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April 3, 2025

Investigating Histone Acetylation in Lipogenic Triple Negative Breast Cancer Cells using Cell Engineering and Bioinformatics

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An abstract of  
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## Abstract

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Triple negative breast cancer (TNBC) is an aggressive breast cancer subtype that has poor outcomes when compounded with obesity. Adipocyte secreted factors (ASFs) activate metabolic pathways that change the level of acetyl-CoA, a substrate involved in histone acetylation and fatty acid synthesis, suggesting an association between epigenetic remodeling and ASFs. Although aberrant histone acetylation is a marker for cancer progression, the relationship between lipogenesis and histone acetylation is yet to be elucidated in TNBC cells. To investigate the association between chromatin structure and tumor suppressor gene (TSG) expression, we used RNA-seq and ATAC-seq to identify if chromosomal accessibility is reduced around the promoter site of downregulated TSGs in lipogenic TNBC cells. We found no significant difference in peak signal value between cells treated with adipocyte-conditioned media (ACM) vs unconditioned media (UCM), suggesting an alternative mechanism for downregulation of TSGs other than chromosomal accessibility. By performing transcription factor enrichment analysis, we found that downregulated TSGs are regulated by a set of TFs that uniquely regulates downDEGs. This suggests downregulation of TSGs is induced by dysregulation of TFs. In future work, we will determine how TNBC aggressiveness is impacted by TFs by treating cells with specific TF inhibitors. To visualize histone acetylation level and nuclear distribution in lipogenic TNBC cells, we use fusion protein technology to engineer plasmids that express histone acetylation reader probes with bromodomain (BRD) dimers fused to fluorescent proteins. We found that BT549 transfection rate with the engineered plasmids was low, likely due to transgene silencing induced by CMV promoter in the GGDestX4 expression vector. In future experiments, we will engineer a new Golden Gate compatible plasmid based on the pSBtetTA-YP\_CFP vector, which has been shown to achieve high transfection rate in BT549 cells. To determine histone acetylation level and its nuclear redistribution in lipogenic TNBC cells, we will re-do transfection with the new expression vector and treat the cells with ACM or UCM.

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## Table of Contents

Abstract.....	1
Background.....	2
Hypothesis.....	6
Research aims.....	6
Methodology.....	7
Results.....	9
Conclusion and Future Directions.....	19
References.....	22

## **Investigating Histone Acetylation in Lipogenic Triple Negative Breast Cancer Cells using Cell Engineering and Bioinformatics**

### **Abstract**

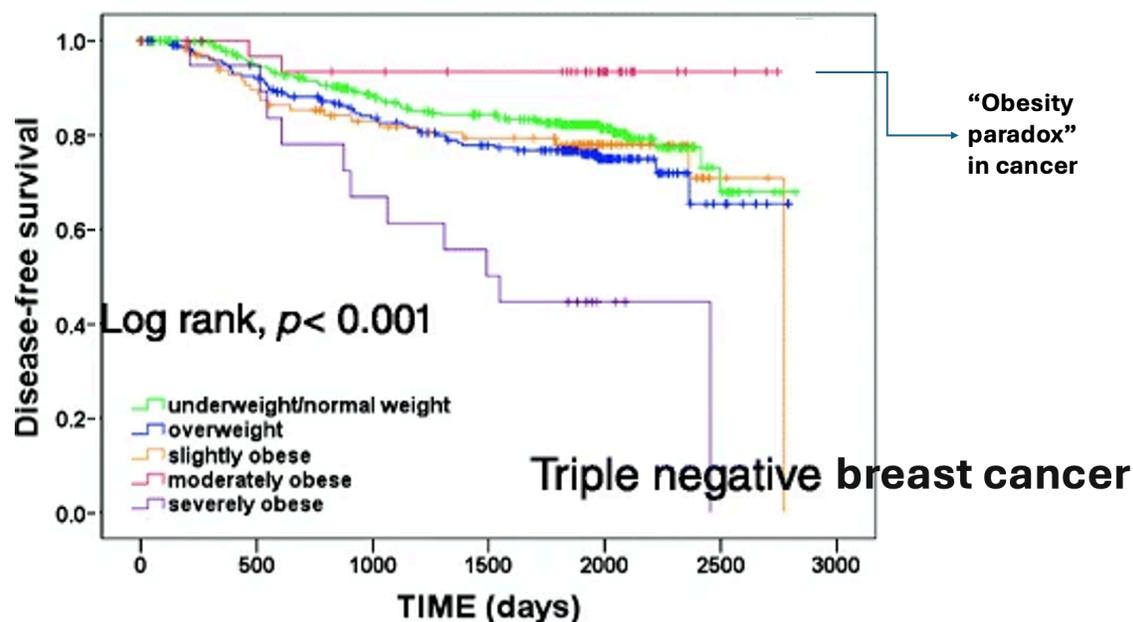
Triple negative breast cancer (TNBC) is an aggressive breast cancer subtype that has poor outcomes when compounded with obesity. Adipocyte secreted factors (ASFs) activate metabolic pathways that change the level of acetyl-CoA, a substrate involved in histone acetylation and fatty acid synthesis, suggesting an association between epigenetic remodeling and ASFs. Although aberrant histone acetylation is a marker for cancer progression, the relationship between lipogenesis and histone acetylation is yet to be elucidated in TNBC cells. To investigate the association between chromatin structure and tumor suppressor gene (TSG) expression, we used RNA-seq and ATAC-seq to identify if chromosomal accessibility is reduced around the promoter site of downregulated TSGs in lipogenic TNBC cells. We found no significant difference in peak signal value between cells treated with adipocyte-conditioned media (ACM) vs unconditioned media (UCM), suggesting an alternative mechanism for downregulation of TSGs other than chromosomal accessibility. By performing transcription factor enrichment analysis, we found that downregulated TSGs are regulated by a set of TFs that uniquely regulates downDEGs. This suggests downregulation of TSGs is induced by dysregulation of TFs. In future work, we will determine how TNBC aggressiveness is impacted by TFs by treating cells with specific TF inhibitors. To visualize histone acetylation level and nuclear distribution in lipogenic TNBC cells, we use fusion protein technology to engineer plasmids that express histone acetylation reader probes with bromodomain (BRD) dimers fused to fluorescent proteins. We found that BT549 transfection rate with the engineered plasmids was low, likely due to

transgene silencing induced by CMV promoter in the GGDestX4 expression vector. In future experiments, we will engineer a new Golden Gate compatible plasmid based on the pSBtetTA-YP\_CFP vector, which has been shown to achieve high transfection rate in BT549 cells. To determine histone acetylation level and its nuclear redistribution in lipogenic TNBC cells, we will re-do transfection with the new expression vector and treat the cells with ACM or UCM.

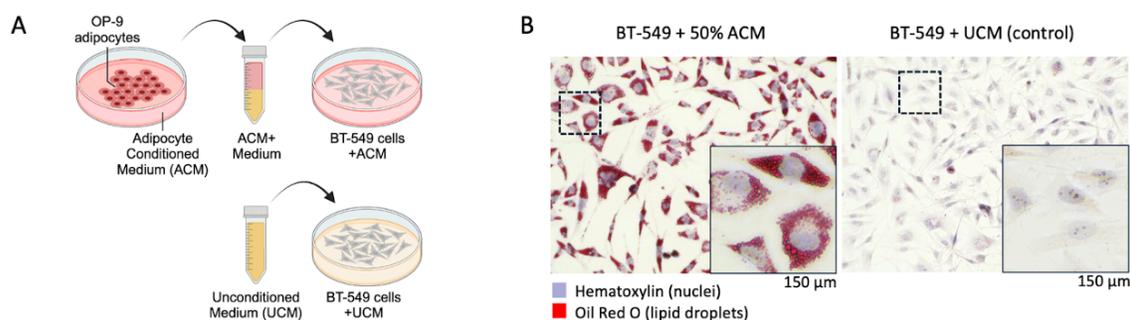
## **Background**

As of 2018, the obesity prevalence rate of US adults aged 20 and older is over 40%<sup>1</sup>. Considered an epidemic<sup>2</sup>, obesity is closely associated with an increased risk of at least 13 types of cancer, including breast cancer<sup>3</sup>. Each year, around 2.1 million women are diagnosed with breast cancer worldwide<sup>4</sup>. Among all breast cancer subtypes, triple-negative breast cancer (TNBC) accounts for 10-20% of all breast cancers and is associated with poor prognosis, high rate of recurrence, and low survival rate<sup>5</sup>. It is reported that premenopausal women with obesity (BMI  $\geq 30$  kg/m<sup>2</sup>) have a 42% higher risk of developing TNBC compared with women with normal weight<sup>6</sup>. However, it is worth noting that obesity, as defined by BMI, does not always have a negative impact on TNBC patient outcome. Based on a study on the effect of BMI on survival outcomes of TNBC patients, moderately obese patients have better survival than severely obese, slightly obese, overweight, and underweight/normal weight patients<sup>7</sup> (**Figure 1**). This finding is known as the “obesity paradox in cancer”. Therefore, it is necessary to take into account other confounding factors that might impact patient outcome, such as race, tumor type, and weight history<sup>8</sup>. In breast cancer patients with obesity, adipocyte secreted factors (ASF) have been shown to promote breast cancer epithelial-mesenchymal transition (EMT), invasion, proliferation, and

lipogenesis in invasive cells<sup>910</sup>. Therefore it is important to identify intracellular factors that respond to ASF signaling and mediate intracellular changes that promote cancer. We prepared adipocyte-conditioned media (ACM) as our in-vitro co-culture obesity model (**Figure 2A**). Our preliminary studies have shown that ACM increases lipid droplet formation in BT549 TNBC cells compared to cells treated with unconditioned media (UCM) (**Figure 2B**). Compared to other traditional models of obesity<sup>1110</sup>, our in-vitro co-culture model creates a system where cancerous cells are directly exposed to secreted factors from adipocytes, while maintaining cell-cell interaction. Moreover, such a model allows for a more controlled and precise simulation of the obese microenvironment, since we are able to decide the amount of ACM treatment for the cells.



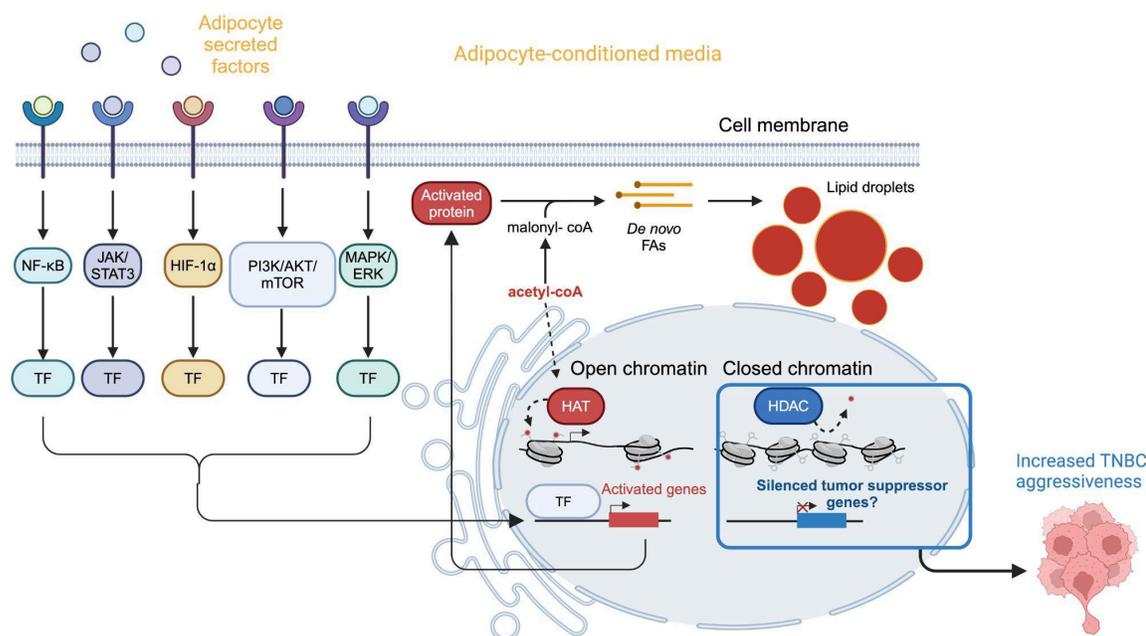
**Figure 1. Kaplan-Meier plot of disease-free survival for TNBC patients based on BMI groups.** Moderately obese TNBC patients have higher survival outcomes than other BMI groups. Figure adapted from Widschwendter et al.<sup>7</sup>



**Figure 2. In-vitro model to study how obesity impacts breast cancer cells.** (A) OP-9 mouse adipocytes are differentiated *in vitro*, the supernatant is collected as adipocyte-conditioned media (ACM) and added to BT549 cells. (B) ACM increases lipid droplet formation in BT549 cells.

Histone acetylation is an epigenetic modification where histone acetyltransferase enzymes (HATs) use acetyl-CoA as substrate to add acetyl groups to histone lysines, decreasing the binding between nucleosome and DNA, and thus activating gene expression<sup>12</sup>. Adipocyte signaling activates pathways including PI3K/AKT/mTOR<sup>13</sup>, NF- $\kappa$ B<sup>14</sup>, JAK/STAT3<sup>15</sup>, hypoxia-induced HIF-1 $\alpha$ <sup>16</sup>, and MAPK/ERK<sup>17</sup>, activating genes that drive metabolic processes, including fatty acid synthesis. In the *de novo* fatty acid (FA) synthesis pathway, acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase 1 (ACC1) for lipogenesis<sup>18</sup>, suggesting an association between lipogenesis and histone acetylation<sup>19</sup> (**Figure 3**). Aberrant histone acetylation is one of the most pervasive epigenetic drivers that promote breast cancer progression<sup>20</sup>. However, to our knowledge, the association between histone acetylation and lipogenesis in TNBC cells is yet to be elucidated. Therefore, we are interested in determining how histone acetylation might

modulate chromatin in lipogenic TNBC cells, which may shed light on how ASF-stimulated chromatin reorganization contributes to cancer aggressiveness.



**Figure 3. Hypothesis model showing the association between lipogenesis through adipocyte secreted factor signaling and histone acetylation.**

Typically, antibodies are used to stain regions in fixed cells that have high levels of specific histone posttranslational modifications (histone PTMs) and other features such as DNA methylation and nonhistone chromatin proteins. Cells naturally express proteins called chromatin readers or reader-effectors that contain chromatin-binding domains (CBDs). Scientists have isolated the minimal CBD sequences needed to selectively bind specific histone PTMs, fused these with fluorescent proteins, and expressed these in living cells to “paint” chromatin regions in the nucleus<sup>21</sup>. This methodology has been used to detect histone acetylation (H3K14ac) in HEK293 cells<sup>22</sup>, but to our knowledge not in lipogenic breast cancer cells. Within the human BRD4 protein, a domain of 119 amino acids known as

BRD (bromodomain) specifically binds acetylated lysines within histones H3 and H4 (H3K14ac, H4K5ac, H4K8ac, H4K12ac, H4K16ac, H4K20ac)<sup>23,24</sup>. Constructs containing dimeric repeats of BRD have been shown to best colocalize with H3K14ac antibody in transfected kidney cells and provide information on the nuclear distribution of histone acetylation<sup>22</sup>. Therefore, we used a one-step DNA assembly method called Golden Gate to build a fluorescent fusion protein that contains a dimeric repeat of BRD fused to fluorescent protein with nuclear localization sequence (NLS) to visualize histone acetylation and its nuclear distribution within the nucleus of lipogenic TNBC cells.

## **Hypothesis**

Lipogenesis in triple negative breast cancer (TNBC) cells drives global loss or redistribution of histone acetylation that results in condensed (closed) chromatin that reduces the expression of tumor suppressor genes (TSGs).

## **Research Aims**

**Aim 1** - Use RNA-seq to determine the transcriptional profile of lipogenic TNBC cells and identify downregulated tumor suppressor genes (TSGs).

**Aim 2** - Use ATAC-seq to determine if chromatin takes on a condensed (closed) state at TSGs and/or other downregulated genes.

**Aim 3** - Use fusion protein technology to engineer histone acetylation reader probe to determine changes in global histone acetylation levels and nuclear distribution.

## **Methodology**

### **Cell Culture**

The BT549 cell line was grown in RPMI1640 media supplemented with 1% L-glutamine, 10% fetal bovine serum (FBS), and 0.0008 mg/mL human recombinant insulin. The BT549 cell media was used as UCM in TNBC experiments.

### **Obesity Model**

Confluent murine OP-9 bone marrow stromal cells were trypsinized and plated at  $10^5$  cells/well in 10-cm plates. Cells were cultured in DMEM media supplemented with 10% FBS. On the following day, media was changed with either fresh DMEM media supplemented with 10% FBS for stromal cell culture or insulin-oleate media (IOM, 1.8mM Oleate bound to BSA with the molar ratio of 5.5:1) for adipocyte differentiation. Supernatants from adipocytes were harvested on day 3 of culture for use as adipocyte-conditioned media (ACM) in TNBC experiments.

### **RNA-Seq**

BT-549 TNBC cells were seeded in unconditioned media (UCM) or 50% ACM, with 3 replicates per condition. We performed RNA extraction and purification for RNA-seq library preparation and deep sequencing at Novogene. RNA-seq was performed with the following parameters: paired-end, 150 bp, Q30  $\geq$  85%. We trimmed raw reads with Trim Galore and performed alignment using STAR<sup>25</sup> with the most recent human genome (GRCh38/hg38). We calculated mRNA levels and generated count matrices with RSEM<sup>26</sup>. Differential gene expression was performed with DESeq2<sup>27</sup>, using a negative binomial

distribution algorithm to recognize genes with a p-value  $\leq 0.05$  and fold change  $\geq 1.5$ . We generated a volcano plot in RStudio to show a transcriptional profile.

### **ATAC-Seq**

We prepared cell samples as in RNA-seq, and sent frozen samples to Novogene for ATAC-seq library preps and deep sequencing. We trimmed raw reads with Trim Galore and performed alignment using BWA-MEM<sup>28</sup> with the most recent human genome (GRCh38/hg38). We removed PCR duplicates using Picard. To adjust read start positions to account for Tn5 chemistry, we aligned all reads as plus (+) strands offset by +4 bp and minus (-) strands offset by -5 bp. We performed peak calling using MACS3<sup>29</sup>. We visually explored our ATAC-seq data on Integrated Genomics Viewer (IGV) to investigate chromosome accessibility near genes of interest.

### **Synthetic Reader Probe Construction**

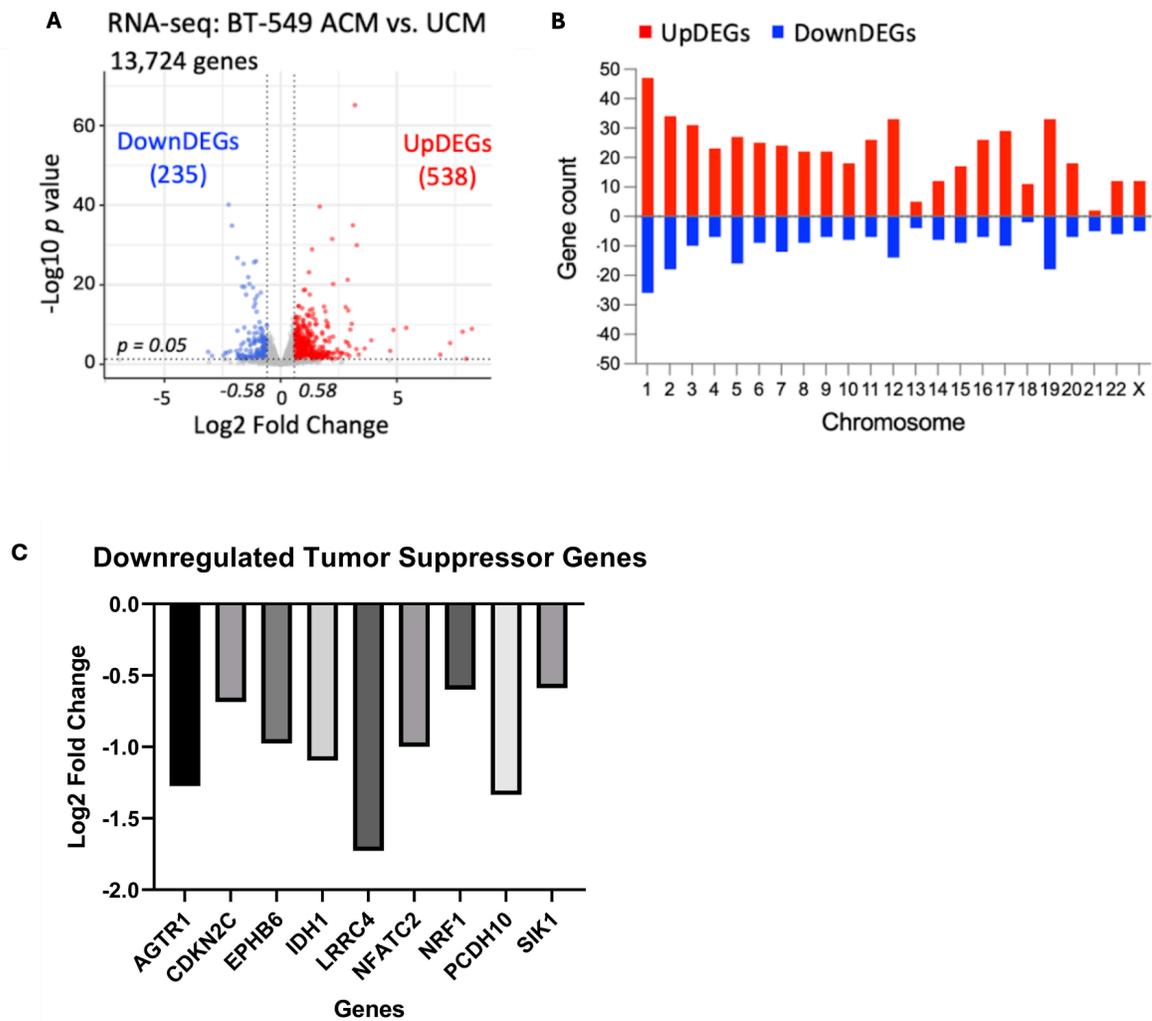
We built a probe containing dimeric BRD repeats fused by a linker, and a fluorescent protein (FP) attached to the nuclear localization sequence (NLS). To determine which fluorescent protein allows for best quantification of histone acetylation, we built four plasmids, each containing a different fluorescent protein: yellow fluorescent protein (YFP), cyan fluorescent protein (CFP), red fluorescent protein (RFP), or green fluorescent protein (GFP). The coding sequence was inserted into an engineered mammalian vector GGDestX4. Under UCM conditions, BT-549 TNBC cells were transfected and selected under G418 to generate a stable clonal cell line. Fluorescence imaging was performed on both the

transiently transfected and stably transfected cells to determine the brightness and nuclear distribution of the fluorescence signals.

## Results

### Downregulation of tumor suppressor genes in lipogenic TNBC cells

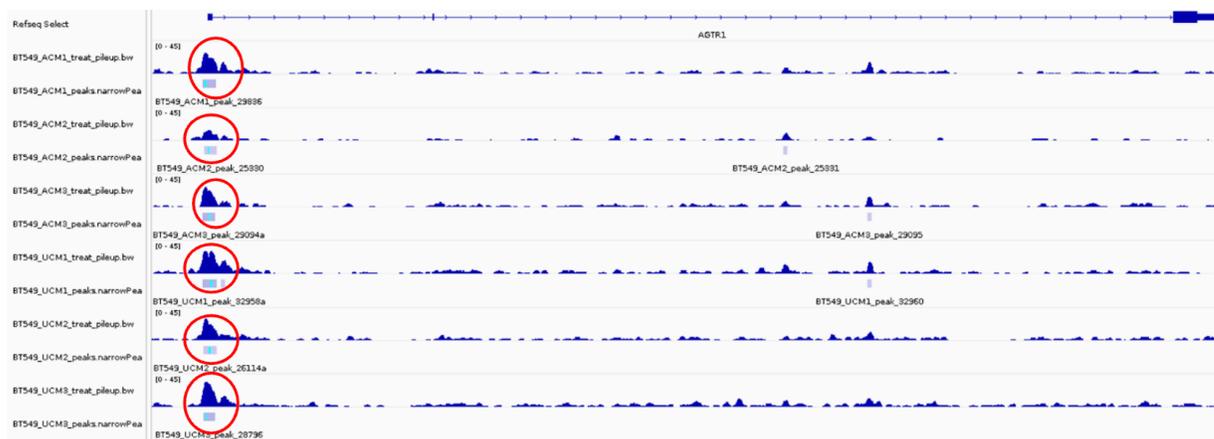
13,724 differentially expressed transcripts of BT549 cells cultured in ACM and UCM were measured using RNA-sequencing analysis. We identified 538 upregulated differentially expressed genes (UpDEGs) in ACM compared to UCM ( $p < 0.05$ ,  $p.adj < 0.05$ , and  $FC > 1.5$ ) and 235 down regulated differentially expressed genes (DownDEGs) ( $p < 0.05$ ,  $p.adj < 0.05$ , and  $FC < -1.5$ ) (**Figure 4A**). DownDEGs are found across all chromosomes, suggesting global repression (**Figure 4B**). We then matched the downDEGs with the breast cancer TSG list BRCA from the Tumor Suppressor Gene Database (TSGene)<sup>3031</sup>. We identified 9 TSGs that were downregulated in cells treated with ACM compared to UCM: AGTR1, CDKN2C, EPHB6, IDH1, LRRC4, NFATC2, NRF1, PCDH10, and SIK1 (**Figure 4C**).



**Figure 4. Adipocyte secretome impacts the transcriptional profile of BT549 cells. (A)** Volcano plot showing upregulated and downregulated differentially expressed genes. **(B)** Differentially expressed gene distribution across all chromosomes. **(C)** Downregulated TSGs identified from RNA-seq.

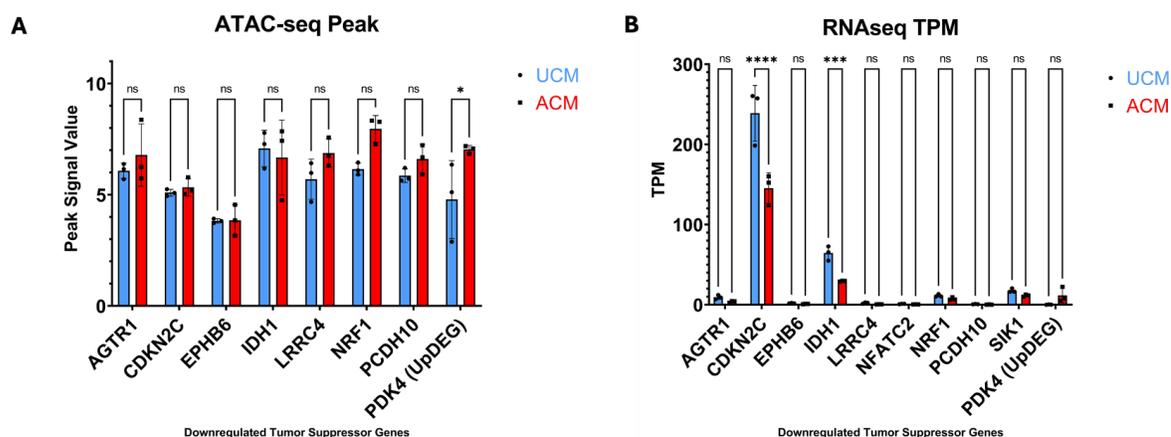
**Downregulation of TSGs due to dysregulation of TFs instead of chromatin structure change**

To determine the chromosomal accessibility around the promoter site of downregulated TSGs, we loaded the six narrowPeak files and the six pileup bigWig files from ATAC-seq analysis as tracks in Integrated Genomics Viewer (IGV)<sup>31,32</sup> and selected Human (GRCh38/hg38) as genome. We navigated the window to promoter sites of all downregulated TSGs. For example, the promoter of AGTR1 is located at chr3:148697903. We looked for consistent peaks near promoter sites across all 6 narrowPeak files and 6 bigWig files (**Figure 5**), and then averaged the peak signal value of 3 ACM replicates and 3 UCM replicates respectively. Two-way ANOVA was performed on the ATAC-seq peak signal values (**Figure 6A**) and the RNA-seq TPM values (**Figure 6B**). Statistical significant difference was determined by p-value < 0.05. The peak signal value significantly increased at the promoter site of PDK4 (one of the upDEGs) for cells treated with ACM, suggesting more chromosomal accessibility for gene activation. This also tracks with RNA-seq transcript expression, as TPM of PDK4 increased in cells treated with ACM. The peak signal values of downregulated TSGs in ACM group are not significantly different from those in UCM group, suggesting no significant change in chromatin structure induced by ASFs at the promoter site of downregulated TSGs.



**Figure 5. Visualization of narrowPeak and bigWig files from ATAC-seq for AGTR1.**

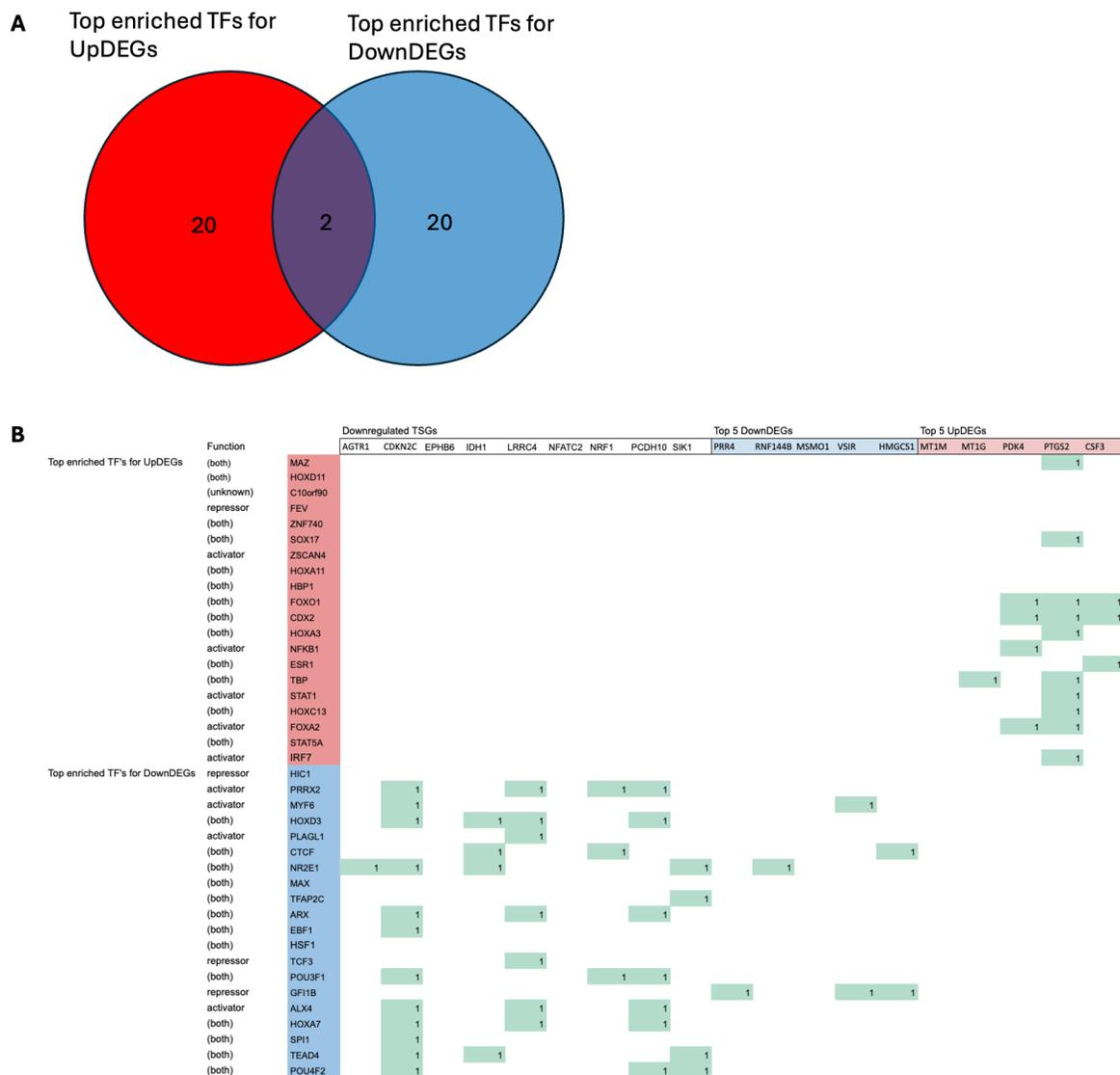
NarrowPeak files provide called peaks of signal enrichment. BigWig files provide dense, continuous data that can be displayed on IGV as graphs. Consistency in peaks is found in all narrowPeak and bigWig files at the promoter site of AGTR1.



**Figure 6. Peak signal values from ATAC-seq and TPM values from RNA-seq for**

**downregulated TSGs.** (A) Peak signal values of ACM replicates are not significantly different from that of UCM replicates for downregulated TSGs. The peak signal value for PDK4 is significantly higher for cells treated with ACM. (B) RNA-seq TPM values showing transcript expression levels for downregulated TSGs and PDK4.

Our ATAC-seq analysis further suggests that the downregulation of TSGs is determined by gene regulation. Therefore, these TSGs could be regulated by a unique set of transcription factors (TFs) that are distinct from TFs that regulate the upDEGs. To test this alternative hypothesis, we performed TF binding sites enrichment analysis on upDEGs and downDEGs using TF\_targets ([https://github.com/cplaisier/TF\\_targets](https://github.com/cplaisier/TF_targets)) and the Transcription Factor Target Gene Database<sup>33</sup>. TFs were ranked based on p-value, which represents the enrichment of input genes (upDEGs or downDEGs) with TF target genes. We selected 22 TFs with most significant p-values and eliminated two TFs (SP1 and ZEB1) that target both upDEGs and downDEGs (**Figure 7A**). We then derived the top 20 most enriched unique TFs for either upDEGs or downDEGs, and determined whether the downregulated TSGs, the top 5 downDEGs and upDEGs are target genes of these TFs. As a result, downregulated TSGs and top 5 downDEGs are only targeted by most enriched TFs for downDEGs, whereas top 5 upDEGs are only targeted by most enriched TFs for upDEGs (**Figure 7B**). This suggests that the downregulation of TSGs is regulated by a specific group of TFs that exclusively regulate downDEGs.



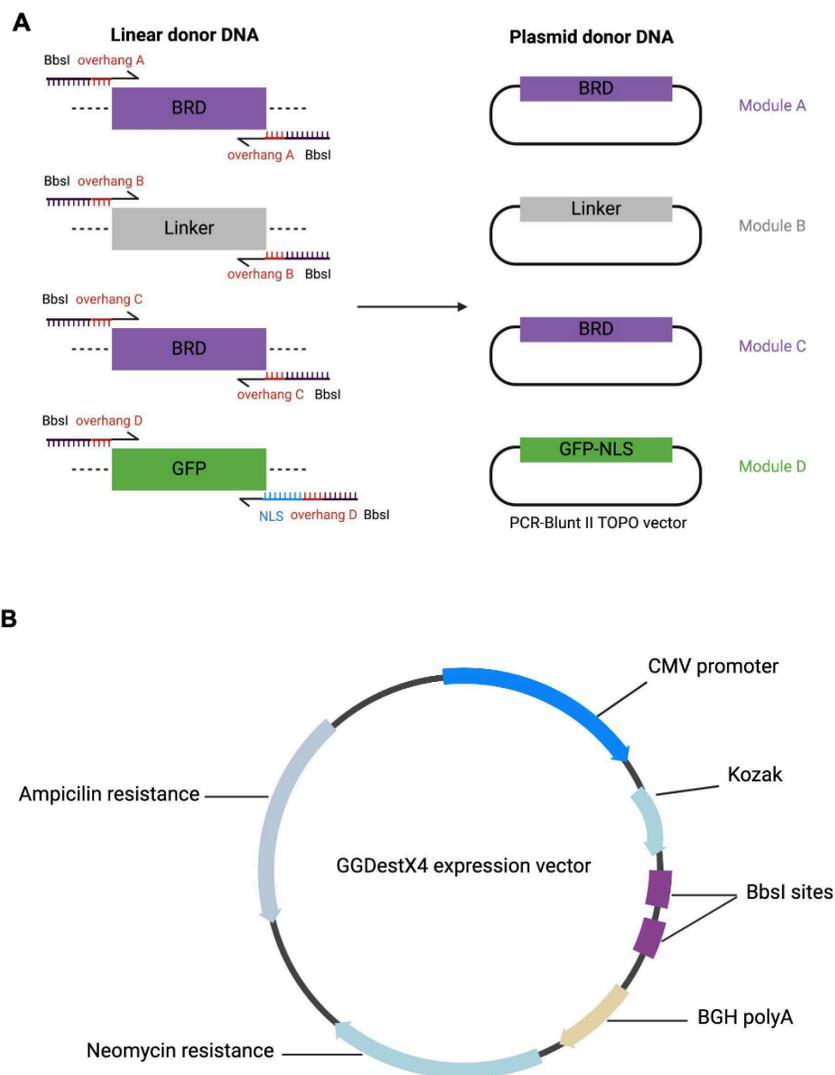
**Figure 7. Downregulated TSGs are targeted by TFs that exclusively regulate downDEGs.** (A) Venn diagram showing top 20 enriched TFs that exclusively regulate upDEGs or downDEGs. (B) DownDEGs and upDEGs are targeted by two distinct groups of TFs. Green box with “1” shows that the specific gene is targeted by that specific TF.

## Engineering Histone Acetylation Reader Probe

To prepare linear DNA donors, we performed Q5 PCR on commercial plasmids (Twist Bioscience) to add recognition sites for the Type IIS restriction enzyme BbsI and 4 bp 5' overhangs to each end of each module's open reading frame (ORF). Using the PCR products, we generated donor plasmids through Zero Blunt™ TOPO™ PCR cloning, which is a system for cloning blunt-end DNA (**Figure 8A**). Such a cloning system has several key advantages. The vector contains a kanamycin resistance gene, which is different from the ampicillin resistance gene in the GGDestX4 expression vector. This allows for the selection of cells transformed with the fully assembled Golden Gate plasmid and kills cells transformed with the donor plasmids. The PCR-Blunt II-TOPO vector also has convenient designs for plasmid donor construction validation, including recognition sites for EcoRI restriction enzyme outside donor ORF, and M13 forward and reverse primer sites for Sanger sequencing or colony PCR screening. In addition, although plasmid donors are not always necessary for Golden Gate assembly<sup>34</sup>, it allows for long-term storage and accurate regeneration of donor parts through bacterial transformation. Successful construction of plasmid donor DNA was verified by restriction digestion and Sanger sequencing.

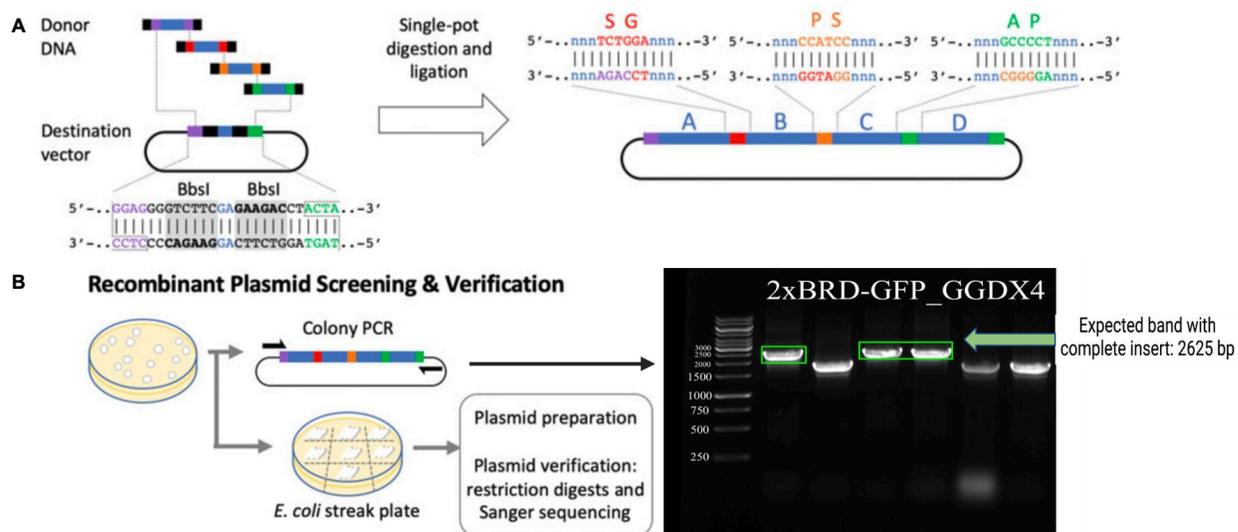
GGDestX4 is an engineered plasmid built from pcDNA™3.1<sup>(+)</sup> neo mammalian expression vector (Thermo Fisher Scientific) that contains two modifications to make it compatible for the Golden Gate assembly (**Figure 8B**). As the expression vector must not contain any BbsI sites, Dr. Karmella Haynes performed site-directed mutagenesis to remove the BbsI site in the bovine growth hormone polyadenylation (BGH polyA) sequence of pcDNA™3.1<sup>(+)</sup> neo. Another key modification that I assisted with was linearizing the pcDNA™3.1<sup>(+)</sup> neo using restriction enzyme, and ligated with an annealed double-stranded oligo (dsOligo) containing a pair of BbsI sites for Golden Gate cloning. The dsOligo also

includes a Kozak sequence that functions as the initiation site for reader probe protein translation in eukaryotic cells<sup>35</sup>. GGDestX4 expression vector has several features that are designed for mammalian cell transfection. The CMV promoter is a strong promoter commonly used to drive transgene expression in mammalian cells<sup>36</sup>. A BGH polyA sequence allows polyA tails to be added to the mRNA transcript to terminate transcription of the reader probe<sup>37</sup>. To select for stable transfection, the GGDestX4 plasmid contains a neomycin resistance marker, which protects stable cells from the toxicity of G418. Traditionally, Golden Gate assembly requires an intermediate shuttle destination vector for the donor fragments to be assembled<sup>34</sup>. Using GGDestX4 bypasses the shuttle vector step and provides a convenient method of assembling donors directly into expression vectors.



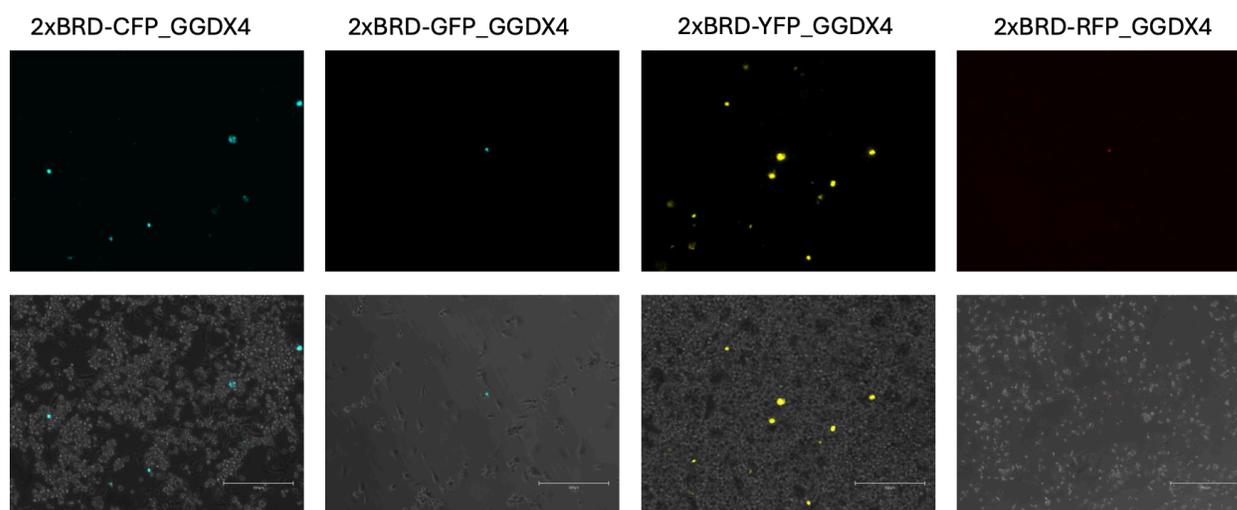
**Figure 8. Construction of Golden Gate plasmid donors and expression destination vector for histone acetylation reader probe. (A) Preparation for linear donors using PCR amplification, and plasmid donors by cloning donor DNA into PCR-Blunt II TOPO vector. GFP is used as an example for the D-part fluorescent protein. (B) Plasmid map of GGDestX4 expression vector.**

We performed Golden Gate single-pot assembly to assemble donor DNA directly into GGDestX4 expression vector. The 4 bp overhang at each end of each module's ORF guides the assembly of the four modules in order. Single-pot digestion using BbsI restriction enzyme allows the donor fragments to be cut out from PCR-Blunt II TOPO vectors, and ligated into the GGDestX4 expression vector in directed assembly (**Figure 9A**). For plasmid verification, colony PCR with MV1 primers and Sanger sequencing were performed (**Figure 9B**). For example, the expected band for plasmid 2xBRD-GFP\_GGDGX4 with complete Golden Gate assembly insert is 2625 bp.



**Figure 9. Single-pot Golden Gate assembly of histone acetylation reader probe and plasmid verification.** (A) Single-pot digestion and ligation allows for directed assembly of donor modules into expression destination vector. Figure adapted from Haynes et al<sup>34</sup>. (B) Plasmid verification for Golden Gate products. The bands showing plasmids with complete Golden Gate insert for 2xBRD-GFP\_GGDGX4 are shown in green box.

The four plasmids expressing histone acetylation probes were then transfected into BT549 cells. Three days after transfection, fluorescence imaging was performed on transiently transfected cells (**Figure 10**). The overall fluorescence expression and transfected cell viability were low. To select for stable transfected cells, we added 250 ug/mL G418 to the cells. We then saw that there was an increase in cell death and the few cells expressing fluorescence did not proliferate.



**Figure 10. Low transient transfection rate across all four histone acetylation probes.**

The first row shows imaging under CFP, GFP, YFP, and RFP channels (from left to right).

The second row shows imaging when the fluorescence channel and trans channel are merged.

## Conclusion and Future Directions

### Downregulation of TSGs induced by adipocyte secretome

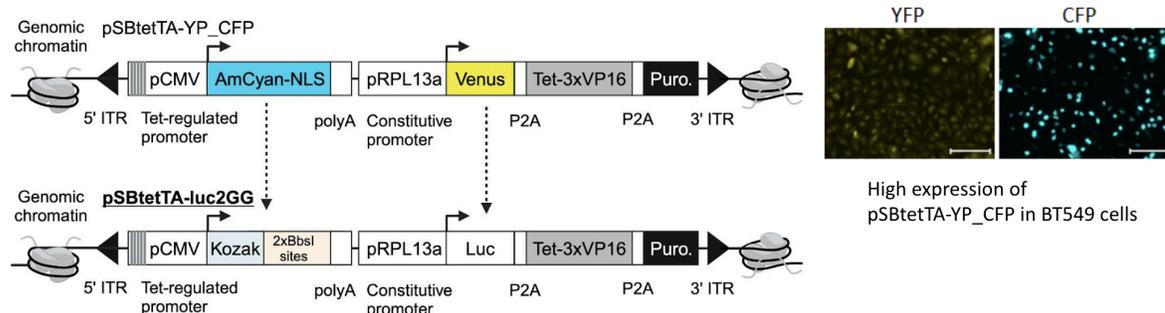
In conclusion, our RNA-seq and ATAC-seq results suggest that downregulation of TSGs is not induced by change in chromosomal accessibility at the promoter site, but rather

regulated by TFs that exclusively target downDEGs. To investigate further regarding the role of TFs in TSG repression, we will treat the cells with inhibitors for specific TF that targets downregulated TSG, and measure changes in terms of cancer proliferation, metastasis, and aggressiveness. In addition, we will work with Dr. David Gorkin to normalize the ATAC-seq results in order to perform differential expression analysis, since current results are only based on signal values after peak calling.

### **Histone acetylation reader probe**

Both of the transiently transfected and stably transfected BT549 cells showed low viability of fluorescence-expressing cells. One possible explanation is that CMV, as a strong viral promoter, induces transgene silencing through CpG methylation<sup>38</sup>. The transgene expression could also be specific to cell type. Our preliminary studies have shown that HEK cells had high transfection rate with GGDestX4, but the histone acetylation probes is our first experiment transfecting GGDestX4 into TNBC cells. Instead of continuing with GGDestX4 expression vector, we will transition to the pSBtetTA-YP\_CFP vector, which is a plasmid engineered by Dr. Karmella Haynes. The pSBtetTA-YP\_CFP vector constitutively expresses YFP signals and expresses CFP when induced by doxycycline (dox) (**Figure 11**). Studies performed by graduate student Ashley Townsel have shown high transfection rates of pSBtetTA-YP\_CFP into BT549 cells under UCM conditions<sup>39</sup>. Therefore, we will make modifications to the pSBtetTA-YP\_CFP to make it compatible for Golden Gate assembly. The Golden-Gate compatible plasmid pSBtetTA-luc2GG will have CFP replaced with a Golden Gate drop-in site by ligating with annealed dsOligo to add a pair of BbsI sites for Golden Gate cloning. The constitutively expressed YFP gene will be replaced by the

colorless luciferase, as YFP's spectrum overlaps with the spectrum of GFP. We will perform site-directed mutagenesis to eliminate the BbsI site in BGH polyA and in luciferase gene.

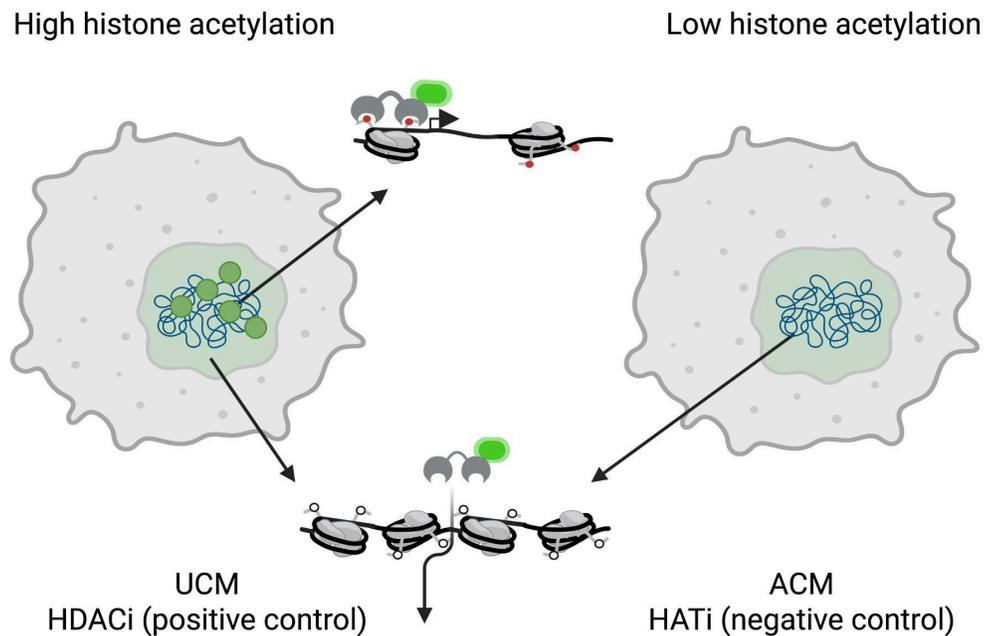


**Figure 11. Engineering design for pSBtetTA-luc2GG based on pSBtetTA-YP\_CFP.**

pSBtetTA-YP\_CFP vector constitutively expresses YFP and contains dox-inducible CFP gene. At the presence of doxycycline, Tet-TA binds to dox and undergoes a conformational change. The complex binds to the pCMV promoter, allowing CFP gene to be expressed.

After assembling the donor modules into pSBtetTA-luc2GG using Golden Gate, we will transfect the plasmids into BT549 cells and treat with dox to induce expression of the histone acetylation probe. We will then select for stable cells by adding puromycin. After generating the stably transfected cells, we will treat them with either ACM or UCM to determine how ASFs impact the fluorescence brightness and nuclear distribution of histone acetylation. We will also have a positive control where cells are treated with histone deacetyltransferase (HDAC) inhibitor, and a negative control where cells are treated with histone acetyltransferase (HAT) inhibitor. We expect to see diffuse fluorescence expression in cells treated with ACM or HAT inhibitor (HATi), as the synthetic reader probes are expressed but do not bind to acetylated histone lysine tails. In cells treated with UCM or HDAC inhibitor (HDACi), we expect to see concentrated fluorescence expression, due to

higher concentration in histone acetylation in some regions of the nucleus than the others (Figure 12).



**Figure 12. Expected results of fluorescence expression in stably transfected BT549 cells treated with different conditions.** Using 2xBRD-GFP\_GGDX4 as an example, we expect to see diffuse GFP expression in cells treated with ACM or HATi, while concentrated GFP expression is expected in cells treated with UCM or HDACi.

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