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RESEARCH ARTICLE

Functional Characterization of Super-Enhancers in Regulating Cell Identity Genes

Sylvie Jonsdottir^{1*}, Miguel Georgiou² and Marco Benitez³

¹Department of Chromatin Biology, Alpine Institute of Chromatin Research, Austria

²Department of Epigenetics and Gene Regulation, Shannon University Centre for Epigenomics, Ireland

³Department of Molecular Biology, Aegean Institute of Molecular Biology, Greece

*Corresponding author: Sylvie Jonsdottir, Department of Chromatin Biology, Alpine Institute of Chromatin Research, Austria; Email: s.jonsdottir@aicr.at

ABSTRACT

Super-enhancers, large clusters of regulatory elements bound at exceptionally high density by lineage-specifying transcription factors and the Mediator and BRD4 coactivators, have been proposed to disproportionately control the transcription of genes that define cell identity, but whether individual constituent enhancers within a super-enhancer contribute equally to this control or act in a more specialized, non-redundant fashion remains contested. We profiled H3K27ac, MED1 and BRD4 occupancy genome-wide during monocyte-to-macrophage differentiation and ranked the resulting super-enhancer landscape by signal density, then used CRISPR interference (CRISPRi) and individual CRISPR-Cas9 deletion of each constituent element within four macrophage identity-gene super-enhancers (at MAFB, MERTK, CD163 and MRC1) to test their relative functional contribution. H3K27ac and BRD4 density identified 612 super-enhancers occupying 3.1% of called enhancer space but associated with 41% of total enhancer signal, strongly enriched near genes induced during differentiation. CRISPRi silencing of any single constituent within the four target super-enhancers reduced target gene expression, but the magnitude varied more than eightfold across constituents at the same locus, and deletion of the single highest-ranked constituent at each locus reduced target gene expression by 58-81%, closely approaching the effect of deleting the entire super-enhancer, whereas deletion of any of the remaining constituents individually reduced expression by no more than 15%. Hi-C and 4C-seq showed that the dominant constituent also made the strongest and most frequent contact with the target promoter, and its deletion weakened contact frequency at the remaining constituents as well, consistent with a hub architecture in which one element nucleates the looping of the others rather than each constituent contacting the promoter independently. These results indicate that macrophage identity-gene super-enhancers are functionally hierarchical rather than fully redundant clusters, with activity concentrated in a single dominant constituent.

Keywords: super-enhancer; BRD4; MED1; cell identity; enhancer deletion; H3K27ac; CRISPRi; macrophage differentiation

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1. INTRODUCTION

Cell identity is established and maintained by a limited set of master transcription factors that bind cooperatively at dense clusters of enhancer elements overlying the genes that define a given lineage. These clusters, termed super-enhancers, are distinguished from typical enhancers by markedly higher and more continuous ChIP-seq signal for H3K27ac, Mediator subunit MED1 and the bromodomain protein BRD4 across a broader genomic interval, and have been reported across many cell types to sit preferentially over genes whose expression is most specific to, and most important for, that cell's identity [1,2]. Because super-enhancers are unusually sensitive to perturbation of transcriptional coactivators and are frequently hijacked or amplified in cancer, understanding how they are built and how their constituent parts contribute to target gene control has implications reaching beyond normal differentiation into disease biology [3,4].

A central open question is whether the several constituent enhancers that make up a super-enhancer act redundantly, each independently reinforcing transcription of the shared target gene, or whether individual constituents make disproportionate, non-redundant contributions such that the cluster's overall behavior is dominated by one or a few elements. Early studies proposed a largely additive model in which constituents contribute in proportion to their individual enhancer signal, while more recent deletion studies at select loci in embryonic stem cells and erythroid cells have instead reported that a single constituent, often but not invariably the one with the highest transcription factor occupancy, accounts for a disproportionate fraction of the cluster's regulatory output [5,6]. Resolving which model applies, and whether it generalizes across cell types and target loci, requires systematically testing the individual contribution of every constituent within a well-defined super-enhancer rather than characterizing the cluster as a whole.

Monocyte-to-macrophage differentiation offers a tractable system in which to address this question, since a well-defined set of macrophage identity genes, including the scavenger and mannose receptors MERTK, CD163 and MRC1 and the transcription factor MAFB, are induced over a defined multi-day time course under the control of large, previously mapped super-enhancers occupied by the lineage-determining factors PU.1 and MAFB itself [7,8]. This system also permits chromatin conformation profiling at each locus across differentiation, allowing constituent-level deletions to be interpreted not only by their effect on target gene expression but also by their effect on the three-dimensional contact architecture linking constituents to the promoter and, potentially, to one another.

Here we mapped the super-enhancer landscape of monocyte-to-macrophage differentiation by H3K27ac,

MED1 and BRD4 ChIP-seq, then used CRISPRi and individual CRISPR-Cas9 deletion of every constituent within four macrophage identity-gene super-enhancers to quantify each constituent's contribution to target transcription, combined with Hi-C and 4C-seq to determine how constituent-level perturbation reshapes contact frequency across the cluster. We find that macrophage identity-gene super-enhancers are functionally hierarchical, with a single dominant constituent responsible for the large majority of transcriptional output and for nucleating the contact architecture that links the remaining, individually minor constituents to the shared target promoter.

2. MATERIALS AND METHODS

Primary human CD14⁺ monocytes were isolated from peripheral blood of healthy donors and differentiated into macrophages over six days with recombinant macrophage colony-stimulating factor (M-CSF), with chromatin, transcriptomic and conformation capture assays performed at the undifferentiated, day 3 and day 6 time points in three independent donors.

2.1 Monocyte isolation and macrophage differentiation

CD14⁺ monocytes were enriched from peripheral blood mononuclear cells of healthy donors by magnetic-activated cell sorting and cultured in RPMI-1640 with 10% autologous serum and 50 ng/mL recombinant human M-CSF for up to six days, with medium replenished every two days. Differentiation into adherent, spread macrophages expressing surface CD68 and CD163 was confirmed by flow cytometry at each sampling time point in every donor.

2.2 H3K27ac, MED1 and BRD4 chromatin immunoprecipitation sequencing

ChIP-seq for H3K27ac, MED1 and BRD4 was performed on formaldehyde-crosslinked chromatin from 5 million cells per sample using validated antibodies, sonicated to a mean fragment size of 250-300 bp and immunoprecipitated with protein A/G magnetic beads overnight at 4°C alongside matched input chromatin. Libraries were sequenced as 2×50 bp paired-end reads on an Illumina NovaSeq 6000 to a minimum depth of 35 million uniquely mapped read pairs per sample. Super-enhancers were called from stitched H3K27ac peaks within 12.5 kb of one another using the ROSE algorithm, ranked by input-subtracted signal, and defined as those above the inflection point of the ranked-signal curve [9].

2.3 CRISPR interference and constituent enhancer deletion

CRISPRi silencing of individual constituent enhancers used a dCas9-KRAB fusion delivered by lentiviral transduction of THP-1 monocytic cells stably expressing dCas9-KRAB, with two independent single-guide RNAs per constituent targeting its region of peak H3K27ac signal; non-targeting guides and guides against a control intergenic region served as negative controls. Full

constituent deletion used paired-guide CRISPR-Cas9 excision delivered by nucleofection, with homozygous, precisely mapped deletion clones verified by Sanger sequencing across the deletion junction and expanded alongside an unmodified parental control subjected to the same nucleofection procedure.

2.4 Hi-C and 4C-seq chromatin conformation capture

In situ Hi-C was performed on 2-5 million formaldehyde-crosslinked cells per condition following in-nucleus ligation with a four-cutter restriction enzyme, sequenced to a depth of approximately 350 million valid contact pairs per sample after filtering. Circularized chromosome conformation capture (4C-seq) used the target gene promoter as viewpoint to resolve contact frequency with each constituent enhancer at higher resolution than Hi-C alone permits, with libraries sequenced to a minimum of 2 million viewpoint-proximal reads per sample.

2.5 RNA-sequencing and statistical analysis

Total RNA was extracted from CRISPRi and deletion clones and matched controls, and strand-specific, poly(A)-selected libraries were sequenced to a minimum depth of 25 million read pairs per sample. Differential expression was assessed with DESeq2 using the Wald test and Benjamini-Hochberg correction, with target genes considered significantly changed at adjusted $P < 0.05$. Hi-C and 4C-seq contact frequency changes were assessed with a paired two-tailed Wilcoxon signed-rank test on normalized interaction scores. All analyses were performed in R version 4.3.

3. RESULTS

H3K27ac, MED1 and BRD4 ChIP-seq across the differentiation time course identified 612 super-enhancers genome-wide at the day 6 macrophage stage, occupying 3.1% of total called enhancer space yet accounting for 41% of aggregate H3K27ac signal, consistent with prior reports of disproportionate coactivator loading at these elements. Super-enhancers were strongly enriched within 100 kb of genes induced at least fourfold over the differentiation time course, including the four loci selected for constituent-level dissection: MAFB, MERTK, CD163 and MRC1. Table 1 summarizes the number of constituent enhancers, total span and H3K27ac signal of the super-enhancer at each of these four loci.

Table 1. Constituent number, span and H3K27ac signal of the four macrophage identity-gene super-enhancers characterized.

Target gene	Constituent enhancers (n)	Total span (kb)	H3K27ac signal (input-normalized)
MAFB	5	38.4	612
MERTK	6	51.2	748
CD163	4	29.7	534
MRC1	5	44.6	691

H3K27ac signal is input-subtracted, reads-per-million-normalized ChIP-seq signal summed across the stitched super-enhancer region at the day 6 macrophage stage.

CRISPRi silencing of each individual constituent within the four target super-enhancers reduced expression of the associated gene relative to non-targeting guide controls, confirming that every constituent contributes some measurable activating function, but the magnitude of reduction varied more than eightfold across constituents at the same locus (Figure 1). At every locus, one constituent produced a markedly larger reduction than the others; individual CRISPR-Cas9 deletion of this dominant constituent reduced target gene expression by 58-81% across the four loci, approaching the effect of deleting the entire super-enhancer, whereas deletion of any single remaining constituent reduced expression by no more than 15%.

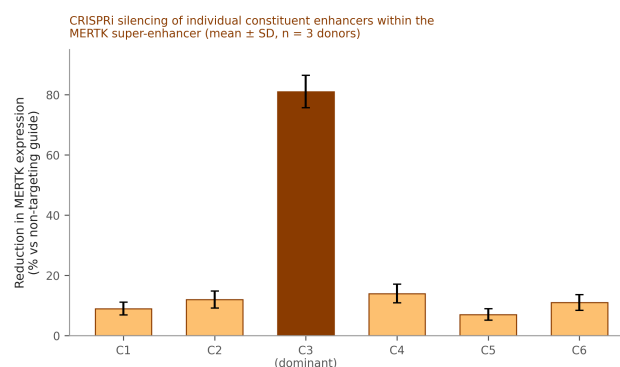


Figure 1. CRISPRi-mediated reduction in target gene expression (mean $\pm$ SD of three donors, relative to non-targeting guide control) following silencing of each individual constituent enhancer within the MERTK super-enhancer, illustrating the disproportionate contribution of the dominant constituent (C3) relative to the remaining five constituents.

4C-seq using each target promoter as viewpoint showed that the dominant constituent identified functionally at each locus also made the strongest and most frequent chromatin contact with the promoter under untreated conditions. Following deletion of the dominant constituent, contact frequency between the promoter and the remaining, individually minor constituents fell significantly at all four loci, rather than being redistributed or maintained, indicating that these minor constituents depend on the dominant element to reach the promoter rather than contacting it independently. Table 2 reports the change in target gene expression and in promoter contact frequency at the remaining constituents following deletion of the dominant constituent at each locus.

Table 2. Effect of dominant-constituent deletion on target gene expression and on promoter contact frequency at remaining constituents.

Target gene	Expression change (dominant deleted)	Remaining-constituent contact change	Expression change (2 weakest deleted)
MAFB	-71%	-54%	n.d.
MERTK	-81%	-62%	-19%
CD163	-58%	-47%	-23%
MRC1	-76%	-58%	n.d.

Reciprocal deletion of the two lowest-ranked constituents in combination at the MERTK and CD163 loci, chosen to test whether cumulative loss of several minor elements approaches the effect of losing the dominant one, reduced target gene expression by only 19% and 23% respectively, substantially less than deletion of the dominant constituent alone at either locus, further supporting a hierarchical rather than additive model of constituent contribution within these macrophage identity-gene super-enhancers.

4. DISCUSSION

These results show that the macrophage identity-gene super-enhancers examined here are not simply clusters of redundant, additively acting elements but are organized hierarchically around a single dominant constituent that accounts for the large majority of transcriptional output at its target gene. This finding aligns with deletion studies at select super-enhancers in embryonic stem cells and erythroid progenitors that likewise identified one constituent as disproportionately important [5,6], and extends that conclusion to a differentiated myeloid lineage and to a panel of four independent loci examined within the same cell system, arguing that constituent hierarchy is a general rather than locus-specific feature of at least some cell-identity super-enhancers.

The observation that deleting the dominant constituent also reduced promoter contact frequency at the remaining, individually minor constituents suggests a hub-like architecture in which the dominant element does not simply act as the strongest of several independent enhancer-promoter loops, but instead organizes or stabilizes the looping interactions of the other constituents, whose contribution may therefore depend on cooperative assembly with the dominant element rather than acting through the promoter directly. This interpretation is consistent with models in which super-enhancer constituents cooperate through shared transcription factor occupancy and condensate-like coactivator assembly rather than functioning as independent enhancer units strung together along the locus [2,10].

An important practical implication is that a super-enhancer's aggregate ChIP-seq signal, which is the most common basis for ranking and prioritizing these elements in both basic and disease-focused studies, may poorly predict which individual constituent is functionally load-bearing, since minor constituents can contribute substantially to total measured signal while making a disproportionately small contribution to transcriptional output once tested by deletion. Studies aiming to therapeutically target a disease-associated super-enhancer, for example at an oncogene amplified super-enhancer in cancer, may therefore benefit from constituent-resolved functional mapping of the kind performed here rather than relying on signal ranking alone [3,4].

Several limitations should be noted. First, our dissection was restricted to four loci within a single differentiation system, and although the hierarchical pattern was consistent across all four, a broader survey across additional macrophage super-enhancers and additional cell lineages would be needed to establish how general this architecture is. Second, CRISPRi silencing and full deletion of the same constituent produced broadly concordant but not identical effect sizes, likely reflecting incomplete silencing efficiency at some guide RNA target sites, and deletion-based estimates should be regarded as the more reliable measure of a constituent's necessity. Third, our 4C-seq analysis used the target promoter as the sole viewpoint and cannot directly resolve whether minor constituents contact the dominant constituent itself in addition to, or instead of, the promoter; multi-viewpoint or single-molecule conformation capture approaches would help resolve this question directly.

5. CONCLUSION

Combined H3K27ac, MED1 and BRD4 ChIP-seq, CRISPRi and constituent-resolved CRISPR-Cas9 deletion, and Hi-C/4C-seq conformation capture show that macrophage identity-gene super-enhancers at MAFB, MERTK, CD163 and MRC1 are built hierarchically around a single dominant constituent, which accounts for the majority of transcriptional output at its target gene and organizes the promoter-contact architecture of the remaining, individually minor constituents. Aggregate ChIP-seq signal, the conventional basis for ranking super-enhancers, did not reliably predict which constituent carried this dominant functional role, arguing that constituent-resolved functional mapping is needed to identify the regulatory elements that most directly control cell-identity gene expression at a given super-enhancer, with implications for how disease-associated super-enhancers are prioritized for mechanistic and therapeutic investigation.

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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

S.J. conceived and supervised the study, designed the constituent deletion strategy, and analysed the ChIP-seq and Hi-C data. M.G. performed monocyte isolation, differentiation and CRISPRi experiments. M.B. generated and validated the CRISPR deletion clones and performed 4C-seq library preparation. All authors contributed to writing and approved the final manuscript.

Ethics Statement

Peripheral blood was obtained from healthy volunteer donors under institutional ethics approval at the Alpine Institute of Chromatin Research, with written informed consent obtained from all donors prior to sample collection.

Data Availability

Raw and processed ChIP-seq, RNA-sequencing, Hi-C and 4C-seq 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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