Systems biology of apoptosis

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Abstract

New approaches are required for the mathematical modelling and system identification of complex signal transduction networks, which are characterized by a large number of unknown parameters and partially poorly understood mechanisms. Here, a new quantitative system identification method is described, which applies the novel concept of 'Sensitivity of Sensitivities' revealing two important system properties: high robustness and modular structures of the dependency between state variables and parameters. This is the key to reduce the system's dimensionality and to estimate unknown parameters on the basis of experimental data. The approach is applied to CD95-induced apoptosis, also called programmed cell death. Defects in the regulation of apoptosis result in a number of serious diseases such as cancer. With the estimated parameters, it becomes possible to reproduce the observed system behaviour and to predict important system properties. Thereby, a novel regulatory mechanism was revealed, i.e. a threshold between cell death and cell survival.

1 Systems biology: paradigm shift from reductionism to holism in biology? The whole is greater than the sum of its parts

It is hard to believe that only 40 years ago Watson and Crick discovered the structure of DNA. Their work was based on a number of studies dating back to the work of Oswald Avery, Erwin Chagraff, and others that discovered the four letters A,C,G,T of the DNA and Chagraff's rule that the amount of nucleotides followed a simple relationship. Since the discovery of the DNA biology was mostly oriented towards a reductionist approach. Triggered by modern high-throughput sequencing technologies the code of life was deciphered letter-by-letter, word-by-word. It is now well known that the early hope of the genomics age could not be met that knowledge of the genetic code would help to comprehensively understand complex biological processes from the function of genes to the pathological mechanisms of genetic diseases such as cancer. Thus, the mistrust in the worldwide sequencing projects by Chagraff, who ironically paved the way for the later discovery of the DNA, has been fulfilled: "Niemand hat uns je gelesen, niemand wird uns je lesen" (Nobody has ever read us, nobody will ever read us). After almost two decades of genome research the leitmotif of reductionism that life is just

chemistry and physics and that once we have found the smallest components of life we will be able to understand the whole is widely rejected.

The obvious limitations of the reductionist's approach to biology has led to a revival of holistic approaches in biology. The approach to biology using the tools of systems engineering is not new (Bertalanffy 1973). In the first half of the last century, Jan C. Smuts laid the foundation for a holistic approach in natural sciences that has even inspired philosophers in their reflection of nature (Smuts 1926). However, success of a holistic approach has only become possible in the postgenomic era since the reductionists have provided information on the basic building blocks of many important cellular processes in a quantitative form. Accordingly, it is probably not correct to name the transition from reductionism to holism a paradigm shift in biology. Before the components of life were deciphered a holistic approach to biology was condemned to be restricted to a mystic holism that was prevalent at the beginning of the 20th century.

With the help of the reductionism, systems biology has now become an analytically rigorous discipline that aims at a description of complex biological processes by mathematical models. Systems biology can, thus, be considered a new discipline in biology that puts the theoretical foundations of system level dissection of living matter into the context of modern high-throughput quantitative experimental data, mathematics and *in silico* simulations in order to gain a holistic view on the complex workings of life. In this chapter, we will describe a mathematical model of programmed cell death called apoptosis. Motivated by this fundamental process prevalent in almost all higher organisms we developed a variety of modern methods for systems biology that are required to tackle complex biochemical pathways involved in cellular signalling.

2 Modelling signal transduction networks

A better understanding of signal transduction networks is one of the most challenging areas in systems biology. Cells show information processing by the biochemical interaction between molecules. Signals of external stimuli are, for example, passed into the nucleus to regulate gene expression, resulting in proliferation, mitosis (nuclear division), changes in metabolism or cell death (Alberts et al. 2002). Interactions like phosphorylation, exchange of smaller molecules, binding or cleavage, are the fundamental mechanisms, which form the signal transduction networks. Complexity arises from the huge number of different molecules and interactions between them. In eucaryotic cells, for example, the steadily growing number of known signalling molecule species is in the order of magnitude of 10^4-10^5.

Different methods of transcribing signal transduction networks into the language of mathematics exist. Dynamic pathway models are constructed using a diversity of mathematical and computational methods. Petri Nets (Reisig 1985) are well suited to describe the state transition process of distributed systems. In agent-based approaches and cellular automata (Wolfram 1994), macroscopic system

properties emerge from the individual properties of the single entities, interacting with each other. Other methods originate from the analysis of biochemical systems ranging from the examination of steady states and flux modes to a large variety of control theories (Kell and Westerhoff 1986; Heinrich and Schuster 1996; Schilling et al. 1999). More recently, theoretical models for describing the signalling behaviour on system levels have been developed, using modular approaches (Kitano 2002; Csete and Doyle 2002; Lauffenburger 2000). Loosely speaking, simulation of signal transduction networks is either based on discrete models describing signalling as information processing, or on continuous models, where the information flux is modelled by a biochemical control system. In the latter approach, which goes back to the pioneering work of Garfinkel and Hess in the mid 60s (Garfinkel and Hess 1964; Garfinkel 1968), the reaction network is translated into a system of ordinary differential equations (Bhalla and Iyengar 1999; Mendes 1997). Today, there is a variety of sophisticated simulation methods to analyze complex biochemical reaction systems (Mendes 1993; Tomita et al. 1999; Sauro and Fell 1991).

3 CD95-induced apoptosis

Programmed Cell Death, called *apoptosis*, is the natural and controlled death of cells, in which the cell and its nucleus shrinks, condenses, and fragments (Alberts et al. 2002; Evan and Littlewood 1998). Apoptosis is one of the most complex signalling pathways and an essential property of higher organisms. Defects in apoptosis result in a number of serious diseases such as cancer, autoimmunity, and neurodegeneration (Krammer 2000; Peter and Krammer 2003). To develop efficient therapies, fundamental questions about molecular mechanisms and regulation of apoptosis remain to be answered. Apoptosis is triggered by a number of factors, including UV-light, γ-radiation, chemotherapeutic drugs, growth factor withdrawal ('death by neglect'), and signalling from the death receptors (Nagata 1999; Ashkenazi and Dixit 1999). Apoptosis pathways can generally be divided into signalling via the death receptors at the membrane (extrinsic pathway) or the mitochondria (intrinsic pathway). Both pathways imply *caspases* as effector molecules (Salvesen 2002). Caspases belong to the family of proteases, which are enzymes that degrade proteins by hydrolyzing some of their peptide bonds (Thornberry and Lazebnik 1998). Caspases mostly exist in their inactive proforms (procaspases) and become active after getting cleaved. Various caspases are involved in both the initiation of the apoptotic process and the execution of the final apoptotic program. CD95-induced apoptosis is one of the best-studied apoptosis pathways. A detailed overview on this mechanism is for example given in Krammer (2000) and Danial and Korsmeyer (2004).

3.1 The CD95-receptor and the DISC

CD95 is a member of the death receptor family, a subfamily of the TNF-receptor superfamily (Nagata 1997). Crosslinking of the CD95-receptor either with its natural ligand CD95L or with agonistic antibodies such as anti-APO-1 induces apoptosis in sensitive cells. Upon CD95 stimulation with CD95L or anti-APO-1, the Death-Inducing Signalling Complex (DISC) is formed. The DISC consists of oligomerized CD95, the death domain-containing adaptor molecule FADD, pro-caspase-8, procaspase-10 and c-FLIP. The interactions between the molecules in the DISC are based on homophilic contacts. The death domain (DD) of CD95 interacts with the DD of FADD, while FADD interacts with procaspase-8 via the so-called death effector domain. Once the DISC is formed, procaspase-8 is autocatalytically cleaved: two procaspase-8 molecules bound at the DISC form the intermediate product p43/p41, followed by generation of an active caspase-8 complex p18/p10 (Lavrik et al. 2003). This process can be inhibited by c-FLIP, which binds to the DISC in various ways and blocks the latter mechanism (Krueger et al. 2001).

3.2 The caspase cascade

After formation of active caspase-8, the apoptotic signalling cascade starts. Caspase-8 cleaves and activates caspase-3 and -7; caspase-3 itself activates caspase-6, which again activates caspase-8, thereby, establishing a self-amplifying activation loop. Caspase-3, -6, and -7 are involved in the execution of the death process, for example, the chromosomal degradation of DNA and, therefore, called *executioner caspases*, whereas the others, responsible for transferring the death signal, are referred to as *initiator caspases*. The DNA degradation plays an important role in the cell death process. It is started after ICAD gets cut off the CAD-ICAD complex by caspase-3 and -7, thereby, terminating the inhibition of CAD, which directly fragments the DNA (Nagata 1999). In parallel, PARP, a molecule, which repairs broken DNA strands is cleaved by executioner caspases as well. Once the DNA fragmentation process is triggered, a complete degradation of the cell starts irreversibly.

3.3 Type I versus type II cells and the regulation of apoptosis

Two different CD95-signaling pathways are established in different cell types (Schmitz et al. 1999; Scaffidi et al. 1998). Type I cells are characterized by intensive DISC formation and mitochondria independent caspase-3 activation. In Type II cells, the formation of the DISC complex is reduced and the activation of caspase-3 occurs downstream of the mitochondria: the active form of caspase-8 cleaves Bid, followed by translocation of the cleavage product tBid to mitochondria, which results in the release of cytochrome-C. Subsequently, apoptosome, a complex consisting of Apaf-1, cytochrome-C and caspase-9, is formed (Zou et al.

2002), leading to the activation of caspase-9, which then activates caspase-3, triggering the subsequent apoptotic events. Here, a feedback loop is established by caspase-2: it is activated by caspase-3 downstream of mitochondria and it cleaves Bid, which in response leads to mitochondrial cytochrome-C release.

Furthermore, CD95-induced signalling is influenced and regulated by many other molecules, which mostly inhibit or amplify the apoptotic process, like XIAP, IAP1/2 and survivin (inhibitors of caspase-3,-7,-9) (Salvesen and Duckett 2002) or the BCL-2 family (Chao and Korsmeyer 1998) consisting of pro-apoptotic (e.g. Bak, Bax) and anti-apoptotic (e.g. BCL-XL) members, regulating the critical cytochrome-C release. An overview about the molecule families, which play an important role in this pathway, is given in (Westphal and Kalthoff 2003).

4 Mathematical models of apoptosis

Despite the steadily increasing number of biological papers on apoptosis, mathematical models of this complex process are very scarce. In a first attempt to theoretically describe apoptotic signalling, a mathematical model including more than 20 reactions was proposed (Fussenegger et al. 2000). However, this model was based on *ad hoc* fixed parameters and, thus, its potential for understanding the regulation of apoptosis remains very limited. More recently, the caspase cascade was translated into a reductionist model and analytical mathematical methods were applied to evaluate the system behaviour within a wide range of parameters (Eissing et al. 2004). System identification methods like parameter estimation (Deuflhard 1983) based on reliably measured time series of data, as it has been successfully applied for chemical reaction system (Bock 1981), are suggested (Swameye et al. 2003). However, system identification is severely impaired by the high number of unknown parameters and the *curse of dimensionality*. Curse of dimensionality refers to the problem that the space of possible parameter value sets grows exponentially with the number of unknown parameters impairing the search for the globally most probable parameter values. The high number of unknown parameters is mainly due to the complexity of signal transduction networks and the absence of reliable quantitative information about the underlying mechanisms. Consequently, data-based studies are typically restricted to small models in which the biochemical interactions are well understood.

Despite the ever-increasing number of studies on CD95-induced apoptosis, a systemic understanding of this complex signalling pathway is still missing. For this reason, we reconstructed the network topology of CD95-induced apoptosis by critically searching databases (Schacherer et al. 2001) and the literature. Molecules and reactions directly or indirectly interacting with the main components of this pathway were incorporated, leading to a network topology with more than 60 molecules and about 100 interactions. This complexity cannot be matched by experimental data at present.
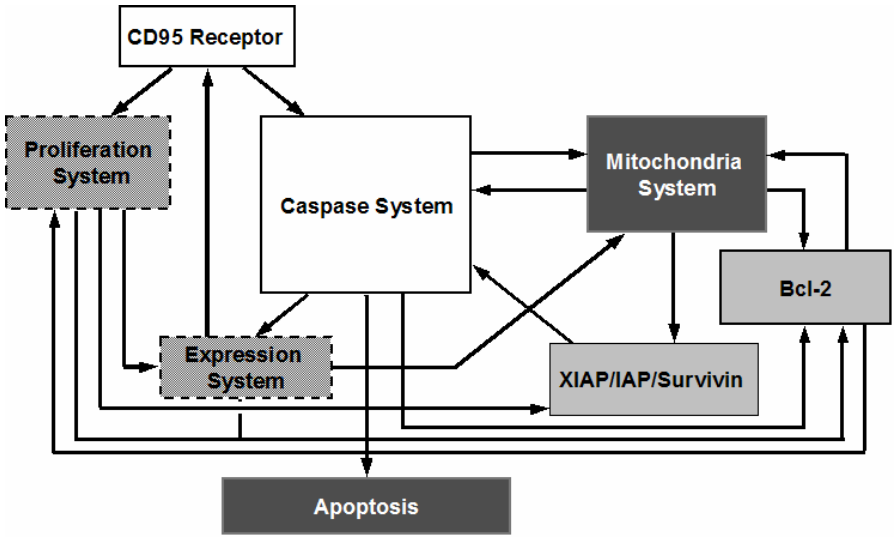
Fig. 1. Model of CD95-induced apoptosis combining subsystems of different information levels. The grey scale level of the boxes corresponds to the information quality (for details see text).

To tackle the high dimensionality of such systems, we developed an approach to large-scale modelling of signal transduction networks, which combines three methods (Bentele et al. 2004). Information on different levels of quality are combined in a unified form leading to the 'Structured Information Models'. Then, a global approach to parametric sensitivity analysis is introduced by which the dimensionality of the system can be significantly reduced. On this basis, a cluster-based and sensitivity-controlled parameter estimation method is set up.

5 Structured information models - The information problem

Information about signal transduction networks can be divided into different levels of information quality (Fig. 1). In most cases, interactions between two molecules are known on the semantic level only (e.g. A inhibits B or A activates B), thereby providing a network topology. For some well-investigated molecules and interactions the biochemical mechanism is also known (e.g. enzymatic process, formation of complexes) allowing quantitative modelling. However, information about the underlying biochemical parameters like reaction rate constants, Michaelis-Menten constants or dissociation rates, is mostly missing. Even if quantitative information is available, its usability is limited if it refers to different experimental settings, cell types or states of cells.

5.1 Network decomposition based on information quality

To reduce the complexity of the model without sacrificing essential components of the network, subsystems of different information qualities were identified and incorporated: subsystems mainly consisting of interactions with well-understood biochemical mechanisms are modelled as chemical reaction systems, whereas all others are modelled as 'black boxes', defined by their experimentally observed input-output behaviour. The subsystems are identified according to the following criteria:

- The input/output behaviour should be measurable.
- The number of input/output variables should be low.
- Subsystems should represent real functional systems (e.g. mitochondria).
- The information within one subsystem should be on the same level.

Notably, the black boxes do not assume knowledge of the exact underlying mechanisms. Instead, they reproduce the behaviour of the respective subsystems in a simplified way. Moreover, minimum sets of state variables and 'effective' parameters are introduced that do not necessarily correspond to molecule concentrations and biochemical parameters. As a consequence, the number of unknown parameters can be drastically reduced a priori.

Note that this concept is motivated by a typical situation in system identification of biochemical networks: due to missing information and limited experimental data, the models should be small and restricted to those network parts which are well understood. On the other hand, parts of biochemical networks can generally not be regarded independently of their environment. Thus, black boxes are introduced to reproduce the relevant effects of the surrounding network parts on a mechanistically well-understood subsystem, rather than for system identification of the surrounding network itself, for which the data basis would be missing anyway.

The degradation process of CD95-induced apoptosis is, for example, modelled as a decay function depending on a virtual state variable describing the 'apoptotic activity', which is influenced by executioner caspases. This function approximates the experimental observations, thereby, requiring a few parameters only.

The decomposition of the complete system into subsystems is an iterative and adaptive process. Based on new information, a subsystem might be split into further subsystems. A great advantage of the so-obtained 'Structured Information Models' is that it combines heterogeneous information in one model instead of dealing with isolated models.

5.2 Combined model definition

For the mathematical description of the mechanistic part of structured information models, interactions are modelled based on reaction rate equations. The state of a system is described by the concentration of l relevant signal transduction molecules $(x_1, \ldots, x_l)$. The reaction rates depend on these concentrations and also on biochemical parameters $(\Phi_1, \ldots, \Phi_r)$ like binding constants. To describe the tem-

poral behaviour, a system of Ordinary Differential Equations is generated as linear combinations of the reaction rates v_j:

$$dx_i / dt = \sum_{j=1}^{n} \nu_{ij} v_j(\vec{x}, \Phi_1,...,\Phi_r),$$

where ν_{ij} denotes the stoichiometric matrix linking the reactions with the molecules affected. Given the initial concentrations, the time evolution of the reaction system can be propagated using an ODE Solver (Deuflhard and Bornemann 2002). Note that the initial concentrations $x_i(t=0)$ are often unknown and are considered unknown parameters as well.

Black boxes are defined by their experimentally observed input-output behaviour. Additional state variables $(x_{l+1}, …, x_m)$ that do not necessarily correspond to molecule concentrations and additional parameters $(\Phi_{r+1}, …, \Phi_s)$ can be introduced. The q-th black box is represented by the functions $f_i^q(x_1, …, x_m, \Phi_1, …, \Phi_s, t)$, $i=(1,…,m)$ that describe the changes of molecule concentrations and other state variables it affects. Boundary conditions like conservation laws have to be taken into account. Thus, the combination of subsystems modelled as chemical reaction systems and black boxes leads to an ODE system, which reads

$$dx_i / dt = \sum_{j=1}^{n} \nu_{ij} v_j(\vec{x}, \Phi_1,...,\Phi_r) + \sum_{q=1}^{N} f_i^q(\vec{x}, \Phi_{r+1},...,\Phi_s) \text{ for } i=(1,...l),$$

$$dx_i / dt = \sum_{q=1}^{N} f_i^q(\vec{x}, \Phi_{r+1},...,\Phi_s) \text{ for } i=(l+1,...m),$$

where n denotes the number of reactions and N the number of black boxes.

5.3 The model of CD95-induced apoptosis

A quantitative model of CD95-induced apoptosis was derived from the network topology, thereby, taking into account the information on all underlying mechanisms (Fig. 2). A detailed reaction mechanism was established for the DISC- and the caspase-system. The mechanisms at the DISC are largely described by elementary reactions, whereas the caspase cleavage process is considered an enzymatic process (e.g. Stennicke and Salvesen 1999). In principle, these interactions could have been modelled in a more simplified way. The influence of caspase-3 on Bid, for example, could have been modelled directly thereby using 'effective' parameters without accounting for the intermediate caspase-2 cleavage. However, since time series about the concentration of caspase-2 have been available, this molecule was kept in the system in order to gain more information for system identification. In contrast, many molecules and interactions with equivalent properties were replaced by 'effective' molecules and interactions based on the analysis of parameter sensitivity correlations. The molecules XIAP, IAP1/2, survivin and their interactions with caspase-3,-7, and -9 are for example reduced to one 'effective' molecule

called IAP and the interactions are described by effective binding parameters. Details about the model are given in Bentele et al. (2004).

5.4 Black boxes

Two subsystems with a significant influence on the signalling system were identified. The death process is described by the degradation of all molecules. It is modelled as an exponential decay-function dependent on the executioner caspase activity. The cytochrome-C release of the mitochondria is based on experimental observations (Goldstein et al. 2000), which describe a complete release within 5 minutes as soon as Bid reaches a certain level in comparison to Bcl-2/Bcl-XL.

To model the degradation process, a virtual state variable called x_{apop} was introduced, which quantifies the 'apoptotic activity', by which the strength of the final death process is characterized. It is assumed that the velocity of cell degradation is directly influenced by this activity. It is also assumed that the activity itself is caused by active caspase-3, -6, and -7 and that the increase of the activity runs in parallel to the experimentally observable PARP cleavage. Thus, x_{apop} represents the activity of the apoptotic processes triggered by executioner caspases. The degradation process is modelled by a decay function depending on x_{apop}, thus, decreasing the concentration of all molecules of the pathway.
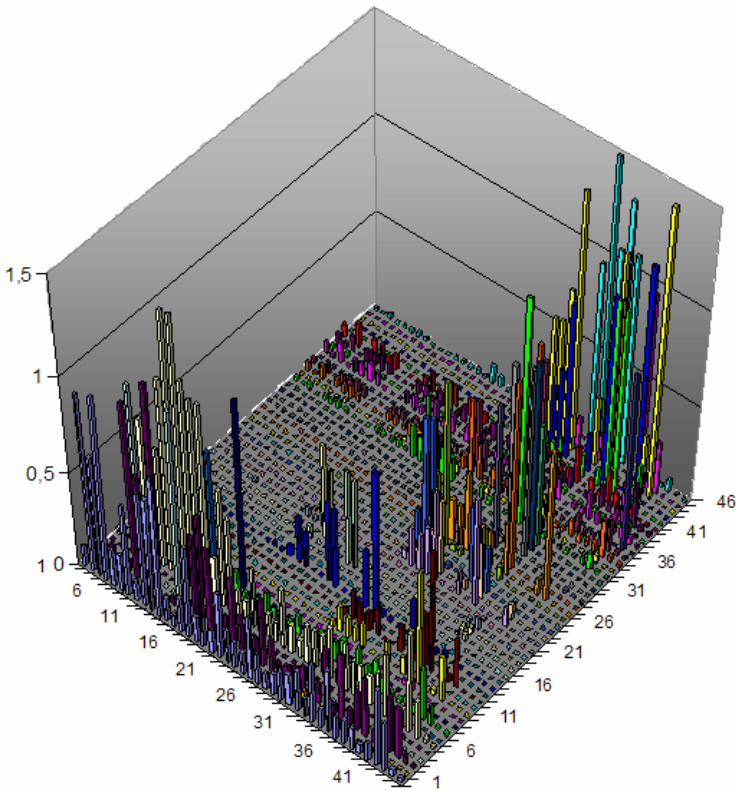
5.5 Experimental data

A set of experiments to measure time series of concentrations of 14 different molecules and complexes (see framed molecules in Fig. 2) after activation of CD95-receptors was designed. Cells were stimulated with different concentrations of agonistic anti-APO-1 antibody, also referred to as ligands in the following, for various periods of time (from 5 minutes to 4 days). Each sample was evaluated by three independent approaches. See Bentele et al. (2004) for experimental details. In a first set of experiments, time series were measured for a 'fast' activation scenario with an oversaturated ligand concentration corresponding to more than one ligand per CD95-receptor. To gain additional information about the system's dynamic, several experiments with much lower ligand concentrations were performed resulting in a slower activation of apoptosis.

6 Model reduction by sensitivity analysis

The above-described model consists of 41 molecules and molecule complexes, 32 reactions, and 2 black boxes. It contains more than 50 missing parameters. Therefore, it is still too complex for reliable system identification and requires further reduction of complexity considering the limited number of data points. Here, we developed an approach for model reduction by sensitivity analysis.

Fig. 2. Structured information model of CD95-induced apoptosis. In the mechanistic part (DISC, caspases, IAP), interactions are modelled as elementary reactions including competitive inhibitions and enzymatic reactions. Receptors are activated by ligands initiating the DISC formation. After binding to the DISC binding site (DISCbs), procaspase-8 is cleaved (initiator caspase), followed by the activation of executioner caspases (3, 6, 7). PARP cleavage was chosen as experimental end-point of the pathway. The mitochondria and the degradation process, which influences all molecules, are modelled as black boxes defined by their input-output behaviour. Experimental time series were measured for the molecules framed in red.

6.1 The sensitivity matrix

Parametric sensitivity analysis determines the changes of the system behaviour as a result of parameter variations (Varma et al. 1999). In a system with m state variables (x_1, …, x_m) and n parameters (Φ_1, …, Φ_n), the relative sensitivities $s_{ij} = (\partial x_i / x_i) / (\partial \Phi_j / \Phi_j)$ describe the relative changes of the state variables as a result of changes of the parameters. In the signal transduction systems, the state variables mostly correspond to molecule concentrations. Note that the sensitivities are time-dependent ($s_{ij} = s_{ij}(t)$) and that the time points, for which sensitivities are

Fig. 3. Sensitivity Matrix. The sensitivity matrix elements $\hat{s}_{ij}$ show the relative changes of each state variable i (left to right), mostly referring to molecule concentrations, with respect to relative changes of each parameter j (front to back). The indices refer to the model definition of (Bentele et al. 2004).

computed, have to be chosen carefully. In *Metabolic Control Analysis* (Kell and Westerhoff 1986; Fell 1992) steady states depending on parameter variations are investigated, whereas in signal transduction systems, the transient behaviour is of high interest to analyse the regulation of a system. As a consequence, the complete time period, during which, for example, a signalling pathway is active and exhibits a dynamical behaviour, is relevant rather than a distinct time point:

$$\hat{s}_{ij} = \frac{1}{\Delta t} \int_{t_0}^{t_0 + \Delta t} s_{ij}(t)dt .$$

The time point t_0 corresponds to the start of the investigated scenario and Δt to the period, in which the system shows reactions to parameter variations. For the apoptosis system, t_0 is the time point at which the pathway becomes activated by CD95-ligands and the time interval ends when the cell is completely degraded.

A sensitivity matrix with elements $\{\hat{s}_{ij}\}$ is visualized in Fig. 3, showing two important facts:

- Sensitivities are low in general indicating high robustness and a lower 'effective' dimensionality of the parameter space since many parameters have only little impact on most molecules.
- Apparently, clusters can be identified that contain a subset of molecules, whose concentrations depend on a subset of parameters only. This inherent system property is an important feature for further modularization.

6.2 Local versus global sensitivity analysis

Usually, sensitivities of huge models can be computed numerically only and a general relation between sensitivities $\hat{s}_{ij}(\vec{\Phi})$ and the parameter set $(\Phi_1, \ldots, \Phi_n)$, at which the sensitivities are determined, cannot be deduced analytically. Instead, sensitivities are determined for specific points in parameter space only and are, therefore, called *local sensitivities*. In this study, however, sensitivity analysis is used as an essential tool for model reduction, which is required for system identification. As a consequence, it has to be performed in a virtual experiment prior to determination of parameters (see stochastic approach to sensitivity analysis below).

Global sensitivity analysis, which provides information about sensitivities for the complete space of possible parameter values, is impaired by the high dimensionality of the parameter space. Although this situation is ubiquitous for complex biological systems, a general solution to this problem does not exist.

6.3 Stochastic approach to global sensitivity analysis

In a virtual experiment, sensitivity analysis is performed for a large number of randomly chosen points in parameter space within specified ranges. The ranges are defined for each parameter type (e.g. bimolecular reaction rate constants, initial concentrations, Michaelis-Menten constants, etc.), unless more precise information was available. Thereby, the concept of 'Sensitivity of Sensitivities' is introduced, which examines the robustness of sensitivities with respect to parameter variations. This is motivated by two important facts:

- Biological systems often keep their system properties constant, although they are subject to high parameter fluctuations (Barkai and S 1997; Meir et al. 2002; Alon et al. 1999) suggesting that at least some sensitivities are insensitive with respect to parameter fluctuations (low Sensitivity of Sensitivities).
- Structure and connectivity of biochemical networks suggests that the influence of some parameters on distant network regions is limited and that the corresponding sensitivities are extremely low - independently of the parameter values.
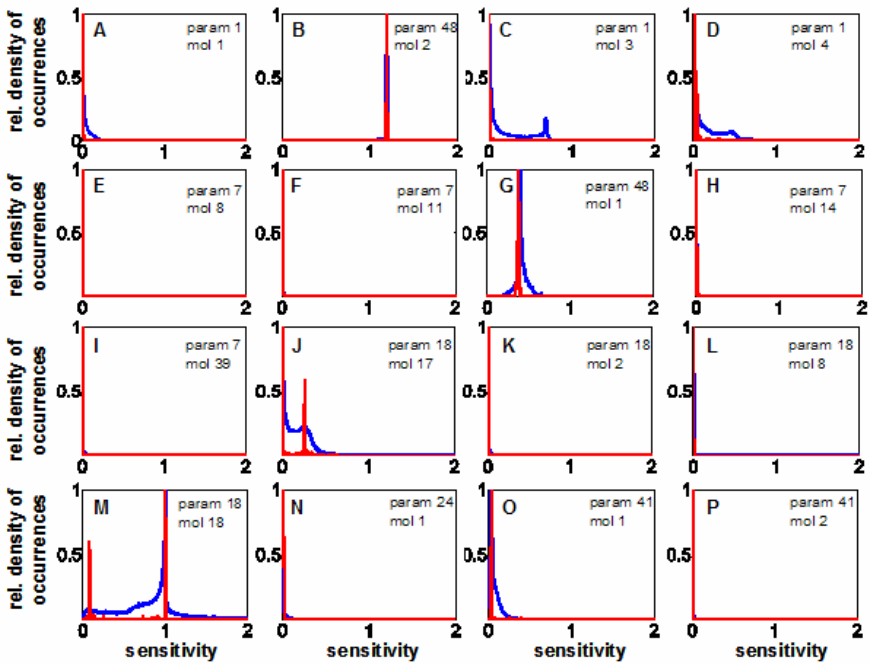
Fig. 4. Sensitivity Histograms: Sensitivity of Sensitivities. Each box shows a histogram for a specific sensitivity $\hat{s}_{ij}$, computed for a large number of randomly chosen points in parameter space. Parameter and molecule indices refer to the model definition of Bentele et al. (2004). The X-axis represents the relative sensitivity values from 0 to 2 and the Y-axis corresponds to the density of occurrences. The blue plot shows the uniformly weighted distribution of sensitivities, whereas for the red one, each contribution was weighted with the Boltzmann factor, resulting in sharper and sometimes slightly shifted peaks. The histograms are exemplary for all matrix elements $\{\hat{s}_{ij}\}$. Typically, histograms show clear peaks close to zero - an important property for further modularization. However, distributions like C, D, J, or M are not informative for modularization.

The distribution of the computed sensitivities $\hat{s}_{ij}(\vec{\Phi})$ for a large number of different points in the parameter space, $\{\vec{\Phi}^q\}$, are plotted in form of histograms for each sensitivity to show their distribution (Fig. 4). The histograms are generated in two different ways. In a first approach, all random parameter sets are equally weighted, independent of how much the resulting systems dynamics deviate from the real system. As an extension, information from experimental data was incorporated by introducing a Boltzmann factor: based on experimental time series of molecule concentrations, an objective function E_q was calculated for each parameter set $\vec{\Phi}^q$ based on the differences between the experimental and simulation data:

$$E_q = \sum_{i,k} \frac{(x_{ik}^{\exp} - x_i^{\mathrm{mod}el}(t_k,\vec{\Phi}^q))^2}{\sigma_{ik}^2} \ ,$$

$x_{ik}^{\exp}$: experimental values of the concentration of molecule i at time t_k,

$x_i^{\mathrm{mod}el}(t_k,\vec{\Phi}^q)$: simulated values of the concentration at time tk for parameter set $\vec{\Phi}^q$,

σ_{ik} : standard deviation according to experimental data,

$\{t_k\}$: time points of experimental data.

Then, it is assumed that the probability p_q that a system with parameter set $\vec{\Phi}^q$ produces the experimental output $\{x_{ik}^{\exp}\}$ follows a Boltzmann distribution with the objective function as energy term:

$p_q \propto \exp(-E_q / kT)$

The assumption is motivated as follows: considering Gaussian random measurement errors, each characterized by σ_{ij}, the probability p_q can be written as product (Gershenfeld 1999)

$$p_q \propto \prod_{i,k} \exp- \frac{1}{2} \frac{(x_{ik}^{\exp} - x_i^{\mathrm{mod}el}(t_k,\vec{\Phi}^q))^2}{\sigma_{ik}^2} \ ,$$

which is equivalent to the upper Boltzmann distribution using the definition of E_q. For generation of sensitivity histograms, the Boltzmann factor was used as weighting factor: instead of counting the number of parameter sets with sensitivities within a certain sensitivity interval, the contribution of each parameter set $\vec{\Phi}^q$ is given by $\exp(-E_q / kT)$. Thereby, the statistical impact of sensitivities for parameter sets that are more consistent with the experimental observations are amplified.

Additional information can be gained by varying the factor kT. If a sensitivity value is insensitive with respect to parameter variations, particularly within the subspace of possible solutions (areas in parameter space with a low objective function), 'cooling down' the system by decreasing kT will result in histograms with a much sharper peak. Sensitivity histograms showing more than one distinct peak when kT is decreased indicate that the respective sensitivity strongly depends on the exact parameter set within the parameter subspace of probable solutions.

6.4 Sensitivity of sensitivities

The most crucial outcome of the 'global' sensitivity analysis approach presented here is the fact that sensitivities of sensitivities are extremely low in most cases, as shown in Fig. 4 for some exemplary sensitivity histograms. Obviously, most distributions show distinct and narrow peaks, indicating high robustness towards

large variations of the parameter values. Whenever a sensitivity value $\hat{s}_{ij}$ is close to zero, an extremely sharp peak indicates that the state variable x_i is likely not to be influenced by parameter Φ_j, regardless of the exact parameter value set. By introduction of the Boltzmann weighting (Fig. 4, red line), most peaks become even sharper. Only in few cases, more than one peak remains or the distribution broadens as a consequence of the 'cooling', indicating that the system runs in different modes for certain areas of the parameters space.

As a consequence of the low sensitivity of sensitivities, subsets of parameters can be determined, which are unlikely to influence certain state variables. Considering the high number of sensitivities with $\hat{s}_{ij} \approx 0$, this step is crucial to reduce the system's dimensionality even without knowledge of the true parameter values. Thus, this method provides a basis for high-dimensional parameter estimation.

7 Sensitivity-controlled parameter estimation

Parameters are estimated based on experimental time series of measurable molecule concentrations using the maximum likelihood estimation (Gershenfeld 1999), which leads to the least-square problem:

$$E(\vec{\Phi}) = \sum_{i,k} \frac{(x_{ik}^{\exp} - x_i^{\mathrm{mod}\,el}(t_k, \vec{\Phi}))^2}{\sigma_{ik}^2} \to \min.$$

Thus, the objective function E, defined as the sum of squares of differences between experimentally measured and simulated molecule concentrations, divided by the standard deviation in order to lower the impact of experimentally less reliable values, has to be minimized. A review about methods commonly applied for biochemical reaction systems is given in Mendes and Kell (1998).

7.1 Cluster-based parameter estimation

This approach takes advantage of the fact that clusters of state variables and parameters can be identified in such a way as to have subsets of state variables whose temporal behaviour depends on a subset of parameters only. Considering the sensitivity matrix $(\hat{s}_{ij})$ of m state variables, n parameters, and an average sensitivity $\langle s \rangle$, it is assumed that sensitivities fulfilling $\hat{s}_{ij} < \Theta$, $\Theta = \Theta_s \cdot \langle s \rangle$, where Θ_s is a low relative threshold (e.g. $\theta_s = 0.01$), indicate that the respective state variable and be considered to be independent of the respective parameter. Whenever a high percentage of sensitivities fulfil this property – and this is the typical case in large signal transduction systems – clusters of the remaining above-
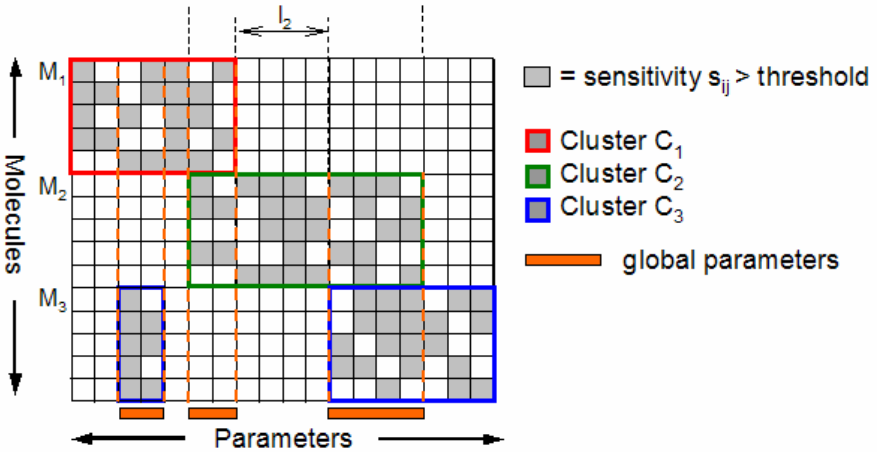
Fig. 5. The sensitivity matrix exemplifies the clustered dependence of state variables (vertical axis) and parameters (horizontal axis). Red bars indicate the global parameters on which state variables of more than one cluster are dependent.

threshold sensitivities can be established after reordering the rows of the sensitivity matrix. A cluster C_q is defined as the sub-matrix of the sensitivity matrix (see Fig. 5):

$$C_q = (\hat{s}_{ij}), \text{ i } \epsilon \ \{M_q,\ldots,M_{q+1}-1\},$$

$$j \ \epsilon \ \{1, \ldots, n \mid \hat{s}_{ij} \geq \Theta \ \text{ for at least one i } \epsilon \ [M_q,\ldots,M_{q+1}-1] \ \},$$

M_q = index of first molecule of cluster q.

Let C_q denote the q-th cluster and l_q the number of 'local parameters' of C_q. Local parameters are defined as those parameters whose sensitivities are below threshold Θ for all state variables outside the cluster they belong to. If all parameters of one cluster influence state variables of the same cluster only, they can be estimated independently from all other clusters leading to a parameter estimation of much lower dimensionality. In general, this is, however, not the case and a cluster-wise estimation would determine the same parameters in the context of different clusters leading to inconsistencies. Therefore, parameters are split into local and global ones: a parameter, on which state variables of more than one cluster depend on, is called a 'global parameter'. Consequently, the total number of global parameters is given by $g = n - \sum l_q$.

Then, a hierarchical approach was designed, in which parameter estimation is performed on two levels. On the upper level, global parameters are estimated by optimising all clusters: for each cluster, parameter estimation is recursively called at the lower level. Thereby, the associated objective function is based on the estimated parameters of the single clusters (lower level). Thus, on the lower level, all remaining (local) parameters are estimated separately for each cluster, depending on the values of the global parameters proposed by the algorithm of the upper

level, but independent of the parameters of all other clusters. This approach significantly reduces the dimensionality from the number of all parameters n to the sum of the number of global parameters g and the maximum number of local parameters ($g+\max(l_q)$). If $W(d)$ denotes the cost for a parameter estimation of dimension d, the total cost W_{tot} of the algorithms can be compared by

Unclustered algorithm: $W_{tot} \sim W(n)$,

Clustered algorithm: $W_{tot} \sim W(g) \bullet \sum W(l_q)$.

Since $W(d)$ strongly increases with dimension d - also known as curse of dimensionality, some methods even show an exponential relationship (Mendes and Kell 1998) - this relation reflects a drastic reduction of computational cost whenever the relative number of global parameters g/n is low, which is typically the case in signal transduction systems. To optimize the computation time for very large systems, it would be adequate to choose the clusters so that $g +\max(l_q)$ is minimized.

Since the clustering is applied as a basis for efficient parameter estimation and thus before parameter values are given, there is no basis to compute a sensitivity matrix, which is, however, required for the clustering. A solution to this problem is given by the 'global' sensitivity analysis approach. Since the clustering method only requires information about which sensitivities are below threshold Θ, the sensitivity histograms are evaluated. The clustering is then based on neglecting those sensitivities, whose histograms indicate that the property $\hat{s}_{ij} < \Theta$ is likely to be fulfilled within the complete space of possible parameters.

7.2 Parameter estimation algorithm

In order to further reduce the dimensionality of the parameter estimation problem sensitivities, which are below the threshold only locally in parameter space, are taken into account by integrating a sensitivity-control in the parameter estimation algorithm. As a robust algorithm for nonlinear least-square problems, the Levenberg-Marquardt method (Gershenfeld 1999) was chosen and combined with a multi-start algorithm. The ranges for the randomly initiated parameter values correspond to those used for sensitivity analysis. For adaptive sensitivity-control, local sensitivity analysis is performed after each iteration step. All those parameters, whose impact on the objective function is significantly below average are kept constant for the next step. This prevents the algorithm from being misguided by 'irrelevant' parameters. Since the distinction between relevant and irrelevant parameters is generally valid for a specific set of parameter values only, the sensitivities are recalculated after each iteration step.
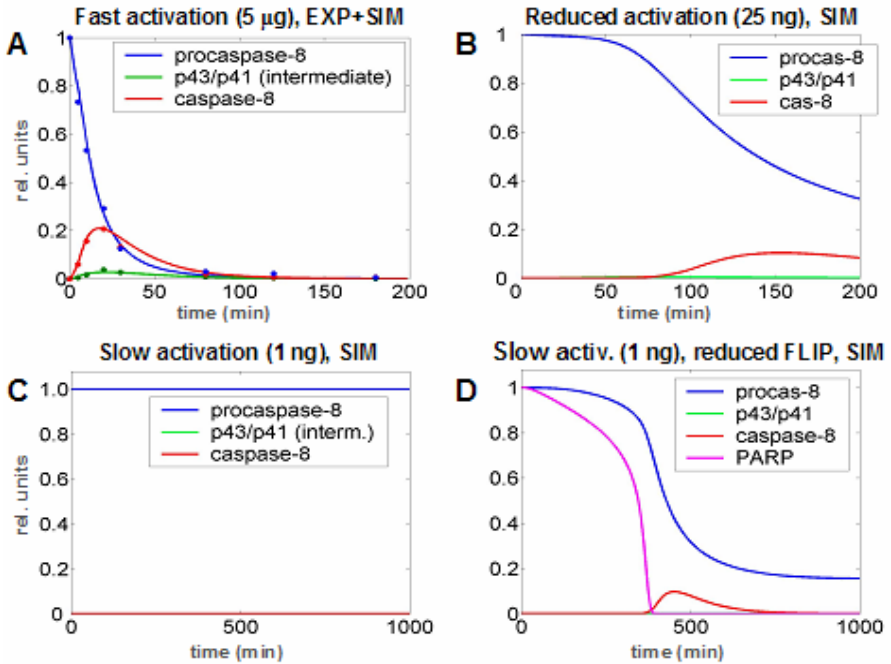
Fig. 6. A: Fast activation scenario: Parameter estimation led to a good fit between model simulations (solid lines) and experimental data (dots). Procaspase-8 is cleaved and active caspase-8 is formed resulting in the activation of executioner caspases, followed by cell death (data not shown). B: The simulation for much lower ligand concentration shows a delay, which is followed by a slow activation of caspase-8, also triggering the death process. C: For a below-threshold ligand concentration, the death process is completely stopped. According to simulation, active caspase-8 cannot be generated in a number sufficiently high to trigger apoptosis. D: Same activation scenario as in C with c-FLIP reduced by 75%. The simulation shows a slow and steady cleavage of procaspase-8, until executioner caspases are generated in a number sufficiently high to trigger the apoptotic process. Thus, the threshold can be shifted by varying the c-FLIP concentration, indicating the crucial role of c-FLIP in CD95-induced apoptosis.

8 Model simulation of apoptosis and experimental results

8.1 Parameter estimation based on multiple scenarios

In a first set of experiments, time series were measured for a 'fast' activation scenario achieved by 5 µg/ml anti-APO-1 corresponding to a ligand-receptor ratio of about 5:1. A good fit between model simulation and experimental data could be achieved reproducing the cleavage of procaspase-8 into its active form (Fig. 6A), followed by activation of the executioner caspases and cleavage of Bid and PARP.

To gain additional information about the system, activation scenarios with lower initial ligand concentrations were measured and the parameter estimation was based on these multiple conditions. It could be shown that the resulting parameters were capable of reproducing several activation scenarios (data not shown). To test the predictability of the model, simulations were then performed for scenarios with even lower initial ligand concentrations.

8.2 Threshold mechanism for CD95-induced apoptosis

The model predictions show that with decreasing ligand concentrations apoptosis is slowed down considerably, but cell death is still achieved (Fig. 6B). However, below a critical concentration corresponding to a ligand-receptor ratio of approx. $1:10^2$, apoptosis is completely stopped (Fig. 6C). This prediction was validated by experiments (Bentele et al. 2004).

   As a next step, the model was used to reveal the underlying mechanism of the threshold. Even for a below-threshold scenario, the number of ligands per receptor is sufficient to cleave procaspase-8, thereby, triggering all subsequent caspases. In the model, the caspase-8 cleavage capacity at the DISC is assumed to be proportional to the number of active CD95 receptors since the DISCs are supposed to remain active after cleaving procaspase-8 molecules. Consequently, it could be assumed that even for a very low ligand concentration apoptosis should not be stopped entirely, but would only be slowed down. Instead, the model simulations show that c-FLIP, which binds to the DISC and which competes with activation of caspase-8 (Krueger et al. 2001), is responsible for completely stopping the death process. According to the parameter estimation, there are many more CD95 receptors and procaspase-8 molecules than c-FLIP molecules. The cleavage rate of procaspase-8 is assumed to be dependent on the number of active receptors. Whenever c-FLIP binds to a DISC, the respective binding site is blocked. Therefore, the simulated scenario with subthreshold concentrations of activating ligands shows a steady decrease of active DISCs until all of them are blocked by c-FLIP. As a consequence, the simulation shows no significant generation of active caspase-8 as a result of the early and complete DISC-blockage. Thus, the c-FLIP mechanism identified in the model can be considered a switch, which blocks the activation of caspase-8 for signals (ligand concentrations) below a critical quantity and passes on the activation signal above this level. As a result, it was predicted that the threshold mechanism is highly sensitive to the concentration of c-FLIP (Fig. 6D). This prediction was also confirmed by experiments (Bentele et al. 2004).

8.3 Delay of apoptosis and point of no return

Another important model prediction addresses the system behaviour above the threshold, where the combination of the c-FLIP mechanism with the amplification loop does not lead to a steadily decreased caspase cleavage rate upon a decreased ligand concentration. Instead, the simulation shows that the caspase cleavage, the

amplification loop and the subsequent death process are delayed but not entirely stopped. As shown in Fig. 6B, there are no observable system changes for up to many hours after activation of the pathway. Then, the death process suddenly starts without any external stimulation of the system. This is due to an extremely slow increase of active caspase concentration, which reaches a critical level upon which the death process is triggered. Thus, for up to many hours, there maybe no phenotypic difference observed for cells, which are not (or insufficiently) stimulated by ligands. However, the death process is irreversibly triggered even for those cells and cannot be stopped anymore (point of no return). The predicted delays have also been verified by experiments.

9 Outlook

The investigation and analysis of complex biological networks and mechanisms in cells is probably one of the most challenging and fastest growing fields of science. The classic experimental approaches, which mostly focus on the investigation of molecule interactions in an isolated context and under specific experimental settings, cannot keep up with the steadily increasing number of potential interaction partners and the diversity and complexity of the real networks. The potential of entirely experimental approaches towards revealing network functionalities is, therefore, limited.

From a theoretical point of view, system identification of networks, characterized by an enormous complexity and a lack of information about the underlying mechanisms on both the qualitative and quantitative level, constitutes a new class of problems, which has not been sufficiently approached yet. Methods to describe complex networks in a qualitative way, for example, as scale free networks (Barabasi and Albert 1999), are promising for revealing general principles like robustness and fragility (Albert et al. 2000; Stelling et al. 2004). Their ability to describe the real system behaviour is, however, limited since single interactions are not quantitatively considered although the properties of real biological networks are often related to very concrete features of single mechanisms.

The emerging field of systems biology, which has been recently started with great enthusiasm, is an important step towards the investigation of biological processes on systems level. However, it has not been proved yet that it also provides qualitatively new methods addressing the new dimension of complexity. Instead, approaches from the field of engineering and numerical mathematics have been widely applied to biological systems, especially to subsystems, where they are expected to be well-suited and promising. Moreover, the majority of studies address specific applications with the goal of answering specific biological questions. Although this is a first important step to establish systematic and theoretical methods in the field of cell biology, it has to be followed by a second, much more challenging step concerning the development of new theoretical approaches for the description and system identification of complex and highly underdetermined biological systems.

It should be noted that numerical parameter identification methods originating from disciplines, where experimental data are generated in huge amounts and with high precision and where the number of unknown parameters is low, cannot be expected to be appropriate for the completely different situation in cell biology. Here, uncertain models and a large number of unknown parameters are facing a low number of experimental data points with high measurement errors. Current numerical methods are based on the assumption that the best parameter fit corresponds to the best solution. For biological systems, a huge solution space is typically obtained and a single parameter fit can be thus considered rather meaningless. One could argue that more experimental data should be generated and more quantitative information concerning the single mechanisms should be obtained to match the high dimensionality of parameter space. Considering the fact that quantitatively reliable *in vivo* measurements like time series of concentrations are still difficult to obtain on a large scale, this demand for a large amount of quantitative data cannot be met in the near future.

It would be fatal though not to attempt extracting information from available quantitative experimental data in the context of existing qualitative knowledge about, for example, network topologies. Therefore, alternative ways disregarding the restrictions of current numerical methods and by-passing the requirement of finding one distinct parameter set as suggested here have to be found. The methods presented here are well suited for theoretically tackling biological systems, as it is well-accepted that biological systems mostly keep their system properties constant although the real parameters are also subject to high variations. Thus, intrinsic biological properties like robustness and the fact that the function of biological systems does not require fine-tuned parameters indicate that the extraction of information is feasible even without exact knowledge of the true parameters. This is a new principle, which was approached here by evaluating randomly chosen parameter sets and by the generation of ensembles of estimated parameter fits based on randomly chosen initial values. The resulting histograms of parameter sensitivities provide a multitude of information, in particular by incorporation of a Boltzmann factor based on experimental data.

The currently developing methods such provided here or in the study of Brown and Sethna (2003) mark the beginning of a new methodology to investigate highly underdetermined systems. Such approaches have to be further refined, for example, by the 'High Dimensional Model Representation' (Li et al. 2001) for a systematic description of an 'effective' parameter space. Furthermore, it has to be extended by the concept of also considering alternative model choices instead of different parameter sets only, leading to model discrimination, which is currently examined by us (Vacheva et al. 2005). For a better understanding of the regulation of programmed cell death, the established loop between modelling, theoretical predictions and experiments has already proven to be highly efficient and has raised a lot of new detailed questions, for example, concerning the influence of spatial aspects on the network function or more detailed investigation of certain key regulatory mechanisms. The modular and hierarchical structure of the presented modelling framework provides a high degree of flexibility for future model extensions in various ways, either by adding additional pathways and systems like

proliferation or gene expression, or by adding more detailed biochemical mechanisms with more information becoming available. Thus, our methods will be well-suited for tackling complex and highly underdetermined networks going far beyond the field of programmed cell death.

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