**Dynamic NF-B and E2F interactions control the priority and timing of inflammatory signalling and cell proliferation**

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**Abstract**

Dynamic cellular systems reprogram gene expression to ensure appropriate cellular fate responses to specific extracellular cues. Here we demonstrate that the dynamics of Nuclear Factor kappa B (NF-κB) signalling and the cell cycle are prioritised differently depending on the timing of an inflammatory signal. Using iterative experimental and computational analyses, we show physical and functional interactions between NF-κB and the E2 Factor 1 (E2F-1) and E2 Factor 4 (E2F-4) cell cycle regulators. These interactions modulate the NF-κB response. In S-phase, the NF-κB response was delayed or repressed, while cell cycle progression was unimpeded. By contrast, activation of NF-κB at the G1/S boundary resulted in a longer cell cycle and more synchronous initial NF-κB responses between cells. These data identify new mechanisms by which the cellular response to stress is differentially controlled at different stages of the cell cycle.

**Introduction**

One of the most important functions in a cell is the accurate interpretation of the information encoded in extracellular signals leading to context-dependent control of cell fate. This is achieved via complex and dynamic signal transduction networks, through which gene expression is re-programmed in response to specific environmental cues ([Barabasi, Gulbahce et al. 2011](#_ENREF_7)). Many signalling systems are subject to temporal changes, involving dynamic alterations to the states of their constituent genes and proteins, with time scales ranging from seconds (Calcium signalling ([Berridge 1990](#_ENREF_9), [Schmidt, Evellin et al. 2001](#_ENREF_51))), hours (DNA damage response ([Lahav, Rosenfeld et al. 2004](#_ENREF_32)), inflammatory response ([Ashall, Horton et al. 2009](#_ENREF_5))), to days (circadian clock ([Welsh, Yoo et al. 2004](#_ENREF_63)), cell cycle ([Sakaue-Sawano, Kurokawa et al. 2008](#_ENREF_50))). Although previous studies have indicated interactions between proteins associated with different dynamical systems ([Wilkins and Kummerfeld 2008](#_ENREF_66), [Bieler, Cannavo et al. 2014](#_ENREF_11), [Feillet, Krusche et al. 2014](#_ENREF_18)), how and when signalling systems are dynamically integrated to determine important cell fate decisions is not well understood.

Nuclear Factor kappa B (NF-κB) is an important signalling system, implicated in many diseases including autoimmune diseases and cancer ([Grivennikov, Greten et al. 2010](#_ENREF_21)). Inflammatory cues such as Tumour Necrosis Factor alpha (TNFα) can trigger the nuclear translocation of the NF-κB RelA subunit and activation of target gene transcription ([Hayden and Ghosh 2008](#_ENREF_23)). Nuclear NF-κB activates feedback regulators, including the IκBα and IκBε inhibitors ([Arenzana-Seisdedos, Turpin et al. 1997](#_ENREF_4), [Kearns, Basak et al. 2006](#_ENREF_28), [Paszek, Ryan et al. 2010](#_ENREF_43)), which bind and transport NF-κB back into the cytoplasm. In response to TNFα, this system shows nuclear-cytoplasmic (N:C) oscillations in the localization of the NF-κB complex associated with out-of-phase cycles of degradation and re-synthesis of IκB proteins ([Nelson, Ihekwaba et al. 2004](#_ENREF_40), [Ashall, Horton et al. 2009](#_ENREF_5), [Lee, Denny et al. 2009](#_ENREF_35), [Sung, Salvatore et al. 2009](#_ENREF_57), [Tay, Hughey et al. 2010](#_ENREF_59), [Turner, Paszek et al. 2010](#_ENREF_62), [Ruland 2011](#_ENREF_49), [Hughey, Gutschow et al. 2015](#_ENREF_26)). Through systems biology and experimental approaches, the frequency of these oscillations has been proposed to be a key parameter that regulates the pattern of downstream gene expression ([Ashall, Horton et al. 2009](#_ENREF_5), [Lee, Walker et al. 2014](#_ENREF_34), [Williams, Timmis et al. 2014](#_ENREF_67)).

NF-κB signalling has also been suggested to have a role in controlling cell division through a number of different mechanisms ([Perkins and Gilmore 2006](#_ENREF_45)). Many NF-κB family members have been characterised as oncoproteins (e.g. c-Rel and Bcl-3 ([Hayden and Ghosh 2008](#_ENREF_23)). Also, a number of cell cycle control proteins have been shown to be NF-κB transcriptional targets, including Cyclin D, ([Guttridge, Albanese et al. 1999](#_ENREF_22), [See, Rajala et al. 2004](#_ENREF_52)) and p21, an inhibitor of Cyclin Dependent Kinase (CDK) activity ([Hinata, Gervin et al. 2003](#_ENREF_24)).

Although interactions between NF-κB and the cell cycle have been reported ([Kundu, Guermah et al. 1997](#_ENREF_31), [Phillips, Ernst et al. 1999](#_ENREF_46), [Perkins and Gilmore 2006](#_ENREF_45)); observing the dynamics of such interactions is challenging via traditional biochemical techniques, which often fail to capture the heterogeneity in a cellular population. Analysis of cell-to-cell heterogeneity has revealed novel regulatory mechanisms for diverse cellular processes ([Pelkmans 2012](#_ENREF_44)) and it has been suggested that this is a fundamental property of the NF-B response ([Paszek, Ryan et al. 2010](#_ENREF_43)).

The E2 Factor (E2F) proteins are differentially expressed during the cell cycle to control cell proliferation ([Bertoli, Skotheim et al. 2013](#_ENREF_10)). They are a family of transcription factors that play a key role in the G1/S cell cycle checkpoint. Previous studies have provided preliminary evidence for physical interaction between NF-κB and E2F proteins ([Tanaka, Matsumura et al. 2002](#_ENREF_58), [Lim, Yao et al. 2007](#_ENREF_37), [Garber, Yosef et al. 2012](#_ENREF_19)) In the current study, a combination of single cell imaging and mathematical modelling was applied to investigate reciprocal co-ordination of the NF-κB response and cell proliferation driven by dynamic interactions between RelA and E2F proteins.

**Results**

**The NF-**κ**B response depends on the cell cycle phase**

We investigated the effect of cell cycle timing on the NF-κB response in HeLa cervical cancer and SK-N-AS neuroblastoma cells. SK-N-AS cells showed repeated oscillations in response to TNFα stimulation that were more damped than those seen in HeLa cells (see Appendix Figure 1 for longer time course data ([Nelson, Ihekwaba et al. 2004](#_ENREF_40), [Ashall, Horton et al. 2009](#_ENREF_5))) In previous studies it was observed that when SK-N-AS cells were treated with a saturating dose of TNFα (10ng/ml) the initial response of NF-κB (i.e. immediate RelA nuclear translocation) was relatively synchronous between cells ([Nelson, Ihekwaba et al. 2004](#_ENREF_40), [Ashall, Horton et al. 2009](#_ENREF_5), [Turner, Paszek et al. 2010](#_ENREF_62)) (Figure 1A; Appendix Figure 1). However, these data showed a variation in timing and amplitude when cells were treated with a lower dose of 30pg/ml TNFα, even though this was functionally close to a saturating dose that gave a strong population-level NF-κB response ([Turner, Paszek et al. 2010](#_ENREF_62)) (Figure 1B). In common with treatment of SK-N-AS cells at 30pg/ml, HeLa cells showed greater heterogeneity in their initial response at a saturating 10ng/ml dose of TNFα, with some cells showing little or no response and others showing a variable delay ([Nelson, Paraoan et al. 2002](#_ENREF_41), [Nelson, Ihekwaba et al. 2004](#_ENREF_40)) (Figure 1C). This is in agreement with data showing heterogeneity of the initial response in other cell types ([Tay, Hughey et al. 2010](#_ENREF_59), [Zambrano, Bianchi et al. 2014](#_ENREF_70)). HeLa cells showed no significant translocation in response to 30pg/ml TNFα (Figure 1D), suggesting that these cell types have differential dynamic NF-κB responses at varying TNFα doses.

We hypothesised that this cell-to-cell heterogeneity in response might be a consequence of cell cycle phase. To test this hypothesis, we investigated the role of cell cycle in both the HeLa and SK-N-AS cells, as these show different dynamic responses to TNFα that are typical of the profile of a wide range of cell lines ([Tay, Hughey et al. 2010](#_ENREF_59), [Turner, Paszek et al. 2010](#_ENREF_62), [Zambrano, Bianchi et al. 2014](#_ENREF_70), [Hughey, Gutschow et al. 2015](#_ENREF_26)). Initially, HeLa cells were treated with 10ng/ml TNFα at various stages of the cell cycle (Figure 1E-H), as they could be easily synchronized at late G1 by a double thymidine block (see Appendix Figure 2). When endogenous RelA was examined using immunocytochemistry, HeLa cells treated with 10ng/ml TNFα in S-phase displayed a reduced nuclear localization, compared to those treated in late G1 (Figure 1E). These results were confirmed using time-lapse imaging of synchronised HeLa cells transiently transfected with RelA-DsRedxp. Cells treated in late G1 showed a strong synchronous translocation of RelA, whereas cells treated in S-phase showed reduced RelA translocation (Figure1F G). These cell cycle-dependent differences following TNFα treatment of synchronized cell populations were supported by alterations in the extent of IκBα degradation and RelA Serine536 phosphorylation at different stages of the cell cycle as measured by western blot (Figure 1H).

To further investigate the effect of cell cycle on the NF-κB response, unsynchronized populations of HeLa and SK-N-AS cells were followed by time-lapse imaging through successive cell divisions. 30 h after the start of this time-course, HeLa cells were stimulated with 10ng/ml TNFα. Cells were assigned to different cell cycle phases based upon their mitosis-to-mitosis and mitosis-to-treatment timings (Figure 2 A and B).

To ensure the accuracy of the inferred cell cycle stage in these experiments, the cycle timing of cells at the point of TNFα treatment was calibrated through control experiments using Fluorescent Ubiquitin-based Cell Cycle Indicators in both HeLa and SK-N-AS cells (FUCCI, Figure 2-Figure Supplement 1 A-B). The G1/S crossing point of Red and Green FUCCI reporters was determined and defined as the G1/S checkpoint The average and distribution of the cell cycle duration in populations of HeLa and SK-N-AS cells was also measured (Figure 2-Figure Supplement 1 C).

The resulting data suggested that HeLa cells treated with TNFα in late G1 (inferred to be G1/S) showed an increase in the translocation amplitude compared to the unsynchronized population average (Figure 2 C). By contrast, cells treated in S-phase appeared to show a damped or delayed response (Figure 2 C), with markedly reduced amplitude of nuclear NF-κB translocation. In G2 phase the NF-κB response appeared to be restored. Analysis of the complete data set confirmed that there was statistically significant higher nuclear translocation amplitude in HeLa cells at G1/S and significantly reduced amplitude in S-phase, compared to G1 and G2 (Figure 2 Supplement 2).

A smaller data set from SK-N-AS cells treated with 30 pg/ml TNFα, showed once again a statistically reduced translocation in S-phase compared to G1-phase. Visually the data are consistent with increased translocation in late G1 and a restored level of translocation in G2- compared to S-phase. However more cells would be required for a statistical analysis of possible differences between these cell cycle phases. (Figure 2 – Figure Supplement 3).

**The effect of NF-κB signalling on cell cycle timing**

We also measured the effect of TNFα treatment on HeLa cell cycle duration (Figure 3). It was found that mean cell cycle duration for cells treated with TNFα showed a small, but statistically significant increase of 1.9 hours (~10%) compared to untreated cells, with the variability in the total population increasing by ~2-fold (Figure 3). Within this TNFα-treated population, cells treated in late G1 were more susceptible to cell cycle elongation with a cell cycle duration that was ~1/3 longer than the untreated population average. TNFα treatment in S-phase had no statistically significant effect on the timing of mitosis. These data suggest a potential direct or indirect role for the NF-κB system in controlling cell cycle duration through an unknown mechanism at the G1/S phase of the cell cycle.

**E2F-1 levels control the dynamics of the NF-**κ**B response**

The mechanism for alteration of NF-κB responses between the late G1- and S-phases of the cell cycle was sought. Previous studies had suggested that E2-Factor-1 (E2F-1) could physically associate with RelA, and/or its major dimer partner p50 ([Kundu, Guermah et al. 1997](#_ENREF_31), [Tanaka, Matsumura et al. 2002](#_ENREF_58), [Lim, Yao et al. 2007](#_ENREF_37)). E2F-1 is the key transcriptional regulator of the cell cycle transition between G1- and S-phase ([Tsantoulis and Gorgoulis 2005](#_ENREF_61)) where its expression is highest. In the presence of ectopically-expressed EGFP-E2F-1, we observed a reduction in the activity of a NF-κB-regulated luciferase reporter (Figure 4A). Moreover, the ability of NF-κB to induce endogenous mRNA levels of IκBα and IκBε was impaired in cells co-expressing EGFP-E2F-1 and RelA-DsRedxp, compared to cells expressing RelA-DsRedxp alone (Figure 4B). E2F-1 target gene transcription was also impaired by RelA expression, as indicated by a reduction in the activity of a Cyclin E luciferase reporter (Figure 4C) and in the mRNA level of E2F-1 itself (Figure 4D). These data support the reciprocal and coordinated control of transcription by E2F-1 and NF-κB.

In transient transfection experiments, a predominantly cytoplasmic localization of RelA-DsRedxp was observed when expressed alone, whereas in cells co-expressing EGFP-E2F-1, both proteins were predominantly nuclear (Figure 4E). In addition we also found that the steady-state cytoplasmic localisation of RelA was restored in cells transiently expressing IκBα-AmCyan in addition to EGFP-E2F-1 and RelA-dsRedxp. These data suggest the hypothesis that IκBα and E2F-1 may compete for the same binding site on RelA, with IκBα perhaps having the higher affinity. Time-series experiments in both SK-N-AS and HeLa cells showed that a decrease in EGFP-E2F-1 expression over time was associated with a re-localization of RelA-DsRedxp from the nucleus to the cytoplasm (for SK-N-AS cells, Figure 4 - Figure supplement 1 A-B; for HeLa cells, Figure 4 Figure supplement 2 A-C). Quantitative analysis showed a strong correlation between the EGFP-E2F1 decay half-life and the delay in RelA-DsRedxp translocation back into the cytoplasm (for SK-N-AS cells, Figure 4 - Figure supplement 1 C; for HeLa cells, Figure 4 Figure supplement 2 D). Initial mathematical modelling of this interacting system (for details of the model see Appendix Section **B**) was able to recapitulate the main features of the observed correlation between E2F-1 levels and RelA localization *in silico* (Figure 4 -Figure supplement 1 D,E).

**Physical and functional interaction between RelA and E2F-1**

These data supported a direct interaction between E2F-1 and RelA. Therefore, the physical interactions between E2F-1 and NF-κB proteins in cells were investigated. Co-localization of E2F-1 and RelA had previously been shown through fluorescence imaging experiments. A clear physical interaction between fluorescently labelled E2F-1 and RelA in the nucleus of living cells was evident using Förster Resonance Energy Transfer (FRET), in conjunction with acceptor photobleaching as a qualitative indicator of intermolecular interaction, and Fluorescence Cross-Correlation Spectroscopy (FCCS) (Figure 5B-D).

In order to further support the interaction between the endogenous proteins, we used co-immunoprecipitation (Co-IP) of endogenous E2F-1 and RelA in HeLa cells that had been synchronized in late G1, when E2F-1 levels are at their peak (Figure 5A). These data confirmed a physical interaction between E2F-1 and RelA, in agreement with previous studies ([Tanaka, Matsumura et al. 2002](#_ENREF_58), [Lim, Yao et al. 2007](#_ENREF_37), [Garber, Yosef et al. 2012](#_ENREF_19)). We were not able to observe a positive co-IP in asynchronous cells (see Appendix Figure 4), suggesting that this interaction was only detectable in HeLa cells at G1/S when E2F-1 is at its highest level. Considered together, all of these different measurements support a significant interaction between these proteins. These data suggest the hypothesis that the interaction between RelA and E2F-1 in the nucleus of G1/S cells, which have been subjected to an inflammatory stimulus, may coordinate differential regulation of NF-κB target gene transcription.

***In-silico* modelling and prediction of NF-κB interaction with E2F-4**

In order to understand and further investigate the dynamic behaviour of TNF-α-mediated NF-κB activation in the presence of E2F-1 (at the G1-S transition), an ordinary differential equation-based mathematical model of the NF-κB system ([Ashall, Horton et al. 2009](#_ENREF_5)) was extended to include the interaction with E2F-1 (see Appendix Section B). In this model, E2F-1 was assumed to compete with IκBα for binding to free NF-κB, but had no effect on the localization of RelA bound to IκBα. Simulations (of nuclear NF-κB levels over time from transfection experiments) using this model, supported the hypothesis that E2F-1 might temporally control the duration of RelA nuclear occupancy through a combination of binding to RelA in the nucleus and inhibition of RelA-dependent IκBα transcription (as suggested by data shown in Figure 4). E2F-1 degradation could allow NF-κB to re-activate IκBα, which in turn could restore RelA to a cytoplasmic localization.

When the initial mathematical model was used to simulate the effect of E2F-1 on the responsiveness of NF-κB to TNFα, the *in silico* simulations predicted that TNF would induce immediate oscillations of free RelA (Figure 6A). In contrast, time-lapse live cell imaging of SK-N-AS cells stimulated with TNFα, showed that in cells expressing RelA-DsRedxp and EGFP-E2F-1 (which initially had nuclear RelA-DsRedxp), there was a delay before the onset of oscillations (Figure 6B and D). The length of this refractory period was on average ~4-fold longer than the peak1:peak2 timing in cells expressing RelA-DsRedxp alone (Figure 6B and ([Ashall, Horton et al. 2009](#_ENREF_5))). Altered model structures were investigated in order to resolve this discrepancy between experimental data and model predictions. One of the simplest altered models predicted that an E2F-1 target gene might stabilize IκBα (keeping NF-κB in the cytoplasm during S-phase (Figure 6C)). In support of this prediction, TNFα treatment of SK-N-AS cell populations transiently expressing EGFP-E2F-1 and RelA-DsRedxp led to reduced levels of phospho-S536-RelA and stabilized levels of IκBα (Figure 6E). Simulations of the response to TNFα from the revised model were consistent with the observed delay in oscillations in single cells expressing ectopic EGFP-E2F-1 (Figure 6B and D) and also with the inhibition or delay in the response during S-phase, but not during G1 or G2 (Figure 2C). Candidates for the E2F-1-regulated component(s) predicted by the revised model were therefore sought.

Previous studies had shown strong structural homology between E2F-1 and other E2F family members ([Tsantoulis and Gorgoulis 2005](#_ENREF_61)). E2F-4 is a transcriptional target of E2F-1 ([Xu, Bieda et al. 2007](#_ENREF_68)) and can be cytoplasmic during S-phase ([Lindeman, Gaubatz et al. 1997](#_ENREF_38)). E2F-4 (together with E2F family members) was therefore considered as a prospective candidate. We confirmed that ectopic expression of E2F-1 in cells resulted in increased E2F-4 expression, consistent with E2F-4 being a transcriptional target of E2F-1 in these cells (Figure 6E). The profile of E2F-4 expression was found to be delayed relative to that of E2F-1 in the cell cycle, peaking in S-phase in synchronized HeLa cells (Figure 6F).

**E2F-4 and RelA physically and functionally interact.**

To further confirm the role of E2F-4 in the suppression of RelA translocation following TNFα treatment during S-phase, the physical and functional interactions between E2F-4 and RelA proteins in cells were investigated. When transiently expressed in either HeLa or SK-N-AS cells, both proteins were located in the cytoplasm (Figure 7 A). Following TNFα treatment, the timing of RelA-DsRedxp translocation to the nucleus in both cell lines was delayed relative to the level of the fluorescent signal from EGFP-E2F-4 (Figure 7 B for dynamic profiles and Figure 7 Supplement 1 for analysis). The physical interaction of endogenous E2F-4 and RelA proteins was supported by Co-IP from HeLa cells synchronized in S-phase (Figure 7 B), where no pull down was possible in cells synchronised in late G1 phase (see Appendix Figure 4). This interaction was confirmed by acceptor photo bleaching FRET and FCCS data obtained from cells transiently expressing ECFP-E2F-4 and RelA-EYFP (for FRET) or RelA-dsRedxp and EGFP-E2F-4 (for FCCS) fluorescent fusion proteins (Figure 7 D and E).These data suggested that members of the E2F family have differing, but functionally linked, roles in the regulation of NF-κB dynamics. The observed dynamics could be represented by a mathematical model that recapitulates data (Figure 5 C) from live cell imaging of the transient expression of the appropriate fluorescent fusion proteins (Figure 5 B and 6 A, for details of modelling see Appendix Section B).

**Analysis of the effect of the cell cycle on the NF-κB response at more physiological expression levels of E2F-1**

The majority of experiments described above utilised transient expression of the E2F and RelA fusion proteins driven from a CMV promoter in a plasmid vector. Previous data had suggested that RelA fusion proteins expressed in a knock-in mouse are functional and fusion protein expression does not perturb the system ([De Lorenzi, Gareus et al. 2009](#_ENREF_15)). Our transcription analyses (Figure 4) suggested that E2F-1 N- and C-terminal fusion proteins also retained functional activity. However, as E2F proteins are normally expressed at specific stages of the cell cycle, ectopic expression from a strong constitutive promoter could give rise to out-of-context expression at inappropriate stages of the cell cycle (i.e. for E2F-1, stages other than late G1 and early S-phase). Therefore, expression of fusion proteins from these vectors might potentially show interactions that were not physiologically relevant. An additional  complication in these experiments was that exogenous expression of E2F-1 (but not E2F-4) fluorescent fusion protein from a CMV promoter caused apoptosis when transfected alone. Interestingly this effect was rescued by co-expression with RelA.

To further validate the functional link between the E2F and RelA proteins, we sought to achieve more physiologically relevant levels and timing of the fluorescent fusion protein expression. To this end, stable HeLa cell lines were generated, with integrated Bacterial Artificial Chromosomes expressing E2F-1-Venus and RelA-DsRedxp under the control of their natural human gene promotors and associated regulatory elements (see Appendix Section C). HeLa cells were chosen for this study based, on their more consistent cell cycle timing (between cells) compared to SK-N-AS cells (as shown in Figure 1 – Figure Supplement 1).

A stable cell line generated with a human E2F-1-Venus BAC construct showed the same pattern of synthesis and degradation of a transiently expressed FUCCI reporter for SCF (SKP-2) activity, indicating normal cell cycle progression (see Appendix Section D). Following the generation of this stable clone, a further step was taken to integrate a RelA-DsRedxp BAC into this cell line. This generated a dual stable clone of E2F-1-Venus and Rel-A-DsRedxp (termed C1-1). This clonal cell line showed a slight increase (~8%) in mean cell cycle length (with similar cell-to-cell variability) in line with wild type HeLa (see Appendix Figure 7). Similar to wild type cells, TNFα treatment in the C1-1 cell line increased the variability in cell cycle timing compared to that of resting cells.

The slight change in mean cell cycle duration (~20 h) in the dual BAC stable clonal cell line C1-1 was taken into account for inference of the dynamics of RelA-DsRedxp translocation at different cell cycle phases. The profile of E2F-1-Venus expression was used for assignment of the cell cycle stage at the time of stimulation cells based upon the time of peak E2F-2-Venus expression. This provided an alternative and faster method of virtual synchronisation to that used in Figure 2, allowing the assignment of G1, S and G2 phases to the data from the simulated BAC stable cells. The level of RelA translocation (Figure 8b and Figure 8- figure supplement 1) was then quantified for cells from each cell cycle phase. In agreement with data from the transiently transfected HeLa and SK-N-AS cells (Figure 2, Figure 2 Supplement 2 and 3), the cells treated in late G1/S-phase showed higher amplitude RelA nuclear translocations. Cells treated in S-phase showed a statistically significant suppression in S-phase RelA translocation (Figure 8 and Figure 8 Figure Supplement 2) compared to cells in early G1- or G2-phases.

Expression of the RelA-DsRedxp and E2F-1-Venus fusion proteins in the stable cell line was quantified through molecular counting of fluorophores via FCS (Figure 8, Figure supplement 4). This gave an estimate of 310,000 ±120,000 molecules of RelA-DsRedxp per cell. This figure was comparable to previous molecular estimates using FCS that had been obtained in stable cell lines generated using lentivirus ([Bagnall, Boddington et al. 2015](#_ENREF_6)), and previous estimates of RelA concentration using analytical chemistry ([Martone, Euskirchen et al. 2003](#_ENREF_39), [Zhao, Widen et al. 2011](#_ENREF_71)). RelA showed an approximate ratio of 3:1 ectopic to endogenous expression based on quantitative analysis of western blot data (see Figure 8, Figure supplement 4A). By contrast, FCS analysis suggested that E2F-1-Venus expression was lower (24,000 ±9,100 molecules of E2F-1-Venus per cell). Western blot analysis (Figure 8, Figure supplement 4B) suggested that there was an approximate ratio of 10:1 endogenous to ectopic levels). This might suggest selective pressure during cloning, as over-expression of E2F-1 has been reported to compromise cell viability ([Crosby and Almasan 2004](#_ENREF_14)). The apparent selective pressure against higher E2F-1 fusion protein expression was also in agreement with our own data that suggested that transient exogenous expression of E2F-1 fusion protein (but not E2F-4) alone caused apoptosis, but that this was rescued by co-expression of RelA. In the same manner observed with low EGFP-E2F1 expression from transient co-expression, the more physiological levels of E2F-1-Venus expression in the stably transfected cells suggested that RelA-DsRedxp remained predominantly cytoplasmic in unstimulated cells.

The interaction between E2F-1-Venus and RelA-DsRedxp following TNFα stimulation was measured by Fluorescence Cross-Correlation Spectroscopy (FCCS). A strong cross-correlation was confirmed in the nucleus (Figure 8 – Figure supplement 4D) indicating that the interaction uncovered by transient transfection with plasmids was not an artefact of over-expression, but was contextually relevant in relation to the cell cycle and RelA activation. Analysis of the dissociation constant (by FCCS) for the RelA-DsRedxp and E2F-1-Venus binding in the nucleus of TNFα–stimulated cells suggested a Kd of 12nM (Figure 8E).

The stable and physiological co-expression of E2F-Venus and RelA-DsRedxp facilitated fluorescently labelled proteins to be observed over the course of a full cell cycle. Cells were virtually synchronized as previously described following stimulation with 10ng/ml TNFα, and translocation of RelA-DsRedxp was plotted against the nuclear expression of E2F-1-Venus (Figure 8 - Figure Supplement 1).

We also investigated the consequences of knocking down both E2F-1 and E2F-4 using siRNA. Imaging experiments showed E2F-1 knockdown did not prevent cell cycle progression, and did not affect the heterogeneity of population response upon TNFα stimulation (data not shown), perhaps indicating compensation by other E2F family members. In addition, our mathematical model predicted that knocking down E2F-1 might not substantially affect the repression of the NF-B response in S-phase, which was instead predicted to be due to the effect of E2F-4 expression. However, knock-down of E2F-4 was found to be lethal to cells ([Crosby and Almasan 2004](#_ENREF_14)) preventing time lapse analysis. A key additional consideration is the overlapping roles of other E2F family members, which makes knock-down of individual E2F proteins unpredictable, due to potentially co-operative and/or redundant functions.

**Discussion**

Biological timing plays a key role in the encoding and decoding of biological information. Of particular interest is the role of biological oscillators, which can have very different cycle periods. A key question is how they may interact to robustly control essential biological processes. Here, we propose a reciprocal relationship between two oscillators, NF-κB signalling and the cell cycle.

TNFα stimulation in S-phase showed a suppressed and delayed translocation of RelA, with no observable perturbation to cell cycle timing. In contrast, stimulation in late G1 showed strong translocation of RelA and led to significant lengthening of the cell cycle (Figure 2 and 3). These data suggest that cells use the G1/S checkpoint to prioritize between inflammatory signalling and the onset of DNA replication prior to cell division (see schematic diagram in figure 9). The presence of a mechanism for prioritization between the important processes of cell proliferation and inflammation suggests that an inflammatory response during DNA replication might be detrimental to the cell.

The data showing that TNFα stimulation alters cell cycle timing in a cell cycle phase-dependent manner is intriguing (Fig. 3). However, our data do not identify a specific mechanism by which TNFα may regulate cell cycle length. The observation that the effect of TNFα stimulation on cell cycle lengthening appears to be specific to G1/S- rather than S-phase suggests that this may occur by delaying transition through the G1/S checkpoint. One hypothesis is that this might occur through NF-κB modulation of E2F family transcriptional activity. At the same time, the system is more complex as NF-κB is known to regulate the expression of other key cell cycle regulating proteins. Important examples include Cyclin D ([Guttridge, Albanese et al. 1999](#_ENREF_22), [Hinz, Krappmann et al. 1999](#_ENREF_25), [See, Rajala et al. 2004](#_ENREF_52)), and p21waf1/cip1 ([Basile, Eichten et al. 2003](#_ENREF_8)). Therefore, there is undoubtedly a more complex set of interactions between NF-κB and the control of cell proliferation and cancer ([Perkins and Gilmore 2006](#_ENREF_45)).

As well as a number of studies that suggest a physical interaction between E2F and NF-B proteins ([Kundu, Guermah et al. 1997](#_ENREF_31), [Chen, Capps et al. 2002](#_ENREF_12), [Tanaka, Matsumura et al. 2002](#_ENREF_58), [Shaw, Yurkova et al. 2008](#_ENREF_54), [Palomer, Álvarez-Guardia et al. 2011](#_ENREF_42)), there have been a few previous studies that have suggested that this interaction might have functional importance. Araki *et al*. described an NF-B-dependent mechanism for growth arrest mediated by a dual mechanism. They suggested that E2F-1-dependent transcription was inhibited by IKK activation and that E2F-4 was phosphorylated directly by IKK resulting in increased activity of the E2F-4/p130 repressor complex ([Araki, Kawauchi et al. 2008](#_ENREF_2)). Their study did not assume direct interactions between the E2F and Rel proteins and did not take into account protein dynamics. Nevertheless, their conclusions are very complementary to the present study.

Another study by Tanaka et al. focused on the combined role of E2F-1 and c-MYC in the inhibition of NF-κB activity ([Tanaka, Matsumura et al. 2002](#_ENREF_58)). This   
study demonstrated interactions between E2F-1 and both RelA and p50. Rather than focusing on cell division, their study showed that inhibition of RelA activity by E2F-1 resulted in increased apoptosis. Since both the NF-κB and E2F families of transcription factors have important roles in the control of apoptosis ([Phillips and Vousden 2001](#_ENREF_47), [Kucharczak, Simmons et al. 2003](#_ENREF_30), [Crosby and Almasan 2004](#_ENREF_14)), it is therefore interesting to speculate that the levels of different E2F proteins at different cell cycle stages may regulate cell fate decision making in collaboration with signaling systems such as NF-κB.

One important conclusion of the current study is the physical interaction of RelA with E2F-1 and E2F-4 proteins. It is however not necessary to assume strong binding and sequestration into different cellular compartments. Instead, control of cross-talk could be a consequence of mutual control of gene expression. We provide some data that suggests that E2F-1 and IκBα may compete for binding to RelA (see Fig. 4E). We suggest that control may be achieved through repression of the IκBα feedback loop (and perhaps other negative feedbacks, such as A20). However, it might be that other genes are differentially activated through the combined action of these transcription factors. In support of this, Garber *et al.* performed a study in dendritic cells where they studied a panel of transcription factors by ChIP-Seq following LPS stimulation. Their data suggested that E2F-1 and RelA are common transcription factor pairs that were bound together at a large set of functionally important gene promoters (see data in Fig. 3B of ([Garber, Yosef et al. 2012](#_ENREF_19))). It therefore seems likely that these proteins mutually regulate patterns of transcriptional activity, controlling the expression of downstream feedback genes, cell proliferation and apoptosis.

We describe a mechanism for E2F-1 that suggests competition with IκBα for NF-κB binding. This was effectively described by the model (see also Figure 9), and was used to predict the role for an E2F-1 target gene, upregulated in S-phase. Our data support E2F-4 as a candidate for this E2F-1 target gene. It should be noted however, that the E2F family of proteins may all play a role in this complex system. A surprising characteristic of E2F-4 is its predominantly cytoplasmic localisation in some cell types. As a result, we were unable to perform a competition localisation experiment (as for E2F1, Figure 4E). We cannot therefore comment on whether E2F-4 also competes with IκBα for RelA binding. Therefore, the model (both mathematical model and schematic model in Figure 9) encode E2F-4 binding as a ternary complex to RelA and IκBα together. We stress that this is only one possible mechanism, but we have used this formulation since it is the simplest model that is consistent with all of our data. As described by Araki *et al.* (see above) there may be other components involved such as IKK-mediated E2F-4 phosphorylation ([Araki, Nakajima et al. 2003](#_ENREF_3)).

Functional and context-dependent coupling between dynamic cellular processes (such as the cell cycle, the circadian clock ([Yang, Pando et al. 2010](#_ENREF_69), [Bieler, Cannavo et al. 2014](#_ENREF_11), [El Cheikh, Bernard et al. 2014](#_ENREF_17)), or p53 ([Toettcher, Loewer et al. 2009](#_ENREF_60))) is emerging as a common theme in intracellular signalling ([Ankers, Spiller et al. 2008](#_ENREF_1), [White and Spiller 2009](#_ENREF_65), [Spiller, Wood et al. 2010](#_ENREF_56)). The present study has characterized a dynamic and functional interaction between NF-B and the cell cycle systems, which each oscillate with different periods. Coupling between cellular processes (e.g. at the G1/S commitment point) can have contrasting effects on cell fate. Such temporal communication between processes represents a way for cells to gate their biological signals and coordinate and prioritize cell fate decisions in response to changes in their environment. In a wider context, understanding how (and when) these dynamic interactions occur, could yield important therapeutic targets for fields such as cancer chronotherapy ([Choong, Yang et al. 2009](#_ENREF_13), [Levi, Okyar et al. 2010](#_ENREF_36)).

**Materials and Methods**

**Materials:** Human recombinant TNFα was supplied by Calbiochem (UK). Tissue culture medium was supplied by Invitrogen (UK) and Fetal Bovine Serum (FBS) was from Harlan Seralab (UK). All other chemicals were supplied by Sigma (UK) unless stated otherwise.

**Plasmids:** All plasmids were propagated using *E. coli* DH5α and purified using Qiagen Maxiprep kits (Qiagen, UK). NF-κB-Luc (Stratagene, UK) contains five repeats of an NF-κB-sensitive enhancer element upstream of the TATA box, controlling expression of luciferase. Luciferase reporter CyclinE-Luc was obtained from Peggy Farnham (University of Wisconsin-Madion, USA). EGFP-E2F-1 and EGFP-E2F-4 contain the Enhanced Green Fluorescent Protein (EGFP) gene (Invitrogen, UK) fused to the N-terminal ends of the human E2F-1 and E2F-4 gene fragments (kind gifts from Emmanuelle Trinh, BRIC, Denmark). Similarly, ECFP-E2F-1 and ECFP-E2F-4 contain the Enhanced Cyan Fluorescent Protein (ECFP) gene (Invitrogen, UK) RelA-DsRedxp contain the optimised DsRed Express protein (DsRedxp) gene (Clontech, CA, USA) fused to the c-terminal end of human RelA gene (described previously in ([Nelson, Paraoan et al. 2002](#_ENREF_41)).RelA-EYFP contain Enhanced Yellow Fluorescent protein (EYFP) gene (Invitogen, UK) fused to the C-terminal end of human RelA gene.

**Cell culture:** SK-N-AS neuroblastoma (cat.no. 94092302) and HeLa cervical carcinoma (cat. no. 93021013) cell lines were obtained from European Collection of Authenticated Cell Cultures (ECACC). Cells were cultured and frozen down from to form a low passage working stock. Subsequent working stocks were used for no more than 10 passages. Working stocks were screened to ensure the absence of mycoplasma every 3 months using LookOut® Mycoplasma PCR Detection Kit (Cat. No. D9307 Sigma, UK). For confocal fluorescence microscopy and immuno-cytochemistry, SK-N-AS and HeLa cells were plated on 35mm glass-bottom dishes (Iwaki, Japan and Greiner, Germany) at 1x105 cells per dish in 3ml medium. HeLa cells were plated at 5x104 cells per dish in 3ml medium. 24h post-plating, the cells were transfected with the appropriate plasmid(s) using Fugene 6 (Boehringer Mannheim/Roche, Germany). The optimized ratio of DNA:Fugene 6 used for transfection of HeLa or SK-N-AS cells was 2µg DNA with 4µl Fugene 6 and 0.8µg DNA with 1.2µl Fugene 6 respectively.

For Co-IP assays, SK-N-AS cells were plated on 100mm tissue culture dishes (Corning, USA) at 4.5x106 cells per dish in 10ml medium. For western blotting, semi-quantitative and quantitative PCR, HeLa and SK-N-AS cells were plated on 60mm tissue culture dishes (Corning, USA) at 5x105 and 1x106 cells respectively per dish in 5ml medium.

**G1/S Cell cycle synchronisation via double Thymidine block**: 24h post-plating, 2mM Thymidine was added to the culture medium. Following a 19h incubation, cells were washed and fresh medium added. Following a 9h incubation, 2mM Thymidine was again added to the culture medium and the cells incubated for a further 16h. Cells were then washed and fresh media added. Following release from Thymidine block, the G1/S-synchronized cells were either imaged or incubated (at 37°C, 5% CO2) for the indicated duration prior to cell lysis or fixation.

**Treatment of cells with TNFα:** For confocal fluorescence microscopy, the cells were treated *in-situ* between imaging acquisitions after an indicated pre-treatment incubation period (usually 24h post-transfection). For western blotting and q-PCR experiments, the cells were treated with TNFα 24h post-plating. The cells were imaged either immediately after treatment, or incubated (at 37°C, 5% CO2) for the indicated duration prior to cell lysis or fixation.

**Fluorescence microscopy:** Confocal microscopy was carried out as described ([Nelson, Ihekwaba et al. 2004](#_ENREF_40)) using either 20x Fluar 0.8 NA or 63x Planapochromat 1.4 NA objectives. CellTracker ([Shen, Nelson et al. 2006](#_ENREF_55), [Du, Marcello et al. 2010](#_ENREF_16)) was used for data extraction. For RelA fusion proteins, mean fluorescence intensities were calculated for each time point for both nucleus and cytoplasm then nuclear:cytoplasmic (N:C) fluorescence intensity ratios were determined. For time lapse microscopy, a modified version of the Autofocus macro (an improved version of the Autotimeseries macro ([Rabut and Ellenberg 2004](#_ENREF_48))) was used.

**Analysis of cell cycle progression:** The cell cycle duration and G1/S timing of SK-N-AS and HeLa cells was analysed using live-cell imaging of successive cell divisions to determine typical cell cycle duration. In addition, the cell cycle dynamics were quantified expressing Fluorescence Ubiquitin-based Cell Cycle Indicators (FUCCI,([Sakaue-Sawano, Kurokawa et al. 2008](#_ENREF_50))) (Figure 2 – Figure supplement 1). The crossing point in fluorescent levels from Fucci markers of APC and SCF E3 ubiqutin ligase was used as an indication of G1/S transition in the cells (Figure 2 Figure supplement 1B). Mitosis to mitosis timings were determined in non-transfected cells, as well as in cells transfected with RelA-dsRedXP and the dual BAC cell line (Appendix Figure 4). For the dual BAC cell line that expressed RelA-dsRedxp and E2F-1-Venus it was only possible to use the single SCF Fucci G1 vector (due to fluorescent protein spectral overlap). As a control it was also shown that overexpression of RelA had no significant effect on cell cycle duration in HeLa cells (Appendix Figure 5).

**Virtual synchronization:** Cells were imaged for ~30h prior to TNFα treatment in order to capture each cell passing through mitosis. The timing of TNFα treatment relative to mitosis for each cell was then calculated. Events following TNFα treatment (i.e. the dynamics of RelA-DsRedxp translocation, or cell cycle duration) could then be correlated to inferred cell cycle phase at the point of treatment. Dual BAC cell lines were imaged for an entire cell cycle. Cells were aligned based upon normalised peak amplitude of E2F-1-Venus, and virtually synchronised based upon alignment of peak E2F-1 expression and the relative timing of TNFα stimulation. Cell cycle boundaries were inferred through characterization of cell cycle progression through transfection of FUCCI G1 phase marker construct (Appendix Figure 4).

**Flow Cytometric DNA Analysis:** HeLa cells were cultured in 100mm dishes. Following trypsinization, and resuspension in 1ml of medium the cells were stained by addition of 250μl of 50 μg/ml propidium iodide, 0.15% TritonX-100, and 150 μg/ml RNase A before analysis in an Altra flow cytometer (Beckman Coulter).

**Förster resonance energy transfer (FRET) Microscopy:** FRET was carried out using a Zeiss LSM510 with “META” spectral detector mounted on an Axiovert 100S microscope with a 63x Planapochromat, 1.4 NA oil-immersion objective (Zeiss). ECFP and EYFP ([Karpova, Baumann et al. 2003](#_ENREF_27)) were excited with 458nm laser light, emitted fluorescence was collected in 8 images each separated by 10nm between 467nm and 638nm in lambda scanning mode. Separation of ECFP and EYFP fluorescence spectra was carried out using the linear unmixing algorithms of the Zeiss LSM510 software (Zeiss), using reference spectra taken from cells expressing the ECFP or EYFP fusion proteins alone or untransfected cells. The fluorescence spectrum was separated into ECFP, EYFP and background signals. FRET was assayed by acceptor (EYFP) photo-bleaching. Bleaching was accomplished using 50 iterations of 514nm laser light with no attenuation from the acousto-optical tuneable filter (AOTF). (For FRET controls see Appendix Section E.)

**Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Cross- Correlation Spectroscopy (FCCS):** FCS and FCCS was carried using either a Zeiss LSM780 or Zeiss 710 with Confocor 3 mounted on an AxioObserver Z1 microscope with a 63x C-apochromat, 1.2 NA water-immersion objective. Zen 2010B software was used for data collection and analysis. EGFP fluorescence was excited with 488nm laser light and emission collected between 500 and 530nm. DsRed-express was excited with 561nm laser light and emission collected between 580 and 630 nm. The protocols as outlined in Kim *et al*. ([Kim, Heinze et al. 2007](#_ENREF_29)) were followed, with 10 x 10s runs used for each measurement. FCS was used to quantify the total number of fluorescent molecules per cell as previously described ([Bagnall, Boddington et al. 2015](#_ENREF_6)). The confocal volume had previously been estimated at 0.59 ± 11fL (mean±SD) using Rhodamine 6G of known diffusion rate, and WT HeLa cells in suspension were imaged by confocal microscopy to give volume estimates of 1420 ± 490fL and 6110 ± 3580fL for nucleus and cytoplasm respectively. (For FCCS controls see Appendix Section E.)

**Co-immunoprecipitation:** HeLa cells synchronized at G1/S or S-phase were washed with room temperature PBS and lysed with modified RIPA buffer (50mMTris-HCl pH 7.4, 150mMNaCl, 1mM EDTA, 1% NP-40) including a 1:100 dilution of Protease Inhibitor cocktail (Sigma, UK), PMSF and phosphatase inhibitor (Phos Stop, Roche). Immunoprecipitation was carried out using Immunoprecipitation kit-Dynabeads® Protein G (Invitrogen).The samples were analyzed by western blotting using anti-E2F-1(Cell Signaling, #3742) or anti E2F-4 (Santa Cruz, C-20 sc-866) antibodies.

**q-PCR**: The RNeasy Mini Kit (Invitrogen, UK) was used to extract mRNA from the cells following manufacturer’s instructions, using the primers: IκBα left TGGTGTCCTTGGGTGCTGAT right GGCAGTCCGGCCATTACA, IκBε left GGACCCTGAAACACCGTTGT right CCCCAGTGGCTCAGTTCAGA, E2F-1 left TGCAGAGCAGATGGTTATGG right TATGGTGGCAGAGTCAGTGG, cyclophilin A left GCTTTGGGTCCAGGAATG right GTTGTCCACAGTCAGCAATGGT.

**Luciferase reporter assay:** Luciferase reporter assay were carried out as described in ([White, Morse et al. 1990](#_ENREF_64)), using a LUMIstar plate reading luminometer (BMG, Germany).

**Immuno-cytochemistry (ICC):** HeLa cells were prepared using combinations of the above techniques, typically involving synchronization and/or TNFα stimulation of cells seeded at appropriate density into 35mm glass-bottomed dishes. Dishes were subsequently washed three times with PBS and fixed with 1ml 4% paraformaldehyde for 15min. Dishes were then washed three times with PBS, and ‘blocked’ to prevent non-specific antibody binding with the addition of 1-2ml of 1% BSA, 0.1% Triton X-100 (in PBS) from 20min up to overnight. The primary antibody (or antibodies for dual-staining), dissolved in Ab Buffer (1% BSA, 0.1% Triton X-100 in PBS), were added to the dishes for 60/90min at a 1:2000 dilution. Dishes were then washed (3x1ml) with Ab buffer for 10min. Secondary Antibody(s) were subsequently added to the dishes (Cy3-anti-mouse, 1:200 dilution (Sigma), FITC Rabbit, 1:200 (AbCam)) for 30/45 min respectively, prior to 3 sequential washes of PBS blocking buffer (described above). Following the addition of fluorescent secondary antibodies, dishes were covered in aluminium foil and left in 2ml PBS prior to imaging.

**Western blotting:** Whole cell lysates were prepared at the indicated times after stimulation. Membranes were probed using the following antibodies: anti-IκBα (#9242, Cell Signaling, MA, USA), anti-RelA (#3034, Cell Signaling, MA, USA), anti-phospho-RelA (Ser 536) (#3031, Cell Signaling, MA, USA), anti-IκBα (#9242, Cell Signaling, MA, USA), anti-E2F-1 (#KH-95, Millipore Biotechnology, USA), anti-E2F-4 (sc-866,Santa Cruz), and anti-cyclophilin A (#07-313, Millipore Biotechnology, USA).

**Figure Legends:**

**Figure 1.**

**NF-κB dynamics following TNFα treatment in HeLa and SK-N-AS cells: Mapping the NF-κB response over the cell cycle in synchronized HeLa cells.** (A,B,C and D) The dynamics of RelA-dsRedxp following 10 ng/ml TNFα treatment in transiently transfected SK-N-AS (A), or following 30 pg/ml TNFα treatment in SK-N-AS, and 10 ng/ml TNFα treatment in HeLa cells (C), and at 30 pg/ml for HeLa (D) cells (n=30 cells analysed per condition). (E) The localization of endogenous RelA in different cell cycle phases, observed by immunocytochemistry at 2 h (G1/S transition), 4 h (mid S-phase), post-release from double thymidine block and with 15 min TNFα treatment. (F and G) The dynamics of RelA-dsRedxp in transiently transfected HeLa cells synchronized by a double thymidine block, following 10 ng/ml TNFα treatment at G1/S (F), or passing through S-phase (G) (n=20 cells analysed per condition). (H) Western blot of Ser536phopho-RelA (p-RelA), IκBα, and cyclophilin-A (cyclo-A) levels in synchronized HeLa cells harvested at 1 h time intervals over the G1/S transition following 15 min treatment with TNFα. Also shown are asynchronous, non-stimulated (ASY NST) and asynchronous, stimulated (ASY ST) controls, harvested at t=0.

**Figure 2.**

**Mapping the NF-**κ**B response over the cell cycle through virtual synchronizsation.**

(A) Selected images from time-lapse imaging of RelA-dsRedxp transiently expressing Hela cells treated with 10ng/ml TNFα. (B) Virtual synchronization of HeLa cells treated with 10ng/ml TNFα. Cells were imaged through two successive divisions (M) allowing correlation of cell cycle timing of TNFα treatment (parameter 1) to RelA dynamics (parameters 2, 3 and 4) and cell cycle duration (parameter 1 and 5). (C) Representative cells of RelA-dsRedxp dynamics following TNFα treatment in asynchronous cells, then virtually synchronized into G1 (n=115), G1/S (n=32), S (n=52) and G2 (n=38) phases.

**Figure 2 - Figure Supplement 1.**

**Analysis of cell cycle duration and G1/S timing in HeLa and SK-N-AS cells.**

(A) Time series for FUCCI expression in single representative HeLa and SK-N-AS cells. White arrows mark cells before and after the fluorescence levels were detectable. (B) Analysis of cells in (A), showing the G1/S crossing point in fluorescence levels from reporters of SCF (SKP2) (Orange) and APC (Green) E3 ubiquitin ligase activity. (C) Analysis of the G1/S crossing point and cell cycle duration in populations of HeLa and SK-N-AS cells transfected with FUCCI vectors (n ≥ 30 cells for all conditions).

**Figure 2 – Figure Supplement 2.**

**Statistical analysis of NF-κB translocation in HeLa cells at inferred cell cycle stages following 10 ng/ml TNFα stimulation.**

(A) Analysis of dynamics of initial RelA-dsRedxp translocation with respect to cell cycle phase, using virtual synchronization in HeLa cells. Data were analyzed using nonparametric Anova analysis with Dunn correction for multiple comparisons. Red lines indicate mean normalised amplitude of NF-κB nuclear translocation for different cell cycle phases, and the population average (dotted line). (B) Analysis of nuclear RelA occupancy assessed in non-synchronized cells expressing RelA-dsRedxp following treatment with 10ng/ml TNFα. Statistical analysis showed significant difference between cell cycle phases with respect to distribution of amplitude of the response (Anova analysis with Dunn correction for multiple comparisons.)).

**Figure 2 – Figure Supplement 3.**

**Statistical analysis of NF-κB translocation in SK-N-AS cells at inferred cell cycle stages following 30 pg/ml TNFα stimulation.**

(A) Correlation of estimated cell cycle timing with RelA-dsRedxp N:C peak amplitude following 30 pg/ml TNFα treatment (n=138). (B) Analysis of dynamics of initial RelA-dsRedxp translocation with respect to cell cycle phase. Statistical analysis showed a difference between G1 and S with respect to distribution of amplitude of the response (Anova analysis with Dunn correction for multiple comparisons).

**Figure 3.**

**Cell cycle length and variability is modified by TNFα addition at G1/S.**

Analysis of the timing and variability of mitosis (parameter 1 and 5 from Figure 2B) following 10 ng/ml TNFα treatment of asynchronous HeLa cells, compared to subsets of those cells stimulated at late G1- or S-phase. Mean durationswere analysed using nonparametric Anova analysis with Dunn correction for multiple comparisons. Variability in the data was analysed using Levene’s test for equality of variance.

**Figure 4.**

**Physical and functional interaction between NF-**κ**B and E2F-1 systems.**

(A) NF-κB-dependent transcription was assessed by luciferase reporter assay (NF-luc), in SK-N-AS cells (n=3, +/- s.d) expressing EGFP-E2F-1, RelA-dsRedxp or both. (B) IκBα and IκBε mRNA levels in SK-N-AS cells (n=3, +/- s.d) following transient expression of EGFP-E2F-1, RelA-DsRedxp or both. (C) E2F-1-dependent transcription as assessed by luciferase reporter assay (CyclinE-luc), in SK-N-AS cells (n=3, +/- s.d) expressing EGFP-E2F-1, RelA-dsRedxp or both. (D) E2F-1 mRNA levels in SK-N-AS cells (n=3, +/- s.d) transiently transfected with RelA-dsRedxp. (E) Representative SK-N-AS cells transiently expressing EGFP-E2F-1 (green), RelA-dsRedxp (red), both fluorescent fusion proteins at different levels, or EGFP-E2F-1, RelA-dsRedxp and IκBα-AmCyan (blue).

**Figure 4 – Figure Supplement 1.**

**E2F-1 modulates NF-**κ**B dynamics in the absence of stimulus in SK-N-AS cells.**

(A) Time-lapse confocal microscopy of representative SK-N-AS cells transiently transfected with RelA-dsRedxp and EGFP-E2F-1. (B) Trajectories of three representative cells expressing different levels of EGFP-E2F-1. (C) Correlation between RelA-dsRedxp T½ nuclear occupancy (NO) time and EGFP-E2F-1 T½ nuclear degradation time, based on data in (A). (D) Recapitulation of the observed dynamics with an *in-silico* model for physical interaction between RelA (*NFkB*) and E2F-1 (*E2F)* (E) Correlation between NF-κB nuclear occupancy time and nuclear E2F-1 degradation time, based on data in (D) (n= 30 cells).

**Figure 4 - Figure Supplement 2.**

**E2F-1 modulates NF-**κ**B dynamics in the absence of stimulus in HeLa cells.**

(A) Representative HeLa cells transiently transfected with combinations of RelA, and E2F-1 fluorescent fusion proteins. (B) Time-lapse confocal microscopy of representative HeLa cells transiently transfected with RelA-dsRedxp and EGFP-E2F-1 representative cells. (C) Trajectories of three representative cells expressing different levels of EGFP-E2F-1. (D) Correlation between RelA-dsRedxp T½ nuclear occupancy (NO) time and EGFP-E2F-1 T½ nuclear degradation time, based on data in (C) (n=20).

**Figure 5.**

**Figure 5. Interaction of E2F-1 with RelA.**

(A) Co-Immunoprecipitation of E2F-1 with RelA pull down in HeLa cells synchronized in late G1. (HeLa cells used for this experiment due to their greater ease of synchronization). (B) Representative cell demonstrating co-localisation of E2F1-EGFP and RelA-dsRedxp upon transient transfection (C) FCCS assay between transiently transfected EGFP-E2F-1 and RelA-RelAdsRedxp (red line) or empty-dsRedxp (blue line) fluorescent fusion proteins in single live SK-N-AS cells (+/- s.e.m based on 10 measurements from 10+ cells per condition). (D) Qualitative FRET assay between transiently transfected ECFP-E2F-1 and RelA-EYFP fluorescent fusion proteins in live SK-N-AS cells. First Negative control between IkB-ECFP and EYFP-E2F1, and second negative control between free ECFP and EYFP fluorophores expressed in an SK-N-AS cell (shown are average ECFP and EYFP signals (+/- s.e.m based on 20 cells per condition normalised to pre-bleach intensity. p.b. indicates the time point at which photo-bleaching occurred).

**Figure 6.**

**Mathematical modelling predicts an additional key component for NF-κB - cell cycle interactions: E2F-4 identified as a putative candidate.**

(A) Model simulations of RelA-dsRedxp dynamics when co-expressed with EGFP-E2F-1 in cells treated with TNFα. (B) Dynamics analysed in representative SK-N-AS cells treated with 10ng/ml TNFα expressing RelA-dsRedxp and EGFP-E2F-1 (C) Model simulation of experimental conditions in B, incorporating interactions between NF-κB complexes and a putative E2F-1-induced target protein, subsequently proposed as E2F-4. (D) Analysis ofaverage timing to second peak of NF-κB translocation following TNFα treatment in SK-N-AS cells expressing RelA-dsRedxp alone or with EGFP-E2F-1 (n=20 cells per condition, error bars show s.d.) (E) Assessment of the extent of RelA Ser536 phosphorylation (p-RelA), E2F-4 and IκBα stability by western blot compared to cyclophilin A (cyclo A) amounts in SK-N-AS cells either untreated or treated with 10ng/ml TNFα and expressing combinations of either untagged or fluorescent proteins RelA-dsRedxp and EGFP-E2F-1. (F) Western blot of E2F-1 and E2F-4 in synchronized HeLa cells, where t=0 is late G1-phase.

**Figure 7.**

**E2F-4 directly interacts with NF-**κ**B and perturbs RelA dynamics in response to TNF**α **stimulation.**

(A) Single cell trajectories from groups of HeLa cells expressing RelA-dsRedxp and different levels of EGFP-E2F-4 showing the dynamics of RelA-dsRedxp after 10ng/ml TNFα treatment (n=60 cells). (B) HeLa cells synchronized in S-phase, co-immunoprecipitated with anti-RelA antibody and probed for E2F-4. Also shown are IgG negative controls and whole cell lysate unsynchronized positive control (ctrl). (C) Representative SK-N-AS cells transiently transfected with RelA-dsRedxp and EGFP-E2F-4. (D) FRET assay in live SK-N-AS cells expressing ECFP-E2F-4 and RelA-EYFP fluorescent fusion proteins (shown are average ECFP and EYFP signals (+/- s.e.m) based on 20 cells per condition normalised to pre-bleach intensity. p.b. indicates the point of photo-bleaching). (E) FCCS assay in cells transiently expressing EGFP-E2F-4 and RelA-dsRedxp (red line) or dsRedxp (blue line) fluorescent proteins in single live SK-N-AS cells (+/- s.e.m based on 10 measurements in each of 10+ cells per condition).

**Figure 7 - Figure Supplement 1.**

**Analysis of RelA-dsRedxp dynamics in HeLa and SK-N-AS cells co-expressing EGFP-E2F-4 following TNFα stimulation.**

The effects of different EGFP-E2F-4 expression levels on the amplitude and timing of the first peak of RelA translocation in HeLa and SK-N-AS cells treated with 10ng/ml and 30pg/ml TNFα, respectively. These data indicate how ectopically expressed EGFP-E2F-4 can inhibit the translocation of RelA-dsRedxp in response to TNFα.

**Figure 8.**

**Effect of cell cycle timing on RelA-dsRedXP translocation in a dual BAC HeLa cells (C1-1 line) that co-expresses E2F-1-Venus fusion protein.**

(A) Selected images from time-lapse experiment of dual BAC HeLa stable clone 1-1 showing translocation of RelA-dsRedXP and E2F-1-Venus expression at different cell cycle phases. Cells were treated with 10ng/ml TNFα. (B) Analysis of the dynamics of initial RelA-dsRedxp translocation in cells ordered at specific cell cycle times with respect to the peak of E2F-1 expression (n = 128). Data were analysed using nonparametric Anova analysis with Dunn correction for multiple comparisons. Red lines indicate mean normalised amplitude of NF-κB nuclear translocation for different cell cycle phases, and the population average (dotted red line). Analysis of nuclear RelA occupancy was assessed in virtually synchronised C 1-1 cells, based on time from cell division and relative to peak E2F-1-Venus expression level. RelA-dsRedxp localization was visualized to allow quantification of translocation, following treatment with 10ng/ml TNFα. The dotted black line shows the spline fitted level of E2F1 at different times and cell cycle stages (see also Figure 8, Figure supplement 1 below). Statistical analysis showed a difference between G1 vs S, and G2 vs S with respect to distribution of amplitude of the RelA translocation response. (c) RelA-dsRedxp dynamics following 10ng/ml TNFα treatment in asynchronous cells (left panel) and cells virtually synchronised into G1, G1/S, S and G2 phases. Cells normalised to T0

**Figure 8 - Figure Supplement 1.**

**Virtually synchronized HeLa C 1-1 cells.**

NormalisedE2F-1-Venus expression at the time of TNFα stimulation of C1-1 cells (data also shown in Figure 8B). E2F-1-Venus expression was normalised to its peak expression. The time axis represents the time of TNFα stimulation related to the peak of E2F-1-Venus for each cell, where time 0 is the peak of E2F-1-Venus expression. Positive times indicate stimulation after the peak of E2F-1-Venus expression and negative values indicate stimulation evens before the peak of E2F-1-Venus expression. The black line shows a spline interpolation of the level of E2F-1-Venus expression. Cell cycle phases were estimated based on measured the E2F1 profile and average cell cycle timing.

**Figure 8 – Figure Supplement 2.**

**Physiological and functional expression of E2F-1-Venus in stable BAC-transduced HeLa cells**.

(a) HeLa cells stably expressing an E2F-1-Venus fluorescent fusion protein from a 5KB endogenous E2F-1 promoter (Green), transiently transfected with a FUCCI reporter for SCF (SKP-2) activity (Orange). Showing the profile of E2F-1 over two consecutive cell cycles (one parent and two daughter cells), with a peak in late G1. E2F-1 levels dropped during S-phase consistent with rapid rise in SCF (SKP-2) activity and a loss of FUCCI fluorescence. (b) Representative cell from the E2F-1-Venus and RelA-DsRedxp stably transfected population of Hela cells through one full cell cycle.

**Figure 8 - Figure Supplement 3.**

**Analysis of the expression of E2F1-Venus and RelA-DsRedxp translocation in single C1-1 HeLa cells stimulated with 10ng/ml TNFα at different cell cycle phases.** Grey line shows the E2F-1-Venus expression level plotted agains the right y-axis. The red, green blue and orange lines show the timecourse of RelA-dsRedxp localization in exemplar cells in the G1, G1/S, S and G2 phases respectively plotted against the left y-axis. The black vertical line represents the point at which cells were treated with TNFα.

**Figure 8 – Figure supplement 4.**

**Expression and interaction of RelA-dsRedxp and E2F-1-Venus**  (A) Western blot of RelA and alpha tubulin levels in dual BAC stable C1-1 and WT HeLa showing degree exogenous expression of RelA from RelA-dsRedxp BAC. (B) Western blot of E2F-1 and cyclophilin A levels in dual BAC stable C1-1 and WT HeLa cells expressing the E2F-1-Venus BAC (C) Total fluorescent molecules per cell for E2F1-Venus at peak expression and RelA-dsRedxp in unstimulated cells (data obtained from Fluorescent Correlation Spectroscopy measurements, and calculated using volume estimates from z-stacked WT HeLa in suspension. (D) FCCS mean correlation curves (+/- s.e.m) between E2F-1-Venus and RelA-dsRedxp (red line, n=46) for TNFα treated BAC stable cells. A comparison to transient empty-dsRedxp is shown (blue line, n=15) (E) Kd determination results using a scatter plot and linear regression (Theil-Sen estimator). The slope of the regression gives the Kd value.

**Figure 9. Schematic representation of NF-κB – E2F interactions.** (A) predicted mechanisms for NF-κB interaction with E2F proteins over the G1/S transition (B) model simulations of single cell behaviour.

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**Competing Interests**

None of the authors have any financial or nonfinancial competing interests.

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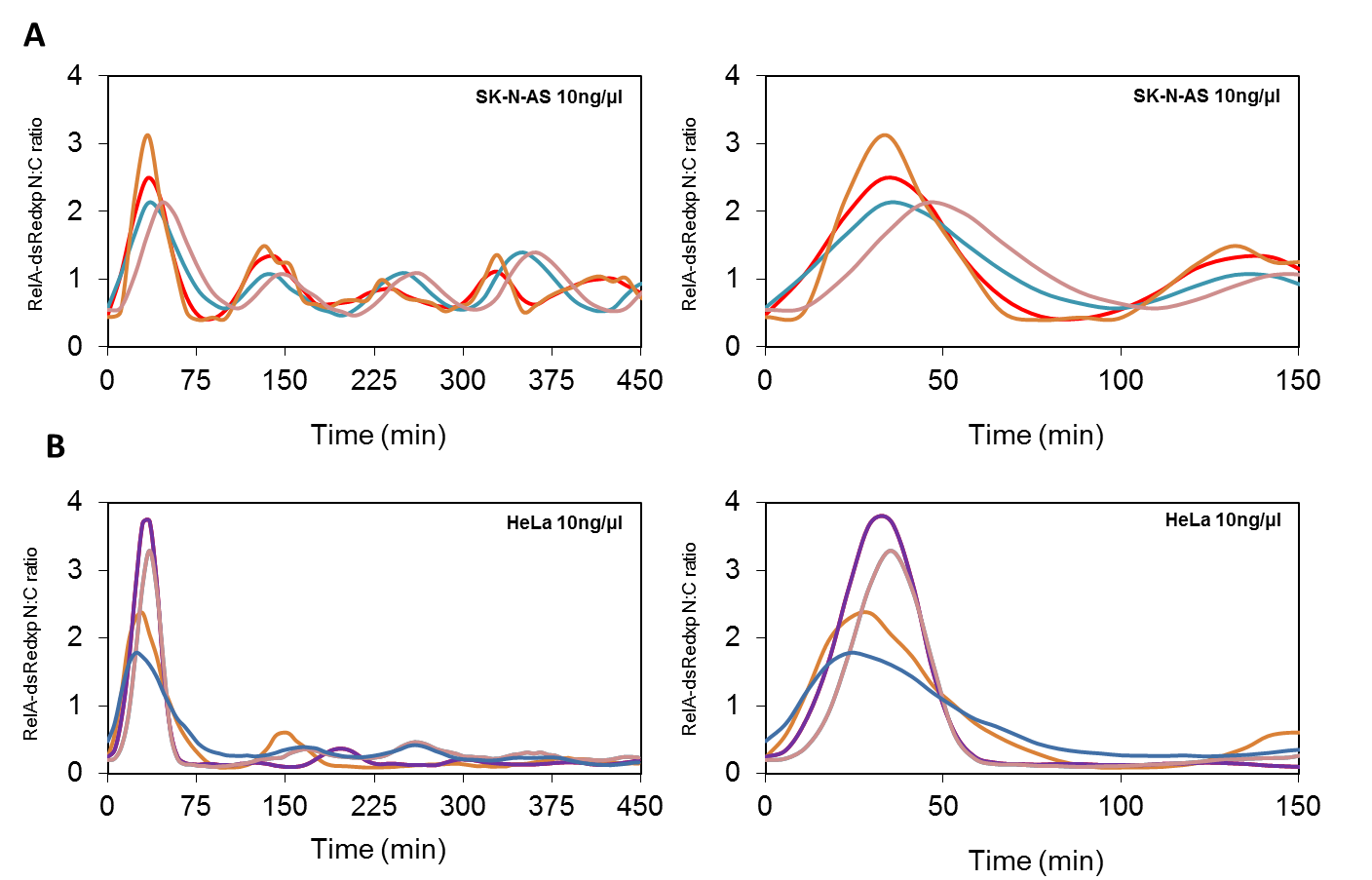
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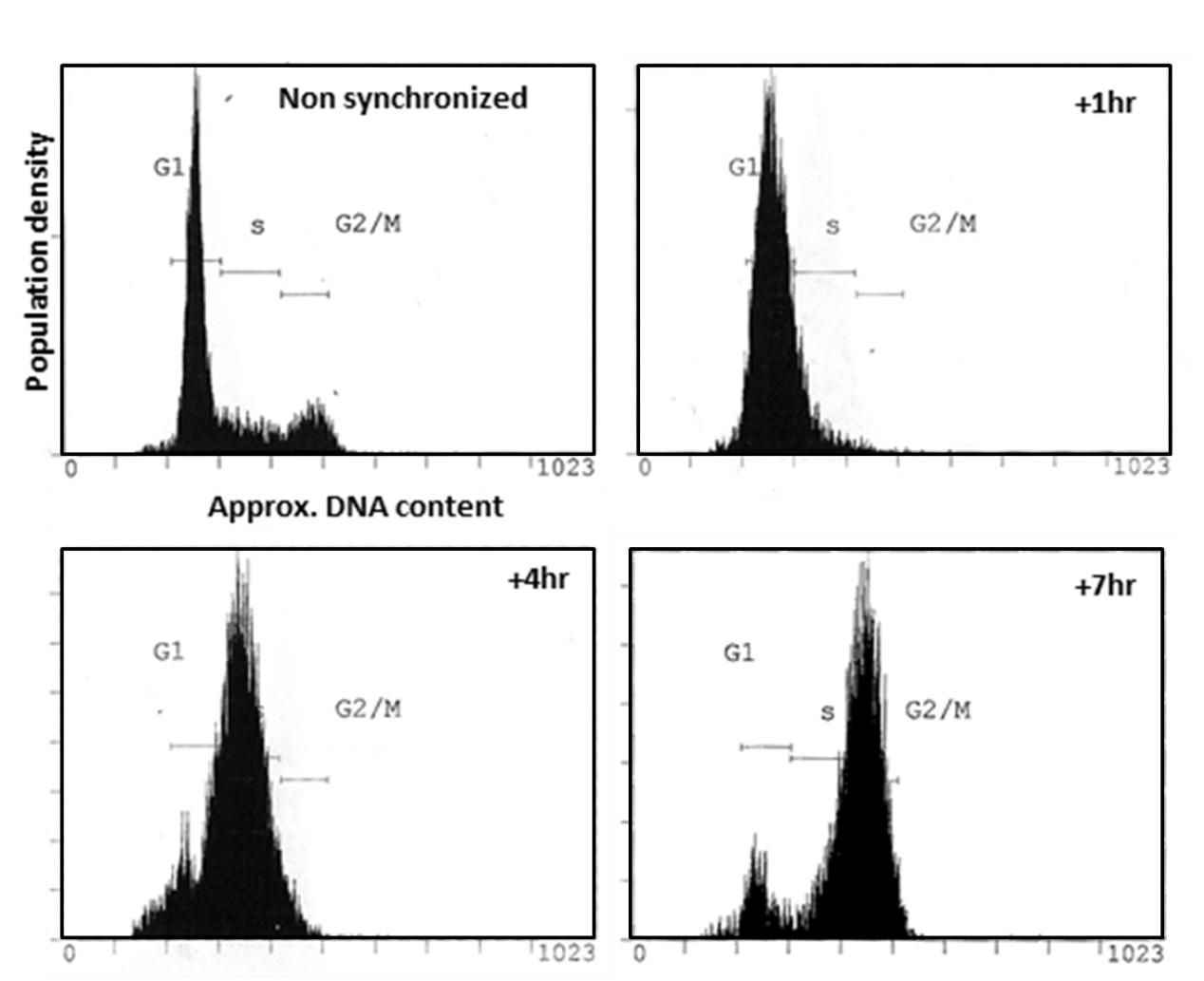
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**Appendix**

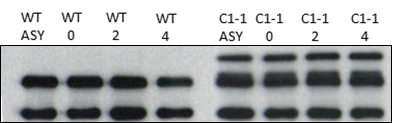
**Section A: Thymidine synchronisation and analysis**

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**Appendix Figure 1.Osscolations in the NF-κB system** (A) Dynamics of RelA-dsRedxp in transient transfected SK-N-AS cells following 10ng/μl TNFα stimulation, plotted over 450 and 150 minutes respectively. (B) Dynamics of RelA-dsRedxp in transient transfected HeLa cells following 10ng/μl TNFα stimulation, plotted over 450 and 150 minutes respectively.

****

**Appendix Figure 2.. Use of double-Thymidine block to synchronize HeLa cells at G1/S.** Flow cytometric analysis of the distribution of DNA content of non-synchronized HeLa cells and cells harvested at relevant times post-release from Thymidine block.



αTubilin

RelA

RelA-dsRedxp

**Appendix Figure 3. Double-Thymidine block on Tagged and endogenous RelA levels HeLa cells.** Endogenous RelA and tagged RelA-DsRedxp expression levels in unsynchronised and synchronised WT-HeLa and double BAC stable cells. Synchronized fractions at 0, 2 and 4 hours post release of thymidine block. Α-Tubulin used as loading control.

E2F-1

Probe

Lysate

CO-IP

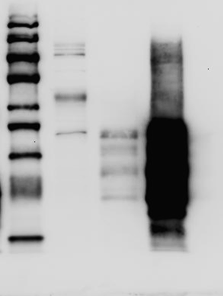
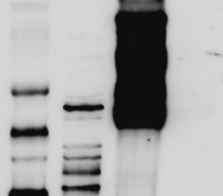
HeLa Asy

RelA

pull-down

IgG

**a**



Lysate

CO-IP

HeLa sync in G1 Phase

RelA

pull-down

IgG

E2F-4

Probe

**B**

**Appendix Figure 4. Negative Co-IP.** (A) Co-Immunoprecipitation of E2F-1 with RelA pull down in HeLa cells asynchronous cells showing no distinct banding. (B) Co-Immunoprecipitation of E2F-4 with RelA pull down in HeLa cells Synchronized in Late G1-Phase cells showing no distinct banding of E2F-4.

**Section B: The NF-κB:E2F mathematical models**

All modelling work was implemented using MATLAB2010 (MathWorks,USA), and simulated using MATLAB ordinary differential equation solver ODE15s. Analysis of simulated time course data was performed in both Microsoft Excel and MATLAB.

Model equations and parameters are shown in SI Appendix Table 2 and 3, respectively. The deterministic model of TNFα-induced NF-κB signalling ([Ashall, Horton et al. 2009](#_ENREF_4)), consisting of a system of ordinary differential equations for species concentrations with respect to time, was extended in two steps. Firstly, the physical interaction between E2F-1 and RelA was included. It was assumed that E2F-1 competes with IκBα for binding of free NF-κB with similar affinity. In addition, free IκBα actively disrupted the NF-κB:E2F-1 complex, while IκBα:NF-κB complex was unaffected by free E2F-1. Secondly, the physical interaction between cytoplasmic NF-B complexes and E2F-4. E2F-4 was modelled as an E2F-1 responsive gene which, upon translation, forms complexes with NF-B and NF-B:IBα, which were not targeted by IKK. This reduced the system sensitivity to TNFα treatment for a prolonged period.

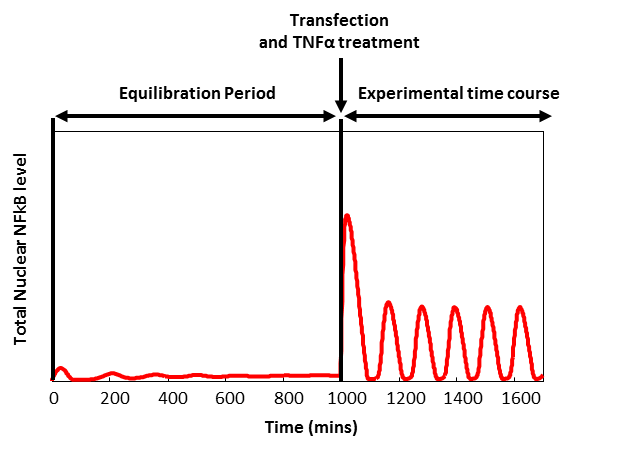
A typical simulation experiment involved three sequential stages: equilibration, transfection and TNFα treatment (shown below):

(1) The model was initialized by setting neutral IKK and cytoplasmic IκBα:NF-κB to 0.1 μM and other variables to 0. The system was then equilibrated for 1000min to reach the untreated steady state.

(2) Initial conditions from the end of the equilibrium stage were amended to mimic cell transfection. For example, in the case of E2F-1 and RelA co-transfection 0.1μM cytoplasmic protein was added to the respective initial conditions.

(3) The equilibrated and transfected model was simulated for 800min (with TR set to either 1 or 0 depending on whether treatment was simulated or not).

Simulations of the full model are shown below. Simulation protocols used throughout the manuscripts are summarized below.



**Appendix Figure 5 A typical simulation protocol of the NF-κB:E2F-1 mathematical model.** Simulation protocol of a live cell imaging experiment involving transfection and TNFα stimulation.

|  |  |  |  |
| --- | --- | --- | --- |
| **Species** | **Biological name** | **Initial Conditions**  **Equilibration stage(μM)** | **Initial Conditions**  **TNFα stimulation**  **(μM)** |
| *NFkB* | Cytoplasmic RelA | *0* | *0.004 (+0.1)* |
| *nNFkB* | Nuclear RelA | *0* | *0.015* |
| *E2F1* | Cytoplasmic E2F1 | *0* | *0 (+0.1)* |
| *nE2F1* | Nuclear E2F1 | *0* | *0* |
| *tIkBa* | IκBα mRNA | *0* | *1e-005* |
| *IkBa* | Cytoplasmic IκBα | *0* | *0.017* |
| *nIkBa* | Nuclear IκBα | *0* | *0.004* |
| *IKKn* | Neutral IKK | *0.1* | *0.1* |
| *IKK* | Active IKK | *0* | *0* |
| *IKKi* | Inactive IKKi | *0* | *0* |
| *tA20* | A20 mRNA | *0* | *1e-005* |
| *A20* | A20 | *0* | *0.001* |
| *pIkBa* | phospho-IκBα | *0* | *0* |
| *pIkBaNFkB* | phospho-IκBα RelA complex | *0* | *0* |
| *NFkBE2F1* | cyto. RelA E2F-1 complex | *0* | *0* |
| *nNFkBE2F1* | nuclear RelA E2F-1 complex | *0* | *0* |
| *IkBaNFkB* | cyto IκBα RelA complex | *0.1* | *0.091* |
| *nIkBaNFkB* | nuclear IκBα RelA complex | *0* | *0.001* |
| *tE2F4* | E2F-1 target E2F-4 mRNA | *0* | *0* |
| *E2F4* | E2F-1 target E2F-4 | *0* | *0* |
| *E2F4NFkB* | E2F-4 RelA complex | *0* | *0* |
| *E2F4IkBaNFkB* | E2F-4 IκBα RelA complex | *0* | *0* |

**Appendix Table 1.** **Initial conditions for the NF-κB:E2F mathematical model.** Shown in red are concentrations of NF-κB and E2F-1 added to mimic cell transfection.

|  |  |
| --- | --- |
|  | (1) |
|  | (2) |
|  | (3) |
|  | (4) |
|  | (5) |
|  | (6) |
|  | (7) |
|  | (8) |
|  | (9) |
|  | (10) |
|  | (11) |
|  | (12) |
|  | (13) |
|  | (14) |
|  | (15) |
|  | (16) |
|  | (17) |
|  | (18) |
|  | (19) |
|  | (20) |
|  | (21) |
|  | (22) |

**Appendix Tablee 2. NF-κB:E2F-1 model equations** Symbol ‘n’ denotes nuclear variables,‘t’ denotes mRNA transcripts, ‘p’ denotes phosphorylated form of IκBα. Symbols denoting cytoplasmic localisation were omitted.

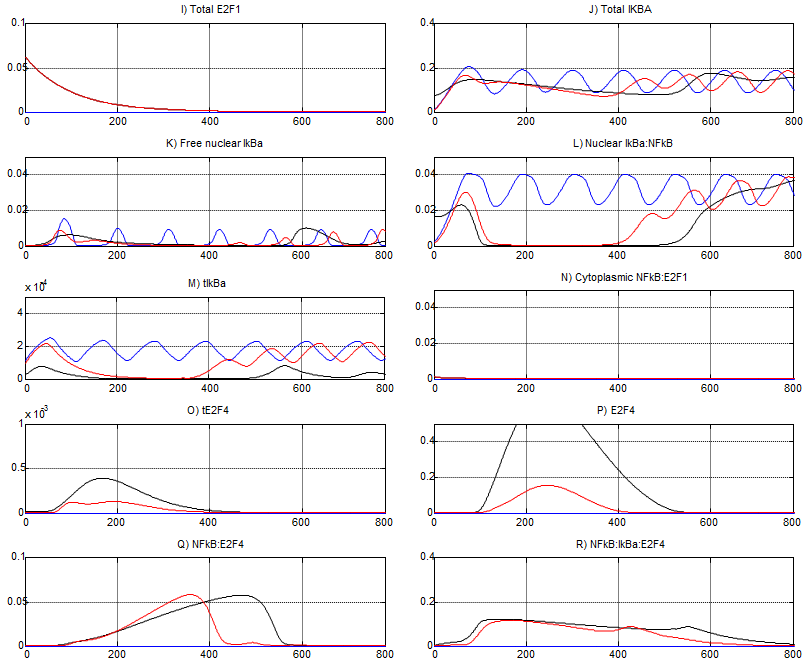
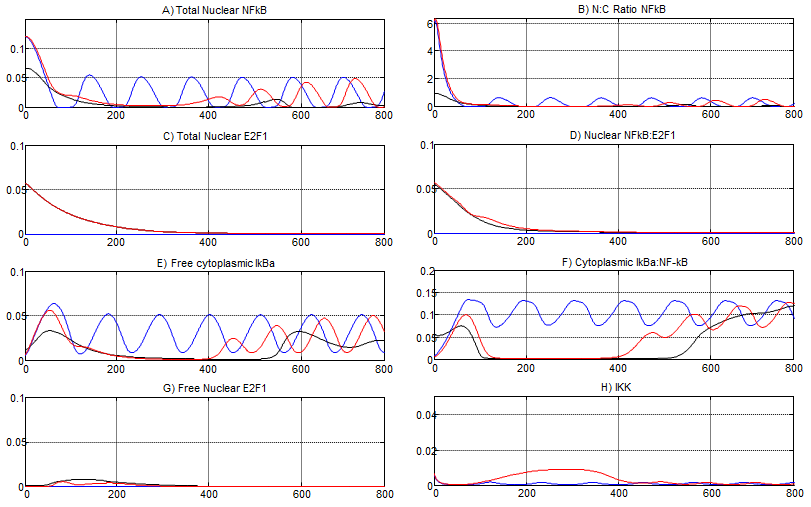
|  |  |  |  |
| --- | --- | --- | --- |
| **Reaction** | **Symbol** | **Value** | **References** |
| ***Spatial parameters*** | | | |
| Total cell volume | *tv* | 2700 μm3 | Measured |
| C:N ratio | *kv* | 3.3 | Measured |
| Conversion to nuclear volume | *nv* | ×(kv+1) | - |
| Conversion to cytoplasmic volume | *cv* | ×(1/kv+1) | - |
| ***Initial concentration*** | | | |
| Total NF-κB | NF | 0.08 μM | Initialized as cytoplasmic IκBα·NF-κB |
| Total IKK | - | 0.08 μM | Initialized as IKKn |
| ***Complex formation & dissociation*** | | | |
| IκBα + NF-κB → IκBα·NF-κB  nIκBα + nNF-κB → nIκBα·NF-κB | *ka1a* | 0.5 μM-1s-1 | ([Hoffmann, Levchenko et al. 2002](#_ENREF_28)) |
| IκBα·NF-κB → IκBα + NF-κB  nIκBα·nNF-κB → nIκBα + nNF-κB | *kd1a* | 0.0005s-1 | ([Hoffmann, Levchenko et al. 2002](#_ENREF_28)) |
| NF-κB + E2F (1 or 4) → NF-κB·E2F  nNF-κB + nE2F → nNF-κB·nE2F | *ka2e* | 0.5 μM-1s-1 | fitted, same as IκBα + NF-κB |
| NF-κB·E2F → NF-κB + E2F  nNF-κB·nE2F → nNF-κB + nE2F | *kd2e* | 0.0005s-1 | fitted, same as IκBα + NF-κB |
| NF-κB·E2F1 + IκBα→ IκBα·NF-κB + E2F1  nNF-κB·nE2F1 + nIκBα → nIκBα·NF-κB + nE2F1 | *kdis* | 0.001s-1 | fitted |
| ***Transport*** | | | |
| NF-κB → nNF-κB | *ki1* | 0.0026 s-1 | Measuredfitting range:  *Average 0.0026 ± 0.0018 s-1* |
| nNF-κB → NF-κB | *ke1* | 0.000052 s-1 | *ki1*/50([Carlotti, Dower et al. 2000](#_ENREF_11)) |
| E2F1→ nE2F1 | *kie* | 0.0026 s-1 | fitted, same as NF-κB |
| nE2F1 → E2F1 | *kee* | 0.000052 s-1 | fitted, same as NF-κB |
| IκBα → nIκBα | *ki3a* | 0.00067 s-1 | Measured fitting range:  Average *0.00043 ± 0.00024 s-1* |
| nIκBα → IκBα | *ke3a* | 0.000335 s-1 | *ki3a*/2([Carlotti, Dower et al. 2000](#_ENREF_11)) |
| nIκBα·nNF-κB → IκBα·NF-κB | *ke2a* | 0.01 s-1 | Fitted |
| NF-κB·E2F1 → nNF-κB·nE2F1 | *kine* | 0.0026 s-1 | fitted, same as NF-κB |
| nNF-κB·nE2F1 → NF-κB·E2F1 | *kene* | 0.000052 s-1 | fitted, same as NF-κB |
| ***Protein synthesis & degradation*** | | | |
| nNF-κB → nNF-κB + tIκBα  *Order of hill function, h=2*  *Half-max constant, k=0.065h(fitted)* | *c1a* | 1.4×10-7 μM-1s-1 | Fitted (constrained): 1.07×10-7 – 8.2×10-7 μM-1s-1 ([Femino, Fay et al. 1998](#_ENREF_20)), ([Cheong, Bergmann et al. 2006](#_ENREF_13)) |
| tIκBα→ tIκBα + IκBα | *c2a* | 0.5 s-1 | ([Lipniacki, Paszek et al. 2004](#_ENREF_41)) |
| NF-κB·IκBα→ NF-κB | *c5a* | 0.000022s-1 | ([Pando and Verma 2000](#_ENREF_49), [Mathes, O'Dea et al. 2008](#_ENREF_44)) |
| nNF-κB·nIκBα→ nNF-κB | *-* | 0 s-1 | Assumed([O'Dea, Barken et al. 2007](#_ENREF_47), [Mathes, O'Dea et al. 2008](#_ENREF_44)) |
| nNF-κB → nNF-κB + tA20  *Order of hill function, h=2 Half-max constant, k=0.065h* | c1 | 1.4×10-7 μM-1s-1 | Assumed to be the same as IκBα |
| nE2F1 → nE2F1 + tE2F4  *Order of hill function, h=2 Half-max constant, k=0.065h* | c1x | 9.8×10-7 μM-1s-1 | Fitted |
| tA20→ tA20 + A20 | c2 | 0.5 s-1 | - |
| tE2F-4→ tE2F-4 + E2F4 | c2x | 0.5 s-1 | - |
| tIκBα→ Sink | *c3a* | 0.0003 s-1 | Fitted (constrained): 0.00077-0.00029 s-1 ([Blattner, Kannouche et al. 2000](#_ENREF_10)) |
| tA20→ Sink | c3 | 0.00048 s-1 | Fitted, constrained >tIκBα turnover([Ashall, Horton et al. 2009](#_ENREF_4)) |
| tE2F4→ Sink | c3x | 0.00048 s-1 | Fitted |
| IκBα→ Sink | *c4a* | 0.0005 s-1 | Fitted (constrained): 0.000105 – 0.002 s-1 ([Pando and Verma 2000](#_ENREF_49), [O'Dea, Barken et al. 2007](#_ENREF_47), [Mathes, O'Dea et al. 2008](#_ENREF_44)) |
| A20 → Sink | c4 | 0.0045 s-1 | Fitted |
| E2F4 → Sink | c4x | 0.00016 s-1 | Fitted |
| E2F1 → Sink | c6e | 0.00016 s-1 | Fitted |
| nE2F1 → Sink | c7e | 0.00016 s-1 | Fitted |
| NF-κB·E2F1 → Sink | c8ne | 0.00016 s-1 | Fitted |
| nNF-κB·nE2F1 → Sink | c9ne | 0.00016 s-1 | Fitted |
| ***TNFα stimulation*** | | | |
| TNFα | TR | 1/0 | on/off ([Lipniacki, Paszek et al. 2004](#_ENREF_41)) |
| ***IKK parameters*** | | | |
| IKKn → IKKa | *ka* | 0.004s-1 | Fitted, as above |
| IKKa → IKKi | *ki* | 0.003 s-1 | Fitted, as above |
| IKKi → IKKn | *kp* | 0.0006 s-1 | Fitted |
| A20 inhibition rate constant | *kbA20* | 0.0018 | Fitted, scales *kp* dependent on receptor state *kbA20×TR* |
| IKKa + IκBα → pIκBα | *kc1a* | 0.074 s-1 | Assumed (0.037×2) ([Heilker, Freuler et al. 1999](#_ENREF_26)) |
| IKKa + IκBα·NF-κB → pIκBα·NF-κB | *kc2a* | 0.37 s-1 | Assumed (0.037×5×2) ([Heilker, Freuler et al. 1999](#_ENREF_26), [Zandi and Karin 1999](#_ENREF_77)) |
| pIκBα → Sink | *kt1a* | 0.1 s-1 | Fitted |
| pIκBα·NF-κB → NF-κB | *kt2a* | 0.1 s-1 | Fitted |

**Appendix Table 3. Model reactions and associated parameters**

|  |  |
| --- | --- |
| **Figure** | **Model conditions** |
| 3E | TR=0, E2F1= (0.05, 0.1, 0.15), E2F4 off |
| 4A | TR=1, E2F1 = 0.1, E2F4 off |
| 4C | TR=1, E2F1 = 0.1, E2F4 on |
| 4I (G1, G2) | TR=1, E2F1= 0, E2F4 on (but unaffected) |
| 4I (G1/S) | TR=1, E2F1 = 0.2, E2F4 on |
| 4I (S) | TR=1, NFkBIkBa = 0.1, E2F1 = 0, E2F4= 0.1 |

**Appendix Table 4. Simulation protocols used throughout the manuscript.**

TNFα stimulation is invoked via TR=0/1. E2F refers to levels of “transfection” (in μM). E2F4 off/on refers to whether its transcription is switched on or off.



**Appendix Figure 6. Simulations from the NF-κB:E2F model.** Blue lines represent *E2F1*= 0, TR= 1. Black lines represent *E2F1*= *NFkB*= 0.1 and TR=0. Red lines represent *E2F1*= *NFkB*= 0.1 and TR=1.

**Section C: Generation of recombinant Bacterial Artificial Chromosome (BAC)**

Bacterial artificial chromosomes (BACs) containing the human E2F-1 gene with 98.7 kb 5′ flanking DNA, 45.5 kb 3′ flanking DNA (RP11-246G11) or the human RelA gene with 82 kb 5′ and 15 kb 3′ flanking DNA (CTD 2116H8) were identified using genome browser (<http://genome.ucsc.edu>) and obtained from Invitrogen/Life technologies. The targeting strategy to create a BACs expressing fusion protein products were based on the seamless recombineering technology developed by Warming *et al,* 2005, with minor modifications. Chimeric primers to amplify the GalK gene tagged with homology arms corresponding to the 50-80bp immediately up and downstream of the stop codon of the gene were used to generate the H-GalK-H recombination cassette for primary targeting. The length of GalK specific portion of the primers was extended to increase Tm and thus the efficiency of the PCR using a two-step PCR method (Phusion high fidelity enzyme, Finnzymes; primer sequences below). The second targeting cassette was generated using chimeric primers with the same homology arms but amplifying the desired fluorescent fusion protein, H-Venus-H for E2F1 or DsRedxp for RelA. Recombination and selection was carried out according to routine protocols ([Warming, Costantino et al. 2005](#_ENREF_68)) and are available at <http://recombineering.ncifcrf.gov>. Clone screening was performed by pulsed field gel electrophoresis, Southern blots and sequencing to confirm in-frame C-terminal insertion of the reporter gene.

Primers used (italics denote homology arm sequence):

E2F1-GalKF

*TCAGAGACCTCTTCGACTGTGACTTTGGGGACCTCACCCCCCTGGATTTC*CCTGTTGACAATTAATCATCGGCATAGTATATCG

E2F1-Galk R *TGCAGAGACAAGGTGAGCATCTCTGGAAACCCTGGTCCCTCCAAGCCCTG*TCAGCACTGTCCTGCTCCTTGTGA

E2F1-Venus F *CCACTTCGGCCTCGAGGAGGGCGAGGGCATCAGAGACCTCTTCGACTGTGACTTTGGGGACCTCACCCCCCTGGATTTC*ATGGTGAGCAAGGGCGAGGAG

E2F1-Venus R *CGGCCAGGGACAGGGGGCTCCAGGGCTGCAGAGACAAGGTGAGCATCTCTGGAAACCCTGGTCCCTCCAAGCCCTG*CTACTTGTACAGCTCGTCCATGCC

RelA-GalKF

*ATGAAGACTTCTCCTCCATTGCGGACATGGACTTCTCAGCCCTGCTGAGTCAGATCAGCTCC*CCTGTTGACAATTAATCATCGGCATAGTATATCG

RelA-Galk R *CAGAATCCGTAAGTGCTTTTGGAGGGCTTCAATCCCCTGCAACCCAGTGCTCTGGGGAGGGCAGGCGTCACCCCC*TCAGCACTGTCCTGCTCCTTGTGA

RelA-DsRedxp F *ATGAAGACTTCTCCTCCATTGCGGACATGGACTTCTCAGCCCTGCTGAGTCAGATCAGCTCC*ATGGCCTCCTCCGAGGACGTC

RelA-DsRedxp R*CAGAATCCGTAAGTGCTTTTGGAGGGCTTCAATCCCCTGCAACCCAGTGCTCTGGGGAGGGCAGGCGTCACCCCCC*TACAGGAACAGGTGGTGGCG

The BAC were initially transiently transfected into HeLa cells using ExGen500 transfection reagent (Fermentas, UK) to confirm fluorescent protein function.

**BAC stable cell line generation:** To generate stable cell lines it was necessary to retrofit the BAC with an appropriate mammalian selection marker. Retrofitting constructs that could universally be applied to any BAC were developed. As the same parent BAC vector construct, pBAC108L, was used to derive the most common BAC vectors, pe3.6 (from the Roslin Park institute library) and the pBeloBAC vector (from the California Institute of Technology) approximately 6kb of the vectors had perfect sequence homology. Within this region the chloramphenicol resistance gene was identified as a suitable target for replacement with a new selection marker as this would not disrupt important bacterial sequences. Restriction site-tagged homology arms 300-400bp in length were amplified from the BAC sequence using the primers (underlined indicates enzyme site):

5’H *Kpn*I F tgtcaaGGTACCGGCAGCCACATCCAG,

5’H *Eco*RI R ggtgccGAATTCTCAACGTCTCATTTTCGC,

3’H *Bam*HI F aatgggGGATCCTGGACAACTTCTTCGCC,

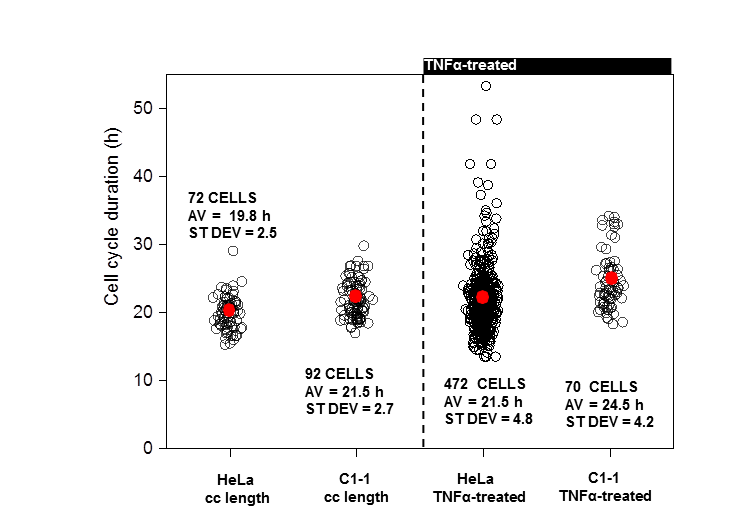
3’H *Sac*II R aatgggCCGCGGGCCGTCGACCAATTCTC

and cloned using the appropriate enzymes into the multiple cloning sites of pL451([Liu, Jenkins et al. 2003](#_ENREF_42)). This resulted in a recombination cassette containing H-pGK-pEM7-Kan/Neo-H. Retrofitting was performed in the same SW102 strain hosting the BAC by heat induction of the bacteria for recombination, transforming with the cassette and plating on LB containing Kanamycin (25µg/ml). Clones were screened by PFGE and >90% recombination efficiency was observed.

**Stable BAC transfection:** BAC DNA was prepared by maxiprep (BAC100 Nucleobond kit, Macherey-Nagel, Germany) and 1µg or 3µg used to transfect 106 cells in a 10cm dish using ExGen500 transfection reagent. Media was changed 3 days post transfection and supplemented with 500µg/ml G418. Media + antibiotic were refreshed every 3-4 days. Colonies formed 2-3 weeks after culturing in selection containing media were ring cloned into individual wells of a 48 well plate and sequentially scaled up to large culture vessels as necessary.

A HeLa cell line stably expressing the E2F-1-venus bacterial artificial chromosome (BAC), was transiently transfected with a FUCCI marker for G1-phase. Shows two consecutive cycles of HeLa cell division for a representative cell. Parent and daughter cells showed cycles of E2F-1 expression, with a peak timing consistent with late G1-phase.

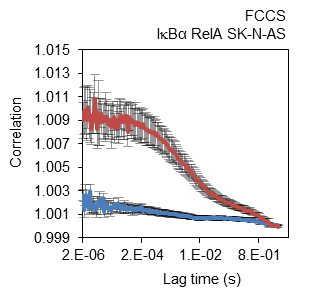
**Section D: BAC Characterisation**

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**Appendix Figure 7. Cell Cycle length of Clonal HeLa BAC population** (A) Analysis of cell cycle duration in populations of dual BAC stable cell line (C1-1) with wild type HeLa cells. (B) Analysis of the effects of TNFα treatment in C1-1 and WT HeLa cells.

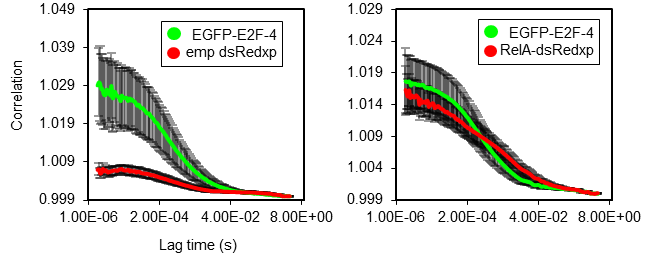
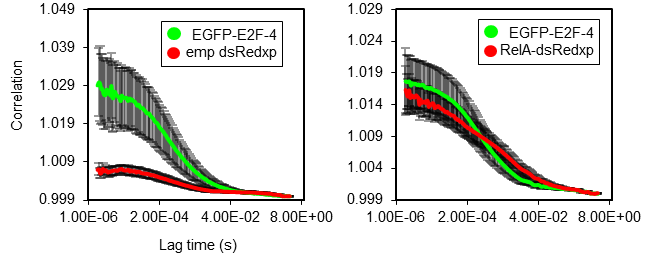
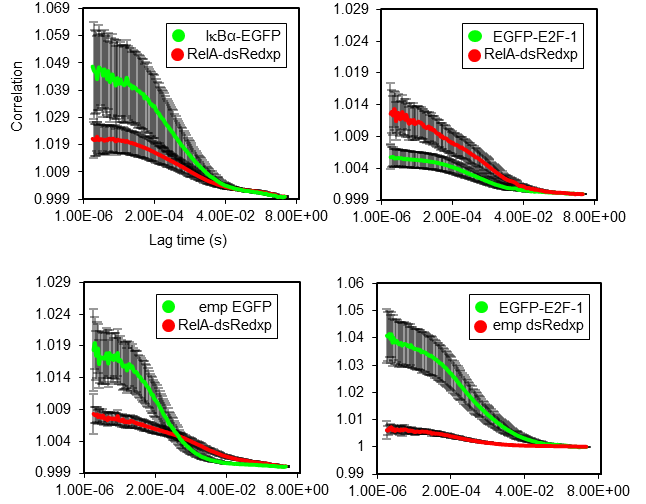
**Section E: Controls for FCCS**

Physical interaction between RelA and IBα was confirmed using FCCS in SK-N-AS cells co-expressing RelA-DsRedxp and IBα-EGFP. FCS exploits fluorescence-intensity fluctuations caused by low numbers of diffusing labelled particles in a diffraction limited confocal volume of light to analyse their concentration and mobility ([Spiller, Wood et al. 2010](#_ENREF_62)). Fluctuations are recorded as function of time and then statistically analysed by autocorrelation analysis. In its dual-colour variant, FCCS, two spectrally distinct fluorophores (such as red and green) are used and the cross-correlation amplitude in conjunction with the auto-correlation amplitudes provides information on molecular binding as well as dynamic co-localisation. In contrast to FRET, FCCS does not depend on the very close proximity of the interacting fluorescent labels.



**Appendix figure 8. FCCS control.** FCCS assays between transiently transfected RelA-dsRedxp and IBα-EGFP (red line) and Empty-DsRedxp and Empty-EGFP (blue line) in single live SK-N-AS cells (+/- s.e.m based on 10 measurements in each of 10+ cells per condition).

Auto-correlation analysis was conducted to show comparable noise between measurements and between controls for RelA/E2F-1 FCCS data



**Appendix Figure 9. FCCS autocorrelation analysis.** Autocorrelation lines for RelA/IkBα, RelA/E2F-1 and RelA/E2F-4 FCCS studies in single live SK-N-AS cells (+/- s.e.m based on 10 measurements in each of 10+ cells per condition).

**Author contributions statement**

JMA designed and performed the experiments, designed the model and wrote the paper. RA performed co-IP experiments, made and analysed experiments with the E2F-1 BAC and cell line. NAJ carried out western blotting, imaging, siRNA knockdown experiments, assisted with FCCS experiments on BAC stable cell lines and wrote the manuscript. JB performed FCCS experiments and edited the paper. SR performed Western blotting and Q-PCR and helped with FRET experiments. ADA led the BAC design and cloning strategy. CVH provided advice at the outset of the project and assisted with qPCR. LB assisted with mathematical model analysis. DGS provided expert help with all imaging experiments and wrote the paper. DAJ assisted with BAC development and advised on and edited the paper. PP provided expert advice on mathematical model building and edited the paper. VS provided expert help on E2F cloning and IP experiments advised on project planning and edited the paper. MRHW directed the project and wrote the paper.

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