Evolution of gene knockout strains of *E. coli* reveal regulatory architectures governed by metabolism

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Biological regulatory network architectures are multi-scale in their function and can adaptively acquire new functions. Gene knockout (KO) experiments provide an established experimental approach not just for studying gene function, but also for unraveling regulatory networks in which a gene and its gene product are involved. Here we study the regulatory architecture of *Escherichia coli* K-12 MG1655 by applying adaptive laboratory evolution (ALE) to metabolic gene KO strains. Multi-omic analysis reveal a common overall schema describing the process of adaptation whereby perturbations in metabolite concentrations lead regulatory networks to produce suboptimal states, whose function is subsequently altered and re-optimized through acquisition of mutations during ALE. These results indicate that metabolite levels, through metabolite-transcription factor interactions, have a dominant role in determining the function of a multi-scale regulatory architecture that has been molded by evolution.
Biological response to gene loss can be evaluated on multiple time-scales. The immediate response to genetic perturbation is studied by measuring an organism’s phenotypic response to a gene knockout (KO)\(^1\)-\(^4\). For example, entire KO strain collections have been generated and used to define essential genes\(^5\)-\(^8\). Besides assessing gene function, gene knockouts can be studied at the systems level through the integration of multi-omics data sets (i.e., metabolomics, fluxomics or network reaction rates, proteomics, and transcriptomics) to better understand the regulatory architecture that relies on the gene product. For example, it has been found that perturbations to the metabolic network are rapidly compensated for by flux re-routing caused by adjustments made at the regulatory level that re-tune enzyme level\(^9\)-\(^11\). Specifically, these studies found that regulatory changes (and in particular, changes in metabolite levels) occurred in proximity to the network lesion that a gene KO created. However, the extent to which distant regulatory changes relative to the location of the network lesion occurred was not discussed\(^1\),\(^5\). In addition, the adaptive consequences of gene loss were not investigated.

The adaptive response to genetic perturbation is studied by measuring changes in physiological function after perturbation and during adaptation\(^10\)-\(^12\), and then characterizing the mutations that are required for the organism to regain the ability to grow optimally under the given conditions\(^3\)-\(^8\),\(^13\)-\(^26\). For example, it has been shown in bacteria and yeast that the likelihood of accumulating compensatory mutations is a function of the fitness cost of the KO\(^22\)-\(^24\). Importantly, compensatory mutations often require the rewiring of existing regulatory networks to regain fitness, thus revealing the role of the lost gene in the regulatory architecture of the biological system\(^26\). Despite the potential to reveal novel insights into the regulatory architecture, to the best of our knowledge, a comprehensive systematic study looking at the rewiring of the regulatory network in response to gene loss has not been performed.

Previous work implemented a novel experimental design that involved gene knockouts (KOs) and adaptive laboratory evolution (ALE) in a pre-evolved *Escherichia coli* K-12 MG1655 strain (Fig. 1) to reveal detailed and mechanistic KO-specific adaptive responses to the loss of a gene\(^27\)-\(^30\). Here, bioinformatics were implemented to reveal commonalities of how biological systems and specifically regulatory networks respond and adapt to gene KO at a systems level. First, the experimental design was confirmed through control evolutions of the pre-evolved strain. Second, multivariate statistical data decomposition methods found that the dominant modes of the data involved the drive towards regaining optimal fitness, while independent replicate evolutions revealed diversity in the adaptive paths selected in pursuit of optimal fitness. In this context, “optimal” indicates the biochemical state that allows for the maximal growth rate that the organism can achieve given the current environmental and genetic conditions. Third, biochemical pathway integration with multi-omic data sets revealed a common model of adaptive evolution. In this schema, network perturbation from gene KO altered metabolic flux, leading to perturbations in metabolite concentrations, which in turn triggered regulatory network responses altering gene expression. Gene expression responses were subsequently modified through mutations selected for during adaptation that improved fitness via ameliorated metabolic flux.

**Results**

**Evolution experiment implementation.** A wild-type *E. coli* K-12 MG1655 strain previously evolved under glucose minimal media at 37 °C\(^31\) (denoted as “Ref”) was used as the starting strain in order to isolate biological changes caused by adaption to the loss of a gene product from those caused by adaption to the growth conditions of the experiment (Fig. 1e). Ref was a non-mutator strain and had the fewest number of mutations among the replicates adaptive laboratory evolution (ALE) endpoints generated.

Perturbations consisting of five separate metabolic gene KOs that were predicted to result in large metabolic rearrangements based on computational metabolic network analysis (see Methods, Supplementary Data 1) were implemented in Ref. Genes (see Methods) encoding enzymes for the reactions of GND (gnd, 6-Phosphogluconate dehydrogenase), GLCptsspp (genes *pts*H, *pts*I, and *crr* corresponding to enzymes HPr, EI, and EIIA, respectively), SUCDi (genes *sucA*, *sucB*, *sucC*, and *sucD* corresponding to the enzyme succinate dehydrogenase), TPI (tpIA, triosephosphate isomerase), and PGI (pgi, phosphoglucose isomerase) were removed to generate strains uGnd, uPtsHcrr, uSdCb, uTpIA, and uPgi, respectively (denoted “unevolved knockout strains” or “uKO”). GND generates d-ribulose-5-phosphate (r5p-D), which is used in nucleotide biosynthesis, and re-charges NADPH, which is used for biosynthesis, in the final step of the oxidative Pentose Phosphate Pathway (oxPPP). *pts*H, *pts*I, and *crr* are primary components of the phosphotransferase system (PTS), which is the primary route for carbon import in *E. coli*, and aids in conserving energy by utilizing phosphoenolpyruvate (pep) to phosphorylate glucose instead of ATP. SUCDi couples the TCA cycle to respiration by charging and donating quinones to the electron transport chain (ETC) via Complex II. TPI avoids bifurcation of lower glycolysis by isomerizing dihydroxyacetone phosphate (dhap) to glyceraldehyde-3-phosphate (g3p) for subsequent enzymatic convert to pyruvate (pyr) via upper glycolysis. PGI converts glucose 6-phosphate (g6p) to fructose 6-phosphate (f6p) in the first committed step through upper glycolysis, thus controlling the flux split between the oxPPP and upper glycolysis.

Replicates of the five knockout strains, as well as Ref, were simultaneously evolved on glucose minimal media at 37 °C in an automated ALE platform\(^31\),\(^32\) denoted “evolved knockout strains” or “eKO” where i denotes the replicate number. The number of replicate endpoints were the following: 2 for “evolved reference strain” (denoted eRef), 3 for eGnd, 4 for ePtsHcrr, 3 for eSdCb, 4 for eTpIA, and 8 for ePgi. Intracellular metabolite levels, gene expression levels, flux levels, and mutations (i.e., system components) were measured for the ref, uKO, and eKO strains during exponential growth. Intracellular metabolite levels consisted of close to 100 absolute and relative quantitative amounts of metabolites from glycolysis, the pentose phosphate pathway, the TCA cycle, energy and redox metabolism, cofactors, nucleotide metabolism, and amino acid metabolism\(^33\),\(^34\). Gene expression levels consisted of relative fold changes from global RNA sequencing. Flux levels consisted of absolute intracellular flux values computed by metabolic flux analysis (MFA) using a genome-scale model from 13C isotope-labeling experiments\(^35\),\(^36\). Mutations consisted of DNA resequencing mapped onto the reference *E. coli* K-12 MG1655 genome.

**Reference strain evolution confirmed the experimental design.** An insignificant fitness change and the fewest number of network changes were found in eRef strains compared to all eKO strains (Fig. 1e). The average numbers of significant component changes per eRef replicate at the metabolite, transcript, and flux levels were 2.0 ± 0.0, 35.0 ± 5.7, and 0.0 ± 0.0 (ave ± stdev, \(n = 2\)), respectively. These changes in systems components were far fewer than in any of the other eKO strains, where the minimum
number of corresponding changes were 19, 341, and 158 (the average number of corresponding changes were 27.7 ± 7.7, 1051.6 ± 513.7, and 307.9 ± 123.2 (ave ± stdev, n = 24)). The average number of mutations per eRef replicate was also the lowest of all lineages, and were primarily found in cell wall biosynthesis genes. The average number of mutations per eRef was 6.5 ± 0.7, while the average number of mutations per all other eKO strains was 12.8 ± 4.5 (ave ± stdev). Overall, these findings demonstrated that the use of a pre-evolved strain minimized the number of confounding component changes.

Evolution to optimal fitness was captured by the data. Multivariate statistical analysis was performed on the data sets generated. Partial least squares discriminatory analysis (PLS-DA) revealed that the primary adaptive response to the gene KO involved a drive towards recovery of the optimal state (i.e., system re-optimization), followed by a secondary adaptive response that described unique alternate states that could be found at the newly evolved state. For almost all cases analyzed, the first most explanatory mode of PLS-DA (Fig. 2) separated Ref and eKO strains from the uKO strain (74% of eKOs from all data types and lineages, see Methods). This result indicated that the primary.

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**Fig. 1** Evolution of knockout (KO) strains from a pre-evolved (i.e., optimized) wild-type strain. a Experimental design using adaptive laboratory evolution (ALE) and enzyme knockouts to investigate system re-optimization following major metabolic perturbations. b An isolated wild-type (wt) E. coli (MG1655 K-12) previously evolved on glucose minimal media at 37 °C31 was used as the starting strain for knockouts of key metabolic genes and subsequent re-evolution, or systems re-optimization. c Reactions disabled by the enzyme knockouts included the phosphotransferase sugar import system (ptsHIcrr), phosphoglucose isomerase (pgi), 6-phosphogluconate dehydrogenase (gnd), triophosphate isomerase (tpi), and succinate dehydrogenase complex (sdhCB). d Adaptive laboratory evolution trajectories of the initial reference knockout and evolved knockout lineages. e Counts of significantly different system components found for each evolved knockout relative to the unevolved knockout. Counts of metabolomic, transcriptomic, and fluxomic data are given as the average and standard deviation of the percent of significant features compared to all features measured for the lineage; counts for mutations are given as the average and standard deviation of the number of significant features (see Methods for criteria for significance).
mode of the data accounted for a dominant transition between the Reference state, perturbed state, and evolved fitness states (i.e., captured systems fitness properties). This result was also reflected in the system component profiles themselves where the majority of component levels were restored or partially restored to reference levels (Fig. 3). For almost all cases analyzed, the second most explanatory mode of PLS-DA separated the Ref and eKO strains. This result indicated that the secondary mode of the data accounted for alternate evolved states (i.e., capturing systems diversity, or a ‘plateau’ in the evolutionary landscape).
alternate states were a result of divergences in trajectory paths that led each replicate evolution towards a unique optimal state. This characteristic was further reflected in the unique distribution of component profiles between each of the eKOs.

**Component profiles reveal systematic variations.** In order to dissect the drive towards fitness (mode 1) and generation of diversity (mode 2) further, changes in each system component (i.e., metabolite, transcript, and flux level) between Ref, uKO, and eKO strains were grouped into six profiles (Fig. 3a, see Methods): novel, overcompensated, partially restored, reinforced, restored, and unrestored. The distribution between these six profiles for each component type are shown with horizontal bar charts in Fig. 3b–d. Several trends were found based on these six profiles.

First, the occurrence of profiles varied between omics data types. Overall, the metabolite levels were the most distributed between the six profiles (i.e., had the least deviation). The ave ± stdev of the relative standard deviation (RSD) between profiles \((n = 12, \pm \text{directions for each of the six profiles})\) and across lineage \((n = 22)\) was 39.9 ± 14.1, 132.1 ± 45.9, and 84.0 ± 12.7% for metabolites, transcript, and fluxes, respectively. In contrast, the transcript levels were dominated by the restored profile, and flux levels were dominated by the restored and unrestored profile. For example, the *pgi* lineages had an ave ± stdev of restored profiles of 50.9 ± 5.0, 80.1 ± 8.3, and 66.9 ± 3.1% for metabolites, transcripts, and fluxes, respectively. The more even metabolite distribution compared to the transcript levels or flux levels indicated that the changes in metabolite levels were less constrained than the gene expression and fluxes.

Second, distribution amongst the profiles varied between KOs. The lineages with the greatest initial loss of fitness had a greater percentage of novel, overcompensated, reinforced, and unrestored profiles than the lineages with a smaller initial loss of fitness. This
The difference was most evident for the transcript levels (ave ± stdv of 2.7 ± 0.4, 8.2 ± 3.7, 31.3 ± 23.4, 20.3 ± 10.9, and 18.6 ± 1.8%), and fitness change across evolution of 11.9 ± 3.9, 11.1 ± 2.9, 365.2 ± 20.0, 337.8 ± 73.8, and 244.3 ± 7.1% for the gnd, sdhCB, pgi, ptsHicrr, and tpiA lineages; Pearson’s R = 0.94, P-value < 0.017, Supplementary Fig. 1). This observation suggests that the larger the loss in fitness, the greater the number of Innovative (as opposed to restorative) network changes required to regain fitness. Future work with larger sample sizes will be needed to confirm this trend.

Third, the distribution amongst profiles also varied between evolved strain lineages. For example, the eight ePgi endpoints had varying levels of fitness (ave ± stdv of 0.68 ± 0.006, 0.61 ± 0.015, 0.65 ± 0.008, 0.72 ± 0.009, 0.64 ± 0.008, 0.69 ± 0.018, 0.67 ± 0.006, 0.69 ± 0.015 h⁻¹), and noticeable differences in the distribution of profiles among endpoints. This highlighted the biochemical differences in evolved network configurations during adaptation to overcome the perturbation.

Finally, a decoupling between degree of fitness change and degree of -omics data change was apparent. The tpiA, pgi, and ptsHicrr lineages incurred the largest loss and recovery of fitness while the gnd and sdhCB lineages incurred only minimal changes in fitness. However, major changes in all -omics data measured between Ref and uKO and between uKO and eKO strains were found in all lineages (Figs. 2 and 3). Interestingly, major changes often occurred in common system components. Major changes could be traced to either perturbed metabolites that act as allosteric or transcriptional regulators (which is consistent with previous studies) or mutations that resulted in alterations to gene expression. The observation about commonly perturbed metabolite levels and mutations coupled with our previous three observations about the profile distributions indicated that changes in fitness and -omics data were independent, given that major alterations in gene expression and protein production could occur as a result of perturbations in relatively few key regulators.

The system component profiles were mapped to the biochemical network of E. coli and analyzed to develop a general framework for understanding evolution at the molecular level. It is important to highlight that the component profiles described above were in all of the analyses presented below. The component profiles were assigned based on statistical criteria. They provided a unitless metric to compare and map multiple data types when quantitative relationships between data types have not been fully established. The component profiles also provided robustness by basing the analysis on change in values between states (i.e., ref, uKO, and eKO) instead of the absolute value found in any one state.

**Changed flux distribution was most prevalent during ALE.** Changes in pathway usage between the Ref, uKO, and eKO strains were calculated, and differences between the flux distribution in the uKO and eKO strains were grouped into changed flux distribution (i.e., the pathway usage was changed) or changed flux capacity (i.e., the same pathway was used but at a higher flux level, see Methods for extended definitions, Fig. 4a–d). Changed flux distribution was found to be more prevalent than changed flux capacity. Changed flux distribution was found to occur 55.6% of the time, while a change in flux capacity was found to occur 22.0% of the time across all perturbations and lineages (Fig. 4e). The remaining 22.4% of cases were unaffected.

For example, flux was initially re-routed through the Entner–Doudoroff (ED) pathway in uGnd (Fig. 4f) in order to generate ribose through the non-oxidative Pentose Phosphate pathway (non-oxPPP). The ED pathway has a net yield of one ATP, NADH, and NADPH per molecule of glucose, whereas glycolysis has a net yield of two ATP and NADH. Instead, the eGnd strains limited the use of the oxidative pentose phosphate pathway (oxPPP) and increased the flux distribution was found to be more prevalent than changed ux capacity. Changed flux distribution was found to occur 55.6% of the time, while a change in flux capacity was found to occur 22.0% of the time across all perturbations and lineages (Fig. 4e). The remaining 22.4% of cases were unaffected.

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indicated that the initial flux distribution of the uKO strains following perturbation were often suboptimal, and required a change primarily in flux distribution and secondarily in flux capacity in order to restore fitness in the eKO strains.

Perturbed metabolite levels triggered TRN responses in uKOs.

Transcriptional regulatory network (TRN) responses in uKOs that were associated with carbon metabolism, nitrogen metabolism, iron regulation, oxidative stress, DNA repair, and other stress responses that control the majority of known functions in *E. coli* were linked to corresponding changes in regulatory metabolite levels (see Methods). Perturbed metabolite levels were traced to known TRN responses by mapping measured metabolite profiles to metabolite-activated transcription factors (TFs). The relationship (i.e., positive or negative) between a metabolite profile, a TF that interacts with the metabolite, and the expression profiles of the transcription units (TUs) regulated by the TF (see Methods, Fig. 5) were compared. Strong evidence (i.e., statistically significant gene expression pattern for genes that are regulated by a single TF, see Methods) for changed TF activation profiles (analogous to the system component profiles, Fig. 2) were identified for 75 TFs (Supplementary Data 2, Fig. 5). These included 7 global TFs (i.e., CRP, Fis, IHF, ArcA, Lrp, FNR, and HNS) and 68 pathway-specific TFs (see Methods). The activation profiles of 15 TFs (which included the 7 global TFs and the 8 pathway-specific TFs ArgR, CpxR, Cra, Fur, NsrR, OxyR, PhoB, and TyrR) were changed across all lineages. The remaining 60 TFs appeared to be changed in a perturbation and lineage-specific manner.

Interestingly, TF activation and TF gene expression was not coincidental (ave ± std 5.4 ± 3.8, 4.1 ± 2.6, and 70.5 ± 6.1% agreement, disagreement, no significant change in expression profile per lineage, respectively, Supplementary Data 3). This result indicated that changed TF activation was mostly attributed to changed concentrations in their metabolic activators as opposed to changed TF gene expression levels. Similar observations have been made for sigma factors and the expression levels of sigma factor DNA binding operons in response to a key rpoB mutation, where alterations in the binding of the regulator subsequently altered gene expression of regulated operons. For example, a changed CRP activation was found in all lineages due to elevated levels of cAMP in the uKOs. CRP was not differentially expressed in any of the lineages, but restored cAMP levels were mirrored by restored gene expression of TUs solely regulated by CRP-cAMP (Supplementary Data 5). ArcA provided another example for global TF activation without a significant gene expression change. The restored activation profile of ArcA and several other iron–sulfur–cluster homeostasis TFs found in all lineages could be linked to changes in TCA cycle intermediates as well as quinone pools (e.g., gnd and sdhcCB). The ArcAB two-component system in particular modulates genes in response to changes in respiratory conditions that are communicated via the intermembrane quinone pools.

Pathway-specific TF activation was also identified in the uKOs. A change in activation of the PurR regulator was found in pgI and several other lineages due to changed levels of purine degradation products. Specifically, the purR dimer binds hypoxanthine and guanine, and regulates genes involved in purine metabolism. The concentration profiles of hypoxanthine and/or guanine matched the expression profile for purR-target genes, while the expression profile for purR itself did not (Supplementary Data 4). In another example, the change in activation of TyrR in many lineages was found to be attributed to the change in levels of L-tyrosine and L-phenylalanine. Another example of pathway-specific TF activation involved the use of small regulatory RNA. A sugar phosphate toxicity response was generated by abnormal elevations in glucose 6-phosphate (g6p) and an imbalance of the glycolytic intermediates in uPgi. SgrR is thought to bind hexose phosphates and induce the expression of the small RNA sgrS55–57 (Fig. 5), which initiates the observed response. It was found that the metabolite concentration profiles matched sgrS expression profiles. SgrS transcriptionally regulates a number of genes that are involved in re-balancing glycolytic intermediates. One target of sgrS attenuation is purR, which explains the opposing purR expression profile compared to its TF activation profile described above. Interestingly, abnormal elevations of g6p and induction of SgrR and SgrR regulons were also found in ptsH1ccr. Additional examples are provided in Fig. 5.

The common perturbation of TFs by small molecules indicated that the majority of transcriptional changes observed may not be beneficial to fitness compensation, but a consequence of “hard-coded” regulatory circuits selected for through evolution that were triggered by perturbations to key metabolite regulators. Many of the hard-coded regulatory circuits were revealed through ALE.
Component profiles revealed competing layers of regulation.

Cells contain multiple levels of counteracting regulatory mechanisms that often overlap. For example, a relatively low agreement between changes in gene expression profiles and metabolic flux profiles (i.e., gene–protein–reaction association, GPR) within each lineage was found (Supplementary Data 2). Specifically, an average agreement of 27.5% (stdev = 17.4%, n = 22) and average disagreement of 11.5% (stdev = 6.8%, n = 22) was found. A similarly low agreement between types of literature-derived regulation were found (Supplementary Data 2). These findings are consistent with previous work and can be explained by the actions of multiple and competing layers of regulation.

Competing levels of regulation can be measured through the disagreement between changes in system components and literature-derived networks of biomolecular interactions (Fig. 5). Disagreements were found to fall into three main groups: (1) counteracting regulatory mechanisms, (2) evidence for inaccurate or incomplete knowledge of regulatory networks, and (3) changes to regulation introduced through fixed mutations. Evidence of competing layers of regulation for 89 regulators (i.e., any biological component that can affect a change in another component, e.g., TF or small-molecule) across 5887 regulated entities (i.e., any biological component that is subject to regulation, e.g., TU or enzyme) were found. Evidence of inaccurate or incomplete knowledge of the regulatory network in 38 regulators across 631 regulated entities was found (Supplementary Data 3). While it is infeasible to investigate each discrepancy here, specific examples are given that illustrate the above three mechanisms.

In an example of counteracting regulatory mechanisms, a hierarchy of TF control over gene expression was recapitulated. The activation profile of Fis54–56 was found to conflict with its consensus activation profile of the pyrD promoter in all of the ptsHIcrr endpoints 1 and 3 (Fig. 5). This indicated that pyrD expression was dominated by PurR regulation. In another example, a restored activation of sgrS found in the ptsHIcrr lineages and a novel activation of sgrS found in the ptsHIcrr endpoints 1 and 3 negated the transcription factor regulation of sgrS target genes57, 58. In another example, the activation profile of cAMP-CRP was found to conflict with its consensus activation profile...
on the gapA promoter in all of the tpiA lineages, whereas the Cra activation profile was found to agree with gapA expression profile (Fig. 5d)69, 79. cAMP-CRP and Cra bind upstream of the promoter region of gapA; CRP-cAMP promotes gapA transcription while Cra inhibits gapA transcription69, 80. This finding indicated that inhibition of gapA expression by Cra was dominant over the promotion of gapA expression by cAMP-CRP, as is consistent with recently reported data71. In another example, the activation profile of the TF Nac, which acts as a global regulator of nitrogen metabolism,72 was found to co-occur with its consensus activation profile for the expression of gabP on the csID promoter in tpiA replicates 1 and 2. Expression of gabP is controlled by cAMP-CRP, CsrI, HNS, and Lrp41, 73. Only the activation profile of Lrp matched, indicating that the expression of gabP was driven by Lrp in those two replicates. In another example, the transcription attenuation by UTP was found to dominate the regulation of pyrLBl operon by ppGpp74, 75.

Unresolved discrepancies in regulatory annotations were found. The expression profiles of regulons that were controlled only by Fur76–78 were found to be inconsistent. Specifically, the expression profiles for entS, exbD, ficl, fepG, fepD, flhuA, flhuE, rybB, and yjiZ, conflicted with that of clp (Fig. 5c). The discrepancies indicated that another TF or transcriptional regulator is present that also controls the transcript levels of these TFs. This finding recapitulated the effects of known regulation, but also revealed the effects of unknown or not fully characterized regulatory mechanisms. The latter provide suggestions for new experimental lines of inquiry.

Mutations altered regulation and enzymatic function. A large number of mutations were identified in the eKOs that changed the effects of global and pathway-specific regulators (discussed above) or targeted specific pathways or imbalances. In total, 673 mutations were found in the eKOs (Supplementary Data 5 and 6). The mutations were found to primarily be single nucleotide polymorphisms (SNPs, 66%), were primarily located in coding regions (48%), and were primarily associated with membrane proteins and transcription factors (27 and 29%, respectively). See Supplementary Data 5 and Fig. 6 for a detailed overview of all mutations found in the eKO strains. The reader is directed to McCloskey et al.27–30 for further in depth characterizations of individual mutations discussed below.

Mutations selected during ALE changed many global regulators. For example, 17% of mutations affected regulators of carbon transport and metabolic processes that appear to offset the activation of operons induced by CRP-cAMP. These included mutations to galR, malT, and crr in the ePgi strains that appeared to negate repression of galR controlled operons. The mutations may give the evolved strains an additional route to import and catabolize glucose because the galactose importer also has the ability to import glucose albeit with lesser affinity than galactose. In addition, the mutation may have improved the fitness of the ePgi strains by increasing the availability of phosphoenolpyruvate (pep) for aromatic amino acid production. Interestingly, mutations in galR or at the galR operon in ePtsHicrr02/04 and in eTpiA01/03 also resulted in the upregulation of GalR controlled genes. The prevalence of galR mutations may indicate that expression of the gal regulon may aid in increasing fitness when the ability to import glucose is impaired or the levels of pep are inadequate for aromatic amino acid production. Additional mutations that affected carbon transport processes included ptsG, galR, and nagC in the ePtsHicrr strains, and ptsG, galR, and nagA, nagC, and nagE in the eTpiA strains.

A series of mutations were also identified that altered protein homeostasis networks, two-component systems, small RNA networks, and the sigma factor networks. These included mutations that altered the Lon protein homeostasis network in ePgi and the two-component system RcsA/RcsB in ePtsHicrr that targeted pathways involved in cell motility, acid resistance, and cell wall biosynthesis. Mutations that altered the SPF small RNA networks, RpoC core RNA polymerase unit, and RpoD sigma factor networks in ePgi were found. Alterations to stress response systems that included SoxS/SoxR in pgI and PhoB/PhoR in tpiA involving oxidative stress and phosphate stress, respectively, were also found.

Mutations were also identified that changed the regulation of pathway-specific TFs. These occurred in a KO-specific manner, and appeared to optimize specific pathways at the regulatory level. For example, the expression of the methylglyoxal pathway in eTpiA strains were altered to more efficiently convert methylglyoxal to lactate through mutations that altered methylglyoxal detox pathway gene expression. These examples of global and pathway-specific regulatory shifts indicated that mutations that affect hubs in complex regulatory networks are common in adaptive evolution37, and provide a fitness advantage by rewiring regulatory network responses that may no longer be optimal for fitness.

Rarer were mutations that introduced innovations that appeared to target-specific metabolite imbalances. For example, the levels of nadph, which is used to drive biosynthesis, was affected in many of the KOs. Mutations were found in the trans hydrogenases in several of the ePgi strains and in all of the eGnd strains to compensate for an overproduction and underproduction of NADPH, respectively. A mutation found in the active site of seven of the eight ePgi endpoints in isocitrate dehydrogenase appeared to alter cofactor specificity to allow for the use of nadh.

Discussion
Taken together, the combination of study design, automated ALE, multi-omic data sets, and statistics and bioinformatics revealed common mechanisms of adaptation whereby imbalances in metabolite levels from altered fluxes triggered a multitude of network responses that were readjusted by mutations selected for during evolution (Fig. 7). The mutations that fixed during adaptation acted to rewire many existing hardwired responses and/or introduce novel network functions that addressed the imbalances that the initial KO lesion created. The findings of this
study represent a step towards developing a fundamental understanding of how cells mechanistically adapt to gene loss from a systems perspective that accounts for proximal and distal relationships in the metabolic and regulatory network. Novel mechanisms and inconsistencies, revealed through adaptation, between measurement and known regulatory mechanisms identified in the case studies present opportunities for future discovery (Supplementary Data 2 and 4). Specific avenues of exploration may include the effect of regulation acting on different time-scales (i.e., transcriptional vs. allosteric regulation) or the effect of RNA and protein stability and degradation that were not addressed in this study.

Methods

**Biological material.** A glucose, 37 °C, evolved *E. coli* K-12 MG1655 (ATCC 700926)\(^{31,32}\) served as the starting strain. Lambda-red-mediated DNA mutagenesis\(^{86}\) was used to create the knockout strains. Knockouts were confirmed by PCR and DNA resequencing. Genes gnd, ptsH, ptsI, crr, sdhC, sdhA, sdhD, sdhC, trpA, and pgI encoding for the reactions of 6-phosphogluconate dehydrogenase (GND), phosphotransferase sugar import (GLCptspp), succinate dehydrogenase complex (SUCDi), triosephosphate isomerase (TPI), and phosphoglucone isomerase (PGI) were removed. PPC was also deleted, but resulted in an auxotrophy for asp-1, and was not included in the study. Genes aceE, aceF, zwf, and atpA encoding for the reactions of PDH, G6PDH2r, and ATPS4rpp could not be removed using the method of Datsenko et al.\(^{86}\). All cultures were grown in unlabeled or labeled glucose M9 minimal media\(^{87}\) with trace elements\(^{88}\) at 25 mL of working volume in a 50 mL autoclaved tube. The cultures were maintained at 37 °C on a heat block and aerated using magnetics.

**Materials and reagents.** Uniformly labeled\(^{13}\)C glucose and \(^{1}\)C glucose were from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA). Unlabeled glucose and other reagents were from Sigma-Aldrich (St. Louis, MO). LC–MS/MS reagents were from Honeywell Burdick & Jackson* (Muskegon, MI), Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich (St. Louis, MO).

**Reaction knockout selection.** iJO1366\(^{89}\) was used as the metabolic model for *E. coli* metabolism; GLPK (version 4.57) was used as the linear program solver. MCMC sampling\(^{90}\) was used to predict the flux distribution of the optimized reference strain. Uptake, secretion, and growth rates were constrained to the measured average value ± SD. Potential reaction deletions were ranked by (1) averaged sampled flux, (2) the number of immediate upstream and downstream metabolites that could be measured, (3) the number of genes required to produce a functional enzyme. Reactions involved in sampling loops, that were spontaneous, were computationally or experimentally essential, or were not actively expressed under the experimental growth conditions were not included in the analysis. Also, reactions that would require more than one genetic alteration to abolish activity

**Results and discussion.** Single-gene knockout strains were generated using Lambda-red-mediated DNA mutagenesis. Knockout strains were generated from the single-gene knockout strains using precise genomic deletion by Red/ET recombination. The functional or structural category of the mutated gene. Categories are based on the “parent class” as found in the EcoCyc database\(^{103}\). The class of mutation. The type of mutation. The location of the mutation. Locations include coding regions, regions associated with cryptic prophages, intergenic regions, and repetitive elements (REP or RIP). The functional or structural category of the mutated gene. Categories are based on the “parent class” as found in the EcoCyc database\(^{103}\). The type of mutation. The location of the mutation. Locations include coding regions, regions associated with cryptic prophages, intergenic regions, and repetitive elements (REP or RIP). The functional or structural category of the mutated gene.

**Fig. 6** Overview of mutation statistics. See Supplementary Data 6 for detailed statistics of each category and categories not shown. **a** The type of mutation. Mutations include amplification (AMP), deletion (DEL), insertions (INS), mobile element aided insertions or deletions (MOB), single nucleotide polymorphism (SNP). **b** The location of the mutation. Locations include coding regions, regions associated with cryptic prophages, intergenic regions, regions two coding genes not classified as an intergenic region (intergenic/intergenic), and repetitive elements (REP or RIP). **c** The class of mutation. Classes include frameshifts, frameshifts resulted in a truncated CDS, missense, non-frameshifts, peptide truncations, and other unclassified mutations. **d** The functional or structural category of the mutated gene.
Mass isotopomer distributions (MIDs) were acquired on the same instrument and processed using MultiQuant software.

Fluxomics. Fluxomics samples were acquired from triplicate cultures (10 mL of cell broth at an OD600 ~ 1.0) using a modified version of the FSF technique as described previously. MIDs were calculated from biological triplicates, each run in analytical duplicates (n = 6). MIDs with an RSD greater than 50% were excluded. In addition, MIDs with a mass that was found to have a signal greater than 80% in unlabeled or blank samples were excluded. A previously validated genome-scale MFA model of E. coli with all MFN minimal alterations was used for alternative reactions using INCA (version 1.4) as described previously. The model was constrained using MIDs as well as measured growth, uptake, and secretion rates. Best flux values that were used to calculate the 95% confidence intervals were estimated from 5000 bootstraps.

The 95% confidence intervals were used as lower and upper bound reaction constraints for further constraint-based analyses. MFA derived constraints that violated optimality were discarded and re-sampled. The descriptive statistics (i.e., mean, median, interquartile ranges, min, max, etc.) for each reaction for each model were calculated from 5000 samples from 5000 steps using optGpSampler (version 1.1), which resulted in an approximate mixed fraction of 0.5 for all models. A permuted P-value < 0.05 and geometric fold-change of sampled flux values > 0.001 were used to determine differential flux levels, differential metabolite utilization levels, and differential subsystem utilization levels between models. Demand reactions and reactions corresponding to unassigned transport; outer membrane porin, transport; inner membrane, inorganic ion transport and metabolism, transport; outer membrane, nucleotide salvage pathway, oxidative phosphorylation were excluded from differential flux analysis. The geometric fold-change of the mean between models and the reference model were used for hierarchical clustering; the median, interquartile ranges, min, and max values of each sampling distribution for each reaction and model were used as representative samples for downstream statistical analyses.

Transcriptomics. Total RNA was sampled from triplicate cultures (3 mL of cell broth at an OD600 ~ 1.0) and immediately added to 2 volumes Qiagen RNA-protect Bacteria Reagent (6 mL), vortexed for 5 s, incubated at room temperature for 5 min, and immediately centrifuged for 10 min at 17,500 RPMs. The supernatant was decanted and the cell pellet was stored in the -80 °C. Cell pellets were then incubated with Readylyse Lysozyme, Supersasel, Protease K, and 20% SDS for 5 min, and immediately centrifuged for 10 min at 17,500 RPMs. The supernatant was added to 50 volumes of 100 mM Tris pH 8.0, 500 mM NaCl, and 10 mM EDTA. RNA was quantified using a Nano Drop and checked for quality using an RNA- nano chip on a bioanalyzer. The RNA was removed using Epicentre’s Ribozyme RNA Purification kit for Gram Negative Bacteria. A KAPA Stranded RNA-Seq Kit (Kapa Biosystems KK8401) was used following the manufacturer’s protocol to create sequencing libraries with an average insert length of around ~300 bp for two of the three biological replicates. Libraries were run on a MiSeq and/or HiSeq (Illumina).

RNA-Seq reads were aligned using Bowtie2 (version 1.1.2 with default parameters). Expression levels for individual samples were quantified using Cufflinks27 (Version 2.2.1, library type fr-firststrand) Quality of the reads was assessed by tracking the percentage of unmapped reads and expression level of genes that mapped to the ribosomal gene loci rrsA-F and rrmA-F. All samples had a percentage of unmapped reads <7%. Differential expression levels for each condition (n = 2 per condition) compared to either the starting strain or initial knockout strain were calculated using Cuffdiff28 (Version 2.2.1, library type fr-firststrand, library norm geometric). Genes with an 0.05 FDR-adjusted P-value < 0.01 were considered differentially expressed. Expression levels for individual samples in all combinations of conditions tested in downstream statistical analyses were normalized using Cuffnorm29 (Version 2.2.1, library type fr-firststrand, library norm geometric). Genes with unmapped reads were imputed using a bootstrapping
approach as coded in the R package Amelia II (version 1.7-4, 1000 imputations). Remaining missing values were filled using the minimum expression level of the data set. Normalized FPKM values for gene expression were log2 normalized to generate an approximately normal distribution prior to any statistical analysis. All replicates for a given condition were found to have a pairwise Pearson correlation coefficient of 0.95 or greater.

DNA resequencing. Total DNA was sample from an overnight culture (1 mL of cell broth at an OD600 of ~2.0) and immediately centrifuged for 5 min at 8000 RPMs. The supernatant was decanted and the cell pellet was frozen in the –80°C. Genomic DNA was isolated using a Nucleospin Tissue kit (Macherey Nagel 740952.50) following the manufacturer’s protocol, including treatment with RNase A. Resequencing libraries were prepared using a Nextera XT kit (Illumina FC-131-1024) following the manufacturer’s protocol. Libraries were ran on a MiSeq (Illumina).

DNA resequencing reads were aligned to the E. coli reference genome (U00996.2, genbank) using Bresq+ (version 0.26.0) as populations. Mutations with a frequency of <0.1, P-value >= 0.01, or quality score <6.0 were removed from the analysis. In addition, genes corresponding to crl, insertion elements (i.e., insH1, insB1, and insA), and the rhs and rtx gene loci were not considered for analysis due to repetitive regions that appear to cause frequent miscalls when using Bresq.

mRNA and peptide sequence changes were predicted using BioPython (https://github.com/biopython/biopython.github.io/). Large regions of DNA (minimum of 200 consecutive indels) were considered to be due to genome duplication.

Structural analysis. Corresponding PDB files for genes with a mutation of interest were downloaded from PDB99, 100. Structural models for genes for which there were no corresponding PDB files were taken from I-TASSER generated homology models101 or generated using the I-TASSER protocol102. The BioPython predicted sequence changes and important protein features as listed in EcoCyc104 were visualized and annotated using VMD105.

System component statistical feature identification analyses. Network components (i.e., RNA-seq, metabolomics, fluxomics, genomics) were pre-processed as described above, and subjected to a feature identification analysis pipeline. Network components for each lineage were first subjected to a differential test (ref vs. KO, KO vs. endpoints, ref vs. endpoints, and endpoints vs. endpoints). The criteria for significance are described above, and subjected to a feature selection pipeline. Metabolites, gene expression and fluxomics features were then used as weights in calculating the shortest path between the reference and knockout from the endpoint. PCA models were then constructed for the reference, knockout, and all endpoints for each network perturbation. The PCA models were validated using cross validation (CV type of Krossmanowski, default 5 segment with 5 CV runs per segment with minimum number of segments equal to the number of samples). Partial Least Squares Discriminatory Analysis (PLS-DA) was implemented using the R package plsd106 (version 2.5, univariate scaling, centering, Canonical Powered Partial Least Squares (cplps) PLS-DA) was used to project replicate samples into component space. PLS-DA models were then first constructed for the reference, knockout, and endpoint for each of the lineages to confirm that the primary component best separated the reference and endpoint from the knockout, and that the second component best separated the reference and knockout from the endpoint. PCA models were then constructed for the reference, knockout, and all endpoints for each network perturbation. The PCA models were validated using cross validation (CV type of Krossmanowski, default 5 segment with 5 CV runs per segment with minimum number of segments equal to the number of samples). Partial Least Squares Discriminatory Analysis (PLS-DA) was implemented using the R package plsd106 (version 2.5, univariate scaling, centering, Canonical Powered Partial Least Squares (cplps) PLS-DA) was used to project replicate samples into component space. PLS-DA models were then first constructed for the reference, knockout, and endpoint for each of the lineages to confirm that the primary component best separated the reference and endpoint from the knockout, and that the second component best separated the reference and knockout from the endpoint. PLS-DA models were then constructed for the reference, knockout, and all endpoints for each network perturbation. The PLS-DA models were validated using cross validation (default 10 segments with minimum number of segments equal to the number of samples). The loadings distance (i.e., the difference in loadings values) between the ref and uKO strain along axis 1 (i.e., model 1) was used as a threshold to determine whether an eKO strain matched the general mode 1 and mode 2 trends identified in section 2a. A relative distance for each eKO strain along axis 1 was calculated as follows: "relative distance = distance(endo)/distance(ref vs. KO) where i = endpoint replicate for a particular KO lineage and j = each KO lineage. An eKO strain with a relative distance greater than 70% along axis 1 was determined to match the trend.

Metabolite, flux, and gene set enrichment analyses. Metabolite and gene set enrichment analyses were conducted using the subsystem categories of jO1366. Flux and metabolite flux sum set enrichment analyses were conducted using the subsystem categories of iDM2015. A P-value < 1e-3 (hypergeometric test) was used as a threshold for enriched subsystems. Genes corresponding to differently expressed genes was also performed using with R package topGO107 with GO annotations for E. coli108. A P-value < 0.05 (Fischer statistic, parent–child algorithm109) was used to test for enriched biological processes and molecular functions.

Network distance and graph analyses. The inverse mean values from sampled distribution that were constrained by the 95% confidence intervals derived from the frequency of nodes. Nodes were weighted as weights in calculating the shortest path from metabolite A to B. The iDM2015 network was deconstructed into a directed acyclic graph with metabolites and reactions composing the nodes and the connections between metabolites and reactions composing the links. Metabolites that did not contain carbon were excluded from the graph network. In addition, metabolites corresponding to CO2, CO, mql8, mql8h2, 2dmmql8, 2dmmql8h2, q8, qh2b, thf, ACP were also excluded. Metabolites corresponding to udpglc, adpglc, agmp were substituted as glycerol_c, uacgam, uacg, respectively, as they were not present in the lumped and reduced iDM2015 network. The A*star algorithm as implemented in the python package networkx (https://github.com/networkx/networkx) (version 1.11) was used to calculate the shortest path of the graph network. The distance from metabolite A to B was calculated as half minus 1 the computed shortest path.

A redistribution of flux was defined as a change in path or path length between the reference and knockout and endpoint from the knockout. A change in flux was defined as a change in flux or path length between the reference and knockout, but not between the knockout and endpoint. Nodes (i.e., metabolites) were categorized as intermediates, carriers, biomass precursors, and/or nucleotide salvage products. The correlation (Spearman R, P-value < 0.05) between path and path length and metabolite level was calculated between intermediates and carriers, carriers and biomass precursors, intermediates and biomass precursors, carriers and nucleotide salvage products, and biomass precursors and nucleotide salvage products.

Biomass to network component correlation analysis. EcoCyc103 subsystems for the following biomass producing pathways were used in the analysis: amines and polyamines biosynthesis, amino acids biosynthesis, nucleosides and nucleotides biosynthesis, fatty acid and lipid biosynthesis, cofactors, pro tease groups, electron transport, cell division, cell cycle, nucleotide biosynthesis, and carbohydrates biosynthesis. Gene identifiers from these pathways were mapped onto iDM2014 via the GPR relation to identify biomass producing reactions and metabolites. The analysis was conducted at the level of individual lineages using the system component profiles of redirected−, novel+, overcompensation+, partially restored−, partially restored+−, reinforced−, reinforced+, restored+, restored−, unreacted−, unreacted+− profiles were encoded in integer form as 1-1-0, 0-0-1, 1-0-2, 1-2-0, 2-0-1, 0-2-1, 2-1-0, 0-1-0, 1-0-0, and 0-1-1. System components were binned into profiles when a Pearson correlation coefficient >0.88 was calculated. Only negligible changes in the assignment of profiles were found when using absolute or relative component units (e.g., mmolGDC3− vs. log2(FC vs. ref)) or different correlation methods (i.e., Spearman).

System component statistical sample trend analysis. Components identified from the differential tests (except for metabolomics) were used for sample trend analyses. Hierarchical clustering was used to diagnose sample groupings and distances between samples (distance metric of Euclidean and linkages method of complete). Principal component analysis (PCA) as encoded in the R package pcamethods110 (version 1.64.0, univariate scaling, centering, SVD PCA) was then used and the representative unsupervised method to project samples into component space, and confirm the relative magnitude and direction of component weights. PCA models were first constructed for the reference, knockout, and endpoint for each of the lineages to confirm that the primary component best separated the reference and endpoint from the knockout, and that the second component best separated the reference and knockout from the endpoint. PCA models were then constructed for the reference, knockout, and all endpoints for each network perturbation. The PCA models were validated using cross validation (CV type of Krossmanowski, default 5 segment with 5 CV runs per segment with minimum number of segments equal to the number of samples). Principal Least Squares Discriminatory Analysis (PLS-DA) was implemented using the R package plsd106 (version 2.5, univariate scaling, centering, Canonical Powered Partial Least Squares (cplps) PLS-DA) was used to project replicate samples into component space. PLS-DA models were then first constructed for the reference, knockout, and endpoint for each of the lineages to confirm that the primary component best separated the reference and endpoint from the knockout, and that the second component best separated the reference and knockout from the endpoint. PLS-DA models were then constructed for the reference, knockout, and all endpoints for each network perturbation. The PLS-DA models were validated using cross validation (default 10 segments with minimum number of segments equal to the number of samples). The loadings distance (i.e., the difference in loadings values) between the ref and uKO strain along axis 1 (i.e., model 1) was used as a threshold to determine whether an eKO strain matched the general mode 1 and mode 2 trends identified in section 2a. A relative distance for each eKO strain along axis 1 was calculated as follows: "relative distance = distance(endo)/distance(ref vs. KO) where i = endpoint replicate for a particular KO lineage and j = each KO lineage. An eKO strain with a relative distance greater than 70% along axis 1 was determined to match the trend.
positively correlated (correlation coefficient > 0.88, Pearson, r) with growth rate or negatively correlated (correlation coefficient < 0.88, Pearson, r) with growth rate were identified.

Inter- and intra-component correlation analysis. A global pairwise correlation between metabolite concentrations, transcript levels, and fluxes was performed by comparing the agreement and disagreement between component profiles of restored+, novel+, overcompensation+, partially restored-, unrestored-, and reinforced+. Components with matching profiles with correlation coefficients > 0.88 (Pearson, R) were correlated; with components matching profiles with correlation coefficients < 0.88 (Pearson, R) were anti-correlated. A similar global pairwise correlation between metabolite concentrations, transcript levels, and fluxes was performed (units of log2(FC vs. ref)) for comparison (data not shown). Components with a correlation coefficient > 0.88 (Spearman, r) were correlated; Components with a correlation coefficient < 0.88 (Spearman, r) were anti-correlated.

Regulation to network component correlation analysis. Significantly correlated components were compared to annotated gene-to-reaction, and metabolite-to-reaction interactions annotations in iJO1366, and to annotated transcription factor-to-gene, metabolite-to-transcription factor, metabolite-to-transcription factor-to-gene, metabolite-to-transcript, and metabolite-to-reaction regulatory interactions annotations in iJO1366, and to annotated transcription factors, nEPs

\[ \text{Regulator activation categorization}. \] A profile for the activation status of each regulator for each knockout evolution was determined. The analysis was first limited to regulated entities that had only a single annotated regulator. The analysis was then expanded to include all regulators and regulated entities. A category weight for each regulated entity for each endpoint was calculated as follows: weight, i\(_j\) = abs(score\(_j\))/(1+abs(score\(_k\))) \[ i = \text{endpoint}, j = \text{category}, k = \text{regulator} \], where \( nEPs \) is the number of endpoints per knockout evolution, corr = correlation coefficient, \( nRegulators \) = number of regulators per regulated entity. A confidence score for each regulator for each knockout evolution was calculated as follows: confidence\(_j\) = sum(abs(weight\(_i\)j)) where \( i = \text{endpoint}, j = \text{endpoint}, k = \text{regulator} \). A higher confidence score indicates a consistently higher correlation to the category across all regulated entities that are regulated by the regulator.

Code availability. Published software used in this study are noted in the Methods. Custom software used for the analyses presented in this study are deposited on Github (https://github.com/dmcloskey).

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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