# Pathway swapping: Toward modular engineering of essential cellular processes

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**Recent developments in synthetic biology enable one-step implementation of entire metabolic pathways in industrial microorganisms. A similarly radical remodelling of central metabolism could greatly accelerate fundamental and applied research, but is impeded by the mosaic organization of microbial genomes. To eliminate this limitation, we propose and explore the concept of "pathway swapping," using yeast glycolysis as the experimental model. Construction of a "single-locus glycolysis" *Saccharomyces cerevisiae* platform enabled quick and easy replacement of this yeast's entire complement of 26 glycolytic isoenzymes by any alternative, functional glycolytic pathway configuration. The potential of this approach was demonstrated by the construction and characterization of *S. cerevisiae* strains whose growth depended on two nonnative glycolytic pathways: a complete glycolysis from the related yeast *Saccharomyces kudriavzevii* and a mosaic glycolysis consisting of yeast and human enzymes. This work demonstrates the feasibility and potential of modular, combinatorial approaches to engineering and analysis of core cellular processes.**

pathway swapping | glycolysis | *Saccharomyces cerevisiae* | modular genomes

Replacement of petrochemistry by bio-based processes is a key element for sustainable development and requires microbes equipped with novel-to-nature capabilities. Recent developments in synthetic biology enable introduction of entire metabolic pathways and, thereby, new functionalities for product formation and substrate consumption, into microbial cells (1). However, industrial relevance of the resulting strains critically depends on optimal interaction of the newly introduced pathways with the core metabolism of the host cell. Central metabolic pathways such as glycolysis, tricarboxylic acid cycle, and pentose phosphate pathways, are essential for synthesis of precursors, for providing free energy (ATP), and for redox-cofactor balancing. Optimization of productivity, product yield, and robustness therefore requires modifications in the configuration and/or regulation of these core metabolic functions.

Engineering of central metabolism is in some respects more challenging than the functional expression of heterologous product pathways. Millions of years of evolution of microorganisms have endowed their metabolic and regulatory networks with a level of complexity that cannot be efficiently reengineered by iterative, single-gene modifications. Enzymes of central metabolism are encoded by hundreds of genes that, especially in eukaryotes, are scattered across microbial genomes. Moreover, inactivation and subsequent replacement of genes involved in central metabolism is complicated by functional redundancy of isoenzymes (2, 3) as well as by the essential role of many of the corresponding biochemical reactions. Microbial platforms in which the configuration of key pathways can be remodelled in a swift, combinatorial manner would provide an invaluable asset for fundamental research and engineering of central metabolism.

Whereas rapid, cost-effective assembly of entire synthetic genomes is becoming a realistic perspective for small bacterial genomes (4, 5), routine synthesis and expression of entire eukaryotic genomes is unlikely to be implemented in the next few years. Here, we propose and experimentally explore a modular approach to the engineering of central metabolism that involves versatile, synthetic microbial strain platforms in which entire metabolic pathways can, in a few simple steps, be replaced by any functional, newly designed configuration. As a proof of principle, we set out to construct a platform that enables swapping of the entire Embden–Meyerhoff–Parnas pathway of glycolysis, a strongly conserved metabolic highway for sugar utilization in the model eukaryote and industrial yeast *Saccharomyces cerevisiae*. Including the reactions leading to the formation of ethanol, the main fermentation product of *S. cerevisiae*, yeast glycolysis encompasses a set of 12 reactions, catalyzed by no fewer than 26 cytosolic isoenzymes. Several of these (e.g., Tpi1, Tdh3, and Adh1) are among the most abundant proteins in yeast cells. The genes encoding glycolytic enzymes are scattered over 12 of the 16 yeast chromosomes. Construction of a platform for glycolysis swapping involved a two-step approach (Fig. 1*A*). In the first step, described in a recent study by our group, the genetic complexity of yeast glycolysis was reduced by deleting the structural genes for 13 of the 26 glycolytic enzymes. Remarkably, a detailed systems analysis revealed that, under laboratory conditions, the phenotype of the resulting minimal glycolysis (MG) strain was virtually identical to that of the parental strain carrying a full complement of glycolytic genes (2). In a next step, the remaining 13 glycolytic genes in MG were expressed from a single chromosomal

## Significance

**Replacement of petrochemistry by bio-based processes requires microbes equipped with novel-to-nature capabilities. The efficiency of such engineered microbes strongly depends on their native metabolic networks, which, forged by eons of evolution, are complex and encoded by mosaic microbial genomes. Absence of a modular organization of genomes tremendously restricts genetic accessibility and presents a major hurdle for fundamental understanding and rational engineering of central metabolism. Using as a paradigm the nearly ubiquitous glycolytic pathway, we introduce a radical approach, enabling the "transplantation" of essential metabolic routes in the model and industrial yeast *Saccharomyces cerevisiae*. This achievement demonstrates that a modular design of synthetic genomes offers unprecedented possibilities for fast, combinatorial exploration, and optimization of the biological function of essential cellular processes.**

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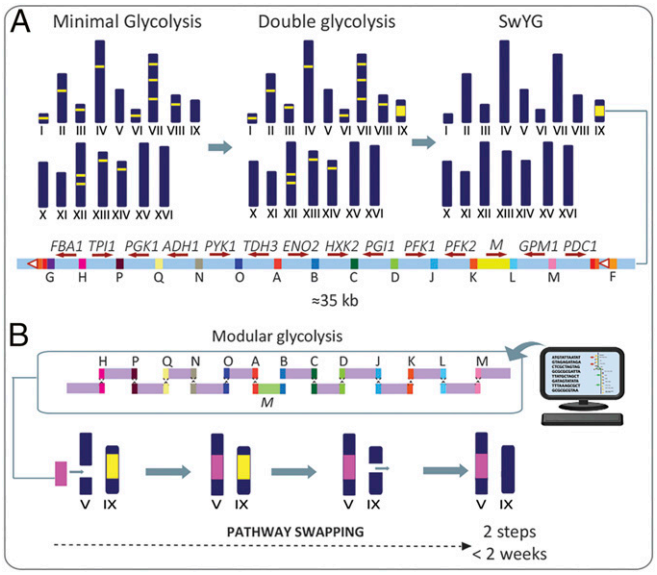


**Fig. 1.** Schematic overview of the glycolysis swapping approach. (*A*) Construction of SwYG that contains a single locus endogenous glycolysis platform for pathway swapping. (*B*) In silico design and in vivo assembly and integration of the glycolytic gene cluster on chromosome V, followed by the removal of the endogenous glycolysis on chromosome IX, leading to a strain with a redesigned glycolysis. *M*, selectable marker.

locus. Finally, the remaining scattered native genes were removed from their original loci, leading to switchable yeast glycolysis (SwYG), a yeast strain carrying a native, minimal single-locus glycolysis on chromosome IX. In SwYG, glycolysis can be swapped in two steps by integration of a new, heterologous, or synthetic glycolytic gene cluster, followed by removal of the minimal single-locus glycolysis that was initially integrated on chromosome IX (Fig. 1*B*).

## Results

**Engineering of a Yeast Platform for Glycolysis Swapping.** A single-locus native glycolysis gene cluster was assembled from "glycoblocks" (Fig. 1 and *SI Appendix*, Fig. S1), 13 DNA cassettes each consisting of a *S. cerevisiae* glycolytic gene, including its native promoter and terminator, flanked by 60-bp synthetic homologous recombination (SHR) sequences (6). SHR sequences share no homology with the *S. cerevisiae* genome and can be used for efficient in vivo assembly and integration, by homologous recombination (HR), of the glycoblocks. Moreover, use of standardized SHR sequences enables flexible design and combinatorial assembly of different glycolytic pathway variants. The single-locus MG cluster was composed of 13 glycoblocks (corresponding to the 13 genes remaining in MG) and of a selectable marker (amdSYM, encoding *Aspergillus nidulans* acetamidase conferring the ability to grow on acetamide as sole nitrogen source) (7) flanked by SHR sequences (Fig. 1*A*). The single-locus native glycolytic cluster was integrated into the yeast genome to promote stable, single-copy expression, using combined assembly and targeted integration (CATI) (8) (*SI Appendix*, Fig. S1). To this end, recognition sequences for the I-SceI homing endonuclease were introduced at the *SGA1* locus on chromosome IX of MG (*SI Appendix*, Fig. S1). The 13 glycoblocks and the amdSYM cassette were cotransformed to the modified MG strain, in which *SCEI* was induced by growth on galactose to introduce a double-strand DNA break at the *SGA1* locus, thereby promoting integration of the glycoblocks (Fig. 2*A* and *SI Appendix*, Fig. S1). Four of five tested transformant colonies harbored the complete 35-kb single-locus native glycolysis (SinLoG-IX) integrated at the *SGA1* locus. In a selected transformant (strain IMX382), next-generation (next-gen) sequencing showed that the in vivo assembled and integrated glycolytic gene cluster was virtually identical to its in silico blueprint (Fig. 2*A*). Six of the nine deviations in nucleotide sequence were found at the HR loci linking the glycoblocks, which may either reveal recombinase-based errors or simply errors in the primers used to construct the HR sequences. Of the glycolytic genes, only *ADH1* was found to contain a mutation that was synonymous (A180A).

*S. cerevisiae* IMX382 was further engineered by deleting the 13 remaining glycolytic genes from their native loci (Fig. 1*A* and *SI Appendix*, Fig. S2). The first five deletions, targeting *PYK1*, *PGI1*, *TPI1*, *TDH3*, and *PGK1*, were performed with standard deletion cassettes, using I-SceI–mediated marker removal to recycle multiple selection markers simultaneously without leaving scars in the genome (9) (*SI Appendix*, Fig. S3). For subsequent engineering, an expression cassette encoding the CRISPR endonuclease Cas9 was integrated at the *PFK2* locus, thereby deleting *PFK2* (*SI Appendix*, Fig. S4). The remaining glycolytic genes, *PFK1*, *GPM1*, *HXK2*, *FBA1*, *ADH1*, and *PDC1*, were deleted from their native loci with the CRISPR/Cas9 system (10). Except for *ENO2*, all glycolytic expression cassettes harbored by the SinLoG-IX cluster were able to complement a null mutation in the corresponding native gene. The native promoter of *ENO2*, designed to be 411 bp long to avoid expressing unwanted ORFs from the glycolytic gene cluster, proved to be too short to drive expression of *ENO2* and was replaced by a longer promoter (1,012 bp) (*SI Appendix*, Table S9). This observation underlines the limited knowledge, even for glycolytic genes, on promotor structure and function in yeast and

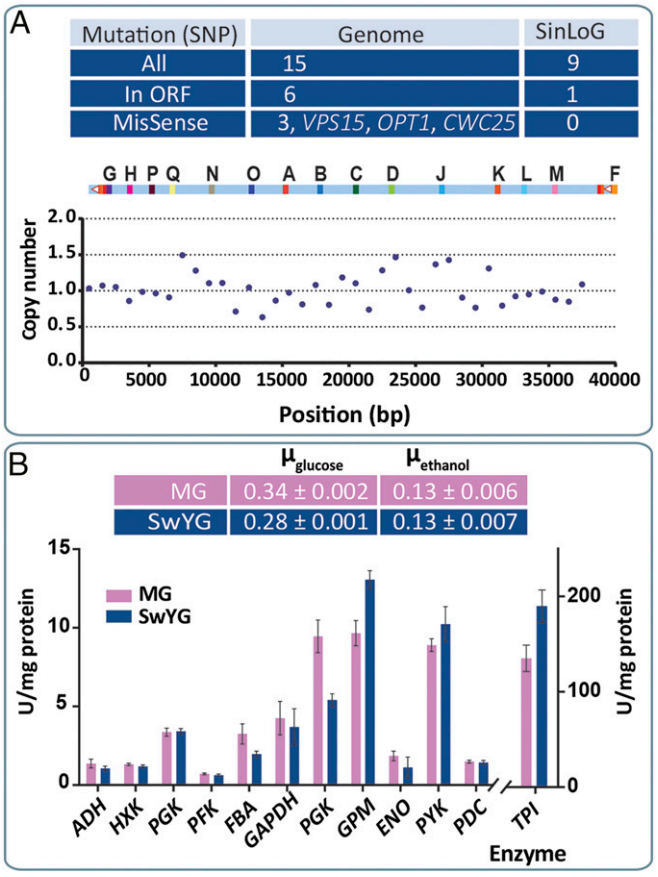


**Fig. 2.** Characterization of SwYG. Next-gen sequencing and copy number analysis of the auxotrophic SwYG (IMX589) containing a clustered set of glycolytic genes (SinLoG-IX) (*A*). Physiological characterization in shake-flask culture using chemically defined medium with glucose as carbon source of IMX606 (prototrophic SwYG strain) and the MG strain (IMX370) (*B*). Growth rates ($hour^{-1}$) and enzyme activity data represent the average and SEM of at least two independent culture replicates.

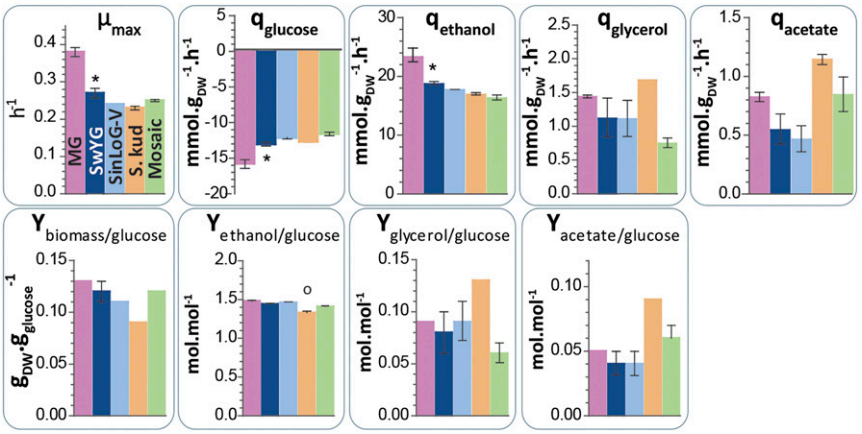


**Fig. 3.** Physiological characterization during aerobic batches in bioreactors of MG (IMX370), SwYG (IMX606), SinLoG-V (IMX605), *Sk*-SinLoG-V (S. kud, IMX652), and mosaic SinLoG-V (Mosaic, IMX645). The strains were cultivated in chemically defined medium with glucose as carbon source. Bars and error bars represent the average and SEM of independent duplicate cultures. Stars indicate that the data from SwYG significantly differ from MG data; empty dots indicate significant differences between SwYG and the SwYG-based strains (*P* value <0.05, two-tailed *t* test, samples with equal variance). DW, biomass dry weight.

highlights the need for systematic design of synthetic promoter. This last genetic modification yielded SwYG (IMX589). Whole-genome sequencing of this strain confirmed: (*i*) the correct sequence of the single-locus native glycolysis (*SI Appendix*, Table S1) and its integration at the *SGA1* locus, (*ii*) deletion of the native glycolytic genes from their original loci (*SI Appendix*, Fig. S5), and (*iii*) absence of duplicated glycolytic genes in the single-locus native glycolysis and in the genome (Fig. 2*A* and *SI Appendix*, Fig. S6). Relative to the ancestor MG strain, only six ORFs in the genome of SwYG contained a nucleotide difference. Three of these caused an amino acid substitution in the encoded protein (Fig. 2*A* and *SI Appendix*, Table S2). None affected glycolytic genes or genes that are known to be otherwise associated with glycolysis. The specific growth rate of the prototrophic SwYG (IMX606) measured in aerobic batch in shake-flask culture, on chemically defined medium using glucose as carbon source, was slightly lower than that of the parental MG strain (17% lower) (Fig. 2*B*). However, more accurate quantification of specific growth rates in tightly controlled bioreactors revealed a stronger impact of the relocalization of the glycolytic gene cluster. The specific growth rate of SwYG was decreased by 28% compared with its parent MG (Fig. 3). Concomitantly the major metabolic fluxes, i.e., the specific glucose uptake, and ethanol production rates, were decreased in SwYG. Remarkably, the biomass and product yields on glucose remained unaffected by the relocalization of the glycolytic genes to chromosome IX (Fig. 3), showing that the growth stoichiometry was conserved and that only the magnitude of fluxes was attenuated in SwYG. These decreased rates of SwYG are unlikely to result from differences in glycolytic capacity as the activities of glycolytic enzymes in cell extracts of these two strains were highly similar (Fig. 2*B*).

**Chromosome Hopping of a Yeast Glycolysis Gene Cluster.** To test the feasibility of glycolysis swapping, we attempted to exchange the single-locus native glycolysis integrated on chromosome IX by a nearly identical copy integrated on chromosome V (*SI Appendix*, Fig. S7). SwYG was transformed with a complete set of glycoblocks (Fig. 4*A*), and with a CRISPR plasmid carrying a guide RNA (gRNA) designed to target Cas9 to the *CAN1* locus on chromosome V, thus enabling in vivo assembly and integration of a second glycolytic gene cluster called SinLoG-V. Colony PCR showed that at least 2 of 12 G418-resistant transformants carried the complete set of genes in this SinLoG-V, correctly inserted at the *CAN1* locus. After curing the *URA3*-carrying CRISPR plasmid, a selected clone was transformed with a 120-bp repair fragment and a new CRISPR plasmid carrying gRNAs targeting Cas9 to sequences positioned at each end of the SinLoG-IX, thereby excising the entire cluster from the genome. All three tested transformants were shown to lack the SinLoG-IX and to have retained the newly inserted single-locus native glycolysis on chromosome V. Whole-genome sequencing of one clone (IMX605) confirmed the successful relocalization of the entire glycolytic gene cluster and

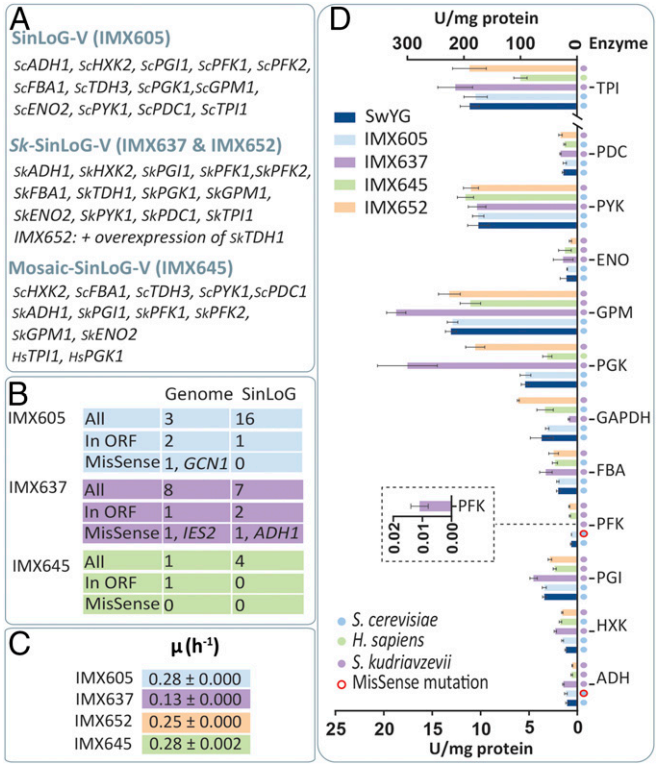


**Fig. 4.** Characterization of yeast strains with a remodelled glycolysis. SinLoG-V (IMX605), *Sk*-SinLoG-V (IMX637), and mosaic SinLoG-V (IMX645) (*A*) were analyzed by next-gen sequencing to identify mutations compared with SwYG (*B*). The maximum specific growth rate on chemically defined medium with glucose as carbon source was measured (*C*), as well as in vitro enzymatic activity of the glycolytic enzymes from cell extracts (*D*). Data represent the average and SEM of at least two independent culture replicates.

that no recombination had occurred between the glycoblocks or the excised single-locus native glycolysis gene cluster and the genome during glycolysis swapping (*SI Appendix*, Fig. S6). IMX605 grew as fast as SwYG in chemically defined medium (Fig. 4*C*) and displayed the same activity of the glycolytic enzymes in cell extracts (Fig. 4*D*). In IMX605, the position of the selectable marker and the *ENO2* glycoblock were reversed compared with the SinLoG-IX cluster in SwYG. However, this different organization did not affect *ENO2* expression (Fig. 4*D*). The similarity in specific growth rate between SwYG and IMX605 was also observed during growth in bioreactors (Fig. 3). These cultures in bioreactor also revealed identical metabolic rates and yields in the two strains (Fig. 3). This lack of locus-specific expression demonstrated that the *CAN1* locus on chromosome V was a suitable "landing pad" for further testing of the pathway swapping concept.

***S. cerevisiae* Expressing a Heterologous Glycolytic Pathway.** Demonstration of the technical feasibility of pathway swapping opened up the way to test whether it is possible to integrally replace yeast glycolysis, an essential, tightly controlled metabolic pathway, by heterologous or synthetic variants. For this purpose, we selected a donor of glycolytic genes within the *Saccharomyces* genus. *Saccharomyces kudriavzevii* is a cold-tolerant close relative of *S. cerevisiae*, recently identified as an important contributor to wine making in cool climates (11, 12). Whereas glycolytic genes and enzymes of *S. kudriavzevii* have not been characterized in detail, its genome sequence is available (13). The complement of putative glycolytic genes in *S. kudriavzevii* and their sequences differ from those of the established *S. cerevisiae* glycolytic genes. However, putative *S. kudriavzevii* glycolytic genes with substantial identity (above 89% at the protein level) with their *S. cerevisiae* orthologs were easily identified by sequence comparison (*SI Appendix*, Table S3). Major glycolytic isoenzymes in *S. kudriavzevii* were selected based on high transcript levels of their structural genes (14). *S. kudriavzevii* does not contain a homolog of *ScTDH3*, the most highly expressed glycolytic gene in *S. cerevisiae* (15), but harbors two other putative *TDH* genes. *SkTDH1* closely resembles *ScTDH1*, whereas *SkTDH2* is more similar to *ScTDH2* and *ScTDH3*. Based on its high expression level during wine fermentation, the gene homologous to *ScTDH1* was selected for construction of a synthetic *S. kudriavzevii* glycolysis cluster. Because promoters within the *Saccharomyces* genus are functional in different species belonging to this group (16, 17), and promoters of *S. kudriavzevii* and *S. cerevisiae* are highly homologous (44–80% identity with an average of 72% for glycolytic promoters), *S. kudriavzevii* glycoblocks were constructed with their native promoters and terminators. Following the approach described above, the single-locus native glycolysis in SwYG was replaced by the *S. kudriavzevii* glycolysis integrated on chromosome V (*Sk*-SinLoG-V) (Fig. 4*A*). Transformants that only retained the *S. kudriavzevii* glycolysis, including a selected clone, IMX637, showed a strongly reduced specific growth rate on glucose, suggesting that the glycolytic function of the integrated set of *S. kudriavzevii* genes was suboptimal (Fig. 4*C*). Because *SkTDH1* was not an ortholog of *ScTDH3*, we hypothesized that insufficient GAPDH activity caused the suboptimal specific growth rate. Indeed, the specific growth rate was almost completely restored to the level of SinLoG-V (IMX605) when *SkTDH1* was overexpressed in the *Sk*-SinLoG-V strain IMX637 (strain IMX652) (Fig. 4*C*). Accordingly GAPDH activity, very low in the *Sk*-SinLoG-V strain, was boosted by overexpressing *SkTDH1* (Fig. 4*D*). Enzyme activity assays also revealed a very low activity of phosphofructokinase in cell extracts of the *Sk*-SinLoG-V strain. Remarkably, this activity was fully restored upon overexpression of *SkTDH1* (Fig. 4*D*). During growth in bioreactor, IMX652, carrying an overexpression of *SkTDH1*, grew nearly as fast as SwYG, but seemed to display a perturbation of its metabolic network (Fig. 3). Most of the observed increase in specific glycerol and acetate production rates and yields in IMX652, however, were not deemed statistically significant compared with SwYG (Fig. 3). Whereas these variations fell within measurement error, they would be consistent with a perturbation of flux distribution at the glyceraldehyde-3-phosphate branch point in favor of glycerol synthesis. Altogether these results illustrate how pathway swapping can identify interesting regulatory phenomena, and interesting leads for follow-up studies.

***S. cerevisiae* Expressing a Mosaic Glycolysis.** To further test the pathway swapping concept, we constructed a mosaic SinLoG composed of a combination of five *S. cerevisiae*, six *S. kudriavzevii*, and two *Homo sapiens* genes. *HsTPI1* and *HsPGK1* can complement null mutations in their *S. cerevisiae* orthologs (18, 19). The most abundant splicing variant of *HsTPI1* (20) and the single splicing variant of *HsPGK1* from muscle were codon optimized for expression in *S. cerevisiae* (*SI Appendix*, Table S4) and each stitched to the promoter and terminator of their respective yeast orthologs. The resulting human glycoblocks were pooled with *HXK2*, *TDH3*, *PYK1*, *FBA1*, and *PDC1* glycoblocks from *S. cerevisiae* and *PGI1*, *PFK1*, *PFK2*, *ENO2*, *GPM1*, and *ADH1* from *S. kudriavzevii* and transformed to SwYG, resulting in the integration of a mosaic glycolysis gene cluster in chromosome V (mosaic SinLoG-V gene cluster). Subsequent removal of the native SinLoG-IX gene cluster yielded strain IMX645, carrying a mosaic single-locus glycolytic gene cluster encoding a set of enzymes capable of supporting the entire glycolytic flux. Whole-genome sequencing confirmed the absence of the SinLoG-IX and the presence of the complete mosaic SinLoG-V. Just a single, silent nucleotide variation was detected within an ORF of the mosaic SinLoG IMX645 strain and its mosaic SinLoG-V cluster was identical to the in silico design (Fig. 4*B* and *SI Appendix*, Fig. S6 and Table S1). Although *HsTPI1* and *HsPGK1* expression was driven by the *ScTPI1* and *ScPGK1* promoters, in vitro activity of both encoded enzymes was approximately 50% lower in the mosaic glycolysis strain than in the native SinLoG-V IMX605 strain (Fig. 4*D*, *t* test, *P* value <0.01). Also the activity of *Sk*ADH was approximately 50% lower in IMX645 than in strain IMX605 (Fig. 4*D*, *t* test, *P* value <0.01). Despite these lower enzyme activities, the strain carrying the mosaic SinLoG-V grew as fast as SinLoG-V IMX605 both in shake flask and bioreactor, and its metabolic fluxes were undisturbed (Figs. 3 and 4), consistent with the notion that most glycolytic enzymes in *S. cerevisiae* have an overcapacity under standard laboratory growth conditions (15).

## Discussion

This study demonstrates how modern genome-editing techniques can make essential biological processes, the partially redundant genetic information for which is scattered over an entire eukaryotic genome, accessible to fast, combinatorial analysis and optimization.

The high efficiency of the pathway swapping approach exceeded our expectations. Pathway swapping involves the transient, simultaneous presence in the yeast nucleus of two SinLoGs sharing high, and in one of the experiments even near-complete, sequence identity. One of these is integrated in chromosome V, whereas a second, 35-kb SinLoG, is excised from chromosome IX. DNA ends are highly recombinogenic and can interact directly with homologous sequences (21) and homologous recombination is the main double-strand break repair mechanism in growing *S. cerevisiae* (22, 23). The excised SinLoG-IX, or fragments generated by unspecific nuclease activity, might recombine with the newly integrated SinLoG-V. In practice, unintended genome rearrangements caused by HR, either between the engineered genetic elements themselves or between engineered genetic elements and the native yeast genome, were not observed. These results highlight the amazing efficiency and versatility of in vivo assembly and CRISPR/Cas9-facilitated genome editing in *S. cerevisiae* (6, 24).

Whereas genetic reduction of the glycolytic pathway did not lead to a detectable phenotype (2), clustering of the entire glycolytic gene set on a single locus resulted in decreased growth rate and

metabolic fluxes. A logical explanation for this phenotype could be a decreased glycolytic capacity, resulting from reduced expression of the clustered and relocalized glycolytic genes; however, the activity of the glycolytic enzymes measured in vitro remained remarkably similar between the SwYG and MG strains. Relocalization of the entire glycolytic gene cluster from chromosome IX to V further substantiated the insensitivity of gene expression to large-scale targeted genome remodelling in *S. cerevisiae*. Whereas it is well documented that the genetic context can strongly influence gene expression, current knowledge does not allow for predictions on how the genomic site at which large synthetic gene clusters are integrated will affect transcription of genes harbored by such clusters. Using single-gene reporter systems, several studies have shown that localization in the vicinity of centromeres and telomeres leads to gene silencing, whereas proximity to autonomously replicating sequences (ARSs) tends to enhance transcription (25, 26). Although not completely understood at a mechanistic level, nucleosome positioning also modulates transcription (27). In the present study, glycolytic genes were concatenated and integrated at two different loci, without noticeable impact on expression. Whereas these rearrangements appear drastic, our design aimed at limiting epigenetic effects by integrating the glycolytic gene clusters in regions distant (>30 kb) from telomeres and centromeres and from active ARSs (>3 kb). Furthermore, nucleosome positioning in promoter regions is important for transcription and is strongly influenced by promoter sequences (28). In the SinLoGs, expression of the glycolytic genes was driven by *S. cerevisiae* promoters or by highly homologous *S. kudriavzevii* promoters. The genetic design of the SinLoGs may therefore explain the remarkable insensitivity of gene expression to relocalization.

Promoters of glycolytic genes are among the strongest in yeast and, consistent with the essentiality of the encoded proteins, glycolytic genes are constitutively expressed (29). Colocalization, in a single locus, of 13 genes that are heavily loaded with RNA polymerase II, might affect the conformation of DNA and thereby locally affect transcription. Moreover, Pol II disruption of chromatin has been proposed to increase sensitivity to several stresses (30) and to affect binding of proteins such as cohesin that play an important role in genetic stability (31). This colocalization of glycolytic genes into a 35-kb transcriptional hotspot had remarkably little impact on the expression of the glycolytic enzymes. Similar to the recently developed "telomerator" (26), SwYG offers an attractive experimental model to systematically explore the impact of broader genomic context and thereby guide the de novo design of synthetic yeast chromosomes.

The present data suggest that the reduced growth phenotype of the SwYG strain lineage is unlikely to result from a reduced glycolytic activity. Several other mechanisms, directly or indirectly related to the pathway swapping concept, could contribute to this slower growth phenotype. A potential epigenetic factor is DNA replication and the requirement for regularly spaced ARSs along chromosomes. Whereas ARSs are typically spaced by 30–40 kb in *S. cerevisiae* (32), insertion of the 35-kb sequence carrying the glycolytic genes resulted in a spacing between adjacent confirmed ARSs (ARS504.2 and ARS507, and ARS912 and ARS913) of 82 and 74 kb for chromosomes V and IX, respectively (33). In their design for a synthetic chromosome III, Annaluru *et al.* kept a conservative approach by maintaining 12 of the 19 native ARSs, with a maximum spacing between ARSs of ∼50 kb (34). Although it has been proposed that 120–300 kb of chromosomal DNA could be replicated from a single replication origin (35), a quantitative evaluation of the impact on the physiology of *S. cerevisiae* of increasing spacing between ARSs would facilitate the design of large chromosomal constructs and synthetic chromosomes. Another factor potentially involved in the slow growth phenotype of SwYG is the secondary function, unrelated to their catalytic role in glycolysis, of three glycolytic enzymes (36), which may be affected by the genetic relocalization. However, involvement of these secondary functions in the slow growth phenotype of SwYG does not appear very likely as the vacuolar role of Fba1 and Eno2 is not expected to lead to visible growth defects under acidic environments (the pH used was ≤6) (37, 38) and the physiological characterization of SwYG did not suggest an altered regulatory activity of Hxk2 (39). Alternatively, we cannot rule out that factors external to the clustering and relocalization of glycolytic genes are responsible for the slower growth of SwYG. For instance three genes, *VPS15*, *CWC25*, and *OPT1*, of which the first two are essential, have a missense mutation in SwYG compared with its parent MG. These mutations may be deleterious for the growth of *S. cerevisiae*. Further research is ongoing to evaluate contribution of these factors in the slow growth phenotype of the SwYG and SwYG-derived strains.

Functional replacement of the entire *S. cerevisiae* glycolysis by that of its close relative, the cold-tolerant yeast *S. kudriavzevii*, provided a proof of principle that pathway swapping can be used to rapidly express and study entire metabolic pathways in a heterologous context. *S. kudriavzevii* and *S. cerevisiae* are sympatric and both show fast, fermentative sugar dissimilation in glucose-rich media (40). Pathway swapping demonstrated that a set of *S. kudriavzevii* glycolytic enzymes can support glycolysis and growth of *S. cerevisiae*. Coevolution in the same ecological niches may have led to similar optima in terms of expression level and transcriptional regulation and explain the highly similar activities observed for most glycolytic enzymes upon replacement of all *S. cerevisiae* glycolytic genes by their *S. kudriavzevii* counterparts, controlled by their native promoters. Functional replacement of the full complement of 26 glycolytic genes in *S. cerevisiae*, deletion of a significant number of which is lethal in wild-type strains, by a set of 13 heterologous variants, demonstrates the potential of the pathway swapping concept for studying essential metabolic pathways. Moreover, it paves the way for modifications and, indeed, complete redesign of other multigene, essential cellular processes.

Approximately 60% of the *S. cerevisiae* genes share significant homology with human genes (41) and, moreover, an estimated 30% (42) of human genes connected with specific diseases have a yeast ortholog. The popularity of *S. cerevisiae* as a model eukaryote is further boosted by its experimental tractability and by the availability of a wide range of tools and technologies, that make this yeast particularly well suited for high throughput studies (43). "Humanized" yeast strains provide powerful models to explore effects of therapeutics, gene dosage, and wild-type or disease-causing variants of human genes on protein function (43). Recent examples include breakthroughs in research on cell-autonomous mechanisms of neurodegeneration and identification of drug candidates against neurodegenerative diseases (44, 45). Recent large-scale studies on the ability of human genes to complement native genes in *S. cerevisiae* demonstrated that complementation of haploid yeast gene knockouts is a reliable approach for functional characterization of human gene variants (43). Although elegant, many such studies are limited to single-gene complementation and require the generation of multiple yeast strains that each contain only a single ortholog of a studied heterologous gene. Pathway swapping enables the systematic analysis of heterologous complementation of entire pathways and should enable humanization of, for example, the complete glycolytic pathway. Availability of strains containing a fully or partially humanized glycolytic pathway will enable testing of the impact of mutations or drugs on human proteins in their natural glycolytic context and thereby identify potential synergetic effects between native human proteins.

The modular pathway swapping approach opens up unprecedented possibilities. Applications ranging from functional analysis of heterologous proteins, testing of kinetic models (now hindered by the multiplicity of paralogs) (46) or screening drugs, to more technical aspects such as exploring the effect of genomic location of highly expressed native pathways, are now within reach. Continued improvements in CRISPR-mediated removal of scattered genes (24) should even further facilitate functional clustering and fast, modular swapping of key pathways/processes.

A worldwide research effort has already led to the first synthetic yeast chromosome and is progressing toward the synthesis of an entire yeast genome (34). The present study demonstrates that a modular design of such synthetic yeast genomes, in which the genetic information for key processes is functionally clustered, offers unprecedented possibilities for fast, combinatorial exploration and optimization of the biological function of multigene, essential cellular functions.

## Materials and Methods

*SI Appendix*, Figs. S1–S12 and Tables S1–S12 provides detailed information about the following: strains and media, molecular biology techniques, construction of glycoblocks and marker cassettes, construction of deletion cassettes and CRISPR-Cas9 plasmids, construction of the SwYG strain, construction of glycolytic gene clusters in the *CAN1* locus, excision of the native SinLoG cassette, SkTDH1 overexpression, sequencing, determination of specific growth rates and in vitro enzyme activities in shake-flask cultures, and cultivation in bioreactors.

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