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Understanding epigenetic regulation: Tracking protein levels across multiple generations of cells

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Abstract. Cells and organisms are remarkably robust: they alter the variety and levels of expressed genes and proteins in response to environmental stimuli, including temperature, chemicals, and the stiffness of their surroundings. Ultimately changes in gene and protein expression can result in a distinct phenotypic state, which in some cases is maintained over multiple generations; the ability to pass on a particular phenotypic state to progeny cells is critical for differentiation. Moreover, epigenetic regulation of phenotype is also thought to provide an evolutionary advantage for a population of cells adapting to a fluctuating environment on faster timescales than the occurrence of genetic mutations. However, simple methods to study patterns of gene and protein expression on multi-generational timescales are sparse. Here we describe a technique to study lineages of single cells over multiple generations using a microfluidic device; this reveals patterns of expression where protein levels are correlated across multiple generations. Such quantitative information of protein expression in the context of pedigree remains hidden when studying the population as an ensemble.

1 Introduction

The life of a cell is random. A cell faces unpredictable environmental changes, such as fluctuations in temperature, nutrient concentrations, and pH. Within the cell many genes are present only in one or few copies, thus the biochemical reaction of transcribing a single gene involves small numbers of molecules, and is inherently stochastic (Fig. 1). Fluctuations in the concentration of proteins and regulators involved in gene expression thus give rise to variability in levels of mRNA, and consequently the numbers of proteins that are produced. Stochasticity in protein expression is elegantly visualized by engineering bacteria cells to express both red and green proteins; because levels of proteins fluctuate over time, some cells appear red, others green, and some are yellow [1] (Fig. 1(b,c)). Cell-to-cell variation in protein expression is also described in many other systems, including yeast and mammalian cells [2–6].

In contrast to the stochastic nature of gene expression, the cell is ordered in both space and time. Higher-order structures such as membranes, filaments, and networks provide internal structure and organization, which is critical for the structure and mechanical properties of cells. For example, cytoskeletal proteins assemble into filaments and networks that provide the cell with mechanical resilience to physical forces [7]. Subcellular organization is also essential for many dynamic processes in the cell, including cell division, motility, and regulation of gene expression via higher-order structures in the nucleus. For example, chromatin is organized in such a way that groups of genes are silenced or activated in response to external cues; global changes in gene expression are initiated during stress response [8] or differentiation [9]. Moreover, cells themselves form higher-order structures, assembling into tissues, organs, and organisms.

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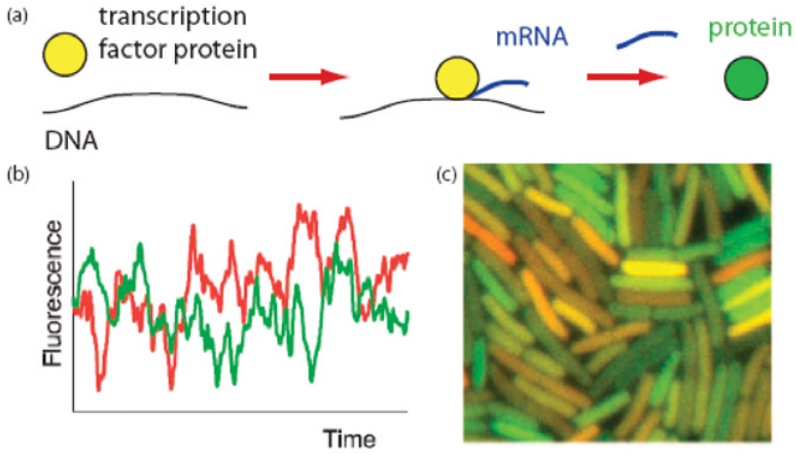


Fig. 1. Biochemical reactions of protein expression. A transcription factor protein binds to DNA; the gene is transcribed to yield mRNA; the mRNA is then transported out of the nucleus into the cytoplasm where it is translated into protein. As many genes are only present in one or very few copies within the nucleus, there is some inherent stochasticity in the expression of genes and proteins, which results in (b) varying levels of proteins over time. (c) Bacteria cells engineered to express both a red and green fluorescent protein under the identical promoter show fluctuations in protein levels over time: some cells are red, some are green, and some are yellow as relative protein levels vary. Figures (b) and (c) are reprinted with permission from [1].

Cell-to-cell variation in protein expression levels can thus result from stochasticity in protein expression, in addition to other factors such as differences in higher-order chromatin structure. Thus, cells that have the same genotype may have different phenotype (Box 1). For example, even though a population of single yeast cells contain the exact same DNA, and are growing in the exact same environment, they express markedly different levels of proteins (Fig. 2). This can be seen by imaging fluorescently-labeled proteins, where the protein levels are proportional to the fluorescence intensity of the cell. Phenotypic variation is also evident in the human body: each cell is genetically identical, yet the properties of the liver differ vastly from the properties of bone. Such differences between cells result when stem cells differentiate and achieve a distinct phenotypic state that is maintained and passed on to progeny cells.

2 A brief introduction to epigenetic inheritance

The ability to pass information on to progeny cells in a non-genetic way is called epigenetic inheritance. Inheritance of a particular state results when a certain phenotype is maintained on timescales longer than the generation time of the cell. One way to regulate the phenotypic state is through the higher-order chromatin structure: genes are less transcriptionally active when tightly packed into a silenced heterochomatin state, compared to when loosely packed in a form accessible to transcription factor proteins [10,11]. A phenotypic state can also be propagated over multiple generations via biochemical networks such as feedback loops. Another possible epigenetic mechanism involves protein aggregation, whereby misfolded proteins are passed on to progeny cells. Termed prions, this form of epigenetic inheritance underlies bovine spongiform encephalopathy, or mad cow disease. [Other references provide a more comprehensive overview of epigenetic mechanisms of inheritance [12,13].]

Why does phenotypic inheritance matter? Robust and reproducible change in phenotypic state is critical in fundamental biological processes such as differentiation. Phenotypic inheritance may also be important for single-celled organisms such as yeast or bacteria: the ability to epigenetically regulate genes involved in stress-response may enable cells to change phenotype

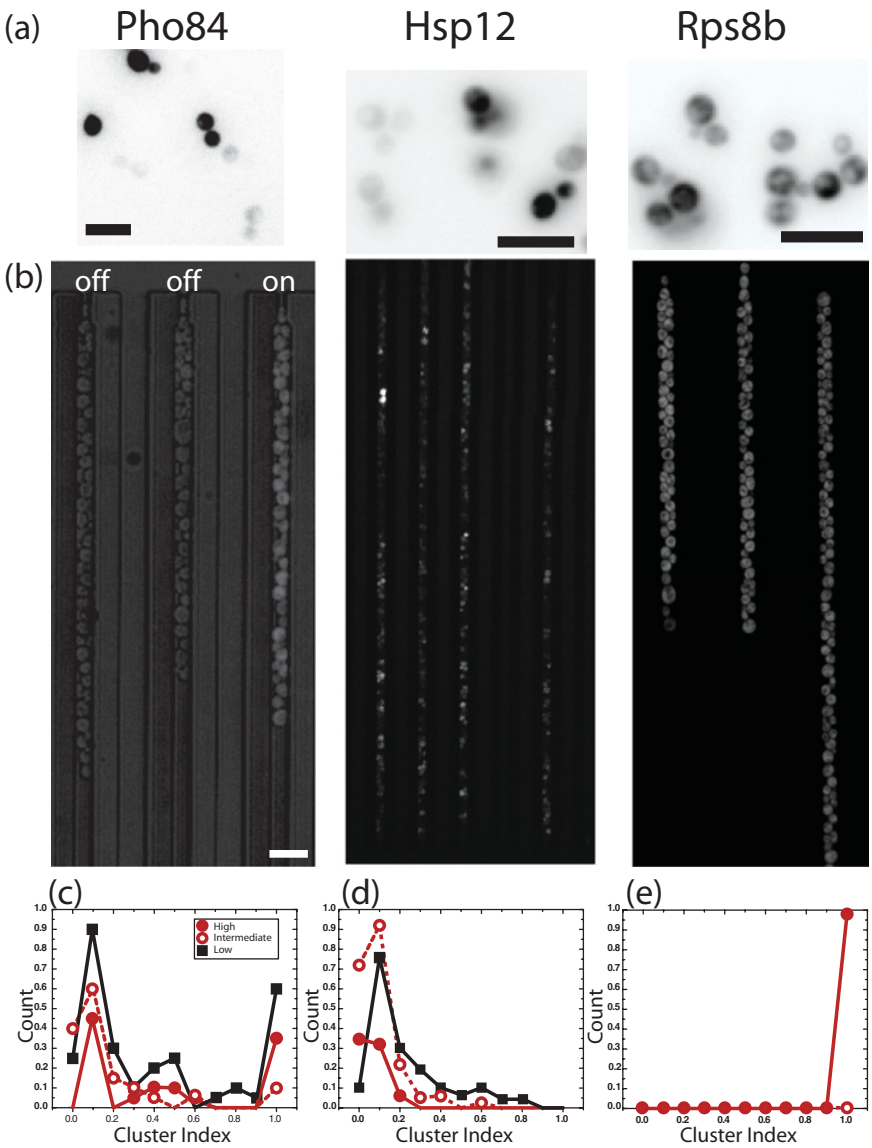
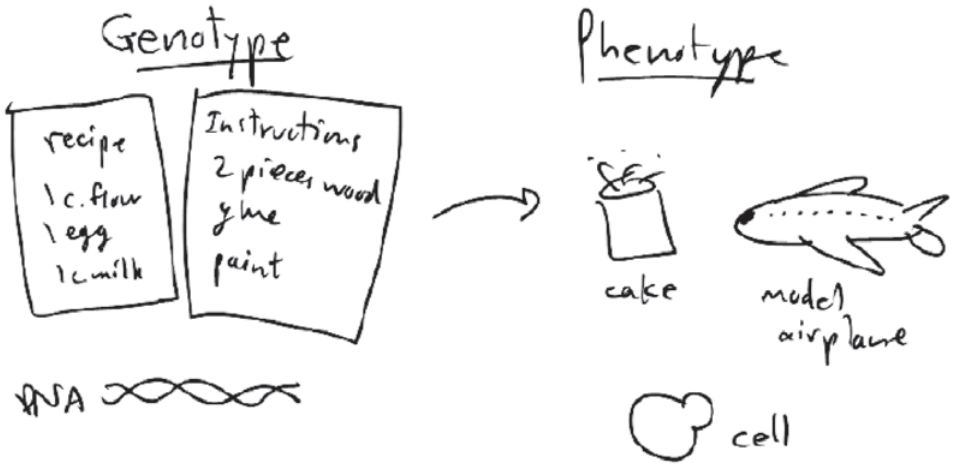


Fig. 2. Persistence time for protein expression varies. Lineages of cells expressing three different proteins reveals timescales of phenotypic variation. **A**: pPho84-GFP, **B**: Hsp12-GFP, and **C**: Rps8b-GFP. (a) Inverted GFP-fluorescence images, and (b) Cells after growth in lineage chambers. Lineages of cells expressing pPHO84-GFP in intermediate phosphate concentration (200 μM phosphate), show lineages that are expressing either high or low levels of protein, suggesting that a certain phenotypic state is maintained during at least five divisions. Smaller clusters of 2–6 cells with similar levels of Hsp12-GFP reveal that levels of this heat shock protein change on a faster timescale. Levels of the ribosomal protein, Rps8b-GFP, remain relatively constant along lineages. Cluster Index (CI) distributions for cells expressing (c) pPHO84-GFP; (d) Hsp12-GFP; (e) Rps8b-GFP. Scale, 10 μm. Adapted from [30].

and adapt on shorter timescales than required for genetic mutations [14]. In yeast, typically about one in 10^6 to 10^8 cells acquires a genetic mutation [15], so the ability to alter phenotype in non-genetic ways, and pass this information on to progeny cells, may offer an evolutionary advantage for a population of cells in a fluctuating environment. While this is an interesting idea, it has not been rigorously shown experimentally.

Box 1. Genotype and Phenotype.



Genotype can be thought of as inherited instruction, like a recipe to make a cake or assemble a model airplane. The 'instructions' encoding the composition of a cell are contained in its DNA.

Phenotype is the physical manifestation or observable characteristics of the genotype. For example, the cake made by following the recipe, the assembled airplane, or the cell.

3 Yeast: Model system

To study questions of phenotypic variation over multiple generations, yeast provides an excellent model system. Compared to stem cells, the budding yeast *Saccharomyces cerevisiae* is much simpler to culture in the laboratory. Moreover, it is a very simple eukaryotic system and cells divide about every 90 minutes, which makes it feasible to study many generations in a single experiment. Because yeast is a unicellular organism, it responds to fluctuations in its environment, and alters its protein expression accordingly. Single yeast cells also grow into colonies that consist of 10^4 to 10^6 cells. In the form of colonies, it is easy to visualize multiple generations of cells, and study factors involved in phenotypic inheritance: for example, colonies of yeast cells have been used to study inheritance by prions [16] and chemical modifications of chromatin [17]. Last but not least, yeast cells are extremely easy to genetically manipulate. Genes can be deleted, or modified so that proteins are tagged with a fluorescent protein for visualization [18]; entire libraries consisting of deleted and fluorescentally-labeled proteins are commercially available. Moreover, fluorescentally-tagged proteins enable protein levels to be monitored by fluorescence intensity.

4 Studying cell-to-cell variations in protein levels

Using fluorescence microscopy, nearly each protein of the yeast proteome can be visualized [18]; this reveals marked variation in the expression levels of particular proteins. To determine a full distribution of protein levels for thousands of cells, flow cytometry is a powerful technique: individual cells are flowed past a laser and detector, allowing interrogation of protein levels in thousands of cells within seconds. Using this method, protein level distributions for the entire yeast proteome have been acquired [19,20]. While this method provides insight into stationary distribution patterns for different proteins, the information is obtained at only a single time point. Sequential runs allow for data collection at multiple timepoints, however, it is impossible to follow individual cells in time. Yet there are many critical questions that require studying

cells over time: what is the frequency of protein level variations over multiple generations? How does expression depend on cell age, history, or pedigree? How does a population of single cells alter their protein expression in response to environmental change?

To track single cells over time, a common method is to place the cells on a gel pad containing nutrients, and then image the cells by microscopy as they grow. However, cells grow out of the focal plane, limiting the number of generations that can be imaged. Also, media cannot be flowed through such a chamber. Another way to study multiple generations of cells is to grow a single cell into a colony of cells. While this method is useful for elucidating epigenetic mechanisms of inheritance, the environment throughout the colony is extremely heterogeneous: there are gradients in the colony height, density of cells, and nutrient concentration. The colony is thus a complex environment, and it is complicated to follow single cells within a colony. To overcome these issues, various flow devices provide media exchange and enable cells to be maintained in a single focal plane during growth [21–27]. However, many of these devices require sophisticated fabrication techniques such as multilayer fabrication with valves [22,24], channel height differences [23], or membranes [21,27]. Furthermore, the initial placement of cells cannot be controlled, which limits the number of lineages that can be studied in a single experiment and reduces the statistical power of these techniques. To robustly and repeatedly trap and culture single cells requires a device that is easy to fabricate and simple to use; this would enable multiple individual cells and their lineages to be spatially organized, as well as data to be collected over many generations of cells in a single experiment.

5 Engineering a microfluidic device for trapping single cells

To follow lineages deriving from individual yeast cells, we developed a device for trapping and culturing single yeast cells [30]. The device is made using soft lithography, and is molded out of a silicon-based polymer (polydimethylsiloxane, PDMS). Using this method, computer-generated designs are inexpensively and rapidly fabricated into devices of any geometry with feature sizes down to microns [28]. The optimal device for trapping single cells, and containing their progeny cells in individual chambers, must fulfill certain geometric requirements. For example, to spatially organize the microcolonies that derive from single cells and force them to grow in a single focal plane, the growth chambers should have a square cross-section that is the width and height of an average single cell, 5 μm, with a constriction of less than 5 μm to trap individual cells. To study multiple lineages in a single experiment, the device should enable simultaneous trapping of many single cells.

To determine the optimal device geometry, we use lumped element modeling, a method that is commonly used to analyze simple electrical circuits. We consider the volumetric flow rate through the channels, Q, which is analogous to electrical current; the pressure drop, ΔP, that is analogous to the voltage drop; and the remaining factors which describe the fluidic resistance that depend largely on the channel geometry. Beside the constricted trapping chamber, we engineer a bypass channel: When the trapping channel is empty, the flow through the bypass channel, Q_2, is less than the flow through the trapping channel, Q_1, but when a single cell is present in the channel, Q_2 is greater than Q_1 so that the majority of the fluid volume, and therefore subsequent cells, flow through the bypass channel and exit the device [29]. We determine the device dimensions by using the Hagen-Poisseuille equation, which describes the relationship between the flow, Q, fluidic resistance, R, and channel geometry, where h, w, l are channel height, width, and length, for the growth chamber (1a), constriction (1b), and bypass (2) channels respectively:

$$\frac{Q_2}{Q_1} = \frac{R_{1a} + R_{1b}}{R_2} = \frac{h_2^3 w_2}{l_2} \left(\frac{l_{1a}}{h_{1a}^3 w_{1a}} + \frac{l_{1b}}{h_{1b}^3 w_{1b}} \right). \tag{1}$$

Thus when a cell flows into the growth chamber and becomes trapped, the fluidic resistance, R_{1a} increases so that subsequent cells preferentially flow around the chamber and exit the device. These flow patterns enable preferential trapping of single cells, and allow us to achieve an array with a higher proportion of single cells than can be achieved by random loading that is

described by Poisson statistics [30]. Importantly, there is still some flow through the chamber; the cells are round while the chambers are square, so we are able to perfuse fluid through the device, enabling cells to divide over the course of the experiment. By fabricating an array of 50 chambers in one device, we can trap multiple cells and monitor their lineages in a single experiment [30].

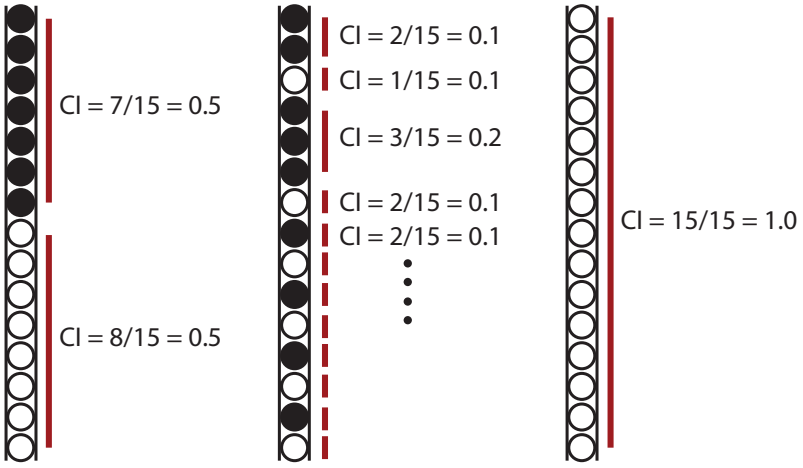
6 Fluctuations in levels of different proteins

Using this technique, we resolve variations in protein levels over time for three representative proteins. Protein **A**, a phosphate transporter (Pho84), shows bimodal distribution at intermediate phosphate concentrations. When cells are starved for phosphate, the expression of this high-affinity phosphate transporter is upregulated in order to bring more phosphate into the cell and increase the intracellular phosphate concentration. By contrast, Pho84 expression is downregulated when phosphate is plentiful, and lower-affinity phosphate transporters are expressed [31]. The bistable behavior observed at intermediate phosphate concentrations is thought to result from an interplay between positive and negative feedback loops in the phosphate-response pathway [31]; such feedback mechanisms are critical for achieving homeostasis, and are ubiquitous motifs in cellular systems to either amplify or dampen signals in biochemical pathways [32–34]. In the case of Pho84 expression, the interplay between feedback loops results in some cells which primarily express the high-affinity transporter, and others that express predominantly the low-affinity transporters [31]. However, the origins of this variation are not entirely understood. Moreover, the number of divisions over which a cell maintains a particular phenotype cannot be determined using flow cytometry. By growing cells in the lineage chamber device, we observe lines of cells that have high or low expression levels (ON or OFF phenotypic state), indicating a particular phenotype can be maintained over multiple generations. Some lineages show clusters of cells with similar phenotype due to a change in expression state as the cells grow and divide. To quantify the size of clusters, we count the number of adjacent cells that share similar phenotype, and define the cluster index, CI, as the number of adjacent cells of similar phenotype divided by the total number of cells in a lineage (Box 2). Thus, lines that are entirely ON or OFF have a cluster index, $CI = 1$ (Fig. 2(c)). We also observe smaller clusters of cells that may result when cells in a single lineage switch phenotype, and occasionally squeeze by each other as they divide within the channel.

Box 2 - Cluster Index (CI) Analysis.

Growing cells in lineage chambers enables *space* to provide a measure of *time*: cells that are most closely related genealogically are located most proximally within a channel.

$$\text{Cluster Index (CI)} = \frac{\text{Number of Adjacent Cells with Similar Intensity}}{\text{Total Number of Cells in Lineage}}$$



Next, we investigate two proteins that exhibit unimodal distributions, but have different variances. Protein **B** is a heat shock protein (Hsp12), which shows a wider distribution as compared to Protein **C**, a ribosomal protein (Rps8b). The width of the distribution is expressed quantitatively by the coefficient of variation, CV = variance/mean; for **B** (Hsp12), CV = 118, while $CV = 35$ for **C** (Rps8b). Perhaps not surprisingly, cells expressing Protein **C**, the ribosomal protein, show lineages of cells that all have similar phenotype, reflecting the tight distribution observed in bulk (Fig. 2(e)). By contrast, cells express varying levels of Protein **B**, the heat shock protein, and it cannot be determined from a stationary distribution how quickly protein levels fluctuate. To address this question, we culture lineages of cells expressing Protein **B**, the heat shock protein, and observe clusters of cells within single lineages that are bright or dark (Fig. 2(d)). Clusters of cells with similar protein levels typically span (2–6) cells. The CI distribution reveals that cells do not maintain phenotype for the entire lineage, suggesting rather that protein levels change frequently as cells grow and divide. While Protein **B** (Hsp12) has 4.5×10^3 molecules per cell [35], other stress-related proteins with higher copy number, such as an enzyme that is involved in glucose metabolism, Hxk1 with 4.8×10^4 molecules per cell, show similar clusters of bright and dark cells in lineages, as well as similar CI distributions. These observations suggest that expression variation is not linked to copy number, but rather to protein function [19,20].

To follow how protein levels change over time, we track the fluorescence intensity of individual cells as they grow, acquiring images at 10-minute intervals and analyzing them using custom software. We then construct a pedigree map, which documents the familial relations of each and every cell, as well as exactly how many times each cell has divided, or its' replicative age (Fig. 3). Plotting the fluorescence intensity levels of each cell divided by the population mean reveals fluctuations in protein levels in single cells over time. Levels of protein **C**, the ribosomal protein, change very little over time, consistent with the role of this "housekeeping" protein in essential cell functions. By contrast, levels of the heat shock protein, protein **B**, show non-periodic fluctuations, with up to 2.5 x increases relative to the population mean. These bursts in protein levels occur under steady-state conditions, in the absence of any applied stress. Each burst is followed by an exponential decay, which can be attributed to a decrease in the concentration of fluorescent protein upon cell growth and division, as well as degradation of mRNA and proteins. Correlation analysis reveals no statistically significant correlation between bursts and replicative age of the cell; instead, the bursts appear to be random. Interestingly, the majority of bursts in protein levels (57%) occur simultaneously in mother and daughter cells; the probability that these correlated bursts are due to random fluctuations is very low (χ_2, $p << 0.001$) [30]. Similar behavior is also observed for the stress-response protein, Hxk1.

What causes these shared expression patterns? Protein expression involves transcription to produce mRNA, followed by translation to generate proteins (Fig. 1). A burst in transcription may thus result in a pool of mRNA that is passed onto progeny cells as they divide, which decays on timescales comparable to cell division. Such non-periodic bursts in expression are observed in living systems including bacteria [6], during differentiation in Dictyoselium [39], and following DNA damage in mammalian cells [40]; they are also predicted from mathematical models of transcription together with stationary distributions obtained by flow cytometry [36] and microscopy [2,37,38]. In addition to mRNA production rate, mRNA stability can be altered in response to environmental stress [41]; the interplay between both transcript production and degradation rates could thus be tuned to regulate protein expression levels, as well as the timescale of phenotypic propagation. Similar expression behavior in mother-daughter pairs could also result from a particular chromatin configuration that is passed on to progeny at cell division, or fluctuations in chromatin structure between active and inactive states [2,4]. Using molecular biology tools, such as deletion mutant strains, and mathematical modeling will help to decipher the physical origins of this behavior.

Our results suggest that particular classes of proteins, such as those involved in stress response, exhibit non-periodic fluctuations in protein expression. These observations are consistent with hypotheses that cells tightly regulate the expression of housekeeping proteins with essential functions [19,20]. The mechanism underlying differences in expression patterns among proteins may be attributed to the architecture of the promoter sequence: while many stress-response genes are enriched with a specific sequence motif (TATA box), which determines the

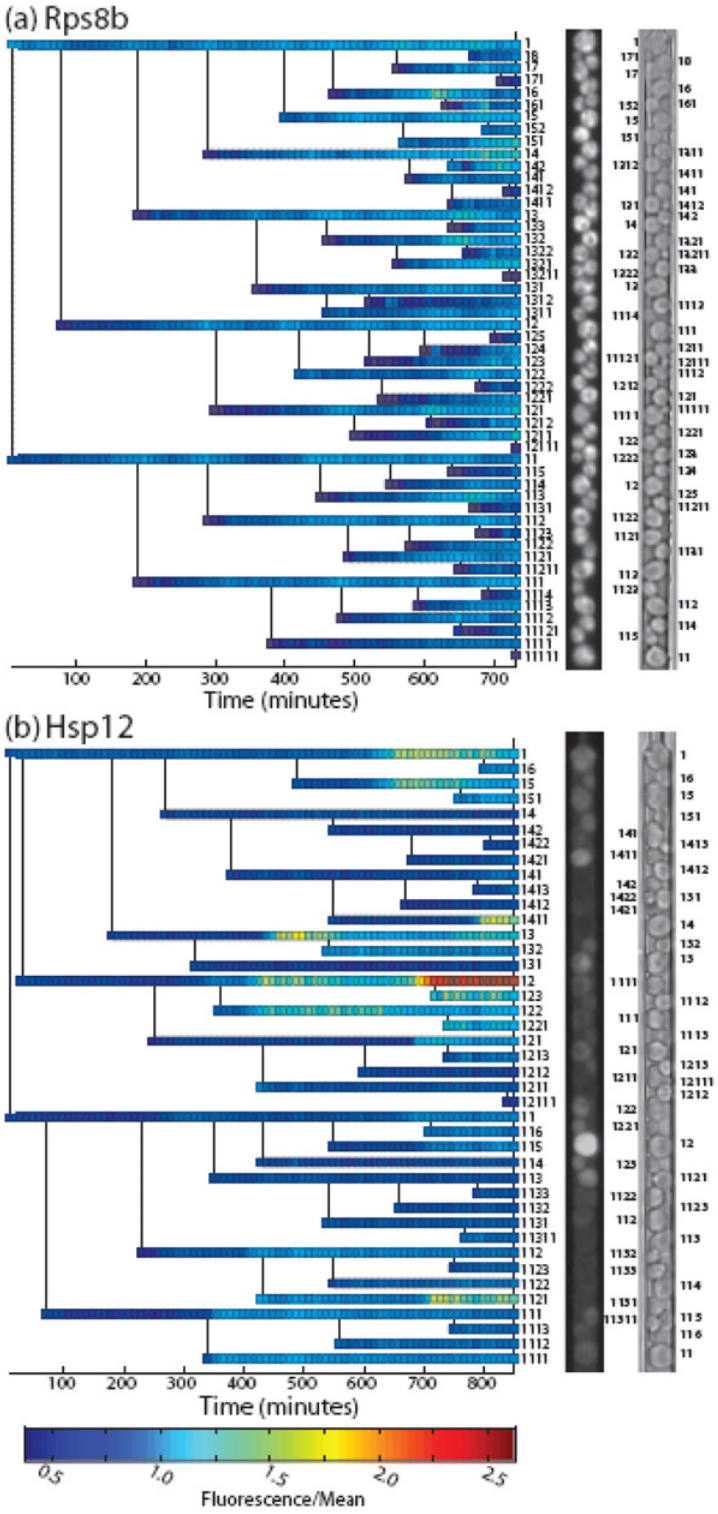


Fig. 3. Dynamic lineage maps show temporal fluctuations in protein levels in the context of pedigree. (a) Protein **C**: Rps8b-GFP; and (b) Protein **B**: Hsp12-GFP. Protein levels are normalized to the mean fluorescence of the population. The genealogical identity of each cell is labeled in the image (right) acquired at the endpoint. Reprinted with permission from [30].

stability of the transcription factor complex, this motif is absent from promoters of essential genes [42–44]. Enhanced binding between proteins and DNA could result in a larger number of transcripts produced per binding event, or faster reinitiation of transcription. Other mechanisms could also explain the observed behavior. For example, the ribosomal protein studied here, Rps8b, is a protein subunit; thus variations in its levels may be buffered by the assembly into its multimeric form. Furthermore, changes in chromatin conformation may play a role in regulating the expression of genes with stress-response function. While these are plausible explanations for the observed differences in protein level variability between stress-response and housekeeping genes, the extent to which these differences impart an evolutionary advantage remains to be seen.

7 Conclusions and perspectives

A general understanding of the timescales of phenotypic variation across multiple generations of cells, as well as the mechanisms underlying fluctuations in protein levels, requires systematic investigation of many types of proteins in varying conditions. Since fluctuations in protein levels of single cells remain masked in the ensemble average, methods to study protein levels must address a population of single cells over time. Using the microfluidic technique to trap individual cells described here, we demonstrate how lineages deriving from single cells can be studied in parallel. We show that levels of different proteins fluctuate on varying timescales. Moreover, we identify patterns in expression that extend across multiple generations, which cannot be seen when studying a population of cells at a single time point. Understanding the mechanisms by which cells can tune the timescales of protein fluctuations to generate epigenetic inheritance will provide insights into fundamental biological questions ranging from differentiation to adaptation.

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References

1. M.B. Elowitz, A.J. Levine, E.D. Siggia, P.S. Swain, Science **297**, 1183 (2002)
2. A. Raj, C.S. Peskin, D. Tranchina, D.Y. Vargas, S. Tyagi, PLoS Biol. **4**, e309 (2006)
3. A. Raj, A. van Oudenaarden, Cell **135**, 216 (2008)
4. J.M. Raser, E.K. O'Shea, Science **304**, 1811 (2004)
5. J.M. Raser, E.K. O'Shea, Science **309**, 2010 (2005)
6. L. Cai, N. Friedman, X.S. Xie, Nature **440**, 358 (2006)
7. K.E. Kasza, A.C. Rowat, J. Liu, T.E. Angelini, C.P. Brangwynne, G.H. Koenderink, D.A. Weitz, Curr. Opin Cell. Biol. **19**, 101 (2007)
8. A.P. Gasch, P.T. Spellman, C.M. Kao, O. Carmel-Harel, M.B. Eisen, G. Storz, D. Botstein, P.O. Brown, Mol. Biol. Cell. **11**, 4241 (2000)
9. E. Meshorer, T. Misteli, Nat. Rev. Mol. Cell. Biol. **7**, 540 (2006)
10. D. Vermaak, K. Ahmad, S. Henikoff, Curr. Opin. Cell. Biol. **15**, 266 (2003)
11. G. Felsenfeld, M. Groudine, Nature **421**, 448 (2003)
12. E. Jablonka, M.J. Lamb, *Evolution in Four Dimensions: Genetic, Epigenetic, Behavioral, and Symbolic Variation in the History of Life* (MIT Press, Cambridge, USA, 2005)
13. O.J. Rando, K.J. Verstrepen, Cell **128**, 655 (2007)
14. M. Lachmann, E. Jablonka, J. Theor. Biol. **181**, 1 (1996)
15. G.I. Lang, A.W. Murray, Genetics **178**, 67 (2008)
16. L. Li, S. Lindquist, Science **287**, 661 (2000)
17. C.M. Smith, Z.W. Haimberger, C.O. Johnson, A.J. Wolf, P.R. Gafken, Z. Zhang, M.R. Parthun, D.E. Gottschling, Proc. Natl. Acad. Sci. USA **99**, 16454 (2002)
18. W.K. Huh, J.V. Falvo, L.C. Gerke, A.S. Carroll, R.W. Howson, J.S. Weissman, E.K. O'Shea, Nature **425**, 686 (2003)

19. J.R. Newman, S. Ghaemmaghami, J. Ihmels, D.K. Breslow, M. Noble, J.L. DeRisi, J.S. Weissman, Nature **441**, 840 (2006)
20. A. Bar-Even, J. Paulsson, N. Maheshri, M. Carmi, E. O'Shea, Y. Pilpel, N. Barkai, Nat. Genet. **38**, 636 (2006)
21. N.Q. Balaban, J. Merrin, R. Chait, L. Kowalik, S. Leibler, Science **305**, 1622 (2004)
22. A. Groisman, C. Lobo, H. Cho, J.K. Campbell, Y.S. Dufour, A.M. Stevens, A. Levchenko, Nat. Meth. **2**, 685 (2005)
23. S. Cookson, N. Ostroff, W.L. Pang, D. Volfson, J. Hasty, Mol. Syst. Biol. **1**, 0024 (2005)
24. F.K. Balagadde, L. You, C.L. Hansen, F.H. Arnold, S.R. Quake, Science **309**, 137 (2005)
25. S. Paliwal, P.A. Iglesias, K. Campbell, Z. Hilioti, A. Groisman, A. Levchenko, Nature **446**, 46 (2007)
26. P.J. Lee, N.C. Helman, W.A. Lim, P.J. Hung, Biotechniques **44**, 91 (2008)
27. G. Charvin, F.R. Cross, E.D. Siggia, PLoS ONE **3**, e1468 (2008)
28. S.K. Sia, G.M. Whitesides, Electrophoresis **24**, 3563 (2003)
29. R.T. Yen, Y.C. Fung, Am. J. Physiol. **235**, H251 (1978)
30. A.C. Rowat, J. Bird, J.J. Agresti, O.J. Rando, D.A. Weitz, Proc. Natl. Acad. Sci. USA **106**, 18149 (2009)
31. D.D. Wykoff, A.H. Rizvi, J.M. Raser, B. Margolin, E.K. O'Shea, Mol. Cell. **27**, 1005 (2007)
32. O. Brandman, T. Meyer, Science **322**, 390 (2008)
33. J.E. Ferrell Jr., Curr. Opin. Cell. Biol. **14**, 140 (2002)
34. A. Novick, M. Weiner, Proc. Natl. Acad. Sci. **43**, 553 (1957)
35. S. Ghaemmaghami, W.K. Huh, K. Bower, R.W. Howson, A. Belle, N. Dephoure, E.K. O'Shea, J.S. Weissman, Nature **425**, 737 (2003)
36. N. Friedman, L. Cai, X.S. Xie, Phys. Rev. Lett. **97**, 168302 (2006)
37. S. Newlands, L.K. Levitt, C.S. Robinson, A.B. Karpf, V.R. Hodgson, R.P. Wade, E.C. Hardeman, Genes Dev. **12**, 2748 (1998)
38. D. Zenklusen, D.R. Larson, R.H. Singer, Nat. Struct. Mol. Biol. **15**, 1263 (2008)
39. J.R. Chubb, T. Trcek, S.M. Shenoy, R.H. Singer, Curr. Biol. **16**, 1018 (2006)
40. G. Lahav, N. Rosenfeld, A. Sigal, N. Geva-Zatorsky, A.J. Levine, M.B. Elowitz, U. Alon, Nat. Genet. **36**, 147 (2004)
41. O. Shalem, O. Dahan, M. Levo, M.R. Martinez, I. Furman, E. Segal, Y. Pilpel, Mol. Syst. Biol. **4**, 1 (2008)
42. W.J. Blake, K.A.M.C.R. Cantor, J.J. Collins, Nature **422**, 633 (2003)
43. J.J. Stewart, L.A. Stargell, J. Biol. Chem. **276**, 30078 (2001)
44. I. Tirosh, A. Weinberger, M. Carmi, N. Barkai, Nat. Genet. **38**, 830 (2006)