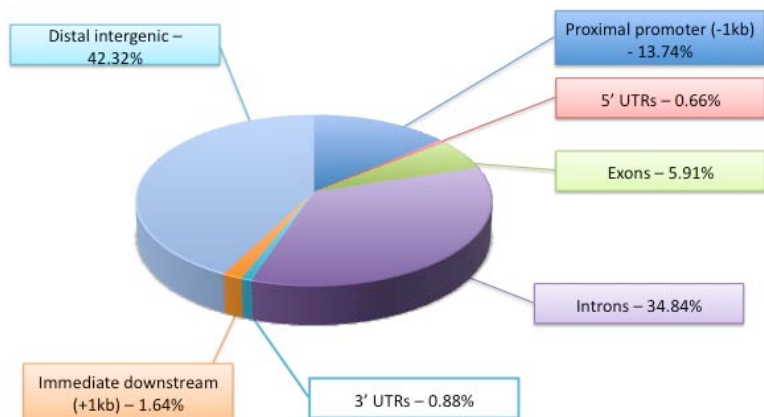


Figure 1 – Representative data of sorted GFP positive cells.
The top 5% of GFP positive cells (boxed events) were selected from the pool of cells infected with the lentiviral vector carrying FLAG-ERbeta and an independently transcribed GFP.

(A) – C4-12-ERbeta, (B) – PC3-ERbeta, (C) – LNCaP-ERbeta

A.



B.

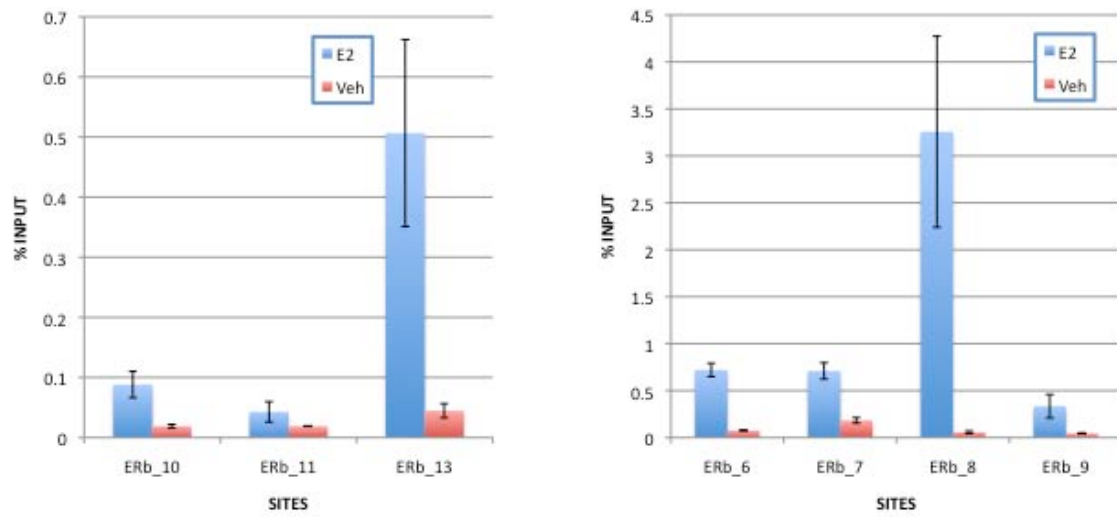


Figure 2 – Analysis of ERbeta genome-wide binding sites in C4-12-ERbeta cells.

(A) Global distribution analysis of ERbeta revealed that 13.74% of binding sites were found closed to the upstream regions of gene transcription start sites; while most binding sites were found within gene introns and distal intergenic regions.

(B) ERbeta binding regions were scanned for enrichment of motifs available in JASPAR motif database. Our analysis have identified a high level of enrichment of ERs binding motif, ERbeta interactors binding motifs as well as those of novel interactors.

A.



B.

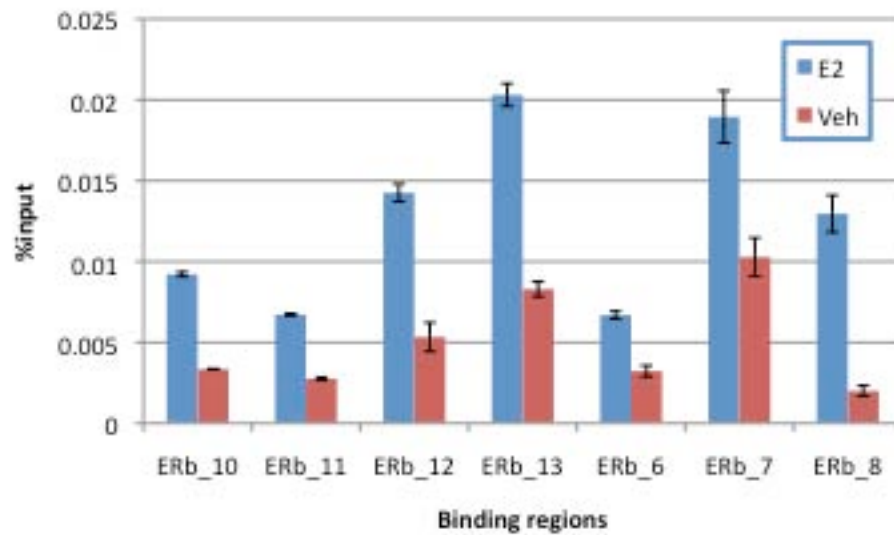


Figure 3 – Validating novel ERbeta binding regions in C4-12-ERbeta cells (A) and in PC3-ERbeta cells (B)

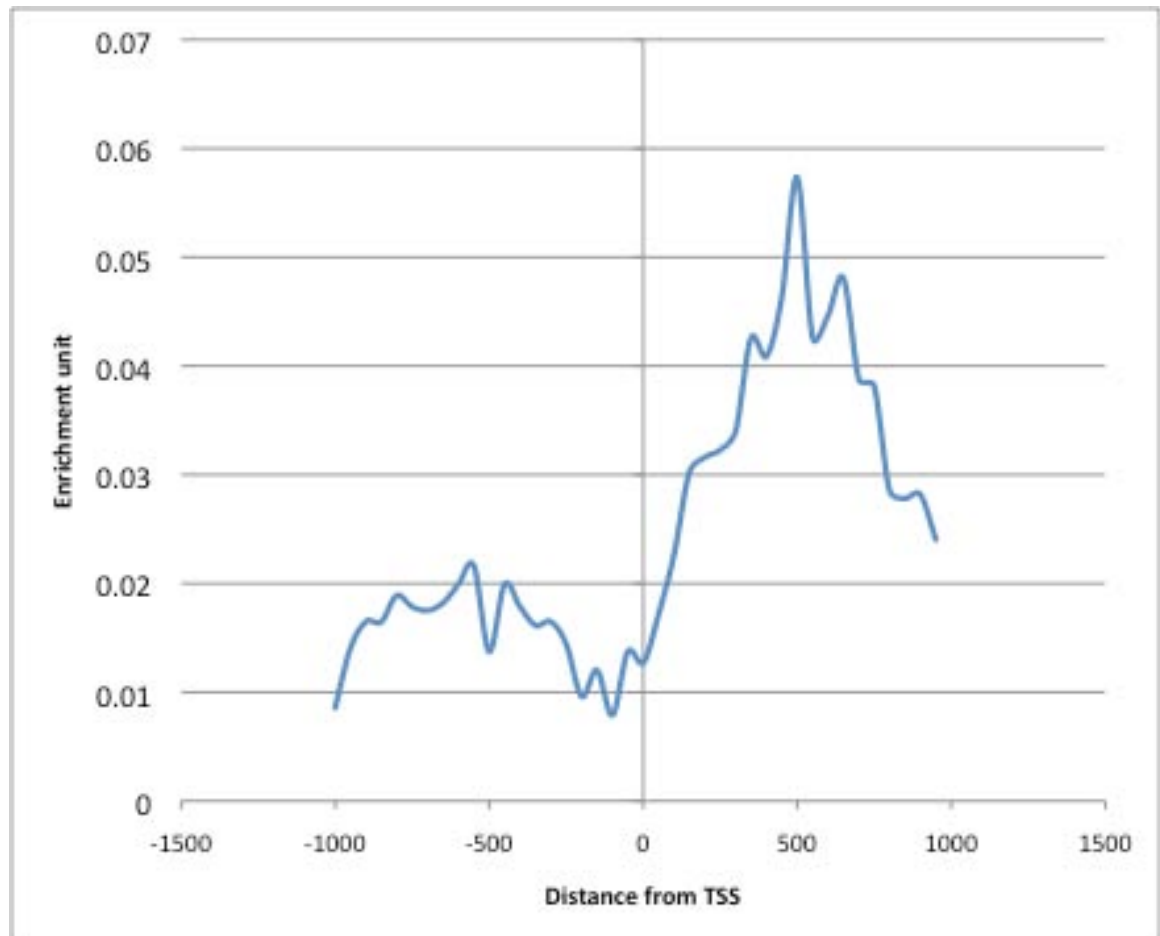


Figure 4 – H3K4me3 binding around TSS in C4-12-ERbeta cells

The enrichment of H3K4me3 around TSS occurs in a bimodal manner around gene TSS. In agreement with other reported study, this finding confirmed our condition to map active promoters in C4-12-ERbeta cells

APPENDICES
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