Cells use a system of chromatin regulators (CRs) and associated histone and DNA modifications to modulate gene expression and establish long-term epigenetic memory (1–4). This system is critical in development (5), aging (6), and disease (7) and could provide essential capabilities for synthetic biology (8). In all of these contexts, the temporal dynamics and cell-to-cell variability of gene expression are critical but have been difficult to study, as current methods usually provide static correlations between chromatin modifications and gene expression or aggregate data across potentially heterogeneous cell populations. Therefore, it has remained unclear how strongly, rapidly, and uniformly each regulator can alter gene expression, and how long these effects persist (Fig. 1A).

To answer these questions, we combined targeted CR recruitment (9–22) with time-lapse microscopy (13) to develop a system to quantitatively track the effects of CRs on a reporter gene in individual cells. More specifically, we fused individual CRs to the reverse Tet repressor (rTetR) (14), which binds to DNA only in the presence of doxycycline (dox), allowing us to control the timing and duration of CR recruitment upstream of a fluorescent reporter gene expressing histone 2B (H2B)-citrine (Fig. 1B). To isolate the system from other genes, the reporter was flanked by insulators (15) and integrated on a human artificial chromosome (HAC) (16). All constructs were stably integrated in Chinese hamster ovary (CHO)-K1 cells, a major model system for synthetic mammalian biology (see supplementary materials and methods). Each cell line constitutively coexpressed H2B-mCherry, thus allowing cell tracking even when the reporter was silenced (Fig. 1, B and C). Control experiments indicated that recruitment of rTetR alone does not repress reporter expression (Fig. S1) and that changes in gene regulation could be detected over time scales as short as 6 hours (fig. S2). Therefore, this system enables analysis of the effects of CR recruitment and release of each CR on gene expression in individual cells.

To compare the capabilities of distinct regulator families, we selected four representative CRs that span a broad range of chromatin modifications: embryonic ectoderm development (EED), Krüppel associated box (KRAB), 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). KRAB functions within >400 zinc finger transcription factors (18), associates with other CRs that write or read histone H3me3 (9), 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 increased significantly.
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 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 reactivation 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,
A 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. S1I). 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 longer 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).

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 $k_f$) depends on recruitment, whereas reactivation (at rate $k_f$) 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 (dts, see materials and methods). Error bars represent the 95% confidence interval of the fit. Curves [(G) and (J)] fits to a Michaelis-Menten–like equation. Lines in (H) are fits to a constant value.
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


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SUPPLEMENTARY MATERIALS

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Materials and Methods

Supplementary Text Figs. S1 to S12

Table S1

References (36–49)

Movies S1 to S16

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