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## RESEARCH ARTICLE

# Histone Acetylation Landscape Changes During Adipocyte Differentiation

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## ABSTRACT

Terminal differentiation of preadipocytes into mature, lipid-storing adipocytes requires coordinated activation of a small set of master transcription factors acting on a chromatin landscape that is remodeled in successive waves, yet the genome-wide dynamics of histone acetylation across this process, and their relationship to super-enhancer formation at adipogenic master regulator loci, remain incompletely mapped at high temporal resolution. Here we profiled H3K27ac and H3K9ac by chromatin immunoprecipitation sequencing (ChIP-seq), chromatin accessibility by ATAC-seq, and gene expression by RNA-sequencing across six time points spanning confluent growth arrest, mitotic clonal expansion and terminal differentiation of 3T3-L1 preadipocytes induced with a standard hormonal cocktail. H3K27ac signal at a defined set of 1,842 genomic regions increased more than four-fold during the first 48 hours of differentiation, preceding peak expression of the early response factors CEBPB and CEBPD, and a substantially larger, largely non-overlapping set of regions gained H3K27ac only after 72 hours, coincident with induction of the terminal adipogenic regulators PPARGgamma and CEBPA. Clustering of dynamic H3K27ac regions by their acetylation trajectory identified a coherent late-gained cluster strongly enriched for super-enhancers overlapping PPARGgamma and CEBPA target genes central to lipid storage and adipokine secretion, and CRISPR interference-mediated silencing of three constituent enhancers within the Fabp4 super-enhancer individually reduced Fabp4 induction by 35-58% without eliminating it, consistent with additive rather than fully redundant enhancer contributions. Pharmacological inhibition of the acetyltransferases p300 and CBP during the mitotic clonal expansion window, but not after its completion, blunted lipid accumulation and terminal marker gene induction, indicating a temporally restricted requirement for acetyltransferase activity during early adipogenic commitment. These results define a two-wave histone acetylation program during adipocyte differentiation and identify a temporally restricted, p300/CBP-dependent window during which the terminal adipogenic super-enhancer landscape is established.

**Keywords:** adipogenesis; histone acetylation; H3K27ac; CEBPA; PPARGgamma; super-enhancer; ChIP-seq

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## 1. INTRODUCTION

Adipocyte differentiation proceeds through a well-defined transcriptional cascade in which growth-arrested preadipocytes re-enter the cell cycle for one or two rounds of mitotic clonal expansion before exiting permanently and activating a terminal differentiation program driven principally by the nuclear receptor PPARgamma and the CCAAT/enhancer-binding protein CEBPA, which together induce the full complement of genes required for lipid uptake, synthesis and storage [1,2]. This transcriptional cascade is accompanied by extensive remodeling of the chromatin landscape, with genome-wide studies establishing that a substantial fraction of PPARgamma and CEBPA binding occurs at genomic regions that only become accessible during differentiation, implying that acquisition of an adipogenic enhancer repertoire is itself a regulated, multi-step process rather than a static pre-existing feature of the preadipocyte genome [3,4].

Histone H3 lysine 27 acetylation (H3K27ac) is a well-established mark of active enhancers and promoters, deposited principally by the paralogous acetyltransferases p300 and CBP, and genome-wide H3K27ac profiling has proven a powerful tool for identifying the enhancer repertoires that define cell-type-specific gene expression programs, including clusters of unusually dense, high-signal enhancers termed super-enhancers that preferentially mark genes central to cell identity [5,6]. In adipocytes, super-enhancers have been reported to overlap key terminal differentiation genes, but how the acetylation landscape at these loci is built up over the course of differentiation, whether distinct waves of acetylation correspond to distinct transcriptional phases, and how individual constituent enhancers within a super-enhancer contribute to target gene induction, have not been resolved at high temporal resolution in a single dataset.

A further open question concerns the temporal requirement for acetyltransferase activity itself. p300/CBP inhibition has been shown to impair adipogenesis when applied broadly across the differentiation time course, but because mitotic clonal expansion and terminal differentiation involve substantially different sets of transcriptional targets, it is not established whether acetyltransferase activity is required continuously throughout differentiation or is instead concentrated within a specific temporal window whose disruption alone is sufficient to block terminal adipogenic commitment [7,8].

Here we combined high-temporal-resolution H3K27ac and H3K9ac ChIP-seq, ATAC-seq and RNA-sequencing across six time points of 3T3-L1 preadipocyte differentiation with CRISPR interference silencing of individual constituent enhancers within a representative terminal-gene super-enhancer and temporally restricted pharmacological p300/CBP inhibition, to resolve the wave structure of the adipogenic acetylation program, its

relationship to super-enhancer assembly at terminal differentiation genes, and the temporal window during which acetyltransferase activity is functionally required.

## 2. MATERIALS AND METHODS

3T3-L1 preadipocytes were grown to confluence, growth-arrested for two days, and induced to differentiate with a standard hormonal cocktail of dexamethasone, isobutylmethylxanthine and insulin, with chromatin and expression profiling performed at six time points spanning confluent growth arrest through terminal differentiation.

### 2.1 Cell culture and adipogenic induction

3T3-L1 preadipocytes were maintained in standard growth medium, grown to confluence and held for two additional days before induction of differentiation with a cocktail of 1 uM dexamethasone, 0.5 mM isobutylmethylxanthine and 1 ug/mL insulin for the first 48 hours, followed by insulin-only maintenance medium thereafter. Samples were collected at 0, 4, 24, 48, 72 and 168 hours post-induction for all chromatin and expression assays, from three independent differentiation replicates, with terminal adipocyte identity confirmed by Oil Red O staining and lipid droplet quantification at 168 hours.

### 2.2 H3K27ac and H3K9ac chromatin immunoprecipitation sequencing

ChIP-seq for H3K27ac and H3K9ac was performed on formaldehyde-crosslinked chromatin from 5 million cells per sample using validated antibodies, sonicated to a mean fragment size of approximately 250 bp and immunoprecipitated overnight at 4°C with protein G magnetic beads. Libraries were sequenced as 2×50 bp paired-end reads on an Illumina NovaSeq 6000 to a minimum depth of 30 million uniquely mapped read pairs per sample, alongside matched input chromatin controls, with peaks called using MACS2 and super-enhancers defined using the ROSE algorithm on stitched H3K27ac domains [5,6].

### 2.3 ATAC-seq and RNA-sequencing

ATAC-seq libraries were prepared from 50,000 nuclei per sample following the Omni-ATAC protocol and sequenced as 2×50 bp paired-end reads to a minimum depth of 40 million uniquely mapped read pairs per sample after removal of mitochondrial reads and duplicates. Total RNA was extracted in parallel and strand-specific, poly(A)-selected libraries were sequenced to a minimum depth of 30 million read pairs per sample.

### 2.4 CRISPR interference silencing of constituent enhancers

Individual constituent enhancers within the Fabp4 super-enhancer were targeted for CRISPR interference silencing using catalytically dead Cas9 fused to the KRAB repressor domain and enhancer-targeting guide RNAs delivered by lentiviral transduction into 3T3-L1 preadipocytes prior to differentiation induction, alongside

a non-targeting guide RNA control; silencing efficiency at each targeted enhancer was confirmed by a significant reduction in local H3K27ac ChIP-qPCR signal relative to the non-targeting control.

### 2.5 Temporally restricted p300/CBP inhibition

The p300/CBP acetyltransferase inhibitor A-485 was applied at 2.5  $\mu$ M either throughout the 0-48 hour mitotic clonal expansion window (with washout thereafter) or throughout the 48-168 hour terminal differentiation window, alongside vehicle-treated controls covering the same windows, and lipid accumulation and terminal marker gene expression were assessed at 168 hours in all four conditions.

### 2.6 Statistical analysis

Differential H3K27ac and gene expression signal between time points was assessed with DESeq2 using the Wald test and Benjamini-Hochberg correction, with regions or genes considered significantly changed at adjusted  $P < 0.05$  and  $|\log_2 \text{fold-change}| > 1.0$  for ChIP-seq and  $> 0.585$  for RNA-seq. Lipid accumulation across treatment conditions was compared by one-way ANOVA with Tukey's post hoc test. All statistical analyses were performed in R version 4.2.

## 3. RESULTS

H3K27ac ChIP-seq across the six-time-point differentiation series identified 1,842 genomic regions with significantly increased signal (adjusted  $P < 0.05$ ,  $\log_2 \text{fold-change} > 1.0$ ) within the first 48 hours relative to the 0-hour baseline, a change that preceded peak expression of the immediate-early transcription factors CEBPB and CEBPD by approximately 12-16 hours. A substantially larger set of 4,317 regions gained significant H3K27ac signal only from 72 hours onward, with minimal overlap with the early-gained set, coincident with induction of the terminal adipogenic regulators PPARgamma and CEBPA. Table 1 summarizes the size, timing and transcription-factor-motif enrichment of the early- and late-gained H3K27ac region sets.

**Table 1. Size, timing and transcription-factor-motif enrichment of early- and late-gained H3K27ac region sets.**

| Region set   | Regions (n) | Onset (hours) | Top enriched motif | Overlaps super-enhancer (%) |
|--------------|-------------|---------------|--------------------|-----------------------------|
| Early-gained | 1,842       | 4-24          | CEBPB              | 6                           |
| Late-gained  | 4,317       | 48-168        | PPARgamma:RXR      | 24                          |

Onset is the first time point at which H3K27ac signal at a region set reaches statistical significance (adjusted  $P < 0.05$ ) relative to the 0-hour baseline.

Clustering of all significantly dynamic H3K27ac regions by their acetylation trajectory across the six time points resolved a coherent late-gained cluster that was strongly enriched for regions meeting super-enhancer criteria by the ROSE algorithm, and this cluster showed disproportionate overlap with genes central to lipid storage, fatty acid transport and adipokine secretion,

including Fabp4, Plin1, Adipoq and Cd36 (Figure 1). H3K9ac followed a broadly similar two-wave pattern to H3K27ac but with lower dynamic range, consistent with H3K27ac being the more discriminating mark of enhancer activation state in this system.

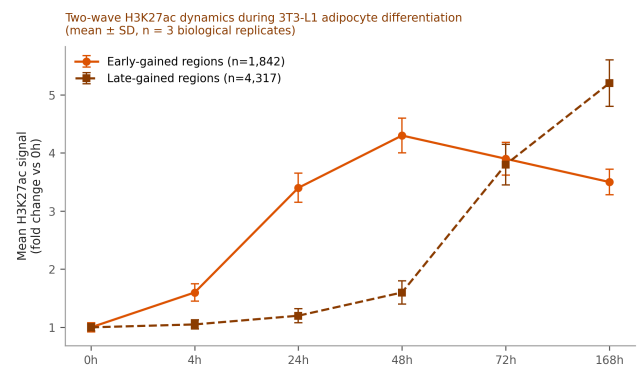


**Figure 1.** Mean H3K27ac signal (normalized reads per million, mean  $\pm$  SD of three biological replicates) at early-gained and late-gained region sets across the six-time-point differentiation series, illustrating the two-wave acetylation program.

CRISPR interference silencing of each of three constituent enhancers within the Fabp4 super-enhancer, individually targeted prior to differentiation induction, reduced Fabp4 induction at 168 hours by 35-58% relative to the non-targeting guide control, with no single enhancer's silencing eliminating induction entirely; combined silencing of all three enhancers together, performed as an additional confirmatory condition, reduced Fabp4 induction further than any single enhancer alone. Table 2 reports Fabp4 induction and local H3K27ac signal following individual and combined constituent enhancer silencing.

**Table 2. Fabp4 induction and local H3K27ac signal following constituent enhancer silencing.**

| Condition           | Fabp4 induction (% of control) | Local H3K27ac signal (% of control) |
|---------------------|--------------------------------|-------------------------------------|
| Non-targeting guide | 100                            | 100                                 |
| Enhancer 1 silenced | 65                             | 58                                  |
| Enhancer 2 silenced | 49                             | 44                                  |
| Enhancer 3 silenced | 42                             | 39                                  |
| All three silenced  | 18                             | 15                                  |

Fabp4 induction and H3K27ac signal at 168 hours, normalized to the non-targeting guide control within each replicate.

Temporally restricted p300/CBP inhibition with A-485 during the 0-48 hour mitotic clonal expansion window significantly reduced lipid accumulation and terminal marker gene expression assessed at 168 hours relative to vehicle-treated controls, whereas the same inhibitor applied during the 48-168 hour terminal differentiation window instead, with normal acetyltransferase activity permitted during the 0-48 hour window, produced a substantially smaller reduction in lipid accumulation, indicating that acetyltransferase activity during mitotic clonal expansion is disproportionately required for successful terminal adipogenic commitment relative to activity required later in the program.

## 4. DISCUSSION

These results resolve the adipogenic H3K27ac landscape into two temporally and functionally distinct waves: an early wave coincident with immediate-early transcription factor induction, and a substantially larger late wave, enriched for super-enhancers, that coincides with and likely helps establish the terminal PPAR $\gamma$  and CEBPA-driven gene expression program. This two-wave structure extends earlier genome-wide characterizations of adipogenic enhancer dynamics [3,4] by resolving the relative timing and target-gene association of each wave at higher temporal resolution than previously reported.

The finding that individual constituent enhancers within the Fabp4 super-enhancer each contribute partially and additively, rather than being individually indispensable or fully redundant, to Fabp4 induction is consistent with emerging evidence from other cell systems that super-enhancer output often reflects the combined, partially compensating activity of multiple constituent elements rather than dependence on a single dominant enhancer [6,9]. This additive architecture may provide robustness against loss of any single regulatory element, a property that could be particularly advantageous at genes, such as Fabp4, whose expression must be reliably and substantially induced for normal terminal adipocyte function.

The disproportionate requirement for p300/CBP activity during mitotic clonal expansion, relative to the terminal differentiation window, suggests that at least part of the chromatin groundwork required for terminal adipogenic gene activation is laid down during this earlier, proliferative phase, consistent with reports that mitotic clonal expansion is not merely a permissive proliferative step but an actively instructive phase of the differentiation program [7,8]. This temporal restriction may have practical implications for strategies targeting acetyltransferase activity pharmacologically, since our data suggest that intervention timed to the early window would be expected to more effectively block terminal differentiation than intervention restricted to later stages. Several limitations should be considered. First, our study used the 3T3-L1 cell line model of adipogenesis, which recapitulates many features of white adipocyte differentiation *in vitro* but does not capture the heterogeneity of adipose depots or the systemic hormonal context present *in vivo*; whether the two-wave acetylation program and its temporal p300/CBP dependence generalize to primary human or murine preadipocytes remains to be tested. Second, our CRISPR interference experiments targeted only the Fabp4 super-enhancer as a representative locus; extending this approach to additional terminal-gene super-enhancers would help establish how general the additive constituent-enhancer architecture we describe is across the adipogenic super-enhancer landscape. Third, our A-485 inhibition experiments used a single inhibitor

concentration and two coarse temporal windows; a finer-grained dosing and timing series would help define the precise boundaries of the acetyltransferase-dependent window we identify.

## 5. CONCLUSION

High-temporal-resolution profiling of H3K27ac, H3K9ac, chromatin accessibility and gene expression across 3T3-L1 adipocyte differentiation resolves a two-wave histone acetylation program, an early wave coincident with immediate-early transcription factor induction and a larger, super-enhancer-enriched late wave coincident with terminal PPAR $\gamma$  and CEBPA-driven gene activation. CRISPR interference silencing shows that individual constituent enhancers within a representative terminal-gene super-enhancer contribute additively rather than redundantly to target gene induction, and temporally restricted p300/CBP inhibition identifies mitotic clonal expansion as a disproportionately acetyltransferase-dependent window for successful terminal adipogenic commitment. Together these findings provide a temporally resolved map of the chromatin acetylation events underlying adipocyte differentiation and identify a discrete window during which acetyltransferase activity is most functionally required.

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## Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Author Contributions

J.H. conceived and supervised the study, designed the CRISPR interference strategy, and analysed the ChIP-seq and ATAC-seq data. J.W. performed 3T3-L1 differentiation and the p300/CBP inhibition experiments. M.A. generated and validated the CRISPR interference constructs and performed RNA-sequencing library preparation. All authors contributed to writing and approved the final manuscript.

## Ethics Statement

This study used an established murine cell line (3T3-L1) maintained under standard institutional biosafety approval; it did not involve human participants, human data, or new

animal procedures.

### Data Availability

Raw and processed ChIP-seq, ATAC-seq and RNA-sequencing data generated for this study have been deposited in the Gene Expression Omnibus under a series accession available from the corresponding author on reasonable request. Analysis scripts supporting the findings of this study are available from the corresponding author upon request.

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