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Model-based identification of TNF α -induced IKK β -mediated and I κ B α -mediated regulation of NF κ B signal transduction as a tool to quantify the impact of drug-induced liver injury compounds

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Drug-induced liver injury (DILI) has become a major problem for patients and for clinicians, academics and the pharmaceutical industry. To date, existing hepatotoxicity test systems are only poorly predictive and the underlying mechanisms are still unclear. One of the factors known to amplify hepatotoxicity is the tumor necrosis factor alpha (TNF α), especially due to its synergy with commonly used drugs such as diclofenac. However, the exact mechanism of how diclofenac in combination with TNF α induces liver injury remains elusive. Here, we combined time-resolved immunoblotting and live-cell imaging data of HepG2 cells and primary human hepatocytes (PHH) with dynamic pathway modeling using ordinary differential equations (ODEs) to describe the complex structure of TNF α -induced NF κ B signal transduction and integrated the perturbations of the pathway caused by diclofenac. The resulting mathematical model was used to systematically identify parameters affected by diclofenac. These analyses showed that more than one regulatory module of TNF α -induced NF κ B signal transduction is affected by diclofenac, suggesting that hepatotoxicity is the integrated consequence of multiple changes in hepatocytes and that multiple factors define toxicity thresholds. Applying our mathematical modeling approach to other DILI-causing compounds representing different putative DILI mechanism classes enabled us to quantify their impact on pathway activation, highlighting the potential of the dynamic pathway model as a quantitative tool for the analysis of DILI compounds.

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INTRODUCTION

Drug-induced liver injury (DILI) is currently one of the most important obstacles during drug development. To date, over 1000 drugs are known to cause DILI,¹ affecting not only a restricted group of patients, but a broad range of medications and treatments.² Current test systems employed by the pharmaceutical industry are poorly predictive since the underlying mechanisms are still unclear. So far, the majority of studies focused on the effects of compounds on hepatocytes, whereas the impacts of synergistic drug–cytokine interactions were rarely considered. Furthermore, due to the complexity of the impact of compounds on the dynamic behavior of the intracellular signaling network, the impacts of multiple factors have to be considered.

One of the top ten DILI-causing compounds is diclofenac (DCF), a commonly used nonsteroidal anti-inflammatory drug. DCF was shown to synergize with tumor necrosis factor alpha (TNF α) by

accelerating apoptosis in primary human hepatocytes (PHH) and HepG2 cells^{3,4} by enhancing endoplasmic reticulum stress as well as oxidative stress.⁵ However, the exact underlying mode of action remained to be elucidated. TNF α signal transduction, apart from being a key mediator of inflammatory responses, plays also a major role in apoptosis. It was observed that there is a tightly regulated and very complex balance between TNF α -induced pro-survival signaling via complex I and death signaling via complex II.^{6,7} The TNFR1-Membrane-Associated Proximal Complex (complex I) is rapidly formed at the plasma membrane and is composed of the receptor itself, TRADD, RIP, TRAF2, and cIAP1, but is devoid of caspase 8 and triggers only the NF κ B response but no apoptotic signaling.⁶ TNF α was reported to enhance cell death^{8,9} if the NF κ B-induced inhibition of apoptotic signaling via JNK or necroptotic signaling via RIP fails.¹⁰ Because NF κ B signal transduction is extremely complex due to a multitude of feedback

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regulators, it has been previously examined by applying mathematical modeling that is a powerful tool to study multi-factorial and complex networks.^{11–15} Since it was proposed that the I κ B kinase (IKK) signaling module is highly relevant for the temporal control of NF κ B signal transduction,¹⁶ several mathematical models included the IKK module.^{11,15,17,18} However, a potential role of IKK in drug-induced hepatotoxicity upon inflammatory responses so far has not been addressed. IKK is a multi-protein complex composed of IKK α , IKK β , and the regulatory IKK γ (NEMO) that phosphorylates I κ B and thereby facilitates degradation of I κ B inhibitors and the subsequent translocation of NF κ B to the nucleus.^{19,20} The activity of the IKK is controlled by positive and negative regulatory phosphorylation cycles modulated by a network of components of the TNF receptor (TNFR) complex.^{19,20} Specifically, activation by TNF α binding to the receptor leads to the phosphorylation of two sites in the activation loop of IKK β , which is essential for the activation of the NF κ B pathway. During this highly active state, IKK β undergoes extensive autophosphorylation at multiple sites at the C-terminus,²¹ which leads to a massive downregulation of its activity. If both the activation loop and the C-terminus are phosphorylated, IKK β is still active, although with almost no catalytic activity. Rather, this state facilitates the recruitment of phosphatases deactivating IKK by dephosphorylation of its activation loop. This in turn creates an inactive state that is refractory to activation by TNF α .²² Therefore, another dephosphorylation event has to take place resulting in the non-phosphorylated state of IKK β that is free to be re-activated by the receptor complex. In sum, the activity of IKK and thereby the activation of NF κ B signal transduction is tightly regulated at the level of IKK by a four-step phosphorylation/dephosphorylation process.²² The latent transcription factor NF κ B is regulated by the inhibitor of NF κ B, I κ B α , which sequesters NF κ B in the cytoplasm. I κ B α in complex with NF κ B is a substrate for the IKK complex.^{23,24} Phosphorylation of I κ B α prompts it for ubiquitination leading eventually to the degradation of I κ B α and the release and nuclear translocation of NF κ B. These steps provide another important regulatory mechanism to control the activation of NF κ B signal transduction downstream of the TNFR complex.

To examine mechanisms responsible for the impact of DCF on TNF α -induced hepatotoxicity, we considered the highly intertwined signaling components and applied a data-based mathematical modeling approach. While the NF κ B pathway has been extensively studied in the past and several dynamic pathway models exist, these mathematical models did not address the effect of DCF on the TNF α -induced NF κ B signal transduction pathway. In the presented study, we generated quantitative data in the human hepatocellular carcinoma cell line HepG2 and in PHHs under standardized conditions. Jointly estimating parameters of our dynamic pathway model from the standardized data enabled us to attribute the observed alterations in the dynamics of TNF α -induced NF κ B signal transduction in response to co-treatment with DCF to specific changes in parameters of the reaction network. To this end, previously established methods of model selection and L_1 regularization^{25,26} were employed to determine the most likely interaction points between DCF and TNF α -induced NF κ B signal transduction. The systematic analyses of the calibrated model revealed the interplay of IKK-mediated and I κ B α -mediated regulation of the dynamics of NF κ B in the nucleus and thereby explained the DCF-induced alterations of NF κ B oscillations in nucleus and cytoplasm. With a mathematical model generalized to explain drug-induced IKK and I κ B α regulation it was possible to quantify the impact of four additional DILI compounds (amiodarone, paracetamol, ximelagatran, and fluralidine) on TNF α -induced NF κ B activation.

RESULTS

Dynamic pathway model of TNF α -induced NF κ B signal transduction

To develop a mathematical model for TNF α -induced NF κ B signal transduction (Fig. 1a), we generated time-resolved data of several pathway components of the canonical NF κ B pathway in the human hepatocellular carcinoma cell line HepG2 and in PHHs. Since it was previously reported that DCF affects the oscillatory behavior of NF κ B, we first examined the dynamics of TNF α -induced nuclear translocation of NF κ B and the corresponding production of the target gene A20. We utilized HepG2 cells stably expressing GFP-tagged RelA, a subunit of NF κ B (further referred to as NF κ B-GFP HepG2) and monitored by life cell imaging the localization of NF κ B in the cytoplasm and the nucleus upon stimulation with TNF α . The NF κ B-GFP intensity was simultaneously quantified in several hundred cells, yielding an average dynamics (Fig. 1b, data points in upper two panels). As shown in Fig. S1, the single-cell dynamics of NF κ B nuclear translocation was highly consistent with the average dynamics, corroborating our approach to analyze the average data and use it for mathematical modeling. Further, we quantified the time course of A20 expression in response to TNF α stimulation in HepG2 cells stably expressing GFP-tagged A20 (A20-GFP HepG2) (Fig. 1b, data points in lower panel). These examinations showed that TNF α -induced nuclear accumulation of NF κ B reaches its first peak after 25 min followed by a second peak at 122 min. The timing of maximal accumulation of NF κ B in the nucleus mirrored the maximal reduction of NF κ B from the cytoplasm showing the expected complementary dynamics. The expression of the target gene A20 was initiated as soon as 50 min past TNF α stimulation, which is shortly after the first peak of nuclear NF κ B, and kept increasing for the entire observation time of 6 h.

To simultaneously examine the dynamics of pathway activation of multiple key components of the canonical NF κ B signal transduction pathway, we used quantitative immunoblotting and determined the dynamics of IKK, I κ B α , and NF κ B phosphorylation and of I κ B α abundance in HepG2 cells that were either stimulated with TNF α or were left untreated. Comparable experiments were performed with PHH that were treated with TNF α . As depicted in Fig. 1c for HepG2 cells and in Fig. 1d for PHH, in both cell types the key components of the canonical NF κ B signal transduction pathway were phosphorylated after 10–20 min of TNF α stimulation. Likewise, the overall dynamics of pathway activation was very comparable in HepG2 cells and PHH, corroborating the use of the HepG2 cell line as model system. However, the extent of I κ B α and NF κ B phosphorylation was slightly lower in PHH compared to HepG2 cells. Further, shortly after the first peak of the IKK, I κ B α , and NF κ B phosphorylation the cytoplasmic levels of I κ B α reached the lowest values after TNF α stimulation at 20 min in both HepG2 cells and PHH. Finally, to quantify the dynamics of phosphorylation events of the NF κ B:I κ B α complex, we performed co-immunoprecipitation experiments (Co-IP) in HepG2 cells that were stimulated with TNF α . Cellular lysates taken at different time points of TNF α stimulation were subjected to immunoprecipitation with antibodies recognizing I κ B α or NF κ B. In immunoblotting experiments we examined the time course of immunoprecipitated I κ B α in the cytoplasm as well as the complex formation with NF κ B and phosphorylated NF κ B. For immunoprecipitated NF κ B the dynamics of NF κ B phosphorylation was monitored as well as the complex formation with I κ B α and phosphorylated I κ B α (Fig. 1e). The obtained results showed that the peak of NF κ B:pI κ B α complex and of pNF κ B:I κ B α slightly preceded the maximum of TNF α -induced NF κ B phosphorylation.

Based on these comprehensive time-resolved examinations an ordinary differential equation (ODE)-based dynamic pathway model of TNF α -induced NF κ B signaling (Fig. 1a) was developed. First, a model with a multitude of independent reaction rates

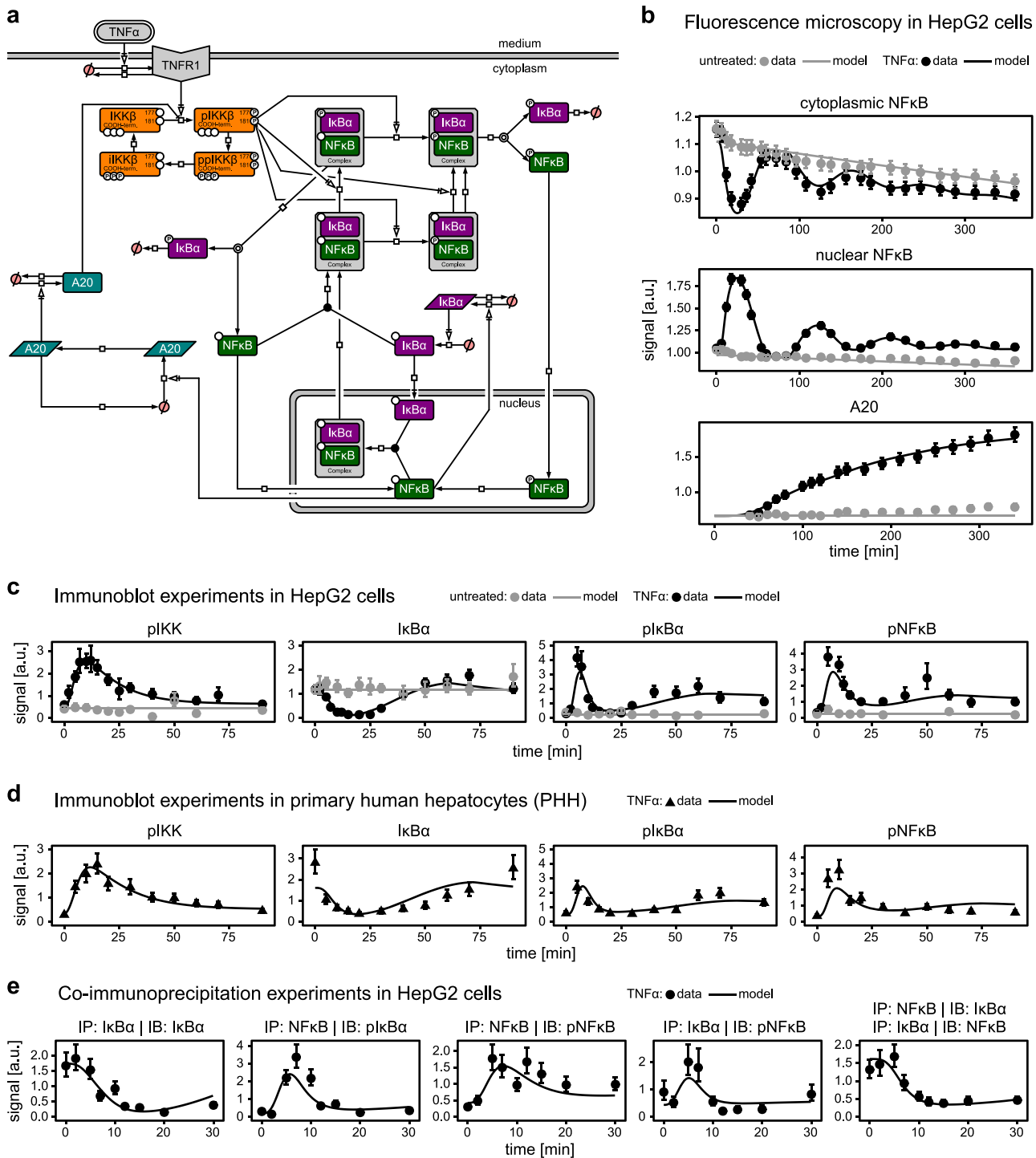


Fig. 1 TNF α -induced NF κ B signaling pathway model for HepG2 cells and primary human hepatocytes (PHH). **a** Schematic representation of the model according to Systems Biology Graphical Notation indicating the considered components and reactions. Arrows indicate biochemical reactions. Boxes with round corners symbolize proteins, parallelograms represent RNA. Slashed circles represent degradation or production. Experimental data was acquired by either live-cell fluorescence microscopy (**b**) of NF κ B translocation (cytoplasm and nuclei, $n = 3$) and of A20 expression dynamics ($n = 1$) in HepG2 cells stably expressing NF κ B-GFP or A20-GFP, respectively, or quantitative immunoblotting (**c–e**) of cytoplasmic lysates of HepG2 cells (**c**, $n = 3–6$, raw data in Figs. S8–S17) and PHHs (**d**, $n = 3$ donors, raw data in Figs. S18–S21) or immunoprecipitations (**e**, $n = 1–3$, raw data in Figs. S22–S29) treated with TNF α (10 ng/ml). Data points with 1σ confidence intervals computed from replicates are indicated by dots with error bars, lines indicate trajectories of the calibrated model

involving IKK, NF κ B, and I κ B α phosphorylation as well as complex formation was established (Fig. S2). Based on this comprehensive model, the major processes contributing to the signaling pathway were identified by inspecting all reaction fluxes (Fig. S3). To

describe the upstream processes leading to the activation of the NF κ B pathway, we included four IKK states in the dynamic pathway model that are denoted as IKK, phosphorylated (p) IKK, multiply phosphorylated (pp) IKK, and inactive (i) IKK. These states

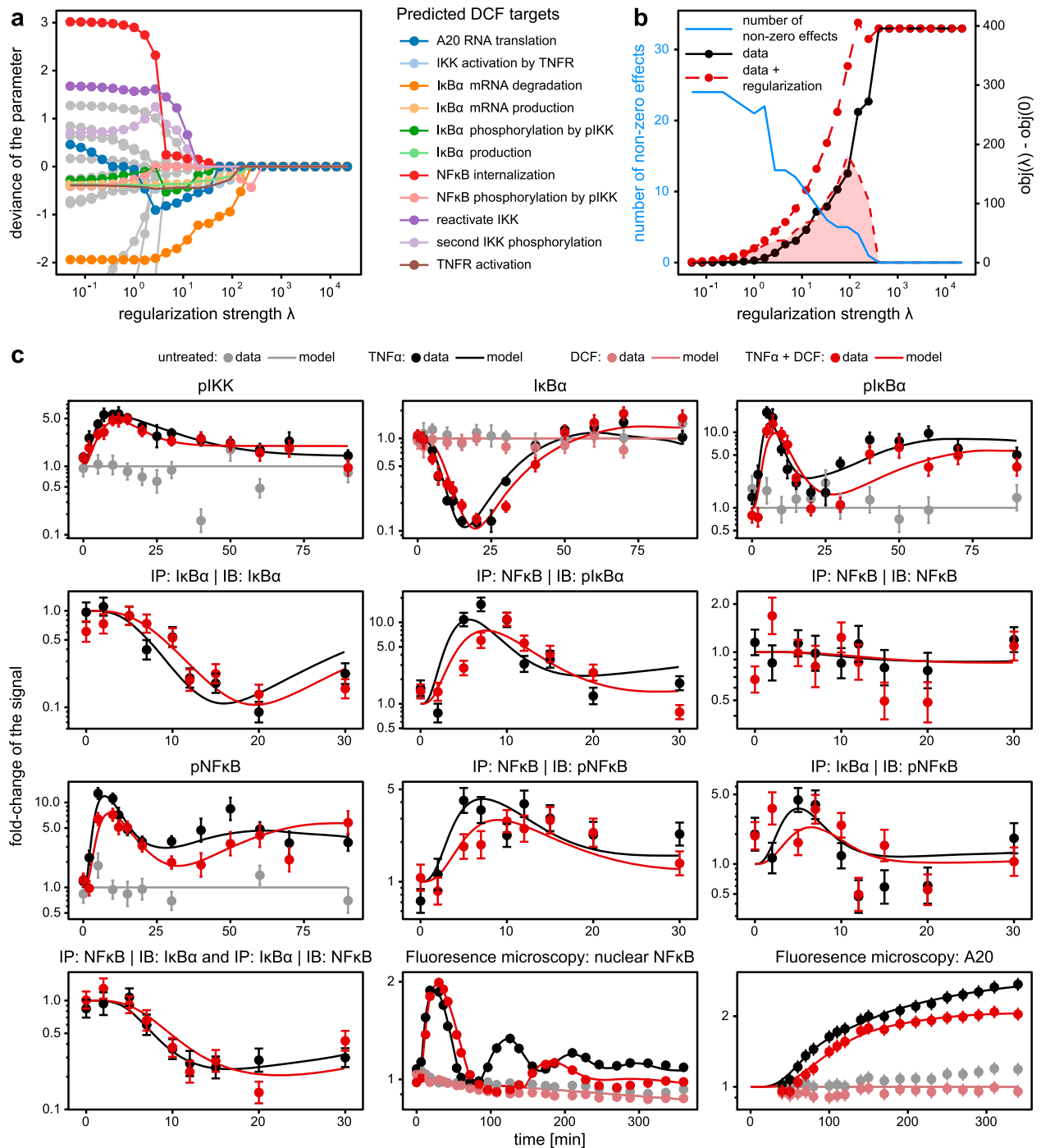


Fig. 2 Identification of diclofenac effects. **a** Increasing regularization strength λ forces parameter differences between the mathematical model describing TNF α -stimulated control data and the model with modified rates based on the data of TNF α and DCF-treated cells to vanish. The eleven parameters disappearing last are indicated as colored paths, early disappearing effects are shown in gray. **b** Every vanishing effect reduces the model complexity by one, shown in blue. Accordingly, the fit quality decreases indicated by larger contributions from the data points to the objective value, indicated in black. The regularization value, shaded area, reaches its maximum when regularization strength and model complexity balance out. Regularization and data contribution sum up to the total objective value, shown as red line. **c** The core model with a total of seven diclofenac-specific rate parameters was fitted to the experimental data. Data points with 1 σ confidence intervals computed from replicates are indicated by dots with error bars, model fits are shown as solid lines. Experimental data was acquired by either live-cell fluorescence microscopy of NF κ B translocation (cytoplasm and nuclei, $n = 3$) and of A20 expression dynamics ($n = 1$) in HepG2 cells stably expressing NF κ B-GFP or A20-GFP, respectively, or by quantitative immunoblotting of cytoplasmic lysates ($n = 3-6$, raw data in Figs. S8-S17) or immunoprecipitations ($n = 1-3$, raw data in Figs. S22-S29) of HepG2 cells, either untreated, treated with TNF α alone (10 ng/ml), treated with diclofenac (500 μ M) alone or co-treated with TNF α (10 ng/ml) and diclofenac (500 μ M)

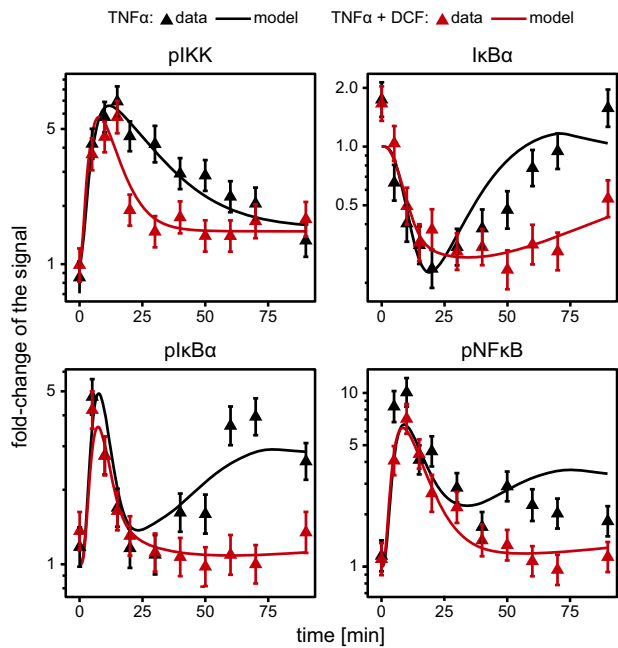


Fig. 3 Primary human hepatocytes data and fit. Experimental data was acquired by quantitative immunoblotting of cytoplasmic lysates of primary human hepatocytes treated with either TNF α alone (10 ng/ml) or with TNF α (10 ng/ml) and diclofenac (500 μ M). Data points with 1σ confidence intervals computed from independent donors ($n = 3$, raw data in Figs. S18–S21) are indicated by triangles and error bars. Only the model parameters specific for DCF treatment, observation-specific scaling and offset parameters and a global time-scale parameter were adjusted to predict the correct IKK, I κ B α , and NF κ B profiles

are based on sequential events induced by TNF α : first, the kinase domain is phosphorylated at the activation loop leading to full kinase activity (pIKK). Second, the C-terminal serines are phosphorylated, while the kinase domain is still phosphorylated (ppIKK). In the ppIKK state, IKK β has very low catalytic activity. Finally, IKK β is inactivated (iIKK) due to dephosphorylation of the activation loop and needs a recovery time before it can be activated again (IKK). Because the antibody applied for measuring the phosphorylation of IKK is detecting the phosphorylation in the activation loop, the immunoblotting measurements represent both pIKK and ppIKK. However, according to our flux analysis, only pIKK has an impact on I κ B α and NF κ B phosphorylation within the complex. Because phosphorylation of I κ B α triggers the dissociation of the NF κ B:I κ B α complex, this results in our model in free NF κ B, either phosphorylated or not. The reaction fluxes showed that both states are necessary and fulfill a different purpose. Inspecting the gain terms of the NF κ B:I κ B α complex we found that complex formation in the cytoplasm of unphosphorylated NF κ B with *de novo* produced I κ B α is almost as strong as the supply by complexes exported from the nucleus. Another finding from the analysis of the reaction fluxes was that the newly synthesized A20 protein, although being known to be a negative feedback on TNF α -mediated IKK activation, has a negligible inhibitory impact on IKK. Based on the analysis of the reaction fluxes a much reduced mathematical model was developed. Further reduction steps were introduced based on the profile likelihood method. The profile likelihood analysis pointed out that several estimated parameters, e.g., nuclear NF κ B dephosphorylation, complex formation and export, are likely to operate on a faster timescale than other reactions, and therefore cannot be constrained by upper confidence bounds based on the data. Finally, the combination of flux and profile likelihood analysis led to a fully identifiable core model capable of describing the dynamics of

TNF α -induced activation of NF κ B signal transduction in HepG2 cells (Fig. 1b, c, e). A list of all reaction equations and parameter values is provided in Supplementary Material 1 (Tables S5–S7).

Identification of DCF targets in the TNF α -induced NF κ B signal transduction pathway

To assess the impact of DCF on the TNF α -induced NF κ B signal transduction pathway, we quantified the dynamics of the respective components in HepG2 cells in the presence or absence of DCF using quantitative immunoblotting and live-cell imaging (Fig. 2c, symbols). To select a suitable DCF dose, the impact of increasing doses of DCF on TNF α -induced I κ B phosphorylation was examined (Fig. S7). In these experiments a major reduction of TNF α -induced I κ B phosphorylation was observed upon treatment with 500 μ M DCF. Since in a previous publication a substantial enhancement of DCF toxicity was observed upon treatment with 500 μ M DCF and TNF α ,⁵ this DCF concentration was selected for all measurements. DCF was added to the cells 30 min prior to stimulation with TNF α . The most prominent effects of co-treatment of TNF α and DCF were a delay of the second NF κ B peak in the nucleus and a reduced A20 production. Specifically, in the presence of TNF α and DCF the first peak of nuclear NF κ B was delayed by 8 min and slightly exceeded the maximum level observed for TNF α treatment alone. The phase of low NF κ B levels was prolonged in TNF α and DCF co-treated HepG2 cells relative to TNF α treatment alone and the second peak of nuclear NF κ B was delayed by 56 min. Also the levels of nuclear NF κ B were lower for TNF α and DCF co-treated cells. These effects were preceded by delayed and reduced concentrations of I κ B α , phosphorylated I κ B α , and NF κ B in the cytoplasm after 30 min of co-stimulation.

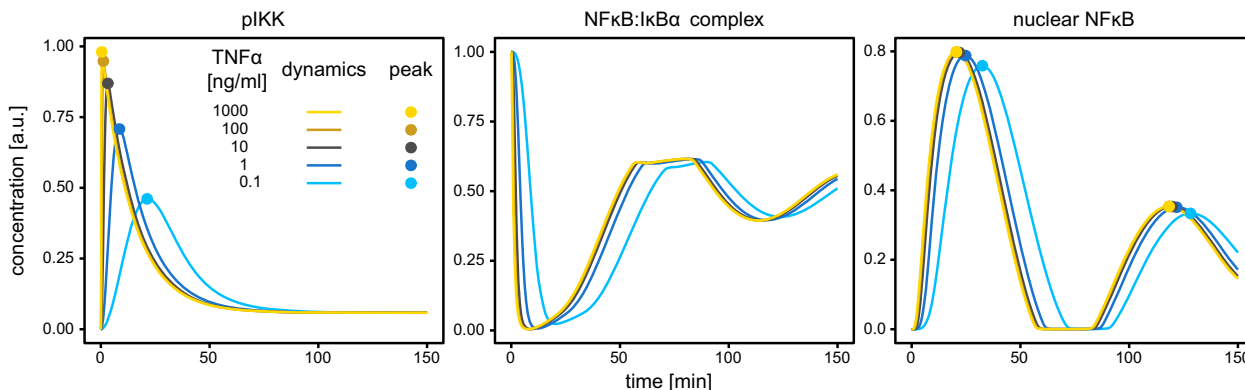
To systematically identify putative targets of DCF in the TNF α signaling network, we utilized the established core model of TNF α -induced NF κ B signal transduction to analyze the time-resolved live-cell imaging and immunoblotting data of the TNF α -stimulated HepG2 cells that were either co-treated with DCF or not (Fig. 2c, data points). For simultaneous parameter estimation in TNF α and DCF co-treated or untreated cells, L_1 regularization was used to gradually reduce the number of hypothesized interactions between DCF and the reaction network. For different values of the regularization strength λ , the regularized objective function $l_1(k, \Delta_k)$ was optimized with respect to the original parameters k and the DCF-specific parameters Δ_k . In Fig. 2a, the estimated Δ -values are shown as function of the regularization strength λ . With increasing λ , more and more parameters were reduced to the baseline indicating that their effect is estimated to be negligible. The 11 parameters that were reduced last to the baseline are indicated by colored lines. These parameters are retained, since they are most essential to maintain a good fit between data and model when the number of non-zero difference parameters is reduced. The decline of non-zero parameters is shown as a blue line in Fig. 2b. For comparison, the objective value with and without the regularization contribution are shown in red and black, respectively. The shaded area, i.e., the regularization contribution, indicates that for high enough regularization strength the number of non-zero Δ -parameters is decreased to an extent that the regularization contribution vanishes. However, at this point the fit between data and model deteriorated by 400 in terms of twice the negative log-likelihood compared to the model with 24 Δ -parameters. According to a likelihood ratio test, deterioration of the likelihood becomes highly significant ($p < 0.001$) when the penalty strength passes the threshold of $\lambda = 10$, corresponding to 11 non-zero effects and a difference of 34 in terms of twice the negative log-likelihood.

The list of non-zero effects revealed that the major targets of DCF are IKK activation and I κ B α production and degradation. In addition, the production of A20 appeared to be directly affected. Of note is that some of the curves depicted in Fig. 2a were

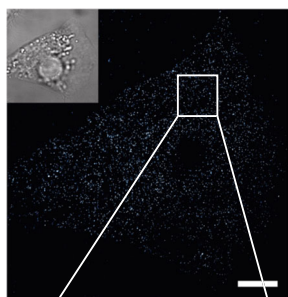
overlapping. For example, IKK activation by the TNFR1 (light blue) and TNFR1 activation (brown) cannot be distinguished in the plot. A change of either rate parameter had the same effect on the model response and therefore these parameters were considered as not independent. Based on the obtained results, seven DCF-specific reaction rates were identified to be independent: the activation of IKK by TNF α , the recovery of iIKK, the

phosphorylation of pIKK, the phosphorylation of I κ B α by pIKK, the production and degradation of I κ B α mRNA, and, finally, the translation of A20 mRNA were introduced into the model yielding the TNF α signaling DCF model. As a result, the data from HepG2 cells treated either with TNF α alone or in with a combination of TNF α and DCF were successfully described by the same model topology (Fig. 2c, solid lines).

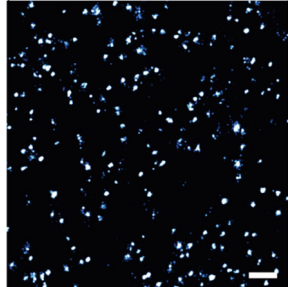
a System response for TNF α



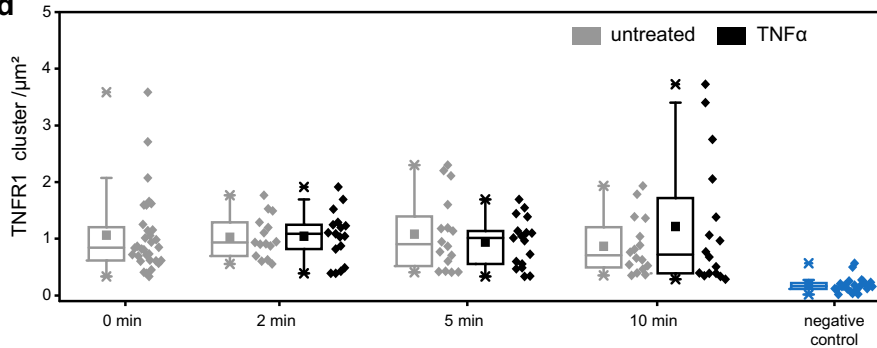
b



c



d



e

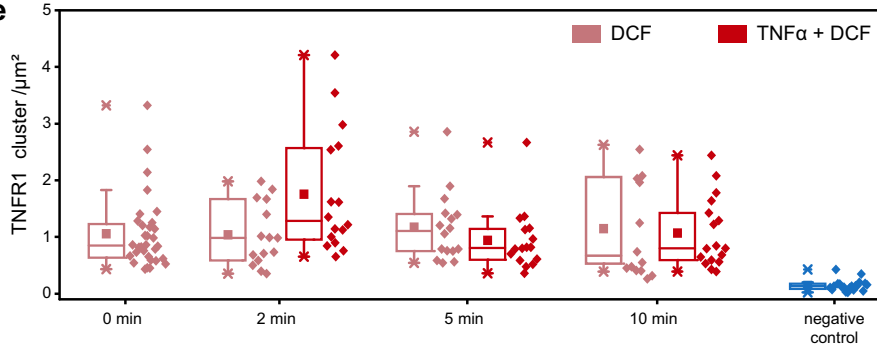


Fig. 4 Impact of DCF on TNF α and TNFR1 interaction. **a** Concentration time courses of pIKK, non-phosphorylated NF κ B-I κ B α complex in the cytoplasm and free, non-phosphorylated NF κ B in the nucleus were simulated for TNF α concentrations between 0.1 and 1000 ng/ml. In the experiment 10 ng/ml were applied. The first maximum of pIKK and the first two maxima of nuclear NF κ B are indicated by dots. The different TNF α levels have a large effect on the peak height and position of pIKK relative to NF κ B-I κ B α complex and nuclear NF κ B that remain almost unchanged for higher TNF α concentrations and exhibit a minorly reduced and delayed response for lower TNF α concentrations. **b–e** dSTORM imaging of TNFR1 in the plasma membrane of HepG2 cells. **b** Representative dSTORM image of TNFR1 labeled via indirect immunocytochemistry on the plasma membrane of HepG2 cells (inset: brightfield image) (scale bar 5 μ m). **c** Magnification of the inset indicated in **b** (scale bar 500 nm). **d** Number of TNFR1 clusters on the cell membrane at the indicated time points before and after induction with TNF α (gray: non-stimulated cells, black: TNF α -stimulated cells, blue: negative control with secondary antibody only). **e** Number of TNFR1 clusters for cells pre-treated with DCF (light red) followed by induction with TNF α (red) (number of cells measured under each condition $n \geq 13$). Stimulation with TNF α shows no significant change to the according unstimulated population. Pre-treatment with DCF shows no significant changes in regard to untreated cells. Box plots in **d** and **e** indicate the median (line in box), lower and upper quartile (box), the mean value (square), and the data range (asterisks). Whiskers represent 1.5 \times the interquartile distance. Statistical analysis was performed using the Kolmogorov–Smirnov test with significance level $\alpha = 0.05$

Impact of DCF on TNF α -induced NF κ B signal transduction in PHHs
To verify that the model-identified impact of DCF on TNF α -induced NF κ B signal transduction holds true in primary liver cells, we tested the TNF α signaling DCF model on data from freshly isolated PHHs, stimulated with TNF α in the presence or absence of DCF (Fig. 3). Since the availability of PHH is very limited, we focused our analysis on immunoblotting experiments and selected time points that were most informative in the analysis of the DCF effects on TNF α -induced NF κ B signal transduction in HepG2 cells. As demonstrated in Fig. 1, the overall dynamics of pathway activation in PHH was highly consistent with the pathway activation dynamics observed in HepG2 cells, but appeared to be slower than in HepG2 cells. To consider this difference in timing, we introduced a global time-scale parameter affecting all reaction rates in the mathematical model. To account for the difference in signal strength and background in the immunoblotting experiments performed with PHH, observation parameters such as signal scale and offset were independently estimated. Furthermore, although we assume that the mechanism by which DCF acts on TNF α signaling in PHH is very comparable to HepG2 cells, the strength of DCF may vary in the primary cells. Taken together, a time-scale parameter, scaling and offset parameters, as well as the DCF-specific parameters were estimated from the PHH data, whereas all other parameter, i.e., reaction rates of the NF κ B signaling network were unchanged. The resulting model trajectories shown in Fig. 3 are in agreement with the experimental data, confirming that our mathematical model can also be applied to data generated in primary cells. Furthermore, experimental data and mathematical model simulation demonstrated that also in PHH DCF has a strong and early impact on TNF α -induced NF κ B signal transduction. In cells co-treated with TNF α and DCF, the activation of IKK was more transient and recovery of I κ B α , phosphorylated I κ B α , and phosphorylated NF κ B were much reduced compared to TNF α -treated cells. These observations underpin the broad applicability of the developed model to quantitatively assess the impact of DCF on TNF α -induced NF κ B signal transduction.

Impact of DCF on the TNF α /TNFR1 interaction in HepG2 cells

The L_1 regularization approach identified seven reaction rates in the signaling network as possible targets of DCF, including IKK activation. In principle, the predicted reduced IKK activation in TNF α and DCF co-treated cells could be mediated by a direct interference of DCF with TNF α binding to its receptor resulting in reduced TNF α levels available for the initiation of signal transduction. To explore this mechanism and evaluate if reduced TNF α levels alone could explain the observed effects of DCF on TNF α -induced NF κ B signal transduction, we utilized our TNF α signaling DCF model and simulated the dynamics of key components of the signaling pathway, pIKK, NF κ B:I κ B α complex, and nuclear NF κ B in response to different TNF α concentrations. We used the experimentally applied 10 ng/ml TNF α as reference and predicting with the model the dynamics for higher (100 and 1000 ng/ml) and lower (1 and 0.1 ng/ml) TNF α doses (Fig. 4a). The model trajectories showed that increasing TNF α concentrations above the experimentally applied 10 ng/ml of TNF α resulted in an increase of IKK phosphorylation levels but not in an increase in the peak height of nuclear NF κ B. This effect can be understood from the predicted dynamics of the NF κ B:I κ B α complex concentration. Already the reference TNF α concentration of 10 ng/ml lead to the dissociation of almost all NF κ B:I κ B α complexes. Even for TNF α concentrations as low as 0.1 ng/ml, more than 90% of all NF κ B:I κ B α complexes dissociated. Accordingly, the peak height of nuclear NF κ B showed only a small variation between different TNF α concentrations. Interestingly, the model simulations revealed only a minor difference in the time of the first and the second peak of nuclear NF κ B upon lowering the TNF α

concentration (dots in Fig. 4a). These predictions were in contrast to the observed impact of DCF (Fig. 2c) characterized by prolonged low levels of NF κ B in the nucleus followed by a much delayed second nuclear peak of NF κ B. Therefore, a reduction in the effective TNF α dose due to interference of DCF with TNF α binding to its receptor apparently would not be sufficient to explain the experimentally observed more transient IKK activation and the shift in the second peak of nuclear NF κ B. To corroborate these model-based insights, we analyzed by single-molecule localization microscopy (SMLM) whether DCF affects the number of TNFR1 complexes present on the plasma membrane of HepG2 cells (Fig. 4b–e). HepG2 cells were stimulated with TNF α for 2, 5, and 10 min or were left unstimulated. The TNFR1 cluster density at the cell membrane of unstimulated HepG2 cells showed a broad distribution with a mean value of 1.1 ± 0.1 clusters per μm^2 (Fig. 4d, Table S10), which is comparable to previous results obtained for HeLa cells.²⁷ The stimulation of HepG2 cells with TNF α did not significantly change the density of TNFR1 clusters at the cell membrane at the indicated time points. Similarly, co-treatment with DCF did not induce a change in the number of TNFR1 clusters (Fig. 4e, Table S10). Additionally, we investigated the size of TNFR1 clusters by analyzing SMLM data using the cluster algorithm DBSCAN.²⁸ We found a homogeneous distribution of TNFR1 cluster size in unstimulated cells with a mean radius of 28.7 ± 0.4 nm. The cluster radius was not altered upon stimulation with TNF α and co-treatment with DCF (see Table S10) indicating that DCF neither changes the number nor the size of TNFR1 clusters in the plasma membrane of HepG2 cells. Together with the model-based insights these results suggest that DCF does not impact the TNFR1, but rather targets intracellular reactions of the NF κ B signaling pathway and thereby shifts the positioning and height of the second peak of nuclear NF κ B.

Mathematical model-based exploration of mechanism explaining the impact of DCF on TNF α -induced NF κ B signal transduction

To identify mechanisms explaining the impact of DCF on intracellular TNF α -induced NF κ B signal transduction, we again utilized our TNF α signaling DCF model to theoretically examine the dynamic behavior of the concentration of unobserved species in response to TNF α stimulation compared to the co-treatment with TNF α and DCF.

The comparison of nuclear NF κ B and I κ B α profiles in response to both treatments indicated that nuclear accumulation of I κ B α and low levels of NF κ B in the nucleus are highly correlated (Fig. 5a). One explanation could be that the lack of nuclear NF κ B prevents complex formation between NF κ B and I κ B α and as a consequence I κ B α accumulates in the nucleus. To test this hypothesis, we examined the reaction fluxes controlling the amount of NF κ B in the nucleus: nuclear NF κ B import (positive flux) and complex formation between NF κ B and I κ B α leading to export of NF κ B to the cytoplasm (negative flux) (Fig. 5b). If the lack of nuclear NF κ B prevented complex formation, the fluxes would drop to zero at some time points due to the absence of NF κ B. However, according to the prediction of the model, the influx of NF κ B into the nucleus and the efflux upon complex formation are different from zero, even if the amount of NF κ B is very small. This indicated that any NF κ B molecule transported into the nucleus will, at the same rate, leave the nucleus in a complex with I κ B α . Since the maximal NF κ B flux is limited, the accumulation of nuclear I κ B α can be traced back to the level of I κ B α in the cytoplasm. If the level rises above a certain threshold, the NF κ B flux is insufficient to counteract the increased I κ B α import and I κ B α will accumulate in the nucleus.

Because the phosphorylation of I κ B α by pIKK as well as the production and degradation of I κ B α mRNA were among the DCF-targeted reaction rates, we investigated I κ B α levels in the cytoplasm (Fig. 5c). Because the transport flux of I κ B α into the

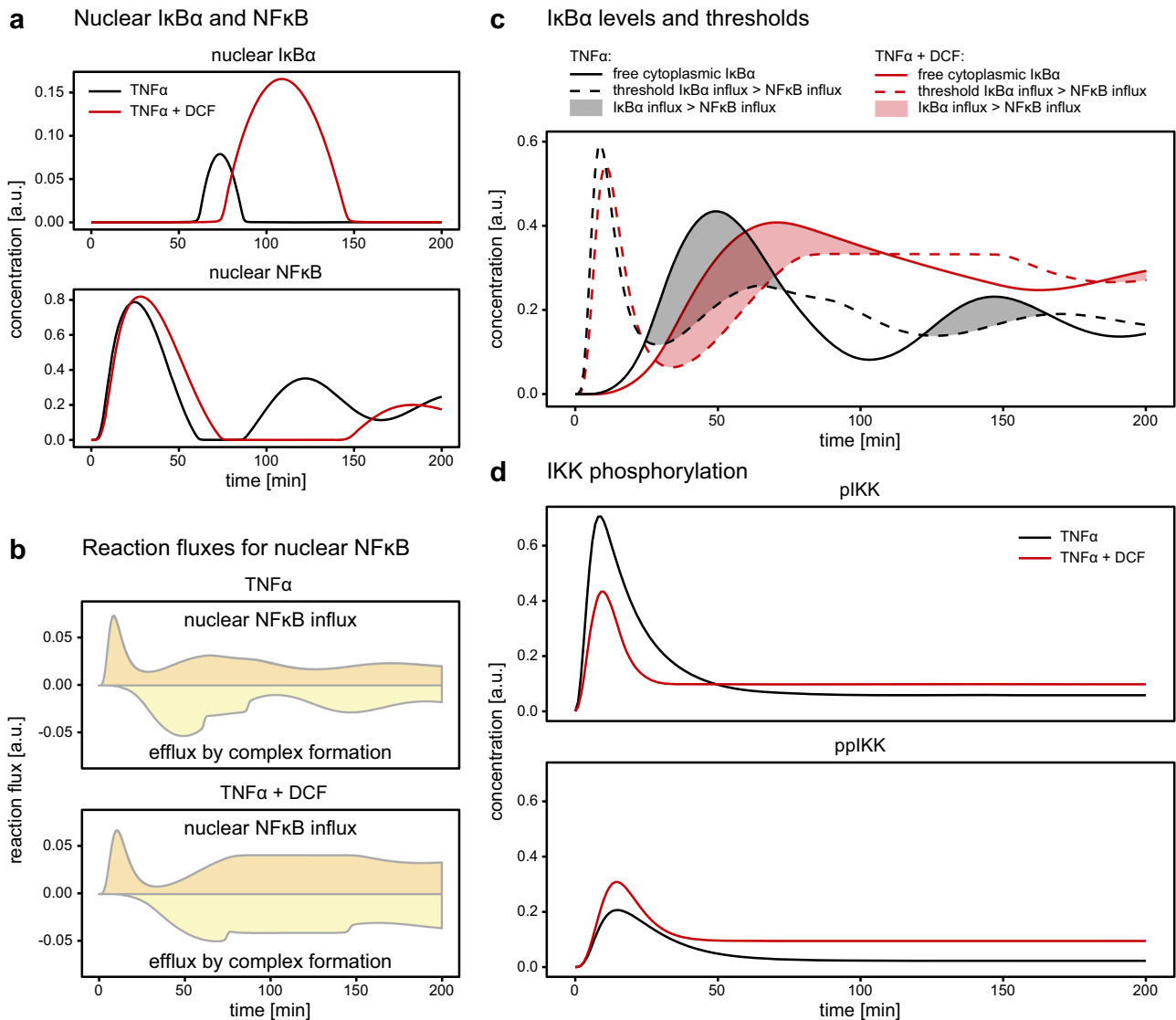


Fig. 5 Model prediction of DCF-induced changes in the NF κ B pathway. **a** Model-predicted time course of nuclear I κ B α and of NF κ B in response to TNF α (black line) and upon TNF α and DCF co-treatment (red line). **b** Model simulation of the time course of the influx to the state of nuclear NF κ B (brown) and efflux due to complex formation (yellow) in response to TNF α stimulation or upon co-treatment of TNF α and DCF. **c** Model-predicted time course of cytoplasmic I κ B α response to TNF α (black solid line) and upon TNF α and DCF co-treatment (red solid line). Depending on the cytoplasmic I κ B α concentration, nuclear NF κ B influx is lower or higher than I κ B α influx. The threshold concentration at which I κ B α influx exceeds the NF κ B influx is shown as dashed black line for TNF α and as dashed red line for TNF α and DCF. Shaded areas indicate phases of higher I κ B α influx leading to I κ B α accumulation in the nucleus. **d** Model-predicted time course of active IKK (pIKK) and of inactive IKK (ppIKK) for time points up to $t = 50$ min. The dynamics of pIKK and ppIKK in the presence of TNF α alone (black) and the presence of TNF α and DCF (red) are displayed. The higher pIKK level for DCF treatment at late time points is reflected in the crossing of I κ B α thresholds at $t = 60$ min

nucleus is proportional to the levels of I κ B α in the cytoplasm, the threshold of when the I κ B α influx balances out the NF κ B influx can be computed (dashed lines in Fig. 5c). The shaded areas in Fig. 5c mark the time frames where cytoplasmic I κ B α levels exceed the computed threshold and more I κ B α than NF κ B is transported to the nucleus, and I κ B α is expected to accumulate in the nucleus.

On the one hand, the comparison between TNF α and DCF co-treated and DCF-untreated cells showed that the I κ B α production and degradation are reduced by DCF co-treatment leading to a slower but prolonged I κ B α aggregation in DCF co-treated cells. Accordingly, I κ B α exceeded the threshold slightly later and intersected it again 110 min after TNF α and DCF co-stimulation, corresponding to the position of the maximum concentration of

nuclear I κ B α (Fig. 5a). On the other hand, the influx of NF κ B to the nucleus depends on its release from the NF κ B:I κ B α complex, and consequently depends directly on the pIKK levels. We hypothesized that lower levels of IKK activation would reduce the I κ B α threshold concentration of when the I κ B α influx into the nucleus exceeds the NF κ B influx. An extended phase of low concentrations of nuclear NF κ B would be the consequence. Therefore, to further examine consequences of the DCF impact on IKK, we predicted the experimentally unobserved concentrations of active pIKK and inactive ppIKK by our dynamic pathway model. The estimated parameters suggested that DCF accelerates the transition from active pIKK to inactive ppIKK (Fig. 5d). Accordingly, the I κ B α threshold shown in Fig. 5c was lowered in TNF α and DCF co-

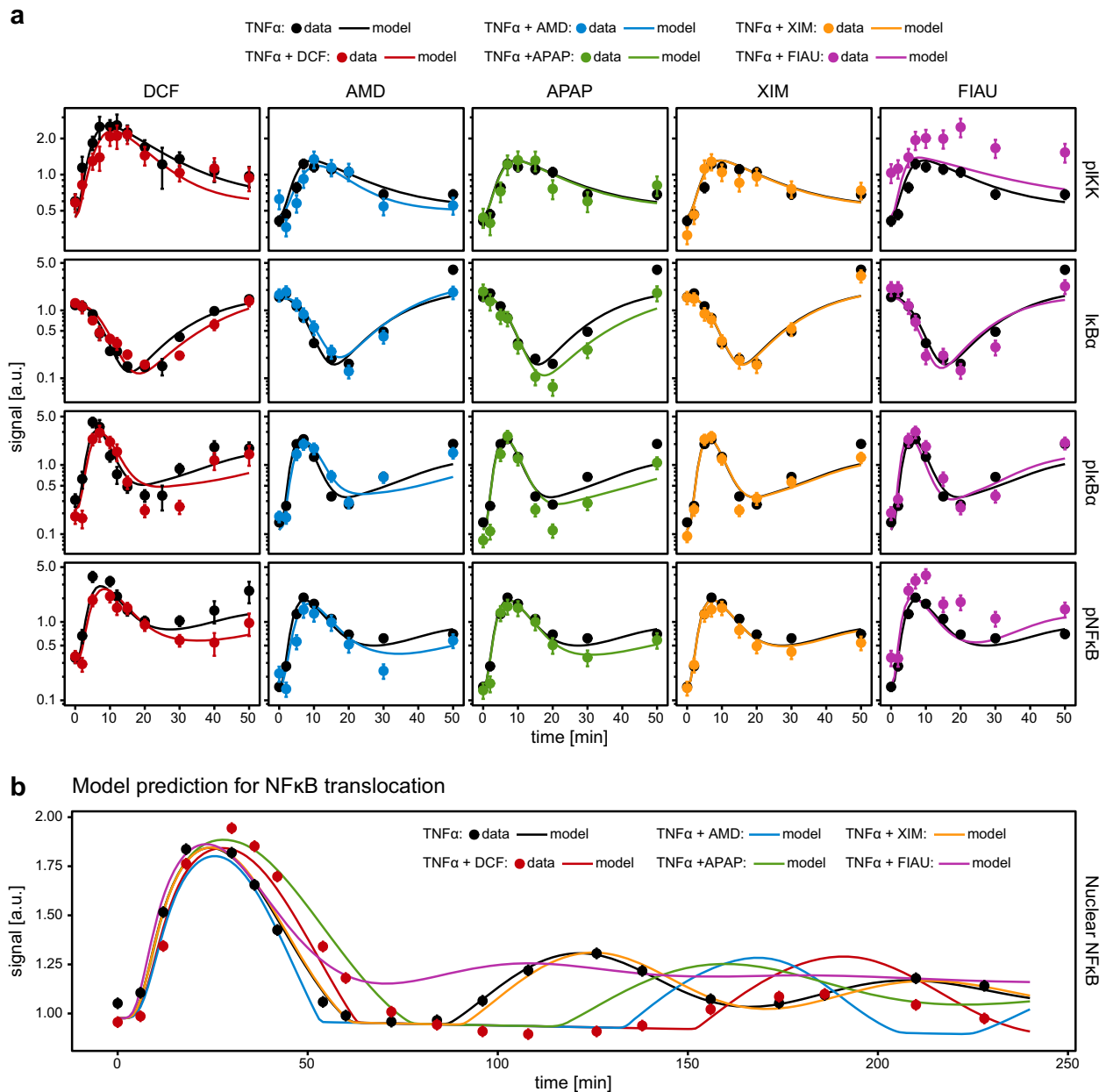


Fig. 6 Application of the dynamic pathway model to additional DILI compound data. **a** Impact of four selected compounds, amiodarone (AMD, 35 μ M), paracetamol (APAP, 10 mM), ximelagatran (XIM, 800 μ M), and fialuridine (FIAU, 500 μ M), on cytoplasmic I κ B α , pI κ B α , pIKK, and pNF κ B levels were measured by quantitative immunoblotting in HepG2 cells treated with either TNF α alone (10 ng/ml) or TNF α (10 ng/ml) and the respective compound ($n = 3-4$, raw data in Figs. S30–S45). Data points with 1σ confidence intervals are shown as dots and error bars in different colors for different compounds, black indicating the TNF α treatment alone. The model fit for the reduced compound-impact model is shown as solid line. Model calibration involves only experiment-specific observation parameters and the two compound-specific effect parameters a and β . **b** The model-predicted dynamics of nuclear NF κ B for a time period of 4 h based on the short-term measurements per compound are shown as solid lines. Data points with 1σ confidence intervals for TNF α -treated cells and cells co-treated with TNF α and DCF are shown as dots with error bars

treated cells, at least during the first hour. At later time points, pIKK in TNF α and DCF co-treated cells was predicted to be above the levels of pIKK in TNF α -treated cells. The model predicted that co-treatment with DCF reduces the peak height of pIKK by around 40% (Fig. 5d), whereas the relative change of experimentally measured IKK phosphorylation was much smaller. The discrepancy can be explained by our four-state IKK model with two IKK states phosphorylated at the activation site, active pIKK, and inactive ppIKK, which cannot be distinguished by the antibody-based

measurement. Therefore, we propose that DCF decreases pIKK and increases ppIKK, resulting in only a small change in the sum of pIKK and ppIKK.

These results indicate that the experimentally observed shift of the position of the second peak of nuclear NF κ B in response to co-treatment with DCF is the integrated result of two regulatory mechanisms: the reduced I κ B α production and degradation rates prolong the presence of elevated levels of I κ B α in the cytoplasm and reduced pIKK levels lower the threshold of when cytoplasmic

I κ B α levels exceed the value necessary to compensate the NF κ B influx into the nucleus.

Model-based analysis of the impact of additional DILI compounds on TNF α -induced NF κ B signal transduction

After identifying IKK phosphorylation and I κ B α production/degradation as major regulatory mechanisms affected by DCF, additional compounds known to cause DILI including amiodarone (AMD), paracetamol (APAP), fialuridine (FIAU), and ximelagatran (XIM) were tested. These compounds, which were previously reported² to represent different mechanisms of DILI, were examined regarding their impact on TNF α -induced NF κ B signal transduction. To select most suitable DILI compound concentrations, we examined, as shown in Fig. S7, the impact of increasing concentrations of AMD, APAP, and XIM on the phosphorylation of I κ B 5 min after co-stimulation with TNF α . Increasing AMD concentrations resulted in rapid reduction of I κ B phosphorylation that was not further increased by high compound concentrations. In line with a recent publication that reported enhanced AMD toxicity upon co-treatment with of 35 μ M AMD and TNF α , we employed a concentration of 35 μ M AMD in our experiments. For APAP no impact on TNF α -induced I κ B phosphorylation was observed at reasonable APAP concentrations and therefore a concentration of 10 mM APAP was selected in line with a multicenter ring trial.²⁹ Since XIM had no effect on TNF α -induced I κ B phosphorylation, we decided based on literature information to apply a concentration of 800 μ M XIM in our experiments. Finally, for FIAU we observed in test experiments elevated IKK β , phosphorylation at 20 min of TNF α treatment. Whereas we observed no major impact of FIAU treatment at 20 min on I κ B phosphorylation, IKK β phosphorylation was increased. We chose a concentration of 500 μ M FIAU, which caused approximately a doubled IKK β phosphorylation signal intensity compared to the control. For each of these compounds, total I κ B α , phosphorylated I κ B α , phosphorylated IKK, and phosphorylated NF κ B in the cytoplasm were measured by quantitative immunoblotting (Fig. 6a, data points). For co-treatment experiments with the selected compounds and TNF α , HepG2 cells were incubated with the compounds for 30 min prior to stimulation with TNF α and then lysed at the indicated time points during an observation time of up to 50 min. To facilitate the analysis of the obtained time course data by our TNF α signaling DCF model, the key mechanisms identified for the impact of DCF were further condensed to describe the possible impact of compounds by only two parameters α and β . The parameter α is associated with IKK phosphorylation. The phosphorylation rates of IKK and pIKK are expressed as $k_{\text{IKK} \rightarrow \text{pIKK}}^* = \alpha^{-1} \cdot k_{\text{IKK} \rightarrow \text{pIKK}}$ and $k_{\text{pIKK} \rightarrow \text{ppIKK}}^* = \alpha \cdot k_{\text{pIKK} \rightarrow \text{ppIKK}}$, respectively. Accordingly, larger values of α shift the IKK phosphorylation from pIKK to ppIKK. The parameter β is associated with the production and degradation rates of I κ B α . The corresponding rates are modified on the mRNA level, $k_{0 \rightarrow \text{mI}\kappa\text{B}\alpha}^* = \beta^{-1} \cdot k_{0 \rightarrow \text{mI}\kappa\text{B}\alpha}$ and $k_{\text{mI}\kappa\text{B}\alpha \rightarrow 0}^* = \beta^{-1} \cdot k_{\text{mI}\kappa\text{B}\alpha \rightarrow 0}$. Accordingly, larger values of β slow down I κ B α mRNA production and degradation for fixed equilibrium constant. The parameters α and β are compound-specific, i.e., they are determined for each compound separately.

First, this simplified model was employed to estimate α and β from the original data obtained for TNF α and DCF co-stimulation. Only the time points and targets that were also measured for the selected compounds were used. Based on these estimates, the dynamics of nuclear NF κ B was predicted (Fig. 6b). The results revealed that the simplified model is capable to correctly predict, based on cytoplasmic measurements restricted to the first 50 min of stimulation, the position of the second peak of nuclear NF κ B at 180 min. The information necessary for this prediction is encoded in the slight time shift of IKK phosphorylation and the decelerated I κ B α dynamics.

Assuming that AMD, XIM, APAP, and FIAU might affect the regulatory mechanisms in a similar way, we used the time course data obtained for these compounds to estimate the compound-specific values for α and β . The analysis revealed that the mathematical model is capable to describe the time course data for AMD, XIM, and APAP, but not for FIAU (Fig. 6a). Experimental data and model trajectories showed that AMD shifted the TNF α -induced IKK phosphorylation similar to DCF, whereas the effect on I κ B α was barely noticeable. The reduced IKK phosphorylation resulted in an extended time frame of low concentrations of nuclear NF κ B (Fig. 6b). In contrast, APAP showed a strong effect on I κ B α but almost no effect on IKK phosphorylation. Consequently, the second NF κ B peak was delayed but the time frame of low nuclear NF κ B concentration was not as extended as for AMD. Finally, XIM neither showed effects on I κ B α nor on IKK phosphorylation. Accordingly, the predicted nuclear NF κ B profile resembled the TNF α -stimulated control data. Because both AMD and APAP seemed to exhibit only one of the identified drug-induced effects, the delay of the TNF α -induced NF κ B peak was not as strong as for DCF. The measurements taken from TNF α and FIAU co-treated cells showed effects on the basal IKK and NF κ B phosphorylation that were not observed for any of the other compounds. These data indicated that an additional mechanism is involved in the case of FIAU that is not covered by our mathematical model.

For DCF the simplified mechanism of action allowed to estimate drug-specific parameters that enable the prediction of the dynamics of nuclear NF κ B. Within the analysis of likely interactions of DCF with the NF κ B pathway, we found that DCF apparently directly modifies the production of A20 (direct impact), whereas the delayed dynamics of nuclear NF κ B (mediated impact) alone is not sufficient to correctly describe the observed A20 dynamics. An evaluation of the possible impact of the other DILI compounds on the cytoplasmic concentration of A20 is presented in Fig. S6, and compares by model simulations a possible direct and an indirect mechanism. The obtained results indicated that AMD is likely to affect the A20 dynamics in a similar way as DCF. APAP and XIM have a smaller inhibitory effect on the A20 concentration in the cytoplasm and FIAU could even increase the production of A20.

In summary, our model-based approach enables us to quantify the impact of DILI compounds on TNF α -induced NF κ B signal transduction using IKK phosphorylation and I κ B α production/degradation as major characteristics of DILI compounds within the TNF α -induced NF κ B signaling pathway.

DISCUSSION

DILI accounts for most of the cases of drug non-approval, withdrawal and abandonment of drugs.^{30,31} In current clinical trials, drug hepatotoxicity still is a major problem because patient-specific effects are unclear and DILI may occur after long latency periods. The heterogeneity of responses suggests that some event during the course of therapy renders the patients particularly sensitive and this could be inflammatory episodes. The histological evaluation of liver biopsies revealed elevated inflammatory scores in samples from DILI patients³² and serum proteomic profiling of patients with DILI showed an increase of components associated with inflammation.³³ In numerous animal studies evidence was provided that even a mild inflammation can decrease the threshold for hepatotoxicity and thereby enhance the sensitivity to chemically induced damage in the liver.^{34–36} Interestingly, it was suggested that the major pro-inflammatory cytokine TNF α is capable of enhancing liver damage in rodents caused by different xenobiotics.^{37–39} An interaction between inflammation and DCF in inducing hepatotoxicity was observed in rats,⁴⁰ suggesting that inflammatory stress is a susceptibility factor for DCF-mediated toxicity. Furthermore, AMD treatment of rats during inflammation led to liver injury and increased TNF α serum

concentrations contributing to hepatotoxicity.⁴¹ For APAP it was observed that TNF α is released in response to APAP intoxication and it was proposed that TNF α is responsible for certain pathological manifestations of APAP-induced hepatotoxicity.⁴² Whereas for these DILI compounds some information is available regarding a potential impact of inflammatory responses on hepatotoxicity, for XIM-induced hepatotoxicity an immune-related mechanism was suggested, and FIAU shows a human-specific mitochondrial toxicity,² and hence the impact of inflammation is less clear. Our model-based approach provides the means to systematically evaluate the impact of DILI compounds on TNF α -induced responses and resolve similarities as well as distinctions.

Current approaches are limited in the ability to cover the variability of patients and there is an urgent need for improved test systems. Mathematical modeling offers a powerful approach to tackle these challenges, because several factors affecting the outcome of drug exposure to hepatocytes can be investigated, the effects can be quantified and unobserved states can be predicted. Thereby, regulatory mechanisms affected by DILI compounds can be elucidated to improve possibilities to predict DILI.

One of the top ten DILI-causing drugs is DCF. It was shown that TNF α enhances the cytotoxicity of DCF⁴ and that DCF interferes with TNF α -induced NF κ B signal transduction⁴ and causes several stress responses.^{5,43} TNF α is a pleiotropic cytokine that promotes proliferative but also cytotoxic responses,^{7,9,44–47} and therefore interference with the tightly regulated balance of responses due to drug exposure could shift the threshold toward hepatotoxicity. However, the underlying mechanisms of how compounds such as DCF affect TNF α -induced NF κ B signal transduction in particular during early time points was previously not resolved. We developed a dynamic pathway model of TNF α -induced NF κ B signal transduction that enabled us to quantitatively examine the perturbations caused by compounds and to analyze the processes at the beginning of a cellular decision, which appear to be crucial for cell survival under drug exposure.

Compared to previous mathematical models of NF κ B signal transduction,^{11–14,17,48} we extended the dynamic pathway model of TNF α -induced NF κ B signal transduction by several aspects. First, we introduced all four states of IKK upon TNF α stimulation into our mathematical model. In the study conducted by Behar and colleagues, the authors suggested a particularly important role of IKK in modulating NF κ B signal transduction. This proposition is supported by our mathematical model, which identified reduced IKK activation as a major effect of DCF. Further, our analysis suggested that DCF enhanced the inactivation of phosphorylated IKK (pIKK) due to phosphorylation at the C terminus (ppIKK). Both effects lead to reduced levels of pIKK, and consequently delayed NF κ B signal transduction. Second, we applied four states of the NF κ B:I κ B α complex. Based on NF κ B and I κ B α phosphorylation data in combination with Co-IP experiments, we were able to distinguish these four states.

The resulting identifiable mathematical model was capable of describing the experimental data generated for HepG2 cells and for PHHs and identified by employing L₁ regularization two major interaction points of DCF in the NF κ B signal transduction pathway. We showed that both regulatory levels, the upstream kinase IKK and the free, cytoplasmic I κ B α , define the hepatotoxic outcome of compound treatments. To confirm the broad applicability of the established dynamic pathway model, we translated the regulatory mechanisms found for DCF to test scenarios with four other DILI-causing drugs with diverse putative mechanisms.² Although their different mechanisms might lead to diverse rate modifications within the NF κ B pathway, it is likely that the key regulators identified for DCF would be affected by the other DILI compounds as well. Model calibration based on the TNF α -induced dynamics of the NF κ B pathway upon co-treatment with amiodarone, paracetamol, ximelagatran, and fialuridine allowed us to quantify the drug-induced effect strengths relative to DCF and predict the

dynamics of nuclear translocation of NF κ B upon co-treatment with these compounds.

Amiodarone is an antiarrhythmic lipophilic compound that was described to accumulate in the liver,⁴⁹ a scenario that is often excluded in pharmacokinetic studies.⁵⁰ It was observed that amiodarone induces severe hepatotoxicity in rats and TNF α contributes to a decrease of the drug toxicity threshold.⁴¹ Furthermore, TNF α co-treatment in Hepa1c1c7 cells revealed that TNF α potentiated the amiodarone-induced cytotoxicity.⁵¹ Concordant with these studies, our studies showed an early effect of amiodarone on TNF α -induced NF κ B signal transduction and therefore highlight the importance of the integration of inflammatory responses in amiodarone-induced liver injury. Interestingly, co-treatment of amiodarone with TNF α caused similar death (caspases) and stress responses as co-treatment with DCF,^{4,5,51} which is reflected by our observed similar effects of DCF and amiodarone on the TNF α -induced dynamics of pIKK, pI κ B α , and pNF κ B (Fig. 6). However, the rather small differences on the dynamics of I κ B α indicated potential differences in the modes of subsequent cell death, as seen in comparison to paracetamol, which does not induce the activation of caspases but rather necroptosis.^{52,53} In contrast to DCF and amiodarone, paracetamol is affecting neither the phosphorylation of IKK nor I κ B α , but is decreasing the levels of I κ B α and the phosphorylation of NF κ B (Fig. 6). The impact of paracetamol could thereby be described by our dynamic pathway model. These observations point to a slightly different mechanism that includes the activation of other pathways, e.g., the JNK by paracetamol.^{54–56} Even though the hepatotoxicity caused by paracetamol is rather complex and other mechanisms might also play a role,² we could detect and integrate the effects caused by paracetamol with our dynamic pathway model.

As seen in the case of fialuridine our model still has limitations. It is proposed that this compound has mitotoxic effects⁵⁷; however, previous *in vitro* assays failed to detect fialuridine as hepatotoxic compound and underlying mechanisms apparently are very complex. Only recently, a 3D PHH spheroid model system was described which facilitated the detection of fialuridine toxicity *in vitro*,⁵⁸ thus providing a starting point for further studies, which can be combined with our approach and used to further improve the established dynamic pathway model.

Ximelagatran showed no effects on TNF α -induced NF κ B signal transduction in our studies, which is in line with previous reports suggesting an immune-mediated mechanism. Extensive studies were performed to unravel mechanisms contributing to the hepatotoxicity of ximelagatran; however, no underlying mechanism could be defined. The metabolism of ximelagatran does not involve the CYP450 system and reactive metabolites are not formed.² Neither did hepatotoxicity by ximelagatran show a dose dependency.⁵⁹ Due to a distinct geographic distribution of liver injury after ximelagatran treatment, the involvement of genetic factors was suggested.⁵⁹ Recently, it was reported based on *in silico* and *in vitro* studies that ximelagatran directly interacts with a human leukocyte antigen strengthening the hypothesis of an immune response mechanism.⁶⁰

DCF is known to generate protein adducts in hepatocytes.⁶¹ In addition, previous studies showed that DCF interferes with TNF α -induced NF κ B signal transduction.^{4,5} Because membrane receptors are the starting point of various signal transduction pathways, we quantified the impact of TNF α and of the co-treatment with DCF on the number of TNFR1 receptor clusters per μ m² and their size at the plasma membrane. A previous study indicated that subtle changes in TNF α -TNFR1 interactions might lead to higher-order clustering.⁶² We showed by SMLM that co-treatment with TNF α and DCF did neither significantly change the number of TNFR1 clusters per μ m² both for stimulated or for non-stimulated cells nor did it change the cluster size. In line with these results our model-based studies revealed that reduced activation of the

TNFR1 due to interference of DCF is not sufficient to explain the impact we detected on the dynamics of TNF α -induced NF κ B signal transduction.

Taken together, the developed mathematical model provides a unique tool to quantitatively assess already in the preclinical phase the impact of compounds on a major aspect of inflammatory signaling such as TNF α -induced NF κ B signal transduction. Our approach enables us to predict the interaction points of several compounds within the TNF α -induced NF κ B signaling pathway even though the tested compounds have different putative toxicity mechanisms.² Apparently, even if the induced death pathways differ between the tested compounds, for example, mainly via apoptosis for DCF^{4,63,64} or via necroptosis as for paracetamol,^{52,53} already rather early effects on TNF α -induced NF κ B signal transduction are detectable. Based on these quantitative data, our dynamic pathway model is capable to quantify the compound-induced impact on nuclear NF κ B and thereby predict how these compounds interfere with NF κ B-induced transcription.

METHODS

Reagents and antibodies

Recombinant human TNF α (210-TA) was acquired from R&D Systems and was reconstituted in sterile PBS containing 0.3% BSA. Diclofenac sodium (DCF, D6899), amiodarone hydrochloride (AMD, A8423), paracetamol (APAP, A7085), and fialuridine (FIAU, SML0632) were obtained from Sigma. Ximelagatran (XIM) was obtained from AstraZeneca. All compounds were dissolved in DMSO (Sigma). The antibodies against pIKK α/β (#2697), IKK β (#2370), IKK α (#2682), pNF κ B (#3031), NF κ B (#6956), pI κ B α (#2859), and I κ B α (#9242) were from purchased from Cell Signaling Technologies. Secondary horseradish peroxidase-coupled antibodies were obtained from Dianova.

Cell lines, cell culture, and cell stimulation

Human hepatoma HepG2 cells (HB8065) were obtained from American Type Culture Collection, cultured in Dulbecco's Modified Eagle Medium (DMEM) without phenol red (Gibco 31053-044) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) Penicillin/Streptomycin (Gibco), 2 mM L-Glutamine (Gibco), and 1 mM Sodium Pyruvate (Gibco) and used for experiments between passage 5 and 20. The HepG2 cell line was authenticated using Multiplex Cell Authentication and the purity of cell line was validated using the Multiplex Cell Contamination Test by Multiplexion (Heidelberg, Germany) as described recently.^{65,66} HepG2 cells were serum-starved over night with DMEM supplemented with 1 mg/ml BSA, 1% (v/v) Penicillin/Streptomycin (Gibco), and 2 mM L-Glutamine (Gibco). DCF was dissolved in DMSO as 200-fold stock and prepared freshly for every experiment. Cells were incubated for 30 min with DCF (final concentration 500 μ M) or the respective compound (final concentration AMD 35 μ M; APAP 10 mM; XIM 800 μ M; FIAU 500 mM) before treatment with TNF α (final concentration 10 ng/ml).

Primary human hepatocytes

Fresh PHHs were isolated as previously described²⁹ from three different donors (104, 105 and, 107 from UoL) with the following characteristics:

| ID | General information | | | | | | | |
|-----|---------------------|-----|-------------|------|-----------|--------|--------------------|--------------------|
| | Sex | Age | Weight (kg) | BMI | Ethnicity | BP | Alcohol units/week | Cigarettes/day |
| 104 | M | 85 | 80.7 | 30 | Caucasian | 178/75 | | Ex smoker 20 years |
| 105 | F | 45 | 70.9 | 25.4 | Caucasian | 132/87 | 10 | Ex smoker |
| 107 | M | 60 | 81.6 | 26.3 | Caucasian | 104/64 | | Ex smoker |

| ID | Patient health | |
|-----|--|---|
| | Diagnosis | Disease |
| 104 | $\times 3$ CRLM (right hepatectomy $\times 1$ VIII, $\times 2$ VI) | Anterior resection (BowelCa T4bN2M0), AF, hypertension, DMT2, osteoarthritis |
| 105 | Bismuth 3A + caudate lobe cholangioCA (radical bile duct excision + en bloc right hemihepatectomy + caudate lobectomy + partial IVC resection) | Hysterectomy |
| 107 | $\times 3$ CRLM (VI resected, III metastasectomy, IV ablated) | Defun ileosotomy, AP resection (rectalCa T4N2M0 R1), relapse lung + liver, lung ablation, liver ablation, lung + liver ablation |

| ID | Medication excluding chemotherapy | | |
|-----|--|-----------------------------|---------------------------------------|
| | Drug | Dose | Frequency |
| 104 | NKDA, metformin, bisoprolol, perindopril | 500 mg/ 1.25 mg/ 4 mg | Three daily/ once daily/once daily |
| 105 | Morphine sensitivity, omeprazole, vitamin B12, paracetamol, piriton, HRT | | |
| 107 | NKDA, loperamide | | PRN |

| ID | Chemotherapy Drug | |
|-----|--|------|
| | Drug | Dose |
| 104 | 5FU | |
| 105 | None | |
| 107 | Chemoradio cetuximab, irinotecan/FOLFOX/FOLFOX | |

Liver resections were received as surgical waste from Aintree Hospital, Liverpool, UK, with full patient consent and ethical approval from the National Research Ethics Service (REC reference: 11/NW/0327). After isolation, cells were seeded onto collagen I coated 6-well plates and after an adhesion time of 3 h medium and cells were left for overnight incubation. Cells were then washed three times the next day and medium was replaced by growth factor depletion medium (Williams Medium E (Biochrome), 2 mM L-Glutamine (Gibco), and 1% (v/v) Penicillin/Streptomycin (Gibco)) for 4 h prior to the experiment.

Time-resolved experiments and cell lysis

For time course experiments, HepG2 cells or PHHs were stimulated with recombinant human TNF α after DCF pre-treatment of 30 min for the indicated times. Cells were then lysed using a cell lysis buffer (150 mM NaCl; 20 mM Tris-HCl pH 7.4; 10 mM NaF; 1 mM EDTA pH 8; 1 mM ZnCl₂ pH 4.0; 1 mM MgCl₂; 1 mM Na₃VO₄; 10% glycerol; 1% (v/v) NP-40; 0.1% Aprotinin; 0.1% 4-(2-Aminoethyl) benzenesulfonyl-fluoride hydrochloride) and lysates were rotated for 20 min at 4 °C, the total protein concentration of the supernatant was measured (bicinchoninic acid assay, Pierce) and aliquots of 30 μ g were made for quantitative immunoblotting.

Immunoprecipitation

For immunoprecipitation with I κ B α antibody (Cell Signaling #9242) and NF κ B antibody (Cell Signaling #6956), 500 μ g of the protein lysate was used. Protein A-Sepharose (GE Healthcare) and indicated antibodies were added to the cell lysates and immunoprecipitation was performed overnight at 4 °C.

SDS-PAGE, quantitative immunoblotting, and data processing

Total cell lysate aliquots or immunoprecipitations were loaded in a randomized order on 10% SDS-polyacrylamide gel electrophoresis gel

(SDS-PAGE), separated by electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore). Primary antibodies were incubated overnight at 4°C and secondary antibodies for 1 h at room temperature. Chemiluminescence detection was performed using enhanced chemiluminescence substrate (GE Healthcare) and charge-coupled device camera-based signal detection was performed using an ImageQuant LAS 4000 system (GE Healthcare). Quantification was performed using ImageQuant TL (GE Healthcare). Appropriate lanes were manually selected and the width of all lanes was fixed. Protein bands of interest were manually selected and the height of the quantification square was adjusted to optimize the coverage of the selected protein band and reduce the amount of background included. Compensations for band distortions were added as necessary. The background was subtracted with the rolling ball method.

Live-cell imaging

HepG2 RelA-GFP (NF- κ B, p65) and A20-GFP BAC reporter cell lines were generated and characterized as described previously.⁶⁷ Accumulation of GFP levels or nuclear translocation, and Hoechst staining was monitored using a Nikon TiE2000 confocal laser microscope (lasers: 640, 540, 488, and 408 nm). This microscope is equipped with an automated stage and perfect focus system at 37°C with humidified atmosphere and 5% CO₂/air mixture. During starvation overnight, HepG2 cells were stained with 50 ng/ml Hoechst₃₃₃₄₂ to visualize the nuclei. The Hoechst medium was replaced with exposure medium containing DCF. After 30 min pre-exposure to compound only, the medium was spiked with TNF α (1:20 dilution in starvation medium) up to a final concentration of 10 ng/ml TNF α . To prevent a delay in TNF α response in the oscillations of the RelA-GFP reporter, TNF α was added at the microscope per well, directly upon imaging of the first image ($t = 0$). Quantitative image analysis was performed with CellProfiler version 2.1.1⁶⁸ with an in house developed module implementing the watershed masked algorithm for improved nuclear segmentation.⁶⁹ Image analysis results were stored as HDF5 files, png images with the segmentation results were stored for quality control. Data analysis and further quality control was performed using the in house developed R package H5CellProfiler. Quantitative data of three independent experiments were used for model input.

Single-molecule localization microscopy and data analysis

For single-molecule microscopy experiments, cells were seeded into 8-well chamber slides (Sarstedt) and grown until confluency was achieved. FCS-free medium containing 0.1% BSA was applied at least 12 h prior to experiments to the cells for starvation. For DCF treatment, cells were either treated with 500 μ M DCF in DMSO (final concentration of DMSO: 0.5% (v/v)) or with DMSO (0.5% (v/v) in culture media) as control. For TNF α (ImmunoTools) treatment, cells were either treated with 10 ng/ml in PBS supplemented with 0.3% BSA (final concentration of BSA 3×10^{-4} % (w/v)) or with BSA only as a control condition (final concentration of BSA 3×10^{-4} %). After incubation with DCF for 30 min, TNF α was added to the medium and co-incubated for 0, 2, 5, or 10 min. After incubation, cells were fixed and prepared for immunostaining.

For fixation, medium was removed and cells were washed with pre-warmed 400 mM sucrose (37°C) (Merck). Cells were incubated in fixation buffer (4% formaldehyde (methanol free; Thermo Scientific), 0.1% glutaraldehyde (Sigma), and 400 mM sucrose) for 15 min. Samples were extensively washed (at least three times) in PBS, followed by blocking in 2% BSA for 30 min. Samples were incubated with a primary antibody solution (monoclonal mouse anti-hTNFR1 (Abcam, clone: H398), 3 μ g/ml in 2% BSA) for 1 h at room temperature. Primary antibody solution was removed by at least three washing steps in PBS. Cells were incubated in secondary antibody solution (Fab'2 fragment-goat anti-mouse labeled with Alexa-Fluor647 (A-21237, Life Technologies), 2 μ g/ml in 2% BSA) for 1 h at room temperature, followed by three washing steps in PBS. After fixation with 4% formaldehyde, cells were washed at least three times with PBS. For negative control measurements, cells were incubated with secondary antibody only.

SMLM experiments were performed with a custom-built setup as described earlier.⁷⁰ Briefly, an inverted microscope (Olympus IX71, Olympus) equipped with a nose piece (Olympus) was used. A read out laser emitting at 643 nm (diode laser, iBEAM smart, Toptica) was coupled into an acousto-optic tunable filter (AAOptics) for illumination intensity adjustment. A second laser emitting at 405 nm (CUBE 405-50C, Coherent) was combined with the 643 nm laser with an appropriate dichroic mirror. Widefield illumination was achieved by focusing the laser beams onto the

back-focal plane of a 100 \times oil immersion objective (PLAPO 100 \times TIRFM, NA ≥ 1.45 , Olympus). A translatable mirror was used for switching between widefield and total internal reflection illumination. Fluorescence emission was detected by the same objective, and fluorescence light was filtered with a bandpass filter (ET 700/75, AHF) and imaged with an EMCCD camera (DU-897U-CSO-#BV, iXon Ultra, Andor). The magnification optics led to an effective image pixel size of 157 nm.

SMLM imaging was performed following the dSTORM protocol.⁷¹ An imaging buffer (10% w/v glucose (Sigma), 40 mg/ml catalase (Sigma), 0.5 mg/ml glucose oxidase (Sigma), and 100 mM β -mercaptoethylamine (MEA; Sigma) in PBS, pH 7.8–8) was added to the sample. Laser intensities of 2–2.5 kW/cm² (643 nm) were used for read out of the fluorescence signal. Appropriate switching rates were achieved by UV illumination ranging between 0–10 W/cm². An image series of 20,000 frames per cell were recorded with an integration time of 30 ms per frame.

Single-molecule data were analyzed with rapidSTORM.⁷² Super-resolved images were generated at a pixel size of 10 nm and further analyzed using the LAMA package⁷³ which can be obtained free of charge (<http://share.smb.uni-frankfurt.de/>).

The localization precision was calculated using a nearest neighbor analysis.⁷⁴ The number of TNFR1 clusters was determined by an image-based analysis routine as described earlier.²⁷ Briefly, a region of interest (ROI) was chosen for each cell and the number of TNFR1 clusters per μ m² was determined using the Fiji Plugin "Analyze Particles".⁷⁵ The size of individual clusters was determined for five ROI per cell, each with a size of $2 \times 2 \mu$ m² using Density-Based Spatial Clustering of Applications with Noise (DBSCAN)²⁸ with input parameters $N_{\min} = 10$ and $\epsilon = 30$ nm. Subsequently, the radius of coextensive clusters with circular shape was calculated. For each condition, at least 13 cells were analyzed from at least 3 different independent experiments. Statistical hypotheses were tested using Kolmogorov–Smirnov test implemented in OriginPro 9.1G (OriginLab). This test allows probing similarity of small, non-normal distributed populations (significance level $\alpha = 0.05$).

Mathematical modeling

The TNF α /NF κ B signaling pathway is modeled by ODEs. Interactions between species are assumed to follow mass-action kinetics. The model topology and details about the model equations are described in Supplementary Material 1. All modeling steps, from model setup to identifiability analysis, parameter estimation, and uncertainty analysis were carried out using the R package dMod available on the Comprehensive R Archive Network.

The dynamic model is described by ODEs $\dot{x} = f(x, p_{\text{dyn}})$ where x denotes the vector of dynamic states such as IKK, NF κ B, I κ B α , etc., and p_{dyn} denotes the vector of model parameters such as activation rates, dissociation rates, etc. Initial values $x_0 = x(t = 0)$ for all dynamic states are treated like model parameters. The dynamic states are linked to experimental observations via an observation function $y = g(x, p_{\text{obs}})$. This function accounts for the fact that (i) only certain combinations of dynamic states can be experimentally observed, that (ii) measurements from fluorescence microscopy suffer from photobleaching and that (iii) observations are evaluated on the log scale.

Identifiability analysis

The structure of the differential equations and the observation function can induce symmetries, i.e., a functional relationship between the model, initial value, and observation parameters that guarantee the invariance of the predicted observation. The TNF α /NF κ B model was systematically scanned for such relationships using a Lie-group approach.⁷⁶ The number of free parameters necessary to describe the model was thereby reduced.

Parameterization and steady states

The same model and observation structure is employed to describe different experimental conditions such as TNF α /no TNF α or DCF/no DCF. However, depending on the condition, parameters are set differently: model, initial value, and observation parameters are obtained by condition-specific functions, $(p_{\text{dyn}}, x_0, p_{\text{obs}})_i = \phi_i(\theta)$ from one single, overarching vector of parameters, θ . Different experimental conditions are denoted by i . The functions ϕ_i used for the TNF α /NF κ B model account for (i) DCF/TNF-specific setting of parameters, (ii) a general log transform of all parameters, and (iii) a steady-state transformation where all initial values x_0 are computed from the dynamic parameters p_{dyn} and total levels of IKK and NF κ B based on the condition $f(x_0, p_{\text{dyn}}) = 0$.

Data preprocessing

Experiments were performed in at least three biological replicates. Samples for quantitative immunoblot analysis from different replicates but equal experimental conditions were partially measured on the same gel, whereas others were measured on different gels. Replicates measured by fluorescence microscopy were analyzed on independent plates on different days. Accordingly, to account for a systematic deviance between equal conditions but different gels/plates, we used the scaling model $S_{i,k,n} = \frac{y_{i,n}}{s_k}$ with the measurements $S_{i,k,n}$ for different conditions i , gels/plates k , and timepoints t_n . The symbols $y_{i,n}$ indicate time course parameters and s_k denotes scaling parameters. The time course parameters were obtained by maximum-likelihood estimation. They represent the average dynamics over all biological replicates. As such they condense the original replicate data and replace it for dynamic modeling.

Parameter estimation by the maximum-likelihood method

The model response, i.e., the dynamics of the pathway species and the corresponding observation are parameterized by the parameters θ . These parameters are estimated by the maximum-likelihood method. Our likelihood is based on the normal distribution, i.e., differences between the model's prediction for the observation at timepoints t_n and the actual observations $y_{i,n}$ are normally distributed: $(g \circ x \circ \phi_i(t_n, \theta) - y_{i,n}) \sim N(0, \sigma^2(\theta))$. Observations for different timepoints are assumed to be statistically independent. The expected variance $\sigma^2(\theta)$ of the residuals is constant per observed target, but unknown, and is therefore expressed by additional parameters collected in θ . From these assumptions, the deviance, i.e., minus twice the log-likelihood is derived:

$$l(\theta) = \sum_{i,n} \left(\frac{g \circ x \circ \phi_i(t_n, \theta) - y_{i,n}}{\sigma(\theta)} \right)^2 + \log[\sigma^2(\theta)]. \quad (1)$$

The function $l(\theta)$ is minimized with respect to θ to obtain parameter estimates $\hat{\theta}$.

Uncertainty analysis and model reduction

Parameter uncertainty was analyzed by the profile-likelihood method.⁷⁷ The method is not only suited to determine parameter uncertainty in nonlinear models, it is also convenient to determine relationships between the model parameters and to propose possible model reductions.⁷⁸ Based on the profile-likelihood approach (Fig. S4) and the experimental data for TNF α -induced NF κ B signaling, the initial model was reduced to a core model (Fig. S5). Within this model, key parameters to be altered to describe the TNF α -induced response of DCF-treated cells were identified.

L₁ regularization

Identification of DCF-specific parameter alterations is based on the LASSO method.²⁶ At first, every reaction rate was considered as a possible target of DCF. The altered rate k^* of any reaction rate k is related to its original value by the equation $k^* = k \cdot r_k$ or, equivalently, applying the logarithm, $\log(k^*) = \log(k) + \Delta_k$, where $\Delta_k = \log(r_k)$. Introducing the L₁ regularization function $\phi_\lambda(\Delta_k) = \lambda \sum_k |\Delta_k|$, the augmented objective function can be expressed as:

$$l_\lambda(k, \Delta_k) = l(k | \text{data}_{\text{control}}) + l(k \cdot e^{\Delta_k} | \text{data}_{\text{DCF}}) + \phi_\lambda(\Delta_k). \quad (2)$$

Here, $l(k | \text{data}_{\text{control}})$ and $l(k \cdot e^{\Delta_k} | \text{data}_{\text{DCF}})$ describe twice the negative log-likelihood of the model given the TNF α -stimulated control data and of the model with modified rates given the data of TNF α and DCF co-treated cells, respectively. First, the objective function $l_\lambda(k, \Delta_k)$ was minimized for $\lambda = 0$. In this setting, the difference parameters Δ_k could be freely estimated, allowing changes in all parameters in response to DCF treatment. Subsequently, λ was gradually increased. Larger values of λ increase the pressure to reduce the Δ -values, eventually leading to zero values. In this case, no parameter differences between the control and the DCF data is allowed.

Data availability

The reactions of the full and the reduced model are shown in Tables S1 and S5, respectively, with the corresponding differential equations in Tables S2 and S6. The observation functions linking the states of the ODE model to the experimentally observed quantities are listed in Table S3. The estimated model parameters are shown in Tables S7–S9. The density of TFNFR1 clusters and corresponding cluster radii are shown in Table S10. Quantitative immunoblots for all the experimental data is shown in Figs.

S8–S45. Data that have been used for mathematical modeling is provided as Data Set csv file.

Code availability

The custom R packages `blott2` and `dMod` used for data preprocessing with a scaling model and parameter estimation in ODE models, respectively, are available on github as open source R packages under the GPL license (<https://github.com/dkaschek/blott2>, <https://github.com/dkaschek/dMod>). The ODE model as being implemented in `dMod` is available on https://github.com/dkaschek/dMod_Examples. The concepts and usage of `dMod` have been introduced previously.⁷⁹

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AUTHOR CONTRIBUTIONS

U.K. designed the project; U.K., J.T., A.O., D.K., and M.S. supervised the project. A.O. conceived and designed the experiments for quantitative immunoblotting. A.O., M.B.-W., F.H., and L.Z. performed cell culture, time courses, and quantitative immunoblotting. S.H. prepared the stable cell lines, designed the live-cell experiments, and performed live-cell imaging and data analysis of microscopy data while B.v.d.W. supervised these experiments. R.S.-Y., F.Z., A.S., R.P.J., and H.M. isolated PHHs and performed the PHH time courses, which were analyzed by A.O. by quantitative immunoblotting. S.M., C.L.K., M.M., and M.H. designed, performed, and analyzed single-molecule localization microscopy experiments while S.M. and M.H. supervised these experiments. D.K. and B.M. performed dynamic pathway modeling and D.K. carried out further model analyses. A.O., D.K., M.S., and U.K. wrote and all authors approved the manuscript.

ADDITIONAL INFORMATION

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