Title: PKA regulatory subunit Bcy1 couples growth, lipid metabolism, and fermentation during anaerobic xylose growth in *Saccharomyces cerevisiae*

Short Title: Growth and fermentation coupling on anaerobic xylose

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Abstract

Organisms have evolved elaborate physiological pathways that regulate growth, proliferation, metabolism, and stress response. These pathways must be properly coordinated to elicit the appropriate response to an ever-changing environment. While individual pathways have been well studied in a variety of model systems, there remains much to uncover about how pathways are integrated to produce systemic changes in a cell, especially in dynamic conditions. We previously showed that deletion of Protein Kinase A (PKA) regulatory subunit BCY1 can decouple growth and metabolism in Saccharomyces cerevisiae engineered for anaerobic xylose fermentation, allowing for robust fermentation in the absence of division. This provides an opportunity to understand how PKA signaling normally coordinates these processes. Here, we integrated transcriptomic, lipidomic, and phosphor-proteomic responses upon a glucose to xylose shift across a series of strains with different genetic mutations promoting either coupled or decoupled xylose-dependent growth and metabolism. Together, results suggested that defects in lipid homeostasis limit growth in the bcy1Δ strain despite robust metabolism. To further understand this mechanism, we performed adaptive laboratory evolutions to re-evolve coupled growth and metabolism in the bcy1Δ parental strain. Genetic mutations in PKA subunit TPK1 and lipid regulator OPI1, among other genes underscored a role for lipid homeostasis, which was further supported by evolved changes in lipid profiles and gene expression. We suggest several models for how cells coordinate growth, metabolism, and other responses in budding yeast and how restructuring these processes enables anaerobic xylose utilization.

Author Summary

All organisms utilize an energy source to generate the cellular resources needed to grow and divide. These individual processes have been well study, but the coordination and crosstalk between the process is not well understood. To study growth and metabolism coupling, we used a yeast strain that was genetically engineered to ferment the sugar xylose but lacked growth on
the sugar. The decoupled growth and metabolism was caused by a single gene deletion in a
highly conserved signaling pathway found in all eukaryotes. While our work is focused on xylose
metabolism, we address the fundamental question of how cells coordinate growth with
metabolism under non-ideal conditions. We identified vast changes in gene expression that
implicated altered regulatory mechanisms involved in lipid metabolism correlating with decouple
growth and metabolism. Our work highlights the complexity of engineering new cellular
functions and that global regulatory modifications, rather than altering individual pathways, may
be required for broad cellular changes.
Introduction

Many physiological processes are essential for growth, but so too is the coordination of those processes to form an integrated cellular system. Actively dividing cells must coordinate metabolism and division with the synthesis and segregation of DNA, proteins, organelles, and other macromolecules, all within a precisely timed cell cycle. Failure to coordinate these processes can jeopardize fitness due to suboptimal cellular composition and energy expenditures. Mechanistically, much remains unknown about how cells coordinate cellular processes. One of the best studied examples is the intimate control of successive cell cycle phases, which depends on interconnected transcriptional and post-translational controls regulated by dispersed checkpoints along the way (1–8). The cell cycle is also coordinated with metabolism; cell-cycle regulators coordinate metabolic flux with cell-cycle phases, which may be related to cell size checkpoints since cells must reach a critical size before a new cell cycle is initiated (2,3,6–8). A critical feature of integrated cellular systems is thus balancing energy demands with division and replication.

Knowing how cells coordinate growth and division with other physiological processes is important for understanding how cells function on a fundamental level, but it also has practical applications. Microbes can be engineered to produce a variety of commodity chemicals and biofuels with high yields to maximize economic returns. Microbial design strategies have considered how cells allocate resources so as to redirect cellular energy toward making compounds of interest (9–15). Redirecting resources away from other processes can improve cellular product yields and thus decrease costs (16,17). However, an added complication is that many industrial processes are stressful for engineered microbes, which mount stress-defense systems that further deplete cellular resources from product formation. The interplay of growth, metabolism, division, and stress defense remain murky, limiting engineering efforts (18,19).

Here, we studied how growth, division, and metabolism are normally coupled in cells by investigating a strain in which these processes have been decoupled. We previously
characterized a series of *Saccharomyces cerevisiae* strains engineered to produce biofuel products from xylose, a pentose sugar abundant in plant biomass but not recognized by *S. cerevisiae* as a fermentable sugar (17,20–22). A major goal for sustainable biofuel production is to utilize xylose and other carbon sources to maximize biomass conversion to products. Past work in our center found that engineering *S. cerevisiae* to ferment xylose anaerobically requires core xylose metabolism genes (encoding xylose isomerase, xylulokinase, and transaldolase (23,24)); however, introducing these genes is not enough to enable fermentation. Many groups have combined strain engineering with adaptive laboratory evolution to evolve xylose fermentation (25–30). Cells require additional null mutations in oxidoreductase GRE3, iron-sulfur (Fe-S) chaperone ISU1, and RAS signaling inhibitor IRA2 (27,31). We previously showed that these mutations help to rewire cellular signaling to unnaturally upregulate the growth-promoting Protein Kinase A (PKA) pathway in conjunction with Snf1 that usually responds to poor carbon sources (32). We propose that activating PKA and Snf1 promotes growth in the context of an otherwise unrecognizable carbon source (32). Coordinated induction of PKA and Snf1 allows cells to recognize xylose as a fermentable carbon source while enhancing growth and metabolism signals.

Although PKA activation is critical for anaerobic xylose growth and metabolism, during that study we made a surprising discovery: the mechanism of PKA up-regulation influences how growth and metabolism are coordinated. PKA can be activated by RAS activity, which stimulates adenylate cyclase to produce the allosteric regulator cAMP that binds and dissociates the PKA regulatory subunit Bcy1 (Fig 1A) (33). In engineered yeast, activating PKA by IRA2 deletion, thus increasing RAS activity, enables rapid anaerobic xylose fermentation and growth on xylose as a sole carbon source. However, activating PKA by deleting the PKA regulatory subunit BCY1 allows rapid anaerobic xylose fermentation but with little to no growth (Figure 1B-C) (32). In both strains, the effect is due to PKA upregulation since inhibition of PKA activity blocked both metabolism and growth (32). Thus, deleting BCY1 in this strain background
decouples growth and xylose metabolism for reasons that are not known. Importantly, other uncoupled biological processes related to PKA function have also been described (34), implying PKA’s central role in process coupling.

**Fig 1. Activation of PKA is needed for xylose fermentation.**

**A.** A brief overview of the PKA signaling pathway.

**B-C.** Average (n = 4 biological replicates) growth (OD600, optical density) (B) and xylose concentration in the medium (C) over time of parental Y184, *ira2Δ*, and *bcy1Δ* strains grown anaerobically on rich medium containing xylose as a carbon source. Asterisks denote significant differences in growth (B) or xylose concentrations (C) (ANOVA).

**D.** Growth on and fermentation capabilities of strains shown in B-C.

Here, we explored phenotypic consequences of *IRA2* and *BCY1* deletions to elucidate how cells normally coordinate growth and metabolism. We integrated transcriptomic, phospho-proteomic, and lipidomic analysis across a suite of strains with different mutations and growth/metabolism phenotypes. The results implicated the importance of lipid metabolism as a
linchpin in the coordination of growth with metabolism: cells lacking BCY1 show unique transcriptomic and lipidomic responses that point to defects in lipid regulation. To uncover causal genes, we also performed adaptive evolution to re-evolve growth coordination in the bcy1Δ strain. Remarkably, the evolved strain acquired mutations in a PKA catalytic subunit TPK1 and phospholipid biosynthesis regulator OPI1, among other genes. These results suggest that PKA-dependent regulation of lipid metabolism is critical for growth, perhaps to coordinate membrane biogenesis and signaling with other cellular processes.

Results

We began by characterizing a suite of strains with different anaerobic xylose growth and fermentation capabilities. Parental strain Y184 harbors the xylose-metabolism gene cassette along with mutations in ISU1 and GRE3 but cannot grow on or metabolize xylose anaerobically (Fig 1B-D). Deleting IRA2 from this strain allows cells to grow on and metabolize xylose anaerobically. In contrast, deletion of BCY1 from Y184 permits rapid anaerobic xylose fermentation but with only minimal growth (Fig 1B-D). We also investigated an ira2Δbcy1Δ double mutant that behaves phenotypically like the bcy1Δ strain (S1 Fig) to understand the relative contribution of both branches of signaling. We started by comparing transcriptomic responses to identify transcripts whose abundance correlates with growth or anaerobic xylose metabolism. Cells were grown in an anaerobic chamber to mid-log phase on rich medium with glucose as a carbon source (YPD) then switched to rich medium containing only xylose (YPX) for three hours, long enough for the ira2Δ strain to resume growing (Fig 2A). We performed short-read sequencing to measure changes in transcript abundance after the glucose-to-xylose shift. To understand strain responses, we compared transcript abundances across strains grown under each condition; we also compared the fold change in transcript abundance within each strain responding to carbon shift. Comparing fold changes in sugar responsiveness captures strain specific differences on both carbon sources: because most of the expression
differences are specific to the xylose condition (Fig 2C), differences in xylose response drive the differences in fold change response (S2A Fig, S1 Table).

Fig 2. Few transcriptomic patterns are associated with anaerobic xylose growth.
A. Experimental overview. Strains were grown anaerobically in rich glucose medium to early/mid-log phase, then switched to anaerobic rich xylose medium for three hours.

B. 65 genes whose \( \log_2 \) (fold change) upon glucose to xylose shift is different (FDR < 0.05) in at least one of the three non-growing strains (Y184, bcy1Δ, ira2Δbcy1Δ) compared to the \( \text{ira2}\Delta \) strain that can grow anaerobically on xylose. Genes (rows) were organized by hierarchical clustering across biological triplicates measured for each strain (columns).

C. Hierarchical clustering of 603 genes whose \( \log_2 \) (fold change) upon glucose to xylose shift is different (FDR < 0.05) between the non-fermenting Y184 strain and all of the xylose fermenters (\( \text{ira2}\Delta, \text{bcy1}\Delta, \text{ira2}\Delta\text{bcy1}\Delta \)). The blue-yellow heatmap on the left represents the \( \log_2 \) (fold change) in expression upon glucose to xylose shift across biological triplicates (columns). The purple-green heatmap on the right represents the abundance of each transcript (rows) in each strain grown on glucose (G) or xylose (X), relative to the average (\( n = 3 \)) abundance of transcript measured in the Y184 YPD sample. Significantly enriched (\( p < 10^{-4} \), hypergeometric test) functional categories are annotated next to Cluster I, II, and III.

Our expectation at the outset was two-fold. On the one hand, we expected to find expression changes common to the three xylose fermenting strains, but discordant in Y184 cells – these expression patterns may relate to xylose metabolism, since Y184 is the only strain incapable of utilizing xylose (Fig 1D). On the other hand, expression patterns unique to the \( \text{ira2}\Delta \) strain – the only strain capable of growing anaerobically on xylose (Fig 1D) – may reflect expression patterns related to growth.

**Few gene expression patterns correlate strictly with growth phenotypes**

Somewhat surprisingly, there were few genes whose expression correlated strictly with growth phenotypes. Only two genes showed xylose-responsive expression changes that were specific to \( \text{ira2}\Delta \) cells compared to the other three strains analyzed as a group (FDR < 0.05; see
Methods): daughter-cell-specific glucanase *DSE4* and L-homoserine-O-acetyltransferase *MET2*. In fact, hierarchical clustering of all genes with a transcriptomic change in response to the carbon shift showed that the *ira2Δ* strain’s response to xylose shift was most similar to that of Y184 cells, even though one strain can grow on and anaerobically ferment xylose and the other cannot (S2A Fig, S1 Table). We next performed pairwise comparisons of the glucose-to-xylose fold-change responses between each strain and the *ira2Δ* strain, then combined the lists of genes identified in all three comparisons. This method identified 65 genes; however, investigating the expression patterns once again indicated that the *ira2Δ* response was most similar to Y184 cells but with weaker magnitudes of change (Fig 2B, S2 Table). This set of 65 genes was enriched for genes induced in the environmental stress response (iESR genes) (*p* = 2x10⁻⁷, hypergeometric test). Many genes induced in the Y184 and *ira2Δ* strains but largely not in *bcy1Δ* strains included genes related to metabolism, including several in the mitochondrial TCA cycle and peroxisomal fatty-acid oxidation pathway, which may reflect that *bcy1Δ* strains are more likely to recognize xylose as a fermentable carbon source. We specifically investigated the set of 65 genes encoding cell-cycle regulators and kinases, since these may be involved in growth kinetics; however only three, six, and eight of these genes were differentially expressed in *ira2Δ* cells compared to Y184, *bcy1Δ*, or *ira2Δbcy1Δ* cells, respectively, in response to xylose shift, respectively (FDR < 0.05). The *ira2Δ* strain showed weak repression of cyclin *CLN2* and anaphase-promoting complex *CDC20*, whereas other strains showed strong gene repression, consistent with reduced cycling in those strains compared to *ira2Δ* cells (S3 Table). Expression of cell-cycle genes did not implicate arrest in a particular cell-cycle stage, consistent with early transcriptomic studies that showed that gene expression during cell-cycle arrest does not parallel expression of cells cycling through those phases (53).

Previous chemostat studies reported that repression of ribosomal protein (RP) and ribosome biogenesis (RiBi) genes is correlated with decreased growth, and these studies proposed that expression of these genes can predict cellular growth rate (54–57). However,
here we saw no correlation of RP and RiBi transcript abundance or response with growth phenotypes. The Y184 strain strongly repressed RP and RiBi genes upon xylose shift, which might be expected for a strain that arrests its growth, but so too did the *ira2Δ* strain, albeit with weaker magnitude of repression. Surprisingly, *bcy1Δ* and *ira2Δbcy1Δ* cells, whose growth is arrested after the xylose shift, showed little change in RP and RiBi transcripts compared to glucose-dependent growth (FDR <0.05, S2B Fig, S4 Table). These results reinforce past work from our lab that the expression of ribosome-associated genes does not necessarily parallel growth rate (58). They further suggest that *bcy1Δ* strains cultured in xylose are unlikely limited by the abundance of RP and RiBi transcripts. Overall, while the non-growing strains have stronger repression of a few cell-cycle regulators when compared to the *ira2Δ* strain, there was not a clear gene expression pattern to describe why *ira2Δ* cells grow and *bcy1Δ* strains do not.

**Few gene expression patterns correlate strictly with metabolism phenotypes**

We next investigated shared gene expression changes related to xylose fermentation. We compared expression in the Y184 strain responding to the xylose shift to the three other strains analyzed as a group and identified 603 differentially expressed genes (FDR < 0.05; S5 Table). Hierarchical clustering revealed that these genes typically had a larger expression change in Y184 that was progressively weaker across the strain series; once again, Y184 and the *ira2Δ* strain were more similar to one another qualitatively than they were to the *bcy1Δ* strain (Fig 2C, S5 Table). Collectively, these genes were heavily enriched for genes in the ESR (*p* = 7.5x10^{-62}, hypergeometric test) and involved in translation (Fig 2C; *p* = 6.43x10^{-32}, hypergeometric test).

Deeper interrogation revealed several small gene clusters of interest. One group of 18 genes (Fig 2C, Cluster II) is the only cluster in which all three xylose-metabolizing mutants showed one pattern, gene repression, whereas Y184 induced gene expression. This group was enriched for genes involved in protein folding (Fig 2C; *p* = 5x10^{-6}, hypergeometric test) and
included several genes whose proteins localize to the mitochondria and have roles in key mitochondrial functions (e.g., heme synthesis, ubiquinone synthesis, tricarboxylic acid cycle).

The purpose of this response is unclear, however mitochondrial function is already implicated in anaerobic xylose utilization (31), and the Hsp90 protein chaperone genes in this cluster has been reported to act as a signal transducer for alternative carbon source metabolism (59).

We next specifically interrogated the 603 genes for those involved in glycolysis, gluconeogenesis, TCA cycle, and carbohydrate storage, predicting that differences in expression would relate to altered xylose metabolism capabilities. This identified 26 genes with functional annotations involved in at least one of these processes (S6 Table). All three xylose fermenting strains shared similar expression compared to Y184 cells at several hallmark genes. For example, Y184 cells strongly induced the hexose transporter HXT7, which is expressed when glucose levels are low, whereas the three mutant strains show a weaker (FDR = 0.02). Genes encoding gluconeogenic enzymes, such as PCK1 and MDH2, and glycogen metabolism genes GSY1 and GLC3, which are induced upon glucose depletion, were induced significantly more in Y184 cells upon xylose shift compared to ira2Δ, bcy1Δ, and ira2Δbcy1Δ cells (FDR < 0.05). These data are consistent with the hypothesis that the three xylose fermenters recognize xylose as a fermentable carbon, whereas Y184 activates a carbon-starvation response.

**Regulatory analysis reveals strain-specific differences in carbon, iron, and lipid gene control**

We next focused on understanding how growth and metabolism are decoupled in the bcy1Δ strain and thus directly compared its expression to that in ira2Δ cells. We focused on genes whose expression changes in response to the xylose shift were in opposing directions (FDR < 0.05; S7 Table) to implicate processes involved in decoupling growth and metabolism (Fig 3A, see Methods). Among the identified genes, we scored enrichment of functional terms (S8 Table) as well as known targets of transcriptional regulators. We also used motif analysis to
discover shared sequence motifs upstream of genes uniquely induced or repressed in the 
bcy1Δ strain, and then matched those to known transcription factor binding sites (see Methods).

We identified 654 genes differentially expressed in bcy1Δ cells in the opposite direction as ira2Δ cells upon the glucose-to-xylose shift (Fig 3A, S7 Table). Importantly, only 82 genes (12.5%) showed significant differences in basal gene expression when cells were grown on glucose (S3A Fig), indicating that the majority of genes are identified due to differences in response to xylose shift.

Fig 3. Genes uniquely expressed in the bcy1Δ strain implicate an integrated response in xylose metabolism and growth coupling.

A. 654 genes whose log₂(fold change) upon glucose to xylose shift is different (FDR < 0.05) between the ira2Δ and bcy1Δ strains and shows expression changes in the opposite direction (see Methods for details). Significant (p < 10⁻⁴, hypergeometric test) functional enrichments are annotated next to the two main clusters.

B. Regulatory relationships between transcription factors whose targets or known binding sites were enriched in (A). Documented PKA-dependent phosphorylation is indicated by a P. See text for details.
The results implicated several regulators, some with prior connections to anaerobic xylose fermentation. 318 genes induced in the bcy1Δ strain shifted to xylose but repressed in the ira2Δ cells were enriched for amino acid and sphingolipid biosynthesis genes, as well as targets of the carbon-responsive Azf1 transcription factor ($p < 10^{-4}$, hypergeometric test).

Previous work from our lab implicated Azf1 in anaerobic xylose fermentation, and indeed, we showed that the over-expression of AZF1 in an ira2Δ strain enhances the rate of anaerobic xylose utilization (32). Additionally, PKA has been implicated in Azf1 phosphorylation (60); together with the fact that the AZF1 gene is uniquely induced in the bcy1Δ strain suggest its functional importance in xylose metabolism (see Discussion).

In contrast, several regulators were implicated by the 336 genes uniquely repressed in the bcy1Δ strain. These included genes harboring upstream binding sites of the iron-responsive Aft1/2 transcription factors (S3B Fig) and known targets of transcriptional activators Ino2/4 that respond to inositol for phospholipid biosynthesis (Fig 3A; see more below). Iron is an important cofactor of many enzymes, including those involved in mitochondrial respiration, lipid biogenesis, and amino acid biosynthesis, all of whose genes were among the differentially regulated genes studied here. Additionally, Aft1/2 regulation and the iron regulon have been linked with PKA activity; however, direct interactions remain to be identified (61). Interestingly, Aft1/2 and Azf1 both are both connected to the regulator Mga2, which controls lipid and hypoxia genes and that we previously showed enhances anaerobic xylose fermentation when over-expressed in ira2Δ cells (32,62) (see Discussion). While we did not find an enrichment of Mga2 targets in any of our comparisons, 42% of the gene targets (11/26 genes) known to be bound by Mga2 differed in expression between bcy1Δ and ira2Δ cells (S3C-D Fig). Furthermore, the canonical Mga2 target OLE1 is strongly induced in ira2Δ cells, with far weaker induction in bcy1Δ and ira2Δbcy1Δ cells (FDR < 0.05). We previously proposed that Mga2 is likely a target of PKA signaling, since deletion of IRA2 increased phosphorylation of a potential PKA phospho-
The presence of many lipid biosynthesis genes in this gene set and the highly regulated role of lipids in cell growth and proliferation prompted a deeper investigation of lipid metabolism genes. The \( \text{bcy1}\Delta \) strain repressed genes involved in ergosterol biosynthesis and targets of \( \text{Ino2}/4 \) that are involved in phospholipid metabolism (Fig 3A). This response is consistent with the model that \( \text{Ino2}/4 \) activity is reduced. However, the \( \text{bcy1}\Delta \) strain also induced many genes involved in the synthesis of phosphatidic acid (PA), which normally promotes \( \text{Ino4} \) activity by sequestering \( \text{Ino4}'s \) inhibitor Opi1 to the ER membrane (63). This response suggests that some connection between PA, Opi1, and \( \text{Ino4} \) is disrupted in the absence of \( \text{BCY1} \). PKA is known to regulate the \( \text{Ino2}/4 \) pathway through direct phosphorylation of Opi1 to increase its inhibitory activity (64). Together, these results raised the possibility that the \( \text{bcy1}\Delta \) strain has important differences in lipid metabolism and perhaps composition, which could be modulated by differences in PKA activity in this strain.

**Lipidomic and phosphoproteomic analyses show disrupted phospholipid metabolism in \( \text{bcy1}\Delta \) cells**

Since the transcriptomic responses implicated differences in lipid metabolism, we investigated the lipidomic composition of our strains. Strains were grown in a similar design as the transcriptomic analysis, where anaerobically glucose-grown Y184, \( \text{ira2}\Delta, \text{bcy1}\Delta, \) and \( \text{ira2}\Delta\text{bcy1}\Delta \) cells were shifted to anaerobic xylose media for three hours before lipids were analyzed by mass spectrometry (see Methods). We detected over 4000 lipid species including 239 that were confidently assigned to a particular lipid class (S9 Table). We again sought to find lipidomic profiles correlated with xylose metabolism and growth, xylose metabolism but no growth, and no xylose metabolism or growth.
We compared the Y184 strain to the three xylose fermenters as a group and identified 18 lipids whose change in abundance upon a shift to xylose significantly differed in Y184 cells. Both confidently classified lipids in this group were phosphatidylserine (PS) species (Fig 4A, S10 Table). Interestingly, all three mutants increased the abundance of these PS species when shifted to xylose, whereas Y184 cells decreased the abundance of one and failed to induce the other to the same degree as the mutants. The gene encoding the PS synthase CHO1 was strongly induced in Y184 cells, indicating that the decrease in PS in Y184 cells is unlikely due to decreased CHO1 expression. Instead, we analyzed previous phosphoproteomic data from our lab and discovered that Cho1 was phosphorylated to a much higher degree in the Y184 strain on serine 46 (|log₂ FC| > 2, Table 1), a known PKA site that inhibits Cho1 activity (65). Together, these results indicate PKA-dependent inhibition of PS synthesis in Y184 cells.

### Table 1. Phosphorylation changes of phospholipid biosynthetic enzymes

<table>
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<tr>
<th>Protein</th>
<th>Residue</th>
<th>Av. logFC (bcy1Δ-)</th>
<th>Av. logFC (ira2Δbcy1Δ-)</th>
<th>Av. logFC (Y184-ira2Δ)</th>
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**Fig 4.** *bcy1Δ* strains show altered phospholipids after anaerobic xylose shift.

**A-B.** Average (n = 3 biological replicates) abundance of lipids (rows) with a significant difference in log₂(fold change) upon anaerobic glucose-to-xylose shift in (A) Y184 compared to all xylose-fermenting strains (*ira2Δ, bcy1Δ, ira2Δbcy1Δ*) analyzed as a group or (B) *ira2Δ* cells compared
to all non-growing strains (Y184, bcy1Δ, ira2Δbcy1Δ) as a group. Lipids of interest are annotated.

C. Partial phospholipid biosynthesis pathway with transcriptomic and lipidomic data represented. Yellow-blue boxes next to each enzyme name represents the average (n=3 biological replicates) log$_2$(fold change) upon glucose-to-xylose shift for each strain (left-right: Y184, ira2Δ, bcy1Δ, ira2Δbcy1Δ), as outlined in the key at the bottom. Significant (FDR < 0.05) differences in log$_2$(fold change) when compared to ira2Δ strain are represented in bolded boxes, whereas insignificant differences are transparent. Colorized arrows (yellow: induced, blue: repressed) represent significantly different (FDR < 0.05) log$_2$(fold change) between the bcy1Δ and ira2Δ strains, where color is based on the predominant expression pattern for enzymes regulating that step. Colored lipidomic boxes represent lipids whose log$_2$(fold change) in abundance upon shift from glucose to xylose are larger (green) in bcy1Δ compared to ira2Δ or smaller (pink) in bcy1Δ compared to ira2Δ, according to the key. Orange lipid box corresponds to a log$_2$(fold change) in abundance upon shift from glucose to xylose that is significantly different in Y184 when compared to the three xylose fermenters (ira2Δ, bcy1Δ, ira2Δbcy1Δ).

D. Average (n = 4 biological replicates) change in OD$_{600}$ of ira2Δ and bcy1Δ grown over time in anaerobic rich xylose medium either in the absence (solid lines) or presence (dashed lines) of inositol (75 µM) and choline (10 mM) ($p = 2.4 \times 10^{-6}$, ANOVA).

We next compared lipidomic profiles in the growing ira2Δ strain shifted to xylose to the bcy1Δ and ira2Δbcy1Δ strains that do not grow. Due to limited statistical power (caused by replicate variation in one of the three bcy1Δ strain replicates), we compared the ira2Δ response to ira2Δbcy1Δ cells, whose response was highly similar to two out of the three bcy1Δ strain replicates. We identified 67 lipids whose fold-change was significantly different in ira2Δbcy1Δ cells upon xylose shift versus ira2Δ cells (FDR < 0.05, Fig. 4B, S11 Table). The analysis
confidently classified six of the lipids, including phosphatidylethanolamines (PE), phosphatidyl dimethylethanolamines (PDME), and cardiolipins (CL).

PE and PDME were more abundant in the ira2Δbcy1Δ strain exposed to the shift compared to ira2Δ cells (FDR < 0.05, Fig 4B). These differences were particularly interesting because PE is further metabolized to PDME and then to phosphatidylcholine (PC), the most abundant phospholipid in the cell, through three consecutive methylation reactions by Cho2 and Opi3, respectively (Fig 4C) (66). While the CHO2 transcript was not differentially expressed between ira2Δ and bcy1Δ strains, OPI3 was: ira2Δ cells shifted to xylose induced OPI3 expression, whereas bcy1Δ and ira2Δbcy1Δ cells repressed it (FDR = 2.45x10\(^{-12}\) and FDR = 6.22x10\(^{-13}\), respectively). Previous studies suggest that blocking PC synthesis through OPI3 deletion, but not CHO2 deletion, inhibits growth due to the accumulation of phosphatidyl monomethylethanolamine (PMME) and insufficient PC production (67). To investigate effects on PC, we analyzed all PC lipid moieties in the dataset; PC lipids were reproducibly lower in abundance after the xylose shift in bcy1Δ cells when compared to ira2Δ cells (\(p = 0.000419\), ANOVA; S 4 Fig, S12 Table). We propose that the bcy1Δ strain experiences a bottleneck in that pathway leading to PC synthesis from PE, which may impact its ability to grow on xylose (see Discussion).

Among other lipids whose abundance was influenced by BCY1 deletion and xylose shift was cardiolipin, a major component of mitochondrial membranes critical for a variety of functions including acetyl coA synthesis, TCA cycle, iron metabolism, arginine metabolism, and protein import (68). Interestingly, cardiolipin abundance was reduced in the ira2Δbcy1Δ strain upon xylose shift compared to ira2Δ cells. The difference is underscored by transcriptomic differences, since several cardiolipin biosynthetic genes were induced in ira2Δ cells but repressed or induced to a weaker extent in bcy1Δ and ira2Δbcy1Δ strains (FDR < 0.05). Additionally, production of PS, PE, and PC is dependent on properly functioning mitochondrial membranes as PS is shuttled into the mitochondria and converted to PE by the
phosphatidylserine decarboxylase Psd1, before PE is shuttled back to the ER. Thus, the effects of cardiolipin reduction in \textit{bcy1}\textsuperscript{Δ} strains are further compounded by impacting other branches of phospholipid biosynthesis.

We expected to see differential abundance of PA in \textit{ira2}\textsuperscript{Δ}bcy1\textsuperscript{Δ} cells versus \textit{ira2}Δ cells, since \textit{bcy1}Δ and \textit{ira2}Δbcy1\textsuperscript{Δ} strains uniquely induced PA biosynthesis genes (Fig 3A). While there were no significant differences in PA moieties between the strains (FDR > 0.05), we did identify altered phosphorylation status of the PA phosphatase enzyme Pah1 (S823; Table 1). Pah1 converts PA to diacylglycerol, which is funneled into storage lipids (66). Phosphorylation of serine 823 is significantly lower in the \textit{bcy1}Δ and \textit{ira2}Δbcy1\textsuperscript{Δ} strains compared to the \textit{ira2}Δ strain (log\textsubscript{2} FC < -1). Interestingly, this serine has not been previously annotated as a phosphorylated residue (BioGRID version 4.4.213) (69), but it is within a potential PKA consensus site (RRxxS/T). PKA is known to phosphorylate Pah1 at another residue not captured in our dataset to inhibit its activity (70). Our results raise the possibility that S823 regulates Pah1 activity in a manner that affects PA in these strains. Overall, the differences seen in PE, PDME, and PC abundances, as well as differences in transcript abundance and phosphorylation status of phospholipid biosynthesis enzymes, suggests a bottleneck in the pathway in the \textit{bcy1}Δ strains that may inhibit their ability to proliferate on xylose.

**Supplementation with phospholipid precursors only modestly improves growth**

We questioned if supplementing xylose medium with phospholipid precursors, particularly inositol and choline that can be funneled into phospholipid biosynthesis via the Kennedy Pathway, may bypass a possible bottleneck and thus rescue the \textit{bcy1}Δ strain’s growth. We therefore grew \textit{bcy1}Δ and \textit{ira2}Δ strains anaerobically in xylose medium with and without 75 \textmu M inositol and 10 mM choline supplementation. While the lipidomics data did not show altered inositol or phosphatidylinositol abundances in the \textit{bcy1}Δ strain, we supplemented inositol due to the repression of \textit{INO1} in the strain (Fig 4C, S5 Table). After 52 hours of growth
in supplementation, \textit{bcy1}\textDelta{} cells experienced a very modest but statistically significant growth improvement ($p = 2.4 \times 10^{-6}$, ANOVA; Fig 4D), whereas the \textit{ira2}\textDelta{} strain did not. While the \textit{bcy1}\textDelta{} strain’s inability to grow anaerobically on xylose cannot be fully explained by a deficiency in phospholipid precursors, the modest improvement implicates it as a contributing factor to the phenotype.

\textit{Growth and metabolism can be genetically recoupled through directed evolution}  

We took a second approach to identify pathways and processes responsible for growth coordination in \textit{bcy1}\textDelta{} strains by conducting adaptive laboratory evolutions to recouple xylose-dependent growth and metabolism. The \textit{bcy1}\textDelta{} strain was first grown anaerobically in rich medium supplemented with 2\% glucose to accumulate mutations (71), then the culture was seeded into fresh anaerobic medium containing 2\% xylose and 0.1\% glucose and passaged periodically for ~35 generations until the culture showed robust changes in cellular density over time (see Methods). Single colonies were isolated and characterized for their growth and fermentation capabilities, and genetic changes were identified through whole genome sequencing (see Methods). Three independent evolutions were performed, and several colonies were selected at different stages of the evolutions.

In all three experiments, we identified mutants with recoupled growth and metabolism despite the absence of \textit{BCY1}, evident by their robust anaerobic growth on xylose medium comparable to that of the \textit{ira2}\textDelta{} strain (Fig 5A, S5A-B Fig). Interrogating the genome sequences identified multiple mutations in each strain, along with copy-number variations and aneuploidy in two of the evolved lines (Table 2). Interestingly, there was no genetic change common to all evolved strains, strongly suggesting multiple routes to recoupling growth and metabolism in the absence of \textit{BCY1}. Four of the characterized strains from the three experiments regained growth rates comparable to and statistically indistinguishable from \textit{ira2}\textDelta{} cells ($p > 0.05$, ANOVA), including EWY55 from the first culture, EWY87-1 and EWY87-3 from the second culture, and
EWY89-3 from the third evolution culture (S6A-B,E Fig). Strains EWY89-1 and EWY9-2 showed modest growth on xylose but did not differ significantly from the bcy1Δ strain ($p > 0.05$, ANOVA; S6C-D Fig). Genetic changes for all evolved strains are listed in Table 2.

**Fig 5. Directed evolution recoupled growth and metabolism on xylose.**

A. Average ($n = 3$ biological replicates) change in OD600 of $ira2\Delta$, $bcy1\Delta$, and EWY55 strains grown anaerobically over time in rich xylose medium ($p < 10^{-4}$, ANOVA).

B. Representative of multiple replicate EWY55 cells lacking $OPI1$ grown anaerobically on solid xylose medium.

C. 233 genes whose log$_2$ YPX abundance was significantly different (FDR < 0.05) in EWY55 and/or $ira2\Delta$ strains when compared to the $bcy1\Delta$ strain. Genes were divided into four clusters.
(A, B, C, D) based on hierarchical clustering. \( \log_2 \) YPX abundance of EWY55 or \( \text{ira2}\Delta \) transcripts, relative to \( \log_2 \) \( \text{bcy1}\Delta \) YPX abundance, are shown in boxplots divided by clusters.

D. Average (\( n = 3 \) biological replicates) abundance of 296 lipids (rows) whose fold change in EWY55 compared to \( \text{ira2}\Delta \) or \( \text{bcy1}\Delta \) strains was at least 1.5X different in all three replicates. Lipids of interest are annotated.

Table 2. Genetic changes in evolved \( \text{bcy1}\Delta \) strains

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Amino acid change</th>
<th>Chromosome Duplications</th>
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<td>( OPI1 )</td>
<td>T715G</td>
<td>S239A</td>
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<td></td>
<td>( TPK1 )</td>
<td>G829C</td>
<td>A277P</td>
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<td></td>
<td>( TOA1 )</td>
<td>T354A</td>
<td>N118K</td>
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<td></td>
<td>( RIM8 )</td>
<td>C1591T</td>
<td>Q531*</td>
</tr>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EWY87-3</td>
<td>( RPA43 )</td>
<td>A751G</td>
<td>S251N</td>
</tr>
<tr>
<td>EWY89-1</td>
<td>( HSC82 )</td>
<td>G235C</td>
<td>D79H</td>
</tr>
</tbody>
</table>
| EWY89-2 | - | - | - | Chr. I, X, XVI, XIV\(^{560217-625584} \)
| EWY89-3 | \( HSC82 \) | G235C | D79H | Chr. I, IX, X, XVI, XIV\(^{560217-625585} \) |
Strain EWY55 was particularly interesting. This strain harbored nonsynonymous mutations in several genes, including PKA catalytic subunit *TPK1*, the negative regulator of phospholipid genes, *OPI1*, described above (Fig 3B), *RIM8* that is required for anaerobic growth (72), and TFIIA large subunit *TOA1* (Table 2). The *OPI1* mutation was especially interesting because Opi1 was implicated in the phospholipid transcriptomic analysis above (Fig 3) and because the mutation changes a known phosphorylation site, serine 239 (73), to alanine (Table 2). CKII has been reported to phosphorylate this site and was previously shown to activate Opi1 (73). This poses the question of whether Opi1 is hyperactive in the *bcy1Δ* strain, and if this is responsible for its lack of growth on xylose.

To identify causal alleles responsible for recoupling growth and metabolism in the EWY55 strain, we performed single allele deletions and allele swaps in the *bcy1Δ* and EWY55 strains (see Methods). This strain background is derived from a wild isolate that is less genetically amendable than laboratory strains (40), and we were unable to recover *TPK1* deletion in either strain despite many efforts. Deletion of *RIM8* or *TOA1* did not impact the growth of EWY55 cells, nor did substituting the parental alleles into the evolved strain (S5C Fig). However, *OPI1* deletion reproducibly reduced growth of the EWY55 strain on solid xylose medium (Fig 5B). Complementation experiments were not successful, since introducing even the empty vector into this strain complemented anaerobic xylose growth on a plate for reasons that are not clear but may suggest that the cells grow differently during drug selection (S5C Fig). While we were not able to confirm the role of the mutant *OPI1* allele, together these results indicate that the route to re-coupling may require several mutant alleles and can occur through multiple routes.

Transcriptomic and lipidomic analysis in the evolved strain implicates relieved phospholipid bottleneck.
To further characterize the evolved EWY55 strain, we performed another transcriptomic and lipidomic experiment as described above (see Methods) with the main goal of identifying if the evolved EWY55 strain reverted its gene expression and lipid composition to that of the \textit{ira2}\textDelta strain. Surprisingly, the EWY55 strain did not recapitulate the \textit{ira2}\textDelta gene expression or lipid abundance profiles at most entities. We identified 297 genes less abundant in EWY55 growing anaerobically on xylose compared to the \textit{bcy1}\textDelta strain, and these were enriched for genes involved in mitochondrial functions, such as electron transport chain, oxidation-reduction, and targets of the HAP2/3/4/5 complex; genes involved in phospholipid metabolism; and genes involved in ergosterol synthesis (FDR < 0.05; S13 Table; Bonferroni corrected \( p < 0.05 \), hypergeometric test, see Methods), representing processes significantly affected in our original comparison of the \textit{bcy1}\textDelta and \textit{ira2}\textDelta strains. Additionally, 93 genes with higher abundance in the EWY55 compared to the \textit{bcy1}\textDelta cells were enriched for ribosomal protein genes and genes involved in translation and sulfate assimilation (FDR < 0.05; S13 Table; Bonferroni corrected \( p < 0.05 \), hypergeometric test, see Methods), processes important for rapid growth. We next asked if these expression changes reverted to \textit{ira2}\textDelta patterns – surprisingly, most did not. Although many of the same genes were affected, the EWY55 strain showed expression in differences compared to \textit{bcy1}\textDelta cells that were even more extreme than \textit{ira2}\textDelta cells compared to the \textit{bcy1}\textDelta strain (Fig 5C). This indicates that expression was progressively higher or lower across the \textit{ira2}\textDelta, \textit{bcy1}\textDelta, and EWY55 strain series. There were a few exceptions, including 22 transcripts of diverse functions in which expression differences in EWY55 recapitulated those seen in \textit{ira2}\textDelta cells compared to the \textit{bcy1}\textDelta strain (Fig 5C, Clusters B and C; S14 Table; FDR < 0.05).

We were particularly interested in phospholipid biosynthesis genes, given all the connections to this pathway throughout our studies. In general, EWY55 cells showed lower transcript abundances of phospholipid biosynthesis genes compared to the \textit{bcy1}\textDelta strain grown anaerobically on xylose (S7 Fig, S15 Table), making its expression even more divergent from
The 

The phospholipid composition further supports the unique changes of the 

The EWY55 strain showed significantly greater induction of storage lipids like 

diacylglycerol (DG) and triacylglycerol (TG), while simultaneously repressing phospholipids like 

PS, PE, PMDE, PC upon carbon shift to xylose compared to 

Fig 5D, S7 Fig, S16 Table; see Methods). Importantly, the reduced levels of PC in EWY55 cells compared 

to 

hypothesis that the evolved EWY55 strain relieved the bottleneck present in 

a distinct method compared to 

Together, our results underscore the complexity of 

responses to xylose growth and metabolism across 

EWY55 (see Discussion).

Discussion

We began this work with two primary goals: to identify signatures of xylose-dependent 

growth and metabolism across a suite of strains with varying capabilities and to elucidate the 

mechanism through which growth and metabolism are decoupled in cells lacking 

One 

key result from our work is that there is no obvious gene expression signature associated with 

the ability to grow anaerobically on xylose (Fig 2B). While we did identify a handful of cell-cycle 

genes whose expression was consistent with cycling in the 

there were no clear 

signatures correlated with growth. This was especially interesting in the case of ribosome-

related genes, since there has been much debate about whether the level of RP transcripts 

underlies growth rate (54–58). In chemostat experiments where growth is limited by nutrient 

restriction, the abundance of RP and RiBi genes correlates with growth rate, consistent with one 

set of long-standing models of growth limitations in bacteria (74–79). However, other seminal 

studies focusing on stress conditions suggest that growth during stress is not limited by 

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ribosome production (58,80–84). Our results show clearly that expression of RP and RiBi genes
is higher in the non-growing bcy1Δ strains than dividing ira2Δ cells (Fig 2C; S2B Fig). In contrast, the EWY55 strain that recovers anaerobic growth on xylose shows higher expression of ribosome-related genes, perhaps supporting rapid division. Together, our results add to a growing body of work that shows that, although production of ribosome components is often correlated with growth rate, division dynamics cannot be universally predicted by RP and RiBi transcript abundances.

However, transcriptomic patterns did implicate an interconnected network of expression differences specific to the bcy1Δ strain, and in turn the evolved EWY55 strain, connected to PKA signaling (Fig 3). The affected network implicates mitochondrial function, iron response, carbon metabolism, and phospholipids. We propose that these processes are normally coordinated by PKA signaling in a manner that requires the regulatory subunit Bcy1. Our results are consistent with prior implications that these processes are involved in anaerobic xylose fermentation. Targets of ER-localized transcription factor Mga2 and carbon-responsive regulator Azf1 were both altered in the bcy1Δ strain upon xylose shift compared to the ira2Δ strain (Fig 3A). We previously showed that altering expression of these transcription factors affects xylose fermentation rates and growth in an ira2Δ strain (32). Our results here strongly suggest that deletion of BCY1 naturally augments the transcription factors’ abundance and/or activity. These factors may indirectly alter mitochondrial and/or iron homeostasis. In fact, deletion of the iron-sulfur scaffold protein ISU1, an important sensor of iron availability, is required for anaerobic xylose metabolism (26,31). Why ISU1 deletion is required for xylose fermentation remains unclear, but one possibility is that it aids in metabolic rewiring influenced by the iron regulon, the Aft1/2 transcription factors, and altered levels of PKA activity (Fig 3, S3B Fig) (61,85–87).

Remarkably, PKA is directly connected to all these processes. Past work implicated PKA in directly phosphorylating Azf1 and Mga2 (32,60). While a direct link between PKA and Aft1/2 activity has yet to be identified, PKA catalytic subunit Tpk2 is required to repress the high-affinity iron uptake pathway under standard conditions (86). Additionally, Ira2 can localize to
mitochondria, suggesting that PKA can also localize to this organelle (88). In fact, PKA is found at the mitochondria of higher eukaryotes (89), suggesting that yeast PKA may also localize to the mitochondria. Together, our results suggest that upregulated PKA activity is required for xylose fermentation and can occur via either IRA2 or BCY1 deletion, but deletion of BCY1 produces stronger effects that underscore its higher per-cell rate of xylose fermentation (32).

A fundamental aspect of BCY1 deletion is that cells can no longer grow robustly despite enhanced xylose metabolism. Our integrated analysis points to a defect in phospholipid flux or metabolism as a major contributor to this decoupling. First, the bcy1∆ strains showed altered gene expression, namely repression of Ino2/4 targets, including their canonical target INO1 (Figs 3A,4C), that pointed to differences in phospholipid metabolism. Second, we found that bcy1∆ strains grown anaerobically on xylose display an altered lipid profile that implicates a key bottleneck in the PE-PDME-PC pathway, along with phosphorylation differences on key phospholipid enzymes (Table 1). Finally, re-evolving a coupling between anaerobic-xylose growth and metabolism in the bcy1∆ parent implicated mutations in PKA subunit TPK1 and the Ino2/4 repressor OPI1 (Table 2), which is known to be directly regulated by PKA phosphorylation (64). Opi1 has complex roles in regulating phospholipids, including during the switch to invasive growth depending on nutrients (90), a process also regulated by the RAS/PKA pathway (91–95). While we were unable to elucidate the exact role of these alleles, our results suggest that the OPI1 mutation may alter Opi1 regulation, especially given that the identified mutation in Opi1 occurs at a known CKII kinase site and is implicated in activating Opi1 (73). Our past network inference across this panel of engineered strains revealed altered phosphorylation of CKII targets (32). Finding that complete deletion of the mutated OPI1 allele reduced growth of the evolved EWY55 strain on xylose (Fig 5B) suggests altered Opi1 activity in the bcy1∆ strain is somehow resolved by mutation of this CKII site. We propose that an interplay between PKA and possibly CKII affect Opi1 regulation in the bcy1∆ strain, and that this interplay is important for growth coupling.
Importantly, phospholipid metabolism is required for growth and division. Cells must generate enough phospholipid to support membrane biogenesis (4,6–101). Furthermore, phospholipids function in inter-organelle communication, connecting the ER and mitochondria via the ER-mitochondria encounter structure (ERMES). Impairment of this structure and inter-organelle communication is known to cause diverse mitochondrial phenotypes and disrupt phospholipid biosynthesis (102,103), connecting phospholipid metabolism to mitochondrial functions, including xylose flux (31). One possibility is that repression of Ino2/4 targets and impaired regulation of Opi1 in \( \text{bcy1}\Delta \) cells disrupt growth in the \( \text{bcy1}\Delta \) strain due to insufficient lipids to support growth. But another possibility is that accumulation of methylated PE intermediates during the conversion to PC create a toxic buildup coupled with insufficient PC (Fig 4C). Ishiwata-Kimata et al. (2022) (67) found that accumulation of PMME leads to a growth defect by triggering the unfolded protein response and growth arrest. Accumulation of PDME in the \( \text{bcy1}\Delta \) strain (Fig 4B-C) may also lead to ER stress preventing growth paired with interfered ER-mitochondrial communication. Importantly, the evolved EWY55 strain does not share the \( \text{bcy1}\Delta \) strain’s accumulation of PMDE, leading us to propose that EWY55 cells have overcome bottleneck in PC synthesis (Fig 5D, S7 Fig).

A major remaining question is how the deletion of \( \text{BCY1} \), but not \( \text{IRA2} \), decouples growth from metabolism specifically under the conditions studied here. One possibility is that \( \text{BCY1} \) deletion upregulates PKA activity to a higher level than deletion of \( \text{IRA2} \), whose activation of PKA is indirect via cAMP regulation (33). PKA activity over some threshold could cause decoupling, as deletion of \( \text{BCY1} \) is well characterized to sensitive cells to environmental stressors (104). An alternate model is that localized cAMP production could influence when and where PKA is active in \( \text{ira2}\Delta \) cells. cAMP exists in concentration gradients in cells to control the subcellular location of active PKA (105–107). It is possible areas with low cAMP concentration locally inactivate PKA in the \( \text{ira2}\Delta \) strain, whereas \( \text{BCY1} \) deletion leads to wholesale activation of PKA throughout the cell. Fitting with this model, \( \text{BCY1} \) deletion inhibits growth and
metabolism on non-fermentable carbon sources, causing cell death during the diauxic shift and
stationary phase, likely from uninhibited PKA (108,109). However, a third possibility is that loss
of BCY1 leads to misdirection of PKA activity. PKA can be directed to subcellular targets in
higher eukaryotes via A-kinase anchoring proteins (AKAP) that bind to and direct localization of
PKA (110). While yeast do not possess orthologs of AKAPs, functional analogs have been
proposed including Bcy1 itself (89,111,112). Anaerobic xylose growth and metabolism may be
decoupled in bcy1Δ strains via disrupted subcellular localization and substrate interactions of
PKA that are coordinated by Bcy1. Additionally, Bcy1 is reported to interact with fatty acid
synthases subunits (Fas1/2) (112), implying a direct, physical connection between PKA and lipid
biosynthesis. While future studies of PKA localization and substrate interactions are needed to
confirm this model, our results show that Bcy1 plays a special role in coordinating PKA activity.

It is evident from this and many other studies that cells have deeply intertwined the
regulation of multiple processes, and disrupting one can have dramatic impacts on many others.
Our results here and in previous work implicate the importance of regulatory rewiring in
decoupling cellular processes. While engineering xylose metabolism pathways is essential to
enable the process, anaerobic xylose fermentation is not enacted without rewiring the regulatory
system to simultaneously activate Snf1 along with PKA (31,32). Here, we propose roles for
several regulators, including Opi1 and Bcy1, among downstream effectors like Azf1, Aft1/2,
Mga2, and Ino2/4, in modulating growth and metabolism decoupling on anaerobic xylose. Our
results strongly suggest that regulatory tinkering rather than altering individual downstream
effectors will be required to optimally engineer new cell functions.

Methods

Media and growth conditions

Cells were grown in YP media (10 g/L yeast extract, 20 g/L peptone) with 20g/L of either
glucose or xylose. Aerobic cultures were grown at 30°C with vigorous shaking. Anaerobic
cultures were grown in a Coy anaerobic chamber (10% CO₂, 10% H₂, 80% N₂) at 30°C with a metal stir bar for mixing. All cultures were inoculated with cells grown aerobically to saturation in YP-glucose and washed one time with the desired growth medium. Anaerobic cultures were inoculated into media incubated in the anaerobic chamber for >16 hours before inoculation. Cell density was monitored by optical density at 600 nm (OD₆₀₀) with an Eppendorf Spectrophotometer. Sugar and ethanol concentrations were measured with HPLC-RID (Refractive Index Detector) analysis (27). Growth on solid media (Fig 5B, Fig S5C) was performed by collecting 1 OD worth of cells from a saturated YP-glucose culture, washing cells with YP-xylose, and plating serial dilutions onto solid YP medium with 2% xylose, with or without 100 µg/mL of nourseothricin. Plates were grown in a Coy anaerobic chamber for seven days before imaging.

Strains and cloning

Saccharomyces cerevisiae strains used in this study are described in Table 3. Gene knockouts were created by homologous recombination with either KanMX or Hph cassettes (35,36) and confirmed with diagnostic PCRs. The KanMX cassette was rescued from the bcy1∆ and EWY55 strains with CRISPR-Cas9 using a gRNA specific for KanMX and a repair template containing the flanking sequence. The bcy1∆ or EWY55 strain’s allele of OPI1, RIM8, or TOA1 was cloned into the pKI plasmid, carrying a nourseothricin [NAT] resistance marker, using standard cloning techniques. Plasmids were verified with Sanger sequencing, then transformed into the appropriate bcy1∆ or EWY55 KAN marker rescued strain using NAT selection.

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Description</th>
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<tr>
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<td>CRB strain with xylose utilization genes (G418-R), gre3::MR isu1::loxP-Hyg (Hyg-R)</td>
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<tr>
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<tr>
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RNA-seq sample collection, RNA extraction, library preparation, and sequencing
Cells from saturated cultures of Y184, *ira2Δ*, *bcy1Δ*, and *ira2Δbcy1Δ* were used to inoculate anaerobic YPD cultures at OD_{600} 0.05. Cultures grew for five hours to early/mid-log phase. 50 mL of the culture was collected, washed with YPX, then used to inoculate anaerobic YPX cultures as described above. Cold 5% phenol/95% ethanol was added to the remaining 50mL YPD cultures, which were harvested by centrifuging at 3000 RPM for 3 minutes and flash frozen in liquid nitrogen. Cell pellets were stored at -80°C until further processing. The YPX cultures grew for 3.5 hours, when the *ira2Δ* strain resumed growth. Cold phenol/ethanol was added to the 50 mL cultures, which were harvested, flash frozen, and stored at -80°C. Samples were collected from three independent replicates performed on different days.

Total RNA was extracted using hot phenol lysis (38) and DNA was digested with Turbo-DNase (Life Technologies, Carlsbad, CA) for 30 minutes at 37°C. RNA was precipitated at 20°C in 2.5 M LiCl for 30 min. rRNA was depleted with EPiCenter Ribo-Zero Magnetic Gold Kit (Yeast) RevA kit (Illumina Inc, San Diego, CA), and the remaining RNA was purified using Agencourt RNACleanXP (Beckman Coulter, Indianapolis, IN) by following the manufacturers’ protocols. RNA-seq libraries were created with the Illuminia TruSeq stranded total RNA kit (Illumina) following the preparation guide (revision C), AMPure XP beads were used for PCR purification (Beckman Coulter, Indianapolis, IN), and cDNA generated with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) as described in the Illumina kit. Libraries were standardized to 2 μM and clusters were generated with standard Cluster kits (version 3) and the Illumina Cluster station. Paired-end 50-bp reads were generated using standard SBS chemistry (version 3) on an Illumina NovaSeq 6000 sequencer.

**RNA-seq data processing and analysis**

RNA-seq reads were processed with Trimmomatic version 0.3 (39) and mapped to the Y22-3 genome (40) using BWA-MEM version 0.7.17 with default settings. Read counts were calculated with HTSeq version 0.6.0(41) using the Y22-3 gene annotations. All raw data were
deposited in the NIH GEO database (accession number pending). Raw sequence counts were normalized using trimmed mean of M-values (TMM) method (42). log₂ fold changes (FC) between YP-xylose and YP-glucose samples for each strain and replicate were calculated, then hierarchical clustered using Gene Cluster 3.0 (43) and visualized with Java Treeview version 1.2.0 (44). Differential expression was analyzed using linear modeling in edgeR version 4.0.3(45) using pairwise and group comparisons, calling significance at < 0.05 Benjamini and Hochberg false discovery rate (FDR) (46). Genes in Fig 2B were identified by pairwise comparisons between The Y184, bcy1Δ, and ira2Δbcy1Δ strains with the ira2Δ strain. Genes in Fig 2C were identified by comparing the ira2Δ, bcy1Δ, and ira2Δbcy1Δ strains as a group with the Y184 strain. Genes in Fig 3A were identified first by pairwise comparison between the ira2Δ and bcy1Δ strains, then subsequently further grouped by genes reproducibly expressed in opposing directions between the two strains (e.g. log₂FC > 0 in bcy1Δ and log₂FC < 0 in ira2Δ). Genes differentially expressed between EWY55 and bcy1Δ strains grown anaerobically on xylose were identified using edgeR version 4.0.3 (45) at FDR (46) < 0.05. Genes were median centered, the log₂ YPX abundance of EWY55 or ira2Δ transcripts, relative to log₂ bcy1Δ YPX abundance were calculated, then divided into four clusters (A, B, C, D) based on hierarchical clustering in Gene Cluster 3.0 (43) and visualized in in boxplots.

Functional gene ontology (GO) term and transcriptional regulator enrichment was performed using SetRank (47); an FDR cutoff of 0.05 was used for transcription target analysis and a p-value cutoff of 10⁻⁴ was used to assess overlapping GO categories. Targets of transcription factors were downloaded from YeasTract (48) using only targets with DNA binding evidence. Upstream regulatory motifs were identified with MEME suite version 5.4.1 (49) and associated transcription factors were implicated using Tomtom (50).

Lipidomics sample collection and preparation
Cells were grown as described previously for the RNAseq collection, flash frozen in liquid nitrogen, then stored at -80°C. On the day of analysis, each sample was removed from -80°C and maintained on dry ice until time of extraction. 240 μL chilled methanol was added to cell pellet samples in their native tubes over dry ice. Native tubes were transferred to ice and then vortexed. Samples were then transferred to 2 mL microcentrifuge tubes over ice. Next 800 μL of chilled methyl tert-butyl ether (MTBE) was added to native tubes followed by vortexing; these samples were also transferred to the microcentrifuge tube. Microcentrifuge tubes were then vortexed for 10 seconds. A 1/32 teaspoon (0.15 mL) of 1,180 μm glass beads (16-25 US sieve) was added to each tube along with 200 μL LC-MS grade water. Tubes were vortexed for 10 seconds. All tubes were centrifuged at 4°C for 2 minutes at 5,000 x g to pellet cell debris. An extraction blank was prepared per sample preparation steps directly into a 2 mL microcentrifuge tube without yeast.

200 μL of the top (lipophilic) layer from each tube was aliquoted into a low volume amber borosilicate glass autosampler vial with tapered insert. For pooled YPD and pooled YPX samples, the 200 μL aliquot was performed in duplicate. Each vial was dried in a vacuum concentrator for approximately one hour. For pooled YPD and pooled YPX samples, resuspension was performed with 50 μL of a 9:1 MeOH:toluene solution on the first of two preparations (“1X”) while the second preparation was resuspended in 25 μL of 9:1 MeOH:toluene (“2X”). Remaining dried samples were resuspended in 50 μL of 9:1 MeOH:toluene. Each vial was vortexed vigorously for 10 seconds to ensure resuspension of the dried contents. Samples were placed in the instrument’s autosampler at 4°C to await injection.

**Lipidomics LC-MS analysis**

LC-MS/MS analysis was performed using an Acquity CSH C18 column (2.1 mm × 100 mm, 1.7 μm particle size, Waters) held at 50°C and a Vanquish Binary Pump (400 μL/mL flow rate; Thermo Scientific, Waltham, MA). Mobile phase A consisted of ACN:H2O (70:30, v/v) with
10 mM ammonium acetate and 0.025% acetic acid. Mobile phase B consisted of IPA:ACN (9:1, v/v) with 10 mM ammonium acetate and 0.025% acetic acid. Initially, mobile phase B was held at 2% for 2 min and increased to 30% over 3 min. In consecutive ramping steps, mobile phase B was increased to 50% over 1 minute, increased to 85% over 14 minutes, and increased to 99% over 1 minute. The gradient was held at 99% mobile phase B for 7 minutes, then decreased to 2% over 0.25 minutes. The column was equilibrated at 2% mobile phase B for 1.75 minutes before the next injection. 10 μL of each extract was injected by a Vanquish Split Sampler HT autosampler (Thermo Scientific, Waltham, MA) in a randomized order.

The LC system was coupled to a Q Exactive HF Orbitrap mass spectrometer (MS) through a heated electrospray ionization (HESI II) source (Thermo Scientific, Waltham, MA). Source conditions were as follows: HESI II and capillary temperature at 350 °C, sheath gas flow rate at 25 units, aux gas flow rate at 15 units, sweep gas flow rate at 5 units, spray voltage at $|3.5 kV|$, and S-lens RF at 60.0 units. The MS was operated in a polarity switching mode acquiring positive and negative full MS and MS2 spectra (Top2) within the same injection. Acquisition parameters for full MS scans in both modes were 30,000 resolution, 1 × 106 automatic gain control (AGC) target, 100 ms ion accumulation time (max IT), and 200 to 2000 m/z scan range. MS2 scans in both modes were then performed at 30,000 resolution, 1 × 105 AGC target, 50 ms max IT, 1.0 m/z isolation window, stepped normalized collision energy (NCE) at 20, 30, 40, and a 10.0 s dynamic exclusion.

**Lipidomics Data Analysis**

The resulting LC–MS data were processed using Compound Discoverer 3.1 (Thermo Scientific, Waltham, MA) and LipiDex, an in-house-developed software suite (51). All peaks between 0.4 min and 21.0 min retention time and between 100 Da and 5000 Da MS1 precursor mass were aggregated into compound groups using a 10-ppm mass, 0.2 min retention time tolerance, a minimum peak intensity of 1x10^5, a maximum peak-width of 0.75 min, and a
signal-to-noise (S/N) ratio of 3. Features were required to be 5-fold greater intensity in samples than blanks. MS/MS spectra were searched against an in-silico generated lipid spectral library. Spectral matches were required to have a dot product score greater than 500 and a reverse dot product score greater than 700. Lipid MS/MS spectra which contain acyl-chain specific fragments and contained no significant interference (<75 %) from co-eluting isobaric lipids were identified at molecular species level. If individual fatty acid substituents were unresolved, then identifications were made with the sum of the fatty acid substituents. Lipid features were further filtered based on 1) presence in a minimum of two raw files, 2) a median absolute retention time deviation of 3.5, and 3) average pooled relative standard deviations of less than 30%.

Differential abundance of lipids was analyzed with linear modeling in edgeR version 4.0.3 using pairwise comparisons and a Benjamini and Hochberg (46) FDR < 0.05 to call significance. After the log$_2$FC between YP-xylose and YP-glucose samples for each strain was calculated, lipids were hierarchically cluster in Gene Cluster 3.0 (43) and visualized in Java Treeview 1.2.0 (44). For all phosphatidylcholine moieties, a paired ANOVA with a cutoff of $p < 0.05$ was performed between $ira2\Delta$ and $bcy1\Delta$ samples. All raw and processed lipidomics data files were deposited in MassIVE database under dataset number MSV000090868.

For EWY55 lipidomics data, differential abundance of lipids was analyzed by calculating the log$_2$(fold change) ratio between YPX and YPD samples for each strain and replicate. The paired log$_2$(fold change) differences between EWY55 and $ira2\Delta$ or $bcy1\Delta$ samples were calculated, and an absolute value difference greater than 1.5 on a log$_2$ scale was called significant. Lipids were hierarchically cluster in Gene Cluster 3.0 (43) and visualized in Java Treeview 1.2.0 (44).

Phosphoproteomics data
Phosphoproteomics data from Myers et al. (2019) (32) was analyzed to compare the phosphorylation of phospholipid biosynthesis enzymes. Reproducible pairwise comparisons between YP-xylose samples of strains with a log2 fold-change >2 were called significant.

**Inositol and Choline Supplementation**

YP-xylose medium was prepared as described above. Myo-inositol (Sigma, Burlington, MA) was added to a final concentration of 75 µM and choline (Thermo Scientific, Waltham, MA) to a concentration of 10 mM. Anaerobic cultures were inoculated from saturated overnight cultures to an OD$_{600}$ of 0.1. Growth, xylose concentration, and ethanol concentration was monitored over 44 hours. A paired ANOVA between YP-xylose and YP-xylose-inositol-choline cultures was performed to determine significant differences between growth using a $p$ value cutoff of 0.05.

**Adaptive Laboratory Evolutions**

$bcy1\Delta$ cells were inoculated in anaerobic YP-glucose medium at an OD$_{600}$ of 0.01 and grown for ~21 generations. This was used to seed a fresh anaerobic YP-glucose culture at an OD$_{600}$ of 0.01, which grew for ~7 generations. From this, a YP-2% xylose 0.1% glucose culture was seeded at an OD$_{600}$ of 0.01, then grown for ~7 generations. This process was repeated four more times before plating the culture on YP-xylose and collecting single colonies capable of growing anaerobically on xylose. Evolutions were performed in three independent cultures.

**Evolved $bcy1\Delta$ strain genome sequencing and analysis**

Evolved $bcy1\Delta$ strains were grown aerobically in YP-glucose and genomic DNA was extracted using the Qiagen (Hilden, Germany) Genomic-tip 20/G kit following manufacturer’s protocol. Genomic DNA was fragmented into ~200 bp fragments using a sonifier with four minutes on and one minute off while incubating on ice, repeated for a total of four cycles. DNA
libraries were made using the NEBNext Ultra II DNA Library Prep Kit for Illumina protocol, using the NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1) (New England Biolabs, Ipswich, MA). Paired-end 300 bp reads were generated on an Illumina MiSeq.

Variants in the parental \( \text{bcy1} \Delta \) strain were identified with GATK version 4.2 (Broad Institute) and substituted into the Y22-3 reference genome as a mapping reference. Reads were mapped to the newly generated \( \text{bcy1} \Delta \) strain genome, and variants were called using GATK version 4.2 and SNPs annotated with SnpEff version 5.0 and vcftools version v0.,1.12b. SNPs resulting in an amino acid change were considered for further analysis and verified with Sanger sequencing.

Acknowledgements

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Supporting Information Captions

S1 Fig. **ira2Δbcy1Δ** strain's growth resembles that of the **bcy1Δ** strain.

A. Average (n = 3 biological replicates) growth (OD600, optical density) of **ira2Δ**, **bcy1Δ**, and **ira2Δbcy1Δ** strains grown anaerobically on rich xylose medium (p < 10^-4, ANOVA).
B. Average (n = 3 biological replicates) xylose concentration in the medium over time for \(ira2\Delta\), \(bcy1\Delta\), and \(ira2\Deltabcy1\Delta\) strains grown anaerobically on rich xylose medium \((p < 10^{-2}, \text{ANOVA})\).

S2 Fig. Transcriptomic profile of strains with varying xylose utilization and growth capabilities show RP transcripts are not limiting.

A. 5834 genes (rows) detected in all four strains \((Y184, \text{ira2}\Delta, \text{bcy1}\Delta, \text{ira2}\Delta\text{bcy1}\Delta)\), organized by hierarchical clustering of \(\log_{2}\) (fold change) upon glucose-to-xylose shift. Each column represents one of three biological replicates of the denoted strain listed above.

B. 374 ribosomal protein genes (rows) in all four strains \((Y184, \text{ira2}\Delta, \text{bcy1}\Delta, \text{ira2}\Delta\text{bcy1}\Delta)\), organized by hierarchical clustering of \(\log_{2}\) (fold change) upon glucose-to-xylose shift.

S3 Fig. Genes expression changes specific to the \(bcy1\Delta\) strain show changes in induction/repression and not basal mRNA abundances.

A. 654 genes whose \(\log_{2}\) (fold change) upon glucose to xylose shift is different (FDR < 0.05) between the \(\text{ira2}\Delta\) and \(\text{bcy1}\Delta\) strains and shows expression changes in the opposite direction (see Methods for details). The yellow-blue heatmap on the left represents the YPX/YPD \(\log_{2}\) (fold change). The green-purple heatmap on the right represents transcript (rows) abundance in anaerobic glucose (G) and anaerobic xylose (X) relative to the average \((n = 3)\) abundance of transcript in the Y184 YPD sample.

B. ~500 base pairs upstream of the ORF for genes repressed in the \(bcy1\Delta\) strain upon shift to xylose were analyzed for enriched motifs (MEME Suite), analyzed for known transcription factor binding sites (TOMTOM), and identified the Aft1/2 consensus site (see Methods for details).

C. 26 genes (rows) reported to be bound Mga2 in the \(\text{ira2}\Delta\) and \(\text{bcy1}\Delta\) strains. Genes are organized by hierarchical clustering of \(\log_{2}\) (fold change) after shift from glucose to xylose.
D. 11 genes (rows) reported to be bound by Mga2 whose log₂(fold change) is significantly (FDR < 0.05) different upon shift to xylose in the *bcy1Δ* strain compared to the *ira2Δ* strain. Genes are organized by hierarchical clustering.

S4 Fig. Phosphatidylcholine abundance upon shift to xylose is lower in *bcy1Δ* cells compared to *ira2Δ* cells.

A. All phosphatidylcholine species (rows) identified show significantly lower log₂(fold change) upon the shift from glucose to xylose in the *bcy1Δ* and *ira2Δbcy1Δ* compared to the *ira2Δ* strain ($p = 0.0015082$, ANOVA).

S5 Fig. Evolved *bcy1Δ* strain recapitulates the *ira2Δ* strain’s phenotype but not transcriptome.

A-B. Average (n = 3 biological replicates) of (A) xylose concentration or (B) ethanol concentration for *ira2Δ*, *bcy1Δ*, and EWY55 strains anaerobically grown in rich xylose medium ($p > 0.05$, ANOVA).

C. Representatives of multiple replicates of EWY55 or EWY55 cells lacking *OPI1* (top panels), *RIM8* (middle panels), or *TOA1* (bottom panels) and complemented with an empty vector or parental or evolved allele grown anaerobically on solid xylose (left) or glucose (right) medium with NAT selection.

S6 Fig. Directed evolution on anaerobic xylose generated multiple evolved strains with varying growth rates.

A-E. Average (n = 3 biological replicates) growth (OD600, optical density) of *ira2Δ*, *bcy1Δ*, EWY55, and (A) EWY87-1, (B) EWY87-3, (C) EWY89-1, (D) EWY89-2, or (E) EWY89-3 strains ($p < 10^{-4}$, ANOVA).
S7 Fig. Evolved bcylΔ strain has lower abundance of phospholipid biosynthesis genes on xylose compared to the bcylΔ strain. Partial phospholipid biosynthesis pathway with transcriptomic and lipidomic data. Yellow-blue boxes next to each enzyme name represents the average (n=3 biological replicates) log₂(fold change) of YPX transcript abundance for (left) EWY55 compared to bcylΔ cells and (right) ira2Δ compared to bcylΔ cells. Significant (FDR < 0.05) differences in log₂(fold change) YPX transcript abundance when compared to bcylΔ strain have bolded/brighter boxes. Colored lipid boxes represent lipids whose log₂(fold change) in abundance upon shift from glucose to xylose are larger (green) in EWY55 compared to bcylΔ or smaller (pink) in EWY55 compared to bcylΔ.

S1 Table. log₂(fold change) (n=3) for all transcripts in dataset.

S2 Table. log₂(fold change) (n=3) for transcripts that significantly differ in fold change upon xylose shift in Y184, bcylΔ, or ira2ΔbcylΔ cells compared to the ira2Δ strain.

S3 Table. Average (n=3) log₂(fold change) of cell cycle kinase and cyclin transcripts.

S4 Table. log₂(fold change) (n=3) for ribosomal protein transcripts.

S5 Table. log₂(fold change) (n=3) for transcripts that significantly differ in fold change upon xylose shift in ira2Δ, bcylΔ, and ira2ΔbcylΔ cells compared to the Y184 strain.

S6 Table. log₂(fold change) (n=3) for transcripts that are annotated in central carbon metabolism and significantly differ in fold change upon xylose shift in ira2Δ, bcylΔ, and ira2ΔbcylΔ cells compared to the Y184 strain.
S7 Table. \( \log_2 \) (fold change) (n=3) for transcripts that significantly differ in fold change and directionality upon xylose shift in \( \text{bcy1} \Delta \) cells compared to \( \text{ira2} \Delta \) cells.

S8 Table. Functional gene ontology enrichments for clusters in Fig 3A.

S9 Table. \( \log_2 \) (fold change) (n=3) for all lipids in dataset.

S10 Table. \( \log_2 \) (fold change) (n=3) for lipids that significantly differ in fold change upon xylose shift in \( \text{ira2} \Delta \), \( \text{bcy1} \Delta \), and \( \text{ira2} \Delta \text{bcy1} \Delta \) cells compared to the Y184 strain.

S11 Table. \( \log_2 \) (fold change) (n=3) for lipids that significantly differ in fold change upon xylose shift in \( \text{ira2} \Delta \text{bcy1} \Delta \) cells compared to the \( \text{ira2} \Delta \) strain.

S12 Table. \( \log_2 \) (fold change) (n=3) for all phosphatidylcholine entities.

S13 Table. Transcripts whose abundance significantly differs in EWY55 cells compared to the \( \text{bcy1} \Delta \) strain in xylose. Transcript abundance differences (n=3) of EWY55 and \( \text{ira2} \Delta \) compared to \( \text{bcy1} \Delta \) cells.

S14 Table. Transcripts (n=3) whose abundance significantly differs in EWY55 and \( \text{ira2} \Delta \) cells compared to the \( \text{bcy1} \Delta \) strain in xylose.

S15 Table. Transcript abundance differences of phospholipid biosynthetic genes in EWY55 and \( \text{ira2} \Delta \) cells compared to \( \text{bcy1} \Delta \) cells on xylose.

S16 Table. \( \log_2 \) (fold change) (n=3) for lipids that significantly differ in fold change upon
xylose shift in EWY55 cells compared to the \textit{bcy1}Δ strain.