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Regulation and control of metabolic fluxes in microbes

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After about ten years of research renaissance in metabolism, the present challenge is to understand how metabolic fluxes are controlled by a complex interplay of overlapping regulatory mechanisms. Reconstruction of various regulatory network topologies is steaming, illustrating that we underestimated the broad importance of post-translational modifications such as enzyme phosphorylation or acetylation for microbial metabolism. With the growing topological knowledge, the functional relevance of these regulatory events becomes an even more pressing need. A major knowledge gap resides in the regulatory network of protein–metabolite interactions, simply because we lacked pertinent methods for systematic analyses – but a start has now been made. Perhaps most dramatic was the conceptual shift in our perception of metabolism from an engine of cellular operation to a generator of input and feedback signals for regulatory circuits that govern many important decisions on cell proliferation, differentiation, death, and naturally metabolism.

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Introduction

Compared to other biological networks, function and topology of metabolism are unusually well understood. Much of the contemporary focus is on the higher level cellular phenomenon of how small molecule fluxes emerge from the interaction between thousands of enzymes and metabolites in this network. Largely driven by advanced experimental [1] and computational methods [2] to estimate not only intracellular fluxes but also metabolite concentrations [3], novel biological insights are generated for microbes [4] and higher organisms [5]. In particular microbial metabolism became a paradigm network for systems biology [6]. In our view, the presently greatest challenge resides in unraveling, in a quantitative sense, the interplay between the various regulatory mechanisms that control metabolic fluxes.

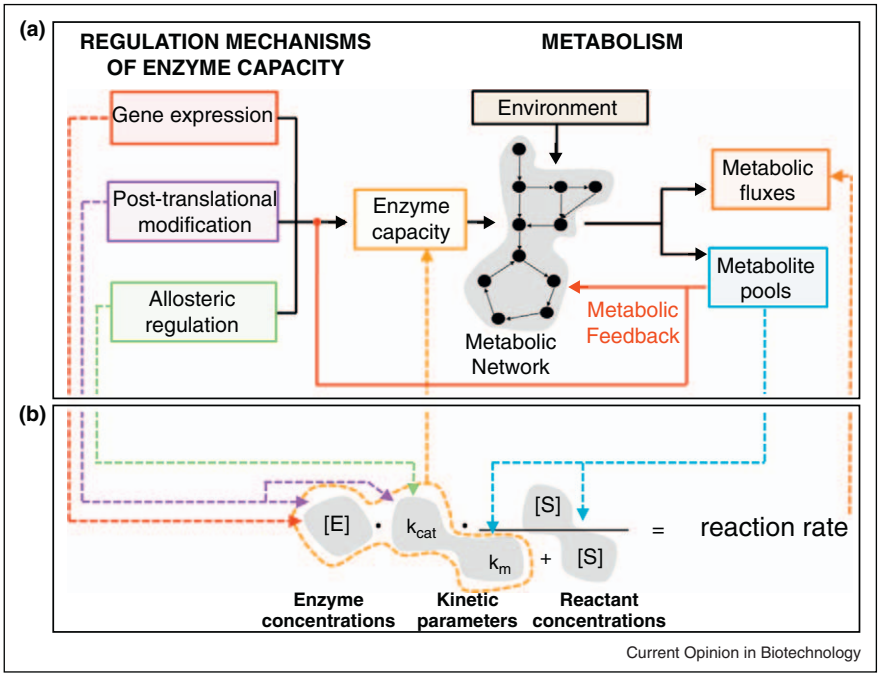
The key problem in understanding how metabolic fluxes are modulated is the complexity arising from multiple overlapping regulatory mechanisms and metabolic feedback into regulatory networks (Figure 1). The actual *in vivo* capacity of an enzyme to realize a catalytic flux is a function of its abundance and kinetic properties. Of the three layers of cellular regulatory mechanisms, *gene expression* acts only on enzyme abundance, *post-translational modification* may modulate abundance and kinetic parameters (e.g. by changing the degradation rate or protein phosphorylation, respectively), while *allosteric regulation* modulates exclusively the kinetic parameters through non-covalent binding of effector molecules to non-catalytic sites. The actual *in vivo* reaction rate (i.e. metabolic flux), however, depends also on the *in vivo reactant concentrations* (Figure 1b). Since these metabolite concentrations are a function of thermodynamics and reaction kinetics, the cell may modulate them only indirectly. Further complexity is caused by the reactant-mediated link to neighboring reactions (i.e. through altered reactant concentrations or competitive inhibition) and through feedback regulation into all three layers of regulatory mechanisms. While current large-scale efforts focus mostly on mapping the interactions between regulators and their targets within a given mechanistic class, for example, transcription [7] or phosphorylation networks [8], understanding metabolic regulation as a whole eventually requires the entire set of regulatory mechanisms to be considered.

Here we review progress since 2009 on metabolism-relevant regulatory networks in microbes, with a focus on publications that provide biological insights rather than new methods. For this purpose, we grouped papers into four categories: i) reconstructing regulatory network topology, ii) unraveling input–output regulatory logics and metabolic feedback, iii) assessing and quantifying the control exerted by regulation events, and iv) understanding the evolution of metabolic regulatory circuits.

Reconstructing the topology of metabolic regulatory networks

Topological knowledge of interactions between regulators and targets, and among regulators, is a prerequisite to understand regulation strategies. Driven by mature experimental methods to quantify gene expression and to map physical interactions between transcription factors and genes (i.e. chip-ChIP microarrays), reconstruction of transcriptional network topologies receives much attention [6].

Figure 1



A systems biology view of metabolism and its regulation. **(a)** Metabolic fluxes and metabolite pools emerge in the metabolic network from the complex interplay between cellular regulation of enzyme capacities, the environment, and metabolite pools themselves. Metabolite pools also provide a feedback mechanism from within metabolism to all modes of enzyme regulation. (b) Metabolic fluxes are net sums of underlying enzymatic reaction rates. Enzymatic reaction rates are themselves integral output of three biological quantities interacting at the level of enzyme kinetics: enzyme concentrations, kinetic parameters, and reactant concentrations, here exemplified by the classical Michaelis–Menten rate law. Enzyme concentrations ([*E*]) can be regulated by gene expression and post-translational modification; kinetic parameters such as turnover (k_{cat}) and substrate affinity (k_m) can be modulated by post-translational modification, allosteric regulation, and competitive inhibition; reactant concentrations ([*S*]) are metabolite pools that emerge from the overall metabolic system.

Curated databases are available for microbial model organisms, some with explicit links to metabolism [9]. Although transcriptional networks are presently the most advanced regulatory network reconstructions, recent investigations of the key *Escherichia coli* transcription factors of central and amino acid metabolism, *Cra* and *Lrp*, revealed 144 and 121 previously not annotated binding sites [10,11]. This 7-fold increase relative to previous knowledge suggests there are still wide gaps in the network topology. Computational analyses of the *E. coli* transcription topology highlight a hierarchical organization of general and specific transcription factors in which the diverse catabolic, anabolic, and central metabolic pathways are targeted by distinct combinations of regulators [12,13]. Notably, reconstruction of transcription networks increasingly embraces the complexity of transcription unit architectures by including regulatory events beyond transcription factors such as alternative sigma factors [7,14], anti-sense transcription [14], and transcriptional attenuation [15]. In contrast to transcription, translational regulation is mostly studied on a case-by-case basis. A recent example draws attention to the role of the small RNA *fnrS* in targeting mRNA of enzymes involved in anaerobic metabolism, acting in cooperation with the transcription factor Fnr [16].

On a different layer of regulation, much progress has been made in reconstructing networks of post-translational modification, primarily by advanced mass spectrometry-based proteomics. The most relevant protein modification is arguably reversible phosphorylation, where phosphoproteome complexity reflects evolutionary progress from few hundred phosphorylated proteins in bacteria (around 5% of all cellular proteins) [17] to thousands in eukaryotes [18]. Frequent phosphorylation of bacterial enzymes suggests a particular relevance for metabolic regulation [17,19,20]. The current focal point for comprehensive mapping of phosphorylation networks is the eukaryotic model organism *Saccharomyces cerevisiae*. An early study with a metabolic focus led to the identification of 80 phosphorylation sites in 48 different mitochondrial proteins, mostly enzymes [21]. Mass spectrometry phosphoproteomics from large-scale genetic perturbations brought a major advance by identifying 8814 regulated phosphorylation events in 124 kinase/phosphatase deletion mutants of yeast [8]. Functional interactions between these kinases and regulatory proteins were mapped by epistatic miniarray profiles from growth rates in pair-wise deletion combinations, revealing rather frequent occurrence in the same complex and/or function in

the same pathway [22]. Typically, however, there is only a partial functional overlap between these interacting pairs of regulators, as was demonstrated by transcriptomics data from 150 single and 20 double kinase/phosphatase mutants [23]. An amazing 1844 of such physical interactions between protein and metabolite kinases with regulatory subunits and phosphatases were identified by mass spectrometry [24•]. These data render the yeast phosphorylation network the currently most comprehensive – nearly 60% of the about 6000 genes encode phosphoproteins [25]. Many metabolism-related phosphorylation events were unraveled, but in particular the Breitkreutz *et al.* [24•] interaction study identified several new effector kinases with relevance in nitrogen and carbon metabolism. In sharp contrast to this great progress in mapping network topology, function of specific phosphorylation events has rarely been demonstrated and quantified, leaving a major challenge for the near future.

Beyond phosphorylation, further post-translational modifications of lysine residues in metabolic enzymes were recently demonstrated in microbes; that is, succinylation [26], acetylation [27,28••], and conjugation with the degradation-regulating, ubiquitin-like protein PUP [29,30]. While succinylation was only described for three *E. coli* enzymes, about 50% of the 191 acetylated *Salmonella* proteins were metabolic enzymes. Functionally, acetylation was shown to control the direction of glycolytic versus gluconeogenic flux and the branching between tricarboxylic acid (TCA) cycle and glyoxylate bypass [28••]. Although functional evidence is lacking, succinylation of the glycolytic glyceraldehyde-3-P dehydrogenase and the TCA cycle isocitrate dehydrogenase in *E. coli* suggests a somewhat similar role [26]. Similarly, the pupylation network of *Mycobacteria* was systematically reconstructed by proteomics, revealing that about half of the 41 identified pupylated proteins were central metabolic or respiratory enzymes [30]. While pupylation has so far been described in only few species and only few succinylation events are known, acetylation represents a more general metabolic regulation mechanism conserved from bacteria [28••] to mammals [27]. At present it remains unclear, however, how specificity in the response to different nutritional conditions can be achieved when only a single pair of lysine acetyltransferase and deacetylase exists like in *Salmonella* [28••].

Finally, reconstruction efforts have begun also to investigate the topology of protein–metabolite interactions. In the absence of a dedicated methodology in the past, we had to rely on serendipitous discovery of allosteric regulation of enzymes or regulatory proteins, although such allosteric interactions are major determinants of dynamic feedback [6,31,32•]. Two recent lines of work demonstrate approaches to systematically identify protein–metabolite interactions, one by starting from lipid metabolites to screen for *in vitro* binding proteins [33,34] and the other starting from affinity-purified proteins to identify the *in vivo* bound metabolites by mass spectrometry [35•]. In particular the *in vivo* analysis revealed an unexpected wealth of binding partners in ergosterol biosynthesis in yeast. Presently, these methods are limited to non-polar metabolites owing to the problem of loosing polar ligands during washing. A more indirect alternative is statistical inference of gene–metabolite interactions through Bayesian integration (calculating probabilities that a hypothesis is true) from coordinated changes in concentration of transcript and metabolite data [36]. Since these interactions encompass direct and indirect relationships, they provide testable hypotheses on regulatory protein–metabolite interactions. Although protein–metabolite interactions are lagging far behind all areas of topology mapping, it is crucial in particular for a better understanding feedback regulation and a start has been made.

For metabolism, all the above network topologies relate to the level or activity of enzymes that modulate *in vivo* fluxes (Figure 1). Beyond the better-known protein localization into different compartments, it hence came somewhat as a surprise to find widespread physical reorganization of metabolic enzymes into large aggregates upon nutrient starvation as a regulatory mechanism, again in yeast [37]. While we have already discussed the importance of protein–protein interactions for phosphorylation-based regulation [24•], here it is the very machines of metabolism themselves that aggregate reversibly into discrete physical structures. The high proportion of central metabolic and biosynthetic enzymes in these large complexes indicates a nutrient-specific function in substrate channeling, flux control, or enzyme storage [37]. Similar proteinaceous microcompartments have been described for ethanolamine utilization in *E. coli* [38], the TCA cycle of *B. subtilis* [39], and the carbon fixation machinery of cyanobacteria [40]. These dynamic enzyme assemblies maintain somewhat separate concentrations of their intermediate, which facilitates fast turnover of labile or toxic compounds and may also prevent undesired crosstalk between different pathways [41].

Unraveling the logic of regulatory networks and metabolic feedback

Beyond interaction topologies, knowledge of how regulatory networks compute input signals into output regulatory responses is necessary to fully understand regulation. This is of particular relevance for the intertwined and cross-talking regulatory networks. Because unraveling of input–output regulatory logics often requires pre-existent topological knowledge, current attempts mainly focus on transcriptional and signaling networks. The simplest and often used modeling abstraction for transcriptional networks is discretization of transcription factors (on or off) and target genes are expressed

according to equations composed of Boolean operators (i.e. AND, OR, NOT). Boolean networks can be easily combined with stoichiometric metabolic models for steady-state flux simulations. Boolean rules link environmental cues and the metabolic state to the transcription of a cell's genome [42] that can be automatically refined through cycles of matching experimental transcript and flux data [43]. Simulations suggest transcriptional regulation to control steady state fluxes in up to 20% of the reactions [44]. Different from the rather crude on/off abstraction of Boolean logic that is applicable to large networks, quantitative models of transcriptional regulation based on kinetic mechanisms or linear approximation can be used for small-sized or medium-sized networks. At the level of individual promoters that integrate different transcription factors, quantitative regulation of enzyme-encoding genes has been investigated by thermodynamic models *in vitro* [45] and *in vivo* [46]. Examples are quantitative input–output functions controlling uptake gene expression upon sugar availability in *E. coli* [47]. Crucial for inference of such quantitative input–output logics in more complex networks is the experimentally cumbersome quantification of transcription factor activities, which must be done by either fluorescent reporter promoters [46] or linear regression analysis of target gene expression on the basis of the known topology [48]. A further major challenge is unraveling the dynamic nature of regulatory responses. By considering temporal motifs, the timing of transcriptional regulation was demonstrated to often follow the topological order of metabolic pathways [49], although this particular case illustrates the danger of missing major mechanistic interrelations when focusing only on one layer of regulation [50]. In combination with modulated transcript half-lives, already the transcriptional network can perform complex tasks such as reliably responding to a slowly changing environment, while effectively ignoring fast fluctuations, as was nicely shown for yeast sugar catabolism [51]. Current efforts highlight transcriptional networks as the regulatory network of preference to study quantitative and temporal aspects of such input–output regulatory logics (see [52•] for a recent review).

Faster than the minute-time scale of transcription network responses, signaling networks simultaneously integrate intracellular and extracellular signals and their input–output logic must additionally consider protein modification and metabolite interactions that occur on a seconds-time scale. Reconstructing such signaling networks at genome-scale comes within reach [53], but current applications are small-scaled. By explicitly including extracellular signaling in a kinetic model, it was demonstrated that *S. cerevisiae* evolved for economic stress protection: almost no response to fast pulse-like osmotic changes but well-adapted to periodic changes at a certain frequency [54]. Considering crosstalk between transcription and signaling is starting to reveal particular input–output logics that control yeast metabolism, such as an incoherent feed forward loop that leads to a transient expression pulse of a glucose transporter [55] and a low-energy check point by the Snf1 kinase that controls transcription of enzymes in central and redox metabolism [56]. Overall, the fast time-scales of signaling networks render unraveling their input–output logics cumbersome, where quantitative and dynamic data are limiting.

While the previous sections dealt with regulatory mechanisms that feed into metabolism, we discuss in the following the opposite direction: feedback signals coming from metabolism into regulatory networks [57]. Frequently, metabolite-binding to transcription factors changes their activity [44,45,46], and metabolite-binding to catalytic RNAs (ribozymes) at so-called riboswitches can also modulate splicing, translation, or RNA stability [58]. The enrichment of kinases and phosphatases in metabolite–protein interaction screens [33,35•] suggests that also post-translational modifications may be subject to metabolic feedback. In some cases, these metabolic feedbacks have been quantified through modeling, such as the intracellular dynamics of fructose-1-6-bisphosphate in *E. coli*, which is hypothesized to signal glucose limitation to central metabolism via the Cra-regulated transcription network [31]. According to control system theory, homeostasis and adaptation is best achieved when internal signals report the systems' state – metabolite levels or flux rates in the case of metabolism – to the regulatory circuits (Figure 1a, red arrow). But how does metabolic feedback through metabolite levels relate to the flux output of metabolism? Kinetic modeling demonstrated that *E. coli* senses concentration changes in few metabolites that report the central metabolic flux state to transcription factors [59••]. Provided that a small amount of new nutrients can enter the cell, this distributed flux-sensing enables to mount appropriate responses to all carbon sources with only a handful of internal signals. Only few metabolites appear to qualify as potential flux sensors (i.e. their concentration is strictly correlated to the pathway flux) and at least some of them appear to be conserved across species boundaries [60]. Typically, such flux sensing is not restricted to transcriptional responses but involves also allosteric feedback at the level of enzymes [61]. First larger reconstructions of regulatory networks indeed highlight the strong involvement of internal metabolites in the general regulation of metabolism [62•,53].

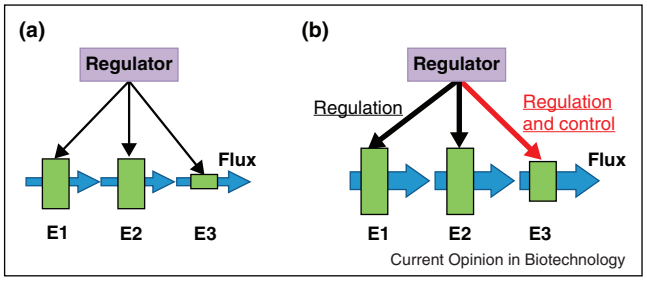
Lastly, even without dedicated regulatory mechanisms, substrate and product levels have a strong impact on the reaction rate and can have feedback-like consequences for the network (Figure 1). Quantitative metabolomics and ordinary-differential-equation-based modeling revealed substrate competition for the active site of saturated enzymes as a key determinant of fluxes in

nitrogen assimilation of *E. coli* [63••]. Such competitive inhibition was shown for *E. coli*, whose metabolite concentrations exceeded the *in vitro*-determined K_m for most of the 100 tested substrate–enzyme pairs [64]. Consistent with the *E. coli* exception of near K_m substrate concentrations in lower glycolysis, not only metabolite concentrations in *S. cerevisiae* glycolysis but also TCA cycle were found to be near the K_m values [65•]. The consequence of near K_m concentrations was an inverse relationship between genetically induced fold-changes in yeast substrate metabolites and their enzymes upon local and global perturbations, suggesting that reaction rates are jointly limited by enzyme capacity and metabolite concentrations [65•]. Hence small variations in enzyme capacity are buffered by a converse change in substrate concentration that maintains a constant reaction flux (homeostasis) over a range of enzyme capacities (Figure 1). Whether indeed near K_m substrate concentrations prevail more generally in central metabolism and above K_m concentrations are the typical situation in the remainder of metabolism will be an issue for the near future.

Assessing and quantifying flux control of regulators

The previous sections summarized current progress in mapping out regulatory networks and the comparatively slower unraveling of their input–output responses to regulatory signals. From knowing that a particular regulation event is happening, however, we cannot infer its functional consequences because regulation is conceptually different from the control it exerts on a given biological functional output; that is, flux in metabolism. Here, the term regulation refers to a mechanism able to modulate enzyme activity by some molecular interaction (Figure 1). Instead, we speak of control when a regulator directly changes the overall pathway flux. Therefore, regulation is a local property influencing individual enzyme activities, while control is a global property effecting fluxes in the overall network (Figure 2) [66]. In metabolism, it is particularly important to distinguish both terms because control of metabolic fluxes is shared among many regulators that act simultaneously at different regulatory layers. Typically, only few of the many co-occurring regulatory events actually exert flux control at a given time and condition, hence it is crucial i) to assess whether a regulatory interaction has an impact on *in vivo* flux and ii) to quantify the extent of its flux control.

Figure 2



Difference between regulation and control of metabolic fluxes. A linear metabolic pathway with three enzymes E1, E2, and E3 overarched by a regulator that modulates enzyme capacities (thickness of green rectangles). **(a)** Despite greater capacity of enzymes E1 and E2, capacity of enzyme E3 limits the overall steady state flux (thickness of blue arrows). **(b)** A regulatory event exerted by the regulator increases the three enzymatic capacities. Of the three observable regulatory events, only upregulation of E3 (red arrow) has an actual effect on the metabolic flux because it constrains the overall steady-state flux.

To address the first point, the most reliable method to determine intracellular fluxes is based on ^{13}C-experiments [1]. Since ^{13}C-flux analysis is now feasible at a throughput of several hundred parallel experiments, it can be used to assess the impact of genetically manipulated regulation events. Precisely this approach has been used to systematically identify transcription factors that control the distribution of flux through different metabolic pathways. Intracellular fluxes were quantified in more than 100 transcription factor deletion mutants of yeast [67•] and *E. coli* [68] under different conditions. Although up to 2/3 of the factors were active on some cellular process based on a detectable growth phenotype, only about a handful of them actually controlled the distribution of flux under a given condition in either organism. Although many transcription factors influence enzyme abundance and fluxes in particular pathways, only up to a handful controlled the distribution of fluxes per condition. The transcriptional control of flux distributions during sugar catabolism focused on the switch between respiratory and fermentative metabolism, which in the case of yeast involved a network of 23 of 119 tested transcription factors that fine-tuned the response under five different conditions [67•]. Since flux data can only reveal the affected pathways, it required 'omics' data from the relevant transcription factor mutants to derive testable hypotheses on the actual target genes that caused the observed flux increase. Rather than perturbing (potential) individual regulation events, the typical approach follows the reverse logic, inferring regulatory relevance from 'omics' data. Two good examples are i) the control of flux direction and branching in central metabolism of *Salmonella* through enzyme acetylation [28••] and ii) unexpectedly strong carbon catabolite repression through the TCA cycle intermediate malate in *B. subtilis* [69]. To extend beyond central metabolism, genome-scale fluxes can be estimated through stoichiometric modeling, albeit with less confidence, and such data have been used to identify the relevant transcript [70] or enzyme [71] changes from flux-transcript (or protein) correlations under different conditions in yeast. While these analyses did not directly reveal the underlying mechanism, they allowed to hypothesize which fluxes are likely to be transcriptionally controlled [70]. The obvious next question is which regulators are responsible for this transcriptional control? Starting

from the regulatory network topology or inferring the most likely transcription factor for observed transcriptional changes [11], it should be possible to design conclusive experiments on flux control.

The second point is to quantify the extent to which a regulation process actually controls a flux under a certain condition. For this we need to know i) how much pathway flux is affected by enzyme activity and ii) how much enzyme regulators affect this activity. Historically, the first question has been addressed in the context of metabolic control analysis and, with a different focus, by biochemical systems theory [66]. The task boils down to estimating numerical coefficients with predictive power from data; that is, numbers quantifying the flux change that will happen upon modulation of enzyme activities at a given steady state. Based on an approximated kinetic model of central metabolism in hepatoma cells, glucose-6-phosphate dehydrogenase and oxidative phosphorylation were shown to exhibit negative and positive flux control, respectively, on the glycolytic flux [72]. Similarly, approximated kinetics highlighted the predominance of enzyme concentration rather than allosteric regulation in controlling glycolytic flux in *Lactococcus lactis* [73]. The second question on which regulator is relevant can only partially be addressed by such approaches because the mechanisms that regulate those putatively controlling reactions typically remain unknown. In this case, the approach of regulation analysis helps delineating which regulatory layer is responsible for establishing fluxes through a given enzyme [74]. In its time-dependent extension, it allows to derive quantitative coefficients for hierarchical (i.e. expression and post-translational modification) and metabolic regulation [75]. For the glycolytic enzymes of yeast, it was thereby shown that there is no particular temporal or enzyme preference for protein degradation or allosteric regulation during the first 4 h of nitrogen starvation. More resolution on the specific regulatory mechanism controlling reactions that limit flux is possible by using detailed kinetic models, when the regulation topology is known and rich data are available. This was done with a differential equation model populated with *in vitro* kinetic parameters of aspartate metabolism in *Arabidopsis*, which revealed allosteric interaction as being the key controlling event to decouple flux rerouting in competing pathways [76]. Using *in vitro* as well as *in vivo* estimated parameters, a kinetic model of ammonia assimilation in *E. coli* surprisingly revealed active-site competitive inhibition of glutamine synthetase by non-substrate metabolites as a key controlling mechanism during dynamic nitrogen perturbations [63••].

Understanding evolution of metabolic regulatory circuits

Are the diverse and overlapping regulatory mechanisms that act on different metabolic pathways in different organisms 'frozen' accidents of evolution or potentially understandable through design principles that would drive evolution towards a particular regulatory configuration? For the simple case of linear pathways, metabolic control theory predicts adaptive evolution to occur first at enzymes with highest flux control, typically in the initial reaction steps [77]. Since flux control is a property specific to a steady state and thus highly condition dependent, evolution theories based on fitness maximization appear to have higher predictive power [78,79•]. For the example of maximizing benefit and cost of protein expression in lactose catabolism of *E. coli*, predictive power was validated experimentally by evolving multiple cost–benefit solutions in different environments [80]. These and other studies suggest that initial regulatory changes occur at the level of gene expression, a view fully consistent with the computationally inferred rate of evolutionary rewiring in various networks from comparative genomics and proteomics data [81]: evolution proceeds continuously slower from transcriptional regulation, phosphorylation, genetic interaction, protein–protein interaction to the slowest changes in metabolism itself. Rapid optimization of metabolic gene expression was empirically shown by transcriptomic and proteomic data from laboratory evolution experiments within 1000 generations [79•]. Overall, the transcriptional network is remarkably robust to rewiring, as was demonstrated by systematic transcription factor knockout studies [67•,68] and addition of 600 rewired links between promoters and transcription factors of *E. coli* [82]. In addition to simply modulating the strength of a given regulatory link, complete absence or addition of entirely novel links can improve condition-specific fitness [68,82] and thus broaden the possibilities for adaptive evolution.

There are clearly common principles underlying the evolution of regulatory circuits that govern metabolism. While our current understanding of these principles is coincidental, many identified architectures optimize properties such as resource allocation [78,79•], robustness-to-noise [83], and response time [55,84] under the framework of evolutionary cost-and-benefit to increase an organisms' fitness. A major obstacle in understanding the evolutionary fitness of regulatory circuits is our limited knowledge of detailed molecular mechanisms and kinetic parameters. Sidestepping the requirement for such detailed knowledge, a recently developed theoretical framework introduces an approach to formally define the design space of biological circuits [85]. By enumerating phenotypes with quantitatively distinct behaviors [32•], it represents a conceptual step towards linking a genotype to the phenotypic advantages it confers to the organisms. Verified through experimental data, this design space framework demonstrated that the non-obvious dimerization rate of the oxygen-sensing transcription factor Fnr of *E. coli* is crucial for optimal regulation of anaerobic metabolism [86].

Conclusions

After about ten years of renaissance in metabolic research, the currently most important contributions are providing novel understanding of how metabolic fluxes are established and regulated. Reconstruction of various regulatory network topologies is steaming. In particular reconstructions of post-translational modification networks such as protein phosphorylation [8,24•] or acetylation [28••], are now in full focus. The past two years clearly showed that we have underestimated the broad importance of such modifications for microbes and their intermediary and energy metabolism. With the growing knowledge of regulatory network topology, the functional relevance of these regulatory events becomes an even more pressing need, and is presently most advanced for transcriptional networks. A major knowledge gap resides in the regulatory network of protein–metabolite interactions, simply because we lacked pertinent methods for systematic analyses – but a start has now been made.

Perhaps most dramatic was the conceptual shift in our perception of metabolism from an engine of cellular operation to an engine that is also steering many processes in microbial [6] and higher cells [87]. Metabolism provides the input and feedback signals for regulatory circuits that govern many important decisions on cell proliferation, differentiation, death, and naturally metabolism itself. Pivotal for a deeper understanding of this active regulatory role are i) methods to detect and quantify protein–metabolite interactions and ii) computational methods to integrate regulatory events dynamically across all relevant levels of molecular interactions.

As we move towards a more quantitative representation of metabolic regulation, we must assess the actual control that an occurring regulatory event exerts on establishing metabolic fluxes. Beyond dynamic component concentrations and flux data – which need also further experimental method development – this requires validated and ready-to-use computational methods to extract control information from dynamic data. Since control is a highly condition-specific property, empirical assessment of control under many different conditions would be an endless task. More effective would be to fit kinetic models to data, perhaps using approximate kinetic laws, from which control can be estimated for all conditions that can be simulated. Lastly, such models may be key in fostering understanding of principles behind the evolution of different regulatory circuits, properly accounting for flux control across environmental scenarios and the fitness advantage it confers.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Sauer U: **Metabolic networks in motion: 13C-based flux analysis**. *Molecular Systems Biology* 2006, **2**:62.
2. Oberhardt MA, Palsson B, Papin JA: **Applications of genome-scale metabolic reconstructions**. *Molecular Systems Biology* 2009, **5**:320.
3. Reaves ML, Rabinowitz JD: **Metabolomics in systems microbiology**. *Current Opinion in Biotechnology* 2011, **22**:17-25.
4. Zamboni N, Sauer U: **Novel biological insights through metabolomics and 13C-flux analysis**. *Current Opinion in Microbiology* 2009, **12**:553-558.
5. Ray LB: **Metabolism. Metabolism is not boring. Introduction**. *Science (New York, NY)* 2010, **330**:1337.
6. Heinemann M, Sauer U: **Systems biology of microbial metabolism**. *Current Opinion in Microbiology* 2010, **13**:337-343.
7. Cho B-K, Zengler K, Qiu Y, Park YS, Knight EM, Barrett CL, Gao Y, Palsson B: **The transcription unit architecture of the *Escherichia coli* genome**. *Nature Biotechnology* 2009, **27**:1043-1049.
8. Bodenmiller B, Wanka S, Kraft C, Urban J, Campbell D, Pedrioli PG, Gerrits B, Picotti P, Lam H, Vitek O *et al.*: **Phosphoproteomic analysis reveals interconnected system-wide responses to perturbations of kinases and phosphatases in yeast**. *Science Signaling* 2010, **3**:rs4-rs14.
9. Keseler IM, Bonavides-Martínez C, Collado-Vides J, Gama-Castro S, Gunsalus RP, Johnson DA, Krummenacker M, Nolan LM, Paley S, Paulsen IT *et al.*: **EcoCyc: a comprehensive view of *Escherichia coli* biology**. *Nucleic Acids Research* 2009, **37**:D464-D470.
10. Shimada T, Yamamoto K, Ishihama A: **Novel members of the Cra regulon involved in carbon metabolism in *Escherichia coli***. *Journal of Bacteriology* 2010, **193**:649-659.
11. Cho B-K, Barrett CL, Knight EM, Park YS, Palsson B: **Genome-scale reconstruction of the Lrp regulatory network in *Escherichia coli***. In *Proceedings of the National Academy of Sciences of the United States of America* 2008, **105**:19462-19467.
12. Seshasayee ASN, Fraser GM, Babu MM, Luscombe NM: **Principles of transcriptional regulation and evolution of the metabolic system in *E. coli***. *Genome Research* 2009, **19**:79-91.
13. Janga SC, Salgado H, Martínez-Antonio A: **Transcriptional regulation shapes the organization of genes on bacterial chromosomes**. *Nucleic Acids Research* 2009, **37**:3680-3688.
14. Mendoza-Vargas A, Olvera L, Olvera M, Grande R, Vega-Alvarado L, Taboada B, Jimenez-Jacinto V, Salgado H, Juárez K, Contreras-Moreira B *et al.*: **Genome-wide identification of transcription start sites, promoters and transcription factor binding sites in *E. coli***. *PLoS ONE* 2009, **4**:e7526.
15. Naville M, Gautheret D: **Transcription attenuation in bacteria: theme and variations**. *Briefings in Functional Genomics* 2010, **9**:178.
16. Durand S, Storz G: **Reprogramming of anaerobic metabolism by the FnrS small RNA**. *Molecular Microbiology* 2010, **75**:1215-1231.
17. Pietack N, Becher D, Schmidl SR, Saier MH, Hecker M, Commichau FM, Stülke J: **In vitro phosphorylation of key metabolic enzymes from *Bacillus subtilis*: PrkC phosphorylates enzymes from different branches of basic metabolism**. *Journal of Molecular Microbiology and Biotechnology* 2010, **18**:129-140.
18. Gnad F, Forner F, Zielinska DF, Birney E, Gunawardena J, Mann M: **Evolutionary constraints of phosphorylation in eukaryotes, prokaryotes, and mitochondria**. *Molecular & Cellular Proteomics: MCP* 2010, **9**:2642-2653.

19. Schmidl SR, Gronau K, Pietack N, Hecker M, Becher D, Stülke J: **The phosphoproteome of the minimal bacterium *Mycoplasma pneumoniae*: analysis of the complete known Ser/Thr kinome suggests the existence of novel kinases**. *Molecular & Cellular Proteomics: MCP* 2010, **9**:1228-1242.

20. Sun X, Ge F, Xiao C-L, Yin X-F, Ge R, Zhang L-H, He Q-Y: **Phosphoproteomic analysis reveals the multiple roles of phosphorylation in pathogenic bacterium *Streptococcus pneumoniae***. *Journal of Proteome Research* 2010, **9**:275-282.

21. Reinders J, Wagner K, Zahedi RP, Stojanovski D, Eyrich B, van der Laan M, Rehling P, Sickmann A, Pfanner N, Meisinger C: **Profiling phosphoproteins of yeast mitochondria reveals a role of phosphorylation in assembly of the ATP synthase**. *Molecular & Cellular Proteomics: MCP* 2007, **6**:1896-1906.

22. Fiedler D, Braberg H, Mehta M, Chechik G, Cagney G, Mukherjee P, Silva AC, Shales M, Collins SR, van Wageningen S *et al.*: **Functional organization of the *S. cerevisiae* phosphorylation network**. *Cell* 2009, **136**:952-963.

23. van Wageningen S, Kemmeren P, Lijnzaad P, Margaritis T, Benschop JJ, de Castro IJ, van Leenen D, Groot Koerkamp MJA, Ko CW, Miles AJ: **Functional overlap and regulatory links shape genetic interactions between signaling pathways**. *Cell* 2010, **143**:991-1004.

24. • Breitkreutz A, Choi H, Sharom JR, Boucher L, Neduva V, Larsen B, Lin ZY, Breitkreutz BJ, Stark C, Liu G *et al.*: **A global protein kinase and phosphatase interaction network in yeast**. *Science* 2010, **328**:1043-1046.

This paper presents a reconstructed protein kinases and phosphatase interaction network for yeast that highlights the role of phosphorylation for many new function in the organism, in particular metabolism in nitrogen and carbon metabolism.

25. Yachie N, Saito R, Sugiyama N, Tomita M, Ishihama Y: **Integrative features of the yeast phosphoproteome and protein–protein interaction map**. *PLoS Computational Biology* 2011, **7**:e1001064.

26. Zhang Z, Tan M, Xie Z, Dai L, Chen Y, Zhao Y: **Identification of lysine succinylation as a new post-translational modification**. *Nature Chemical Biology* 2010, **7**:58-63.

27. Zhao S, Xu W, Jiang W, Yu W, Lin Y, Zhang T, Yao J, Zhou L, Zeng Y, Li H *et al.*: **Regulation of cellular metabolism by protein lysine acetylation**. *Science (New York, NY)* 2010, **327**:1000-1004.

28. •• Wang Q, Zhang Y, Yang C, Xiong H, Lin Y, Yao J, Li H, Xie L, Zhao W, Yao Y *et al.*: **Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux**. *Science (New York, NY)* 2010, **327**:1004-1007.

After mapping a large number of acetylated proteins, the work demonstrates also the functional relevance of several of these protein modifications for controlling major fluxes in central metabolism of Salmonella.

29. Pearce MJ, Mintseris J, Ferreyra J, Gygi SP, Darwin KH: **Ubiquitin-like protein involved in the proteasome pathway of *Mycobacterium tuberculosis***. *Science (New York, NY)* 2008, **322**:1104-1107.

30. Poulsen C, Akhter Y, Jeon AH-W, Schmitt-Ulms G, Meyer HE, Stefanski A, Stühler K, Wilmanns M, Song Y-H: **Proteome-wide identification of mycobacterial pupylation targets**. *Molecular Systems Biology* 2010, **6**:386.

31. Hardiman T, Meinhold H, Hofmann J, Ewald JC, Siemann-Herzberg M, Reuss M: **Prediction of kinetic parameters from DNA-binding site sequences for modeling global transcription dynamics in *Escherichia coli***. *Metabolic Engineering* 2010, **12**:196-211.

32. • Savageau MA: **Biomedical engineering strategies in system design space**. *Annals of Biomedical Engineering* 2011, **39**:1278-1295.

This works propose a systematically and robust mathematical approach to map complex quantitative genotypes into coarse-grained informative phenotypic output.

33. Gallego O, Betts MJ, Gvozdenovic-Jeremic J, Maeda K, Matetzki C, Aguilar-Gurrieri C, Beltran-Alvarez P, Bonn S, Fernández-Tornero C, Jensen LJ *et al.*: **A systematic screen for protein–lipid interactions in *Saccharomyces cerevisiae***. *Molecular Systems Biology* 2010:6.

34. Tagore R, Thomas HR, Homan EA, Munawar A, Saghatelian A: **A global metabolite profiling approach to identify protein–metabolite interactions**. *Journal of the American Chemical Society* 2008, **130**:14111-14113.

35. • Li X, Gianoulis TA, Yip KY, Gerstein M, Snyder M: **Extensive in vivo metabolite–protein interactions revealed by large-scale systematic analyses**. *Cell* 2010, **143**:639-650.

This works tackles with a novel approach the task of systematically identifying metabolite–protein regulatory interactions.

36. Bradley PH, Brauer MJ, Rabinowitz JD, Troyanskaya OG: **Coordinated concentration changes of transcripts and metabolites in *Saccharomyces cerevisiae***. *PLoS Computational Biology* 2009, **5**:e1000270.

37. Narayanaswamy R, Levy M, Tsechansky M, Stovall GM, O'Connell JD, Mirrielees J, Ellington AD, Marcotte EM: **Widespread reorganization of metabolic enzymes into reversible assemblies upon nutrient starvation**. In *Proceedings of the National Academy of Sciences of the United States of America* 2009, **106**:10147-10152.

38. Tanaka S, Sawaya MR, Yeates TO: **Structure and mechanisms of a protein-based organelle in *Escherichia coli***. *Science (New York, NY)* 2010, **327**:81-84.

39. Meyer FM, Gerwig J, Hammer E, Herzberg C, Commichau FM, Völker U, Stülke J: **Physical interactions between tricarboxylic acid cycle enzymes in *Bacillus subtilis*: evidence for a metabolon**. *Metabolic Engineering* 2010, **13**:18-27.

40. Savage DF, Afonso B, Chen AH, Silver PA: **Spatially ordered dynamics of the bacterial carbon fixation machinery**. *Science (New York, NY)* 2010, **327**:1258-1261.

41. Møller BL: **Plant science. Dynamic metabolons**. *Science (New York, NY)* 2010, **330**:1328-1329.

42. Gianchandani EP, Joyce AR, Palsson B, Papin JA: **Functional states of the genome-scale *Escherichia coli* transcriptional regulatory system**. *PLoS Computational Biology* 2009, **5**:e1000403.

43. Barua D, Kim J, Reed JL: **An automated phenotype-driven approach (GeneForce) for refining metabolic and regulatory models**. *PLoS Computational Biology* 2010, **6**:e1000970.

44. Shlomi T, Eisenberg Y, Sharan R, Ruppin E: **A genome-scale computational study of the interplay between transcriptional regulation and metabolism**. *Molecular Systems Biology* 2007, **3**:101.

45. Krishna S, Orosz L, Sneppen K, Adhya S, Semsey S: **Relation of intracellular signal levels and promoter activities in the gal regulon of *Escherichia coli***. *Journal of Molecular Biology* 2009, **391**:671-678.

46. Davidson CJ, Narang A, Surette MG: **Integration of transcriptional inputs at promoters of the arabinose catabolic pathway**. *BMC Systems Biology* 2010, **4**:75.

47. Kaplan S, Bren A, Zaslaver A, Dekel E, Alon U: **Diverse two-dimensional input functions control bacterial sugar genes**. *Molecular Cell* 2008, **29**:786-792.

48. Brynildsen MP, Liao JC: **An integrated network approach identifies the isobutanol response network of Escherichia coli**. *Molecular Systems Biology* 2009, **5**:277.

49. Chechik G, Oh E, Rando O, Weissman J, Regev A, Koller D: **Activity motifs reveal principles of timing in transcriptional control of the yeast metabolic network**. *Nature Biotechnology* 2008, **26**:1251-1259.

50. Ralser M, Wamelink MMC, Latkolik S, Jansen EEW, Lehrach H, Jakobs C: **Metabolic reconfiguration precedes transcriptional regulation in the antioxidant response**. *Nature Biotechnology* 2009, **27**:604-605.

51. Bennett MR, Pang WL, Ostroff NA, Baumgartner BL, Nayak S, Tsimring LS, Hasty J: **Metabolic gene regulation in a dynamically changing environment**. *Nature* 2008, **454**:1119-1122.

52. • Kim HD, Shay T, O'Shea EK, Regev A: **Transcriptional regulatory circuits: predicting numbers from alphabets**. *Science (New York, NY)* 2009, **325**:429-432.

This review presents the current state-of-the-art modeling tools to represent and interpret transcriptional regulatory networks. In particular, it highlights the steps needed to bring quantitative models from small-scale to large-scale.

53. Hyduke DR, Palsson B: **Towards genome-scale signalling-network reconstructions**. *Nature Reviews. Genetics* 2010, **11**:297-307.

54. Zi Z, Liebermeister W, Klipp E: **A quantitative study of the Hog1 MAPK response to fluctuating osmotic stress in *Saccharomyces cerevisiae***. *PLoS ONE* 2010, **5**:e9522.

55. Kuttykrishnan S, Sabina J, Langton LL, Johnston M, Brent MR: **A quantitative model of glucose signaling in yeast reveals an incoherent feed forward loop leading to a specific, transient pulse of transcription**. In *Proceedings of the National Academy of Sciences* 2010, **107**:16743-16748.

56. Usaite R, Jewett MC, Oliveira AP, Yates JR, Olsson L, Nielsen J: **Reconstruction of the yeast Snf1 kinase regulatory network reveals its role as a global energy regulator**. *Molecular Systems Biology* 2009, **5**:319.

57. Grüning N-M, Lehrach H, Ralser M: **Regulatory crosstalk of the metabolic network**. *Trends in Biochemical Sciences* 2010, **35**:220-227.

58. Zhang J, Lau MW, Ferré-D'Amaré AR: **Ribozymes and riboswitches: modulation of RNA function by small molecules**. *Biochemistry* 2010, **49**:9123-9131.

59. •• Kotte O, Zaugg JB, Heinemann M: **Bacterial adaptation through distributed sensing of metabolic fluxes**. *Molecular Systems Biology* 2010, **6**:355.
A large kinetic model shows how metabolite concentrations can be flux reporters for pathway usage or directionality in a distributed flux sensing architecture that triggers transcriptional and allosteric regulation.

60. Christen S, Sauer U: **Intracellular characterization of aerobic glucose metabolism in seven yeast species by (13) C flux analysis and metabolomics**. *FEMS Yeast Research* 2011, **11**:263-272.

61. Kremling A, Bettenbrock K, Gilles ED: **A feed-forward loop guarantees robust behavior in *Escherichia coli* carbohydrate uptake**. *Bioinformatics (Oxford, England)* 2008, **24**:704-710.

62. • Goelzer A, Bekkal Brikci F, Martin-Verstraete I, Noirot P, Bessières P, Aymerich S, Fromion V: **Reconstruction and analysis of the genetic and metabolic regulatory networks of the central metabolism of *Bacillus subtilis***. *BMC Systems Biology* 2008, **2**:20.
A manually curated reconstruction of the diverse regulatory layers of central metabolism in *B. subtilis* that encompasses regulatory mechanisms often ignored in other reconstructions and highlights the role of metabolites as regulatory components.

63. •• Yuan J, Doucette CD, Fowler WU, Feng X-J, Piazza M, Rabitz HA, Wingreen NS, Rabinowitz JD: **Metabolomics-driven quantitative analysis of ammonia assimilation in *E. coli***. *Molecular Systems Biology* 2009, **5**:302.
This work is a prime example on how iterations of data fitting and hypothesis generation by means of mathematical modeling can unravel kinetic regulatory mechanisms controlling metabolic operation.

64. Bennett BD, Kimball EH, Gao M, Osterhout R, Van Dien SJ, Rabinowitz JD: **Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli***. *Nature Chemical Biology* 2009, **5**:593-599.

65. • Fendt S-M, Buescher JM, Rudroff F, Picotti P, Zamboni N, Sauer U: **Tradeoff between enzyme and metabolite efficiency maintains metabolic homeostasis upon perturbations in enzyme capacity**. *Molecular Systems Biology* 2010, **6**:356.
This work tackles the open question of how metabolite and enzyme concentrations are coordinated in central metabolism. It strongly proposes that reaction rates are usually jointly controlled by both metabolites and enzymes.

66. Westerhoff HV, Kolodkin A, Conradie R, Wilkinson SJ, Bruggeman FJ, Krab K, van Schuppen JH, Hardin H, Bakker BM, Moné MJ *et al.*: **Systems biology towards life in silico: mathematics of the control of living cells**. *Journal of Mathematical Biology* 2009, **58**:7-34.

67. • Fendt S-M, Oliveira AP, Christen S, Picotti P, Dechant RC, Sauer U: **Unraveling condition-dependent networks of transcription factors that control metabolic pathway activity in yeast**. *Molecular Systems Biology* 2010, **6**:432.
The largest functional screen of transcription factor knockout mutants for ^{13}C-derived fluxes that identified key, flux distribution-controlling factors. Transcriptomics and proteomics data of these key transcription factor mutants then provided insights into which target genes actually caused the observed flux increase.

68. Haverkorn van Rijsewijk BRB, Nanchen A, Nallet S, Kleijn RJ, Sauer U: **Large-scale 13C-flux analysis reveals distinct transcriptional control of respiratory and fermentative metabolism in *Escherichia coli***. *Molecular Systems Biology* 2011, **7**:477.

69. Kleijn RJ, Buescher JM, Le Chat L, Jules M, Aymerich S, Sauer U: **Metabolic fluxes during strong carbon catabolite repression by malate in *Bacillus subtilis***. *The Journal of Biological Chemistry* 2010, **285**:1587-1596.

70. Bordel S, Agren R, Nielsen J: **Sampling the solution space in genome-scale metabolic networks reveals transcriptional regulation in key enzymes**. *PLoS Computational Biology* 2010, **6**:e1000859.

71. Costenoble R, Picotti P, Reiter L, Stallmach R, Heinemann M, Sauer U, Aebersold R: **Comprehensive quantitative analysis of central carbon and amino-acid metabolism in *Saccharomyces cerevisiae* under multiple conditions by targeted proteomics**. *Molecular Systems Biology* 2011, **7**:464.

72. Maier K, Hofmann U, Reuss M, Mauch K: **Dynamics and control of the central carbon metabolism in hepatoma cells**. *BMC Systems Biology* 2010, **4**:54.

73. Rossell S, Solem C, Jensen PR, Heijnen JJ: **Towards a quantitative prediction of the fluxome from the proteome**. *Metabolic Engineering* 2011, **13**:253-262.

74. Daran-Lapujade P, Rossell S, van Gulik WM, Luttik MAH, de Groot MJL, Slijper M, Heck AJR, Daran J-M, de Winde JH, Westerhoff HV *et al.*: **The fluxes through glycolytic enzymes in *Saccharomyces cerevisiae* are predominantly regulated at posttranscriptional levels**. In *Proceedings of the National Academy of Sciences of the United States of America* 2007, **104**:15753-15758.

75. van Eunen K, Bouwman J, Lindenbergh A, Westerhoff HV, Bakker BM: **Time-dependent regulation analysis dissects shifts between metabolic and gene-expression regulation during nitrogen starvation in baker's yeast**. *The FEBS Journal* 2009, **276**:5521-5536.

76. Curien G, Bastien O, Robert-Genthon M, Cornish-Bowden A, Cárdenas ML, Dumas R: **Understanding the regulation of aspartate metabolism using a model based on measured kinetic parameters**. *Molecular Systems Biology* 2009, **5**:271.

77. Wright KM, Rausher MD: **The evolution of control and distribution of adaptive mutations in a metabolic pathway**. *Genetics* 2010, **184**:483-502.

78. Molenaar D, Berlo R, van Ridder D, de Teusink B: **Shifts in growth strategies reflect tradeoffs in cellular economics**. *Molecular Systems Biology* 2009, **5**:323.

79. • Lewis NE, Hixson KK, Conrad TM, Lerman JA, Charusanti P, Polpitiya AD, Adkins JN, Schramm G, Purvine SO, Lopez-Ferrer D *et al.*: **Omic data from evolved *E. coli* are consistent with computed optimal growth from genome-scale models**. *Molecular Systems Biology* 2010:6.
This fruitful combination of modeling and large scale data demonstrates optimized gene expression as a key target of metabolic evolution.

80. Dekel E, Alon U: **Optimality and evolutionary tuning of the expression level of a protein**. *Nature* 2005, **436**:588-592.

81. Shou C, Bhardwaj N, Lam HYK, Yan K-K, Kim PM, Snyder M, Gerstein MB: **Measuring the evolutionary rewiring of biological networks**. *PLoS Computational Biology* 2011, **7**:e1001050.

82. Isalan M, Lemerle C, Michalodimitrakis K, Horn C, Beltrao P, Raineri E, Garriga-Canut M, Serrano L: **Evolvability and hierarchy in rewired bacterial gene networks**. *Nature* 2008, **452**:840-845.

83. Shinar G, Rabinowitz JD, Alon U: **Robustness in glyoxylate bypass regulation**. *PLoS Computational Biology* 2009, **5**:e1000297.

84. Chin C-S, Chubukov V, Jolly ER, DeRisi J, Li H: **Dynamics and design principles of a basic regulatory architecture controlling metabolic pathways**. *PLoS Biology* 2008, **6**:e146.

85. Savageau MA, Coelho PMBM, Fasani RA, Tolla DA, Salvador A: **Phenotypes and tolerances in the design space of biochemical systems**. In *Proceedings of the National Academy of Sciences of the United States of America* 2009, **106**:6435-6440.

86. Tolla DA, Savageau MA: **Phenotypic repertoire of the FNR regulatory network in *Escherichia coli***. *Molecular Microbiology* 2010, **79**:149-165.

87. Buchakjian MR, Kornbluth S: **The engine driving the ship: metabolic steering of cell proliferation and death**. *Nature Reviews. Molecular Cell Biology* 2010, **11**:715-727.