Supplementary Note 2: Flux Balance Simulations of Bacterial Metabolism

The *Escherichia coli* flux balance analysis (FBA) model is a compilation of known metabolic pathways, comprising ~950 genes representing enzymes or transporters, with ~540 metabolites between them[1] (Suppl. Data 2). Because the fluxes for most reactions in the network are unknown, FBA performs a linear fitting to determine the reaction rates that most closely match the optimal growth levels given a specified input profile (nutrients and other ingredients to be consumed) for each growth condition. The solution is further constrained to be consistent with known reaction rates when they are available, to satisfy the conservation of mass through each reaction, and to prefer glycolysis when sufficient ingredients are present.

We optimized the network fluxes for growth under two conditions, minimal acetate media with oxygen present[2], and minimal glucose media in the absence of oxygen[3], chosen to activate very different pathways in the metabolic network. The simulated phenotype is modeled by means of a "growth" reaction connected to 47 metabolites in the network representing the production levels needed for growth. We simulated growth under aerobic respiration with minimal acetate + O_2 media[2] and fermentation with minimal glucose + CO_2 media[3].

Responses to drug inhibition were modeled by restricting target enzymes singly or in pairs, by putting upper limits on their reaction rates, and for each inhibition level, we re-optimized the fluxes using minimization of metabolic adjustment[4] to determine the network's response. First, we determined the concentration of each agent singly that produced 90% inhibition in each phenotype. We then sampled each agent's curve with two-fold dilutions for two concentrations above and four points below the EC90, resulting in 8-point response curves, including the untreated point (Suppl. Data 3). For each pair of inhibitors, we also generated a fixed dosing ratio response curve by adding both single agent doses along the combined curve.

This procedure was used to inhibit all ~550 enzymes, and 491 of these agents had successfully converged simulations and sufficient overlap between the two conditions to enable accurate selectivity calculations. We then simulated all 120,295 pairwise combinations of those 491 inhibitors as fixed ratio dose series determined by the ratio of effective concentrations in each assay. For 8,906 combinations (~7%) the combined simulations failed to converge. Synergy $\boldsymbol{S}$ and selectivity $\boldsymbol{SI}$ were measured for each combination, using fermentation as the test and aerobic growth as the control phenotype (Suppl. Data 3).

Average responses were calculated across all combinations in each of 136 multi-target mechanism groups, derived from 16 broad mechanistic classes for FBA model targets (Fig. 2), or for 990 multi-target groups from all 44 pathways represented in the *E. coli* FBA model. The resulting synergy and selectivity profiles (Suppl. Data 4), when grouped by mechanistic classes (Figs. S5-6), highlight pathways that separate fermentation from aerobic metabolism. The selectivity is most striking for citric acid cycle and pyruvate metabolism, and to a lesser extent for glutamate metabolism within the amino acids. The selectivity profiles highlight these differences, and the overall selectivity bias ($\boldsymbol{B}$ ~ 0.6) for the top 1% of synergistic combinations corresponds to almost a fourfold increase in potency over the single agents (Fig. 2).

The $\boldsymbol{SI}$ distributions were compared for the top synergies, the full set of combinations, and the single agents, both for the "forward" (ferment – aerobic) and the "reverse" (aerobic – ferment) endpoint comparisons (Fig. S7). For both assay pairs, we used a synergy cutoff of 1, corresponding to the top ~0.4% for the forward and top ~1% for the reverse pair. Although the comparisons differed considerably in detail, both had significant selectivity biases, with $\boldsymbol{B}_{fwd} = 0.596 \pm 0.005$ and $\boldsymbol{B}_{rev} = 0.894 \pm 0.007$ respectively, suggesting that there were synergies in each that were unrelated to the other endpoint.

References

1. Duarte, N.C., Herrgard, M.J. & Palsson, B.O. Reconstruction and validation of Saccharomyces cerevisiae iND750, a fully compartmentalized genome-scale metabolic model. *Genome Res.* **14**, 1298-1309 (2004).
2. Edwards, J.S., Ibarra, R.U. & Palsson, B.O. In silico predictions of Escherichia coli metabolic capabilities are consistent with experimental data. *Nat Biotechnol* **19**, 125-130 (2001).
3. Edwards, J.S. & Palsson, B.O. Metabolic flux balance analysis and the in silico analysis of Escherichia coli K-12 gene deletions. *BMC Bioinformatics* **1**, 1 (2000).
4. Segrè, D., Deluna, A., Church, G.M. & Kishony, R. Modular epistasis in yeast metabolism. *Nat Genet* **37**, 77-83 (2005).

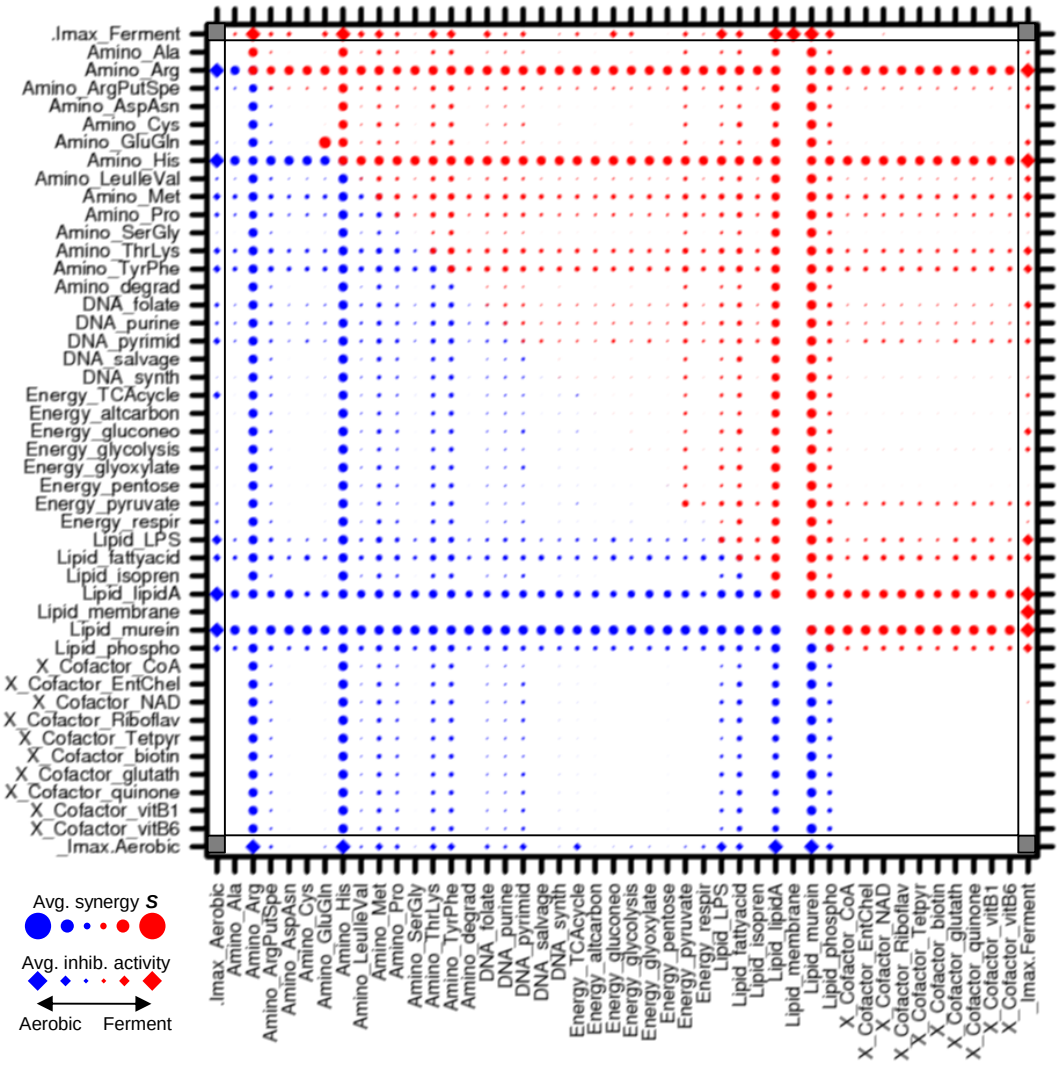

Figure S5. FBA simulations of synergy in *Escherichia coli* metabolism. Growth under aerobic minimal acetate or minimal glucose fermentation conditions was simulated using flux balance analysis (FBA). Enzyme inhibition was modeled by limiting the maximum flux of a target reaction and measuring the effect on the growth reaction. Each enzyme was restricted at a series of concentrations sampling the transition to activity and each combination was simulated as a fixed ratio series by simultaneously inhibiting both targets together. Single inhibitor responses show the average maximum inhibition level (diamonds) within each pathway, and the average synergy score (circles) for each pair of pathways in the *E. coli* FBA model. Results for aerobic conditions are shown in blue (lower left), and for fermentation are shown in red (upper right). Most synergy occurs between pathways with active single inhibitors, in proportion to the single inhibitor activity. The most striking differences between conditions occur with the citric acid cycle and pyruvate metabolism.

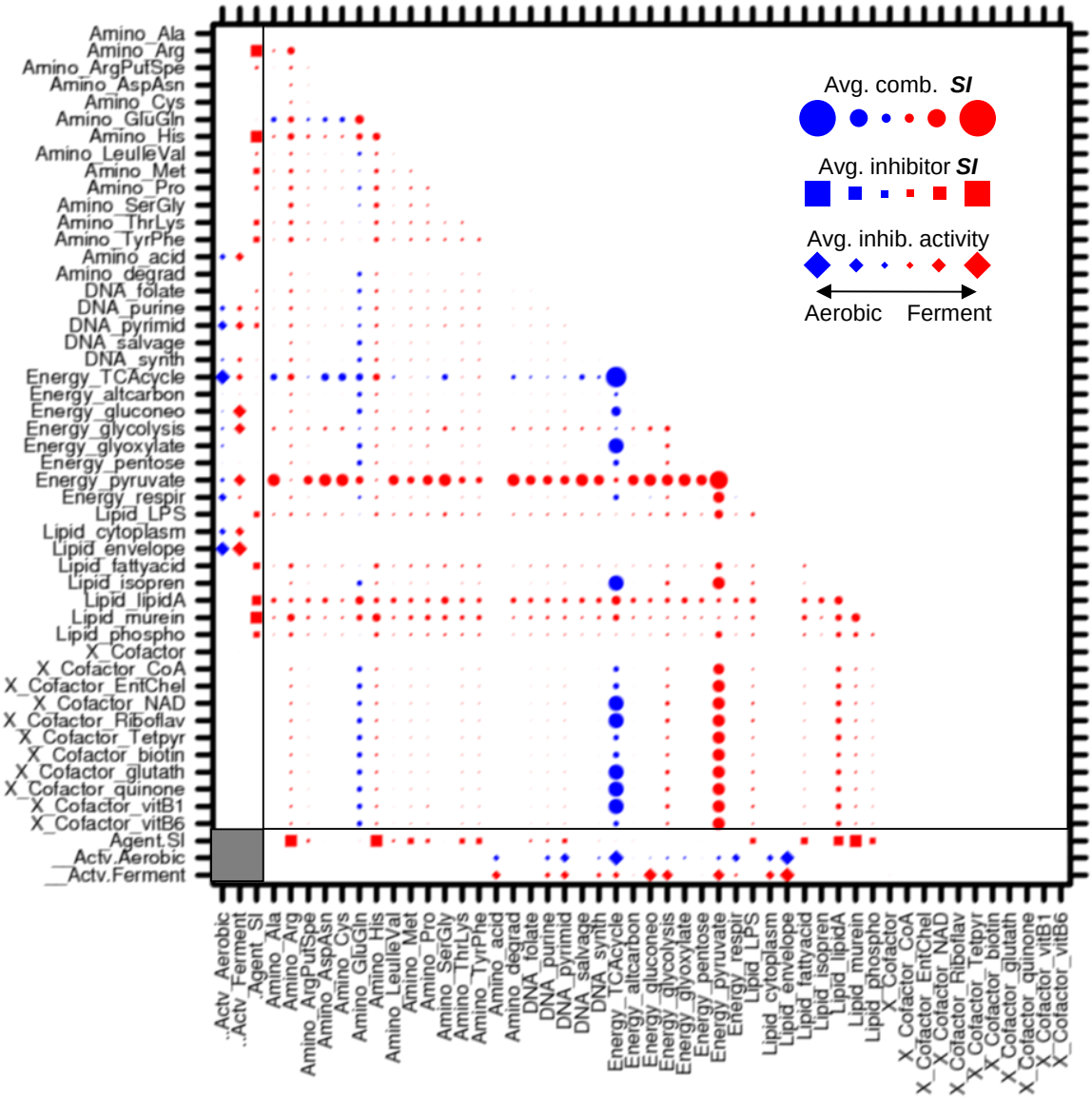

Figure S6. Selectivity for simulated *E. coli* metabolism. Single inhibitor responses show the activity area $\boldsymbol{A} = \boldsymbol{Z}_{max}(6-\log_{10}\mathrm{EC50})$ as diamonds and selectivity index ***SI*** as squares, averaged across all agents in each pathway. The activity area gives an indication of both maximum activity and potency for an inhibitor. For each pair of pathways, the average combination ***SI*** is shown as circles. Selectivity favoring essentiality for aerobic or fermentation conditions is shown in blue or red respectively. The transition from reliance upon citric acid cycle to pyruvate metabolism is much more apparent with the ***SI*** displays than it was from the synergy scores. Also apparent are more subtle effects, such as the importance of glutamate metabolism for aerobic respiration, resulting from that amino acid's connection to citric acid metabolism.

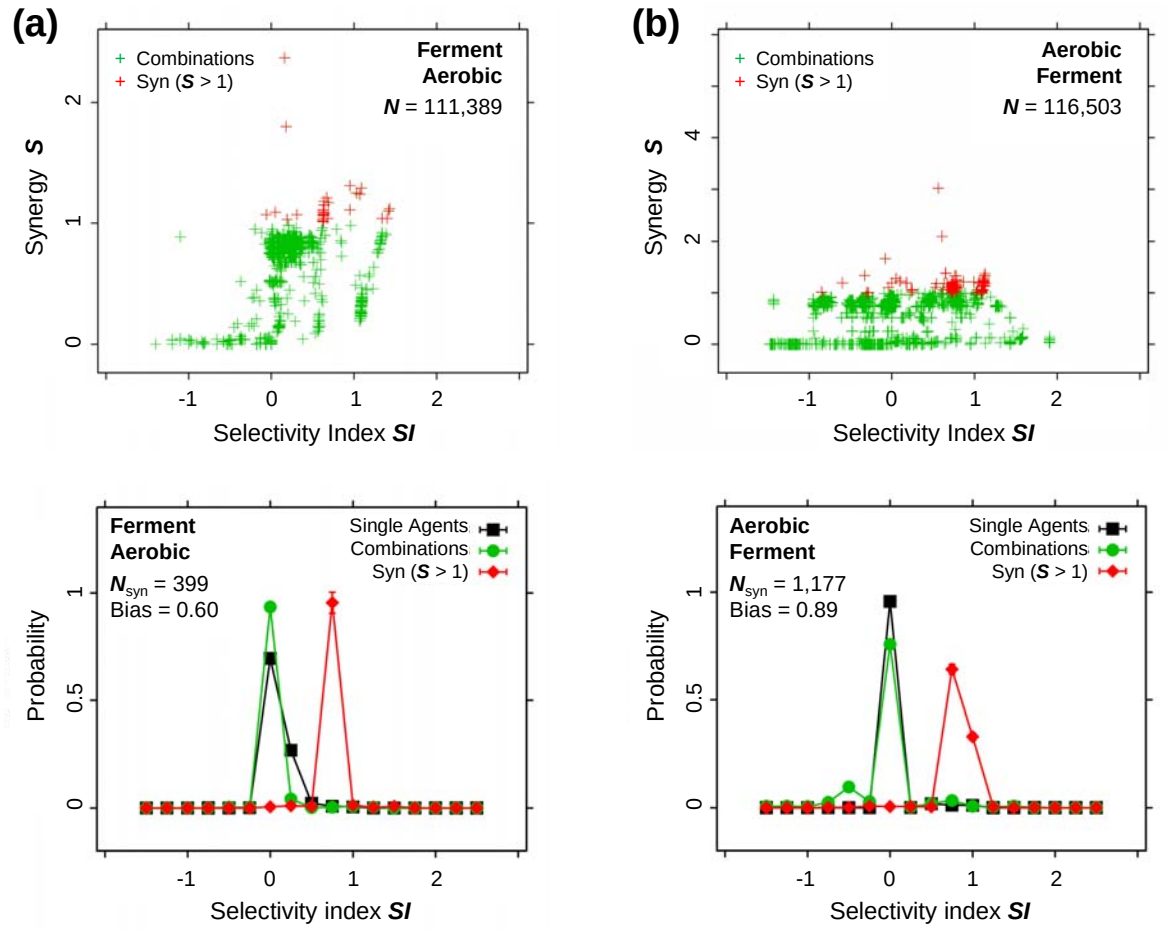

Figure S7. Selectivity bias for simulated *E. coli* metabolism, showing the "forward" (a) and "reverse" (b) endpoint comparisons, relative to that shown in Fig. 2. The upper panels show the distribution of ***S*** and ***SI*** values for all combinations (green) compared to the synergies (red), and the lower panels compare the ***SI*** distributions for the synergies (red), all combinations (green) and the single agents (black, from the more selective of the two inhibited genes in each combination). Although the ***S***/***SI*** distributions are very different, both show strong biases, with $\boldsymbol{B}_{fwd} = 0.596 \pm 0.005$ and $\boldsymbol{B}_{rev} = 0.894 \pm 0.007$ respectively, suggesting that there are numerous synergies in each that are not involved with the other.