Regulatory Dynamics of Cell Differentiation Revealed by True Time Series From Multinucleate Single Cells

Anna Pretschner1, Sophie Pabel1, Markus Haas1, Monika Heiner2 and Wolfgang Marwan1*

1 Magdeburg Centre for Systems Biology and Institute of Biology, Otto von Guericke University, Magdeburg, Germany,
2 Computer Science Institute, Brandenburg University of Technology Cottbus-Senftenberg, Cottbus, Germany

Dynamics of cell fate decisions are commonly investigated by inferring temporal sequences of gene expression states by assembling snapshots of individual cells where each cell is measured once. Ordering cells according to minimal differences in expression patterns and assuming that differentiation occurs by a sequence of irreversible steps, yields unidirectional, eventually branching Markov chains with a single source node. In an alternative approach, we used multi-nucleate cells to follow gene expression taking true time series. Assembling state machines, each made from single-cell trajectories, gives a network of highly structured Markov chains of states with different source and sink nodes including cycles, revealing essential information on the dynamics of regulatory events. We argue that the obtained networks depict aspects of the Waddington landscape of cell differentiation and characterize them as reachability graphs that provide the basis for the reconstruction of the underlying gene regulatory network.

Keywords: single cell time series, gene regulatory network, Petri net, Markov chain, systems biology, Waddington landscape

INTRODUCTION

Single-cell analyses revealed complex dynamics of gene regulation in differentiating cells (Spiller et al., 2010; Junker and van Oudenaarden, 2014; Paul et al., 2015; Marr et al., 2016; Plass et al., 2018). It is believed that dynamic effects possibly superimposed by stochastic fluctuations in gene expression levels may play crucial roles in cell fate choice, commitment, and reprogramming (Graf and Enver, 2009; Huang et al., 2009; Zhou and Huang, 2011; Ferrell Jr., 2012; Il Joo et al., 2018; Bornholdt and Kauffman, 2019). Changes in gene expression over time have not been directly measured in single mammalian cells as cells are - for technical reasons - sacrificed during the analysis procedure and hence can be measured only once. Instead, algorithms have been developed to infer the gene expression trajectory of a typical cell in pseudo-time from static snapshots of gene expression states in a cell population, resulting in Markov chains of states (Bendall et al., 2014; Cannoodt et al., 2016; Chen et al., 2019; Saelens et al., 2019; Setty et al., 2019). Most trajectory inference algorithms are based on the assumption that differentiation is unidirectional (Bendall et al., 2014; Haghverdi et al., 2016; Street et al., 2018; Saelens et al., 2019) and that the probability of transiting from one state to the next similar state is independent of the individual history of
a cell (Setty et al., 2019). The inference of trajectories has been used to create pseudo-time series for differentiation (Marco et al., 2014; Moignard et al., 2015; Shin et al., 2015; Macaulay et al., 2016), cell cycle (Kafri et al., 2013), and the response to perturbation (Gaublomme Jellert et al., 2015). As any given distribution of expression patterns could result from multiple dynamics, the reconstruction of trajectories from snapshots faces fundamental limits (Weinreb et al., 2018). Even though regulatory mechanisms cannot be directly and rigorously inferred from snapshots (Weinreb et al., 2018), dynamic analyses may be of immediate importance to resolve competing views on basic mechanisms and the role of stochasticity in cell fate decisions (Moris et al., 2016).

True single cell time series can be obtained in *Physarum polycephalum* by taking multiple samples of one and the same giant cell. *Physarum* belongs to the amoebozoa group of organisms. It has a complex, prototypical eukaryote genome (Schaap et al., 2016) and forms different cell types during its life cycle (Alexopoulos and Mims, 1979).

Giant, multi-nucleate cells, so-called plasmodia provide a source of macroscopic amounts of homogeneous protoplasm with a naturally synchronous population of nuclei, which is continually mixed by vigorous shuttle-streaming (Guttes and Guttes, 1961, 1964; Rusch et al., 1966; Dove et al., 1986). The differentiation of a plasmodium into fruiting bodies involves extensive remodeling of signal transduction and transcription factor networks with alterations at the transcriptional, translational, and post-translational level (Glöckner and Marwan, 2017).

In starving plasmodial cells, the formation of fruiting bodies can be experimentally triggered by a brief pulse of far-red light received by phytochrome as photoreceptor (Starostzik and Marwan, 1995b; Lamparter and Marwan, 2001; Schaap et al., 2016). Retrieving small samples of the same plasmodial cell before and at different time points after an inductive light pulse allows to follow how gene expression changes over real time. Because cell cycle, cell fate choice, and development are synchronous throughout the plasmodium (Rusch et al., 1966; Starostzik and Marwan, 1995a; Hofmann et al., 2012; Walter et al., 2013; Rätzel and Marwan, 2015), single-cell gene expression trajectories can indeed be constructed from time series. By assembling finite state machines made from trajectories we have constructed Petri net models for the state transitions that predict Markov chains as variable developmental routes to differentiation (Werthmann and Marwan, 2017; Rätzel et al., 2020) which may be considered as trajectories through the Waddington landscape (Waddington, 1957; Huang et al., 2009). These Petri nets also predict reversible and irreversible steps, commitment points, and meta-stable states in cells responding to a differentiation stimulus. However, the computational approach for the construction of Petri nets from time series has been originally developed with data sets of a coarse resolution in time and the structural resolution of the nets was accordingly limited. Nevertheless, the approach turned out to be useful for capturing the dynamics of the process. For this paper, we developed a method for retrieving smaller samples from even larger plasmodial cells and showed that these cells provide a homogeneous source for samples to be taken. This allowed us to considerably improve the time resolution as compared to previous studies. Sampling cells at higher time resolution, allowed the construction of Petri nets with enhanced structural and dynamic resolution. Structural complexity, highly connected nodes, parallel pathways, reversible reactions, and Petri net places representing meta-stable states in the developmental network, as revealed by the new data sets, characterize the differentiation response as complex and dynamic in contrast to a smooth, continuous process. We describe the graph properties of the Waddington Petri nets and conclude that the gene expression dynamics revealed by our analysis most likely emerge from the non-linear dynamic behavior of the underlying regulatory network rather than from stochastic fluctuations in the concentration of regulatory molecules.

**MATERIALS AND METHODS**

**Plasmodial Strain, Growth of Cells, Sample Preparation, and Gene Expression Analysis**

Sporulation-competent plasmodial cells of wild type strain LU897 × LU898 (Starostzik and Marwan, 1998) were obtained as previously described (Starostzik and Marwan, 1998; Rätzel et al., 2020). A total of 2.8 gram of plasmodial mass was applied to a 14 cm Ø Petri dish that contained 90 ml of semi-rich Golderer agar (Golderer et al., 2001), based on a salt solution of 0.01% (w/v) niacin, 0.01% (w/v) niacinamide, 0.1% (w/v) CaCO₃, and 0.14 mM CuCl₂, supplemented with 5 g peptone from meat (Sigma Aldrich), 0.75 g yeast extract (Becton, Dickinson & Co.), and 3.9 mM glucose per liter, adjusted to pH 4.6 with concentrated HCl. After starvation for 7 days at 22°C in complete darkness, sporulation was induced with a 15 min pulse of far-red light (λ ≥ 700 nm, 13 W/m²) (Starostzik and Marwan, 1998). Before and at 1-h time intervals after the start of the far-red pulse, samples were taken in duplicate at arbitrarily chosen but distant positions on the plate. Each sample was obtained by picking an agar plug of 1.13 cm² with the cut bulb of a disposable Pasteur pipette (EA62.1; Carl Roth, Karlsruhe, Germany). The plasmodial mass on the agar plug was scraped off with a pipet tip and, by cutting the tip, transferred into a vial of glass beads immersed in liquid nitrogen (Figure 1). After extraction of RNA and removal of contaminating DNA (Marquardt et al., 2017), the relative abundance of the mRNAs of 35 genes, differentiation marker and reference genes (Hoffmann et al., 2012; Supplementary Table 2) was analyzed by gene expression profiling (GeXP), a multiplex RT-PCR method (Hayashi et al., 2007) as previously described (Rätzel and Marwan, 2015; Marquardt et al., 2017).

**Data Analysis Pipeline and Automated Generation of Petri Nets**

To correct for differences in the concentration of total RNA and in the efficiency of the RT-PCR reaction, the gene expression values were normalized to the median of the estimated relative concentrations of mRNAs of the 35 genes in each RNA sample. Each normalized expression value was subsequently normalized.
FIGURE 1 | Experimental protocol for taking time series by repeated sampling of individual plasmodial cells and time course of light-induced sporulation. (A) Each Petri dish contained one individual plasmodial cell supported by an agar substratum. Before and at 1-h time intervals after stimulation of the cell with a pulse of far-red light, samples were taken in duplicate by picking an agar plug at arbitrarily chosen but distant positions on the plate. The cell mass was scraped off from the agar plug with a pipet tip and transferred into a vial containing glass beads and liquid nitrogen. After purification of RNA, the gene expression pattern was estimated twice in each sample, each with two independent multiplex RT-PCR reactions (see section “Materials and Methods” for details). For dark controls, the far-red light stimulus was omitted. (B) Time frame of light-induced sporulation of a plasmodial cell. At about four to 6 h after stimulation with a pulse of far-red light, the cell is irreversibly committed to sporulation by crossing the point of no return (PNR) while there is no obvious change in the plasmodial morphology. Morphogenesis then starts at about 11 h after the stimulus by the formation of nodules that subsequently culminate to form the fruiting bodies. Panel B was taken from Glöckner and Marwan (2017).

to the geometric mean of all values obtained for a given gene, and this was performed separately for each gene.

Data were analyzed and processed with a revised and extended pipeline written in R (R Core Team., 2016), based on the previously described script (Rätzel et al., 2020). The normalized gene expression data were clustered and significant clusters were determined with the help of the Simprof algorithm (Clarke et al., 2008) as provided by the clustsig package (Whitaker and Christman, 2014). Expression patterns were visualized in the form of a heatmap generated by the heatmap.2 function, provided as part of the gplots package (Warnes et al., 2016). Changes in gene expression over time were visualized by multidimensional scaling based on Euclidean distance (Gower, 1966) with the help of the cmdscale function provided as part of the stats package v3.5.1 (R Core Team., 2016). Petri nets were constructed from single cell trajectories of gene expression as previously described (Rätzel et al., 2020). Each trajectory is a temporal sequence of gene expression states, where each state corresponds to a Simprof significant cluster. Petri nets specified in ANDL format (Abstract Net Description Language) (Heiner et al., 2013) were imported into Snoopy (Rohr et al., 2010) and graphically displayed by running the Sugiyama layout algorithm (Sugiyama et al., 1981). Petri net places, each representing a gene expression state, were colored according to the relative temporal stability
of the expression state or according to the relative frequency with which each gene expression state occurred. Petri net transitions, corresponding to transits between states were colored according to the frequency with which each transit occurred or according to the data subset in which the transit occurred. These parameters were computed and coloring was performed by automatic editing of Snoopy files encoded in xml format, again with the help of a R script. The raw data and the complete computational pipeline used in this study is provided as part of the Supplementary Material.

RESULTS

Even Large Plasmodia Provide a Source of Homogeneous Cell Material for True Time Series Analysis of Gene Expression

In previous studies we have shown that the gene expression pattern in samples taken at the same time from different sites of a plasmodium covering a standard Petri dish (9 cm Ø) did not change within the limits of accuracy of the measurements. Accordingly, repeated sampling of the same plasmodal cell yields true time series (Rätzel, 2015; Werthmann and Marwan, 2017). To allow more samples to be taken without consuming too much of the plasmodal mass, we now prepared plasmodia on 14 cm Ø Petri dishes, increasing the surface area covered by the plasmodal mass by 2.4-fold, and took smaller samples by punching agar plugs of 1.13 cm$^2$ per sample, to harvest a small portion of the initial total plasmodal mass. To test whether the homogeneity in gene expression is impaired or even lost in the larger plasmodia, we took 9 or 16 samples at the same time from approximately evenly spread sites of a plasmodium, and estimated the gene expression pattern twice in the RNA of each sample, to obtain one technical replicate of each measurement (Aselmeyer, 2019; Driesch, 2019). This allowed to estimate the biological variation in gene expression within a plasmodium as compared to the technical accuracy of the measurements. In order to correct for potential differences in the efficiency of the RT-PCR between reactions, the expression value for each gene was normalized to the median of the expression values of all genes measured in the sample (for details see Materials and Methods). To estimate the technical accuracy of the measurements, the relative deviation of first and second measurement from the mean of the two measurements was estimated for each assayed gene in each of the retrieved plasmodial samples. The frequency distribution of all values was almost symmetrical with a tail consisting of a small number of low values, obviously as a result of inefficient RT-PCR reactions. To estimate the degree of homogeneity in gene expression within a plasmodial cell, we asked to which extent the expression values for the 9 or 16 samples taken from the same plasmodial cell deviated from their median. To restrict the influence of technical artifacts on the result, we considered the subset of the data where first and second measurement of the same plasmodial sample deviated not more than two-fold from the mean of the two values. In three of the total of 46 analyzed plasmodia (30 far-red stimulated; 16 dark controls), individual samples deviated from the rest of the samples of the same plasmodium by more than a factor of two. As errors in sample preparation could not be ruled out, these three plasmodia were excluded and the remaining data set of 43 plasmodia (28 far-red stimulated; 15 dark controls) was analyzed taking the mean of 1st and 2nd measurement for each gene in each sample. Among the total of 7160 values, 98% of the symmetric frequency distribution (Supplementary Figure 1) were between 0.48- and 2.10-fold deviation of the median of all values of the respective plasmodium (Supplementary Table 1). There was no obvious difference between dark controls and far-red stimulated plasmodia which were measured at 6 h after the light pulse when genes were already differentially regulated (Supplementary Figure 1 and Supplementary Table 1), indicating that even during the period where the mRNA abundance changed in time, the homogeneity in gene expression levels is maintained. Visual inspection of outliers within the distribution did not reveal any candidates for specific genes that might be inhomogeneously expressed.

In summary, the gene expression values throughout a plasmodium deviated not more than approximately two-fold from the median of all samples from the same plasmodium and were thus within the limits of the technical accuracy of the measurements, even under conditions where genes were in the process of being up- or down-regulated. These differences measured between samples were minor as compared to the differential regulation where the expression level of genes changed in the order of ten to more than hundred-fold (Supplementary Figure 2). These results are consistent with the results of the time-series experiment, where for each time point, two samples were retrieved and analyzed from the same plasmodial cell (see below).

Sampling of Plasmodia at 1 h Time Interval

As the assayed genes were evenly expressed and changed evenly in time throughout the large plasmodium, at least within the limits of accuracy of the measurements, we took time series at 1 h time intervals. In order to assay, in each experiment, for the homogeneity and synchrony in gene expression throughout the plasmodium, we took two samples at each time point from different, arbitrarily chosen but distant sites of the plasmodium (Figure 1). In far-red stimulated plasmodia, the first samples (referred to as the 0 h samples) were taken at the start of the experiment, i.e., immediately before application of the 15 min pulse of far-red light. All subsequent samples were taken at 1 h time intervals until 10 h after the start of the experiment [At 5 to 6 h after the far-red pulse cells have passed the commitment point, while visible morphogenesis starts several hours later by entering the transient nodulation stage at about 11 h after the pulse (Hoffmann et al., 2012)]. In the dark controls, the far-red stimulus was omitted. Gene expression in each plasmodal sample taken at a given time point was analyzed twice by GeXP-RT-PCR, where the measurement and the corresponding technical replicate are referred to as 1st and 2nd measurement for sample #1, and 3rd and 4th measurement for sample #2, respectively. Data were normalized as described above.
The technical quality of the measurements was estimated separately for the two data sets, each comprising the data of the samples collected at the 11 time points of the time series. For each plasmodial sample, the relative deviation of the two measurements (1st and 2nd, or 3rd and 4th) from the mean of the two measurements was estimated. The frequency distributions of the deviations and corresponding quantile values indicated that the technical qualities of 1st and 2nd, as well as 3rd and 4th measurement were virtually identical with 95% of the values differing less than a factor of two from each other (Figure 2 and Table 1).

The degree of spatial variability of gene expression within a plasmodium was estimated by combining the data sets for the first and the second sample of a plasmodium taken at each time point of the time series. The frequency distribution of the deviation of each measurement from the mean of 1st, 2nd, 3rd, and 4th measurement of the two samples taken from each plasmodium at any time point was virtually identical to the frequency distributions obtained for the technical replicates, indicating that gene expression within the analyzed plasmodia varied at maximum within the limits of accuracy of the measurements (within a factor of 2 in 95% of the samples). This conclusion is based on the comparison of the quantile distributions of the data sets (Figure 2 and Table 1) considering a total of 36,540 data points.

**Multi-Dimensional Scaling Analysis**

With this data set, we investigated how expression changes as a function of time in the individual plasmodial cells. The gene expression pattern of a plasmodial cell at a given time point was obtained as the mean of the four expression values of each gene measured in the two plasmodial samples picked at that time point.

For visual representation of the data set and of single-cell trajectories of gene expression, we performed multidimensional scaling (MDS) to obtain a data point for the expression pattern of each cell at each time point. Single-cell trajectories of gene expression are shown in Figure 3. Notably, the gene expression patterns of un-stimulated cells (dark controls) changed as a function of time with the highest variability along coordinate 2 of the MDS plot Figures 3A,B. Trajectories of far-red stimulated cells (Figures 3C,D) moved from the left side to the right side of the plot, while the shape of individual trajectories varied to a certain extent, indicating that the response of the cells was similar though not identical. Obviously, the trajectories of six of the eight far-red-stimulated plasmodia of experiment #1 traversed a considerably larger area of the MDS plot (Figure 3C) as compared to the other stimulated cells, indicating a larger variation in gene expression during the response to the stimulus. The extent of variation is accordingly obvious when the bulk of data points is placed in the same plot (Figure 4A). To search for genes that may account for the scattering along coordinate 2, we visually inspected the individual time series displayed in the form of a heat map (Supplementary Figure 2). In addition to the genes that were clearly up- or down-regulated in response to the stimulus, the messages of four genes, hstA, nhpA, pcnA, and uchA,
in the following called pcnA-group genes, changed over time in some of the plasmodia, but there was no obvious consistent relationship to the time point of stimulus application. When only genes were included in the analysis that were clearly up- or down-regulated in response to the stimulus (Supplementary Figure 2, see also Figure 8), the data points of the MDS plot were indeed less scattered (Figure 4B). A qualitatively similar result was obtained plotting the up-regulated and the down-regulated genes separately (Figures 4C,D), with some more variation in the expression of the down-regulated genes. However, expression of the pcnA-group over time (Figure 4E) was clearly different from the up- or down-regulated genes. Expression of the pcnA-group genes was different between cells from experiments #1 and #2 as seen from the trajectories of the cells (Supplementary Figure 3), suggesting that ongoing internal processes in cells of experiment #1 might even influence their response to the light stimulus. Indeed, according to the corresponding MDS plots of the bulk data points (Supplementary Figure 4) the response of the up- and down-regulated genes was less uniformly in cells of experiment #1 (Supplementary Figure 4C) as compared to those of experiment #2 (Supplementary Figure 4D).

Construction and Graph Properties of Waddington Landscape Petri Nets

For a further analysis, we performed hierarchical clustering of the expression data for all assayed 35 genes, differentiation marker and reference genes (Supplementary Table 2), and identified significantly different clusters of expression patterns with the help of the Simprof algorithm (Clarke et al., 2008; Supplementary Figure 5). The temporal sequences of gene expression patterns classified as Simprof significant clusters defined a trajectory for each individual cell and revealed significant differences between cell trajectories (Table 2). To relate gene expression states and trajectories we constructed a Petri net (bipartite graph) as previously described (Werthmann and Marwan, 2017; Rätzel et al., 2020), by representing each gene expression state by a place and the temporal transit between two states by a transition (Figure 5). A single token marking one place of the Petri net indicates the current gene expression state of a cell. The token moves from its place to a downstream place when the transition, connecting the two places through directed arcs, fires. As each transition is connected to exactly two places (one pre-place and one post-place), tokens are neither formed nor destroyed when moving through the net, so the gene expression state of the cell remains unequivocally defined at any time. The coherent Petri net obtained this way represents a state machine predicting possible developmental trajectories in terms of Markov chains of gene expression states (Rätzel et al., 2020).

The basic modeling principles are summarized in Table 3. We observe the following structural properties of the model which we call ‘Waddington landscape Petri net’:

- Each transition has exactly one pre-place and one post-place.
- There are places having more than one post-transition. These post-transitions are in conflict. But, because every transition has exactly one preplace, each conflict is a free choice conflict, meaning the token is free to choose which route to take, predicting a corresponding free choice for the cell (see Discussion).
- There are places having more than one pre-transition, i.e., alternative paths may re-join. Thus, the Petri net structure does not form a tree.
- There are cycles: a cell may switch back to previous states or oscillate between states as defined by the expression patterns of the set of observed genes.

For technical reasons we add immediate transitions starting alternative trajectories, in order to get a statistical distribution of states in which the experiments have started or will start with a given probability.

In contrast to most state-of-the-art pseudo-time series approaches found in the literature (Saelsens et al., 2019), the structure of the Waddington landscape Petri net is not restricted to a partial order, meaning it is neither restricted to a directed acyclic graph nor to a tree. Instead we obtain what is known in Petri net theory as ‘state machine,’ also called in other communities ‘finite state machine’ or ‘finite automata,’ which may involve cycles.

A state machine with one token and its reachability graph, or Markov chain for stochastic Petri nets, are isomorphic (i.e., have the same structure, there is a 1-to-1 correspondence); to put it differently: our (stochastic) Petri net represents the

| TABLE 1 | Quantil distributions of the x-fold deviation (x) of a value from the mean of two or four values, characterizing the reproducibility of measurements as estimated through technical and biological replicates, respectively. |
|---|---|---|---|---|---|---|---|
| Measurements | 1st and 2nd | 3rd and 4th | 1st to 4th |
| Percent of values | Quantile (Log2 (x)) | Quantile (x) | Quantile (Log2 (x)) | Quantile (x) | Quantile (Log2 (x)) | Quantile (x) |
| 1% | -1.308 | 0.404 | -1.383 | 0.383 | -1.349 | 0.392 |
| 5% | -0.555 | 0.681 | -0.592 | 0.663 | -0.574 | 0.672 |
| 25% | -0.169 | 0.890 | -0.169 | 0.890 | -0.169 | 0.890 |
| 50% | -0.010 | 0.993 | -0.008 | 0.995 | -0.009 | 0.994 |
| 75% | 0.137 | 1.100 | 0.142 | 1.103 | 0.139 | 1.101 |
| 95% | 0.414 | 1.333 | 0.415 | 1.334 | 0.415 | 1.333 |
| 99% | 0.750 | 1.682 | 0.746 | 1.677 | 0.747 | 1.679 |

The table quantitatively characterizes the frequency distributions shown in Figure 2.
FIGURE 3 | Single cell trajectories of gene expression displayed after multidimensional scaling (MDS) of the expression patterns. Panels (A,B) show the trajectories of unstimulated cells of experiment #1 and experiment #2, respectively. Panels (C,D) show the trajectories of far-red stimulated cells of the two experiments. Each data point represents the gene expression pattern of a cell at a given time point at 1 h time intervals. The start position (0 h) of each trajectory is encoded in pink and the endpoint (10 h) in red. The number displayed in each subpanel refers to the ID number of the plasmodial cell (P1 to P24) as listed in Table 2. All cells of the dark controls did not sporulate while all far-red irradiated cells sporulated. All plots are displayed at the same scale.
FIGURE 4 | Gene expression patterns of all analyzed cells displayed for different sub sets of genes. Multidimensional scaling was performed for the complete set of 35 genes (A), the subset of the up- and the down-regulated genes (B), or exclusively for up-regulated (C), down-regulated (D) or the pcnA-group of genes (E). Each data point represents the expression pattern of an individual cell at a given time point. Time is encoded by color (0 h, pink; 1 h, 2 h, black; 3 h, 4 h, blue; 5 h, 6 h, green; 7 h, 8 h, ochre; 9 h, 10 h, red). Developmental destiny, cell number (as assigned in Table 2), and time is given for each data point. The label + P12_1 h, for example, indicates that the data point refers to the expression pattern of plasmodium number 12 as measured at 1 h after the start of the experiment (corresponding to the onset of the far-red light stimulus in light-stimulated cells) and that the plasmodium had sporulated (+) in response to the stimulus (+, sporulated; -, not sporulated). The percent of variance is given for each coordinate.

Markov chain of states the cells assume in the course of their developmental trajectory and accordingly on their walk through the Waddington landscape. We assume that the Petri net represents the corresponding region of the Waddington landscape predicting possible developmental paths a single cell can follow, which of course yields a state machine.

Representing Markov chains as Petri nets comes with a couple of advantages.

First, Petri nets are equipped with the concept of T-invariants, which belong to the standard body of Petri net theory from very early on (Lautenbach, 1973). We consider T-invariants as crucial in terms of biological interpretation of the generated net structures (Sackmann et al., 2006; Heiner, 2009). The computation of T-invariants is rather straightforward for state machines; due to their simple structure it holds:

- each cycle in a state machine defines a T-invariant, and
- each elementary cycle (no repetition of transitions) is a minimal T-invariant.

Second, modeling the differentiation-inducing stimuli, what we have not done so far, would turn some of the free choice conflicts into non-free choice conflicts, which involves, technically speaking, leaving the state machine net class. To unequivocally identify transits that are stimulus-dependent, we need a higher data density which we will hopefully achieve in one of our next experiments. With stimulus-dependent transitions, the constructed Petri nets and their Markov chains do not coincide anymore, instead the Markov chains as well as the reachability graph are directly derived from the Petri nets and may be analyzed by standard algorithms. Finally, our Petri net approach paves the way for the actual ultimate goal of our future work - reconstructing the underlying gene regulatory networks based on the reachability graphs encoded by the Waddington landscape Petri nets.

Characterization of Petri Nets

Construced From Gene Expression Data

Figure 6 displays a Petri net assembled from cell trajectories considering the set of 35 genes. The graphical representation laid out using the Sugiyama algorithm emphasizes the directionality of concurrent processes (Sugiyama et al., 1981). The net indicates that cells started in different states (connected to C0; see Legend to Figure 5 for details) and, after stimulation by far-red light, proceeded to a small set of terminal states (places C71, C76,
C77, C78, C79, C95) via multiple, more or less highly connected intermediate states. To facilitate the interpretation of the Petri net, we have colored the places and transitions according to different criteria. In Figure 6A, the transitions being specific to cells of experiments #1 or #2 and for dark controls or light-stimulated cells in the respective experiments, are colored differently. Each place is colored according to the relative frequency of its corresponding gene expression state, indicating that some states occurred more frequently than others. From this representation it is obvious that cells of experiments #1 and #2 form different branches, in part projecting onto different terminal states, reflecting accordingly different developmental trajectories to commitment and sporulation. Figure 6B displays the same Petri net, but with a different color coding. Here, transitions are colored according to how frequent the corresponding transitions occurred, indicating that some paths were more frequently taken than others. Places are colored according to their relative stability, defined as the average residence time of a cell in the respective state (see Methods for details). Coloring indicates that cells reached a terminal state through states of different stability, e.g., meta-stable intermediates.

Un-stimulated cells (Table 2, dark controls) spontaneously switched between significantly different states of gene expression. Their trajectories gave three disconnectedPetri nets (Figure 7A); the three nets were connected to C0 for technical reasons, see legend to Figure 5.

Petri nets of Figures 6A, B, 7A indicate that the expression pattern in both, stimulated and un-stimulated cells developed predominantly in forward directions while there were some transits back to previous states creating so-called transition-invariants (T-Invariants; Figure 5). We asked whether stimulus-independent temporal expression differences like those observed in the subset of the pcnA-group of genes might have added to this directedness. Therefore, we constructed a Petri net from trajectories based on significant clusters, this time exclusively clustering the subset of up- and down-regulated genes. Basic features found in the Petri nets of up- and down-regulated genes were similar to the ones found in the Petri nets for the full set of 35 genes: Trajectories formed parallel main branches, there were intermediate nodes of different stability, of different connectedness, and hence states that occurred with different frequency (Supplementary Figure 6). In contrast, there was a high number of minimal T-invariants that heavily involved places representing gene expression states that occurred in un-stimulated cells. A Petri net built by considering only transits that occurred in un-stimulated cells (Figure 7B) was nearly covered with T-Invariants, indicating spontaneous, reversible alterations in the expression of up- and/or down-regulated genes. Again, states of gene expression displayed different stability. Considerable variation in the expression level of the up-and down-regulated genes is even most obvious from the heat map of initial states from which trajectories emerged and of terminal states that were observed during the experiment (Figure 8). The high density of T-invariants in the un-stimulated cells suggests that similarly, the T-invariants involving places corresponding to light-stimulated cells are due to gene expression changes that do spontaneously occur before cells are caught by a new attractor formed in response to the far-red stimulus (see Discussion).

Figure 7 also shows that selecting sets or subsets of genes for hierarchical clustering and subsequent Petri net construction may yield Petri nets of different structure delivering accordingly non-redundant information on corresponding subsets. This is also shown in Table 4 for the set of 35 genes and for subsets, the
down-regulated, up-regulated, down- and up-regulated, and the \textit{pcnA}-group of genes. Except for the \textit{pcnA}-group, the number of places per gene was approximately the same. The number of transitions per gene however became less with more genes considered. This suggests that up-regulation, down-regulation and even expression of the \textit{pcnA}-group of genes are at least partly coordinated or co-regulated processes. The average number of minimal T-invariants per gene was approximately the same. The number of transitions per gene however became less with more genes considered. This suggests that up-regulation, down-regulation and even expression of the \textit{pcnA}-group of genes are at least partly coordinated or co-regulated processes. The average number of minimal T-invariants per gene compared for the different groups of genes (Table 4) suggests that reversibility observed for the subsets vanishes when more genes are considered, obviously due to combinatorial effects.

**Table 2** | Single cell trajectories of gene expression.

| Experiment | Cell | 0.0 h | 1.0 h | 2.0 h | 3.0 h | 4.0 h | 5.0 h | 6.0 h | 7.0 h | 8.0 h | 9.0 h | 10.0 h |
|------------|-----|------|------|------|------|------|------|------|------|------|------|-------|
| Exp #1, Dark | P1  | 60   | 56   | 59   | 26   | 35   | 34   | 34   | 28   | 28   | 32   | 31    |
| Exp #1, Dark | P2  | 57   | 57   | 60   | 63   | 35   | 35   | 35   | 14   | 32   | 32   | 32    |
| Exp #1, Dark | P3  | 59   | 56   | 26   | 36   | 34   | 34   | 28   | 13   | 31   | 32   | 32    |
| Exp #1, Dark | P4  | 37   | 37   | 37   | 33   | 28   | 28   | 28   | 15   | 29   | 30   | 30    |
| Exp #2, Dark | P5  | 24   | 52   | 52   | 52   | 49   | 52   | 52   | 52   | 48   | 47   | 48    |
| Exp #2, Dark | P6  | 25   | 45   | 45   | 44   | 44   | 43   | 43   | 21   | 7    | 5    | 1     |
| Exp #2, Dark | P7  | 3    | 3    | 3    | 11   | 12   | 63   | 63   | 63   | 60   | 60   | 61    |
| Exp #2, Dark | P8  | 46   | 45   | 45   | 42   | 42   | 42   | 42   | 21   | 7    | 6    | 1     |
| Exp #1, Far-red | P9 | 33  | 23   | 27   | 16   | 101  | 111  | 105  | 87   | 88   | 70   | 79    |
| Exp #1, Far-red | P10 | 36   | 23   | 27   | 16   | 88   | 111  | 105  | 89   | 80   | 75   | 78    |
| Exp #1, Far-red | P11 | 10   | 53   | 54   | 54   | 15   | 96   | 100  | 100  | 89   | 91   | 93    |
| Exp #1, Far-red | P12 | 53   | 55   | 54   | 54   | 15   | 100  | 100  | 100  | 89   | 91   | 92    |
| Exp #1, Far-red | P13 | 8    | 4    | 4    | 12   | 53   | 69   | 69   | 69   | 68   | 66   | 65    |
| Exp #1, Far-red | P14 | 22   | 4    | 2    | 9    | 53   | 69   | 69   | 69   | 68   | 66   | 65    |
| Exp #1, Far-red | P15 | 22   | 4    | 2    | 9    | 96   | 96   | 69   | 69   | 67   | 64   | 94    |
| Exp #1, Far-red | P16 | 62   | 55   | 58   | 54   | 97   | 99   | 89   | 90   | 93   | 93   | 75    |
| Exp #2, Far-red | P17 | 50   | 40   | 45   | 19   | 104  | 103  | 105  | 105  | 87   | 82   | 72    |
| Exp #2, Far-red | P18 | 51   | 41   | 25   | 19   | 104  | 102  | 110  | 85   | 81   | 72   | 77    |
| Exp #2, Far-red | P19 | 20   | 20   | 20   | 17   | 18   | 102  | 109  | 83   | 82   | 72   | 77    |
| Exp #2, Far-red | P20 | 44   | 40   | 45   | 19   | 103  | 108  | 110  | 84   | 82   | 73   | 77    |
| Exp #2, Far-red | P21 | 51   | 39   | 45   | 19   | 103  | 106  | 110  | 84   | 82   | 74   | 76    |
| Exp #2, Far-red | P22 | 52   | 39   | 45   | 19   | 103  | 107  | 110  | 86   | 81   | 73   | 76    |
| Exp #2, Far-red | P23 | 46   | 39   | 45   | 19   | 103  | 107  | 110  | 86   | 81   | 73   | 77    |
| Exp #2, Far-red | P24 | 24   | 38   | 45   | 19   | 104  | 108  | 110  | 84   | 81   | 74   | 77    |

Trajectories are displayed as temporal sequences of gene expression states. Each state is given by the cluster ID number to which it was assigned by the Simprof algorithm. The two experiments, Exp #1 and Exp #2, were performed on two different days, respectively, with the same strain (LU897 × LU898) and under virtually identical experimental conditions. All cells of the dark controls did not sporulate while all far-red irradiated cells sporulated.

**Table 3** | Terminology, basic modeling principles, and Petri net elements.

| Place | Cellular state as defined by a significant cluster of gene expression patterns. |
|-------|--------------------------------------------------------------------------------|
| Transition | The cellular state in which an experiment (recording of a time series) starts. There can be various source nodes, depending on the particular state in which a cell is in the moment when the experiment starts. |
| Source place (no predecessor node) | Place with no outgoing arc, indicating a terminal state of gene expression reached at the end of the experiment. There can be multiple sink nodes. |
| Sink node (no successor node) | Forward branching places modeling bifurcation. |
| Conflict | The token indicates the cell and its current gene expression state; there is always just a single token. |
| Path | A single path from a source node to a sink node represents a possible developmental trajectory of a single cell. But a trajectory may not necessarily involve a source node and/or a sink node. |
| Petri net | The entire Petri net gives, for the genes analyzed, that part of the Waddington landscape through which cells passed and accordingly all corresponding developmental trajectories a single cell may undergo. |

**Single Cell Trajectories Reveal Qualitatively Different Patterns in Differential Gene Regulation**

We have argued that Petri net modeling disentangles the complex response of cell reprogramming (Rätzel et al., 2020) and predicts feasible developmental pathways through the Waddington landscape, resulting in significantly distinct single cell trajectories. To reveal similarities and differences of the expression kinetics of individual genes, we plot the geometric mean of the concentration values of the mRNA in a cluster.
FIGURE 6 | Two copies of the same Petri net, automatically constructed from the single cell trajectories of gene expression as displayed in Table 2. Places and transitions in the two nets were colored according to different criteria. Arcs as part of T-invariants are highlighted in blue. (A) As indicated by the rainbow color key, places are colored according to the relative frequency with which respective gene expression states occurred in the data set. Transitions are colored according to whether corresponding transits occurred in experiments #1 or #2, and whether cells were far-red stimulated or un-stimulated (dark controls), respectively, as indicated by the panel lower right. (B) Places are color coded according to the relative stability of the states of gene expression they represent. This relative stability indicates how long a cell on average resided in a certain state. The color of transitions indicates, in absolute numbers, how frequent a corresponding transit occurred in the data set.

logarithmically, normalized to its concentration at the start of the experiment \((t = 0 \, \text{h})\) as a function of time for any single cell trajectory. In this kind of plot, the time course of the mRNA of each gene starts at the same point, while the slope of the curve indicates the x-fold change in mRNA abundance over time. Plotting subsets of genes suggests that trajectories through different regions of the Petri net of Figure 6 indeed emerge from qualitatively different expression kinetics, and that genes are also differently regulated relative to each other when different trajectories are compared. In the example shown in Figure 9, \(pldA\) is early up-regulated in quite a number of trajectories, followed by \(pwiA\) and finally by \(ligA\) and \(rgsA\) that appear strongly correlated at least in some of the plots. Qualitatively different patterns of regulation relative to each other are also evident for the three phospholipase D-encoding genes (Supplementary Figure 7). The \(pldA\) gene is up-regulated while \(pldB\) and \(pldC\) are down-regulated. In some of the trajectories, the initial change in the concentration of the \(pldA\) and \(pldC\) mRNAs is inverse as compared to the overall time course. A more comprehensive representation with more genes displayed makes similarities and differences between trajectories even more obvious (Supplementary Figure 8). Here, we observe a phenomenon, which is also seen in Table 2, namely that cells remain in a certain state for some time. This occurs...
FIGURE 7 | Petri nets constructed for different sets of genes from the gene expression trajectories of un-stimulated cells. (A) Trajectories considering the complete set of 35 genes yield three disconnected Petri nets containing a low number of T-invariants (as indicated by arcs highlighted in blue). (B) When only those genes are considered that are up- or down-regulated in response to far-red stimulation (omitting the pcnA-group of genes), the trajectories of un-stimulated cells give one coherent Petri net which is almost covered with T-invariants. Color coding of places indicates the relative frequencies of states of gene expression in un-stimulated cells. Differences in gene expression patterns of un-stimulated cells between the cells of experiment #1 and #2 are indicated by the appearance of experiment-specific transits and accordingly differently colored transitions.

predominantly in the unstimulated cells but is also seen in some of the light stimulated cells, e.g., in those that proceed to state C95 (Figures 6A,B). Cells seemingly are trapped in a metastable state (e.g., C96, C69, C100, etc.; Figure 6B) for some time until the developmental program proceeds. We presumably will need more data to see whether this is an artifact which occurs by the discretization of gene expression through clustering. Conversely, discretization might help to identify tipping points for the differential regulation of gene expression as the plots in Supplementary Figure 8 suggest.

DISCUSSION

We have analyzed the gene expression dynamics in response to a differentiation-inducing stimulus pulse in true time by repeatedly taking samples of large, multinucleate plasmodial cells. Control experiments have demonstrated that the gene expression patterns in samples simultaneously retrieved from different sites of a large plasmodial cell did not deviate within the range of the technical accuracy of the measurements. This again confirms that the plasmodial cytoplasm, at least at the level of macroscopic sampling, can be considered as a homogeneous reaction volume.

The injuries caused by multiple sampling of a plasmodial cell heal spontaneously and cutting the plasmodial mass neither induces nor prevents sporulation (Starostzik and Marwan, 1994, 1995b, 1998; Rätzel and Marwan, 2015). This is also confirmed for the large plasmodia used in this study through the dark controls that did not sporulate (Table 2 and Supplementary Figure 1), while the far-red light induced plasmodia sporulated. The changes in gene expression patterns observed in the dark controls seem to be spontaneous and not caused by repeated
FIGURE 8 | Heat map visualizing the variability of initial and terminal states of gene expression. The initial states displayed in this panel are the start points of trajectories of un-stimulated cells as listed in Table 2. The terminal states are endpoints of trajectories of far-red stimulated cells corresponding to terminal places of the Petri net of Figure 6. Color-coded expression values correspond to the logarithm to the base 10 of the geometric mean of all expression values of a respective cluster with its ID number indicated on the right side of the panel. For clarity, expression of the pcnA-group of genes is displayed as a separate block.

sampling for the following reasons. First, plasmodia are in different states of gene expression already at the start of the experiments (Figure 8 and Table 2), i.e., at the moment before the first sample is taken, so sampling cannot be the reason for this heterogeneity. Second, the trajectories of unstimulated cells developed in different directions although the sampling procedure was the same in all of these cells (Figures 3A,B), indicating that the shift in gene expression was not directed with respect to the start of the experiment. Third, the Petri net considering the differential regulation of the subset of light-regulated genes, the expression of which changed to some extent even in unstimulated cells, is almost completely covered with T-invariants (Figure 7B), indicating no directed change in the expression of this set of light-regulated genes, neither with respect to the start of the experiment nor with respect to the initial states in which the cells resided before the first sample was taken. We cannot exclude that certain genes might be differentially regulated in response to injury, e.g., genes involved in membrane biosynthesis, as leaks in the membrane readily heal. However, there seems to be no systematic effect on the differential expression of the genes analyzed in the present study.

In contrast to a typical mammalian cell, which has a relatively small cytoplasmic volume, while many genes are present in two copies only, the plasmodial cell contains many millions of nuclei. These nuclei are suspended in a large cytoplasmic volume which continually mixes by the vigorous shuttle streaming. The T-invariants in the Petri nets of Figure 7B and Supplementary Figure 6 indicating the up- and down-regulation of genes were due to changes in the mRNA concentration that occurred at distant located sites of the plasmodium at the same time. Hence, it seems unlikely that these changes are stochastic gene expression noise. Instead, the T-invariants, presumably do reflect the (non-linear) dynamics of the system. Non-linear, switch-like behavior, bifurcations, multistability, or oscillations all certainly do have fundamental biological and functional implications, and the T-invariant analysis of true single cell time series can help to identify them.

Gene expression states of the cells were defined by hierarchical clustering and discretized by assigning each gene expression pattern to a Simprof significant cluster (Clarke et al., 2008; Rätzel et al., 2020). Trajectories of subsequent discrete states were then assembled into a state machine implemented as a Petri net. In the Petri net, each gene expression state is represented by a place and each transit between two states is represented by a transition. The Petri net, as it has been defined in this and previous studies depends on the data pre-processing by clustering of the data...
It models gene expression trajectories as Markov chains (Gagniuc, 2017), which assumes that each subsequent state only depends on the current state of a cell and not on its previous states, i.e., it does not depend on the individual history of a cell. This assumption is commonly made by computing pseudo-time series from snapshots of individual mammalian cells (Bendall et al., 2014; Shin et al., 2015; Haghverdi et al., 2016; Marr et al., 2016; Street et al., 2018; Weinreb et al., 2018; Chen et al., 2019; Setty et al., 2019). It follows the principle of parsimony in making not more assumptions than necessary and giving the simplest possible explanation for an observed phenomenon. Practically this means that any path which a token can take through the Petri net, by stochastic firing of the transitions, translates into a feasible trajectory of an individual cell. Hence, firing of a transition does only depend on the marking of the pre-place of this transition and not on the identity of any upstream places from which the token originally came. Defining the cell’s state of gene expression by measuring more genes might well diversify places and hence change the structure of the Petri net. This has been demonstrated by constructing nets from subsets of genes. In the examples provided, the structure of the Petri net changed and the number of T-invariants increased drastically upon reduction of the number of considered genes (Table 4).

If the structure of the Petri net does depend on the set of genes analyzed, what is its actual value? The actual value is that it reveals the behavior of states defined by sets or subsets of genes. Limiting the analysis to the chosen subset of up- and down-regulated genes, as we have done here, revealed extensive on- and off-switching of the genes in unstimulated cells that are differentially regulated in response to a differentiation-inducing stimulus. This became immediately obvious through structural analysis of the Petri net by determining the number of minimal T-invariants. Displaying the net in Sugiymama representation revealed another phenomenon with respect to this subset of genes. The light stimulus caused directed development toward a small number of terminal states reducing the overall number of alternative states in which the cells resided. This suggests that a cellular attractor is formed in response to the stimulus causing the commitment to differentiation.

Coloring the transitions of the Petri net according to the frequency by which transits occurred, allows identification and visualization of main paths, i.e., paths which the system preferably took. Coloring places according to the relative stability of the states they represent indicated metastable states that were not necessarily identical to highly connected places. Places having many pre-transitions (many incoming arcs) represent states, the system is likely to assume, like a corrie in the metaphor of the Waddington landscape, through which the system will pass. Places having many post-transitions (many outgoing arcs) represent branching points from which the system has multiple options to proceed.

Our analysis has confirmed former observations (Rätzel et al., 2020), now at considerably larger resolution in time, that unstimulated cells spontaneously and reversibly change their expression pattern. These changes involved the expression of genes that are differentially regulated in response to a differentiation-inducing stimulus. Spontaneous switching of gene expression patterns is at least one reason why stimulated cells started their way to commitment and differentiation from quite different states, indicating substantial heterogeneity in the population of cells. In other words, cells can start differentiation while being in various different states. The differentiation-inducing stimulus then collects or focusses these cells onto a narrow set of states like an attractor of a dynamic system would do. This phenomenon is graphically revealed by the funnel- or cone-like appearance of the Petri net in the Sugiymama layout (Supplementary Figure 6).

The response of a cell to a differentiation-inducing stimulus seems to depend on the cell’s current internal state. Figure 6A revealed distinct main branches (visible through transitions of different color) for cells from experiment #1 as compared to experiment #2, suggesting that the response of the cell in terms of its developmental pathway did indeed depend on the initial physiological or gene expression state in which the cell resided while receiving the stimulus. The cells proceeded to slightly different terminal states that however might belong to the same cellular attractor.

One might be tempted to suspect a certain structure in the list of subsequently recorded trajectories (Table 2). Changes in the initial state of the plasmodium until the time of stimulus application might have occurred as the experiment proceeded. Similarities in subsequently recorded trajectories may be by chance and we cannot draw any final conclusion because the number of analyzed plasmodia is by far too low. With more plasmodia analyzed and more genes measured, we might discover that the individual history of a cell indeed matters, meaning that the Markov assumption is wrong. Even if this should be the case, the Petri net representation would still be valid, however with the firing probability of certain transitions meaning that the Markov assumption is wrong. Even if this should be the case, the Petri net representation would still be valid, however with the firing probability of certain transitions depending on which path the token came from. Technically, this dependency could be implemented in the form of a colored Petri net. In this context it is trivial and at the same time

| Gene set          | Down | Up  | Up and down | All 35 | pcnA etc. |
|-------------------|------|-----|-------------|--------|-----------|
| Genes             | 10   | 10  | 20          | 35     | 4         |
| Places            | 35   | 44  | 69          | 111    | 24        |
| Transitions       | 105  | 114 | 144         | 159    | 59        |
| P/Gene            | 3.5  | 4.4 | 3.5         | 3.2    | 6.0       |
| T/Genes           | 10.5 | 11.4| 7.2         | 4.5    | 14.8      |
| Time points       | 11   | 11  | 11          | 11     | 11        |
| Cells             | 24   | 24  | 24          | 24     | 24        |
| P/(Genes × tps × cells) | 0.013 | 0.017 | 0.013 | 0.012 | 0.023 |
| T/(Genes × tps × cells) | 0.040 | 0.043 | 0.027 | 0.017 | 0.056 |
| T/P               | 3.00 | 2.59| 2.09        | 1.43   | 2.46      |
| T-lnv             | 4,413| 1,371| 1,063       | 7      | 732       |
| T-lnv/Gene        | 441  | 137 | 53          | 0.20   | 183       |

P: places; T: transitions; tps: time points; T-lnv: T-invariants.
FIGURE 9 | Gene expression kinetics as derived from single cell trajectories. Each panel represents the trajectory of one single cell as characterized by subsequent states of expression of the same, arbitrarily chosen set of genes. For each gene, the logarithm to the base 10 of the geometric mean of the expression values of each cluster was plotted against time. Plotting the geometric mean instead of individual expression values results in discretization of the data while comparing different trajectories.
important to note that the identity of a place (i.e., state) is always defined by the set of genes that have been measured. Measuring more genes might split any place into more places or even into a separate Petri net with an increased overall number of places. This holds not only for states defined by gene expression but also for cellular states defined by the covalent modification of proteins, etc.

We have previously argued that the Petri net depicts aspects of the topology of the Waddington landscape (Waddington, 1957; Huang et al., 2009) with respect to and limited to the set of observed genes (or measured molecular entities) (Werthmann and Marwan, 2017; Rätzel et al., 2020). Then, the token in the Petri net corresponds to the marble rolling down the Waddington landscape as developmental processes unfold. Each Petri net place represents, albeit implicitly, a significantly distinct gene expression state. Despite this implicit representation, the temporal information on each gene for each cell trajectory is available and we have used this information to reveal the temporal hierarchy of differentially regulated genes. The Petri net representation disentangles accordingly the complex gene expression response and identifies alternative regulatory programs or routes. Using this information, the underlying regulatory network can be inferred by applying appropriate algorithms (Marwan et al., 2008; Durzinsky et al., 2011, 2013). This is a possible next step to go.

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DATA AVAILABILITY STATEMENT
The raw data supporting the conclusions of this article are provided as part of the Supplementary Material.

AUTHOR CONTRIBUTIONS
AP and SP performed single cell time-series experiments and gene expression analyses and evaluated their results. MHa supervised the experimental work and developed the sample preparation method together with SP. MHe essentially contributed the analysis of the graph-theoretical properties of Petri nets and wrote a corresponding section of the manuscript. WM conceived and supervised the study, performed computational analyses including the automated generation of the Petri nets and wrote the manuscript. All authors read and approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.612256/full#supplementary-material

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