SI Appendix

1. C13-based reaction flux determination.

Metabolic fluxes were determined by minimizing the difference between model simulated and measured mass isotopomer distributions of biomass amino acids(1). In short, the following constrained least-squares minimization problem was solved:

$$(1) \qquad \min_{v} \Phi = \left(x(v) - x^{obs}\right)^{T} \cdot \Sigma_{x}^{-1} \cdot \left(x(v) - x^{obs}\right) \quad \text{s.t.} \quad v \geq 0$$

Where the objective function Φ is the covariance-weighted sum of squared residuals, v is the vector of assumed fluxes, x(v) is the vector of simulated measurements, x^{obs} is the vector of measured mass isotopomer abundances, and Σ_x is the measurement covariance matrix. Eq. (1) is nonlinear and requires an iterative solution scheme. We applied successive quadratic programming search method to find the minimum(1). To ensure that a global optimum was found, flux estimation was repeated at least ten times starting with random initial values for all fluxes. At convergence, accurate confidence intervals of fluxes were calculated by evaluating the sensitivity of the objective function with respect to individual fluxes.

$$(2) \qquad \frac{\Phi(u)\big|_{v_i} - \Phi(\hat{u})}{\Phi(\hat{u}) / (n-p)} = F(1, n-p)$$

The statistic in Eq. (2) follows an F-distribution with (1, *n*-*p*) degrees of freedom, where *n* is the number of measurements, and *p* is the number of estimated fluxes. Statistical validation of the fit was accomplished by a statistical test for the goodness-of-fit (i.e., chi-square test for model adequacy), and a normality test for the weighted residuals(1).

(3) $$\Phi(u)\big|_{v_i=v_{i0}} \leq \left(\Phi(\hat{u}) + \chi^2_{1-\alpha}(1)\right)$$

Mass isotopomer measurements were simulated at each iteration using the elementary metabolite units (EMU) modeling framework. The EMU method produces an efficient simulation model for a given biochemical reaction network system(2). To simulate the isotopic measurements in the EMU framework, we solve a cascade of linear subproblems of the form:

(4a) $$A_1(v) \cdot x_1 = B_1(v) \cdot y_1(y_1^{in})$$

(4b) $$A_2(v) \cdot x_2 = B_2(v) \cdot y_2(y_2^{in}, X_1)$$

...

(4c) $$A_z(v) \cdot x_z = B_z(v) \cdot y_z(y_z^{in}, X_{z-1}, ..., X_1)$$

Where A(v) and B(v) are isotopomer transition matrices that are strictly functions of fluxes v, x is the vector of simulated measurements, and y is the vector of substrate labeling. We have shown previously that the EMU method always computes a unique and stable solution for simulated measurements for any given metabolic fluxes, substrate labeling and network model(2).

2. C13-based reaction flux determination in follow up, shake flask experiments.

Analogous to flux determination in chemostat cultures described above, metabolic fluxes were determined in shake-flask experiments by minimizing the difference between model simulated and measured mass isotopomer distributions of biomass amino acids. Because no external fluxes were measured in shake-flasks, metabolic fluxes were normalized to protein biosynthesis flux assuming constant protein composition. As such, these fluxes measure the contribution of central carbon metabolic pathways to the production of proteinogenic amino acids from glucose (and methionine). For experiments with additional methionine in the culture medium, the contribution of exogenous methionine to protein

production was quantified from the fractional labeling of proteinogenic amino acids as described in Antoniewicz et al (3):

amino acid labeling = G x (model predicted amino acid labeling from 13C-glucose) + (1-G) x (unlabeled amino acid)

Here, the G-value quantifies the fractional contribution of glucose to amino acid production, i.e. a G-value of 1 indicates that 13C-glucose contributed 100% to amino acid production, whereas a value of 0 corresponds to unlabeled methionine as being the sole precursor.

3. mRNA-flux model.

Flux changes were predicted individually for each biosynthetic reaction (see Supporting Spreadsheet) using

(5) $\Delta\text{flux} = \exp\left(-p_1 \cdot d_{\text{interaction}}\right)\frac{\Delta\text{mRNA}}{p_2}$, where

- $d_{\text{interaction}}$, the metabolite-enzyme interaction density for the individual reaction node (see Supporting Spreadsheet)
- ΔmRNA, the median change in mRNA for the reaction node (see Supporting Spreadsheet)
- p_1, the optimized metabolite-enzyme interaction density
- p_2, the optimized mRNA scaling term

These flux changes predicted for each reaction individually in Eq. (5) were then subject to a least squares linear optimization in which they were constrained to the metabolic network in Eq. (6). Here, overbars indicate the vector set of fluxes and S is the stoichiometric matrix (see Supporting Spreadsheet):

(6) $\min\left(\overline{\Delta\text{flux}} - \overline{\Delta\text{flux}_{\text{individual}}}\right)^2$, subject to

- $S \cdot \overline{\Delta\text{flux}} = 0$

Furthermore, the following constraints were made on these network-constrained fluxes:

- $\Delta\text{flux} = 0$ for glucose uptake, carbohydrate output, CO2 output, and net RNA output in accordance with our measurements confirming equivalent macroscopic phenotype
- $\sum_{\substack{\text{amino-acid drain} \\ \text{rates to biomass}}} \Delta\text{flux} = 0$ in accordance with our measurements confirming no change in *overall* protein output
- $\Delta\text{flux} = 0$ for glutamate and histidine drain rates to biomass in accordance with our understanding of glutamate biomass flux as central regulatory node not reasonably able to vary significantly at equivalent macroscopic growth
- $-0.25 \cdot \text{abs}(\text{flux}) < \Delta\text{flux} < 0.25 \cdot \text{abs}(\text{flux})$ for all amino acid drain rates to biomass in accordance with our understanding that an amino acid content in protein should be allowed to fluctuate but kept within physically reasonable bounds (see Supplementary Spreadsheet for absolute amino acid drain rates)

Given this model to predict flux changes, we conducted a global optimization of the two parameters p_1 and p_2 above in Eq. (5) to minimize deviations from the measured changes in fluxes.

(7) $\min\left(\overline{\Delta\text{flux}_{\text{measured}}} - \overline{\Delta\text{flux}_{\text{predicted}}}\right)^2$ varying

- p_1, the optimized metabolite-enzyme interaction density (found as -8)
- p_2, the optimized mRNA scaling term (found as 5)

Of particular interest, p_1 was found to be negative which indicates that increasing flux control (e.g., decreasing deviations in flux) is associated with increasing metabolite-enzyme interaction density.

Note. Constraining predicted fluxes to the same reaction network statistically verified by the flux measurement data set partially accounts for the improved agreement. However, the underdetermined reaction network contains many degrees of freedom by which predicted fluxes vary. Thus, the mathematical framework is not circular.

4. Discussion of metabolite-enzyme interaction density in aromatics, arginine, and lysine pathways

The model illustrates that flux control (i.e. invariance to perturbation) increases as metabolite - enzyme interaction density increases. In other words, greater interaction density lessens transcriptional control and gives metabolites an increasing role in regulating the flux phenotype. This model supports the body of literature on feedback inhibition in that increased metabolite regulation of enzyme activity results in greater metabolic control. Specific biosynthesis pathways in the expanded network model provide illustrative foils for this principle (Fig. 3). Aromatics biosynthesis comprises 10 feedback inhibition and 3 feedforward activation interactions among 14 aromatic enzymes involving various pathway metabolites (Fig. 3a, 4a). Even though most mRNA and end product metabolite levels increase, high metabolite interaction density ($d_{interaction} = 13/14$) results in unchanged fluxes. This is visualized by upregulated (green) enzyme and end product metabolite nodes and unchanging (white) flux nodes (Fig. 3a). The isoleucine-leucine-valine superpathway, $d_{interaction} = 7/11$, (Fig. 3b) corroborates this hypothesis. Despite 7 biosynthetic mRNAs increasing significantly (gene-wise False Discovery Rate (FDR) < 2%), pathway fluxes remain unchanged (within the 3% deviation noise region). For the high metabolite interaction densities in Fig. 3a and 3b, we hypothesize that because end products are not being increasingly used in protein synthesis, the metabolite levels build up and cause feedback inhibition at the enzyme level, resulting in tightly regulated flux.

In contrast, the metabolic control structures for arginine and lysine are not governed by high metabolite interaction densities (Fig. 3c, 3d, 4a). Having only 1 of 10 enzymes regulated by enzyme-metabolite feedback inhibition (Arg5,6p) ($d_{interaction} = 1/10$), the arginine biosynthetic pathway conforms to the null hypothesis model motivated by the central dogma. That is, activations propagate from mRNA to flux (30% deviation) to end-product metabolites, as shown

by the green nodes in Fig. 3c. Like arginine, the lysine pathway flux, $d_{interaction} = 2/7$, increases markedly (here, 7% deviation) as a result of increasing mRNA levels, indicating less tightness in flux control (Fig. 3d, although the log scale does not color the flux node perceptibly green**)**. Instead of high metabolite interaction density, arginine and lysine biosynthetic controls use alternative strategies. To compensate for unnecessary metabolite build-up, these pathways rely on preferential vacuolar localization of products and activation of transcription factor regulators separate from Gcn4p. These include the ArgR-Mcm1 repressor complex in the case of arginine and co-inducers Lys14p and α-aminoadipate semialdehyde (AASA) in the case of lysine.

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