# Response to Comment on "Oscillations in NF-κB Signaling Control the Dynamics of Gene Expression"

Single-cell oscillations of signaling proteins can only be detected by time-lapse imaging of fluorescent proteins. We discussed the functionality of our NF-κB (RelA) and IκBα fusion constructs in Section A of the supplementary material in (*1*), and it is clearly important to control for and to minimize overexpression. We previously showed that the IκBα:RelA ratio affects the timing of translocation responses (*2*) and that sensitive parameters such as NF-κB–dependent IκBα transcription significantly affect the system (*1*, *3*). The ability to relate expression level to behavior of the system [see figure 3 in (*1*)] allowed us to identify when ectopic expression perturbs the system and provides information to improve computational models.

Using the Hoffmann model (*4*), we concluded by sensitivity analysis (*3*) that RelA overexpression would minimally perturb the system; therefore, we used RelA localization as our principal output. We estimated that average overexpression of RelA fusion proteins was 3 to 5 times that of endogenous RelA levels in transfected cells, with a distribution in the cell population. Using simulations, Barken *et al.* (*5*) suggest that RelA, IκBα, or RelA and IκBα expression (within the range suggested by our data) maintains oscillations but alters oscillation frequency and amplitude. They suggest [figure 1 in (*5*)], that simulated 4-fold overexpression of RelA delays the second peak of nuclear localization by 3.5 hours (normal 2-hour peak delayed to 5.5 hours). This does not fit our experimental data, because in both cell lines used, the second peak of nuclear localization with RelA overexpression alone was at around 3 hours [see figure 1 and supplemental material in (*1*)]. Reanalysis of our data from SK-N-AS cells (Fig. 1) and HeLa and Swiss 3T3 cells (data not shown) demonstrates no correlation between RelA-DsRed expression level and successive peak timing (also of amplitude, not shown) with cellular fluorescent levels that vary up to ~20 fold. This clear inconsistency between their in silico results and our experimental data suggests that their computational model cannot faithfully reproduce all aspects of the system. Altering the computational model (*1*, *4*) by replacing the second-order term for NF-κB–induced IκBα synthesis with a linear expression [reaction 28 in table S1 in (*1*)] results in a new model that reproduces all of the fundamental characteristics of our experimental data. This model shows much reduced sensitivity of period to RelA concentration (Fig. 2), which demonstrates that continued refinement of the Hoffmann model (*6*, *7*) may well bring the simulations closer to observed biological phenomena.

Barken *et al.* (*5*) show immunocytochemistry (ICC) analysis of serum-starved 3T3 fibroblasts after TNFα stimulation [figure 2 in (*5*)]. We have already described ICC analysis of localization of endogenous RelA in HeLa cells after TNFα stimulation [figure S3 in (*1*)]. These data showed that the nuclear (N) to cytoplasmic (C) ratio was entirely consistent with the oscillations that we saw using fluorescent protein imaging in the same cells. Barken *et al.* interpret their ICC data as suggesting that synchronous and

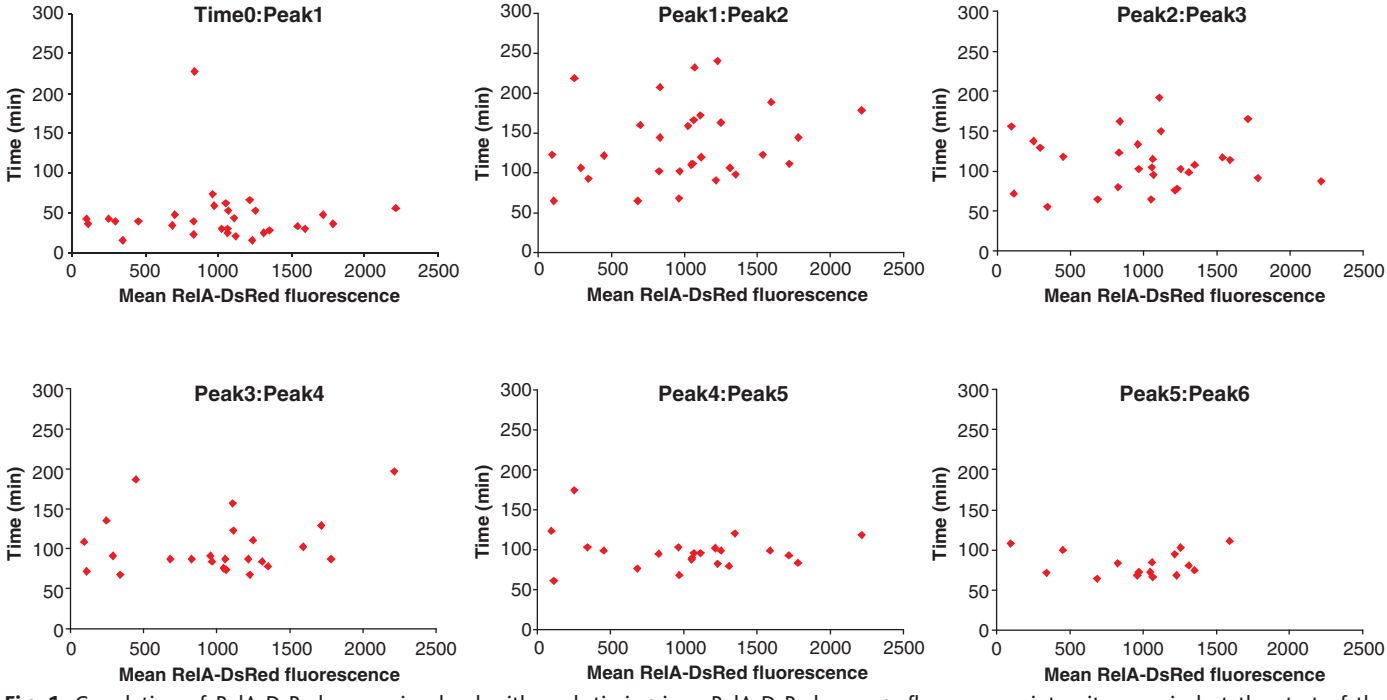


**Fig. 1.** Correlation of RelA-DsRed expression level with peak timing in single SK-N-AS cells. SK-N-AS cells were transfected with a RelA-DsRed expression vector together with a control EGFP-N1 construct (Clontech). At 24 hours after transfection, cells were treated with 10 ng/ml TNFα and imaged by time-lapse confocal microscopy, as described (*1*). Peak timings (expressed as the time difference between successive peaks) from 30 cells from three separate experiments were plotted against the RelA-DsRed average fluorescence intensity per pixel at the start of the experiment. The number of cells between graphs decreases primarily due to cells that divided during the course of the experiments. These data showed no statistically significant correlation (by linear regression) between initial RelA-DsRed expression level and peak timing. Analysis of absolute peak timing (rather than the difference between peaks) also showed no observable correlation.

highly damped oscillations occur in their fibroblast cells and that the RelA N:C average ratios become less variable. Our data suggested that oscillations in the SK-N-AS cells were also heavily damped. Surprisingly, Barken *et al*. comment that "[i]f the responses of individual cells are asynchronous and show undamped oscillations, one would expect higher variance at late times than at early times and lower averages due to prolonged phases between peaks." However, both our previous data and their new data clearly show damped oscillations. The anticipated change in variance is therefore irrelevant. It is also not appropriate to directly compare data from different cell lines, because damping may differ [e.g., stronger damping in HeLa compared with SK-N-AS cells, as shown in (*1*)].

Barken *et al*. show experimental data [figure 3 in (*5*)] from genetically engineered cells: IκBβ$^{-/-}$ε$^{-/-}$ and IκBα$^{-/-}$ε$^{-/-}$ knockout mouse embryonic fibroblasts expressing only IκBα or IκBβ, respectively. Cells expressing only IκBα showed synchronous oscillations (by nuclear electromobility shift assay), whereas cells in which this negative feedback loop was removed (expressing only IκBβ) showed a single extended phase of NF-κB–DNA binding. Using RNAse protection analysis of five NF-κB–regulated genes, they showed that IκBβ-expressing cells exhibit similar kinetics of transcription as IκBα-expressing cells over an 8-hour period. However, these cells lack two IκB isoforms, which leads to a significantly altered NF-κB signaling network. The classical NF-κB heterodimer (p65/p50) is predominantly, although not exclusively, regulated by IκBα. Mice lacking IκBα show runting and skin defects and die within 8 days after birth, which demonstrates that NF-κB signaling is far from normal in these animals (*8*, *9*). The IκBα-deficient mice display increased, but not constitutive, p65/p50 DNA binding activity, which suggests that other mechanisms regulate the activity of this dimer. It is possible that compensatory changes have occurred to allow the continued growth of these mutant cell lines, and we cannot exclude the possibility that the activities of some of their NF-κB–regulating kinases/phosphatases may be altered. Redundancy in the NF-κB pathway might allow other members of the family that are not regulated through IκBα to counteract the mutation. Therefore, as with our experiments using leptomycin B to block nuclear export (thus blocking oscillations), the experiments of Barken *et al*. are an experimental compromise. Other approaches will help to further elucidate the functional role of oscillations.

We do not claim that oscillations are essential for gene expression at all NF-κB–regulated promoters in response to all NF-κB–activating stimuli. In the same way that calcium oscillations are not a prerequisite for functional calcium signaling, we propose that oscillations are one feature of NF-κB signaling that may be functionally important. One of the major unanswered questions in the field is how an apparently identical signal can lead to very different transcriptional and cell-fate responses. Oscillations may lead to differential control at different promoters, and this may vary depending on the rate of NF-κB inactivation (e.g., by nuclear dephosphorylation). A lowered rate of nuclear inactivation might mean that oscillations could be less important. We find that the rate of dephosphorylation of RelA Ser536 is markedly different between HeLa and SK-N-AS cells [supplementary material in (*1*)]. The presumption that bulk translocation is absolutely linked to transcription is disputed, and there are examples of normal translocation of NF-κB without transcriptional up-regulation (*10*, *11*). If the modification of NF-κB is the key activation step, then if NF-κB is activated, but IκBα is not degraded, a low level of endogenous shuttling into the nucleus without bulk translocation could activate transcription. Alternatively, IκBα degradation without NF-κB activation could lead to movement of nonfunctional (or differentially functional) NF-κB into the nucleus (*12*).

As discussed by Lahav (*13*), the negative feedback intrinsic to this system seems wasteful compared with leaving the NF-κB in the nucleus for as long as the TNFα is present. One possible explanation is that a message could reside in the oscillation frequency or oscillations might allow robust independent sampling of the state of the transcription factor at several time points (*13*). We do consistently observe that the period is more robust than the amplitude. The use of fluorescent-protein imaging has been a very important tool for the analysis of the dynamics of protein translocation, oscillations, and transcription in cells (*14*–*16*). Only such single-cell measurements can quantify the period, amplitude, and phase of oscillatory signals. When these are used, we find that oscillations in NF-κB expression are substantial and appear to exert a controlling influence on the dynamics of gene expression.

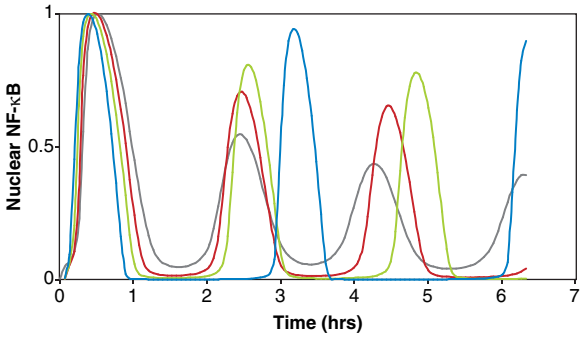


**Fig. 2.** Dependence of oscillation period on RelA concentration using a modified computational model. Simulated nuclear NF-κB activity over time normalized to peak 1. NF-κB concentrations were increased by factors of 1 (black), 1.5 (red), 2 (green), and 4 (blue) in the model. Simulations were performed using the computational model as in (*1*) with reduction of the term describing the induced synthesis of IκBα by NF-κB to a linear form [reaction 28 in table S1 in (*1*)]. [This second-order term has been removed in other variations of the model (*5*, *6*) and is unlikely to be relevant at higher RelA concentrations.] To produce oscillatory behavior, which was representative of the experimental data, appropriate fitting of a single parameter was performed, resulting in parameter tr2 set to 0.0582 min$^{-1}$. All other parameters were unchanged.

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