

## Review Article

# Quantitative metabolic fluxes regulated by trans-omic networks

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Cells change their metabolism in response to internal and external conditions by regulating the trans-omic network, which is a global biochemical network with multiple omic layers. Metabolic flux is a direct measure of the activity of a metabolic reaction that provides valuable information for understanding complex trans-omic networks. Over the past decades, techniques to determine metabolic fluxes, including <sup>13</sup>C-metabolic flux analysis (<sup>13</sup>C-MFA), flux balance analysis (FBA), and kinetic modeling, have been developed. Recent studies that acquire quantitative metabolic flux and multi-omic data have greatly advanced the quantitative understanding and prediction of metabolism-centric trans-omic networks. In this review, we present an overview of <sup>13</sup>C-MFA, FBA, and kinetic modeling as the main techniques to determine quantitative metabolic fluxes, and discuss their advantages and disadvantages. We also introduce case studies with the aim of understanding complex metabolism-centric trans-omic networks based on the determination of metabolic fluxes.

## Introduction

Cellular metabolism controls the storage of nutrients, production of energy, and synthesis of cellular component precursors. Cells change their metabolism to maintain metabolic homeostasis, or in response to internal and external conditions by altering the metabolic reaction rates of metabolites [1–3]. Metabolism has been investigated in various research areas such as biochemistry, bioengineering, cellular physiology, and biomedicine.

Metabolic flux, the rate of turnover of metabolites through a metabolic reaction, is a direct measure of the quantitative activity of the reaction, often irrespective of the concentration of that metabolite. Metabolic fluxes provide valuable information on cellular metabolisms, such as the quantitative flow of carbon and energy [4,5], which can be further used for unraveling key regulatory interactions [6–9], understanding mechanisms of diseases [10–12], identifying drug targets [13–15] and designing and optimizing strains for microbial bioproduction [16–19]. Since metabolic fluxes are not physical entities that can be directly measured, researchers may measure a concentration of a metabolite that is associated with a reaction of interest and attempt to capture the metabolic flux or activity involved. However, changes in concentration do not necessarily dictate metabolic flux changes or their direction, because an increase in the concentration of a metabolite can indicate greater activity of reactions consuming the metabolite, according to many reaction kinetics, or decreased activity of the same reactions, according to the mass balance of the metabolite. Therefore, techniques have been developed to determine quantitative metabolic fluxes, including <sup>13</sup>C-metabolic flux analysis (<sup>13</sup>C-MFA), flux balance analysis (FBA), and kinetic modeling [20–22]. These techniques have determined cellular metabolic fluxes for various cells and organisms, such as bacteria, yeast, plants, Chinese hamster ovary

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(CHO) cells, cancer cell lines, and mammalian organs [23–27]. One goal of these techniques is to reveal quantitative metabolic fluxes in a cell for a specific condition, but with different assumptions and parameters to be estimated.

Cellular metabolism is a complex biosystem for study. Cellular metabolism is realized by a global network consisting of many molecules including metabolites, proteins, and transcripts, and the biochemical interactions among these molecules. Global networks that span multiple omic layers are called ‘trans-omic’ networks [28–34]. Comprehensive measurement techniques for each omic layer are now becoming available, such as metabolomics and proteomics by mass spectrometry, and genomics and transcriptomics by next-generation sequencing [35–39]. Multi-omic data can be used for constructing trans-omic networks based on prior knowledge of molecular networks or statistical associations [28].

Metabolic flux through a specific reaction can be influenced by many reaction species including enzymes and metabolites as substrates, products, and cofactors (Figure 1A). Post-translational modification and allosteric enzyme regulation can direct enzyme activities, which in turn influence metabolic flux. Thus, in a trans-omic network, a metabolic reaction with a metabolic flux can be a node in the reaction layer that connects metabolites and enzymes that are associated with the reaction (Figure 1B) [40]. Mathematically, the connections of metabolic fluxes with metabolite and enzyme concentrations are defined as mass balance and reaction kinetic equations. Recent studies that acquire quantitative metabolic flux, metabolomic, and proteomic data have greatly advanced quantitative understanding and prediction of metabolism-centric trans-omic networks.

Here we present an overview of  $^{13}\text{C}$ -MFA, FBA, and kinetic modeling as the main techniques for determining quantitative metabolic fluxes, and discuss their advantages and disadvantages. We also introduce case studies toward an understanding of the complex metabolism-centric trans-omic networks based on metabolic fluxes.

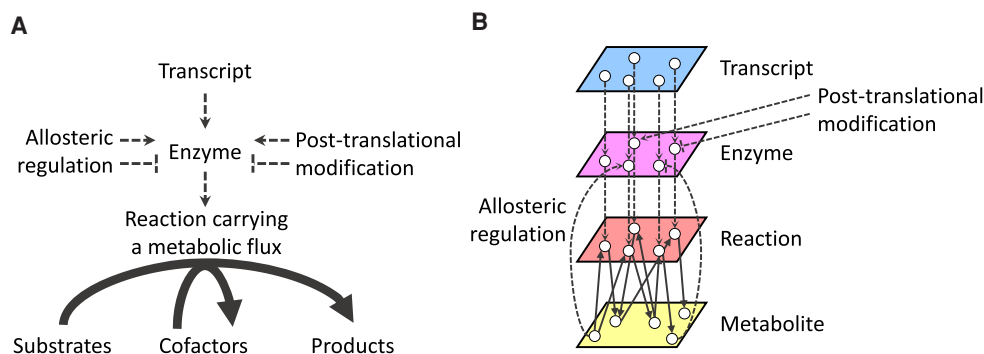
## Techniques to determine metabolic fluxes

### General mathematical formulation for metabolic systems

Prior to introducing techniques to determine metabolic fluxes, we describe the general mathematical formulation of a metabolic system, including metabolites and metabolic fluxes. The dynamic behavior of this system is represented by a set of ordinary differential equations (ODEs) describing the mass balances for the reacting species in the system:

$$\frac{dc}{dt} = S \cdot v(E, c, \theta) \quad (1)$$

$$c(t_0) = c_0,$$



**Figure 1. Complex regulation of metabolic flux.**

(A) A regulatory structure of a metabolic reaction with a metabolic flux. Metabolic flux through a reaction can be influenced by enzymes and metabolites as substrates, products, and cofactors. Post-translational modifications and allosteric regulations of enzymes can also regulate enzyme activities, which in turn influence the metabolic flux. (B) A trans-omic network across the transcript, enzyme, reaction, and metabolite layers. Metabolic flux can be represented as the size of nodes in the reaction layer. Solid lines represent the conversion of metabolites through reactions, while dashed lines represent regulations.

where  $\mathbf{S}$  denotes the stoichiometric matrix, with the element  $S_{ij}$  at row  $i$  and column  $j$  as the stoichiometric coefficients of metabolite  $i$  in reaction  $j$ , and  $\mathbf{v}$  is the vector of metabolic fluxes. The metabolic flux through a reaction is described as a reaction kinetic equation and depends on the metabolite concentrations ( $\mathbf{c}$ ), enzyme concentrations ( $\mathbf{E}$ ), and the kinetic parameters ( $\boldsymbol{\theta}$ ) associated with the reaction;  $\mathbf{c}_0$  is the initial conditions of the metabolite concentrations at the time  $t_0$ . This ordinary differential equation can be applied to metabolic systems with short time scales where the time variations of enzyme concentrations can be negligible.

In the following sections, we present an overview of  $^{13}\text{C}$ -MFA, FBA, and kinetic modeling (Figure 2). All three techniques to determine metabolic fluxes are based on Eq. (1), but with different assumptions and parameters. We also discussed the advantages and disadvantages of each technique (Table 1).

### $^{13}\text{C}$ -metabolic flux analysis ( $^{13}\text{C}$ -MFA)

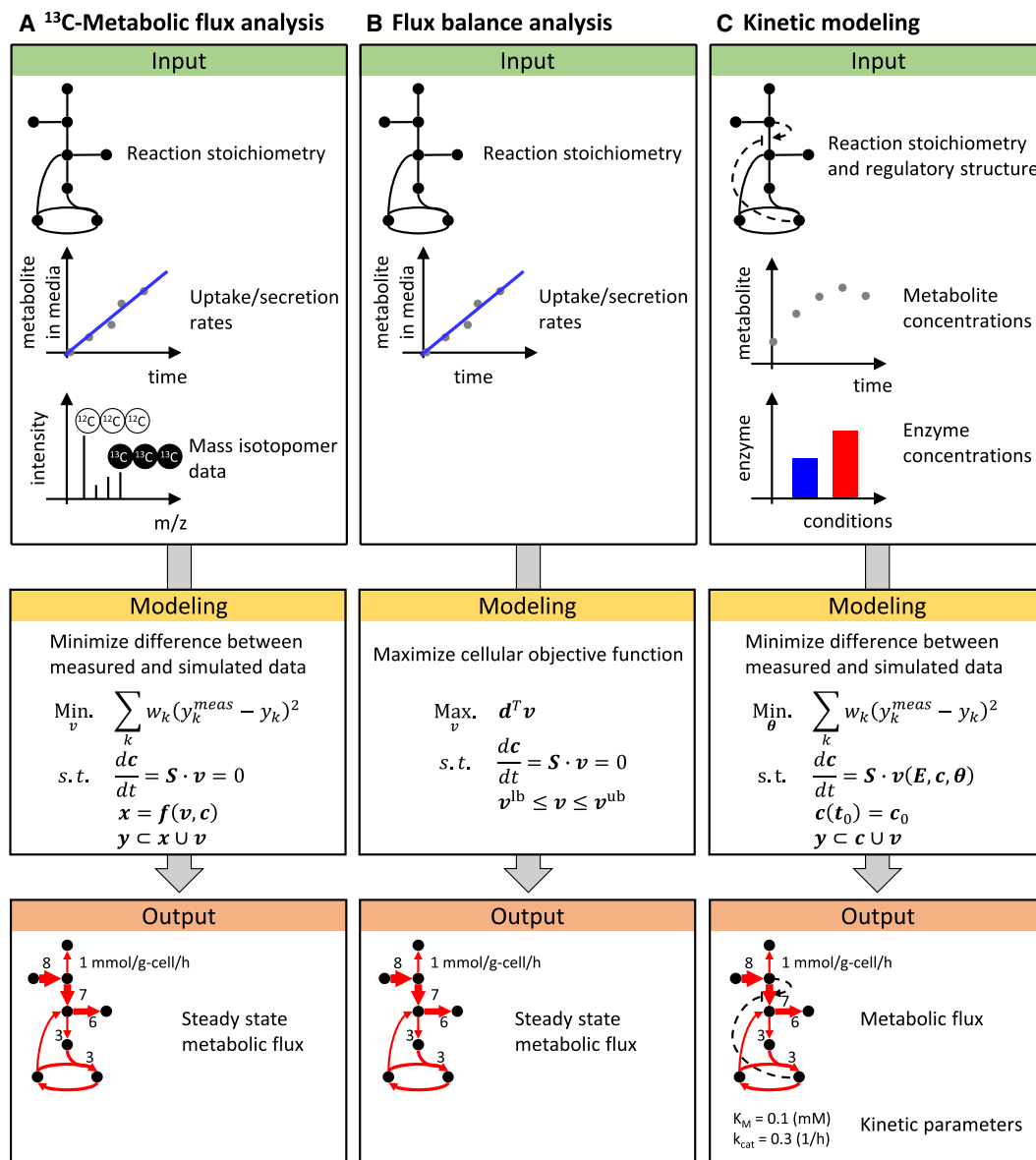
The gold standard to determine cellular metabolic fluxes in microorganisms and mammalian cell lines in a metabolic steady-state has been the  $^{13}\text{C}$ -MFA method [20,41]. With  $^{13}\text{C}$ -MFA, one or more labeled substrates (e.g.  $^{13}\text{C}$ -glucose,  $^{13}\text{C}$ -glutamate) is administered to the cell, causing the labeling of intracellular metabolites and macromolecules (e.g. proteins), and the isotopic labeling patterns of these molecules are then quantified using analytical techniques such as mass spectrometry [42,43], and nuclear magnetic resonance (NMR) [44]. The labeling patterns are highly dependent on metabolic fluxes in cells; therefore, the metabolic fluxes that best explain these patterns can be determined. In addition to isotopic labeling patterns, external fluxes (uptake or secretion rates of metabolites) are also measured for the calculation of metabolic fluxes in microorganisms and mammalian cell lines (Figure 2A).

In fundamental  $^{13}\text{C}$ -MFA, the following least-squares problem is solved for calculating cellular metabolic fluxes:

$$\begin{aligned} \text{Min.}_{\mathbf{v}} \quad & \sum_k w_k (y_k^{\text{meas}} - y_k)^2, \\ \text{s.t.} \quad & \frac{d\mathbf{c}}{dt} = \mathbf{S} \cdot \mathbf{v} = 0, \\ & \mathbf{x} = \mathbf{f}(\mathbf{v}, \mathbf{c}), \\ & \mathbf{y} \subset \mathbf{x} \cup \mathbf{v}, \end{aligned} \quad (2)$$

where  $y_k$  denotes the  $k$ -th labeling pattern or external flux; the superscript ‘meas’ is the measured value; and  $w_k$  denotes the weighting of the  $k$ -th residual, which is often the inverse of the variance of the measured values. Changes in metabolite concentrations over time ( $d\mathbf{x}/dt$ ) are equal to zero because cells are assumed to be in a metabolic steady-state with  $^{13}\text{C}$ -MFA. The vector of functions ( $\mathbf{f}$ ) to calculate labeling patterns from metabolic fluxes and metabolite concentrations is defined based on mass balance equations for isotopomers. The function  $\mathbf{f}$  is usually non-linear [45–49], thus a non-linear optimization method is necessary to solve the least-squares problem. Therefore, the scale of the metabolic network to which most of the  $^{13}\text{C}$ -MFA frameworks can be applied is limited to the central carbon metabolic pathway.

The standard metabolic systems to which  $^{13}\text{C}$ -MFA is applied are cells maintained at metabolic steady-state for a sufficient duration to reach an isotopic steady-state. The  $^{13}\text{C}$ -MFA framework for these metabolic systems is currently routinely applied to quantify metabolic fluxes, especially in the field of metabolic engineering. Isotopic labeling patterns in an isotopic steady-state are independent of metabolite concentrations, and metabolic fluxes can be calculated from measured isotopic labeling patterns and external fluxes. Over the past decades, advanced  $^{13}\text{C}$ -MFA frameworks have been developed to analyze fluxes in isotopic non-steady-state [50,51] or metabolic non-steady-state systems [6,52–54]. In these systems, ODEs for isotopic labeling patterns are not necessarily equal to zero and need to be solved numerically, which requires additional computational costs than those for isotopic steady-state systems. Moreover, metabolic fluxes can change over time in a metabolic non-steady-state system. Although a metabolic flux is inherently a non-linear function of time determined by reaction kinetics based on mechanistic interactions between metabolites and enzymes, a metabolic flux in  $^{13}\text{C}$ -MFA frameworks for metabolic non-steady-state systems is expressed as an approximate function of time, such as a piecewise linear function [6,55,56] or a B-spline function [54,57]. The development of these  $^{13}\text{C}$ -MFA frameworks enabled the determination of metabolic fluxes in photosynthetic microorganisms that consume  $^{13}\text{C}$ -CO<sub>2</sub> as the sole carbon source [58], as well as mammalian cell lines that dynamically respond to hormones [6,54].



**Figure 2. Three main techniques to determine metabolic fluxes.**

(A) <sup>13</sup>C-Metabolic flux analysis (<sup>13</sup>C-MFA). Metabolic fluxes are determined by minimizing the difference between simulated and measured isotopic labeling patterns obtained from <sup>13</sup>C-tracer experiments. (B) Flux balance analysis (FBA). Metabolic fluxes are calculated by maximizing a cellular objective function, such as cellular growth rate. (C) Kinetic modeling, metabolic fluxes, described as reaction kinetic equations, are determined by minimizing the difference between simulated and measured metabolite concentrations and fluxes. All three techniques use the stoichiometry matrix for ODEs describing the mass balances for the reacting species in the system ( $dc/dt = S \cdot v$ ). Formulations of only the basic methods for each technique are shown, and not those of advanced frameworks.

An important characteristic of <sup>13</sup>C-MFA is that metabolic fluxes are determined from mass balance equations of metabolites and their isotopomers, but not from reaction kinetics. Therefore, metabolic fluxes determined by <sup>13</sup>C-MFA are dependent on the reaction stoichiometry of a metabolic network of interest, and are independent of the regulation of enzyme and metabolite. Many unknown or unclear regulations would be involved in cellular metabolisms, even in the well-studied central carbon metabolism in model organisms [8,9,59], compared with those of the reaction stoichiometry [60]. The calculation of metabolic fluxes by

**Table 1 A comparison of techniques to determine metabolic flux**

	<sup>13</sup> C-metabolic flux analysis	Flux balance analysis	Kinetic modeling
Scale of metabolic network	Central carbon metabolism	Genome-scale metabolism	Small scale — central carbon metabolism
Metabolic system	Steady-state	Steady-state	Dynamic
Advantages	<ul style="list-style-type: none"> <li>• Independent of reaction kinetics and regulations</li> <li>• The most standard technique to determine metabolic fluxes</li> </ul>	<ul style="list-style-type: none"> <li>• Independent of reaction kinetics and regulations</li> <li>• Large scale</li> <li>• Low computational cost</li> </ul>	<ul style="list-style-type: none"> <li>• Mechanistic</li> <li>• Dynamic</li> <li>• Detailed prediction</li> </ul>
Disadvantages	<ul style="list-style-type: none"> <li>• High computational cost</li> <li>• Tracer experiments and isotopic measurements required</li> <li>• Unavailable for prediction</li> </ul>	<ul style="list-style-type: none"> <li>• Dependent on biological objective function</li> </ul>	<ul style="list-style-type: none"> <li>• High computational cost</li> <li>• Difficult parameterization</li> <li>• Much experimental data required</li> </ul>

Characteristics of the basic methods for each technique are shown and not that of advanced frameworks.

<sup>13</sup>C-MFA depends on relatively reliable reaction stoichiometries. In addition, on a scale of the central carbon metabolism, it is usually possible to obtain a sufficiently large number of isotopic labeling measurements to determine metabolic fluxes. Ultimately, this large amount of measurement data translates into a high flux precision and enhanced confidence in the accuracy of the calculated metabolic flux [61]. However, the throughput of calculating metabolic fluxes by <sup>13</sup>C-MFA is low compared with that of FBA, because of data availabilities and computational costs. In addition, the scale of metabolic networks for which fluxes can be calculated by <sup>13</sup>C-MFA is limited to the central carbon metabolism. Other disadvantages of <sup>13</sup>C-MFA are the requirements for substantial optimization of tracer experiments (e.g. appropriate isotopic tracers) and the selection of metabolites and isotopomers to be measured, which greatly affect the flux precision.

The mathematical formulation of <sup>13</sup>C-MFA does not include information on the regulation of metabolic flux, such as enzyme concentrations, enzyme post-translational modifications, and allosteric regulations. Therefore, for the integration of metabolic flux with other omic layers (e.g. metabolite and enzyme layers) to construct a metabolism-centric trans-omic network, reaction kinetics should be introduced to explicitly define relationships between metabolic flux and molecules in other omic layers.

## Flux balance analysis (FBA)

The first method for biological predictions using constraint-based modeling of genome-scale metabolic models (GEMs) is FBA [21,24,62]. The mathematical formulation of FBA is based on the hypothesis that the metabolic fluxes in a cell are the result of maximization or minimization of a metabolic objective, subject to the constraints imposed by genotypes and environments (Figure 2B). A common cellular objective to determine steady-state metabolic fluxes in wild-type microorganisms by FBA is the maximization of the cellular growth rate [63,64]. Metabolic fluxes calculated by maximizing the cellular growth rate are consistent with those in wild-type microorganisms determined by <sup>13</sup>C-MFA [65,66].

For FBA, cellular metabolic fluxes in a steady-state can be determined by solving the following linear optimization problem:

$$\begin{aligned}
 & \text{Max. } \mathbf{d}^T \mathbf{v}, \\
 & \text{s.t. } \frac{d\mathbf{c}}{dt} = \mathbf{S} \cdot \mathbf{v} = 0, \\
 & \quad \mathbf{v}^{\text{lb}} \leq \mathbf{v} \leq \mathbf{v}^{\text{ub}}.
 \end{aligned} \tag{3}$$

Column vector  $\mathbf{d}$  of Equation 3 denotes the linear coefficients representing the weight of the fluxes in the objective function. In a GEM, cell growth is expressed as a reaction in which the cellular contents (e.g. protein, RNA, and lipids) and energy (e.g. ATP) are consumed in a specific ratio, and the coefficient corresponding to the metabolic flux through this reaction is 1 when the cellular growth rate is maximized.  $\mathbf{v}^{\text{lb}}$  and  $\mathbf{v}^{\text{ub}}$  denote the lower and upper bounds of metabolic fluxes, respectively, and define reaction reversibility (i.e.  $\mathbf{v}_j^{\text{lb}} = 0$  if

reaction  $j$  is irreversible). These metabolic flux boundaries can be also used for defining nutrient availability, fixing external fluxes to measurement values, and specifying reactions associated with deleted genes (i.e.  $v_j^{\text{lb}} = v_j^{\text{ub}} = 0$ ).

Although FBA only considers metabolism, several advanced modeling frameworks have recently been reported that incorporate protein concentrations (or transcript concentrations) into FBA [67–69]. Advanced FBA frameworks of a GEM with Enzymatic Constraints using Kinetic and Omics data (GECKO) [70] and Metabolic Modeling with ENzyme kinETics (MOMENT) [71] impose constraints on the upper bounds of metabolic fluxes by the product of enzyme concentrations and their activities (i.e. turnover rate). In addition to constraints on metabolic fluxes, detailed descriptions of the protein expression process are incorporated in an integrated model of metabolism and macromolecular expression (ME-Model) [72,73], as well as Expression and Thermodynamics-enabled FLux models (ETFL) [74], and Resource Balance Analysis (RBA) [75,76]. Other advanced FBA frameworks include Gene Inactivity Moderated by Metabolism and Expression (GIMME) [77] and Integrative Network Inference for Tissues (INIT) [78], in which metabolic fluxes are determined by maximizing high-expression reaction use over that of low expression reactions. Integration of protein expression and metabolism in these modeling frameworks enabled the reproduction of the overflow metabolism (e.g. Crabtree effect in yeast) [70] and the construction of tissue-specific GEMs [78], which could not be achieved by simply applying FBA to GEMs.

Since FBA can be formulated as a linear optimization problem, using FBA to calculate metabolic fluxes requires a low computational cost. Therefore, metabolic networks applied to by FBA can include thousands of reactions and even genome-wide metabolic fluxes. Additionally, FBA can provide quantitative flux predictions of microorganisms with various genotypes and environmental conditions. This method has been used in various ways, such as for computational strain design in metabolic engineering [18,79–81], and the prediction of essential genes of pathogenic bacteria and cancer cells [15,82,83]. However, metabolic fluxes calculated by FBA rely on the validity of the hypothesis that the cellular metabolic fluxes in a cell are the result of optimization of a cellular objective function. Although previous reports showed that metabolic fluxes calculated by maximizing the cellular growth rate were consistent with those in wild-type microorganisms determined by  $^{13}\text{C}$ -MFA [65,66], this does not imply the theoretical correctness of solving the system as an optimization problem. The maximization of cellular growth would not be appropriate for especially mutant strains, non-growing cells, or mammalian cells. Therefore, the reliability of the metabolic fluxes calculated by FBA should be carefully considered.

The original FBA is applied to GEMs where Boolean logic is used to describe gene-protein-reaction associations, and metabolic enzymes simply determine active or inactive states of the reactions (e.g. constraining metabolic flux through a reaction associated with deleted genes to zero). Therefore, when we attempt to quantitatively understand the metabolism-centric trans-omic network using the original FBA, introducing new relationships between metabolic flux and molecules in other omic layers through reaction kinetics should be included. On the other hand, advanced modeling frameworks, including GECKO, MOMENT, ME-model, ETFL, and RBA, impose constraints on metabolic flux by enzyme concentrations and their activities, and the quantitative relationship between metabolic flux and molecules in other omic layers is included in the modeling framework itself. Therefore, a model constructed by the advanced modeling frameworks can be regarded as a trans-omics network that considers both metabolic flux and enzymes.

## Kinetic modeling

The kinetic modeling of metabolism attempts to provide a mechanistic description of enzymatic activities regulating metabolic fluxes and mass balances for all reactions and metabolites in the network (Figure 2C) [22,23,84]. Kinetic models explicitly describe metabolic fluxes as a function of metabolite and enzyme concentrations, enabling dynamic and quantitative investigations of metabolomic, fluxomic, and proteomic data.

A basic optimization problem to calculate metabolic fluxes and kinetic parameters in a kinetic model can be described by

$$\begin{aligned} \text{Min.}_{\boldsymbol{\theta}} \quad & \sum_k w_k (y_k^{\text{meas}} - y_k)^2, \\ \text{s.t.} \quad & \frac{d\mathbf{c}}{dt} = \mathbf{S} \cdot \mathbf{v}(\mathbf{E}, \mathbf{c}, \boldsymbol{\theta}), \\ & \mathbf{c}(t_0) = \mathbf{c}_0, \\ & \mathbf{y} \subset \mathbf{c} \cup \mathbf{v}. \end{aligned} \quad (4)$$

Equation 4 considers a parameter estimation to minimize the weighted residual sum of squares between the measured and simulated metabolite concentrations and external fluxes. In some cases, algebraic equations or additional ODEs are incorporated into this basic equation to reflect total concentrations of conserved moieties, dynamics of enzyme concentrations, changes in cellular volume, constraints in thermodynamics, or to fix a part of a parameter or variable. Unlike  $^{13}\text{C}$ -MFA and FBA, the metabolic flux is explicitly described as a reaction kinetic equation of a function of enzyme concentrations  $E$ , metabolite concentrations  $x$ , and parameters  $\theta$ . There are various reaction kinetics used in kinetic modeling [23,85], including mass action, Michaelis–Menten rate law, lin-log kinetics [86], convenience kinetics [87], and modular rate law [88]. The types of parameters required for modeling depend on the reaction kinetics and can include enzymatic reaction turnover rates, equilibrium constants, dissociation constants of substrates, products, and allosteric effectors bound to the enzyme, and elasticity coefficients. Kinetic modeling can be also applied to non-steady-state conditions where metabolite concentrations and metabolic fluxes change over time, and numerical solutions of ODEs are often required.

Reaction kinetic equations are explicitly described in kinetic modeling so that we can simulate dynamic changes in metabolite concentrations and metabolic fluxes in response to perturbations in parameters and variables. Metabolic control analysis (MCA), one of the sensitivity analyses commonly used in metabolic modeling, quantitatively evaluates the sensitivities of metabolite concentrations and metabolic fluxes to small perturbations in parameters and enzyme concentrations [89–91]. Analyses of the Jacobian matrix of ODEs in a kinetic model allow for the appraisal of the dynamic properties of the system such as the stability, oscillations, and bifurcations [92,93]. These analyses have contributed to the identification of rate-limiting metabolic reactions in a metabolic pathway for microbial bioproduction of useful compounds [16], the understanding of regulations that enhance the stability of a metabolic system [92,93], and the evaluation of drug effects on metabolism [13], which cannot be achieved with  $^{13}\text{C}$ -MFA or FBA alone.

The most challenging task for the kinetic modeling of metabolism is the estimation of parameters for enlarged networks [94,95]. Reaction kinetics used for mechanistic kinetic modeling are usually non-linear functions of parameters; thus, the estimation of parameters needs to be solved using a non-linear optimization problem, in which optimization can be readily trapped in a local optimum. Moreover, a metabolic system can be described by multiple model variants, and choosing the superior variant is also challenging [9,96,97]. Different model variants can vary in their reactions, regulations on metabolic fluxes, and kinetic rate laws. A model that is too complex for the data cannot accurately estimate the parameters and the estimated parameters would be sensitive to data errors, while a model that is too simple would not be able to estimate the parameters that reproduce the data well. Therefore, kinetic modeling requires statistical model selection methods [96], such as cross-validation and statistical criteria (e.g. Akaike information criterion (AIC) [98]). In spite of the above difficulties, advanced frameworks of kinetic modeling have been developed, such as Structural Kinetic Modeling (SKM) [92], Optimization and Risk Analysis of Complex Living Entities (ORACLE) [99], Mass Action Stoichiometric Simulation (MASS) framework [100,101], Ensemble Modeling (EM) [102], General Reaction and Assembly Platform (GRASP) [103], and Omics-Based Metabolic Flux Estimation without Labeling for Extended Trans-omic Analysis (OMELET) [12], which offer novel capabilities for kinetic model analysis.

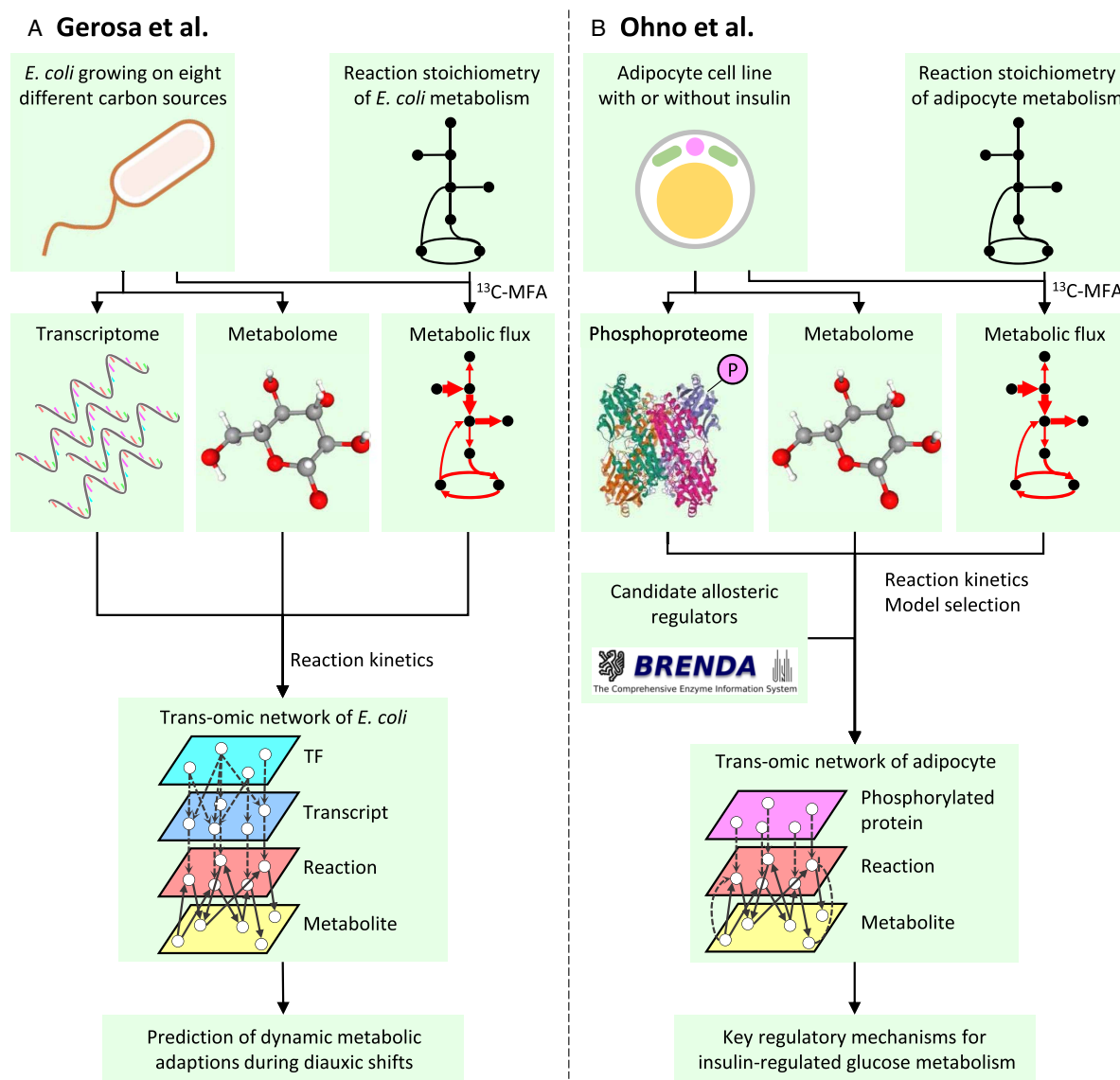
When the enzyme concentration is explicitly included in the reaction kinetics, the kinetic model not only calculates metabolic flux, but also provides mechanistic interactions among enzymes, metabolites, and metabolic fluxes, which differs from  $^{13}\text{C}$ -MFA and FBA. Therefore, such kinetic models themselves can represent a metabolism-centric trans-omics network.

## Case studies that determine metabolic fluxes to understand metabolism-centric trans-omic networks

In the following, we review several case studies that aim to understand metabolism-centric trans-omic networks through the measurement of quantitative metabolic fluxes.

### Approaches based on $^{13}\text{C}$ -MFA to understand metabolism-centric trans-omic networks

Gerosa et al. [104] determined metabolic fluxes in *Escherichia coli* during exponential growth on eight different carbon sources, and inferred regulatory events in metabolisms that drive adaptation of metabolic fluxes between two carbon courses (Figure 3A). Those authors obtained metabolic fluxes by applying  $^{13}\text{C}$ -MFA to a



**Figure 3. Approaches based on  $^{13}\text{C}$ -MFA to understand metabolism-centric trans-omic networks.**

(A) Gerosa et al. [104]. Metabolic fluxes in *E. coli* during exponential growth on eight different carbon sources were determined by  $^{13}\text{C}$ -MFA, and regulatory events in metabolisms that drive adaptation of metabolic fluxes between two carbon courses were inferred. (B) Ohno et al. [6]. Metabolic flux changes over time in cultured adipocytes with or without insulin were determined by advanced  $^{13}\text{C}$ -MFA applicable to non-steady-state conditions, and regulatory events in the metabolism that drive insulin-induced changes in metabolic flux in adipocytes were inferred.

well-studied system of central carbon metabolism in *E. coli*. They also measured transcript amounts and metabolite concentrations using microarrays and targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS), respectively. The determined metabolic fluxes through 34 reactions were described by reaction kinetic equations including the transcript amounts, substrate concentrations, and Gibbs energies as thermodynamic potentials. By transforming the kinetic equations for each reaction, the authors calculated the contributions of transcript, substrate, and thermodynamic regulatory effects to the differences in metabolic fluxes between two carbon sources [105–107]. They also estimated the non-measurable activities of transcription factors (TFs) by network component analysis [108], and calculated the contribution of TFs to the difference in transcript amounts between the carbon sources. These analyses revealed that metabolic fluxes in the TCA cycle are



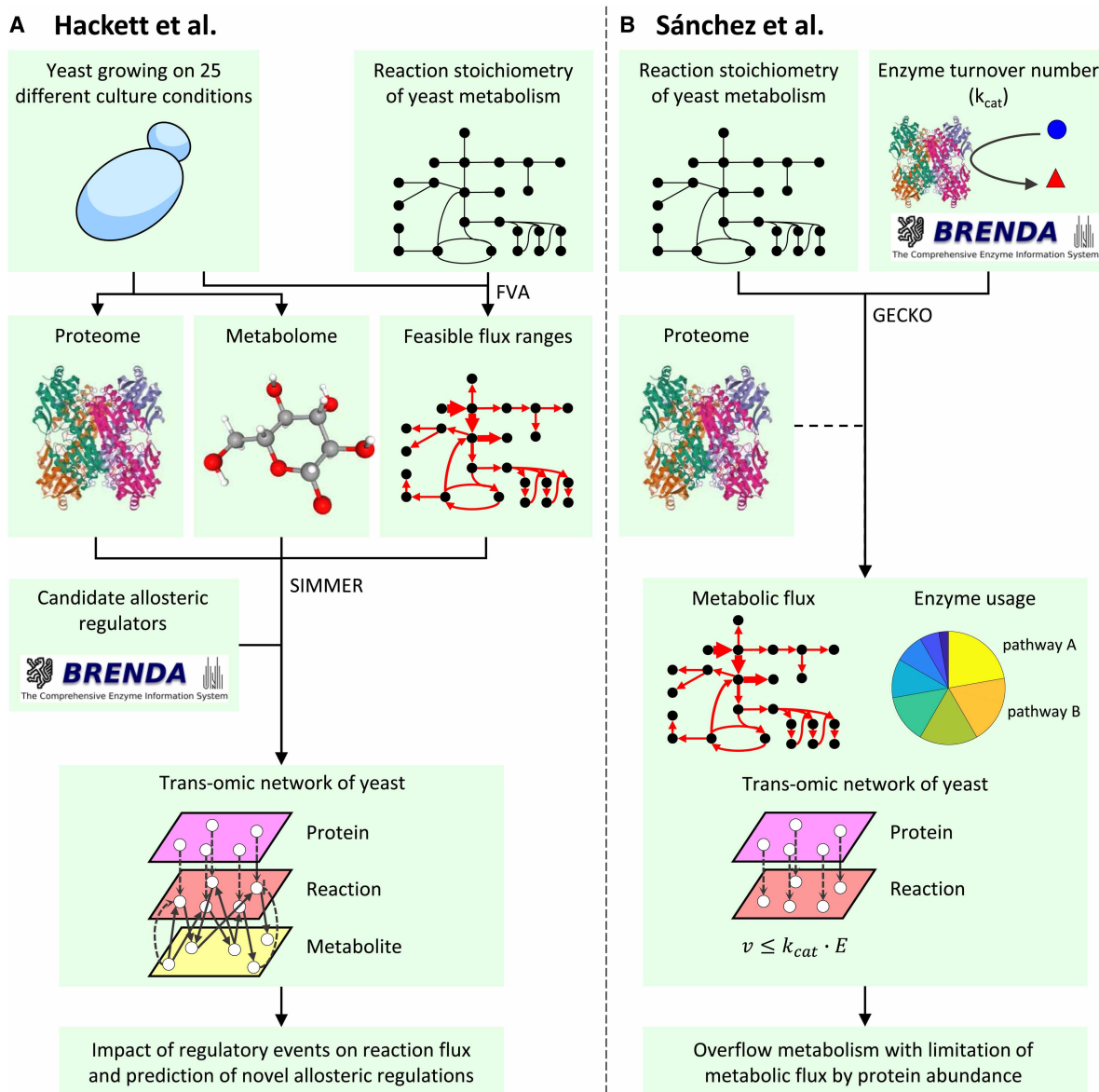
regulated by gene expression, while those in the glycolysis are regulated by substrates. Gerosa et al. [104] predicted that regulatory events with large contributions to metabolic fluxes would drive the dynamic changes in metabolism during adaptation from one carbon source to another, and experimentally verified some of these predictions. Multiple steady-state observations of  $^{13}\text{C}$ -resolved fluxes, metabolites, and transcripts were used to infer which regulatory events induced the metabolic adaptations following environmental transitions, and this was referred to as ‘pseudo-transition analysis.’ Since metabolic fluxes in a scale of the central carbon metabolism and comprehensive transcriptomic and metabolomic data were connected via reaction kinetics, this study can be regarded as integrating the transcript, metabolic flux, and metabolite to reveal differences in trans-omics networks of *E. coli* between nutritional conditions. Metabolic fluxes determined by  $^{13}\text{C}$ -MFA allowed for the mechanistic connection between metabolites and transcripts.

Ohno et al. [6] determined metabolic flux changes over time in cultured adipocytes with or without insulin, and inferred regulatory events in the metabolism that drive insulin-induced changes in adipocyte metabolic flux using these metabolic fluxes as well as metabolomic and phosphoproteomic data [6,109,110] (Figure 3B). The authors refer to this approach as ‘kinetic trans-omic analysis.’ Conventional  $^{13}\text{C}$ -MFA is applicable to a steady-state metabolism; however, the adipocyte glucose metabolism dynamically responds to insulin. Therefore, the authors developed  $^{13}\text{C}$ -MFA under non-steady-state conditions by describing the metabolic fluxes as piecewise linear functions in time [55,56]. These metabolic fluxes were subsequently described as reaction kinetic equations of modular rate law [88], and the contributions of enzyme phosphorylation, allosteric effectors, substrates and products, and other unaccounted regulators to insulin-induced changes in metabolic fluxes were calculated. The authors assumed that changes in metabolic enzymes were almost constant and did not contribute greatly to insulin-induced flux changes, because this study focused on relatively short time scales of within 60 min after insulin treatment. In addition, measured enzyme phosphorylation and literature-based allosteric regulation do not necessarily affect insulin-induced flux changes in adipocytes; thus, the authors used AIC to select plausible enzyme phosphorylation and allosteric regulation to be included in the reaction kinetic equations. This kinetic trans-omic analysis revealed that most reactions in the glucose metabolism, in which metabolic fluxes largely change as a result of insulin, are regulated by substrates and products, whereas a few reactions, including the glucose transporter and ATP-citrate lyase in the fatty-acid synthesis pathway, are regulated by enzyme phosphorylation or allosteric effectors. Although the approach proposed by Ohno et al. [6] is similar to that of Gerosa et al. [104], the former approach can be applied to dynamically changing metabolic systems in response to external stimuli. Moreover, that approach handles uncertainty in the calculated metabolic fluxes and kinetic parameters due to experimental and estimation errors.

Both studies describe the metabolic fluxes determined by  $^{13}\text{C}$ -MFA as reaction kinetic equations, which are helpful for understanding trans-omics networks. Once precise metabolic fluxes are determined with  $^{13}\text{C}$ -MFA, describing metabolic fluxes as reaction kinetic equations can be done independently for each reaction. Therefore, lower computational costs are required for describing metabolic fluxes as reaction kinetic equations, including those related to the calculation of kinetic parameters, selection of plausible enzyme phosphorylation and allosteric regulations, and calculation of the contribution of regulatory events to flux differences between conditions, compared with the kinetic modeling in which reaction kinetic equations for all reaction are simultaneously estimated directly from multi-omic data. However, the availability of data and high computational cost for  $^{13}\text{C}$ -MFA limit the scale of metabolic networks for which fluxes can be determined, and the throughput of determining fluxes is relatively low compared with that of FBA. In addition,  $^{13}\text{C}$ -MFA requires tracer experiments using isotopes, which often require different experimental conditions than other omics measurements. Moreover, the calculated kinetic parameters are independent on the stability of the metabolic system, and detailed and quantitative prediction of metabolite concentrations and metabolic fluxes in a different genetic or experimental condition could be challenging.

## Approaches based on FBA to understand metabolism-centric trans-omic networks

Hackett et al. [8] calculated feasible ranges of metabolic fluxes in yeast during exponential growth in 15 different cultivation conditions and identified physiologically relevant regulations supporting the metabolic reactions in yeast (Figure 4A). The authors referred to their method as systematic identification of meaningful metabolic enzyme regulation (SIMMER). Feasible ranges for genome-wide metabolic fluxes were calculated using flux variability analysis (FVA) [111], a method derived from FBA under constraints of the weighted residual sum of



**Figure 4. Approaches based on FBA to understand metabolism-centric trans-omic networks.**

(A) Hackett et al. [8]. Feasible ranges of metabolic fluxes in yeast during exponential growth in 15 different cultivation conditions were calculated by FVA, a method derived from FBA, and physiologically relevant regulations supporting metabolic reactions in yeast were identified. (B) Sánchez et al. [70]. The authors developed GECKO, a method that enhances a GEM to account for enzymes so that each metabolic flux ( $v$ ) could not exceed its maximum capacity, equal to the product of the enzyme concentration ( $E$ ) and the turnover number ( $k_{cat}$ ). GECKO was applied to a yeast GEM and found overflow metabolism with limitation of metabolic flux by protein abundance.

squares (RSS) between the measured and calculated uptake or secretion rates of metabolites added with the L1 norm of all metabolic fluxes below a certain value, as well as the constraint of the steady-state of the metabolic system. Those authors identified physiologically relevant regulations supporting reactions using a Bayesian approach with calculated metabolic flux ranges and measured metabolite and enzyme concentrations, and by statistical variable selection using the Akaike information criterion with a correction for finite sample sizes (AICc) [112,113]. Out of 46 reactions for which kinetic equations of convenience rate law [87] were described using the metabolic flux ranges and measured enzyme and metabolite concentrations, 17 did not include any

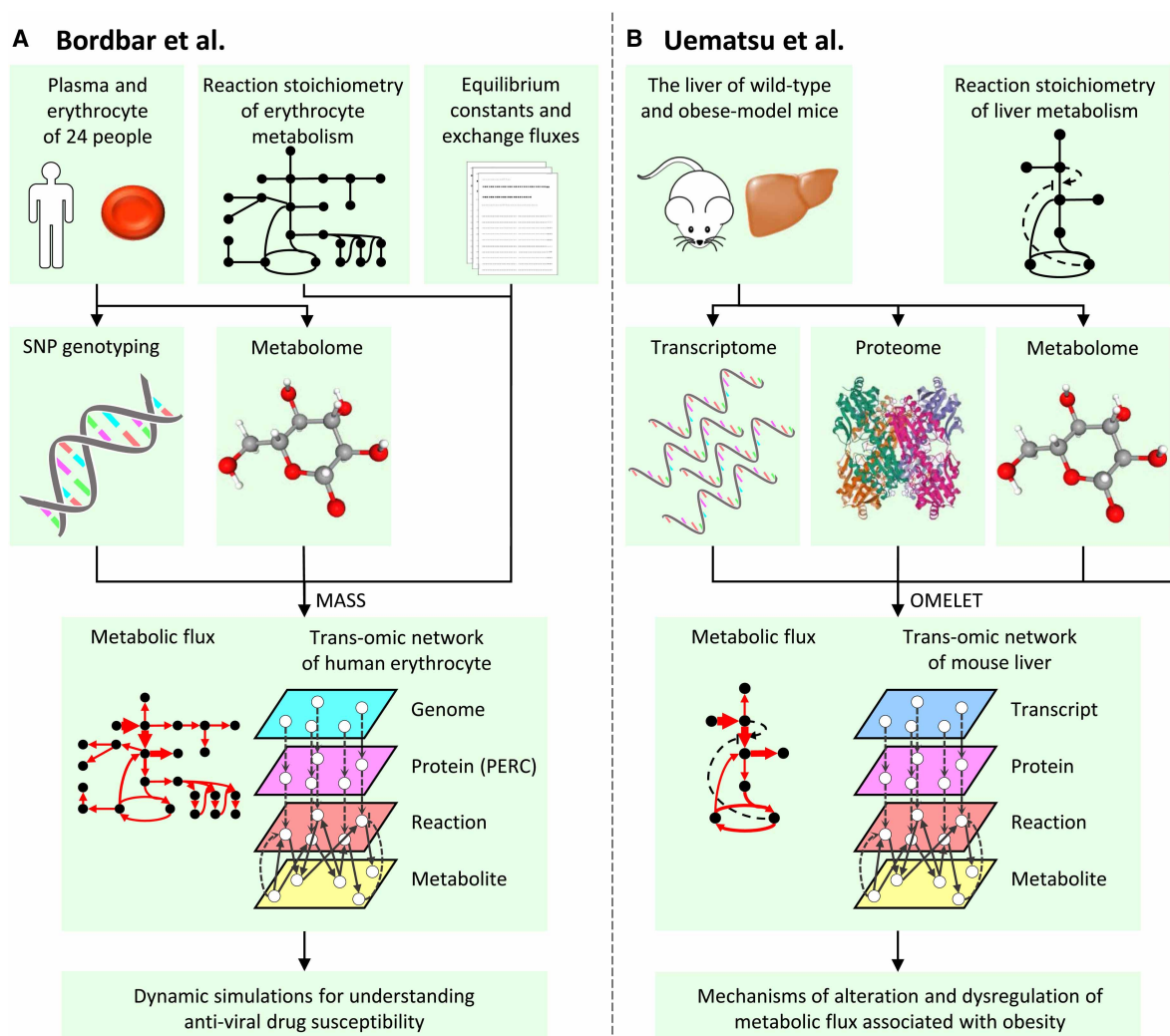
regulation and 29 were regulated by one to two allosteric regulators. Three previously unrecognized regulatory interactions, such as the allosteric inhibition of pyruvate kinase by citrate, were validated biochemically. In addition to revealing physiologically relevant regulations, the authors assessed the impact of physiological variation in enzyme and metabolite concentrations on metabolic flux, by partitioning the total variability in flux across conditions into the contributions of individual regulators. They found that substrates are the most important determinant of fluxes in general, with enzymes and allosteric regulators having a comparably important role in the case of physiologically irreversible reactions. Physiologically relevant interactions of metabolic enzymes and metabolites with genome-wide metabolic fluxes can be identified with SIMMER via reaction kinetics, and this study can be regarded as revealing a trans-omic network in yeast that integrates enzymes, reactions, and metabolites under different yeast culture conditions. Metabolic flux ranges calculated by an FBA-derived method cover genome-wide metabolic reactions, and the resulting trans-omic network is not limited to the central carbon metabolism, although this is dependent on the coverage of metabolite and protein measurements.

Sánchez et al. [70] developed GECKO, a method that enhances a GEM to account for enzymes, so that each metabolic flux cannot exceed its maximum capacity, which is equal to the product of the enzyme concentration and the turnover number (Figure 4B). GECKO is an extension of FBA and can both simulate protein concentrations and incorporate measured protein concentrations as inputs. The authors applied GECKO to a yeast GEM, and the resulting model consisted of 6741 reactions, 3388 metabolites, and 764 enzymes. Using the GECKO model, the authors calculated feasible ranges of metabolic fluxes by FVA, with the objective function value close to the optimal, and demonstrated that GECKO correctly describes an overflow metabolism (or Crabtree effect) in which fermentative metabolism with the production of ethanol occurs under aerobic conditions. They found that this overflow metabolism is caused by the replacement of enzymes in the oxidative phosphorylation pathway with glycolytic enzymes when yeast cells grow above a critical specific growth rate. The authors also showed phenotypes such as yeast coping with temperature stress and overexpressing a specific pathway, which was consistent with experiments. GECKO models represent a kind of trans-omic network consisting of enzyme and reaction layers, in that GECKO uses enzyme concentrations to limit the upper bounds of metabolic fluxes. When the metabolic flux through a reaction reaches its upper bound in a GECKO model, the enzyme concentration is the main regulator of the metabolic flux rather than the substrate and product metabolites. The authors suggested that GECKO could be used for metabolic engineering, and in a later study by another group, the key enzyme that regulates lysine production flux in *E. coli* was predicted by GECKO and experimentally verified [114]. Although GECKO may only be effective for species rich in known enzyme turnover numbers, it is an approach that successfully incorporates concentrations of metabolic enzymes into the FBA framework. The GECKO modeling is described as a linear optimization problem that can be solved with a low computational cost.

Both studies used an FBA-based approach for calculating metabolic fluxes. Unlike <sup>13</sup>C-MFA, metabolic fluxes and their regulations can be discussed for entire metabolic pathways, including amino acid and nucleotide metabolisms, as well as the central carbon metabolism. One difference between the approaches is that Hackett et al. [8] first calculated metabolic flux ranges and then used reaction kinetics to integrate metabolic enzymes, metabolites, and metabolic fluxes, while Sánchez et al. [70] incorporated enzyme concentrations to limit the upper bounds of metabolic flux to calculate metabolic fluxes, and regulations by metabolites are not explicitly described. The metabolic fluxes in both studies were not uniquely determined, due to insufficient data, and the authors investigated their feasible ranges. In addition, various constraints and objective functions are used in frameworks derived from FBA, and we should carefully consider whether assumptions adopted in an FBA-derived framework are appropriate for understanding the specific trans-omic network of interest.

## Approaches based on kinetic modeling to understand metabolism-centric trans-omic networks

Bordbar et al. [13] constructed personalized whole-cell kinetic models of erythrocyte metabolism for 24 healthy individuals based on metabolomic data from plasma and erythrocytes, and exchange fluxes and equilibrium constants from experimental literature (Figure 5A). They used MASS [100,101] for the construction of the kinetic models. Briefly, starting from a linear approximation of ODEs of metabolite concentrations around a reference state, the Jacobian of the ODEs can be decomposed into the stoichiometric matrix of the metabolic network, the diagonal matrix including phenomenological kinetic constants (pseudo-elementary rate constants, PERCs), and the gradient matrix that incorporates the metabolite concentrations and equilibrium constants.



**Figure 5. Approaches based on kinetic modeling to understand metabolism-centric trans-omic networks.**

(A) Bordbar et al. [13]. Personalized whole-cell kinetic models of erythrocyte metabolism for 24 healthy individuals were constructed using MASS, an advanced kinetic modeling framework, and dynamic simulations for understanding anti-viral drug susceptibility were performed. (B) Uematsu et al. [12]. The authors developed OMELET, which estimates metabolic fluxes based on the reaction kinetics and Bayesian theory from metabolite, enzyme, and transcript amounts. By applying OMELET to the glucose metabolism in the liver of fasted mice, they revealed the difference in metabolic fluxes between wild-type mice and obese-model *ob/ob* mice, and identified regulatory events that mainly contribute to the difference in metabolic flux.

The authors built a baseline model using the average metabolite concentrations of the human plasma and erythrocytes, constructed an ensemble of personalized kinetic models for each individual by substituting their measured metabolite concentrations for the baseline levels, and calculating a personalized set of phenomenological kinetic constants by resolving the equations. The PERC is a bulk approximation of traditional enzymatic parameters (e.g.  $K_M$ ,  $k_{cat}$ , and enzyme concentrations) and depends on the DNA sequence. Thus, the authors assessed the dependences of calculated PERCs in personalized models on genetic variants occurring in the gene region of the metabolic genes (exon, intron, and promoter regions). They found statistical enrichment for all genetic variant types (non-coding, synonymous exon, non-synonymous exon), suggesting that the PERC, in part, depends on the genotype. In addition, temporal decomposition of the models elucidated that the first timescale ( $\sim$ millisecond) is the coordination of fast reactions near equilibrium, such as isomerases, and the second time scale, where most individual variation was detected, is the coordination of fluxes into parts of key erythrocyte pathways, including lower glycolysis, the oxidative pentose phosphate pathway, and glutathione

recycling. Finally dynamic simulations of the personalized models generated novel predictions about susceptibility to hemolytic anemia induced by ribavirin, an anti-viral drug used against the hepatitis C virus. MASS is an approach to construct a kinetic model in which metabolic fluxes and metabolites are connected by reaction kinetic equations with PERCs depending on genetic variants. Therefore, MASS can be used to construct a trans-omic network consisting of genome, protein (PERC), reaction, and metabolite layers. The linear approximation of ODEs around a reference state enables the construction of large-scale personalized kinetic models. However, such simplified linear reaction kinetics would lead to inconsistency with mechanistic non-linear reaction kinetics when the metabolic state is distant from the reference state [23,85,86], and accurate predictions of the system behavior against relatively large metabolic perturbations could not be achieved by this approach.

Uematsu et al. [12] developed OMELET, which estimates metabolic fluxes based on the reaction kinetics and Bayesian theory from metabolite, enzyme, and transcript amounts (Figure 5B). By applying OMELET to the glucose metabolism in the livers of fasted mice, those authors revealed the difference in metabolic fluxes between wild-type mice and obese-model *ob/ob* mice, and identified the main regulatory events that contribute to the difference in metabolic flux. OMELET estimates the posterior probability of parameters including metabolic fluxes using prior probabilities updated with the likelihoods of measured enzyme and transcript amounts. The prior probability of metabolic fluxes is assumed to follow a multivariate normal distribution under a pseudo-steady-state condition. Estimated enzyme and transcript amounts for the likelihoods are calculated based on lin-log reaction kinetics [86] and enzyme turnover, respectively. Once metabolic fluxes are estimated using OMELET, the contribution of regulators, such as enzymes, substrates, products, and allosteric effectors, to differences in metabolic fluxes between conditions can be readily calculated based on the propagation of uncertainty of the regulator amounts to metabolic flux. The authors integrated measured multi-omic data, estimated metabolic fluxes, and calculated contributions to construct a quantitative trans-omic network. The nodes in the trans-omic network are molecules of transcripts, proteins, reactions, and metabolites, and the edges are regulations. The node sizes represent differences in amounts of molecules or metabolic fluxes between conditions, while the edge sizes represent the contributions of regulations. The constructed trans-omic network showed that increased metabolic flux through gluconeogenesis by obesity resulted primarily from increased transcripts, whereas increased metabolic flux through the pyruvate cycle resulted from both increased transcripts and changes in substrates of metabolic enzymes. Although OMELET is a kind of kinetic modeling, it can quantitatively integrate several omic layers based on reaction kinetic equations. Such multi-omic integration into one mathematical framework is still challenging. Additionally, OMELET can handle missing values in omics data, which often occurs due to the low amounts or physical properties of molecules to be measured. Kinetic modeling including such molecules increases the uncertainty of the parameters. In contrast, OMELET does not require the description of reaction kinetics for all the reactions in the metabolic network of interest, and only a subset of reactions is required to be described. This reduces the uncertainty of the parameters and the error of the estimated metabolic fluxes, as well as the contribution of regulations, although this prevents OMELET from predicting the change in metabolite amounts and metabolic fluxes from the perturbation. Furthermore, based on Bayesian theory, OMELET can provide the uncertainty in the calculated metabolic fluxes and the contribution of regulations depending on experimental errors.

Both studies constructed kinetic models in which kinetic parameters were calculated to reproduce the measured omics data well. Interactions among enzymes, metabolic fluxes, and metabolites are explicitly described as reaction kinetic equations in kinetic models, thus kinetic modeling itself can be regarded as an approach to construct a trans-omic network. An ideal kinetic model of a trans-omic network should be able to represent many data and phenotypes under various genetic and environmental conditions, and predict novel regulatory interactions and metabolic states; however, construction of such an ideal kinetic model is generally difficult, especially due to the uncertainty of the regulatory interactions and kinetic parameters. Various kinetic modeling frameworks, including MASS by Bordbar et al. [13] and OMELET by Uematsu et al. [12], resolve some of the challenges, but also lose some of the advantages of kinetic models [23]. Therefore, we should carefully consider what insights we aim to attain from the trans-omics network using kinetic modeling, and select an appropriate kinetic modeling framework based on those goals.

## Conclusion

We reviewed three techniques to determine quantitative metabolic fluxes, <sup>13</sup>C-MFA, FBA, and kinetic modeling, and introduced several studies which aimed at understanding metabolism-centric trans-omic networks. As

mentioned, all three techniques and all approaches for understanding metabolism-centric trans-omic networks have advantages and disadvantages. Therefore, we should carefully consider which techniques would be appropriate for the metabolic system of interest and what insights we aim to obtain from the trans-omics network.

The advancement of omics measurement technologies, modeling frameworks, and computational instruments enables us to determine metabolic fluxes more comprehensively, precisely, and rapidly, in larger scale metabolic networks, which in turn will facilitate an understanding of metabolism-centric trans-omic networks and a prediction of their responses to arbitrary perturbations. Such advances will make the determination of metabolic fluxes essential and standard in the study of metabolism-centric trans-omic network for many fields, including microbial engineering, metabolic physiology, and systems medicine.

### Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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### Abbreviations

<sup>13</sup>C-MFA, <sup>13</sup>C-metabolic flux analysis; AIC, Akaike information criterion; AICc, Akaike information criterion with a correction for finite sample sizes; CHO, Chinese hamster ovary; EM, Ensemble Modeling; ETFL, Expression and Thermodynamics-enabled FLux models; FBA, flux balance analysis; FVA, flux variability analysis; GECKO, GEM with Enzymatic Constraints using Kinetic and Omics data; GEM, genome-scale metabolic model; GIMME, Gene Inactivity Moderated by Metabolism and Expression; GRASP, General Reaction and Assembly Platform; INIT, Integrative Network Inference for Tissues; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MASS, Mass Action Stoichiometric Simulation; MCA, metabolic control analysis; ME-Model, integrated model of metabolism and macromolecular expression; MOMENT, MetabOlic Modeling with ENzyme kineTics; NMR, nuclear magnetic resonance; ODE, ordinary differential equation; OMELET, Omics-Based Metabolic Flux Estimation without Labeling for Extended Trans-omic Analysis; ORACLE, Optimization and Risk Analysis of Complex Living Entities; PERC, pseudo-elementary rate constants; RBA, resource balance analysis; RSS, residual sum of squares; SIMMER, systematic identification of meaningful metabolic enzyme regulation; SKM, structural kinetic modeling; SNP, single nucleotide polymorphism; TF, transcription factor.

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