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ACKNOWLEDGMENTS

Data from Project Achilles and the Cancer Cell Line Encyclopedia can be accessed at www.broadinstitute.org/achilles and www.broadinstitute.org/ccle/home, respectively. We thank members of the Garraway laboratory for helpful discussion. We thank D. Hernandez for assistance with seeding cell lines for metabolomics studies. We gratefully acknowledge the Carlos Slim Foundation for providing access to shRNA screening data (funded in part by the Slim Initiative in Genomic Medicine for the Americas project) for validation studies. Funding for this work was provided by the Novartis Institutes for BioMedical Research, the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation, the Melanoma Research Alliance, a NIH P01 Research Program Project grant (each to L.A.G.), an Integrative Cancer Biology Program grant (U54 CA112962 to T.R.G.), and an NIH U01 CA176058 (to W.C.H.). F.H.W. is supported by a Conquer Cancer Foundation of the American Society of Clinical Oncology Young Investigator Award, the 2014 AACR-Bristol-Myers Squibb Oncology Fellowship in Clinical Cancer Research (grant 14-40-15-WLS), a grant from the Karin Grunebaum Cancer Research Foundation, and a KL2/Catalyst Medical Research Investigator Training award (an appointed KL2 award) from Harvard Catalyst/The Harvard Clinical and Translational Science Center (National Center for Research Resources and the National Center for Advancing Translational Sciences, NIH Award KL2 TR001100). G.V.K., A.T., F.V., B.A.W., and C.M.B. performed computational analysis of data sets generated from Project Achilles and CCLE under the supervision of J.S.B., T.R.G., W.C.H., and L.A.G. G.S.C., D.E.R., and W.C.H. supervised the generation of Project Achilles screening data. F.H.W. and S.E.M. performed experiments to validate the computational shRNA screening findings. G.V.K., F.H.W., S.E.M., J.M.S., C.D., and C.B.C. performed metabolomic studies and analyzed data. Isogenic cell line pairs were generated and characterized by F.H.W. and J.R.R. J.R.R. conducted cell viability IC₅₀ experiments and the MTA methyltransferase selectivity assay. J.P. performed the MTA mechanism of action study under the supervision of J.E.B. M.E.F. and M.T. synthesized EP2015666 under the supervision of J.E.B. F.H.W., G.V.K., J.R.R., and L.A.G. wrote the manuscript. L.A.G. is a paid consultant for Novartis and Boehringer Ingelheim; he holds equity in, and is a paid consultant for, Foundation Medicine; and he is a recipient of a grant from Novartis. W.C.H. is a paid consultant for Novartis and recipient of a grant from Novartis. M.T. is a visiting scientist from Mitsubishi Tanabe Pharma Corporation (MTPC) and a recipient of nonresearch support from MTPC. The Broad Institute and the authors (L.A.G., F.H.W., G.V.K., and J.R.R.) have filed a patent application (BI2015/040 46783.00.2109 US PRO Pending 62/131,825) relating to the use of PRMT5 inhibitors to treat MTAP-deficient tumors.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/351/6278/1214/suppl/DC1
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7 October 2015; accepted 1 February 2016
Published online 11 February 2016
10.1126/science.aad5214

TRANSCRIPTION

Measurement of gene regulation in individual cells reveals rapid switching between promoter states

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In vivo mapping of transcription-factor binding to the transcriptional output of the regulated gene is hindered by probabilistic promoter occupancy, the presence of multiple gene copies, and cell-to-cell variability. We demonstrate how to overcome these obstacles in the lysogeny maintenance promoter of bacteriophage lambda, P_{RM} . We simultaneously measured the concentration of the lambda repressor CI and the number of messenger RNAs (mRNAs) from P_{RM} in individual *Escherichia coli* cells, and used a theoretical model to identify the stochastic activity corresponding to different CI binding configurations. We found that switching between promoter configurations is faster than mRNA lifetime and that individual gene copies within the same cell act independently. The simultaneous quantification of transcription factor and promoter activity, followed by stochastic theoretical analysis, provides a tool that can be applied to other genetic circuits.

Sequence-specific transcription factors drive the diversity of cell phenotypes in development and homeostasis (1). For each target gene, alternative transcription-factor binding configurations (by different transcription factors or by multiple copies of the same one) result in varied transcriptional outputs, in turn leading to alternative cell fates and behaviors (2, 3). Elucidating the relations between transcription-factor configurations [which can number in the hundreds (4–6)] and the resulting transcriptional activity remains a challenge. Application of traditional genetic and biochemical approaches usually requires a genetically modified system or assays of purified components in vitro (7). Ideally, however, one would like to map transcription-factor configuration to promoter activity inside the cell, with minimal perturbation to the endogenous system.

Multiple factors hinder such direct measurement. First, individual cells vary in both transcription-factor concentration and the resulting transcriptional activity (8, 9); averaging over many cells thus filters out details of the regulatory relation. Second, even within the single cell, more than one copy of the regulated gene is typically present, with each copy individually regulated (10). Finally, even at the level of a single gene copy, multiple binding configurations are possible at a given

transcription-factor concentration (11, 12). The relative probabilities of these different configurations and the rate of switching between them will define the stochastic activity of the regulated promoter (13).

We simultaneously measured, in individual cells, the concentration of a transcription factor and the number of mRNAs produced from the regulated gene. We also measured how the gene copy number changes through the cell cycle. We then analyzed the full single-cell data using a theoretical model, which allowed us to identify the contributions of different transcription-factor binding configurations to the stochastic activity of the promoter.

Specifically, we examined the lysogeny maintenance promoter of phage lambda, P_{RM} . The regulation of this promoter by its own gene product, the lambda repressor (CI), is a paradigm for how alternative binding configurations drive transcriptional activity and the resulting cell fate—stable lysogeny or lytic induction resulting in cell death (7). The number of possible CI configurations is very large [>100 (4, 5)]. Briefly, as CI concentration increases, CI dimers gradually occupy three proximal (O_{R1-3}) and three distal (O_{L1-3}) operator sites, leading first to activation, then repression, of P_{RM} (Fig. 1A). Cooperative CI binding, and looping of DNA between the O_R and O_L sites, play important roles in shaping the P_{RM} (CI) regulatory curve (14).

In a lysogen (a bacterium carrying a prophage), CI concentration is believed to be such that P_{RM} fluctuates between the activated and repressed states (15) (Fig. 1A), and this has been suggested to stabilize the lysogenic state against random fluctuations in CI levels (14). However, the nature of the lysogenic “mixed state” (activated/repressed) is unknown: Are the promoter fluctuations slow

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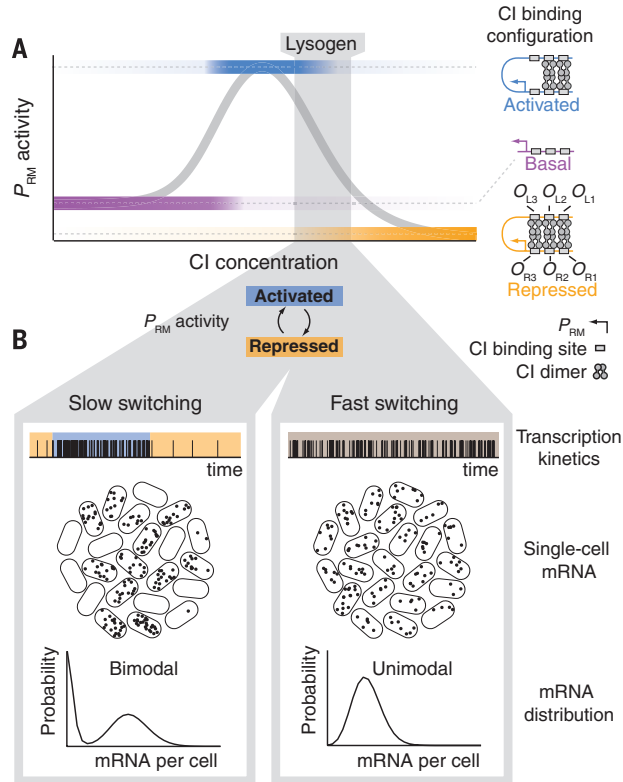
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enough, such that two distinct cell populations coexist, exhibiting high and low P_{RM} expression, respectively? Alternatively, are promoter fluctua-

tions fast, such that all cells exhibit an intermediate, well-defined level of P_{RM} expression (Fig. 1B)?

Fig. 1. Schematic of P_{RM} regulation by CI.

(A) As the concentration of CI increases, the probabilities of different CI binding configurations at the O_R and O_L operators change (color shading), resulting in varying P_{RM} activity (gray curve). Three configurations, expected to be the most probable, are depicted. In lysogenic cells, P_{RM} has comparable probabilities of being in the activated and repressed promoter states (gray shading). (B) The rate of switching between activated and repressed states drives the stochastic activity of P_{RM} in lysogenic cells. Two alternative hypotheses are illustrated: If switching is slow relative to the mRNA lifetime (left), two subpopulations of cells will exist, with low and high mRNA levels. If switching is fast (right), the mRNA distribution will be unimodal.

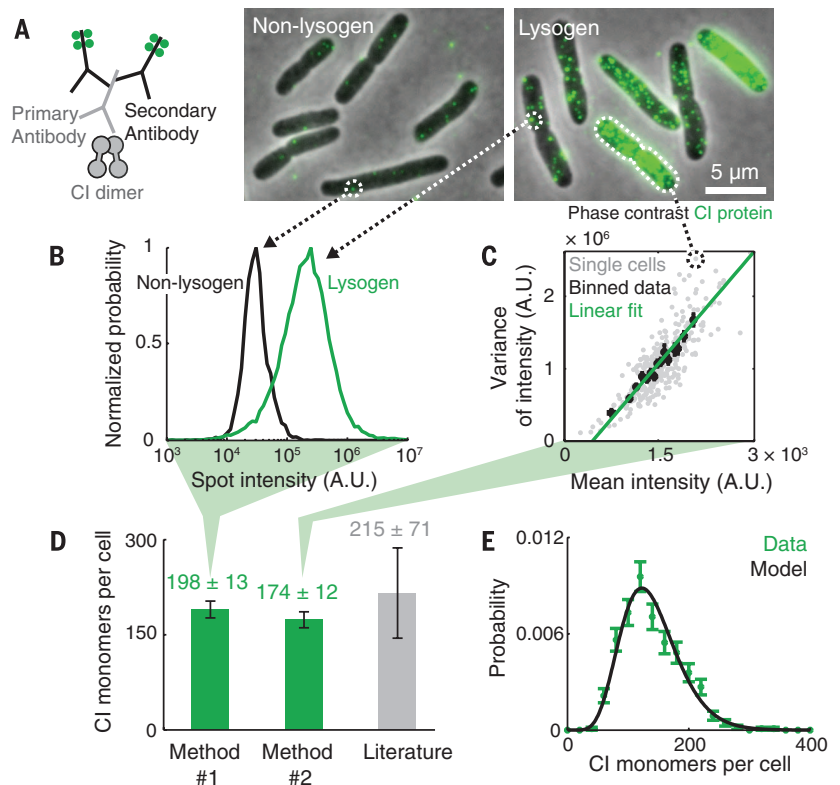


To measure CI concentration in individual cells, we used antibody labeling (immunofluorescence). Lysogenic cells (see table S1) exhibited a strong CI signal, whereas nonlysogenic (uninfected) cells showed only a weak background signal (Fig. 2A and fig. S1). To verify that the antibody signal reliably represents CI levels, we expressed a CI-yellow fluorescent protein (YFP) fusion protein (16) in nonlysogenic cells and compared the YFP fluorescence to the signal exhibited by the antibody to CI in each cell. The two signals were linear with each other (fig. S2A), and single-molecule imaging revealed that most YFP molecules were colocalized with an antibody to CI, as expected (fig. S2B).

To convert the antibody signal to CI concentration in each cell, we needed to know the fluorescence value corresponding to a single antibody-labeled CI molecule [a CI dimer, which is the dominant species in the cell (17)]. To obtain this calibration constant, we used two methods (18) (Fig. 2, B and C): In the first method, we used automated image analysis to identify individual fluorescent particles (spots, fig. S3). These spots displayed a well-defined intensity value, distinct from the corresponding signal found in negative samples (Fig. 2B). We identified the positive-sample spot intensity as corresponding to individual CI dimers (fig. S4A) (each one decorated by ~20 fluorescent dyes, due to the stoichiometry of antibody labeling; fig. S5) and used it to convert cell fluorescence to CI concentration. In the second method, we used the fact that the Poisson statistics of random protein positions within the cell lead to a linear relation between the

Fig. 2. Measuring the number of CI molecules in individual cells.

(A) CI proteins were labeled with antibodies to CI and fluorescently labeled secondary antibodies (left). Under the microscope, lysogenic *E. coli* cells exhibited a strong CI signal (right) whereas nonlysogens showed a weak background signal (center). (B) Method 1 for measuring the number of CI proteins per cell. The typical fluorescence of a single CI dimer was obtained from the spot intensity distribution in lysogenic cells (green, $N = 23,631$ spots), distinguishable from that of the negative sample (black, $N = 1764$ spots). (C) Method 2 for measuring the number of CI proteins per cell. The variance versus the mean of pixel intensity in individual cells (gray, $N = 324$) was fitted to a linear function (green). The slope of this line was used to estimate the fluorescence intensity of a single CI dimer. (D) The estimated number of CI molecules in a lysogen, obtained with the two single-cell methods (green, mean \pm SEM from six experiments, 327 to 704 cells each). Also shown is the value reported in the literature [gray, mean \pm SD from three studies (19–21)]. (E) The distribution of CI copy number in lysogenic cells (green; $N = 560$ cells). The data are described well by a gamma distribution (black).



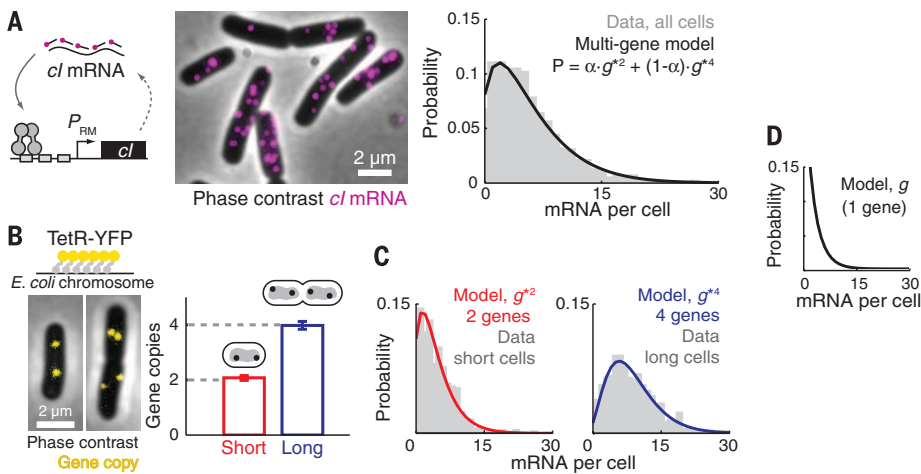


Fig. 3. Measuring the transcriptional activity of a single P_{RM} copy. (A) (Left and center) cI mRNA in lysogenic cells was labeled by smFISH. (Right) The measured distribution of cI mRNA number per cell from the whole population (gray, $N = 2893$ cells) consists of contributions from cells containing two and four gene copies [black; see (B) to (D)]. α is the fraction of cells with two copies of the P_{RM} - cI gene. (B) Estimating the number of P_{RM} - cI gene copies in lysogenic cells. TetR-YFP binds to an array of $tetO$ sites inserted next to the gene locus, resulting in visible foci (left). (Right) Newborn cells (length percentile 5 to 20, “short,” red, $N = 493$) contained two copies of the P_{RM} - cI locus, whereas cells about to divide (length percentile 80 to 95, “long,” blue, $N = 493$) contained four copies. Error bars indicate SEM. (C) The measured distributions of cI mRNA numbers for short (left) and long (right) cells. Both were well fitted by a model assuming independent stochastic activity of each gene copy. (D) The theoretical fit from (C) was used to reconstruct the cI mRNA distribution from a single gene copy. This distribution was then used to predict the distribution for the whole population (A).

fluorescence mean and the pixel-to-pixel variance within each cell (Fig. 2C and fig. S6). Measuring the slope of this line allowed us to identify the fluorescence corresponding to a single labeled protein (fig. S7). Using either method to estimate CI concentration in lysogens gave similar results (Fig. 2D and fig. S4B). These measured values also agreed with those reported in the literature (19–21) (Fig. 2D and table S2).

The two imaging-based methods allowed us to measure CI numbers in individual lysogenic cells (Fig. 2E). Fitting the CI distribution to a stochastic model of protein production (22) indicated that, on average, the ~200 CI monomers in the cell are produced in ~10 random bursts, of ~20 proteins each, during the 30-min cell cycle (table S3). The estimated burst frequency is consistent with a (more accurate) value that we obtained from cI mRNA statistics (Fig. 3). It is also consistent with the measured stability of the lysogenic state [which depends exponentially on the CI burst frequency (23)].

To measure the activity of the P_{RM} promoter in individual lysogenic cells, we used single-molecule fluorescence in situ hybridization (smFISH) (24, 25) to label and count cI mRNAs, produced from P_{RM} (Fig. 3A). Fluorescent spots were identified by means of an automated algorithm (25) (fig. S3), and the fluorescence intensity corresponding to a single mRNA was identified (fig. S8). We used this intensity to convert the total spot intensity in each cell to the number of cI mRNAs (25). The copy-number distribution of cI mRNA in a lysogen (Fig. 3A) represents the combined contribution from multiple copies of the P_{RM} - cI

gene in each cell (26). To identify the contribution of a single gene copy, we first examined how the cI gene copy number varies during the cell cycle. We engineered an array of 140 Tet operators ($tetO$) (27) into the gal locus of *Escherichia coli* (~16 kb away from the lambda integration site). The gene locus was detected through the binding of a Tet repressor (TetR)-YFP fusion (27) (Fig. 3B). We used automated image analysis to count the number of YFP foci in each cell. Gating the cell population by length, we found that newborn cells had on average 2.1 ± 0.1 (mean \pm SEM) foci per cell. Cells about to divide had 4.0 ± 0.1 foci per cell (Fig. 3B). These values are in good agreement with the expected copy number of the cI locus under our experimental conditions (26). We used these measured copy numbers to delineate the transcriptional activity of individual gene copies. If the stochastic activity of each copy is independent of the other copies in the same cell, then the cI mRNA distribution for cells having two gene copies will be given by the autoconvolution of the distribution for a single gene copy (a distribution that we cannot measure directly). Similarly, the mRNA distribution for four-copy cells will be equal to the one-copy distribution taken to the fourth convolution power. The experimental histograms agreed well with these predictions (Fig. 3C and fig. S9). Furthermore, knowing the fraction of cells in the population that have two and four copies allowed us to then predict the cI mRNA distribution for the whole population. The predicted distribution agreed well with the experimentally measured one (Fig. 3A).

Analyzing the single-gene mRNA distribution (Fig. 3D) revealed that a single copy of P_{RM} produces a burst of cI mRNA every ~6 min on average (table S4). When accounting for the presence of two to four gene copies per cell (Fig. 3B), this value is consistent with the burst frequency estimated from the CI protein histogram (Fig. 2E). Comparing the protein and mRNA data also allowed us to directly calculate the number of CI proteins produced from each cI mRNA, ~6 on average (table S3). This value is in good agreement with a previous theoretical calculation (23).

To measure the regulatory relation between CI concentration and P_{RM} activity, we used a reporter system in which the autoregulatory feedback from CI to P_{RM} existing in the lysogen is broken: CI is expressed from an inducible promoter, whereas P_{RM} transcribes the $lacZ$ gene rather than cI (14) (Fig. 4A). To simultaneously measure CI concentration and P_{RM} activity in the same cell, we combined immunofluorescence (using antibody to CI) with smFISH (using $lacZ$ probes) (18) (Fig. 4B and fig. S10) and measured the corresponding protein and mRNA numbers as described above. Performing this measurement over a range of CI levels, then plotting $lacZ$ mRNA numbers versus CI concentration from many individual cells, produced highly scattered data (Fig. 4C), as expected from the stochasticity of the regulation and transcription processes (9). Averaging within finite windows of CI concentration revealed the mean regulatory relation between CI and P_{RM} , known as the gene regulation function (16) (Fig. 4C and fig. S11). The shape of the regulation function agreed with that from previous reports, with P_{RM} activity first increasing, then decreasing, with CI concentration (4, 14, 28). However, our measurement provides the absolute numbers for both the input (CI concentration) and output (mRNA numbers), rather than relative expression levels (4, 5, 14, 28). The absolute values are crucial for the subsequent steps in our analysis of P_{RM} regulation.

As the first step in this analysis, we wrote down a theoretical model in which the probabilities of different CI binding configurations are given by their thermodynamic weights (15) (fig. S12A). This thermodynamic model successfully reproduced the regulation function (Fig. 4C and fig. S13). In performing this procedure, most free-energy values used in the model were identical to those reported (15) (table S5). The model also provided the probabilities of observing the different promoter activity states—basal, activated [with the DNA between O_R and O_L either looped or unlooped (15)], and repressed—as a function of CI concentration (Fig. 4D). The overlap between the different states underlines the challenge in identifying the transcriptional signature of a single promoter state: For example, the probability of P_{RM} being in the activated state does not surpass ~50%.

To reveal the activity of individual promoter states, we introduced a stochastic version of the theoretical model (Fig. 4E and fig. S12). In the model, the CI binding configurations are grouped based on the expected promoter activity: basal, activated unlooped, activated looped, and repressed

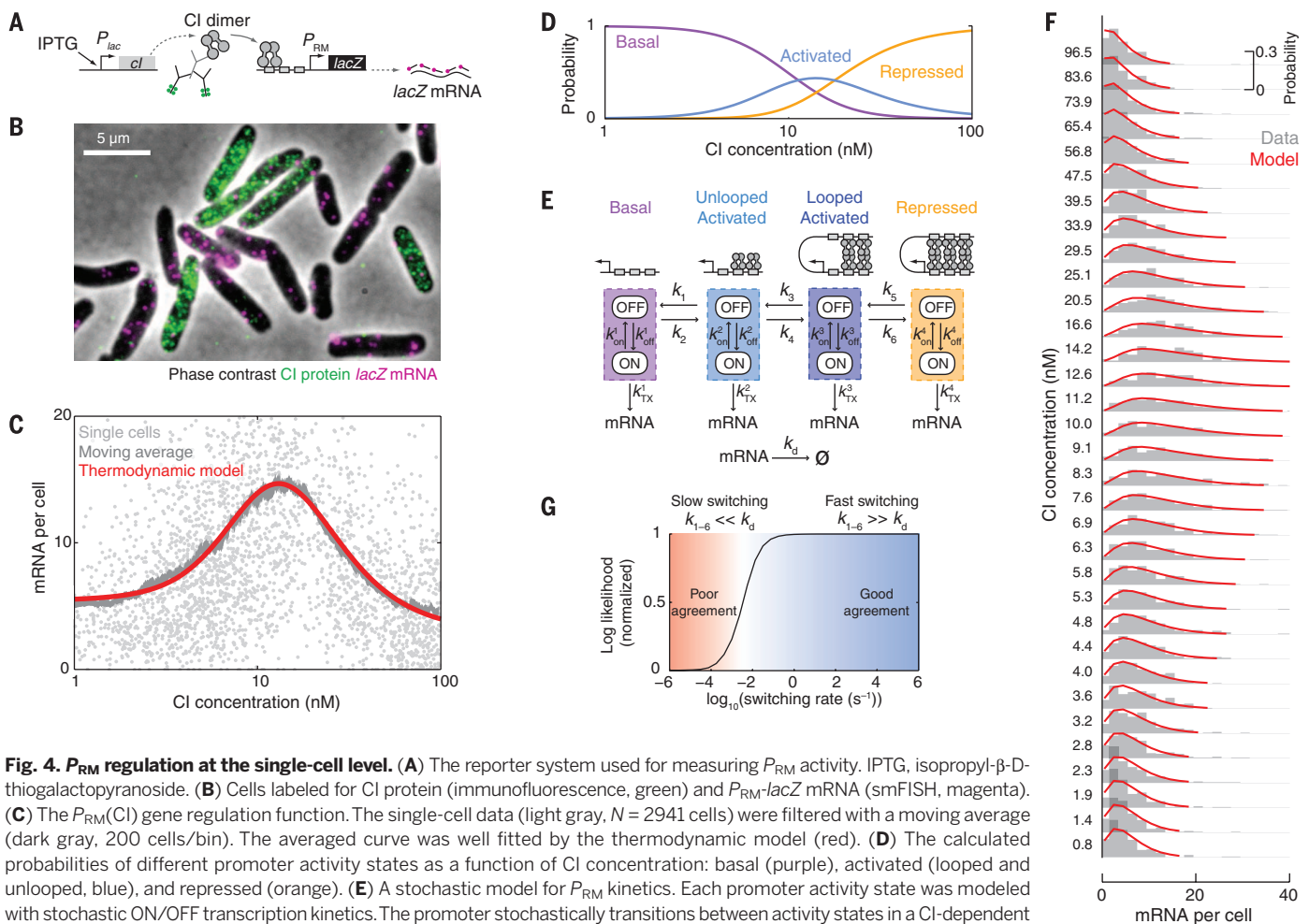


Fig. 4. P_{RM} regulation at the single-cell level. (A) The reporter system used for measuring P_{RM} activity. IPTG, isopropyl- β -D-thiogalactopyranoside. (B) Cells labeled for Cl protein (immunofluorescence, green) and P_{RM} -*lacZ* mRNA (smFISH, magenta). (C) The P_{RM} (Cl) gene regulation function. The single-cell data (light gray, $N = 2941$ cells) were filtered with a moving average (dark gray, 200 cells/bin). The averaged curve was well fitted by the thermodynamic model (red). (D) The calculated probabilities of different promoter activity states as a function of Cl concentration: basal (purple), activated (looped and unlooped, blue), and repressed (orange). (E) A stochastic model for P_{RM} kinetics. Each promoter activity state was modeled with stochastic ON/OFF transcription kinetics. The promoter stochastically transitions between activity states in a Cl-dependent manner. (F) The stochastic model successfully described the P_{RM} (Cl) single-cell data. The experimental data (C) were binned into 100-cell histograms (gray) and fitted to the model (red) by maximum likelihood estimation. (G) Consistency of measured mRNA statistics with rapid switching between promoter states. Solving the stochastic model for different switching rates, and fitting the model results to the measured mRNA statistics, resulted in a good fit for fast switching (right, blue); slow switching yielded a poor fit (left, red).

(15). Each promoter activity state is described by stochastic bursty kinetics of mRNA production (29). P_{RM} stochastically switches between its four activity states. The switching rates are initially unknown, but the thermodynamic model above provides us with the equilibrium constant (ratio between switching left and right) for each pair of states, at a given Cl concentration. For each set of parameters, the stochastic model can be solved to yield the expected mRNA copy-number distribution for the population of multicopy cells.

We used the stochastic model to analyze the full P_{RM} (Cl) single-cell data set (Fig. 4C). Applying maximum-likelihood estimation, we found good agreement between the experimental and theoretical mRNA distributions over the full range of Cl concentrations (Fig. 4F, fig. S14, and movie S1). The fitting procedure allowed us to extract the mRNA statistics corresponding to the different activity states of P_{RM} (fig. S15). The calculated distributions were in good agreement with those obtained with genetic controls: cells expressing no CI (basal), and cells overexpressing CI in wild-type P_{RM} (repressed) and in a mutant lacking the

O_L operator (activated unlooped) (14) (fig. S15B and table S6). The stochastic kinetics of each promoter state exhibited a similar relation between expression level and burst size to that measured in other *E. coli* promoters (29) (fig. S15C).

Even though the measured mRNA distribution at each Cl concentration represents a mixture of multiple promoter states, each of the histograms is unimodal and can be described by a simple kinetic model with a single burst size and frequency (Fig. 4F and fig. S16). The parameter that determines the shape of the “mixed state” mRNA distribution is the rate of switching between promoter states (Fig. 1B). Previous *in vitro* studies of O_R - O_L looping suggested that the switching between looped and unlooped promoter configurations is fast (~seconds) (30), but similar studies of looping in the cell left the question open (31). Our stochastic model predicts that if promoter switching is very slow relative to mRNA lifetime [here ~2 min (29)], the observed mRNA distribution will be the weighed sum of the underlying single-promoter-state distributions. Our experimental data strongly disagreed with this

prediction (Fig. 4G). By contrast, if switching is fast, the observed distribution will be given by a (weighed) convolution of the underlying single-promoter-state distributions, and if the underlying states can each be described by simple bursty kinetics, the new mixed state can be as well. This is indeed what we observed (Fig. 4, F and G, and fig. S16). Thus, P_{RM} switches rapidly between different promoter states, resulting in a stochastic signature that (at a given Cl concentration) is indistinguishable from that of a single promoter state, but with renormalized kinetic parameters. Our finding of rapid switching explains why, in the lysogen, we did not detect distinct “active” and “repressed” populations in either the protein (Fig. 2E) or mRNA (Fig. 3A) histograms, but instead both data sets indicated a single, well-defined promoter activity.

Precise single-cell measurements, accompanied by theoretical analysis, can reveal new features even in well-studied model systems. When combined with genetic and synthetic-biology approaches (13), this strategy may allow prediction of the stochastic characteristics of promoter activity,

a prediction that remains a challenge to our understanding of gene regulation (9, 32).

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ACKNOWLEDGMENTS

We are grateful to the following people for generous advice and for providing reagents: I. Dodd, M. Elowitz, L. Finzi, H. Garcia, T. Gregor, T. Kuhlman, L. McLane, R. Phillips, A. Raj, E. Rothenberg, A. Sanchez, K. Shearwin, R. Singer, S. Skinner, L-H. So, A. Sokac, L. Zeng, and C. Zong. Work in the Golding lab is supported by grants from NIH (R01 GM082837), NSF (PHY 1147498, PHY 1430124 and PHY 1427654), The Welch Foundation (Q-1759), and The John S. Dunn Foundation (Collaborative Research Award). H.X. is supported by the Burroughs Wellcome Fund Career Award at the Scientific Interface. We gratefully acknowledge the computing resources provided by the Computational and Integrative Biomedical Research Center of Baylor College of Medicine.

SUPPLEMENTARY MATERIALS

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20 July 2015; accepted 25 January 2016
10.1126/science.aad0635

Measurement of gene regulation in individual cells reveals rapid switching between promoter states

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Science **351** (6278), 1218-1222.
DOI: 10.1126/science.aad0635

Stochastic properties of phage promoter

Full understanding of regulated gene expression requires characterization of stochastic variation in the activity of individual promoters. To avoid cell-to-cell variability and variation between the activity of specific gene copies, Sepúlveda *et al.* investigated the behavior of the lysogeny maintenance promoter of phage lambda in individual *Escherichia coli* cells. They measured the concentration of transcription factor and the actual number of mRNAs produced, and used mathematical modeling to discern the stochastic activity of the regulated promoter. The promoter underwent switching between configurations that occurred more rapidly than the lifetime of mRNA molecules produced, and individual copies of the same gene functioned independently in the same cell. Such studies can reveal new aspects of systems that have been well studied by more conventional techniques.

Science, this issue p. 1218

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