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In Search of Lost Time: Enhancers As Modulators of Timing in Lymphocyte Development and Differentiation

Jonathan Chu[1,2,3], **Nicholas Pease**[1,2,3], **Hao Yuan Kueh**[1,2,3]

[1]Department of Bioengineering, University of Washington

[2]Molecular and Cellular Biology Program, University of Washington

[3]Institute for Stem Cell and Regenerative Medicine

Summary

Proper timing of gene expression is central to lymphocyte development and differentiation. Lymphocytes often delay gene activation for hours to days after the onset of signaling components, which act on the order of seconds to minutes. Such delays play a prominent role during the intricate choreography of developmental timing and during the execution of an effector response. Though a number of mechanisms are sufficient to explain timing at short timescales, it is not known how timing delays are implemented over long timescales that may span several cell generations. Based on the literature, we propose that a class of *cis*-regulatory elements, termed "timing enhancers", can explain how timing delays are controlled over these long timescales. By considering chromatin as a kinetic barrier to state switching, the timing enhancer model explains experimentally observed dynamics of gene expression where other models fall short. In this review, we elaborate on features of the timing enhancer model and discuss evidence for its generality throughout development and differentiation. We then discuss potential molecular mechanisms underlying timing enhancer function. Finally, we explore recent evidence drawing connections between timing enhancers and genetic risk for immunopathology. We argue that the timing enhancer model is a useful lens through which to understand how *cis*-regulatory elements control the central dimension of time in lymphocyte biology.

Introduction

In the immune system, regulatory events that control cell states and cell fates unfold over a staggeringly wide-range of timescales (Figure 1). During an immune response, B and T lymphocytes activate signaling pathways within seconds, but differentiate into effector and memory cells only days to weeks later. Similarly, in response to differentiation signals, lymphocyte progenitors proceed through an intricate choreography of developmental stages, each of which is orchestrated over many days. The timing of these stages allows progenitors to perform developmental functions, such as antigen receptor rearrangement, repertoire selection, and expansion into a mature, selected cell population (Figure 1). Despite its implicit importance, much remains unknown about how timing is set during lymphocyte

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development and differentiation. In particular, how timing is specified at extended timescales, ranging from days to months, is a mystery. While specific examples of lymphocyte timing control have been elucidated[1], general principles remain unclear.

By drawing upon older and more recent literature, we present evidence for a distinct class of *cis*-regulatory elements, which we term "timing enhancers", that control the timing of gene expression and cell fate transitions. Enhancers are non-coding DNA elements that mediate the establishment of lineage-specific gene programs during cell differentiation. Their importance for optimal immune function is underscored by the large enrichment of disease-associated polymorphisms within their sequences[2]. While enhancers are often assumed to control the expression levels of genes[3,4], there is evidence that enhancers can control the timing at which genes switch on, and do so over extended timescales spanning many days and cell generations.

Here, we first provide a definition of timing enhancers that distinguishes them from other types of enhancer control. We then review evidence for timing enhancers in lymphocyte development and differentiation. Next we discuss potential molecular mechanisms underlying timing enhancer function. Finally, we review evidence that disruptions to timing enhancers, by altering gene activation kinetics, can perturb immune response dynamics and contribute to immunopathology.

Timing enhancers: a definition

Enhancers are non-coding DNA sequences that act in *cis* to promote the expression of their target genes. Their action is critical to the proper establishment of cell lineage-specific gene expression programs during multicellular development and function. Soon after their discovery in the early 1980s, it was observed from single-cell measurements that enhancers could impact gene expression dynamics in different ways[5]. In particular, some enhancers increase the amplitude of expression of their target genes[6,7], whereas others increase the likelihood of their activation in an all-or-none manner[8,9].

Tremendous progress has since been made to elucidate the molecular basis of enhancer action[10–14], and to define and classify enhancer types based on distinguishing molecular features. However, as these newer classifications mostly arise from bulk-averaged measurements, it remains unclear how they map onto dynamic mechanisms of enhancer control observed at the single-cell level. Here we elaborate on a formal definition of enhancer types based on their dynamic modes of gene expression control. This framework will provide a lens through which one can discern these distinct types of enhancer function in lymphocytes.

Based on older and more recent studies in single cells, there is growing evidence that enhancers can modulate either the expression levels of their target genes or the timing at which these genes become expressed. As such, we propose classifying enhancers into two types: amplitude enhancers and timing enhancers (Figure 2). We note that a single enhancer can sometimes have both timing and amplitude control functions. While discussing

enhancers with mixed functionality, our classification scheme allows one to discern distinct modes of dynamic control.

Timing enhancers control the timing at which their target genes switch from a silent to an active chromatin state, which is then accessible to the transcriptional machinery. Switching is initiated by the binding of *trans*-factors to the enhancer; however, even with full *trans*-factor induction and binding, switching does not occur rapidly. Rather, switching occurs only after an extended time delay that can range from hours to days, and possibly longer (Figure 2, top left). In some cases, this time delay can span multiple cell generations, implying that some genes can switch between stable chromatin states that are heritable across cell division. This ability to control switching between different heritable states links timing enhancers to chromatin-based mechanisms that can stably propagate across cell division[15–17], a point we discuss further below. It also accounts for chromatin state as a kinetic barrier that can explain the delay between *trans*-factor action and gene activation.

As seen in a range of studies[6,7,18], gene activation delays generated by timing enhancers are inherently probabilistic, such that cells in a uniform population turn on the target gene at different times, even when they concurrently upregulate *trans*-factors that bind the enhancer. As a result of this variability, initially homogeneous cell populations could generate multiple subpopulations with or without target gene expression, also termed "variegated expression" in the earlier literature (Figure 2, top right). This heterogeneity arises even upon exposure to uniform signals. Variability in activation is likely linked to the probabilistic nature of chromatin state switching, as observed across diverse systems[19–21].

The ability of timing enhancers to control chromatin states links them to "locus control regions" (LCRs). LCRs are extended *cis*-regulatory elements that can establish active chromatin states at proximal regions to drive cell-type specific gene expression[22]. LCRs are in turn closely related to a more recently defined class of *cis*-regulatory elements, termed "super-enhancers" or "stretch enhancers"[23,24]. These elements typically contain multiple modules of transcription factor binding sites that are distributed over an extended genomic region. Individual modules within LCRs may harbor timing control functions; however, we note that timing enhancers do not necessarily reside within LCRs or super-enhancers[25,26].

Amplitude enhancers, on the other hand, modulate the expression magnitude of their target genes. To do so, they increase the transcription rate of their target genes, by recruiting *trans*-factors that load or drive elongation of RNA polymerase II at an already accessible locus (Figure 2, bottom left). Like chromatin state switching, transcription initiation is also a stochastic process[27–29], occuring in intermittent bursts of polymerase loading and release from a gene promoter. Amplitude enhancers appear to primarily control burst initiation frequencies[30–33], though they may also control burst duration[34]. However, unlike chromatin state switching as controlled by timing enhancers, transcriptional bursting is transient, and occurs over fast timescales, ranging from seconds to tens of minutes[34]. As a result, the primary effect of an amplitude enhancer is to generate graded changes in expression magnitude within a single population (Figure 2, bottom right). This is in contrast to timing enhancers, which generate distinct, stable subpopulations with discrete levels of target gene expression. Thus, amplitude enhancers, by controlling bursting kinetics, would modulate

mean expression levels of their target gene across a single population, and do so relatively rapidly in response to *trans*-factor binding. Amplitude enhancers are important for maintaining proper levels of expression after a gene locus has been activated, and we contrast them with timing enhancers here to distinguish between the functional properties of these regulatory elements; however, in keeping with the focus of this review, we do not discuss them further here.

Potential functions of timing enhancers

What functional roles could timing enhancers play in immune development and function? Broadly speaking, timing enhancers endow lymphocytes with an ability to regulate the timescales of immune regulatory events in a cell-autonomous manner (Figure 3A). Immune cells execute carefully choreographed functions while being mobile and broadly distributed throughout tissues and organs. Thus, they may benefit from the use of cell-autonomous timekeeping mechanisms to set the pace and order of fate transitions independently of environmental signals[35]. For instance, upon antigen stimulation, T cells enter an activated state, where they proliferate explosively before either re-entering a quiescent state or undergoing cell death. The duration of this activated state, and the degree of cell proliferation it undergoes, is set by a cell-autonomous timer that persists upon antigen withdrawal[36,37]. Autonomous timekeeping mechanisms may play roles in controlling other cellular transitions in lymphocyte development and function; however, for most processes it remains to be determined how autonomous timers contribute. By controlling the activation of lineage-specifying genes, timing enhancers could enable cells to delay their differentiation upon receiving instructive signals, as observed in a number of other developmental systems[38,39]. On the other hand, by controlling the activation of cytokines or other effector genes, timing enhancers could control the pace or duration of immune effector responses.

In the absence of competing regulation, all cells that await activation of a gene controlled by a timing enhancer would eventually turn it on, given enough time (Figure 3A). Indeed, complete gene activation in a cell population is sometimes observed[25]. However, in some cases, a gene may be competent for activation only during a limited time window, or may be blocked from activation by the expression of an antagonistic regulator, such that only a fraction of cells end up activating and expressing the gene (Figure 3B). In this case, the strength of the timing enhancer not only affects the initial kinetics of gene induction, but also controls the final fraction of cells in the end population that stably express the target gene. In many of the examples discussed below, perturbations of candidate timing enhancers that control lineage-specifying genes are linked to changes in the proportions of immune cell types[26,40,41]. Thus, timing enhancers could have integral roles in maintaining different cell populations at proper sizes and proportions for optimal immune system function.

Timing enhancers in lymphocytes

Early experiments studying enhancers at the single-cell level established the concept that enhancers can modulate the probability of gene expression in an all-or-none manner[6,7,42]. However, many questions remained unresolved from this work. Because these early studies

were performed by transfection of reporter genes, it was unclear whether enhancers at endogenous loci also work in similar ways. Additionally, it remained unclear whether such enhancers could have functional roles, such as timing control of cell-fate specifying genes to alter population fractions. Finally, despite subsequent work that has since shed light on the first two questions, it remained unresolved whether heterogeneity in gene activation is indeed caused by variability in the timing of gene activation via the direct action of enhancers or whether it may instead reflect variability in cell states that indirectly affect enhancer activity. This uncertainty lingered, in part, because of the challenges in directly measuring gene activation dynamics at single gene loci in single cells.

Enhancers loop over to regulate their target genes on the same chromosome, in *cis*; thus, to definitely show that they control the activation timing of their target genes, it is first necessary to establish that time delays in gene activation can occur in *cis*, at individual gene loci. In most standard models of gene regulation[43–45], the timing of gene activation is assumed to be regulated in *trans*, through changes in the levels or activity of upstream transcription factors[43,46,47]. In such a model, transcription factor levels would need to reach a certain threshold before changing the expression state of a target gene. On the other hand, if gene activation delays occur in *cis*, as predicted by the timing enhancer model, they can arise even when upstream transcription factors are already fully present[21,48,49].

Do the enhancers seen in earlier experiments change gene expression probability by acting in *cis*, as predicted by the timing enhancer model? Or do they simply integrate changes in *trans*-factors levels, which are the determinants of gene activation timing? To answer these questions clearly, one would need to distinguish *cis* and *trans* mechanisms. In general, it has been challenging to measure the relative contribution of of *cis*-acting steps to gene activation, independently from the effects of evolving transcription factor levels.

A powerful way to distinguish these two modes of control is by separately monitoring both copies of a gene in single cells[50] (Figure 4). Transcription factors affect both alleles of a gene simultaneously in *trans*, whereas epigenetic chromatin regulation can function independently at each gene copy in *cis*. If the rates of gene activation or silencing were controlled solely by transcription factor levels, changes in the expression state of each gene copy should occur nearly simultaneously. However, if the rates of gene activation or silencing are additionally controlled by epigenetic chromatin regulation, each copy could adopt distinct expression states even within the same nucleus (Figure 4A). Therefore, by tracking the activation dynamics of single gene copies in single cells over time, one can investigate the role for epigenetic chromatin regulation in controlling the timing of cell fate decisions. Such an approach would build on earlier findings of dynamic enhancer control by demonstrating that an endogenous timing enhancer could act in *cis* to control the delay of a cell-fate specifying gene through epigenetic chromatin regulation.

To this end we and others have generated transgenic models, in which distinguishable fluorescent proteins are separately inserted into the two endogenous copies of a cell-fate specifying gene[51–53]. Using this approach, we found direct evidence for a *cis*-acting mechanism that generates a long, multi-day delay in the activation of *Bcl11b,* which encodes a transcription factor essential for T-cell lineage commitment[54].

Bcl11b

Bcl11b encodes a transcription factor that is required for the development of T cells and type 2 innate lymphoid cells (ILC2s) in the thymus and bone marrow, respectively[41]. Upon entering the thymus, T-cell progenitors progress through discrete developmental stages that are accompanied by restriction of alternative lineage potential and progenitor expansion. Switch-like expression of *Bcl11b* at the DN2 progenitor stage induces complete T-cell lineage commitment through the silencing of multipotency genes and restriction of alternative lineage potential[55–57]. Similarly, multipotent common lymphoid progenitors of the bone marrow progress through sequential stages of lineage restriction. The onset of *Bcl11b* expression occurs at the common helper innate lymphoid progenitor stage to induce ILC2 lineage commitment[58]. Thus, *Bcl11b* activation is carefully orchestrated during lymphocyte development to properly specify T-cell and ILC2 lineages.

Observations of *Bcl11b* activation during T-cell development reveal a strikingly long time delay incompatible with the prediction that *trans*-factors solely control gene activation dynamics. A collection of transcription factors known to bind and regulate *Bcl11b* expression are expressed in early thymic progenitors; however, the onset of *Bcl11b* expression occurs only after a multi-day time delay after entry into the subsequent DN2 stage[59,60]. This delay suggests that the levels of *trans*-factors are not sufficient to explain the delay in *Bcl11b* activation, and that a *cis*-acting epigenetic mechanism may contribute. Indeed, the *Bcl11b* locus undergoes dramatic changes in chromatin state and conformation during activation, including changes in nuclear positioning, DNA methylation, histone modifications, and long-range chromatin looping interactions[61–63].

To determine if these epigenetic mechanisms contribute to timing of *Bcl11b* activation, the locus was studied using the dual-color biallelic reporter approach described above (Figure 4A). By separately tracking expression of each *Bcl11b* allele in single cells using live imaging, it was revealed that each allele turns on independently during T-cell development, with one allele frequently activating multiple days and cell divisions before another[51]. Activation occurred with equal probabilities per unit time for the two alleles, consistent with *Bcl11b* activation timing being controlled by a stochastic, rate-limiting step that is regulated in *cis*. These findings suggest the presence of rate-limiting epigenetic regulation at the locus consistent with a timing enhancer mechanism.

Previous studies had identified a putative enhancer that lies 850 kb downstream of *Bcl11b*[59]. To determine whether this enhancer is involved in regulating *Bcl11b* activation timing, we deleted it from a single allele in the dual-allele reporter model[51] (Figure 4B, left). Mice harboring this single-allele enhancer deletion displayed a reduced fraction of DN2 progenitors expressing the mutated allele while expression of the wild-type allele remained unaffected. However, the majority of cells at later stages of thymocyte development not only expressed the deleted-enhancer allele but expressed it at levels that were indistinguishable from that of the wild-type allele, suggesting that this enhancer controls the timing of *Bcl11b* expression, rather than its expression magnitude.

To characterize this element as a bona fide timing enhancer, DN2 progenitors mono-allellically expressing the wild-type allele but not yet expressing the enhancer-deleted allele

were sorted and re-cultured *in vitro* to compare the time delay to that of a wild-type allele[51] (Figure 4B, right). These mono-allelically expressing cells necessarily express the required *trans*-factors at levels sufficient for activation. They thus provide an excellent way to isolate and characterize the rate-limiting epigenetic step at inactive alleles independently of events occurring in *trans*[51,64]. Consistent with predictions made for a timing enhancer, the enhancer-deleted allele switched to an active state more slowly than the wild-type allele. Furthermore, when bi-allelically expressing DN2 progenitors were re-cultured *in vitro*, the maintenance and amplitude of expression from the deleted-enhancer allele was identical to that of the wild-type allele. This suggests that the enhancer functions to accelerate initiation of *Bcl11b* expression, but is not required for its maintenance.

Due to its ability to regulate the timing of *Bcl11b* activation independently of expression magnitude, the *Bcl11b* timing enhancer enabled investigation of the functional impact of altered timing control during lymphocyte development. How does altering the timing of *Bcl11b* activation affect T cell and ILC2 production *in vivo*? Mice with the timing enhancer removed from both *Bcl11b* alleles have roughly half the total number of ILC2 cells compared to wild-type mice[41]. Similarly, mice lacking the *Bcl11b* timing enhancer have a reduced number of thymocytes (*Pease* et al. unpublished), indicating that proper control of *Bcl11b* activation timing is important for specifying T-cell population sizes.

Collectively, these findings provide evidence for an endogenous enhancer that behaves according to the predictions of the timing enhancer model. It controls gene expression primarily through alterations of cell fractions, without affecting expression magnitude. It appears to alter gene expression by altering the onset of stochastic switching between heritable chromatin states. It is able to explain how time delays over long timescales could be controlled where a *trans*-factor model is insufficient. Finally, perturbations of this enhancer alter immune cell output according to the predictions of a timing enhancer mechanism.

Based on their ability to alter gene expression kinetics and cell population fractions without altering magnitude, we argue that timing enhancers are prevalent throughout lymphocyte development and differentiation. However, few studies have perturbed endogenous regulatory elements and tracked their effects in *cis* as has been done for *Bcl11b*. Therefore, based on the insights that we have gained above, we define a list of distinguishing functional features to identify putative timing enhancers in the lymphocyte literature:

1. Timing enhancers, when disrupted, primarily lead to changes in the fraction of cells that express their target genes in an all-or-none fashion. In contrast, perturbations to amplitude enhancers change the expression magnitude of their target genes.
2. Timing enhancers are important for controlling the kinetics of gene activation, but are usually dispensable for maintaining expression once the gene has been turned on; however, there are notable exceptions where an enhancer can have both timing and amplitude control functions, or may also be required for preventing locus re-silencing caused by heterochromatin spreading[20,65].

3. Gene expression states established by timing enhancers are stable over time, and can be inherited through cell division. This property links timing enhancers to stable chromatin modifications associated with epigenetic states, such as DNA methylation or histone H3K27-trimethylation. However, the existence of these chromatin modifications does not necessarily imply heritability or timing enhancer action.

Evidence for generality

As mentioned previously, the strongest evidence for timing enhancers comes from studies that resolve gene expression at the single-cell and single-allele level. Across studies that have perturbed endogenous *cis*-elements and made single-cell measurements, there are a number of examples that are most consistent with the predictions of a timing enhancer mechanism. Many of these examples come from immunology, where extensive single-cell analysis by flow cytometry has enabled these observations. We note, however, that timing enhancers have been proposed to function across a range of developmental processes. For example, the *MyoD* "core enhancer" regulates the timing of *MyoD* expression in muscle lineages during embryonic development [66]. Removal of this timing enhancer delays the onset of *MyoD* expression and myocyte differentiation for about two days. Similarly, enhancers at the *Hoxc8* and *Hoxd11* loci regulate the onset of their expression during embryonic development, but have no effect on the expression levels[67–69]. Removing the timing enhancer at the *Hoxc8* locus results in morphological changes to the axial skeleton whereas mutations in the *Hoxd11* timing enhancer result in premature *Hoxd11* expression and an anterior shift of vertebrae[68]. While many of these studies lack single-cell resolution of expression states, they provide evidence that timing enhancers are pervasive throughout development and are important for shaping tissue size and composition.

Below, we provide examples from lymphocyte development and differentiation that show some of the strongest evidence for timing enhancers. Flow cytometry shows how perturbation of these *cis*-elements can primarily affect population fractions without altering magnitude, a key prediction of a timing enhancer mechanism. A few studies provide evidence for additional predictions of a timing enhancer mechanism such as: 1) an involvement with initiation but not maintenance, 2) a change in heritable gene activation states, and 3) an involvement of chromatin modifications associated with heritable gene activation states (e.g. DNA methylation or H3K27me3). We also discuss potential functions of these timing enhancers. In addition to these examples, we also provide a more comprehensive list of candidate timing enhancers that we have identified from the literature (Table 1).

Cd8

During T-cell development, $CD4^-CD8^-$ double negative (DN)3 cells productively rearrange their T-cell receptor (TCR) β-chain and proliferate for 2–4 days before entering the $CD4^+CD8^+$ double positive (DP) stage, whereupon they rearrange their TCR α-chain and undergo selection[70,71]. During progression from the DN to DP stage, CD4 and CD8 co-receptors must turn on in a timely manner, to ensure that cells are equipped to engage TCR signaling for productive selection.

The *Cd8* locus, which contains both the *Cd8a* and *Cd8b* genes, contains an intricate system of interacting *cis*-regulatory elements that together regulate *Cd8* expression during T-cell development[72]. Within this system, there is evidence of multiple *cis*-regulatory elements that work, either singly or in combination, as timing enhancers for *Cd8* activation during the DN to DP transition. In one study, combined deletion of two enhancer elements, $E8_I$ and $E8_{II}$, led to a population of cells that did not activate *Cd8* expression during the DN to DP transition[73], resulting in a population of $CD8^-CD4^+$ single-positive cells with an immature phenotype. However, cells that did turn on *Cd8* expressed it at levels similar to normal cells, consistent with a role for these two populations in controlling all-or-none *Cd8* activation. In another study, deletion of the $E8_V$ enhancer also generated an immature population of *Cd8* non-expressing cells, consistent with delayed *Cd8* activation during the DN to DP transition[74]. Using mice with two different *Cd8* alleles, the authors determined the expression of *Cd8* with or without the enhancer deletion and further confirmed that the delay in *Cd8* activation is controlled in *cis*. Disruption of these *Cd8* enhancers reduced the fractions of $CD8^+$ cells in both the thymus and the periphery, suggesting that control of *Cd8* activation timing is important for ensuring that $CD8^+$ cells are generated in proper numbers and fractions. Together, these studies support a view, where multiple timing enhancers collaborate to ensure proper CD8 co-receptor activation and mature CD8 T cell generation during T-cell development.

Cd4

After TCR α-chain rearrangement, DP thymocytes undergo positive selection, then silence expression of either the CD4 or CD8 co-receptors to commit to the MHC-class I restricted $CD8^+$ cytotoxic T-cell lineage or the MHC-class II restricted $CD4^+$ helper T-cell lineage[72]. How DP thymocytes enter the $CD4^+$ and $CD8^+$ lineages in response to selection on the same co-receptors is explained by the kinetic signaling model[75]. According to this model, DP thymocytes reduce *Cd8* expression after positive selection to enter a $CD4^+CD8^{low}$ state. In this intermediate state, MHC-class II restricted cells maintain TCR signaling due to continued *Cd4* expression, resulting in $CD4^+$ lineage commitment. On the other hand, MHC-class I restricted cells extinguish TCR signaling due to the decrease in *Cd8* expression. TCR signal extinction leads to *Cd4* silencing, *Cd8* re-activation, and $CD8^+$ lineage commitment. Implicit in this model is a requirement for proper timing of lineage choice: to prevent spurious commitment in response to transient fluctuations in TCR signals, $CD4^+CD8^{low}$ thymocytes must integrate information about TCR signal duration, such that they commit to the $CD4^+$ (or $CD8^+$) lineages only upon continuation (or cessation) of TCR signals of a sufficient length[71].

The timing of CD4 and CD8 expression during this process may be regulated by timing control *cis*-regulatory elements at these loci. Pioneering work by Littman and coworkers identified an intronic silencer element, termed S4, responsible for terminating *Cd4* expression during CD8 lineage commitment[76]. Though not an enhancer, we include it in this review as it represents a paradigmatic example that can inform work in other systems. Subsequent follow-up studies showed that this silencer bears many functional characteristics of a timing control element as defined above[77–79]: First, deletion of the S4 silencer led to an additional population of peripheral $CD8^+$ T cells that failed to silence *Cd4* expression[78]. *Cd4*

expression levels in this cell population were similar to those in peripheral $CD4^+$ T cells, suggesting that S4 controls the timing of probabilistic *Cd4* silencing, and not its expression magnitude[76,77]. Second, by inducing S4 deletion before or after the silencing stage[77], it was found that S4 is important for initiating *Cd4* silencing during $CD8^+$ lineage commitment, but is dispensable for maintaining a silent state after it has been established. Finally, upon S4 deletion in mature $CD8^+$ T cells, cells maintained the *Cd4*-silent state over multiple cell divisions, consistent with this element inducing a switch between chromatin states that are heritable over cell division. Together, these studies suggest that S4 acts as a "timing silencer" that generates a time delay in *Cd4* silencing in response to withdrawal of TCR signaling. This time delay set by S4 may ensure that cells do not spuriously commit to the $CD8^+$ lineage when TCR signals drop transiently, but are able to effectively silence *Cd4* to maintain functionality of MHC-class I restricted cells.

Foxp3

The forkhead box protein P3 (FoxP3) transcription factor is essential for the development of regulatory T cells (Tregs)[80,81]. *Foxp3* turns on in $CD4^+$ T cells in either the thymus or the periphery to give rise to two Treg lineages with distinct functions: natural Tregs (nTregs) and induced Tregs (iTregs)[80]. Work from Rudensky and coworkers identified multiple *cis*-regulatory elements within the *Foxp3* locus, in which there exists two candidate timing enhancers, CNS1 and CNS3. Deletion of the CNS3 element results in a decreased fraction of $FoxP3^+$ Tregs in the thymus and periphery, without affecting *Foxp3* expression magnitude[26]. This suggests that CNS3 may act as a timing enhancer to control the kinetics of *Foxp3* activation in nTregs and iTregs. In contrast, CNS1 deletion has no effect on *Foxp3* induction in thymic Tregs, but does affect *Foxp3* activation in peripheral iTregs. Mice harboring a CNS1 deletion exhibit reduced fractions and numbers of $FoxP3^+$ $CD4^+$ T cells in peripheral tissues and display a wide-range of phenotypes including excessive Th2 inflammation of mucosal tissues, increased rates of embryo resorption, and spontaneous diabetes with severe insulitis[82–84]. These findings suggest that distinct timing enhancers at the *Foxp3* gene are important for regulating the size of the Treg pool, and that their disruption alters immune homeostasis and leads to pathology.

Zbtb16

The PLZF transcription factor, encoded by *Zbtb16*, is required for optimal development and function of natural killer T cells (NKTs)[85]. A careful study of the functionality of putative regulatory elements at the *Zbtb16* locus identified a timing enhancer, +21/23, that controls the onset of *Zbtb16* transcription during NKT development[40]. By measuring PLZF expression at each stage of NKT development, Bendelac and coworkers found that mice harboring a deletion of +21/23 showed reduced fractions of $PLZF^+$ cells at the early stages of NKT development, but expressed normal levels of PLZF at later developmental stages and in mature NKT. This delay in PLZF expression caused a moderate delay in NKT development in the thymus and resulted in dramatic decreases in the number of mature NKT cells found in peripheral tissues. Interestingly, the delay in NKT development had differential effects on different NKT subtypes, with NKT2 cell numbers being unaffected by the timing enhancer deletion. This suggests that in addition to regulating the total number of NKT committed cells that emerge during development, the timing of *Zbtb16* activation is

also important for determining the relative proportions of NKT subtypes, possibly by altering the activation timing of downstream cell fate genes.

Cytokines

After development and migration to peripheral tissues, $CD4^+$ T helper (Th) cells further differentiate into Th subsets which possess distinct functional responses upon TCR stimulation. Two cytokines, encoded by *IFNG*/*Ifng* and *Il4*, are critical for the differentiation and functionality of the Th1 and Th2 subsets, which are responsible for orchestrating type I and II responses, respectively. Over the past decade, both the *IFNG/Ifng* and *Il4* loci have been finely dissected to identify functional non-coding regulatory elements. Remarkably, many functionally defined elements display the hallmarks of timing enhancers. For example, when CNS-4 or CNS+20 are removed from the *IFNG* locus, a smaller fraction of Th1 polarized cells will express IFN-γ following stimulation; however, those cells that do activate *IFNG* express it at normal levels[86]. Similarly, when CNS-22 is removed from the *Ifng* locus in mice, IFN-γ expression is delayed in naive $CD4^+$ T cells following TCR stimulation, but IFN-γ is ultimately expressed at normal levels at the single-cell level[87].

The *Il4* locus also harbors at least two candidate timing enhancers, each of which plays a critical role in mounting proper type 2 immune responses and pathogen clearance *in vivo*. Deletion of the hypersensitivity site 2 (HS-2) at the *Il4* locus decreases the fraction of Th2 polarized cells that express IL-4 following stimulation[88]. This delayed activation of *Il4* does not affect the overall production of other type 2 cytokines, suggesting that this timing enhancer primarily functions by controlling the rate at which Th2 cells begin producing IL-4. In contrast, deletion of HS-V at the *Il4* locus reduces the percentage of both IL-4 and IL-13 producing cells when Th2 polarized cells are stimulated[89]. This suggests that delay of IL-4 activation in this case may also affect Th2 polarization efficiency by reducing positive feedback signals from secreted IL-4. More recently, candidate timing enhancers have also been identified at other cytokine gene loci including *Il9* and *IL3*/*Il3*[90,91]. This suggests that timing enhancers are likely prevalent features of effector genes, and may be critical for ensuring that effector cell responses occur at proper contexts and timescales.

Mechanistic basis for timing enhancers

How do timing enhancers induce all-or-none switches in the expression states of their target gene loci that occur many hours or days after induction of upstream *trans*-factors? Transcription factors bind to genomic loci rapidly, within seconds, and initiation of transcription by RNA polymerase also occurs rapidly, within tens of minutes (Figure 1). Thus, transcription processes alone are unlikely to fully account for the significantly long delays associated with timing enhancer action. This disparity in timescales instead points to an involvement of chromatin mechanisms that heritably maintain silent or active states at gene loci over many cell generations.

Drawing upon literature evidence, we describe a model where genes are initially held in a stable and heritable repressed state through an epigenetic mechanism that involves repressive histone modifications. This state can switch in an all-or-none manner to an active expressing state upon prolonged exposure to enhancer-bound transcription factors (Figure 5). Because

of the stability of the repressed state, *trans*-factors that attempt to reverse these states do so inefficiently, such that state switching occurs with only low probabilities per unit time.

We discuss this model primarily in the context of our work on understanding the timing enhancer controlling *Bcl11b* activation; however, the mechanisms described here are more general and likely underlie switching at other loci. We note that in order to initiate chromatin remodeling at their target gene promoters, enhancers first need to search for and establish contacts with their target genes, sometimes over very long genomic distances. Recent work has identified cohesin-mediated loop extrusion as a candidate mechanism for organizing the genome to shape enhancer-promoter interactions[94] (Figure 5, top). While proper establishment of enhancer-promoter contacts is critical for any enhancer, including timing enhancers, we do not discuss recent advances in this area, and instead refer the reader to these excellent reviews[11,92–96].

Maintenance of a repressive chromatin state.

Prior to activation, genes must be held in a repressed state that is stable over days and persists through cell division. Two types of histone modifications, histone 3 lysine 27 trimethylation (H3K27me3) or histone 3 lysine 9 di/tri-methylation (H3K9me2/3)[15,97–99], can give rise to repressed states that are heritable over cell division. Both histone modifications have the ability to propagate a repressed state over time and across cell division, though underlying mechanisms are not completely clear. Earlier work showed that both H3K9me2/3 or H3K27me3 methyltransferases can bind the mark they write at an allosteric site, and be induced to write the same modification in its vicinity[100,101]. This creates a positive feedback that can potentially underlie stability of the silent state. More recently, H3K9me2/3 and H3K27me3 were both shown to bind reader proteins that undergo liquid-liquid phase separation[102–104]. These phase separated droplets may generate compacted chromatin assemblies that are inaccessible to the transcriptional machinery, and can also show remarkable stability[105], providing a separate mechanism to explain how repressive states can persist over time.

Disruption of a repressive chromatin state by transcription factors.

To switch a gene on from a repressed state, enhancers must deliver transcription factors to the gene to reverse its stable, repressive chromatin state. Pioneer factors, a class of transcription factors that are capable of binding to repressed chromatin regions[106–108], may act on timing enhancers to induce state switching (Figure 5, middle). Pioneer factors are defined by their ability to bind both nucleosomes and their cognate DNA motifs[109]. Upon binding, pioneer factors can open repressed chromatin, either by directly evicting nucleosomes in their vicinity, or through the recruitment of chromatin remodeling complexes[110,111].

While these biochemical activities facilitate the dissolution of compacted chromatin assemblies, they do not directly aid in the removal of repressive H3K27me3 modifications. One possibility is that there are separate enhancer-bound factors that serve to recruit H3K27me3 and H3K9me2/3-specific demethylases to the target gene promoter[112,113] (Figure 5, middle). An alternative possibility, discussed further below, is that there is an

intrinsic coupling between chromatin compaction and histone methylation, such that changes in compaction state would affect methylation activities, and vice versa. We discuss this possibility in our model below. The *Bcl11b* timing enhancer binds two transcription factors that have previously been shown to have pioneering functions, Gata3 and TCF-1[61,114]. Gata factors can bind and open repressive chromatin in a variety of cell types[115]. Strikingly, TCF-1 also binds to H3K27me3-marked regions, and, when expressed in fibroblasts, is sufficient to open a H3K27me3 repressed chromatin region at the *Bcl11b* promoter to induce reprogramming to the T-cell lineage[116]. Similar to the timing enhancer itself, both Gata3 and TCF-1 are required for initiation of *Bcl11b* expression, but do not regulate *Bcl11b* expression magnitude, nor are they required for maintenance of expression after activation[60]. Furthermore, depletion of these factors in early thymic progenitors delays the onset of *Bcl11b* expression to a similar degree as removal of the enhancer itself, suggesting that they act through a common mechanism to antagonize the repressive chromatin at *Bcl11b* promoter[51,60].

Generation of extended switching time delays.

Transcription factors bound to timing enhancers are ultimately responsible for initiating chromatin state switches at target genes; yet, switching events occur only long after these transcription factors are up-regulated in the cell. Where is this temporal bottleneck? As transcription factors bind DNA rapidly, typically over timescales of seconds[117–119], these slow, rate-limiting steps would most likely involve downstream chromatin modification or remodeling events. This view agrees with multiple studies on time courses of inducible pioneer factor binding and chromatin remodeling: upon transcription factor induction, binding sites for these factors became occupied rapidly[48,113,120]; however, chromatin opening events and activating histone modifications around these binding sites did not appear until hours or even days later. Similarly, when histone-modifying enzymes were inducibly targeted to genomic loci, they bound rapidly, but generated chromatin and gene expression state changes only hours to days later[19,21].

Bound transcription factors may initiate chromatin state changes after long delays, because they act on chromatin states that are highly stabilized and not easily altered. To better understand the biophysical basis of chromatin state stability, and to determine its impact on switching control, we developed a mathematical model for gene activation at the *Bcl11b* locus[64]. In this model, the locus is an array of nucleosomes that can either be reversibly methylated, or can associate with other nucleosomes. This nucleosomal array initially exists in a compacted, silent state, but switches into an open, extended state in response to transcription factor binding. Transcription factors associated with timing enhancers can either recruit chromatin-modifying enzymes to remove histone modifications or directly displace nucleosomes upon binding.

We found that this model, where histone methylation and chromatin compaction are coupled to each other, recapitulates essential dynamic properties of timed chromatin state switching, as observed for *Bcl11b* and other gene loci. Gene loci switch from silent to active states in an all-or-none manner, and do so with a probabilistic time constant spanning multiple days and cell divisions. Transcription factors can tunably modulate switching times, and can do so

through either demethylase recruitment or nucleosome eviction. Notably, activation time constants in our model were unaffected by changes in cell cycle speed, in agreement with experimental measurements, implying that activation time delays set by the *Bcl11b* enhancer are set independently from cell division. The methylation-compaction model will still need to be further tested, both for *Bcl11b* and in other gene regulatory systems; nonetheless, this model provides a framework that can explain, on a biophysical level, how timing enhancers may execute their function and control delays over extended timescales.

If a timing enhancer functions by promoting chromatin decompaction at target genes, as predicted from the model, one would expect that removal of such a timing enhancer would increase repressive histone modifications and decrease DNA accessibility at the locus. Consistent with this idea, removal of the HS-V *Il4* timing enhancer increases the repressive H3K27me3 modification at the *Il4* locus[121]. Similarly, removal of the *Il9* timing enhancer decreases the enrichment of the active H3K27Ac modification and transcription factor binding at the *Il9* promoter[90]. In a variation of such a mechanism, removal of the putative timing enhancer at the *IL3* locus results in the inability of a second *IL3* enhancer to become accessible, suggesting that some timing enhancers may function by promoting chromatin state changes at additional enhancers which may themselves directly interact with the target gene promoter[91].

Delayed gene activation by DNA demethylation.

The chromatin decompaction mechanism, described above, can generate long, enhancer-modulated delays in gene activation; however, alternative mechanisms involving DNA demethylation may also generate delayed enhancer-mediated switches in chromatin state. DNA methylation, occurring primarily on CpG sites in the genome, is a heritable epigenetic mark associated with gene repression[17,122]. Its removal from gene promoters is important for gene expression. Timing enhancers could potentially drive delayed gene activation by facilitating removal of DNA methylation at gene loci. For instance, the combined $E8_I$ and $E8_{II}$ enhancer, which may regulate *Cd8* activation timing as discussed above, may work by facilitating demethylation of the *Cd8* locus[123]. Similarly, the $E4_p$ proximal enhancer, which regulates *Cd4*, also works by promoting DNA demethylation[79]. There are multiple candidate mechanisms through which DNA demethylation could proceed with slow kinetics at gene loci. One possibility is that removal of DNA methylation marks, as initiated by enhancers, may involve passive dilution through DNA replication[124–126]. In such a model, long activation delays could ensue if multiple cell divisions are needed for methylation mark levels to fall below a threshold required for gene expression. Another possibility is that DNA methylation may cooperate with repressive histone methylation marks to drive silencing, such that mechanisms involving chromatin compaction, as described above, may ultimately underlie the rate-limiting barrier to activation. Consistent with this idea, gene silencing at the *Cd4* locus is enhanced by HP-1[78], a protein required for heterochromatin formation and gene silencing. HP-1 associates with both the maintenance DNA methyltransferase DNMT1 as well as H3K9me2/3 modifications[127], and could therefore work together with these different modifications to create a chromatin barrier for activation timing control.

Timing enhancers in disease

The studies discussed suggest an importance of proper timing enhancer activity in the control of immune response kinetics and cell type proportions. Consequently, perturbations to timing enhancers, caused by genetic alterations, could disrupt immune function and lead to disease. Here, we explore specific examples where timing enhancers can be associated with immunopathological outcomes. While it is not yet established that timing contributes to disease risk, these findings point to the usefulness of viewing *cis*-elements through the lens of timing enhancer function.

A major finding from genome-wide association studies (GWAS) has been the identification of many disease-associated variants in putative enhancers[128,129]. Some of the strongest signals come from immune-related diseases, suggesting a significant role for genetic risk in immunopathology. It remains challenging to discern how enhancer-associated SNPs contribute to disease risk. While many SNPs change expression magnitude, some could act through alterations of gene expression kinetics.

IL2RA

The *IL2RA* locus encodes the α subunit of the IL-2 receptor, IL-2Rα, and confers the receptor with a high binding affinity for its ligand, the cytokine IL-2[130]. IL-2 has pleiotropic roles in the control of T-cell differentiation. It is expressed upon activation of naive $CD4^+$ T cells and drives differentiation of Th1, Th2, and iTreg cells, while suppressing differentiation of Th17 cells. IL-2 is also required for the development of nTregs in the thymus.

IL-2 binding to IL-2 receptor, mediated by IL-2Rα, is critical for development and differentiation of Tregs and contributes to suppression of autoimmunity. IL-2Rα is constitutively expressed on nTregs where it is required for survival, whereas it is induced upon activation of naive $CD4^+$ T cells to drive differentiation of iTregs. Thus *IL2RA* expression is critical for producing iTregs in balance with other T-cell effectors also induced by IL-2.

GWAS have identified SNPs in non-coding regions at the *IL2RA* locus associated with risk for several autoimmune diseases[131]. A CRISPR-Cas9 based activation screen revealed a stimulation-responsive intronic enhancer, named CaRE4[25]. This locus harbors a SNP associated with increased risk for Crohn's disease and decreased risk for type 1 diabetes (T1D). Interestingly, enhancer deletions or SNP mutations do not affect steady-state expression of *Il2ra* in peripheral nTreg populations. Upon *in vitro* stimulation of naive T cells, the authors observed a decrease in the fraction of $IL2RA^+$ cells after one day. However, after three days, *Il2ra* expression fraction and magnitude reached wildtype levels, consistent with its role as a timing enhancer. Similarly, *in vivo* stimulation of enhancer-deleted mice revealed decreased *Il2ra* expression in naive $CD4^+$ T cells upon induction, but no significant changes in steady-state *Il2ra* expression magnitude in either mature nTreg or iTreg populations. Together, these results suggest that CaRE4 primarily affects gene expression kinetics during the induction stage rather than gene expression magnitude at

steady-state, consistent with its action as a timing enhancer to control the kinetics of *Il2ra* induction.

Il2ra expression is critical for iTreg differentiation from naive $CD4^+$ T cells. A delay in *Il2ra* induction could alter polarization towards alternative cell fates. This could lead to functional consequences if there were increased differentiation of Th17 cells, which are normally suppressed by IL-2. Th17 activity is associated with increased risk for autoimmune diseases such as Crohn's disease. To determine the functional consequences of an *Il2ra* delay, the authors stimulate naive $CD4^+$ T cells *in vitro* and measure the resulting balance between Th17 and iTreg cells. A CaRE4 deletion results in increased polarization towards the Th17 fate under conditions of low IL-2. This may occur if a delay in *Il2ra* and low IL-2 frees naive $CD4^+$ T cells to differentiate to Th17 cells, whereas high amounts of *Il2ra* and IL-2 would induce formation of iTregs.

A more recent study tracked the occurrence of T1D in Non-obese Diabetic (NOD) mice with a CaRE4 deletion. The authors find that CarRE4 deletion protects against T1D[132]. The proposed model for this protection is that delayed *IL2RA* induction frees IL-2 for surrounding nTregs to suppress effector functions. These results suggest that subtle effects on gene expression timing, mediated through enhancer SNPs, may contribute to genetic risk for autoimmune diseases.

Lrrc32/GARP

A recent study attempted to characterize an intergenic SNP associated with increased risk for asthma, T1D, allergy, and Crohn's disease[133]. The SNP localizes in an enhancer region that influences expression of the *Lrrc32* gene, which encodes the protein glycoprotein A repetitions predominant (GARP). GARP is a transmembrane glycoprotein that anchors TGF-β on the surface of Tregs, where its release contributes to the immunosuppressive function of Tregs[134,135]. Enhancer-deleted mice show decreased fractions of $GARP^+$ Tregs in the thymus, spleen, and mesenteric lymph nodes. Notably, the magnitude of GARP expression does not appear to change, suggesting that the SNP may act through alterations of timing enhancer function. These mice proceed to develop loss in body mass, reduced colon length, and histopathologic features upon dextran sulfate sodium (DSS) induced colitis. Though a time course is needed to directly test timing enhancer function, this study suggests another case where alteration of timing enhancer function by a disease-associated SNP potentiates disease.

Beyond autoimmunity

Given the widespread involvement of the immune system throughout the body, it is possible that SNPs in timing enhancers may contribute to a wider range of diseases beyond autoimmune conditions. For example, given the increasing evidence for lymphocyte involvement in the brain, it is possible that timing enhancer SNPs could contribute to neuropsychiatric disorders[136]. Additionally, given the involvement of the immune system with cancer response, timing enhancers could underlie genetic predisposition to cancer or cancer immunotherapy responses[137]. Thus, continued study of timing enhancers may not only highlight novel mechanisms of timing in the immune system, but may also reveal

insight to the contribution of genetic risk in a wide range of diseases. In general, to establish a role for SNPs in altering gene induction timing, we will need to track gene expression over time at the single-cell level. Further studies designed to detect the contribution of timing enhancers to immune function may reveal the basis by which some SNPs contribute to immunopathology.

Conclusions and future directions

An ability to regulate the timing of cell state and lineage transitions is critical for immune system development and function. By drawing upon specific examples in lymphocyte biology, we have defined a distinct class of *cis*-regulatory elements, termed "timing enhancers", that can modulate the timing of gene activation over hours, days, and possibly longer timescales. Timing enhancers control the activation times of lineage-specifying genes in immune development and function[26,40,51,65,78], and could consequently set the sizes and fractions of different immune cell lineages that emerge. They can also control the expression kinetics of effector genes, such as cytokines, and thereby regulate the speed and the overall strength of an immune response[88,90]. From these examples, we suggest that timing enhancers could be more broadly utilized to control the temporal dynamics of the immune system.

Moving forward, it will be useful to systematically identify timing enhancers in lymphocytes, and determine their roles in immune development and function. To identify candidate timing enhancers, it will be useful to know what chromatin and genome architectural features distinguish timing enhancers from other types of *cis*-regulatory elements. Heritable chromatin features, such as DNA methylation and histone H3K27 trimethylation, may indicate timing enhancer function, though we will need further study of the gene regulatory mechanisms operating at specific timing enhancer loci to reveal definitive distinguishing molecular features. Timing enhancers can also be identified by the effects of their disruption on gene activation and target cell population fractions. Such studies have mostly involved analysis of individual candidate elements through generation of mice with targeted enhancer deletions. Cas9-based approaches[138,139], by enabling disruption of genomic elements in primary cells[140,141], could enable a more high-throughput analysis. Ultimately, to conclusively demonstrate that an enhancer acts in *cis* to generate an activation delay, it will be necessary to make time course measurements of gene activation at the level of single alleles in single cells. The dual allelic reporter approach we used for *Bcl11b* could also be broadly used to reveal timing enhancer action for other genes.

Elucidating the physical basis by which timing enhancers control chromatin and gene expression will deepen our understanding of eukaryotic gene regulation and provide a basis for engineering immune gene regulatory circuits for therapy. We now have a fairly detailed parts list of genes and proteins that participate in chromatin regulation; however, it is not apparent how these parts can together implement switching dynamics over long timescales that are tunable by enhancer action. To ultimately explain these physical properties, we will need to develop physics-based models of gene activation[64,142]. We will then need to iteratively test these models using quantitative measurements of gene expression at the resolution of single alleles in single cells[143,144]. Ultimately, to elucidate the molecular basis

of timing enhancer action, we will also need to resolve chromatin modifications and conformational states at the single-allele, single-cell level. Such endeavours will be aided by recent advances in super-resolution microscopy and live-cell imaging which enable simultaneous visualization of chromatin-associated proteins and the chromatin accessibility state at endogenous genomic loci[145–147]. Ultimately, gaining a physical understanding of gene regulation in lymphocytes and other mammalian cell types will open the door to efforts to manipulate and engineer gene regulatory circuits for cell-based therapies.

In addition to forward engineering of immune cell circuits, a timing enhancer model may allow us to make sense of the vast amounts of genetic information collected for clinical use. Millions of DNA variations occur on average between individuals, with several thousand in coding-regions and only several hundred currently clinically actionable[148]. Non-coding SNPs are thought to underlie the bulk of genetic risk, but much of the effort to understand them assumes an effect on gene expression magnitude[149]. By drawing attention to their potential effects on gene expression kinetics, one may design experiments to detect the subtle effects of many SNPs that currently escape attention. A whole new landscape of genetic risk may be revealed by viewing gene expression control with the added dimension of time.

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Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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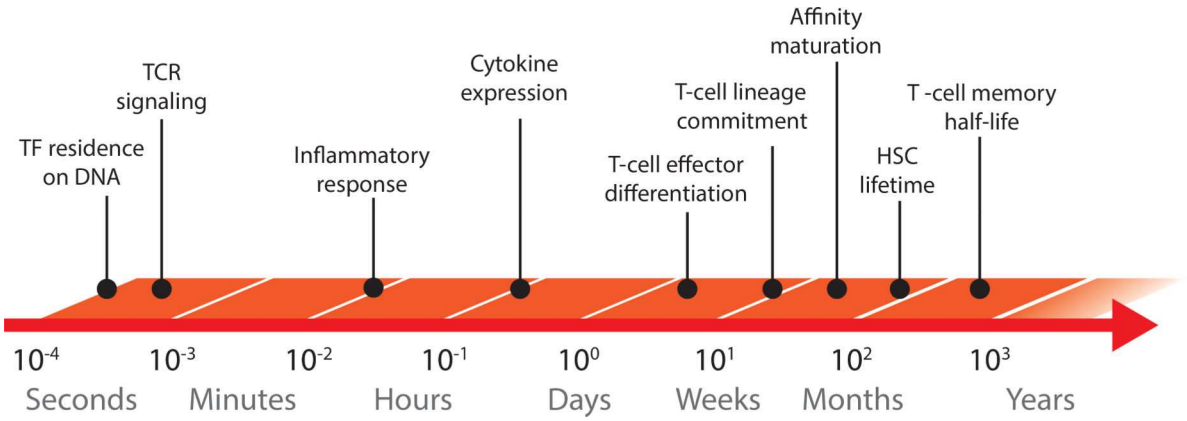

Figure 1. Timescales in lymphocyte biology.

From left to right: Transcription factor (TF) residence on DNA (seconds to minutes)[117]; T-cell receptor (TCR) signaling (seconds to minutes)[150]; Inflammatory response (minutes to hours)[151]; Cytokine activation (hours)[151]; T-cell effector differentiation (3 – 5 days)[152]; T-cell lineage commitment (1 – 2 weeks)[71]; Affinity maturation (3 – 4 weeks)[151]; Hematopoietic stem cell (HSC) lifetime (months)[71]; T-cell memory half-life in mice (> 60 days)[153].

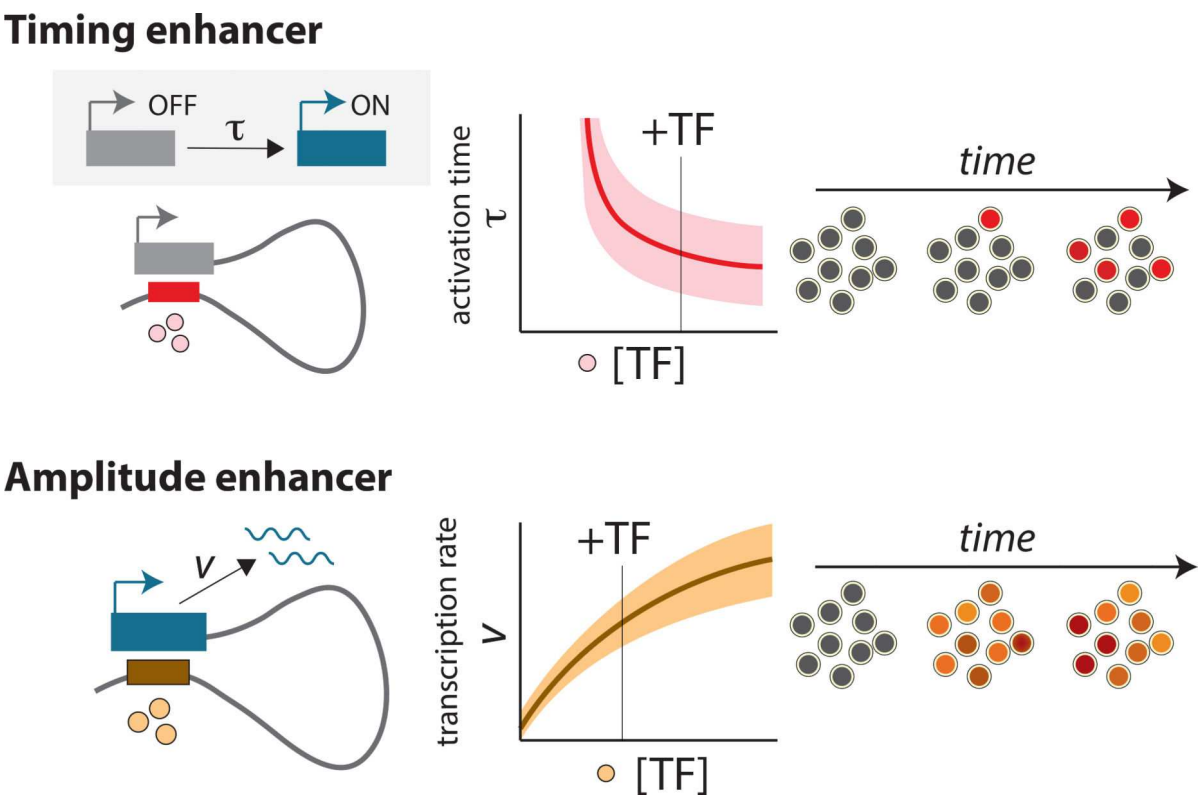

Figure 2. Timing enhancer versus amplitude enhancer.

(A) A timing enhancer alters the activation time (τ) for a gene locus to switch from an inactive to an active expression state. An increase in transcription factor concentration [TF] shortens activation time as its primary action. A timing enhancer is predicted to produce stable subpopulations of cells with discrete gene expression states. Modulations in timing enhancer activity change the probability that these subpopulations arise over time, without affecting gene expression magnitude. **(B)** An amplitude enhancer alters the transcription rate (*v*), which measures transcript production as a result of RNA polymerase II loading and elongation. An increase in [TF] increases transcription rate. An amplitude enhancer is predicted to give rise to a single population of cells with graded changes in expression magnitude. Modulations in amplitude enhancer activity change expression magnitude without altering timing.

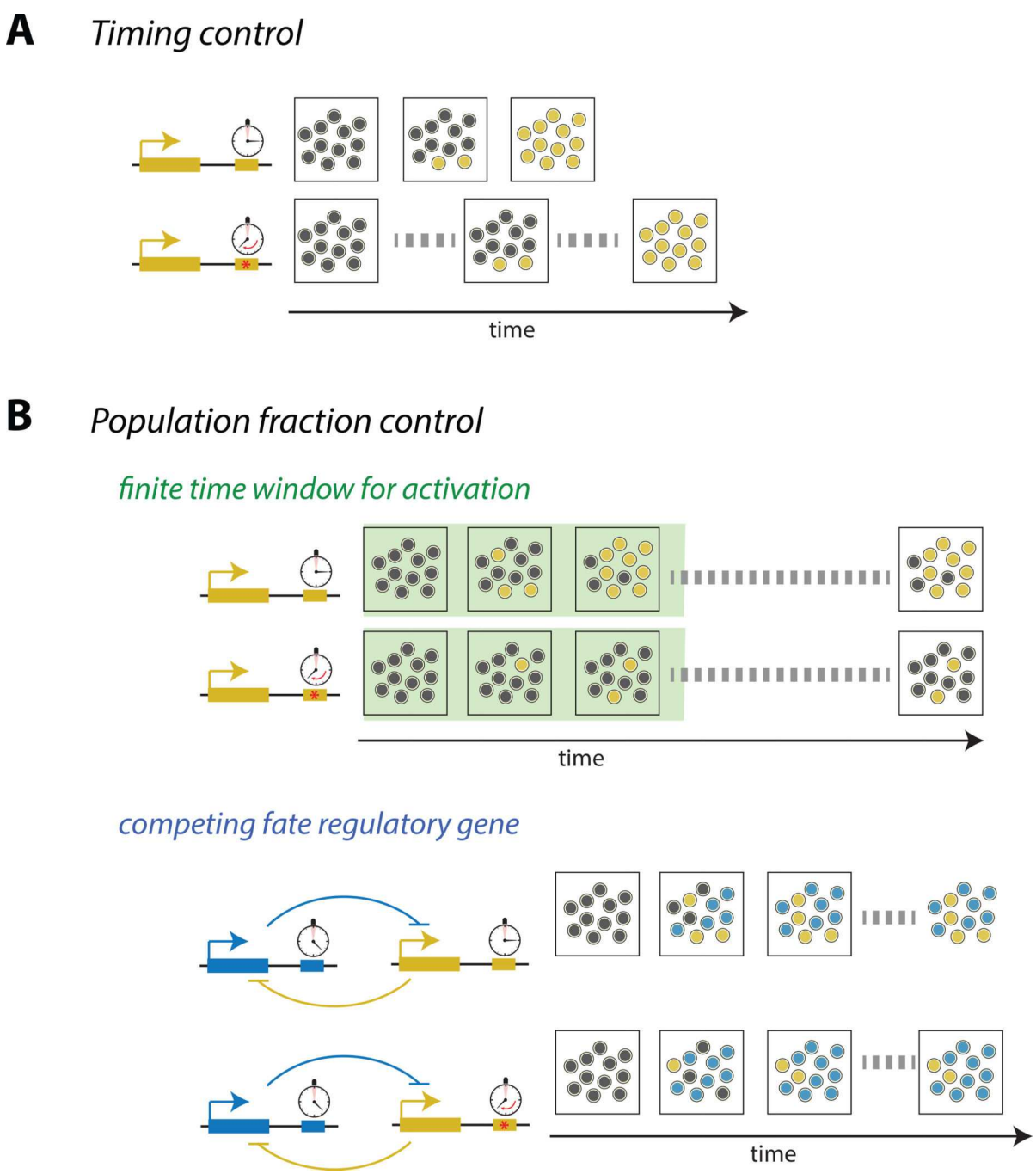

Figure 3. Timing enhancers could modulate the timing of regulatory events and differentiated cell population sizes.

(A) When gene activation is unconstrained by competing factors, changes in timing enhancer strength would have no effect on the fraction of output cells, because all cells would eventually turn on the target gene. **(B, top)** When gene activation timing is limited to a finite time window, changes in timing enhancer strength could alter the final fraction of cell fates. **(B, bottom)** When two competing cell fate genes express antagonistic regulators,

changes in timing enhancer strength at one gene would simultaneously alter the fraction of both cell fates.

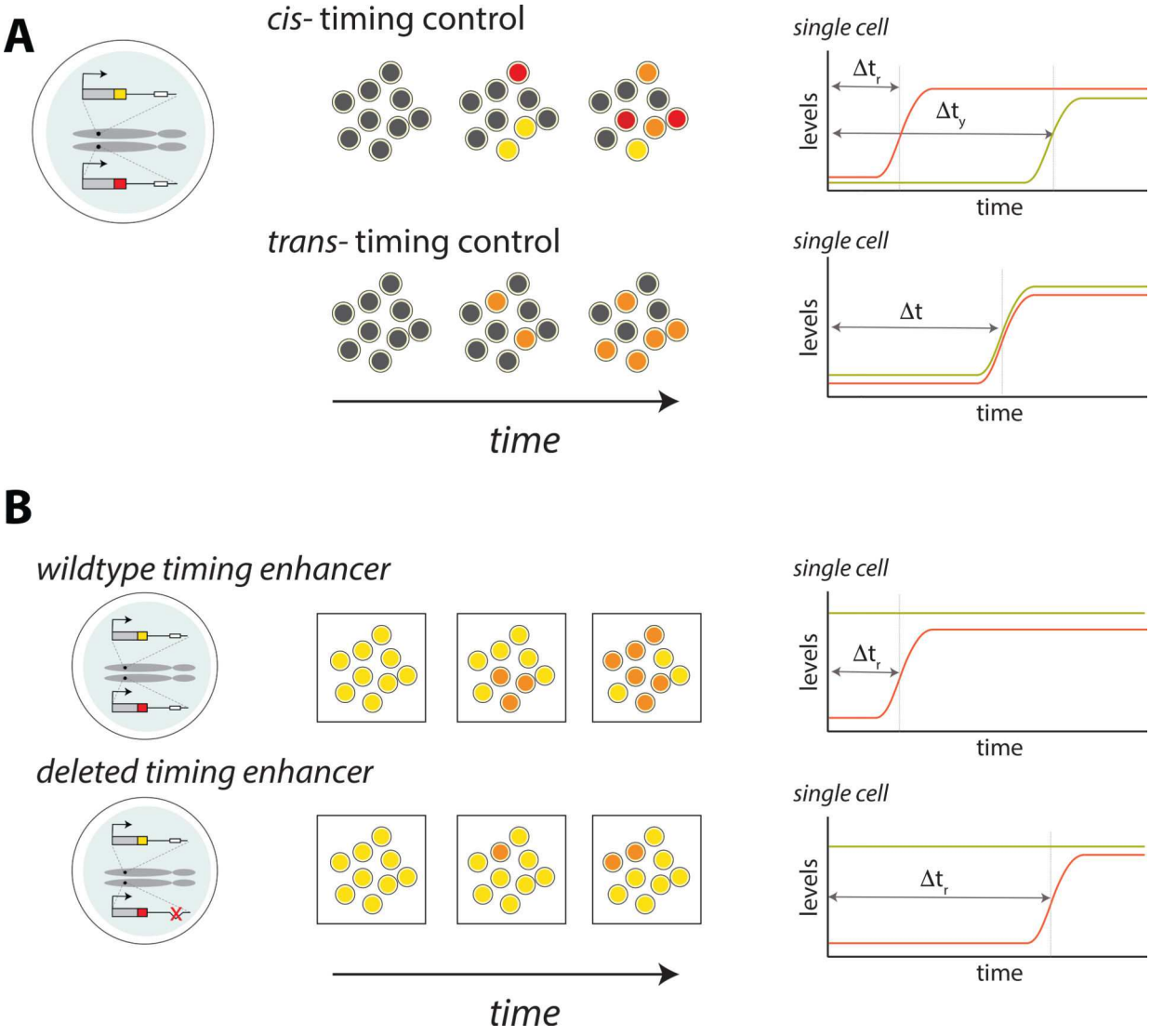

Figure 4. Tracking two copies of the same gene in single cells reveals *cis*-timing control and timing enhancer action.

(A) Separately tracking the activation of two gene copies, marked with distinguishable fluorescent reporters, distinguishes *cis* versus *trans* control of gene activation timing. When activation is limited by *cis* events at single loci, the two alleles turn on asynchronously in single cells, with time differences that can span extended timescales. In contrast, when activation is limited by *trans* events occurring in the nucleus, the two alleles turn on synchronously. **(B)** Single-allele perturbations of non-coding regulatory elements enable the unperturbed wild-type allele to serve as a same-cell internal control to ensure all *trans*-factors necessary for gene expression are present. To interrogate the function of a candidate enhancer, single-cells expressing the unperturbed allele (yellow) can be isolated and re-cultured. This allows monitoring of the perturbed allele (red) by live-cell imaging. Single-cell, single-allele tracking enables quantification of the *cis*-activation timing delay and uncovers the function of the candidate enhancer.

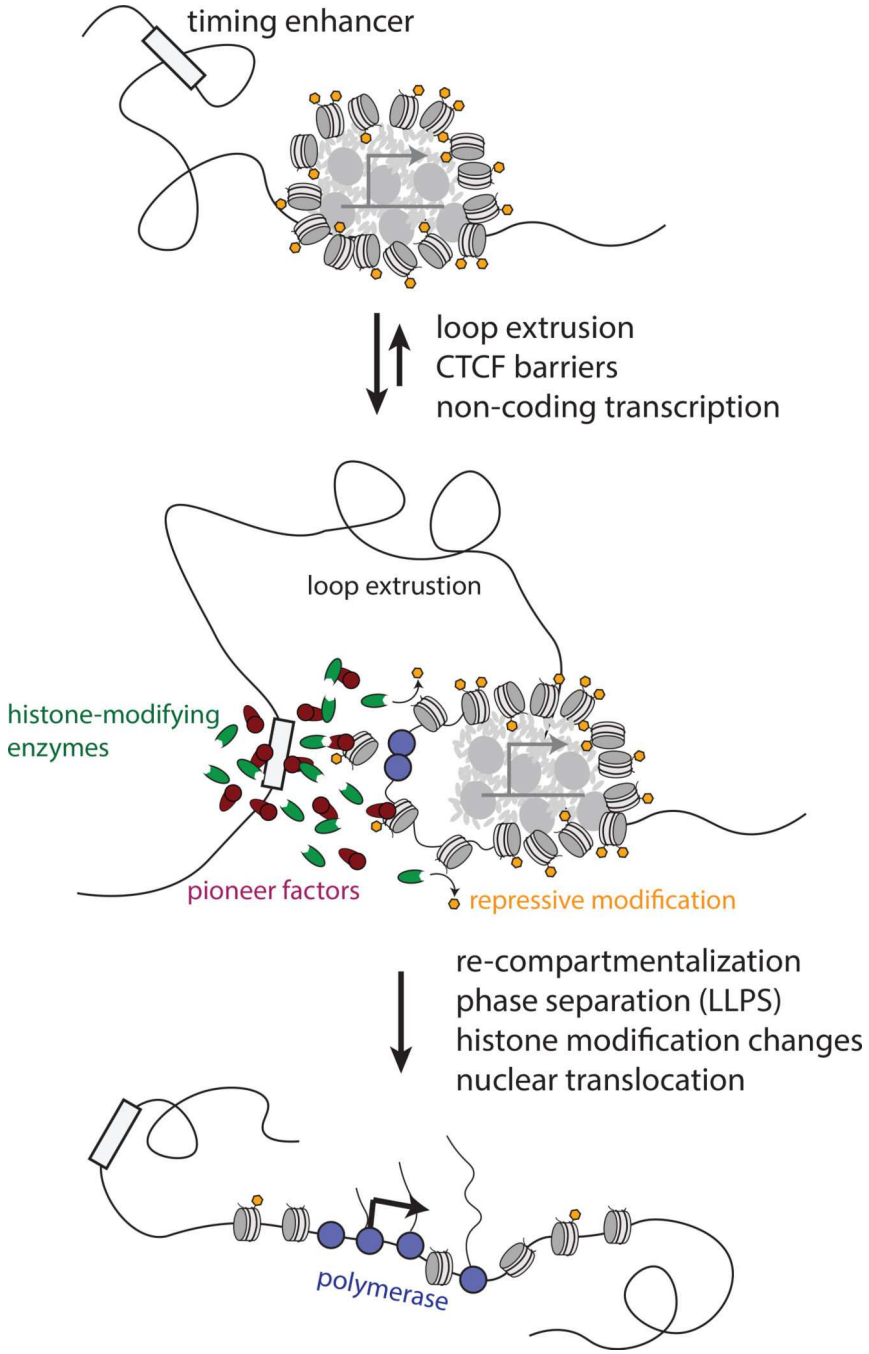

Figure 5. Model for timing enhancer control of gene activation.

Gene loci reside in stable, inactive chromatin states that must be overcome in order for them to switch to accessible and transcriptionally active chromatin states. Conformational changes at inactive gene loci, possibly driven by loop extrusion, increase the probability or duration that a timing enhancer physically interacts with its target gene promoter. Timing enhancers bind and localize pioneer transcription factors and chromatin-remodeling factors that disrupt condensed chromatin states. Disruptions to the condensed chromatin state can lead to all-or-

none switching to an active chromatin state that is accessible to the transcriptional machinery.

Table 1:

Candidate timing enhancers from the literature. As discussed in the main text, these enhancers were selected based on the observed effects of enhancer perturbation on (1) population sizes of cells expressing the target gene, (2) initiation, but not maintenance of expression, and (3) the stability of enhancer-controlled inactive and active states over cell division. Details for individual studies are provided above.

Target Gene	Candidate Timing Enhancer	Location	Effect of Deletion	References
Cd4	S4		Mutation of individual binding motifs within intronic silencer S4 results in variegated derepression of CD4 in lymph node T cells. This leads to a reduced fraction of $CD8^+$ T cells in periphery. Suggests that the silencer regulates the locus by tuning the probability of heterochromatin silencing. A time course of CD4 expression could reveal whether the element affects the timing of CD4 silencing.	78
Cd8	E8I/E8II E8V	*Cd8* enhancers lie in a 36 kb intergenic region between *Cd8b* and *Cd8a*.	Combined deletions result in variegated expression of *Cd8* in thymocytes during DN-DP transition. This leads to a reduced fraction of $CD8^+$ T cells in lymph nodes, although *Cd8* magnitude remains unaffected. A time course suggests a delay in *Cd8* activation. Deletion results variegated expression of *Cd8* in thymocytes during DN-DP transition. This leads to a reduced fraction of $CD8^+$ T cells in lymph nodes, although *Cd8* magnitude remains unaffected.	73 74
Foxp3	CNS1 CNS3 MML4BS	−1 intron downstream *Foxp3* promoter +1 intron downstream *Foxp3* promoter −8.5 kb upstream *Foxp3*	CNS1-KO does not affect the percentage of $FoxP3^+$ Tregs in the thymus but results in a reduced percentage of $FoxP3^+$ induced Tregs found in the periphery and also show a decrease in the fraction of $FoxP3^+$ cells upon *in vitro* stimulation of naive T cells without affecting expression magnitude. CNS1-KO mice display a wide-range of phenotypes including excessive Th2 inflammation of mucosal tissues, increased rates of embryo resorption, and spontaneous diabetes with severe insulutis. Deletion of CNS3 decreases the fraction of $FoxP3^+$ thymocytes without affecting expression magnitude. CNS3-deficient mice have reduced fraction of $FoxP3^+$ cells upon *in vitro* TGF-β stimulation without affecting magnitude. Increased $Ki67^+FoxP3^+$ thymocytes and peripheral cells in CNS3-KO mice suggests compensation to maintain T-cell compartment size. Deletion of this MLL4 binding region delays the onset of *Foxp3* expression and reduces the fraction of $FoxP3^+$ $CD4^+$ lymphocytes following stimulation. Mice with this element removed exhibited an increase in the percentage of Treg precursor cells found in the thymus, but a decrease in mature Treg cells found in the peripheral lymphoid organs.	26,82–84 26 154
Bcl11b	Major Peak	+850 kb downstream *Bcl11b*	Deletion of 2 kb region lying 850 kb downstream of *Bcl11b* delays expression of *Bcl11b* in *cis*. The affected allele eventually expresses *Bcl11b* at wildtype magnitude. Evidence that an enhancer regulates the timing of expression without affecting magnitude. Deletion of this element results in a decline of ILC2 output.	51,59
Tcf7	Region 3	−30 kb upstream *Tcf7*	Deletion of a 1 kb region impedes initiation of TCF-1 expression in T-cell and ILC progenitors. TCF-1 eventually gets expressed at later stages at lower magnitude, suggesting that the enhancer has properties of both timing and amplitude enhancers. Deletion results in a decline of thymic and peripheral T-cell output. This region harbors a SNP, rs244689.	155
Igh	LCR	VDJ-Cμ intron	Deletion of the LCR leads to variegated expression of *Igh* in a hybridoma cell line. These all-or-none states are heritable and partially mediated in *cis*. By controlling the delay in *Igh* expression in *cis*, these elements are proposed to contribute to allelic exclusion of antigen receptor genes.	156,157
Il2ra	CaRE4	+1 intron downstream *Il2ra* promoter	SNP rs61839660C:T in the intronic CaRE4 region delays induction of *Il2ra* in naive T cells from 1 to 3 days. *Il2ra* eventually expresses at wildtype magnitude. 12-nucleotide deletion of the region surrounding rs61839660 results in a stronger delay effect. *In vitro*, the deletion skews polarization toward Th17 cells, possibly contributing to increased risk for Crohn's disease. In NOD mice the deletion protects against T1D, suggesting that the delay factors Treg suppression in response to IL-2 signals.	25,132

Target Gene	Candidate Timing Enhancer	Location	Effect of Deletion	References
Lrrc32 (encodes GARP protein)	*Lrrc32* enhancer	+69.8 kb downstream *Lrrc32*	Deletion of 2.3 kb region containing SNP rs11236797 results in reduced fraction of GARP$^+$ Treg. Mice with deletion show loss in body mass, reduced colon length, and histopathologic features upon dextran sulfate sodium (DSS) induced colitis. A time course could confirm whether deletion alters GARP expression kinetics without affecting magnitude.	133
Zbtb16	+21/23	+1 intron 21 kb downstream *Zbtb16*	Deletion of +21/23 intronic region reduces the fraction of Zbtb16$^+$ NKT precursor cells at Stage 0 but has no effect on *Zbtb16* expression levels at later stages of NKT development. Mice carrying a +21/23 deletion display a moderate delay in thymic NKT development and have reduced numbers of NKT cells found in peripheral tissues.	40
Il4	HS-2 HS-V	+2 intron downstream *Il4* 3' of *Il4*	Removal of HS2 enhancer decreases the percentage of Th2 polarized cells that produce IL-4 in response to TCR stimulation. Mice harboring an HS2 deletion exhibit diminished IgE production and eosinophil generations resulting in airway pathogenesis in response to OVA immunization. Removal of HS-V decreases the fraction of Th2 polarized cells that produce IL-4 in response to PMA stimulation. Mice harboring HS-V deletion display diminished IgE production and increased parasite burden following *Leishmania* infection.	88 89
Ifng	CNS-22, CNS-4, CNS+20	−22, −4, +20 kb to *Ifng* TSS	Deletion of either CNS-22, CNS-4, CNS+20 decreases the fraction of Th1 polarized lymphocytes that express *Ifng* following stimulation. Expression levels of IFN-γ in IFN-γ$^+$ cells are unaffected.	86,87
Il9	CNS-25	−25 kb upstream *Il9*	Removal of CNS-25 decreases the fraction of Th9 polarized CD4$^+$ lymphocytes that produce IL-9 following PMA stimulation. CNS-25 deficient mice have a reduced percentage IL9$^+$ CD4$^+$ lymphocytes and less mast cell and mucus production in response to *Aspergillus fumigatus* challenge.	90
IL3	−34 DHS	−24 kb upstream *IL3*	Deletion of −34 DHS in Jurkat cells results in delayed *IL3* transcription following PMA stimulation.	91