

1 An analog to digital converter creates nuclear localization pulses in yeast calcium signaling

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6 Abstract:

7 Several examples of transcription factors that show stochastic, unsynchronized pulses of nuclear  
8 localization have been described. Here we show that under constant calcium stress, nuclear  
9 localization pulses of the transcription factor Crz1 follow stochastic variations in cytoplasmic  
10 calcium concentration. We find that the size of the stochastic calcium pulses is positively  
11 correlated with the number of subsequent Crz1 pulses. Based on our observations, we propose a  
12 simple stochastic model of how the signaling pathway converts a constant external calcium  
13 concentration into a digital number of Crz1 pulses in the nucleus, due to the time delay from  
14 nuclear transport and the stochastic decoherence of individual Crz1 molecule dynamics. We find  
15 support for several additional predictions of the model and conclude that stochastic input to  
16 nuclear transport may produce digital responses to analog signals in other signaling systems.

17 Keywords: stochastic pulsing transcription factor, Crz1, time delay model, mathematical  
18 modeling, calcineurin pathway

19 Introduction:

20 Cells transmit information through signaling pathways. Rather than simple “ON” or “OFF”  
21 responses, several key pathways (p53, NF- $\kappa$ B, and others) are now appreciated to encode  
22 information in the dynamics of the signaling response(1–9). Here we focus on the calcium  
23 signaling pathway in yeast, which controls gene transcription through frequency modulation  
24 (FM) of the transcription factor Crz1(9). In this system, the analog external calcium  
25 concentration is converted into the frequency of digital pulses of nuclear localization (discrete  
26 rapid rising and falling of nuclear concentration on the order a few minutes).

27 Mechanistic models of FM pulsatile transcription factors have relied on negative feedback  
28 coupled with positive feedback(7,8,10,11) or delayed negative feedback(11,12). However,  
29 whether there is a negative feedback loop in calcium signaling pathway that can generate Crz1  
30 pulses is unclear (discussed further below). Furthermore, in each cell, during each Crz1 nuclear  
31 localization pulse, approximately 500 Crz1 molecules are transported in and out of the nucleus in  
32 a coordinated fashion(13), but these pulses are stochastic (and not synchronized between cells).  
33 To our knowledge, no mechanistic model of this process has yet been proposed.

34 One possibility is that Crz1 nuclear localization pulses are connected to variation in cytoplasmic  
35 calcium concentration ( $[Ca^{2+}]_{cyt}$ ) since calcineurin is activated by calcium(14). Calcium pulses  
36 have been observed in many cell types(15–19), but the connections between cytoplasmic calcium  
37 concentration and Crz1 localization have not been analyzed in single cells. Mechanistic models  
38 of Crz1 regulation through calcium signaling do not predict external calcium concentration  
39 ( $[Ca^{2+}]_{ext}$ )-induced oscillation of  $[Ca^{2+}]_{cyt}$ (20). Further, although average Crz1 nuclear  
40 localization increases when  $[Ca^{2+}]_{ext}$  increases(9),  $[Ca^{2+}]_{cyt}$  is known to be under tight  
41 homeostatic control: the average  $[Ca^{2+}]_{cyt}$  remains similar under a wide range of  $[Ca^{2+}]_{ext}$ (21,22).  
42 Variation in  $[Ca^{2+}]_{cyt}$  is unlikely to follow the frequency of Crz1 pulsatility, which increases

43 when  $[Ca^{2+}]_{ext}$  increases(9). Thus, the relationship between calcium and Crz1 pulses remains  
44 unclear.

45 In this study, we examined the connection between  $[Ca^{2+}]_{cyt}$  and Crz1 pulsatile dynamics through  
46 dual fluorescence time-lapse microscopy(23). We found that cytoplasmic calcium concentration  
47 varies stochastically at the single cell level, showing pulses on the timescale of 10-100 seconds.  
48 We observed overshoots of the calcium concentration, strongly implicating calcium channels in  
49 these pulses. We found that Crz1 pulses tend to follow these calcium pulses, but that the  
50 relationship is not simple: multiple Crz1 pulses may follow each calcium pulse, and the number  
51 of Crz1 pulses depends on the size of the calcium pulse. We modulated calcium channel activity  
52 and found much larger calcium pulses, which led to greater numbers of Crz1 pulses. To explain  
53 how  $[Ca^{2+}]_{cyt}$  affects Crz1 nuclear localization, we developed a stochastic model of Crz1 nuclear  
54 localization and tested predictions in the experimental data. In general, stochastic pulses in  
55 signaling dynamics may be generated by time-delayed responses to fluctuations in second  
56 messenger concentration.

## 57 Results

### 58 *Calcium pulses are observed when yeast cells are under calcium stress*

59 In order to study the relationship between the dynamics of  $[Ca^{2+}]_{cyt}$  and Crz1 nuclear  
60 localization, we constructed a dual Crz1-Calcium reporter strain and measured dynamics using  
61 time-lapse microscopy. We tagged Crz1 with the mCherry fluorescent protein in a strain with a  
62 cytoplasmically expressed calcium sensor GCaMP3(16), and recorded movies on a confocal  
63 fluorescence microscope (see Methods). As in previous studies(9), we observed stochastic and  
64 rapid increases and decreases of  $[Ca^{2+}]_{cyt}$  when yeast are under calcium stress (Figure 1 A,

65 supplementary video 1). We noticed that these “calcium pulses” (defined by a threshold ratio  
66 above background, see Methods) are followed by an overshoot of calcium concentration below  
67 the resting level (average of 50 largest calcium local maxima in a representative time lapse  
68 movie is shown in Figure 1B left panel), whose depth is positively correlated to the height of the  
69 pulse (Figure 1B, right panel,  $R^2 = 0.37$ ). This overshoot cannot fit an exponential curve and  
70 suggests negative feedback on  $[Ca^{2+}]_{cyt}$ , which is consistent with the predictions of calcium  
71 models constructed in previous studies of homeostasis(24,25).

### 72 *Quantification of calcium pulses and Crz1 pulses suggests an analog to digital converter*

73 Individual cell trajectories do not show a simple relationship between  $[Ca^{2+}]_{cyt}$  and Crz1 nuclear  
74 localization (Figure 1A), so we next sought to understand how Crz1 pulses (see Methods for  
75 definition of Crz1 pulses) are affected by calcium pulses. We analyzed the distribution of the  
76 time differences between a calcium pulse and following Crz1 pulse(s) (using so-called pulse-  
77 triggered averaging(23)). The coherence of the first and second pulses suggested to us that one or  
78 more Crz1 pulses follows a single calcium pulse (Figure 2A). We therefore compared the time  
79 until the first Crz1 pulse within 10 minutes of a calcium pulse to the time until the first Crz1  
80 pulse within 10 minutes from a randomly chosen cell that may or may not contain a calcium  
81 pulse. Consistent with our hypothesis, we found that the time until the first Crz1 pulses after  
82 calcium pulses shows reduced standard deviation (122.47 seconds vs. 161.25 seconds, F-test,  
83  $p < 0.005$ ,  $n = 168$ ) and occurs sooner than observed in randomly chosen cells ( $83.01 \pm 9.58$   
84 seconds vs.  $210.62 \pm 28.89$  for random, two-tailed t-test,  $p < 10^{-15}$ ,  $n = 193$ ). A similar range of  
85 time differences is also observed in cross correlation analysis (supplementary figure 1).  
86 Furthermore, the distributions of time differences disperse when the order of Crz1 pulses  
87 increases (Figure 2A, e.g., 122.47 seconds,  $n = 81$  for first pulses vs. 181.66 seconds,  $n = 45$  for

88 second pulses, F-test,  $p < 0.005$ ), suggesting that the effect of the calcium pulse on Crz1 dynamics  
89 decreases over time. These results suggest that one calcium pulse can lead to multiple Crz1  
90 pulses.

91 We sought to identify factors that determine the number of Crz1 pulses that follow each calcium  
92 pulse. We found that the number of Crz1 pulses after a calcium pulse is positively correlated to  
93 the height of that calcium pulse (Figure 2B, generalized linear model regression with Poisson  
94 distribution, slope =  $0.16 \pm 0.10$ ). To test whether a calcium pulse causes Crz1 pulses, we tested  
95 whether there is a correlation between calcium pulse height and the number of Crz1 pulses  
96 before a calcium pulse. If an (unmeasured) third factor affects both the height of a calcium pulse  
97 and the number of the Crz1 pulses in a cell, we expect more Crz1 pulses both before and after  
98 large calcium pulses. We found that the number before is not correlated with calcium pulse size  
99 (slope =  $-0.10 \pm 0.13$ , generalized linear model regression with Poisson distribution). This result  
100 is consistent with the idea that calcium pulses can cause more than one Crz1 pulse, and suggests  
101 that calcium pulse heights are converted into digital numbers of Crz1 pulses.

### 102 *Artificially increased calcium pulse height supports the analog to digital converter model*

103 The hypothesis of an analog to digital converter between calcium pulse height and Crz1 pulse  
104 number predicts that the average number of Crz1 pulses following calcium pulses could be made  
105 larger by artificially inducing larger calcium pulses. To test this prediction, we treated cells with  
106 nifedipine (See Methods). By doing so, we reliably induced synchronized calcium pulses  
107 (supplementary video 2) that were on average twice as large as the stochastic pulses observed at  
108 steady state in 0.2M calcium treatment alone (Figure 3A, compared to Figure 1B). The majority  
109 of these large calcium pulses are followed by at least 4 Crz1 pulses that disperse over time  
110 (Figure 3B). As predicted by the model, the number of Crz1 pulses after a calcium pulse is

111 positively correlated to calcium pulse height, and the range is larger than untreated range (Figure  
112 3C, compared to Figure 2B, generalized linear model regression with Poisson distribution, slope  
113 =  $0.37 \pm 0.11$ ). In individual cells, nuclear Crz1 now clearly appears to oscillate while no  
114 oscillations are observed in cytoplasmic calcium concentration (supplementary figure 2). These  
115 results confirm that single calcium pulses are followed by multiple Crz1 pulses, and support the  
116 idea that an analog-to-digital converter in the calmodulin/calcineurin signaling pathway converts  
117 calcium pulse height into Crz1 pulse number.

118 *A simple time delay model can reproduce the properties of Crz1 pulses after a calcium pulse*

119 To explain the mechanism of the analog to digital converter, we considered a two-step process in  
120 single cells (Figure 4A). The first step is that external calcium concentration leads to cytoplasmic  
121 calcium pulses (4A, blue trace) through stochastic channel opening, and the second step is that a  
122 calcium pulse leads to nuclear Crz1 pulses (4A, red trace) through the calcineurin pathway.

123 A negative feedback loop in the calcineurin pathway could lead to oscillation of calcineurin  
124 activity and drive Crz1 pulses, but we decided not to include one in our model for two reasons.  
125 First, the known negative feedback loop in the calcineurin pathway through Rcn1 does not  
126 appear to affect Crz1 pulsatility. Rcn1 is an inhibitor of calcineurin that is degraded when  
127 phosphorylated and is dephosphorylated by activated calcineurin (26,27), thus leading to  
128 negative feedback. However, the negative feedback loop is thought to be controlled by the  
129 protein abundance of Rcn1, as phosphorylation does not prevent Rcn1's inhibition of  
130 calcineurin(26). Since Crz1 pulsatility occurs when protein synthesis is inhibited by  
131 cycloheximide (supplementary figure 3) we consider it unlikely that Rcn1 provides negative  
132 feedback through changes in protein abundance. Second, models with feedback mechanisms

133 seem incompatible with the observation that the frequency but not the amplitude of Crz1 pulses  
134 increases when the affinity of calcineurin docking site on Crz1 is enhanced(9). This is opposite  
135 to the expectation if Crz1 nucleocytoplasmic transition were driven by a feedback mechanism.  
136 Therefore, we worked toward models that do not include a feedback mechanism.

137 Previously, Crz1 nuclear localization dynamics were explained with a conformational switch  
138 model(22). This model assumes that the large number of phosphorylation sites on Crz1 leads to a  
139 sigmoid function relating calcineurin activity to Crz1 nuclear localization, so when calcineurin  
140 activity swings above and below a threshold, Crz1 sensitively reads out the perturbation in  
141 calcineurin activity and switches fully nuclear or cytoplasmic (22,28). This model would predict  
142 that calcium concentration crosses a threshold before each Crz1 pulse, and pulses stop once  
143 calcium oscillations decay below the threshold. However, calcium is not observed to pass a  
144 threshold before each Crz1 pulse in our data (supplementary figure 2D for examples).

145 We therefore considered another single cell model. Inspired by the observations on the  
146 population level that Crz1 pulses tend to occur within 100 seconds after calcium pulses and then  
147 disperse over time, and that larger calcium pulses lead to more Crz1 pulses, we constructed a  
148 discrete-time stochastic model that explains single cell Crz1 nuclear localization based on time  
149 delays during nuclear import and export with variation among Crz1 molecules. Time delay  
150 models have been constructed through different approaches, including deterministic and  
151 stochastic delay differential equations with a fixed or variable delay periods(12,29–31). We used  
152 a discrete-time Markov chain because it is simple to simulate trajectories. In the model, Crz1  
153 molecules transit between the nucleus and the cytoplasm in a coordinated manner (show pulsing  
154 dynamics on average) only when calcineurin activity is very high due to a recent calcium pulse.

155 As calcineurin activity slowly returns to its basal level, the coordinated transport of 500 Crz1  
156 molecules in a single cell decoheres.

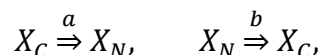
157 We model Crz1 nuclear signal by aggregating the states of individual Crz1 molecules after an  
158 increase in cytoplasmic calcium concentration of a single cell. As the input to the model, we  
159 provide calcium concentration,  $Ca$ , as a function of time,  $t$ , which can be obtained from  
160 experimental data (using GCaMP reporter fluorescence as a proxy).

161 We assume that calcineurin activity at time  $t$ ,  $Cn(t)$ , has an activation rate proportional to  
162 calcium concentration and a constant rate of decay. The discrete time dynamics of  $Cn(t)$  is  
163 described by

$$164 \Delta Cn \equiv Cn(t + 1) - Cn(t) = (Cn_{base} - Cn(t)) D + \max[0, Ca(t)] A,$$

165 where  $Cn_{base}$  is the basal activity of calcineurin,  $D$  is the decay rate of calcineurin activity, and  
166  $A$  is the activation rate by cytoplasmic calcium. Although calcium concentration can never be  
167 negative, our GCaMP reporter data is normalized such that baseline fluorescence level is defined  
168 as 0. When calcium pulses overshoot, we obtain negative values, and hence include  
169  $\max[0, Ca(t)]$  in the equation above. The probability of a Crz1 molecule being imported is  $Cn(t)$   
170 multiplied by the probability of dephosphorylation by an active calcineurin molecule (see  
171 Methods for details). Thus, when calcineurin activity increases, the probability of a Crz1  
172 molecular being imported increases.

173 Once a Crz1 molecule is imported into the nucleus, it returns to the cytoplasm after it is  
174 phosphorylated in the nucleus, which we assume occurs at a constant rate. These chemical  
175 reactions can be formulated using a standard biochemical rate approach as





176 where  $X_C$  and  $X_N$  are cytoplasmic and nuclear Crz1 molecules, respectively, and  $a$  and  $b$  are the  
177 rates of delayed transports (denoted as thick arrows). In our Markov chain framework, we  
178 assume that transports are multistage, so the delay time follows a Gamma distribution with the  
179 two parameters related to the number of states and the transition probability (see Supplementary  
180 text for details).

181 This model can qualitatively reproduce Crz1 pulses after a calcium pulse (Figure 4B). The  
182 number of Crz1 pulses after a calcium pulse is positively correlated to the height of that calcium  
183 pulse (Figure 4C, generalized linear model regression with Poisson distribution, slope = 0.29+-  
184 0.13), while the number of that before a calcium pulse is not (slope = 0.02+-0.16).

185 *Other predictions of the model are found in the experimental data*

186 The time delay model also predicts other properties of Crz1 pulsatility. A first prediction is that  
187 the periodicity of a Crz1 trajectory is correlated with calcium pulse size, such that a Crz1  
188 trajectory after a larger calcium pulse keeps oscillating longer. To test this prediction, we  
189 quantified the periodicity of Crz1 dynamics after the largest calcium pulse of each cell using a  
190 Gaussian Process model (see Methods), which computes the log-likelihood ratio (LLR)  
191 comparing a periodic to an aperiodic kernel. The LLR of post-calcium-pulse trajectories is  
192 correlated to the height of calcium pulses (Figure 5A). Calcium pulses larger than 0.11 show  
193 LLR significantly larger than that of the rest (two tailed t-test, sample sizes are 92 and 95,  $p < 10^{-5}$ ),  
194 which means larger calcium pulses lead to Crz1 dynamics that can be better described by a  
195 periodic Gaussian process. As a control, we also computed the LLR for pre-calcium-pulse  
196 trajectories, and found that the pre-calcium-pulse trajectories of for calcium pulse height above  
197 0.11 are not statistically more periodic ( $p > 0.1$ ). Periodic dynamics after large calcium pulses

198 would also be predicted by conformational switch model, where, after a larger calcium pulse,  
199 calcium oscillates longer and has more peaks crossing a threshold to trigger Crz1 pulse  
200 (supplementary figure 4).

201 Additional predictions of the time delay model are that, after a calcium pulse that is followed by  
202 at least two Crz1 pulses, the second Crz1 pulse is shorter and wider than the first Crz1 pulse  
203 because the coordinated transport of Crz1 molecules disperses across time. Although both the  
204 conformational switch model and the time delay model predict shorter second Crz1 pulses, the  
205 conformational switch model would predict a narrower second Crz1 pulse as the calcium  
206 oscillations decay (Figure 5C). To test these predictions, we identified the calcium pulses that are  
207 followed by two Crz1 pulses, and fit them to a “logistic pulse model” (Figure 5B, median  $R^2 =$   
208  $0.85$ , mean  $R^2 = 0.79$ , See Methods) to estimate pulse height and narrowness. We found that the  
209 height of the second pulse is significantly smaller (Figure 5D, paired t-test,  $p < 0.005$ ,  $n = 42$ ),  
210 and that the mean narrowness of the second pulses is significantly smaller than that of the first  
211 pulses (Figure 5D, paired t-test,  $p < 10^{-8}$ ,  $n = 42$ ), supporting the time delay model to the  
212 exclusion of the conformational switch model.

213 Thus, the data support three additional predictions of a simple stochastic model of Crz1 nuclear  
214 import and export. Together with the explanation of the analog to digital converter, our results  
215 support the idea that coordination of Crz1 localization (and thus pulsatility) is the result of a  
216 time-delay in nuclear import and export (see Discussion).

## 217 Discussion

218 Our results show that  $[Ca^{2+}]_{cyt}$  is linked to Crz1 pulsatility through an analog to digital  
219 conversion: larger calcium pulses lead to more Crz1 pulses. Through this model, we can explain

220 Crz1 pulses found after calcium pulses and provide a possible link between irregular  $\text{Ca}^{2+}$   
221 oscillation and transcription(17). However, in our cells, Crz1 fluctuations are also found without  
222 preceding calcium pulses. These Crz1 fluctuations are aperiodic and do not show correlations  
223 between number of pulses and calcium size. We recorded movies at four different  $[\text{Ca}^{2+}]_{\text{ext}}$ , and  
224 found that, although the frequency of calcium pulses is correlated with external calcium  
225 concentration, the increase in calcium pulsing frequency is not comparable to the increase in  
226 Crz1 pulsing frequency, and the average size of calcium pulses does not increase significantly.  
227 Therefore, our model cannot fully explain the calcium concentration dependence of Crz1  
228 fluctuations. However, cross-correlation analysis shows that these Crz1 dynamics have only a  
229 small correlation with  $[\text{Ca}^{2+}]_{\text{cyt}}$  dynamics (supplementary figure 1). We suggest that other  
230 intrinsic environmental fluctuations that affect Crz1 localization, such as light-(32), osmotic  
231 pressure-(33), or glucose-(34,35) induced Crz1 regulation might be involved in producing these  
232 fluctuations.

233 One of the interesting properties of pulsatile dynamics is that the pulses in individual cells are  
234 not synchronized, despite cells experiencing the same environmental stress(36). The analog-  
235 digital converter model explains this aspect of Crz1 pulsatility by arguing that the  $[\text{Ca}^{2+}]_{\text{cyt}}$   
236 among individual cells at a given time point is stochastic, perhaps due to spontaneous  $\text{Ca}^{2+}$   
237 transients(37). This model predicts that, if calcium pulses among cells could be synchronized in  
238 time, then Crz1 pulsatility should be synchronized immediately after, and that this induced  
239 synchrony would gradually decay. Consistent with this, in our nifedipine treated cells where  
240 large calcium pulses were induced in every cell immediately after calcium was added to the  
241 media, the Crz1 pulses following these synchronized calcium pulses are also synchronized, and  
242 this synchrony decays with time (Figure 3B). However, by simulating Crz1 dynamic many times

243 with identical parameter values (Table 1), we found that our stochastic time delay model does  
244 not predict this loss of synchrony at the population level. This suggests that additional sources of  
245 cell-to-cell variability are likely missing from the stochastic time delay model.

246 Nevertheless, our stochastic time delay model has several advantages over a conformational  
247 switch model that assumes Crz1 nuclear localization sensitively reads out  $[Ca^{2+}]_{cyt}$  when  $[Ca^{2+}]_{cyt}$   
248 passes through a threshold(22). Once a damped calcium oscillation is present, the conformational  
249 switch model can generate Crz1 pulses as a readout of  $[Ca^{2+}]_{cyt}$  passing through a threshold  
250 (supplementary figure 4). Although we do not observe calcium oscillations passing a threshold in  
251 our movies, it is possible that our calcium sensor is not sensitive enough to distinguish these  
252 dynamics from background noise. Both our model and the conformational switch model require  
253 no negative feedback in the calmodulin/calcineurin signaling pathway. However, our model  
254 predicts the width of the second Crz1 pulse to be wider than the first, while the conformational  
255 switch model predicts the opposite: a narrower second Crz1 pulse because it is reading out a  
256 smaller fluctuation in  $[Ca^{2+}]_{cyt}$ . The comparison of pulse widths (Figure 5D) supports the time  
257 delay model. We also note that the stochastic model is simpler (fewer parameters needed to  
258 generate pulses and no assumption a sensitive threshold), and can directly explain the  
259 coordination of the subcellular localization of the ~500 Crz1 molecules in the cell through time-  
260 delay in nuclear transport.

261 One crucial assumption in our model for the coordination among Crz1 molecules is the  
262 deactivation rate of calcineurin. Previous studies show that calcineurin has a deactivation rate *in*  
263 *vitro* of 0.08 fold change per minute while both calcium ions and calmodulin are presented, and  
264 has an even slower deactivation rate when either of them is not presented(38–40). The  
265 deactivation rate is slow enough to maintain the synchronous translocation of each Crz1

266 molecule in our model, which only requires calcineurin to return to baseline activity around 5  
267 minutes after a calcium pulse, a length of time that has been reported *in vitro*(38–40). A  
268 conclusive test of our model would be a mutation in calcineurin that solely affects the  
269 deactivation rate, but no such mutant is available to our knowledge.

270 Previous work on Crz1 pulsatility suggested that Crz1 pulses are actively generated rather than  
271 passively reading out the fluctuation in  $[Ca^{2+}]_{cyt}$ (9). If our model is correct, then it suggests a  
272 third possibility: individual Crz1 molecules read out  $[Ca^{2+}]_{cyt}$  with a time delay. This possibility  
273 can explain the observation that higher affinity of calcineurin docking site on Crz1 leads to  
274 higher pulsing frequency(9), because higher affinity allows Crz1 to be dephosphorylated by a  
275 lower fraction of activated calcineurin and, therefore, oscillate longer after a calcium pulse. The  
276 time delay is assumed to be created by the transport between cytoplasm and nucleus, which  
277 because it requires a complicated series of steps, leads to a transport rate in the order of  
278 minutes(41). This model can be generalized to relocalization of other pulsatile transcription  
279 factors and macromolecules that have dynamics on the order of minutes, and can explain how  
280 signals that are short and fluctuating are converted into the frequency of pulses without a  
281 negative feedback loop.

282

283 Materials and methods

284 *Yeast cell strain and growth conditions*

285 BY4741 was used to construct the dual Crz1-Calcium reporter strain. Plasmids expressing  
286 GCaMP3 calcium reporter were constructed using Gibson assembly protocol(42) and gel

287 purification. The calcium reporter gene was assembled between the promoter of ribosomal  
288 protein L39, RPL39, and the ADH1 terminator. pRPL39-GCaMP3-tADH1 was integrated at the  
289 *HO* locus using a selectable marker (LEU2) and confirmed by Sanger sequencing. Four  
290 replicates were performed and all showed expected GCaMP3 expression(15,16). To tag Crz1  
291 with mCherry at the C terminus, genomic integration of pCrz1-ymCherry was done at the *CRZ1*  
292 locus using a selectable marker (URA3) and confirmed by PCR. All transformations were  
293 performed using the standard lithium acetate procedure(43).

294 All the time-lapse imaging experiments were started when cells were in log-phase (4 hours after  
295 being diluted from overnight liquid culture). Cells were grown in synthetic complete (SC) media  
296 lacking leucine and uracil to maintain section of markers. Carbon source was 2% glucose. For  
297 artificially increased calcium pulse experiments, 200 $\mu$ m Nifedipine were added during the 4 hour  
298 inoculation.

### 299 *Spinning-disk Confocal Microscopy and image analysis*

300 Nikon CSU-X1 was utilized for time-lapse imaging at room temperature (22° C). For GCaMP3,  
301 488 nm laser was applied with time resolutions of 6 sec/frame, exposure time of 100 msec, and  
302 25% laser intensity; for mCherry, 561 nm laser was applied with time resolution 30 sec/frame,  
303 exposure time of 700 msec, and 50% laser intensity. Bright field images with out-of-focus black  
304 cell edge were acquired every minute for cell segmentation and tracking.

305 Cells were attached to glass-bottom dishes with 0.1 mg/ml Concanavalin-A as a binding agent  
306 using a standard protocol(44,45). For each experiment, a time-lapse image series without  
307 calcium stress induction was recorded as a negative control. At the beginning of each time-lapse  
308 image series, an area of the dish that had not been exposed to laser was recorded in order to

309 avoid blue light stress, which is known to induce Crz1 nuclear localization(32). Calcium chloride  
310 solution was added to the dish to a final concentration of 0.2M through a syringe within 20  
311 seconds. To record the dynamics during steady state, time-lapse movies of 30 min or 1 hour were  
312 recorded after more than 1 hour of calcium stress induction for two to four time-lapse movies in  
313 each experiment. 27 replicates of time-lapse movies (18 hours in total) were recorded. Every  
314 analysis was done in both 1 hour and 30 minute time-lapse experiments.

315 Segmentation was automatically performed by identifying the area within cell edge through  
316 MATLAB Image Segmentation Toolbox, and cell tracking was performed by identifying 90%  
317 overlapping cell areas between two time frames. Mis-segmented and mis-stracked objects were  
318 manually removed. 23-87 cells were identified in each time-lapse movie. Single cell  
319 photobleaching correction was conducted after single cell reporter intensities were quantified  
320 (see below) using bi-exponential regression(46): for GCaMP3 intensity, correction was  
321 performed according to baseline intensity; for Crz1 expected nuclear signal, correction was  
322 performed according to Crz1 expected cytoplasmic signal. Baseline was normalized to 0 after  
323 photobleaching correction.

#### 324 *Osmotic shock reduction*

325 Crz1 localizes into the nucleus for 10 to 15 minutes after an osmotic shock (33). In the  
326 experiments where we artificially increased calcium pulses (nifedipine treatment), the effect  
327 from osmotic shock was undesirable because the calcium pulses occur immediately after addition  
328 of calcium. Change in osmotic pressure due to 0.2M calcium chloride is around 3.4 Pa, so prior  
329 to the experiment, sodium chloride solution was added (to reach 0.4M) to increase osmotic  
330 pressure to 6.1 Pa. When calcium chloride solution was added (so final concentrations of both

331 sodium chloride and calcium chloride were 0.2M), the final osmotic pressure was now around  
332 6.5 Pa, reducing the change in osmotic pressure before and after addition of calcium to around  
333 0.5 Pa.

334 *Reporter intensity quantification*

335 GCaMP3 intensity for each time point was estimated as average pixel intensity for all pixels in  
336 the cell.

337 Nuclear localization for each time point was quantified by fitting a mixture of a Gaussian  
338 distribution and a uniform distribution, and the parameters of distributions were estimated using  
339 expectation-maximization on the pixel data from each cell (see supplementary text for more  
340 details and derivation of the algorithm).

341 *Peak finding, pulse analyses and periodicity*

342 Local maxima/minima were identified with Matlab function findpeaks. To smooth fluctuations  
343 shorter than 4 time points, Savitzky-Golay filtering was applied on each trajectory before  
344 defining the Crz1 pulse threshold, identifying Crz1 pulses, and quantifying calcium overshoot  
345 depth

346 To define the threshold for pulses, every local maximum in all cells growing in standard liquid  
347 culture (no additional calcium) was identified with a minimum distance of 60 seconds.

348 Thresholds were then chosen to filter out most of the background noise: we chose the top 0.5%  
349 of the peak height (0.09) for calcium pulses, and the top 5% of both the peak height (0.30) and  
350 prominence (0.15) for Crz1 pulses.



351 For the analysis of the relationship between calcium pulse height and number of Crz1 pulses, the  
352 Crz1 pulses following a calcium pulse were counted until the next calcium pulse or the end of  
353 the time series, and the Crz1 pulses before a calcium pulse were counted until the previous  
354 calcium pulse or the beginning of the time series.

355 For every cell that has its largest calcium pulse after 5 minutes from the beginning or before 5  
356 minutes from the end of the time-lapse experiments, its Crz1 trajectory was separated into pre-  
357 calcium-pulse and post-calcium-pulse trajectories. Each part was quantified if a trajectory prefers  
358 an aperiodic Gaussian process model or a periodic Gaussian process model with log likelihood  
359 ratio (LLR) using established method and MATLAB scripts(47).

#### 360 *Logistic pulse fitting*

361 A least squares method is developed to quantify Crz1 pulse height and width based on the  
362 analytic solution of logistic curve (see supplementary text for more details and derivation).

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#### 369 Conflict of interest

370 The authors of this manuscript do not have affiliation with or involvement in any organization or  
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### 373 Author contributions

374 I. S. H. performed the experiments. B. S. and S. P. provided experimental training and support. I.  
375 S. H. and A. M. developed the computational models. A. M. supervised the project. I. S. H. and  
376 A. M. wrote the paper.

### 377 References

- 378 1. Purvis JE, Karhohs KW, Mock C, Batchelor E, Loewer A, Lahav G. p53 dynamics control  
379 cell fate. *Science*. 2012 Jun 15;336(6087):1440–4.
- 380 2. Ashall L, Horton CA, Nelson DE, Paszek P, Harper CV, Sillitoe K, et al. Pulsatile  
381 stimulation determines timing and specificity of NF-kappaB-dependent transcription.  
382 *Science*. 2009 Apr 10;324(5924):242–6.
- 383 3. Hansen AS, O’Shea EK. Limits on information transduction through amplitude and  
384 frequency regulation of transcription factor activity. *eLife*. 2015 May 18;4:e06559.
- 385 4. Hansen AS, O’Shea EK. Encoding four gene expression programs in the activation  
386 dynamics of a single transcription factor. *Curr Biol CB*. 2016 Apr 4;26(7):R269-271.
- 387 5. Levine JH, Lin Y, Elowitz MB. Functional roles of pulsing in genetic circuits. *Science*.  
388 2013 Dec 6;342(6163):1193–200.
- 389 6. Nandagopal N, Santat LA, LeBon L, Sprinzak D, Bronner ME, Elowitz MB. Dynamic  
390 Ligand Discrimination in the Notch Signaling Pathway. *Cell*. 2018 Feb 8;172(4):869-  
391 880.e19.
- 392 7. Albeck JG, Mills GB, Brugge JS. Frequency-modulated pulses of ERK activity transmit  
393 quantitative proliferation signals. *Mol Cell*. 2013 Jan 24;49(2):249–61.
- 394 8. Locke JCW, Young JW, Fontes M, Hernández Jiménez MJ, Elowitz MB. Stochastic pulse  
395 regulation in bacterial stress response. *Science*. 2011 Oct 21;334(6054):366–9.
- 396 9. Cai L, Dalal CK, Elowitz MB. Frequency-modulated nuclear localization bursts coordinate  
397 gene regulation. *Nature*. 2008 Sep 25;455(7212):485–90.

- 398 10. Jiang Y, AkhavanAghdam Z, Tsimring LS, Hao N. Coupled feedback loops control the  
399 stimulus-dependent dynamics of the yeast transcription factor Msn2. *J Biol Chem.* 2017  
400 28;292(30):12366–72.
- 401 11. Geva-Zatorsky N, Rosenfeld N, Itzkovitz S, Milo R, Sigal A, Dekel E, et al. Oscillations  
402 and variability in the p53 system. *Mol Syst Biol.* 2006;2:2006.0033.
- 403 12. Longo DM, Selimkhanov J, Kearns JD, Hasty J, Hoffmann A, Tsimring LS. Dual delayed  
404 feedback provides sensitivity and robustness to the NF- $\kappa$ B signaling module. *PLoS Comput*  
405 *Biol.* 2013;9(6):e1003112.
- 406 13. Kulak NA, Pichler G, Paron I, Nagaraj N, Mann M. Minimal, encapsulated proteomic-  
407 sample processing applied to copy-number estimation in eukaryotic cells. *Nat Methods.*  
408 2014 Mar;11(3):319–24.
- 409 14. Cyert MS. Calcineurin signaling in *Saccharomyces cerevisiae*: how yeast go crazy in  
410 response to stress. *Biochem Biophys Res Commun.* 2003 Nov 28;311(4):1143–50.
- 411 15. Carbó N, Tarkowski N, Ipiña EP, Dawson SP, Aguilar PS. Sexual pheromone modulates  
412 the frequency of cytosolic Ca<sup>2+</sup>bursts in *Saccharomyces cerevisiae*. *Mol Biol Cell.* 2017  
413 Feb 15;28(4):501–10.
- 414 16. Tian L, Hires SA, Mao T, Huber D, Chiappe ME, Chalasani SH, et al. Imaging neural  
415 activity in worms, flies and mice with improved GCaMP calcium indicators. *Nat Methods.*  
416 2009 Dec;6(12):875–81.
- 417 17. Song S, Li J, Zhu L, Cai L, Xu Q, Ling C, et al. Irregular Ca(2+) oscillations regulate  
418 transcription via cumulative spike duration and spike amplitude. *J Biol Chem.* 2012 Nov  
419 23;287(48):40246–55.
- 420 18. Sneyd J, Han JM, Wang L, Chen J, Yang X, Tanimura A, et al. On the dynamical structure  
421 of calcium oscillations. *Proc Natl Acad Sci.* 2017 Feb 14;114(7):1456–61.
- 422 19. Tsien RW, Tsien RY. Calcium channels, stores, and oscillations. *Annu Rev Cell Biol.*  
423 1990;6:715–60.
- 424 20. Cui J, Kaandorp JA, Ositelu OO, Beaudry V, Knight A, Nanfack YF, et al. Simulating  
425 calcium influx and free calcium concentrations in yeast. *Cell Calcium.* 2009  
426 Feb;45(2):123–32.
- 427 21. Miseta A, Fu L, Kellermayer R, Buckley J, Bedwell DM. The Golgi apparatus plays a  
428 significant role in the maintenance of Ca<sup>2+</sup> homeostasis in the vps33Delta vacuolar  
429 biogenesis mutant of *Saccharomyces cerevisiae*. *J Biol Chem.* 1999 Feb 26;274(9):5939–  
430 47.
- 431 22. Cui J, Kaandorp JA. Mathematical modeling of calcium homeostasis in yeast cells. *Cell*  
432 *Calcium.* 2006 Apr;39(4):337–48.

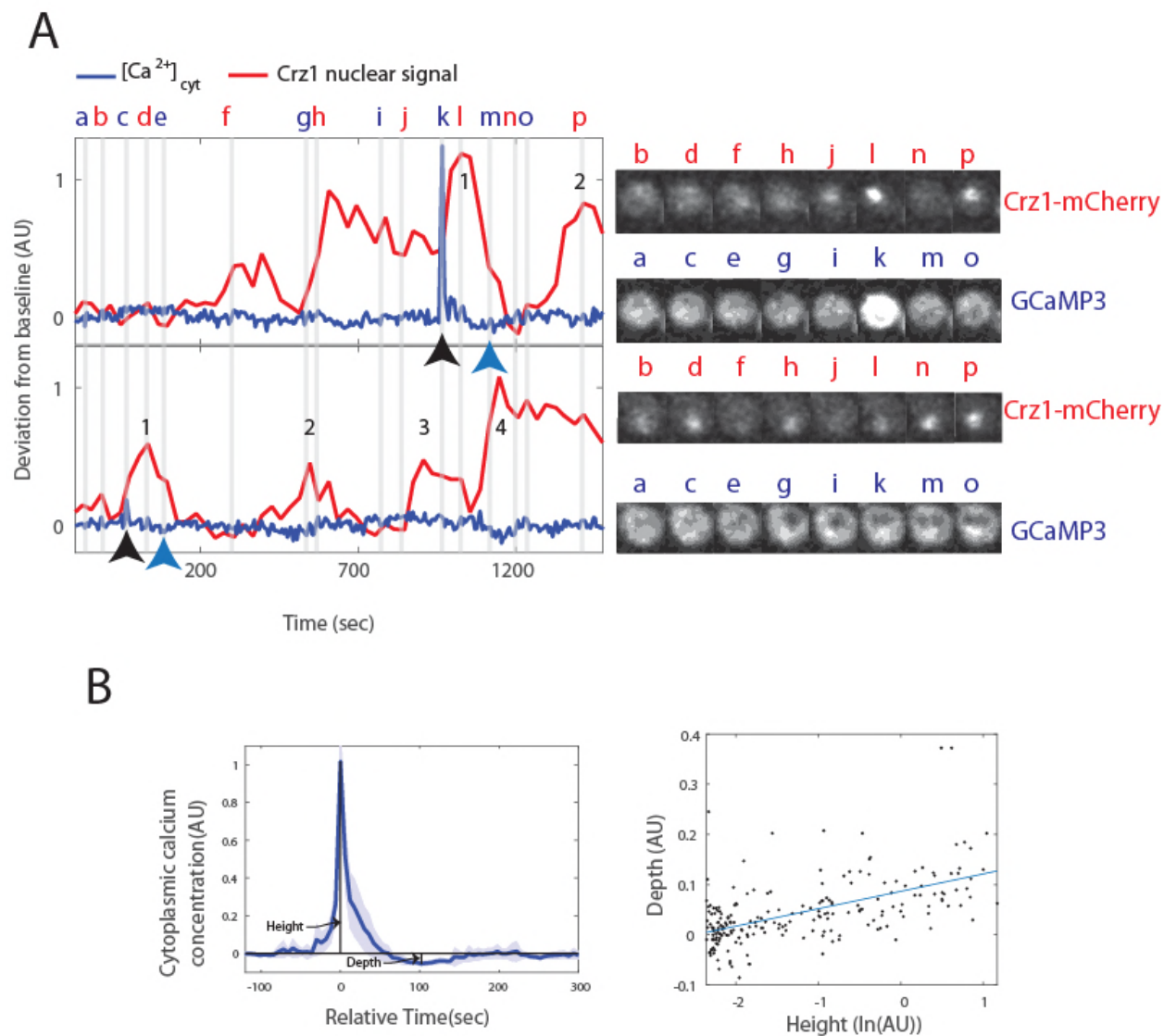
- 433 23. Lin Y, Sohn CH, Dalal CK, Cai L, Elowitz MB. Combinatorial gene regulation by  
434 modulation of relative pulse timing. *Nature*. 2015 Nov 5;527(7576):54–8.
- 435 24. Ni XY, Drengstig T, Ruoff P. The control of the controller: molecular mechanisms for  
436 robust perfect adaptation and temperature compensation. *Biophys J*. 2009 Sep  
437 2;97(5):1244–53.
- 438 25. Thorsen K, Agafonov O, Selstø CH, Jolma IW, Ni XY, Drengstig T, et al. Robust  
439 concentration and frequency control in oscillatory homeostats. *PloS One*.  
440 2014;9(9):e107766.
- 441 26. Kishi T, Ikeda A, Nagao R, Koyama N. The SCFCdc4 ubiquitin ligase regulates calcineurin  
442 signaling through degradation of phosphorylated Rcn1, an inhibitor of calcineurin. *Proc*  
443 *Natl Acad Sci*. 2007 Oct 30;104(44):17418–23.
- 444 27. Rodríguez A, Roy J, Martínez-Martínez S, López-Maderuelo MD, Niño-Moreno P, Ortí L,  
445 et al. A conserved docking surface on calcineurin mediates interaction with substrates and  
446 immunosuppressants. *Mol Cell*. 2009 Mar 13;33(5):616–26.
- 447 28. Salazar C, Höfer T. Allosteric regulation of the transcription factor NFAT1 by multiple  
448 phosphorylation sites: a mathematical analysis. *J Mol Biol*. 2003 Mar 14;327(1):31–45.
- 449 29. Bratsun D, Volfson D, Tsimring LS, Hasty J. Delay-induced stochastic oscillations in gene  
450 regulation. *Proc Natl Acad Sci U S A*. 2005 Oct 11;102(41):14593–8.
- 451 30. Mittler JE, Sulzer B, Neumann AU, Perelson AS. Influence of delayed viral production on  
452 viral dynamics in HIV-1 infected patients. *Math Biosci*. 1998 Sep;152(2):143–63.
- 453 31. Galla T. Intrinsic fluctuations in stochastic delay systems: theoretical description and  
454 application to a simple model of gene regulation. *Phys Rev E Stat Nonlin Soft Matter Phys*.  
455 2009 Aug;80(2 Pt 1):021909.
- 456 32. Bodvard K, Jörhov A, Blomberg A, Molin M, Käll M. The yeast transcription factor Crz1 is  
457 activated by light in a Ca<sup>2+</sup>/calcineurin-dependent and PKA-independent manner. *PloS*  
458 *One*. 2013;8(1):e53404.
- 459 33. Denis V, Cyert MS. Internal Ca<sup>(2+)</sup> release in yeast is triggered by hypertonic shock and  
460 mediated by a TRP channel homologue. *J Cell Biol*. 2002 Jan 7;156(1):29–34.
- 461 34. Bouillet LEM, Cardoso AS, Perovano E, Pereira RR, Ribeiro EMC, Trófia MJM, et al. The  
462 involvement of calcium carriers and of the vacuole in the glucose-induced calcium  
463 signaling and activation of the plasma membrane H<sup>(+)</sup>-ATPase in *Saccharomyces*  
464 *cerevisiae* cells. *Cell Calcium*. 2012 Jan;51(1):72–81.
- 465 35. D’hooge P, Coun C, Van Eyck V, Faes L, Ghillebert R, Mariën L, et al. Ca<sup>(2+)</sup>  
466 homeostasis in the budding yeast *Saccharomyces cerevisiae*: Impact of ER/Golgi Ca<sup>(2+)</sup>  
467 storage. *Cell Calcium*. 2015 Aug;58(2):226–35.

- 468 36. Dalal CK, Cai L, Lin Y, Rahbar K, Elowitz MB. Pulsatile dynamics in the yeast proteome.  
469 *Curr Biol* CB. 2014 Sep 22;24(18):2189–94.
- 470 37. Baker SA, Drumm BT, Saur D, Hennig GW, Ward SM, Sanders KM. Spontaneous Ca(2+)  
471 transients in interstitial cells of Cajal located within the deep muscular plexus of the murine  
472 small intestine. *J Physiol*. 2016 15;594(12):3317–38.
- 473 38. Kincaid RL, Martensen TM, Vaughan M. Modulation of calcineurin phosphotyrosyl protein  
474 phosphatase activity by calmodulin and protease treatment. *Biochem Biophys Res*  
475 *Commun*. 1986 Oct 15;140(1):320–8.
- 476 39. Pallen CJ, Wang JH. Stoichiometry and dynamic interaction of metal ion activators with  
477 calcineurin phosphatase. *J Biol Chem*. 1986 Dec 5;261(34):16115–20.
- 478 40. King MM, Huang CY. The calmodulin-dependent activation and deactivation of the  
479 phosphoprotein phosphatase, calcineurin, and the effect of nucleotides, pyrophosphate, and  
480 divalent metal ions. Identification of calcineurin as a Zn and Fe metalloenzyme. *J Biol*  
481 *Chem*. 1984 Jul 25;259(14):8847–56.
- 482 41. Ribbeck K, Görlich D. Kinetic analysis of translocation through nuclear pore complexes.  
483 *EMBO J*. 2001 Mar 15;20(6):1320–30.
- 484 42. Gibson DG. Enzymatic assembly of overlapping DNA fragments. *Methods Enzymol*.  
485 2011;498:349–61.
- 486 43. Schiestl RH, Gietz RD. High efficiency transformation of intact yeast cells using single  
487 stranded nucleic acids as a carrier. *Curr Genet*. 1989 Dec;16(5–6):339–46.
- 488 44. Pemberton LF. Preparation of yeast cells for live-cell imaging and indirect  
489 immunofluorescence. *Methods Mol Biol Clifton NJ*. 2014;1205:79–90.
- 490 45. Zarin T, Tsai CN, Nguyen Ba AN, Moses AM. Selection maintains signaling function of a  
491 highly diverged intrinsically disordered region. *Proc Natl Acad Sci U S A*. 2017 Feb  
492 21;114(8):E1450–9.
- 493 46. Vicente NB, Zamboni JED, Adur JF, Paravani EV, Casco VH. Photobleaching correction in  
494 fluorescence microscopy images. *J Phys Conf Ser*. 2007;90(1):012068.
- 495 47. Phillips NE, Manning C, Papalopulu N, Rattray M. Identifying stochastic oscillations in  
496 single-cell live imaging time series using Gaussian processes. *PLoS Comput Biol*. 2017  
497 May;13(5):e1005479.

498

499

500 Figure legends:



501

502 Figure 1. Calcium pulses with overshoots are found in yeast cells under calcium stress. A) Left

503 panel shows examples of single cell trajectories for Crz1 (red trace) and calcium (blue trace) and

504 snapshots at representative time points from two cells close to each other in the original field of

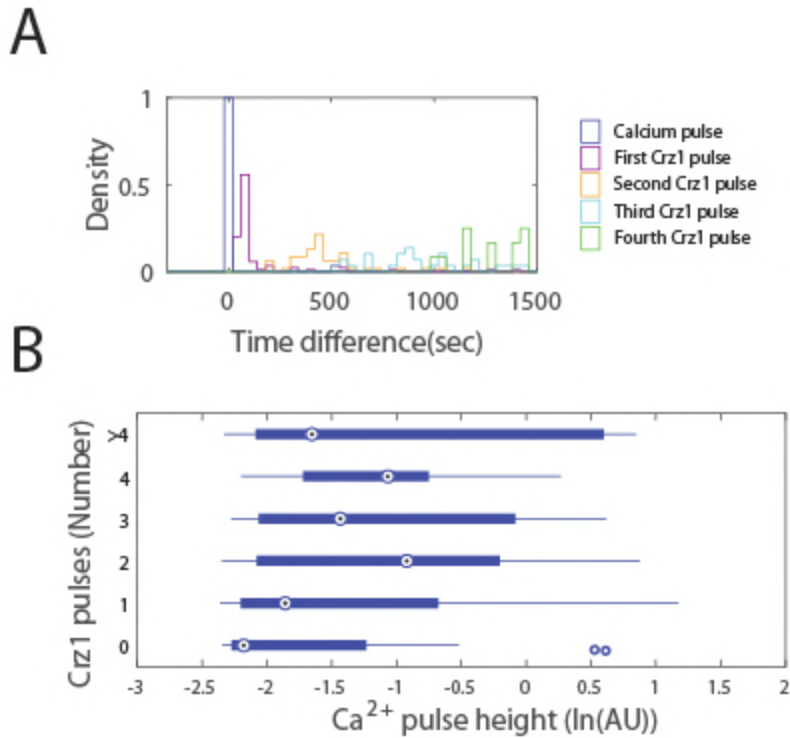
505 view. Numbers along the red trace indicate the Crz1 pulses identified following a calcium pulse

506 (black arrow below blue trace). Blue arrow indicates the local minimum (so-called overshoot)

507 following the calcium pulse. Images in the right panel show the mCherry channel and GCaMP3

508 channel at points indicated in the left panel. B) Left panel shows the average trace of 50 calcium  
509 pulses. Shaded area shows 95% CI of the average trace. Maxima of calcium pulses are aligned to  
510 time = 0 sec (Relative time). An overshoot can be found around time = 100 sec. In the right  
511 panel, each dot represents a single calcium pulse. The x-axis is in natural log of peak height,  
512 while the y-axis is the depth of the overshoot. Blue line shows a linear fit ( $R^2 = 0.37$ ).

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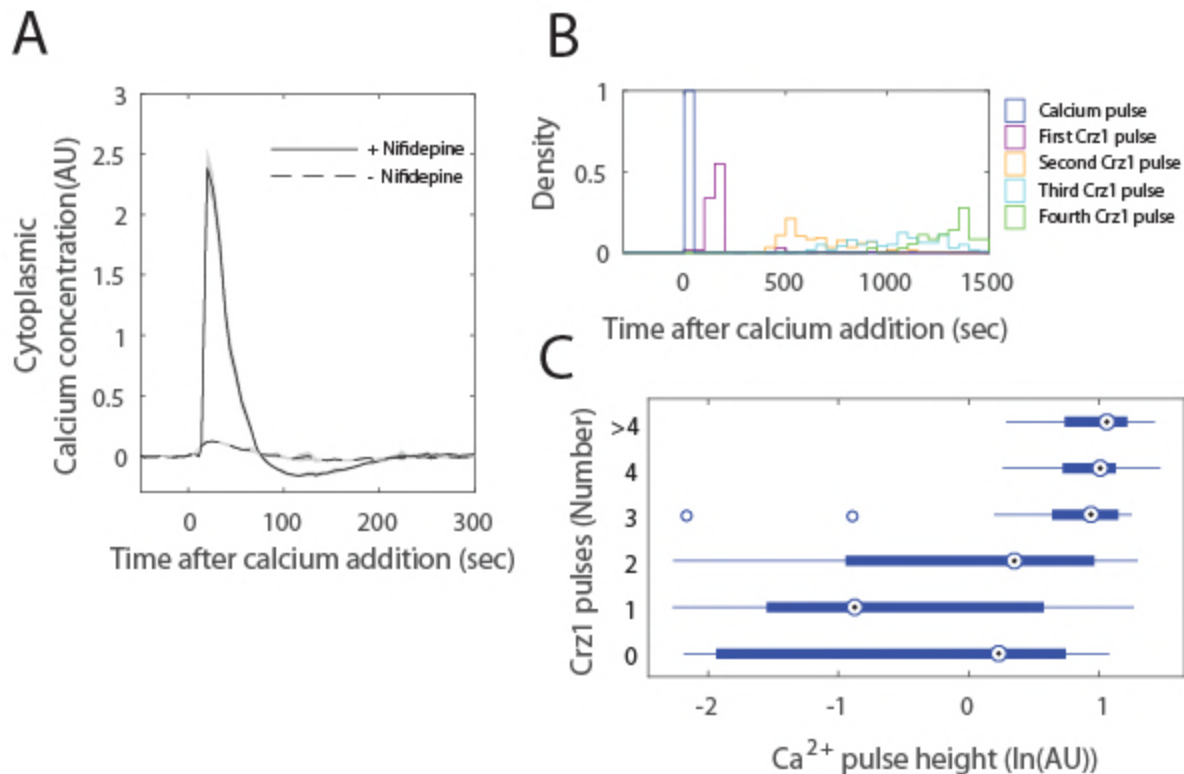


514

515 Figure 2. A calcium pulse is followed by multiple Crz1 pulses, and the number of Crz1 pulses is  
516 positively correlated to the height of the calcium pulse. A) The density of first, second third and  
517 fourth Crz1 pulses (purple, yellow, cyan and green, respectively) is plotted as a function of the  
518 time they occur after the calcium pulse (blue). B) Sample sizes of each number of Crz1 pulses  
519 are 29 for 0, 73 for 1, 43 for 2, 21 for 3, 16 for >3. Blue boxes indicate the 25% - 75% range,  
520 large circles represent the mean, lines represent the range of the data, and individual points show  
521 the locations of two outliers.

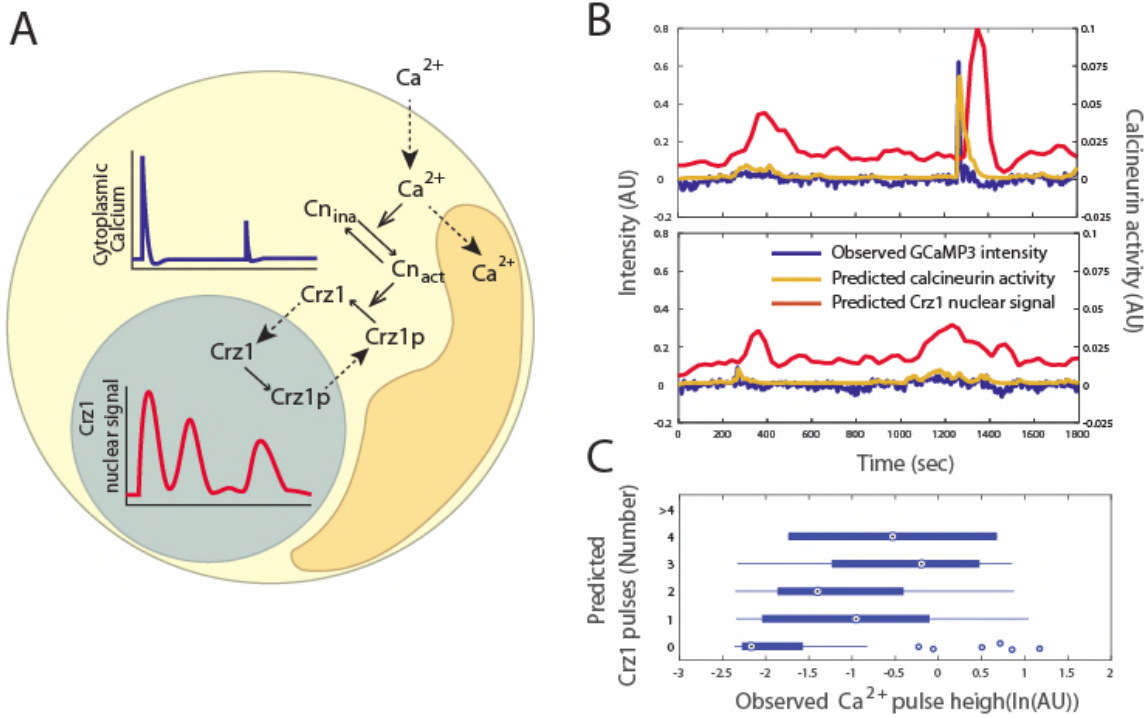
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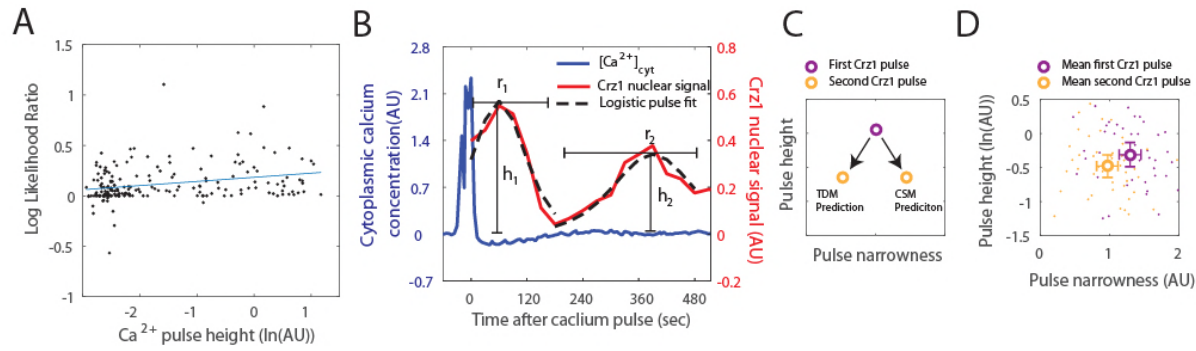
523

524 Figure 3. Artificially induced calcium pulses with large average height are followed by more  
525 Crz1 pulses. A) Average single cell trajectory (without aligning the maxima) after calcium  
526 addition shows synchronized calcium pulses in cells treated with nifedipine (solid line is mean  
527 GCaMP3 signal), not in untreated cells (broken line is the mean GCaMP3 from untreated cells.  
528 Shaded areas show the 95% CI of mean. B) The density of first, second third and fourth Crz1  
529 pulses (purple, yellow, cyan and green, respectively) is plotted as a function of the time they  
530 occur after the calcium pulse (blue). C) Sample sizes of each number of Crz1 pulses are 16 for 0,  
531 36 for 1, 36 for 2, 30 for 3, 48 for 4, 14 for >4. Blue boxes indicate the 25% - 75% range, large  
532 circles represent the mean, lines represent the range of the data, and individual points show the  
533 locations of outliers.



534

535 Figure 4. A time delay model for Crz1 nuclear pulsing can qualitatively reproduce Crz1  
 536 dynamics after calcium pulses. A) In this single cell model,  $Ca^{2+}$  in the cytoplasm (yellow  
 537 shaded area) is controlled through a two-channel system (dotted arrows crossing from outside the  
 538 cell to inside, and crossing from yellow shaded area to orange shaded area representing the  
 539 vacuole). Calcineurin is activated ( $Cn_{ina}$  to  $Cn_{act}$ ) by cytoplasmic calcium, and leads to Crz1  
 540 dephosphorylation ( $Crz1p$  to Crz1). Dephosphorylated Crz1 is imported (dotted arrow) from  
 541 cytoplasm (yellow shaded area) to nucleus (grey shaded area) and phosphorylated Crz1 is  
 542 exported (dotted arrow from nucleus to cytoplasm). B) The Crz1 pulses produced by the model  
 543 (red trace) following calcium pulses (blue trace). Gold trace shows the calcineurin activity  
 544 predicted by the model. C) Blue boxes indicate the 25% - 75% range, large circles represent the  
 545 mean, lines represent the range of the data, and individual points show the locations of outliers.  
 546 Sample sizes of each number of Crz1 pulses are 68 for 0, 72 for 1, 31 for 2, 9 for 3, 2 for 4, 0 for  
 547  $>4$ .



548

549 Figure 5. Predictions of the time delay model are confirmed by experimental data. A) Log  
 550 likelihood ratio between a periodic and an aperiodic Gaussian process model shows an  
 551 increasing preference towards the periodic process when calcium pulse height increases (blue  
 552 line shows a linear fit,  $R^2 = 0.07$ ). Each dot represents the Crz1 trajectory following a calcium  
 553 pulse. B) shows an example of logistic pulse fit (dotted trace) to experimental data (red trace) for  
 554 estimating the narrowness ( $r_1$  and  $r_2$ ) and the heights ( $h_1$  and  $h_2$ ) of the first and second Crz1  
 555 pulses after a calcium pulse. C) Conformational switch model (CSM) predicts that the second  
 556 Crz1 pulse is shorter and narrower than the first Crz1 pulse, while the time delay model (TDM)  
 557 predicts that the second Crz1 pulse is shorter and less narrow than the first. D) Unfilled circles  
 558 indicate the mean height and narrowness of Crz1 pulses. The first and second Crz1 pulses are  
 559 purple and yellow, respectively. Each dot corresponds to a single Crz1 pulse identified following  
 560 a calcium pulse.