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Temporal competition between differentiation programs determines cell fate choice

Anna Kuchina[1,2], Lorena Espinar[3], Tolga Çağatay[1,2], Alejandro O Balbin[1,2], Fang Zhang[1,2], Alma Alvarado[1,2], Jordi Garcia-Ojalvo[3] and Gürol M Süel[1,2,*]

[1] Green Center for Systems Biology, University of Texas Southwestern Medical Center, Dallas, TX, USA, [2] Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX, USA and [3] Departament de Física i Enginyeria Nuclear, Universitat Politècnica de Catalunya, Edif. GAIA, Terrassa, Spain
* Corresponding author. Department of Pharmacology, University of Texas Southwestern Medical Center, 6001 Forest Park Ln, Dallas, TX 75390, USA.
Tel.: +1 214 645 6369; Fax: +1 214 645 6333; E-mail: gurol.suel@UTSouthwestern.edu

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Multipotent differentiation, where cells adopt one of several possible fates, occurs in diverse systems ranging from bacteria to mammals. This decision-making process is driven by multiple differentiation programs that operate simultaneously in the cell. How these programs interact to govern cell fate choice is poorly understood. To investigate this issue, we simultaneously measured activities of the competing sporulation and competence programs in single *Bacillus subtilis* cells. This approach revealed that these competing differentiation programs progress independently without cross-regulation before the decision point. Cells seem to arrive at a fate choice through differences in the relative timing between the two programs. To test this proposed dynamic mechanism, we altered the relative timing by engineering artificial cross-regulation between the sporulation and competence circuits. Results suggest a simple model that does not require a checkpoint or intricate cross-regulation before cellular decision-making. Rather, cell fate choice appears to be the outcome of a 'molecular race' between differentiation programs that compete in time, providing a simple dynamic mechanism for decision-making.
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Introduction

Cellular decision-making underlies diverse biological processes such as multipotent differentiation, and is typically described as a complex process that proceeds over time and involves many biochemical interactions (Errington, 1993; Bertrand and Traver, 2009; Parra, 2009; Perkins and Swain, 2009). Even though many of the components comprising differentiation programs may be known, how cells reach a mutually exclusive decision between alternative fates over time remains poorly understood. Numerous studies have suggested that competing differentiation programs are tightly coupled through cross-regulation which controls decision-making and ensures that cell fate outcomes are mutually exclusive (Grossman, 1995; Hahn *et al*, 1995; Berka *et al*, 2002; Veening *et al*, 2006; Schultz *et al*, 2009). However, it is not known if cross-regulation is required before the decision point, or if the main role of cross-regulation is to ensure reliable execution once the decision has been made.

Whether competing differentiation programs operate with a degree of independence or if they are intricately coupled before cell fate choice, can suggest distinct decision-making strategies. In one scenario, multiple cross-regulatory interactions can progressively favor one cell fate and in turn decrease the probability of the alternative over time. Such regulation could give rise to checkpoints that allow the cell to continue progression toward one cell fate only if the state of the alternative differentiation program permits it (Hosoya *et al*, 2010). The opposite scenario would be that cross-regulation is not required for cells to reach a decision. Even if potential cross-regulatory interactions have been identified (Bai *et al*, 1993; Hoa *et al*, 2002; Hamoen *et al*, 2003; Smits *et al*, 2007), when these particular links are active in individual cells and what specific role they have during decision-making is commonly not known. Perhaps, these potential links are not active in all cells and at all times, and may not be necessary for decision-making. Cell fate choice could then simply be the outcome of the independent dynamics of competing differentiation programs. For example, recent studies have suggested that the cellular decision-making process in mammalian cells to undergo apoptosis or slippage during cell-cycle checkpoint activation may be the result of competition in time among independently operating genetic programs (Huang *et al*, 2009, 2010). In these systems, the main role of cross-regulation may be to prevent interference among alternative programs after the decision point.

A critical determinant of cell fate choice is the activity of key molecules, often referred to as master regulators, which are expected to be the target of cross-regulation (Grossman, 1995; van Sinderen *et al*, 1995; Fujita and Losick, 2005; Kalmar *et al*,

2009). In the bacterial model organism *Bacillus subtilis*, such master regulators have been identified and well characterized for the competing processes of sporulation and competence (van Sinderen *et al*, 1995; Fujita and Losick, 2005). In stressful environments, the majority of *B. subtilis* cells form spores that survive environmental extremes (Hoch, 1976; Losick *et al*, 1986; Piggot and Hilbert, 2004), while ∼3% of cells transiently differentiate into the alternative cell fate of competence allowing uptake of extracellular DNA (Grossman, 1995; Dubnau, 1999). The master regulator of sporulation is the phosphorylated transcription factor Spo0A, while competence is controlled by the master transcription factor ComK. Recent single-cell measurements revealed cell-to-cell variability in ComK and Spo0A expression that generated heterogeneity at the population level (Suel *et al*, 2006; Chastanet *et al*, 2010; de Jong *et al*, 2010). Despite these recent advances, it is not well understood how the activities of ComK and Spo0A govern cellular decision-making as a function of time in individual cells.

Even though competence and sporulation programs have often been characterized separately (Losick *et al*, 1986; Losick and Stragier, 1992; Stragier and Losick, 1996; Suel *et al*, 2006, 2007; Cagatay *et al*, 2009), several studies have identified potential cross-regulatory links between these programs (Bai *et al*, 1993; Hoa *et al*, 2002; Veening *et al*, 2006; Smits *et al*, 2007; Schultz *et al*, 2009). However, it is not known whether these links are active in individual cells before, or at the time of cell fate choice. In general, the activity of a gene or protein is rarely characterized in single cells and over time to reveal how it contributes to cellular behavior. Therefore, the relationship between the competing differentiation programs of competence and sporulation and the resulting dynamics of cell fate choice remain unclear. Specifically, it is not known how an individual cell makes a cell fate decision over time and when cross-regulation between competing differentiation programs has a role. Here, we measured competing differentiation programs simultaneously in single *B. subtilis* cells over time, to determine if intricate cross-regulation between programs is required before cell fate choice, or if the dynamics of competing differentiation programs can dictate cellular decision-making.

Our investigation of how cells arrive at a cell fate choice uncovered that competence and sporulation programs progress independently early on, suggesting the absence of active cross-regulation before decision-making in *B. subtilis*. After the decision point, cross-regulatory links did exhibit activity presumably to ensure reliable execution of the differentiation choice. However, the actual decision-making appears to be the result of a simple dynamic mechanism based on the temporal competition between distinct programs, referred to here as a molecular race. We tested the molecular race model using engineered cross-regulatory interactions between sporulation and competence that allowed us to precisely control the relative timing of competing differentiation programs in single cells without the need to synchronize inherent cell-to-cell variability. These experiments showed that cellular decision can be controlled by altering the relative timing of competing differentiation programs. These findings draw parallels between bacterial and mammalian decision-making systems, suggesting that perhaps other biological processes such as cell-cycle regulation or differentiation may also be governed by the relative timing of competing events (Huang *et al*, 2010; Sprinzak *et al*, 2010). Together, these results suggest that complex behaviors such as cellular decision-making can arise from simple dynamic behaviors of the underlying genetic programs.

Results

We began by establishing the dynamics of the progression to spore formation. Using fluorescent reporter constructs and time-lapse microscopy, we measured the activity of the promoter for Spo0A (P_{spo0A}), the master regulator of sporulation (Fujita and Losick, 2003; Molle *et al*, 2003). Phosphorylated Spo0A has a high affinity for its own promoter and positively regulates its own expression (Strauch *et al*, 1992; Asayama and Kobayashi, 1993; Molle *et al*, 2003). Utilizing a second spectrally distinct fluorescent reporter, we simultaneously measured expression of the SpoIIR promoter (P_{spoIIR}) to establish a reference point for spore formation. Initiation of P_{spoIIR} expression occurs only in the precursor of the spore known as the forespore, and indicates the point of irreversible commitment to sporulation (Karow *et al*, 1995; Dworkin and Losick, 2005). These measurements showed that the population average of P_{spo0A} expression steadily increased and reached its maximum near forespore formation (Figure 1A). As expected, we found that the conditional probability of spore formation increased with the increased P_{spo0A} activity (Supplementary Figure S3A). Additionally, we observed an identical increase in the probability of spore formation as a function of the activity of the Spo0F promoter (P_{spo0F}), which is governed by phosphorylated Spo0A (Supplementary Figure S3B; Lewandoski *et al*, 1986; Strauch *et al*, 1993). Therefore, P_{spo0A} expression provides a measure for the gradual progression to spore formation.

Initiation of competence is independent from the progression to spore formation

We next asked how the probability of the alternate fate of competence changed as cells approached spore formation, to establish if cross-regulation between the sporulation and competence programs takes place before decision-making. Specifically, we simultaneously measured P_{spo0A} and comG promoter (P_{comG}) expression with single-cell resolution (Figure 1B). Expression of P_{comG} is a well-established competence reporter as it is exclusively regulated transcriptionally by ComK, the master regulator for competence (van Sinderen *et al*, 1995), with no other direct inputs. Therefore, by simultaneously measuring P_{spo0A} and P_{comG} expression we were able to track the sporulation and competence master regulators together in the same cell. Using this data, we determined the amplitude of P_{spo0A} expression at the initiation of competence events (Figure 1B). Competence was initiated over a wide range of P_{spo0A} expression amplitudes and at variable times before spore formation (Figure 1C). These results are consistent with previous studies that suggest a stochastic initiation mechanism for competence (Maamar *et al*, 2007; Suel *et al*, 2007). Calculations based on our measurements directly show that the conditional probability of competence remains nearly constant during the progression

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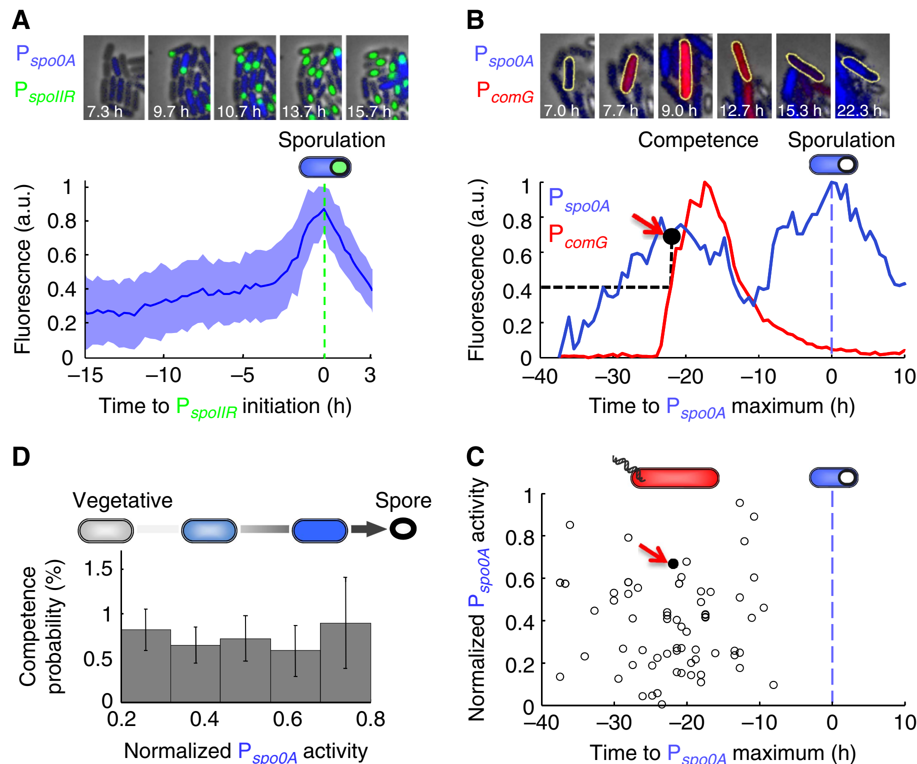

Figure 1 Competence initiation during the progression to spore formation. (**A**) Average fluorescence time trace of sporulating cells from 0A-IIR strain with P_{spo0A}-YFP (blue) and P_{spoIIR}-CFP (green). Individual traces (n=32) are normalized in amplitude and aligned in time with respect to P_{spoIIR} initiation (60% of peak activity) before averaging. The blue area corresponds to standard deviation. A representative filmstrip of sporulating microcolony of 0A-IIR strain is shown above. For strain definitions, see Supplementary Table S2. We note that the drop of fluorescence signal after the maximum is due to lysis of the mother cell during the spore formation process. (**B**) Sample quantitative traces illustrating how the activity of sporulation was determined at the initiation of competence. Specifically, the panel shows a single cell (0A-comG strain) undergoing a competence event followed by sporulation. Competence reporter P_{comG} activity is measured by CFP fluorescence intensity (shown in red) and P_{spo0A} activity is measured by YFP fluorescence intensity (in blue). The competence and sporulation events are highlighted by the cartoon and the filmstrip on top. The traces are aligned in time with respect to maximum P_{spo0A} activity at sporulation (set as zero time point). These data are utilized to determine P_{spo0A} activity (black circle highlighted by red arrow) at initiation of competence (defined by $>30\%$ P_{comG} activity relative to maximum P_{comG} amplitude at competence). For comparison, both time traces are normalized with respect to amplitude. (**C**) P_{spo0A} activity (black circles) at initiation of competence measured in single cells, n=67, from 0A-comG strain, as shown in (B). The filled circle highlighted by red arrow corresponds to the measurement shown in (B). The traces are aligned with respect to maximum P_{spo0A} activity at sporulation as described in (B). (**D**) Conditional probability of competence as a function of normalized P_{spo0A} activity. P_{spo0A} expression at onset of competence was measured in single cells from the 0A-comG strain as shown in (B) and (C). Conditional probability of competence is calculated as the number of competence initiations observed at specified values of P_{spo0A} expression normalized by the number of total surrounding cells with the specified P_{spo0A} amplitude (Supplementary Figure S1; Supplementary information, S2.4.3). Only data for P_{spo0A} activities between 20 and 80% are shown, because data $<20\%$ is within error and cells $>80\%$ are too close to spore formation ((A)). Error bars represent counting error.

to spore formation as defined by P_{spo0A} expression (Figure 1D; Supplementary Figure S1; see also Supplementary information, S2.4.3). Similarly, the probability of competence initiation was also found to be independent of P_{spo0F} expression (Supplementary Figures S2 and S3). In addition, we indeed found that sister cells appeared to be able to diverge and commit to alternative cell fates, even though they shared the same cytoplasm and starting P_{spo0A} expression level (Supplementary Figure S4). Together, these results show that the independence of competence initiation from the progression state of sporulation argues against active cross-regulation that can predispose cell fate choice before the actual decision point.

Expression of cross-regulatory genes before the decision does not predict cell fate outcome

We wondered if the implied independence of competence and sporulation before cell fate choice was reflected in the expression of genes involved in cross-regulation. If cross-regulatory genes have an active role before the decision point, they could exhibit distinct activity just before the decision that could predict cell fate outcomes. Specifically, we examined the cross-regulators *abrB* and *sinI*, which are both regulated by Spo0A (Zuber and Losick, 1987; Strauch *et al*, 1990; Hahn *et al*, 1995; Shafikhani *et al*, 2002). While Spo0A represses P_{abrB}, it induces expression from P_{sinI}. SinI has been suggested to repress competence indirectly through antagonizing SinR, a transcriptional inducer of ComK (Bai *et al*, 1993). AbrB, on the other hand, is a transcription factor that has been proposed to either activate or repress ComK expression, in a concentration-dependent manner (Hamoen *et al*, 2003). Therefore, if these cross-regulators have a critical role leading up to decision-making, their activity levels before initiation of competence or spore formation should differ. Using three spectrally distinct fluorescence reporters, we performed three-color measurements to simultaneously track activities of P_{comG}, P_{spo0A} and either *abrB* (P_{abrB}) or *sinI* (P_{sinI}) promoters in single cells.

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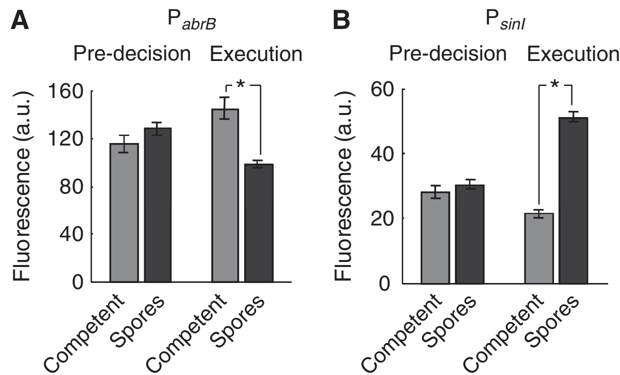

Figure 2 Cross-regulation genes expression in single cells displays a cell fate-specific pattern only after cell fate choice. Panels (**A**) and (**B**) show mean P_{abrB}-YFP (A) and P_{sinI}-YFP (B) fluorescence measured in single cells of strains 0A-comG-abrB (n=10 competent and 40 spores), and 0A-comG-sinI (n=10 competent and 25 spores), respectively. The fluorescence measurements were taken from time points either 40 min before initiation of respective cell fate reporter (30% of maximum value for P_{comG}, 90% of maximum value for P_{spo0A}), labeled 'pre-decision', or 40 min after the maximum expression of respective cell fate reporter, labeled 'execution'. Error bars represent standard error of the mean (s.e.m.). $^{*}P<0.001$.

Analysis of P_{abrB} and P_{sinI} activities before initiation of competence or spore formation revealed no significant difference in absolute expression amplitudes for either gene (Figure 2A and B). These results show that expression of these cross-regulatory genes before the decision point is not predictive of cell fate outcomes, further indicating the absence of cross-regulation before cell fate choice.

Cross-regulatory genes exhibit specific activity only after the decision point

Next, we measured average P_{abrB} and P_{sinI} activities during the execution of competence or spore formation and found their absolute amplitudes to be distinct. Specifically, P_{abrB} activity was lower in spore forming cells compared with those executing competence, while the opposite was observed for P_{sinI} (Figure 2A and B). These findings also indicate that sporulation appears to be inhibited in cells undergoing competence. Indeed, measurements of P_{spo0A} showed that its average activity reached a local minimum during competence, indicating that progression to sporulation is inhibited during competence (Supplementary Figure S5A). In contrast to their expression profiles before the decision point, we thus find that cross-regulatory genes exhibit specific activity during the execution of competence and spore formation (see also Supplementary Figure S5B–E).

Dual-activity cell state indicates the absence of cross-regulation and checkpoints during cell fate choice

The apparent absence of cross-regulation before the decision point is intriguing, because competence and spore formation are mutually exclusive cell fates (Grossman, 1995). Lack of cross-regulation implies that ComK expression could initiate even as cells near the irreversible point of commitment to spore formation. Indeed, we identified a small percentage ($0.11\pm0.04\%$, N=10 561) of cells in which competence is initiated just as spores are forming. Cells in this conflicted state, referred to here as dual-activity (DA) cells, appear to initiate competence by expressing ComK, but proceed directly to form normal spores (Supplementary Figure S6). The small percentage of cells captured in this state is not surprising. In particular, the DA state is defined by the simultaneous occurrence of two independent low probability events in the same cell: (1) stochastic initiation of ComK expression and (2) close proximity to the decision toward spore formation. Therefore, we estimated from our data that any regular cell has a 0.1% chance of becoming a DA cell, which is statistically indistinguishable from the experimentally observed frequency (Supplementary information, S2.4.4).

Initially, DA cells are similar to those that enter regular competence events (Figure 3A) in that they express P_{comG}, the reporter for the competence master regulator ComK (van Sinderen *et al*, 1995; Figure 3B). In contrast to competent cells, however, DA cells do not undergo elongation during ComK activity (Figure 3B). Instead, they begin forespore formation (as indicated by the appearance of an asymmetric septum) despite increasing ComK activity. Ultimately, DA cells progress to lysis of the mother cell (Figure 3B), as is common for all sporulating cells. Therefore, DA cells behave like spore forming cells and thus are very distinct from typical competence events, in which cells must exit competence before spore formation (Suel *et al*, 2006, 2007; Supplementary Figure S6). The apparently futile expression of ComK seems to be the only difference between DA and regular spore forming cells. This is further supported by measurements of cross-regulatory gene activities in DA cells which, in contrast to competent cells, display the same P_{abrB} and P_{sinI} activity patterns as sporulating cells (Supplementary Figure S7, compare with Figure 2 and Supplementary Figure S5). The very existence of cells in this DA state thus demonstrates that expression of the competence master regulator is not inhibited by the progression to sporulation, and vice versa, that sporulation is not inhibited by expression of ComK. These findings further demonstrate that active cross-regulation or checkpoints do not have a critical role before cell fate choice.

Identification of the relative temporal location of the competence exclusion point

To better understand the inability of DA cells to access competence and prevent spore formation despite ComK expression, we further examined these cells. DA cells do not correspond to a novel physiological state with distinct function, but rather serve to reveal the timing and dynamics of the cell fate choice. In particular, DA cells simultaneously express both the sporulation and competence master regulators Spo0A and ComK, respectively. However, competence is excluded while spore formation proceeds uninterruptedly. Therefore, quantitative measurements of competence (P_{comG}) and sporulation (P_{spo0A}) reporters in DA cells allowed us to determine the precise dynamics of cellular decision-making (Figure 3C). In contrast to regular competent cells, P_{comG}

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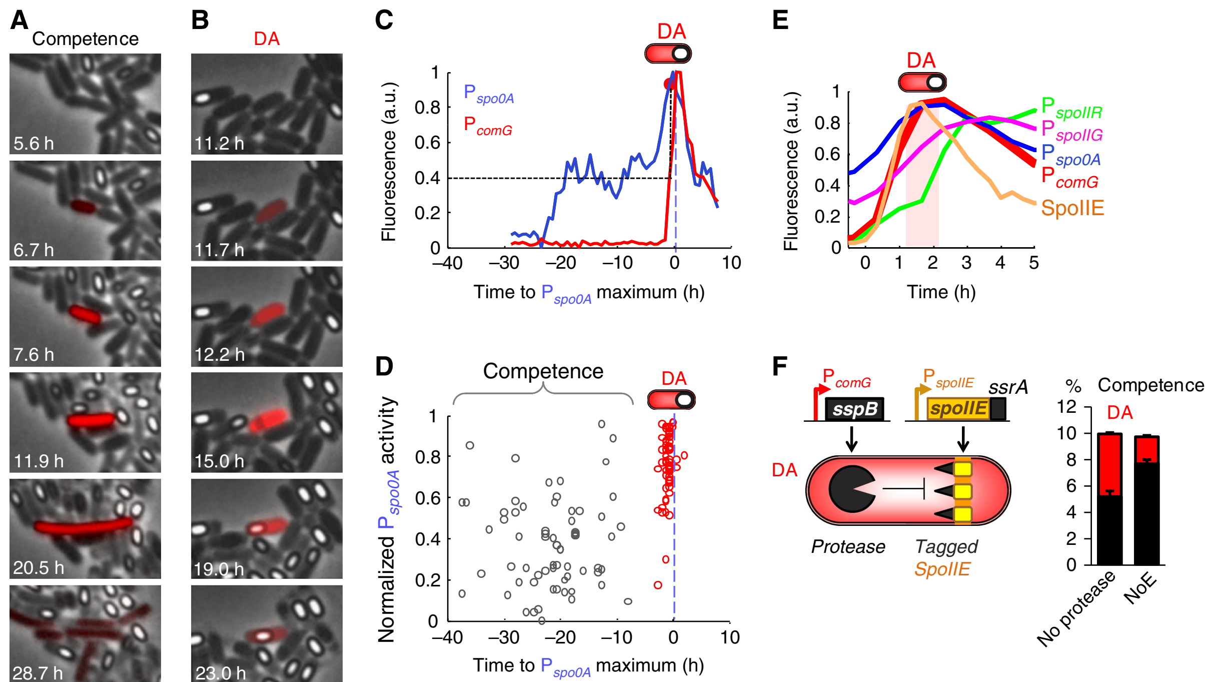

Figure 3 Cells identified in the DA state suggest lack of cross-regulation before cell fate decision. (**A**) Filmstrip of a representative competent cell visualized by competence reporter P_{comG}-CFP (red) (see also Supplementary Figure S6A). (**B**) Filmstrip of a representative DA cell expressing the same reporter (P_{comG}-CFP) as shown in (A) (see also Supplementary Figure S6B). (**C**) Sample time traces illustrating how the activity of sporulation was determined specifically in DA cells. The panel shows a single DA cell (0A-comG strain), in which P_{comG} activity is measured by CFP fluorescence intensity (shown in red) and P_{spo0A} activity is measured by YFP fluorescence intensity (in blue). The traces are aligned in time with respect to maximum P_{spo0A} activity at sporulation (set as zero time point). There data are utilized to determine P_{spo0A} activity (red circle) at initiation of P_{comG} expression in DA cells (defined by $>30\%$ P_{comG} activity relative to maximum observed P_{comG} amplitude), similarly to Figure 1B. For comparison, both time traces are normalized with respect to amplitude. (**D**) P_{spo0A} activity measured in single DA cells (red circles), $n=67$. The data were combined from measurements of 0A-comG, $[0A\text{-}comG]^{0A-K}$, $[0A\text{-}comG]^{IIG-K}$ strains (see Supplementary Figure S8 and the manuscript below). The traces are aligned with respect to maximum P_{spo0A} activity at sporulation as described in (C). P_{spo0A} activity at the onset of competence (black circles) determined in Figure 1C is shown for comparison. For strain definitions, see Supplementary Table S2. (**E**) Average time traces of single DA cells measured in strains containing pairwise combinations of each of the indicated sporulation reporters together with P_{comG}-CFP; strains are as follows: 0A-comG ($n=15$), IIE-comG ($n=7$), IIG-comG ($n=13$), IIR-comG ($n=4$). All traces are normalized in amplitude and aligned in time with respect to P_{comG} activity (30% of peak activity). See also Supplementary Figure S8A. (**F**) Left: The design of the NoE strain. An *E. coli* protease tag ssrA was translationally fused to SpoIIE protein, and a specific *E. coli* protease factor SspB that targets this tag for degradation was expressed from P_{comG} promoter. Simultaneous activation of both differentiation programs, a characteristic of DA state, in engineered NoE cells would result in targeted degradation of SpoIIE. For details, see Supplementary information, S2.3. Right: Competent and DA cell frequencies observed in a strain lacking the NoE modification ('No protease') compared with the NoE strain. Black bars show competent cells fraction, while red bars indicate DA cells fraction. Both strains have enhanced background DA frequency for easier analysis as a result of ectopic ComK expression from P_{spoIIG} promoter (see Figure 5 and Supplementary Table S1). Error bars represent standard error of the mean (s.e.m.).

expression in DA cells was restricted to a narrow time window just before spore formation at which the amplitude of P_{spo0A} activity was high and less variable (Figure 3D). Therefore, DA cells provided a reference point for cell fate choice that uncovered the timing of the competence exclusion point.

To study the competence exclusion point identified in DA cells in greater detail, we determined its temporal location relative to the distinct steps of sporulation. To that end, we measured the activity of four sporulation reporters (P_{spo0A}, P_{spoIIG}, SpoIIE and P_{spoIIR}) simultaneously with the competence reporter P_{comG} in DA cells (Figure 3E). Utilization of multiple sporulation reporters is critical, since P_{spo0A} does not exclusively report the functionally active phosphorylated state of Spo0A. To that end, we utilized a functional fluorescent protein translational fusion to SpoIIE, a protein phosphatase that activates the forespore-specific transcription factor (σ^F) and whose expression requires high concentrations of phosphorylated Spo0A (Duncan *et al*, 1995; Arigoni *et al*, 1996; Fujita *et al*, 2005). In addition, localization of SpoIIE to the asymmetric septum is the first morphological marker for forespore formation (Barak *et al*, 1996). Expression dynamics of each reporter pair, averaged over all analyzed cells, were aligned with respect to P_{comG} activity, which served as a common temporal anchor point (Supplementary Figure S8). These measurements showed that the DA state is observed after P_{spo0A} activation, but before P_{spoIIG} and P_{spoIIR} activation (Figure 3E). In particular, the DA state overlaps in time with localization of SpoIIE to the asymmetric septum.

The temporal overlap of P_{comG} activation with SpoIIE localization was further investigated in DA cells. In particular, we interfered with SpoIIE localization and activity through targeted proteolytic degradation, exclusively in cells expressing ComK (Figure 3F, left panel; Supplementary Figure S9; Supplementary information, S2.3). This conditional and targeted degradation of SpoIIE resulted in 40% decrease in DA cell frequency and a corresponding increase in competence frequency (Figure 3F, right panel). Partial decrease in the

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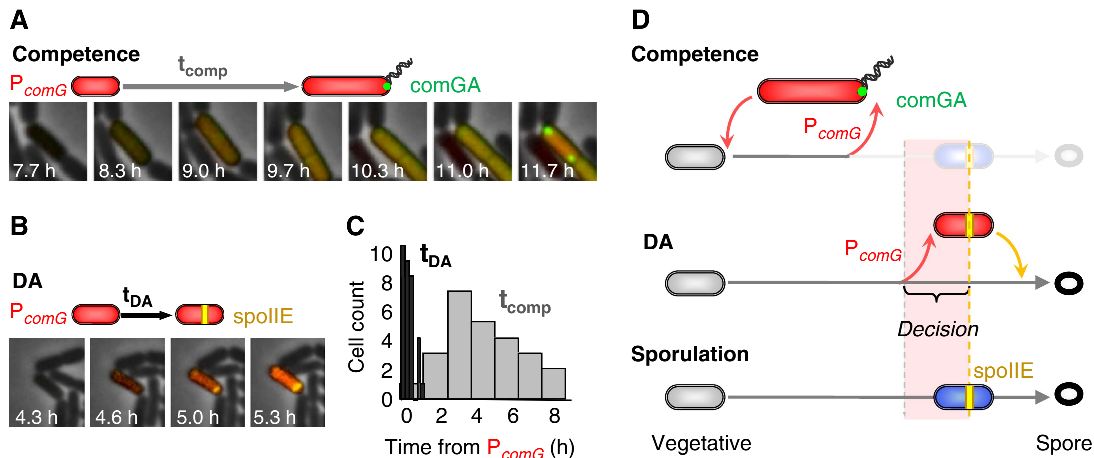

Figure 4 Temporal competition between differentiation programs determines cell fate. (**A**) Filmstrip showing a typical competent cell from the strain expressing P_{comG}-CFP (red) and ComGA-YFP protein fusion (green). Time between P_{comG} initiation (>30% maximum) and ComGA localization to cell poles (11.7 h) is indicated as t_{comp}. (**B**) Filmstrip of a representative DA cell visualized by P_{comG}-CFP (red) and SpoIIE-YFP (orange). Time between P_{comG} initiation (>30% maximum) and SpoIIE localization to asymmetric septum (>40% maximum, 5.3 h) is indicated as t_{DA}. (**C**) Histogram of time between P_{comG} initiation defined as in (A) (set as zero time point) and either ComGA localization to cell poles in competent cells (t_{comp}, n=27), or SpoIIE localization to asymmetric septum in DA cells (t_{DA}, n=32) measured in single cells as described in (A) and (B). (**D**) Diagram of the 'molecular race' hypothesis. Competence (top panel) occurs if ComK expression begins early enough to give sufficient time for ComGA localization (green dot) before SpoIIE (yellow line). However, functional competence cannot develop if ComK is expressed within the critical time window (termed 'Decision' and shown in pink) close to SpoIIE localization, resulting in DA state where sporulation takes over (middle panel). Sporulation is shown in the bottom panel for reference.

number of DA cells was expected since we used an unstable protease to target SpoIIE for degradation. Utilization of a stable protease completely eliminated DA cells, but also interfered with sporulation (Supplementary Figure S9C). We note that SpoIIE degradation did not result in an increased frequency of ComK expressing cells, but rather a corresponding redistribution of DA and competence frequencies (Figure 3F, right panel). Together, these results further demonstrate that while the competence exclusion point overlaps in time with the localization of SpoIIE, there is no active inhibition of competence initiation in cells with SpoIIE localization. Furthermore, shortly after SpoIIE localization, DA cells undergo sporulation and the associated lysis of the mother cell, which eliminates the possibility of additional competence initiation.

DA cells lack sufficient time to develop functional competence

We examined if DA cells had sufficient time to develop functional competence before mother cell lysis during spore formation. Specifically, we determined how long it took, after initiation of ComK expression, for competence to reach the ability to take up extracellular DNA. As a marker for functional competence, we utilized the localization of the membrane protein ComGA to the cell pole, as part of the ATP-dependent complex required for uptake of extracellular DNA (Chung and Dubnau, 1998; Hahn *et al*, 2005). Furthermore, ComGA localization has also been suggested to participate in the blocking of chromosome replication during competence (Haijema *et al*, 2001). In typical competence events, ComGA localization occurred on average 4 h after initiation of ComK expression (Figure 4A and C). In contrast, SpoIIE localization in DA cells occurred on average only 40 min after initiation of ComK expression, which was then directly followed by lysis of the mother cell (Figure 4B and C). Therefore, DA cells reached SpoIIE localization and consequent spore formation in less time than is needed for the localization of the DNA uptake machinery during competence (Figure 4C). Accordingly, we found that none of the DA cells exhibited localization of this critical downstream ComGA machinery required for the biological function of competence (Supplementary Figure S10). It, thus, appeared that when DA cells reached SpoIIE localization, competence had simply run out of time, leaving spore formation as the only possible outcome.

Based on these results, we hypothesized that a simple molecular race between the sporulation and competence programs determines cell fate (Figure 4D). In other words, the relative timing of competing differentiation programs is a critical determinant of cellular decision-making (Figure 4D). If ComK expression is initiated immediately before SpoIIE localization, functional competence lacks sufficient time to develop, giving rise to the DA state and spore formation. In contrast, competence is possible if ComK expression occurs much earlier than SpoIIE localization. Such temporal competition can ensure mutually exclusive cell fates, even if both programs were initiated at the same time, since only one of them has sufficient time to differentiate into its functional state.

Relative timing of competing differentiation programs determines cell fate outcomes

To test the molecular race hypothesis, we investigated how the timing of ComK expression relative to the sporulation program affected cell fate outcome. Specifically, we utilized the sporulation promoters P_{spo0A}, P_{spoIIG}, and P_{spoIIR} to ectopically express a copy of *comK* at three distinct stages of sporulation

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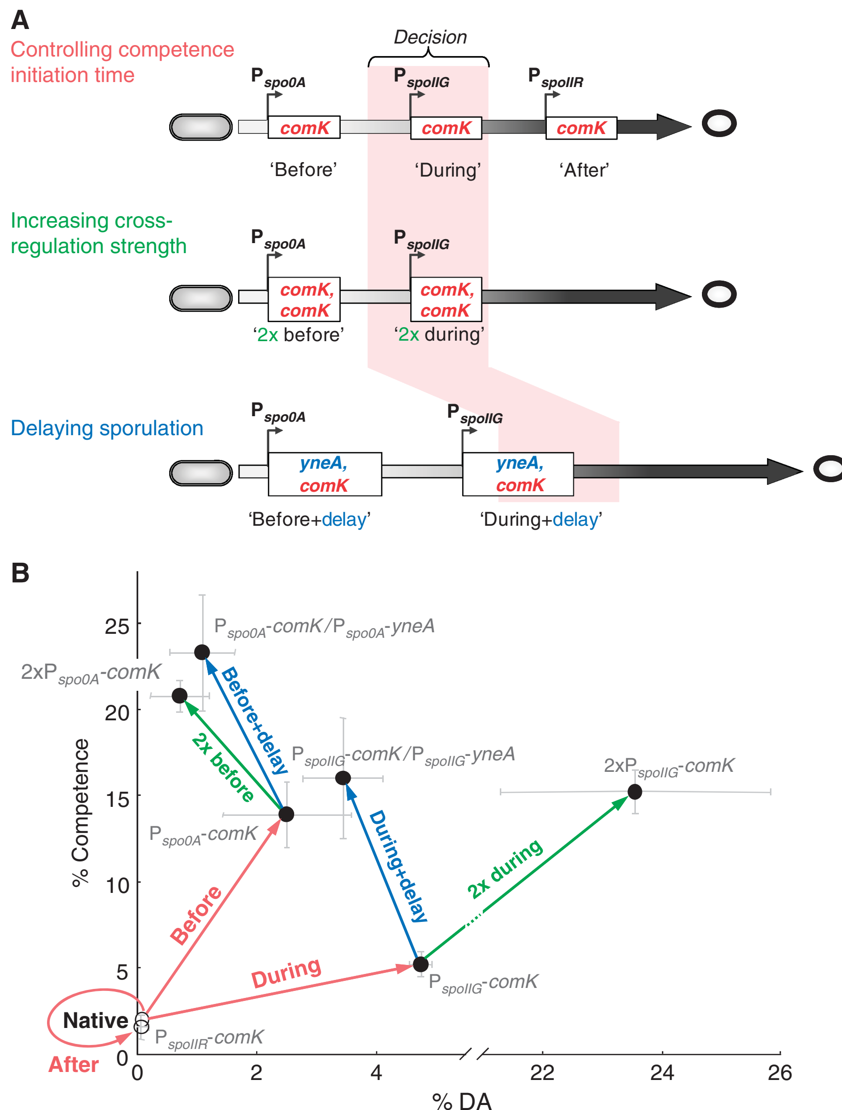

Figure 5 Perturbation of timing between sporulation and competence changes cell fate outcome. (**A**) Engineered cross-regulatory links between sporulation and competence circuits designed to test the 'molecular race' hypothesis. Top panel, *comK* is ectopically expressed either from the early stage of sporulation (P_{spo0A} promoter) before the decision time window discussed in Figure 4D ('Decision', pink area), the later stage close in time to decision (P_{spoIIG} promoter) or past the decision window (P_{spoIIR} promoter). Middle panel, two independent copies of *comK* gene are ectopically expressed from the indicated promoters (P_{spo0A} and P_{spoIIG}). Bottom panel, *yneA* is ectopically expressed together with *comK* from either early (P_{spo0A}) or later (P_{spoIIG}) stage of sporulation. (**B**) Competence and DA fractions measured in the strains expressing *yneA* and/or *comK* from early and late stage-specific sporulation promoters as described in (A). All strains are also expressing P_{comG}-CFP to report ComK activity. Each dot indicates the labeled specific strain. 'Native' denotes the *PY79 B. subtilis* strain expressing only the P_{comG} reporter. 'Before' and 'During' expression corresponds to ectopic expression from P_{spo0A} and P_{spoIIG} promoters, respectively, as shown in (A). Red arrows indicate *comK* expression. Green arrows point to strains expressing two copies of P_{spo0A}-comK ('2 × before') and P_{spoIIG}-comK ('2 × during') denoted '2 × P_{spo0A}-comK' and '2 × P_{spoIIG}-comK', respectively. Blue arrows show *yneA* expression from either P_{spo0A} or P_{spoIIG} promoter, termed 'before + delay' and 'during + delay', respectively. Detailed statistics for all strains can be found in Supplementary Table S1. Error bars represent standard deviation.

(Figure 5A, upper panel). The use of native sporulation promoters takes advantage of the cell intrinsic timing of the progression to sporulation, bypassing the need to synchronize cell–cell variability within the population (Supplementary Figure S8B and C). As expected, *comK* expression from P_{spoIIR}, which is activated after the decision point (Figure 3E), did not alter cell fate choice relative to wild type (Figure 5B). On the other hand, *comK* expression from the early sporulation promoter P_{spo0A} biased cell fate choice toward competence, increasing competence frequency from 2% (s.d.=0.3%) to 13.9% (s.d.=1.9%) (Figure 5B). Since P_{spo0A} activity is not limited to the early stages of sporulation, we also observed an anticipated increase in DA frequency. In contrast, *comK* expression from the later sporulation promoter P_{spoIIG} only slightly increased competence frequency to 5.2% (s.d.=0.7%), while DA cell frequency rose from 0.11% (s.d.=0.04%) to 4.8% (s.d.=0.2%), a 60-fold increase over wild type (Figure 5B). These results show how the timing of ComK expression relative to the sporulation program can alter cellular decision-making (Supplementary Table S1).

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We also investigated the contribution of the strength of ectopic ComK expression by introducing a respective second copy of ComK expression to each strain (Figure 5A, middle panel). In cells with two copies of P_{spo0A}-*comK*, the frequency of competence over DA increased compared with its single copy counterpart. Specifically, the ratio of competence to DA cells rose from 5.5 to 29.3 with the addition of the second copy of P_{spo0A}-*comK* as more cells adopted the competence fate, while fewer DA cells were observed (Figure 5B). On the other hand, cells with two copies of P_{spoIIG}-*comK* increased the DA frequency 5-fold relative to their single copy counterpart, reaching 23.6% (s.d.=2.3%), which is a 300-fold increase relative to the wild-type strain. However, doubling of P_{spoIIG}-*comK* only increased competence frequency by <3-fold (Figure 5B). These experiments showed that the timing-dependent bias for competence and DA cells remains even when ectopic ComK expression is doubled. While additional ComK expression increased the overall frequency of cells expressing ComK, as expected, the timing of ComK expression appears to dictate cell fate choice.

The molecular race hypothesis would also predict that delaying sporulation would favor competence. To test this prediction, we delayed sporulation by ectopically expressing *yneA* from the above-described sporulation stage-specific promoters (Figure 5A, bottom panel). The *yneA* gene is normally expressed during competence and has been shown to delay growth and suppress cell division by delaying FtsZ ring formation, which is required for both symmetric and asymmetric septation (Haijema *et al*, 1996; Kawai *et al*, 2003). In turn, septum formation by FtsZ is also required for SpoIIE localization and thus spore formation (Ben-Yehuda and Losick, 2002). Therefore, the expression of *yneA* should delay progression to spore formation (Kawai *et al*, 2003). We confirmed the expected delay in single-cell measurements, which showed that cells with ectopic *yneA* expression exhibited prolonged periods of high P_{spo0A} and P_{spoIIG} expression relative to native cells (Supplementary Figure S11). We ectopically expressed *yneA* in the above-described strains with ectopic *comK* expression (Figure 5A, bottom panel). Specifically, *yneA* and *comK* were expressed from copies of the same promoter. Thereby, the delay of sporulation and activation of *comK* expression occurred synchronously, allowing precise control of the timing between the two differentiation programs. These results show that *comK* expression and delay of sporulation by *yneA* expression have distinct effects on cell fate choice. Contrary to *comK*, ectopic expression of *yneA* from either early or later sporulation stage promoters increased competence, but not DA cell frequency (Figure 5B). In fact, we observed a decrease in DA cell frequency. These findings further support the molecular race hypothesis by demonstrating that a delay of sporulation at either stage favors competence events by giving competence development extra time.

Discussion

Our findings suggest that the initiation probability of competence is independent from the state of the sporulation program. This independence argues against the active participation of cross-regulation and checkpoints before cell fate choice. In many other systems, checkpoints have been well characterized in cellular decision-making processes. For example, progression of the cell cycle can be halted at specific checkpoints, if conditions such as replication of DNA are not met (Truong and Wu, 2011). However, recent studies have also shown that even in these systems certain cellular decisions such as the choice between apoptosis and slippage upon cell-cycle disruption may not require such checkpoints (Huang *et al*, 2010). Similarly, we found no evidence that checkpoints regulate the progression toward cell fate choice in our bacterial model system. On the contrary, the identification of cells in the conflicted DA state, where both programs are simultaneously active, demonstrates the absence of such checkpoints. Instead, we found that DA cells give rise to a mutually exclusive cell fate outcome based on the temporal competition between competence and sporulation. Once sporulation reaches asymmetric septum formation and the associated localization of SpoIIE, there is insufficient time for cells to reach the functional state of competence, regardless of ComK expression. This temporal competition can be costly due to futile expression of competence proteins in DA cells. However, only a small percentage of cells initiate competence and an even smaller fraction ever access the DA state. Therefore, the costs associated with this temporal race only burden a tiny fraction of the population, while the costly expression and regulation of a complex network of cross-regulation would be active in all cells. Therefore, different cell fate choice strategies may be favorable depending on whether cells exhibit a bias in adopting alternative cell fates. In addition, independence of differentiation programs might be more advantageous given the noise-induced heterogeneity inherent to the population stress response (Chastanet *et al*, 2010; de Jong *et al*, 2010), which would interfere with reliable cross-regulation. It will, thus, be interesting to determine in future studies if a general link exists between the strategy of cell fate choice, the asymmetry and noise-induced heterogeneity in population cell fate outcomes.

The molecular race-described here constitutes a simple mechanism for precise cellular decision-making that does not require complex cross-regulatory interactions between differentiation programs before cell fate choice. These results suggest a degree of independence and thus simplification for the operation of competing programs leading up to cell fate choice, but not a complete absence of cross-regulation. In fact, several *B. subtilis* genes clearly provide cross-regulation between competence and sporulation such as the transcriptional regulator AbrB (Banse *et al*, 2008). However, our single cell measurements show that regulators such as AbrB exhibit specific activity only during the execution of cell fate choice, after the decision has already been made. While these regulators may be critical for the overall cell fate outcome after the decision, we argue that they do not actively participate in the processes leading up to the decision point in any given individual cell. Quantitative single-cell measurements as a function of time can thus provide insights complementary to other approaches such as genetics and reduce the potential complexity assumed to underlie biological processes.

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The simple dynamic mechanism based on the temporal competition appears to be a very effective decision-making process in single cells. Therefore, our findings emphasize the importance of the dynamics and relative timing between competing differentiation programs. Specifically, by engineering artificial cross-regulatory interactions, we demonstrated that perturbation of the relative timing between competence and sporulation can control cell fate outcome. This perturbation of differentiation dynamics allowed for finer control of cell fate choice compared with traditional perturbations like gene deletions. In addition to the mammalian cell-cycle decision mentioned above, analogous molecular races may also govern the dynamics and operation of other biological systems, such as differentiation of microorganisms and decision-making in mammalian signaling pathways (Eldar *et al*, 2009; Sprinzak *et al*, 2010; Zeng *et al*, 2010; Balazsi *et al*, 2011). It may, thus, be possible to apply our methods to these systems to control diverse cellular decision-making processes.

Materials and methods

Strain construction

Supplementary Table S2 lists *B. subtilis* strains isogenic to wild-type *B. subtilis* PY79 strain. Promoter-fluorescent proteins or *comK* fusions were generated using fusion PCR techniques and cloned into *B. subtilis* chromosomal integration vectors utilizing standard cloning techniques. The vectors used in this study are as follows: pSac-Cm, integrating into the *sacA* locus (constructed by R Middleton and obtained from the Bacillus Genetic Stock Center); pLD30 designed to integrate into the *amyE* locus (kind gift from Jonathan Dworkin, Columbia University); pGlt-Kan, designed to integrate into the *gltA* locus (Middleton and Hofmeister, 2004) (constructed by R Middleton and obtained from the Bacillus Genetic Stock Center); per449, a generic integration vector constructed for integration into the gene of interest (kind gift from Wade Winkler, UT Southwestern); and the bifunctional cloning plasmid pHP13 carrying the replication origin of the cryptic *B. subtilis* plasmid pTA1060 (five copies per genome) (Haima *et al*, 1987). Standard *B. subtilis* transformation protocols were followed to transform *B. subtilis* strain PY79 with these constructs.

Growth and imaging conditions

For imaging, *B. subtilis* cells were grown at 37°C in LB with appropriate selection. Antibiotics for selection were added to the following final concentrations: 5 μg/ml chloramphenicol, 5 μg/ml neomycin, 5 μg/ml erythromycin, and 100 μg/ml spectinomycin. After reaching OD 1.8, the cells were resuspended in 0.5 volume of Resuspension Media (RM; see Supplementary information, S2.4.1) supplemented with 0.02% glucose. The cells were incubated at 37°C for 1 h, then diluted 10-fold in RM and applied onto a 1.5% low-melting agarose pad placed into a coverslip-bottom Willco dish for imaging. This protocol is optimized for time-lapse microscopy.

Time-lapse microscopy

Growth of microcolonies was observed with fluorescence time-lapse microscopy at 37°C with an Olympus IX-81 inverted microscope with a motorized stage (ASI) and an incubation chamber. Image sets were acquired either every 40 min, or every 20 min with a Hamamatsu ORCA-ER camera. The imaging time has been optimized in order to prevent phototoxicity (Suel *et al*, 2006). Custom Visual Basic software in combination with the Image Pro Plus (Media Cybernetics) was used to automate image acquisition and microscope control.

Image analysis

Time-lapse movie data analysis was performed by custom software developed with MATLAB image processing and statistics toolboxes (The Mathworks) described in Rosenfeld *et al* (2005) and Suel *et al* (2006).

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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Author contributions: GMS and JGO conceived the project; AK, LE, TC, JGO and GMS designed experiments; AK, LE, TC, AOB, FZ and AA performed experiments; AK, TC and FZ analyzed the data; AK, JGO and GMS wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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