Chapter 27

Modeling of Receptor Tyrosine Kinase Signaling: Computational and Experimental Protocols

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Abstract

The advent of systems biology has convincingly demonstrated that the integration of experiments and dynamic modelling is a powerful approach to understand the cellular network biology. Here we present experimental and computational protocols that are necessary for applying this integrative approach to the quantitative studies of receptor tyrosine kinase (RTK) signaling networks. Signaling by RTKs controls multiple cellular processes, including the regulation of cell survival, motility, proliferation, differentiation, glucose metabolism, and apoptosis. We describe methods of model building and training on experimentally obtained quantitative datasets, as well as experimental methods of obtaining quantitative dose-response and temporal dependencies of protein phosphorylation and activities. The presented methods make possible (1) both the fine-grained modeling of complex signaling dynamics and identification of salient, course-grained network structures (such as feedback loops) that bring about intricate dynamics, and (2) experimental validation of dynamic models.

Key words Computational model, Mathematical modeling, Model validation, Cell signaling, Receptor tyrosine kinase, Phosphorylation, Cellular networks, Semi-quantitative analysis, Kinetics, Perturbations

1 Introduction

Mechanistic modeling uses the data on reaction mechanisms and kinetic parameters of the rates of all reactions involved in a model. The receptor tyrosine kinase (RTK) family involves more than 50 cell-surface receptors with intrinsic tyrosine kinase activity. Mechanisms of RTK activation include homo- and hetero-dimerization, as well as formation of oligomer clusters. Following activation, RTKs auto- and trans-phosphorylate multiple tyrosine residues in their cytoplasmic domains. Subsequently, these phosphotyrosines bind multiple adaptor proteins and enzymes, which contain characteristic protein domains, such as Src homology (SH2 and SH3), phosphotyrosine

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binding (PTB), and pleckstrin homology (PH) domains. These interactions recruit adaptor proteins and enzymes to the plasma membrane (PM), which in turn triggers further propagation of phosphorylation and dephosphorylation reactions through a tangled network of interconnecting cytoplasmic kinase and phosphatase cascades to the nucleus [1]. Although multiple signaling interactions are described, many of these processes are incompletely kinetically characterized. Therefore, any mechanistic model bears a great uncertainty of precise reaction mechanisms and kinetic parameters, which are not directly measured in situ. Thus, a model has to be trained using a training set of the time course and dose-response biochemical data. Predictions of a trained model are subsequently validated experimentally, using a variety of perturbations, such as siRNA and small molecule inhibitors. Quantitative methods of experimental calibration and validation of kinetic signaling models are described in the second part of this chapter.

2 Materials

2.1 Modeling a Simplified Reaction Kinetic Scheme

1. A complete list of reactions to be modelled is required [2]. This list could also be provided in the form of a reaction kinetic diagram.
2. Implementing and simulating the model requires a reaction kinetic modelling software such as Copasi [3] (free software), or the Data2Dynamics [4] (free) or Systems Biology Toolbox 2 [5] (free) for Matlab (commercial software), or a general mathematical programming language, such as Matlab or Mathematica (both commercial) or Scilab [6] and GNU Octave [7] (both free software).

2.2 Modeling Complex Interaction Networks with Multiple Phosphorylation Sites and Interaction Domains

1. A complete list of the processes (such as protein-protein interactions, phosphorylation states, and (de)phosphorylation reactions) is required.
2. Alternatively, one can use a list of so-called rules, simulated using a rule-based modeling software such as BioNetGen [8] or RuleBender [9] (both free software).

2.3 Using Core Models to Describe Salient System Properties

1. Same as Subheading 2.1.

2.4 Parameter Estimation

1. Estimating the parameters requires a modeling software that supports this functionality such as Copasi [3] (free software) and PEPSSBI [10] (free) or the Data2Dynamics [4] (free) or Systems Biology Toolbox 2 [5] (free, now also called IQM Tools Lite) or PotterWheel Toolbox [11] (commercial) for Matlab.
2. Estimation of rule-based models is possible with BioNetFit [12] (free).

2.5 Cell Growth

1. Adherent mammalian cell line(s) of choice (*see* **Note 1**).
2. *Complete medium* with supplements (serum, antibiotics, and/or growth factors) for routine cell culturing (*see* **Note 2**).
3. Cell *starvation medium* (medium without serum and/or growth factors, but with antibiotics).
4. Humidified 5% CO_2 incubator.
5. Tissue culture flasks *to maintain cells* (we use 75 cm^2 flasks with vent cap, referred as T75).
6. Tissue culture dishes *to extract proteins in whole cell lysates* [we use 60 × 15 mm or 100 × 20 mm dishes with grip ring (Santa Cruz Biotechnology, Dallas, TX)] (*see* **Note 3**).
7. Tissue culture dishes t*o extract proteins from subcellular fractions* (we use 100 × 20 mm dishes).
8. Tissue culture dishes t*o extract protein-protein complexes or detect phosphorylation of immunoprecipitated protein*s (we use TPP 150 × 20 mm tissue culture dishes) (MidSci, Valley Park, MO).
9. Hemocytometer or another cell counting device.
10. Borosilicate glass disposable Pasteur pipets, Serological 2, 5, 10 and 25 mL pipettes and pipettor.
11. Sterile-filtered 0.25% Trypsin-EDTA or TrypLE™ Express solution [Thermo Fisher Scientific (TFS), Grand Island, NY].

2.6 Cell Stimulation and Whole Cell Lysis

1. Lyophilized ligand(s) of choice (e.g., recombinant human EGF) (*see* **Note 4**).
2. Protease inhibitor cocktail solution or tablets (e.g., Roche's Complete ULTRA or Mini Tablets).
3. Phosphatase inhibitor cocktail solution or tablets (e.g., Roche's PhosSTOP).
4. *Whole cell lysis (WCL) buffer* for protein extraction from entire cells: 150 mM NaCl, 50 mM HEPES (pH 7.4), 1 mM EGTA, 1% Triton-X-100, and 10% glycerol diluted in dH_2O (*see* **Note 5**). Store at +4 °C. Immediately before use, supplement with protease and phosphatase inhibitor cocktails.

5. Colorimetric of fluorimetric protein quantification assay (e.g., colorimetric 600 nm protein assay or BCA-Reducing Agent Compatible Assay or fluorescent EZQ™ Protein Quantitation Kit from TFS) for assessment of total protein content per sample.
6. 1.5 and 2 mL flat-top safe-lock regular and siliconized micro-centrifuge tubes.
7. 15 and 50 mL Greiner conical centrifuge tubes.
8. Adjustable-volume pipettes: 0.5–10, 10–100, 20–200, and 100–1000 μL ranges.
9. Distilled reagent-grade water (dH_2O).
10. Calcium and magnesium-free 1× Dulbecco's Phosphate Buffered Saline (DPBS) or 1× Phosphate-buffered saline (PBS).
11. Cell scrapers.
12. Bucket(s) prefilled with ice pellets.
13. Refrigerated high-speed centrifuge and tabletop mini-centrifuge.
14. Rocking platform, rotator, and nutator.
15. Vacuum suction device.
16. Dry heat bath with heat blocks.
17. Ice machine.
18. Vortex/tube shaker.
19. Media bottles and measuring glassware.
20. Timers.

2.7 Additional Materials Required for Pharmacological Protein Inhibition or Suppression by RNA Interference (RNAi)

1. Small molecule inhibitors or activators (*see* **Note 6**).
2. Small interfering RNAs (siRNAs) (*see* **Note 7**).
3. Cell transfection reagent for protein suppression by RNAi (e.g., Ingenio Electroporation solution by Mirus Bio, Madison WI for or Lipofectamine 2000 by TFS).
4. Electroporation/nucleofection device with accessories [e.g., Amaxa's Nucleofector II (Lonza Cologne AZ, Basel, Switzerland) (optional, used for electroporation/nucleofection of cells)].

2.8 Additional Materials Required for Protein Immunoprecipitation (IP)

1. *Immunoprecipitation (IP) buffer* for purification of a single antigen from a complex protein mixture in whole cell lysates using a specific antibody attached to a beaded support: 150 mM NaCl, 25 mM HEPES (pH 7.4), 1% Triton-X-100, and 10% glycerol in dH_2O. Store at +4 °C.
2. *HNTG buffer* for gentle wash of immunoprecipitated complexes: 150 mM NaCl, 20 mM HEPES (pH 7.4), 0.1% Triton-X, and 10% glycerol. Store at +4 °C.

3. Recombinant protein A-, G-, or L-coupled Sepharose/agarose or magnetic beads.
4. Primary immunoprecipitation antibody against the POI.
5. Non-relevant antibody (negative control) (*see* **Note 8**).

2.9 Additional Materials Required for Subcellular Fractionation

1. *Digitonin-based cell fractionation (CF) buffer* for separation of crude membrane and cytoplasmic subcellular fractions: 1 M HEPES (pH 7.4), 150 mM NaCl, 150 μg/mL Digitonin, 1 mM EGTA, and 10% glycerol. Store at +4 °C.
2. *Modified RIPA buffer:* WCL buffer supplemented with 0.5% sodium deoxycholate (SOD), 0.1% Sodium-dodecyl sulfate (SDS), and 70 mM *n*-Octyl-β-D-glucoside (OG).
3. *Subcellular fractionation (SF) buffer* for separation of subcellular fractions by ultracentrifugation: 250 mM Sucrose, 20 mM HEPES (pH 7.4), 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, and 1 mM EGTA. Before use, add 10 μL of 1 mM DTT (per 50 mL of buffer) and phosphatase/protease inhibitors.
4. Ultracentrifuge (optional, used for centrifugation-based subcellular fractionation).
5. 25 Gauge needles and 2 mL syringes (optional, used for centrifugation-based subcellular fractionation).
6. Sonicator (optional, used for centrifugation-based subcellular fractionation).

2.10 Lithium-Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (LDS-PAGE)

1. Freshly prepared or thawed and centrifuged whole cell lysates.
2. Electrophoresis units for mini gels: XCell SureLock Mini-Cell (TFS, #EI0001) or similar.
3. Precast or hand-poured multi-well gels (*see* **Note 9**). Store at +4 °C or at RT.
4. Pre-stained molecular weight marker: Precision Plus ProteinTM All Blue standards (Bio-Rad, Hercules, CA, #161-0373) (*see* **Note 10**). Store at −20 °C.
5. *4× LDS Sample Buffer* (pH 8.5) (TFS, #NP0007 or #84788): 40% glycerol, 4% LDS, 0.8 M triethanolamine-Cl (pH 7.6), 0.025% phenol red, 0.025% Coomassie G250, and 2 mM EDTA disodium salt in ultrapure H_2O. Store at RT.
6. *10× Sample Reducing Agent*: 50 mM DL-Dithiothreitol (DTT) solution. Prepare fresh each week and store at +4 °C in foil-wrapped or amber centrifuge tubes.
7. *20× MOPS-SDS Running buffer* (TFS, #NP0001): 50 mM 3-(N-morpholino)-propanesulfonic acid (MOPS), 50 mM Tris, 1 mM EDTA (pH 7.7), and 0.1% (w/v) SDS (*see* **Note 11**). Mix the reagents in 800 mL of dH_2O, and prior to adding SDS, adjust the pH to 7.7. Adjust volume to 1 L with dH_2O. For LDS-PAGE, dilute the buffer to 1× with dH_2O. Store at RT.

8. 1× *Antioxidant solution* (TFS, #NP0005): 15% (w/w) Sodium Bisulfite and 10% (w/w) *N,N*-Dimethylformamide. Store at +4 °C in amber 50 mL centrifuge tube.
9. Gel-loading pipet tips.

2.11 Western Blotting (WB)/ Immunoblotting (IB)

2.11.1 Protein Transfer

1. 0.22 μm porous nitrocellulose membrane (*see* **Note 12**).
2. Filter paper (FP) sheets: (a) extra-thick [7 × 10 × 0.248 cm (W × L × H), 320 grade], for mini-gels cut to 7 × 9 cm; (b) thin (7.5 × 10 × 0.083 cm, grade 222), for mini-gels cut to 7.5 × 9 cm.
3. Blotting units: iBlot® Dry Blotting System (TFS, #IB21001), XCell II™ Blot wet transfer module (TFS, #EI9051), or similar (*see* **Note 13**).
4. iBlot® Nitrocellulose Regular Stacks. One stack fits two whole mini-gels or two multi-gel-strip assemblies at once (optional, for iBlot® Dry Blotting System only) (*see* **Note 14**).
5. Sponge pads (for XCell II™ Blot only, TFS, #EI9052) (4 pads per each blot).
6. Gel cutting knife (e.g., TFS, #EI9010).
7. Flat and upward bent tip tweezers (e.g., Electron Microscopy Sciences, #78336-36A).
8. Gel/Blot assembly trays (e.g., Bio-Rad, #170-4089) and blotting roller (e.g., TFS, #LC2100).
9. *20× Transfer buffer* (TFS, #NP0006-1): 25 mM bicine, 25 mM Bis-Tris, 1 mM EDTA (pH 7.2), and 0.05 mM chlorobutanol. Store at RT. For transfer, dilute 50 mL of 20× buffer with 850 mL dH_2O, add 100 mL methanol (final concentration 10% w/w), and supplement with 1 mL of *1× Antioxidant solution*. The pH of the 1× buffer should be 7.2, but do not adjust. Refrigerate.
10. *1× Soaking buffer*: 25 mM Tris, 192 mM Glycine, 0.1% SDS, 20% (v/v) Methanol in dH_2O.
11. SimplyBlue SafeStain (TFS) for gel staining following transfer (optional).
12. Ponceau S red staining solution (Sigma Aldrich, St. Louis, MO) for rapid and reversible membrane staining following transfer: 2% Ponceau S powder (w/v) in 30% TCA (trichloroacetic acid) and 30% sulfosalicylic acid (optional).

2.11.2 Protein Detection

1. Square Petri Dishes with Grid (Fisher Scientific, #FB0875711A).
2. *10× TBS-T buffer*: 87.66 g of NaCl, 100 mL of 1 M Tris (pH 8.0), 5 mL of Triton-X-100, and dH_2O up to 1 L. Store

at RT. To make *1× TBS-T buffer*, take 100 mL of 10× TBS-T buffer and add 900 mL of dH_2O. Store at +4.

3. *Blocking buffer*: 3% (w/v) bovine serum albumin (BSA) in 1× TBST buffer.
4. *Primary antibody (1^0Ab) solution*: unconjugated or horseradish peroxidase (HRP)-conjugated 1^0Ab of choice diluted in 1× TBST buffer (*see* **Note 15**).
5. *Secondary antibody (2^0Ab) solution*: secondary HRP-linked antibodies of choice diluted in 1× TBST buffer (*see* **Note 15**).
6. SNAP i.d.® Protein Detection system (EMD Millipore, Upstate, NY) (optional) (*see* **Note 16**).
7. Enhanced chemiluminescence (ECL) reagent to detect proteins bands on blots (e.g., SuperSignal West Dura Chemiluminescent substrate (TFS, #34075).
8. 3 M Scotch Magic Transparent Tape and clean titanium-bonded scissors.
9. Avery® Diamond Clear Heavyweight Quick-Load *Sheet Protectors*. Cut each sheet horizontally into three or four pieces, depending on the width of the blot to be protected during its visualization.
10. Imaging system with CCD sensor and zoom and dedicated software for densitometric analysis: e.g., Image Station 440CF (Eastman Kodak Scientific Imaging Systems, New Haven, CT).
11. Data plotting and statistical analysis software (e.g., Microsoft Excel, Systat SigmaPlot, GraphPad Prism, or similar) and image-editing software (e.g., Adobe Photoshop or similar).

3 Methods

3.1 Modeling a Simplified Reaction Kinetic Scheme of EGFR Activation and Competitive Adaptor Protein Binding

Modeling of biological systems with ordinary differential equations (ODEs) relies on two simplifying assumptions. These assumptions are based on two mathematical facts. First, ODEs consider only one independent variable, usually time. Thus, spatial effects are neglected (*see* **Note 17**). Second, the dependent variables (species concentrations) are continuous functions of time. Thus, the discrete nature of the molecule numbers (1, 2, … , 1000, etc.) is neglected. Whether these assumptions are appropriate has to be considered on a case to case basis and depends on the scales of time and space involved (*see* **Note 18**). Deriving these ODEs is based on the principle of mass balance as follows. For each species, we have to track what produces it (collect the reactions where it acts as a product) and what consumes it (collect the reactions where it acts as a substrate). Summing this processes up, while respecting their stoichiometry (*see* **step 3**

below), gives the corresponding rate of change of this species. Loosely speaking this can be expressed as follows [2]:

$$\begin{aligned}\text{Rate of change of } x = &\text{ total rate of } x \text{ production} \\ &- \text{total rate of } x \text{ consumption}\end{aligned}$$

Here the total rate is the sum of the reaction rates that produce or consume x.

1. *List all reactions, including their substrates, products, and modifiers.* The model will be built on a network of biochemical reactions (*see* **Note 19** and Fig. 1 *for an example)*. Generally, these reactions are of the following form

$$\alpha_1 S_1 + \ldots + \alpha_{n_s} S_{n_s} \rightarrow \beta_1 P_1 + \ldots + \beta_{n_p} P_{n_p}, \tag{1}$$

   where S_i denote substrates that are transformed into the products P_i and α , β are stoichiometric coefficients.

2. *Assign a reaction kinetic law for each reaction.* To describe how fast each reaction operates, a reaction kinetic law must be assigned. Mathematically, these laws use so-called kinetic parameters to express the rate at which the reaction proceeds as a function of the concentrations of substrates, products, and modifiers (Fig. 2). The basic law of elementary reaction steps is the law of mass action, in which the rate is proportional to all substrate concentrations raised to their stoichiometric coefficient:

$$v = k\, s_1^{\alpha_1} \cdots s_{n_s}^{\alpha_{n_s}},$$

   where v is the rate of the reaction (usually in nM/s) and k is a rate parameter. There is a wide range of reaction kinetic laws to choose from. Which one is appropriate depends on the modeled process (Fig. 2). For example, the Michaelis-Menten rate law, which is commonly used for enzyme-mediated reactions, can be derived by formally assuming that association and dissociation of the enzyme-substrate complex are in thermodynamic equilibrium (*see* **Note 20**). Other, more complicated reactions are often modeled phenomenologically. For example, the expression rate of a gene usually depends on its transcription factor in a sigmoidal fashion and can thus be modeled using the Hill equation.

3. *Formulate a system of ordinary differential equations.* Based on the stoichiometry and the rate laws, a system of ordinary differential equations can be constructed using the principle of mass balance. The principle of mass balance states that the overall rate of change of a state x is the sum of all reactions contributing to this state according to their stoichiometry. Reactions where x is a substrate consume x and therefore contribute

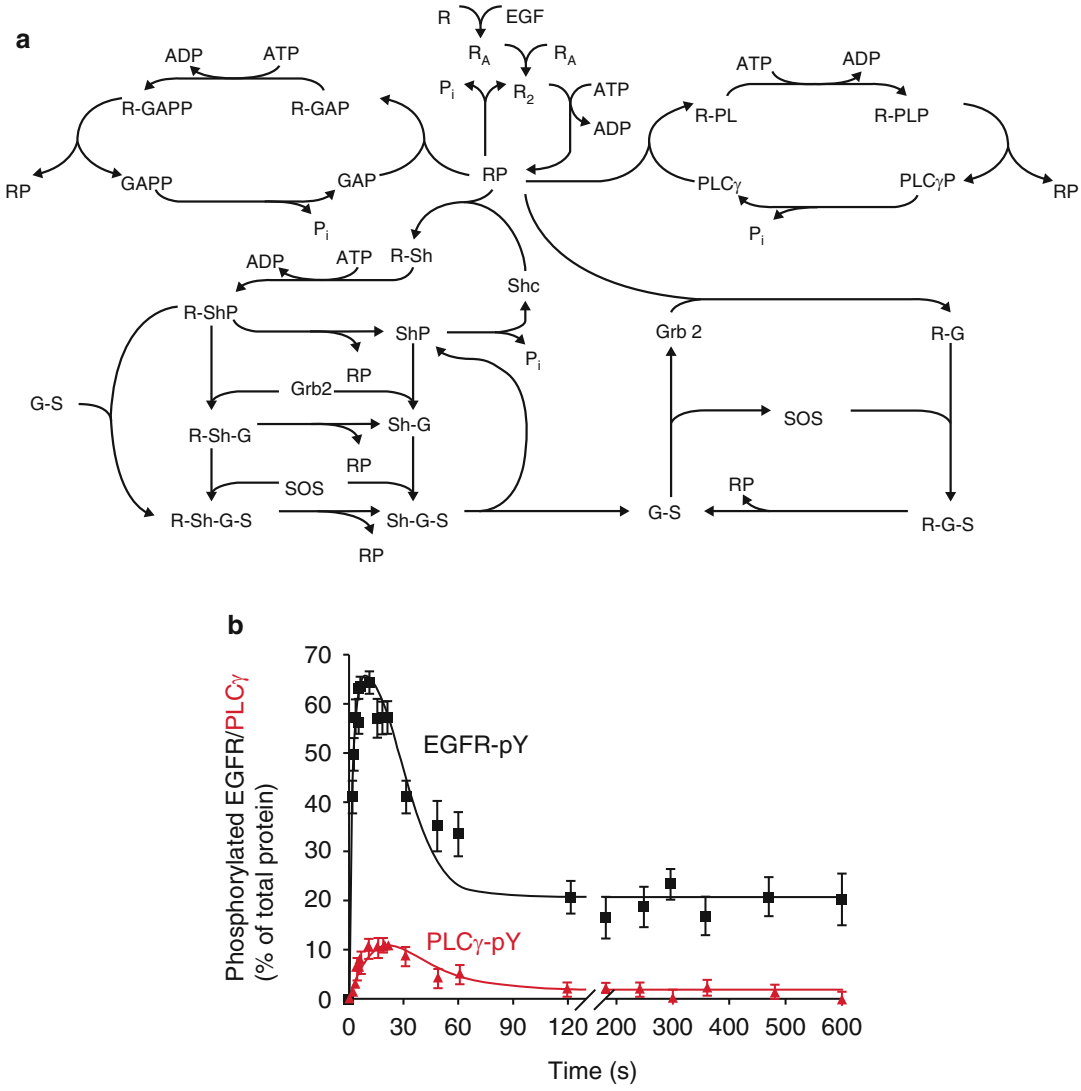

Fig. 1 A simplified kinetic model of the EGF receptor signaling [2]. (**a**) Illustration of the model as a reaction kinetic diagram. (**b**) Time course simulations (*solid lines*) and experimental data (markers and error bars, showing mean and standard error for several biological replicates)

negatively to the overall balance; reactions where x is a product contribute positively. For example, the reaction given in Eq. 1 consumes α_2 molecules of s_2, which means

$$\frac{d}{dt}s_2 = \ldots - \alpha_2 \cdot v + \ldots$$

Here, the dots indicate that there might be other reactions that consume or produce s_2. Adding up the contributions of all reactions, we arrive at the following system of ODEs,

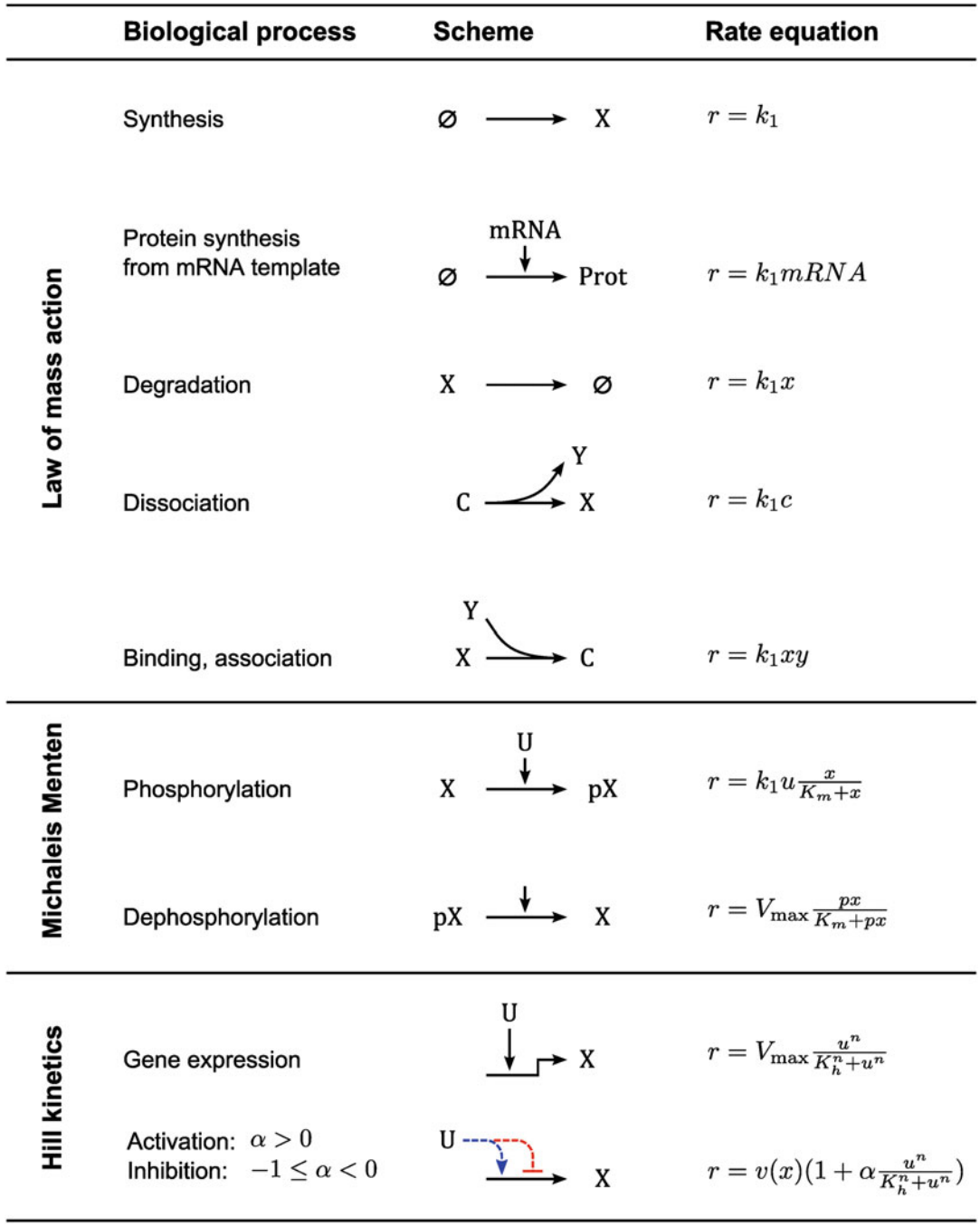

Fig. 2 Commonly modeled biochemical processes and their corresponding kinetic rate laws

$$\frac{d}{dt}x = Nv(x, p), \tag{2}$$

where $x \in \mathbb{R}^{n}_{\geq 0}$ is the vector of species concentrations analyzed in the model, $p \in \mathbb{R}^{n_p}_{>0}$ the parameter vector, and $v \in \mathbb{R}^{n}_{\geq 0} \times \mathbb{R}^{n_p}_{>0} \mapsto \mathbb{R}^{m}$ the vector of rate laws. The stoichiometric matrix $N \in \mathbb{R}^{n \times m}$ depends on the stoichiometric coefficients and possible factors compensating for different compartment volumes and units. Kinetic equations are usually written in terms of concentrations (not of mole numbers), because the reaction rates are functions of concentrations. If the same reaction contributes to molecule changes in different compartments, the effective concentration change will be different depending on the volume of the corresponding compartment [2]. For example, we can consider reaction 9 in Fig. 1a (Grb2 binding to RP) to take place in the cytosol, then its effect on the concentration change of RP on the membrane must be scaled by V_m/V_c, where V_m denotes the surface area of the cell membrane and V_c denotes volume of the cytosol.

4. *Assign the initial conditions and parameter values, and simulate the system*. To simulate the ODE system given by Eq. 2, numerical values for the parameters k and initial conditions x_0 must be assigned (beware of non-physiological parameter choices such as association rate constants over the diffusion limit or violation of the detailed balance, *see* **Note 21**). The initial condition specifies the starting point for the simulation, that is, the concentrations at time $t = 0$. Starting from the initial condition, $x_0 = x(0)$, the simulation calculates the change of the species in the model over time, $x(t)$, using numerical integration schemes (*see* **Notes 22** and **23**). The result is usually plotted as a function of time in a graph, with time on the x-axis and the concentration(s) on the y-axis (Fig. 1).

A significant challenge in the modeling of cell signaling is the combinatorial increase of the number of states that can arise from multi-site proteins forming large complexes [13]. An example is the epidermal growth factor receptor (EGFR), which can bind several downstream proteins simultaneously [14]. Because the assembly of

3.2 Modeling Complex Interaction Networks: Rule-Based Modeling for RTK Receptors with Multiple Phosphorylation Sites and Interaction Domains

such large complexes can arise in any order, for example, EGFR may bind Shc first, then PLCγ (or Grb2), or the other way around; a computational description of the system dynamics has to keep track of all the possible combinations. Mathematically this leads to a large system of ODEs with many states (species), one for each possible complex. Even seemingly simple systems, such as a receptor that can bind a ligand, dimerize, phosphorylate itself, and bind a scaffold (Fig. 2), can turn out to be much more complex than initially thought, resulting in 102 combinatorial reactions. For a more detailed treatment of how to build rule-based models, we refer to [8, 12, 13].

3.3 Using Core Models to Describe Salient System Properties That Arise for Wide Parameter Ranges

A good model is built with the purpose to gain insights into the system's behavior and answer certain questions. As such, a model is never a complete replica of a biological system but highlights certain aspects of reality while neglecting others. Thus, when modeling a particular system, one has to carefully consider the scope and the level of detail of the model. By scope we mean the boundaries of the model, what components and processes to include or not to include. By the level of detail, we mean the accuracy at which the included processes are described (Fig. 3). Even simple models of growth factor signaling that neglect most of the complexity arising from the assembly of large multi-protein complexes at the receptor level (*see* Subheading 3.2) can be very useful, for example, for explaining an interesting, experimentally observed phenomenon [15, 16]. In particular, the notion of feedback is one of the most fundamental concepts in biological control. Positive and negative feedback loops can destabilize steady states, thereby causing complex dynamic behaviors. For example, depending on the parameter values, negative feedback can lead to oscillations, and positive feedback can cause bistability [17, 18]. It is therefore critical to model the feedforward and feedback structures accurately.

1. *Identify the feedforward and feedback structures within the system*. Feedforward and feedback structures are the most important factors determining the dynamic behavior of a system. They can be often identified by drawing a reaction kinetic diagram, and visually inspecting this diagram, paying special attention to branching points (diverging and converging branches) and cycles (a subset of reactions forming a closed loop).
2. *Build a simple model that preserves the feedforward and feedback structures*. In contrast to diverging and converging feedforward and feedback loops, a linear sequence of events can often be described in simplified terms. It is relatively straightforward to lump a linear sequence of processes into a single step and assign

BioNetGen model

```
begin model

begin parameters
   kf 1
   kr 0.1
   ka 1
   kd 0.1
   kp 1
   kdp 0.1
end parameters

begin molecule types
   R(LB,Dm,Y1~0~P)
   L(RB)
   S(RB,B1,B2)
end molecule types

begin seed species
   R(LB,Dm,Y1~0) 100
   L(RB) 100
   S(RB,B1,B2) 50
end seed species

begin observables
   Molecules R_dimer   R(Dm!1).R(Dm!1)
   Molecules RS        R(Y1!1).S(RB!1)
end observables

begin reaction rules
# L binding to any R form
R(LB) + L(RB)<->R(LB!1).L(RB!1) kf,kr
# R dimerization
R(LB!1,Dm).L(R!1) + R(LB!2,Dm).L(RB!2) <->
R(LB!1,Dm!3).L(R!1).R(LB!2,Dm!3).L(RB!2) ka,kd
# Phosphorylation of Y1 in the complex
R(Dm!1).R(Dm!1,Y1~0) <-> R(Dm!1).R(Dm!1,Y1~P) kp,kdp
#S binding to Y1~P
R(Dm!1).R(Dm!1,Y1~P) + S(RB) <-> R(Dm!1).R(Dm!1,Y1~P!2).S(RB!2) ka,kd

end reaction rules

end model
```

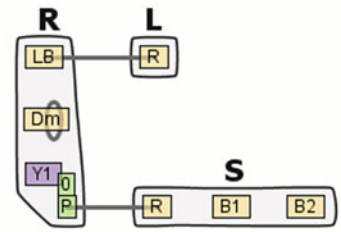

Fig. 3 BioNetGen model of a receptor tyrosine kinase (RTK). RTK can bind a ligand, which results in receptor dimerization, following by phosphorylation of a tyrosine (Y1) residue and binding of a scaffold protein. Notations: *R* receptor, *L* ligand, *S* scaffold, *RB* receptor binding domain, *LB* ligand binding domain, *Dm* domain for receptor dimerization, *Y1* residue that can be unphosphorylated (0) or phosphorylated (P) and bind the scaffold; B1 and B2, two binding domains of the scaffold (not used in this model)

a rate law. But choosing the appropriate rate law can be complicated depending on whether formal model reduction techniques or simple phenomenological expressions are used (Fig. 3). Ideally, the feedforward and feedback structures should be described by a few tunable and biologically relevant parameters.

3. *Explore the dynamic systems behavior in simulations*. We can characterize the parameter space into regions of different behaviors by simulating the model with different parameter values and observing the associated dynamic behavior. There are two possible aims. Firstly, in the absence of any experimental data, the aim is to identify an interesting model prediction that can subsequently be validated. Secondly, when data are available, the aim is to explain these data, *see* **step 4** below.
4. *Compare simulated systems behavior to experimental data*. By comparing the simulated predictions back to experimental data, we can identify not only the interaction structures but also the interaction strengths that are critical for explaining the experimentally observed phenomenon. For example, the model in ref. [19] first predicted oscillations in ERK signaling arising from a negative feedback loop from ERK to Ras or Sos. These oscillations were later discovered experimentally [20, 21]. In another example by Ryu et al. [15], the addition of a feedforward loop regulating the strength of the feedbacks was required to explain the ERK dynamics following single-cell, pulse stimulation experiments with two different growth factors, EGF and NGF.

3.4 Parameter Estimation to Reproduce Experimental Data

Direct measurements of parameters such as rate constants are often not available or even infeasible. Armed with a hypothesis about the structure of a biochemical system incorporated into a model, matching the model output with experimental data usually involves tweaking unknown parameters to determine which set of parameters is most likely to have produced the data. The preferred method is to do this parameter tuning systematically using formal parameter estimation procedures.

1. *Build a model* (*see* Subheadings 3.1–3.3).
2. *Decide which parameters are known and unknown* (*see* **Note 21**) *and which parameters can be controlled in* experiments (such as the EGF concentration), *and collect experimental data* (*see* Subheading 3.5). The resolution and amount of the required experimental data depend on the number of parameters that must be estimated and the acceptable amount of uncertainty in the model predictions [22]. Usually, a large number of time course responses (data at several time points following a stimulation or perturbation) and dose-responses (data using several growth-factor or drug concentrations) under several

experimental conditions (pretreatments with a kinase inhibitor or siRNA, gene knockouts, etc.) are required to obtain accurate parameter estimates [23–25].

3. *Specify the models' input/control parameters and initial conditions to match the experimental setup.* If several experimental conditions have to be simulated, one such model setup for each condition is required. For example, if data were collected in the presence of a MEK inhibitor, the parameter corresponding to MEK catalytic activity in the model should be set to zero or a low value (*see* **Note 24**).
4. *Estimate the unknown parameters using optimization algorithms.* This requires simulating all experimental conditions, computing the error between simulation and measurement, and minimizing this error in iterative steps (*see* **Note 25**). Many software packages for parameter estimation, such as Copasi, PottersWheel, and Systems Biology Toolbox 2, automate this process and also offer a variety of different optimization algorithms. For details, we refer to the original software documentations. A particularly useful software package is PEBSSBI (short for Parameter Estimation Pipeline for Systems and Synthetic Biology [10]) that has been developed to address practical aspects of parameter estimation that received limited attention in other software packages. In particular, relative data normalization and data handling are automated, and there is native support of multi-condition experiments.

3.5 Quantitative Methods of Experimental Calibration and Validation of Kinetic Signaling Models

The methods below describe the most common experimental in vitro approaches to obtain the parameters needed for calibration and validation of computational models.

3.5.1 Assessment of the Dose-Response and Temporal Protein Activation Profiles in Whole Cell Lysates Under Physiological Normal Growth Conditions

Total levels and post-translational modification states of proteins in whole cell lysates or specific subcellular locations can be estimated under both normal (physiological) or perturbed conditions. In RTK signaling studies, most widely used proteins of interest (POIs) are listed in Fig. 4. An example of quantitative measurement of RTK epidermal growth factor receptor (EGFR) activation and response of downstream readout protein in dose- and time-dependent manner following Subheadings 3.5.1.1–3.5.1.5 is shown in Fig. 5.

Cell Stimulation and Harvest

1. Grow the cells of choice in T75 cell culture flasks and use between passages 3 and 10 when they reach 70–80% confluence (*see* **Note 26**).

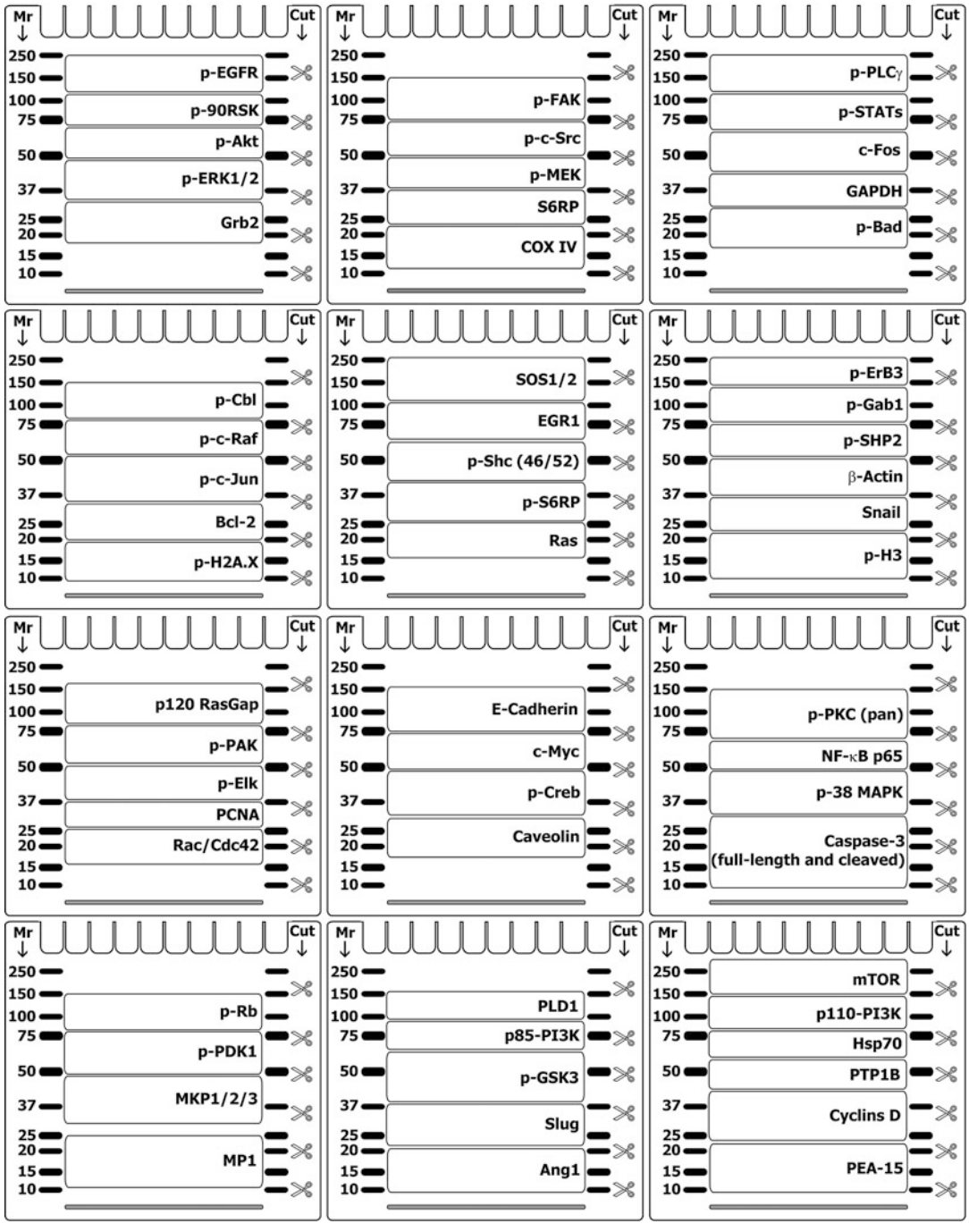

Fig. 4 Most common POIs implicated in EGFR signaling network and their migration range during electrophoresis. Suggested gel cutting guidelines to isolate particular POIs for MSWB are provided

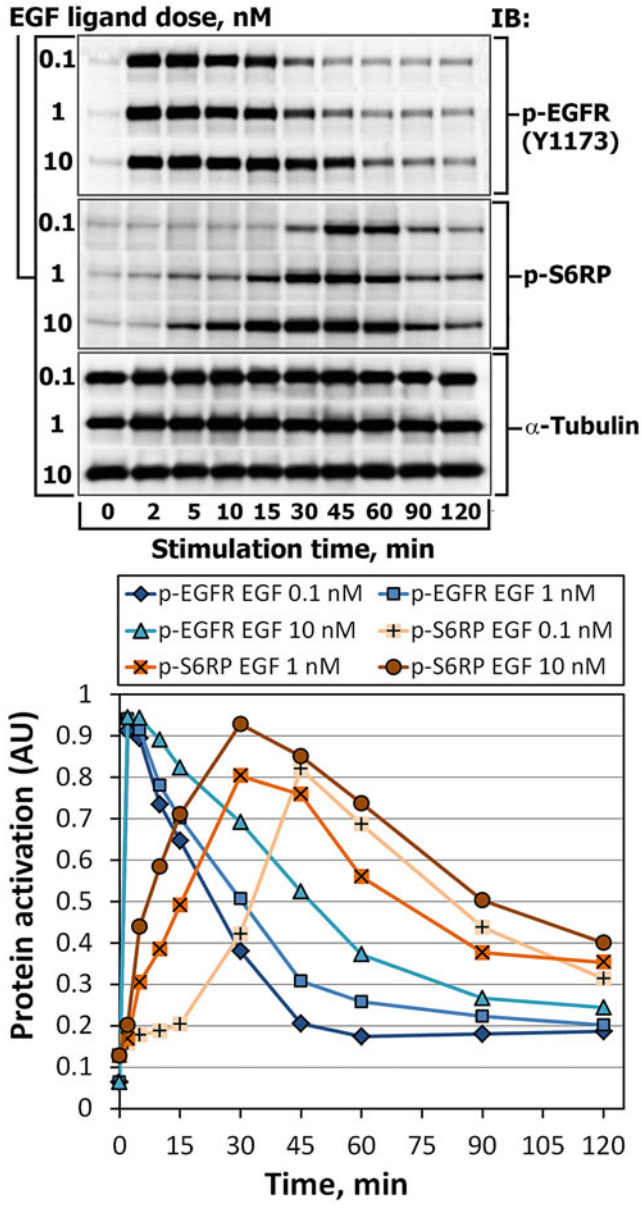

Fig. 5 (*Upper panel*) Blot showing the dose-response kinetics of EGF receptor (EGFR) autophosphorylation and the subsequent activation of a downstream POI (S6 ribosomal protein, S6RP). Measurement of housekeeping protein alpha-tubulin signal was used as a loading control. (*Lower panel*) The signal net intensities of p-EGFR and p-S6RP bands shown above were normalized to corresponding α-tubulin signals and represented as scatter plot. Such plot should include standard deviation (SD) or standard error (SE) of the mean, when the experimental values are obtained from three or more biological replicates

2. Trypsinize the cells and dilute to desired concentration with complete medium.
3. Plate the cells into 60 × 15 mm or 100 × 20 mm cell culture dishes and grow until 70–80% confluence (*see* **Note 26**) in a humidified 5% CO_2 incubator at +37 °C. Use one dish per data point.
4. Replace the complete medium with cell starvation medium and incubate cells for 6–16 h (*see* **Note 27**).
5. Stimulate the cells by adding the ligand dropwise to the dish. For dose-response experiment, different amounts of ligand should be diluted to final concentration in medium. If testing a more complex model which predicts the outcome of interactions between two or more signaling networks [26], expose the cells to individual ligands as well as to their mixture (*see* **Note 28**).
6. Leave at least one dish for each time-series unstimulated (control) to assess the baseline levels of protein activation (*see* **Note 29**).
7. Gently mix the medium by circular movements of dish and immediately start the timer countdown from a selected time point (e.g., 10 min). Place the dish onto +37 °C dry heat bath block surface, or if stimulating cells for more than 3 min, place the dish back to incubator.
8. Ten seconds before the end of cell stimulation, lift the dish, remove the medium by vacuum suction, and place the dish onto the ice pellets.
9. Immediately add 0.6 mL or 1–1.2 mL ice-cold *WCL buffer* to the 60 or 100 mm dishes, respectively, and scrape the cells with sterile plastic cell scraper.
10. Collect cell lysate into 1.5 mL microcentrifuge tube, vortex extensively, and keep on ice until the end of experiment. Similarly, prepare cell lysates for other data points.

Preparation of Samples

1. Centrifuge tubes with collected cell lysates at 12,000 × *g* for 10 min at +4 °C to remove detergent-insoluble material.
2. Measure total protein content in the samples (*see* **Note 30**).
3. Prepare Laemmli samples for protein separation by LDS-PAGE: mix the supernatant of each cell lysate with *4× LDS Sample Buffer* and *10× Sample Reducing Agent* in a ratio of 65:25:10 in pre-labeled 1.5 mL microcentrifuge tubes, heat at 75 °C for 5 min, and cool to RT (*see* **Note 31**).

Electrophoresis

1. Carefully remove a comb from each gel, rinse its wells and the whole gels under a running stream of dH_2O, and place into electrophoresis unit. Fill both upper and lower chambers with cool *1× MOPS-SDS* or other *running buffer* (*see* **Note 11**).

2. Prior to sample loading, supplement the running buffer in the upper (cathode) chamber of electrophoresis unit with 0.5 mL *1× Antioxidant solution*.
3. Using gel loading tips, load 5–10 μL of protein molecular weight marker (M) on both sides of the gel, then load even amount of prepared Laemmli samples onto the rest wells of the gel. For 1.0 mm thickness 10-well NuPAGE mini-gel, maximum sample load volume is 30 μL, for the same thickness 12-well gel, 20 μL, and for 15-well gel, 15 μL. The number of gels to be loaded depends on the number of data series and the number of samples within each series to be analyzed (*see* **Note 32**). If necessary, load the samples in technical replicates.
4. Separate the proteins by electrophoresis. If using NuPAGE Bis-Tris 4–12% gradient gels, run at 120–150 V constant voltage.
5. When the dye front reaches the bottom of each gel, stop electrophoresis. At 130 V constant voltage, the proteins are fully separated within 1 mm thickness NuPAGE mini-gel in ~1.5 h. Remove gel cassette out of apparatus, rinse under a stream of dH_2O, and gently open with a gel knife. Note that upon opening the cassette, the gel can adhere on either side (*see* **Note 33**).

Western Blotting/ Immunoblotting (IB)

1. Perform either conventional or modified IB procedure, referred to as Multi-Strip Western Blotting (MSWB). MSWB is based on simultaneous electrophoretic transfer of proteins from multiple strips of polyacrylamide gels to a single membrane sheet. It allows concurrent comparison of protein activation temporal profiles obtained in response to varying stimuli or perturbations and therefore is a preferred choice to obtain parameters for model fitting. The detailed steps and tips how to perform this procedure correctly are provided in a separate protocol [27].
2. For MSWB, cut the desired strips containing POI from each gel with gel cutting knife, and assemble the strips onto a single sheet of extra-thick (if used in Western sandwich for XCell II™ Blot) or thin (if used in iBlot® Dry Blotting System) filter paper. The number of strips to be cut out from one gel depends on the number of distinct proteins to be analyzed. *See* Fig. 4 for the migration zones and suggested gel cutting guidelines of most widely used POIs in EGFR signaling studies.
3. Fill one side of gel/blot assembly tray with 500 mL of refrigerated *1× Setup buffer*, while another side—with 400 mL of *1× Transfer buffer*.
4. If using the XCell II™ Blot, presoak sponge pads in *1× Setup buffer* and sheets of the membrane in *1× Transfer buffer* at least

for 5 min before assembling into Western sandwich. Do not presoak the sponge provided in iBlot® Nitrocellulose Regular Stacks, moisten only the membrane.

5. Form Western sandwich (*see* **steps 6** and **7** below) and transfer the proteins.
6. *For iBlot® Dry Blotting System*, put the bottom anode stack, place the moistened membrane, flip the thin filter paper with assembled gel strips so that they face the membrane, place one more thin filter paper, and add cathode stack. Put the provided sponge near copper cathode on the top and close the lid. Select the optimal program and transfer the proteins (*see* **Note 34**).
7. *For XCell II™ Blot*, place two wet sponge pads into the cathode (−) core of the blot module. Place an extra-thick filter paper with assembled gel strips on the top. Cover the surface of gel strips with a sheet of membrane. Remove any trapped air bubbles by rolling a blotting roller over the membrane surface. Place three moistened extra-thick filters onto the surface of the membrane followed by a tandem of wet sponge pads. Place the anode (+) core on the top of the pads. Slide the blot module into the rails on the lower chamber of XCell SureLock Mini-Cell. Lock the gel tension lever. Fill the blot module with *1× Transfer buffer* until the blotting sandwich is completely submerged. If there is no leakage, fill the outer chamber with refrigerated dH_2O, add a lid on the buffer core, and connect the unit to power supply. If using gradient 4–12% Bis-Tris gels, transfer at 30 V constant for 90 min.
8. Once the transfer is over, remove the membrane with transferred proteins out of the blot module and attach to the middle of a square Petri dish by sticking the membrane's corners with a transparent tape. Label the lid (indicate the *1^0Ab* antibody type to be used for given blot, ID#, and if desired, the sequence of sample loading).
9. Equilibrate the membrane in dH_2O for 3–5 min, then replace dH_2O with 30–50 mL of *blocking buffer*, and incubate for 1 h at RT on a horizontal rotating platform.
10. Decant *blocking buffer*, add appropriate *1^0Ab solution*, and incubate the membrane with agitation for 6 h at RT or overnight at +4 °C.
11. Collect *1^0Ab solution* into appropriately labeled 50 mL tubes and store at +4 °C (*see* **Note 35**). Rinse each membrane with dH_2O and subsequently wash in 1× TBST (four times, 7 min each).
12. Incubate the membrane with appropriate *2^0Ab solution* for 1–2 h at RT.

13. Discard *2^0Ab solution,* wash the blot with running dH_2O, and incubate with 1× TBST (four times, 7 min each). Make a final fifth wash in dH_2O to remove the residual TBST. Keep the blot in dH_2O until protein detection step.

Protein Detection

1. Prepare 25–30 mL of ECL reagent working solution in 50 mL conical tube immediately before use. Pour the solution into the clean Petri dish and label the lid "ECL reagent."
2. Using tweezers, gently remove the tape from the corners of the blot. Take out the blot from the dish with dH_2O and place it into the dish with ECL reagent. Make sure the reagent covers the whole blot and incubate for ~5 min.
3. Place the membrane in the precut piece of sheet protector and place upside down in the imaging system. Visualize and capture the protein bands (*see* **Note 36**).
4. Save original images of blots. Export images as TIFF or JPG files.
5. Quantify the net signal intensity of each detected protein band using densitometry analysis software. Export data file as TXT or Excel file.
6. Normalize each data point to the signal of housekeeping protein (e.g., GAPDH, β-Actin, α-Tubulin, Grb2) and/or to the signal of corresponding total protein.
7. Plot normalized dose-time series data to generate temporal protein activation curves.
8. Provide the data for modeling fitting analysis.
9. Repeat the same experiment at least three times to obtain biological replicates.

3.5.2 Assessment of Temporal Protein Translocation and Activation Profiles at Specific Cellular Compartments

Some computational models will predict protein recruitment and activation or inhibition at the specific cellular location, such as plasma membrane, cytoplasm, nucleus, mitochondria, etc. This requires estimating protein levels and their post-translational modification states in different subcellular fractions. Procedure for separating nuclear, mitochondrial, membrane, and cytoplasmic cell fractions can involve centrifugation methods as well as using specific detergents, such as saponin or digitonin [28–31]. An example of an experiment to obtain the temporal protein expression and activation profiles induced by two-ligand combination in nuclear cell fractions is shown in Fig. 6. Please note that there are many commercially available cell fractionation kits.

Preparation of Crude Soluble and Insoluble Fractions Using Digitonin

1. Plate the cells into 100 × 20 mm cell culture dishes and grow until 70–80% confluence in a humidified 5% CO_2 incubator at +37 °C. Use one or two dishes per data point.
2. Follow **steps 2–8** under Subheading 3.5.1.1.

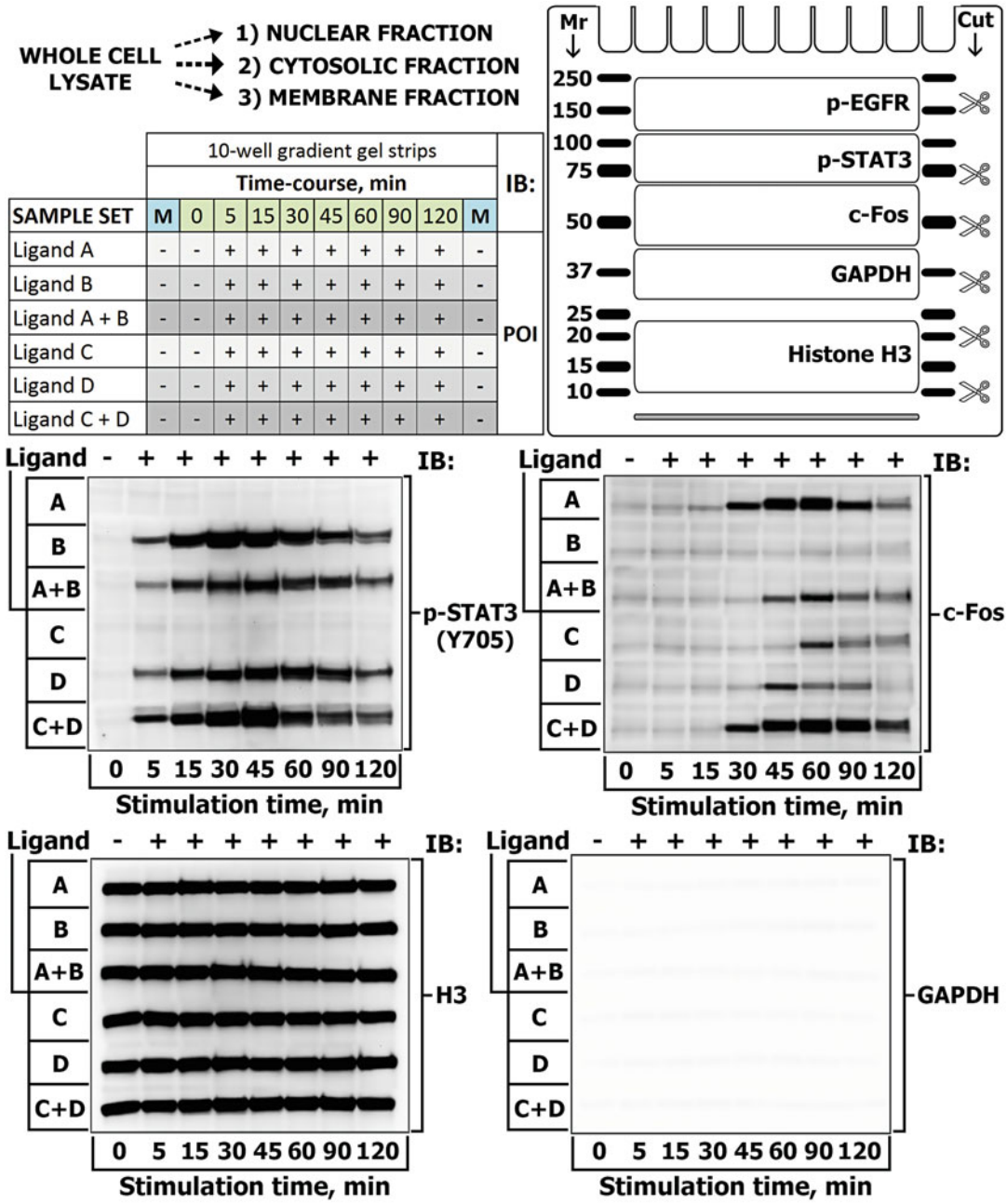

Fig. 6 Suggested experimental layout for testing the impact of different ligands and their combinations on induction and/or activation of POIs. Different subcellular fractions were isolated from stimulated and unstimulated (control) cells using Subcellular Protein Fractionation Kit for Cultured Cells (TFS, #78840) and then separated by LDS-PAGE. Indicated POIs were cut out of each gel according to their migration zones. The gel strips containing identical POI were assembled onto a single membrane sheet and subjected to MSWB. Blots were probed with anti-p-EGFR (Y1173) (membrane protein, not shown), c-Fos (nuclear protein), p-STAT3 (Y705) (cytosolic and nuclear), GAPDH (primarily cytosolic protein), and histone 3 (H3) (nuclear chromatin-bound protein) antibodies. Chemiluminescent signals were detected by KODAK Image Station 440CF. Nearly complete absence of GAPDH in nuclear fraction indicates low cross-contamination among fractions

3. Immediately add 1.6 mL ice-cold CF *buffer*, gently rotate to cover the bottom of the dish with the buffer, and place the dish onto ice pellets. Incubate for 10–15 min (*see* **Note 37**).
4. Gently scrape the cells into 2.0 mL microcentrifuge tube and spin the tube for 30–60 s at highest microcentrifuge speed.
5. Transfer all supernatant to another 2.0 mL microcentrifuge tube and label "CYT"—this is *cytosolic protein fraction*. Do not disrupt the pellet.
6. Add 200–500 μL of *Modified RIPA buffer* onto the pellet. Pipette up and down several times to break the pellet, then vortex until the pellet completely dissolves. Label the tube as "NUC/MEM"—this is crude integral and nuclear membrane protein fraction.
7. Keep both tubes on ice until the end of experiment. Similarly, prepare subcellular fractions for other time/dose data points.
8. Follow the steps listed under Subheadings 3.5.1.2–3.5.1.5.

Preparation of Subcellular Fractions Using Ultracentrifugation

1. Plate the cells into 100 × 20 mm cell culture dishes and grow until 70–80% confluence in a humidified 5% CO_2 incubator at +37 °C. Use two dishes per data point.
2. Follow the **steps 2–8** under Subheading 3.5.1.1.
3. Immediately add 1.6 mL ice-cold *SF buffer*, gently rotate to cover the bottom of the dish with the buffer, and place the dish onto ice pellets. Incubate on ice for 20 min.
4. Gently scrape the cells into pre-chilled 2.0 mL syringe with attached 25 gauge needle.
5. Pass cell suspension through the needle ten times.
6. Collect the suspension into 2.0 mL microcentrifuge tube and leave on ice for 15–30 min.
7. Centrifuge the sample at 720 × *g* (3000 rpm) for 5 min at 4 °C.
8. Transfer supernatant into a separate tube, labeled as MIT (it contains cytoplasm, membrane and mitochondria) and keep it on ice. Label the tube with remaining pellet as NUC (nuclei).
9. Do the same steps for a second dish.
10. Disperse nuclei-containing pellet with a pipette in 500 μL of *SF buffer*.
11. Combine nuclei-containing pellets from the first and second dish. Collect suspension into 1 mL syringe and pass through a 25 gauge needle ten times.
12. Centrifuge the suspension at 720 × *g* (3000 rpm) for 10 min at 4 °C.

13. Discard the supernatant and keep the pellet that contains nuclei.
14. Resuspend the pellet in 250–500 µL of *Modified RIPA buffer*. Adding more buffer to the sample will decrease sample protein concentration. Vortex the sample. It contains *nuclear fraction*.
15. If the sample is too viscous, sonicate the suspension briefly to shear genomic DNA. Alternatively, add DNAse I solution (TFS) to digest and remove unwanted DNA from samples.
16. Continue with MT-labeled sample tubes. Centrifuge each MT tube at 10,000 × *g* (8000 rpm) for 7 min at 4 °C. Pellet contains *mitochondrial fraction*.
17. Transfer the supernatant into a fresh tube labeled MEM (it contains both cytoplasm and membrane fractions).
18. Take mitochondria-containing pellet and process it as described in **steps 10–14**.
19. Continue with MEM-labeled sample tubes. To obtain a membrane fraction, centrifuge tubes in an ultracentrifuge at 100,000 × *g* (40,000 rpm) for 1 h at 4 °C.
20. Transfer a supernatant into a fresh tube labeled CYT. It contains *cytoplasmic fraction* (*see* **Note 38**).
21. Resuspend the remaining pellet in MEM tube in 400 µL of *SF buffer*.
22. Combine membrane-containing pellets from the first and second dish. Collect suspension into 1 mL syringe and pass through a 25 gauge needle three times.
23. Re-centrifuge MEM samples for 45 min.
24. Resuspend the membrane pellet in the same buffer as used for the nuclei. It contains *membrane fraction*.
25. Follow the steps listed under Subheadings 3.5.1.2–3.5.1.5.

3.5.3 Assessment of Temporal Protein-Protein Interaction Profiles

Some computational models will predict the kinetics of protein binding to other proteins at the specific cellular location, such as plasma membrane, cytoplasm, nucleus, etc. To validate such predictions, protein complexes must be isolated out of the unstimulated and stimulated whole cells of their specific subcellular fractions by immunoprecipitation (IP) technique.

Protein Immunoprecipitation

1. Plate the cells into 100 × 20 mm or 150 × 20 mm cell culture dishes and grow until 70–80% confluence in a humidified 5% CO_2 incubator at +37 °C. Use one dish per data point.
2. Follow **steps 2–8** under Subheading 3.5.1.1.

3. Immediately add 1.3 or 1.8 mL ice-cold *IP buffer* to the 100 and 150 mm dishes, respectively, and scrape the cells with sterile plastic cell scraper.
4. Collect cell lysate into 1.5 or 2.0 mL regular microcentrifuge tube, vortex extensively, and keep on ice until the end of experiment. Similarly, prepare cell lysates for other data points.
5. Centrifuge the tubes with collected cell lysates at 10,000 × *g* for 10 min at +4 °C to remove detergent-insoluble material.
6. If using anti-phosphotyrosine-conjugated or antibody-conjugated agarose beads, proceed to **step 9**. If using small G protein activation assays, refer to manufacturer's protocol.
7. Transfer 0.65–1 mL of supernatant into siliconized microcentrifuge tube and add 1^0Ab antibody at appropriate dilution (generally we use 5 μg/per such sample).
8. Incubate the tubes for 2–6 h at RT, gently mixing the sample on a nutator.
9. Add 60–120 μL of agarose/Sepharose conjugate suspension (approx. 30–60 μL beads/bed volume) to sample and incubate for 1–3 h at 4 °C on a nutator.
10. Collect immunoprecipitated complexes by centrifugation at 3000 × *g* for 2 min at 4 °C. Discard supernatant.
11. Wash bead pellet with 1 mL of ice-cold *HNTG buffer* by resuspension and centrifugation in tabletop mini-centrifuge. Repeat this step at least three times, and thereafter wash twice with ice-cold PBS.
12. With gel loading tip, carefully absorb PBS from the bead pellet and immediately resuspend it in 100–200 μL Laemmli sample buffer prepared with certain volume of PBS as a substitute for cell lysate. Heat samples at 95 °C for 5 min.
13. Centrifuge the samples for 30 s at 12,000 × *g* at RT. Collect supernatant (IP sample).
14. Follow the steps listed under Subheadings 3.5.1.2–3.5.1.5.

3.5.4 Assessment of the Dose-Response and Temporal Protein Activation, Protein-Protein Interaction, or Protein Translocation Profiles Under the Perturbed Conditions

Some mathematical models will predict the behavior of the proteins under certain physiological conditions, e.g., when the enzymatic activity of certain protein in the very same or distinct signaling pathway is completely absent, is downregulated (e.g., at 50%) or upregulatged (e.g., at 150%), or when the concentration of certain enzyme, adaptor, or structural protein is variable. The validation of such model predictions can be achieved by cellular perturbations. These include activation or inhibition of specific enzyme (typically protein kinases or phosphatases) by selective pharmacological small molecule inhibitor/activator (I/A). Some inhibitors may selectively or non-selectively target more than one protein [32]. Protein-protein

or protein-lipid interactions may be prevented by cell treatment with peptides and compounds that specifically block protein domains (e.g., SH2 or PH) [33–35] or by introducing dominant-negative proteins. Both protein levels and activation can be modulated by RNA interference (RNAi) and cell treatment with microRNA or their inhibitors (anti-miRs) [36–38]. Clustered regularly interspaced short palindromic repeats (CRISPR) technology allows systematically to disable each individual human gene [39]. Finally, transient or stable (constitutive) expression of wild-type or mutated (gain-of-function or loss-of-function) gene can be achieved by cell transfection with specifically constructed plasmid vectors [40]. Newer strategies for reversible modulation of protein activity include site-specific conjugation of small molecule and polymer [41].

Protein Inhibition or Activation by Pharmacological Inhibitors or Activators (I/A)

1. In the preliminary experiment, determine the optimal I/A dose required to activate or inhibit POI without nonspecific or off-target side effects, and use this concentration for subsequent analysis (*see* **Note 39** and Fig. 7).
2. Perform procedures as described in **steps 1–4** under Subheading 3.5.1.1.
3. Before stimulation with a ligand of choice at different concentrations, pre-incubate the serum-starved cells with optimal I/A dose for predetermined time interval. You may also want to evaluate the impact of different I/A at fixed ligand dose, as seen in experimental layout of Fig. 8.
4. Pre-incubate control serum-starved cells with solvent only, i.e., solution that was used to dissolve the I/A (e.g., DMSO, ethanol, etc.). This step is required for verification of solvent-mediated effects on cellular signaling.
5. Perform **steps 5–8** listed under Subheading 3.5.1.1.
6. For the dose-response and temporal protein activation in whole cell lysates, proceed to **step 9** listed under Subheading 3.5.1.1.
7. For the dose-response, protein translocation, and activation at specific cellular compartments, *see* Subheading 3.5.2.
8. For the dose-response and protein-protein interactions, *see* Subheading 3.5.3.

Protein Suppression by RNAi

1. In the preliminary experiment, determine the optimal concentration of siRNA and incubation time required to maximally suppress the expression levels of POI (not only its mRNA) in the cell line of choice without nonspecific or off-target side effects.
2. Plate cells of choice in an appropriate complete (supplemented with serum, supplementary growth factors, and/or antibiotics)

A.

	10-well gel strips									
	Time-course, min									IB:
SAMPLE SET	M	0	5		15		30		0	M
EGF + I/A 0.1 nM	-	-	-	+	-	+	-	+	+	-
EGF + I/A 1 nM	-	-	-	+	-	+	-	+	+	-
EGF + I/A 10 nM	-	-	-	+	-	+	-	+	+	-
EGF + I/A 100 nM	-	-	-	+	-	+	-	+	+	-
EGF + I/A 1 μM	-	-	-	+	-	+	-	+	+	-
EGF + I/A 10 μM	-	-	-	+	-	+	-	+	+	-
	Treatment with I/A									

POI

	10-well gradient gel strips									
	Time-course, min									IB:
SAMPLE SET	M	0	2	5	10	15	30	45	60	M
EGF 0.1 nM	-	-	+	+	+	+	+	+	+	-
EGF 1 nM	-	-	+	+	+	+	+	+	+	-
EGF 10 nM	-	-	+	+	+	+	+	+	+	-
EGF 0.1 nM + I/A	-	-	+	+	+	+	+	+	+	-
EGF 1 nM + I/A	-	-	+	+	+	+	+	+	+	-
EGF 10 nM + I/A	-	-	+	+	+	+	+	+	+	-

POI

B.

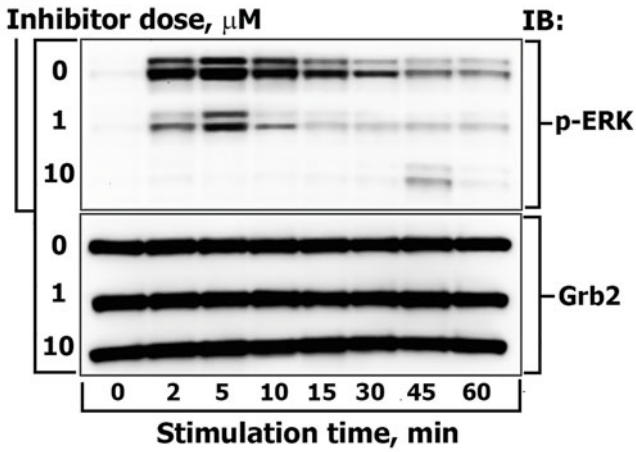

Fig. 7 (**a**) *Upper panel*. Suggested experimental layout for evaluation of effective inhibitor/activator (I/A) dose on protein of interest (POI) abundance, activation (e.g., through phosphorylation), translocation (if samples loaded are subcellular fractions), or interactions with other POI (for IP samples only) at constant ligand (e.g., EGF) signal strength. *Bottom panel*. Suggested layout for assessing the impact of fixed dose I/A on POI functions at variable signal strength. (**b**) The final MSWB-generated blot of phosphorylated (p-) ERK1/2 and housekeeping adaptor protein Grb2 in IGF-1-stimulated MCF-7 cells pretreated with varying doses of IGF1 receptor inhibitor

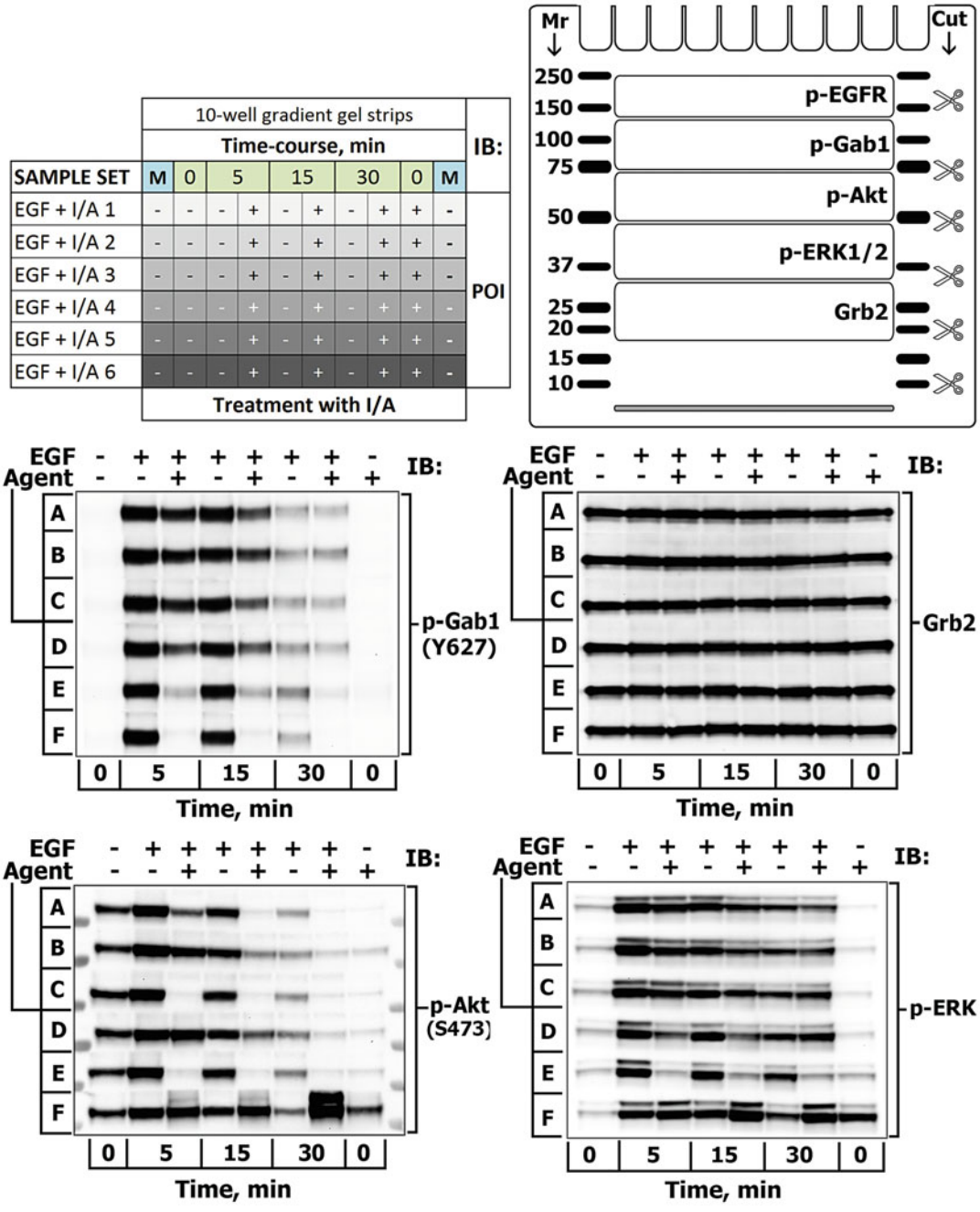

Fig. 8 Comparison of impact of lipid raft/caveola disruption by different inhibitory agents (I/A 1 through 6) on signaling downstream of the EGF receptor. Samples were separated by electrophoresis and indicated POI were cut out of each gel according to their migration zones. The gel strips containing identical POI were assembled onto a single membrane sheet and subjected to MSWB. Blots were probed with anti-p-EGFR (Y1173) (not shown), p-Gab1 (Y627), p-Akt (S473), p-p44/p42 MAPK (ERK1/2) (Thr202/Tyr204), and Grb2 antibodies. Chemiluminescent signals of each blot were detected by KODAK Image Station 440CF

cell culture medium in 60 mm cell culture dishes and grow until 70–80% confluence in a humidified 5% CO_2 incubator at 37 °C. Use one or two dishes per data point.

3. If using Lipofectamine 2000 or similar transfection reagent, which transfects already adherent cells even in the presence of serum and/or antibiotics, proceed to **step 9** below.
4. For cell electroporation, trypsinize cells with sterile 0.25% Trypsin-EDTA solution and resuspend in antibiotic-free complete media.
5. Aliquot at least 1.2×10^6 cells per sample into microcentrifuge tubes, and centrifuge at $90 \times g$ for 10 min at RT.
6. Remove supernatant and resuspend cell pellet in 100 μL of Ingenio Electroporation solution containing 100 nM (or optimized amount) of test siRNA of your choice or selected control siRNA.
7. Electroporate cell suspensions containing siRNA using the appropriate program on Amaxa's Nucleofector II device following manufacturer's instructions.
8. Immediately after electroporation, add 0.5 mL of the pre-equilibrated antibiotic-free complete medium to the cuvette and transfer the cell suspension into 60 × 15 mm plate (final volume 2.0 mL media per dish).
9. Allow cells to attach the surface of the dish before adding antibiotic solution.
10. When the inhibitory effect of siRNA has reached the maximum level of protein knockdown (e.g., at 24, 48, or 72 h post-transfection) (*see* **Note 40**), stimulate the specific siRNA- and non-targeting siRNA-transfected cell series with a ligand or a mixture of ligands. The stimulation may be performed in the presence or absence of other I/A.
11. For the dose-response and temporal protein activation in whole cell lysates, proceed to **step 9** listed under Subheading 3.5.1.1.
12. For the dose-response, protein translocation, and activation at specific cellular compartments, *see* Subheading 3.5.2.
13. For the dose-response and protein-protein interactions, *see* Subheading 3.5.3.

4 Notes

1. The provided experimental cell stimulation protocol is for adherent cells.
2. Follow the recommendations of the provider how to culture and freeze specific cell lines.

3. We strongly advise against using multi-well plates for signaling experiments because some cell lines are very susceptible to signaling caused by flow shear stress which occurs and compounds during repetitive handling of the such plate.
4. Centrifuge the vial with lyophilized ligand and reconstitute in solution recommended by a manufacturer to a concentration of 0.1–1.0 mg/mL. This stock solution can be further diluted into other aqueous buffers and stored at 4 °C for a week or −20 °C for future use.
5. To obtain more denaturing cell lysis buffer, add 0.5% sodium deoxycholate (SOD), 0.1% Sodium-dodecyl sulfate (SDS) and 70 mM *n*-Octyl-β-D-glucoside (OG) (optional). Ionic detergents SOD and SDS disrupt nuclear membranes. Non-ionic detergent OG effectively dissolves the lipid rafts and solubilizes integral membrane proteins. Caution: this enhanced buffer may denature some kinases.
6. Reconstitute the I/A in solvent recommended by a manufacturer. This stock solution can be further diluted into other aqueous buffers and stored at 4 °C or −20 °C for future use.
7. You will need several types of siRNA: (a) Individual or pooled siRNA against specific protein of interest (POI); (b) negative control siRNA (non-targeting siRNA with nonsense/scrambled sequence); (c) positive control siRNA (e.g., fluorescently tagged (e.g., GFP) siRNA to confirm transfection of cells) (optional); and (d) "mock" control siRNA [siRNA against another protein (e.g., GAPDH) to check that RNAi is not affecting overall cell function] (optional).
8. Alternatively, the investigator may use specific commercially available antibody-bead conjugate (e.g., Grb2-conjugated agarose beads).
9. We recommend using 10-well or 12-well NuPAGE Novex 4–12% gradient Bis-Tris, 1.0 mm thickness, 10 × 10 cm Minigels (TFS, #NP0321BOX and #NP0322BOX, respectively).
10. Reference bands of MW marker other than that used in this protocol will have different migration patterns, generating a different number of designated protein migration zones depicted in this procedure that may also vary in width.
11. Other running buffers (MES-SDS or Tris-Acetate-SDS) will change the width and quantity of designated protein migration zones depicted in this procedure and will require optimization.
12. If desired, PVDF or nylon membranes can be also used, but be aware of pore size.
13. Choose the type of apparatus for protein transfer that is suitable for the size of your gels.

14. We replace the NC membrane provided in gel transfer stacks with Bio-Rad NC membrane due to the noticed significant differences in their protein-binding efficacy.
15. We routinely use quite concentrated 1^0Ab solution with minimum of 1:1000 dilution, horse Anti-Mouse HRP-linked IgG 2^0Ab (Cell Signaling, Danvers, MA, #7076) at 0.1 μL/mL concentration, and Pierce Goat Anti-Rabbit HRP-conjugated IgG (H + L) 2^0Ab (TFS, #31460) at 0.025 μL/mL concentration.
16. Although SNAP system significantly shortens the time of membrane blocking and/or incubation with primary/secondary antibodies, it requires using heavily concentrated 1^0Ab solution.
17. Neglecting special effects is not appropriate when reactions are spatially separated, and the diffusion of substrates or products has to be considered. For example, a species might participate in a fast reaction before it can diffuse throughout the cell. When space and diffusion have to be considered, partial differential equations can be used [42].
18. The continuity assumption is usually satisfied for receptor systems, who typically contain 10,000–20,000 receptor molecules on the cell surface [43]. But it is violated for low molecule numbers. Essentially, biochemical reactions are stochastic with random fluctuations that scale inversely to the root of the molecule numbers: $CV = 1/\sqrt{N}$, where CV is the coefficient of variation defined as the standard deviation over the mean and N is the number of molecules. For numbers below $N = 300$, the noise is around 5%, and the chemical master equation [44] and stochastic simulations, for example, using the Gillespie algorithm [45], should be used. Most tools such as BioNetGen/NFsim allow for both deterministic and stochastic simulations.
19. Here one often relies on simplifying assumptions. For example, the scheme in Fig. 1 implies that the direct binding of Grb2, Shc, and PLCγ to the receptor dimer is competitive (mutually exclusive). That is, for example, if Shc has bound, PLCγ can no longer bind. The binding of two Shc molecules to two different protomers in the receptor dimer is also not possible in this model. These are simplifying assumptions. If two or more molecules could simultaneously bind to different binding sites on the receptor, Subheading 3.2 has to be used.
20. Consider the following enzymatic process, consisting of the formation of an enzyme substrate complex, and the subsequent conversion, and dissociation of the product

$$X + E \rightleftharpoons C \rightharpoonup Y + E,$$

where X, E, C, and Y denote substrate, enzyme, enzyme-substrate complex, and product, accordingly. To simplify this two-step process and describe the rate of product formation in a single step, we will make two assumptions. The first is that the association and dissociation reactions are in thermodynamic equilibrium, that is, the forward and the reverse rate are balanced: $k_1 \; x \; e = k_2 \; c$, which is also often called the *rapid equilibrium* approximation. The second assumption is that the total amount of enzyme in the system does not change: $E_{\text{total}} = e + c$. Eliminating the free enzyme concentration e in these two equations gives $c = E_{\text{total}} \frac{k_1 \; x}{k_1 \; x + k_2}$, which can be used to describe the rate of product formation $v = k_3 c$ as a function of the substrate concentration and the total enzyme concentration: $v = V_{\max} \frac{x}{x + K_{\text{m}}}$, where $V_{\max} = k_3 E_{\text{total}}$ is the maximal reaction velocity and $K_{\text{m}} = \frac{k_2}{k_1}$ is the Michaelis-Menten constant. Note that this derivation follows the original works of Michelis and Menten [46, 47]. Briggs and Haldane [48] also provided an alternative derivation based on the quasi steady state assumption of the complex dynamics: $\frac{d}{dt} c = k_1 \; x \; e\text{-}k_2 c\text{-}k_3 c = 0$.

21. The detailed balance is a constraint on the allowable parameter values arising from the laws of thermodynamics. If the model contains one or more reversible reaction cycles in which no energy, for example, in the form of ATP, is produced or consumed, then the parameter values of the participating reactions have to be chosen such that the cycle can be in thermodynamic equilibrium. This means that in steady state, the forward flux of the cycle must equal the reverse flux of cycle (*see* [49] for details).
22. Numerical solvers use recursive schemes to solve differential equations iteratively starting from the initial condition. It is therefore necessary to specify the initial condition. An ODE together with a specified initial condition is called an *initial value problem*.
23. At each discrete time step, numerical solvers introduce errors. Depending on the numerical solver used, these errors can quickly accumulate, especially for so-called stiff systems consisting of both very fast and very slow changing variables. Stiff solvers, such as implicit Runge-Kutta methods, minimize the risk of large errors and should thus be the preferred method of choice for biological systems, which are often stiff.
24. Prior knowledge can come in the form of hard constraints (decay rates cannot be negative) or soft constraints (e.g., we might think that it is somewhat unlikely that a given rate constant is faster than a millisecond and extremely unlikely that it is faster than a nanosecond). Hard constraints can be dealt with by explicitly disallowing parameters that do not meet

the constraints. Soft constraints (known as *priors* in the language of Bayesian inference) can be incorporated with extra terms in the goodness-of-fit function that discourage extreme parameters.

25. Parameter estimation procedures minimize a so-called *goodness-of-fit function* (often also called *cost function* or *objective function*) that measures how well a model describes the experimental data. Popular choices for the goodness-of-fit function are the sum of squared residuals:

$$\mathrm{SSR}(p) = \sum_i^N \left(y_i - y(t_i, u_i, p)\right)^2,$$

where y_i denotes the experimentally measured data (usually the average over three or more replicates) and $y(t_i, u_i, p)$ the corresponding simulated data point (with the time t_i and the known control parameters u_i matching the time-point and conditions at which the experimental data was collected; or the chi-square function

$$X^2(p) = \sum_i^N \left(\left(y_i - y(t_i, u_i, p)\right)^2 / \sigma_i^2\right),$$

where σ_i denotes the standard deviation of the experimental data at point y_i.

26. Be consistent throughout all experimental series and do not allow cells to overgrow to prevent contact inhibition of division, as it can affect RTK activation and signaling kinetics [50–52].
27. The length of cell starvation depends on the metabolic activity of given cell line. Some tissue cells (e.g., skin fibroblasts) divide rapidly and consume more nutrients from the media than others. Generally, overnight starvation suppresses the baseline activation levels of most proteins. However, in some cells, long starvation can activate or alter the quantity of certain proteins as well as induce anoikis-cell-detachment-induced apoptosis. We advise to compare the baseline levels of protein phosphorylation between unstimulated cells grown under starvation and complete media conditions to ensure that the duration of starvation is sufficient. Some proteins harbor activating mutations. In such case, cell starvation will not affect or significantly reduce their phosphorylation levels.
28. Stimulate the first series of cells by adding the first ligand of choice for required time intervals. Apply the second ligand to the second group of cells. Add both ligands simultaneously to the plates of the third cell group. In this experimental setup, the signals should be obtained and compared under identical conditions!
29. If not sure, whether a chosen ligand can induce the detectable phosphorylation of POI, include the positive control: lysate of the

cells stimulated with a ligand that has a proven ability to stimulate the phosphorylation of POI. For instance, for phosphorylated STAT3 on Tyr-705 residue, a good positive control is IL-6-stimulated (2 nM for 10 min) Panc-1 or BxPc-3 cells.

30. It is advisable to know the protein concentration range in the samples. If cell growth rate, scraping, and lysis time are consistent, the protein content should not differ among the same series of samples or between technical and biological replicates.
31. Run samples within a 2-week period. For extended storage, store the samples at +4 °C (reheat prior to loading). Unused amount of cell lysates can be stored for further use at −20 °C/−80 °C.
32. If running more than two gels, make an interval of at least 5–10 min before loading the next tandem of gels and powering on the electrophoresis unit. This will reserve enough time for follow-up steps. Also, you may want to attach the sticky notes on the electrophoresis apparatus, helping to identify the gels (e.g., Gel 1 sample loading sequence—FRONT, Gel 2 sample loading sequence—BACK).
33. If the gel remains on a shorter (notched) side of the plate, a sequence of sampling should be rewritten in the laboratory notebook in a reversed order. The gel strips that will be derived from such reversed gel during MSWB procedure will inevitably need to be flipped horizontally by imaging software. To avoid this, reverse the entire gel or the strips during assembly step onto the filter paper.
34. The efficacy of protein transfer from the gel onto a membrane can be verified by staining the gel following transfer in Coomassie Dye or SimplyBlue SafeStain (TFS). After 1 h, discard the stain and replace with new load. It will improve staining accuracy. Alternatively, use more sensitive Imperial or PageBlue Protein Stains (TFS). The membrane can be rapidly and reversibly stained using Ponceau S red staining solution.
35. *1° Ab solution* can be reused multiple times if supplemented with 0.05–0.1% (w/v) sodium azide. Caution: the inclusion of sodium azide is to be avoided in all steps that use HRP-conjugated antibodies. If precipitation occurs, filter the solution through 0.22 μm filter, and supplement the solution with extra amount (e.g., 10 μL) of primary antibody.
36. For comparison of the signals from different blots, the capture time and number of frames should be equal for each separately exposed membrane.
37. The duration of cell exposure to CF buffer containing either digitoning or saponin detergents needs to be optimized for each separate cell line [31].

38. One can concentrate the supernatant by centrifuging through the filter unit. This concentrates the cytosol fraction down to approximately 50–75 μL/per tube.
39. It may be necessary to optimize the treatment time of tested I/A, which may range from several minutes to several hours. A 30–60 min preincubation period is often used. Also, it is important to verify that selected I/A dose maintains target protein in inactive/active state throughout the full time course of experiment. Thus, at least three data time points (early, middle, and late) should be included in the analysis of I/A efficacy. For instance, if I/A will be used for 1-h long kinetics, one may take a readout at 5, 15, and 30 min (*see* experimental layout in Fig. 7a, upper panel). Because of nonlinear network responses, it is not sufficient to obtain dose-response behavior of POI at single time point in the presence of I/A, so full time course perturbation experiment is required (Fig. 7a, lower panel). See actual experimental layout to assess inhibitor's effect on downstream POI in Fig. 7b.
40. The time of maximum knockdown of POI depends on transfection efficiency, initial concentration, and turnover of POI. There are several methods to determine protein half-life [53].

References

1. Lemmon MA, Schlessinger J (2010) Cell signaling by receptor tyrosine kinases. Cell 141 (7):1117–1134
2. Kholodenko BN et al (1999) Quantification of short term signaling by the epidermal growth factor receptor. J Biol Chem 274 (42):30169–30181
3. http://copasi.org/. Cited 17 Dec 2015
4. Raue A et al (2015) Data2Dynamics: a modeling environment tailored to parameter estimation in dynamical systems. Bioinformatics 31 (21):3558–3560
5. Schmidt H, Jirstrand M (2006) Systems biology toolbox for MATLAB: a computational platform for research in systems biology. Bioinformatics 22(4):514–515
6. http://www.scilab.org/. Cited 17 Dec 2015
7. http://www.gnu.org/software/octave/. Cited 17 Dec 2015
8. Blinov ML et al (2004) BioNetGen: software for rule-based modeling of signal transduction based on the interactions of molecular domains. Bioinformatics 20(17):3289–3291
9. Smith AM et al (2012) RuleBender: integrated modeling, simulation and visualization for rule-based intracellular biochemistry. BMC Bioinformatics 13(Suppl 8):S3
10. https://bitbucket.org/andreadega/systems-biology-compiler. Cited 15 Dec 2015
11. Maiwald T, Timmer J (2008) Dynamical modeling and multi-experiment fitting with PottersWheel. Bioinformatics 24(18):2037–2043
12. Thomas BR et al (2016) BioNetFit: a fitting tool compatible with BioNetGen, NFsim and distributed computing environments. Bioinformatics 32(5):798–800
13. Hlavacek WS et al (2006) Rules for modeling signal-transduction systems. Sci STKE 2006 (344):re6
14. Blinov ML et al (2006) A network model of early events in epidermal growth factor receptor signaling that accounts for combinatorial complexity. Biosystems 83(2–3):136–151
15. Ryu H et al (2015) Frequency modulation of ERK activation dynamics rewires cell fate. Mol Syst Biol 11(11):838
16. Santos SD, Verveer PJ, Bastiaens PI (2007) Growth factor-induced MAPK network topology shapes Erk response determining PC-12 cell fate. Nat Cell Biol 9(3):324–330
17. Kholodenko BN, Hancock JF, Kolch W (2010) Signalling ballet in space and time. Nat Rev Mol Cell Biol 11(6):414–426

18. Kholodenko BN (2006) Cell-signalling dynamics in time and space. Nat Rev Mol Cell Biol 7(3):165–176
19. Kholodenko BN (2000) Negative feedback and ultrasensitivity can bring about oscillations in the mitogen-activated protein kinase cascades. Eur J Biochem 267(6):1583–1588
20. Shankaran H et al (2009) Rapid and sustained nuclear-cytoplasmic ERK oscillations induced by epidermal growth factor. Mol Syst Biol 5:332
21. Nakayama K et al (2008) FGF induces oscillations of Hes1 expression and Ras/ERK activation. Curr Biol 18(8):R332–R334
22. Gutenkunst RN et al (2007) Universally sloppy parameter sensitivities in systems biology models. PLoS Comput Biol 3(10):1871–1878
23. Kreutz C, Timmer J (2009) Systems biology: experimental design. FEBS J 276(4):923–942
24. Banga JR, Balsa-Canto E (2008) Parameter estimation and optimal experimental design. Essays Biochem 45:195–209
25. Moles CG, Mendes P, Banga JR (2003) Parameter estimation in biochemical pathways: a comparison of global optimization methods. Genome Res 13(11):2467–2474
26. Borisov N et al (2009) Systems-level interactions between insulin-EGF networks amplify mitogenic signaling. Mol Syst Biol 5:256
27. Aksamitiene E, Hoek JB, Kiyatkin A (2015) Multistrip Western blotting: a tool for comparative quantitative analysis of multiple proteins. Methods Mol Biol 1312:197–226
28. Kiyatkin A et al (2006) Scaffolding protein Grb2-associated binder 1 sustains epidermal growth factor-induced mitogenic and survival signaling by multiple positive feedback loops. J Biol Chem 281(29):19925–19938
29. Aksamitiene E et al (2011) Prolactin-stimulated activation of ERK1/2 mitogen-activated protein kinases is controlled by PI3-kinase/Rac/PAK signaling pathway in breast cancer cells. Cell Signal 23(11):1794–1805
30. Rockstroh M et al (2011) Cell fractionation – an important tool for compartment proteomics. J Integr OMICS 1(1):135–143
31. Holden P, Horton WA (2009) Crude subcellular fractionation of cultured mammalian cell lines. BMC Res Notes 2:243
32. Cozza G et al (2014) Cell-permeable dual inhibitors of protein kinases CK2 and PIM-1: structural features and pharmacological potential. Cell Mol Life Sci 71 (16):3173–3185
33. Sierecki E et al (2010) Discovery of small molecule inhibitors of the PH domain leucine-rich repeat protein phosphatase (PHLPP) by chemical and virtual screening. J Med Chem 53(19):6899–6911
34. Quartararo JS, Wu P, Kritzer JA (2012) Peptide bicycles that inhibit the Grb2 SH2 domain. Chembiochem 13(10):1490–1496
35. Mahadevan D et al (2008) Discovery of a novel class of AKT pleckstrin homology domain inhibitors. Mol Cancer Ther 7(9):2621–2632
36. Agrawal N et al (2003) RNA interference: biology, mechanism, and applications. Microbiol Mol Biol Rev 67(4):657–685
37. Wilson RC, Doudna JA (2013) Molecular mechanisms of RNA interference. Annu Rev Biophys 42:217–239
38. Stenvang J et al (2012) Inhibition of microRNA function by antimiR oligonucleotides. Silence 3(1):1
39. Laufer BI, Singh SM (2015) Strategies for precision modulation of gene expression by epigenome editing: an overview. Epigenetics Chromatin 8:34
40. Prelich G (2012) Gene overexpression: uses, mechanisms, and interpretation. Genetics 190 (3):841–854
41. Wang L et al (2014) New strategy for reversible modulation of protein activity through site-specific conjugation of small molecule and polymer. Bioconjug Chem 25(7):1252–1260
42. Brown GC, Kholodenko BN (1999) Spatial gradients of cellular phospho-proteins. FEBS Lett 457(3):452–454
43. Lodish HF (2000) Molecular cell biology, 4th edn. W.H. Freeman, New York. xxxvi, 1084, G-17, I-36p
44. Gillespie DT (1992) A rigorous derivation of the chemical master equation. Physica A 188 (1–3):404–425
45. Gillespie DT (1976) A general method for numerically simulating the stochastic time evolution of coupled chemical reactions. J Comput Phys 22(4):403–434
46. Michaelis L et al (2011) The original Michaelis constant: translation of the 1913 Michaelis-Menten paper. Biochemistry 50 (39):8264–8269
47. Michaelis L, Menten ML (1913) Die kinetik der invertinwirkung. Biochem Z 49 (333–369):352
48. Briggs GE, Haldane JB (1925) A note on the kinetics of enzyme action. Biochem J 19 (2):338–339

49. Ederer M, Gilles ED (2007) Thermodynamically feasible kinetic models of reaction networks. Biophys J 92(6):1846–1857
50. Swat A et al (2009) Cell density-dependent inhibition of epidermal growth factor receptor signaling by p38alpha mitogen-activated protein kinase via Sprouty2 downregulation. Mol Cell Biol 29(12):3332–3343
51. Curto M et al (2007) Contact-dependent inhibition of EGFR signaling by Nf2/Merlin. J Cell Biol 177(5):893–903
52. Zi Z, Klipp E (2007) Cellular signaling is potentially regulated by cell density in receptor trafficking networks. FEBS Lett 581 (24):4589–4595
53. Zhou P (2004) Determining protein half-lives. Methods Mol Biol 284:67–77