

Bringing biological networks to life with ANIMO

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ABSTRACT

Motivation: Computational modelling is a vital part of the empirical cycle, formalizing existing knowledge and experimental data in a systematic way. Existing methods are often parameter intensive, or supporting tools are not accessible enough to the biological community. These factors hamper widespread application of modelling in biological research.

Results: We present an approach to modelling based on a series of abstractions that limit the number of parameters in the model. To support this approach, we developed a user-friendly software tool, ANIMO (Analysis of Networks with Interactive MOdelling). An ANIMO model of signal transduction events downstream of TNF α and EGF in HT-29 human colon carcinoma cells is described as a case study. This model gives a formal description of crosstalk between the pathways at different cellular levels and led to the formulation of novel testable hypotheses.

Availability and implementation: ANIMO is implemented as a plug-in to the network visualization tool Cytoscape and can be downloaded from <http://fmt.cs.utwente.nl/tools/animo>. Additional information and a step-by-step installation guide can be found in Supplementary Section 1. A user manual is provided in Supplementary Section 2.

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1 INTRODUCTION

In living cells, processes are regulated by networks of interacting molecules. Aberrations in these networks underlie a wide range of pathologies. The development of new therapies requires a thorough insight in the functioning of these networks. Obtaining such insight can be a challenging task. Feedback loops and crosstalk between pathways lead to an intricate wiring of the network. Hence, it is necessary to study the ensemble of molecules involved, because the behaviour of individual molecules is not sufficient for a complete understanding. Since the human brain is ill-suited to grasp the non-linear dynamics of these complex networks and the entailed emergent properties, the role of computational support is increasing in molecular biology.

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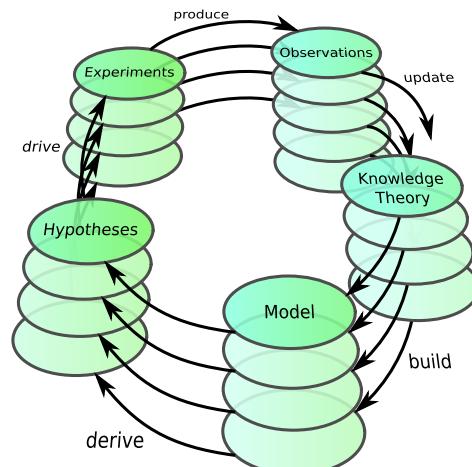


Fig. 1: The empirical spiral: applying the empirical cycle in successive rounds leads to a gradual build-up of knowledge.

The systems biology approach to understanding biological systems starts off from a scientific question and then follows an empirical cycle - or rather a positive spiral - of knowledge/theory → model → hypotheses → experiments → observations → update and/or refinement of knowledge/theory, until an answer to the original question is found (Figure 1). A model plays a pivotal role in this cycle:

1. to organize data and store knowledge,
2. to structure reasoning and discussion
3. to perform *in silico* experiments and derive hypotheses.

An *in silico* model is always a simplified representation of biological reality and is never the aim in itself. Rather, it is a powerful means in the process of gaining an understanding of the biological system. Given its role in the empirical cycle, the process of modelling is especially effective when applied by the experts with respect to a certain biological system. Biologists usually have a good sense of cause-and-effect relationships of molecular interactions. In addition, they are the most knowledgeable on the network topology and the dynamics of the biological system they are studying. Since they also benefit most from the generation of hypotheses and from an efficient

experimental design, biologists would be the primary candidates to construct models of their research topic.

As models are a formalization of knowledge or theories, an underlying formalism is needed to express this knowledge. Different formal methods have been successfully applied to construct representations of biological systems. Among these methods are Boolean logic (Mendoza et al., 1999; Shmulevich and Zhang, 2002), ordinary differential equations (ODEs, reviewed by de Jong, 2002), interacting state machines (Fisher et al., 2005; Efroni et al., 2003), process calculi (Dematté et al., 2008; Ciocchetta and Hillston, 2009), Timed Automata (Siebert and Bockmayr, 2008; Bartocci et al., 2009; Batt et al., 2007) and Petri nets (Reisig, 2011; Bonzanni et al., 2009). Most of these formal methods have been implemented into software tools to aid the process of modelling. Due to the lack of such a supporting tool, Timed Automata have remained a less frequently applied method.

Timed Automata have been developed to model the dynamic behaviour of systems with processes running in parallel (Alur and Dill, 1994). As such, Timed Automata have been applied in communication protocols and industrial control engineering (Bengtsson et al., 2002; Hessel and Pettersson, 2007; Marques Jr. et al., 2013). The parallels between these application areas and regulatory processes in cells have triggered the step towards their use in biology. Siebert and Bockmayr (2008) use Timed Automata to extend a classical modelling paradigm (Thomas, 1973), allowing to add temporal dynamics to gene network models. Bartocci et al. (2009) describe a model of biological oscillators and test synchronization properties in this dynamic system. A discretization of ODEs to Timed Automata is proposed by Batt et al. (2007), applying a translation between the two formalisms to an example gene regulatory network. Two different approaches to transforming a Petri net model into Timed Automata are presented by Nakano and Yamaguchi (2011), who also address the important issue of state space explosion in their paper. Finally, Man et al. (2011) propose an *ad hoc* Timed Automata model of a radiation treatment system, which is then validated through UPPAAL.

Each of these approaches has been successfully validated, demonstrating the potential of Timed Automata in biological applications. However, these approaches were all limited to simple or specific examples and none of these modelling methods has led to a tool implementation of the proposed method to encourage a broader use of Timed Automata in molecular biology.

Mastery of most existing modelling tools requires training and experience in mathematical modelling. In this respect, a lack of tradition in quantitative reasoning and formal methods within the biological community at large is still a stumbling block for widespread application of modelling of biological systems. Here, we present an intuitive method for the construction of formal *in silico* models of the dynamics of molecular networks, supported by a novel, user friendly modelling tool, ANIMO (Analysis of Networks with Interactive MOdelling, Schivo et al., 2012). Timed Automata are used as the underlying mathematical formalism.

In the Methods section, we will explain how choosing a suitable abstraction level can make the construction of models more intuitive. We will then show how ANIMO is designed to support the modelling process following this approach. Construction of a small model based on experimental data will exemplify the method that we propose. In the Results section, we first show an ANIMO

model of the genes and proteins that constitute the circadian clock network in *Drosophila Melanogaster*. The remainder of that section is dedicated to illustrate how a single modelling iteration in the empirical cycle is used to compile prior knowledge and experimental data into a model, perform *in silico* simulations and derive meaningful testable hypotheses. These hypotheses are supported by literature on interactions in different cell types.

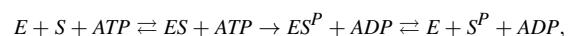
2 METHODS

2.1 Modelling abstractions

In living cells, cascades of chemical and physical interactions enable propagation of signals through molecular networks. In this process, the activity of upstream molecules induces a change in the concentration or activity of downstream molecules. For many reactions, the values of the kinetic parameters are unknown or difficult to collect. This lack of knowledge hampers the feasibility of computational models that describe molecular networks in fine mechanistic detail, especially for larger networks. As a solution to this problem, we propose the construction of models at a higher level of abstraction, thereby reducing the number of parameters involved. In choosing a suitable abstraction level, it is important to retain enough descriptive power to give a meaningful formal description of the topology and the associated dynamic behaviour of biological networks.

As a first abstraction in ANIMO models, the active and inactive forms of each network component are represented together by a single node in the network. Each of these nodes is characterized by its *activity level*, which represents the fraction of active molecules of that molecular species. When a molecule is known to be constitutively active, changes in concentrations of that molecule are treated as changes in its activity level. Activity levels are discretized into integer variables with a user-defined granularity, ranging from Boolean (2 levels) to near-continuous (100 levels).

Detailed biochemical reaction mechanisms are abstracted to *interactions*, which can represent either activations (\rightarrow) or inhibitions (\leftarrow). This aggregation of elementary reactions into single interaction steps reduces the number of kinetic parameters involved, while preserving cause-and-effect relationships. For example, consider a reaction in which enzyme E phosphorylates and activates substrate S , transferring a phosphate group from a molecule of ATP to a molecule of S . Biochemically, this reaction can be represented as



with conservation condition $S + S^P = \text{constant}$ and $ATP + ADP = \text{constant}$. Under the assumption of ATP constantly being replenished by the cell, this reaction is abstracted in ANIMO to the corresponding interaction



Each occurrence of the interaction $E \rightarrow S$ will increase the activity level of S by one discrete step. Since the activity level is defined as the active fraction of a molecular species, an increase in the active fraction implies a decrease in the inactive fraction. Hence, the original conservation condition is automatically satisfied. The interaction rate, R , depends on the activity levels of the reactants involved and on a single kinetic parameter k that is set by the user. The three available interaction scenarios can be interpreted as abstracted kinetic rate laws:

1. $R = k \times [E]$: the interaction rate depends only on the activity level of the upstream node.
2. $R = k \times [E] \times [1 - S]$ (activations) or $R = k \times [E] \times [S]$ (inhibitions): the rate depends on the activity levels of both the upstream and downstream participants. Activations depend on the presence of inactive substrate, $[1 - S]$, whereas inhibitions depend on the level of active substrate, $[S]$.

3. $R = k \times [E_1] \times [E_2]$: this scenario can be used when the activation or inhibition of a downstream node depends on the simultaneous activity of two upstream nodes. This scenario is comparable to an *AND-gate* in Boolean logic.

We will show in Section 3 that the abstraction proposed here preserves ample expressivity to capture the dynamic behaviour of a biological network.

2.2 Modelling interactions with Timed Automata

Timed Automata have been shown to be a powerful formalism to model biological processes (Siebert and Bockmayr, 2008; Bartocci et al., 2009; Batt et al., 2007). A timed automaton consists of locations and transitions between these locations (see Fig. 2), and a system of timed automata can be used to model a system of interacting molecules. At any time, each automaton is in a specific location, and together these locations represent the current state of the biological system. Each timed automaton can have one or more local clocks associated to it, allowing temporal control of transitions between locations. These transitions are used to represent interactions between molecules. Fast interactions take less time than slow interactions to perform an activation or inhibition step. We have previously described in detail how the scenarios presented in Section 2.1 can be used to calculate the timing of molecular interactions to give a description of network dynamics (Schivo et al., 2012). Figure 2 presents a small example that illustrates the basic properties of TA. This model describes the activation of ERK by MEK¹.

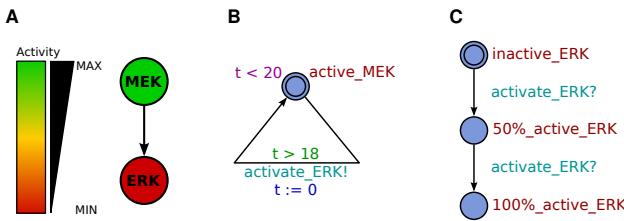


Fig. 2: Formalization of an activation interaction into a TA model. (A) Classical depiction of a well-studied intracellular signal transduction interaction: MEK activates downstream protein ERK. (B) A TA model of MEK, consisting of a single location (circle, active_MEK) and a single transition (arrow). In this example, MEK activity is not regulated and MEK is always active. $t < 20$ is termed an invariant on the location, allowing residence in this location as long as local clock time t is smaller than 20 units. $t > 18$ is termed a guard on the transition, allowing the transition to take place when local clock t is greater than 18 units. Together, the invariant and guard ensure that the transition must take place within the continuous time interval $18 < t < 20$. When the transition takes place, the action `activate_ERK!` is performed and the local clock is reset, $t := 0$. (C) A TA model of ERK, consisting of three locations, `inactive_ERK` (the starting location, indicated by a double circle), `50%_active_ERK` and `100%_active_ERK`, and two transitions between the locations. Here, ERK has three activity levels: completely inactive, halfway active and completely active. A transition will take place when it is possible to synchronize with the corresponding action `activate_ERK!` in the MEK automaton. Each synchronization on channel `activate_ERK` represents the occurrence of the activating interaction between MEK and ERK, and allows ERK to eventually become completely active. If we replace the time constraints for the occurrence of `activate_ERK!` with variables depending on scenario 1 ($R = k \times [MEK]$), the second activation step would have the same time constraints as the first activation step, since the interaction rate only depends on MEK. If we use scenario 2 ($R = k \times [MEK] \times [1 - ERK]$) instead, the time constraints are doubled after the first activation step, because only 50 % of inactive ERK is left. The second activation step would then take twice the time of the first step.

¹ All acronyms used in this paper and their corresponding UniProt IDs are listed in Suppl. Sect. 3.

2.3 ANIMO

The modelling approach described in Section 2.1 and Section 2.2 is implemented in the software tool ANIMO (Analysis of Networks with Interactive MOdelling, Schivo et al. 2012) as a plug-in to the network visualization tool Cytoscape (Killcoyne et al., 2009). The visual interface of Cytoscape makes the construction, expansion and rewiring of a network topology a fast and user-friendly process.

When a new node is added to the network, it has to be initialized with the number of activity levels and its initial activity. For each interaction, a scenario needs to be selected, together with the corresponding kinetic parameter and the interaction type: activation or inhibition. All settings can be readily adapted by double clicking, or via a table of nodes or interactions.

ANIMO automatically translates the user input to a TA model, which is then simulated with the model checking tool UPPAAL (Larsen et al., 1997). The results are subsequently parsed and translated to a graph that shows the dynamic behaviour of nodes in the network. A schematic overview of this process is given in Supplementary Section 5. No training or prior knowledge on the use of TA or UPPAAL is needed in order to benefit from ANIMO. Nevertheless, the TA model and the model checking process in UPPAAL can be accessed when desired by the user.

The dynamic behaviour of a model can be interactively explored by moving a time slider underneath the graph to highlight time points in a simulation. In the network view, each node will be coloured according to its activity level at the selected time point. Experimental data can be compared to the model by importing and superposing these data upon an output graph from the model (Figure 3 B,D,F). The ANIMO user workflow and the features described above are illustrated in Suppl. Video 1.

2.4 Using ANIMO to build a model based on data

To illustrate the process of modelling with ANIMO, we show the construction of a small model based on a literature compendium of signal transduction events in HT-29 human colon carcinoma cells (Gaudet et al., 2005). This data set comprises triplicate measurements of 11 different protein activities or post-translational modification states at 13 time points after treatment with different combinations of TNF α , EGF and insulin. The data set contains relative protein levels and activities, which is a typical situation in biochemistry.

As a first step, we normalized measurements for each protein to the maximum value in the complete experiment. This normalization results in a nondimensionalized data set that is suitable for use with ANIMO.

In Figure 3, we show the stepwise construction of a model of a small part of the network that is able to account for measured variations in activity of IKK, JNK1, MK2, Casp8 and Casp3 upon stimulation with 100 ng/ml TNF α . In this example we aimed for inclusion of a minimum number of nodes in the network, while preserving biological relationships. Multi-step cascades were aggregated into a single step when possible. Parameters for all interactions were set manually, and a close match was obtained between the model and the patterns that are present in the dataset.

A more comprehensive model based on the same dataset is presented in Section 3.2.

3 RESULTS

3.1 Expressivity of ANIMO models

Results obtained with ANIMO are comparable to results with other modeling approaches. To demonstrate this, Figure 4 represents an ANIMO model of the circadian clock in *Drosophila Melanogaster*, based on the work by Fathallah-Shaykh et al. (2009), where ordinary differential equations (ODEs) were used. The cyclic behavior of the circadian clock is based on the alternating formation and destruction of the CYC/CLK protein complex. Concentration levels of this complex are in turn regulated by a series of proteins which are produced as a consequence of CYC/CLK formation. The CWO

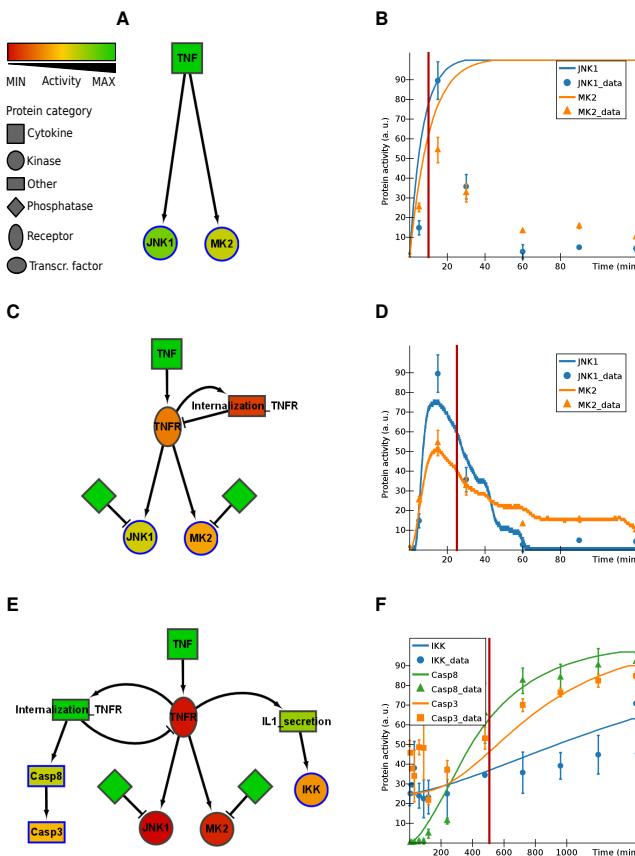


Fig. 3: Incremental construction of an ANIMO model of signal transduction events in human colon carcinoma cells upon stimulation with 100 ng/ml TNF α . Each construction step (top to bottom) is simulated in ANIMO, giving intermediate feedback useful for the piecewise refinement of the model. The graphs on the right show the dynamic behaviour of the corresponding models on the left, comparing it to the measured activity values by Gaudet et al. (2005) (error bars represent the standard deviation). On the vertical axis, “100” represents the maximum protein activity in the complete experiment. The red vertical line in each graph indicates a selected time point in the time course. Nodes in the corresponding network representation are coloured according to their activity at that time point. All images in this figure are taken from the ANIMO user interface. (A, B) Basic model showing direct activation of JNK1 and MK2 by TNF α . No peak dynamics are observed because no inactivating processes are present. (C, D) The model after addition of inactivating phosphatases and a negative feedback loop that down-regulates TNFR. Note that adding TNFR internalization or phosphatases alone would not be enough to reproduce activity peaks. (E, F) The model after addition of IKK, IL1-secretion (abstracting the autocrine IL-1 signalling described by Janes et al., 2006), Casp8 and Casp3, showing the late response to TNF α signalling. The _data suffix identifies experimental data; all other series are computed by ANIMO. As the data set did not contain values for cleaved caspase-3, but only for its non-cleaved precursor pro-caspase-3, we computed the Casp3_data series as 100% – [pro-Casp3].

protein is central to the functioning of the network, as it degrades the mRNA for most of the involved proteins. As such, CWO act as an inhibitor that counterbalances the effect of CYC/CLK. The positive influence of the light-regulated cryptochromes CRY on the degradation of TIM is a consequence of the passage between day and night, allowing the circadian clock to synchronize to a time zone (see Suppl. Sect. 6.1).

The output of the ANIMO model in Figure 4 closely matches the original ODE model. In particular, the oscillations in both models show the same periods and phases (see Suppl. Fig S9). Due to

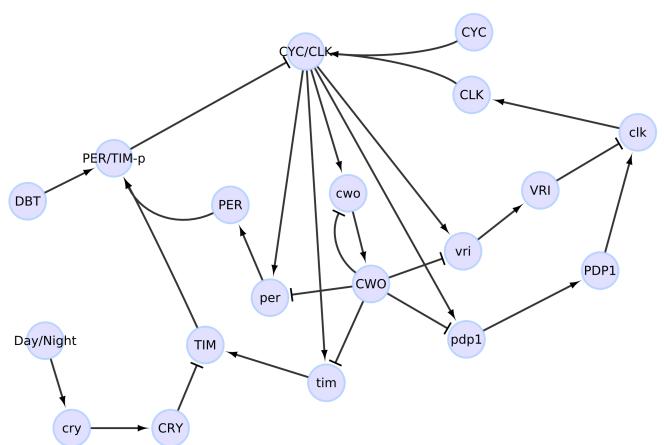


Fig. 4: ANIMO model of the circadian clock in *Drosophila Melanogaster*. Autoregulatory negative feedback loops are present on each of the nodes of the network, following the original model by Fathallah-Shaykh et al. (2009). These feedback loops ensure that protein levels decrease over time when activating inputs are absent. The feedback loops are not represented here for cosmetic reasons and clarity. Naming conventions follow the same rules as in the original model, with lower-case names representing mRNA, and upper-case names indicating proteins.

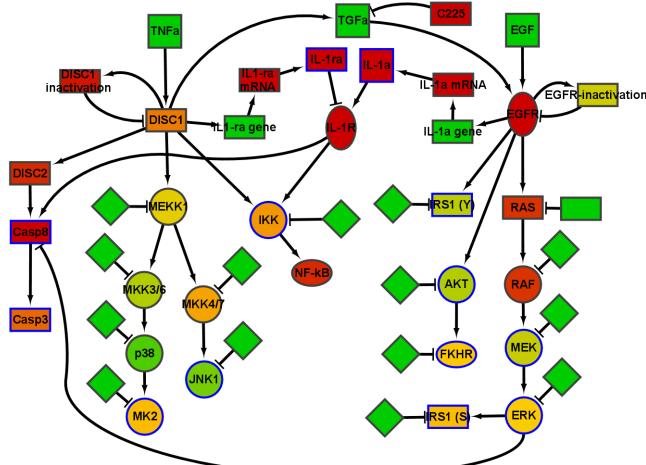
the compositional nature of Timed Automata ANIMO allows for intuitive *in silico* knock-out experiments, by right-clicking a node in the model and disabling it. Such experiments have been done before (Fathallah-Shaykh et al., 2009) and give similar results in our model.

3.2 Case study: using ANIMO to generate hypotheses

In order to validate our modeling approach, we constructed a larger model of the signaling network downstream of TNF α and EGF, formalizing the crosstalk that takes place between the pathways at different levels of cellular regulation. We first modeled the two pathways in isolation (Suppl. Figs. S10, S11), using information on protein interactions from the KEGG (Kanehisa and Goto, 2000) and phosphosite (Hornbeck et al., 2004) databases. These models were fitted to experimental data from studies by Gaudet et al. (2005) and Janes et al. (2006). We then merged the two pathways into a single model and added autocrine crosstalk between the pathways that has been described by Janes et al. (2006). Briefly, stimulation with TNF α leads to a rapid release of TGF α (TGF α in the model), which activates the EGF receptor (EGFR). This activation causes secretion of IL-1 α (IL-1 α) at later time points. The effect of IL-1 α is down-regulated by the secretion of IL-1 receptor antagonist (IL-1ra) downstream of TNF α . The resulting model (Fig. 5A) was compared to the experimental data for treatments with 100 ng/ml TNF alone and 100 ng/ml EGF alone (data not shown) (Gaudet et al., 2005).

At this point, the behavior of the model deviated from the data for some of the nodes. This is an interesting situation, as it requires modifications to the model, that can be interpreted as new hypotheses. Below, we give two examples and show how adaptation of the model can be used to generate novel testable hypotheses.

Experimentally, treatment with TGF α alone does not lead to secretion of IL-1 α . Instead, a co-stimulation with TGF α and TNF α is required (Janes et al., 2006). However, in the first version of the model, treatment with TGF α was sufficient for IL-1 α expression (Fig. 5B). Given the time delay until secretion of IL-1 α , it can

A**Protein category**

■ Cytokine ● Kinase ■ Other
 ♦ Phosphatase ○ Receptor ● Transcr. factor

Activity

Min Max

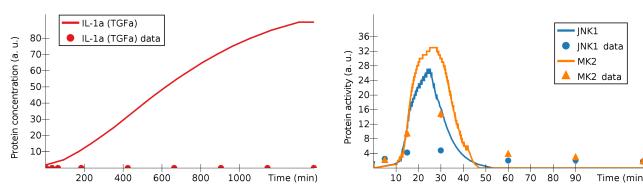
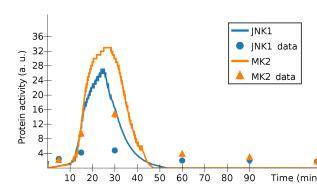
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Fig. 5: Signalling network downstream of $\text{TNF}\alpha$ and EGF in human colon carcinoma cells. (A) The model for the merged $\text{TNF}\alpha$ and EGF pathways. Node colours represent the activity level of the corresponding modelled reactants at time $t = 15$ minutes after a stimulation of 100 ng/ml $\text{TNF}\alpha$ + 100 ng/ml EGF . (B) Modelled production of $\text{IL-1}\alpha$ after stimulation with 100 ng/ml $\text{TGF}\alpha$ (24 hours). (C) Modelled activation of JNK1 and MK2 after stimulation with 5 ng/ml $\text{TNF}\alpha$ + 10 $\mu\text{g}/\text{ml}$ C225 (2 hours).

The .data suffix identifies experimental data; all other series are computed by ANIMO.

be expected that *de novo* synthesis of $\text{IL-1}\alpha$ is required and that both $\text{TNF}\alpha$ and $\text{TGF}\alpha$ are needed to activate transcription of the $\text{IL-1}\alpha$ gene. JNK1 and ERK signal downstream of $\text{TNF}\alpha$ and $\text{TGF}\alpha$, respectively, and are known to affect the activity of multiple transcription factors. We altered the model to make activation of $\text{IL-1}\alpha$ expression dependent on both JNK1 activity and ERK activity (Suppl. Fig. S12, arrows linking JNK1 and ERK to $\text{IL-1}\alpha$ gene). After this modification to the model, $\text{IL-1}\alpha$ was no longer secreted upon stimulation with $\text{TGF}\alpha$ alone, which greatly improved the fit between the measured $\text{IL-1}\alpha$ levels and the model (Fig. 6B). This hypothesis could now be used to design a new experiment to validate $\text{IL-1}\alpha$ as a target of combined JNK1 activity and ERK activity in HT-29 cells. For example, kinase inhibitors specific to JNK1 and ERK could be used to confirm that activity of both kinases is required for expression and secretion of $\text{IL-1}\alpha$. Performing the experiment is beyond the scope of this study, but this hypothesis finds support in literature. Transcription factors c-Jun and c-Fos together form a heterodimer known as AP-1 and are activated by JNK1 and ERK, respectively (Davis, 2000; Bannister et al., 1994). AP-1 has been reported to bind to the promoter of $\text{IL-1}\alpha$, providing

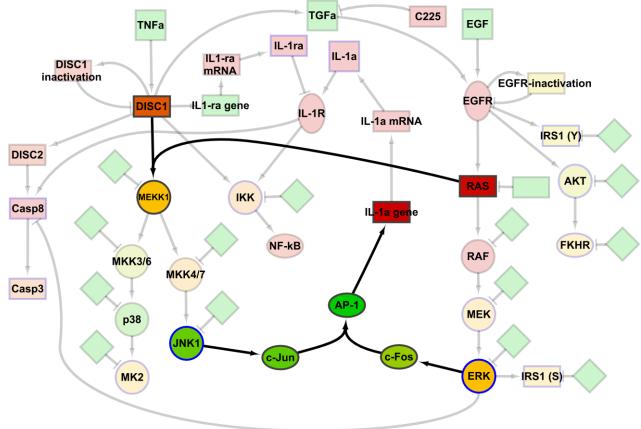
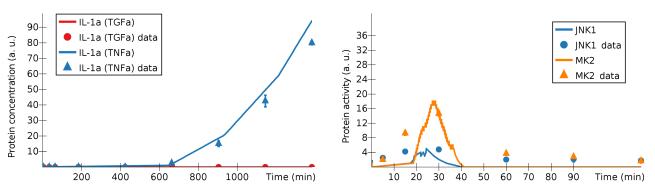
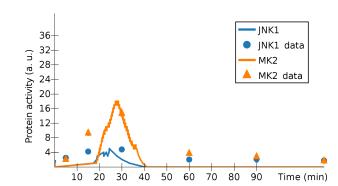
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Fig. 6: (A) The model for the merged $\text{TNF}\alpha$ and EGF pathways after addition of the two hypotheses (highlighted). Hypothesis 1 assumes $\text{IL-1}\alpha$ expression to depend on AP-1 activity, which in turn requires both c-Jun en c-Fos to be activated by JNK1 and ERK, respectively. Hypothesis 2 assumes RAS as an activator of MEKK1. Node colours represent the activity levels 15 minutes after stimulation of 100 ng/ml $\text{TNF}\alpha$ + 100 ng/ml EGF . (B) After the addition of the first hypothesis (activation of $\text{IL-1}\alpha$ production depending both on JNK1 and ERK): production of $\text{IL-1}\alpha$ after stimulation with 100 ng/ml $\text{TNF}\alpha$ (series $\text{IL-1}\alpha$ (TNFα)) compared with stimulation with 100 ng/ml $\text{TGF}\alpha$ (series $\text{IL-1}\alpha$ (TGFα)) (24 hours). (C) After the addition of the second hypothesis (activation of MEKK1 downstream of EGFR): stimulation with 5 ng/ml $\text{TNF}\alpha$ + 10 $\mu\text{g}/\text{ml}$ C225 (2 hours). Suppl. Sect. 6.3 explains how the dosage of 5 ng/ml $\text{TNF}\alpha$ was represented in the model. The .data suffix identifies experimental data; all other series are computed by ANIMO.

evidence for a role in the regulation of $\text{IL-1}\alpha$ expression (Bailly et al., 1996). Based on these findings in literature we included c-Jun and c-Fos in our model as transcriptional activators of $\text{IL-1}\alpha$ (Fig. 6A).

As a second example, we considered the behaviour of JNK1 and MK2. In the model, both proteins were located downstream of $\text{TNF}\alpha$ but not $\text{TGF}\alpha$ or EGF . Hence, the model did not show an effect of C225, a pharmacological inhibitor of ligand-EGFR binding, on activation of JNK1 or MK2 after stimulation with $\text{TNF}\alpha$. However, experimental data show that C225 strongly reduces activation of JNK1 and MK2 upon stimulation with $\text{TNF}\alpha$ (Janes et al., 2006). This fact is indicative of a role for EGFR in activation of JNK1 and MK2. Since both JNK1 and MK2 are located downstream of MEKK1, we hypothesized that activation of MEKK1 is dependent on both $\text{TNF}\alpha$ -signalling and $\text{TGF}\alpha$ -signalling. In the model we added a new hypothetical node Hyp 2 (hypothesis 2) to link EGFR to MEKK1 (Suppl. Fig. S12). This addition led to an improved fit of the model to the data upon treatment with $\text{TNF}\alpha$ + C225: activation of both MK2 and JNK1 was strongly suppressed by C225 (Fig. 6C). Stimulation

with EGF alone did not lead to activation of JNK1 and MK2. These data support the validity of the modification to the model. Further support for a link between EGFR and MEKK1 was found in literature. Specifically, Ras has been reported as a direct activator of MEKK1 (Russell et al., 1995). EGFR is a well-known and potent activator of Ras, which is why it was already in our network (Kanehisa and Goto, 2000). Other studies also report activation of JNK1 and phosphorylation of c-Jun downstream of Ras, which is consistent with an interaction between Ras and MEKK1 (Bannister et al., 1994; Dérijard et al., 1994). Based on these findings, we adapted our model by removing the Hyp 2 node and creating a direct interaction between Ras and MEKK1 (Fig. 6A). Experimentally, the role of Ras could be confirmed by using a pharmacological inhibitor of Ras activity, and measuring the effect of this inhibitor on the activation of JNK1 and MK2. Together, our model suggests that EGFR activity is required but not sufficient for activation of JNK1 and MK2 in HT-29 cells.

There are other nodes for which the experimental data deviates from the model in one or more of the experimental conditions. A comparison between model and experimental data can be found in Figures S13, S14 and S15. A complete deciphering of the signalling events in this biological system is outside the scope of this paper. Instead, we illustrated how interactive modelling of the dynamic behaviour of a signal transduction network can be used to extend previous pathway topologies and can lead to the generation of novel hypotheses.

4 DISCUSSION

The ultimate aim of research projects is to solve a problem or get the answer to a scientific question. In biology, an in-depth understanding of the relevant biological system is an important step towards this goal. Successive repetitions of the empirical cycle result in a stepwise increase in understanding, until the goal is reached. For complex biological systems, computational modelling is indispensable in this process. The mere act of creating a computational model based on prior knowledge, experimental data and hypotheses assists in gaining more insight in the system.

We developed a new modelling approach by proposing a series of abstractions from the detailed molecular mechanisms of biological systems. These abstractions reduce the need for kinetic parameters, while preserving enough expressivity for a useful description of the dynamic behaviour of biological networks. A novel modelling tool, ANIMO, allows effective use of this approach and enables an intuitive construction of formal models.

ANIMO is not the first modelling tool to provide an interface to a modelling formalism. Such interfaces exist in many other tools (see Suppl. Tab. S4). With its focus on user-friendliness and intuitive modelling, ANIMO's main contribution lies in making computational modelling more accessible to experts in biology. Making use of the visual interface provided by Cytoscape, network representations subscribe to biological conventions. Model parameters are kept to a minimum and can be directly accessed by mouse-clicking on nodes and edges. Because of the automatic translation of the network topology and user-defined parameters into an underlying formal model, training in the use of formal methods is not needed. In Supplementary Section 7, a more in-depth comparison between ANIMO and other modeling tools is

given. For this comparison we selected a tool for each of the most commonly used formalisms, and used criteria with a strong focus on user-friendliness.

In Section 3.1, we described the construction of an ANIMO model of the circadian clock in *Drosophila Melanogaster*. This model captured the dynamics of the regulatory network and led to similar conclusions as an ODE model that had been published previously (Fathallah-Shaykh et al., 2009). This finding supports the use of the series of modelling abstractions that we proposed. The biggest difference between the construction of these models is that the model by Fathallah-Shaykh et al. (2009) is constructed by writing a system of mathematical equations, together with an algorithm for simulation. In ANIMO, instead, a number of network nodes is drawn for the molecules involved. These nodes are then linked by directed interactions that represent cause-and-effect relationships, with a single parameter that defines the strength of each interaction. This is a more intuitive approach to construct a model. Further contributing to an interactive modelling process is the compositionality of the model. Each node in the network can be disabled at any time by the user, or extra nodes can be added, without having to change any of the existing interactions.

In Section 3.2, we showed the construction of an executable model of signalling events downstream of TNF α and EGF in human colon carcinoma cells. This data set has been used for previous modelling studies, based on partial least-squares regression and fuzzy logic (Janes et al., 2005; Aldridge et al., 2009). The partial least-squares regression model describes an abstract data-driven model that uses statistical correlations to relate signal transduction events to various cellular decisions. This type of modelling is very useful in uncovering new and unexpected relations. It is also successful in making predictions, but gives little direct in the dynamic behaviour of the network. Fuzzy logic analysis led to a model that gave a better fit to the dynamic network behaviour than discrete logic (Boolean) models. Inspection of the inputs to the logical gates that were used to model protein behaviour led to the prediction of novel interactions between proteins, showing the usefulness of this approach. For most of the proteins, such as JNK1, time was used as an input parameter. For example, the logical gates "if TNF α is high AND time is low, then JNK1 is high" and "if TNF α is high AND time is high, then JNK1 is low" were used to describe the dynamic behaviour of JNK1. Although this leads to a representative description of the dynamic behaviour of JNK1, peaks in protein activity at early time points were not reproduced by the fuzzy logic model. Moreover, it gives no insight in the molecular interactions that are involved in activation or inhibition.

In this study, we used the same data set and performed a single round of the empirical cycle. This cycle starts off with the experiments carried out by Gaudet et al. (2005). We used the resulting experimental data, together with knowledge from curated databases (Kanehisa and Goto, 2000; Hornbeck et al., 2004) to construct an executable model of the biological system. In contrast with the two approaches described above, ANIMO is aimed at the construction of more mechanistic models, mimicking biochemical interactions *in silico*. This way of modelling gives a different type of insight. In the process of model construction, we extended a prior-knowledge network with time-dependent extracellular crosstalk that has been reported previously (Janes et al., 2006). To come up with possible explanations for a disagreement between the model and the experimental data, two additional layers of crosstalk were

introduced, at the signal transduction and transcriptional level. These modifications improved the fit of the model to the data and can be interpreted as novel testable hypotheses. Finally, we proposed new experiments that could be carried out to test these hypotheses, closing the empirical cycle. Together, our model sheds more light on the intricate entanglement between the TNF α and EGF pathways at multiple cellular levels. But above all, the model provides an excellent starting point for further investigation. Every new round in the empirical cycle will lift the understanding of the system to a higher level, leading to an incremental build-up of knowledge and an upward empirical spiral. Being intuitively accessible, ANIMO models facilitate sharing knowledge within and between groups and encourage collaborations.

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Conflict of interest: none declared.

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Bringing biological networks to life with ANIMO

Additional Materials

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1 REQUIREMENTS AND INSTALLATION

In order to run ANIMO, a desktop or laptop computer is needed with the following software installed:

- Java: see Section 1.1
- Cytoscape (Killcoyne et al., 2009): see Section 1.2
- UPPAAL (Larsen et al., 1997): see Section 1.3

The software to run Java-based programs is provided for free by Oracle. More information is available on the java.com website. Cytoscape is an open source project released under the terms of the GNU Lesser General Public License. UPPAAL is developed by a collaboration by the universities of Uppsala (Sweden) and Aalborg (Denmark), and is free for non-commercial applications in academia only.

All of these softwares work under Windows () Mac-OS () and all most common GNU/Linux () distributions. If the requirements are already met, ANIMO can be directly installed following the instructions in Section 1.4.

Note: when required to type something, the text to input will be represented “like this”: the quotation marks are not intended to be typed.

In case of problems accessing a web site with Microsoft Internet Explorer, we advise to try with a different web browser (such as Mozilla Firefox or Google Chrome) or to update Internet Explorer.

1.1 Java

1. In order to check that Java is installed, open a console

 Windows 7: press Windows button and type “cmd”, then press Return. Previous versions: in the Start menu find *All programs* → *Accessories* → *Command Prompt*.

 Go to *Applications* → *Utilities* → *Terminal*.

 Under Gnome, press Alt-F2, type “gnome-terminal”, then press Return. Under KDE, press the KMenu button, type “konsole” and click Konsole. Under Unity, press the home button (the one with the Ubuntu logo: ) type “terminal” and click the Terminal icon.

2. Type “java” and press Return. If a brief error message like “unknown command” is shown, Java needs to be installed: please proceed to step 3. Otherwise, please continue to Section 1.2.

3.  Point your web browser to java.com.

Click *Free Java Download*, then on *Agree and Start Free Download*.

If asked for permission to run the installer, grant that permission.

After the download, double click the installer and install Java following the guided steps.

To check that Java has been correctly installed, a web page is automatically opened at the end of the installation process. Click on *Verify Java version*. If a *Congratulations!* message is shown, Java has been successfully installed. Otherwise, please try again or contact Java support.

 Go to *Applications* → *Utilities* → *Java Preferences*.

If the Java Preferences window is shown, Java is already installed, otherwise the system will prompt you to install it. Follow the instructions and Java will be correctly installed at the end of the procedure.

 If you run Ubuntu, open the Software centre, search for “java” and select *OpenJDK Java 6 Runtime*. An *Install* button will appear next to the name of the package: click that button and

Java will be correctly installed.

If you run another distribution, use your package manager in a similar way. If you cannot find OpenJDK, there may be the possibility to install *Oracle Java Development Kit (JDK)* instead.

1.2 Cytoscape

Cytoscape can be found at the address www.cytoscape.org/download.php: an automatic installer program can be downloaded. Please note that you need to register and accept Cytoscape's terms of use before being able to start the download. Choose the latest 2.x version (at least 2.8.3, on the left column), possibly using a platform specific installer. For Windows, you can choose *64bit* only if you know that your computer can run 64bit programs, otherwise it is safe to choose *32bit*.

1.3 UPPAAL

1. Point your browser to www.uppaal.org.

Note: UPPAAL is free only for academic use. Information and contacts for commercial licenses can be found on the web site.

2. Click the *Download* link, and choose the latest *development* version (at least 4.1) for your operating system.

3. Fill in the required contact information and click the *Accept and download* button to download UPPAAL.

Note: problems with the registration on UPPAAL website have been reported when using some versions of Microsoft Internet Explorer. If the registration is unsuccessful, please consider updating Internet Explorer or changing your web browser.

4. Unzip the downloaded file to a known location: UPPAAL will be installed there.

5. Complete UPPAAL installation.



Open the UPPAAL installation location in Finder, drop the *UPPAAL.App* icon in your *Applications* folder, and copy the *verifyta* executable file to a known location. The installation of UPPAAL is complete: go to Section 1.4.



Open a console (this was done in Sec. 1.1, step number 1), type “`cd PATH_TO_THE_UPPAAL_DIRECTORY`” and press Return; `PATH_TO_THE_UPPAAL_DIRECTORY` is the path to the directory where you installed UPPAAL. It can be for example “`c:\Users\myuser\Desktop\uppaal-4.1.15`”, or “`/home/myuser/programs/uppaal-4.1.15`”.

Note: some Windows users may have access only to specific partitions (D:, Z:,...): in that case, please first change to the corresponding drive letter where the downloaded file was extracted. For example: if UPPAAL is located in `d:\myuser\Programs\uppaal-4.1.15`, the two commands to be entered are
“`d:`”
“`cd \user\Programs\uppaal-4.1.15`”

6. Type “`java -jar uppaal.jar`” and press Return.

7. The license for UPPAAL will be automatically acquired, and the main window of UPPAAL user interface will appear: you may now close that window.

1.4 Installing ANIMO

1. ANIMO is **free only for academic use**. For commercial licenses, please contact us.
2. Run Cytoscape.
3. Click the menu command *Plugins → Manage Plugins*: the *Manage Plugins* window will open.
4. Select the *Settings tab* and press the *Add* button.
5. Insert this *Name*: “ANIMO”, and this *URL*: “<http://fmt.cs.utwente.nl/tools/animoplugins.xml>” (please note: the “`http://`” is required), then confirm with *OK*.
6. From the *Download Sites* list in the upper part of the *Manage Plugins* window, select *ANIMO* (you may need to scroll down: it should appear after *Cytoscape*).
7. The panel on the left shows a smaller list of plugins: under *Available for Install → Analysis*, select *ANIMO v1.37*.
8. Click the *Install* button. The ANIMO tool will be automatically downloaded and installed.
9. The tool will ask you to indicate the position of the *verifyta* executable, which is the tool to verify Timed Automata models. You can find it in the *bin* (*bin-Linux*, *bin-Win32*, ...) depending on your operating system) directory inside the UPPAAL installation directory, or where it was copied at step 5 for  in Section 1.3.
10. Click the *Close* button to close the *Manage Plugins* window.
11. ANIMO is correctly installed and ready to be used.

2 ANIMO USER'S MANUAL

We will now present a step-by-step sequence to obtain an example model with ANIMO, which will allow us to illustrate the main features of the tool.

2.1 Modelling a small network

1. Run Cytoscape.
2. If Cytoscape is already running and there are open documents, please make sure that the current work is saved before proceeding.
3. From the *File* menu, select *New → Session*. Answer positively to the question “*Current session (all networks/attributes) will be lost. Do you want to continue?*”.
4. From the *File* menu, select *New → Network → Empty Network*.
5. In the *Control Panel* find the *Editor* tab. If you cannot find it, click the black arrows on the top right of the panel to search through the available tabs. Click the name of the tab to activate it.
6. Add 5 nodes to the empty network by Ctrl-clicking on empty areas of the *Network* window.
Note: *Ctrl-clicks* are obtained as follows. While holding the *Ctrl* key down, click with the left mouse button, then release the *Ctrl* key. The *Ctrl* key is usually located in the lower left or lower right corner of the keyboard. Apple keyboards may have the *⌘* symbol instead of *Ctrl*.
7. The *Edit reactant* dialogue window is opened when a new node is added, or when you right click an existing node and then select the *[ANIMO] Edit reactant...* item from the menu. Use that window to set the properties of the nodes as indicated in Table S1, taking the setting in Figure S1 for node A as reference. When the properties of a node have been inserted, confirm the choice with the *Save* button.

Table S1. The settings for the nodes (signalling network components) in the example.

Name	Total act. levels	Initial act. level	Molecule type	Enabled?	Plotted?
A	15	15	Cytokine	Yes	No
B	15	0	Receptor	Yes	No
C	15	0	Other	Yes	No
D	100	0	Kinase	Yes	Yes
E	1	1	Phosphatase	Yes	No

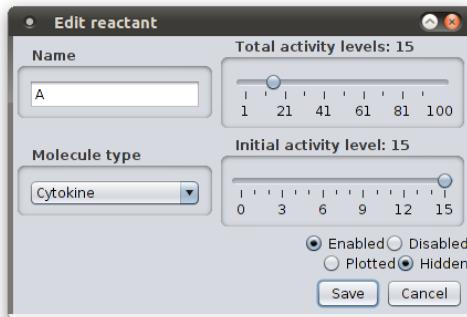


Figure S1: The *Edit reactant* window: modifying the properties of node A.

8. In order to add edges to the network, make sure that the *Editor* tab is still active in the *Control Panel*, and add the following edges by Ctrl-clicking the source and then clicking the target: A → B, B → C, C → B, B → D, E → D.
9. The *Edit reaction* dialogue window is opened when you add a new edge, or when you right click an existing edge and then select the *[ANIMO] Edit reaction...* item from the menu. Use that window to set the parameters of the edges as indicated in Table S2. The settings for the edge A → B should reflect the ones shown in the *Edit reaction* window in Figure S2.

Note: In order to insert a qualitative parameter like the ones required by the example network, click once the slider in the *parameter* box to activate it, and then move the slider to match the requested value.

Table S2. The settings for the edges (interactions) in the example.

Interaction	Influence	Scenario	Parameter value
A → B	Activation	1	Medium
B → C	Activation	1	Slow
C → B	Inhibition	1	Fast
B → D	Activation	1	Slow
E → D	Inhibition	2	V. Slow

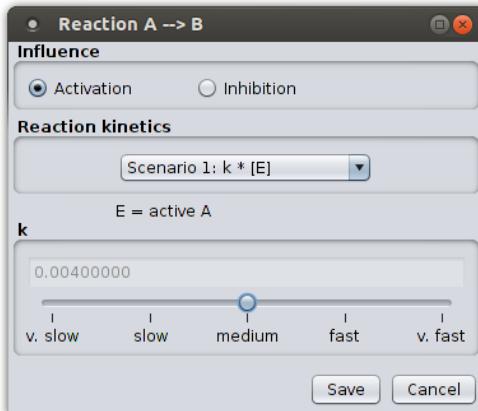


Figure S2: The *Edit reaction* window: modifying the properties of edge A → B.

10. In the *Control Panel* activate the *ANIMO* tab by clicking its title.
11. Click the *Choose seconds/step* button. A new dialogue window will appear: you can safely choose a time resolution of 1 second per step and click *OK*.
12. Click the *Analyse network* button.
13. After a few seconds the *Results Panel* should appear on the right, showing a plot of the activity level of reactant D over a time course of 120 minutes. Figure S3 shows the resulting network and graph plot.

2.1.1 Managing simulation data and activity levels plots Each time a simulation result is obtained, a new tab is added to the *Results Panel* (see the right part of Fig. S3) in which we identify three buttons, a plot of the activity levels of the selected reactants and a time slider.

Clicking on the button *Change title* allows to select a new title for the tab: this can be useful e.g. when comparing different simulations made on similar configurations of the same network. Button *Save simulation data...* allows to save the simulation data of the current tab on a *.sim* file, which can then be loaded and inspected in the future. The *Load simulation data...* button in the *Control Panel* above the *Simulation* box can be used for this purpose. Please note that the best results are obtained only when loading a *.sim* file when the *Network* window contains the same network on which the simulation data are based. If no network is currently opened, a network will *not* be opened by loading a *.sim* file. The *Close* button is used to close the currently displayed results tab.

Right clicking inside the graph area will bring up a menu that allows to perform some basic operations with the graph and its data:

- *Add data from CSV*: superpose the graph with other data series found in a *.csv* (comma separated values) file. This file type can be obtained for example by exporting data from the default Excel format. If you want the data in the *.csv* file to be rescaled so that its maximum Y value coincides with the maximum in the plot, the data file needs to contain a column named (exactly) *Number_of_levels*, on the first row of which the maximum of the scale for the *.csv* data needs to be put. For example, if the data in the *.csv* file are on a 0-100 scale, the value for *Number_of_levels* will be 100.

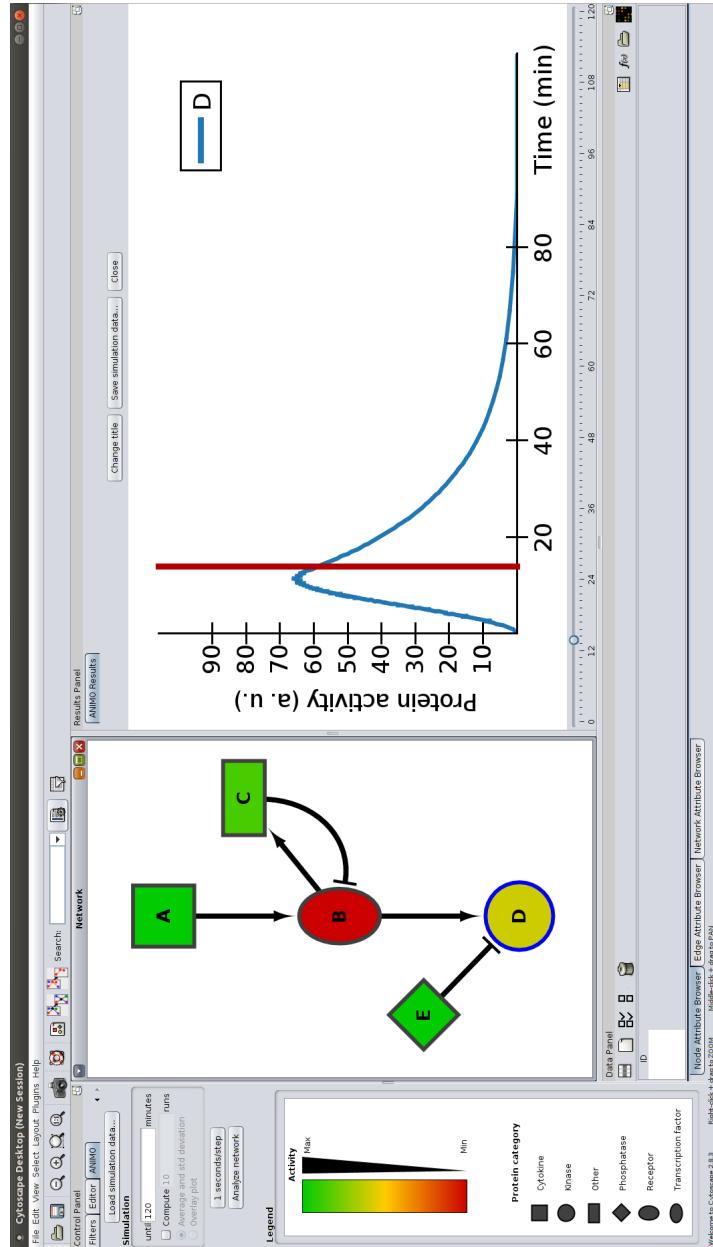


Figure S3: The completed example, where also the feature that allows to view the activity levels of reactants at chosen simulation times is demonstrated: the vertical red bar in the graph on the right can be moved through the slider under the graph, and indicates the point in the time series on which the colouring of the nodes in the *Network* window is based. The legends for colours and shapes can be found in the *Legend* panel.

- *Save as PNG*: save the graph as it is shown in a *.png* image file. This file format can be opened by most image editors.
- *Export visible as CSV*: export to a *.csv* file all the series that are currently visible (i.e., not hidden) in the graph.
- *Clear Data*: clear the contents of the graph, removing all series. This can be useful for plotting a *.csv* file without superposing it to the current graph, or for loading a file in which all hidden data were removed (exporting the visible graph to a *.csv* with the previous command).
- *Graph interval*: change the lower and upper bounds for X and Y axes.
- *Zoom rectangle*: zoom the graph around a user-chosen rectangular area. After selecting this command the shape of the mouse cursor changes into a cross. The area of the plot to be zoomed can then be selected by dragging a rectangular selection around it (see the definition of *rectangular selection* on page 8).
- *Zoom extents*: bring the zoom level back to default, cancelling the effects of any *Zoom rectangle* command.

Whenever the result of one or more simulations is shown as a graph, it is possible to use the slider under the graph to move through the entire simulation, showing the activity levels of all reactants represented with different node colouring in the *Network* window on the left. For an example, see Figure S3: the vertical red line in the graph represents the time instant on which the colours of the nodes in the *Network* window are based, and can be moved with the slider over which the mouse cursor is drawn.

2.2 Additional tips

2.2.1 Editing a network in Cytoscape Nodes and arcs can be placed in the network as shown previously: with the *Editor* tab selected in the *Control Panel*, Ctrl-click (click while holding the `Ctrl` or `⌘` key) in an empty place to add a node; Ctrl-click the source node and click the target node to add an arc. It is also possible to drag and drop the node/arc icons from the *Control Panel* into the *Network* window. *Note*: to perform *drag and drop* move the mouse cursor over the icon in the *Control Panel*, click with the left mouse button and, without releasing the button, drag the mouse cursor on the *Network* window where you want to place the symbol; then release the left mouse button.

In order to delete a node/edge, select it by clicking or grouping them in a larger rectangular selection, and then press the *Delete* key on the keyboard or select the *Edit → Delete Selected Nodes and Edges* menu command.

Note: in order to obtain a *rectangular selection*, left click in the *Network* window where the upper-left corner of the rectangle should be and, without releasing the left mouse button, drag the mouse cursor to where the lower-right corner of the rectangular selection should be; then release the left mouse button. All the entities which were even partially touched by the rectangle are now selected.

Navigation inside the *Network* window can be performed by clicking and dragging the centre mouse button, while zooming can be done by either rotating the mouse wheel or clicking and holding the right mouse button while moving the mouse in a vertical direction.

Finally, note that the colours used to represent node activity can be changed using the *VizMapper* interface provided by Cytoscape, changing the setting for *Node colour*, shown in Figure S4. To change the node colours, activate the *VizMapperTM* tab in the *Control Panel*, and find the entry named *Node Colour* in the *Visual Mapping Browser* box. Click the arrow-shaped icon directly to the left of *Node Colour*: the current setting for the node colours should appear: click the coloured bar to open the window shown in Figure S4. The *activityRatio* on which the node colouring is based is the ratio between the

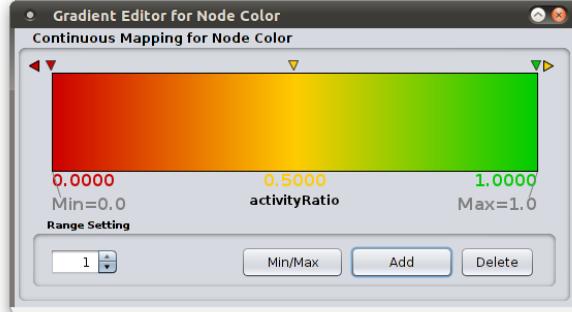


Figure S4: Changing the colours used to represent node activity in an ANIMO model.

current activity level of a node and its number of activity levels. The coloured arrows pointing downward on the upper border of the coloured rectangle can be dragged along the length of the $[0, 1]$ interval, thus changing the point at which a particular colour appears. New arrows can be added with the *Add* button, while clicking an arrow and pressing the *Delete* button will remove one. To change the colour of a point in the interval, double click the corresponding arrow, and a new window will open, allowing you to choose a new colour: clicking *OK* will accept the new colour. The modifications made in the *Gradient Editor for Node Colour* window should be automatically reflected in the model: when the gradient is as wanted, simply close the window. If the node colours seem not to have been updated, please move the slider under an existing graph in the *Results Panel*.

2.2.2 ANIMO features Nodes and edges (and groups of nodes/edges highlighted via a rectangular selection) can be disabled by choosing *[ANIMO] Enable/disable* from the right-click menu: they will be represented with less saturated colours and can be re-enabled by performing the same action. Moreover, a node can be enabled/disabled directly in its properties window, where it is also possible to add/remove the node from the list of series appearing in the graph resulting from a simulation of the network by selecting *Plotted* or *Hidden* (see also Figure S1): nodes that will be plotted are circled in blue. Every enabled node will be taken into account when computing the evolution of the system, but only nodes marked as *Plotted* will appear in a graph.

Each plot in an *ANIMO Results* tab contains by default a legend, which can be used to modify which series are displayed and how they are displayed. Clicking with the central mouse button on a series name will hide it from the graph, while the same centre-click the coloured line beside the series name will change the colour of that series, cycling through a predefined set of available colours. The entire legend can be hidden by clicking with the central mouse button anywhere on the graph (not inside the legend), or it can be dragged around by clicking and holding the left mouse button, releasing it when the preferred position is reached. Rotating the mouse wheel will allow the thickness of all the graph lines, and the size of the text, to grow or decrease: this feature can be useful when the window containing the graph is very large.

As the model is non-deterministic, i.e. its evolution will not be exactly the same for every single simulation run, it is possible to ask ANIMO to perform a number of simulation runs in a batch, plotting the averages of the activity levels over the runs together with a standard deviation value, or showing a so-called *overlay plot* where all runs are plotted over each other. The controls that allow to ask for multiple simulation runs can be found in the *Control Panel*, inside the *Simulation* box.

Standard deviation may be represented in the graph: it is normally shown as vertical bars, but its aspect can be cycled through five possibilities (vertical bars, shading, both bars and shading, bars and symbols, none) by right-clicking the corresponding line in the legend. Symbols associated to a representation of standard deviation can be changed by Shift-right-clicking (holding down the *Shift* key, right click) on the corresponding line in the legend. Standard deviation values can be obtained when asking for multiple simulations in the network analysis, but they can also be present in a *.csv* file, e.g. when the file contains averages of experimental data. In a *.csv* file, the column containing the standard deviation values for column *A* should be named *A_StdDev* for the program to recognize it and properly display the data series with the associated error values.

2.2.3 Parameter settings The application of some basic strategies when setting the parameters for a network allows the less experienced users to considerably shorten the modelling time. First of all, it is important to proceed in a *top-to-bottom* order, trying to match a component to the corresponding data before inserting the components downstream thereof. Second, when choosing the kinetic parameter for an interaction, we advise to first use the qualitative settings (very slow, slow, medium, fast, very fast): this allows to define the relative speeds of the interactions as soon as possible, leaving the more precise parameter setting procedure as a follow-up step. Finally, as can be seen from the parameter settings of Section 2.1, in order to obtain a peak behaviour it is particularly important that a negative feedback is present (as an example, see the interactions involving B and C in Tab. S2), and that the inactivating interaction in the loop is faster than the ones activating the target node.

A final note on the *seconds/step* button. This button allows to define the time granularity of the simulations, but it is not strictly necessary to choose a very precise value. If the current value for *seconds/step* is too high (or too low) to allow the network to be properly simulated, ANIMO will automatically choose (respectively) the highest (lowest) value that still allows to avoid rounding problems. It will be possible to notice such a change in the value of time scale when the number on the *seconds/step* button changes.

2.2.4 Updating ANIMO To check whether a new version of ANIMO has been published, run Cytoscape and ask for an update of all plug-ins via the menu command *Plugins → Update Plugins*. After some seconds during which Cytoscape will contact all the providers of the installed plug-ins, the system should report the list of updatable plug-ins.

Note: a window with the message *Attempting to connect to XYZ...* may appear and disappear multiple times: it is the normal behaviour.

If an updated version of ANIMO is available, it will appear under the category *Updatable Plugins → Analysis → ANIMO v1.37*. If no plug-in can be updated, a message stating *No updates available for currently installed plug-ins*. will be shown.

3 NAMING CONVENTIONS

Table S3 explains the abbreviations used in the paper.

Table S3: Explanation of the abbreviated names referring to molecular species in the main text.

Abbreviation	Full name	
Akt	protein kinase B	P31749
AP-1	activator protein 1	heterodimer of c-Jun and c-Fos
Casp3	caspase 3	P42574
Casp8	caspase 8	Q14790
c-Fos	proto-oncogene-protein c-fos	P01100
c-Jun	Jun activation domain binding protein	P05412
CLK	clock	O61735
CRY	cryptochrome	O77059
CWO	clockwork orange	Q9VGZ5
CYC	cycle	O61734
CYC/CLK	cycle-clock complex	
DBT	double-time kinase	O76324
DISC1	death-inducing signalling complex 1	
DISC2	death-inducing signalling complex 2	
EGF	epidermal growth factor	P01133
EGFR	EGF receptor	P00533
ERK	extracellular regulated kinase	P27361
FKHR	forkhead box protein O1	Q12778
IKK	inhibitor of nuclear factor kappa-B kinase	O14920
IL-1a	interleukin 1 α	P01583
IL-1R	interleukin 1 receptor	P14778
IL-1ra	interleukin 1 receptor antagonist	P18510
IRS1 (S)	insulin receptor substrate 1 (Serine 636)	P35568
IRS1 (Y)	insulin receptor substrate 1 (Tyrosine 896)	P35568
JNK1	c-Jun N-terminal kinase 1	P45983

Continued on next page

Table S3 - continued from previous page

Abbreviation	Full name	
MEK	MAPK ERK kinase	Q02750
MEKK1	MAPK/ERK kinase kinase 1	Q13233
MK2	mitogen-activated protein kinase-activated protein kinase 2	P49137
MKK3/6	dual specificity mitogen-activated protein kinase kinase 3/6	P46734 / P52564
MKK4/7	dual specificity mitogen-activated protein kinase kinase 4/7	P45985 / O14733
NF-kB	nuclear factor kappa-B	P19838
p38	mitogen-activated protein kinase p38	Q16539
PDP1	par-domain protein 1	Q9TVY0
PER	period	P07663
PER/TIM-p	phosphorylated period-timeless complex	
RAF	Raf	P04049
RAS	Ras GTPase-activating protein	P01112
TGF α	transforming growth factor α	P01135
TNF α	tumour necrosis factor- α	P01375
TNFR	TNF receptor	P19438
TIM	timeless	P49021
VRI	vrlle	O18660

4 NORMALIZING EXPERIMENTAL DATA FOR USE WITH ANIMO

Document S1 in the Supplemental Data of the work by Janes et al. (2006) contains three tables, named **Replicates**, **Averages** and **DPLSR dataset**. The data we use to compare the results computed with ANIMO are based on the values from the **Averages** table. In particular, we compute activity data by performing a normalization on a 0 . . . 100 scale using this formula

$$v_{\text{norm}} = \frac{v}{v_{\text{max}}} \times 100$$

where v is the datum to be normalized taken from the column v in the **Averages** table, v_{norm} is the normalized value and v_{max} is the maximum value over the whole column v in the **Replicates** table. For each series, we compute also the standard deviation using the triplicate measurements present in table **Replicates**. The standard deviation is also normalized using the formula presented for the average. Each table produced with this process contains a subset of the columns from the **Averages** table, and refers to one treatment condition only. A column with time references is added to the table in first position. Finally, a column named *Number_of_levels* containing only the value 100 (see the instructions on page 6) is added at the rightmost position. The tables are all exported in .csv format to be used with ANIMO, and are included in the *Model_and_data.zip* file in the additional materials of the present work.

5 ANIMO AND TIMED AUTOMATA

5.1 Timed Automata model

The Timed Automata (TA) model underlying an ANIMO network is generated whenever an analysis is requested by the user. Starting from the network represented in the Cytoscape-based user interface, ANIMO automatically generates a TA model to be used with UPPAAL. The analysis result is then parsed and properly presented to the user, for example as a graph of reactant activity levels. This workflow is described in Figure S5.

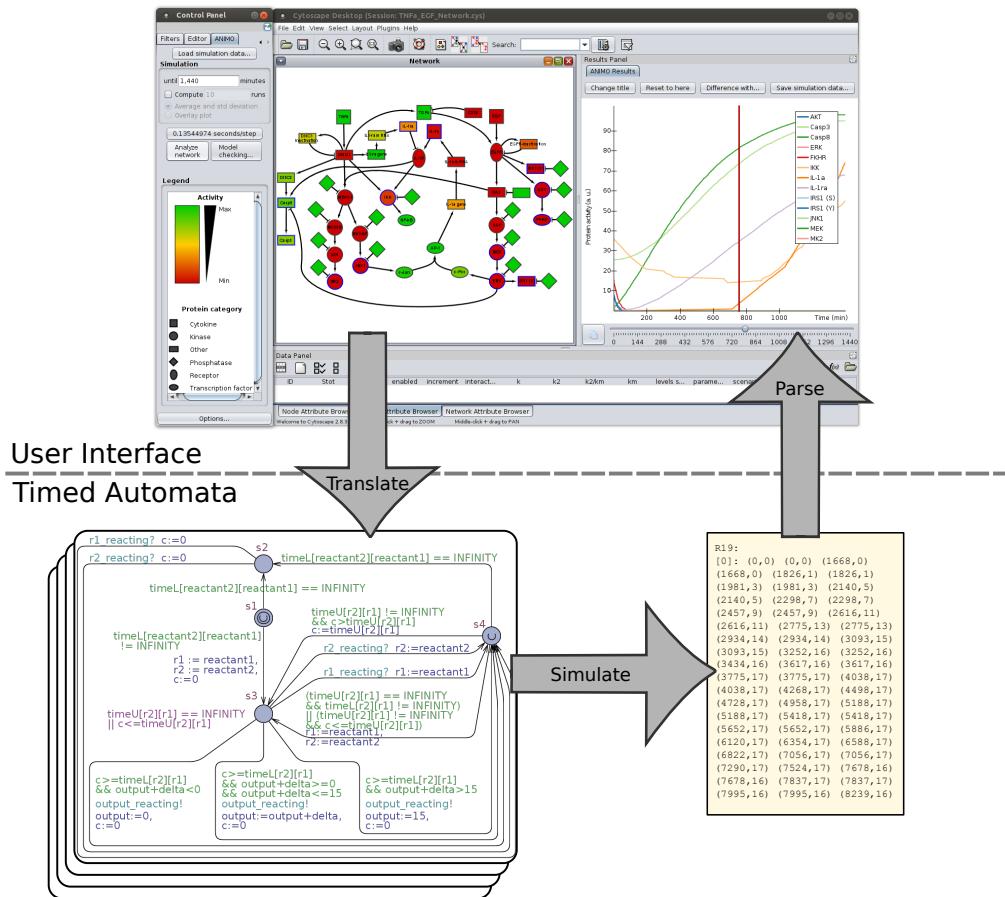


Figure S5: The passages intercurring between the press of the *Analyse network* button and the generation of an activity level graph. A simulation produces, for each selected node, a series of pairs (t, a) , with t a time instant along the simulation and a an activity level. These data are then parsed and translated into a graph.

Each TA model generated by ANIMO contains one automaton for each interaction (activation or inhibition) in the network. A TA representing an interaction performs a cyclic series of steps, continuously updating the target of the interaction it represents, and adapting the timing of the next update according to the user-defined dynamics. Synchronizations between different automata occur when the activity level of a network component (e.g. ERK) changes: this allows the automata depending on that component to update their time settings.

The abstract behaviour of the interaction $\text{MEK} \rightarrow \text{ERK}$ in the TA model used in ANIMO is described in Figure S6. There, the activity levels of MEK and ERK are represented by variables called, respectively, $\text{MEK}_{\text{activity}}$ and $\text{ERK}_{\text{activity}}$. A more detailed description of the TA model underlying ANIMO has been presented at the IEEE conference BIBE 2012 (Schivo et al., 2012).

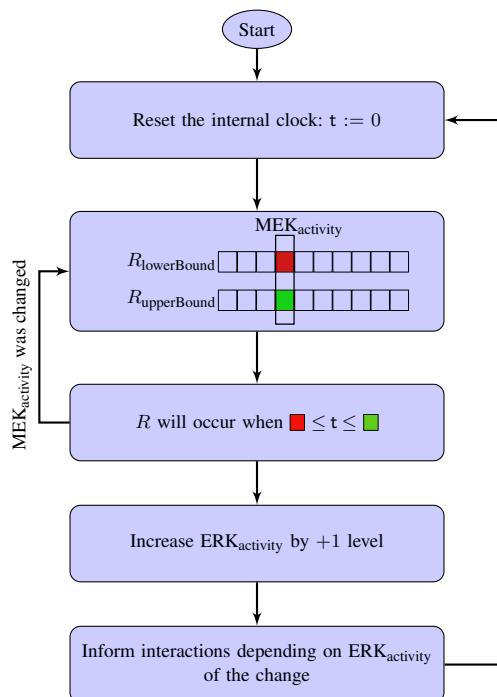


Figure S6: Schematic overview of the steps taken during a simulation run by a Timed Automaton modelling an interaction R that increases $\text{ERK}_{\text{activity}}$ and depends only on $\text{MEK}_{\text{activity}}$. In this example, MEK has 10 activity levels. After resetting the internal clock t , the automaton sets the time constraints for the interaction. $\text{MEK}_{\text{activity}}$ is used as the index inside the time tables $R_{\text{lowerBound}}$ and $R_{\text{upperBound}}$, which contain pre-computed lower- and upper-bounds for the interaction timing. Once the bounds have been identified, R can occur when t reaches a value inside the continuous time interval $[\text{red square}, \text{green square}]$. When it occurs, R increases the value of $\text{ERK}_{\text{activity}}$ by 1. All interactions that depend on $\text{ERK}_{\text{activity}}$ are notified of the change (via a synchronization on a specific channel), so that the associated time bounds are updated accordingly. After resetting the clock t , the process can restart. If $\text{MEK}_{\text{activity}}$ was changed by another automaton before the occurrence of R , the time bounds are updated according to the new activity level of MEK.

5.2 Granularity of an ANIMO network node

Figure S7 shows the differences between different choices for the number of levels of a node. This allows to adapt a model to the quality of experimental data.

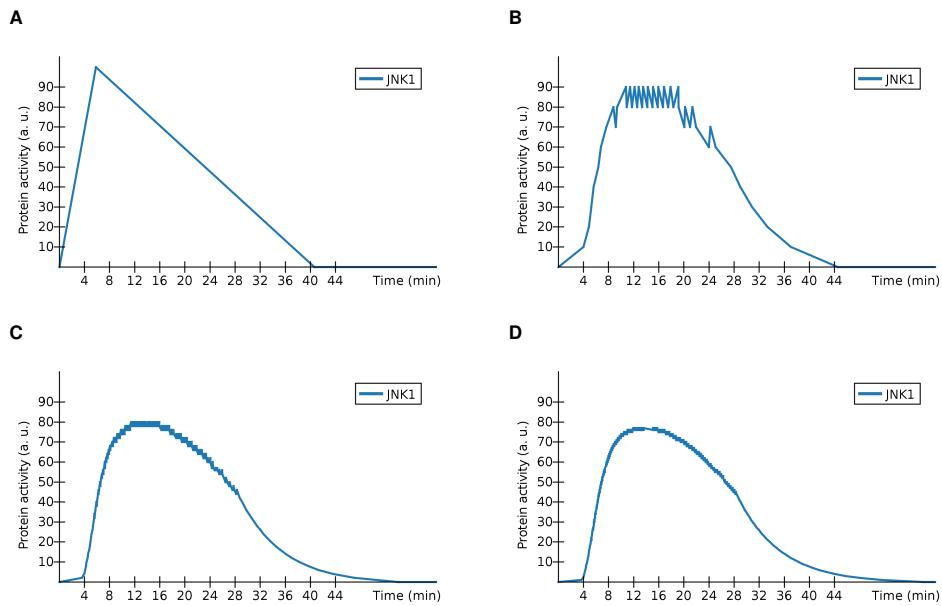


Figure S7: Comparing different reactant granularity settings. (A) 2 levels, (B) 10 levels, (C) 50 levels, (D) 100 levels. The JNK1 series is computed from the model presented in Figure 6A, considering 100 ng/ml TNF α as treatment condition over a period of 60 minutes.

6 ADDITIONAL NOTES

6.1 Simulating the day-night cycle

The model presented in Figure 4 contains a node labelled Day/Night. That node abstracts our representation of the cyclic alternation of day and night, which causes the variations in cryptochrome (*cry*): these oscillations allow the network to synchronize to a time zone. Note that the network oscillates also when the node *cry* is not included in the model.

The alternation between day and night is represented in our model with a repressilator-like (Elowitz, 2000) subnetwork, as can be seen in Figure S8. In the model by Fathallah-Shaykh et al. (2009) a specific function was introduced in the equations to approximate the experimental data from Kadener et al. (2007).

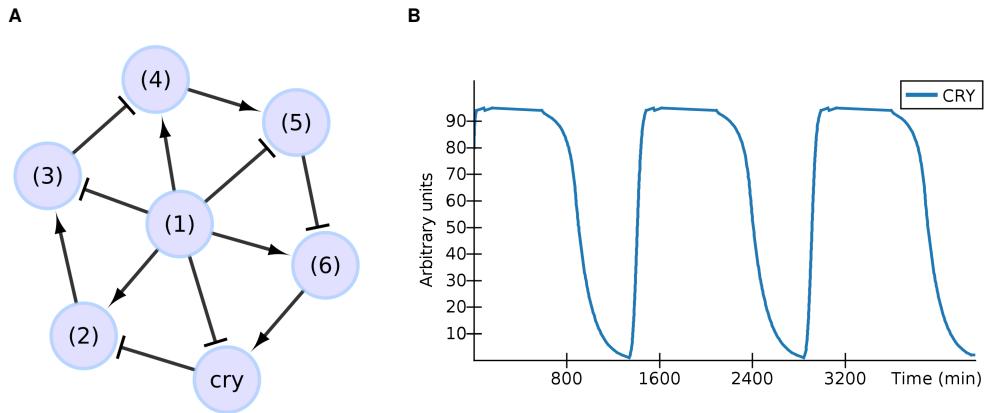


Figure S8: (A) The repressilator-like subnetwork used to represent the alternation between day and night that cause the oscillations in CRY concentrations in the network modelled in Section 3.1. (B) A graph plotting the oscillations in CRY along a period of three days.

6.2 ANIMO model of the Drosophila circadian clock

We compared the simulation results from the ANIMO model presented in Figure 4 with the ODE model described by Fathallah-Shaykh et al. (2009). The raw data coming from the two models were aligned to have a roughly close initial point, and all amplitudes were normalized following the procedure described in Suppl. Sect. 4. The results of this comparison can be seen in Figure S9.

Most of the molecules represented in the two models evolve with the same period and phase. CLK and clk in the ANIMO model have a small oscillation range (their values change by around 10% during a simulation), so their behaviour match the continuous model less precisely.

6.3 Note on the parameters in the TNF α -EGF model

The parameters in the model in Figure 6A have been set by fitting the model to the experimental data for conditions with 100 ng/ml TNF α . In the model we have set the starting level of TNF α at 100 out of 100 for these conditions. This level is a dimensionless quantity that indicates the maximum activity level in the data set. We found that setting the initial level of TNF α at level 8 out of 100 gave slightly better results for the condition with 5 ng/ml TNF α than level 5 out of 100. We believe that this has to do with the fact that 100 ng/ml is a highly supra-physiological concentration of TNF α , that will rapidly cause activation of all receptors present. Fitting the model to this experimental condition may have resulted in slight deviations

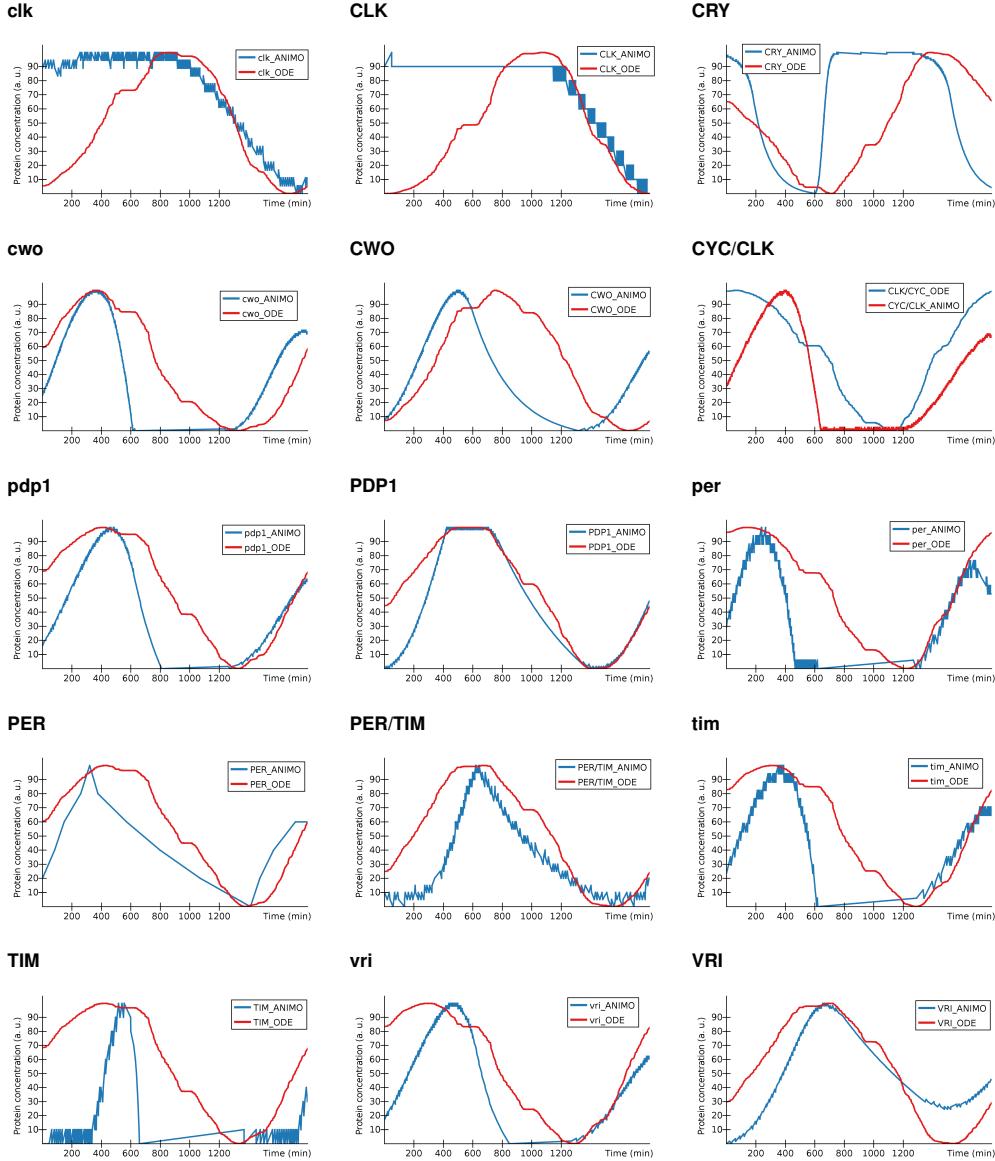


Figure S9: Comparing the simulation results from the ANIMO and ODE models of *Drosophila Melanogaster* circadian clock. Blue lines (_ANIMO series) represent data from the ANIMO model in Figure 4, while red lines (_ODE series) represent data from the ODE model by Fathallah-Shaykh et al. (2009).

in the parameter values. Nevertheless, the modelling results illustrate that building a model with basic kinetic rate laws can give useful predictions over a range of concentrations. Figures 5C and 6C show the modelling results with TNF set at 8 out of 100.

7 COMPARISON BETWEEN ANIMO AND OTHER MODELLING TOOLS

Different formalisms are in use in the field of computational modelling of biological systems, each with their specific characteristics. Many of these formalisms have been implemented into software tools to support modelling efforts. In order to compare ANIMO with existing tools, we have selected a number of mathematical formalisms, each connected to a supporting tool. With an emphasis on the modelling process rather than the final model, we compared these tools on the basis of the following parameters:

1. **Hidden formalism:** a knowledge of the underlying formalism is not required in order to use the tool
2. **Visual modelling:** the tool allows the user to model using a visual interface, and is not exclusively founded on formula-, text- or table-based input forms
3. **Qualitative parameters:** parameters for reactions can be input as approximated estimations, and not exclusively as numbers
4. **Tight coupling with topology:** models are tightly and clearly coupled to the networks they represent, showing the visual representation of the model in a shape similar or comparable to the representation currently used by biologists for signalling pathways
5. **User-chosen granularity:** if discretization is applied during the modelling process, the user can change the granularity with which such discretization is made, possibly for each component of the model separately

Table S4 shows the comparison between ANIMO and the selected tools.

Table S4. Comparison between ANIMO and some existing approaches to modelling biological systems. A “Yes” under a column indicates that the modelling tool (mostly) fulfils the parameter, “No” indicates very limited or no fulfilment.

Tool	Formalism	Hidden formalism	Visual modelling	Qualitative parameters	Tight coupling with topology	User-chosen granularity
ANIMO (Schivo et al., 2012)	Timed Automata	Yes	Yes	Yes	Yes	Yes
Bio-PEPA Workbench (Ciocchetta et al., 2009)	Bio-PEPA	No	No	No	No	Yes
Cell Illustrator (Nagasaki et al., 2011)	Petri Nets	Yes	Yes	No	Yes	No
COPASI (Mendes et al., 2009)	ODE, stochastic models	No	No	No	No	No
COSBILAB ¹	BlenX	Yes	Yes	No	Yes	No
GINsim (Chaouiya et al., 2003)	Boolean Networks	No	Yes	Yes	Yes	Yes ²
GNA (de Jong et al., 2003)	ODE	No	Yes	Yes	Yes	No ³
Rhapsody ⁴	Statecharts	No	Yes	Yes	No ⁵	No

¹ COSBILab web page <http://www.cosbi.eu/index.php/research/cosbi-lab>

² The user can choose the number of levels for each reactant, allowing to define multi-level models based on Boolean reaction dynamics.

³ When discretizing an ODE model, the granularity depends on the mathematical features of the model, and not directly on the user's choice.

⁴ IBM Rational Rhapsody web page <http://www-01.ibm.com/software/rational/products/rhapsody/designer>

⁵ Statecharts represent more closely the so-called *transition system* of the model as opposed to the components and interactions occurring among them.

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SUPPLEMENTARY FIGURES

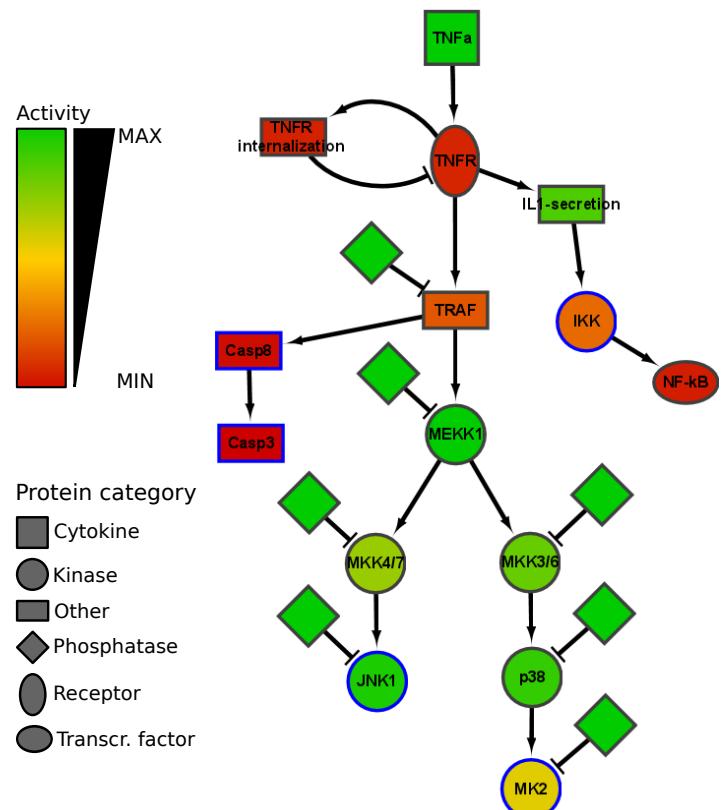


Figure S10: The model for the TNF α pathway in isolation. Node colours represent the activity level of the corresponding modelled reactants at time $t = 10$ minutes after a stimulation of 100 ng/ml TNF α .

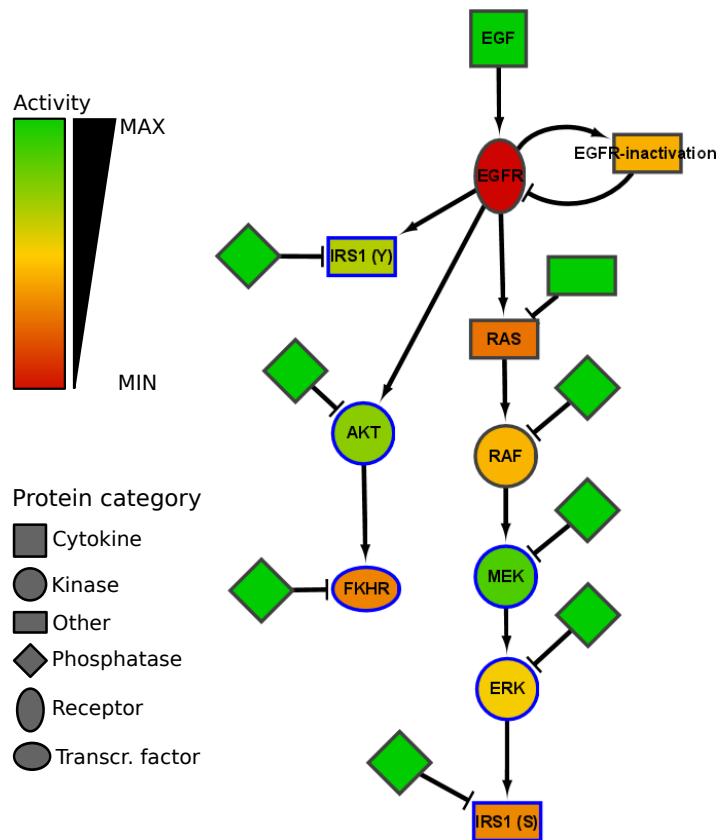


Figure S11: The model for the EGF pathway in isolation. Node colours represent the activity level of the corresponding modelled reactants at time $t = 5$ minutes after a stimulation of 100 ng/ml EGF.

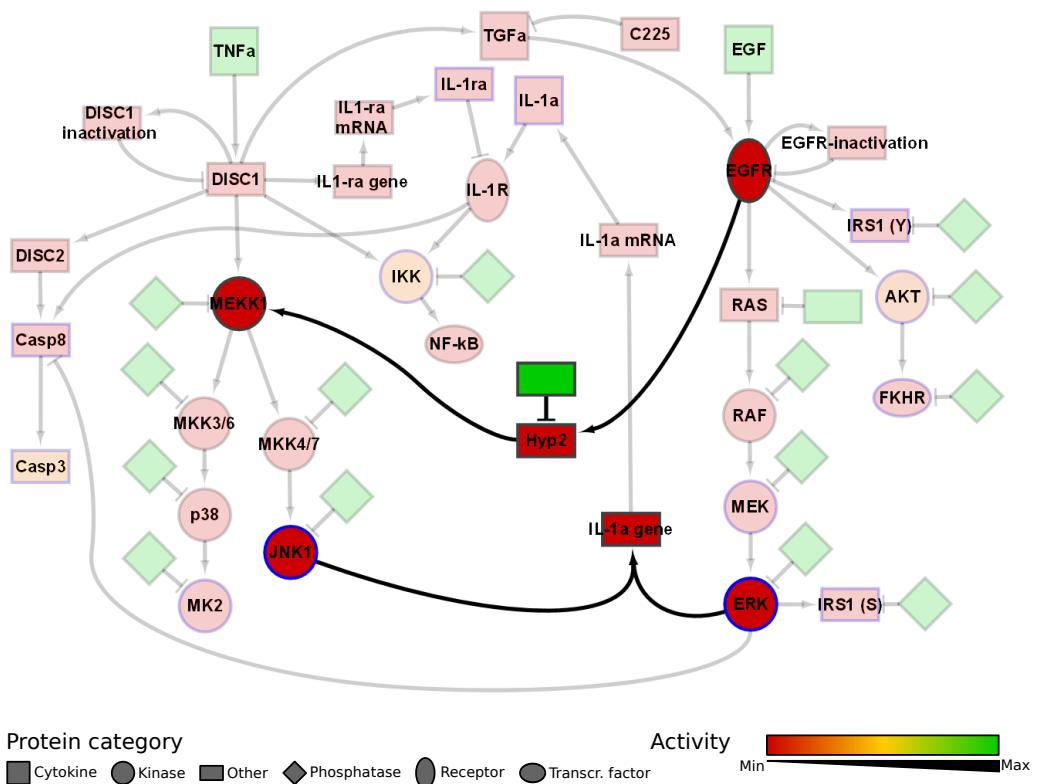


Figure S12: The merged model for the TNF α -EGF pathway in which the two hypotheses are highlighted. The first hypothesis is the dependence IL-1 α expression on the combined activity of ERK and JNK1. The second hypothesis assumes an as yet unidentified protein (Hyp2) to link EGFR to MEKK1. Node colours represent initial activity levels.

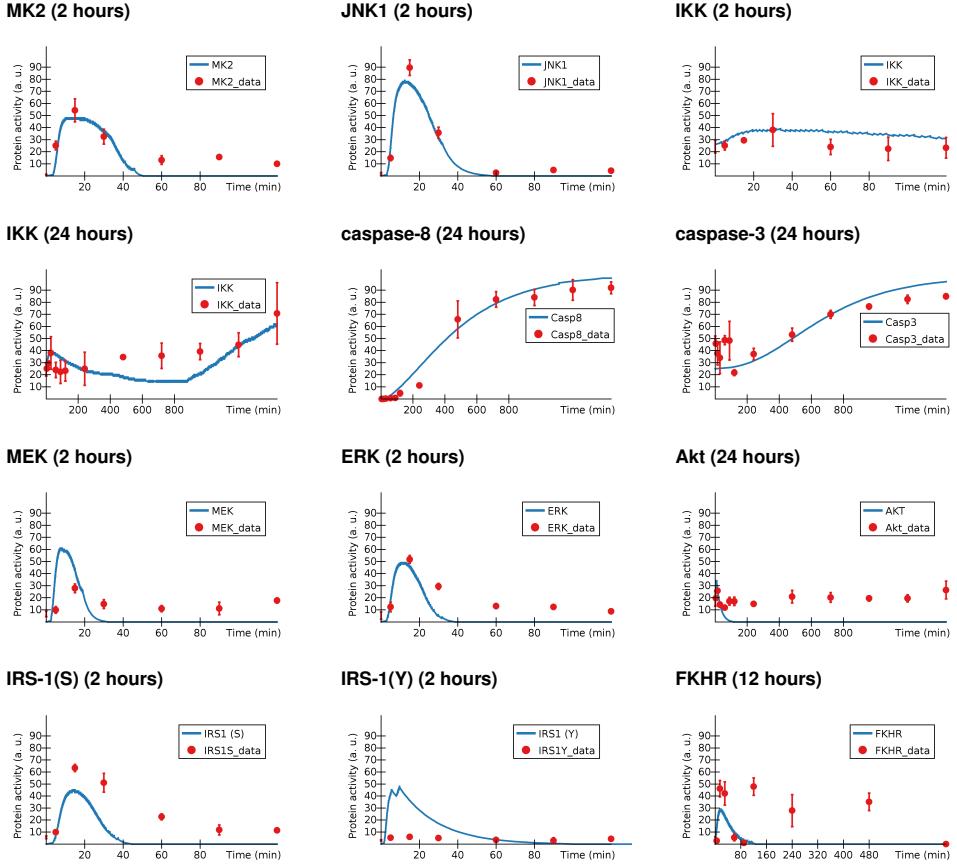


Figure S13: Comparison between the ANIMO model in Figure 6A and experimental data. Treatment condition: 100 ng/ml TNF α . In order to ease the comparison for earlier responses, the time span for those cases is less than 24 hours.

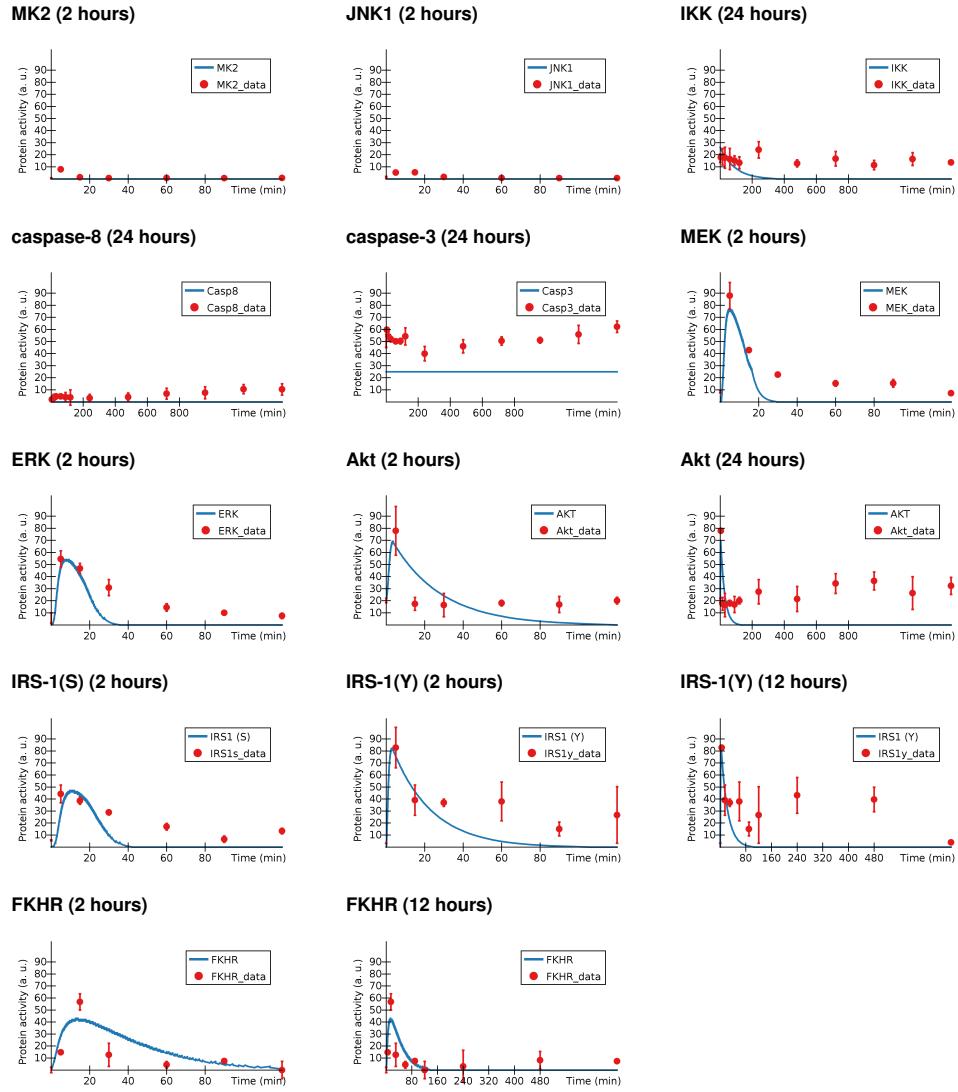


Figure S14: Comparison between the ANIMO model in Figure 6A and experimental data. Treatment condition: 100 ng/ml EGF. In order to ease the comparison for earlier responses, the time span for those cases is less than 24 hours.

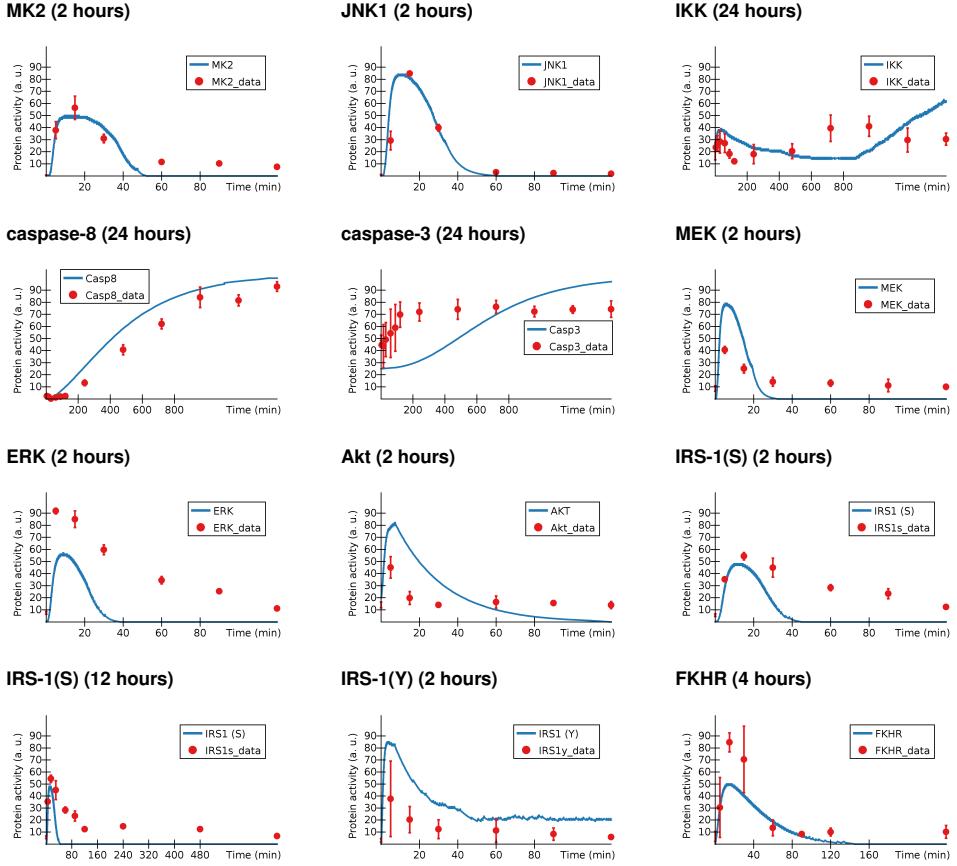


Figure S15: Comparison between the ANIMO model in Figure 6A and experimental data. Treatment condition: 100 ng/ml TNF α + 100 ng/ml EGF. In order to ease the comparison for earlier responses, the time span for those cases is less than 24 hours.