Interfering with Glycolysis Causes Sir2-Dependent Hyper-Recombination of *Saccharomyces cerevisiae* Plasmids

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## Abstract

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key metabolic regulator implicated in a variety of cellular processes. It functions as a glycolytic enzyme, a protein kinase, and a metabolic switch under oxidative stress. Its enzymatic inactivation causes a major shift in the primary carbohydrate flux. Furthermore, the protein is implicated in regulating transcription, ER-to-Golgi transport, and apoptosis. We found that *Saccharomyces cerevisiae* cells null for all GAPDH paralogues (Tdh1, Tdh2, and Tdh3) survived the counter-selection of a GAPDH–encoding plasmid when the NAD⁺ metabolizing deacetylase Sir2 was overexpressed. This phenotype required a fully functional copy of Sir2 and resulted from hyper-recombination between *S. cerevisiae* plasmids. In the wild-type background, GAPDH overexpression increased the plasmid recombination rate in a growth-condition dependent manner. We conclude that GAPDH influences yeast episome stability via Sir2 and propose a model for the interplay of Sir2, GAPDH, and the glycolytic flux.

## Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a central metabolic regulator named for its enzymatic conversion of glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate in the sixth step of glycolysis [1]. The glycolytic activity of GAPDH can be modified by a variety of conditions. For instance, GAPDH is redox sensitive and is inactivated in oxidant-treated bacteria, yeast, nematode, mammalian, and plant cells [2–8]. Moreover, alterations in GAPDH activity have been detected in a variety of disorders, including cancer, diabetes, Huntington’s disease, and Alzheimer’s disease [9–11].

Oxidation of cysteine residues in the active site of GAPDH replenishes its activity under oxidative stress [2,3]. Several other modifications, including S-nitrosylation, S-thiolation, carboxylation, and ADP-ribosylation, have also been reported to contribute to or accompany this process [4–7,12,13]. Interestingly, the inactivation of GAPDH causes a re-direction of metabolic flux from glycolysis into the pentose phosphate pathway [14]. This metabolic re-configuration results in the recycling of NADPH, a major redox cofactor in the antioxidant machinery and the source of redox power for glutathione recycling [14,15].

In addition to being an important catabolic enzyme, GAPDH is a key regulatory modulator in a variety of processes. For instance, GAPDH can act as a protein kinase by phosphorylating the long intracellular loop of the GABA(A) receptor alpha 1 subunit, thereby regulating synaptic transmission in neurons [16], and influences the viral lifecycle by phosphorylating the hepatitis B virus core protein [17]. GAPDH can also contribute to the initiation of apoptosis (reviewed in [13]), for instance, by binding to the ubiquitin ligase Siah1 in response to cellular stress and translocating into the nucleus. There, the complex targets nuclear proteins for degradation [18]. GAPDH also participates in ER vesicle-to-Golgi transport. Upon activation via tyrosine phosphorylation by Src, GAPDH is recruited by Rab2 to the vesicular-tubular clusters of the ER, where it helps to form COPI vesicles [19].

Finally, GAPDH can activate transcription. GAPDH and lactate dehydrogenase are part of the OCA-S transcriptional coactivator complex that links the metabolic state to gene transcription [20]. Moreover, the yeast GAPDH parologue Tdh3 interacts genetically with Sir2 [21], a member of the Sirtuin family of proteins, which function as NAD⁺-dependent protein deacetylases. Sirtuins have a well-established role in deacetylating histones and are essential for gene silencing and chromatin stability [22]. Like GAPDH, Sirtuins are conserved metabolic regulators [23] and play a still controversial role in the cellular aging process [24].

Evidence for an interaction between GAPDH and Sir2 include data from a *S. cerevisiae* screen for multicopy suppressors of lethality caused by GAL1-promoter driven overexpression of Sir2 [21]. In addition to histone 4, two ribosomal proteins, and the sphinganine C4-hydroxylase Sur2, overexpression of the predominant yeast GAPDH parologue, Tdh3, suppressed Sir2-induced lethality. Moreover, a large scale study revealed that Sir2 and Tdh3 were present in a protein complex purified by a TAP-tagging strategy [25]. These results suggest a close relationship between GAPDH and Sir2, although the details of their genetic and biochemical interactions are not understood.

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Results and Discussion

We generated a yeast model for studying different GAPDH species by deleting the genes encoding the three GAPDH isozymes (Tdh1, Tdh2 and Tdh3) in a commonly used S. cerevisiae strain (BY4741). To prevent the synthetic lethal phenotype of Δtdh1Δtdh2Δtdh3 triple deletion mutants [26], we introduced a counter-selectable plasmid carrying the E. coli GAPDH paralogue EcoGAP (79% amino acid similarity to Tdh3 by Blossum62) into the parent strain before deletion of the genomic loci.

We used this strain to study GAPDH activity by performing classic 5′fluoroorotic acid (5′FOA) plasmid shuffle assays, in which a second plasmid carrying a HIS3 marker and the gene to be studied were introduced into cells. Then, clones containing both the URA3 and the HIS3 plasmids were selected on synthetic media lacking histidine and uracil (SC-HIS-URA), grown overnight, and spotted in a five-fold dilution series on synthetic complete media with or without 0.15% 5′FOA. Only yeast cells deficient for uracil synthesis (cells that have lost the GAPDH-encoding URA3 plasmid) are able to grow on the 5′FOA containing media; thus, only cells in which the HIS3 plasmid compensates for the loss of the GAPDH plasmid are viable.

A typical experiment is illustrated in Figure 1A. Yeast cells expressing Tdh3 or EcoGAP from the HIS3 episome were viable on 5′FOA media. In turn, yeast cells harboring the empty HIS3 plasmid or a plasmid encoding for Kluyveromyces lactis Gdp1, were not viable. This demonstrates that ectopic expression of GAPDH isoforms can rescue for the loss of the chromosomally encoded GAPDH genes. Kluyveromyces lactis Gdp1 is highly homologous to Tdh3 (76% similarity), and both enzymes catalyze the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate; the only difference is that the redox cofactor: Gdp1 depends on NADP(H) [27]. The fact that Gdp1 expression does not compensate for the loss of GAPDH underscores the high specificity of the assay.

Surprisingly, we discovered that Δtdh1Δtdh2Δtdh3 cells transformed with a plasmid overexpressing Sir2 also grew on 5′FOA media (Figure 1A), indicating functional rescue of GAPDH by Sir2. However, Sir2 has been extensively studied at the molecular level, and there has been no indication that Sir2 catalyzes NAD(H)-dependent oxidative phosphorylation of glyceraldehyde-3-phosphate. To exclude the possibility that this result was an artifact of the 5′FOA assay, for instance through a Sir2-dependent increase in the 5′FOA resistance of the yeast, we plated the counter-selected cells on SC-HIS and SC-HIS-URA plates. We found that they were viable on SC-HIS but unable to grow on SC-HIS-URA, confirming that the URA3 plasmid was indeed counter-selected (data not shown).

Next, to test for the specificity of the Sir2-GAPDH interaction, we generated a yeast strain in which an unrelated enzyme, the ribulose-5-ketol-isomerase Rki1, was deleted and tested for rescue by Sir2. Like GAPDH, Rki1 is essential for cytoplasmic carbohydrate metabolism and is highly conserved. Rki1 is encoded by a single yeast gene and has no other paralogues in S. cerevisiae; the Rki1 strain was kept viable by expression of the human Rki1 orthologue Rpi1 from an URA3 plasmid. We performed a counter-selection assay with this strain, as illustrated in Figure 1B. Yeast cells carrying the HIS3 plasmid encoding Rpi1 were viable on 5′FOA media, whereas cells carrying the empty vector were not, confirming that human Rpi compensates for the loss of its yeast orthologue. However, yeast cells with plasmids encoding EcoGAP or Sir2 did not grow on 5′FOA media, indicating that EcoGAP and Sir2 cannot rescue cells with the RPI1 deletion. Thus, the rescue of Δtdh1Δtdh2Δtdh3 cells by Sir2 overexpression was specific.

GAPDH and Sir2 are both dependent on the metabolic cofactor NAD+. Whereas GAPDH reduces NAD+ to NADH during glycolysis, Sir2 transfers an acetyl group to the ADP-ribose part of NAD+, forming O-acetyl-ADP-ribose. To determine whether the rescue of Δtdh1Δtdh2Δtdh3 cells by Sir2 overexpression is a direct or indirect consequence of this metabolic relationship, we cloned two mammalian cytoplasmic Sirtuins, SirT1 and SirT2, into a HIS3-containing centromeric plasmid with a TEF1 promoter. SirT1 is the direct mammalian orthologue of Sir2, whereas SirT2 targets other acetylated substrates, but is nonetheless a NAD+ dependent, O-acetyl-ADP-ribose-forming deacetylase. As illustrated in Figure 1C, overexpression of human SirT1, human SirT2, and mouse SirT2 failed to rescue Δtdh1Δtdh2Δtdh3 cells after counter-selection for the GAPDH-encoding plasmid. Thus, overexpression of other NAD+-dependent deacetylases was not sufficient to promote survival of Δtdh1Δtdh2Δtdh3 cells.

We next used site-directed PCR mutagenesis to perform structure-function studies of Sir2 in Δtdh1Δtdh2Δtdh3 yeast cells overexpressing EcoGAP. One mutation substitutes a tyrosine for a phylogenetically invariant histidine residue, thereby abolishing the deacetylase activity of Sir2 (Sir2H346Y) [28]. As illustrated in Figure 1D, Δtdh1Δtdh2Δtdh3 cells expressing Sir2H346Y are unable to grow on media containing 5′FOA. These results indicate that the enzymatic activity of overexpressed Sir2 is essential for its ability to rescue cells lacking GAPDH. We also generated another mutant, Sir2P314I, which has normal catalytic activity but is deficient in homotrimerization [29]. Sir2P314I is not able to rescue the GAPDH-deficient cells (Figure 1D). Together, these results show that the NAD+-dependent deacetylase activity of Sir2 is necessary but not sufficient to rescue Δtdh1Δtdh2Δtdh3 cells.

GAPDH acts as an enzymatic metabolic switch; once inactivated, the cytoplasmic carbohydrate flux re-routes from glycolysis to the pentose phosphate pathway [14]. This alters the redox state of the cell and is required for cellular survival under oxidative stress [4,14,30]. Inactivation of Triose phosphate isomerase (Tpi), the enzyme that catalyzes the glycolytic step preceding the one catalyzed by GAPDH, has similar metabolic consequences as the inactivation of GAPDH. Hence, TPI1 mutants can be used to distinguish between direct (enzymatic or other direct activities of GAPDH) and indirect (metabolic alterations in the carbohydrate flux) consequences of GAPDH inactivation.

To determine whether Sir2 overexpression has similar effects on yeast cells ectopically expressing Tpi1, we tested a Δtpi1 yeast strain (MR110) that expresses S. cerevisiae Tpi1 from a URA3 plasmid [31]. As illustrated in Figure 1E, neither the empty vector, nor K. lactis Gdp1, Rpi1, or SirT1 overexpression allowed Δtpi1 cells to grow on 5′FOA. As expected [31], human Tpi1 complemented for the growth phenotype of Δtpi1. However, overexpression of Sir2 allowed the Δtpi1 cells to grow on 5′FOA-containing media. These results indicate that rescue of GAPDH-deficient cells by Sir2 overexpression depends on the glycolytic and other direct activities of GAPDH and indirect (metabolic alterations in the carbohydrate flux) consequences of GAPDH inactivation.

Glycolysis is interconnected with the pentose phosphate pathway (PPP). Although glucose equivalents can be fully metabolized in the PPP, use of this pathway alone does not support cellular survival. For instance, deletion of the phosphoglucone-isomerase gene PGI1 blocks the entry of sugar phosphates into glycolysis and is lethal under standard conditions, even though glucose equivalents can still be metabolized in the PPP [32].
However, PGI1 mutant yeast are viable when they overexpress Kluyveromyces lactis Gdp1 or the glutamate dehydrogenase Gdh2 [27,32]. To test whether Sir2 overexpression results in a metabolic reconfiguration that allows cellular survival without glycolysis, we generated a quadruple-mutant in which all three GAPDH genes and the Glucose-6-phosphate dehydrogenase ZWF1 are deleted.

Figure 1. Δtdh1Δtdh2Δtdh3 yeast survives counter-selection of a GAPDH-encoding plasmid when Sir2 is overexpressed. (A) The Δtdh1Δtdh2Δtdh3 strain MR173 containing the plasmid p(URA3)-EcoGAP was transformed with the indicated HIS3 plasmids. Transformants were selected, grown overnight, and spotted in a five-fold serial dilution on SC media containing 5 FOA for counter-selection of the URA3 plasmid or on SC media as control. Plates were then incubated for 3 days at 30°C. (B) Similar experiment as described in (A), but using Δrki1 yeast expressing the human RKI1 orthologue (Rpi1) from the URA3 plasmid. (C) Δtdh1Δtdh2Δtdh3 yeast overexpressing Sir2 or its mammalian homologues, human SirT1, human SirT2, and mouse SirT2, were processed as in (A). (D) Similar to (C), but overexpressing the mutant proteins Sir2H364Y and Sir2P394L. (E) The yeast strain MR110, in which chromosomal TPI1 is deleted and yeast TPI1 is expressed from an URA3 episome, was transformed with the indicated HIS3 plasmids and processed as described above. (F) Similar experiment using the quadruple deletion strain Δtdh1Δtdh2Δtdh3Δzwf1. (G) Similar to (E), but using yeast strain MR101, which is isogenic to MR110, but expresses human instead of yeast Tpi1 from the URA3 episome.

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Deletion of the Zwf1 enzyme prevents the shunting of glucose equivalents from glycolysis into the PPP. As illustrated in Figure 1F, Δdih1Δdih2Δdih3Δtdh1 yeast overexpressing Sir2 showed the same amount of growth on 5′FOA media as the isogenic ΔWFI1-wild-type strain. Thus, Sir2 overexpression does not promote survival of cells lacking GAPDH by redirection the metabolic flux through the PPP.

We next performed similar experiments in yeast strains with reduced Tpi activity. Human Tpi has 68% amino acid similarity to yeast Tpi [Blossum62] and complements for the growth phenotypes of Δtpi1 cells [14]. Surprisingly, in strains expressing human Tpi, Sir2 overexpression does not permit cell growth on 5′FOA media (Figure 1G). We also performed the experiment in a yeast strain expressing a Tpi mutant, TpiΔI170Val, that has only 30% activity compared to wild-type human Tpi [31], and obtained similar results (data not shown). First, this finding indicates that human Tpi is not able to complement to 100% for yeast Tpi. For instance the binding affinities of regulatory Tpi interactors could be different. This is consistent with our previous findings showing that yeast cells expressing human Tpi have only 70% overall Tpi activity compared to a wild-type strain, and display an increased resistance against the oxidant diamide [14,31].

Still, this result is very surprising, because the strains we used were direct descendants of the same parent strain (MR100) and should therefore not differ after counterselection of the Tpi encoding URA3 plasmid. Possible explanations for this unforeseen result include epigenetic differences between the two strains or the presence of the different Tpi isoforms in the cell even after counter-selection of the URA3 plasmid.

Therefore, we re-isolated HIS3 plasmids from counter-selected Δdih1Δdih2Δdih3 and Δtdh1 yeast. The plasmids were re-transformed into E. coli for amplification and subsequently analyzed by restriction digest with EcoRI/SalI, because the original HIS3 plasmid was constructed by ligating the Sir2 coding sequence into the EcoRI/SalI sites of the vector p413TEF. As illustrated in Figure 2A (left panel), this produced the expected 1689 bp Sir2-containing band. Surprisingly, however, this DNA fragment was not detectable in the HIS3 plasmids that were re-isolated from the counter-selected yeast.

We next digested the plasmids with KpnI, which produces a 954 bp band due to an internal KpnI site in the HIS3 marker (Figure 2A, middle panel). This band was found in digests of the original Sir2 vector and plasmids re-isolated from counter-selected Δdih1Δdih2Δdih3 and Δtdh1 cells, but not in digests of the original URA3 plasmids encoding EcoGAP or Tpd1. Hence, the plasmid isolated from the counter-selected yeast strains was neither the original plasmid harboring Sir2, nor the plasmid used to express GAPDH or Tpi.

Other KpnI restriction fragments detectable in pURA3-EcoGAP or pURA3-TPI1 were observed from plasmids isolated from Δdih1Δdih2Δdih3 or Δtdh1 yeast. This suggested that the re-isolated plasmids were recombined hybrids of the original URA3 and HIS3 vectors. To test this, we performed a third digest using BamHI and XhoI. This produced a 996 bp fragment corresponding to the EcoGAP coding sequence from pURA3-EcoGAP and a 747 bp fragment corresponding to the TPI1 coding sequence from pURA3-TPI1, but produced multiple fragments from the original Sir2-encoding plasmid (Figure 2A, right panel). Indeed, the plasmid isolated from the Δdih1Δdih2Δdih3 strain produced a BamHI/XhoI fragment corresponding to the EcoGAP coding sequence, whereas the Δtdh1 strain produced a fragment corresponding to yeast Tpi1. Thus, the plasmid isolated from the Δdih1Δdih2Δdih3 strain contained a HIS3 marker and the EcoGAP coding sequence. Similarly, the plasmid isolated from Δtdh1 yeast encoded HIS3 and Tpd1. Hence, the new plasmids were recombined hybrids of the two original plasmids.

The original plasmids shared a number of sequence features. In Figure 2C, the plasmid regions with 100% identity (e.g. the ampicillin-resistance gene, the E. coli replication-origins, and the ARS sequences) are highlighted; more than 30% of the sequence was found in all plasmids. The high homology of the shared plasmid features and the fact that all extracted plasmids showed a similar restriction pattern suggests that homologous recombination between vectors caused the observed phenomena.

To further dissect the putative recombination events, we re-transformed the original and re-isolated Tpi1-encoding plasmids into the yeast strain BY4741. As illustrated in Figure 2B, yeast transformed with pURA3-yeast TPI1 grew on SC–URA plates, but not on SC–HIS plates. The Tpi1 encoding plasmid re-isolated from the Δtdh1 strain resulted in colonies on SC–HIS, but not on SC–URA. Identical results were obtained with the plasmids encoding EcoGAP. Therefore, the viability of the counter-selected Δdih1Δdih2Δdih3 and Δtdh1 cells can be explained by plasmid recombination; after expressing Sir2, these strains contained a new HIS3 plasmid encoding either GAPDH or Tpi.

Other laboratories have found that the lack of Sir2 results in hyper-recombination of rDNA repeat units [33,34]. The most obvious difference between our studies and theirs is that our experiments were performed in yeast strains not wild-type for glycolysis. Consequently, we extended our investigations by studying plasmid recombination in a wild-type background. For this, we developed a plasmid-recombination assay using the E. coli β-galactosidase gene lacZ as a reporter. We PCR-amplified 5′ and 3′ fragments of the lacZ gene from E. coli genomic DNA (strain GM2929) that overlapped by 564 bp. The 5′ fragment was cloned into the 2 μ URA3 expression vector p426GPD under the control of the constitutive GPD1 promoter, and the 3′ fragment was cloned into the LEU2 containing 2 μ vector pRS425. Neither plasmid alone is able to produce a functional lacZ enzyme, but upon plasmid recombination, a functional lacZ gene is reconstituted and yeast cells turn blue in an X-GAL assay (Figure 2D).

To test the influence of GAPDH and Sir2 overexpression on plasmid recombination, we transformed both lacZ reporter plasmids into the yeast strain BY4741. Single colonies were selected and transformed with a third, HIS3-containing plasmid encoding Sir2 or GAPDH. Each transformation was performed in triplicate, and the transformation mixture was plated directly on nylon membranes for the lacZ assay.

As illustrated in Figure 3A, about 45% of the yeast colonies transformed with the empty HIS3 vector turned blue, indicating that these colonies contained yeast cells expressing a functional β-galactosidase protein. The number of blue colonies was very similar in the Sir2-overexpressing cells. Remarkably, the number of blue colonies was greatly increased in the EcoGAP-overexpressing cells. These results indicate that GAPDH overexpression increases recombination between yeast plasmids in the wild-type background.

In general, the number of lacZ positive colonies was very high, probably because the lacZ assay is highly sensitive and only a few β-galactosidase-expressing cells are required to cause a color shift of the whole colony. To analyze, if the recombination events occurred before or during colony formation, we re-spread colonies on a nylon membrane and tested for β-galactosidase activity. In no case, all colony descendants were lacZ positive, indicating that recombination predominantly occurred during colony formation. We next set up a similar assay permitting an authentic quantification of plasmid recombination events. We used a
marker cassette from the pAG25 plasmid that confers resistance against the antibiotic nourseothricin [35]. As for the lac\_Z assay, we generated two vectors, one containing the 5' and the other containing the 3' region of the \textit{natMX4} cassette, with an overlap between the fragments of 160 bp. Both vectors were transformed into BY4741 cells, and double-transformants were selected in SC-LEU-URA media. These transformants were regrown and transformed with the third plasmid encoding the protein to be studied.

In the first experiment, we pooled 50 individual clones from each transformation, grew them overnight in 50 ml YPD, and plated a dilution series in triplicate on SC-LEU-URA-HIS or YPD containing 75 \(\mu\)g/ml nourseothricin (NTC, Jena Bioscience). As illustrated in Figure 3B (middle panel), we tested several plasmids and counted the number of colonies growing on the NTC-containing media. Cultures containing the empty \textit{HIS3} vector produced the lowest number of NTC-resistant colonies (24 NTC-resistant clones per 10^5 cfu's on SC-LEU-URA-HIS media). The number of NTC-resistant colonies was four times higher in \textit{EcoGAP} expressing cells, and about 10 times higher in \textit{Tdh3}-expressing cells. Expression of \textit{Sir2} and \textit{SirT1} increased the number of recombinants as well, but not with the magnitude

Figure 2. Deregression of the glycolysis/Sir2 equilibrium causes plasmid recombination. (A) Plasmids isolated from S’FOA counter-selected, Sir2-overexpressing \textit{Δtpi1} and \textit{Δtdh1Δtdh2Δtdh3} (\textit{ΔGAPDH}) cells were amplified in \textit{E. coli} and digested with EcoRI/Sall (left panel), \textit{KpnI} (middle panel) or \textit{BamH}/Xhol (right panel) and compared with equivalently-treated \textit{p(HIS3)-SIR2}, \textit{p(URA3)-yeast TPI1}, or \textit{p(URA3)-EcoGAP}, respectively. (B) \textit{p(URA3)-TPI1} and the \textit{Tpi}-encoding plasmid isolated from S’FOA counter-selected, Sir2-overexpressing \textit{Δtpi1} yeast were transformed into BY4741, plated on SC-\textit{HIS} and SC-\textit{URA} media and incubated at 30 °C. (C) Plasmid sequences of \textit{p(URA3)-EcoGAP} and \textit{p(URA3)-yeast Tpi1} were aligned to the sequence of \textit{p(HIS3)-SIR2}. Identical regions between \textit{p(URA3)-EcoGAP} and \textit{p(HIS3)-SIR2} are highlighted in green, regions shared between \textit{p(URA3)-yeast TPI1} and \textit{p(HIS3)-SIR2} in blue.

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observed with the GAPDH paralogues. Finally, expression of the Sirtuin paralogue Hst2 had a stronger effect on the plasmid recombination rate than its homologues, but was still weaker than yeast GAPDH Tdh3. These results confirm that overexpression of GAPDH paralogues significantly affect the plasmid recombination rate.

Interestingly, the results differed slightly from those obtained from the lacZ-based assay. Sir2 had no effect in the lacZ assay, but slightly increased the number of NTC-resistant recombinants in the natMX4 assay. The two assays differ in two ways: first, transformants are exposed to positive growth selection in antibiotic-containing media in the natMX4 assay, but not in the lacZ assay. Second, the clones in the natMX4 assay were grown in liquid culture, whereas the lacZ assay was performed with plate-grown colonies.

To determine whether these differences could account for the observed phenotypes, we modified our natMX4 assay by spreading the clones on the NTC-containing media without re-growing them in liquid media. As illustrated in Figure 3B (right panel), the number of NTC-positive clones carrying empty vector and overexpressing Sir2 or Hst2 was very similar in both experiments, indicating that the different growth conditions did not significantly affect the recombination rate in these transformants. In contrast, SirT1 produced more NTC-resistant colonies compared to the empty vector in the liquid-growth experiment, but showed a slightly lower number of clones in the plating assay.

Interestingly, the growth conditions had a major effect on cells overexpressing GAPDH. EcoGAP increased the recombination frequency by a factor of four in the liquid culture assay, whereas the recombination frequency was increased by about two-fold in the direct-plating assay. This phenomenon was even stronger with yeast GAPDH: Tdh3 overexpression caused a 10-fold higher rate of recombination in the liquid culture, but only a three-fold higher rate in the direct-plating assay.
The differences between the two conditions might be surprising, but are reasonable. Glycolysis is responsible for fermentative energy production during the exponential growth phase, and since GAPDH is a major regulator of this pathway, it makes sense that its influence varies with the rate of glycolytic activity.

Conclusions
Sir2 plays an essential role in gene silencing and in maintaining chromatin structure. Null mutants of Sir2 are deficient in heterochromatin formation and have defects in chromatin structure. Extra copies of Sir2 have been shown to increase the stability of yeast rDNA cycles and to extend the average and maximum lifespan of this single-cell eukaryote [22–24,36].

Sir2 has previously been associated with plasmid stability. Silencing mediated by the 2 μ autonomous replicative sequence is Sir2-dependent [37], and Sir2 null mutants accumulate negative plasmid supercoils. Overexpression of Sir2 leads to positively supercoiled plasmid topoisomers, an effect that depends on Histone 4-K16 acetylation and the Sir2 expression level [38]. It is therefore likely that an increase in Sir2 activity destabilizes episomes and therefore triggers homologous recombination between plasmids.

Here, we show that yeast cells with deregulated glycolysis exhibit an elevation in plasmid recombination rate that is independent of plasmid origin (both 2 μ and centromere-containing plasmids were affected). This increased plasmid recombination rate occurs both in GAPDH-deficient yeast cells overexpressing Sir2 and in wild-type yeast cells overexpressing GAPDH. Although GAPDH is a multifunctional protein, this effect seems to depend on its glycolytic function, since modulating the activity of Tpi, which simulates the metabolic consequences of GAPDH inactivation [14], has a strong effect on the phenotype.

The effect of Sir2 on chromatin structure depends on its enzymatic activity as a protein deacetylase. Sir2 requires oxidized NAD+ for forming O-acetyl-ADP-ribose by transferring an acetyl group to its ADP-ribose part [28,39]. Consequently, Sir2 depends on the glycolytic redox cofactor, a fact that represents a direct link between carbohydrate catalysis and chromatin silencing.

Remarkably, the nicotinamide concentration has been shown to be the limiting factor for Sir2 activity [40]. Moreover, Sir2 and GAPDH are present in the same protein complex [25]. Indeed, these facts propose a mechanism for the interplay of GAPDH and Sir2 (Figure 4): In close proximity to Sir2, GAPDH can provide the required NAD+ by oxidizing NADH. However, this is dependent on the 1,3-bisphosphoglycerate/glyceraldehyde-3-phosphate ratio. In case of high glycolytic activity, glyceraldehyde-3-phosphate is produced at much higher rates, enforcing the reverse reaction: GAPDH would metabolize NAD+ rather than providing it.

Thus, under conditions of low glycolytic activity, GAPDH could enhance Sir2 activity, but under conditions with high glycolytic activity, the opposite is expected to occur. Consequently, dependent on the metabolic activity, GAPDH might be able to both, activate and inhibit Sir2.

This finding is consistent with earlier observations that link Sir2 and glycolysis with chromatin structure; reducing the glucose concentration in the media from 2% to 0.5% dramatically extends the replicative lifespan of S. cerevisiae. Lin and colleagues reported that cells deleted for Sir2 lack this phenotype [40,41]. Conversely, extra copies of Sir2 extended the replicative lifespan in full glucose media [36].

An interesting future question regards the biological role of the very close relation of carbohydrate metabolism and recombination. One might speculate that under strong nutrient deprivation and stress conditions (both influencing the glycolytic flux) a higher rate of recombination increases the genetic diversity of a given yeast population, thus, facilitating the evolutionary adoption to a changing environment [42]. Supportingly, earlier studies also observed a correlation between yeast’s nutritional states and meiotic recombination [43]. Of course, much more work is required to fully understand how glycolysis affects cellular processes and what the direct and indirect targets of Sir2 are. Our findings, as well as the recombination assay presented here, provide a good platform for answering these questions.

Materials and Methods
Yeast growth conditions
Yeast was grown in yeast peptone dextrose (YPD) or synthetic complete (SC) media containing 2% glucose as a carbon source.
URA3 counter-selection was performed on SC media containing 0.15% fluoroorotic acid (5′-FOA, Fluorochem, UK). Cells carrying the natMX4 marker were grown on YPD containing 75 μg/ml nourseothricin (Jena Bioscience, Germany).

Plasmid generation

Plasmids were generated by classic restriction/ligation procedures and are listed in Table 1. The vector backbones p423GPD, p416GPD, p413TEF and p413GPD were described by [44], and pRS425 by [45]. Human Rpi1 and SirT1 coding sequences were amplified from a human fetal cDNA library (Clontech), mouse SirT1 from a mouse testis cDNA library (Clontech), E. lactis GDP1 from p1696 [46], SIR2 from pAR14 [47], Hst2 from BY4741-, and EcoGAP and lacZ from E. coli genomic DNA (strains Xllblue and GM2929, respectively). Sir2 mutants, Sir2H364Y and Sir2H364L, were generated by site-directed PCR mutagenesis; all cloning experiments involving a PCR were verified by sequencing.

Table 1. Plasmids used in this study.

| Name | Vector backbone | S.c. origin | Aux. | S.c. prom | Encoded prot. (species) | Cloning sites | Reference |
|------|----------------|-------------|------|----------|-------------------------|--------------|-----------|
| p(HIS3)-EcoGAP | p423GPD | 2 μ | HIS3 | GDP1 | EcoGAP (E. coli) | BamHI/XhoI | [14] |
| p(URA3)-EcoGAP | p423GPD | 2 μ | URA3 | GDP1 | EcoGAP (E. coli) | BamHI/XhoI | This study |
| p(URA3)-Rpi1 | p416GPD | cen | URA3 | GDP1 | Rpi1 (H. sapiens) | BamHI/XhoI | This study |
| p(HIS3)-Rpi1 | p413GPD | cen | HIS3 | GDP1 | Rpi1 (H. sapiens) | BamHI/XhoI | This study |
| p(URA3)-human TPI1 | p416GPD | cen | URA3 | GDP1 | Tpl1 (H. sapiens) | BamHI/XhoI | [31] |
| p(URA3)-yeast TPI1 | p416GPD | cen | URA3 | GDP1 | Tpl1 (S. cerevisiae) | BamHI/XhoI | [31] |
| p(HIS3)-TDH3 | p423GPD | 2 μ | HIS3 | GDP1 | Tdh3 (S. cerevisiae) | BamHI/XhoI | [14] |
| p(URA3)-TDH3 | p426GPD | 2 μ | URA3 | GDP1 | Tdh3 (S. cerevisiae) | BamHI/XhoI | This study |
| p(HIS3)-SIR2 | p413TEF | cen | HIS3 | TEF1 | Sir2 (S. cerevisiae) | EcoRI/SalI | This study |
| p(HIS3)-SIR2H364Y | p413TEF | cen | HIS3 | TEF1 | Sir2H364Y (S. cerevisiae) | EcoRI/SalI | This study |
| p(HIS3)-SIR2F394L | p413TEF | cen | HIS3 | TEF1 | Sir2F394L (S. cerevisiae) | EcoRI/SalI | This study |
| p(HIS3)-SirT1 | p413TEF | cen | HIS3 | TEF1 | SirT1 (H. sapiens) | EcoRI/SalI | This study |
| p(HIS3)-SirT2 | p413TEF | cen | HIS3 | TEF1 | SirT2 (H. sapiens) | EcoRI/SalI | This study |
| p(HIS3)-SirT2mm | p413TEF | cen | HIS3 | TEF1 | SirT2 (M. musculus) | EcoRI/SalI | This study |
| p(HIS3)-Hst2 | p413TEF | cen | HIS3 | TEF1 | Hst2 (S. cerevisiae) | EcoRI/SalI | This study |
| p(HIS3)-GDP1 | p413GPD | cen | HIS3 | GDP1 | Gdp1 (K. lactis) | BamHI/XhoI | This study |
| p426GPD-lacZ-NT | p426GPD | 2 μ | URA3 | GDP1 | 5′ region of lacZ bp 1-843 (E. coli EG10527) | BamHI/ClaI | This study |
| pRS425-lacZ-CT | pRS425 | 2 μ | LEU2 | none | 3′ region of lacZ bp 280-3075 (E. coli EG10527) | BamHI/XhoI | This study |
| p426GPD-natMX4-5′ | p426GPD | 2 μ | URA3 | internal(TEF1) | 5′ region of natMX4 from pAG25 | SacI/SalI | This study |
| pRS425-natMX4-3′ | pRS425 | 2 μ | LEU2 | none | 3 region of natMX4 from pAG25 | SacI/SalI | This study |

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Plasmid recombination assays

**lacZ-based recombination assay.** First, the yeast strain BY4741 was transformed with the plasmids p426GPD-lacZ-NT and pRS425-lacZ-CT. Then, respective transformants were selected on SC-URA-LEU and re-grown in liquid SC-URA-LEU media, and transformed in triplicate with the HIS3 plasmids to be studied. The transformation mixture was plated directly on nylon media, and transformed in triplicate with the HIS3 plasmids to be studied. The transformation mixture was plated directly on nylon membranes (“Magna Charge” nylon transfer membrane, Micron Separation, USA) placed on SC-URA-LEU-HIS agar and incubated at 30°C until yeast colonies were grown. Then, the membranes were detached from the agar, shock-frozen in liquid nitrogen, and placed on Whatman paper saturated with buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 0.15% X-Gal, and 10 mM DTT; pH = 7.0). After 4–5 hours of incubation at 37°C, membranes were air-dried in the dark before blue and white colonies were counted.

**natMX4-based recombination assay.** The natMX4 based recombination assay was performed in a similar fashion as the lacZ assay. Two overlapping fragments of the 5′ and 3′ regions of natMX4 were amplified from pAG25 [35] and cloned into the URA3 (p426GPD) and LEU2 (pRS425) vectors, respectively. Then, yeast cells were transformed with these plasmids and grown on SC-URA-LEU media. Resulting clones were transformed with a third plasmid encoding the protein to be studied, and triple transformants were selected on SC-URA-LEU-HIS. Then a) 50 clones from each transformation were pooled and grown overnight in 50 ml YPD and spread on YPD+75 μg/ml NTC (Jena Biotech) and...
SC-URA-LEU-HIS or b) spread on YPD-NTC and SC-URA-LEU-HIS without re-growth. Colonies were counted after 3 days incubation at 30°C.

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Author Contributions
Conceived and designed the experiments: MR. Performed the experiments: MR UZ. Analyzed the data: MR UZ. Contributed reagents/materials/analysis tools: HL. Wrote the paper: MR HL.