EPIGENETICS

Dynamic epigenetic regulation at the single-cell level

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Chromatin regulators play a major role in establishing and maintaining gene expression states. Yet how they control gene expression in single cells, quantitatively and over time, remains unclear. We used time-lapse microscopy to analyze the dynamic effects of four silencers associated with reprogramming: DNA methylation, histone deacetylation, and histone methylation. For all regulators, silencing and reactivation occurred in all-or-none events, enabling the regulators to modulate the fraction of cells silenced rather than the amount of gene expression. These dynamics could be described by a three-state model involving stochastic transitions between active, reversibly silent, and irreversibly silent states. Through their individual transition rates, these regulators operate over different time scales and generate distinct types of epigenetic memory. Our results provide a framework for understanding and engineering mammalian chromatin regulation and epigenetic memory.

To compare the capabilities of distinct regulators, we selected four repression CRs that span a broad range of chromatin modifications: embryonic ectoderm development (EED), Krüppel associated box (KRB), DNA methyltransferase 3B (DNMT3B), and histone deacetylase 4 (HDAC4). EED functions as part of the Polycomb repressive complex 2 (PRC2), which methylates histone H3 at lysine 27 (H3K27me3) (17). KRB functions within >400 zinc finger transcription factors (18), associates with other CRs that write or read H3K9me3 (19), and is often used in genetic engineering (10, 20). DNMT3B causes de novo methylation of cytosine-guanine dinucleotides (CpGs) (21). HDAC4 removes acetyl groups from histones H3 and H4 (22). All of these CRs have been shown to silence gene expression during development, and their molecular mechanisms have been dissected in diverse studies (19, 23–25). However, their dynamic operational behaviors have not been analyzed in single cells and compared side-by-side at the same target gene.

To analyze how recruitment of each CR alters gene expression, we used time-lapse microscopy to follow silencing in individual cells after the addition of dox (Fig. 1C, movies S1 to S8, and materials and methods). Recruitment of each CR strongly and specifically silenced H2B-citrine expression (Fig. 1D and fig. S3, B and C). Silencing occurred in an all-or-none fashion in individual cells for all four CRs (Fig. 1D) at varying times after recruitment (Fig. 1E). During the silencing
event, median production rates dropped below 20% of their presilencing value within ~20 hours, or about one cell cycle (Fig. 1, F and G; see also fig. S4 for deviations from this behavior). This all-or-none response is similar to that observed upon recruitment of heterochromatin protein 1 (HP1) (9) and is consistent with previous reports of chromatin-related gene silencing (26, 27).

In contrast to the overall similarity in silencing event profiles, the timing of the silencing events we observed varied widely between cells, and the rate of silencing depended strongly on the CR used (Fig. 1, E and H). Silencing by KRAB and HDAC4 was rapid, with all cells silenced within one cell cycle (~20 hours), whereas EED and DNMT3B exhibited slower rates of silencing, with 50% of cells silenced at 35 and 62 hours, respectively. For these CRs, the broad cell-to-cell variability in Toff (defined as the delay between dox addition and silencing) (Fig. 1E) and the lack of a strong correlation of silencing behavior between sister cells (fig. S5) indicate that chromatin silencing is a stochastic process. In fact, after a relatively short time lag, the fraction of silenced cells varied widely between cells, and the rate of silencing depended strongly on the CR used (Fig. 1, E and H).
cells as a function of time is well described by a single-rate process for each CR (solid lines in Fig. 1H). Together, these results strongly suggest that silencing occurs through stochastic all-or-none events at distinct rates for each CR.

We next asked how the CRs differed in terms of reactivation dynamics and epigenetic memory. After 5 days of recruitment, we washed out dox to release the CRs and tracked the resulting changes in gene expression using time-lapse movies (Fig. 2A, fig. S6, and movies S9 to S16). For EED, KRAB, and HDAC4, reactivation occurred in stochastic all-or-none events, resembling silencing events in reverse (Fig. 2B). In contrast, we observed no reactivation events in cells silenced by DNMT3B recruitment, up to 80 hours after dox removal, after which cell density became too high for tracking.

To extend these measurements to longer durations, we switched to flow cytometry analysis. As expected for all-or-none reactivation, distributions of total fluorescence were bimodal (Fig. 2C and fig. S7, A to C), allowing us to quantitatively track the fraction of silent cells as a function of time (Fig. 2D and fig. S7D). The CRs produced qualitatively different modes of epigenetic memory (Fig. 2D), associated with distinct sets of chromatin modifications, as measured by DNA methylation and chromatin immunoprecipitation and quantitative polymerase chain reaction (fig. S8). HDAC4 imparted short-term memory: Upon its release, silencing was lost in all cells within 5 days, consistent with rapid dynamics of histone acetylation and deacetylation (28). In contrast, DNMT3B produced long-term memory: Cells were stably silenced for the duration of the experiment (30 days), in agreement with reports that DNA methylation is stably inherited (4). Finally, both EED and KRAB enabled a distinct type of hybrid memory that is not associated with DNA methylation (fig. S8B). For these CRs, a fraction of cells fully reactivated within 2 to 3 weeks, whereas the remaining fraction remained completely silenced for at least a month.

The hybrid memory could be explained by a three-state model (Fig. 3A) in which recruitment of a silencing CR causes cells to stochastically advance from an actively expressing state (A) to a reversibly silent state (R) and then to an irreversibly silent state (I). We assume that after the end of recruitment, the forward silencing rates become negligible, allowing cells in the R state to revert to the A state, reactivating gene expression, whereas cells in the I state remain silenced.

This three-state model predicts that longer durations of recruitment should increase the fraction of irreversibly silenced cells. To test this prediction, we systematically varied the duration of recruitment and analyzed the subsequent reactivation dynamics (Fig. 3B). For both EED and KRAB, the fraction of cells remaining silent 30 days after CR release increased with the duration of the initial recruitment, as predicted (Fig. 3, C and D). Similar increases in the stability of silencing with recruitment duration were also reported for HP1 (9). Aside from a relatively small time lag before the onset of reactivation (1 to 2 days), all data for a given CR could be fit to the three-state model with a single set of rate constants across the entire range of recruitment durations (solid lines in Fig. 3, C and D; see also materials and methods). Moreover, simplified forms of this model can also explain the behavior of HDAC4 and DNMT3B,
Fig. 3. A three-state model explains gene expression dynamics across different recruitment durations and strengths. (A) Proposed model based on stochastic transitions between actively expressing (A), reversibly silent (R), and irreversibly silent (I) states. Silencing (at rates \( k_s \) and \( k_d \)) depends on recruitment, whereas reactivation (at rate \( k_r \)) is independent of recruitment. (B) Experimental strategy: The duration of recruitment was varied from 1 to 5 days (colored arrows). After removal of dox, the fraction of cells remaining silenced was measured for up to 30 days. (C to F) Flow cytometry measurements show the fraction of silent cells over time after CR release. Colors indicate recruitment duration, as in (B). Data from two or more independent experiments are shown. Each set of solid lines represents a single fit of all data for that factor to the model, with rate constants indicated above each panel (see materials and methods for details of fitting). (G to I) Silencing and reactivation dynamics are measured at different dox concentrations. For each concentration, these data are fit with the corresponding model for each CR to extract the kinetic rates indicated in the diagram (diots, see materials and methods). Error bars represent the 95% confidence interval of the fit. Curves [(G) and (I)] are fits to a Michaelis-Menten–like equation. Lines in (H) are fits to a constant value.

A key parameter in these experiments is the recruitment strength of the CR, which is controlled by the dox concentration. To understand how recruitment strength affects silencing and reactivation capabilities, we analyzed the effects of 5 days of CR recruitment for a range of dox concentrations. Qualitatively, each CR produced the same number and type of states across dox concentrations (compare Fig. 3, C to F, to fig. S9, B to E). Quantitatively, recruitment strength modulated the silencing rates, but not the reactivation rates, which depended only on the identity of the silencing CR (Fig. 3, G to I). Together, these data provide a comprehensive view of how the dynamic effects of each CR on gene expression depend on recruitment duration and strength.

The three-state model (Fig. 3A) provides a unifying framework for comparing the operational capabilities of different CRs. More specifically, each CR traces a distinct curve within the parameter space defined by the three rate constants of the model over a range of recruitment strengths (Fig. 4A). Going forward, it will be critical to determine how these operational parameters depend on promoter architecture, the chromatin state of the locus, and the specific set of chromatin regulatory components expressed in different cell types. Moreover, it will be important to determine how the phenomenological states and transitions associated with each CR emerge from underlying molecular states and biochemical processes. Although the stochastic nature of silencing is consistent with simple models of spreading of chromatin modifications (9) (supplementary text and fig. S10), other processes—such as chromatin compaction and translocation to the nuclear lamina—may be involved.

Despite their differences, the CRs analyzed here were all capable of regulating gene expression through duration-dependent fractional control. In this mode, the duration of CR recruitment controls the fraction of cells in which the target gene is silenced in all-or-none fashion. This is possible when the lifetime of the reversible silenced state is long compared with the lifetimes of mRNA and protein (supplementary text and fig. S11).

Duration-dependent fractional control can be contrasted with other transcriptional regulation systems, in which more rapid dynamics enables the occupancy of a transcription factor at the promoter to control protein expression levels in a graded manner (29–31). Because of their different parameters, each CR generates a distinct control mode (Fig. 4B): DNMT3B faithfully records the duration or strength of recruitment. HDAC4 enables fast and reversible fractional control at maximum recruitment strengths but can also lead to graded changes in protein levels at lower ones (fig. S11). EED and KRAB, due to their hybrid memory, enable regulation across multiple time scales. For example, with these CRs, pulses of recruitment of different durations that both silence the entire population in the short term can establish different degrees of permanent memory in the long term (Fig. 4C), similarly to the classical example of PRC2-mediated silencing of the flowering locus during vernalization (32). These types of fractional control strategies could be used to integrate signals for cellular decision-making (33, 34).
It is now possible to use the framework developed here to classify the operational capabilities of other CRs, as well as to determine how their behaviors depend on biological context and how they interact combinatorially to provide additional capabilities. More generally, this approach should help us to understand why specific CRs are employed in particular natural genetic circuits and to enable the design of synthetic gene circuits that take advantage of the inherent temporal control and memory capabilities of chromatin-mediated regulation.

REFERENCES AND NOTES


SUPPLEMENTARY MATERIALS

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