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BRIEF COMMUNICATION

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Early Chromatin Remodeling Events in Acutely Stimulated CD8$^+$ T Cells

Bryan McDonald[a,b,1], Brent Y. Chick[c,d,1], Diana C. Hargreaves[c], and Susan M. Kaech[a,*]

[a]*NOMIS Center for Immunobiology and Microbial Pathogenesis, Salk Institute for Biological Studies, La Jolla, CA, USA;* [b]*Biomedical Sciences Graduate Program, University of California San Diego, La Jolla, CA, USA;* [c]*Molecular and Cell Biology Laboratory, Salk Institute for Biological Studies, La Jolla, CA, USA;* [d]*Biological Sciences Graduate Program, University of California at San Diego, La Jolla, California, USA*

T cells undergo extensive chromatin remodeling over several days following stimulation through the T cell receptor. However, the kinetics and gene loci targeted by early remodeling events within the first 24 hours of T cell priming to orchestrate effector differentiation have not been well described. We identified that chromatin accessibility is rapidly and extensively remodeled within 1 hour of stimulation of naïve CD8$^+$ T cells, leading to increased global chromatin accessibility at many effector T cell-associated genes that are enriched for AP-1, early growth response (EGR), and nuclear factor of activated T cells (NFAT) binding sites, but this short duration of stimulation is insufficient for commitment to clonal expansion *in vivo*. Sustained 24-hour stimulation led to further chromatin remodeling and was sufficient to enable clonal expansion. These data suggest that the duration of antigen receptor signaling is intimately coupled to chromatin remodeling and activation of genes involved in effector cell differentiation and highlight a potential mechanism that helps CD8$^+$ T cells discriminate between foreign- and self-antigens.

INTRODUCTION

CD8$^+$ T cells are critical for host defense against intracellular pathogens and against tumors. Following T cell receptor (TCR)-dependent recognition of cognate peptides bound to major histocompatibility complex class I (MHC-1) on antigen presenting cells, naïve CD8$^+$ T cells initiate protein tyrosine kinase signaling cascades, including ZAP-70 (signaling via MAP kinase cascades), and phospholipase Cγ1 (signaling via second messengers to activate protein kinase C (PKC) and regulate intracellular Ca^{2+} levels) [1]. These signaling pathways convey information to the nucleus via nuclear accumulation of the transcription factors (TFs) NFAT, NFkB, and the AP-1 family of TFs [2,3], leading to activation of biosynthesis pathways, epigenetic remodeling, increased global tran-

*To whom all correspondence should be addressed: Susan M. Kaech, Director, NOMIS Center for Immunobiology and Microbial Pathogenesis, Salk Institute for Biological Studies, La Jolla, CA; Email: skaech@salk.edu; ORCID: 0000-0003-1674-1420.

Abbreviations: TF, Transcription factor; EGR, Early growth response; NFAT, Nuclear factor of activated T cells; TCR, T cell receptor; PKC, protein kinase C; NFkB, Nuclear factor κB; pMHC, peptide major histocompatibility complex 1; BAF, BRG/BRM associated factors; DC, Dendritic cell; ATAC-seq, assay for transposable chromatin with sequencing; OCR, open chromatin region; PRC2, polycomb repressive complex 2.

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[1]These authors contributed equally

scription and translation, rewiring of metabolic pathways to sustain energetic expenditure, activation of cytotoxic and other functional effector genes, and entry into the cell cycle and rapid clonal expansion that gives rise to a large effector cell pool capable of mediating host defense [4,5].

In order to prevent aberrant T cell responses directed against host tissues, peripheral T cells must discriminate between foreign and host antigens, and both TCR signal strength and duration are key factors regulating this discriminatory property. On short timescales (seconds), one proposed underlying mechanism is the "kinetic proofreading" antigen recognition model: local TCR-proximal signaling events (eg, tyrosine phosphorylation, signaling complex recruitment, which occur within ~3-4 seconds of TCR-pMHC engagement [6,7]), propagate more slowly than individual TCR-pMHC interaction lifetimes, while dissociation rates of nonspecific complexes are sufficiently rapid as to prevent premature systemic secondary messenger activity, therefore necessitating sustained high affinity (low K_{OFF} rate) TCR-pMHC interactions for successful T cell activation [8,9]. Indeed, ~6 hours of continuous antigenic stimulus are minimally required for CD8+ T cells to enter into programmed, or "autopilot" proliferation [10-12]. Similarly, the BAF (BRG/BRM associated factors) chromatin remodeling complex is rapidly recruited to chromatin within minutes of activation by TCR crosslinking or by PKC stimulation [13]. *In vivo,* naïve CD8+ T cells were observed to undergo priming in phases, initially by scanning DCs and forming transient suboptimal interactions for ~8 hours [14], followed by prolonged ~8-20 hour continuous interactions by high affinity TCR-pMHC interactions with DCs [15]. If antigen receptor signaling can induce rapid BAF complex recruitment (and presumably activity, which occurs within minutes of recruitment *in vitro* [16,17]), then what longer timescale mechanisms exist to help naïve T cells discriminate between spurious short-term (<6 hours) and longer (>6 hours) antigen-derived signals to either prevent or enable effector cell differentiation and programmed proliferation?

There is mounting evidence that CD8+ effector cell fates are established early following activation—even prior to the first cell division *in vitro* [18] and during infection *in vivo* [19,20]. However, the extent to which effector cell fates become epigenetically imprinted via chromatin remodeling in this timeframe, particularly within the first hour of activation, has not been fully resolved. Therefore, to address this gap we investigated how the chromatin landscape is influenced by stimulation signal duration (1 hour versus 24 hour). Chromatin was rapidly remodeled within 1 hour of stimulation, defined by simultaneous *de novo* gain of open chromatin regions (OCRs) enriched for bZIP (AP-1), EGR, and NFAT TF binding motifs that neighbored genes involved in effector functions, cell growth, and *Myc*-related genes. However, 1 hour stimulated cells could not undergo programmed proliferation when transferred into antigen-free hosts. In summary, these data define early temporal kinetics of chromatin accessibility dynamics of recently activated CD8+ T cells.

MATERIALS AND METHODS

Mice

C57BL/6J were purchased from Jackson Laboratories. P14 mice [21] have been previously described. Animals were housed in specific-pathogen-free facilities at the Salk Institute, La Jolla, CA. All experimental studies were approved and performed in accordance with guidelines and regulations implemented by the Salk Institute Animal Care and Use Committee.

Cell Isolation

Spleens and lymph nodes were mechanically dissociated with 1mL syringe plungers over a 70um nylon strainer. Cells were incubated in ammonium chloride potassium (ACK) buffer for 5 minutes to lyse red blood cells. P14 cells were isolated by negative selection with biotinylated antibodies against CD4, CD19, B220, MHCII, CD11b, CD11c, and CD49b in MACS buffer, and MojoSort beads were added at 5% v/v for 5 minutes before placing the cell suspension on a magnet and collecting the supernatant.

In Vitro Stimulations

24-well plates were coated with anti-Armenian Hamster IgG (Jackson ImmunoResearch #127-005-099) at 30μg/mL in PBS at 4°C overnight. Purified P14 CD8+ T cells were stimulated at $1x10^6$ cells in 300μL complete RPMI (RPMI with 10% FBS, Pen/strep, L-glutamine, 50uM β-ME) with 2μg/mL anti-CD3e (BD #567114), anti-CD28 (BD #567110), and 100U/mL recombinant human IL-2 (PeproTech #212-02).

Adoptive Transfer and Immunization

P14 cells were labeled with CellTrace Violet in warm PBS for 8 minutes, and then washed with excess cold complete RPMI (RPMI with 10% FBS, Pen/strep, L-glutamine, 50uM β-ME). Recipient mice received 0.8-$1.5x10^6$ cells in 100μL PBS via retroorbital injection. Immunized mice received an i.p. injection containing 5μg GP33 peptide and 25μg CpG-B (ODN-1826) in 100μL PBS at the time of P14 transfer.

Flow Cytometry

Cell suspensions were first incubated with eBiosci-

ence Fixable Viability Dye eFluor 780 for 5 minutes at room temp. Cells were stained with primary surface antibodies in PBS with 2% FBS, 0.1% NaN_3 for 20 minutes on ice.

ATAC-Seq Library Preparation and Sequencing

ATAC-seq was performed as previously described [22]. Briefly, 5,000-50,000 viable cells were washed with cold PBS, collected by centrifugation, then lysed in resuspension buffer (RSB) (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2) supplemented with 0.1% NP40, 0.1% Tween-20, and 0.01% digitonin. Samples were incubated on ice for 3 min, then washed out with 1 ml RSB containing 0.1% Tween-20. Nuclei were pelleted by centrifugation at 500g for 10 min at 4°C then resuspended in 50 ul transposition mix (25ul 2x TD buffer, 2.5 ul transposase (100 nM final), 16.5 ul PBS, 0.5 ul 1% digitonin, 0.5 ul 10% Tween-20, 5 ul H_2O) and incubated at 37°C for 30 min in a thermomixer with 1000 RPM mixing. DNA was purified using a Qiagen MinElute PCR cleanup kit, then PCR amplified using indexed oligos. The optimal number of amplification cycles for each sample was determined by qPCR. Libraries were size selected using AmpureXP beads and sequenced using an Illumina NextSeq500 for 75bp paired-end reads.

Data Availability

All sequencing data from this paper are available in GEO under accession series GSE236306.

ATAC-Seq Analysis

Paired-end reads were aligned to the *Mus musculus* mm10 genome using STAR [23] with default parameters. ATAC-seq peaks were called using HOMER [24] findPeaks.pl using parameters `-style dnase`. Peaks were called when enriched >4.0-fold over local tag counts. Reads mapped to peaks were quantified using featureCounts [25]. Differentially accessible regions were identified using DESeq2 [26] with fold change ≥2.0 or ≤−2.0, FDR < 0.05. Peaks sets were annotated with HOMER, and visualizations were created using deepTools v3.5.1 [27].

Transcription Factor Binding Motif Analysis

Sequences within 100 bp of peak centers were compared to known motifs in the HOMER database using the findMotifsGenome.pl command with default parameters. Random GC content-matched genomic regions were used as background. Enriched motifs are statistically significant motifs in input over background by a P value of less than 0.05 using a cumulative binomial distribution.

RESULTS

Chromatin is Remodeled Rapidly After T Cell Activation

To better understand the dynamics of T cell chromatin remodeling within short timeframes, we activated purified naïve P14 TCR-transgenic $CD8^+$ T cells (which recognize the GP_{33-41} epitope from the lymphocytic choriomeningitis virus (LCMV) glycoprotein) with immobilized antibodies directed against CD3 and CD28 with recombinant hIL-2 for either 1 hour or 24 hours, and then isolated nuclei and performed assay for transposable chromatin with sequencing (ATAC-seq) to assess genome-wide chromatin accessibility profiles. Principal component analysis (PCA) of all replicates revealed that 84.6% of variance within the dataset was explained by the duration of antigenic stimulus (Figure 1a). Importantly, this analysis indicated that 1 hour stimulated cells were epigenetically distinct from unstimulated cells, which was further underscored by quantification of differentially accessible regions that showed that ~9500 sites gained *de novo* accessibility, and ~4500 sites lost accessibility following 1 hour of stimulation *in vitro* (Figure 1b). Cells stimulated for 24 hours continued to both acquire and lose several thousand OCRs (Figure 1b), indicating that chromatin remodeling is a dynamic and evolving process in $CD8^+$ T cells responding to antigenic stimulus over a 24-hour period. In spite of the fact that 1 hour stimulated cells undergo robust chromatin remodeling and upregulate activation markers like CD44, most of these cells failed to divide at all when transferred into congenic C57BL/6 mice, while a small subset underwent a single round of division (Figure 1c-d), altogether indicating that this epigenetic rewiring was not sufficient to engage "autopilot" proliferation *in vivo*. Importantly, however, 1 hour stimulated cells that were transferred into mice that were subsequently immunized with GP_{33-41} peptide along with CpG-B (ODN-1826) adjuvant successfully underwent multiple rounds of division similar to 24 hour stimulated cells (Figure 1d), indicating that acute cessation of antigen signaling after 1 hour does not prevent the cells from responding to antigen at a later time (ie, the cells do not become anergic).

Early Activated Accessible Regions are Enriched in Cell Cycle and Effector Response-Associated Genes

To better define the biological processes that are regulated by the changes in chromatin accessibility, we first annotated the genomic positions of OCRs and then clustered the OCRs by temporal dynamics (Figure 2a). This revealed various patterns of accessibility dynamics, including I) rapid and progressively closed OCRs, II)

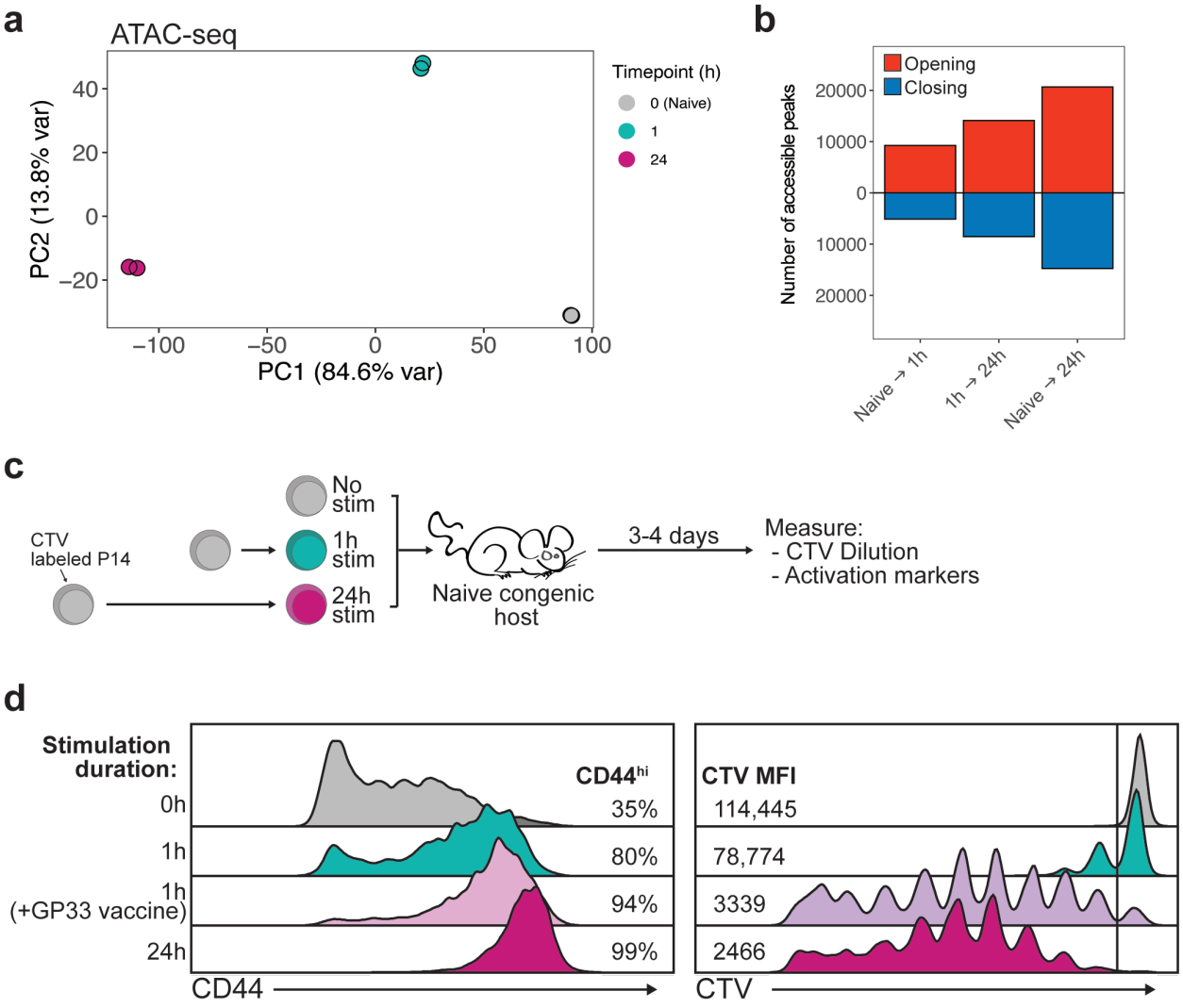

Figure 1. **Chromatin is remodeled rapidly after T cell activation**. (**a**) Principal component analysis of ATAC-seq from naïve, 1 hour, or 24 hour stimulated P14 CD8⁺ T cells. (**b**) Number of OCRs gained and lost following activation. (**c**) Experimental design and (**d**) representative CD44 and CellTrace Violet (CTV) histograms from transferred P14 cells 3 days after adoptive transfer.

slow closing OCRs, III) transient, 1 hour activation-specific OCRs, IV) rapid induced OCRs, and V) late induced OCRs. Clusters I and II were strongly and similarly enriched for ETS, FORKHEAD, LEF1, and TCF7 binding motifs, consistent with the known roles of these TF families in naïve CD8⁺ T cells. In contrast, all induced clusters (clusters III-V) were strongly enriched for bZIP and NFAT family motifs. Early induced clusters (III-IV) were also enriched in EGR motifs, while late induced cluster V OCRs were enriched for IRF, PRDM1, and T-box binding motifs. All five dynamic OCR clusters were predominantly located in non-promoter (ie, intronic and intergenic) regions (Figure 2b). Genes annotated to early induced OCRs at 1 hour were enriched for Gene Ontology (GO) terms related to transcription and translation, cell development, and nuclear receptor binding (Figure 2c), suggesting that cells are preparing to enter the cell growth phase, though no GO terms related to cell division or proliferation were significantly enriched (data not shown).

To more finely resolve the genes that may be regulated by opening and closing of OCRs, we focused on several classes of genes with established roles in regulating T cell responses or cell cycle dynamics and analyzed both chromatin accessibility at each locus as well as corresponding protein abundance [28] (Figure 2d, 2e). Genes exhibiting early induced OCRs included functional effector molecule *Ifng* and the high affinity IL-2 receptor

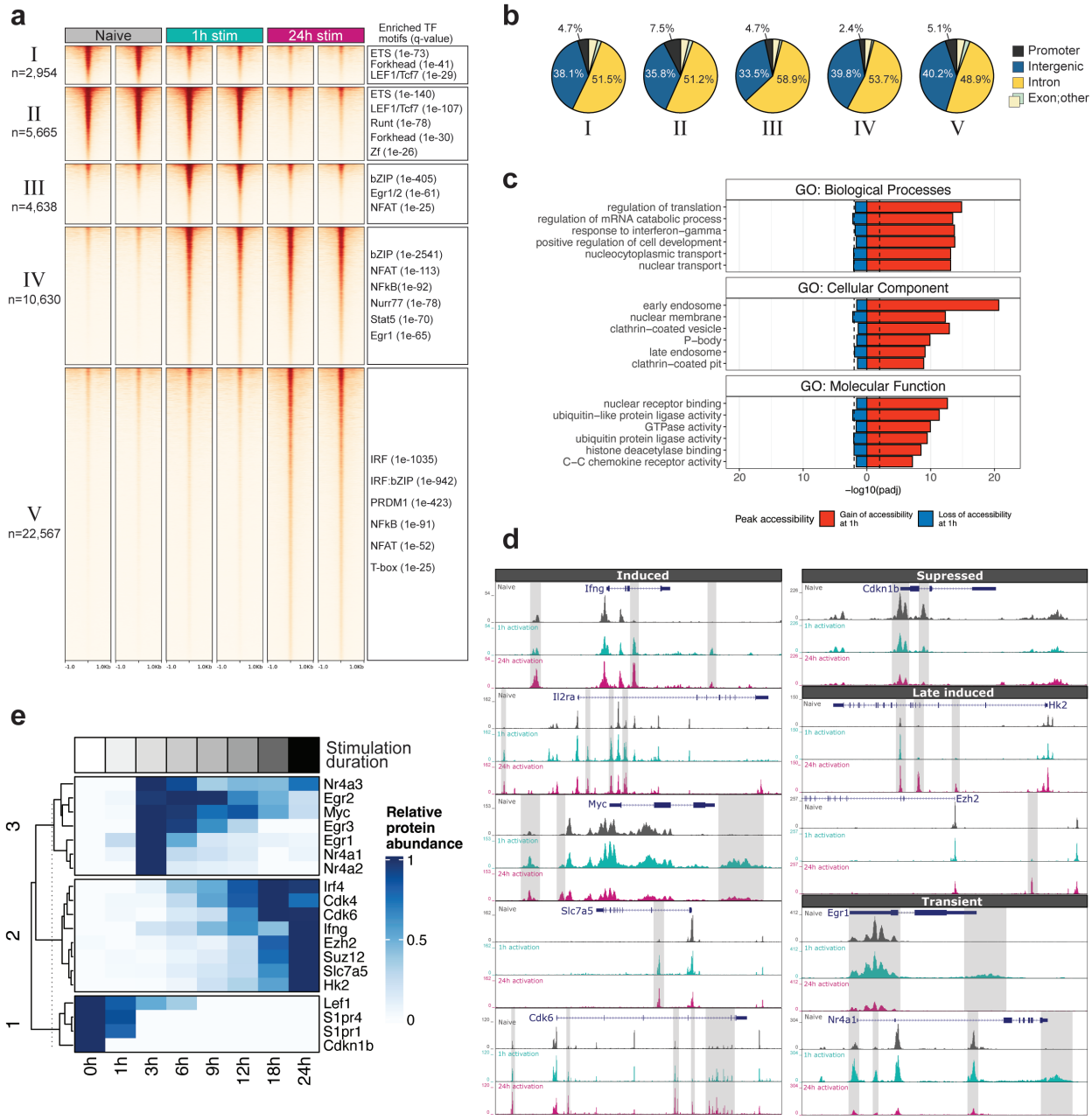

Figure 2. **Early activated accessible regions are enriched in cell cycle and effector response-associated genes**. (**a**) ATAC-seq signal tracks and clusters. Highly enriched TF binding motifs and associated enrichment q-values for each cluster are indicated. (**b**) Gene Ontology enrichment analysis of protein-coding genes annotated to differential OCRs between 1 hour-stimulated and naïve cells. (**c**) Gene Ontology (GO) enrichment of OCRs gained or lost in 1 hour stimulated cells. (**d**) ATAC-seq signal tracks at *Ifng*, *Il2ra*, *Myc*, *Slc7a5*, *Cdk6*, *Cdkn1b*, *Hk2*, *Ezh2*, *Egr1*, and *Nr4a1* loci. (**e**) Heatmap of Immpres T cell receptor activation timecourse proteomes [28].

Il2ra, the critical transcriptional regulator of cell growth *Myc* and its target gene *Slc7a5* [29] which is critical for amino acid transport in activated T cells. Several intronic regions at the positive cell cycle regulator *Cdk6* locus increased and maintained accessibility by 1 hour, while the key negative cell cycle regulator *Cdkn1b* ($p27^{Kip1}$) locus exhibited an overall decrease in accessibility over time. *Hk2* (hexokinase 2), a critical regulator of glucose metabolism and the rate limiting factor for aerobic glycolysis [30], increased in accessibility at one intron within 1 hour, and two additional intronic regions by 24 hours. *Ezh2*, a component of polycomb repressive complex 2 (PRC2),

showed *de novo* accessibility at an upstream intergenic site. Finally, transcription factor genes *Egr1* and *Nr4a1* exhibited transient locus-wide increases in accessibility which sharply decreased by 24 hours, consistent with the kinetics of protein levels of these factors (Figure 2e).

DISCUSSION

Our study reveals that chromatin remodeling activity is rapidly induced following antigen receptor-dependent activation, but that this remodeling activity continues for at least 24 hours in the presence of continuous antigenic stimulation. Sets of genes underwent remodeling with distinct kinetics. For example, early response genes like, Myc, EGR1/2 and NR4A1/2/3 that are transiently expressed shortly after activation exhibit similar kinetics of transient increases in open chromatin after 1 hour but decreasing or baseline accessibility at 24 hours. Conversely, late response genes, like *Cdk6*, *Ezh2*, and *Hk2*, with peak protein abundance between 18-24 hours post-activation required sustained activation before full remodeling was achieved across each respective locus.

We and others previously demonstrated that *Ezh2* was required to suppress the memory-associated genetic program in order for cells to terminally differentiate [31,32]. Our analysis identified a potential upstream *Ezh2* enhancer that was present at 24 hours, but not 1 hour after activation, pointing to a potential time-dependent *cis*-regulatory mechanism controlling EZH2 expression. Indeed, EZH2, EED, and SUZ12 protein abundance reached peak values after 18 hours of activation, suggesting that a sustained antigenic stimulation was required to induce expression and allow for PRC2 activity, possibly to suppress the naïve T cell epigenetic program via polycomb repression only under conditions of sustained antigen receptor signaling.

Interestingly, positive cell cycle regulator genes (eg, *Cdk6*) increased accessibility at multiple putative intronic enhancers. Given that T cells undergoing programmed proliferation divide every 6-8 hours [33], we speculate that generational inheritance of an accessible *Cdk6* locus allows for rapid re-engagement of the cell cycle immediately after mitotic exit in order to sustain this high proliferative rate. On the other hand, while negative regulation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (*Cdkn1b*) has largely been ascribed to its proteolytic degradation [34], our analysis points to an potential additional mechanism by which *Cdkn1b* levels may be regulated via the loss of accessible chromatin at its regulatory sequences.

In summary, we have outlined early temporal kinetics of chromatin remodeling in acutely stimulated naïve $CD8^+$ T cells and mapped genome-wide patterns of opening and closing of *cis*-regulatory regions. Chromatin remodeling is extensive in the first hour following stimulation, and while insufficient to enable effector cell fate commitment, this early response likely prepares the cells for eventual proliferative and effector gene programming that can only proceed to completion in the presence of subsequent continuous stimulation. By analogy to the kinetic proofreading model, where 1) short-duration antigen receptor engagement (1 hour) represents non-specific, or perhaps non-pathogenic interactions, and 2) long-duration (>6 hour) stimuli represents specific or pathogenic interactions, and 3) assuming that naive and proliferation-competent effector-primed cells represent distinct quantized functional states; we propose that the rate of chromatin remodeling may be sufficiently slow so as to allow for discrimination between these two lengths of stimulation, and ensure proper proliferative responses by optimally stimulated cells while averting proliferation and host tissue-directed responses by sub-optimally stimulated T cells. In fitting with such a model, certain assumptions will need to be formally demonstrated: namely, that early activation-induced chromatin states revert back to naïve baseline states if antigen signaling is halted before ~6 hours. Though our study focused exclusively on T cell differentiation, this model may serve as a generalizable framework to understand how differentiation and cell fates are regulated in all types of cells responding to extrinsic signals.

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