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Report

Nucleocytoplasmic Oscillations of the Yeast Transcription Factor Msn2: Evidence for Periodic PKA Activation

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Summary

At intermediate intensities, stress induces oscillations in the nucleocytoplasmic shuttling of the transcription factor Msn2 in budding yeast [1]. Activation by stress results in a reversible translocation of Msn2 from the cytoplasm to the nucleus [2]. This translocation is negatively controlled by the cAMP-PKA pathway through Msn2 phosphorylation [2, 3]. Here we show that the nuclear localization signal (NLS) of Msn2 is necessary and sufficient to promote the nucleocytoplasmic oscillations of the transcription factor. Because the NLS is controlled by protein kinase A (PKA) phosphorylation, we use a computational model to investigate the possibility that the cAMP-PKA pathway could function as an oscillator driving the periodic shuttling of Msn2. The model indicates that sustained oscillations of cAMP can indeed occur in a range bounded by two critical values of stress intensity, owing to the negative feedback exerted by PKA on cAMP accumulation. We verify the predictions of the model in mutants by showing that suppressing this negative-feedback loop prevents the oscillatory shuttling but still promotes the stress-induced nuclear localization of Msn2. The physiological significance of Msn2 oscillations is discussed in the light of the frequency encoding of cellular rhythms

Results and Discussion

The Oscillatory Behavior Is Mediated by the Msn2-NLS

In a previous report [1], we distinguished two classes of mechanism capable of producing oscillations in the nucleocytoplasmic shuttling of Msn2. The first involves a biochemical oscillator independent of Msn2 that would drive oscillations in its nucleocytoplasmic shuttling. The second relies on an oscillator that directly involves Msn2. At the time, in the absence of cues about the underlying mechanism and of indications of an external oscillator, we proposed a hypothetical model assuming that Msn2 could be part of the feedback loop producing the oscillatory behavior. To test the possible role of the interaction of Msn2 with the transcriptional machinery [4], we showed that deleting the part of Msn2 that encodes the DNA-binding domain did not prevent the oscillations [1]. We now show that removing the major part of the transcriptional-activation domain [5] does not affect the periodic shuttling of Msn2 (Figure 1C). Eliminating these two domains still allows oscillatory shuttling of the truncated protein (Figure 1D). These results indicate that the mechanism of oscillation is independent from the formation of the transcriptional complex.

To examine the possible involvement of the nuclear localization signals (NLSs) and nuclear export signals (NESs) located within the remaining active portion of Msn2 (Figure 1A), we first replaced the NLS domain of Msn2 within Msn2-GFP with the NLS from the T antigen of SV40 [6]. This construct still responds to stress by accumulating and remaining in the nucleus, but it was unable to reinitiate any shuttling (data not shown); this suggests that the Msn2-NLS could be necessary for oscillatory shuttling. We then replaced the Msn2-NES by fusing the NES-PKI [7] to the N-terminal part of Msn2 containing only the NLS region and the DNA-binding domain fused to the green florescent protein (GFP) (Figure 1A). As illustrated in Figure 1E, this construct was able to respond to stress and reinitiate shuttling. The oscillatory pattern, which occurs in fewer cells, is quite irregular, and the time spent in the nucleus might also be longer as if the NES-PKI was less efficient than the Mns2-NES, but the occurrence of shuttling demonstrates that it contains elements sufficient to promote oscillatory behavior. Because the DNA-binding domain in this construct is not required for oscillations, the result indicates that the NLS region contains the main determinant for oscillatory behavior.

The NLS is recognized by karyopherins [8], which mediate transport into the nucleus. Although karyopherin Kap123 was reported to be involved in nuclear translocation of Msn2 [9], we found that translocation was prevented only in the double karyopherin mutant *pse1^{ts}(kap121)*, *kap123Δ* (unpublished data). The interaction of NLS with karyopherin usually requires basic residues, all of which represent protein kinase A (PKA)-recognition sites within the region encompassing the NLS of Msn2 (Figure 1F). Thus, the serine residues adjacent to the arginines are targets of PKA. These serine residues are natural candidates for sensing a modulated signal triggered by an external oscillator. Such a view is supported by the observation that inactivation of the cAMP-PKA system leads to the permanent localization of Msn2 within the nucleus [1–3]. In addition to the NLS, the NES might also be involved in the oscillatory response because it also contains a PKA-phosphorylation site. The Msn2 carrying the NES-PKI appears indeed to be less efficiently exported from the nucleus than the native Msn2.

A Computational Model for the cAMP-PKA Pathway in Yeast Predicts Oscillations of cAMP

What is the nature of the oscillator affecting the phosphorylation state of Msn2? The oscillator could involve

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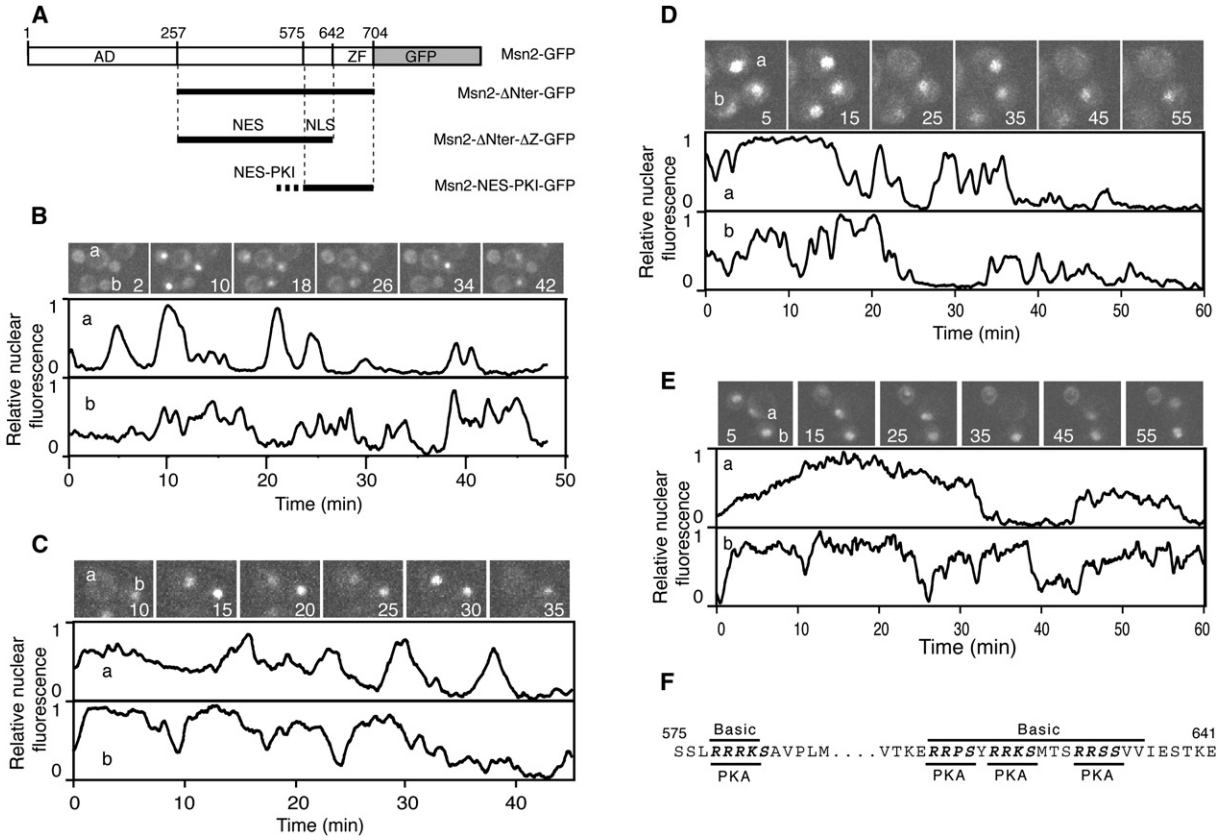

Figure 1. Minimal Region of Msn2 Involved in Oscillatory Behavior

(A) Schematic representation of Msn2 and the truncated forms used in this figure. The position of the DNA-binding domain ("ZF"), activation domain ("AD"), NLS, and NES are indicated.

(B–E) W303 cells were transformed with plasmids containing Msn2-GFP (B), Msn2-ΔNter-GFP (residues 257–704 of Msn2 fused to GFP) (C), Msn2-ΔNter-ΔZ-GFP (residues 257–642 of Msn2 fused to GFP) (D), and Msn2-NES-PKI-GFP (residues 575–704) (E). Cells were observed when growing in the exponential phase in yeast nitrogen base (YNB) medium supplemented with the required amino acids. Typical kinetics of the nuclear translocation with high-resolution time-lapse video microscopy on single cells are presented. For each construct, a sequence of photo frames taken at the time indicated (min) is shown on top. The curves below have been drawn from the fluorescence intensity of the nucleus of the indicated cells ("a" and "b"), taken every 10 s, and measured with ImageJ software. They are smoothened by taking the average between three consecutive values. For each construct, two curves are presented to illustrate the usual response heterogeneity.

(F) The amino acid sequence of the NLS region of Msn2 showing the PKA-phosphorylation sites and the basic residues characteristic of the NLS.

the PKA, the phosphatase acting on Msn2, or both. We deleted the two PP2A phosphatase genes involved in the stress response of Msn2, *PPH21* and *PPH22* [10], and found that the light-induced nuclear translocation was abolished (data not shown). A direct demonstration of oscillations in Msn2 phosphorylation and dephosphorylation being out of reach in single yeast cells, and in the absence of sufficient knowledge on the control of the phosphatases, we examined the propensity of the cAMP-PKA pathway, regulated by a strong negative-feedback loop [11], to undergo sustained oscillations. To this end, we built a computational model to investigate whether the cAMP-PKA system could produce oscillations over a wide range of parameter values that match at least some experimentally determined values. The model is schematized in Figure 2 and presented in more detail in the Supplemental Data available online.

In yeast, cAMP is produced by an adenylate cyclase (product of *CYR1*) [12] that is activated by Ras proteins (Ras1 and Ras2), which are themselves activated by association with GTP that is catalyzed by the guanine exchange factor (GEF) Cdc25 [13]; Ras is reset to its GDP form by the GTPase-activating proteins (GAP) Ira1 and Ira2 [14]. cAMP binds to the inhibitory subunit of PKA, the product of *BCY1* [12]. The binding of cAMP to this regulatory subunit results in the dissociation and concomitant activation of the two catalytic subunits of PKA (C) that are encoded by one or two of three different genes: *TPK1*, *TPK2*, and *TPK3* [15]. cAMP is hydrolyzed by the two phosphodiesterases Pde1 and Pde2, of low and high affinity, respectively [16]. A strong negative-feedback loop is exerted on cAMP synthesis by PKA, as evidenced by the thousand-fold increase in cAMP in cells in which one of the three *TPK* genes is mutated (tpk^{w}) and produces a catalytic subunit of very low activity while the two other *TPK* genes are deleted [11]. In the model (see Figure 2 and Supplemental Data), for the sake of simplicity, we consider as single units the redundant functional units RAS, GEF, GAP, PDE, and C. Negative feedback is applied both on the phosphodiesterase PDE and on the GTPase-activating protein GAP, to hold with the original finding that the fully deregulated level of cAMP found in $tpk2^{w}$ mutants was only observed in cells containing the $RAS2^{val19}$ mutation yielding

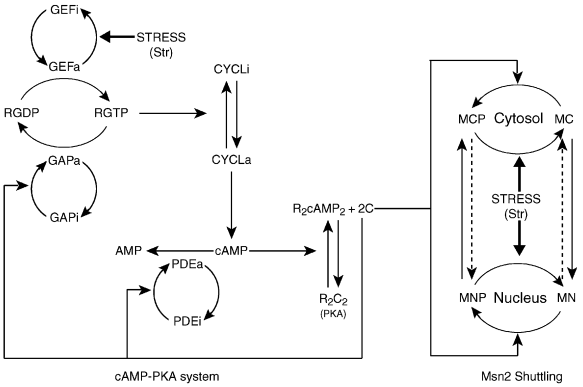

Figure 2. Model for Nucleocytoplasmic Shuttling of Msn2 Coupled to Oscillations in the cAMP-PKA System

Scheme of the model showing the different interactions between the components of the cAMP-PKA system and the coupling to Msn2 shuttling between cytosol and nucleus in yeast. The variables of the model are the following: the fractions GEFa and GAPa of active GEF (Cdc25) and GAP proteins (Ira1 and Ira2); the fraction Ras-GTP (RGTP) of Ras proteins (Ras1 and Ras2) bound to GTP; the fraction CYCLa of adenylate cyclase (Cyr1) in the active state; the concentration of cAMP; the fraction of active phosphodiesterase PDEa (Pde1 and Pde2); the fraction R_2C_2 of PKA in the form of a holoenzyme complex between the regulatory subunit Bcy1 (R) and the three catalytic subunits, free of cAMP (C). R_2cAMP_2 denotes the holoenzyme with a cAMP molecule bound to each of the two regulatory subunits. We assume that stress (of intensity Str) elicits the inactivation of GEF and the dephosphorylation of Msn2 both in the nucleus and in the cytosol.

constitutively active Ras, and in which the two phosphodiesterases were deleted [11].

Coupling of Msn2 to cAMP variations is mediated through by PKA-induced phosphorylation, which promotes cytoplasmic localization of the transcription factor. Msn2 can be phosphorylated by PKA both in the cytoplasm and in the nucleus [2] because the kinase is present in both compartments in glucose-grown cells [17] and cAMP is small enough to diffuse rapidly to the nucleus. Although the NLS is controlled by four phosphorylation sites [2], we consider only one phosphorylation-dephosphorylation event in our simplified model because this is sufficient to couple the shuttling of Msn2 to the dynamics of the cAMP-PKA system. The effect of stress is likely manifold: We assume that it controls the cAMP-PKA pathway through the inactivation of GEF (Cdc25) [18] and enhances the activity of phosphatases acting on Msn2, as can be deduced from the experimental results presented in the next subsection, where Msn2 still responds to stress by entering the nucleus in the absence of any variation in cAMP.

Depending on the stress intensity measured by parameter Str, the model predicts that an oscillatory regime separates different types of steady-state situations corresponding to low or high levels of cAMP and PKA activity and associated with distinct patterns of Msn2 subcellular localization (Figure 3). At low stress values, the system reaches a stable steady state that corresponds to a high level of cAMP and leads to the predominantly cytoplasmic localization of Msn2 (Figure 3A, blue curves). Beyond a critical value of stress intensity, oscillations of cAMP occur spontaneously (Figures 3A and 3B). These oscillations are accompanied by oscillations of all variables of the cAMP-PKA pathway, such as RGTP and PKA, and by the periodic shuttling of Msn2 between the cytoplasm and the nucleus (Figure 3A, green curves). Finally, above a second, higher critical value of stress intensity, cAMP reaches a stable, low steady-state level, and Msn2 ceases to oscillate and becomes predominantly located in the nucleus (Figure 3A, red curves; Figure 3C).

As shown in Figure 3B, the model predicts that the period of Msn2 oscillations increases by about a factor of two with the intensity of stress. Presumably, this result stems from the fact that increased stress further inhibits GEF and, subsequently, adenylate cyclase, so that cAMP takes more time to accumulate up to the level at which the negative-feedback loop mediated by PKA begins to operate. It is somewhat difficult to compare these predictions with experimental observations because of the irregular nature of oscillations within a given cell.

Impairing the Negative Feedback on cAMP Synthesis Prevents Oscillations of Msn2

To test the prediction that the periodic shuttling of Msn2 is driven by a cAMP oscillator, we used a genetic approach based on the assumption that the Msn2-GFP localization reflects the level of cAMP and examined whether the oscillatory behavior is lost in mutants lacking the feedback regulation. To perform these experiments, we took advantage of new *tpk2^w* mutants, generously provided by J. Broach, in which the residual PKA activity suffices to maintain Msn2-GFP in the cytoplasm in the absence of stress. We examined the kinetic behavior of 67 cells from two different strains and found that Msn2-GFP responds to stress by accumulating in the nucleus in 60% of them, but none of the cells ever presented an oscillatory behavior. One example of the stress response in a *tpk2^w* mutant is shown in Figure 4A. To alter the negative feedback, we also used a strain lacking the two RAS genes and containing in the gene *CYR1*—which encodes adenylate cyclase—a mutation that renders it constitutively leaky [19]. In this strain, the negative feedback mediated by Ras is therefore abolished. Msn2-GFP did not display an oscillatory behavior in any of the examined cells (n = 71). For at least 50% of the cells, a stress-induced nuclear localization was observed, and in few cases (n = 5), Msn2-GFP was exported back to the cytoplasm, as illustrated in Figure 4B. This last result might reflect the transient nature of the stress response that adapts after a time [20]. The observation that the oscillatory behavior of Msn2 is lost when the negative-feedback loop on cAMP accumulation is impaired holds with the prediction that cAMP oscillations due to this negative feedback play a key role in the periodic shuttling of Msn2.

Link with cAMP Oscillations in Other Cell Types

Oscillations of cAMP have already been characterized in several organisms. The most thoroughly studied example is that of the slime mold *Dictyostelium discoideum*, for which aggregation following starvation is governed by cAMP oscillations of a period of 5–10 min [21]. The origin of cAMP oscillations involves the interplay between a positive and a negative feedback exerted on adenylate cyclase by extracellular cAMP upon binding to a membrane receptor [22]. Recent experiments

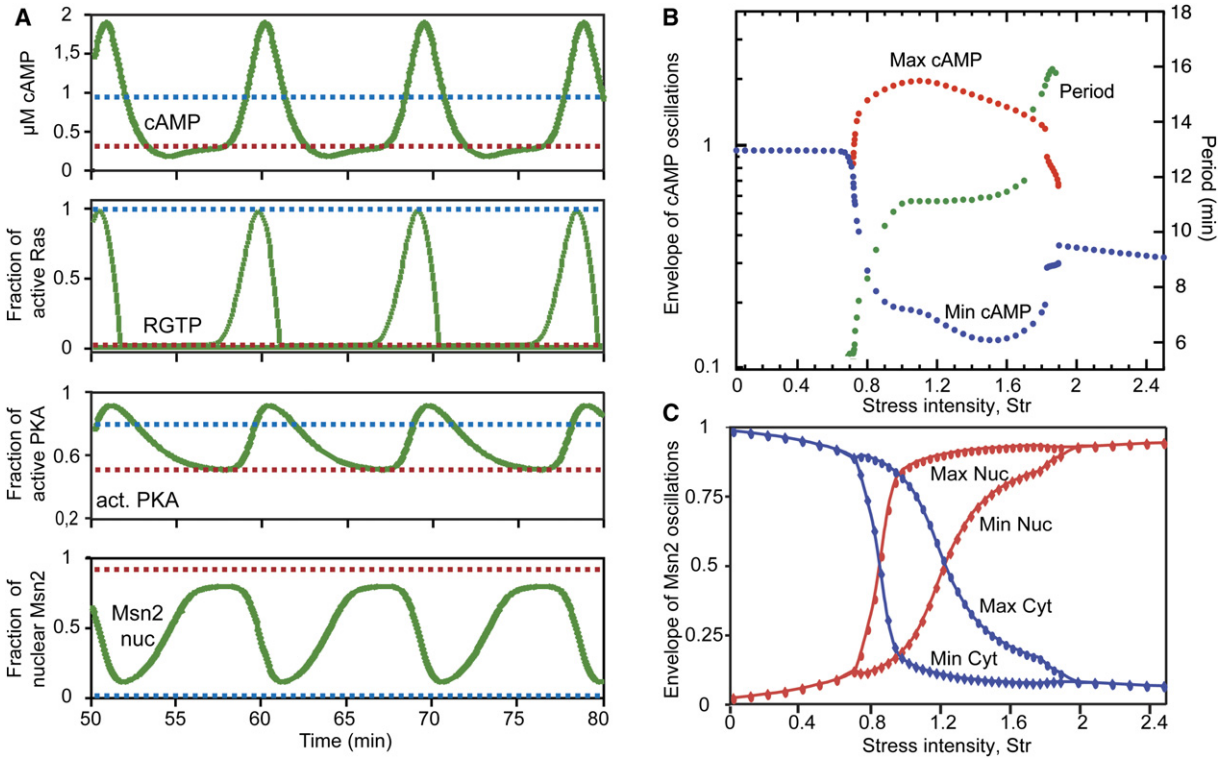

Figure 3. Dynamic Behavior Predicted by the Model

(A) Time evolution of RGTP, cAMP, active PKA, and nuclear Msn2 predicted by the model for three different values of Str, the dimensionless parameter measuring stress intensity. At low value, Str = 0 (dotted blue curves), and at high value, Str = 2.5 (dotted red curves), a steady-state level is observed for the different components, whereas at intermediate value, Str = 1 (green curves), sustained oscillations occur. The curves show the oscillatory behavior after the elimination of transients.

(B) Envelope of cAMP oscillations as a function of stress intensity showing the maximum (red curve) and the minimum (blue curve) values during sustained oscillations. The variation of periodicity (green dots) is also shown.

(C) Envelope of oscillations in Msn2 subcellular localization. The curves show the maximum values (Max) and minimum values (Min) for cytoplasmic Msn2 (in blue) and nuclear Msn2 (in red). Outside of the oscillatory range, the system reaches a stable steady state.

The curves have been obtained with the Berkeley Madonna program, by numerical integration of Equations S1–S4. Parameter values are given in Table S2. Initial conditions were as follows: GEFa 0.36, GAPa 0.5, RGTP 0.1, CYCLa 0.1, cAMP 1, R_2C_2 0.5, MC 0.25, MN 0.25, MCP 0.25, MNP 0.25, and PDEa 0.5.

suggest that the mechanism of cAMP oscillations in *Dictyostelium* might also rely on the intracellular negative regulation of adenylate cyclase, mediated by PKA and MAP kinase [23]. The model that we consider is closely related to the one investigated by Laub and Loomis for *Dictyostelium* [24], but it differs in its details and is more complex. Both models predict cAMP oscillations based on negative feedback on adenylate cyclase via PKA, and the periodicity is in the same time range. Evidence for intracellular oscillations of cAMP has also been obtained in relation to Ca^{2+} spiking in neurons [25] and in hormone-stimulated insulin-secreting β cells [26]. A role for intracellular oscillations of cAMP has also been invoked in relation with the pulsatile release of the hormone GnRH, with a periodicity of about 30 min, in a mouse cell line of immortalized GnRH-secreting cells [27]. The present results point to the occurrence of cAMP oscillations in yet another cell type by suggesting that stress induces cAMP oscillations in yeast.

What Could Be the Function of Stress-Induced Oscillatory Shuttling of Msn2?

The physiological significance of Msn2 oscillations deserves to be addressed in future experiments, but insights into the possible function of the phenomenon can already be gained by comparing it with other examples of periodic behavior in cell signaling that show that information might be encoded in terms of the frequency of the oscillatory process [28]. In *Dictyostelium* cells, only signals of cAMP delivered at the physiological frequency of one pulse every 5 min promote aggregation and differentiation [29]. Many hormonal rhythms are frequency-encoded [30], as exemplified by GnRH pulses [31] and by the pulsatile secretion of growth hormone [32]. Frequency encoding also occurs for Ca^{2+} oscillations in a variety of cell types upon stimulation by a hormone or a neurotransmitter [33]. The number of spikes of p53 triggered by DNA damage increases with the intensity of the radiation [34]. Oscillations in NF-KB are thought to modulate gene expression according to their frequency, which itself is controlled by the extent of cell stimulation by tumor necrosis factor (TNF) [35]. All of these examples of the frequency encoding of oscillations in signal transduction raise the possibility that the oscillatory nucleocytoplasmic shuttling of Msn2 allows the yeast cell to adapt the level of activation of this transcription factor, and hence its transcriptional activity, to the intensity of stress perceived by the cell.

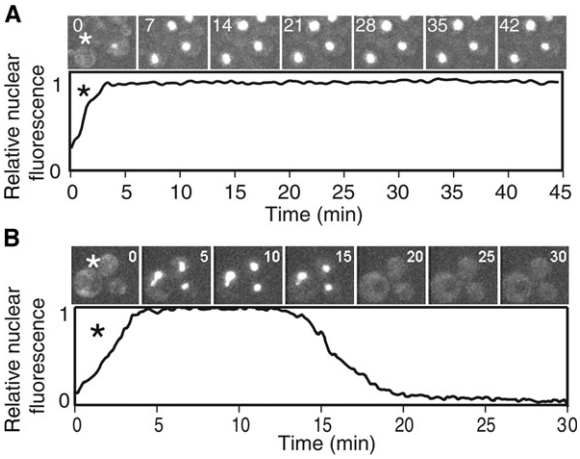

Figure 4. Lack of Oscillations in Mutants with Impaired Regulation of the cAMP-PKA Pathway

(A) Dynamics of Msn2-GFP in a mutant *tpk2*$^{w(E235Q)}$. The Y3399 strain has been transformed with plasmid pJL42 coding for MSN2-GFP. Similar results were obtained with the strain Y2857 [*tpk2*$^{w(V218G)}$], whereas in the strain Y3398 [*tpk2*$^{w(Q138E)}$], Msn2-GFP was always in the nucleus, presumably because the residual activity of PKA was lower. The sequence of pictures (above) and the kinetic curve (below) are given as in Figure 1, except that pictures were taken every 20 s.

(B) Dynamics of Msn2-GFP in a mutant *ras1*Δ, *ras2*Δ *CRI4* (B). The F1D strain has been transformed with plasmid pGR213 coding for Msn2-GFP. The sequence of pictures (above) and the kinetic curve (below) are given as in Figure 1.

Supplemental Data

Experimental Procedures, model, one figure, four tables, and five movies are available at http://www.current-biology.com/cgi/content/full/17/12/1044/DC1/.

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