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Steady-State Differential Dose Response in Biological Systems

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ABSTRACT In pharmacology and systems biology, it is a fundamental problem to determine how biological systems change their dose-response behavior upon perturbations. In particular, it is unclear how topologies, reactions, and parameters (differentially) affect the dose response. Because parameters are often unknown, systematic approaches should directly relate network structure and function. Here, we outline a procedure to compare general non-monotone dose-response curves and subsequently develop a comprehensive theory for differential dose responses of biochemical networks captured by non-equilibrium steady-state linear framework models. Although these models are amenable to analytical derivations of non-equilibrium steady states in principle, their size frequently increases (super) exponentially with model size. We extract general principles of differential responses based on a model's graph structure and thereby alleviate the combinatorial explosion. This allows us, for example, to determine reactions that affect differential responses, to identify classes of networks with equivalent differential, and to reject hypothetical models reliably without needing to know parameter values. We exemplify such applications for models of insulin signaling.

INTRODUCTION

Dose-response curves are a classical tool for relating the dose of a biochemically active agent, such as a ligand, enzyme, drug, or toxicant, to its biochemical (e.g., receptor activation in a cell), physiological (concentration of a chemical in a body compartment), or even population-level (mortality) effect (1). Experimentally obtained dose-response curves frequently associate a given dose with its time-independent, steady-state effect and they often have a sigmoid shape when the dose is plotted in log scale (see Fig. 1). Their standard analysis aims to retrieve important characteristics such as the baseline and maximal responses as well as the dose that produces a response halfway between baseline and maximum as a measure of the agent's potency, alternatively denoted as effective concentration (EC_{50}), inhibitory concentration (IC_{50}), or infectious dose (ID_{50}). Such measures allow one to analyze relative differences between a reference and a perturbed dose-response curve, which we call a "differential response" (or "differential" for short). One can, for example, compare how dose affects different system responses to find an optimal trade-off between therapeutic efficiency and toxicity via the therapeutic index (2). Natural systems also exploit differential responses. For example, insulin receptors recognize various natural ligands, such as insulin and insulin-like growth factor 1 (IGF-1), that have different binding affinities to the receptor to trigger appropriate differential (metabolic or mitogenic) responses (3).

More generally, it is often of interest to understand how perturbations such as mutations, drugs, or natural variations affect dose-response relations. Shifts in quantities such as the EC_{50} easily capture the differential between sigmoid dose-response curves. However, biphasic dose-response curves with low-dose stimulation and high-dose inhibition, so-called hormetic curves, have received renewed attention (4–6), and non-monotonic dose-response curves with several peaks have been experimentally obtained (7). A recent study of drug responses in cancer cells illustrates that "non-standard" dose-response relations occur in substantial numbers: among 11,650 experimental dose-response curves, 28% were best described by a non-monotonic model, and 12% required two inflection points (6). However, it is unclear how to compare dose-response curves of (potentially) any shape with each other (see Fig. 1).

Like any perturbation experiment, differential analysis can probe the functioning of a biological system to identify the underlying mechanisms of the system's (steady-state)

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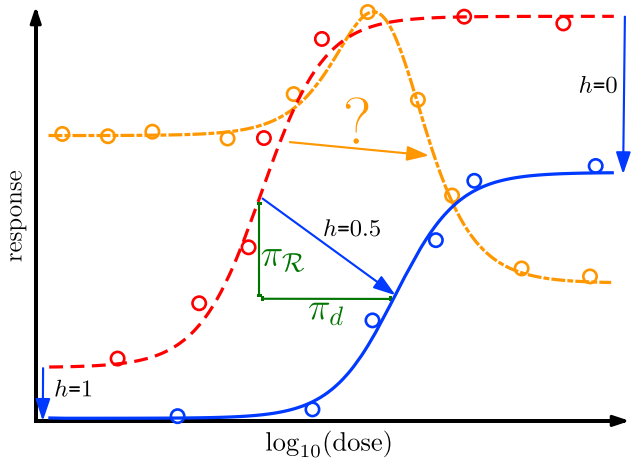

FIGURE 1 Relations between sigmoid reference (*red*, *dashed line*), perturbed sigmoid (*blue*, *solid line*), and hormetic (*orange*, *dash-dotted line*) dose-response curves and hypothetical experimental data (*circles*) and fitted empirical models (*lines*). When perturbations preserve the shape of the dose-response curve (*blue*, *solid line* versus *red*, *dashed line*), the effect of a perturbation can be quantified by the shifts in baseline response and maximal response, and by the difference in the dose required for half-maximal response, as indicated by blue arrows. Analogously, we quantify the differential in the dose (π_d, *green*) and the response ($\pi_{\mathcal{R}}$) components via the distance between points on a reference curve and points on a perturbed curve that have the same proportion of response between the minimum ($h = 1$) and maximum ($h = 0$; correspondence indicated by *blue arrows*). As indicated by the question mark, such a quantification is not straightforward for dose-response curves with different shapes. To see this figure in color, go online.

behavior. However, relating the observed effects to biochemical mechanisms is a key open challenge. Mathematical models in dose-response analysis are frequently empirical (for data interpolation to estimate the characteristics described above), and not mechanistic in the sense of incorporating (hypothesized) relationships between biochemical entities that give rise to the experimental data. There exists no comprehensive mechanistic model-based theory to describe differential responses, despite their fundamental nature and importance in all fields of biology. This is not surprising: many relevant biological network models are non-linear and cannot be analyzed symbolically for non-trivial network sizes to derive design principles. Thus, the characterization of differential responses has to resort to numerical simulations that depend on often unknown network structures and parameter values. Current parameter-free methods such as chemical reaction network theory, which uses the network (model) structure to obtain a qualitative understanding of biological systems by determining their capabilities in terms of number of steady states (8,9), are not directly applicable to differential responses, because we are interested in quantitative features (e.g., shifts in EC_{50}).

To tackle such limitations, and to extract general principles of biological systems, we concentrate on models in the graph-theoretical "linear framework" introduced in (10), which we call "linear framework models". These models represent linear molecular-state transitions but can also explicitly account for certain non-linear kinetics (10). They can be interpreted as deterministic (linear ordinary differential equation (ODE), or linear algebraic for the steady state) or stochastic (master equations of Markov processes) models of biochemical systems (11). The "linear framework" has provided multiple promising applications (12–14) and it unifies results across many biological areas such as enzyme kinetics, G-protein-coupled receptors, and gene regulation (10). Importantly, the framework connects the symbolic derivation of steady-state expressions to combinatorial objects on graphs (10). This relation between network function and structure makes the framework appealing to analyze principles of differential responses.

Linear framework models result from demonstrated or, more frequently, assumed timescale separation, where a part of a biochemical system reaches a steady state because it operates much faster than the rest of the system (15). Dose-response analysis with mechanistic models, for instance in pharmacology, often assumes that the system is at thermodynamic equilibrium (1). There, the principle of detailed balance imposes structural (all reactions must be microscopically reversible) and parametric (the so-called "cycle condition") constraints on linear framework models. It leads to simple "history-independent" equilibrium derivations that contain only products of equilibrium constants along any path in the model graph (10). However, for systems that dissipate energy, such as cell signaling pathways and eukaryotic gene regulation, this formalism does not apply. Energy dissipation keeps systems away from equilibrium, thus abolishing detailed balance. As a result, non-equilibrium steady states become "history dependent" and algebraically substantially more complex (14). A paramount challenge in working with non-equilibrium steady states of linear framework models symbolically is their frequently (super)exponentially growing size with model size—making even apparently "small" systems practically intractable.

Here, we develop a comprehensive theory and practically scalable computational methods for studying non-equilibrium steady-state differential dose-response relationships to pinpoint the mechanisms leading to experimentally observed behaviors. We extend the classic comparison of sigmoid dose-response curves and formally define a general notion of the differential. We then exploit connectivity properties of directed graphs representing linear framework models to address challenges such as determining the reactions that affect differential responses, identifying equivalence classes of networks according to their differential, and reliably rejecting hypothetical models without needing to know parameter values. Specifically, the theory helps determine which reactions take part in the differential and how perturbations such as variation of parameter values, deletions and additions of states and reactions affect the differential. In our approach, realistic practical applications are

possible, because we do not actually need to derive complete steady-state expressions for quantitative and qualitative symbolic analysis. Our computationally efficient graph algorithms (16; unpublished data) yield compact, factorized steady-state expressions. We illustrate the application of the framework for insulin signaling, covering aspects such as model building and analysis, model rejection, experimental design, and (numerical) bounds on differential dose-response relations.

MATERIALS AND METHODS

Linear framework models

We focus on the deterministic interpretation of linear framework models, that is, on biochemical reaction networks governed by Laplacian dynamics and modeled by systems of linear ODEs. Consider the example network in Fig. 2 *A*, in which all reactions follow first-order mass-action kinetics. It comprises three species and four reactions: a receptor, R, can transition to (from) its ligand-bound state, RL, with rate constant r_1 (r_2), or the receptor can become irreversibly phosphorylated as RLp with rate constant r_3, and RLp can transition to R with rate constant r_4. This system is a simple model of insulin receptor activation, and its dynamics can be expressed as the ODE system

$$\frac{d}{dt}\begin{pmatrix} x_{\mathrm{R}} \\ x_{\mathrm{RL}} \\ x_{\mathrm{RLp}} \end{pmatrix} = \underbrace{\begin{pmatrix} -r_1 & r_2 & r_4 \\ r_1 & -(r_2+r_3) & 0 \\ 0 & r_3 & -r_4 \end{pmatrix}}_{\mathcal{L}} \begin{pmatrix} x_{\mathrm{R}} \\ x_{\mathrm{RL}} \\ x_{\mathrm{RLp}} \end{pmatrix}, \tag{1}$$

where x denotes the concentration of the respective species in the subscript and $\mathcal{L}$ is called the Laplacian matrix of the system.

The dynamics of linear framework models can be represented as a diffusion process on directed graphs corresponding to their reaction schemes. Formally, a simple directed graph, $G = (V, E)$, consists of a set of vertices, $V(G)$, and a set of edges (ordered pairs of distinct vertices), $E(G)$; it has no self loops and no multiple parallel edges. For conciseness, we use "graph" as shorthand for "directed graph" throughout. Here, we concentrate on linear framework models of biological networks corresponding to "strongly connected" graphs, that is, graphs G in which, for any two distinct vertices $u, v \in V(G)$, there exists a directed path from u to v and from v to u. For example, the graph in Fig. 2 *A* is strongly connected (whereas the graph in Fig. 2 *D* is not). Strong connectivity is not a restrictive assumption for our theory, but rather mirrors the cell signaling models we consider. Note that non-strongly-connected graphs could emerge during the analysis procedures, but these are not linear framework models.

We consider the reaction scheme in Fig. 2 *A* as a labeled graph, G, with $V(G) = \{v_{\mathrm{R}}, v_{\mathrm{RL}}, v_{\mathrm{RLp}}\}$, where v denotes the vertex corresponding to the species in the subscript, and $E(G) = \{v_{\mathrm{R}}v_{\mathrm{RL}}, v_{\mathrm{RL}}v_{\mathrm{RLp}}, v_{\mathrm{RL}}v_{\mathrm{R}}, v_{\mathrm{RLp}}v_{\mathrm{R}}\}$. The reaction rate constants are labels of the corresponding edges in G. In such a labeled graph G, we can associate each vertex, $v_i \in V(G)$, to a non-negative species concentration x_i and each edge to a mass-action reaction. Hence, we obtain a dynamical system in which species concentrations hosted on the vertices of G flow in the direction of the edges at rates proportional to the concentrations on the edges' source vertices.

Proportionality of reaction rates is defined by edge labels, which have units of inverse time. We denote the label of an edge $uv \in E(G)$ by $\ell(uv)$. For example, $\ell(v_{\mathrm{R}}v_{\mathrm{RL}}) = r_1$. Edge labels can also host complex algebraic expressions of species concentrations and kinetic parameters. They can exactly account for non-linearities, for example, by containing concentrations of slow species resulting from timescale separation, provided that the uncoupling condition holds that prohibits concentrations corresponding to species from $V(G)$ in the edge labels (10). Note that here we mostly take the labels $\ell(e)$ as uninterpreted symbols, that is, we ignore the algebraic expressions to which they correspond and regard them as unique edge names. The only exceptions are edges affected by the dose (of a ligand), because we need explicit dose dependencies to determine differentials.

Linear framework models can be "closed," not exchanging matter with the environment (as in the illustrative example), or "open," when synthesis and degradation reactions are present. In general, the dynamics of closed models can be expressed in the form:

$$\frac{dx}{dt} = \mathcal{L}(G)x, \tag{2}$$

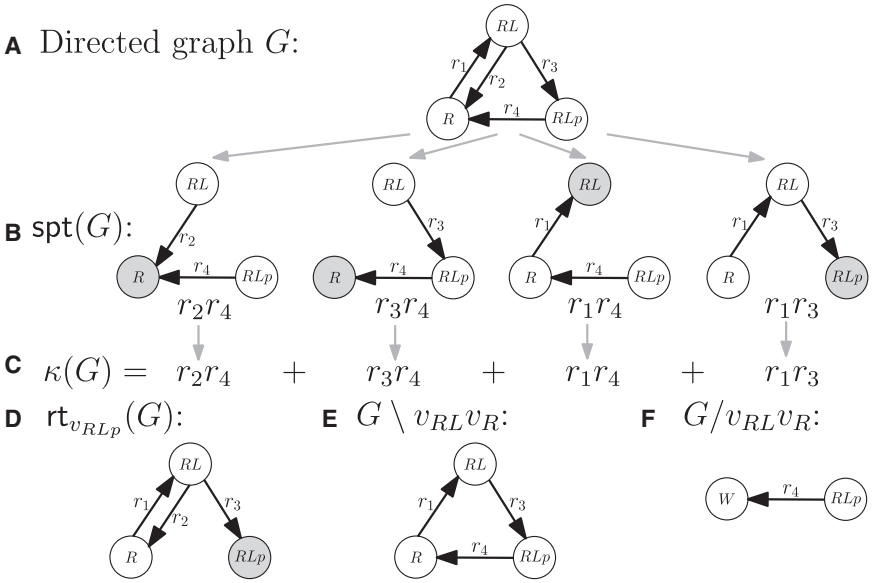

FIGURE 2 Example model of insulin receptor activation. (*A*) Graph (kinetic scheme) G. (*B*) All spanning trees of graph G rooted at each vertex. (*C*) The corresponding Kirchhoff polynomial. (*D*) The graph obtained by rooting G at v_{RLp}. (*E*) The edge-deleted graph, $G \setminus v_{\mathrm{RL}}v_{\mathrm{R}}$. (*F*) The edge-contracted graph, $G/v_{\mathrm{RL}}v_{\mathrm{R}}$. Labels on vertices denote names of species represented by them and W denotes a vertex obtained after the application of graph operations. Gray vertices are roots of the corresponding spanning tree and of all spanning trees when rooting a graph, respectively.

where $x = (x_1, \ldots, x_n)^T$ is the vector of species concentrations corresponding to each vertex $v_1, \ldots, v_n \in V(G)$ and $\mathcal{L}(G)$ is the Laplacian matrix of G, defined as

$$\mathcal{L}(G)_{ij} = \begin{cases} \ell(v_j v_i) & \text{if } i \neq j, \\ -\sum_{r \neq j} \ell(v_j v_r) & \text{if } i = j, \end{cases} \quad (3)$$

and $\ell(v_j v_i) = 0$ when $v_j v_i \notin E(G)$. In closed models, the total amount of material x_t is conserved and there is a single conservation law, $x_1 + \cdots + x_n = x_t$. The system reaches a unique stable steady state that can be derived symbolically for any species from x_t and the kernel of the Laplacian matrix. Analogous definitions for open models can be found in the Supporting Material, and for more details, proofs, and derivations, see (10,11,17).

Spanning trees

Non-equilibrium steady states of linear framework models can always be derived in symbolic form, but, in practice, the length of the symbolic steady-state expressions grows (super) exponentially with the size of graph G. To cope with this growth, we introduce concepts intimately connected to both the structure of linear framework models and their steady states. Namely, a certain class of subgraphs, so-called "rooted directed spanning trees," can be used to generate Kirchhoff polynomials (see next section).

Formally, a graph H is a subgraph of a graph G if $V(H) \subseteq V(G)$ and $E(H) \subseteq E(G)$, where every edge in G between vertices in H is also an edge in H. A strongly connected component (SCC) of G is any largest (w.r.t. vertex inclusion) strongly connected induced subgraph of G. It can be shown that no two distinct SCCs can share a vertex, and thus, the SCCs $G_1, \ldots, G_k$ of a graph G induce a unique partition, $V(G_1), \ldots, V(G_k)$ of $V(G)$. Additionally, for two distinct SCCs G_i and G_j, there can be a directed path from G_i to G_j, or from G_j to G_i, but not both. The existence of such paths between SCCs naturally induces a unique partial order on the SCCs $G_1, \ldots, G_k$.

A rooted directed spanning tree (spanning tree, for short) A is a subgraph of G spanning its vertex set such that a root vertex is reachable from all vertices along a unique directed path. By $\mathsf{spt}(G)$ we denote the set of all spanning trees of G, and by $\mathsf{spt}_v(G)$ the set of all spanning trees rooted at vertex v. All spanning trees of the example graph are shown in Fig. 2 *B*: two spanning trees are rooted at v_{R}, one is rooted at v_{RL}, and another is rooted at v_{RLp}. Let rt be the graph rooting operation such that $\mathsf{rt}_v(G)$ is the graph constructed from G by removing all edges outgoing from v (see Fig. 2 *D*). All spanning trees of $\mathsf{rt}_v(G)$ are necessarily rooted at v. We will say that a graph G is rooted at a vertex v if v has no outgoing edges and v is reachable from every other vertex in G. Observe that $\mathsf{spt}_v(G) = \mathsf{spt}(\mathsf{rt}_v(G))$. Note also that a spanning tree of G exists iff the partial order of the SCCs has exactly one maximal element (no other SCC is reachable from a maximal SCC). Such an SCC is called a terminal *SCC*.

Kirchhoff polynomials and steady states of linear framework models

A spanning tree A of a graph G with n vertices has $n-1$ edges, $e_1, \ldots, e_{n-1} \in E(G)$ (for a concise notation, we denote edges with the symbol e when not referring to the pairs of vertices defining them). A spanning tree can also be represented as a monomial $\ell(e_1)\ell(e_2)\cdots\ell(e_{n-1})$ in the edge labels $\ell(e_1), \ell(e_2), \ldots, \ell(e_{n-1})$, when the edge labels are taken as uninterpreted symbols denoting unique edge names. Correspondingly, one can represent the set of all spanning trees in G by a homogeneous multivariate polynomial over the variables $\ell(e_i)$, $e_i \in E(G)$. This polynomial is called the Kirchhoff polynomial, $\kappa(G)$:

$$\kappa(G) = \sum_{A \in \mathsf{spt}(G)} \prod_{e_i \in E(A)} \ell(e_i).$$

The Kirchhoff polynomial of the example graph is shown in Fig. 2 *C*. Observe that if G is disconnected or has more than one terminal SCC, then $\kappa(G) = 0$, and if G consists of a single vertex, then $\kappa(G) = 1$.

Kirchhoff polynomials establish a direct connection between model structure, in terms of spanning trees, and function, in terms of steady-state expressions. Briefly, the steady state of a linear framework model can be symbolically obtained from initial conditions and the kernel of the Laplacian matrix by employing Tutte's Matrix-Tree Theorem (18), which links the minors of the Laplacian matrix to the spanning trees in the model's graph and their representation as Kirchhoff polynomials (see (10,11,17) for details and proofs).

Here, we are interested in the final results, namely that the steady-state concentration x_i^{SS} of species i in a closed system associated to a vertex v_i can be expressed as a fraction of Kirchhoff polynomials:

$$x_i^{\mathrm{SS}} = \frac{\kappa_{v_i}(G)}{\kappa(G)} x_t, \quad (4)$$

where we denote the Kirchhoff polynomial of all spanning trees rooted at vertex v by $\kappa_v(G)$ ($\kappa_v(G)$ is a shorter notation for $\kappa(\mathsf{rt}_v(G))$). Thus, the steady-state concentration of species RLp associated to vertex v_{RLp} in our example system is (see Fig. 2, *B–D*)

$$x_{\mathrm{RLp}}^{\mathrm{SS}} = \frac{\kappa_{v_{\mathrm{RLp}}}(G)}{\kappa(G)} x_t = \frac{r_1 r_3}{r_3 r_4 + r_2 r_4 + r_1 r_4 + r_1 r_3} x_t.$$

Although these expressions look simple, the number of spanning trees in a graph G often grows (super) exponentially with the size of G (19). Symbolic steady-state expressions of linear framework models as expressed by Kirchhoff polynomials, therefore, face the problem of combinatorial explosion, which makes manipulation and generation of such expressions challenging.

Manipulation of Kirchhoff polynomials

The manipulation of combinatorially complex Kirchhoff polynomials is facilitated by establishing a relation between procedures for their algebraic simplification and operations on their corresponding graphs (unpublished data). Graph operations such as edge deletion-contraction and prime decomposition (16) allow a Kirchhoff polynomial to be written as a sum and a product, respectively, of other Kirchhoff polynomials without the need of explicit Kirchhoff polynomial generation.

For a graph G with $e \in E(G)$ and $v \in V(G)$ we denote edge deletion by $G \setminus e$, i.e., the graph obtained from G by deleting e (see Fig. 2 *E* for an application to the example graph). Further, for a graph G and an edge $e = v_i v_j \in E(G)$, we denote by G/e the edge-contracted graph that is constructed from G by 1) removing the edge $v_j v_i$, if it exists, and all outgoing edges from v_i, i.e., $v_i u \in E(G)$, and 2) fusing vertices v_i and v_j into a single new vertex, w (see Fig. 2 *F*). Edge contractions may yield graphs with multiple parallel edges between two vertices. We resolve this by replacing m multiple parallel directed edges $e_1, e_2, \ldots, e_{\mathrm{m}}$ going from u to v with a single edge $e = uv$ such that $\ell(e) = \ell(e_1) + \ell(e_2) + \ldots + \ell(e_{\mathrm{m}})$.

The classic deletion-contraction formula for an edge $e \in E(G)$ partitions $\mathsf{spt}(G)$ into two sets, one in which e participates in no spanning trees and one in which e participates in all spanning trees. Equivalently, it decomposes $\kappa(G)$ into a sum of Kirchhoff polynomials (20):

$$\kappa(G) = \kappa(G \setminus e) + \ell(e)\kappa(G/e). \quad (5)$$

We call a Kirchhoff polynomial, P, a "factor" of another Kirchhoff polynomial, Q, if there exists a Kirchhoff polynomial R such that $Q = PR$. A Kirchhoff polynomial P that cannot be factorized into non-trivial factors is called "prime". We extend these definitions to graphs by calling G' a component (a prime component) of G if $\kappa(G')$ is a factor (a prime factor) of $\kappa(G)$. The work in (16) provides graph decomposition rules that correspond to factorization steps of the Kirchhoff polynomial and also gives necessary and sufficient primality conditions of the resulting factors expressed by connectivity properties of the corresponding decomposed components. In particular, the exhaustive application of two decomposition rules to a graph G yields in linear time graphs whose Kirchhoff polynomials are prime factors of the Kirchhoff polynomial of the original G:

$$\kappa(G) = \prod_{i=1}^{n} \kappa(P_i),$$

where P_i are the prime components of G. A prime component P_i can be either 1) strongly connected or 2) rooted at v such that $P_i \setminus v$ is strongly connected and P_i does not have any non-trivial vertex dominators (using the definition that a vertex u dominates a vertex w if every path from w to v goes through u). Note that the prime factorization is conditional on label uniqueness—when the labels are not unique or contain expressions, the factorization is not guaranteed to be prime. This is why we regard edge labels as unique uninterpreted symbols when defining Kirchhoff polynomials on linear framework models. Further, we call an edge $e \in E(G)$ a "prime bridge" if the edge deleted graph $G \setminus e$ has more non-trivial prime components than the original graph G. To efficiently manipulate and generate Kirchhoff polynomials we used the Python package KirchPy (unpublished data).

RESULTS

Formalizing differential responses

Differentials describe how a perturbation transforms a reference dose-response curve. For monotone curves, we can simply quantify this transformation by the difference between points on the reference and the perturbed curve with identical percentage of response between baseline and maximum, such as the distance between EC_{50} values. However, there exists no established approach for comparing dose-response curves of different functional form (sigmoid or multiphasic). Comparisons are particularly ambiguous when they involve non-monotone curves (see Fig. 1), but they are essential, because non-monotone curves have been experimentally observed and receive increasing attention (4,7).

To provide a general formal procedure for comparison, let $\mathcal{R}^{\alpha}(d)$ and $\mathcal{R}^{\beta}(d)$ be the functions generating the reference and the perturbed dose-response curves, respectively. Here and in the following, the superscripts α and β denote the specific dissimilar (parametric and structural) features of the systems generating the two curves. Without explicitly specifying α or β, we refer to both identifiers at the same time. To quantify the differential, we propose the following procedure (see the Supporting Material for details):

1) Subdivide the curves into monotone segments. To obtain monotone segments, we subdivide the dose-response curves along the dose coordinate at their critical points, that is, at the doses for which $\mathcal{R}(d)$'s first derivative is zero.
2) Decide which segments to compare. If the curves have equal numbers of segments, it is reasonable to assume that the perturbation shifts and scales the segments such that they can be compared in the order defined by the critical points: the first segment of $\mathcal{R}^{\alpha}(d)$ maps to the first segment in $\mathcal{R}^{\beta}(d)$, and so on. When the perturbed curve has more or fewer segments than the reference curve, and without information about which segments fuse or split, we compare the segments in all possible ways while preserving their order. For example, comparing a sigmoid and a biphasic curve amounts to mapping the sigmoid to each of the two monotone segments of the biphasic curve.
3) Determine corresponding points in compared pairs of segments. As for monotone dose-response curves, we relate the doses that have the same proportion h, $h \in [0, 1]$, of response between the minimal and maximal response in each pair of segments.
4) Quantify the displacement of corresponding points. We quantify the displacement between points with identical proportion of response h for all pairs of mapped segments. In particular, we calculate the dose differential, $\pi_d(h)$, and the response differential, $\pi_{\mathcal{R}}(h)$, as the difference in the dose and the response components of corresponding points:

$$\pi_d(h) := \log_{10}\frac{\mathrm{d}_h^{\alpha}}{\mathrm{d}_h^{\beta}} \text{ and } \pi_{\mathcal{R}}(h) := \mathrm{R}_h^{\alpha} - \mathrm{R}_h^{\beta},$$

with doses d and responses R. The dose differential is expressed in log scale to easily identify fold differences. These quantities have clear biological interpretations. For example, for corresponding segments, $\pi_d(h = 0.5)$ is the difference between EC_{50} log values and $\pi_d(h = 0)$ is the difference between maximal responses.

Differentials cannot be obtained in closed form in general, but we will show next that one can often determine differentials analytically when functional relations between dose and response originate from steady-state expressions of linear framework models.

Differential systems

To derive general expressions for the dose (π_d) and response $(\pi_{\mathcal{R}})$ differentials, we need to define dose, response, and perturbation in linear framework models (see Materials and Methods for formal concepts), leading to the notion of differential linear framework models.

Here, we assume that the dose variable affects one or more reactions proportionally, which can be interpreted as an input changing the rate constant gradually, or as an input species with constant concentration binding to the educt of

the reaction; for example, the effect of a ligand with constant external concentration that binds to a receptor incorporated in this way via the law of mass action. Formally, the input dose variable, d, partakes in the mathematical expressions labeling w edges in the model's graph G, $I(G) := \{e_{d,1}, \ldots, e_{d,w}\}$. We call members of the set $I(G)$ "dose edges" of G and their labels are expressions proportional to the input dose variable, d:

$$\ell(e_{d,i}) = g_i(p)d,$$

where $g_i(p)$ are functions that do not contain d (see Fig. 3 *A* for the insulin receptor example in which we assume that the dose affects receptor-ligand binding).

In our analysis, the response $\mathcal{R}$ is a linear combination of the steady-state concentrations at chosen output vertices (such as the phosphorylated receptor-ligand complex, RL_p, in Fig. 3 *A*). The q species eliciting the response are associated with a set of output vertices $O(G) := \{v_1, \ldots, v_q\}$ in closed systems. Then, if we use Eq. 4 to derive a general expression for the steady-state response of closed models using Kirchhoff polynomials, $\kappa(G)$, we obtain

$$\mathcal{R}_{O(G)} = \frac{\sum_{v_i \in O(G)} a_i \kappa_{v_i}(G)}{\kappa(G)} x_t,$$

where $a_i \geq 0$ designates the weight given to the steady-state concentration associated with vertex v_i. The response function for open models is similar (see the Supporting Material). This implies that steady-state dose-response curves of linear framework models are rational functions of the dose variable, for example, $\mathcal{R}_{O(G)}(d)$.

Perturbations are any changes in the model structure (additions and deletions of species and reactions), parameters (having different values in the reference and perturbed model; such parameters we call "differential parameters"), number and position of the edges affected by the input variable, number and position of the output vertices, and parametrization of the output function. We capture these perturbations by defining two linear framework models for a reference (α) and a perturbed (β) condition, each of which consists of a graph, G, a set of parameters, p (from G's labels and x_t for closed models), a set of dose edges, I (whose labels contain the dose variable, d), and a set of output vertices, O, the concentrations associated to which are weighted by a to obtain the observed response $\mathcal{R}$. Formally, a differential system is then an ordered pair, $\mathcal{D} = ((G^\alpha, p^\alpha, I^\alpha, O^\alpha, a^\alpha), (G^\beta, p^\beta, I^\beta, O^\beta, a^\beta))$.

We use our example insulin receptor model from Fig. 2 *A* to define a first differential system, $\mathcal{D}$ (Fig. 3 *A*). Let the receptor, R, transition to its ligand-bound state, RL, upon activation by a ligand with constant concentration d. We account for the dose variable d in the transition rate by changing the label of edge $v_R v_{RL}$ to $r_1 d$. To construct a differential Laplacian system, we consider a reference model with graph topology as the example graph, G, dose edge $e_d = v_R v_{RL}$, i.e., $I = \{e_d\}$ with $\ell(e_d) = r_1 d$, a single output vertex, $O = \{v_{RLp}\}$, weighted by 1, and parameters $p = \{r_1, r_2, r_3, r_4, x_t\}$. The perturbed model is identical to the reference one, with the exception of the values of parameters r_1 and r_2, which we will denote as r_1^α, r_2^α (r_1^β and r_2^β) in the reference (perturbed) system. These differential parameters correspond to stimulation of the system with two different ligands that have different affinities to the receptor R, such as insulin and IGF-1. Since reference and perturbed model are identical except for their differential parameters, we can illustrate the differential system by a single graph and highlight the differential parameters as shown in Fig. 3 *A*.

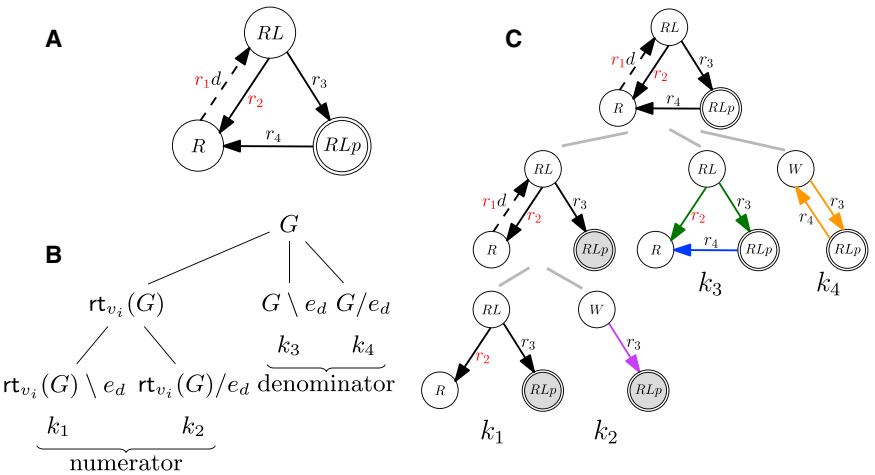

FIGURE 3 Differential analysis for the simple insulin receptor activation model. (*A*) Graph for the differential system. The dose edge is marked by a dashed arrow, red symbols correspond to differential parameters (those with different values in the reference and perturbed model), and doubly encircled vertices mark the output vertices. (*B*) Tree scheme for a general graph G, showing how to obtain the relevant graphs participating in the coefficients k_i of the dose-response relationship in closed systems (for reference and perturbed models through the graph operations rooting, deletion, and contraction). Note that there are also additional terms contained in the steady-state coefficients. (*C*) The tree scheme for decomposition of the insulin receptor activation model. Gray-shaded vertices denote that the graphs are rooted at them; different edge colors in the leaves of the tree mark the prime components (same color means same prime component also among different graph leaves), and black edges (when present) are not part of any prime component. To see this figure in color, go online.

Symbolic derivation of the differential and its properties

For tractability, we are first interested in deriving analytical expressions for the differential of systems with a constant input that influences exactly one edge proportionally in both reference and perturbed models, ($I(G) = \{e_d\}$ and $\ell(e_d) = g(p)d$), to model, for example, a ligand binding once to a receptor.

To express the steady-state response, $\mathcal{R}$, explicitly as a function of the dose variable, d, we apply the deletion-contraction property from Eq. 5 to partition the set of spanning trees from the numerator and denominator of the corresponding rational function into two categories—those that contain the dose d in one of their labels and those that do not. This is equivalent to factoring out the edge labels from the monomials in the corresponding Kirchhoff polynomial that contain the dose variable d. After simplification, we obtain the general form of dose-response expressions for models with a graph G:

$$\mathcal{R}_O(d) = \frac{k_1 + k_2 d}{k_3 + k_4 d}, \tag{6}$$

where for closed models (see the Supporting Material for open models),

$$k_1 = x_t \sum_{v_i \in O(G)} a_{v_i} \kappa_{v_i}(G \backslash e_d),$$

$$k_2 = x_t g(p) \sum_{v_i \in O(G)} a_{v_i} \kappa_{v_i}(G / e_d),$$

$$k_3 = \kappa(G \backslash e_d),\ k_4 = g(p)\kappa(G / e_d).$$

We call the k_i terms "steady-state coefficients," although they are symbolic expressions involving parameters and Kirchhoff polynomials of specific graphs obtained from G. The coefficients k might be zero if spanning trees do not exist in the respective graphs.

How to obtain the relevant graphs participating in the coefficients for closed models can be seen in the tree scheme from Fig. 3 *B* (see the tree scheme for open models in Fig. S1), and an application to the example differential system can be found in Fig. 3 *C*. With only one edge containing the dose d, the numerator and denominator are at most first degree in d, and thus, the tree schemes in Fig. 3, *B* and *C*, have four leaves. Note that the tree scheme is the same for the reference and perturbed systems, except for the differential parameters. The coefficients for the example are $k_1 = x_t \kappa(\mathrm{rt}_{\mathrm{RLp}}(G) \backslash e_d)$, $k_2 = x_t r_1 \kappa(\mathrm{rt}_{\mathrm{RLp}}(G) / e_d)$, $k_3 = \kappa(G \backslash e_d)$, and $k_4 = r_1 \kappa(G / e_d)$. By generating the Kirchhoff polynomials in the respective graphs, we obtain

$$k_1 = 0,\ k_2 = x_t r_1 r_3,$$

$$k_3 = r_4(r_2 + r_3),\ \text{and}\ \ k_4 = r_1(r_3 + r_4).$$

Following the procedure for deriving the differential expressions π_d and $\pi_{\mathcal{R}}$ (for details, see the Supporting Material), we obtain

$$\begin{aligned} \pi_d &= \log_{10} \frac{k_3^\alpha k_4^\beta}{k_3^\beta k_4^\alpha} \quad \text{and} \\ \pi_{\mathcal{R}}(h) &= h\left(\frac{k_1^\alpha}{k_3^\alpha} - \frac{k_1^\beta}{k_3^\beta}\right) + (1-h)\left(\frac{k_2^\alpha}{k_4^\alpha} - \frac{k_2^\beta}{k_4^\beta}\right). \end{aligned} \tag{7}$$

When either k_1 or k_2 is zero, the response differential $\pi_{\mathcal{R}}$ has a simpler form but the dose differential π_d is not affected. The differential can be degenerate, e.g., when $\mathcal{R}(d)$ is always zero, implying that $k_1 = k_2 = 0$, or undefined, e.g., when $\mathcal{R}(d)$ is either constant or unbounded, leading to $k_3 = 0$ or $k_4 = 0$.

The dose differential π_d in Eq. 7 depends not on h, k_1, and k_2 but on the Kirchhoff polynomials contained in the coefficients k_3 and k_4. It is therefore independent of the choice of output vertices in O, of the vertex weights, a, and of the total conserved amount, x_t, in closed systems (the synthesis reactions, s_i, in open systems) for the reference and perturbed models. The dose differential can be simplified to the logarithm of an irreducible fraction by obtaining the prime factorizations of the numerator and denominator and dividing them by the greatest common divisor, $\gcd(k_3^\alpha k_4^\beta, k_3^\beta k_4^\alpha)$. Then, the necessary and sufficient condition for a reaction to participate in π_d is to be part of a prime component of the relevant graphs that is different in the reference and perturbed models, and not the same in the dose-edge-deleted and dose-edge-contracted graphs of the same condition. To illustrate these points, consider π_d for closed differential systems:

$$\pi_d = \log_{10} \frac{\kappa\left(G^\alpha \backslash e_d^\alpha\right) g^\beta(p^\beta) \kappa\left(G^\beta / e_d^\beta\right)}{\kappa\left(G^\beta \backslash e_d^\beta\right) g^\alpha(p^\alpha) \kappa\left(G^\alpha / e_d^\alpha\right)}.$$

The polynomials $\kappa(G \backslash e_d)$ are factorizable if and only if e_d is a prime bridge. Edge contraction for $\kappa(G / e_d)$ could also lead to a factorizable Kirchhoff polynomial if any of the deleted edges during the procedure is a prime bridge. The factor $g(p)$ we assume to be prime as part of the label of a single edge. Overall, thus, whether the dose differential is reducible depends exclusively on the perturbation, the connectivity of the dose edge, e_d (in G^α and G^β), and the connectivity of the edges in the immediate neighborhood of e_d. Note that the reducibility characterization of π_d for open differential systems is analogous but includes an additional dependency on the location and connectivity of the synthesis edges in G^α and G^β.

The response differential, $\pi_{\mathcal{R}}$, in Eq. 7 is a sum of ratios dependent on all coefficients k_i and includes the conserved x_t in each ratio for closed systems, the synthesis reactions in open systems, and the mapping variable h. However, it

does not depend on the dose-edge label function, $g(p)$, because $g(p)$ always cancels in the fraction k_2/k_4 (for both reference and perturbed coefficients). To illustrate, let us focus on the response differential, $\pi_{\mathcal{R}}$ (see Eq. 7), for closed systems when $h = 0$. To simplify the expression, first, the common factors between k_2 and k_4 are canceled (keeping in mind that k_2 is a linear combination of prime factorized Kirchhoff polynomials) to obtain $\overline{k_2}$ and $\overline{k_4}$. The response differential can be further reduced if $\gcd(\overline{k_4}^{\alpha}, \overline{k_4}^{\beta}) \neq 1$ by combining the fractions under a common denominator. Note that the response differential could be zero due to the minus sign. The characterization of the response differential in the general case for h not fixed and for open systems is analogous.

For the example differential system, we find that $\pi_d = \log_{10}(r_1^{\beta}/r_1^{\alpha})(r_2^{\alpha} + r_3)/(r_2^{\beta} + r_3)$ and $\pi_{\mathcal{R}} = 0$, where the response component of the differential vanishes because $k_1 = 0$ and because the differential parameters r_1 in k_2 and k_4 cancel each other. Also, the dose differential is independent of the rate constant r_4.The example illustrates that by deriving the general form of the differentials π_d and $\pi_{\mathcal{R}}$, which are algebraic expressions of Kirchhoff polynomials in linear framework models, we establish a direct connection between the structure of the differential system and its function.

Analyzing the insulin receptor life-cycle

Insulin signaling in response to various ligands determines differential cellular responses through complex and incompletely understood mechanisms (3,21). To unravel the processes at play, it is important to comprehend the role of reactions and species in the observed cellular functions. To illustrate applicability of our theory to such an investigation, we extend the example insulin model stepwise to a model incorporating insulin receptor binding, recycling, and phosphorylation. The resulting model is a subsystem of a more comprehensive insulin receptor signaling model from (22). This subsystem is appropriate for our analysis, because it is a linear framework model, it is away from equilibrium since receptor recycling and phosphorylation dissipate energy (as indicated by irreversible reactions), and the steady state of phosphorylated insulin receptors determines insulin signal transduction (23).

Here, we do not explicitly consider the molecular details of all reactions of this model but adopt a more general structural analysis approach by equating each reaction rate constant to a unique symbol. Specifically, we take the reaction rate constants r_4, r_7, and r_{16} (see Fig. 6 *A*) as unique symbols, although they actually depend on the concentration of protein tyrosine phosphatase (PTP) and are thus coupled. This still allows us to analyze the role of each reaction in the differential, but it precludes algebraic simplifications for the concentration of PTP; if more molecular details are of interest, they have to be explicitly included. Again, we define the differential system by assuming that the reference and the perturbed model differ only in the values of a subset of parameters—the differential parameters corresponding to ligands with different affinity toward the insulin receptor—and that all other elements are identical.

We extend the example model (Fig. 2) with two states, an internalized phosphorylated ligand-bound receptor, $\mathrm{RL_{pi}}$, and an internalized receptor, $\mathrm{R_i}$ (Fig. 4). These states can be reached by reversible reactions from their non-internalized (membrane) counterparts, RLp and R, to represent endocytosis and receptor recycling, and $\mathrm{RLp_i}$ can be dephosphorylated to R by an irreversible reaction. We regard the phosphorylated ligand-bound receptors as output species, such that $O(G) = \{v_{\mathrm{RLp}}, v_{\mathrm{RLpi}}\}$, with unit weights $(a_{\mathrm{RLp}} = a_{\mathrm{RLp_i}} = 1)$.

The resulting differential system is what we call a "basic signaling system." It is a differential system with a closed graph that contains a reversible reaction for which the forward and reverse rates differ between the reference and perturbed model, and for which the forward rate is affected by the dose variable, d. More precisely, we have a graph G with $I(G) = \{e_{\mathrm{on}}\}$, $\ell(e_{\mathrm{on}}) = g_{\mathrm{on}}(p)d$, and $\ell(e_{\mathrm{off}}) = g_{\mathrm{off}}(p)$. Also, the reference and perturbed models have no structural differences; they differ only by the functions $g_{\mathrm{on}}(p)$ and $g_{\mathrm{off}}(p)$ (and the parameters contained in them). In such basic signaling models, the dose-edge-contracted graph G/e_{on} is the same for the reference and perturbed systems; therefore, its Kirchhoff polynomial always cancels in the dose-differential fraction. The dose differential for closed systems (results and definitions are analogous for open systems) is

$$\pi_d = \log_{10} \frac{\kappa(G^{\alpha} \backslash e_{\mathrm{on}})}{\kappa(G^{\beta} \backslash e_{\mathrm{on}})} \frac{g_{\mathrm{on}}^{\beta}(p^{\beta})}{g_{\mathrm{on}}^{\alpha}(p^{\alpha})}.$$

The response component of the differential for $h = 0$ will always be zero under the stated assumptions, since $(k_2^{\alpha}/k_4^{\alpha}) = (k_2^{\beta}/k_4^{\beta})$. When $h = 1$, the response component is not affected by the assumptions for basic signaling systems.

Although the graph of the extended system contains more states and reactions and the steady-state expression is more complicated (see the Supporting Material), the differential is identical to that of our initial example without endocytosis, because the newly added reactions take part in prime components that do not contain differential parameters and therefore cancel out (see Fig. 4). We can formally describe such differential systems giving rise to identical differential expressions by employing the mathematical concept of "equivalence classes." Let $\mathfrak{D}$ be a set of differential systems and define the equivalence relation, $\sim_{\pi}$, on $\mathfrak{D}$ such that $\mathcal{D}_1 \sim_{\pi} \mathcal{D}_2$, $\mathcal{D}_1, \mathcal{D}_2 \in \mathfrak{D}$, iff $\pi_d(\mathcal{D}_1) = \pi_d(\mathcal{D}_2)$ and $\pi_{\mathcal{R}}(\mathcal{D}_1) = \pi_{\mathcal{R}}(\mathcal{D}_2)$. Thus, the initial and extended models of insulin signaling belong to the same equivalence class with respect to their differential. The concept of equivalence classes has direct applications for model selection and

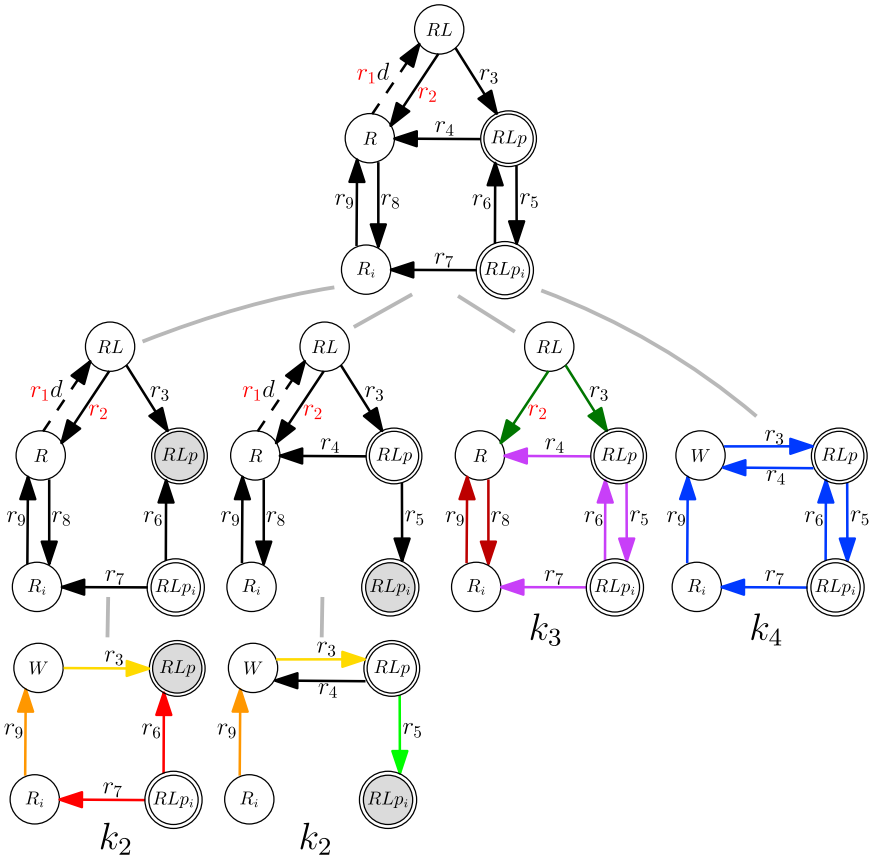

FIGURE 4 Tree scheme for the differential system in Fig. 3 extended with internalized species RLp_i and R_i. Note that only graphs belonging to non-zero coefficients are shown. To see this figure in color, go online.

experimental design. For example, assume that we have experimental evidence that changes in the rate constant of a reaction such as r_4, in both the reference and perturbed models, affect the dose differential. If this reaction does not take part in the dose-differential expression of the equivalence class of models we are studying (as for the equivalence class for insulin signaling defined above), we can directly reject all members of the equivalence class, because they are incompatible with the experimental observation. Similarly, we can use the analytic framework to answer questions such as what second perturbation to design (or how to change the first perturbation) to differentiate between models in the same equivalence class (see the Supporting Material).

Next, to analyze long-term effects of insulin receptor trafficking, we need to account for receptor synthesis and degradation. This converts the model from a closed system to an open system. Let us consider a model extension in which the non-internalized free receptor, R, is synthesized and degraded. Then, however, the differential is not defined because the steady-state coefficient k_4 is zero. Fig. 5 *A* shows that the contraction of the dose edge eliminates the degradation edge $e_{r_{10}}$ and, additionally, that the rooting at the environment vertex $v_\emptyset$ eliminates the synthesis edge $e_{r_{11}}$; this results in a disconnected graph with no spanning trees in k_4. Hence, we can reject this extension just based on structural considerations. We therefore add synthesis and degradation reactions for R_i as a more biologically plausible way to capture receptor trafficking (Fig. 5 *B*). The steady-state expression changes in this case (see the Supporting Material), but the differential is still identical to that of the initial example model from Fig. 2; the extended model belongs to the same equivalence class due to cancellation of prime factors that do not contain differential parameters in both reference and perturbed models (see Fig. 5 *B*). Overall, our structural analysis predicts that differential responses for ligands with different affinities always exhibit a zero response differential and a dose differential dependent on the ligand affinities. This result is corroborated by the observation that most natural and modified insulin receptor ligands shift sigmoid dose-response curves leftward or rightward (relative to insulin) but have an identical maximal response (24).

Extension: two dose edges

To extend the framework to cases in which hormesis is possible, we consider that the input dose acts proportionally and simultaneously on two edges, i.e., $I(G) = \{e_{d,1}, e_{d,2}\}$, $\ell(e_{d,1}) = g_1(p)d$, and $\ell(e_{d,2}) = g_2(p)d$. To derive the general form of dose-response expressions for closed and

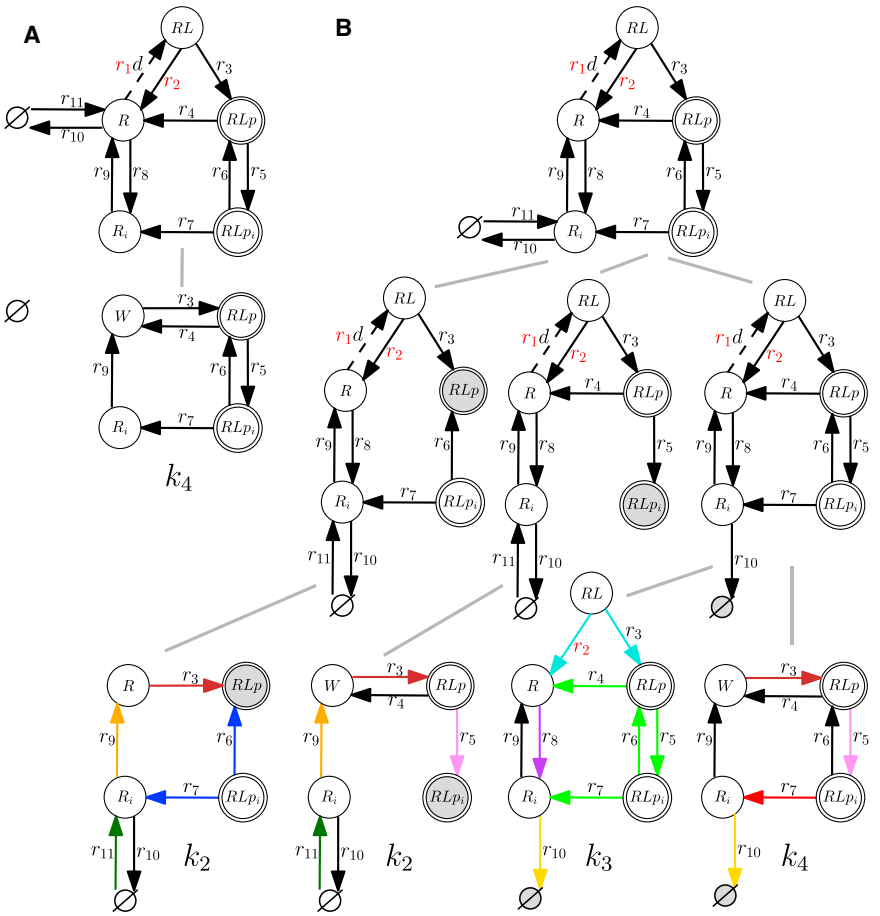

FIGURE 5 Extended insulin model with receptor degradation (open system). (*A*) The dose and response differential for the system with synthesis and degradation of membrane-bound receptor R (for reference and perturbed graph) is not defined, since the steady-state coefficient $k_4 = 0$ (the contained graph is disconnected and therefore does not contain any spanning trees). (*B*) Tree scheme for a differential system containing synthesis and degradation of the internalized receptor, R_i. Graphs without spanning trees are not shown. To see this figure in color, go online.

open systems, we apply the deletion-contraction formula to partition the set of spanning trees from the numerator and denominator of the response function, $\mathcal{R}$, into four categories—those containing no input edges, those containing $e_{d,1}$ but not $e_{d,2}$, those containing $e_{d,2}$ but not $e_{d,1}$, and those containing both $e_{d,1}$ and $e_{d,2}$. After simplification, we obtain

$$\mathcal{R}_O(d) = \frac{k_1 + k_{23}d + k_4 d^2}{k_5 + k_{67}d + k_8 d^2}, \tag{8}$$

where $k_{23} := k_2 + k_3$ and $k_{67} := k_6 + k_7$ (see the Supporting Material for all details). For two dose edges, the numerator and denominator polynomials in the dose d can be at most of degree two; for an input acting on w edges simultaneously, the maximal degree is w. However, the spanning tree partitioning determines the graphs contained in the coefficients k (see, for definitions, the tree scheme in Fig. S3). The exponential increase of the number of relevant graphs with number of dose edges (2^w graphs) makes systems with multiple inputs complex.

Depending on the coefficients k_i, two or three critical points define a sigmoid or a biphasic/hormetic dose-response relationship, respectively. A necessary and sufficient condition for a biphasic dose-response function, which we call the "Hormesis condition," is

$$\begin{aligned}&(k_{67} = 0 \wedge k_{23} \neq 0) \vee \\ &\left(k_{67} \neq 0 \wedge \left(\frac{k_{23}}{k_{67}} < \frac{k_4}{k_8} \leq \frac{k_1}{k_5} \vee \frac{k_{23}}{k_{67}} < \frac{k_1}{k_5} < \frac{k_4}{k_8} \vee \right.\right. \\ &\left.\left. \frac{k_4}{k_8} \leq \frac{k_1}{k_5} < \frac{k_{23}}{k_{67}} \vee \frac{k_1}{k_5} < \frac{k_4}{k_8} < \frac{k_{23}}{k_{67}}\right)\right).\end{aligned}$$

Because the values of the coefficients k_i are not known in general, the number of critical points cannot be determined unambiguously, and we need to distinguish cases depending on the number of segments in the reference and perturbed curves that are mapped to each other to derive the differential π_d and $\pi_{\mathcal{R}}$ (see the Supporting Material for details).

The differential expressions have a more complicated form (for example, they may involve square roots), and multiple conditions have to be considered, but the expressions are symbolic and (more involved) symbolic analysis with efficient methods for the generation and manipulation of Kirchhoff polynomials is applicable. For example, general properties of the differentials in systems with two dose

edges are 1) in contrast to systems with a single dose edge, the dose differential, π_d, depends on the choice of output vertices and their weights; 2) π_d depends on the proportion variable, h, and the synthesis reactions in open systems, but not on the conserved x_t in closed systems; 3) the response differential, $\pi_{\mathcal{R}}$, depends on all eight partitions of the set of spanning trees of G, and it includes x_t in closed systems; and 4) the differential can also be degenerate or undefined.

To apply the theory to insulin signaling, we consider a more detailed model for receptor trafficking that includes binding of a second ligand molecule to the receptor, as well as the relevant reactions and internalized species shown in Fig. 6 *A*. It is a receptor-level subsystem of the more comprehensive model of insulin signaling from (22). The motivation for the analysis is twofold. First, designed insulin analogs that trigger hormetic responses could potentially influence insulin differential signaling, for example, by alleviating mitogenic effects at high ligand doses. Second, biphasic dose-response behaviors of insulin receptor phosphorylation were experimentally observed in cells stimulated with an insulin analog, the peptide S961 (24). We therefore asked if there exist sets of species that need to be active to achieve a robust hormetic response in the model. We derived the steady-state coefficients for all 127 combinations of output vertices in O and used the hormesis condition to classify the resulting models into four groups. The first three groups contain models with robust dose-responses (same qualitative shape irrespective of parameter values), namely, 1) constant response for models with output vertex $\{v_{\mathrm{Ri}}\}$; 2) sigmoid for models with output vertices $\{v_{\mathrm{RLLp}}\}$, $\{v_{\mathrm{RLLpi}}\}$, and $\{v_{\mathrm{RLLp}}, v_{\mathrm{RLLpi}}\}$; and 3) hormetic for models with output vertices $\{v_{\mathrm{RLp}}\}$, $\{v_{\mathrm{RLpi}}\}$, and $\{v_{\mathrm{RLp}}, v_{\mathrm{RLpi}}\}$ (see Fig. 6 *B*). The fourth group contains the remaining 120 combinations of output vertices; the shapes of dose-response curves generated by these models depend on parameter values (see the Supporting Material for examples of analysis results that account for parameter dependencies). In particular, outputs producing robust hormetic responses comprise only phosphorylated receptors bound to a single ligand molecule, implying that doubly bound phosphorylated receptors should not signal if we are to obtain a robust hormetic dose response. This is consistent with the proposed mechanism of action of S961 in (24), namely, that the first binding of S961 cross-links the receptor, leading to agonist activity, and the second binding results in receptor inactivation.

Extension: numerical analysis

Numerical methods can augment the symbolic analysis when quantitative prior knowledge or experimental data are available. For example, we can directly incorporate known parameter values (or their ratios) to simplify symbolic expressions. We may also account for prior knowledge by defining plausible intervals of parameter values and determining whether the model's thus bounded differential is consistent with an experimentally observed differential, thereby assessing whether the model agrees with the observations.

Formally, we denote the range of the differential as the interval $\mathbb{D} = \{\pi(p^\alpha, p^\beta) \in \mathbb{R} \mid p^\alpha, p^\beta \in \mathcal{I}\}$, where $\mathcal{I} \subseteq (\mathbb{R}_{\geq 0})^N$ is a box in parameter space. Let a and b be the infimum and supremum of $\mathbb{D}$. We want to determine tight outer bounds of the differential, finding $\widehat{a}$ and $\widehat{b}$ of $\mathbb{D}$ over the parameter box $\mathcal{I}$, where $\widehat{a} \leq a$ and $\widehat{b} \geq b$. In systems with a single dose edge, with edge labels that contain rational expressions, and regarding all parameters as variables, bounding the differential translates to finding the global extrema of a multivariate rational function. This is an NP-hard problem (25), but several numerical methods give certificates for global optimality or find bounds based on polynomial optimization. Here, we focus on Bernstein enclosures of rational functions (26) whose implementation in the Kodiak package, for example, was shown to produce tighter outer bounds for a dose differential expression with 12 free variables compared to the established method of interval arithmetic (27).

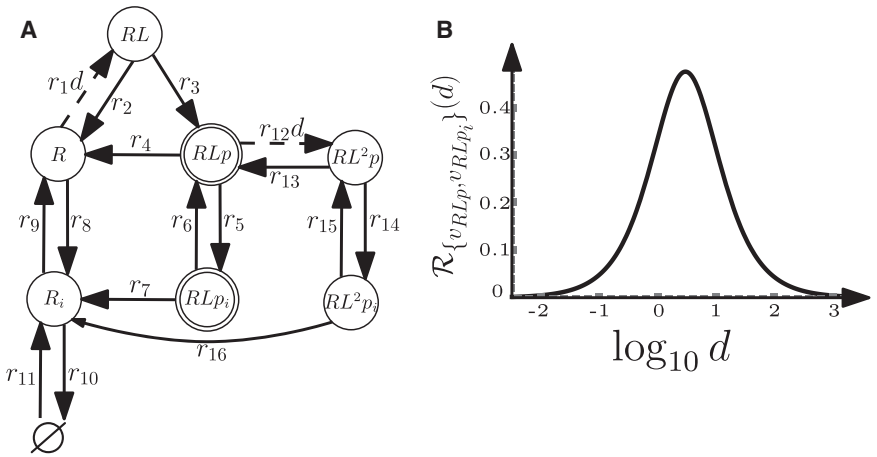

FIGURE 6 Extended insulin model with two dose edges. (*A*) Model of insulin receptor binding, recycling, and phosphorylation with notation as in Fig. 3 *A*. (*B*) A hormetic dose-response curve generated by the model for output vertices $O = \{v_{\mathrm{RLp}}, v_{\mathrm{RLpi}}\}$ and all parameters fixed to 0.5.

To illustrate the approach for the example model from Fig. 3 *A* (for a two-dose-edge example, see the Supporting Material), we analyze how the bounds of the dose differential change when a parameter is altered; we call the resulting plots "profile differential bounds". Specifically, we fix each of the free parameters taking part in the differential, r_2^β and r_3, for every value in their interval of definition, and calculate the bounds of the dose differential (Fig. 7 *A*). This analysis shows the capacity of individual parameters to control and constrain the possible magnitude of the differential: independent of the value of the other free parameter, r_2^β can significantly change the lower bound of the differential for values smaller than $\sim 10^{-2}$, and for higher values it starts decreasing the upper bound. If the observed differential is outside the calculated bounds, one can reliably reject the model (and the parameter box $\mathcal{I}$), because no parametrization (in $\mathcal{I}$) can reproduce the observed differential. Conversely, this analysis can help confine $\mathcal{I}$ such that all considered parameter values are consistent with the experimental observations.

The non-linear differential expressions' algebraic structures also imply that not every differential value is equally likely when we sample parameter values uniformly from $\mathcal{I}$. The non-uniformity of the differential can be interpreted as parametric robustness, because random changes of parameters may not lead to random magnitudes of the differential. For our simple model from Fig. 3 *A*, uniform sampling of the dose differential yields a few magnitudes of variability ($\widehat{\mathbb{D}_{\pi_d}} = [-4, -2]$) but two small regions of most probable values with a marginal density peaked at -2 and -4 (Fig. 7 *B*). The profile differential distributions in Fig. 7 *A* show how the free parameters r_2^β (ligand dissociation rate in the perturbed model) and r_3 (receptor phosphorylation) affect not only the bounds but also the peak of the marginal distribution, revealing their potential to control the differential. Hence, the differential system induces an important structural prior on the behavior—system structures (and parameter intervals) define what magnitude of the differential can be expected. This information could be exploited for more detailed model selection against experimental data.

DISCUSSION

Here, we aimed to develop a theory for steady-state dose-response relationships in linear framework models of biochemical reaction networks that is analytic, and therefore also applicable when parameter values are largely unknown. We formalized the concept of differential responses to establish a comprehensive parameter-free framework for analyzing relative responses. It helps to study system behaviors upon perturbations of many features of reaction networks, such as network topology, parameters, and choice and number of inputs and outputs. In particular, the algebraic and numerical methods allow us to explore possible network topologies and perturbations to arrive efficiently at a set of candidate models that are consistent with prior knowledge and experimental data. For example, if it is known from experiments that a particular perturbation leads to a significant dose differential, we can reliably reject all potential models for which the differential expression does not depend on the perturbed variable. Numerical bounding over a predefined region in parameter space provides us with limits for all possible differential magnitudes—which we can use as a certificate to reliably reject models that can never reproduce quantitative experimental data. Another application of our methodology is in experimental design, namely, to determine (optimal) perturbations of a reference system that lead to a desired differential, to invalidate a model, to discriminate between equivalence classes of networks, or for applications such as finding optimal (combinations of) drug targets. Note also that the differential analysis framework extends to applications beyond the ones we covered: in general, every element of the

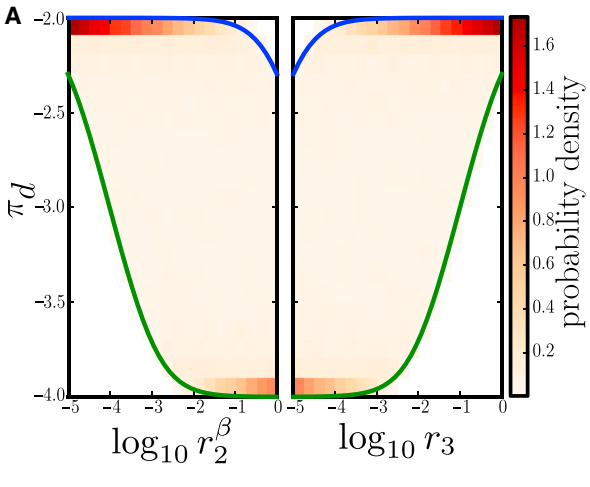

FIGURE 7 Numerical analysis of the example single-dose-edge model from Fig. 3 *A*. (*A*) Profile bounds (*blue*, upper outer bound; *green*, lower outer bound) superimposed on the profile differential distribution (density) for the free parameters r_2^β and r_3. (*B*) Marginal probability distribution of the dose differential magnitude. Densities were obtained from uniform samples of the parameter box $\mathcal{I}$ defined by $r_1, r_2, r_3, r_4 \in [10^{-5}, 1]\, s^{-1}$, assuming $x_t = 1\text{nM}$, $r_1^\alpha / r_1^\beta = 100$, and $r_2^\alpha / r_2^\beta = 0.01$. To see this figure in color, go online.

differential system can be perturbed and labels for dose edges can be formulated in a more general form.

The most obvious limitation of our framework is the restriction to systems with Laplacian dynamics; it has to rely on prior, often parameter- and state-dependent simplifications from higher-order kinetics, such as those obtained via timescale separation. In terms of scalability, the exponential growth of the number of graphs to be considered for the analysis and the resulting high-degree polynomials with increasing number of reactions influenced by the dose is a challenge. Our examples demonstrate that systems with up to two dose edges can be analyzed efficiently due to the linear scaling of the prime factorization algorithm and the few relevant graphs. General algebraic solutions for the roots of polynomial equations of degree five or higher with arbitrary coefficients—corresponding to the dose acting proportionally on five or more edges—do not exist. However, depending on the particular graph structure and form of the label expressions, such cases, or cases with labels that are non-linear functions of the dose, could be analytically tractable if the polynomials simplify to a lower degree. Similarly, obtaining exact bounds might only be feasible for simple differential expressions with few variables and convex properties. In practice, therefore, bounding methods provide us with inner bounds, which do not guarantee reliable model rejection. Because bounding approaches are often employed in control theory (28) and methods are being continuously improved (27,29), these limitations may be less pertinent.

Extensions of the framework could further build on the central insight that prime factors and components (and their similarity in perturbed graphs) are the units, the very characterization, of the differential response function in steady-state linear framework models. For example, additions and deletions of vertices and edges in certain parts of both the reference and perturbed graph may never have an effect on the differential if they belong to a prime component that always cancels in the differential expression. In general, the positions (connectivity) of additions and deletions within the graph and the size distribution of the induced prime components play an important role in the change of the differential upon structural perturbations. This could be exploited to develop a notion of "structural robustness" of the differential to random changes of the network topology. Finally, many of our graph theoretical notions subsume graph theoretical concepts such as distributions of sizes of SCCs, strong bridges, and strong articulation points that are actively researched in computer science; efficient algorithms for their characterization (30–34) could help to extend the scope of our framework.

In terms of applications, our analysis of the insulin signaling network demonstrates first steps in a direction we believe will become increasingly important: a systematic analysis of differential dose responses in biochemical reaction networks despite prevailing uncertainties as to the networks' quantitative features. In particular, we showed how to characterize the dose responses of natural ligands, such as insulin and IGF-1, where different affinities influence the dose but not the maximal response component, and we recovered previously hypothesized modes of action of the insulin analog S961. We envisage that our framework will be most useful for the systematic study of mechanisms underlying hormesis, a phenomenon in toxicology and cell signaling that is receiving increasing attention. For example, empirical evidence favored hormetic over threshold models for dose-response relationships in a large-scale yeast anticancer drug screen (35), and hormetic phenomena are frequently observed in stress responses and their relations to aging (36). Corresponding theoretical work has only (re-)started very recently, showing, for example, that non-monotonic dose-response relationships can arise through non-obvious pathway interactions, and that network structures impose fundamental constraints on options for pharmacological treatment (37).

SUPPORTING MATERIAL

Supporting Materials and Methods, Supporting Results, and five figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(17)35042-7.

AUTHOR CONTRIBUTIONS

P.Y. and J.S. designed research, P.Y. performed research, and P.Y. and J.S. wrote the manuscript.

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Supplemental Information

Steady-State Differential Dose Response in Biological Systems

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Steady-State Differential Dose-Response in Biological Systems —Supporting Material—

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Open Linear Framework Models

Graphs G representing open linear framework models are obtained by adding a vertex $v_\varnothing$ denoting the environment to a *core graph* $\overline{G}$ (akin to closed models, the core graph is composed of all non-synthesis and non-degradation reactions) and by introducing directed edges from $v_\varnothing$ to the synthesized species in $\overline{G}$ with labels s_i and edges labeled d_i from the degraded species to $v_\varnothing$. The dynamics of open linear framework models are defined in general form as:

$$\frac{dx}{dt} = \mathcal{L}(\overline{G})x - \Delta x + S,$$

where $\mathcal{L}(\overline{G})$ is the Laplacian matrix of the core graph, Δ is a diagonal matrix with $\Delta_{ii} = \delta_i$ the degradation rate constants of the species with index i, and S is a vector $S_i = s_i$ comprising the synthesis rate constants for all species. If a species does not have a degradation or a synthesis reaction then $s_i = 0$ or $\delta_i = 0$, respectively. In open models, the total amount of matter is not conserved but the rates at which matter enters and leaves the system determine the final distribution of steady-state concentrations. In particular, synthesis and degradation at steady-state are balanced: $\delta_1 x_1 + \ldots + \delta_n x_n = s_1 + \ldots + s_n$. Similarly to closed models, but assuring that the steady-state concentration at $v_\varnothing$ is always 1, the unique stable steady-state for vertex v_i ($v_i \neq v_\varnothing$) can be symbolically derived.

The general form of the steady-state concentration x_i^{SS} for open systems and a vertex v_i ($v_i \neq v_\varnothing$) is given by:

$$x_i^{SS} = \frac{\kappa_{v_i}(G)}{\kappa_{v_\varnothing}(G)}.$$

For more details, proofs, and derivations on open linear framework models we refer to (1, 2).

In open models, the q species eliciting the response are associated with a set of output vertices $O(\overline{G})$. Then the general expression for the steady-state response of open models using Kirchhoff polynomials reads:

$$\mathcal{R}_{O(\overline{G})} = \frac{\sum_{v_i \in O(\overline{G})} a_i \kappa_{v_i}(G)}{\kappa_{v_\varnothing}(G)},$$

where $a_i \geq 0$ is the weight given to the steady-state concentration associated with vertex v_i.

The denominator in the steady-state expression of open strongly connected models contains the strongly connected G rooted at the environment vertex $v_\varnothing$ which can yield graphs with factorisable Kirchhoff polynomials. Hence, the Kirchhoff polynomial corresponding to $\mathsf{rt}_{v_\varnothing}(G)$ is non-trivially factorisable when any synthesis reaction s_i is a prime bridge during the sequential deletion of s_i resulting from the rooting operation at $v_\varnothing$ (s_1 might not be a prime bridge in G but in $G \setminus s_2 \ldots \setminus s_n$). Likewise, the numerator of the steady-state expression for open models consists of the linear combination of rooted polynomials, each of which could be factorisable. In this case, there could exist prime factors shared between the numerator and the denominator which can be canceled out. Thereby, in open systems there exist equivalence classes of models with different graphs G but the same steady-state expressions. A necessary and sufficient condition for a reaction to take part in the steady-state expression is that it is part of a prime component that does not get canceled.

Applying the deletion-contraction property from Eq. 5 to express the steady-state response $\mathcal{R}$ as a function of the dose variable d, we obtain the general form of dose-response expression for open models with a graph G:

$$\mathcal{R}_O(d) = \frac{k_1 + k_2 d}{k_3 + k_4 d}, \tag{1}$$

$$\text{with steady-state coefficients:} \quad \begin{aligned} k_1 &= \textstyle\sum_{v_i \in O(\overline{G})} a_{v_i} \kappa_{v_i}(G \backslash e_d), \\ k_2 &= g(p) \textstyle\sum_{v_i \in O(\overline{G})} a_{v_i} \kappa_{v_i}(G / e_d), \\ k_3 &= \kappa_{v_\varnothing}(G \backslash e_d), \\ k_4 &= g(p) \kappa_{v_\varnothing}(G / e_d). \end{aligned}$$

Fig. S1 shows the tree scheme for generating the steady-state coefficients of open models.

Formalizing Differential Responses

Here we give details about the proposed procedure for quantifying the differential response, i.e. how a reference dose-response curve transforms into a perturbed one. Our definition of the differential employs established concepts for comparisons between monotone dose-response curves to generalise comparisons between non-monotone curves. Note that other definitions could be more appropriate

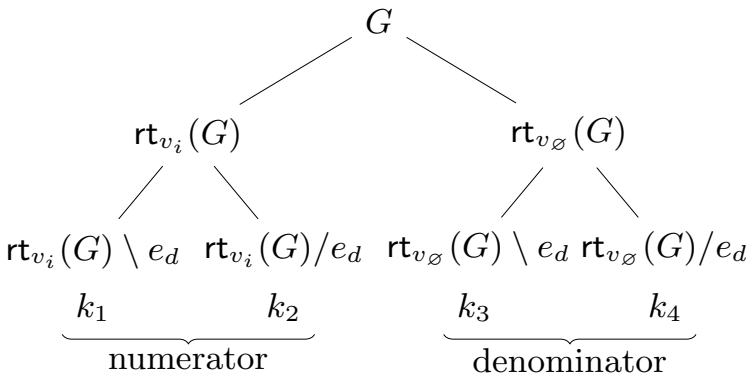

Figure S1: Tree scheme for a general graph G for obtaining the relevant graphs participating in the coefficients k_i of the dose-response relationship in open models (for reference and perturbed systems) through the graph operations rooting, deletion, and contraction. Note that there are also additional terms contained in the coefficients.

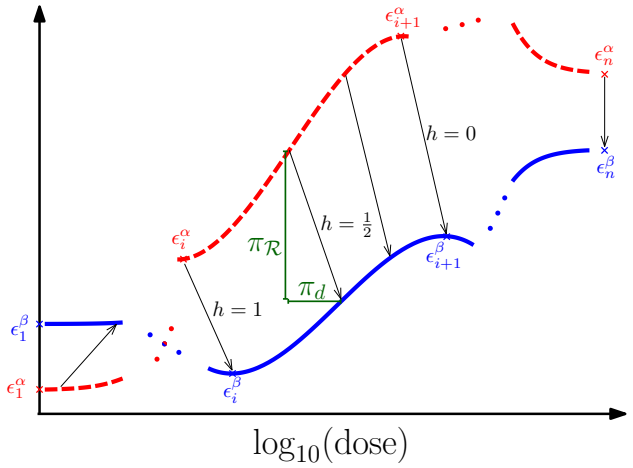

Figure S2: Definition of the differential response as the length of displacement of a reference dose-response curve (red, dashed, marked with the α superscript) to a perturbed curve (blue, solid, marked with the β superscript), generated by functions $\mathcal{R}^\alpha(d)$ and $\mathcal{R}^\beta(d)$, respectively, both with n critical points $\epsilon^{\{\alpha,\beta\}}_{1\ldots n}$. The curves are subdivided along their critical points to obtain monotone segments. The resulting segments are related through a map $\mathcal{M}$ that preserves the order of critical points and segments. Points on a pair of mapped segments with the same proportion of response $h \in [0, 1]$ between the minimum and the maximum are related to each other (corresponding points indicated by black arrows). Distances in the dose and the response dimensions between corresponding points are called the *dose differential* and the *response differential*, and denoted as $\pi_d(h)$ and $\pi_\mathcal{R}(h)$ (green), respectively.

when specific knowledge on the curve transformation contradicts the assumptions we make.

Let dose-response curves be generated by functions $\mathcal{R} : \mathbb{R}_{\geq 0} \to \mathbb{R}_{\geq 0}$, that are continuous, smooth, and bounded (unbounded responses are not biologically feasible). We denote the functions generating the reference and the perturbed curve as $\mathcal{R}^\alpha(d)$ and $\mathcal{R}^\beta(d)$, respectively, where d is the dose variable. A point on a dose-response curve, $(\mathtt{d}, \mathtt{R})$, consists of a dose component $\mathtt{d}$ and a response component $\mathtt{R}$ such that $\mathtt{R} = \mathcal{R}(\mathtt{d})$.

We express the differential through distances between corresponding points on $\mathcal{R}^\alpha(d)$ and

$\mathcal{R}^\beta(d)$. To find the correspondence between points and quantify the distance between corresponding points we follow the procedure (also see Fig. S2):

(i) *Subdivide the curves into monotone segments.*

We subdivide the dose-response curves at their critical points (suprema, infima, extrema, and stationary points of inflection that are identified by the functions' first derivatives) to obtain monotone segments for further comparison.

Assume that $\mathcal{R}^\alpha(d)$ and $\mathcal{R}^\beta(d)$ have, respectively, n and m critical points and denote them by $\epsilon_i \in \mathcal{E}$, where $\mathcal{E}$ is the set of all critical points for the relevant dose-response curve and i is their index ($i \in \{1, \ldots, n\}$ for ϵ_i^α and $i \in \{1, \ldots, m\}$ for ϵ_i^β). Due to the functional relation between dose and response and by considering any two or more identical critical points as a single one, the critical points follow a strict total order in their dose component $\mathtt{d}_{\epsilon_i}$ (e.g. for $\mathcal{R}^\alpha(d)$, $\mathtt{d}_{\epsilon_1^\alpha} < \ldots < \mathtt{d}_{\epsilon_n^\alpha}$), which we use to define a strict total order of the critical points (e.g. for $\mathcal{R}^\alpha(d)$, $\epsilon_1^\alpha < \ldots < \epsilon_n^\alpha$).

From the boundedness requirement on $\mathcal{R}(d)$ it follows that the first and the last critical points are reached when the dose goes to zero and infinity, respectively. The intermediate critical points are defined by doses for which the first derivative of f is zero. Thus the critical points of $\mathcal{R}^\alpha(d)$ are:

$$\epsilon_1^\alpha := \left(0, \lim_{d\to 0} \mathcal{R}^\alpha(d)\right), \epsilon_i^\alpha := \{(\mathtt{d}_{\epsilon_i}, \mathcal{R}^\alpha(\mathtt{d}_{\epsilon_i}))|D_d\mathcal{R}^\alpha(\mathtt{d}_{\epsilon_i}) = 0\},$$
$$\text{and } \epsilon_n^\alpha := \left(\infty, \lim_{d\to\infty} \mathcal{R}^\alpha(d)\right),$$

where $i \in \{2, \ldots, n-1\}$ indexes the intermediate critical points and D_d denotes the first derivative with respect to the dose variable d.

The critical points of $\mathcal{R}^\alpha(d)$ partition its domain into $n-1$ monotone segments σ_j, $j \in \{1, \ldots, n-1\}$. Each segment is defined by two consecutive critical points:

$$\sigma_j^\alpha : \mathcal{R}^\alpha(d), \text{ for } d \in \left[\mathtt{d}_{\epsilon_j^\alpha}, \mathtt{d}_{\epsilon_{j+1}^\alpha}\right],$$

where the domain of the segment is semi-open for the last critical point since it has a dose component at infinity. Let us denote the set of all segments as Σ^α. The definitions of critical points and segments for $\mathcal{R}^\beta(d)$ are analogous.

(ii) *Decide which segments to compare.*

A map $\mathcal{M}$ defines the correspondence between the monotone segments from the reference curve and the monotone segments from the perturbed curve. The definition of the map can be application-specific. Here, in the absence of specific knowledge on the transformation between the curves, we propose that $\mathcal{M}$ preserves the order (and succession, i.e. no critical point is missed out) of critical points and segments. Let us assume, w.l.o.g., that $\mathcal{R}^\alpha(d)$ has less or equal number of critical points than $\mathcal{R}^\beta(d)$ ($n \leq m$). Then, we define $\mathcal{M}$ to map all consecutive segments of $\mathcal{R}^\alpha(.)$ to all possible n consecutive segments of $\mathcal{R}^\beta(.)$,namely:

$$\mathcal{M}(i;\Sigma^\alpha,\Sigma^\beta): \begin{cases} \sigma_1^\alpha \to \sigma_i^\beta \\ \sigma_2^\alpha \to \sigma_{i+1}^\beta \\ \quad \vdots \\ \sigma_{n-1}^\alpha \to \sigma_{i+n-2}^\beta \end{cases},$$

where $i \in \{1,2,\ldots,1+m-n\}$. Notice that in the case when $n=m$ the map is bijective and it does not depend on the index $i=1$.

(iii) *Determine corresponding points in compared pairs of segments.*

In each pair of mapped segments $\sigma_i^\alpha \mapsto \sigma_j^\beta$ we relate the points having the same proportion h ($h \in [0,1]$) of response between the minimal and maximal response, as is customary for monotone dose-response curves. The minimal and maximal responses in each segment are determined by the response components of the critical points enclosing it. Let $\zeta(h;x,y)$ be the *proportional response function* which gives the response for a proportion h and response components x and y of the critical points enclosing the segment of interest. Then, we can obtain the response components of the related points within the segments:

$$\mathtt{R}_{\sigma_i^\alpha,h} \mapsto \mathtt{R}_{\sigma_j^\beta,h},$$

where $\mathtt{R}_{\sigma_i^\alpha,h} = \zeta\left(h;\mathtt{R}_{\epsilon_i^\alpha},\mathtt{R}_{\epsilon_{i+1}^\alpha}\right)$ and $\mathtt{R}_{\sigma_j^\beta,h} = \zeta\left(h;\mathtt{R}_{\epsilon_j^\beta},\mathtt{R}_{\epsilon_{j+1}^\beta}\right)$.

We recover the dose components of the related points from the dose-response function:

$$\mathtt{d}_{\sigma_i^\alpha,h} \mapsto \mathtt{d}_{\sigma_j^\beta,h},$$

where $\mathtt{d}_{\sigma_i^\alpha,h} = {\mathcal{R}^\alpha}^{-1}\left(\zeta\left(h;\mathtt{R}_{\epsilon_i^\alpha},\mathtt{R}_{\epsilon_{i+1}^\alpha}\right)\right)$, ${\mathcal{R}^\alpha}^{-1}(.)$ is the inverse function of $\mathcal{R}^\alpha$ in the interval $\left[d_{\epsilon_i^\alpha}, d_{\epsilon_{i+1}^\alpha}\right]$ (the interval is semi-open for the last critical point since it has a dose component

at infinity). The inverse exists due to continuity and monotonicity of the segment σ_i^α.

The following two definitions can serve as a proportional response function:

$$\zeta_1(h;x,y) := \begin{cases} hx + (1-h)y, & \text{if } x \neq y \\ x, & \text{when } x = y \end{cases}, \text{ and}$$

$$\zeta_2(h;x,y) := \begin{cases} hx + (1-h)y, & \text{if } x > y \\ (1-h)x + hy, & \text{if } x < y \\ x, & \text{when } x = y \end{cases},$$

which are simplifications, respectively, of:

$$\zeta_1(h;x,y) := \frac{1}{2}\left(\left(1 + \frac{x-y}{|x-y|}\right)h + \left(1 - \frac{x-y}{|x-y|}\right)(1-h)\right)|x-y| + \min(x,y) \qquad \text{and}$$

$$\zeta_2(h;x,y) := h\,|x-y| + \min(x,y)\,,$$

where, again, $h \in [0,1]$ is the proportion of response and x and y are the response coordinates (corresponding to dose coordinates $\mathtt{d}_x$ and $\mathtt{d}_y$, $\mathtt{d}_x < \mathtt{d}_y$) defining a segment σ. Note also that we are not interested in the case when $x = y$ in a segment since it leads to a trivial differential expression.

The differences between these very similar definitions become evident when one of the compared segments is monotonically increasing and the other one is monotonically decreasing; otherwise the definitions are identical up to the parametrisation of h. We choose to use ζ_1, hence, calling it only ζ, due to the simpler expressions it yields in our subsequent derivations. Note that $\zeta_1(0;x,y) = y$ and $\zeta_1(1;x,y) = x$, which means that for $h = 1$ the response coordinates of smaller dose coordinates correspond to each other while for $h = 0$ the correspondence is between the response coordinates of larger dose coordinates.

(iv) *Quantify the displacement of corresponding points.*

The last step to derive the differential is to quantify the displacement between corresponding points on the reference and perturbed curves for all mapped segments. For each pair of points we derive the displacement in dose, what we call the *dose differential*, and the displacement in response – the *response differential*. Formally, to identify fold differences in the dose variable, we define the *dose differential* as the difference of logarithms of the dose components of

corresponding points:

$$\pi_d(h; \sigma_i^\alpha, \sigma_j^\beta) := \log_{10} \frac{\mathtt{d}_{\sigma_i^\alpha, h}}{\mathtt{d}_{\sigma_j^\beta, h}}$$

and the response differential as the difference in response between corresponding points:

$$\pi_{\mathcal{R}}(h; \sigma_i^\alpha, \sigma_j^\beta) := \mathtt{R}_{\sigma_i^\alpha, h} - \mathtt{R}_{\sigma_j^\beta, h}.$$

The set of all these displacements constitutes the differential between the curves.

Note that for monotone dose-response curves the differential reduces to the established comparison of points with the same percentage of response. For non-monotone curves each segment is compared to another one at least once allowing to quantify the relative difference between dose-response curves. Note that even when dose-response curves can be derived in closed form, the differential can be symbolically derived only when the critical points and dose-response functions' inverses can be found symbolically.

Derivation of the Differential for One Dose Edge

We derive the differential for dose-response curves generated by functions of the form of Eq. 6 using the procedure outlined in the previous section. We have only fixed to have a single dose edge, all other features of the reference and perturbed models can be arbitrary.

(i) *Subdivide the curves into monotone segments.*

The steady-state function $\mathcal{R}_O(d)$ does not have extrema when varying the dose d since the first derivative is nowhere zero (apart from infinity and the dose independent case when $k_2 k_3 = k_1 k_4$). There are only two critical points and the dose-response curve is a sigmoid in log scale. The critical points are:

$$\mathcal{E} = \left\{ \epsilon_1 = \left(0, \frac{k_1}{k_3}\right), \epsilon_2 = \left(\infty, \frac{k_2}{k_4}\right) \right\}.$$

In each of the reference and the perturbed curve there exists only one segment defined between the dose components of ϵ_1 and ϵ_2, which we call σ.

(ii) *Decide which segments to compare.*

Since each of the compared curves consists of a single segment the mapping is trivial:

$$\mathcal{M} : \sigma^{\alpha} \to \sigma^{\beta}.$$

(iii) *Determine corresponding points in compared pairs of segments.*

We relate the response coordinates of points on σ^{α} and σ^{β} with identical percentage of response:

$$\zeta\left(h; \frac{k_1^{\alpha}}{k_3^{\alpha}}, \frac{k_2^{\alpha}}{k_4^{\alpha}}\right) \mapsto \zeta\left(h; \frac{k_1^{\beta}}{k_3^{\beta}}, \frac{k_2^{\beta}}{k_4^{\beta}}\right) \;\Leftrightarrow\; h\frac{k_1^{\alpha}}{k_3^{\alpha}} + (1-h)\frac{k_2^{\alpha}}{k_4^{\alpha}} \mapsto h\frac{k_1^{\beta}}{k_3^{\beta}} + (1-h)\frac{k_2^{\beta}}{k_4^{\beta}},$$

where the superscripts α and β indicate that the coefficients k have been obtained from the reference or perturbed system, respectively.

To relate the dose components of corresponding points, $\mathtt{d}_{\sigma^{\alpha},h} \mapsto \mathtt{d}_{\sigma^{\beta},h}$, we find the inverse of the single dose edge dose-response function and plug in the proportional response function:

$$\mathtt{d}_{\sigma,h} = \mathcal{R}^{-1}\left(\zeta\left(h; \frac{k_1}{k_3}, \frac{k_2}{k_4}\right)\right) = \frac{k_1 - \zeta\left(h; \frac{k_1}{k_3}, \frac{k_2}{k_4}\right) k_3}{\zeta\left(h; \frac{k_1}{k_3}, \frac{k_2}{k_4}\right) k_4 - k_2},$$

which reduces to:

$$\mathtt{d}_{\sigma,h} = \begin{cases} \frac{1-h}{h}\frac{k_3}{k_4} & \text{if } \frac{k_1}{k_3} \neq \frac{k_2}{k_4}, \\ \text{not defined} & \text{if } \frac{k_1}{k_3} = \frac{k_2}{k_4}. \end{cases}$$

Ignoring the trivial case when $\frac{k_1}{k_3} = \frac{k_2}{k_4}$ we obtain the following correspondence between the dose components of related points:

$$\frac{1-h}{h}\frac{k_3^{\alpha}}{k_4^{\alpha}} \mapsto \frac{1-h}{h}\frac{k_3^{\beta}}{k_4^{\beta}}.$$

(iv) *Quantify the displacement of corresponding points.*

Having determined the correspondence between points, we obtain general expressions for the dose and response differentials in differential systems $\mathcal{D}$ with a single dose edge (also Eq. 7):

$$\pi_d = \log_{10}\frac{k_3^{\alpha}k_4^{\beta}}{k_3^{\beta}k_4^{\alpha}} \qquad \text{and} \qquad \pi_{\mathcal{R}}(h) = h\left(\frac{k_1^{\alpha}}{k_3^{\alpha}} - \frac{k_1^{\beta}}{k_3^{\beta}}\right) + (1-h)\left(\frac{k_2^{\alpha}}{k_4^{\alpha}} - \frac{k_2^{\beta}}{k_4^{\beta}}\right).$$

Steady-state Expressions for the Investigated Single Dose Edge Models

The steady-state expression (before plugging in the differential parameters) for the example from Fig. 4 reads:

$$\mathcal{R}_O(d) = \frac{r_1 r_3 r_9 \left(r_5 + r_6 + r_7\right) d}{\left(r_2 + r_3\right)\left(r_8 + r_9\right)\left(r_4\left(r_6 + r_7\right) + r_5 r_7\right) + \\ + r_1\left(r_5\left(r_3 r_9 + r_7\left(r_3 + r_9\right)\right) + r_9\left(r_3 + r_4\right)\left(r_6 + r_7\right)\right) d} x_t.$$

The steady-state expression for the example from Fig. 5B is:

$$\mathcal{R}_O(d) = \frac{r_1\left(r_3 r_5 r_9 r_{11} + r_3\left(r_6 + r_7\right) r_9 r_{11}\right) d}{r_8 r_{10}\left(r_2 + r_3\right)\left(r_4\left(r_6 + r_7\right) + r_5 r_7\right) + r_1 r_3 r_5 r_7 r_{10} d}.$$

Applications for Experimental Design

To discriminate between models in the same equivalence class, a logical next question is what second perturbation to design (or how to change the first perturbation) in order to differentiate between models in the same equivalence class. In other words, we want to divide the class into smaller equivalence classes, and ultimately identify a single model that represents the biological process. More specifically, a second perturbation could change the prime factors, for example, by adding or deleting new species and reactions, or by changing the input edge. We illustrate the theory's capabilities by deciding which reaction rate constant to alter in the perturbed system for the model from from Fig. 5B. For example, we ask whether to experimentally perturb r_8 or r_5 to obtain the largest effect in the dose differential. For the steady-state coefficient k_3, we observe that r_8 is alone in a prime component while r_5 has three more reaction constants in the same prime component. This means that, if we perturb r_8, we will obtain a factor in the dose differential corresponding to $\frac{k_3^\alpha}{k_3^\beta} = \frac{r_8^\alpha}{r_8^\beta}\frac{r_2^\alpha + r_3}{r_2^\beta + r_3}$ where our perturbation will have a multiplicative effect on the dose differential. On the other hand, if we perturb r_5 we obtain $\frac{k_3^\alpha}{k_3^\beta} = \frac{r_4(r_6+r_7)+r_5^\alpha r_7}{r_4(r_6+r_7)+r_5^\beta r_7}\frac{r_2^\alpha + r_3}{r_2^\beta + r_3}$, where the perturbation is dampened by the other reaction rates—the change in the dose differential upon this perturbation might become experimentally indistinguishable. Hence, perturbing elements of smaller factors has a more direct effect on the observed dose differential, and a corresponding experimental design is more likely to help determining whether the model under consideration is appropriate.

General Form of Dose-Response Curves Generated by Two Dose Edge Models

We consider the case in which the input dose acts proportionally and simultaneously on two edges, i.e. $I(G) = \{e_{d,1}, e_{d,2}\}$, $\ell(e_{d,1}) = g_1(p)d$, and $\ell(e_{d,2}) = g_2(p)d$.

We apply the deletion-contraction formula to partition the set of spanning trees from the numerator and denominator of the response function $\mathcal{R}$ into four categories – those containing no input edges, those containing $e_{d,1}$ but not $e_{d,2}$, those containing $e_{d,2}$ but not $e_{d,1}$, and those containing both $e_{d,1}$ and $e_{d,2}$. Simplifying, we obtain the general form of dose-response expressions for closed and open systems:

$$\mathcal{R}_O(d) = \frac{k_1 + k_{23}d + k_4 d^2}{k_5 + k_{67}d + k_8 d^2}, \tag{2}$$

where $k_{23} := k_2 + k_3$ and $k_{67} := k_6 + k_7$, $\mathcal{R}_O(d)$ is bounded (the degree of the numerator is not higher than the degree of the denominator) and of second degree, and the steady-state coefficients are:

for closed models

$k_1 = x_t \sum_{v_i \in O(G)} a_{v_i} \kappa_{v_i}(G \backslash e_{d,1} \backslash e_{d,2})$,
$k_2 = x_t g_2(p) \sum_{v_i \in O(G)} a_{v_i} \kappa_{v_i}(G \backslash e_{d,1} / e_{d,2})$,
$k_3 = x_t g_1(p) \sum_{v_i \in O(G)} a_{v_i} \kappa_{v_i}(G / e_{d,1} \backslash e_{d,2})$,
$k_4 = x_t g_1(p) g_2(p) \sum_{v_i \in O(G)} a_{v_i} \kappa_{v_i}(G / e_{d,1} / e_{d,2})$,
$k_5 = \kappa(G \backslash e_{d,1} \backslash e_{d,2})$,
$k_6 = g_2(p) \kappa(G \backslash e_{d,1} / e_{d,2})$,
$k_7 = g_1(p) \kappa(G / e_{d,1} \backslash e_{d,2})$,
$k_8 = g_1(p) g_2(p) \kappa(G / e_{d,1} / e_{d,2})$,

for open models

$k_1 = \sum_{v_i \in O(\overline{G})} a_{v_i} \kappa_{v_i}(G \backslash e_{d,1} \backslash e_{d,2})$,
$k_2 = g_2(p) \sum_{v_i \in O(\overline{G})} a_{v_i} \kappa_{v_i}(G \backslash e_{d,1} / e_{d,2})$,
$k_3 = g_1(p) \sum_{v_i \in O(\overline{G})} a_{v_i} \kappa_{v_i}(G / e_{d,1} \backslash e_{d,2})$,
$k_4 = g_1(p) g_2(p) \sum_{v_i \in O(\overline{G})} a_{v_i} \kappa_{v_i}(G / e_{d,1} / e_{d,2})$,
$k_5 = \kappa_{v_\varnothing}(G \backslash e_{d,1} \backslash e_{d,2})$,
$k_6 = g_2(p) \kappa_{v_\varnothing}(G \backslash e_{d,1} / e_{d,2})$,
$k_7 = g_1(p) \kappa_{v_\varnothing}(G / e_{d,1} \backslash e_{d,2})$,
$k_8 = g_1(p) g_2(p) \kappa_{v_\varnothing}(G / e_{d,1} / e_{d,2})$.

The spanning tree partitioning determines the graphs contained in the coefficients k, which can be seen in the tree scheme from Fig. S3. The numerator and denominator polynomials in the dose variable d can be at most of degree two, where the highest degree corresponds to spanning trees containing both $e_{d,1}$ and $e_{d,2}$. We see that even though the degree of the polynomials grows by one, the number of graphs to consider grows exponentially. In the general case, for an input acting on w edges simultaneously, the numerator and denominator are at most of degree w and the graphs giving rise to the coefficients of the polynomials are 2^w and, therefore, the tree scheme has 2^{w+1} leaves. Again, it could happen that spanning trees do not exist for some graphs resulting to simpler, trivial, or unbounded dose-response relationships.

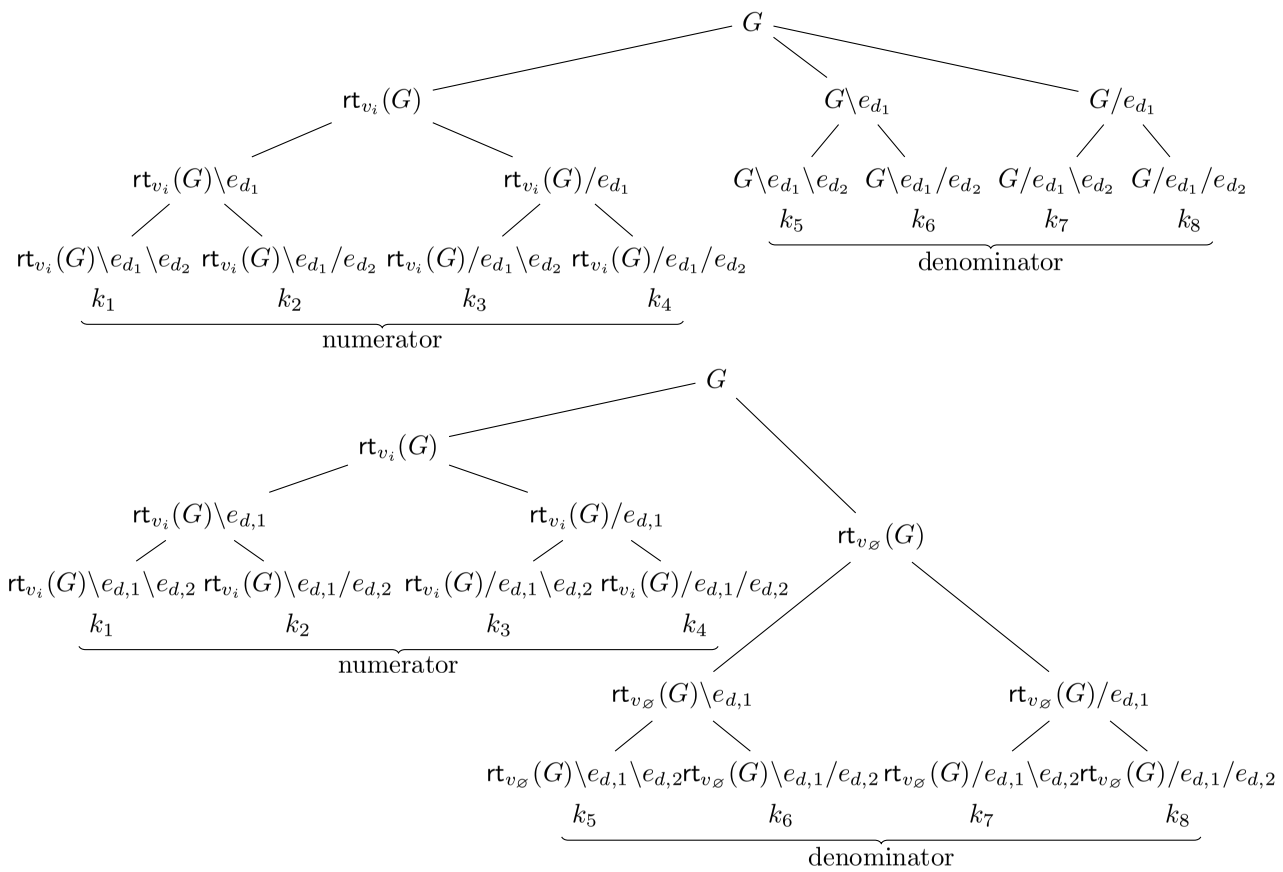

Figure S3: Tree scheme for obtaining the relevant graphs generating the coefficients k_i in the dose-response relationship when the dose acts simultaneously on two edges for (**above**) closed and (**below**) open systems through the graph operations rooting, deletion, and contraction. Note that there are also additional terms contained in the coefficients.

Derivation of the Differential for Two Dose Edges

We derive the differential for the case when the reference and perturbed dose-response curves are both generated by functions having the form of Eq. 2.

Hormesis condition. First, we are interested in deriving conditions guaranteeing positivity of an extremum and thus ensuring that hormesis is present. The steady-state dose-response function $\mathcal{R}_O(d)$ could have at most two extrema when varying the dose variable since its first derivative can become zero for two values of d.

$$D_d \frac{k_1 + k_{23}d + k_4 d^2}{k_5 + k_{67}d + k_8 d^2} = 0 \Leftrightarrow$$

$$\frac{k_5 k_{23} - k_1 k_{67} + 2(k_4 k_5 - k_1 k_8)d + (k_4 k_{67} - k_8 k_{23})d^2}{(k_5 + k_{67}d + k_8 d^2)^2} = 0.$$

The denominator of the condition is never zero for positive doses d and k_i leading to non-degenerate systems (not all k_i being zero). The doses for which the numerator equals zero are the ones corresponding to extrema in the dose response, namely:

$$\mathtt{d}^{(1,2)} = \frac{k_1 k_8 - k_4 k_5 \pm \sqrt{U}}{k_4 k_{67} - k_8 k_{23}},$$

where $U = (k_1 k_8 - k_4 k_5)^2 + (k_1 k_{67} - k_5 k_{23})\,(k_4 k_{67} - k_8 k_{23})$ and $k_4 k_{67} \neq k_8 k_{23}$.

Of interest are only the positive real roots since they are the extrema of the dose response relationships we study. The two roots can never be positive at the same time for non-negative values of the coefficients k_i, which means that the dose-response curves can be at most biphasic. This fact becomes clear after employing Vieta's formulas for second degree polynomials and requiring that the sum and the product of the roots are positive, namely:

$$\mathtt{d}^{(1)} + \mathtt{d}^{(2)} = \frac{-2(k_4 k_5 - k_1 k_8)}{k_4 k_{67} - k_8 k_{23}} > 0 \quad \wedge \quad \mathtt{d}^{(1)}\mathtt{d}^{(2)} = \frac{k_5 k_{23} - k_1 k_{67}}{k_4 k_{67} - k_8 k_{23}} > 0,$$

which is equivalent to:

$$\left(\frac{k_{23}}{k_{67}} < \frac{k_4}{k_8} < \frac{k_1}{k_5} \vee \frac{k_1}{k_5} < \frac{k_4}{k_8} < \frac{k_{23}}{k_{67}}\right) \wedge \left(\frac{k_1}{k_5} < \frac{k_1}{k_5} < \frac{k_4}{k_8} \vee \frac{k_4}{k_8} < \frac{k_{23}}{k_{67}} < \frac{k_1}{k_5}\right).$$

It is evident that for non-negative coefficients k_i there exists no solution for the logical expression of the set of inequalities. More precisely, according to Vietta's formula sum condition the ratio $\frac{k_4}{k_8}$

needs to have a value between the ratios $\frac{k_1}{k_5}$ and $\frac{k_{23}}{k_{67}}$, and according to Vietta's product condition $\frac{k_4}{k_8}$ has to be either the largest or the smallest among the ratios. The two conditions can not hold simultaneously and thus the two roots can not be positive at the same time.

Two possibilities to obtain biphasic dose-response remain:

Condition 1: One root is negative and the other one is positive, yielding the condition:

$$k_4k_{67} \neq k_8k_{23} \ \wedge \ \mathtt{d}^{(1)}\mathtt{d}^{(2)} = \frac{k_5k_{23} - k_1k_{67}}{k_4k_{67} - k_8k_{23}} < 0,$$

which implies $U > 0$.

Condition 2: One root is positive and the other one is zero, translating to the condition:

$$k_5k_{23} = k_1k_{67} \ \wedge \ k_4k_5 \neq k_1k_8 \ \wedge \ k_4k_{67} \neq k_8k_{23} \ \wedge \ \mathtt{d} = \frac{-2(k_4k_5 - k_1k_8)}{k_4k_{67} - k_8k_{23}} > 0$$

The dose-response coefficients are non-negative, which means that they could also be zero. However, to have bounded dose-response curves we require that $k_5 \neq 0 \ \wedge \ k_8 \neq 0$.

Let us first examine the case when $k_{67} = 0$. Then *Condition 1* is satisfied when $\frac{k_5k_{23}}{-k_8k_{23}} < 0$ or equivalently when $k_{23} \neq 0$, while *Condition 2* is never satisfied. In the case when $k_{67} \neq 0$, *Condition 1* could equivalently be written as:

$$\frac{k_{23}}{k_{67}} < \frac{k_4}{k_8} \leq \frac{k_1}{k_5} \ \vee \ \frac{k_{23}}{k_{67}} < \frac{k_1}{k_5} < \frac{k_4}{k_8} \ \vee \ \frac{k_4}{k_8} \leq \frac{k_1}{k_5} < \frac{k_{23}}{k_{67}} \ \vee \ \frac{k_1}{k_5} < \frac{k_4}{k_8} < \frac{k_{23}}{k_{67}},$$

while *Condition 2* again never holds.

We summarize the derived necessary and sufficient conditions for having a positive extremum of the dose-response function, which we call the *Hormesis condition* as:

$$(k_{67} = 0 \ \wedge \ k_{23} \neq 0) \ \vee \ \Bigg(k_{67} \neq 0 \wedge \left(\frac{k_{23}}{k_{67}} < \frac{k_4}{k_8} \leq \frac{k_1}{k_5} \ \vee \ \frac{k_{23}}{k_{67}} < \frac{k_1}{k_5} < \frac{k_4}{k_8} \ \vee \ \frac{k_4}{k_8} \leq \frac{k_1}{k_5} < \frac{k_{23}}{k_{67}} \ \vee \ \frac{k_1}{k_5} < \frac{k_4}{k_8} < \frac{k_{23}}{k_{67}} \right) \Bigg).$$

Note that even when the Hormesis condition is satisfied the biphasic behavior might be weak and experimentally not evident.

Derivation of the differential. After having derived the Hormesis condition, we can proceed to derive the differential following the procedure:

(i) *Subdivide the curves into monotone segments.*

Combining the critical points at zero and infinite dose with the positive extremum we obtain a set of critical points $\mathcal{E}$:

$$\mathcal{E} = \left\{ \epsilon_1 = \left(0, \frac{k_1}{k_5}\right), \epsilon_2 = \left(\frac{k_1 k_8 - k_4 k_5 \pm \sqrt{U}}{k_4 k_{67} - k_8 k_{23}}, \frac{k_{23} k_{67} - 2(k_1 k_8 + k_4 k_5) \pm 2\sqrt{U}}{k_{67}^2 - 4 k_5 k_8} \right), \epsilon_3 = \left(\infty, \frac{k_4}{k_8}\right) \right\},$$

where the sign in front of $\sqrt{U}$ in ϵ_2 depends on the steady-state coefficients:

$$\begin{aligned} &k_{67} = 0 \wedge k_{23} \neq 0 \wedge : && -, \\ &k_{67} \neq 0 \wedge \left(\tfrac{k_{23}}{k_{67}} < \tfrac{k_4}{k_8} \leq \tfrac{k_1}{k_5} \vee \tfrac{k_{23}}{k_{67}} < \tfrac{k_1}{k_5} < \tfrac{k_4}{k_8} \right) : && +, \\ &k_{67} \neq 0 \wedge \left(\tfrac{k_4}{k_8} \leq \tfrac{k_1}{k_5} < \tfrac{k_{23}}{k_{67}} \vee \tfrac{k_1}{k_5} < \tfrac{k_4}{k_8} < \tfrac{k_{23}}{k_{67}} \right) : && -. \end{aligned}$$

When the Hormesis condition is satisfied for a dose-response curve all three critical points are relevant (depending on the conditions, the roots with the appropriate sign have to be selected), thus the curve has two segments – σ_1 (defined by ϵ_1 and ϵ_2) and σ_2 (defined by ϵ_2 and ϵ_3). When the Hormesis condition does not hold, we consider only ϵ_1 and ϵ_3 which define the single segment σ. In general, the values of the coefficients k_i are not known and the number of critical points cannot be determined unambiguously.

(ii) *Decide which segments to compare.*

Thus, three cases, depending on the number of segments in the compared reference and perturbed curves need to be considered (assuming, w.l.o.g., that the reference curve has less or equal critical points than the perturbed one), namely:

Case 1: The Hormesis condition holds neither for the reference nor for the perturbed curve.

Hence, the single segment σ^α of the reference is mapped to the single segment σ^β of the perturbed curve, i.e. $n = m = 2$:

$$\mathcal{M}(i = 1; \Sigma^\alpha, \Sigma^\beta) : \sigma^\alpha \to \sigma^\beta.$$

Case 2: The Hormesis condition does not hold for the reference but holds for the perturbed curve.

Hence, the single segment σ^α of the reference curve is mapped to the two segments σ_1^β

and σ_2^β of of the perturbed curve, i.e. $n = 2$ and $m = 3$:

$$\mathcal{M}(i = 1; \Sigma^\alpha, \Sigma^\beta) : \sigma_1^\alpha \to \sigma_1^\beta, \qquad \mathcal{M}(i = 2; \Sigma^\alpha, \Sigma^\beta) : \sigma_1^\alpha \to \sigma_2^\beta.$$

Case 3: The Hormesis condition holds for both the reference and the perturbed curve.

Hence, the two segments σ_1^α and σ_2^α of the reference curve are mapped to the two segments σ_1^β and σ_2^β of of the perturbed curve, i.e. $n = m = 3$:

$$\mathcal{M}(i = 1; \Sigma^\alpha, \Sigma^\beta) : \begin{cases} \sigma_1^\alpha \to \sigma_1^\beta \\ \sigma_2^\alpha \to \sigma_2^\beta \end{cases}.$$

(iii) *Determine corresponding points in compared pairs of segments.*

The correspondence between points for the three cases is obtained by plugging in the appropriate arguments in the proportion function. The derivation of the related dose components is, however, more involved since the inverses of the segments $\mathcal{R}(d)$ have a more complicated form. In the general case, the parametrised dose component inside a segment ($h \neq 0, 1$) reads:

$$\mathtt{d}_{\sigma,h}^{(1,2)} = \frac{k_{67}\zeta\left(h; x, y\right) - k_{23} \pm \sqrt{W(h; x, y)}}{2\left(k_4 - k_8\zeta\left(h; x, y\right)\right)},$$

where $W(h; x, y) = \left(k_{67}\zeta\left(h; x, y\right) - k_{23}\right)^2 - 4\left(k_1 - k_5\zeta\left(h; x, y\right)\right)\left(k_4 - k_8\zeta\left(h; x, y\right)\right)$, $\mathtt{d}_{\sigma,h}^{(1)}$ is the solution with $+\sqrt{W(h; x, y)}$ and $\mathtt{d}_{\sigma,h}^{(2)}$ with $-\sqrt{W(h; x, y)}$.

The relevant solution should be positive for all $h \in (0, 1)$ and belong to the dose interval of definition of the desired segment σ (defined by the doses corresponding to x and y) when $W(h; x, y) \geq 0$. Solution positivity leads to:

$$\frac{k_{23}}{k_{67}} \mp \frac{\sqrt{W(h; x, y)}}{k_{67}} > \zeta\left(h; x, y\right) > \frac{k_4}{k_8} \;\vee\; \frac{k_{23}}{k_{67}} \mp \frac{\sqrt{W(h; x, y)}}{k_{67}} < \zeta\left(h; x, y\right) < \frac{k_4}{k_8}.$$

(iv) *Quantify the displacement of corresponding points.*

Depending on the particular mapped segments σ_i^α and σ_j^β, the differential expressions π_d and $\pi_\mathcal{R}$ have the general form:

$$\pi_d\left(h\right) = \log_{10} \frac{k_4^\beta - k_8^\beta\zeta\left(h; x^\beta, y^\beta\right)}{k_4^\alpha - k_8^\alpha\zeta\left(h; x^\alpha, y^\alpha\right)} \frac{k_{67}^\alpha\zeta\left(h; x^\alpha, y^\alpha\right) - k_{23}^\alpha \pm \sqrt{W^\beta(h; x^\alpha, y^\alpha)}}{k_{67}^\beta\zeta\left(h; x^\beta, y^\beta\right) - k_{23}^\beta \pm \sqrt{W^\beta(h; x^\beta, y^\beta)}}$$

and

$$\pi_{\mathcal{R}}(h) = \zeta(h; x^{\alpha}, y^{\alpha}) - \zeta\left(h; x^{\beta}, y^{\beta}\right).$$

Note that when $h = 0$ or $h = 1$ the dose differential is derived by mapping the dose components of the respective critical points.

In particular, the differential expressions are different with respect to the number of segments in each dose-response curve:

Case 1: The condition for positivity of the dose component solutions for all $h \in (0,1)$ when $W(h; \frac{k_1}{k_5}, \frac{k_4}{k_8}) \geq 0$ can be reduced to:

$$\frac{k_{23}}{k_{67}} + \frac{\sqrt{W(h; \frac{k_1}{k_5}, \frac{k_4}{k_8})}}{k_{67}} > h\frac{k_1}{k_5} + (1-h)\frac{k_4}{k_8} > \frac{k_4}{k_8} \vee \frac{k_{23}}{k_{67}} - \frac{\sqrt{W(h; \frac{k_1}{k_5}, \frac{k_4}{k_8})}}{k_{67}} < h\frac{k_1}{k_5} + (1-h)\frac{k_4}{k_8} < \frac{k_4}{k_8},$$

which corresponds to the non-hormesis conditions $\frac{k_4}{k_8} \leq \frac{k_{23}}{k_{67}} \leq \frac{k_1}{k_5}$ and $\frac{k_1}{k_5} \leq \frac{k_{23}}{k_{67}} \leq \frac{k_4}{k_8}$, and the solutions $\mathtt{d}^{(2)}_{\sigma,h}$ and $\mathtt{d}^{(1)}_{\sigma,h}$, respectively.

To see why, let us show that $\frac{k_{23}}{k_{67}} - \frac{\sqrt{W(h; \frac{k_1}{k_5}, \frac{k_4}{k_8})}}{k_{67}} > \zeta\left(h; \frac{k_1}{k_5}, \frac{k_4}{k_8}\right) > \frac{k_4}{k_8}$ never holds. We rearrange the inequality to:

$$\frac{k_{23}}{k_{67}} - \zeta\left(h; \frac{k_1}{k_5}, \frac{k_4}{k_8}\right) > \sqrt{\left(\frac{k_{23}}{k_{67}} - \zeta\left(h; \frac{k_1}{k_5}, \frac{k_4}{k_8}\right)\right)^2 - 4\frac{\left(k_1 - k_5\zeta\left(h; \frac{k_1}{k_5}, \frac{k_4}{k_8}\right)\right)\left(k_4 - k_8\zeta\left(h; \frac{k_1}{k_5}, \frac{k_4}{k_8}\right)\right)}{k_{67}^2}},$$

and notice that due to the non-hormesis, $\frac{k_4}{k_8} < \zeta\left(h; \frac{k_1}{k_5}, \frac{k_4}{k_8}\right) < \frac{k_1}{k_5}$, which means the inequality never holds since

$$\left(k_1 - k_5\zeta\left(h; \frac{k_1}{k_5}, \frac{k_4}{k_8}\right)\right)\left(k_4 - k_8\zeta\left(h; \frac{k_1}{k_5}, \frac{k_4}{k_8}\right)\right) < 0.$$

The considerations are analogous for the other inequality in the positivity condition. This simplification shows that, depending on which positivity condition is met after α and β specifics are applied, only one solution $\mathtt{d}_{\sigma,h}$ is relevant for the differential.

Now, ignoring the trivial case when $\frac{k_1}{k_5} = \frac{k_4}{k_8}$ for any differential structure and value of the differential parameters, the dose and the response component mappings read:

$$\mathtt{d}^{(1,2),\alpha}_{\sigma,h} \mapsto \mathtt{d}^{(1,2),\beta}_{\sigma,h} \qquad \text{and} \qquad h\frac{k_1^{\alpha}}{k_5^{\alpha}} + (1-h)\frac{k_4^{\alpha}}{k_8^{\alpha}} \mapsto h\frac{k_1^{\beta}}{k_5^{\beta}} + (1-h)\frac{k_4^{\beta}}{k_8^{\beta}}.$$

In this case, we have already expressed the relevant critical points through the dose-

response coefficients. Thus we can write the differential as:

$$\pi_d(h) = \log_{10} \frac{k_8^\beta}{k_8^\alpha} \frac{k_4^\beta k_5^\beta - k_1^\beta k_8^\beta}{k_4^\alpha k_5^\alpha - k_1^\alpha k_8^\alpha} \frac{k_5^\alpha k_8^\alpha \left(-k_{23}^\alpha \pm \sqrt{W(h; \frac{k_1^\alpha}{k_5^\alpha}, \frac{k_4^\alpha}{k_8^\alpha})}\right) + k_{67}^\alpha \left(h k_1^\alpha k_8^\alpha + (1-h) k_4^\alpha k_5^\alpha\right)}{k_5^\beta k_8^\beta \left(-k_{23}^\beta \pm \sqrt{W(h; \frac{k_1^\beta}{k_5^\beta}, \frac{k_4^\beta}{k_8^\beta})}\right) + k_{67}^\beta \left(h k_1^\beta k_8^\beta + (1-h) k_4^\beta k_5^\beta\right)}$$

and

$$\pi_{\mathcal{R}}(h) = h \left(\frac{k_1^\alpha}{k_5^\alpha} - \frac{k_1^\beta}{k_5^\beta}\right) + (1-h) \left(\frac{k_4^\alpha}{k_8^\alpha} - \frac{k_4^\beta}{k_8^\beta}\right).$$

Note that the sign in front of the square root can be determined only by the positivity conditions, i.e. if it is not known which one is satisfied for the reference and perturbed curve all combinations have to be considered.

Case 2: The dose and response differential for the different segment mappings in this case are:

$$\pi_d(h; i=1) = \log_{10} \frac{k_4^\beta - k_8^\beta \zeta\left(h; \frac{k_1^\beta}{k_5^\beta}, \mathtt{R}_{\epsilon_2}^\beta\right)}{k_4^\alpha - k_8^\alpha \zeta\left(h; \frac{k_1^\alpha}{k_5^\alpha}, \frac{k_4^\alpha}{k_8^\alpha}\right)} \frac{k_{67}^\alpha \zeta\left(h; \frac{k_1^\alpha}{k_5^\alpha}, \frac{k_4^\alpha}{k_8^\alpha}\right) - k_{23}^\alpha \pm \sqrt{W(h; \frac{k_1^\alpha}{k_5^\alpha}, \frac{k_4^\alpha}{k_8^\alpha})}}{k_{67}^\beta \zeta\left(h; \frac{k_1^\beta}{k_5^\beta}, \mathtt{R}_{\epsilon_2}^\beta\right) - k_{23}^\beta \pm \sqrt{W(h; \frac{k_1^\beta}{k_5^\beta}, \mathtt{R}_{\epsilon_2}^\beta)}}$$

and

$$\pi_{\mathcal{R}}(h; i=1) = \zeta\left(h; \frac{k_1^\alpha}{k_5^\alpha}, \frac{k_4^\alpha}{k_8^\alpha}\right) - \zeta\left(h; \frac{k_1^\beta}{k_5^\beta}, \mathtt{R}_{\epsilon_2}^\beta\right),$$

$$\pi_d(h; i=2) = \log_{10} \frac{k_4^\beta - k_8^\beta \zeta\left(h; \mathtt{R}_{\epsilon_2}^\beta, \frac{k_4^\beta}{k_8^\beta}\right)}{k_4^\alpha - k_8^\alpha \zeta\left(h; \frac{k_1^\alpha}{k_5^\alpha}, \frac{k_4^\alpha}{k_8^\alpha}\right)} \frac{k_{67}^\alpha \zeta\left(h; \frac{k_1^\alpha}{k_5^\alpha}, \frac{k_4^\alpha}{k_8^\alpha}\right) - k_{23}^\alpha \pm \sqrt{W(h; \frac{k_1^\alpha}{k_5^\alpha}, \frac{k_4^\alpha}{k_8^\alpha})}}{k_{67}^\beta \zeta\left(h; \mathtt{R}_{\epsilon_2}^\beta, \frac{k_4^\beta}{k_8^\beta}\right) - k_{23}^\beta \pm \sqrt{W(h; \mathtt{R}_{\epsilon_2}^\beta, \frac{k_4^\beta}{k_8^\beta})}}$$

and

$$\pi_{\mathcal{R}}(h, i=2) = \zeta\left(h; \frac{k_1^\alpha}{k_5^\alpha}, \frac{k_4^\alpha}{k_8^\alpha}\right) - \zeta\left(h; \mathtt{R}_{\epsilon_2}^\beta, \frac{k_4^\beta}{k_8^\beta}\right).$$

Choosing the relevant solution from $\mathtt{d}_{\sigma,h}^{(1,2)}$ when deriving the dose differential depends on the Hormesis condition and the particular segment (the solution needs to be in the dose domain of the segment).

Case 3: The dose and response differential for the corresponding segments are:

$$\pi_d(h) = \begin{cases} \log_{10} \dfrac{k_4^\beta - k_8^\beta \zeta\left(h; \frac{k_1^\beta}{k_5^\beta}, \mathtt{R}_{\epsilon_2}^\beta\right)}{k_4^\alpha - k_8^\alpha \zeta\left(h; \frac{k_1^\alpha}{k_5^\alpha}, \mathtt{R}_{\epsilon_2}^\alpha\right)} \dfrac{k_{67}^\alpha \zeta\left(h; \frac{k_1^\alpha}{k_5^\alpha}, \mathtt{R}_{\epsilon_2}^\alpha\right) - k_{23}^\alpha \pm \sqrt{W(h; \frac{k_1^\alpha}{k_5^\alpha}, \mathtt{R}_{\epsilon_2}^\alpha)}}{k_{67}^\beta \zeta\left(h; \frac{k_1^\beta}{k_5^\beta}, \mathtt{R}_{\epsilon_2}^\beta\right) - k_{23}^\beta \pm \sqrt{W(h; \frac{k_1^\beta}{k_5^\beta}, \mathtt{R}_{\epsilon_2}^\beta)}} \\ \log_{10} \dfrac{k_4^\beta - k_8^\beta \zeta\left(h; \mathtt{R}_{\epsilon_2}^\beta, \frac{k_4^\beta}{k_8^\beta}\right)}{k_4^\alpha - k_8^\alpha \zeta\left(h; \mathtt{R}_{\epsilon_2}^\alpha, \frac{k_4^\alpha}{k_8^\alpha}\right)} \dfrac{k_{67}^\alpha \zeta\left(h; \mathtt{R}_{\epsilon_2}^\alpha, \frac{k_4^\alpha}{k_8^\alpha}\right) - k_{23}^\alpha \pm \sqrt{W(h; \mathtt{R}_{\epsilon_2}^\alpha, \frac{k_4^\alpha}{k_8^\alpha})}}{k_{67}^\beta \zeta\left(h; \mathtt{R}_{\epsilon_2}^\beta, \frac{k_4^\beta}{k_8^\beta}\right) - k_{23}^\beta \pm \sqrt{W(h; \mathtt{R}_{\epsilon_2}^\beta, \frac{k_4^\beta}{k_8^\beta})}} \end{cases},$$

and

$$\pi_{\mathcal{R}}(h) = \begin{cases} \zeta\left(h; \frac{k_1^\alpha}{k_5^\alpha}, \mathtt{R}_{\epsilon_2}^\alpha\right) - \zeta\left(h; \frac{k_1^\beta}{k_5^\beta}, \mathtt{R}_{\epsilon_2}^\beta\right) \\ \zeta\left(h; \mathtt{R}_{\epsilon_2}^\alpha, \frac{k_4^\alpha}{k_8^\alpha}\right) - \zeta\left(h; \mathtt{R}_{\epsilon_2}^\beta, \frac{k_4^\beta}{k_8^\beta}\right) \end{cases}.$$

Again, the choice of an appropriate solution from $\mathtt{d}_{\sigma,h}^{(1,2)}$ has to comply with the Hormesis condition and the relevant segment.

It is evident that the obtained differential expressions have a more complicated form than in the case for a single dose edge. Also, multiple conditions depending on the ratios between the dose-response coefficients have to be considered. However, the expressions are symbolic and symbolic analysis can be applied.

Two Dose Edge Example: Insulin Receptor Life-Cycle Model

Robust Hormetic Response

For active species corresponding to the vertices $O = \{v_{RLp}, v_{RLpi}\}$ in the more detailed insulin receptor life-cycle model from Fig 6A we obtain the steady-state coefficients (in polynomial form):

$$\begin{aligned} k_1 &= 0, \\ k_{23} &= r_1 r_3 (r_5 + r_6 + r_7) r_9 r_{11} (r_{13}(r_{15} + r_{16}) + r_{14} r_{16}), \\ k_4 &= 0, \\ k_5 &= (r_2 + r_3) r_8 r_{10} (r_4(r_6 + r_7) + r_5 r_7)(r_{13}(r_{15} + r_{16}) + r_{14} r_{16}), \\ k_{67} &= r_{10}((r_2 + r_3)(r_6 + r_7) r_8 r_{12} r_{14} r_{16} + r_1 r_3 r_5 r_7 (r_{13}(r_{15} + r_{16}) + r_{14} r_{16})), \\ k_8 &= r_1 r_3 (r_6 + r_7) r_{10} r_{12} r_{14} r_{16}. \end{aligned}$$

We can see that the Hormesis condition $k_{67} \neq 0 \wedge \frac{k_4}{k_8} \leq \frac{k_1}{k_5} < \frac{k_{23}}{k_{67}}$ ($k_{67} \neq 0 \wedge 0 \leq 0 < \frac{k_{23}}{k_{67}}$) holds for all possible positive values of the reaction rate constants. Thus the model generates a robust

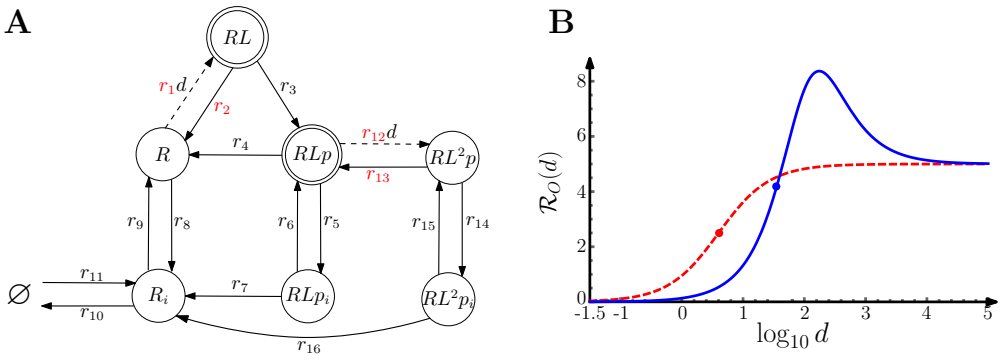

Figure S4: The extended insulin model with two dose edges with output vertices $O = \{v_{RL}, v_{RLp}\}$ exhibits parameter-dependent hormetic dose-response. (**A**) Graph corresponding to the subsystem of insulin receptor binding, recycling, and phosphorylation from (3) with notation as in Fig. 3A; differential parameters are shown in red. (**B**) Sigmoid reference (dashed red) and hormetic perturbed (blue) dose-response curves. The half-maximal response points ($h = 0.5$) for which the dose differential was analyzed are marked with a red and a blue dot on the reference and the first segment of the perturbed curve, respectively. The differential parameters were fixed to $r_1^\alpha = 0.03\, nM^{-1}s^{-1}$, $r_{12}^\alpha = 0.1\, nM^{-1}s^{-1}$, $r_2^\alpha = 0.1\, s^{-1}$, $r_{13}^\alpha = 0.001\, s^{-1}$, $r_1^\beta = 0.002\, nM^{-1}s^{-1}$, $r_{12}^\beta = 0.001\, nM^{-1}s^{-1}$, $r_2^\beta = r_{13}^\beta = 0.01\, s^{-1}$. Other parameters were fixed to $r_9 = 0.5\, s^{-1}$, $r_4 = r_6 = r_{14} = 0.2\, s^{-1}$, $r_3 = r_7 = r_8 = r_{15} = r_{10} = r_{11} = r_{16} = 0.1\, s^{-1}$, and $r_5 = 0.01\, s^{-1}$.

hormetic response.

Parameter-dependent Hormetic Response

Here, we demonstrate how to analyze the differential of models generating dose-response curves with shapes depending on parameter values. Let us consider the more detailed model for insulin receptor trafficking (Fig. S4A). We assume that we measure the singly ligand-bound receptor species on the cell surface, RL and RLp, thus $O = \{v_{RL}, v_{RLp}\}$, and obtain two dose-response curves by stimulating the system with two ligands that differ in their affinity to the receptor—ligand α with reaction rate constants r_1^α, r_2^α, r_{12}^α, r_{13}^α, and ligand β with r_1^β, r_2^β, r_{12}^β, r_{13}^β. Suppose that the dose-response curve for α (reference) is sigmoidal and the curve for β (perturbed) is hormetic (biphasic) (differential as in *Case 2*). We aim to derive the dose differential between the reference curve and the first segment of the perturbed curve at $h = 0.5$ as well as the response differential between the reference curve and the second segment of the perturbed curve at $d \to \infty$, i.e. $h = 0$.

Steady-state coefficients. For conciseness, we analyze the steady-state coefficients in polynomial form instead of graph form:

$$
\begin{aligned}
k_1 &= 0,\\
k_{23} &= r_1 r_9 r_{11}((r_3+r_4)(r_6+r_7)+r_5 r_7)(r_{13}(r_{15}+r_{16})+r_{14}r_{16}),\\
k_4 &= r_1(r_6+r_7)r_9 r_{11}\, r_{12}\, r_{14} r_{16},\\
k_5 &= (r_2+r_3)r_8 r_{10}(r_4(r_6+r_7)+r_5 r_7)(r_{13}(r_{15}+r_{16})+r_{14}r_{16}),\\
k_{67} &= r_{10}((r_2+r_3)(r_6+r_7)r_8\, r_{12}\, r_{14}r_{16}+r_1 r_3 r_5 r_7(r_{13}(r_{15}+r_{16})+r_{14}r_{16})),\\
k_8 &= r_1 r_3(r_6+r_7)r_{10}\, r_{12}\, r_{14} r_{16},
\end{aligned}
$$

where the differential parameters are marked in red.

We can use the *Hormesis condition* to find parametrizations such that the reference dose-response curve is sigmoidal, whereas the perturbed curve is hormetic (Fig. S4B). Due to $k_1 = 0$, the only non-hormesis condition holding for the reference curve α is $\frac{k_1}{k_5} = 0 \leq \frac{k^\alpha_{23}}{k^\alpha_{67}} \leq \frac{k^\alpha_4}{k^\alpha_8}$ and the only Hormesis condition holding for the perturbed curve β is $\frac{k_1}{k_5} = 0 < \frac{k^\beta_4}{k^\beta_8} < \frac{k^\beta_{23}}{k^\beta_{67}}$. This indicates that the perturbation should flip the inequality sign between the non-zero steady-state coefficient ratios. Also noting that $\frac{k^\alpha_4}{k^\alpha_8} = \frac{k^\beta_4}{k^\beta_8}$, these conditions enforce the following condition on the parameters:

$$
\begin{aligned}
\frac{r_1^\alpha r_3((r_3+r_4)(r_6+r_7)+r_5 r_7)(r^\alpha_{13}(r_{15}+r_{16})+r_{14}r_{16})}{(r_2^\alpha+r_3)(r_6+r_7)r_8 r^\alpha_{12} r_{14}r_{16}+r_1^\alpha r_3 r_5 r_7(r^\alpha_{13}(r_{15}+r_{16})+r_{14}r_{16})} &\leq 1\\
&< \frac{r_1^\beta r_3((r_3+r_4)(r_6+r_7)+r_5 r_7)(r^\beta_{13}(r_{15}+r_{16})+r_{14}r_{16})}{(r_2^\beta+r_3)(r_6+r_7)r_8 r^\beta_{12} r_{14}r_{16}+r_1^\beta r_3 r_5 r_7(r^\beta_{13}(r_{15}+r_{16})+r_{14}r_{16})}. \quad (3)
\end{aligned}
$$

This implies that the values of r_9, r_{10}, and r_{11} (free receptor externalisation, degradation, and synthesis, respectively) do not affect whether or not the response is hormetic.

Derivation of the differential.

(i) *Subdivide the curves into monotone segments.*

We derive the two critical points of the non-hormetic reference curve as:

$$
\mathcal{E}^\alpha = \left\{ \epsilon_1^\alpha = (0,0)\,, \epsilon_2^\alpha = \left(\infty, \frac{r_9 r_{11}}{r_3 r_{10}}\right) \right\}.
$$

When deriving the second critical point of the perturbed hormetic curve we comply with the

Hormesis condition by choosing the solution that contains $-\sqrt{U^\beta}$, leading to:

$$\mathcal{E}^\beta = \Bigg\{ \epsilon_1^\beta = (0,0)\,,$$

$$\epsilon_2^\beta = \Bigg(\frac{r_{13}^\beta(r_{15}+r_{16})+r_{14}r_{16}}{r_{12}^\beta r_{14}r_{16}(r_6+r_7)} \frac{r_{12}^\beta r_{14}r_{16}r_8(r_2^\beta+r_3)(r_4(r_6+r_7)+r_5r_7)+\sqrt{\underline{U^\beta}}}{r_1^\beta r_3(r_3+r_4)(r_{13}^\beta(r_{15}+r_{16})+r_{14}r_{16})-r_{12}^\beta r_{14}r_{16}r_8(r_2^\beta+r_3)},$$

$$\frac{r_{11}r_1^\beta r_9(r_{13}^\beta(r_{15}+r_{16})+r_{14}r_{16})}{r_{10}} \frac{\begin{array}{r}(r_7(r_3+r_4+r_5)+r_6(r_3+r_4))\Big(r_{12}^\beta r_{14}r_{16}r_8(r_2^\beta+r_3)(r_6+r_7)\\ +r_1^\beta r_3r_5r_7(r_{13}^\beta(r_{15}+r_{16})+r_{14}r_{16})\Big)-2(r_6+r_7)\sqrt{\underline{U^\beta}}\\ -2r_{12}^\beta r_{14}r_{16}r_8(r_2^\beta+r_3)(r_6+r_7)(r_4(r_6+r_7)+r_5r_7)\end{array}}{\begin{array}{r}\Big(r_{12}^\beta r_{14}r_{16}r_8(r_2^\beta+r_3)(r_6+r_7)+r_1^\beta r_3r_5r_7\big(r_{13}^\beta(r_{15}+r_{16})\\ +r_{14}r_{16}\big)\Big)^2-4r_{12}^\beta r_{14}r_{16}r_1^\beta r_3r_8(r_2^\beta+r_3)(r_6+r_7)\big(r_{13}^\beta(r_{15}+r_{16})\\ +r_{14}r_{16}\big)(r_4(r_6+r_7)+r_5r_7)\end{array}} \Bigg),$$

$$\epsilon_3^\beta = \left(\infty, \frac{r_9r_{11}}{r_3r_{10}}\right) \Bigg\},$$

where $\underline{U^\beta}$ denotes U^β with squared factors taken out of the square root and has the form:

$$\underline{U^\beta} = r_{12}^\beta r_{14}r_{16}r_3r_8(r_2^\beta+r_3)(r_4(r_6+r_7)+r_5r_7)\Big(-r_{12}^\beta r_{14}r_{16}r_8(r_2^\beta+r_3)(r_6+r_7)$$
$$+r_1^\beta(r_3+r_4)(r_{13}^\beta(r_{15}+r_{16})+r_{14}r_{16})(r_7(r_3+r_4+r_5)+r_6(r_3+r_4))\Big).$$

This leads to the following observations: (i) the first and last critical points of the reference and perturbed curves are identical; (ii) the last critical points depend only on the four reaction rates r_3, r_9, r_{10}, and r_{11}; and (iii) the dose component of the second critical point of the perturbed system ϵ_2^β does not depend on r_9, r_{10}, and r_{11}.

(ii) *Decide which segments to compare.*

See *Case 2* of the two dose edge differential derivations.

(iii) *Determine corresponding points in compared pairs of segments.*

See the general two dose edge differential derivations.

(iv) *Quantify the displacement of corresponding points.*

It is straightforward to see that the response differential between the reference curve and the second segment of the perturbed curve at $d \to \infty$ is always zero, independent of the

magnitude of the perturbation and of the reaction constants' values:

$$\pi_{\mathcal{R}}\left(h=0,i=2\right)=\zeta\left(h=0;0,\frac{k_4^{\alpha}}{k_8^{\alpha}}\right)-\zeta\left(h=0;\mathtt{R}_{\epsilon_2}^{\beta},\frac{k_4^{\beta}}{k_8^{\beta}}\right)=\frac{r_9r_{11}}{r_3r_{10}}-\frac{r_9r_{11}}{r_3r_{10}}=0.$$

The expressions in the previous section also allow us to identify feasible perturbations to alter the dose-response behavior. For example, if we were to design a new perturbation, different from applying a ligand with modified affinity, that again leads to a hormetic perturbed dose-response, but to a non-zero response differential, it has to target parameters r_9, r_{11},r_3, or r_{10}. However, since hormesis is not affected by r_9, r_{11}, and r_{10}, r_3 need to be perturbed.

To find the dose differential between the reference curve and the first segment of the perturbed curve with $h = 0.5$, we need to select the appropriate solution from $\mathtt{d}_{\sigma,h}^{(1,2)}$. The relevant solution for the reference curve is $\mathtt{d}_{\sigma,h}^{(1)}$ since it corresponds to the non-hormesis condition $\frac{k_1}{k_5}\leq\frac{k_{23}}{k_{67}}\leq\frac{k_4}{k_8}$. Furthermore, when choosing the relevant solution, there are two cases of interest: (i) when the solutions have different signs we take the larger (positive) solution, and (ii) when the two solutions are positive we consider the smaller solution, which corresponds to the first segment of the hormetic curve. According to Vietta's formulas, the solutions have different signs when $\frac{-k_5^{\beta}\zeta(h;x,y)}{k_4^{\beta}-k_8^{\beta}\zeta(h;x,y)}<0$, which translates to $k_4^{\beta}-k_8^{\beta}\zeta\left(h;x,y\right)>0$, indicating that the relevant larger solution is $\mathtt{d}_{\sigma,h}^{(1)}$. Accordingly, both solutions are positive when $-\frac{k_{23}^{\beta}-k_{67}^{\beta}\zeta(h;x,y)}{k_4^{\beta}-k_8^{\beta}\zeta(h;x,y)}>0$ and $\frac{-k_5^{\beta}\zeta(h;x,y)}{k_4^{\beta}-k_8^{\beta}\zeta(h;x,y)}>0$, implying $k_4^{\beta}-k_8^{\beta}\zeta\left(h;x,y\right)<0$ and $k_{23}^{\beta}-k_{67}^{\beta}\zeta\left(h;x,y\right)>0$. This satisfies the Hormesis condition and settles the smaller positive solution to be $\mathtt{d}_{\sigma,h}^{(1)}$ again.

Thus, we select the solution $\mathtt{d}_{\sigma,h}^{(1)}$ for the reference and the perturbed curve, which gives:

$$\pi_d\left(h=\frac{1}{2};i=1\right)=\log_{10}\frac{2k_4^{\beta}-k_8^{\beta}\mathtt{R}_{\epsilon_2}^{\beta}}{k_4^{\alpha}k_8^{\alpha}}\frac{k_4^{\alpha}k_{67}^{\alpha}-2k_{23}^{\alpha}k_8^{\alpha}+2k_8^{\alpha}\sqrt{W(\frac{1}{2};0,\frac{k_4^{\alpha}}{k_8^{\alpha}})}}{k_{67}^{\beta}\mathtt{R}_{\epsilon_2}^{\beta}-2k_{23}^{\beta}+2\sqrt{W(\frac{1}{2};0,\mathtt{R}_{\epsilon_2}^{\beta})}},$$

with $W(\frac{1}{2};0,y)=\left(\frac{k_{67}y}{2}-k_{23}\right)^2+k_5y\left(2k_4-k_8y\right)$.

After substituting the steady-state coefficients, we find the symbolic expression for the dose differential. By looking at the greatest common divisor of the separate terms in the numerator and denominator of the expression, again the reaction rate constants r_9, r_{10}, and r_{11} cross out. Therefore, both the dose and the response differential are invariant with respect to these parameters.

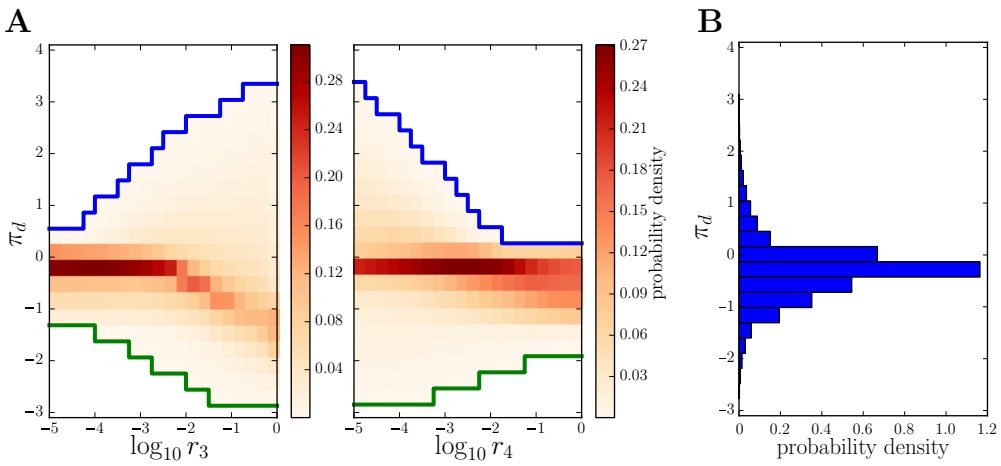

Figure S5: Numerical analysis of the two dose edge insulin model from Fig. S4A. (**A**) Profile bounds (blue – upper inner bound, green – lower inner bound) superimposed on the profile differential distribution (density) for the free parameters r_3 and r_4. (**B**) Marginal probability distribution of the dose differential magnitude. Densities were obtained using the values for the differential parameters from Fig. S4B and uniformly sampling the remaining n parameters from the parameter box $\mathcal{I} = [10^{-5}, 1]^n$; note that $n = 12$ in (B) and $n = 11$ in (A) since one additional parameter is fixed at a time.

Numerical analysis. For the insulin model with two dose edges (Fig. S4A), if we assume only the affinities of the two ligands to be known parameters, uniform sampling of the dose differential yields a few magnitudes of variability ($\widehat{\mathbb{D}_{\pi_d}} = [-2.8, 3.1]$) but a small region of most probable values with a marginal density peaked around -0.25 (Fig. S5B). The profile differential distributions in Fig. S5A show how the free parameters r_3 (receptor phosphorylation) and r_4 (receptor dephosphorylation) affect the bounds as well as the peak of the marginal distribution, revealing their potential to control the differential.

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