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Evidence of Noisy Oscillations of cAMP under Nutritional Stress Condition in Budding Yeast

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Abstract: The Ras/cAMP/PKA pathway regulates responses to nutrients' availability and stress in budding yeast. The cAMP levels are subjected to negative feedback, and we have previously simulated a dynamic model of this pathway suggesting the existence of stable oscillatory states depending on the symmetrical and opposed activity of the RasGEF (Cdc25) and RasGAPs (Ira proteins). Noisy oscillations related to the activity of this pathway were reported by looking at the nuclear localization of the transcription factor Msn2, and sustained oscillations of the nuclear accumulation of Msn2 under the condition of limiting glucose were observed. We were able to reproduce the periodic accumulation of Msn2-GFP protein in a yeast cell under the condition of limiting glucose, and we also detected oscillations of cAMP. We used a sensor based on a fusion protein between YFP-Epac2-CFP expressed in yeast cells. The FRET between CFP and YFP is controlled by the cAMP concentration. This sensor allows us to monitor changes in cAMP concentrations in a single yeast cell over a long time. Using this method, we were able to detect noisy oscillations of cAMP levels in single yeast cells under conditions of nutritional stress caused by limiting glucose availability.

Keywords: *Saccharomyces cerevisiae*; cyclic AMP; FRET; protein kinase A; oscillations

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1. Introduction

In the yeast *Saccharomyces cerevisiae*, the cAMP/PKA pathway plays a relevant role in the control of metabolism, stress resistance, and proliferation [1–5]. The key component of this pathway is adenylate cyclase, whose activity is controlled by two G-proteins, the Ras proteins and the Gα protein Gpa2 [6,7]. Cyclic AMP is synthesized by adenylate cyclase, encoded by *CYR1* gene, and induces the activation of the cAMP-dependent protein kinase (PKA). When activated, PKA phosphorylates many proteins involved in key cellular processes. The whole signaling cascade is tightly regulated, and experimental evidence indicates that multiple feedback mechanisms operate within the pathway through the generation of a complex interplay between the cascade components [8–10].

Ras proteins are positively regulated by the activity of Cdc25, which stimulates the GDP-GTP exchange, and are negatively regulated by Ira1 and Ira2, which stimulate the intrinsic GTPase activity. The degradation of cAMP is carried out by phosphodiesterases that constitute the major feedback mechanism in the pathway [8], although Colombo et al. [9] demonstrated that the feedback inhibition mechanism also acts by changing the Ras2 protein activation state. PKA phosphorylates Cdc25, reducing its exchange activity [11], and Ira proteins likely regulate its activity [12,13]. A symmetrical action between Ras activators and inhibitor is required in order to maintain a steady state.

We have previously developed and simulated a dynamic model of the whole pathway, and our results suggest the existence of stable oscillatory states that depend on the activity of the Ras activator (Cdc25) and of Ras inhibitors (Ira proteins) [10]. Moreover, we found

that stable oscillatory regimes of intracellular cAMP levels can only be established when a feedback operating on Ira proteins is activated and that the insurgence of the oscillations is also deeply affected by the intracellular GTP/GDP ratio, linking the transition between stable steady states and oscillations to reduced nutritional conditions. Indeed, under these conditions, the asymmetry between the activity of the activator (Cdc25) and of inhibitors (Ira proteins) is probably related to the insurgence of the oscillations, also triggered by significant molecular noise [10]. Sustained oscillations of the cAMP level have never been observed in budding yeast, although in some cases damped oscillations were reported after the addition of glucose to glucose-derepressed cells [14] in yeast populations.

However, the existence of sustained oscillatory states can be inferred indirectly by looking at the nuclear localization of the transcription factors Msn2 and Msn4. In fact, noisy irregular oscillations related to the activity of the PKA pathway were reported by looking at the nuclear localization of the transcription factors Msn2 and Msn4 [15–17]. In particular, Medvedik et al. [17] reported sustained oscillations of the nuclear accumulation of Msn2 under conditions of a limited glucose availability.

Although the cAMP/PKA pathway has been extensively studied in yeast and both upstream and downstream elements are known, the changes in cAMP levels and the activity of this pathway have usually been measured in cell populations over a very short time. Therefore, there are very few data on the spatiotemporal variation of cAMP and PKA activity in single cells. Some years ago, Nikolaev et al. developed Fluorescence Resonance Energy Transfer (FRET) probes that were EPAC-based in order to monitor cAMP levels in vivo in single mammalian cells [18]. These sensors consist of part of the cAMP-binding protein Epac1 or Epac2 sandwiched between cyan and yellow fluorescent proteins (CFP and YFP). The construct unfolds upon binding of cAMP to the EPAC moiety, and cAMP increases are thus easily monitored as a drop in FRET [18]. We previously adapted these Epac-based probes for expression in yeast vectors, and we found a specific FRET signal that was clearly related to changes of cAMP on single yeast cells using a confocal microscope [19]. Here, we used this FRET-based cAMP sensor to evidence the presence of a sustained oscillation of cAMP in single yeast cells under the condition of limiting glucose (0.1%), i.e., under nutritional stress.

2. Materials and Methods

2.1. Yeast Strains and Plasmid

Strains used in this study: SP1 (*MATa his3 leu2 ura3 trp1 ade8 can1*) [20]; BY4741-YMR037C (*MATa his3 leu2 ura3 MSN2*-GFP) (Invitrogen, Carlsbad, CA, USA), SP1 [pYX212-YFP-Epac2-CFP] [19]. To obtain the pYX212-YFP-Epac2-CFP plasmid, we used the following strategy. The YFP-Epac2-CFP fragment, obtained by digesting the pcDNA3- YFP-Epac2-CFP vector (kindly provided by Dr. V.O. Nikolaev, University of Wuerzburg, Wuerzburg, Germany) [18] with *Xho*I and *Hind*III restriction endonucleases, was ligated into the expression vector pYX212, digested with the same enzymes [19].

2.2. Media and Growth Conditions

Yeast cells (SP1 and BY4741-YMR037C) were grown in synthetic complete media (SD) containing 2% glucose, 6.7 g/L YNB *w/o* amino acids (supplied by Formedium, Hunstanton, UK) and 0.8 g/L CSM (Complete Synthetic Medium + adenine, DCS0021 supplied by Formedium, Hunstanton, UK) at 30 °C in shaken flasks.

The yeast strain SP1 transformed with pXY212-YFP-Epac2-CFP plasmid was grown in SC-ura glucose medium (2% glucose, 6.7 g/L YNB *w/o* aminoacids) and 0.77 g/L CSM drop-out (without uracil) (DCS0161, CSM, Single Drop-Out-Ura (Formedium, Hunstanton, UK)).

Culture density was measured with a Coulter Counter (Coulter mod. Z2) on mildly sonicated samples.

2.3. Fluorescence Microscopy and FRET Determination

Cells were grown in SC-ura medium containing 2% glucose at 30 °C until the exponential phase, collected by centrifugation, washed two times with SC-ura medium containing 0.1% glucose, suspended in SC-ura + 0.1% glucose-medium at a density of 5×10^7 cells/mL and incubated at 30 °C for at least 1 h. Subsequently, 40 µL of cell suspension were seeded on concanavalin A (ConA) (Sigma-Aldrich, Milano, Italy)-coated cover glass for 10 min [19]. The cover glass was washed four times with SC-ura + 0.1% glucose medium, mounted on a custom chamber and covered with 500 µL of the same medium. Time stacks of images (512 × 512 pixels, typical field of view 150 µm × 150 µm, 400 Hz scanning frequency) were acquired by means of a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) through a 40× oil objective (HCX PL APO CS 1.30). The pinhole was set at 150–170 µm in order to detect a higher signal from each cell and to avoid losing the focal plane in long time acquisitions. Cyan Fluorescent Protein (CFP) was excited at 458 nm and its emission was detected in the range 468–494 nm, while Yellow Fluorescent Protein (YFP) emission was detected in the range 530–600 nm. The acquisition (one image every 2.6 s) started without interruption for at least 60 min. We previously showed that under these conditions, the fluorescence signal was stable for a long time with minimal photobleaching [19].

For each sample, image time series have been acquired while selecting a field of view populated with more than 50 cells. After acquisition, data were analyzed by means of the Leica Application Suite Software (version 4.0, Leica Microsystem, Wetzlar, Germany). A ROI (Region of Interest) was selected that included each cell, and the CFP and YFP fluorescence signals in the two acquisition channels have been saved together with their ratio versus time. In this way, both the single cell behavior and average values were calculated for each sample. The raw data were then further elaborated with Excel.

For the analysis of Msn2-Green Fluorescent Protein (GFP), nuclear localization yeast cells (BY4741-YMR037C) expressing the Msn2-GFP fusion protein were grown until the exponential phase in synthetic medium (SD + 2% glucose). Cells were collected by centrifugation (5 min at 3000 rpm), washed two times with SD medium containing 0.1% glucose and suspended in SD + 0.1% glucose medium at a density of 5×10^7 cells/mL. After incubation for 60 min at 30 °C, an aliquot (20 µL) of cell suspension was seeded on a coverslip coated with concanavalin-A [19] and put on top of a Thoma chamber. Images were acquired with a Nikon Eclipse 90i fluorescence microscope equipped with a 60× oil immersion objective with GFP adapted filters, and photographs were taken at specific time intervals. To avoid bleaching, the fluorescence images were acquired for 1 s every 2.5 min, and the shutter was kept off in the meantime. Images were analyzed with ImageJ software (https://imagej.nih.gov/ij/index.html, version for Windows 64 bit (accessed on 30 July 2023)) in order to calculate the ratios of average nuclear intensity versus average cytoplasmic intensity of green fluorescence. Briefly, for each cell, a ROI (elliptical area) that comprised the whole cell allowed for the calculation of the average fluorescence intensity. Then, a smaller ROI, which comprised only the nucleus, allowed for the calculation of the average nuclear fluorescence. The ratio was calculated, and if the fluorescence of the nucleus was equal or lower than the whole cell, the ratio would be equal to or less than 1, while if the fluorescence of the nucleus was higher, the ratio would be higher than 1.

2.4. Analysis of Periodicity

Spectral analysis of raw data was conducted using the software Kyplot 5 ™, running on a PC (Windows 7) (http://www.kyenslab.com/en/kyplot.html (accessed on 30 July 2023)). A noise reduction (smoothing) was sometimes performed using a moving average of 20 experimental points in Excel. The raw or smoothed data were also used for Recurrence analysis [21] (Visual Recurrence Analysis Software: http://www.visualization-2002.org/VRA_MAIN_PAGE_.html (accessed on 30 July 2023)). Simulated cAMP data were obtained with the software BioSimWare [10], using a personal computer running on Windows XP. All stochastic simulations were performed by exploiting the tau-leaping algorithm [22].

2.5. Abbreviations Used

A list of used abbreviations is enclosed in the Supplementary Materials Section.

3. Results and Discussion

3.1. Nutritional Stress Condition Induces Stochastic Periodic Nuclear Localization of Msn2 Protein

We have previously developed and simulated a dynamic model of the Ras/cAMP/PKA pathway in budding yeast, and our results suggest the existence of stable oscillatory states that depend on the activity of the Ras activator (Cdc25) and Ras inhibitors (Ira proteins) [10]. An example of these oscillatory regimes is shown in the Supplementary Materials (Figure S1).

In addition, we have shown that cAMP levels and PKA activity can be measured in single yeast cells using specific FRET probes [19]. Therefore, we investigated the possibility of evidencing sustained cAMP oscillations in single yeast cells using the YFP-Epac2-CFP probe [18,19]. In preliminary experiments, we tested the conditions required to induce oscillations in the nuclear localization of Msn2 protein. To this end, we used a yeast strain expressing a fusion of Msn2 protein with eGFP. In this strain, the green fluorescence was diffused in the cytoplasm when the cells were growing in glucose medium and relocalized in the nucleus after glucose starvation [17]. Under conditions of limited glucose availability (0.1% glucose, i.e., in nutritional stress), we were able to evidence the insurgence of asynchronous oscillations (Figure 1 and Supplementary Movie S1), in agreement with data reported previously by Medvedik et al. [17]. As shown in Figure 1, each cell behaves in a different and asynchronous way, with a periodicity in the range of 5–10 min.

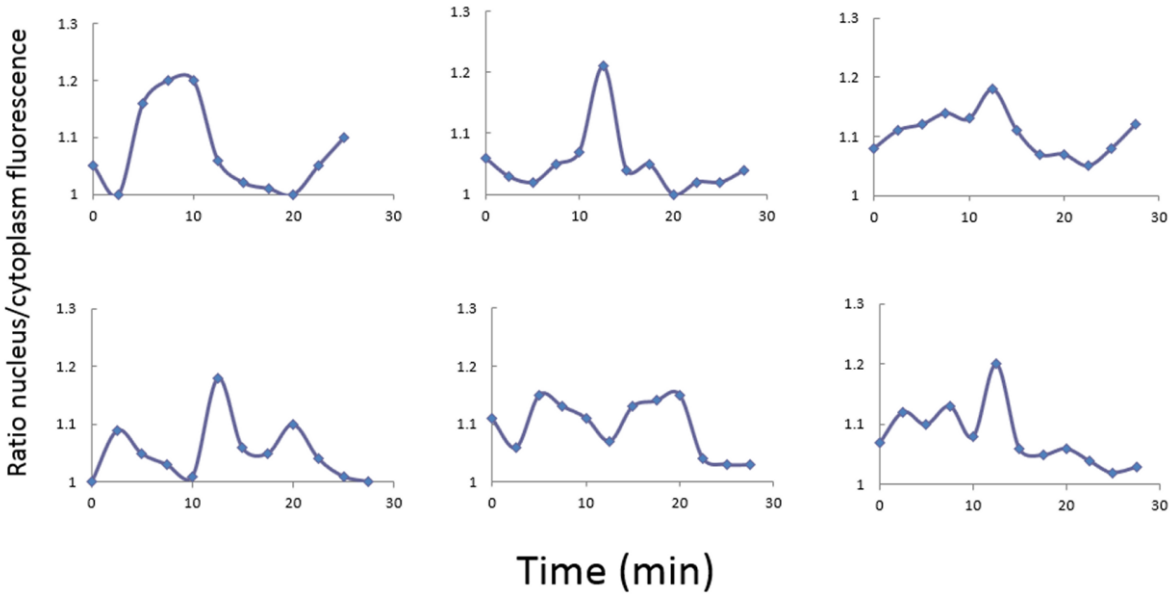

Figure 1. Time course of localization of Msn2-eGFP protein in the nucleus of six yeast cells. The yeast strain BY4741-YMR037C expressing a fusion protein Msn2-eGFP was used, and the oscillations were observed after resuspension of yeast cells in low-glucose medium (0.1% glucose), as described in Materials and Methods. The ratio nucleus/cytoplasm fluorescence was estimated by the intensities of GFP fluorescence measured in the two compartments on microscopic images taken every 2.5 min.

3.2. Nutritional Stress Induces Noisy Oscillation of cAMP in Single Yeast Cells

To evaluate the presence of oscillations of cAMP levels in yeast cells, we used an SP1 strain expressing the FRET probe YFP-Epac2-CFP [19]. This strain was grown exponentially in 2% glucose synthetic selective (SC-ura) medium, and then the yeast cells were resuspended in the same medium containing a reduced amount of glucose (0.1% *w*/*v*) and treated as described in Materials and Methods. The yeast cells, incubated in a small chamber, were observed with a Confocal Inverted Microscope, and time-stacked

images were acquired, as reported in Materials and Methods. An example of the acquired images is shown in Supplementary Figure S2. After acquisition of a time series of images, a Circular Region (ROI) was selected that included a single yeast cell, and the CFP and YFP fluorescence signals in the two acquisition channels were measured and saved together with their ratio versus time. In this way, both the single cell behavior and average values were calculated for each sample.

We collected time series for more than 100 single cells, and we found a lot of random noise in the fluorescence ratio used for FRET analysis (YFP/CFP fluorescence); an example of raw data is shown in Figure 2. We used two different approaches to filter the noise in order to evidence the eventual presence of oscillations: a simple moving average window that is sufficient to reduce the noise, and a more sophisticated Spectral Analysis of raw data (performed on 76 single cells) that evidences the presence of low-frequency components that are well-separated from higher-frequency noise. Both methods evidenced a high heterogeneity in the behavior of single yeast cells. In many of them (50 out of 76 cells), only a noise was apparent, without any specific low-frequency oscillations (examples are shown in Figure 3); in other single cells, low-frequency oscillations with a period in the range of 4–6 min were detectable immediately after the start of data acquisition (Figure 4) (12 cells).

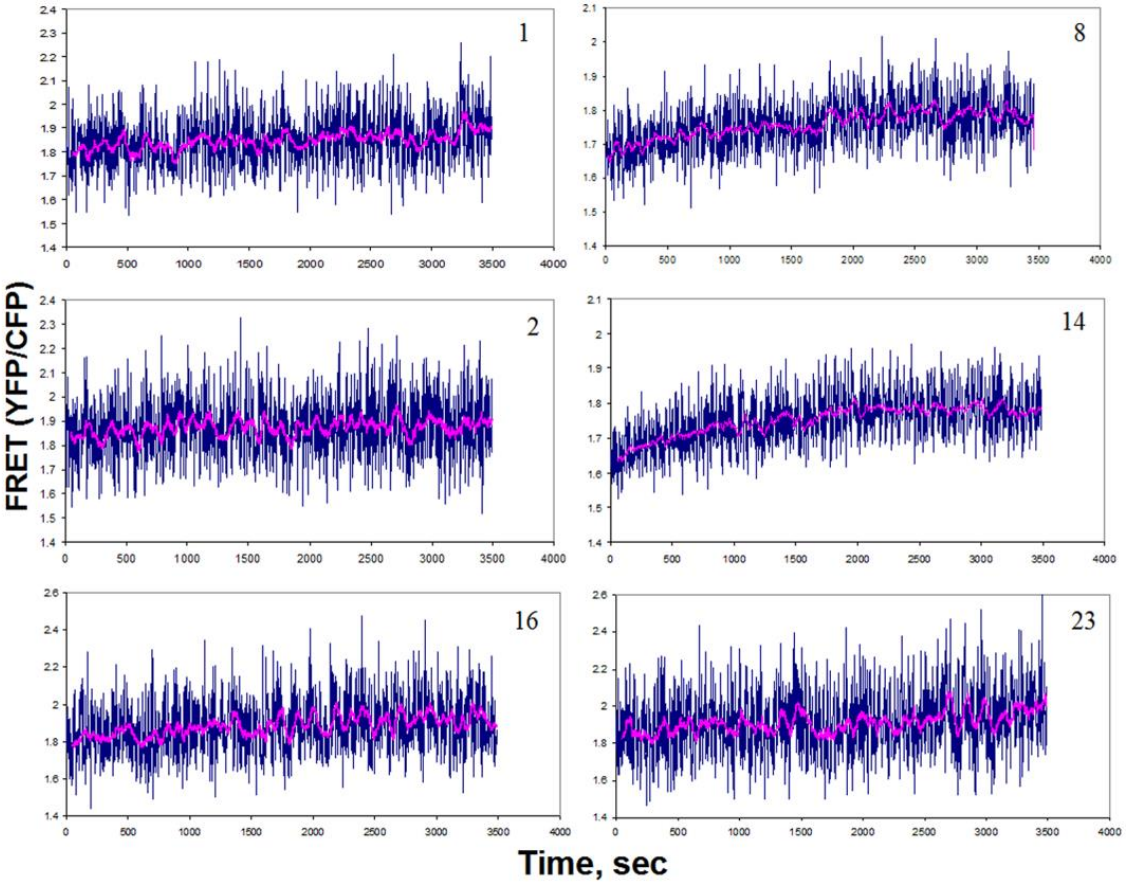

Figure 2. Example of FRET measured in single yeast cell every 2.6 s for a total of 3490 s. The raw data (YFP/CFP fluorescence ratio) are reported in blue, while a moving average of 20 points is reported in pink. The numbers (1, 2, 8, 14, 16, 23) indicate the ROI of the measured cell (see Supplementary Figure S2).

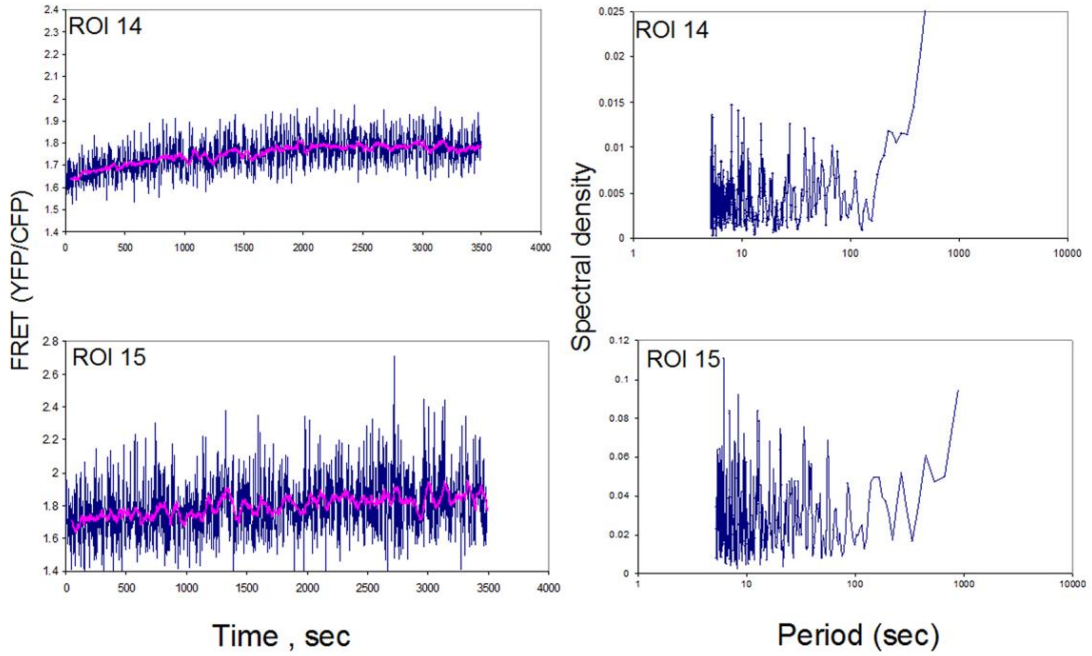

Figure 3. FRET time course and spectral analysis of two cells that fail to exhibit low-frequency oscillations. (**Left panels**) FRET values reported as YFP/CFP fluorescence ratio; the raw data are reported in blue, while a moving average of 20 points is reported in pink. (**Right panels**) Spectral analysis performed on the raw data; the spectral analysis does not evidence low-frequency components emerging over the noise.

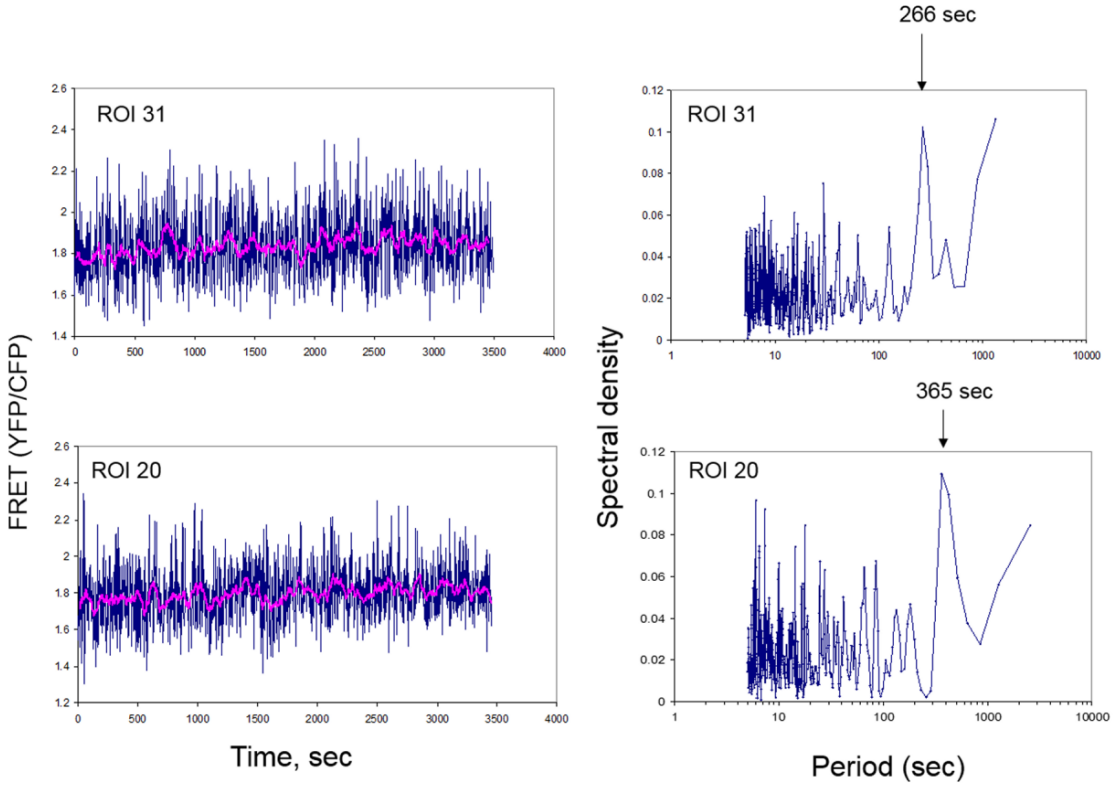

Figure 4. Example of cells that exhibit oscillations from the beginning. (**Left panels**) FRET values reported as YFP/CFP fluorescence ratio; the raw data are reported in blue, while a moving average of 20 points is reported in pink. (**Right panels**) Spectral analysis performed on the raw data; the spectral analysis evidences low-frequency components with a period of 266 s and 365 s (indicated by arrows).

Finally in some other cells (14 cells), a more interesting behavior was observed after a variable period without oscillations: at a given point, low-frequency oscillations appeared (Figures 5 and 6). In all these cells, the FRET signal steadily increased during the first non-oscillatory period, suggesting that the intracellular level of cAMP decreased until the insurgence of oscillations. In these cases, the spectral analysis, performed on the whole series of raw data (from 1 to 3490 s), failed to evidence the presence of low-frequency oscillations. However, oscillations were markedly evident when the analysis was performed on the second half of the data (from 1500 to 3490 s) (Figures 5 and 6).

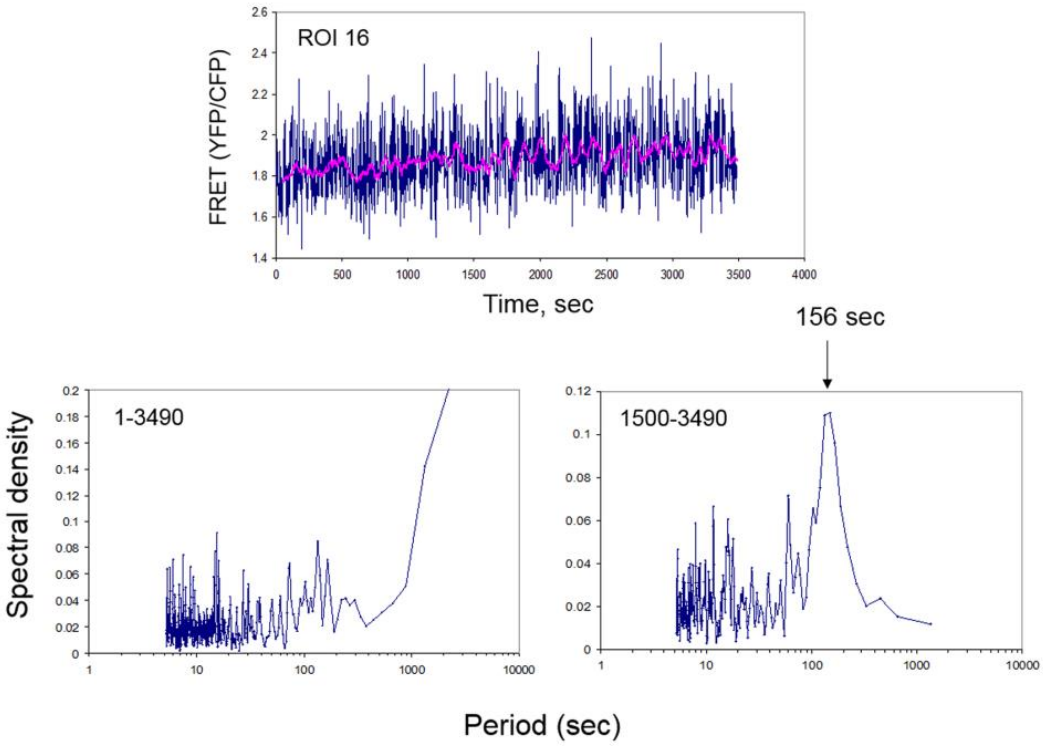

Figure 5. Example of a cell that only exhibits oscillations during the second part of the experiment (after 25 min). Upper panel: FRET values (raw data in blue and a moving average of 20 points reported in pink) of the cell named ROI 16. Lower panels: Spectral analysis of the raw data performed on the full data (from 1 to 3490 s) (**left panel**) or spectral analysis performed on data collected after 1500 s (1500 to 3490 s) (**right panel**). In this second case, a low-frequency component with a period of 156 s (indicated by arrow) is markedly evident.

An example of this behavior is also markedly evident for the cell analyzed as ROI 8 (Figure 6). The presence of oscillations in the second half of the data (points from 1500 to 3490 s) is barely evident on raw data, but it is clear after smoothing (using a moving average window of 20 experimental points). The apparent period is 4 min, in agreement with spectral analysis performed on the same raw data. The reverse of FRET (i.e., CFP/YFP fluorescence signal) is directly related to the intracellular cAMP concentration, which steadily decreases in the first 600 points (equivalent to 26 min) and then starts to oscillate in the second part for at least 30 min (Supplementary Figure S3).

Taking into account the affinity of the EPAC2-based probe for cAMP (Kd = 0.8–1 μM) [18,23], we estimated that the intracellular cAMP concentration oscillates between 0.2 and 0.8 μM (about 6000–25,000 molecules/cell) in the range reported in ref. [14] (Supplementary Figure S4).

A similar behavior was observed for other cells (ROI 16, for example, in Figure 5), and the insurgence of an oscillatory state can also be evidenced by a recurrence analysis software, as shown in Figure 7, where a change of pattern and the appearance of many lateral diagonals are markedly visible, indicating a periodicity.

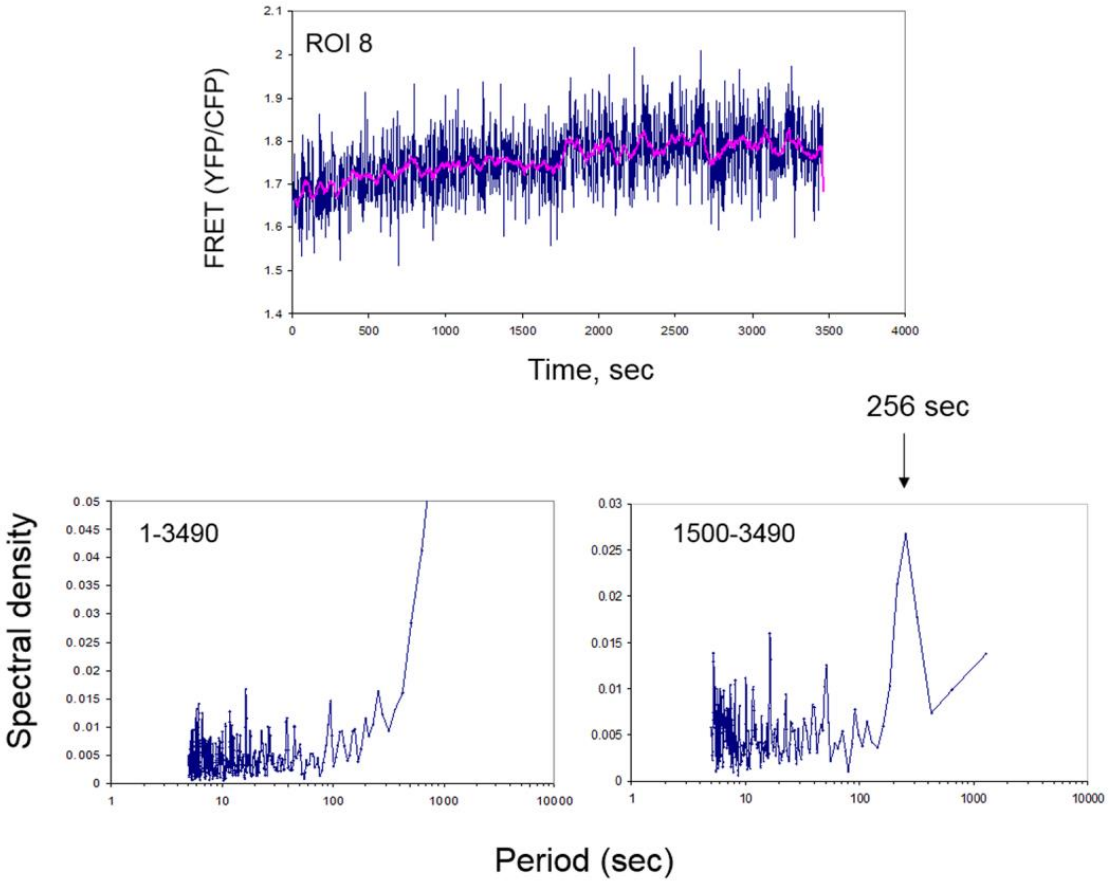

Figure 6. The cell identified as ROI 8 showed clear oscillations starting after 26 min. Upper panel: FRET values (raw data in blue and a moving average of 20 points reported in pink). Lower panels: Spectral analysis of the raw data performed on the full data (from 1 to 3490 s) (**left panel**) or spectral analysis performed on data collected after 1500 s (1500 to 3490 s) (**right panel**). In this second case, a low-frequency component with a period of 256 s (indicated by arrow) is markedly evident.

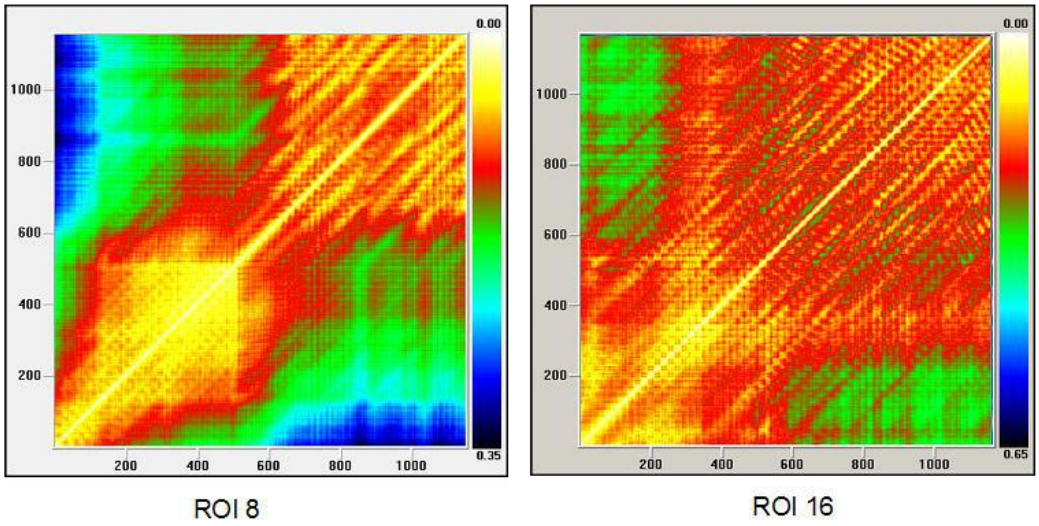

Figure 7. Recurrence analysis [21] performed with the software Visual Recurrence—VRA (version 4.2, http://www.visualization-2002.org/VRA_MAIN_PAGE_.html (accessed on 30 July 2023))—using the smoothed data (moving average of 20) of the cells ROI 8 and ROI 16. A change in pattern in the second part of the data, corresponding with the insurgence of the oscillations, is clearly evident.

4. Conclusions

This is the first time that spontaneous sustained oscillations in cAMP levels have been observed in budding yeast. The oscillations were simultaneously observed in several yeast cells under conditions of nutritional stress (i.e., low glucose availability). Each cell behaved in a different way, i.e., many cells did not show any oscillations, others showed oscillations from the beginning of the experiment, while other cells presented oscillations later during the experiment. About 34% of the cells analyzed with spectral analysis showed an oscillatory pattern with an oscillation period varying from 3 to 6 min. Moreover, the oscillations were not synchronous and were partially masked by high-frequency noise. This oscillatory behavior of the cAMP/PKA pathway was predicted by simulations performed with a stochastic model of this pathway [10,16] and could explain the nucleo-cytoplasmic shuttle of Msn2 transcription factor [15–17]. It is interesting to note that in previous experiments using the same strain of SP1 expressing the YFP-Epac2-CFP probe, we found damped oscillations of cAMP after adding glucose to derepressed starved cells [19]. We re-analyzed these data, and spectral analysis showed a periodicity of 250 s, close to the period of sustained oscillations observed here. This suggests that the oscillatory behavior in the Ras/cAMP/PKA pathway is related to complex feedback mechanisms embedded in this signaling pathway. Sustained oscillations only occurred under nutritional stress conditions, but damped oscillations were also evident after a strong perturbation of the pathway (i.e., glucose addition to starved cells) [14,19], and it is interesting to underline that a similar period of 4–6 min was observed.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/sym15091793/s1, Figure S1: Simulation of cAMP oscillations in single cell; Figure S2: Example of imaging of yeast cells expressing the cAMP probe; Figure S3: Elaboration of raw data of cell named ROI 8; Figure S4: Intracellular concentration of cAMP estimated from FRET data of Figure S3; Table S1: Raw data used for Figures 2–6; Video S1: Nucleo-cytoplasmic oscillations of Msn2-GFP; Abbreviations used.

Author Contributions: Conceptualization, E.M. and S.C.; methodology, S.C., M.C. and L.D.; software, M.C., G.C. and E.M.; investigation, S.C. and L.D.; resources, E.M. and G.C.; data curation, M.C. and E.M.; writing—original draft preparation, E.M.; writing—review and editing, E.M., S.C. and G.C.; supervision, E.M. and G.C. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data supporting the reported results can be found in Supplementary Table S1.

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Conflicts of Interest: The authors declare no conflict of interest. The founders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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