A catabolic block does not sufficiently explain how 2-deoxy-D-glucose inhibits cell growth

Markus Ralser[a,1], Mirjam M. Wamelink[b], Eduard A. Struys[b], Christian Joppich[a], Sylvia Krobitsch[a], Cornelis Jakobs[b], and Hans Lehrach[a]

[a]Max Planck Institute for Molecular Genetics, Ihnestrasse 73, 14195 Berlin, Germany; and [b]Department of Clinical Chemistry, Metabolic Unit, VU Medical Center, de Boelelaan 1117, 1081 HV Amsterdam, The Netherlands

Edited by David E. Housman, Massachusetts Institute of Technology, Cambridge, MA, and approved September 29, 2008 (received for review April 1, 2008)

The glucose analogue 2-deoxy-D-glucose (2-DG) restrains growth of normal and malignant cells, prolongs the lifespan of *C. elegans*, and is widely used as a glycolytic inhibitor to study metabolic activity with regard to cancer, neurodegeneration, calorie restriction, and aging. Here, we report that separating glycolysis and the pentose phosphate pathway highly increases cellular tolerance to 2-DG. This finding indicates that 2-DG does not block cell growth solely by preventing glucose catabolism. In addition, 2-DG provoked similar concentration changes of sugar-phosphate intermediates in wild-type and 2-DG-resistant yeast strains and in human primary fibroblasts. Finally, a genome-wide analysis revealed 19 2-DG-resistant yeast knockouts of genes implicated in carbohydrate metabolism and mitochondrial homeostasis, as well as ribosome biogenesis, mRNA decay, transcriptional regulation, and cell cycle. Thus, processes beyond the metabolic block are essential for the biological properties of 2-DG.

cell growth inhibition | glycolysis | off-target effect | pentose phosphate pathway | carbohydrate metabolism

2-Deoxy-D-glucose (2-DG) is a stable glucose analogue that is actively taken up by the hexose transporters and phosphorylated but cannot be fully metabolized. 2-DG-6-phosphate accumulates in the cell and interferes with carbohydrate metabolism by inhibiting glycolytic enzymes. 2-DG-6-phosphate inhibits phosphoglucose isomerase (PGI) in a competitive, and hexokinase (HXK) in a noncompetitive, manner (1–3). On the cellular level, 2-DG provokes rapid growth inhibition and results in altered glycosylation that is highly dependent on catabolic glucose intermediates (4, 5).

2-DG has been used in numerous studies focused on the effects of reduced metabolic rates. Recently, 2-DG has been used in this study of glycolytic inhibition with respect to calorie restriction and aging. 2-DG increases the lifespan of *C. elegans*, an effect that is reversed by antioxidants (6, 7). Moreover, 2-DG mitigates disease progression in a murine model of temporal lobe epilepsy (8), possibly due to the repression of the BDNF promoter. 2-DG shows possibilities as an antiviral drug, as it targets gene expression in papillomavirus (9). Finally, 2-DG has been thoroughly studied in regard to cancer biology. Malignant cells exhibit increased glucose metabolism compared with surrounding tissue (10) and, therefore, increased 2-DG uptake. 2-DG efficiently inhibits tumor progression and is a focus of clinical trials (reviewed in ref. 11).

In general, it is assumed that the biological effects of 2-DG are the consequence of a block in carbohydrate catalysis, implying that 2-DG treated cells, unable to metabolize glucose, stop growing as a result of a lack of energy and metabolic intermediates. However, several observations bring this assumption into question. First, only a moderate decline in ATP has been observed in 2-DG-treated eukaryotes (7, 12). Second, a current study reveals that tumor cells react differently to 2-DG than to its close analogue 2-fluorodeoxy-D-glucose (2-FDG). Although 2-FDG seems to be the more potent glycolytic inhibitor, 2-FDG was not toxic to all tumor cells that reacted to 2-DG (13). And finally, the effectiveness of the anticancer properties of 2-DG correlates with changes in the glycosylation pattern of the target tissue (14).

Here, we focus on glycolysis and the pentose phosphate pathway (PPP) in cells treated with 2-DG. Interrupting the interplay of these pathways increased, rather than decreased, cellular tolerance to 2-DG. Moreover, although 2-DG provoked concentration changes of several sugar-phosphate intermediates of both pathways, we observed no linear decrease of glucose catabolites. The metabolic shift was similar in the wild-type and two 2-DG resistant yeast strains. Finally, we screened a collection of ~5,150 knockouts and identified 19 2-DG-resistant mutants. The identified genes were implicated in carbohydrate metabolism and its regulation, cell cycle control, signaling, mRNA decay, redox control, protein degradation, ribosome biogenesis, and DNA repair. Thus, we conclude that a metabolic block in the classic sense does not sufficiently explain how 2-DG impacts eukaryotic cell growth, bringing into question the current assumptions concerning how 2-DG interferes with biological systems.

Results

Glycolysis begins with the phosphorylation of glucose to glucose-6-phosphate by HXK. It is then either converted by phosphoglucose-isomerase (PGI) and enters glycolysis, or it is metabolized by glucose-6-phosphate dehydrogenase (G6PD; Fig. 1*A*) and enters the PPP. 2-DG inhibits HXK and PGI, and the inhibition of PGI is competitive (1, 3). It is likely that cells with impaired glucose metabolism due to the inhibition of PGI have an increased influx of glucose-6-phosphate into the PPP. This is true for yeast with deletions of PGI (15). Thus, if glycolysis is inhibited at a step at or after PGI, the glucose equivalents can still be metabolized in the PPP. Consequently, deletion of the unique G6PD gene would prevent the entrance of glucose-6-phosphate into the PPP and, consequently, should increase the sensitivity of the cells to 2-DG. A stronger 2-DG phenotype in the G6PD mutant would also be expected, if reduced activity of the PPP would contribute to the cytotoxicity of 2-DG (as suggested in ref. 16).

Surprisingly, the deletion of the G6PD gene (*ZWF1*) in an S288c derivate (BY4741) resulted in cells that were highly resistant to 2-DG (Fig. 1*B*). To exclude the possibility that this finding was a background-specific artifact, *ZWF1* was deleted in the W303 derivate Y2546 as well, but this strain was also resistant to 2-DG [supporting information (SI) Fig. S1*A*].

Author contributions: M.R., E.A.S., and C. Jakobs designed research; M.R., M.M.W., E.A.S., and C. Joppich performed research; S.K. and C. Jakobs contributed new reagents/analytic tools; M.R., M.M.W., and H.L. analyzed data; and M.R., C. Jakobs, and H.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: ralser@molgen.mpg.de.

This article contains supporting information online at www.pnas.org/cgi/content/full/0803090105/DCSupplemental.

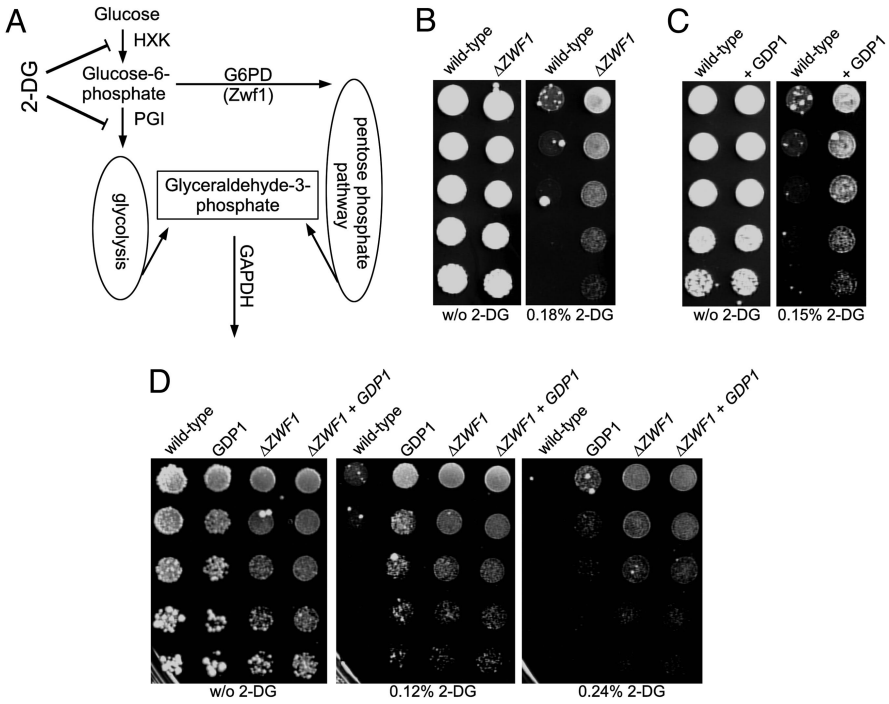


Fig. 1. Yeast cells interrupted for the interplay of glycolysis and the PPP are 2-DG resistant. (*A*) Simplified overview of glycolysis, the PPP, and the enzymatic targets of 2-DG. (*B*) Wild-type and Δ*ZWF1* yeast were spotted as 5-fold serial dilution on SC media with or without 2-DG and incubated at 30 °C. (*C*) Similar to (*B*), but with yeast cells expressing *K. lactis GDP1*. (*D*) Wild-type and Δ*ZWF1* yeast transformed with a *K. lactis GDP1* expression plasmid were spotted on SC^{-URA} media with or without 2-DG

GDP1 of *Kluyveromyces lactis* is a phosphorylating GAPDH, unique among nonplant eukaryotes, that can rescue the lethal phenotype of the *PGI1* deletion in *S. cerevisiae* (17). When growing on pentoses, *K. lactis* avoids carbon exhaust by inducing the expression of this enzyme; *GDP1* interferes with the interplay of glycolysis and the PPP by switching the glycolytic redox-cofactor from NAD(H) to NADP(H) (17, 18). 2-DG tolerance was examined in the presence of *GDP1*. The wild-type strain was transformed with a *GDP1* expression plasmid and tested for 2-DG resistance. As illustrated in Fig. 1*C*, BY4741 cells expressing *GDP1* were resistant to 2-DG. *GDP1* transformants of the unrelated Y2546 strain expressed a similar phenotype (Fig. S1*B*). Finally, *GDP1* was expressed in the Δ*ZWF1* strain (Fig. 1*D*). However, *GDP1* expression did not further increase the 2-DG tolerance of the Δ*ZWF1* strain. That the effects were not additive indicates the possibility of a shared mechanism.

Next, we investigated how 2-DG impacts the metabolite homeostasis of glycolysis and the PPP in the wild-type as well as in the resistant mutants. To determine the optimum 2-DG concentration for these analyses, BY4741 cells were grown in yeast peptone dextrose (YPD) without 2-DG or supplemented with 0.05, 0.10, or 0.20% 2-DG (Fig. 2*A*). 2-DG strongly inhibited cell growth at all concentrations. As expected, 0.20% 2-DG had the strongest effect, although growth was only minimally decreased compared with 0.10% 2-DG; both were more effective than 0.05% 2-DG. The cells treated with 0.10% 2-DG were profiled (Fig. 2*B*). 2-DG treatment provoked quantitative

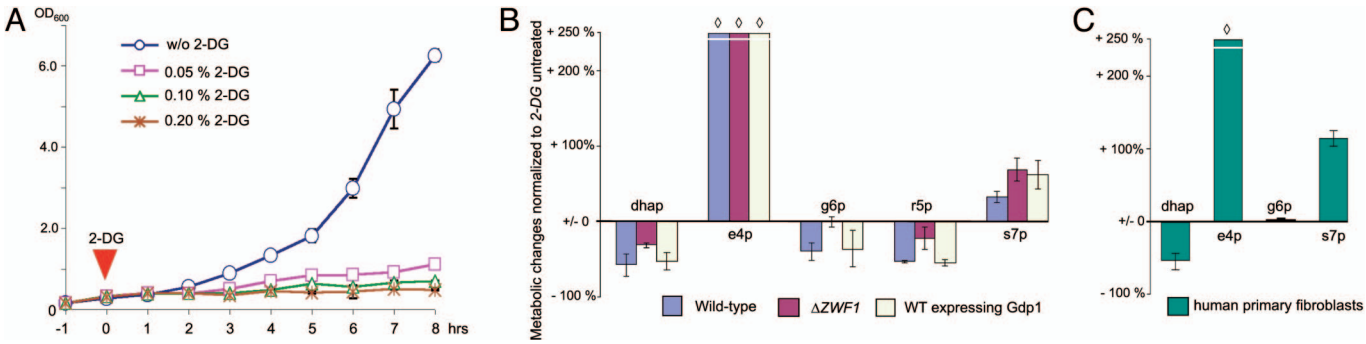


Fig. 2. Metabolic profiling of eukaryotic cells treated with 2-DG. (*A*) Growth of yeast batch cultures supplemented with 2-DG at different concentrations. (*B*) Quantitative changes of metabolites in wild-type, Δ*ZWF1*, and *GDP1*-expressing yeast treated with 2-DG. Shown are the relative increases or decreases in percentage compared with the respective untreated strain. ◇, e4p was below detection limit in the 2-DG-untreated cells, but strongly increased (to 6.5 ± 0.55 mM/(OD_{600} × ml) [WT yeast], 9.9 ± 0.76 mM/(OD_{600} × ml) [Δ*ZWF1*], and 6.5 ± 0.67 mM/(OD_{600} × ml) [*GDP1*] in the 2-DG-treated sample). (*C*) Human primary fibroblasts were incubated with 4 mM 2-DG and processed for sugar-phosphate analyses. ◇, e4p increased to 9.70 nmol/$mg_{total\ protein}$. (*B* and *C*) Error bars indicate the normalized standard deviation of the 2-DG-treated cells.

changes in all assayed metabolites of glycolysis and the PPP. As glucose-6-phosphate and fructose-6-phosphate could not be distinguished by the chosen methodology, their combined concentration is given as g6p. In agreement with previous observations that 2-DG blocks the upstream enzyme HXK, g6p concentration decreased by 39.4% in the 2-DG-treated wild-type strain. Another glycolytic metabolite, the three-carbon sugar dihydroxyacetone phosphate (dhap), also decreased (−57.6%). Dhap is converted by triosephosphate isomerase to glyceraldehyde-3-phosphate, the GAPDH metabolite. Remarkably, ribose-5-phosphate (r5p) was diminished by 52.8%, ruling out a general increase of PPP intermediates in 2-DG-treated cells. Other metabolites, however, behaved differently. Sedoheptulose-7-phosphate (s7p), a 7C intermediate of the PPP, increased (+32.4%). Erythrose-4-phosphate (e4p), an intermediate of the nonoxidative branch of the pentose phosphate pathway and a product of transaldolase, exhibited the most striking change. Whereas e4p of untreated yeast was below the detection limit, in 2-DG-treated cells e4p concentrations were comparable to the most abundant metabolites (dhap and g6p).

The metabolic profiles of the two resistant strains (Δ*ZWF1* and WT expressing *GDP1*) were also examined. Although the absolute concentrations differed (see Table S1), the relative changes caused by 2-DG were similar (Fig. 2*B*). The Δ*ZWF1* strain exhibited similar quantitative tendencies for dhap, e4p, r5p, and s7p; only g6p was not altered. Remarkably, the PPP metabolites r5p and s7p shifted as in the wild-type strain, even though entrance into the oxidative branch of the PPP is prevented in the mutant. The *GDP1* expressing strain was qualitatively and quantitatively like the wild-type strain and showed similar tendencies for every assayed metabolite. Notably, the s7p concentration correlated with the 2-DG resistance. To clarify if this molecule plays a key role in the underlying mechanism, we tested two yeast strains deleted for s7p metabolizing enzymes, *TAL1* (encoding transaldolase) and *TKL1* (encoding the major transketolase isoform). Although both mutants are known to have altered s7p levels, both strains showed no difference in 2-DG resistance compared with wild type (Fig. S2). Thus, s7p seems not directly involved in the mechanism promoting 2-DG tolerance.

Overall, with the exception of e4p, the metabolic changes were relatively moderate and did not reveal a linear decrease of the intermediates. Mutations reducing triosephosphate isomerase activity or eliminating transaldolase activity, for instance, are known to provoke larger metabolic changes without strongly impacting cell growth (18, 19). Remarkably, the 2-DG treatment provoked similar metabolic changes in both the resistant strains and the wild type. This indicates that the resistance of both strains is not based on a reduced 2-DG uptake, and that glycolysis is targeted in these strains as in the wild type.

Next, the effects of 2-DG on the carbohydrate intermediates in mammalian cells were examined. Human primary fibroblasts were treated with 2-DG and the changes in sugar-phosphate concentration were monitored. As illustrated in Fig. 2*C*, 2-DG treatment resulted in a decrease in dhap, a strong increase in e4p (the e4p concentration of the native fibroblasts was below the detection limit), and an increase in s7p. Only g6p was unaffected by 2-DG. Unfortunately, we were unable to determine the change in r5p as the absolute r5p concentration was too low in these cells for adequate quantification. Thus, 2-DG caused similar metabolic changes in yeast and mammalian cells.

These results raised the possibility that regulatory events rather than classic metabolic inhibition are central for the growth-inhibiting properties of 2-DG, suggesting that the respective target pathways could be identified in a chemical genetic screen. Therefore, we tested a complete collection of viable yeast gene deletion strains (~5,150 strains of the *MAT*a knockout collection, including the version 2 supplemental set) for 2-DG resistance. Knockouts that reproducibly tolerated high concentrations of 2-DG were re-grown and verified by sequencing of the genetic barcode. Nineteen 2-DG-resistant knockouts were identified. Surprisingly, the Δ*ZWF1* strain, deleted for the G6PD, was not among them; but a subsequent verification of the respective strain taken from our copy of the knockout collection revealed that it was wild type for this gene. As illustrated in Fig. 3, all identified strains were highly resistant; most of them tolerated more than 0.40% 2-DG. Three candidates were deleted for metabolic enzymes, including *TPS2*, which catalyzes one step in trehalose synthesis. Like glycogen, trehalose functions as a storage carbohydrate, but also protects cells from desiccation and stress (20). In addition, at least three of the identified genes are implicated in the regulation of glycogen storage (*REG1*, *PCL8*, and *DGR2*).

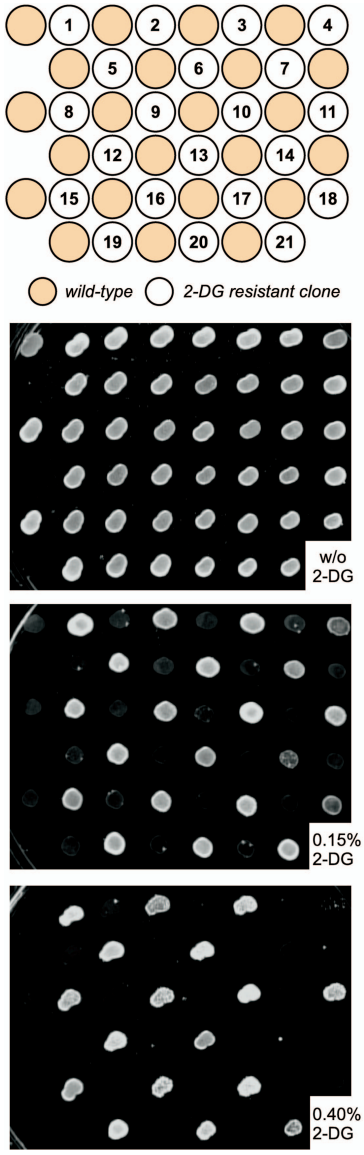
Several knockouts were implicated in mRNA decay (*LSM6*, *EDC2*, and *DHH1*), subunits of the RNA pol II mediator complex (*ROX3*), or involved in 60S ribosomal subunit biogenesis (*NOP16*). The mRNA decay is compartmentalized to so-called P-bodies, sites that are also important for mRNA stability control and translation (21), indicating that the control of mRNA turnover could be targeted by 2-DG.

Moreover, two deletion mutants of yet unassigned function (YNL130C-A [termed *DGR1* for 2-deoxy-glucose-resistant-1] and YKL121W [*DGR2*]) were 2-DG tolerant. The protein encoded by *DGR2* has several homologues in different species and contains a WD40 domain found in proteins that function as adaptor/regulatory modules in signal transduction, pre-mRNA processing, and cytoskeleton assembly. In contrast, *DGR1* encodes for a putative small polypeptide (49 residues), but neither nucleotide nor protein (PHI- and PSI-) BLAST searches identified any putative orthologues.

Remarkably, the strain deleted for hexokinase-2 (*HXK2*) was 2-DG resistant. *HXK2* is predominant in yeast growing on glucose, whereas the other glucose-phosphorylating enzymes (*HXK1* and *GLK1*) are induced on nonfermentable carbon sources (22). This observation is a strong indication that a block of HXK by 2-DG does not explain how 2-DG restrains cell growth. However, *HXK2* mutants are deficient for glucose repression (23), as is another candidate, Δ*REG1*, a regulator of the protein phosphatase type 1 (PP1; *GLC7*) (24). Moreover, the identified cyclin *PCL8* interacts with the Cdk Pho85, which itself phosphorylates the PP1 regulator Glc8 (25, 26). Taken together, these findings suggest that the prevention of glucose repression mediates 2-DG resistance, indicating that 2-DG-mediated growth inhibition is a regulatory consequence.

Discussion

Here, we provide abundant evidence that inhibition of carbohydrate catalysis in not sufficient to explain how 2-DG restrains cell growth. First, inhibition of the PPP, the alternate pathway by which glucose analogues enter the Krebs cycle, increases the cellular tolerance to 2-DG. Second, metabolic profiling did not show a linear decrease of downstream catabolic intermediates and, moreover, two 2-DG resistant strains had a similar metabolic profile as the wild type. Finally, 2-DG-resistant knockouts were implicated in various pathways and included the major hexokinase isoform as well as genes involved in mRNA decay and different regulatory pathways. Thus, the biological response to 2-DG involves pathways that are beyond the catabolic block. Consequently, the current assumptions concerning how 2-DG acts as an anticancer therapeutic, improves temporal lobe epilepsy in mice, and increases the lifespan of *C. elegans* are called into question. Nonetheless, it remains plausible that metabolic changes are central to the effects of 2-DG. The strong increase in e4p, for example, could be involved in yet unknown regulatory mechanisms and feedback control systems. In concordance, recent



Nr.	LOCUS	NAME	FUNCTION
			Carbohydrate Metabolism
13	YLR307W	*CDA1*	Chitin deacetylase, mutants are sensitive to hydrolyrtic enzymes, ether, and heat shock
17	YGL253W	*HXK2*	the predominant Hexokinase; catalyzes the phosphorylation of glucose in the cytosol
21	YDR074W	*TPS2*	Phosphatase subunit of the trehalose-6-phosphatesynthase/phosphatase complex
			Signaling-, Cell cycle-, and Transcriptional Regulators
1	YPL219W	*PCL8*	CycLin, interacts with Pho85p cyclin-dependent kinase (Cdk), regulates glycogen synthase
6	YDR200C	*VPS64*	required for cytoplasm to vacuole targeting; involved in pheromone-induced cell cycle arrest
10	YDR028C	*REG1*	Regulatory subunit of type 1 protein phosphatase Glc7p, regulator of glucose-repression
14	YOR275C	*RIM20*	PalA/AIP1/Alix family member, regulator of Ime2p and Rim101p, binds the ESCRT-III subunit Snf7p
18	YBL093C	*ROX3*	Subunit of the RNA polymerase II mediator complex
			mRNA decay
7	YDL160C	*DHH1*	Cytoplasmic DExD/H-box helicase, stimulates mRNA decapping
16	YER035W	*EDC2*	activates mRNA decapping directly by binding to mRNA; enhances Dcp1p and Dcp2p activity
19	YDR378C	*LSM6*	involved in mRNA decay; part of U6 snRNP and involved in processing tRNA, snoRNA, and rRNA
			Mitochondria and Redox Control
4	YJR118C	*ILM1*	involved in mitochondrial DNA maintenance
8; 15	YOR018W	*ROD1*	confers resistance to the GST substrate o-dinitrobenzene as well as to zinc and calcium
			Other Processes
2; 3	YKL010C	*UFD4*	E3-Ubiquitin ligase; interacts with Rpt4p and Rpt6p
5	YER007W	*PAC2*	Microtubule effector; required for tubulin heterodimer formation
9	YER002W	*NOP16*	Constituent of 66S pre-ribosomal particles, involved in 60S ribosomal subunit biogenesis
12	YDR097C	*MSH6*	required for mismatch repair in mitosis and meiosis
			Newly assigned
11	YNL130C-A	*DGR1*	Putative protein of unknown function
20	YKL121W	*DGR2*	Putative protein of unknown function; increased accumulation of glycogen

Fig. 3. Identification of 2-DG-resistant yeast knockouts. 2-DG-resistant yeast strains identified in the genome-wide screen were spotted, alternating with the wild-type strain, on SC media with or without 2-DG, as indicated. Growth was monitored after incubation for 3 days at 30°C.

work highlights the correlation between glycosylation patterns and the properties of 2-DG (5, 14), even though no protein known to be involved in this process has been identified in the screen.

Remarkably, the cellular adenosine triphosphate (ATP) sensor target of rapamycin (TOR) is known to be inactivated in 2-DG-treated cells (27). Because TOR activation is able to block the cell cycle in G_1 (28), we assayed yeast strains deleted for genes involved in the G_1 checkpoint (*CLN1*, *CLN2*, *CLN3*, *CLG1*, *PCL1*, and *WHI2*) for a potential 2-DG phenotype. Although one Pho85 cyclin, *PCL8*, was identified in the resistance screen, none of these knockouts showed altered sensitivity to 2-DG (Fig. S3). This suggested that 2-DG toxicity is not primarily based on a TOR-mediated G_1 block. Supporting, recent work showed that the TOR inhibitor CCI-779 (a rapamycin analogue) sensitizes, not protects, HIF1-α-expressing tumor cells against 2-DG (29).

Moreover, it is feasible that the cellular redox state, especially of the NADP(H) pool (because targeted by *GDP1*), could be involved in the effects of 2-DG. Our investigations failed to support the idea that cells stop growing due to a lack of $NADP^+$ (data not shown). But, as *GDP1* is a close homologue to the three yeast GAPDH paralogues *TDH1*, *TDH2*, and *TDH3*, the substance might target other processes influenced by these central metabolic regulators. GAPDH is implicated in many cellular processes; for instance, it acts as metabolic switch, receptor kinase, transcription factor, and apoptosis regulator (18, 30–32). Accordingly, it is of viable interest to reevaluate the mechanisms by which 2-DG treatment increases lifespan and blocks tumor and disease progression. Enhanced understanding will extend the wide-ranging pharmacological properties of 2-DG and could lead to the identification of novel targets.

Methods

Cell Culture, Yeast Cultivation, Screening, and Strain Generation. Primary fibroblasts were isolated and cultivated as described (19), and treated with 4 mM 2-DG. Yeast was cultivated in YPD or synthetic complete media (SC; Difco) with 2% glucose. Yeast strains deleted for *ZWF1* were generated by genomic integration of the *kanMX4* module, and strains deleted for *TKL1* by integrating *natMX4*. *GPD1* promoter-driven *K. lactis GDP1* was integrated into the *HIS3* locus by using

a NheI-digested pRS303 vector. Details of the strain and plasmid generation are given in *SI Text*; genotypes of the yeast strains used are listed in Table S2.

For the chemical genetic screening, the *MAT*a deletion collection was grown in multiwell plates, transferred three times to SC media containing 0.25% or 0.20% 2-DG, and incubated at 30 °C. Strains that were identified at least twice were selected and retested for 2-DG resistance. The positive clones were verified by sequencing of the genetic barcode and rearrayed for comparative 2-DG tests as shown in Fig. 3.

Metabolic Profiling. Sugar phosphates were extracted and measured by LC-MS/MS operating in negative multiple reaction monitoring, as described previously (18, 19).

ACKNOWLEDGMENTS. We thank E. Jansen (VU Medical Center) and G. Kustatscher (European Molecular Biology Laboratory, Heidelberg) for technical support and M. Breitenbach (University of Salzburg, Austria) for interesting discussions. This work was supported by the Max Planck Society.

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