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The induction of HAD-like phosphatases by multiple signaling pathways confers resistance to the metabolic inhibitor 2-deoxyglucose

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Abstract

Anti-cancer strategies that target the glycolytic metabolism of tumors have been proposed. The glucose analog 2-deoxyglucose (2DG) is imported into cells and after phosphorylation becomes 2DG-6-phosphate, a toxic by-product that inhibits glycolysis. Using yeast as a model, we performed an unbiased mass spectrometry-based approach to probe the cellular effects of 2DG on the proteome and study resistance mechanisms to 2DG. We found that two phosphatases that target 2DG-6-phosphate were induced upon exposure to 2DG and participated in 2DG detoxication. Dog1 and Dog2 are HAD (haloacid dehalogenase)-like phosphatases, which are evolutionarily conserved. 2DG induced Dog2 by activating several signaling pathways, such as the stress response pathway mediated by the p38 MAPK ortholog Hog1, the unfolded protein response (UPR) triggered by 2DG-induced ER stress, and the cell wall integrity (CWI) pathway mediated by the MAPK Slt2. Loss of the UPR or CWI pathways led to 2DG hypersensitivity. In contrast, mutants impaired in the glucose-mediated repression of genes, were 2DG-resistant because glucose availability transcriptionally repressed *DOG2* by inhibiting signaling mediated by the AMPK ortholog Snf1. The characterization and genome resequencing of spontaneous 2DG-resistant mutants revealed that *DOG2* overexpression was a common strategy underlying 2DG resistance. The human Dog2 homolog HDHD1 displayed 2DG-6-phosphate phosphatase activity in vitro and its overexpression conferred 2DG resistance in HeLa cells, suggesting that this 2DG phosphatase could interfere with 2DG-based chemotherapies. These results show that HAD-like phosphatases are evolutionarily conserved regulators of 2DG resistance.

Introduction

Most cancer cells display an altered metabolism, with an increased glucose consumption to support their proliferative metabolism that is based on aerobic glycolysis (Warburg effect) (1, 2). Inhibiting glycolysis has been proposed as a strategy to target cancer cells and various metabolic inhibitors have been considered (3, 4).

2-deoxy-D-glucose (2DG) is a derivative of D-glucose that is imported by glucose transporters and is phosphorylated by hexokinase into 2-deoxy-D-glucose-6-phosphate (2DG6P), but cannot be further metabolized due to the 2-deoxy substitution, triggering a decrease in cellular ATP content in tumors (5). Mechanistically, 2DG6P accumulation hampers glycolysis by inhibiting hexokinase activity in a non-competitive manner (6, 7), as well as by inhibiting phospho-glucose isomerase activity in a competitive manner (8). Because cancer cells rely on an increased glycolysis rate for proliferation, 2DG has been of interest for cancer therapy, particularly in combination with radiotherapy or other metabolic inhibitors (9-11). These features led to a phase I clinical trial using 2DG in combination with other drugs to treat solid tumors (12). Its derivative ¹⁸Fluoro-2DG is also used in cancer imaging (PET scans) because it preferentially accumulates in tumor cells due to their increased glucose uptake (13). Additionally, due to its structural similarity to mannose, 2DG (which could also be referred as to 2-deoxymannose, because mannose is the C2 epimer of glucose) also interferes with N-linked glycosylation and causes ER stress (14-16), which has been proposed to be the main mechanism by which 2DG kills normoxic cells (17). 2DG toxicity has also been linked to the depletion of phosphate pools following 2DG phosphorylation (18). Finally, interference of 2DG with lipid metabolism and calcium homeostasis through unknown underlying mechanisms have been described (19). Resistance to 2-deoxyglucose has been detected in cell cultures (20).

Because these metabolic and signalling pathways are evolutionarily conserved, simpler eukaryotic models such as the budding yeast *Saccharomyces cerevisiae* can be used to understand the mode of action of 2DG. Moreover, yeast is particularly well-suited for these studies because of its peculiar metabolism (21). Akin to cancer cells, *Saccharomyces cerevisiae* preferentially consumes

1 glucose through glycolysis over respiration, regardless of the presence of oxygen. This occurs through
2 glucose-mediated repression of genes involved in respiration and alternative carbon metabolism,
3 which operates at the transcriptional level. This glucose-mediated repression mechanism is relieved
4 upon activation of the yeast ortholog of 5'AMP-activated protein kinase (AMPK), Snf1, which
5 phosphorylates the transcriptional repressor Mig1 and leads to its translocation out of the nucleus (22-
6 25). Mutations that render yeast cells more tolerant to 2DG have been identified (26-31). These
7 findings suggest the existence of cellular mechanisms that can modulate 2DG toxicity, which are
8 important to characterize if 2DG is to be used therapeutically.

9 In yeast, 2DG was initially used to identify genes involved in glucose repression because 2DG,
10 like glucose, causes Snf1 inactivation and thus prevents the use of alternative carbon sources (32-34).
11 The characterization of mutants that can grow in 2DG-containing sucrose medium has allowed the
12 identification of components of the glucose repression pathway (26, 29, 35) and has revealed that
13 mutations in *HXK2*, which encodes hexokinase II, also render yeast cells more tolerant to 2DG, perhaps
14 by limiting 2DG phosphorylation and 2DG6P accumulation (29-31, 36, 37). Finally, several 2DG-
15 resistant mutants display increased 2DG6P phosphatase activity, which could detoxify the cells of this
16 metabolite and dampen its negative effects on cellular physiology (38, 39). Indeed, two 2DG6P
17 phosphatases named *DOG1* and *DOG2* have been cloned, and their overexpression triggers 2DG
18 resistance and prevents 2DG-mediated repression of genes (33, 40, 41).

19 The toxicity of 2DG has also been studied in the context of cells grown in glucose-containing
20 media, which may be more relevant for the understanding its mode of action in mammalian cells.
21 Under these conditions, 2DG toxicity is independent of its effect on the glucose repression of genes
22 and involves different mechanisms, such as a direct inhibition of glycolysis and other cellular pathways
23 (30, 42, 43). Accordingly, several mutations leading to 2DG resistance in cells grown in sucrose medium
24 do not lead to resistance in cells grown in glucose medium (30, 44). Deletion of *REG1*, which encodes
25 a regulatory subunit of protein phosphatase 1 (PP1) that inhibits Snf1 (45, 46) leads to 2DG resistance
26 (30). The resistance of the *reg1Δ* mutant depends on the presence of Snf1, and the single deletion of

1 *SNF1* also renders yeast hypersensitive to 2DG (31), thus demonstrating that Snf1 activity is crucial for
2 2DG resistance. A model has been proposed in which the 2DG sensitivity displayed by the *snf1Δ* mutant
3 involves misregulated expression and localization of the low-affinity glucose transporters Hxt1 and
4 Hxt3 (47). Additionally, the deletion of *LSM6*, which encodes a component of a complex involved in
5 mRNA degradation, also leads to 2DG resistance in a Snf1-dependent manner, but the mechanism by
6 which this occurs is unknown (31). Thus, many aspects of the pathways mediating 2DG sensitivity or
7 resistance remain to be explored.

8 In the present study, our unbiased, mass-spectrometry-based approach in yeast revealed that
9 the main 2DG6P phosphatase, Dog2, was induced upon exposure to 2DG and participated in 2DG
10 detoxification in glucose medium. We found that 2DG induced Dog2 (and Dog1, to a certain extent),
11 by triggering UPR and MAPK-based stress-responsive signaling pathways. Moreover, the expression of
12 *DOG2* was additionally regulated by Snf1 and the glucose-repression pathway through the action of
13 downstream transcriptional repressors and contributed to the resistance of glucose-repression
14 mutants to 2DG. The partial characterization of 24 spontaneous 2DG-resistant mutants revealed that
15 most mutants displayed increased *DOG2* expression, suggesting a common strategy used to acquire
16 2DG resistance. Particularly, genome resequencing identified that mutations in *CYC8*, which encodes
17 a transcriptional corepressor, caused 2DG resistance through increased Dog2 expression. The
18 identification of a potential human homolog of the Dog1/2 proteins, HDHD1, which displays 2-
19 deoxyglucose-6-phosphate (2DG6P) phosphatase activity in vitro and which caused 2DG resistance in
20 HeLa cells upon overexpression, suggests that HAD-like phosphatases are conserved regulators of 2DG
21 resistance.

22

Results.

A proteomic assessment of the cellular response to 2DG reveals increased expression of metabolic enzymes

As a first step to characterize the cellular response of yeast cells to 2-deoxyglucose (2DG) treatment in an unbiased and quantitative manner, we performed proteome-wide mass spectrometry-based proteomics on WT cells treated with 0.2% 2DG, a concentration that prevents growth of WT cells on plates (30). Untreated cells were used as a negative control. Overall, 78 proteins were significantly more abundant, whereas 18 proteins were less abundant after 2DG treatment (False Discovery Rate (FDR) of 0.01) (Fig. 1A, Data File S1). Among the up-regulated candidates, proteins involved in various metabolic processes were significantly enriched, including those involved in the metabolism of glucose, glucose-6-phosphate and other carbohydrates (Fig. 1B, fig. S1), in line with 2DG interfering with glycolysis (5).

These proteomics data revealed an increase in the abundance of the 2-deoxyglucose-6-phosphate phosphatases Dog1 or Dog2, which could not be discriminated at the mass spectrometry level because of their high sequence identity (92%). Although these phosphatases can promote 2DG resistance (33, 40, 41), the increased expression of Dog1 and/or Dog2 in response to 2DG was intriguing because it raised the question of how exposure to this synthetic molecule could trigger an adaptive resistance mechanism in yeast. To first confirm that 2DG induced Dog1 and Dog2, we GFP-tagged each at their chromosomal locus to maintain endogenous regulation. Western blotting revealed that both Dog1 and Dog2 expression levels were increased in the presence of 2DG, and that Dog2-GFP was more abundant than Dog1-GFP (Fig. 1C).

To evaluate the contribution of transcription in the regulation of *DOG1* and *DOG2* genes by 2DG, we fused the corresponding promoters to a β -galactosidase reporter. 2DG increased the activity of the *DOG1* and *DOG2* promoters (Fig. 1D), suggesting that the expression of Dog1 and Dog2 was at least partially due to increased transcription. The deletion of *DOG2* sensitized yeast cells to 2DG, but that of *DOG1* had little effect (Fig. 1E), indicating that Dog2 is functionally more important than Dog1, perhaps

1 due to its higher expression level (Fig. 1C). These results indicate that Dog2 participates in the natural
2 tolerance of WT cells to low concentrations (0.05%) of 2DG.

3 The expression of Dog2, whose endogenous function in yeast metabolism is unknown, is induced
4 by various stresses, such as oxidative and osmotic stresses, through the stress-responsive mitogen
5 activated protein kinase (MAPK) ortholog of p38, Hog1 (48). The deletion of *HOG1* prevented the
6 maximal induction of *pDOG2-LacZ* expression (Fig. 1F) and Dog2 induction (Fig. 1G-H) without affecting
7 the induction of Dog1 (fig. S2A). When we tested the effect of 2DG on Hog1 phosphorylation using an
8 antibody directed against the phosphorylated form of mammalian p38 (49), we found that Hog1
9 appeared partially activated upon 2DG addition, but to a lower extent compared to the activation
10 induced by hyperosmotic shock (fig. S2B). These data show that the stress-activated protein kinase
11 Hog1 participates in Dog2 induction but also that the maximal expression of Dog1 and Dog2 by 2DG
12 involves at least one additional level of regulation.

13

14 **The expression of Dog1 and Dog2 is induced by the unfolded protein response pathway through** 15 **2DG-induced ER stress**

16 Exposure of cancer cells to 2DG interferes with N-linked glycosylation, likely because of the
17 structural similarity of 2DG with mannose, a constituent of the N-glycan structures (14). This
18 interference results in endoplasmic reticulum (ER) stress and consequently, in the induction of the
19 unfolded protein response (UPR) pathway in mammalian cells (14). Treatment of yeast with 2DG also
20 induced a defect in the glycosylation of carboxypeptidase Y (CPY) (Fig. 2A), a vacuolar (lysosomal)
21 protease whose membrane-anchored precursor is N-glycosylated in the course of its intracellular
22 trafficking (50). This defect was not as extensive as that observed upon treatment of cells with
23 tunicamycin, an inhibitor of the first step of glycosylation that also causes ER stress and is a strong UPR
24 inducer (51, 52) (Fig. 2A). The addition of exogenous mannose in the medium suppressed the
25 glycosylation defects caused by 2DG, but not those caused by tunicamycin (Fig. 2A). This result

1 supports the idea that 2DG mimics mannose and interferes with its incorporation in N-glycans, as has
2 been proposed in mammalian cells (14, 17).

3 In yeast, the UPR pathway is initiated by a multifunctional ER membrane protein named Ire1. Upon
4 sensing ER stress and after dimerization, Ire1 splices pre-mRNAs encoding the transcription factor
5 Hac1, leading to its translation and the trans-activation of Hac1 targets carrying an UPRE (UPR Element)
6 in their promoter (Fig. 2B) (53). To address whether 2DG can induce the UPR pathway in yeast, we
7 used an UPRE-driven reporter, UPRE1-LacZ (52). 2DG elicited the expression of this reporter in a
8 manner dependent on Hac1, confirming that 2DG is a bona fide UPR inducer (Fig. 2C).

9 We then tested whether 2DG-mediated induction of Dog2 involved the UPR pathway. First, we
10 found that tunicamycin strongly induced the *pDOG2*-LacZ reporter (Fig. 2D), suggesting that
11 glycosylation defects and/or the ensuing ER stress promotes *DOG2* expression. Moreover, the
12 induction of this reporter by 2DG was reduced by more than 2-fold in *hac1Δ* mutant cells, suggesting
13 that UPR contributed to this induction (Fig. 2E). Comparable results were obtained for *pDOG1*-LacZ
14 (fig. S3, A and B). These results were confirmed at the protein level for Dog2 (Fig. 2F-G).

15 UPR-compromised mutants such as *hac1Δ* and *ire1Δ* were both hypersensitive to 2DG (Fig. 2H),
16 suggesting that they cannot cope with ER stress caused by 2DG. The addition of exogenous mannose
17 in the medium, which can alleviate the glycosylation defects caused by 2DG (Fig. 2A), restored 2DG-
18 tolerance of these mutants to a comparable level as that of WT cells (Fig. 2H) but not that of *snf1Δ*, a
19 2DG-hypersensitive mutant (31). Together, these results confirm that 2DG induces ER stress by
20 interfering with N-glycosylation, that the subsequent activation of the UPR pathway increased Dog1
21 and Dog2 expression, and that UPR mutants display an increased sensitivity to 2DG which correlates
22 with a lower level of Dog1 and Dog2 expression. This was further supported by the ability of Dog2
23 overexpression to restore the growth of *hac1Δ* or *ire1Δ* cells at various concentrations of 2DG (Fig. 2I).

24

25 **2DG also activates the MAPK-based cell-wall integrity pathway which additionally contributes to**
26 **Dog1 and Dog2 expression**

1 To address a possible additional contribution of other signaling pathways that would contribute to
2 the 2DG-induced expression of Dog1 and Dog2, we ran a bioinformatics analysis on the proteins that
3 were more abundant upon 2DG treatment (**Fig. 1A** and **Data File S1**) using YEASTRACT (54) to evaluate
4 whether the observed variations in protein expression could reveal a potential transcriptional
5 signature. The best hit (corresponding to 35/78 candidates, p -value=0) was the MADS-box
6 transcription factor Rlm1, a downstream target of the cell wall integrity (CWI) signaling pathway (55).
7 The CWI pathway is activated by several stresses such as cell wall damage, and involves plasma
8 membrane-localized sensors, the GTPase Rho1 and its cofactors (guanine nucleotide exchange factors
9 [GEFs] and accessory proteins), protein kinase C, and a cascade leading to the activation of the MAPK
10 Slt2 and downstream transcription factors, such as Rlm1 (**Fig. 3A**) (55). There are well-established links
11 between CWI signaling and ER stress (56-60); in particular, the CWI pathway is required for viability
12 upon tunicamycin-induced ER stress. Moreover, 2DG causes cell wall defects (43, 61-63) and as such
13 might trigger CWI pathway activation. We found that the deletion of many genes in the CWI pathway
14 that act upstream of Rlm1, such as those encoding the sensors *MID2* and *WSC1*, caused an increase in
15 2DG sensitivity (**Fig. 3B**). Thus, much like the UPR pathway, the CWI pathway is required for 2DG
16 tolerance. 2DG treatment induced the phosphorylation of the MAPK Slt2 within the first hour of
17 exposure (**fig. S4A**). 2DG also triggered the induction of a reporter consisting of an Rlm1-regulated
18 promoter fused to LacZ (**fig. S4B**). Thus, the CWI pathway is activated by 2DG treatment.

19 We thus questioned whether CWI activation by 2DG contributed to Dog1 and Dog2 induction.
20 Indeed, the activity of both promoters was decreased in the *slt2Δ* mutant (**Fig. 3C** and **S4C**), which was
21 confirmed for Dog2 by analyzing the expression levels of Dog2-GFP (**Fig. 3D-E**). Thus, similarly to
22 tunicamycin (58), 2DG induces cell-wall integrity signaling and promotes increased Dog1 and Dog2
23 expression, in addition to inducing the UPR pathway. These effects may in turn contribute to 2DG
24 tolerance. However, the addition of exogenous mannose did not improve tolerance of CWI mutants
25 to 2DG (**Fig. 3F**). Thus, ER stress relief is not sufficient to suppress 2DG toxicity in these mutants,
26 suggesting that 2DG has other cellular effects beyond triggering ER stress.

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A third pathway inhibits the expression of Dog2, but not Dog1, by glucose availability and participates in the resistance of glucose-repression mutants to 2DG

2DG inhibits the activity of hexokinase and phosphoglucose isomerase (6-8), thereby impairing glycolysis and leading to energetic stress. Accordingly, 2DG treatment activates AMPK in mammals (64). Several lines of evidence indicate that the activity of the yeast AMPK orthologue Snf1 is important for 2DG tolerance. Whereas the *snf1Δ* mutant is hypersensitive to 2DG, the *reg1Δ* mutant, in which Snf1 is hyperactive, displays increased 2DG resistance that depends on Snf1 activity (31). We questioned whether some of the resistance or sensitivity phenotypes associated with this pathway (Fig. 4A) could be due to an altered level of Dog1 or Dog2 expression, particularly because Snf1 promotes Dog2 expression (48). To confirm the effect of Snf1 on Dog1 and Dog2 expression, glucose-grown cells were transferred to a medium containing lactate as a sole carbon source, which should trigger Snf1 activation and consequently, the de-repression of glucose-repressed genes (65) (Fig. 4A). As expected, switching cells to lactate medium induced Snf1 activation, as determined using antibodies directed against the activated (phosphorylated) form of human AMPK α , which cross-reacts with yeast Snf1 (66) (Fig. 4B). The expression of Dog2, but not that of Dog1, was increased under these conditions in a Snf1-dependent manner (Fig. 4B). In line with these data, the *pDOG2-LacZ* reporter was induced in cells transferred to lactate medium, but this response was decreased in the *snf1Δ* mutant, suggesting that *DOG2* is repressed by glucose and is thus constantly repressed in the *snf1Δ* mutant (Fig. 4C), which may explain why the *snf1Δ* mutant is sensitive to 2DG. Although Dog1/2 overexpression in a *snf1Δ* mutant has been reported to not rescue resistance to 2DG (47), we thought that this might be because the authors used a high-copy plasmid in which Dog2 expression was still under the control of its endogenous, Snf1-regulated promoter. Indeed, when overexpressed using a strong and constitutive promoter (*pGPD*), Dog2 rescued Snf1 growth in 2DG-exposed cells (Fig. 4D).

In contrast, Dog2 expression was increased in glucose-repression mutants which display increased Snf1 activity, such as mutants lacking the hexokinase Hxk2 or the PP1 regulatory phosphatase Reg1

1 (44, 67), both at the promoter and the protein levels (Fig. 4E-F). In addition, lack of the Snf1-regulated
2 transcriptional repressors Mig1/Mig2 (Fig. 4A) also led to an increase in Dog2 expression (Fig. 4E-F). A
3 regulatory sequence in the promoter that is present between -250 and -350 bp relative to the ATG
4 start codon, combined with a proposed Mig1-binding site located at ~200 bp (40, 48), was critical for
5 this glucose-mediated repression (fig. S5, A and B). The *reg1Δ* or *hvk2Δ* mutants, which are more
6 tolerant to 2DG than WT (30, 31), showed increased Dog2 expression (Fig. 4E-F), which was also the
7 case for the double mutant *mig1Δ mig2Δ* (Fig. 4G). We deleted *DOG1* and *DOG2* in these mutants to
8 evaluate their contribution to 2DG resistance. The absence of Dog1 and Dog2 sensitized all strains to
9 2DG (Fig. 4G); in particular, the *mig1Δ mig2Δ dog1Δ dog2Δ* showed a level of sensitivity that was
10 comparable to that of the WT, demonstrating that the resistance of the *mig1Δ mig2Δ* mutant was due
11 to increased *DOG* expression. In contrast, the *reg1Δ dog1Δ dog2Δ* and the *hvk2Δ dog1Δ dog2Δ* strains
12 remained more resistant to 2DG than the WT, suggesting additional mechanisms of resistance. Finally,
13 we observed that the *snf1Δ mig1Δ mig2Δ* mutant was resistant to 2DG despite the absence of Snf1, in
14 line with the idea that *snf1Δ* is 2DG-sensitive because of the constitutive repression of Mig1/Mig2
15 target genes, such as Dog2 and possibly other genes (Fig. 4F). Overall, we conclude that Dog2 is also
16 regulated by glucose availability through Snf1 activity, which contributes to the resistance of glucose-
17 repression mutants to 2DG.

18

19 **Increased expression of *DOG2* is frequently observed in spontaneous 2DG-resistant clones**

20 Previous screens have identified 2DG-resistant mutants (26, 29, 35). The initial purpose of these
21 screens was to identify mutants that were insensitive to the repressive effect of 2DG, or that were
22 impaired for glucose phosphorylation (because only 2DG6P is toxic to cells), and consequently, were
23 performed on media containing other carbon sources than glucose. The mechanisms involved in 2DG
24 resistance on glucose medium were tackled later by screening of the deletion library (30), which led
25 to the identification of resistant mutants, some of which were subsequently confirmed (31). In the
26 course of our experiments, we often found that 2DG-resistant clones could spontaneously arise from

1 WT or even 2DG-susceptible strains (Fig. 1E). Yeast acquires 2DG resistance at a high frequency, which
2 can be accompanied by an increase in 2DG-6-phosphatase activity (28, 38). To study whether Dog2,
3 which is functionally critical for 2DG resistance, was upregulated during the emergence of spontaneous
4 2DG-resistant mutants, we spread $\sim 2 \times 10^6$ cells on glucose-based medium containing 0.2% 2DG and
5 selected clones that had appeared after 6 days of growth. In total, 24 clones were obtained whose
6 resistance to 2DG was confirmed (fig. S6A). Using the pDOG2-LacZ reporter, we found that 13 clones
7 displayed significantly increased *DOG2* promoter activity as compared to WT strains (Fig. 5A),
8 suggesting that Dog2 overexpression is a frequent feature of 2DG-resistant clones. Among those, we
9 expected to isolate *reg1* and *hvk2* mutants because both are resistant to 2DG in glucose medium (See
10 Figure 5G; 30, 31). To identify them, we first tested whether some of the isolated resistant clones
11 displayed phenotypes typical of *reg1* mutants, such as sensitivity to tunicamycin (68, 69) or selenite
12 (which enters the cell through the glucose-repressed transporter Jen1: 70, 71). Indeed, three of the
13 isolated clones displayed these phenotypes (fig. S6A). Sequencing of the *REG1* coding sequence in
14 these clones revealed the presence of nonsense mutations which likely explain Reg1 loss of function
15 (fig. S6B). To identify potential *hvk2* mutants, we performed a complementation test by crossing the
16 2DG-resistant clones with either a WT strain or an *hvk2* Δ strain and tested the ability of the resulting
17 diploids to grow on 2DG. All diploids generated by the cross with the WT strain lost their ability to grow
18 on 2DG, except for two (clones #23 and #24) (fig. S6C), indicating that 2DG resistance was generally
19 caused by a recessive mutation(s). In contrast, 12 diploids obtained by the cross with the *hvk2* Δ strain
20 maintained their ability to grow on 2DG (fig. S6C), suggesting that 2DG resistance of the initial strains
21 was caused by a deficiency in *HVK2* function. Sequencing of the *HVK2* ORF in these 12 mutants (fig.
22 S6D) revealed that two did not display any mutation in the *HVK2* ORF (clones #1 and #8) and may
23 represent regulatory mutants in *cis*, possibly in regions that were not sequenced. In favor of this
24 hypothesis, we found that their 2DG sensitivity was restored by re-expression of *HVK2* using a
25 multicopy, genomic clone (fig. S6E). The other 10 mutants carried at least one mutation in the *HVK2*
26 coding sequence (fig. S6D). Four mutants acquired nonsense mutations (clones #12, #15, #16 and #20),

1 5 mutants (#2, #3, #11, #13 and #14) carried a missense mutation in residues conserved across all
2 hexokinase sequences tested (**fig. S7, alignment**) and one mutant (#21) bore two missense mutations.
3 All of these missense mutations occurred in residues involved in glucose or ATP binding or were located
4 near such residues (**fig. S7**) (72-74).

5 Of the 2DG-resistant clones which expressed *pDOG2-LacZ* at a high level, 2 clones (#9 and #10)
6 lacked mutations in either *REG1* or *HXK2*, suggesting that their resistance involved a mechanism that
7 did not involve these proteins. Whole genome resequencing of the genomic DNA isolated from these
8 clones and comparison with that of the parent strain revealed several SNPs (**Table S1**) including a
9 nonsense mutation in *CYC8* (C⁹⁵⁸>T) that was identified in both strains, causing a premature stop codon
10 at position 320 (**Fig. 5B**). The *CYC8* gene is also known as *SSN6* (*Suppressor of snf1*) and mutations in
11 this gene lead to constitutive expression of the glucose-repressed gene encoding invertase (*SUC2*),
12 even in a *snf1* mutant (75, 76). Indeed, *CYC8* encodes a transcriptional co-repressor which controls the
13 expression of glucose-regulated genes (77). Because the repression of *DOG2* expression by glucose is
14 controlled by Snf1 and Mig1/Mig2 (**Fig. 4, E and F**), we hypothesized that *CYC8* could also take part in
15 *DOG2* regulation, such that a mutation in *CYC8* could lead to increased 2DG resistance through *DOG2*
16 overexpression. We observed that indeed, mutants #9 and #10 expressed invertase even in when
17 grown in glucose medium (repressive conditions), in agreement with a mutation in *CYC8* (**Fig. 5C**). We
18 also observed that these mutants displayed increased adhesion, or persistence of colony structures
19 upon washing the plate (78) that is often associated with mutants that are prone to flocculation, such
20 as *cyc8* mutants (79, 80) (**Fig. 5D**). Introduction of a low copy (centromeric) plasmid containing *CYC8*
21 under the control of its endogenous promoter suppressed the 2DG resistance of mutants #9 and #10
22 as well as their adhesion to agar plates (**Fig. 5D**). This was not the case when mutants #9 and #10
23 expressed a truncated version of *CYC8* identified in this screen (**Fig. 5D**), although these mutants did
24 not display slow growth in contrast to the *cyc8Δ* mutant, suggesting that it is at least partially active
25 for other functions. Finally, we tested whether the strong activity of the *DOG2* promoter observed in
26 mutants #9 and #10 (**Fig. 5A**) was also due to the lack of a functional *CYC8*. Indeed, the re-introduction

1 of low-copy vector containing *CYC8* led to a decreased reporter expression in these mutants (Fig. 5E).
2 This result was confirmed by examining Dog2-GFP expression at the protein level (Fig. 5F-G).
3 Therefore, *DOG2* expression is repressed by Cyc8 and spontaneous *cyc8* mutants display an increased
4 Dog2 expression. The increased resistance of these mutants to 2DG also depended on the expression
5 of Dog1 and Dog2 as the deletion of *DOG1* and *DOG2* restored their sensitivity to 2DG (Fig. 5H).
6 Together, this initial characterization revealed that *DOG2* overexpression is a common phenomenon
7 within spontaneous 2DG-resistant clones, both in known 2DG-resistant mutants (*reg1* and *hvk2*) and
8 in the 2DG-resistant *cyc8* mutants that we isolated.

9

10 **HDHD1, a human member of the HAD-like phosphatase family, is a 2DG-6-P phosphatase** 11 **involved in 2DG resistance**

12 The increase in Dog2 expression in various spontaneously 2DG-resistant mutants reminded us of
13 an earlier study in HeLa cells showing increased 2DG6P phosphatase activity in isolated 2DG resistant
14 clones (20). Dog1 and Dog2 belong to the family of HAD (haloacid dehalogenase)-like phosphatases
15 which are conserved from bacteria to human (Fig. 6A). The bacterial homolog of Dog1/Dog2, named
16 YniC, can also dephosphorylate 2DG6P in vitro (81) and we found that the expression of YniC in the
17 double *dog1Δ dog2Δ* yeast mutant also restored 2DG resistance (Fig. 6B). We used this phenotype to
18 identify potential human homologs (Fig. 6B). A PSI-BLAST analysis (82) on the human proteome using
19 the Dog2 protein sequence retrieved HDHD1-isoform a (NP_036212.3) as the candidate with highest
20 (39%) homology. HDHD1 (for Haloacid Dehalogenase-like Hydrolase Domain containing 1; also named
21 PUDP for pseudouridine-5'-phosphatase) is a HAD-like phosphatase with in vitro activity towards
22 phosphorylated metabolites such as pseudouridine-5'-phosphate (83). When expressed in in the
23 double *dog1Δ dog2Δ* mutant, HDHD1 partially rescued growth on 2DG containing medium (Fig. 6B).
24 Yeast growth on 2DG was not restored by the expression of HDHD4 (also named NANP, for N-
25 acylneuraminate-9-phosphatase; 84), which belongs to the same subfamily as HDHD1 within the HAD-
26 phosphatase family (37% homology), or of PSPH (phosphoserine phosphatase; 85), another close

1 family member (fig. S8A,B). Moreover, among the four predicted isoforms of HDHD1 (isoforms 1-4),
2 only HDHD1-1 rescued the growth of the *dog1Δ dog2Δ* on 2DG-containing medium (fig. S8C) although
3 all isoforms were expressed in yeast, as confirmed by Western blotting using HDHD1 antibodies (fig.
4 S8D).

5 The mutation of conserved aspartate residues (Asp¹² and Asp¹⁴; Fig. 6A) predicted to be essential
6 for the catalytic activity of HAD phosphatases (86) abolished the ability of HDHD1 to restore growth of
7 the *dog1Δ dog2Δ* mutant on 2DG (Fig. 6C and fig. S8E), suggesting it may act as a 2DG-6P phosphatase.
8 This notion was confirmed by the ability of purified recombinant HDHD1 (Fig. 6D) to dephosphorylate
9 2DG-6P in vitro, an activity that required its putative catalytic residues (Fig. 6E). We then tested
10 whether HDHD1 overexpression in HeLa cells could lead to an increased resistance to 2DG. Low
11 concentrations of 2DG (5 mM) in the presence of glucose (25 mM) were sufficient to inhibit the growth
12 of HeLa cells transfected with an empty vector, whereas those that overexpressed HDHD1 were
13 insensitive to 2DG treatment (Fig. 6F). These results suggest that dysregulated expression of HDHD1
14 could modulate 2DG resistance in human cells.

Discussion

1
2
3 Although the first studies examining the effect of 2DG on glycolysis in yeast and in normal and
4 cancer tissues were published more than 60 years ago (87-89), the mode of action for 2DG is not fully
5 understood. 2DG can be considered to be a general competitor of glucose (90) and a glycolysis inhibitor
6 because of its inhibition of glycolytic enzymes (6-8). However, this view has been challenged because
7 exposure of cancer cells to 2DG interferes with N-linked glycosylation, likely because of the structural
8 similarity of 2DG with mannose (91), resulting in ER stress and UPR induction in mammalian cells (14,
9 16). Therefore, 2DG interferes with other cellular functions beyond glycolysis.

10 In this study, we used an unbiased mass spectrometry approach to analyze the effects of 2DG on
11 the total cellular proteome. We found that the abundance of many glycolytic enzymes was increased,
12 likely because of impaired glycolysis, as well as many genes regulated by the MAPK-based cell wall
13 integrity pathway, suggestive of its activation. Using the list of 2DG-upregulated proteins, we also
14 identified the phosphatases Dog1 and/or Dog2, which have been shown to play a role in 2DG
15 resistance (33, 40, 41). Dog2 induction upon 2DG treatment was intriguing because it was not clear
16 how a synthetic molecule such as 2DG could trigger a resistance mechanism. It should be noted that
17 the cellular functions of Dog1 and Dog2, beyond 2DG dephosphorylation, are unknown. They can
18 dephosphorylate various phosphorylated sugars in vitro (40), in agreement with the ability of other
19 members of the HAD family of phosphatases to accommodate various substrates (81). Therefore, it
20 seemed plausible that Dog1/2 induction was a response to one or more of the cellular consequences
21 of 2DG treatment, rather than to 2DG itself. Our study revealed that the expression of Dog1 and Dog2
22 was actually controlled by multiple signaling pathways, each of which are activated as a response to
23 2DG (Fig. 7).

24 This study also probed the signaling pathways that are activated upon 2DG exposure and
25 determined the causes for these activations. First, we confirmed that 2DG triggered ER stress and the
26 onset of the UPR pathway. This finding was not unexpected given the proposed mode of action of this
27 drug and the available data in the literature on mammalian cells (14, 16), but had not been formally

1 proven in yeast with current tools. The induction of an UPR reporter upon 2DG treatment and the
2 hypersensitivity of UPR mutant strains such as *hac1Δ* or *ire1Δ* support this conclusion. The latter
3 phenotype was alleviated by restoring N-glycosylation through the addition of mannose to the culture
4 medium. The consolidation of the links between 2DG and ER stress, notably the identification of UPR
5 mutants as 2DG-hypersensitive mutants suggests that drugs targeting the UPR may synergize with 2DG
6 in a glycolytic cancer background. We also showed that Dog1 and Dog2 are induced by ER stress, in
7 line with high-throughput studies suggesting that Dog1 and Dog2 are UPR-induced genes (92).

8 Second, we showed that 2DG activates the MAPK-based CWI pathway, which mediates the cellular
9 response to cell wall alteration and other stresses. Several studies reported connections between ER
10 stress signaling and the CWI pathway (56-60), and 2DG-elicited N-glycosylation defects and ER stress
11 may have repercussions on the CWI pathway. For instance, CWI pathway mutants are sensitive to
12 tunicamycin because they lack an ER surveillance-pathway which normally prevents the inheritance of
13 stressed ER to the daughter cell during cell division (58). Many CWI mutants were indeed sensitive to
14 low concentrations (0.05%) of 2DG but unlike UPR mutants, their growth was not restored by the
15 addition of exogenous mannose, suggesting that an N-glycosylation defect is not the sole reason why
16 CWI mutants are hypersensitive to 2DG. However, 2DG interferes with the synthesis of structural
17 polysaccharides that make up the yeast cell wall because 2DG acts as an antagonist of mannose and
18 glucose incorporation into these polymers (42, 61-63). 2DG exposure leads to yeast cell lysis at sites of
19 growth, which is where glucan synthesis occurs (43) and where the major glucan synthase, Fks1, is
20 localized (93). 2DG-induced weakening of the cell wall could trigger the CWI pathway (Fig. 7), which
21 would explain why the main sensors responsible for sensing cell wall damage, Wsc1 and Mid2
22 (reviewed in 55), are required for growth on 2DG. In addition, we report that the expression of Dog1
23 and Dog2 was decreased in CWI mutants, which could further sensitize these strains to 2DG. The effect
24 of 2DG on cell wall synthesis suggests an interference of 2DG with UDP-glucose metabolism that is
25 likely to be conserved in metazoans (94), where it may affect metabolic pathways involving these
26 precursors, such as glycogen synthesis (95, 96).

1 Our data also revealed that Dog2 expression was regulated at the transcriptional level by glucose
2 availability through the glucose-repression pathway, which is controlled by the kinase Snf1 and the
3 regulatory subunit of the PP1 phosphatase, Reg1. These findings explain why *reg1* mutants have
4 increased 2DG6P phosphatase activity (28, 38, 39), because the glucose-mediated repression of Dog2
5 is defective in these mutants. The negative regulation of Dog2 by this pathway may also contribute to
6 the 2DG resistance of *hvk2* and *mig1* mutants, which participate in the glucose-repression pathways
7 and have been identified in previous screens (29, 35). Indeed, the 2DG resistance displayed by the
8 *reg1Δ* and *hvk2Δ* strains partially depended on increased expression of Dog2, because even when
9 these mutants lacked both *DOG* genes, they still grew better than a WT strain on 2DG-containing
10 media. In the *hvk2Δ* mutant, the lack of hexokinase 2 is compensated for by the expression of other
11 glucose-phosphorylating enzymes (such as Hvk1 and the yeast glucokinase homolog, Glk1) (97) which
12 may be less prone to phosphorylate 2DG (36), and thus could lead to a lower accumulation of 2DG6P
13 in the cell. Hvk2 also has non-metabolic roles beyond sugar phosphorylation (reviewed in Ref. 98), and
14 loss of these non-metabolic roles may additionally contribute to this phenotype. However, many of
15 the *hvk2* point mutants isolated during the characterization of spontaneous 2DG-resistant clones were
16 affected at or near glucose-binding residues, suggesting a primary metabolic role for Hvk2 in 2DG
17 resistance. Concerning the *reg1Δ* strain, additional mechanisms of resistance beyond an increased
18 expression of Dog2 also remain to be investigated. Previous work suggested that the resistance of the
19 *reg1Δ* mutant depends on Snf1 hyperactivity, because the additional deletion of *SNF1* in this
20 background restores 2DG sensitivity to the *reg1Δ* strain (31). Thus, additional Snf1-dependent
21 mechanisms cooperate with the increased expression of *DOG2* to allow *reg1Δ* cells to resist 2DG.

22 After 2DG import and phosphorylation, the amount of 2DG6P in cells has been reported to exceed
23 that of G6P by up to 80-fold (99), although this number should probably be confirmed by more direct
24 and modern methods. Overexpression of Dog2 can revert the repressive effect of 2DG (33), suggesting
25 that it can clear the 2DG6P pool up to a point at which 2DG6P is no longer detected by a yet unknown
26 cellular glucose-sensing mechanism. Thus, Dog2 overexpression appears to be a good strategy for

1 acquiring 2DG resistance, and indeed, Dog2 was overexpressed in the majority of the spontaneously
2 resistant mutants we isolated, including strains with mutations in *REG1* and *HXK2*. Additionally, we
3 identified two mutants carrying a point mutation in the gene encoding the transcriptional repressor
4 Cyc8, leading to a truncated protein at codon 320, within its 8th predicted tetratricopeptide (TPR)
5 repeat. The TPR repeats are involved in the interaction of Cyc8 with its co-repressor Tup1 and in its
6 recruitment to specific promoters, possibly through pathway-specific DNA-binding proteins (100).
7 Point mutations in TPR units 9 and 10 affect Cyc8 function in a similar manner as the mutation we
8 isolated (namely, an effect on glucose repression but not global growth), suggesting that alterations at
9 this region only affect a subset of Cyc8 functions (101). This finding confirms the differential
10 requirement of TPR repeats for the various functions of Cyc8, and in particular the involvement of the
11 TPR repeats 8-10 in glucose repression (102). Together, these data describe *DOG2* overexpression as
12 a successful strategy to overcome 2DG toxicity.

13 Based on sequence similarity, we identified HDHD1 as an enzyme displaying in vitro 2DG6P
14 phosphatase activity and whose overexpression in both yeast and HeLa cells allowed resistance to
15 2DG. Whether HDHD1 is responsible for the 2DG resistance previously reported in human cells (20)
16 remains to be investigated.

17 Together, our work shows that 2DG-induced activation of multiple signaling pathways can rewire
18 the expression of endogenous proteins that target 2DG6P to promote 2DG tolerance, and whose
19 increased expression can lead to 2DG resistance. Because of the existence of endogenous genes that
20 can confer 2DG resistance when overexpressed, such as *HDHD1*, and of the perturbation of several
21 cellular pathways in mammalian cells by 2DG, it is possible that similar resistance strategies occur in
22 human cells. Such strategies likely superimpose on other resistance mechanisms that should be
23 scrutinized in the future.

24

25

Material and Methods

Yeast strain construction and growth conditions

All yeast strains used in this study derive from the *Saccharomyces cerevisiae* BY4741 or BY4742 background and are listed in **Table S2**. Apart from the mutant strains obtained from the yeast deletion collection (Euroscarf) and the fluorescent GFP tagged strains originating from the yeast GFP clone collection (103), all yeast strains were constructed by transformation with the standard lithium acetate-polyethylene glycol protocol using homologous recombination and verified by PCR on genomic DNA prepared with a lithium acetate (200mM) / SDS (0.1%) method (104).

Yeast cells were grown in YPD medium (Yeast extract-Peptone-Dextrose 2%) or in synthetic complete medium (SC, containing 1.7g/L yeast nitrogen base (MP Biomedicals), 5g/L ammonium sulfate (Sigma-Aldrich), the appropriate drop-out amino acid preparations (MP Biomedicals) and 2% (w/vol) glucose unless otherwise indicated). Alternatively, SC medium could contain 0.5% lactate as a carbon source (from a 5% stock adjusted to pH=5; Sigma Aldrich). Pre-cultures of 4mL were incubated at 30°C for 8 hours and diluted in fresh medium on the evening to 20mL cultures grown overnight with inoculation optical densities (OD₆₀₀) of 0.0003 for YPD and 0.001 for SC medium, giving a culture at mid-log phase the next morning.

For glucose depletion experiments, cultures were centrifuged and resuspended in an equal volume of SC/lactate medium and incubated at 30°C during the indicated times. For 2DG, NaCl and tunicamycin treatments, the compounds were added to mid-log phase yeast cultures grown overnight to respective final concentrations of 0.2% (w/v), 400mM and 1µg/mL and incubated for the indicated times. 2-deoxyglucose and tunicamycin were purchased from Sigma-Aldrich. The mannose-supplemented medium (Fig. 2A) consisted of an SC medium that contained all the elements indicated above plus 2% (w/vol) mannose.

Plasmid construction

All the plasmids presented in this study are listed in **Table S3** and were directly constructed in yeast using plasmid homologous recombination (105). DNA inserts were amplified by PCR using 70-mer primers containing 50nt homology overhangs and Thermo Fisher Phusion High-fidelity DNA Polymerase and receiver plasmids were digested with restriction enzymes targeting the insertion region. Competent yeast cells rinsed with lithium acetate were incubated for 30 minutes at 30°C with 20µL of the PCR product and 1µL of the plasmid digestion product, followed by a heat shock at 42°C for 20 minutes and a recovery phase in rich medium (YPD) for 90 minutes at 30°C and plated on synthetic medium without uracil. The pDOG1/2-LacZ vectors were generated using the pJEN1-LacZ vector obtained from Bernard Guiard (106) and cloning the pDOG1 or pDOG2 promoters (1kb) at *Bg*III and *Eco*RI sites. The *DOG1*, *DOG2*, *HDHD1* (all four isoforms), *HDHD4*, *PSPH* and *yniC* overexpression

1 vectors were obtained by digesting a pRS426 vector (2 μ , *URA3*) containing the p*GPD* (glyceraldehyde-
2 3-phosphate dehydrogenase gene, *TDH3*) (107) promoter with *EcoRI* and *BamHI* enzymes. In parallel,
3 inserts were PCR-amplified using the following DNA templates: *DOG1* and *DOG2* from yeast genomic
4 DNA preparations (primers: oSL1166/1167 and oSL1141/1142), the *yniC* ORF from *Escherichia coli*
5 DH5 α cells (oSL1172/1173) and the human gene ORFs (Uniprot identifiers: HDHD1=Q08623-1/2/3/4,
6 HDHD4=Q8TBE9 and PSPH=P78330) from DNA sequences generated by gene synthesis after codon
7 optimization for yeast expression (Eurofins Genomics) (*HDHD1*: oSL1170/1171 for all isoforms except
8 isoform 3: oSL1170/1216; *HDHD4*: oSL1214/1215; *PSPH*: oSL1212/1213). These PCR products were
9 designed to include a 50-bp overlap with the digested plasmid to enable their cloning by homologous
10 recombination in yeast after co-transformation. The p*GPD*-*HDHD1*-DDAA vector was obtained by PCR
11 amplification (oSL1155/1171) of the *HDHD1* DNA sequence obtained by gene synthesis using a specific
12 5' primer carrying two D>A mutations and insertion of this insert into the pRS426 vector digested with
13 *EcoRI* and *BamHI* as described above. This construct was verified by sequencing. The DDAA mutant
14 was PCR amplified (oSL1297/oSL1298) and subcloned at *NdeI*/*BamHI* sites into pET15b-6His-*HDHD1*, a
15 kind gift of Dr. E. Van Schaftingen. The wild-type *CYC8* gene and the mutant allele present in mutant
16 #9 were obtained by PCR amplification on the corresponding genomic DNAs with primers
17 (oSL1369/1370) containing a 50-bp overlap with a pRS415 vector (*CEN*, *LEU2*). The resulting PCR
18 products were digested with *XbaI* and *BamHI*, and co-transformed for cloning by homologous
19 recombination in yeast as described above. The plasmids generated in yeast were rescued by
20 extraction (lithium acetate/SDS method (104)) and electroporation in bacteria, then amplified and
21 sequenced before being re-transformed in the appropriate strains.

22 **Mass spectrometry and proteomics analyses**

23 Samples used for the proteome-wide analysis of 2-deoxyglucose treatments were prepared from six
24 liquid cultures (WT strain, BY4741) growing overnight at 30°C in 100mL of rich medium with 2% glucose
25 to mid-log phase. On the next morning, 2-deoxyglucose was added to three of the cultures to a final
26 concentration of 0.2% in order to obtain triplicates treated with 2DG and triplicates without drug
27 treatment (negative control). After 2.5 hours of incubation at 30°C, the six cultures were centrifuged
28 at 4000*g* for 5 minutes at 4°C, resuspended in 500 μ L of 10% trichloroacetic acid (TCA, Sigma-Aldrich)
29 and lysed by shaking after addition of glass beads (0.4-0.6mm, Sartorius) for 10 minutes at 4°C. Cell
30 lysates were retrieved by piercing under the 1.5mL tubes and brief centrifugation. Precipitated
31 proteins were centrifuged at 16000*g* for 10 minutes at 4°C, supernatants were discarded and pellets
32 were rinsed 4 times in 1mL of 100% cold acetone.

33 Proteins were then digested overnight at 37°C in 20 μ L of 25 mM NH_4HCO_3 containing sequencing-
34 grade trypsin (12.5 μ g/mL; Promega). The resulting peptides were sequentially extracted with 70%
35 acetonitrile, 0.1% formic acid. Digested samples were acidified with 0.1% formic acid. All digests were

1 analyzed by an Orbitrap Fusion equipped with an EASY-Spray nanoelectrospray ion source and coupled
2 to an Easy nano-LC Proxeon 1000 system (all from Thermo Fisher Scientific, San Jose, CA).
3 Chromatographic separation of peptides was performed with the following parameters: Acclaim
4 PepMap100 C18 pre-column (2 cm, 75 μm i.d., 3 μm , 100 \AA), Pepmap-RSLC Proxeon C18 column (50
5 cm, 75 μm i.d., 2 μm , 100 \AA), 300 nl/min flow, using a gradient rising from 95 % solvent A (water, 0.1
6 % formic acid) to 40 % B (80 % acetonitrile, 0.1% formic acid) in 120 minutes, followed by a column
7 regeneration of 20 min, for a total run of 140 min. Peptides were analyzed in the orbitrap in full-ion
8 scan mode at a resolution of 120,000 (at m/z 200) and with a mass range of m/z 350-1550, and an AGC
9 target of 2×10^5 . Fragments were obtained by higher-energy C-trap dissociation (HCD) activation with
10 a collisional energy of 30 %, and a quadrupole isolation window of 1.6 Da. MS/MS data were acquired
11 in the linear ion trap in a data-dependent mode, in top-speed mode with a total cycle of 3 seconds,
12 with a dynamic exclusion of 50 seconds and an exclusion duration of 60 seconds. The maximum ion
13 accumulation times were set to 250 ms for MS acquisition and 30 ms for MS/MS acquisition in
14 parallelization mode.

15 Raw mass spectrometry data from the Thermo Fisher Orbitrap Fusion were analyzed using the
16 MaxQuant software (108) version 1.5.0.7, which includes the Andromeda peptide search engine (109).
17 Theoretical peptides were created using the *Saccharomyces cerevisiae* S288C proteome database
18 obtained from Uniprot. Identified spectra were matched to peptides with a main search peptide
19 tolerance of 6ppm. After filtering of contaminants and reverse identifications, the total amount of
20 yeast proteins identified among the six samples was equal to 3425. Protein quantifications were
21 performed using MaxLFQ (110) on proteins identified with a minimum amount of two peptides with a
22 False Discovery Rate threshold of 0.05. LFQ values were then analyzed using Perseus (version 1.5.0.15).
23 For the statistical analysis of yeast proteomes treated with 2-dexoglyucose compared to negative
24 control samples, each group of triplicates was gathered into a statistical group in order to perform a
25 Student's t-test. Results are presented in the form of Volcano-plots (111) and significantly up-regulated
26 and down-regulated candidates were determined by setting an FDR of 0.01 and an S_0 of 2.

27 **GO-term analyses of proteomics data**

28 The 79 significantly up-regulated candidates obtained in the proteomics analysis were used as input
29 for the FunSpec web interface (<http://funspec.med.utoronto.ca/>) with default settings and a p-value
30 cutoff of 0.01 in order to determine the Gene Ontology biological processes that are enriched in this
31 list of 79 genes compared to the total *Saccharomyces cerevisiae* genome annotation (number of total
32 categories=2062). The complete list of enriched GO biological processes with a p-value<0.01, as well
33 as the genes included in each category, are displayed in Fig. 1B.

34 **Protein extracts and immunoblotting**

1 Yeast cultures used for protein extracts were all grown in synthetic complete medium. For each protein
2 sample, 1.4mL of culture was incubated with 100 μ L of 100% TCA for 10 minutes on ice to precipitate
3 proteins, centrifuged at 16000g at 4°C for 10 minutes and broken for 10 minutes with glass beads, as
4 described for LC-MS/MS sample preparation. Lysates were transferred to another 1.5mL tube and
5 centrifuged 5 minutes at 16000g at 4°C, supernatants were discarded and protein pellets were
6 resuspended in 50 μ L*(OD₆₀₀ of the culture) of sample buffer (50 mM Tris-HCl pH6.8, 100 mM DTT, 2%
7 SDS, 0.1% bromophenol blue, 10% glycerol, complemented with 50mM Tris-Base pH8.8). Protein
8 samples were heated at 95°C for 5 minutes and 10 μ L were loaded on SDS-PAGE gels (4-20%Mini-
9 PROTEAN TGX Stain-Free, BioRad). After electrophoresis, gels were blotted on nitrocellulose
10 membranes for 60 minutes with a liquid transfer system (BioRad), membranes were blocked in 2%
11 milk for 20 minutes and incubated for at least two hours with the corresponding primary antibodies.
12 Primary and secondary antibodies used in this study as well as their dilutions are listed in **Table S4**.
13 Membranes were washed three times for 10 minutes in Tris-Borate-SDS-Tween20 0.5% buffer and
14 incubated for at least an hour with the corresponding secondary antibody (coupled with Horse Radish
15 Peroxidase). Luminescence signals were acquired with the LAS-4000 imaging system (Fujifilm). Rsp5
16 was used as a loading control; alternatively, total proteins were visualized in gels using a trihalo
17 compound incorporated in SDS-PAGE gels (stain-free TGX gels, 4–20%; Bio-Rad) after 1 min UV-
18 induced photoactivation and imaging using a Gel Doc EZ Imager (Bio-Rad).

19 β -galactosidase assays

20 β -Galactosidase assays were performed using 1mL of mid-log phase yeast cultures carrying the *p_{DOG1}-*
21 *LacZ* or *p_{DOG2}-LacZ* plasmids, grown overnight in SC medium without uracil with glucose 2% and
22 switched to the specified conditions. The OD (600 nm) of the culture was measured, and samples were
23 taken and centrifuged at 16000g at 4°C for 10 minutes. Cell pellets were snap frozen in liquid nitrogen
24 and resuspended in 800 μ L of Buffer Z (pH=7, 50mM NaH₂PO₄, 45mM Na₂HPO₄, 10mM MgSO₄, 10mM
25 KCl and 38mM β -mercaptoethanol). After addition of 160 μ L of 4mg/mL ONPG (ortho-nitrophenyl- β -D-
26 galactopyranoside, Sigma-Aldrich), samples were incubated at 37°C. Enzymatic reactions were
27 stopped in the linear phase (60min incubation for *p_{DOG2}-LacZ* and 120min incubation for the *p_{DOG1}-LacZ*
28 plasmid, as per initial tests) by addition of 400 μ L of Na₂CO₃, and cell debris were discarded by
29 centrifugation at 16000g. The absorbance of clarified samples was measured with a
30 spectrophotometer set at 420nm. β -Galactosidase activities (arbitrary units, AU) were calculated using
31 the formula $1000*[A_{420}/(A_{600} * t)]$, where A_{420} refers to the enzyme activity and A_{600} is the turbidity
32 of the culture, and t the incubation time. Each enzymatic assay was repeated independently at least
33 three times.

1 **Drop tests**

2 Yeast cells grown in liquid rich or synthetic complete medium for at least 6 hours at 30°C were adjusted
3 to an optical density (600nm) of 0.5. Serial 10-fold dilutions were prepared in 96-well plates and, using
4 a pin replicator, drops were spotted on plates containing rich or SC medium containing 2% (w/v) agar
5 and when indicated, 2DG (0.05%, or 0.2%, w/vol), sodium selenite (200µM) or tunicamycin (1µg/mL).
6 Mannose plates (Fig 2I and 3F) were prepared as regular SC plates containing 2% mannose (w/vol) in
7 addition to glucose. Plates were incubated at 30°C for 3 to 4 days before scanning. For the adhesion
8 test (Fig. 5D), the plates were scanned and the colonies were then washed with tap water under a
9 constant flow of water for 1 min as previously described (78). Excess water was removed before the
10 plates were scanned again.

11 **Isolation of spontaneous mutants and characterization.**

12 WT cells transformed with a *pDOG2-LacZ* plasmid (pSL410) were grown overnight in SC-Ura medium
13 and ca. 2×10^6 cells were spread on SC-Ura plates containing 0.2% 2DG and grown for 6 days at 30°C.
14 The clones obtained (24 clones) were restreaked on SC-Ura to isolate single clones. Resistance to 2DG
15 was confirmed by drop tests (**Fig. S6A**). β -galactosidase enzyme assays and total protein extracts were
16 performed on cultures grown to the exponential phase. For β -galactosidase assays, the results were
17 statistically tested using an unpaired t-test with equal variance, assuming a normal distribution of the
18 values.

19 Diploids were obtained by crossing each resistant mutant with a WT or *hxx2Δ* strain of the opposite
20 mating type (BY4742, *Mat α*) and selecting single diploid clones on selective medium (SC-Met-Lys).
21 Sequencing of the *REG1* and *HXX2* loci were done after PCR amplification on genomic DNA isolated
22 from the corresponding clones. For whole genome sequencing, genomic DNA of the WT, clone 9 and
23 clone 10 was purified using the Qiagen genomic DNA kit (Genomic-tip 20/G) using 30 OD equivalents
24 of material following the manufacturer's instructions after zymolyase treatment (Seikagaku). A PCR-
25 free library was generated from 10 µg of gDNA and sequenced at the Beijing Genomics Institute (Hong
26 Kong) on Illumina HiSeq 4000. The mutations in each clone were identified through comparative
27 analysis of the variants detected by mapping their reads to the reference genome (BY4741) (112, 113)
28 and those detected by mapping the WT reads to the reference. The differential variants were filtered
29 by quality (vcf QUAL>1000) and manually inspected through IGV for validation (114).

30 **Cell culture and transfection.**

31 HeLa cells were maintained at 37°C and 5% CO₂ in a humidified incubator and grown in Dulbecco's
32 modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum. Cells were regularly split
33 using Trypsin-EDTA to maintain exponential growth. HeLa cells were transfected with plasmid pCMV-

1 Sport6-HDHD1 and pCS2 (empty control) using Lipofectamine 2000 according to the manufacturer's
2 instructions. All culture media reagents were from Thermo Fisher Scientific. 2DG (Sigma) was used at
3 a final concentration of 5 mM and tunicamycin (from *Streptomyces sp*; Sigma) was used at a final
4 concentration of 5 µg/mL. For 2DG resistance assays, cells were grown in 10 cm² flask and split in a
5 24-well plate in the absence or presence of 5 mM 2DG. Cells were counted each day with a
6 hemocytometer after trypsinization and labeling with Trypan Blue. Total extracts were prepared by
7 incubating cells (10 cm²) on ice for 20 min with 400 µL TSE Triton buffer (50 mM Tris- HCl pH 8.0, NaCl
8 150 mM, 0.5 mM EDTA, 1% Triton X-100) containing protease inhibitors (cOmplete protease inhibitor
9 cocktail, EDTA-Free, Roche Diagnostics). Cells were then lysed mechanically with scrapers, and the
10 lysate was centrifuged at 13,000 g, 4°C for 30 min. Proteins were assayed in the supernatant using the
11 Bio-Rad Protein Assay reagent (Bio-Rad) and 40 µg proteins were loaded on SDS-PAGE gels.

12 **Recombinant His-tagged HDHD1 and HDHD1-DDAA protein purifications**

13 *E. coli* BL21 bacteria were transformed with plasmids allowing the expression of His-tagged HDHD1 or
14 HDHD1-DDAA . A 100mL-preculture was grown overnight in LB+Ampicillin (100 µg/mL), diluted 50-fold
15 into 1L culture. The OD reached 0.7-0.9. IPTG (1 mM) was then added to induce the recombinant
16 protein and cells were further grown at 25°C for 3 hours. Cells were harvested, the pellet was frozen
17 in liquid N₂ and thawed on ice. The pellet was resuspended in 20 mL of lysis buffer (HEPES 25mM pH
18 6.7, 300mM NaCl, imidazole 15 mM, β-mercaptoethanol 2mM, glycerol 10% v/v and protease inhibitor
19 cocktail [cOmplete protease inhibitor cocktail, EDTA-Free, Roche Diagnostics]). Cells were then
20 sonicated and Triton X-100 was added to a final concentration of 1%. The lysate was centrifuged at
21 12,000rpm in a SW-32 rotor (Beckman Coulter) for 15min at 4°C, and then the supernatant was further
22 centrifuged at 35 000 rpm for 1h at 4°C. The supernatant was incubated with 800 µL of Ni-NTA bead
23 slurry (Qiagen) and rotated overnight at 4°C. The beads were collected by centrifugation (1000g, 2min,
24 4°C), resuspended in lysis buffer, and washed with 50 mL of lysis buffer at 4°C, and then washed again
25 with 50 mL thrombin cleavage buffer (Hepes 50 mM, CaCl₂ 5 mM, NaCl 100mM, glycerol 10%) at 4°C.
26 The His-tag was removed by cleavage with 16 U of thrombin (Ref 27-0846-01, Sigma) added directly
27 onto the beads for 2h at 25°C. The eluate was then collected and incubated with 500 µL benzamide-
28 sepharose 6B (GE Healthcare) at room temperature for 30 min to remove thrombin. The supernatant
29 was collected and protein content was assayed by SDS-PAGE and colloidal blue staining (Brilliant Blue
30 G-colloidal, Sigma), and protein concentration was assayed by the Bradford method (Bio-Rad protein
31 assay, Bio-Rad).

32 **Enzyme assays**

33 2DG6P phosphatase assays were performed in 250 µL of reaction containing 1.5mM 2DG6P (#17149,
34 Cayman Chemicals, Ann Arbor, Michigan, USA) in 50 mM HEPES pH 6.7, 10 mM MgCl₂ and 10% glycerol

1 and 30 μg recombinant HDHD1 or HDHD1-DDAA. Samples were incubated at 37°C for various times (0,
2 5, 10, 15min) and the reaction was stopped by adding 150 μL EDTA (0.5M). Then, the 2DG generated
3 was assayed by adding 500 μL of glucose assay reagent (GAGO20, Sigma) and further incubating at 37°C
4 for 30min. The reaction was stopped by adding 500 μL H_2SO_4 (12N) and the absorbance of the reaction
5 was measured at 540 nm. A slope (A540 over time) was calculated to assess enzyme activity and to
6 make sure that the reaction was in the linear range. The measurements were repeated 3 times.

7 **Statistical analysis**

8 Mean values calculated using a minimum of three independent measurements from three biological
9 replicates and are plotted with error bars representing standard error of the mean (SEM). Statistical
10 significance was determined using a t-test for paired variables assuming a normal distribution of the
11 values, as follows: *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; ns: $P > 0.05$.

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Supplementary Materials

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- 3 **Fig. S1. Characterization of other candidates showing increased abundance after 2DG treatment.**
- 4 **Fig. S2. Hog1 signaling responds to 2DG but *DOG1* expression is not regulated by Hog1.**
- 5 **Fig. S3. *DOG1* expression is regulated by the UPR pathway**
- 6 **Fig. S4. Slt2 participates in the regulation of *DOG1* expression**
- 7 **Fig. S5. *Cis* regions involved in the regulation of the *DOG2* promoter by glucose**
- 8 **Fig. S6. Identification of *reg1* and *hvk2* mutants within the isolated spontaneous 2DG-resistant**
- 9 **mutants**
- 10 **Fig. S7. Multiple protein sequence alignment of Hvk2 orthologs and positions of the mutations**
- 11 **identified.**
- 12 **Fig. S8. HDHD1 but not its close homologues HDHD4 or PSPH allow resistance to 2DG**
- 13
- 14 **Table S1. Single nucleotide variants in clones #9 and #10 as compared to the WT strain, as**
- 15 **identified by whole genome resequencing.**
- 16 **Table S2. Yeast strains used in this study.**
- 17 **Table S3. Plasmids used in this study.**
- 18 **Table S4. Antibodies used in this study.**
- 19
- 20 **Data File S1. Proteomic response to 2DG treatment.**
- 21

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Figure Legends

Figure 1. Proteomics analysis of the response to 2DG in yeast reveals transcriptional induction of the 2DG6P phosphatases Dog1 and Dog2.

(A) Volcano-plot representing changes in protein abundance in total protein extracts of wild-type (WT) yeast in response to 2DG (0.2%), obtained by mass spectrometry-based proteomics and analyzed with MaxQuant software. The x axis corresponds to the \log_2 value of the abundance ratio (LFQ: Label-Free Quantification) between 2DG treatment and the negative control. The y axis represents the $-\log_{10}$ of the p -value of the statistical t-test for each quantified protein ($n=3$ independent biological replicates). Lines: threshold with a False Discovery Rate of 0.01. (B) Gene Ontology (GO) analysis of the proteins identified as upregulated in response to 2DG treatment along with their p -value and the proteins included in each category. (C) Western blot on total protein extracts of yeast cells expressing endogenously tagged Dog1-GFP or Dog2-GFP, before and after 2DG addition for the indicated times, using an anti-GFP antibody. A longer exposure is displayed for Dog1-GFP cells to highlight the higher abundance of Dog1 after 2DG addition. Rsp5, whose levels did not change upon 2DG addition in all of our experiments, is used as a loading control. ($n=2$ independent experiments.) (D) β -galactosidase assays of wild-type yeast cells expressing *LacZ* under the control of the *pDOG1* or *pDOG2* promoters, before and after 2DG treatments for 3 hours (\pm SEM, $n=3$ independent experiments, t-test). (E) Serial dilutions of cultures from the indicated strains were spotted onto SD plates containing no DG or 0.05% 2DG and grown for 3 days at 30°C. ($n=2$ independent experiments.) (F) β -galactosidase assays of wild-type and *hog1 Δ* strains expressing *LacZ* under the control of the *pDOG2* promoter, before and after 2DG treatments for 3 hours (\pm SEM, $n=3$ independent experiments, t-test). (G) Western blot on total protein extracts of WT and *hog1 Δ* cells endogenously expressing a Dog2-GFP fusion, before and after 2DG addition for 3h, using an anti-GFP antibody. Total protein was visualized in gels using a trihalo compound. Glc, glucose. (H) Relative expression of Dog2-GFP in the same conditions as (G) after normalization to total protein and using WT/untreated as a reference (\pm SEM, $n=3$ independent experiments, t-test).

Figure 2. 2DG treatment induces Dog2 expression through glycosylation defects that trigger ER stress and the Unfolded Protein Response.

(A) WT cells were grown overnight to mid-log phase in SC medium, centrifuged and resuspended in SC-medium containing mannose (2%) or not, and treated with 0.2% 2DG or 1 μ g/mL tunicamycin (Tm) for 4 hours. Total protein extracts were Western blotted for Carboxypeptidase Y (CPY). ($n=3$ independent experiments.) Glc, glucose. Glycosyl., glycosylation. (B) Schematic of the UPR signaling

pathway in yeast showing how ER stress triggers Ire1-mediated splicing of the pre-mRNA encoding the transcription factor Hac1 and the subsequent induction of UPR target genes. (C) β -galactosidase assays on WT and *hac1* Δ cells expressing *LacZ* under the control of an UPR-inducible promoter (*pUPRE1*) and treated with 0.2% 2DG or 1 μ g/mL tunicamycin for 3 hours (\pm SEM, $n=3$ independent experiments, t-test). (D) β -galactosidase assays on WT cells expressing *LacZ* under the control of the *DOG2* promoter and treated as in (C) (\pm SEM, $n=3$ independent experiments, t-test). (E) β -galactosidase assays on WT and *hac1* Δ cells expressing *LacZ* under the control the *DOG2* promoter, before and after 3h 2DG treatments (\pm SEM, $n=3$ independent experiments, t-test). (F) Western blot for GFP on total protein extracts of WT and *hac1* Δ cells endogenously expressing a Dog2-GFP fusion, before and after 3h treatment with 2DG or tunicamycin. (G) Relative expression of Dog2-GFP under the same conditions as (F) after normalization to total protein and using WT/untreated as a reference (\pm SEM, $n=3$ independent experiments, t-test). (H) Serial dilutions of cultures from the indicated strains were spotted onto SC plates (supplemented with 2% mannose when indicated) containing no DG or 0.05% 2DG, and were grown for 3 days at 30°C. ($n=2$ independent experiments.) (I) Serial dilutions of cultures from the indicated strains overexpressing *DOG2* (*pGPD-DOG2*) or not (\emptyset) were spotted onto SC-Ura plates containing 0, 0.05% or 0.2% 2DG. The plates were scanned after 3 days of incubation at 30°C. ($n=2$ independent experiments.)

Figure 3. 2DG activates the MAPK-based CWI pathway, which is required for 2DG tolerance and additionally contributes to the regulation of Dog2 expression.

(A) Schematic of the CWI pathway showing the various components and their requirement for growth on 2DG (see color code in the inset) based on drop tests shown in (B). (B) Serial dilutions of cultures from the indicated deletion strains were spotted onto SD plates containing no DG or 0.05% 2DG and grown for 3 days at 30°C. ($n=2$ independent experiments.) (C) β -galactosidase assays on WT and *slt2* Δ cells expressing *LacZ* under the control the *DOG2* promoter, before and after 3h 2DG treatments (\pm SEM, $n=3$ independent experiments, t-test). (D) Western blot on total protein extracts of WT and *slt2* Δ cells expressing an endogenously tagged Dog2-GFP fusion, before and after 3h treatment with 2DG, using an anti-GFP antibody. (E) Relative expression of Dog2-GFP in the same conditions as (D) after normalization to total protein and using WT/untreated as a reference (\pm SEM, $n=3$ independent experiments, t-test). (F) Serial dilutions of cultures from the indicated strains were spotted onto SC plates (supplemented with 2% mannose when indicated) containing no DG or 0.05% 2DG and were grown for 3 days at 30°C. ($n=2$ independent experiments.)

Figure 4. *DOG2*, but not *DOG1*, is negatively controlled by glucose availability through transcriptional repression by Mig1-Mig2 and the kinase Snf1.

(A) Schematic of the glucose repression pathway showing how Snf1 (the yeast homolog of AMPK), PP1 (composed of Glc7 and Reg1 subunits) and their downstream transcriptional repressors Mig1/Mig2 regulate glucose-repressed genes in response to glucose availability or absence (such in the presence of lactate). (B) WT and *snf1Δ* strains, both expressing endogenously tagged Dog1-TAP and Dog2-GFP fusions, were grown overnight in SC medium and then either treated with 0.2% 2DG or switched to an SC-lactate medium for 4 hours. Dog1-TAP was detected with the peroxidase-anti-peroxidase (PAP) complex and Dog2-GFP with anti-GFP antibodies, phosphorylated (p) Snf1 with anti-phospho-AMPK and total Snf1 with an anti-polyHis tag (because Snf1 contains a stretch of 13 histidine residues that can be used for its detection) (n=2 independent experiments.) (C) β -galactosidase activity on WT and *snf1Δ* cells expressing *LacZ* under the control the *DOG1* or the *DOG2* promoter, before and after 3h growth in lactate. The fold-induction after transfer to lactate is indicated for each promoter in each strain (\pm SEM, n=4 independent experiments). (D) WT and *snf1Δ* strains, transformed with either a genomic clone containing both *DOG1* and *DOG2* under the control of its own promoter (*pend:DOG*), or with a vector containing *DOG2* under the control of the strong *GPD* promoter (*pGPD:DOG2*) were grown, serial-diluted and spotted onto SC plates (SC-Leu or SC-Ura) with or without 0.2% DG, and grown for 3 days at 30°C. (n=2 independent experiments.) (E) β -galactosidase assays on WT and the indicated deletion mutants expressing *LacZ* under the control the *DOG2* promoter after overnight growth in SC medium (exponential phase) (\pm SEM, n=6 independent experiments). (F) The indicated strains, all expressing an endogenously tagged Dog2-GFP fusion, were grown overnight in SC medium (to exponential phase). Total protein extracts were immunoblotted with anti-GFP antibodies. Rsp5 was used as a loading control. (n=2 independent experiments.) (G) Serial dilutions of cultures of the indicated mutants were spotted on YPD plates containing 0, 0.2% or 0.5% of 2DG. Plates were scanned after 3 days of growth at 30°C. (n=3 independent experiments.)

Figure 5. The characterization of spontaneous 2DG-resistant strains identifies mutants showing increased Dog2 expression, including a new mutant allele of *CYC8*.

(A) 24 clones showing a spontaneous resistance to 0.2% 2DG were isolated. The β -galactosidase activity of these mutants, due to the expression of the *LacZ* reporter driven by the *DOG2* promoter, was measured after overnight growth in SC medium (to exponential phase) (\pm SEM, n=4 independent experiments, t-test). Colors represent the identity of the mutants as determined in [fig. S6 A-B](#) for *reg1* and [fig. S6 C-E](#) for *hxx2* and [B-G](#) for *cyc8*. (B) Schematic of the domain organization of the Cyc8 protein, showing the Poly(Q) and Poly(QA) repeats as well as the N-terminal TPR repeats. Red: mutation identified by whole genome resequencing of the spontaneous 2DG-resistant mutants #9 and #10. (C)

A WT strain, the mutant strains #9 and #10 and the *reg1Δ* mutant (used as a positive control) were grown in SC medium (to exponential phase). Total protein extracts were immunoblotted for invertase (invertase is heavily glycosylated and migrates as a smear (116)). (n=2 independent experiments.) (D) WT and mutants #9 and #10 were transformed with low-copy (centromeric) plasmid either empty or containing WT *CYC8* or mutant *cyc8* (Gln^{320*}), and were spotted on SC-Leu or SC-Leu + 0.2% 2DG medium, and grown for 3 days at 30°C. Middle panel: the control plate was scanned and then washed for 1 min under a constant flow of water, and then scanned again. (n=2 independent experiments.) (E) β-galactosidase activity on WT and mutants #9 and #10 expressing *LacZ* under the control the *DOG2* promoter and transformed with an empty vector or a low-copy vector containing WT *CYC8*, after growth in SC medium (normalized to the value of the WT, ± SEM, n=3 independent experiments, t-test). (F) Western blot on total protein extracts of WT and mutants #9 and #10 cells expressing an endogenously tagged Dog2-GFP fusion and transformed with either an empty plasmid or a low-copy (centromeric) plasmid containing WT *CYC8* after growth in SC medium, using an anti-GFP antibody. (G) Relative expression of Dog2-GFP in the same conditions as (F) after normalization by total proteins and using the WT control as a reference (± SEM, n=3 independent experiments, t-test). (H) Serial dilutions of cultures of the indicated mutants were spotted on SC medium or SC + 0.2% 2DG medium and grown for 3 days at 30°C (n=2 independent experiments). mut, mutant.

Figure 6. The human phosphatase HDHD1 has a 2DG6P phosphatase activity and its overexpression leads to 2DG resistance in HeLa cells.

(A) Multiple protein sequence alignment of yeast Dog1, Dog2, *Escherichia coli* yniC and the human proteins HDHD1, HDHD4 and PSPH aligned with ClustalX 2.0. The highly conserved catalytic aspartates are displayed in yellow. The six first amino-acids of PSPH were truncated to optimize the N-terminal alignment of its catalytic aspartates with the other phosphatases. (B) Serial dilutions of WT and *dog1Δdog2Δ* strains transformed with the indicated plasmids were spotted on SC-Ura medium with or without 0.05% 2DG and were scanned after 3 days of growth at 30°C. (n=2 independent experiments.) (C) Serial dilutions of *dog1Δdog2Δ* strains transformed with an empty vector or vectors allowing the expression of a HDHD1 or its predicted catalytic mutant, HDHD1-DD>AA (in which the N-terminal catalytic aspartates were mutated to alanines) were spotted on SC-Ura medium with or without 0.05% 2DG and were scanned after 3 days of growth at 30°C. (n=2 independent experiments.) (D) Recombinant, His-tagged HDHD1 and HDHD1-DD>AA were expressed in bacteria and purified for in vitro enzymatic tests. 0.7μg were loaded on a gel to show homogeneity of the protein purification. (E) In vitro 2DG6P phosphatase activity of HDHD1 and HDHD1>DDAA as measured by assaying glucose release from 2DG6P. (n=3 independent experiments.) (F) Growth of HeLa cells transfected with an empty vector (□) or with a construct allowing the overexpression of HDHD1 (○) over time in the

absence (open symbols) or presence (filled symbol) of 5mM 2DG. The number of cells is normalized to that of the untransformed/untreated cells after 3 days (\pm SEM, $n=3$ independent experiments, t-test).

Figure 7. Working Model.

Glucose phosphorylation triggers the onset of the glucose-repression pathway in which PP1 inactivates Snf1. This leads to the lack of phosphorylation of Mig1 and Mig2, which remain in the nucleus to mediate the glucose-repression of genes such as *DOG2*. The deletion of *REG1*, *HXK2*, or *MIG1* and *MIG2* or a mutation in *CYC8* leads to 2DG resistance, which is at least partially mediated through increased expression of *DOG2*, leading to the dephosphorylation of 2DG-6-P. In contrast, the deletion of *SNF1* causes an increased sensitivity to 2DG, which can be rescued by the deletion of *MIG1* and *MIG2* or by Dog2 overexpression. In parallel, 2DG6P causes (i) ER stress and triggers the UPR pathway, which stimulates *DOG2* expression through the transcription factor Hac1, and (ii) the CWI pathway, likely through interference with polysaccharide and cell wall synthesis, which also induces *DOG2* through the transcription factor Rlm1.

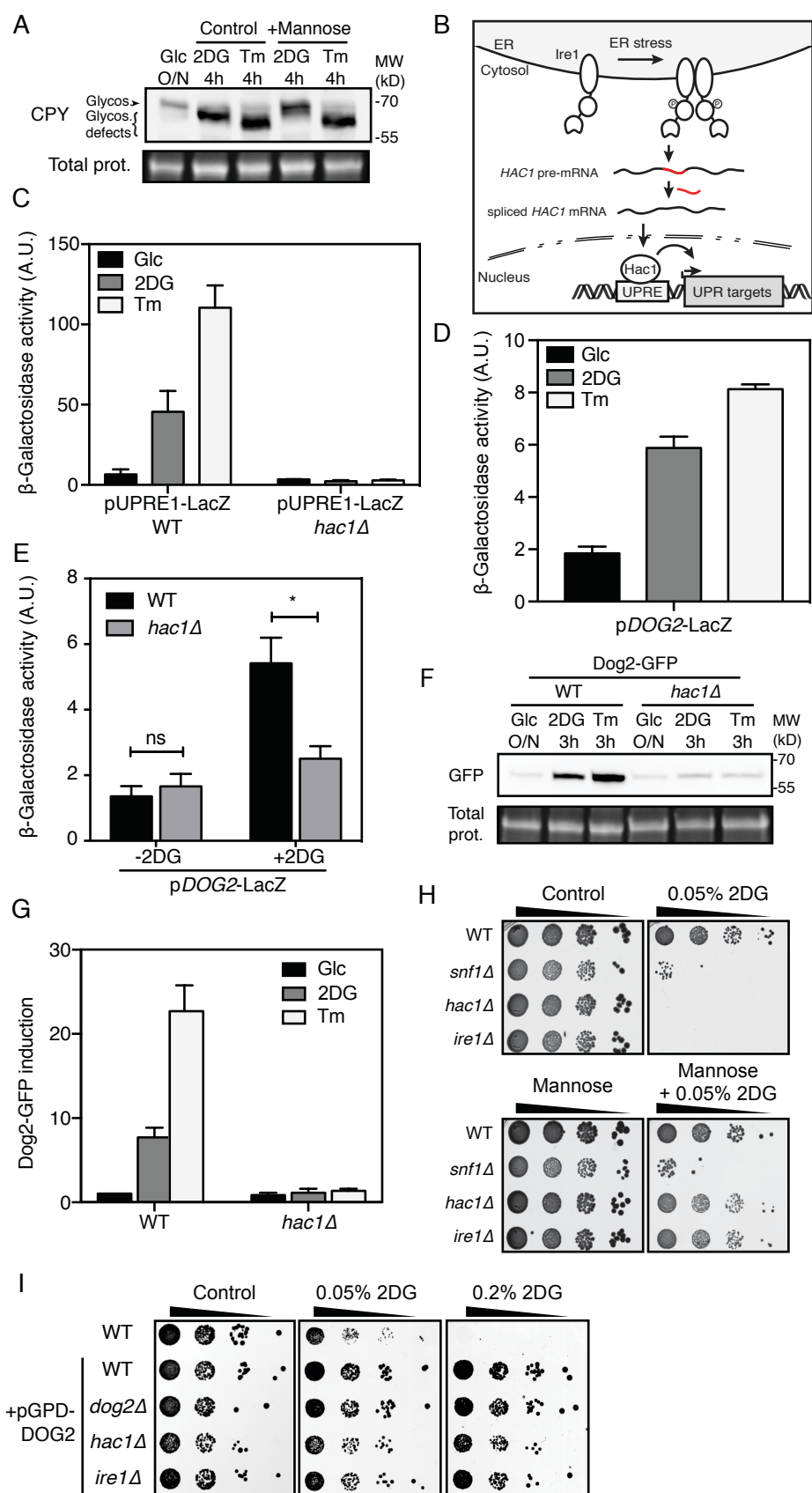


Figure 2; Defenouillère, Verraes et al.

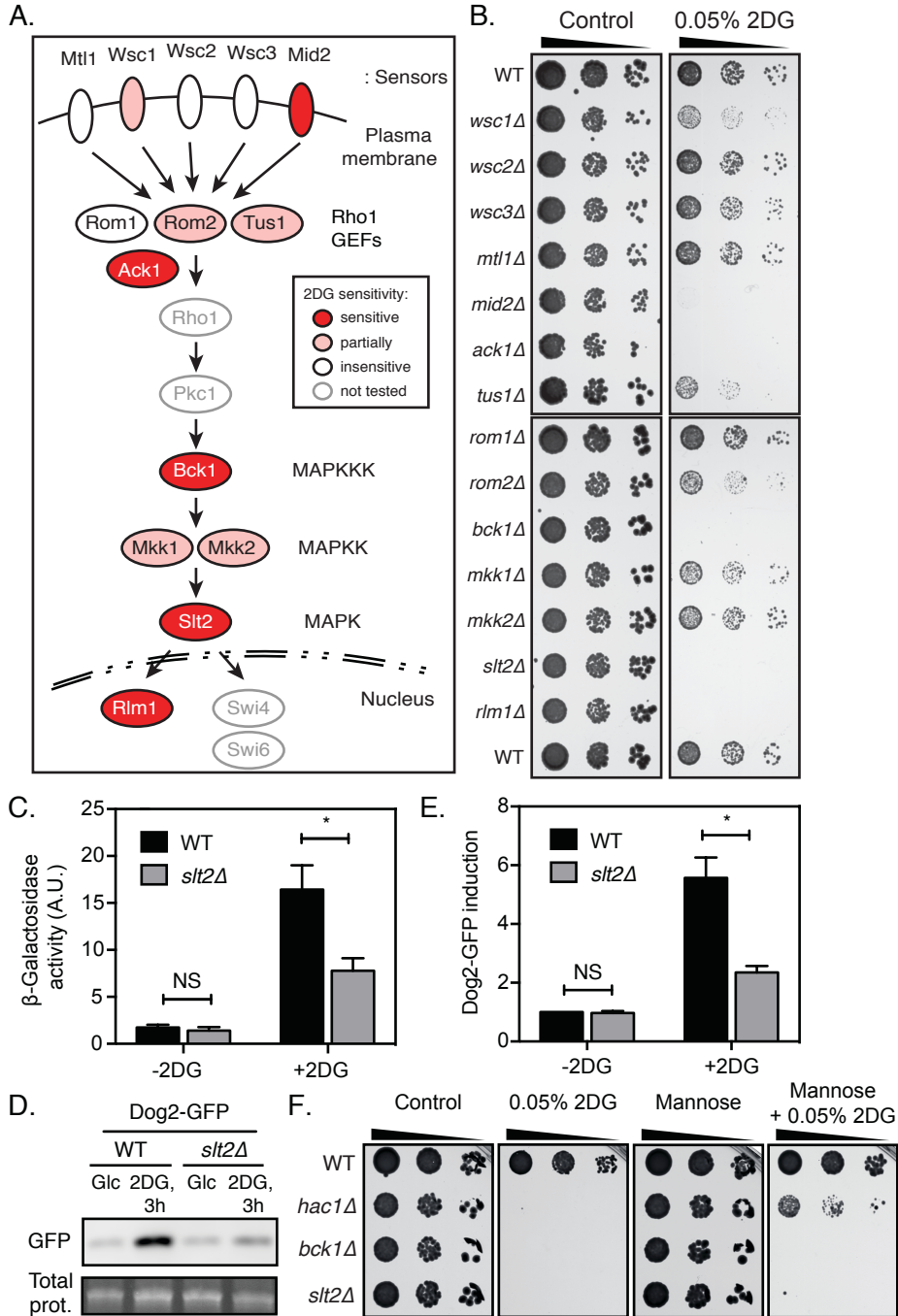


Figure 3; Defenouillère, Verraes et al.

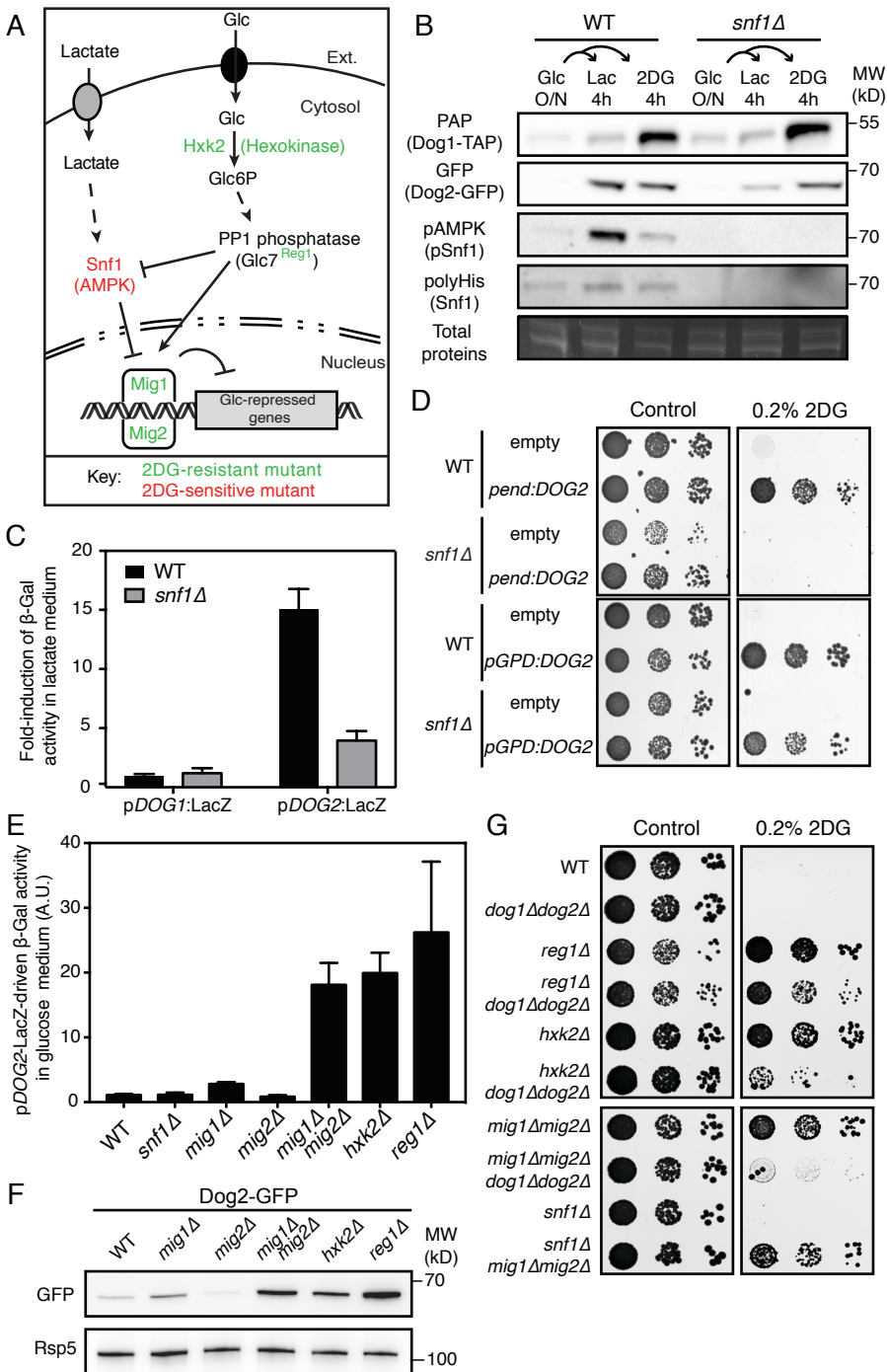


Figure 4; Defenouillère, Verraes et al.

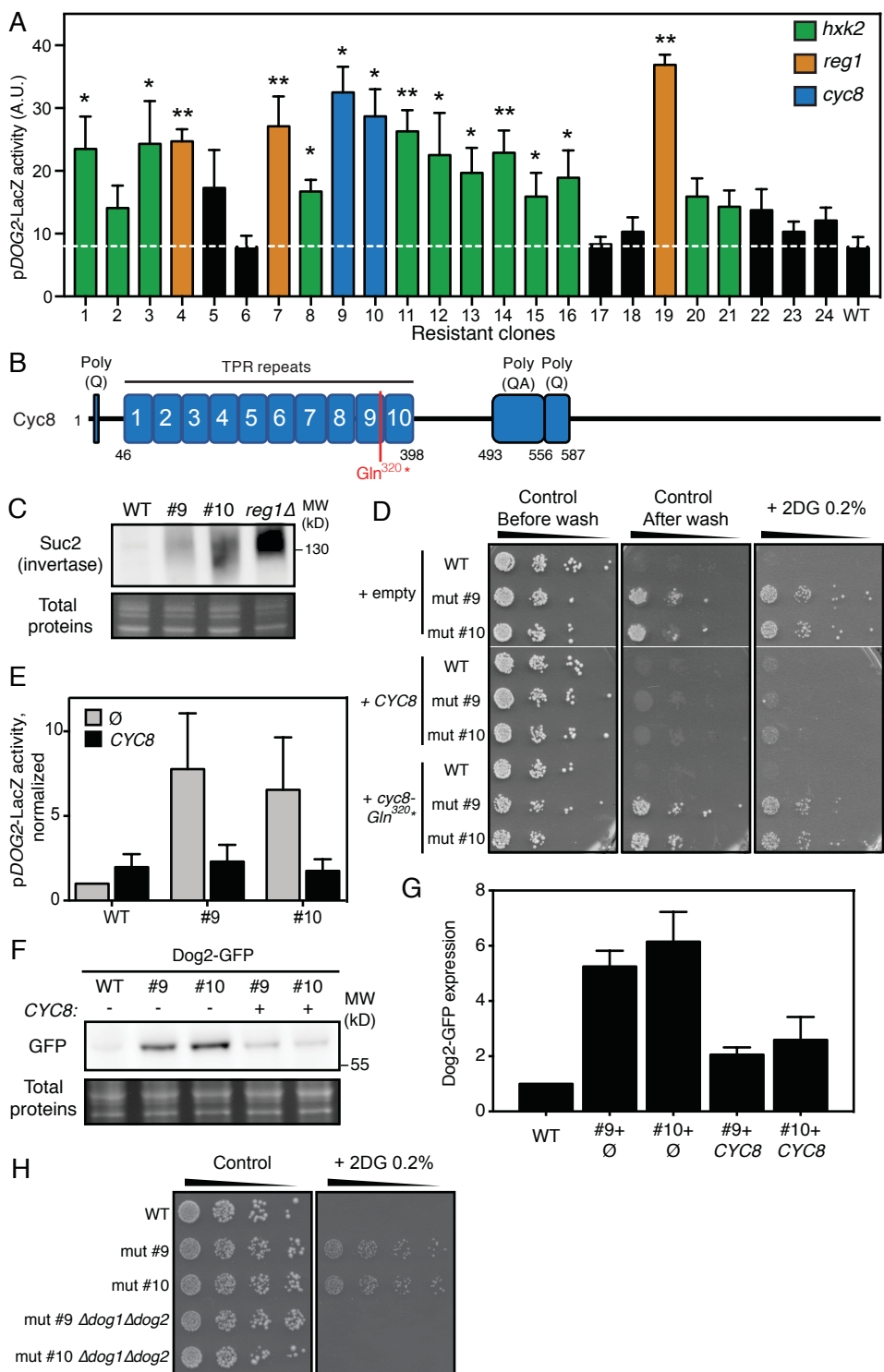


Figure 5; Defenouillère, Verraes et al

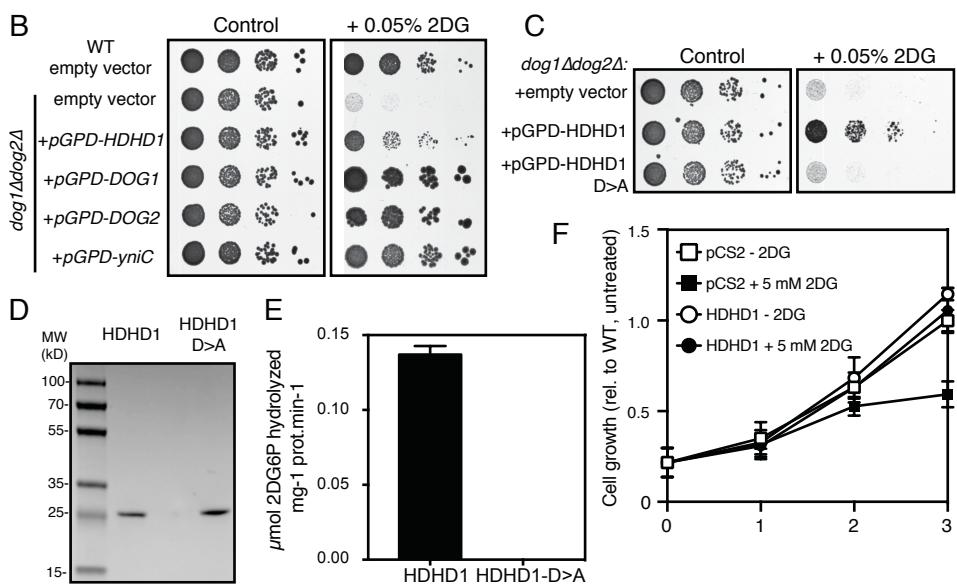
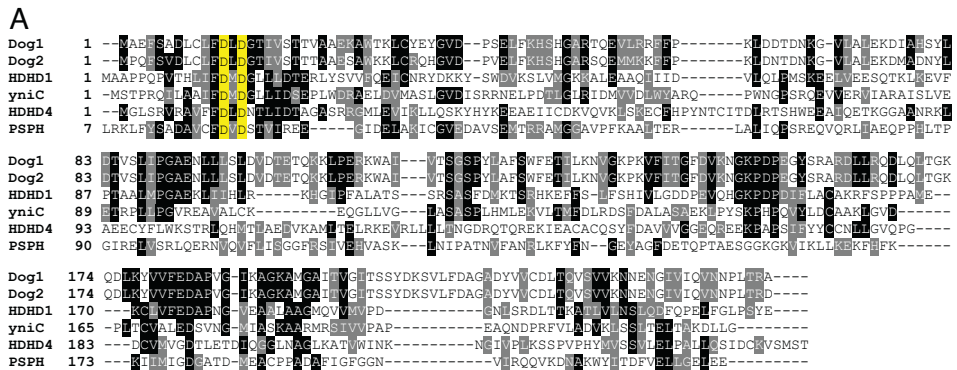


Figure 6; Defenouillère, Verraes et al.

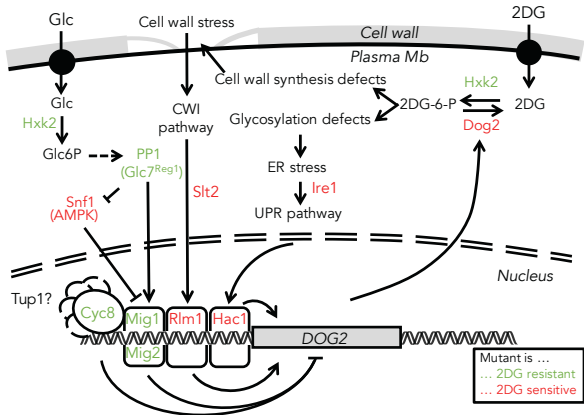


Figure 7; Defenouillère, Verraes et al.

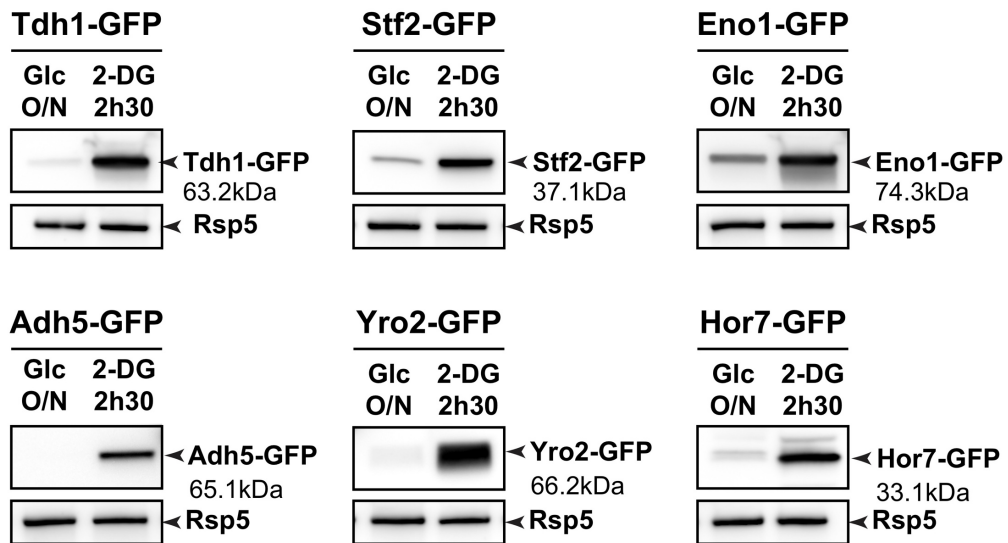


Figure S1. Characterization of other candidates showing increased abundance after 2DG treatment.

Western blot for GFP on total protein extracts of yeast cells expressing the indicated endogenously GFP-tagged proteins, before and after 2.5h 2DG addition. Rsp5 is used as a loading control. (n=3 independent experiments.)

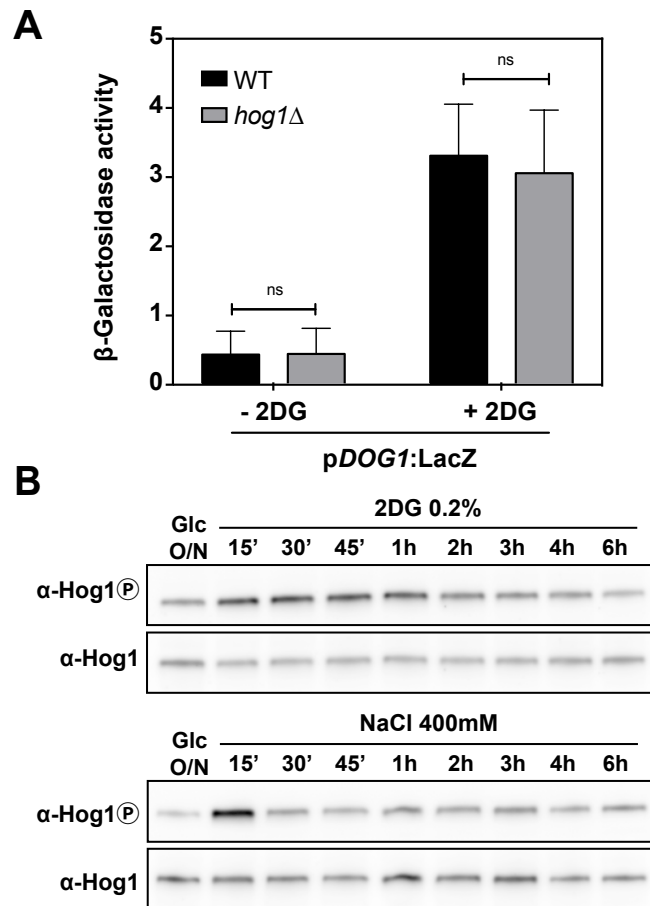


Figure S2. Hog1 signaling responds to 2DG but *DOG1* expression is not regulated by Hog1.

(A). β -galactosidase assays of wild-type and *hog1* Δ strains expressing *LacZ* under the control of the *pDOG1* promoter, before and after 2DG treatments for 3 hours. Error bars: SEM ($n=3$ independent experiments, t-test). (B). Western blot on total extracts of wild-type yeast cells grown overnight in SD medium and treated with 0.2% 2DG or 400mM NaCl for the indicated times. The anti-phospho-p38/Hog1 antibody enables the detection of phosphorylated Hog1 and Hog1 is used as a control for Hog1 abundance throughout the time course. ($n=2$ independent experiments.)

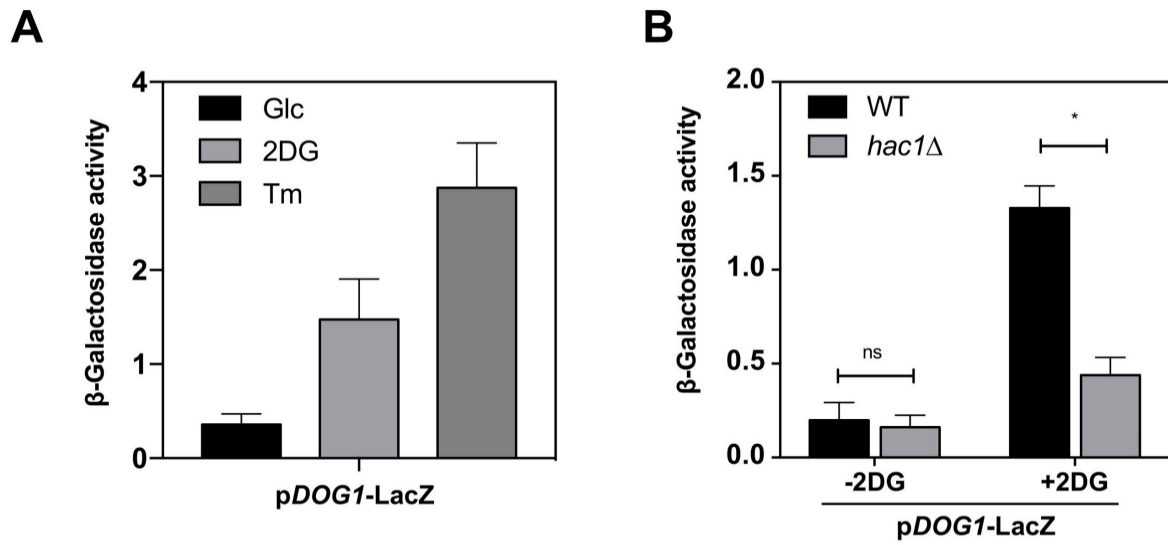


Figure S3. *DOG1* expression is regulated by the UPR pathway.

(A). β -galactosidase assays on WT cells expressing *LacZ* under the control of the *DOG1* promoter and treated with 0.2% 2DG or 1 μ g/mL tunicamycin for 3 hours (\pm SEM, $n=3$ independent experiments, t-test). (B). β -galactosidase assays on WT and *hac1* Δ cells expressing *LacZ* under the control of the *DOG1* promoter, before and after 3h 2DG treatments (\pm SEM, $n=4$ independent experiments, *: $p<0.05$).

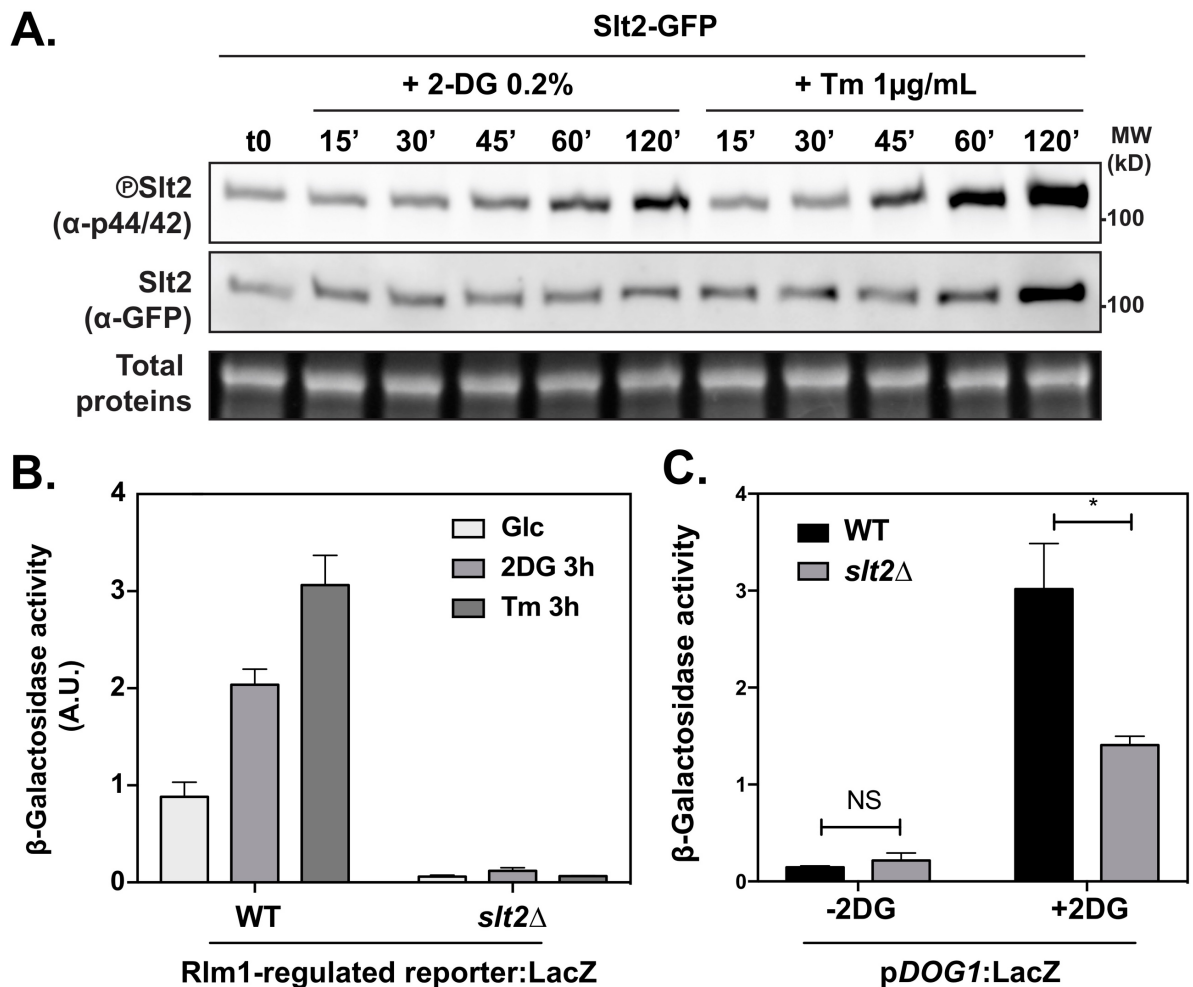


Figure S4. Slt2 participates in the regulation of *DOG1* expression.

(A) A WT strain expressing an endogenously-tagged Slt2-GFP construct was grown in SC medium and treated with 2DG (0.2%) or tunicamycin (1 µg/mL). Total protein extracts were prepared at the indicated times and blotted with the following antibodies: anti-p44/42, to reveal activated (phosphorylated) Slt2, and anti-GFP to reveal total levels of Slt2. (n=2 independent experiments.) (B) β-galactosidase assays on WT and *slt2Δ* cells expressing *LacZ* under the control of an Rlm1-regulated promoter, before and after 3h 2DG (0.2%) or tunicamycin (1 µg/mL) treatments (± SEM, n=3 independent experiments, t-test). (C) β-galactosidase assays on WT and *slt2Δ* cells expressing *LacZ* under the control the *DOG1* promoter, before and after 3h 2DG treatments (± SEM, n=3 independent experiments, t-test).

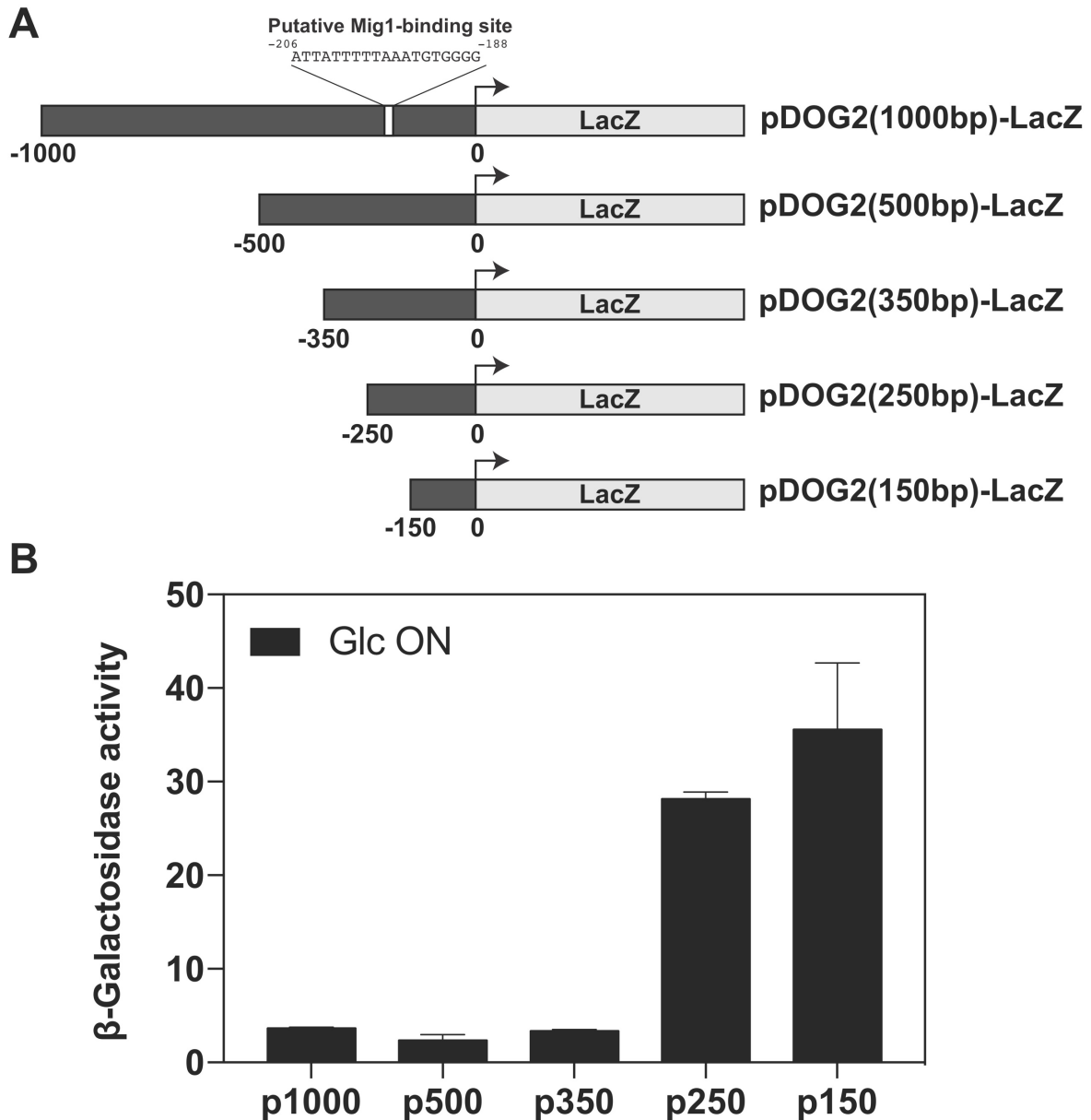


Figure S5. Cis regions involved in the regulation of the *DOG2* promoter by glucose

(A) Schematic showing the various constructs generated to study *DOG2* promoter regulation by glucose availability. (B) β -galactosidase assays on WT cells expressing *LacZ* under the control of the indicated truncated version of the *DOG2* promoter after overnight growth in glucose medium (\pm SEM, $n=3$ independent experiments, t-test).

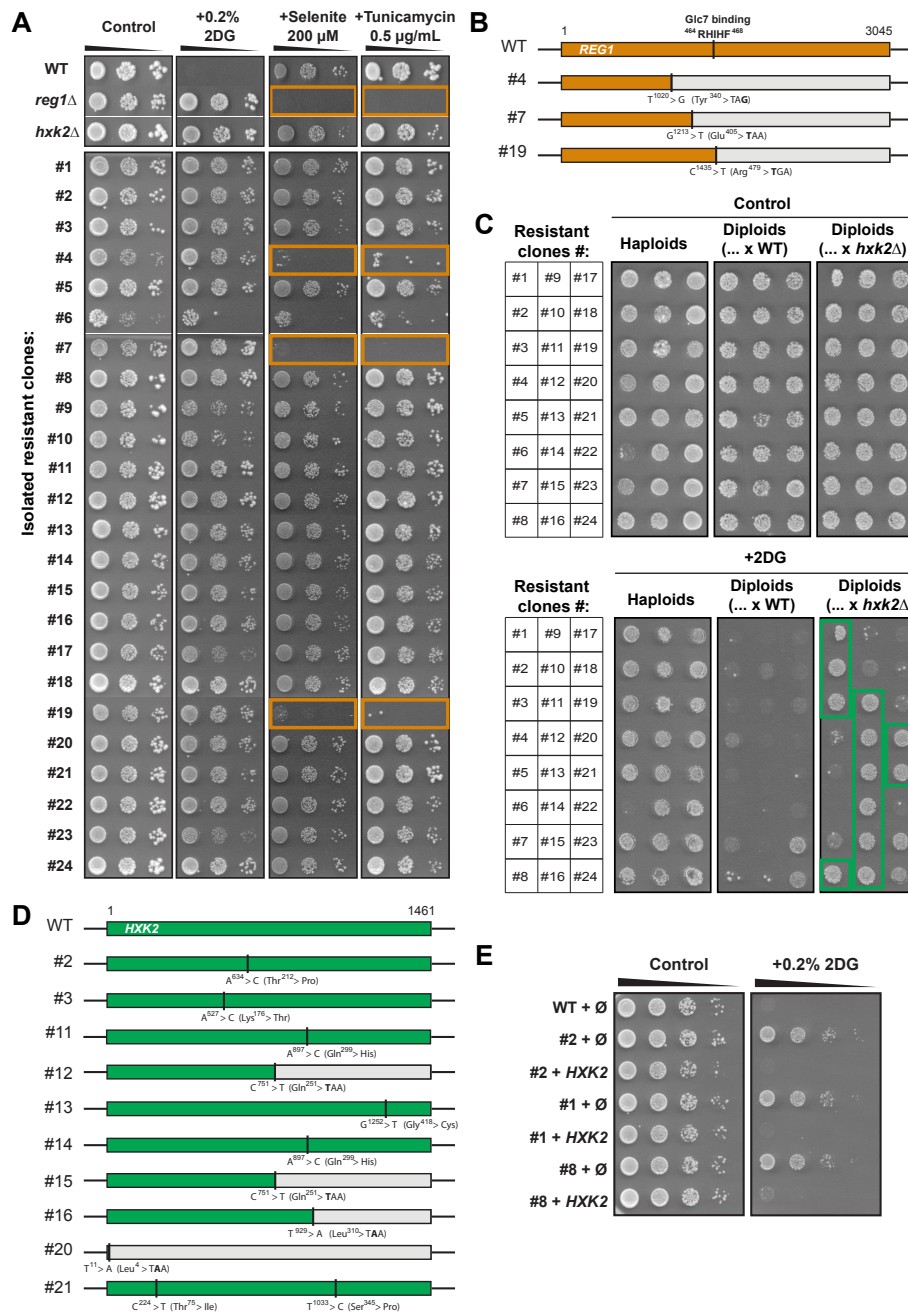


Figure S6. Identification of *reg1* and *hvk2* mutants within the isolated, spontaneous 2DG-resistant mutants

(A) Serial dilutions of cultures from the indicated deletion strains and resistant clones were spotted onto SC plates containing no DG or 0.2% 2DG, as well as 200 μ M selenite or 0.5 μ g/mL tunicamycin and grown for 3 days at 30°C. Note that clone #6 grows very slowly even in absence of 2DG. Orange squares: identified *reg1* mutants. (B) Schematic of the identified mutations within the *REG1* ORF in the indicated mutants as obtained by sequencing. (C) The 24 resistant clones were crossed with a WT or an *hvk2* Δ strain of the opposite mating type, and cultures of the haploid resistant clones (see matrix, left) or the resulting diploids were spotted onto SC medium with or without 0.2% DG. Green squares: identified *hvk2* mutants. (n=2 independent experiments.) (D) Schematic of the identified mutations within the *HXK2* ORF in the indicated mutants as obtained by sequencing. (E) Growth test showing that the 2DG sensitivity of the 2DG-resistant clones #1 and #8 can be restored by expression of a multicopy, genomic clone containing *HXK2*. Clone #2 (which displays a mutation in *HXK2*, see D-E) is used as a positive control. (n=2 independent experiments.)

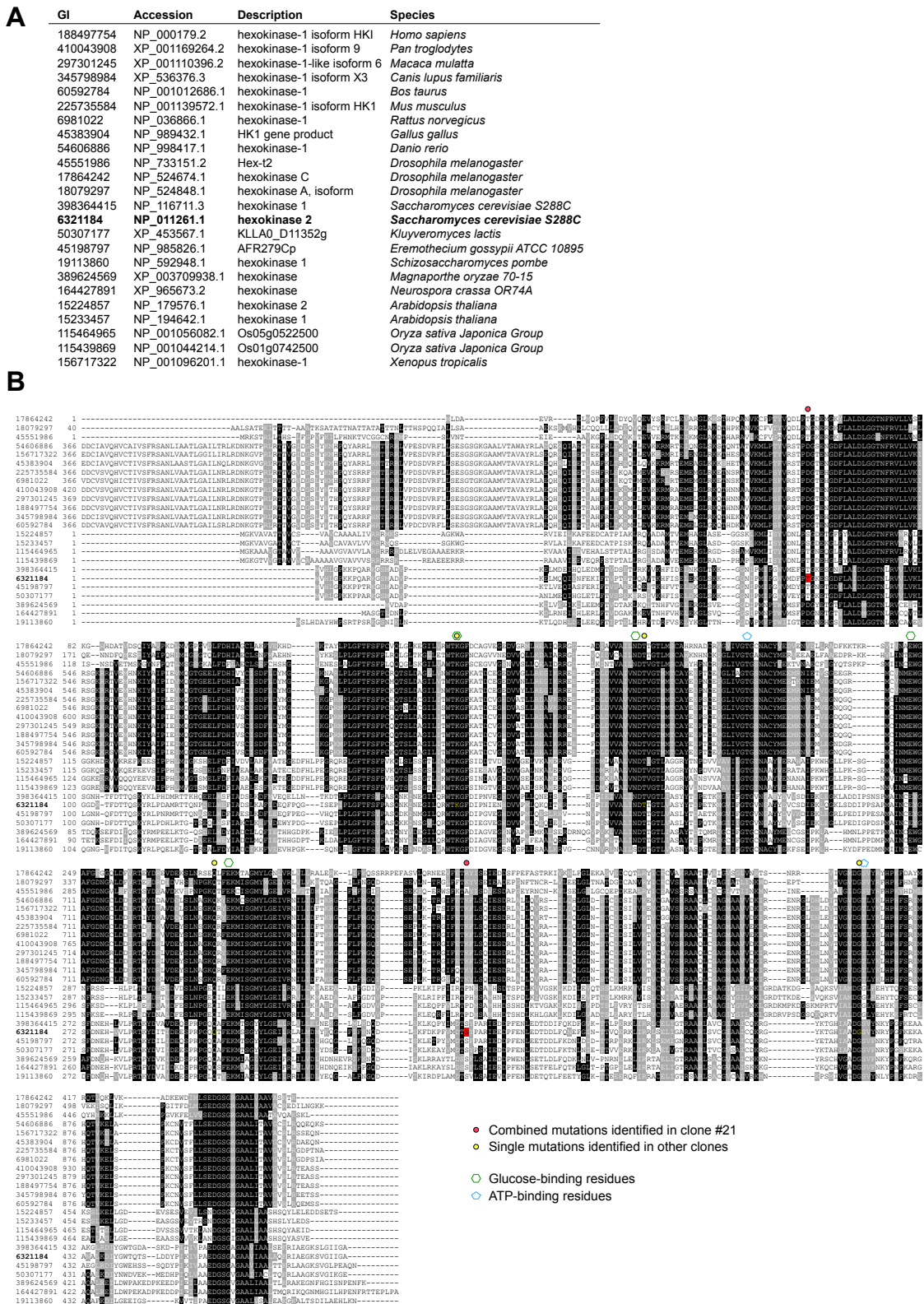


Figure S7. Multiple protein sequence alignment of Hxk2 orthologs and positions of the mutations identified.

(A) List of Hxk2 orthologues used for the alignment, as identified using HomoloGene entry #100530 (NCBI). (B) The sequences (numbers refer to their GI numbers) were aligned using ClustalX 2.1 (17) and formatted using BoxShade server (v.3.21). After the alignment, some sequences were truncated in the N-terminus as indicated. The sequence in bold corresponds to that of *S. cerevisiae* Hxk2.

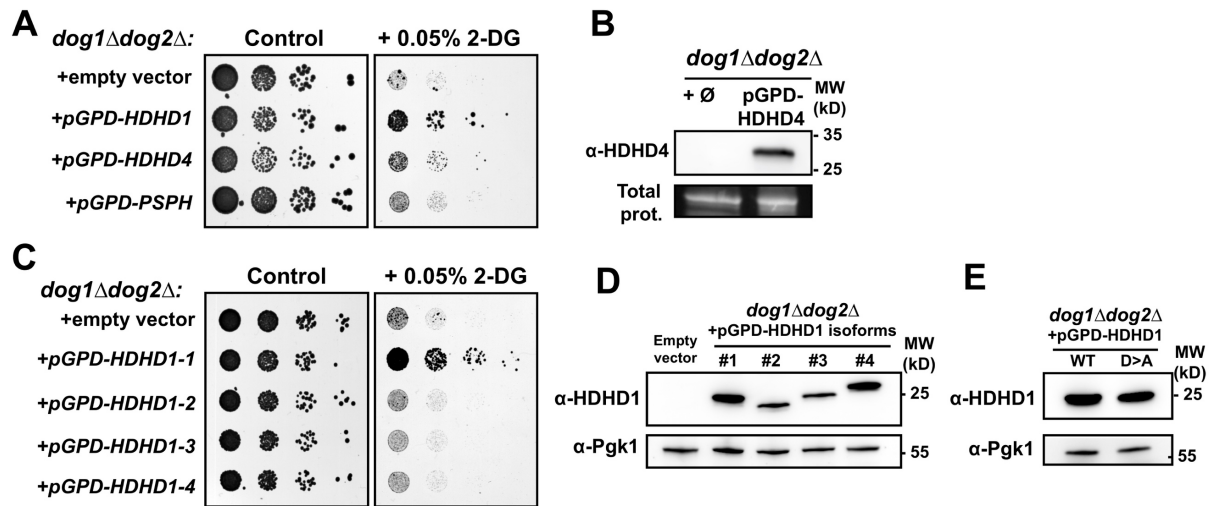


Figure S8. HDHD1 but not its close homologues HDHD4 or PSPH allow resistance to 2DG.

(A) Serial dilutions of *dog1Δdog2Δ* strains transformed with the indicated plasmids were spotted on SC-Ura medium with or without 0.05% 2DG and were scanned after 3 days of growth at 30°C. (n=2 independent experiments.) (B) Total proteins extracts of *dog1Δdog2Δ* cells transformed with an empty vector or a vector allowing the overexpression of HDHD4 were blotted with an anti-HDHD4 antibody. (n=2 independent experiments.) (C) Serial dilutions of *dog1Δdog2Δ* strains transformed with an empty vector or the indicated vectors allowing the expression of the indicated HDHD1 isoforms were spotted on SC-Ura medium with or without 0.05% 2DG and were scanned after 3 days of growth at 30°C. (n=2 independent experiments.) (D) Total protein extracts prepared from the same strains as in (C) were immunoblotted for HDHD1. Pgk1 (phosphoglycerate kinase) was used as a loading control. (E) Total protein extracts prepared from the same strains as in Fig 8C were immunoblotted for HDHD1. Pgk1 was used as a loading control. (n=2 independent experiments.)

Table S1. Single nucleotide variants in clones #9 and #10 as compared to the WT strain, as identified by whole genome resequencing

Clone	Chrom.	Position	Reference	Mutation	Gene	Mutation in coding region
#9:	chrII	464813	G	A	<i>CYC8</i> (<i>YBR112C</i>)	c.958C>T (p.Gln320X)
	chrXI	570553	C	G	<i>BET3</i> (<i>YKR068C</i>)	c.357G>C (p.Leu119Phe)
	chrXII	969100	C	G	<i>YLR422W</i>	c.3204C>G (p.Tyr1068X)
#10	chrII	464813	G	A	<i>CYC8</i> (<i>YBR112C</i>)	c.958C>T (p.Gln320X)
	chrIV	283345	T	A	Intergenic region <i>GET3-BUG1</i> (<i>YDL100C-YDL099W</i>)	
	chrVIII	303712	T	C	<i>TRA1</i> (<i>YHR099W</i>)	c.952T>C (p.Leu318Leu)
	chrIX	368278	G	C	<i>PAN1</i> (<i>YIR006C</i>)	c.1631C>G (p.Thr544Ser)
	chrXI	570553	C	G	<i>BET3</i> (<i>YKR068C</i>)	c.357G>C (p.Leu119Phe)
	chrXIV	381266	A	C	<i>DGR1</i> (<i>YNL130C-A</i>)	c.126T>G (p.Cys42Trp)
	chrXV	499715	G	A	<i>YOR093C</i>	c.2738C>T (p.Ser913Leu)

Table S2. Yeast strains used in this study.

Name/description	Genotype	Origin/reference	Figures/Panels
BY4741 (WT)	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0</i>	(118)	All
ySL2289 Dog1-GFP	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 DOG1::GFP-HIS3MX6</i>	This study	1C
ySL2290 Dog2-GFP	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 DOG2::GFP-HIS3MX6</i>	(103)	1C, 1G, 1H, 4E
ySL2195 dog1Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 dog1Δ::KanMX</i>	Euroscarf deletion collection	1E
ySL2196 dog2Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 dog2Δ::KanMX</i>	Euroscarf deletion collection	1E, 2J
ySL2197 dog1Δ dog2Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 dog1Δ-dog2Δ::LEU2MX</i>	This study	1E, 6B-C, S7A-E
ySL2339 Tdh1-GFP	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 TDH1::GFP-HIS3MX6</i>	(103)	S1
ySL2340 Stf2-GFP	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 STF2::GFP-HIS3MX6</i>	(103)	S1
ySL2341 Eno1-GFP	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 ENO1::GFP-HIS3MX6</i>	(103)	S1
ySL2342 Adh5-GFP	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 ADH5::GFP-HIS3MX6</i>	(103)	S1
ySL2343 Yro2-GFP	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 YRO2::GFP-HIS3MX6</i>	(103)	S1
ySL2344 Hor7-GFP	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 HOR7::GFP-HIS3MX6</i>	(103)	S1
ySL2315 hog1Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 hog1Δ::KanMX</i>	Euroscarf deletion collection	1F, S2A

ySL2416 <i>hog1</i>Δ Dog2-GFP	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 hog1Δ::KanMX DOG2::GFP-HIS3MX6</i>	This study	1G, 1H
ySL2204 <i>hac1</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 hac1Δ::KanMX</i>	This study	2C, 2E-I, 3G, S3B
ySL2205 <i>ire1</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 ire1Δ::KanMX</i>	Euroscarf deletion collection	2H, 2I
ySL567 <i>snf1</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 snf1Δ::KanMX</i>	Euroscarf deletion collection	2H, 4B-D
ySL488 Slt2-GFP	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 SLT2::GFP-HIS3MX6</i>	(103)	S4A
ySL1961 <i>slt2</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 slt2Δ::KanMX</i>	Euroscarf deletion collection	3B-F, S4B-C
ySL557 <i>wsc1</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 wsc1Δ::KanMX</i>	Euroscarf deletion collection	3B
ySL2380 <i>wsc2</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 wsc2Δ::KanMX</i>	Euroscarf deletion collection	3B
ySL2379 <i>wsc3</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 wsc3Δ::KanMX</i>	Euroscarf deletion collection	3B
ySL2386 <i>mtl1</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 mtl1Δ::KanMX</i>	Euroscarf deletion collection	3B
ySL2387 <i>mid2</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 mid2Δ::KanMX</i>	Euroscarf deletion collection	3B
ySL481 <i>ack1</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 ack1Δ::KanMX</i>	Euroscarf deletion collection	3B
ySL2384 <i>rom1</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 rom1Δ::KanMX</i>	Euroscarf deletion collection	3B
ySL590 <i>rom2</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 rom2Δ::KanMX</i>	Euroscarf deletion collection	3B
ySL2385 <i>tus1</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 tus1Δ::KanMX</i>	Euroscarf deletion collection	3B
ySL2405 <i>bck1</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 bck1Δ::KanMX</i>	Euroscarf deletion collection	3B, 3F
ySL2382 <i>mkk1</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 mkk1Δ::KanMX</i>	Euroscarf deletion collection	3B
ySL2383 <i>mkk2</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 mkk2Δ::KanMX</i>	Euroscarf deletion collection	3B
ySL2381 <i>rlm1</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 rlm1Δ::KanMX</i>	Euroscarf deletion collection	3B
ySL2297 Dog1-TAP Dog2-GFP	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 DOG1::TAP-KanMX, DOG2::GFP-HIS3MX6</i>	This study	4B
ySL2298 Dog1-TAP Dog2-GFP <i>snf1</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 DOG1::TAP-KanMX, DOG2::GFP-HIS3MX6, snf1Δ::hphNT1</i>	This study	4B
ySL2292 Dog2-GFP <i>mig1</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 DOG2::GFP-HIS3MX6, mig1Δ::KanMX</i>	This study	4F
ySL2293 Dog2-GFP <i>mig2</i>Δ	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 DOG2::GFP-HIS3MX6, mig2Δ::KanMX</i>	This study	4F

ySL2294 Dog2-GFP <i>mig1Δ mig2Δ</i>	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 DOG2::GFP-HIS3MX6, mig1Δ::LEU2MX, mig2Δ::KanMX</i>	This study	4F
ySL2295 Dog2-GFP <i>hvk2Δ</i>	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 DOG2::GFP-HIS3MX6, hvk2Δ::KanMX</i>	This study	4F
ySL2291 Dog2-GFP <i>reg1Δ</i>	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 DOG2::GFP-HIS3MX6, reg1Δ::LEU2MX</i>	This study	4F
ySL2192 <i>mig1Δ</i>	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 mig1Δ::KanMX</i>	Euroscarf deletion collection	4E
ySL2193 <i>mig2Δ</i>	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 mig2Δ::KanMX</i>	Euroscarf deletion collection	4E
ySL2194 <i>mig1Δ mig2Δ</i>	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 mig1Δ::KanMX, mig2Δ::HIS3MX6</i>	This study	4E, 4G
ySL2200 <i>hvk2Δ</i>	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 hvk2Δ::KanMX</i>	Euroscarf deletion collection	4E, 4G, 5B
ySL2447 <i>hvk2Δ</i>	<i>MATalpha, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 hvk2Δ::KanMX</i>	Euroscarf deletion collection	5D
ySL2199 <i>reg1Δ</i>	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 reg1Δ::HIS3MX6</i>	This study	4E, 4G, 5C, S6A
ySL2201 <i>reg1Δ dog1Δ dog2Δ</i>	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 reg1Δ::HIS3MX6, dog1Δ-dog2Δ::LEU2MX</i>	This study	4G
ySL2202 <i>hvk2Δ dog1Δ dog2Δ</i>	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 hvk2Δ::KanMX, dog1Δ-dog2Δ::LEU2MX</i>	This study	4G
ySL2203 <i>mig1Δ mig2Δ dog1Δ dog2Δ</i>	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 mig1Δ::KanMX, mig2Δ::HIS3MX6, dog1Δ-dog2Δ::LEU2MX</i>	This study	4G
ySL2317 <i>mig1Δ mig2Δ snf1Δ</i>	<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0 mig1Δ::KanMX, mig2Δ::HIS3MX6, snf1Δ::NatMX</i>	This study	4G
ySL2474 mut. #9	Spontaneous mutant arising from BY4741 strain (<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0</i>) selected on 0.2% 2DG	This study	5A, 5C-F, S6A
ySL2475 mut. #10	Spontaneous mutant arising from BY4741 strain (<i>MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0</i>) selected on 0.2% 2DG	This study	5A, 5C-F, S6A
ySL2476 mut. #9-Dog2-GFP	ySL2474 <i>DOG2::GFP- HIS3MX6</i>	This study	5F-G
ySL2477 mut. #10-Dog2-GFP	ySL2475 <i>DOG2::GFP- HIS3MX6</i>	This study	5F-G
ySL2614 mut. #9-<i>dog1Δ dog2Δ</i>	ySL2474 <i>dog1Δ-dog2Δ::HphNTI</i>	This study	5H
ySL2615 mut. #10-<i>dog1Δ dog2Δ</i>	ySL2475 <i>dog1Δ-dog2Δ::HphNTI</i>	This study	5H

Table S3. Plasmids used in this study.

Name	Description	Origin/reference
pSL209	pGP564: empty vector control for genomic tiling collection, 2 μ , <i>LEU2</i>	(119)
pSL409	<i>p_{DOG1}(1000bp):lacZ</i> , 2 μ , <i>URA3</i> (Yep58-based)	This study
pSL410	<i>p_{DOG2}(1000bp):lacZ</i> , 2 μ , <i>URA3</i> (Yep58-based)	This study
pSL405	<i>p_{UPRE1}:lacZ</i> , 2 μ , <i>URA3</i> (pGA1695-based)	P. Walter's lab, pJC5 (52)
pSL385	<i>p_{CYC(2xRlm1)}:lacZ</i> , 2 μ , <i>URA3</i> (pLG Δ -312-based)	D. Levin's lab, p1434 (120)
pSL412	<i>p_{GPD}:DOG2</i> , 2 μ , <i>URA3</i> (pRS426-based)	This study
pSL425	pGP564-based genomic clone YGPM10o10 containing <i>DOG1</i> and <i>DOG2</i> ; 2 μ , <i>LEU2</i> (genomic tiling collection - chr.VIII:188052-198738)	(119)
pSL438	<i>p_{DOG2}(150bp):lacZ</i> , 2 μ , <i>URA3</i> (Yep58-based)	This study
pSL439	<i>p_{DOG2}(250bp):lacZ</i> , 2 μ , <i>URA3</i> (Yep58-based)	This study
pSL440	<i>p_{DOG2}(350bp):lacZ</i> , 2 μ , <i>URA3</i> (Yep58-based)	This study
pSL441	<i>p_{DOG2}(500bp):lacZ</i> , 2 μ , <i>URA3</i> (Yep58-based)	This study
pSL411	<i>p_{GPD}:DOG1</i> , 2 μ , <i>URA3</i> (pRS426-based)	This study
pSL413	<i>p_{GPD}:yniC</i> , 2 μ , <i>URA3</i> (pRS426-based)	This study
pSL414	<i>p_{GPD}:HDHD1-isoform 1</i> , 2 μ , <i>URA3</i> (pRS426-based)	This study
pSL492	<i>p_{GPD}:HDHD1-isoform 2</i> , 2 μ , <i>URA3</i> (pRS426-based)	This study
pSL493	<i>p_{GPD}:HDHD1-isoform 3</i> , 2 μ , <i>URA3</i> (pRS426-based)	This study
pSL494	<i>p_{GPD}:HDHD1-isoform 4</i> , 2 μ , <i>URA3</i> (pRS426-based)	This study
pSL495	<i>p_{GPD}:HDHD4</i> , 2 μ , <i>URA3</i> (pRS426-based)	This study
pSL496	<i>p_{GPD}:PSPH</i> , 2 μ , <i>URA3</i> (pRS426-based)	This study
ySL2335	<i>p_{GPD}:HDHD1-isoform 1-DD>AA</i> , 2 μ , <i>URA3</i> (pRS426-based)	This study
pSL422	<i>pCMV-SPORT6-HDHD1</i>	Dharmacon (CloneId:4478358)
pSL458	<i>pCS2</i> (empty vector - expression in mammalian cells)	(121)
pSL431	<i>pET15b-6His-HDHD1</i>	E. van Schaftigen (83)
pSL459	<i>pET15b-6His-HDHD1(DD>AA)</i>	This study
pSL460	pGP564-based genomic clone YGPM24j02 containing <i>HXK2</i> ; 2 μ , <i>LEU2</i> (genomic tiling collection - chr.VII: 23019-35747)	(119)
pSL465	pGP564-based genomic clone YGPM2h11 containing <i>CYC8</i> ; 2 μ , <i>LEU2</i> (genomic tiling collection - chr. II: 458726-473023)	(119)
pSL466	<i>CYC8</i> (-1000/+300) ; CEN, <i>LEU2</i> (pRS415-based)	This study
pSL467	<i>cyc8</i> (C ⁹⁵⁸ >T) (-1000/+300) ; CEN, <i>LEU2</i> (pRS415-based)	This study

Table S4. Antibodies used in this study.

Name	Description	Dilution	Reference/Origin
α GFP	Mouse monoclonal against GFP, clones 7.1 and 13.1	1/5000	11814460001 - Roche
α Rsp5/Nedd4	Rabbit polyclonal antibody against Nedd4	1/5000	ab14592 - Abcam
α @p38/@Hog1	Mouse monoclonal against Tyr182-phosphorylated p38 (E-1)	1/1000	sc166182 - Santa Cruz
α p38/Hog1	Mouse monoclonal against human p38 (D-3)	1/1000	sc165978 -Santa Cruz
α CPY	Rabbit polyclonal against Carboxypeptidase Y (PRC1)	1/2000	ab34636 - Abcam
PAP	Peroxidase-anti-Peroxidase complex	1/5000	P1291-Sigma Aldrich
α @AMPK/@Snf1	Rabbit polyclonal against Thr172-phosphorylated human AMPK α	1/1000	#2535 -Cell Signaling Technology
α @ p44/42 MAPK (Erk1/2) (T202/Y204)	Rabbit monoclonal against a synthetic phosphopeptide corresponding to residues surrounding Thr202/Tyr204 of human p44 MAP kinase	1/2000	#4370 - Cell Signaling Technology
α polyHis tag	Mouse monoclonal antibody used to reveal endogenous Snf1 which contains a stretch of 13 His residues	1/2000	# H1029 - Sigma
α HDHD1	Rabbit polyclonal against human HDHD1. To visualize HDHD1 in lysates of yeast or bacteria overexpressing HDHD1.	1/2000	SAB2700505 - Sigma
α HDHD4	Mouse monoclonal anti-HDHD4 (NANP) Antibody (D8). To visualize HDHD4 in lysates of yeast overexpressing HDHD4.	1/5000	sc-374637-Santa Cruz
α -CD147	Mouse monoclonal anti-CD147 (Clone HIM6)	1/5000	555961-BD Bioscience
α -PGK	Rabbit polyclonal against yeast 3-phosphoglycerate kinase	1/10000	NE130/7S Nordic MUBio
α -Suc2 (invertase)	Rabbit polyclonal against invertase	1/5000	C. Stirling
α Rabbit IgG	Goat secondary antibody against Rabbit IgG	1/5000	A6154 - Sigma Aldrich
α Mouse IgG	Goat secondary antibody against Mouse IgG	1/5000	A5278 - Sigma Aldrich

Data File S1. Proteomic response to 2DG treatment.