Functional Requirements of Cellular Differentiation: Lessons from *Bacillus subtilis*

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Abstract

Successful execution of differentiation programs requires cells to assess multitudes of internal and external cues and respond with appropriate gene expression programs. Here, we review how *Bacillus subtilis* sporulation network deals with these tasks focusing on the lessons generalizable to other systems. With feedforward loops controlling both production and activation of downstream transcriptional regulators, cells achieve ultrasensitive threshold-like responses. The arrangement of sporulation network genes on the chromosome and transcriptional feedback loops allow coordination of sporulation decision with DNA-replication. Furthermore, to assess the starvation conditions without sensing specific metabolites, cells respond to changes in their growth rates with increased activity of sporulation master regulator. These design features of the network enable cells to robustly decide between vegetative growth and sporulation.

Introduction

Cellular differentiation is a generic term for the processes by which cells specialize phenotypically and morphologically to adapt to their environmental conditions or to perform specific functions [1]. This ability to generate specialized cell types from multipotent precursor cells is certainly central to the development of multi-cellular eukaryotes [1-3] but it is also ubiquitous in single cell organisms [4-7]. Over decades of prior research, it has become evident that cell differentiation primarily results from specialization of the gene expression repertoire [8-11]. This specialization in turn is controlled by Gene Regulatory Networks (GRNs) that sense changes in the cellular environment, detect and process differentiation and stress related signals and execute the appropriate gene expression response with a combination of transcriptional and post-translational controls [3,12]. Therefore to mechanistically understand cell differentiation programs we have to study the structure and function of the underlying GRNs.

Sporulation in *Bacillus subtilis* has long been used as a model for prokaryotic differentiation. This differentiation program enables *B. subtilis* cells to produce metabolically inert and stress-resistant spores capable of surviving long periods of starvation (Fig. 1A). From decades of molecular genetic studies, a large and complex network controlling sporulation has been uncovered [5,13,14](Fig. 1B). However, as with most GRNs controlling cellular differentiation (hereafter, differentiation GRNs), from a cursory glance the sporulation network appears an inscrutable mess of interconnected components impeding mechanistic analysis. However, more recent and careful examinations of the network structure show that it is in fact hierarchically organized into functionally distinct modular sub-networks [13,15]. At the top of the hierarchy is the phosphorelay - a four component version of the typical bacterial two component system [16]. The phosphorelay comprises histidine kinases (KinA-E), which can auto-phosphorylate in response to environmental signals. These kinases then transfer their phosphoryl groups to the sporulation master regulator Spo0A via phosphotransferases Spo0F and Spo0B. Increase in phosphorylated Spo0A (an active form, hereafter Spo0A~P) activates transcription of a multitude of genes, including those encoding two alternative sigma factors σ^F and σ^E , and promotes asymmetric division [17]. Subsequently, σ^F and σ^E are activated post-translationally in a compartment-specific manner in the forespore and the mother cell respectively (Fig. 1A). Following this break of symmetry, the two compartments initiate their separate gene expression programs eventually resulting in spore formation [14,17,18].

This type of modular organization is not unique to sporulation and is, in fact, a shared structural pattern across a wide range of biological networks. Many differentiation GRNs similarly contain at least three modules (Fig. 1B): (i) A signal-sensing/encoding module (such as the phosphorelay) that senses all the relevant intracellular and extracellular cues for the specific differentiation program and integrates all the information into a level and activity of the master regulator; (ii) One or a few differentiation master regulators (such as Spo0A in *B. subtilis*) that orchestrate the network response, i.e. encode all the relevant signals and dictate the cell-fate decision; and (iii) signal decoding/execution modules (such as σ^F and σ^E activation networks) that operate downstream of master regulators and convert the activity level of the master regulator into a go-no-go binary decision whether to activate differentiation-specific genes.

Figure 1. Ultrasensitive decision making ability of the sporulation network. **A.** Schematic overview of the cell-fate decision between vegetative division and sporulation made by *B. subtilis* cells during starvation. **B.** Diagram of the sporulation network that senses starvation and controls the cell-fate decision. **C.** Modeling predictions for the responses of σ^F and σ^E activation. Solid lines and shaded areas represent the means and standard deviations of the response calculated from stochastic simulations. **D.** Single cell fluorescence measurements of the responses of the σ^F and σ^E activation modules as a function of Spo0A activity in wildtype cells. A triple reporter strain was used to measure Spo0A and both sigma factors activities simultaneously in wildtype cells grown in starvation media. Cells were binned based on CFP fluorescence for Spo0A activity (PspollA-cfp), and the fractions of cells in each bin that are σ^F -active and σ^E -active (based on appropriate PspollQ-mCherry and PspollD-yfp fluorescence thresholds) are shown in the upper and lower panels respectively. The solid lines represent Hill equation fits. These fits indicate that, as predicted by modeling results, fractions of cells displaying both σ^F and σ^E activities increase ultrasensitively at different Spo0A activity thresholds.

The similarity in structure of different GRNs is unsurprising, since these regulatory networks are the result of evolutionary selective pressures to successfully execute their respective differentiation programs, which often share functional requirements [2,19,20]. This structure-function-relationship view suggests that if we parse all the regulatory tasks of a particular differentiation GRN, we should be able to understand the structural design of that enables these tasks. Moreover the similarity of network structures implies that uncovering structure-function relationships for one particular differentiation GRN can yield general *design principles* – rules relating functional requirements of network with its designs. Such principles can enable our understanding of a GRN structures in the wider context [19,21]. In this review, using the *B. subtilis* sporulation network, we demonstrate how a combination of mathematical modeling and quantitative experiments enables understanding of design principles for differentiation GRNs.

Design principles of *Bacillus subtilis* sporulation network

Switch-like gene expression control

Cell differentiation normally requires the activation of a one set of genes and the down-regulation of another [8,11]. Thus the key requirement for a cellular differentiation GRN is the ability to dramatically shift gene expression profiles within a cell. Many studies of GRNs focus specifically on elucidating the mechanisms of this switch-like behavior [22]. Two types of GRN responses are known to achieve this switch-like behavior: ultrasensitive (i.e. highly nonlinear) and bistable (i.e. toggle-switch-like) responses

[22,23] (Box 1). Both ultrasensitive and bistable systems convert continuous signals into all-or-none responses based on a signal threshold [24-26]. However, unique dynamical properties of each mechanism can explain their selection for a particular differentiation program (Box 1).

Ability of differentiation GRNs such as the sporulation network to achieve switch-like behavior is usually easily demonstrated from the distinctly bimodal pattern of differentiation-specific genes downstream of the master regulator (e.g., σ^F -controlled genes *spoIIQ* or σ^E -controlled *spoIID* [29] σ^G -controlled *sspB* in sporulation). Moreover engineered strains, in which the master regulator activity is artificially controlled, can determine if a threshold activity is essential for differentiation commitment. Indeed, a threshold amount of Spo0A~P is required for sporulation [31,32]. But which thresholding mechanism - ultrasensitivity or bistability - is the basis for the all-or-none pattern of sporulation activation? Furthermore it is unclear whether this pattern originates at the level of the master regulator activity (e.g., Spo0A~P) or those in the downstream signal-decoding modules (e.g., σ^F or σ^E activation).

For sporulation, several earlier studies hypothesized that the positive feedback loops in the phosphorelay network (Fig. 1B) make it into a bistable switch resulting in bimodal patterns of Spo0A activity [33]. This would explain the observed all-or-none sporulation commitment response. However subsequent single-cell measurements ruled out this hypothesis by demonstrating broad and unimodal distributions of Spo0A activity with significant overlap between sporulating and non-sporulating cells [34]. Mathematical modeling suggests that the phosphorelay operating as bistable switch would be very slow to activate potentially hindering successful sporulation [29]. This result offers a possible reason why bistable design wasn't evolutionarily selected in the case of sporulation.

The exclusion of Spo0A~P bistability led into further inquiry whether activation of the σ^F and σ^E modules can explain the robust threshold of Spo0A activity and the all-or-none expression of downstream sporulation genes [29]. Mathematical modeling of activation of σ^F and σ^E modules showed that each of these modules respond ultrasensitively to changes in Spo0A~P [29,35]. In the case of σ^F , the formation of a dead-end complex between the SpoIIAB (anti- σ^F factor) and SpoIIAA (anti-anti- σ^F factor) makes the ultrasensitive post-translational activation of σ^F as a function of the level of Spo0A~P-regulated phosphatase SpoIIE. For σ^E activation, the competition between Spo0A~P-activated production of the unstable active form of σ^E and its rapid but saturable proteolytic degradation leads to an ultrasensitive response via a zero-order mechanism. Furthermore, σ^F is controlled directly and indirectly by Spo0A~P, forming a feedforward loop with one branch controlling σ^F protein level and another one controlling its activation (Fig. 1B). Similarly, activation of σ^E is also controlled by Spo0A~P both directly and indirectly (through σ^F), forming another feed-forward loop. An integrated model of σ^F and σ^E activation predicted that the cascaded structure of these feed-forward loops combines the ultrasensitivity of the individual modules, resulting in a further increase in the sensitivity of sigma-factors activity to Spo0A~P level (Fig. 1C). This prediction was confirmed by investigating single-cell activity of Spo0A, σ^F , and σ^E with a 3 distinct fluorescent reporters. With this strain, it was shown that, in single cells, the probability of activating downstream sigma factors increases ultrasensitively as a function of Spo0A activity (Fig. 1D). These experiments also confirmed another model prediction - that the threshold of Spo0A activity required for σ^F activation is less than that required for σ^E activation and consequently that σ^E activation is the point of sporulation commitment (Fig. 1C). Indeed, experiments with both wild-type and engineered strains showed that the fraction of cells activating σ^F exceeds the fraction activating σ^E and sporulating (Fig. 1D). Thus, σ^F activation does not ensure completion of the sporulation program and therefore σ^E activation is the decision point in the sporulation network.

When cell-fate decisions are made in decoding modules downstream of the master regulator, it allows the master regulator itself to be regulated more dynamically. This design enables master regulators like Spo0A~P to encode more information about differentiation signals and the cellular environment than 1 bit maximally possible value for switch-like steady-state responses. As described below, investigations of the dynamics turn out to be critical for the sporulation network.

Dynamic cell-cycle coupling

Given that DNA replication and cell division are often essential for generation of different cell-types during differentiation, the underlying GRNs need to be coordinated with the cell cycle [2,36-39]. In some model systems, especially eukaryotes, the regulatory connections between cell-cycle and differentiation networks are well established [38-40]. Nevertheless, the detailed mechanisms of coupling between these are still incompletely understood. The problem arises because the cell-cycle by definition is a highly dynamic process. Accordingly, coordination with the cell-cycle requires the regulatory control to also be time-dependent. As a result, the standard approaches of understanding GRN function that focus on static diagrams of network structure fail to capture the underlying regulatory logic.

For sporulating *B. subtilis*, coordination of sporulation commitment with DNA replication is critically important [41]. Successful sporulation requires each compartment formed after asymmetric septation to quickly acquire its own chromosome in order to initiate the compartment specific sporulation gene expression programs [17]. Consequently, asymmetric cell division should only occur once DNA replication is complete (Fig. 2A). Since asymmetric septation is controlled by Spo0A~P [42], this requirement can only be met by temporally coordinating Spo0A activation with DNA replication cycle. In line with this requirement, single-cell measurements of Spo0A activity revealed that it is indeed under dynamic control. In fact, recent experiments showed that during every cell cycle in starvation, Spo0A exhibits a single pulse of activity [43,44]. These Spo0A activity pulses always follow the completion of DNA replication [43-45]. Therefore, the sporulation network achieves coordination of Spo0A activation (and Spo0A~P dependent septation) with completion DNA replication by using pulsatile activation.

The key to explaining Spo0A~P pulsing and its cell-cycle coordination turns out to be a seemingly unrelated feature of the phosphorelay network. Mathematical model of the phosphorelay predicted that the inhibition of KinA autophosphorylation in the presence of excess phosphotransferase Spo0F – a feature of the phosphorelay that has been observed both *in vitro* [46] and *in vivo* [34,44] – plays a critical role in regulating Spo0A~P dynamics [44]. First of all, since inhibition of KinA by Spo0F requires stoichiometric binding between the two, the phosphorylation flux can be very sensitive to the relative concentration of KinA and Spo0F. Second, the transcriptional activation of *spo0F* by Spo0A~P together with the substrate inhibition results in a delayed negative feedback loop (Fig. 2B). Such network motifs are known to respond with pulsatile responses/overshoots [47,48]. Third, the *spo0F* gene is located near the origin of chromosome replication whereas *kinA* gene is near the terminus leading to transient imbalance in their production during replication. Taken together, the model predicted that KinA:Spo0F imbalance during replication would inhibit Spo0A activation. Following replication Spo0A~P increases and due to the delayed negative feedback loop overshoots its steady state thereby producing a pulse (Fig. 2C). In other words – two design features of the network are required for pulsing – gene dosage imbalance and transcriptional feedback loop.

Figure 2. Cell-cycle coupling of the sporulation network. **A.** Spo0A~P activation has to be coordinated with the completion of DNA replication for successful sporulation: activation of Spo0A~P before completion of DNA replication leads to failed sporulation or cell death. **B.** Simplified view of the negative feedback architecture of the sporulation network. **C.** Mechanism of DNA replication coupled Spo0A~P pulsing. Transient decrease in *kinA/spo0F* gene copy-number-ratio during DNA replication represses Spo0A~P level. After the ratio returns to 1 at the end of DNA replication, negative feedback in the phosphorelay produces an overshoot pulse of Spo0A~P. **D-F.** Single cell time-lapse microscopy measurements of Spo0A activity in wildtype and two mutant strains. Trans-0F^{gltA} (*spo0F* translocated *gltA* locus at DNA-replication proximal region – no transient changes in *kinA/spo0F* ratio) and i0F^{gltA} (*spo0F* expressed at *gltA* locus from IPTG regulated *P_{hsp}* promoter – no negative feedback).

To test these predictions, single-cell measurements of Spo0A activity were performed in the wild-type strain as well as engineered strains in which the transcriptional feedback was eliminated by transcribing *spo0F* from inducible promoter and/or gene dosage imbalance was perturbed by translocation of either *spo0F* or *kinA* gene to a different locus (Fig. 2D-F). The results confirmed all model predictions – Spo0A~P pulsing, accumulation and consequently sporulation were eliminated in the strain in which *spo0F* was expressed from its native promoter at a terminus-proximal locus (Fig. 2E). However, if *spo0F* was expressed from the inducible promoter at low levels, starving cells show non-pulsatile increase in Spo0A activity and can eventually reach Spo0A-threshold levels and sporulate (Fig. 2F). These observations raised an interesting question about the physiological role of pulses given that they are not absolutely required for sporulation. Careful analysis of single-cell phenotypes in time-lapse movies demonstrated that the engineered non-pulsatile strain showed a three-fold increase in sporulation defects. All the defects can be associated with attempts to commit to sporulation before completion of DNA-replication [44].

The major implication of these results is that GRNs can be implicitly coupled to cell cycle and DNA replication by proper chromosomal arrangement of network genes. This design feature can be exploited by differentiation GRNs when coupling is physiologically necessary. However, it can also unnecessarily entrain differentiation programs to chromosome duplication thereby making it essential for GRNs to include appropriate buffering mechanisms [49,50]. Notably, a different phosphorelay kinase, KinC can in certain genetic backgrounds bypass Spo0F [51] so activation of Spo0A by KinC may not be pulsatile. Given that this kinase that is active under conditions that do not produce spores, the same phosphorelay may be able to produce distinct dynamical responses activating alternative cell-fates. Such dynamic flexibility in the regulation of the master regulator may be a recurring feature for GRNs that control multiple differentiation programs. It should also be noted that many differentiation GRNs, including sporulation, are not only subject to regulation by the cell-cycle but also control the cell-cycle themselves [52]. Whether this type of mutual regulation enhances the robustness of coordination may be an interesting avenue for further studies.

Signal integration and sensitivity to cell growth

The traditional paradigm for understanding cell-fate decisions in cellular differentiation assumes that cells evaluate a signaling molecule (e.g. morphogen) concentration and respond with activation of certain gene expression programs. In many cases, such responses are assumed to involve a sensor protein that specifically binds the signaling molecule and transduces this information to downstream network components. This view tends to isolate the differentiation program from the milieu of metabolic, growth, and other cell-cycle processes within which it necessarily operates. However, variation in intrinsic physiological parameters such as growth rate is a major contributor to gene expression variability [53] and cell-fate heterogeneity [54,55]. Despite this evidence, we lack a clear understanding of how differentiation GRNs handle perturbations in physiological processes such as cell growth.

This deficiency is especially apparent for stress-activated differentiation programs which operate under conditions of suboptimal cell growth. Sporulation is a classic model of stress-activated programs. Starvation in *B. subtilis* can be triggered by a wide variety of environmental perturbations that cannot be defined by the level of a single metabolite [16,56,57]. To appropriately respond to starvation, the

sporulation network must therefore solve two problems. First, it must be able to detect the availability of a variety of nutrients and integrate this information into the cellular response. Second, it has to be able to properly operate despite the inevitable consequences of starvation: slowdown of cell growth and its effects on gene expression and cell cycle.

Prior studies of *B. subtilis* sporulation postulated that the design of the phosphorelay network offers multiple entry points for putative signals, e.g. via multiple kinases and phosphatases [16,58,59]. Consequently, the focus was identification of specific signaling molecules that regulate the phosphorelay response. In contrast, recently, a completely different hypothesis was investigated – whether the

sporulation network can actually exploit one starvation related problem, i.e. growth-slowdown-related changes in gene expression, to solve another problem, i.e. integration of multiple nutrient levels into a single starvation response [60].

This hypothesis was motivated by single-cell time-lapse microscopy results showing that the amplitude of Spo0A~P pulses is inversely correlated with cell growth (Fig. 3A). Furthermore, fates of over 85% cells can be explained by a simple model postulating that only cells growing slower than a threshold value sporulate (Fig. 3A). The same model also correctly predicted the distribution of sporulation deferral times during starvation based solely on the growth-rate distribution in each generation. But how can the sporulation network detect the cell growth rate?

To uncover a plausible mechanistic relationship between the growth rate and sporulation, the mathematical model of phosphorelay was used to uncover two complementary mechanisms (Fig. 3B). The model showed that as cell growth decreases, the cellular concentration of the rate-limiting phosphorelay kinase KinA increases as its dilution slows down and cell volume decreases. In addition, slowdown of growth leads to a longer time of DNA replication and also longer intervals between subsequent rounds of replication. Both effects can be quantified from the single-cell data and incorporated into the mathematical model of Spo0A~P pulsing. The resulting model shows amplitude-modulation of Spo0A~P comparable to that of the experiments (Fig. 3A, C). Furthermore, experiments with engineered strain, in which GFP-fused KinA is induced from an IPTG-inducible promoter, demonstrated that increasing KinA production can trigger sporulation in faster growing (less starving) cells. However, regardless of the starvation conditions the single-cell amount of KinA required for sporulation remains the same (Fig.3D). Therefore, in the conditions tested, KinA activity is not starvation-dependent and plays no role in controlling sporulation. Thus, in these experiments, growth-slowdown-controlled KinA level is the primary starvation signal. On the other hand, under different conditions, additional signals that may also affect the phosphorelay and either serve as checkpoints prohibiting sporulation in certain conditions or modulating the growth-rate threshold required for sporulation.

The critical advantage of using cell growth as a starvation signal is that it is a naturally modulated by cellular metabolism. As such, cell growth integrates all the necessary nutrient information relevant for sporulation into a single signal. Consequently, growth sensitivity of the phosphorelay turns out to be a useful design feature of sporulation network. In contrast, for other stress-related differentiation programs, growth-rate sensitivity may represent a major functional challenge. Recent studies have demonstrated how changes in cell growth can impact the partitioning of cellular resources such as RNA polymerase and ribosomes [61-63] that are shared among all cellular processes including differentiation. Understanding the impact of cell growth on differentiation GRNs for a wide range of model systems represents a major direction for future studies of cellular differentiation.

Conclusions

The remarkable complexity and diversity of differentiation GRNs represents a daunting obstacle to our understanding of their functionality. We hope that the examples from the studies of sporulation illustrate how a combination of mathematical modeling and single-cell experiments can be used to tackle these networks. Critically, modeling enables the systematic reduction of complexity by

abstraction of networks by uncovering their core functional structure (e.g., phosphorelay as a delayed negative feedback loop; σ^F activation network as a zero-order ultrasensitivity module). From this perspective, it becomes far easier to relate the network design to functional properties and requirements (e.g. cell-cycle coordination by the pulse-generating negative feedback loop or binary cell-fate decisions by the ultrasensitive σ^F and σ^E activation networks). These approaches also enable the identification of critical design features that are rarely evident on a network wiring diagram, such as chromosomal gene arrangement and growth sensitivity, which could be recurrent features across different differentiation programs with seemingly little in common.

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Graphical Abstract (for review)