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Switch-like reprogramming of gene expression after fusion of multinucleate plasmodial cells of two *Physarum polycephalum* sporulation mutants

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ABSTRACT

Nonlinear dynamic processes involving the differential regulation of transcription factors are considered to impact the reprogramming of stem cells, germ cells, and somatic cells. Here, we fused two multinucleate plasmodial cells of *Physarum polycephalum* mutants defective in different sporulation control genes while being in different physiological states. The resulting heterokaryons established one of two significantly different expression patterns of marker genes while the plasmodial halves that were fused to each other synchronized spontaneously. Spontaneous synchronization suggests that switch-like control mechanisms spread over and finally control the entire plasmodium as a result of cytoplasmic mixing. Regulatory molecules due to the large volume of the vigorously streaming cytoplasm will define concentrations in acting on the population of nuclei and in the global setting of switches. Mixing of a large cytoplasmic volume is expected to damp stochasticity when individual nuclei deliver certain RNAs at low copy number into the cytoplasm. We conclude that spontaneous synchronization, the damping of molecular noise in gene expression by the large cytoplasmic volume, and the option to take multiple macroscopic samples from the same plasmodium provide unique options for studying the dynamics of cellular reprogramming at the single cell level.

1. Introduction

It has been suggested that dynamic processes play an important role in the regulatory control of the reprogramming of stem cells, germ cells, and somatic cells [1,2]. Here we provide experimental evidence that multinucleate plasmodial cells of *Physarum polycephalum* obtained by fusion of two genetically and physiologically different individuals choose between two clearly different gene expression patters suggesting switch-like control mechanisms.

P. polycephalum like *Dictyostelium discoideum* and *Entamoeba histolytica* belongs to the amoebozoa group of organisms [3–7]. The life cycle of *P. polycephalum* comprises several cell types of specific morphology, function, and gene expression pattern that occur in temporal order as regulated by environmental conditions [8,9]. One stage is the so-called plasmodium, a multinucleate single cell. Since the early 1970s the *Physarum* plasmodium was used as a model organism to study different cell biological phenomena including cell cycle, cell differentiation, DNA replication, and cancer [10,11]. Plasmodia can be easily grown to a diameter of 10 cm. Even such large cells which then may contain 10^7–10^8 nuclei display natural synchrony in cell cycle and differentiation [12,13]. Plasmodia of compatible fusion type [14] spontaneously fuse upon cell contact and the cellular content mixes through the vigorous rhythmic cytoplasmic shuttle streaming [15]. When two fusing plasmodia are in different phases of the cell cycle, the nuclear populations synchronise over the entire plasmodium within a short period of time [12]. By quantitative evaluation of these phenomena the regulatory control of the cell cycle has been studied early on [13].

Upon starvation, macroscopically visible plasmodia (macroplasmodia) develop into a veined network [16]. Also in this physiological state, the cytoplasm is vigorously pumped forth and back through the veins by the rhythmic contraction of actin–myosin fibres [15,17,18]. Starved plasmodia are competent for being induced to sporulation by brief pulses of blue light [19,20] or far-red light received by specific blue light or phytochrome-like photoreceptors, respectively [21,22].

When a light stimulated plasmodium is fused with an unstimulated plasmodium, the developmental decision to sporulate depends on the physiological states of the two fusion partners and will be all-or-none for the entire plasmodium [21,22].

When two mutant plasmodia that are unable to sporulate in response to light fuse with each other, the ability to sporulate can be recovered as one fusion partner contributes the wild type gene product which is missing in the other partner and *vice versa* [23,24]. Fusion of plasmodia of different genotype in general leads to the formation of so-called heterokaryons in which genetically different populations of nuclei stably coexist. The different genetic identity of the nuclei is preserved as the nuclear membrane

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remains intact even during the cell cycle which occurs in the form of a closed mitosis [8].

Although we have previously shown that experimental fusion of a light stimulated plasmodium with an unstimulated plasmodium results in an all-or-none response in terms of sporulation [25–27], molecular events that are associated with the commitment of the cell were unknown. Accordingly, it was unclear to which extent the processes triggered by the light signal spread over the entire heterokaryon. In the present work we show that plasmodial fusion of two different sporulation-deficient mutants, one plasmodium light-stimulated the other not stimulated, results in the establishment of a common cytoplasmic state as characterised by the switch-like expression of developmentally regulated genes.

2. Materials and methods

2.1. Growth and preparation of plasmodia

For stock cultures, plasmodia of wild-type and mutant strains were hatched from spherules and taken into liquid shaken culture to be grown in the form of microplasmodial suspensions at 24 °C as described [24]. Subculturing was performed every 3.5 days by inoculating 50 ml Daniel and Baldwin liquid medium [28] in a 500 ml baffled flask with cotton plug. Plasmodia for gene expression studies were grown in a 5 L fermenter (Minifors, Infors HT, Bottmingen, Switzerland) with 1.5 L of growth medium [28] which was inoculated with 2 vol.% of a 3.5 days old shaken culture. Plasmodia were grown for four days at 24 °C, supplied with 1 L of air per minute, and stirred at 250 rpm with a marine propeller. Microplasmodia were harvested, washed twice with salt medium, and applied to starvation agar plates (9 cm diameter) with niacin and niacinamide [29] as described [30]. A ring of 1 g of cell paste (fresh weight) was applied to the centre of each plate with the help of a motor-driven 50 ml syringe coupled to an automatic device for rotating the agar plate around its axis. Plates were incubated for 8 days at 22 °C in complete darkness. During this time period, one multinucleate macroplasmodium develops on each plate. During the starvation period, wild type plasmodia become competent for sporulation.

2.2. Light stimulation of plasmodia and preparation of samples for RNA isolation

For stimulation with light, plasmodia were exposed to a 30 min pulse of far-red light (30 min, $\lambda \geqslant 700$ nm, 13 W/m^2), which was generated by Concentra Weißlicht lamps (Osram, Munich, Germany) and passed through an Orange 478 combined to a Blue 627 plexiglass filter (Röhm, Darmstadt, Germany) [27]. After irradiation, plasmodia were returned to the dark and incubated 22 °C. At approximately 6 h after the onset of the light pulse the plasmodia were harvested with a small glass spoon (Roth, Karlsruhe, FRG) and each individual plasmodium was separately shock-frozen in liquid nitrogen and stored at −80 °C for RNA isolation and gene expression analysis. Control plasmodia were treated identically except that the light pulse was omitted (dark controls). All manipulations were done under sterile conditions and under dim green safe light as described [31].

2.3. Fusion of mutant plasmodia

Plasmodia of strain PHO26 starved for 8 days in the dark were exposed to a 30 min pulse of far-red light. At 5.5 h after the onset of the pulse, the agar plates carrying the plasmodia were cut into 8 sectors each using a scalpel. In parallel, PHO1 plasmodia, which had not been irradiated, were also cut into 8 equal sectors. One sector carrying a PHO26 plasmodium was transferred into an empty Petri dish and placed adjacent to a sector carrying a PHO1 plasmodium so that the two agar slices touched each other and that large veins were brought into close proximity as much as possible. The sectors were marked according to the strain they were carrying and the plates were incubated in the dark for another 5 h. Subsequently, the two sectors in each plate were separated again and all plasmodial halves were frozen separately in liquid nitrogen for gene expression analysis. All manipulations were done under sterile conditions and under dim green safe light.

Table 1
Genes encoding transcripts that were quantified in the present study and their orthologs in the Uniprot database. For further details see [32].

Gene	Similarity	Uniprot entry
anxA	Annexin-B12	P26256
ardA	Actin, plasmodial isoform	P02576
arpA	Probable basic-leucine zipper transcription factor G	Q54RZ9
cdcA	Cell division control protein 31	P06704
cudA	Putative transcriptional regulator cudA	O00841
damA	DNA damage-binding protein 1a	Q9M0V3
dspA	Dual specificity protein phosphatase 12	Q9JIM4
ehdA	EH domain-containing protein 1	Q641Z6
gapA	Probable GTPase-activating protein 8	Q8H100
hcpA	Histone chaperone ASF1A	Q2KIG1
hstA	Probable histone H2B 4	Q27876
ligA	Checkpoint protein hus1 homolog 1 (LigA)	Q54NC0
meiB	Meiosis protein mei2	Q64M78.1
nhpA	Non-histone chromosomal protein 6	Q4PBZ9
pakA	Serine/threonine-protein kinase pakC	Q55GV3
pcnA	Proliferating cell nuclear antigen	Q43124
pikB	Phosphatidylinositol 3-kinase 2	P54674
pikC	Phosphatidylinositol 4-kinase beta	Q49GP3
pksA	Serine/threonine-protein kinase phg2	Q54QQ1
pldA	Phosphatidylinositol-glycan-specific phospholipase D	Q8R2H5
pldB	Phosphatidylinositol-glycan-specific phospholipase D	P80108
pldC	Phospholipase D	Q9LRZ5
pptA	Phosphatase DCR2	Q05924
pptB	Protein phosphatase 2C POL	Q8RWN7
psgA	Physarum specific gene	
pumA	Pumilio homolog 2	Q80U58
pwiA	Piwi-like protein 1	Q96J94
ralA	Circularly permutated Ras protein 1	Q75J93.1
rasA	Ras-related protein RABD2a	P28188
rgsA	Regulator of G-protein signaling 2	O08849
ribA	60S ribosomal protein L38	Q1HRT4
ribB	60S ribosomal protein L4-2	Q54Z69
spiA	Protein spire	Q9U1K1
tspA	Tumor suppressor p53-binding protein 1	P70399
uchA	Programmed cell death protein 2	Q2YDC9

2.4. Isolation of RNA and GeXP multiplex reverse transcription polymerase chain reaction (RT-PCR)

RNA was isolated from the plasmodial samples according to a standard protocol [30]. After reverse transcription, the transcripts of 35 genes were coamplified in each sample by PCR and the fluorescently labelled fragments separated and quantified on a Beckman Coulter 8-capillary sequencer (CEQ 8800) exactly as described previously [32]. With this method, repeated measurements of the same sample give identical results as the abundances of the individual transcripts are determined relative to each other [30,32].

3. Results

3.1. Gene expression patterns in the non-sporulating mutants PHO1 and PHO26 are altered as compared to the wild type

Two strains of *Physarum polycephalum*, PHO1 and PHO26, isolated in screens for sporulation-deficient mutants display a strongly reduced probability to sporulate [27,30] in response to a

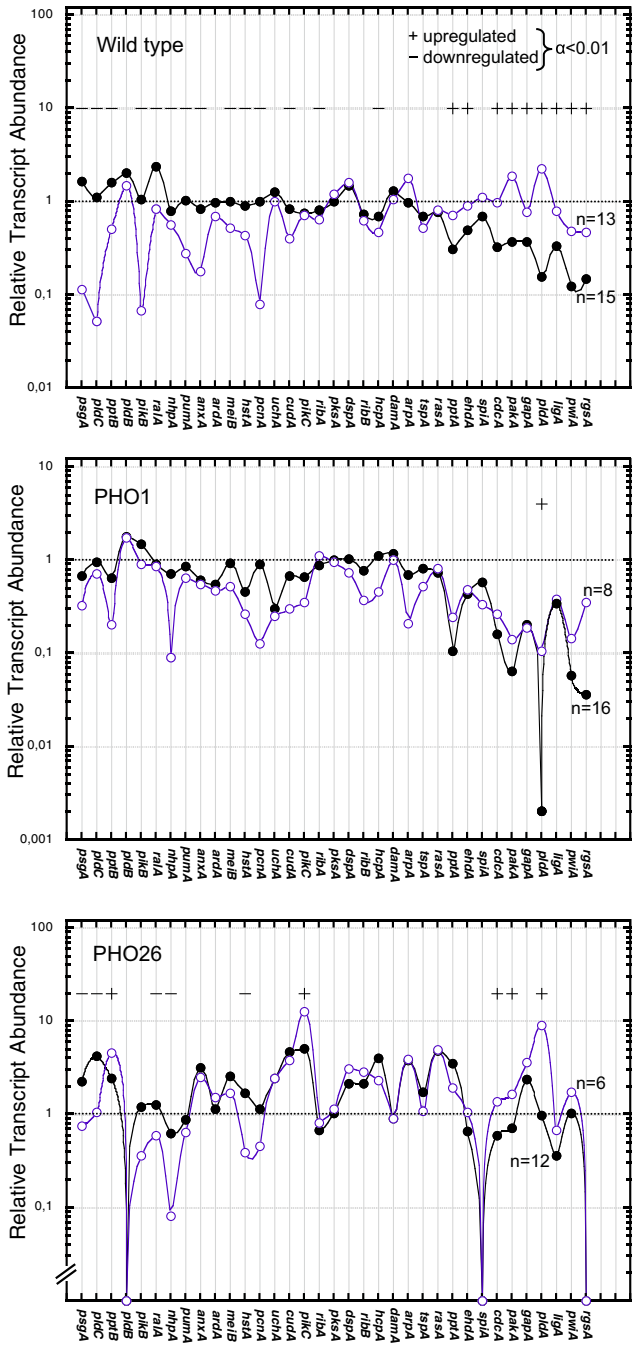

Fig. 1. Differential gene expression in wild type plasmodial cells and sporulation deficient mutants in response to far-red light. Plasmodia were exposed to a 30 min pulse of far-red light and the gene expression patterns measured at approximately 6 h after stimulus application. At this time, the wild type is already committed to sporulation. The relative abundance of each transcript as normalised to the spike-in RNA [32] and the *pksA* transcript was plotted logarithmically for the light-stimulated (○) and the unstimulated (●) plasmodia and fitted by spline just to guide the eye. Data points represent the mean values of the transcript abundance measured in n plasmodia. Data for the wild type were taken from [32], normalised and replotted. The Wilcoxon rank sum test indicates (−)down- or (+) upregulation for each gene in response to far-red light at 6.5 h (wild type), 6.0 h (PHO1), and 5.5 h (PHO26) after the pulse with an error probability of $\alpha < 0.01$. In PHO26 plasmodia, the transcripts of *pldB*, *spiA*, and *rgsA* were not detectable.

far-red light pulse which is highly saturating in causing sporulation of the wild type. To characterise the mutants at the molecular level, the expression patterns of 35 marker genes (Table 1) selected for being involved in diverse cellular processes [32] were quantitatively analysed with GeXP, a multiplex RT-PCR method [32,33]. For the wild type and the mutants, individual plasmodial cells were exposed to a far-red light pulse and returned to the dark. At approximately six hours after the pulse, when wild type cells are committed to sporulation, the cells were harvested individually for isolation of RNA and gene expression analysis.

The relative abundance of each transcript relative to a spike-in RNA was averaged over all plasmodial cells of a sample, normalised to the abundance of the *pksA* message which did not show any detectable changes in response to light, and plotted logarithmically (Fig. 1). In unstimulated PHO1 and PHO26 mutant strains, the messages of *pldA* (in PHO1) and of *pldB*, *spiA*, and *rgsA* (in PHO26) were drastically reduced as compared to the wild type. In addition, the response of the mutants to stimulation far-red light was reduced as compared to the wild type. In Fig. 1, the genes that are significantly upregulated or downregulated as compared to the dark controls ($\alpha < 0.01$; Wilcoxon rank-sum test) are marked with (+) or (−), respectively. In PHO1, the mRNA of the *pldA* gene encoding a protein with similarity to phospholipase D was strongly up-regulated by light. Despite this light response the *pldA* expression level only approached the level which is found in unstimulated wild type cells.

We have previously shown that PHO1 and PHO26 can complement each other with respect to the ability to sporulate in response to far-red when plasmodia of the two mutants are fused with each other [24]. We now wanted to analyse to which extent complementation of the two mutations can be detected at the molecular level when two mutant plasmodia are fused with each other.

3.2. Fusion of light-stimulated PHO26 cells with unstimulated PHO1 cells

PHO26 plasmodia were exposed to a far-red light pulse and at $\Delta t_1 = 5.5$ h after light exposure each plasmodium was cut into eight slices of equal size together with the supporting agar substratum. Each slice was transferred to a new Petri dish and combined with an equally sized slice cut from a PHO1 plasmodium which had not been irradiated (Fig. 2A). The slices were adjusted relative to each other so that large veins of the two plasmodia were placed in close proximity to facilitate fusion. Cutted plasmodia form pseudopodia at the cutted sites that reach out to actively find adjacent plasmodial cells to fuse with. Neither cutting nor fusion *per se* causes or prevents the sporulation of a plasmodium [25,26]. In about 30 min after physical adjustment of the agar slices, major veins formed and vigorous protoplasmic shuttle streaming through these veins caused the mixing of the cytoplasmic volumes of the two plasmodia. The cytoplasms of PHO1 and PHO26 were allowed to mix and after an incubation time of $\Delta t_2 = 5$ h the two agar slices were separated again and the two plasmodial halves were frozen separately in liquid nitrogen for the isolation of RNA (Fig. 2A).

We have found previously, that in pairs of mutants in which plasmodial fusion caused the complementation of the mutations, the entire plasmodium developed into fruiting bodies [23,24]. However it was not clear to which extent the gene expression pattern in the two plasmodial halves became similar. Therefore we analysed the patterns in the two plasmodial halves for all fused plasmodia. Although there were no significant differences between the halves taken from different plasmodia in *pksA*, *dspA*, and *ribB* expression levels, there were clear and highly significant differences in some of the genes that are down- or up-regulated in the wild type (Fig. 2B and C). Based on the expression levels of these genes (Fig. 2C, asterisks) the plasmodial halves formed two clearly distinct groups. Differential regulation of genes in response to the light stimulus suggests that genetic complementation between the two mutants indeed occurred. However, it obviously occured only in some of the fused plasmodial cells and not for all transcripts that are differentially regulated in the wild type. Those plasmodia in

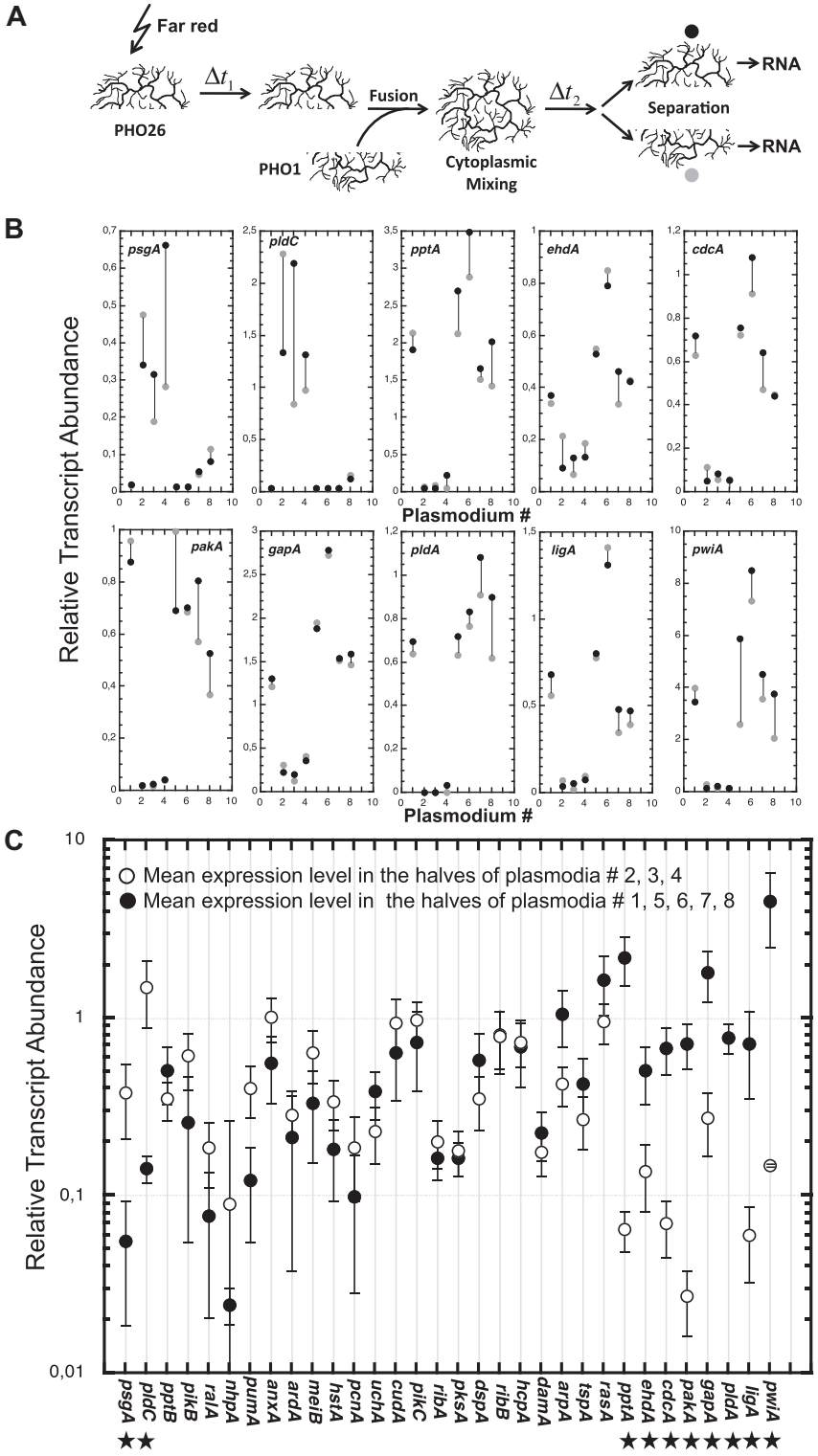

Fig. 2. Gene expression after fusion of light-stimulated PHO26 with unstimulated PHO1 plasmodial cells. (A) PHO26 plasmodia were exposed to a 30 min pulse of far-red light, returned to the dark and after $\Delta t_1 = 5.5$ h allowed to fuse with a PHO1 plasmodium which had not been exposed to light. After an incubation time of $\Delta t_2 = 5$ h, the plasmodia were separated again and the gene expression pattern was determined in each of the two plasmodial halves. (B) The relative transcript abundance in the two halves of plasmodium number 1–8 is displayed for selected genes (as marked by asterisk in C). Where appropriate, data points representing the expression values of the two halves of a plasmodium were connected by vertical lines to guide the eye. In those cases where there is only one data point visible for a given plasmodium and gene, the expression values measured in the two plasmodial halves match exactly. (C) Plasmodia were separated into two groups according to the expression level (high or low) of the genes indicated with the asterisk and the relative transcript abundance as measured in the corresponding plasmodial halves was averaged. The transcripts of *pldB*, *spiA*, and *rgsA* were not detectable in the heterokaryon. Error bars indicate the standard deviation.

which transcripts were upregulated in response to light stimulation showed also a response of some genes that are down regulated in the wild type. Expression levels of those differentially regulated transcripts were similar in being either high or low when the two halves of each plasmodium were compared to each other (Fig. 2B). Pairwise comparison revealed for each transcript shown in Fig. 2B

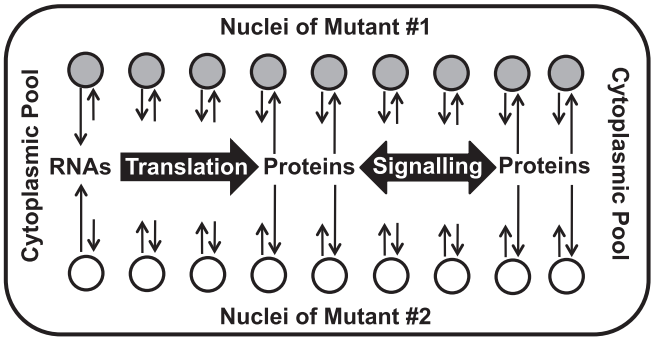

Fig. 3. Schematic presentation of the flows of RNA and protein between nuclei and cytoplasm in a heterokaryon obtained by fusion of two genetically different plasmodial cells. For clarity, the nuclei of the two mutants were drawn on opposite sides.

that its expression level was similar in the two halves of a plasmodium as compared to the overall variation of the considered transcript. This indicates a switch like response to the light stimulus: no matter to which of the two groups (with transcripts high or low, respectively) a plasmodium belonged, the gene expression patterns found in the two halves spontaneously synchronized upon fusion.

4. Discussion

We have analysed how plasmodial fusion of two mutant strains influences the gene expression pattern in the resulting heterokaryon after light stimulation. Although it was known that fusion of a light stimulated plasmodium with an unstimulated one results in an all-or-none response in terms of sporulation [22,25–27], it was unclear to which extent the biochemical processes triggered by the light signal spread over the entire heterokaryon. For obtaining experimental evidence, we have irradiated PHO26 plasmodia, allowed the downstream signalling events to proceed for a certain time (5.5 h) and then fused the stimulated plasmodium with an unstimulated PHO1 plasmodium. The cytoplasms of the two plasmodial cells were allowed to mix driven by the vigorous cytoplasmic shuttle streaming for several hours. Then the two halves were separated and analysed for gene expression.

There were two major findings which shall be discussed in the following: (1) the expression values for a number of genes fell into two categories, low or high, respectively, and (2) the two halves retrieved from individual plasmodia after fusion were highly similar in gene expression as compared to the range of values measured in all plasmodial halves. Bifurcation between high and low on one hand and the similarity in gene expression between two corresponding halves on the other, occurred despite the fact that the two fusion partners were of different genotype and in different physiological state, i.e. one stimulated with light, the other not stimulated.

Obviously, mixing of the two cytoplasms, each in a different state, led to one of two alternative gene expression patterns which was spontaneously established in the two halves of any given plasmodium. The fact that after spontaneous synchronisation a common group of transcripts were either high or low suggests the occurrence of switch-like mechanisms that become globally active.

How can spontaneous synchronisation be explained? When two plasmodial mutants fuse with each other, their cytoplasms mix and accordingly, the two populations of nuclei that are suspended in the cytoplasms mix as well (Fig. 3). Because the cytoplasmic volume of a plasmodium is by orders of magnitude larger than the volume of most typical eukaryotic cells, freely diffusible proteins will define a concentration in encountering each nucleus, even if this concentration in terms of protein copy number per nucleus is low. Although the uptake of low copy number regulatory molecules into an individual nucleus may occur stochastically, the high number (in the range of 10^7–10^8) of nuclei in the plasmodium in some sense averages over this stochasticity: Even in case each individual nucleus stochastically delivers specific mRNA molecules only at low copy number into the continuously flowing cytoplasm, the protein molecules translated from the resulting pool of mRNAs again will define a cytoplasmic concentration damping stochasticity in the interaction with other cytoplasmic regulatory molecules (Fig. 3). Gene expression and other molecular processes in the plasmodium hence are expected to reflect the effects of concentrations rather than the effects of stochastic molecular events. All-or-none responses (like the developmental decision to sporulation or switching of gene expression) observed in the plasmodium should therefore reflect the activity of circuits with switch-like behaviour rather than being caused by stochastic protein copy number fluctuation. Accordingly, the large cytoplasmic mass of a plasmodium, the phenomenon of natural synchrony, and the option to take multiple samples as a function of time provides a unique system for studying the dynamics of cellular reprogramming at the transcriptomic and proteomic level.

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