Can specific tuning of single-gene expression be deduced from a local measurement of that gene alone?

Comment on the paper by Freddolino et al.: "Stochastic tuning of gene expression enables cellular adaptation in the absence of pre-existing regulatory circuitry"

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Abstract

The living cell is a complex system in which metabolism, gene expression and regulation, protein and other molecular interactions are interconnected, leading to strong cross-talks between different parts of the genome. Modularity and specificity are sometimes found in cellular responses to external cues such as nutrient change or a common stress response. However, under more general stressful conditions with no pre-existing regulatory program, ad-hoc solutions must be invoked, which likely involve multiple components of the system. Experiments have shown that indeed cells exhibit a remarkable ability to overcome such unforeseen challenges without any pre-defined program (1, 2). While the underlying mechanisms are not well understood yet, the phenomenon can be quantitatively characterized in terms of growth patterns (3) gene expression (4-6) and more. Such measurements have demonstrated that large portions of the genome are involved in the response to the challenge, with coherence across the genome and with a stochastic non-repeatable nature. Many features of this global response remain to be studied.

A recent paper by Freddolino *et al*. (7), proposes that under similar stressful conditions improved fitness is achieved by the stochastic tuning of an individual gene. Their experiments follow precisely our methodology developed to understand the adaptation of cells to an unforeseen challenge (1). However, in contrast to our experimental approach which consists of global measurements of the genome over extended time scales, they focused on local measurements of a single gene of interest and one other control gene, at two time points. The results are used to deduce an underlying mechanism for cellular adaptation, proposed to rely on the stochastic tuning of a single gene which in turn determines cellular fitness.

The question whether the expression of a gene reflects its own tuning, or is a part of a more global response, cannot be answered by local measurements on the gene of interest alone. This is a general statement on experimental methodology that follows simply because anything that is measured locally is consistent with both local and global effects. We therefore argue that as a necessary stage, prior to interpretation of mechanisms, the phenomenon needs to be characterized on a global scale to determine its extent across the genome and its characteristic timescales. In this case, the conclusion drawn from local measurements by Freddolino *et al*. is found to contradict previously published global data.

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Introduction

The living cell is a complex system in which many of the underlying processes are dynamically interconnected. In particular, gene regulation is strongly interconnected with metabolism, protein interactions and other processes in the cell. This implies that, in general, the expression of any gene is in not isolated from other genes; they are coupled through metabolic and other processes which integrate the activity of different parts of the genome. Understanding the intricate relations across the genome and their integration into a metabolic function is still not well understood and constitutes a major challenge of biological research.

While it is true that under some conditions, specifically under known external conditions shaped in evolution, certain genes react in a modular way and their response can be somewhat isolated from the rest of the genome, this may not represent a common behavior. Adaptation of a cell to a stressful challenge, e.g. to a novel and unforeseen challenge for which there is no pre-defined regulatory program, represents a generic situation where the complex nature of the cell will be reflected in its response (1). As a result, one cannot *a-priori* assume that genes respond in a local way disconnected from the response of other parts of the genome.

The question whether a particular gene reacts in isolation, or alternatively its reaction is part of a more global response, can only be answered experimentally by a global observation of the system over extended time scales. This is simply because a local measurement by itself, without a global view, might be consistent with both types of reactions, local or global; in contrast, a global view will distinguish between these two possibilities.

Beyond the phenomenological description of a cellular response, stands the question of biological function (e.g. cell growth and division) and how it relates to individual genes. We argue that, in general, one may not assume *a-priori* which component is relevant for the functioning of the cell and conduct experiments that zoom only on that single component. Yet, in many cases the complexity of the cell is ignored by the motivation to search for a simplistic explanation for complex phenomena.

The paper "Stochastic tuning of gene expression enables cellular adaptation in the absence of pre-existing regulatory circuitry" by Freddolino *et al*. (7) takes exactly this simplistic approach. It describes the idea that “hard-wired” regulatory pathways cannot account for the adaptation of cells to environments never encountered before. This idea is not new and indeed was proposed and studied by us as the basis of the ability of cells to adapt to unforeseen challenges and evolve (1). The authors propose a specific mode of gene regulation that might be in operation under such challenges: individual genes achieve optimal expression through a ‘stochastic tuning’ that leads to improved fitness. The paper describes computer simulations demonstrating this idea, followed by a set of experiments to support it. Yeast cells were engineered to have the metabolic gene *URA3* detached from its native regulation and placed under different foreign promoters. The adaptation of cells is manifested in their ability to grow on uracil-deficient medium in which *URA3* is essential. Based on analyzing the expression levels of the rewired *URA3* gene at two time points (alone or in comparison to another non-essential gene), and on genomic analysis around the rewired locus, the authors conclude that the observed adaptation is carried out by the suggested mechanism of stochastic tuning demonstrated in the simulations.

The purpose of this comment is to argue that the proposed mechanism of stochastic tuning of individual genes cannot explain the observed adaptation as it contradicts a host of previous results from experiments on the same phenomenon. First, from the general considerations mentioned above, measuring only the response of one metabolic gene (even if this gene is central to the specific metabolic process) while not testing the rest of the system, as done by Freddolino *et al*., does not provide enough support to the claim for a specific tuning. More importantly, as explained below, previous experiments on yeast cells utilizing a similar type of challenge and using precisely the same methodology, showed that adaptation of the cells to the challenge involved large parts of the genome and an intricate complex interplay of metabolism and gene regulation. These response also have a 'stochastic' element to them, but the data manifestly contradicts the mechanism of specific tuning of a gene as described in Freddolino *et al*. Unfortunately these data and their contradiction with the hypothesized mechanism were altogether ignored by the new article.

Adaptation of yeast cells to unforeseen challenge: experimental methodology and growth characteristics

The idea that cells can adapt to an unforeseen challenge, never before encountered in their history, is not new. Starting in 2006, our lab has been developing the concepts and experimental methodology for studying it (1). Our approach involved rewiring an essential metabolic gene in yeast cells, detaching it from its native regulatory system and placing it under a foreign promoter. Growing these cells in an environment where expression of the rewired gene is essential, while its natural regulation is compromised, are the basic ingredients of this paradigm (2). Our first experiments were focused on the *HIS3* gene, an essential metabolic enzyme in an environment lacking histidine, rewired to a promoter of the GAL system which is repressed in a glucose environment. Further pressure was applied in some of the experiments by supplying the medium with the HIS3p inhibitor, *3AT*. Similar experiments were repeated by rewiring *HIS3* to various cell-cycle promoters, with essentially similar results (4). Despite the severe regulatory challenge, yeast cells adapted and established stable growth, in a process that was consequently studied by us in the following decade (reviewed in (1)). These experiments had already established some generality to the phenomenon of adaptation to an unforeseen challenge in rewired yeast cells, with quantitative details depending on the promoter to which the essential gene is rewired.

The work of Freddolino *et al*. (7) follows this paradigm step-by-step, with two differences: rewiring of *URA3* instead of *HIS3* (and using its corresponding inhibitor *6AU* instead of *3AT*), and rewiring to several different natural and semi-synthetic promoters. They report the same phenomenology of cellular adaptation as our previous work, suggesting its robustness also to these differences.

More specifically, all typical growth patterns of yeast cells adapting to the regulatory challenge on plates, reported in Freddolino *et al*., reproduce our previous results: (1) A significant delay (~100 hours) of growth on plates following exposure to the challenging environment (compare ref (2), Fig. 1 and ref (4) Fig. 1 to Freddolino *et al*. (7), Fig. 3.). (2) A temporary arrest of growth at variable colony size (between few to hundreds of cells), and for variable arrest times. This results in a broad distribution of adaptation times leading to colony growth, and a broad distribution of colony sizes at a given time (compare ref (2), Fig. 1 and ref (8), Fig.1 to Freddolino *et al*. (7) Fig. 3). (3) Appearance of multiple growth centers in parallel in growth-arrested colonies (compare ref (8), Fig. 1 and Freddolino *et al*. (7) Fig. 3). (4) Eventually a fraction of yeast cells resume normal growth and division in the face of the unforeseen challenge, within a relatively short timescale (compared to evolutionary timescales). The exact fraction and adaptation time depends on promoter and level of inhibitor (0.5-0.8 in our experiments with the GAL

promoter (2, 9), and a broader range with cell-cycle promoters, including the case of no observed adaptation after 25 days (4); 10^{-3}-0.1 in Freddolino *et al*. (7)). (5) The adapted state was stably inherited across generations (2, 4, 7, 9). This is a set of unusual growth characteristics, which we view as the hallmarks of yeast cells adapting to unforeseen regulatory challenge (see Fig. 1 in this comment).

In their paper, Freddolino *et al*. note that the adaptation phenomenon they observe is common to a range of different synthetic and natural promoters, with different quantitative features (e.g. fraction of adapting cells). Taken together with our previous results, it appears that the experimental results of Freddolino *et al* (7) belongs to the same class of adaptation of yeast cells to regulatory challenge that has been studied by us for over a decade, from different angles and by different growth techniques (chemostat, batch culture and plates; see (1)). Indeed our previous results had shown that such adaptation is generally characterized by a high level of variability, multiple possible routes to adaptation, and non-repeatability of details (see the review (1)).

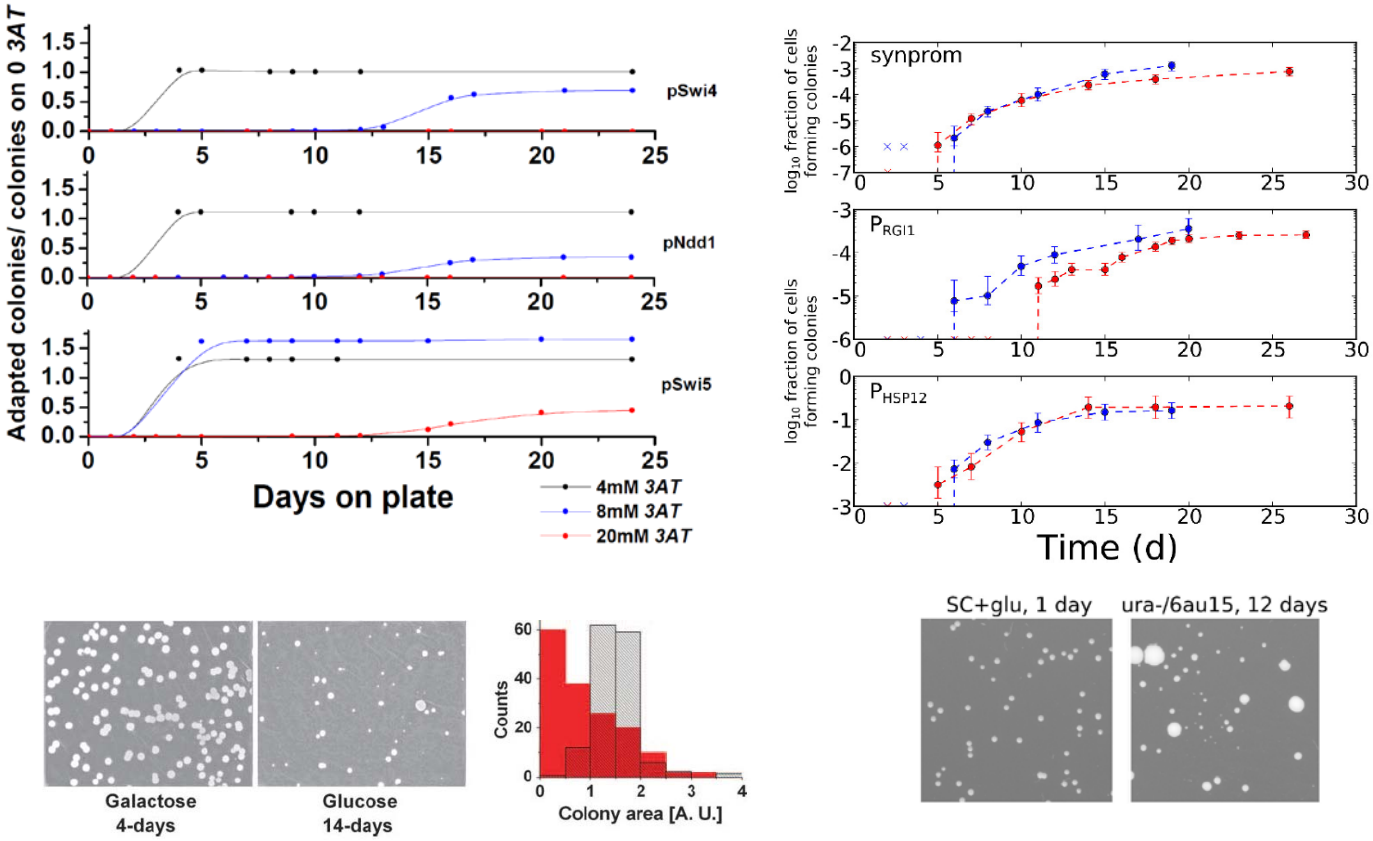

Fig. 1: Identical growth patterns in yeast cells adapting to regulatory challenge following gene recruitment protocol. **Top**: growth of rewired cells on plates shows extended incubation of several days before adapted colonies appear. (left: from ref (4) ; right: Freddolino et al. 2018). **Bottom**: Colonies of adapting cells appear at highly variable times, resulting in a broad distribution of colony sizes (left: from ref (2); right: Freddolino et al., 2018).

Measuring gene expression in the adaptation dynamics: global versus local response

We next discuss the experimental characterization of gene expression in the adaptation dynamics described above. In their Fig. 4, Freddolino *et al*. (7) show the results of flow-cytometry measurements, comparing the expression levels of the rewired *URA3*-mRuby to another marker attached to a non-essential gene on a sister chromatin, *DHFR*-GFP. The measurements are taken at two time points, before and after the transition into the growth phase. The differential increase of *URA3* in coordination with the time at which cells resume growth, is taken as support for their proposed mechanism of individual stochastic tuning as driving adaptation.

However, extensive previous measurements of genome-wide expression at multiple time-points during adaptation reveal a completely different picture which is incompatible with this interpretation. DNA micro-arrays show that hundreds of genes exhibit a strong and coherent response, with dynamics coordinated with the adaptation process ((4, 5)). Moreover, real-time PCR measurements of tens of genes involved in metabolism and regulation at high temporal resolution, show the temporal complexity of the gene expression response over extended timescales during adaptation (6). Thus, adaptation involves global gene expression dynamics, including also the rewired gene *HIS3*, **showing similar and coordinated response with numerous other genes**. This response cannot be deduced from measurement of one or two genes at two time points. Since the rewired gene responds in coordination with numerous other genes, residing on different parts of the genome, the isolated measurement of its response can lead to the misleading interpretation that its expression is specifically tuned toward functional needs of the cell to overcome the challenge.

By constructing a flow-cytometer online with the chemostat, we have further characterized the *HIS3*-GFP expression dynamics (the rewired gene which is challenged) in real time, at high temporal resolution over extended periods throughout the adaptation process (3). These measurements revealed that an increase in expression at the onset of population adaptation, is actually followed by non-monotonic dynamics, converging to steady state only after a long period of ~100 generations (see Fig. 2 in (3)). The expression response of the rewired gene was thus found to be an integral part of the global response (6). Moreover, it also shows that measuring the response at only two time points is not enough; an extended-time measurements are needed to separate transient and long term responses toward adaptation.

Taken together, these analyses show that the hypothesis of stochastic tuning – which Freddolino *et al*. base on a limited set of gene expression measurements – stands in contradiction to more extensive data in term of number of genes and timespan. The simultaneous complex dynamics of numerous genes across the genome, which are in coordination with that of the rewired gene and in correlation with the adaptation dynamics, show that specific tuning the expression of the rewired gene by itself is not the mechanism supporting adaptation. Measuring the response of the rewired gene by itself in comparison to a non-functional gene at the same locus on the sister chromatin is simply not enough of a support to deduce a local response. Note that this conclusion is completely detached from the question of the true underlying mechanism. We still do not understand the complex behavior of the living cell to a degree that allows detailed interpretation of the global response; this is a topic of ongoing investigation (10). However, irrespective of the interpretation of the global response, the fact that numerous genes show coordinated dynamics with the rewired gene (the gene of interest for the metabolic challenge) means that specific tuning of this particular gene cannot serve as an explanation for the cell response.

The role of genetic mutations in the adaptation process

Finally, we remark on the role of genetic mutations in the adaptation of rewired yeast cells to an unforeseen regulatory challenge. Freddolino *et al*.(7) first claim that, if genetic mutations underlie adaptation, the high expression of *URA3* should remain high even when switching to a ura+ medium. However, the level of gene expression by itself cannot be a test for the presence of mutation; it is widely accepted that any given genotype can give rise to multiple patterns of gene expression. Second, they performed whole-genome sequencing, but scanned only the region within 25-kb of the rewired gene. In this region, they found variability between replicate adapting lineages, where some are completely free of mutations while others show a variety of mutations that cannot explain a fitness increase. They conclude that mutations can arise during the adaptation process but cannot be the cause of the process. These results are consistent with our extensive genetic study of the adapting populations (8, 11), demonstrating explicitly that adaptation can proceed ***without the involvement of mutations at all***; that when mutations do arise, they can arise in different parts of the genome, not necessarily in cis; that they are insufficient to induce adaptation so other mechanisms must be involved; and that they emerge very late during the growth of the colony, further demonstrating that at least in some cases these mutations are induced by the process rather than causing it (8). Importantly, a genome-wide sequencing analysis of the entire genome in complete linages, clearly proves that there is ***no hypermutation*** involved in the adaptation process (8).

Freddolino *et al*. were aware of our work when writing their paper: in their discussion, they state that our experiments are irrelevant to their case because "… genetic mutations are the primary mechanism of adaptation, possibly driven by hypermutability of the genes involved in the response of interest…". This statement is clearly false and misrepresents our findings in different experiments as presented above. This claim certainly cannot be used to avoid true discussion of our contradictory data to their interpretation, of very similar experimental results utilizing exactly the same methodology. We hope this comment will encourage a thorough and honest discussion of the points raised in it and will benefit the community, as they touch deep issues related to our quest for understanding the living cell.

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