Mutations in a single signaling pathway allow growth on a different solvent than water

Caroline Kampmeyer¹, Jens V. Johansen², Christian Holmberg¹, Magnus Karlson³, Sarah K. Gersing¹, Heloisa N. Bordallo⁴, Birthe B. Kragelund¹, Mathilde H. Lerche³, Isabelle Jourdain⁵, Jakob R. Winther¹ and Rasmus Hartmann-Petersen^{1,*}

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1: The Linderstrøm-Lang Center, Department of Biology, University of Copenhagen, Ole Maaløes Vej 5, DK-2200 Copenhagen, Denmark.

2: Biotech Research and Innovation Centre, University of Copenhagen, Ole Maaløes Vej 5, DK-2200, Copenhagen, Denmark.

3: Technical University of Denmark, Department of Electrical Engineering, Ørsted Plads, Bld. 349, DK-2800 Kgs. Lyngby, Denmark.

4: Niels Bohr Institute, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen, Denmark.

5: College of Life and Environmental Sciences, University of Exeter, Geoffrey Pope Building, Stocker Road, Exeter EX4 4QD, United Kingdom.

*Corresponding author: R.H.-P. (rhpetersen@bio.ku.dk).

25 Abstract

26 Since life is completely dependent on water, it is difficult to gauge the impact of solvent change. To
 27 analyze the role of water as a solvent in biology, we replaced water with heavy water (D₂O), and
 28 investigated the biological effects by a wide range of techniques, using the fission yeast
 29 *Schizosaccharomyces pombe* as model organism. We show that high concentrations of D₂O lead to
 30 altered glucose metabolism, growth retardation, and inhibition of meiosis. However, mitosis and
 31 overall cell viability were only slightly affected. After prolonged incubation in D₂O, cells displayed
 32 gross morphological changes, thickened cell walls as well as aberrant septa and cytoskeletal
 33 organization. RNA sequencing revealed that D₂O causes a strong downregulation of most tRNAs and
 34 triggers activation of the general stress response pathway. Genetic screens identified several D₂O
 35 sensitive mutants, while mutants compromised in the cell integrity pathway, including the protein
 36 kinase genes *pmk1*, *mkh1*, *pek1* and *pck2*, that control cell wall biogenesis, were more tolerant to
 37 D₂O. We speculate that D₂O affects the phospholipid membrane or cell wall glycans causing an
 38 activation of the cell integrity pathway. In conclusion, the effects of solvent replacement are
 39 pleiotropic but the D₂O-triggered activation of the cell integrity pathway and subsequent increased
 40 deposition of cell wall material and septation problems appear most critical for the cell growth
 41 defects.

42

43 Introduction

44 Although in principle life might be possible in other solvents such as ammonia or formamide [1], life,
 45 as we know it, is completely dependent on water. As solvent, water provides a liquid phase that
 46 facilitates chemical reactions by allowing reactants to encounter each other, but also ensures that
 47 various biomolecules, organelles and tissues can maintain a functional structure. Importantly, water
 48 also actively partakes in chemical reactions as a reactant (e.g. hydrolysis) or as a product (e.g.
 49 condensation). To some degree, organic solvents, such as DMSO, fulfill some of these requirements.
 50 However, these solvents are often toxic already at low concentrations [2] and do not generally engage
 51 in chemical reactions.

52 ^1H is by far the most abundant isotope of hydrogen and while ^2H or deuterium (D) and D_2O (heavy
 53 water) are fairly similar to ^1H and light water, respectively, the chemical effects of isotope substitution
 54 is far stronger than seen for most other chemical elements [3]. Thus, when substituting water as
 55 solvent, there are very significant differences between light and heavy water. Both compounds are
 56 non-radioactive, but D_2O is denser (1.1 vs. 1.0 g mL^{-1}) and more viscous (1.25 vs. 1.00 mPa s at 20
 57 $^{\circ}\text{C}$) than H_2O . It has a higher melting point (3.82 vs. 0 $^{\circ}\text{C}$) as well as a higher boiling point (101.4 vs.
 58 100 $^{\circ}\text{C}$), consistent with the deuterium bonds in D_2O being stronger than the corresponding hydrogen
 59 bonds in H_2O . Accordingly, D_2O is known to influence the conformational and functional properties
 60 of proteins and other macromolecules *in vitro* [4, 5].

61 Although the natural abundance of deuterium in water is less than 0.02%, it is interesting to study the
 62 cellular effects of D_2O because its unique properties allow for a complete solvent replacement, and
 63 may therefore highlight the importance of the biophysical properties of H_2O as solvent for biological
 64 processes. However, the cellular consequences of exchanging H_2O with D_2O have so far not been
 65 systematically addressed. When D_2O replaces H_2O in the cell, it affects reaction kinetics and the
 66 structure of macromolecules, giving rise to the so-called “solvent isotope effect” [5]. However, the

deuterium atoms in D₂O can also metabolically replace hydrogen atoms in biomolecules. When deuterium is metabolically incorporated into proteins and other biomolecules, the properties of C-D bonds in particular, which do not readily exchange with H, become important. Thus, C-D bonds may affect the structure and dynamics of macromolecules, resulting in the “deuterium isotope effect” [5]. Since hydrogen is much lighter than other biologically-relevant elements, the proportional isotope effects of deuterium are significantly stronger than e.g. those of ¹³C (vs. ¹²C) or ¹⁸O (vs. ¹⁶O) [3].

Soon after its discovery and large-scale manufacture, the first biological effects of D₂O were described (for review see [5]). Today we know that small amounts of D₂O are not toxic [6], and D₂O can be used to measure the metabolic rate in humans [7, 8], or as a tracer for compliance in drug trials [9]. However, biological studies on D₂O have shown that at high levels (>25% of body weight) D₂O is toxic to animals and causes sterility [5]. In addition, heavy water has been reported to affect the period of circadian oscillations in *Drosophila* [10], while studies in rodents have shown that high D₂O concentrations causes anemia and early death [11, 12]. In plant and animal cells, D₂O inhibits mitosis [13, 14], which may be linked to increased microtubule stabilization [15, 16]. Bacteria can adapt to grow in 100% D₂O [17-19], while budding yeast cells can tolerate up to 90% heavy water [20]. However, heavy water sensitivity has been described as a conditional phenotype in budding yeast and a D₂O hypersensitive mutant in the *ASP5* gene, encoding a cytosolic aspartate aminotransferase involved in nitrogen metabolism, has been isolated [20]. Recently, it was shown that the amount of deuterated metabolites decline in yeast cells during aging, and supplementing the growth medium with 50 % D₂O increases the lifespan [21], presumably by slowing the metabolism.

As a solvent, D₂O has been reported to marginally increase the heat stability of macromolecules [4], including double stranded DNA [22, 23] and some proteins [24-26]. In addition, D₂O can function as a chemical chaperone for misfolded proteins [27]. However, on the cellular level, D₂O may lead to a decrease in heat tolerance [28].

91 Here, we analyzed the biochemical and physiological effects of D₂O on the fission yeast
 92 *Schizosaccharomyces pombe*, a well characterized and genetically tractable eukaryotic model
 93 organism [29]. We found that the *solvent exchange* effect rather than the *isotope* effect caused a
 94 strongly reduced growth rate at high concentrations of D₂O. Although the overall cell viability
 95 remained largely unaffected, D₂O inhibited the glucose metabolism and caused gross morphological
 96 changes, thickened cell walls and abnormal cell septation. Transcriptomic analyses revealed that D₂O
 97 triggered differential expression of numerous genes, including a strong reduction of tRNAs, and
 98 activation of the general stress response pathway. Genetic screens identified several D₂O sensitive
 99 mutants, including a deletion in the *rga7* GTPase activating protein (GAP). Surprisingly, mutants in
 100 *pck2*, *mkh1*, *pek1*, and *pmk1*, that encode kinases of the cell integrity pathway, which controls cell
 101 wall biogenesis, were tolerant to the solvent exchange.

102

103 Results

104

105 *Dramatically reduced growth of S. pombe exposed to high concentrations of D₂O*

106 The D₂O was purchased as 99.8% D₂O/0.2% H₂O. For simplicity, when all regular deionized water
107 in the growth medium was replaced with the 99.8% pure D₂O we will in the following refer to these
108 experiments as performed at 100% D₂O, and similarly for experiments conducted at lower D₂O
109 concentrations. Importantly, the other components (e.g. glucose) in the media were, unless stated
110 otherwise, not deuterated. Hence, even in our 100% D₂O media some non-exchangeable protons are
111 available to the cells and will be incorporated in biomolecules.

112 As a first step in our cell physiological analyses of D₂O, we followed the growth of wild type *S.*
113 *pombe* cells exposed to different concentrations of D₂O. Pre-cultures, prepared in standard rich
114 medium (YES medium with H₂O), were washed and diluted with YES medium with 100%, 75%,
115 50%, 25% and 0% heavy water and growth was monitored by measuring the turbidity of the cultures.
116 At D₂O concentrations >50%, the cell growth rate was strongly reduced (Fig. 1AB) and the onset of
117 growth inhibition occurred rapidly, already within the first generation (Fig. 1C). The effect was
118 dosage dependent leading to an increase in doubling time from approximately 2.5 hours in 0% D₂O
119 to around 7 hours at 100% D₂O (Fig. 1B). The observed growth retardation was not caused by cell
120 death, since cell viability was only marginally affected after prolonged incubation with D₂O (Fig.
121 1D).

122 Since bacteria can be adapted to growth at 100% D₂O [17, 19], we tested if pre-incubating the cells
123 with different amounts of D₂O would allow for increased growth rates in the presence of D₂O on solid
124 rich medium. However, this was not the case for fission yeast. Hence, for all tested pre-incubations
125 no changes in growth were observed (Fig. 1E), although we noted that when grown on solid media,
126 wild type cells eventually formed colonies even at 100% D₂O.

127 The observed effects of D₂O may be caused by either incorporating deuterium into macromolecules
 128 or by replacing the solvent (H₂O) with D₂O or by a combination of the above. Thus, we compared
 129 the growth of cells on media prepared with normal glucose or deuterated glucose (glucose-C-d₇). On
 130 rich media, there was no obvious effect of exchanging glucose with deuterated glucose (Fig. 1F).
 131 Since amino acids are plentiful in this medium, significant protein deuteration of non-solvent-
 132 exchangeable protons is not expected. On minimal medium, a slight growth inhibitory effect was
 133 observed (Fig. 1F). Since glucose here also serves as a carbon source for amino acid synthesis, the
 134 slightly reduced growth is, at least in part, likely to be ascribed to deuteration of proteins and other
 135 biomolecules. However, since the effect of deuterated glucose is minor, the observed effect of D₂O
 136 is mainly caused by the solvent replacement. Accordingly, the growth retardation observed on media
 137 prepared with both deuterated glucose and D₂O were similar to that observed on media with
 138 hydrogenated (normal) glucose and D₂O (Fig. 1F). We therefore conclude that the growth inhibitory
 139 effect of D₂O largely can be attributed to the *solvent isotope effect* rather than an effect of deuterium-
 140 induced malfunction of various biomolecules.

141

142 *D₂O causes gross morphological changes, increased cell wall thickness and aberrant cell septation*

143 The reduced cell growth was accompanied by an elongated and swollen cell morphology, which was
 144 more apparent after prolonged incubation in D₂O (Fig. 2A). Cell septation was analyzed by calcofluor
 145 staining. This revealed a clear dosage-dependent septation defect, which appeared gradually upon
 146 shifting the cells to D₂O medium. Hence, after 5 hours in the exponential growth phase, roughly 20%
 147 of the cells in 0% D₂O were septated, while in 100% D₂O this was increased to 60% of the cells (Fig.
 148 2B). After 24 hours, the cells grown in 0% D₂O had entered stationary phase (Fig. 1A) and were
 149 therefore not septated (Fig. 2AB). However, at 100% D₂O about 60% of the cells were still septated
 150 (Fig. 2B) and about 20% displayed multiple septa (Fig. 2AB). In addition, an increased thickness of

151 septa was visible already after 5 hours in D₂O, which was succeeded by unevenly shaped septa after
152 24 hours (Fig. 2A).

153 By transmission electron microscopy we confirmed that cells grown for 24 hours in D₂O appeared
154 swollen and displayed thickened cell walls (Fig. 2CD). Accordingly, cells incubated for 24 hours in
155 D₂O-containing medium were resistant to the cell wall degrading enzyme β -glucanase, supporting
156 that, unlike the situation for bacteria [19], D₂O causes cell wall thickening of yeast cells (Fig. 2E).

157 The *S. pombe* septum consists of primary (outer) and secondary (inner) septa that are made up of
158 different glucans [30]. The secondary septum will eventually form the cell wall at the tip of the
159 daughter cell. Both the primary septa (white layer) and secondary septa (grey layers flanking the
160 primary septum) were thicker for cells grown for 24 hours in D₂O (Fig. 2CD). In general, septum
161 formation appeared abnormal in D₂O. For example, in some of the D₂O-treated cells we observed an
162 asymmetrical septum growth from only one side (Fig. 2D, panels e, g, and k), while for the control
163 cells, the septa always grew uniformly inwards (Fig. 2D, panels a-c). Once the septa formed, their
164 closure appeared normal, but for the cells in D₂O, septum material continued to build up, leading to
165 strongly thickened secondary septa (Fig. 2D, panels d-k). Since the primary septa were stabilized in
166 D₂O, the cells failed to detach and remained associated with sister cells from previous generations
167 (Fig. 2D, panel j). However, rather than reinitiating another cell cycle and forming a chain of cells,
168 most of the multiseptated cells appeared to have formed the second or third septum near the original
169 septum (Fig. 2D, panels e, f, and j), and not between nuclei, indicating that no further nuclear division
170 occurred. This may suggest that D₂O affects the septation initiation network [31]. Moreover, in some
171 cells we observed that the second or third septum seemed to branch from the original septum, or to
172 grow perpendicular to it (Fig. 2D, panels i and j).

173

174 *D₂O affects both the actin and tubulin cytoskeleton*

175 To determine if the cytoskeleton was affected, wild type cells carrying the LifeAct-GFP actin marker
 176 and GFP-tagged α -tubulin (Atb2) were exposed to D₂O and analyzed by fluorescence microscopy.
 177 In *S. pombe* the actin cytoskeleton typically appears as cortical patches located mainly at the growing
 178 cell tips during interphase and as a contractile ring, which forms during mitosis [32]. The tubulin
 179 cytoskeleton is composed of cables that extend between the cell ends in interphase and as a dense
 180 spindle between the sister chromatids during mitosis [32].
 181 Upon treatment with D₂O, we observed that the actin patches were no longer concentrated at the cell
 182 tips, but rather appeared more evenly distributed throughout the cells (supporting information, Fig.
 183 S1A). The formation of the contractile ring, however, appeared normal (supporting information, Fig.
 184 S1A). In response to D₂O, the tubulin cables were shorter, more numerous, and in some cells
 185 perpendicular rather than parallel to the cell (supporting information, Fig. S1B). However, similar to
 186 the situation with the actin cytoskeleton in mitosis, the mitotic spindle appeared unaffected by D₂O
 187 (supporting information, Fig. S1B). Thus, unlike the situation in mammalian cells [16], the mitotic
 188 spindle was formed and appeared normal even after prolonged incubation in D₂O.

189

190 *D₂O inhibits meiosis*

191 To test whether the solvent replacement affected cell cycle progression, cells exposed to different
 192 concentrations of D₂O were analyzed for DNA content by DNA staining and automated microscopy.
 193 In *S. pombe*, cytokinesis normally occurs in S phase and cells in G₁ and S phase are therefore binuclear
 194 and, by DNA staining, indistinguishable from the cells in G₂. Accordingly, the DNA content profile
 195 presents as a single 2N peak. After five hours of exponential growth in D₂O, the DNA peak remained
 196 unchanged. However, we note the appearance of a small population with 4N DNA content
 197 (supporting information, Fig. S2A), which may represent cells that have re-replicated DNA without
 198 prior mitosis or cells that have failed to undergo cytokinesis. After 24 hours, when the cultures with

low concentrations of D₂O had entered stationary phase with some cells arrested in the G₁ phase, the 1N peak was still not evident for cells grown at 75% or 100% D₂O (supporting information, Fig. S2A). This may indicate a reduced progression through mitosis, whereas the sharp 2N peak indicates that the cells spend a relatively short time period in S phase, suggesting that progression through S phase is not strongly affected. To more directly test whether mitosis was affected, the number of cells displaying spindles was determined using the Atb2-GFP strain. After 24 hours in D₂O, we observed a small increase in the number of cells with spindles (supporting information, Fig. S2B), indicating that D₂O only causes a slightly slowed progression through mitosis, and the reduced cell growth of *S. pombe* cells in D₂O is therefore most likely caused by an overall slowing of the cell cycle. To test if meiosis was affected, a wild type strain (*h⁹⁰*) was starved for nitrogen in the presence of H₂O or D₂O. After two days, multiple asci were evident for the cells grown on the H₂O control medium, whereas for cells incubated on D₂O medium we were never able to detect any asci (supporting information Fig. S3). Accordingly, iodine stained the starch in spore walls dark for the cells on the H₂O medium, but failed to stain the cells on the D₂O medium (supporting information Fig. S3AC), suggesting that zygote formation and/or meiosis is severely inhibited by D₂O. Using diploid cells, we observed numerous asci when the cells were incubated in H₂O, but only a few asci for cells on D₂O medium (supporting information Fig. S3BC). Collectively, these data indicate that D₂O inhibits mating and meiosis. An obvious hypothesis is that the mating defect, at least in part, is caused by the D₂O-induced cell wall thickening.

218

219 *D₂O blocks glucose metabolism through inhibition of glucose-6-phosphate isomerase*

220 Since the isotope effects of D₂O are likely to disturb metabolism [33], we decided to investigate
221 whether D₂O affects the kinetics of enzymes in the glycolytic pathway on a short timescale. This was
222 accomplished using hyperpolarized NMR (signal enhanced nuclear magnetic resonance) [34] which

allows detailed metabolic mapping of glycolysis. A ^{13}C -labelled glucose tracer was fed to live cells in either 100% D_2O or 100% H_2O MES buffer for 2 or 10 minutes, after which the metabolism was quenched in acid and the soluble metabolites extracted. This extract was then hyperpolarized and analyzed with single spectrum ^{13}C -NMR. Examples of an NMR spectra from cells fed with the glucose tracer in D_2O and H_2O , respectively, are shown in Fig. 3A. The crowded spectral region from 60-100 ppm contains signals from the substrate glucose. The singlet signal at 24 ppm originates from an added standard, which allows quantification and direct comparison between the metabolite signals in the different spectra [35]. This comparison clearly revealed that several metabolic changes appear when *S. pombe* is fed glucose in D_2O . In particular, we noted the accumulation of a large pool of α -glucose-6-phosphate (α -G6P) (Fig. 3A, blue insert), suggesting that the α anomer is being metabolised more slowly in D_2O . Whereas β -G6P is readily used as a substrate in the pentose phosphate pathway (PPP), α -G6P must be converted to β -G6P for use in PPP or to β -fructose-6-phosphate (β -F6P) for use in glycolysis. A single enzyme, glucose-6-phosphate isomerase (GPI, EC 5.3.1.9), is responsible for both types of isomerase activities (<https://www.genome.jp/kegg/>). The PPP was indeed active, as demonstrated by the signals from 6-phosphogluconate (6PGA) (Fig. 3A, red insert). The 6PGA pool was approximately twice as large in D_2O as in H_2O . That the activity of PGI could be negatively affected by the exchange of H_2O for D_2O is plausible since the mechanism of catalytic isomerization involves several proton transfer steps [36].

The doublet signal at about 179.4 ppm, assigned to gluconate (GA) (Fig. 3A), confirms that the gluconate shunt is active in *S. pombe* cells [37, 38]. In 30-sec. time-resolved data (supporting information, Fig. S4) only gluconate could be detected and the gluconate shunt seemed to be unaffected by D_2O .

The accumulation of α -G6P in the D_2O -exposed cells is already apparent after 2 minutes of incubation with the glucose tracer (supporting information, Fig. S5). However, in order to facilitate

quantification and comparison of the amount of G6P under the two studied conditions, exposure times of 10 minutes were used, revealing an about 8 times larger pool of G6P in D₂O exposed cells (Fig. 3B). In summary, glycolysis as well as the pathway leading to PPP are affected negatively by an inhibition of α -G6P isomerization in *S. pombe* cells exposed to D₂O (Fig. 3C). We conclude that, under these conditions, *S. pombe* cells use a highly active gluconate shunt to produce PPP metabolites from glucose, which allow the cells to bypass GPI.

D₂O induces a cellular stress response

Recently, it was reported that deuterium causes dramatic changes in the *E. coli* proteome [19]. To determine if the D₂O-triggered growth inhibition was accompanied by differential gene expression, total RNA was purified from *S. pombe* cells under each condition in quadruplicates, and analyzed by next-generation sequencing. The four repeats of each condition clustered in separate groups (Fig. 4AB). In total, we could assign read counts to 6992 of 7015 annotated genes in the *S. pombe* genome. By setting a significance cut-off of absolute log₂ fold change ≥ 2 between the groups and adjusted *p* values $\leq 5\%$ (false discovery rate, FDR), we identified 178 genes that were significantly up-regulated, while 38 genes were significantly down-regulated after five hours with D₂O. After 24 hours in D₂O 284 genes were significantly up-regulated and 51 genes were significantly down-regulated. The identified differentially expressed genes are listed in the supporting material (supporting material file 1). The sequencing data has been uploaded to Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>; accession no. GSE119785). To further test the quality of the dataset, we quantified the mRNA by real-time PCR of selected up-regulated genes. In agreement with the RNA sequencing, the expression of these genes was up-regulated (supporting information, Fig. S6).

270 When analyzing the differentially expressed genes, we noted a strong D₂O-dependent down-
 271 regulation of several cytosolic and mitochondrial tRNAs (Table S1). This is in line with earlier
 272 observations in *E. coli* [39] and the reduced abundance of translational proteins recently observed
 273 by proteomics [19]. Comparison of our datasets with previous transcriptomic analyses in fission
 274 yeast (Chen et al., 2003; Poulsen et al., 2017; Vjestica et al., 2013) indicated that D₂O causes a
 275 general stress response in the cells. Thus, the D₂O-triggered gene expression pattern resembled those
 276 observed upon heat shock, deletion of the Hsf1 transcriptional repressor Mas5 or the Hsp70
 277 chaperone Ssa2, or overexpression (OE) of the co-chaperone Bag101 (Fig. 4CD). We therefore
 278 tested if prior activation of the stress response pathway conferred any fitness advantage to the cells
 279 in the presence of D₂O. To this end, wild type cells were given a 30 minute heat shock at 43 °C
 280 immediately prior to testing growth in the presence of D₂O on solid media. However, rather than
 281 protecting the cells from D₂O, this revealed a slight, but reproducible, decreased D₂O tolerance (Fig.
 282 4E), suggesting that induction of molecular chaperones, oxidoreductases and other stress-relieving
 283 proteins are unable to counteract the adverse effects of D₂O. Similarly, applying oxidative stress via
 284 a pre-treatment of cells for 1 hour with 0.7 mM H₂O₂ also did not provide protection from D₂O (Fig.
 285 4F). Conversely, pre-incubating cells for 5 hours with 100% D₂O led to an increased survival of
 286 cells after a severe heat shock (Fig. 4G) and an increased growth in the presence of H₂O₂ (Fig. 4H).
 287 This suggests that the D₂O-triggered induction of the stress-response genes leads to increased
 288 tolerance towards heat-shock and oxidative stress conditions.

289

290 *Screening the S. pombe gene deletion library for D₂O sensitive or D₂O resistant mutants*

291 Previous studies in budding yeast have shown that heavy water sensitivity is a conditional phenotype
 292 and that a loss-of-function mutant in *ASP5*, encoding an aspartate aminotransferase, is hypersensitive
 293 to D₂O [20]. As a next step, we therefore explored the genetic requirements for D₂O tolerance by

294 individually screening 3233 different mutants from the fission yeast haploid gene deletion collection
295 for hypersensitivity to D₂O on solid media.

296 Stationary phase cells were replica plated in 384-pin format onto YES plates with 0% or 100% D₂O.
297 This concentration of D₂O was used because it completely blocked growth of a deletion strain in the
298 *ASP5* orthologue in fission yeast, *caal1*, but also impaired the growth of wild type cells (Fig. 5A), so
299 both D₂O hypersensitive and resistant mutants could be scored. We selected mutants with reduced
300 growth on D₂O medium, but excluded mutants with slow growth on 0% D₂O medium. This led to 39
301 mutants scored as D₂O sensitive (Table 1). We noted that the positive control, *caal1Δ*, was among
302 these. We also isolated one mutant, *pmk1Δ*, deleted for the gene encoding the Pmk1 MAP kinase that
303 appeared resistant to D₂O.

304 To assess the validity of the screen, we tested selected D₂O sensitive mutants and the *pmk1Δ* strain
305 in growth assays on solid media. This revealed that the *pmk1Δ* strain was slightly resistant to D₂O,
306 while the selected sensitive mutants all displayed a reduced D₂O tolerance (Fig. 5B). In addition,
307 using the BioGRID database (<https://thebiogrid.org/>) [40] we noted several previously identified
308 genetic interactions between many of the D₂O hypersensitive/resistant strains, including ones
309 between the D₂O tolerant *pmk1Δ* strain and the D₂O sensitive *cch1Δ* [41, 42], *rga7Δ* [43, 44], *cph2Δ*
310 [45] and *swd2Δ* [45] (Fig. 5C).

311

312 *D₂O activates the cell wall integrity pathway*

313 To obtain further insight into the biological effects of the solvent replacement, we set up a screen for
314 spontaneous D₂O resistant mutants. Wild type cells were inoculated in 100% D₂O rich medium in 20
315 separate tubes and incubated at 30 °C. After about one week, slight growth was apparent in four of
316 the tubes, suggesting that independent spontaneous mutations that allowed for D₂O tolerance had
317 occurred in these cultures. To avoid selecting clones, cells from each culture were spread on 100%

318 D₂O plates and only one well-isolated large colony from each plate was selected. This led to the
319 isolation of three independent heavy water resistant (*hwr*) mutants (*hwr1-3*), that all displayed a
320 markedly increased growth in the presence of D₂O, but otherwise appeared normal (Fig. 6A). The
321 increased tolerance to D₂O was also evident on 100% D₂O minimal media (supporting information,
322 Fig. S7). However, the *hwr* mutants were not resistant to the slight growth inhibitory effect conferred
323 by deuterated glucose on minimal medium (supporting information, Fig. S7), indicating that the
324 isolated *hwr* mutants were specifically tolerant to the solvent replacement effect.

325 Since the isolated *hwr* strains were the result of spontaneous mutations, as opposed to mutagenesis
326 induced by chemicals or irradiation, we reasoned that their genome sequences were likely highly
327 similar with the exception of the mutations causing D₂O resistance. We therefore analyzed the *hwr*
328 strains and their wild type parent by whole genome sequencing using Illumina HiSeq2000 high-
329 throughput sequencing technology. The average sequence coverage was greater than 1000 reads/bp
330 for all samples. The full sequencing datasets are deposited at the Sequence Read Archive
331 (<https://www.ncbi.nlm.nih.gov/sra>; accession no. SRP161455). Comparison of obtained sequences
332 from the *hwr* strains and the wild type control revealed a match, except for the listed changes (Table
333 2, and supporting material file 2).

334 When studying the mutated genes in the *hwr1-3* strains (Table 2), we noticed that several have been
335 linked to tRNA metabolism (*gta2* and *trm72*) and cell wall formation (*pck2*, *mkh1* and *pek1*). The
336 kinases Pck2, Mkh1 and Pek1 were mutated in the *hwr3*, *hwr2* and *hwr1* strains, respectively. These
337 kinases function as upstream activators of Pmk1 in the so-called cell integrity pathway [46, 47], which
338 is activated by cell wall damage to upregulate synthesis of various cell wall components [48], while
339 the Rho2 GAP, Rga7, restricts signaling through this pathway (Fig. 6B) [43, 44]. None of these genes
340 are essential, but except for the D₂O-sensitive *rga7*Δ and D₂O-tolerant *pmk1*Δ, the deletion mutants
341 were not included in the screened knock-out library. The *pek1* and *mkh1* mutations both result in

342 frame shifts in regions encoding the respective kinase domains of Pck1 and Mkh1 that we predict will
 343 cause loss of function, while *pck2* contained a missense mutation (supporting information, Table S2).
 344 These changes were confirmed by PCR and Sanger sequencing (supporting information, Fig. S8-
 345 S10). We therefore continued to test if the D₂O tolerant phenotype was also evident in the independent
 346 *pck2-8*, *mkh1Δ*, and *pek1Δ* strains. Indeed, in growth assays, these strains appeared more resistant to
 347 D₂O than wild type cells (Fig. 6C), thus phenocopying the *hwr* strains. In agreement with this, the
 348 cell septation problems observed for wild type cells in D₂O were reduced in the *hwr* strains (Fig. 6D).
 349 The conclusions above suggest that D₂O activates the cell integrity pathway and blocking this
 350 pathway leads to an increased fitness at high concentrations of D₂O, presumably by limiting synthesis
 351 of glycans and/or other cell wall components. To test this prediction, we directly analyzed the
 352 activation of the cell integrity pathway in response to D₂O in a wild type strain, expressing HA-tagged
 353 Pmk1. This revealed a marked increase in the level of phosphorylated Pmk1 after 5 and 24 hours in
 354 D₂O-containing media (Fig. 6E), which further suggests that the solvent replacement activates the
 355 cell integrity pathway. In turn, blocking the Pck2-Mkh1-Pek1-Pmk1 signaling axis, at least partially,
 356 alleviates the D₂O-triggered cell wall and growth defects, while activating it by deletion of *rga7*
 357 increases D₂O sensitivity (Fig. 6B).

358

359 Discussion

360 In the present study, we have systematically addressed the cellular and biochemical consequences of
 361 exchanging H₂O for D₂O in eukaryotic cells using *S. pombe* as a model organism. The effect of D₂O
 362 cannot directly be compared to any other insults that a cell can experience because it entails an
 363 almost complete substitution of solvent. This possibility of solvent change for biological reactions
 364 and life as such is unique. In some ways it can be compared to changing pH, which also implies
 365 global solvent effects, but contrary to shifts in pH, which are common in nature and has well-defined
 366 biological responses, a shift to D₂O is completely abiotic and something that we can say with
 367 absolute certainty that cellular responses have not experienced before. Thus, querying the effects of
 368 D₂O on cellular growth and metabolism highlights subtleties of the biophysical properties of H₂O as
 369 solvent for biological processes. *A priori* one would expect that the toxic effects of replacing H₂O
 370 with D₂O would be the sum of multiple smaller pleiotropic effects, and it is therefore surprising that
 371 this work demonstrates that modification of a single signaling pathway is sufficient to overcome the
 372 growth defect induced by the solvent replacement.

373 In agreement with early studies using human cells and other model systems [5], we observed that
 374 high concentrations of D₂O strongly reduced cell growth. In principle, this effect can be the result of
 375 deuteration of various biomolecules or the solvent exchange effect. When D is incorporated into
 376 biomolecules, this can occur either *de novo*, where D₂O donates D atoms during *de novo* biosynthesis,
 377 or by exchange of loosely bound hydrogens in preexisting biomolecules. Hydrogen covalently bound
 378 to carbon does not exchange, and generation of C-D bonds therefore requires *de novo* synthesis. Since
 379 we only observed minor effects of exchanging glucose with deuterated glucose on minimal medium,
 380 metabolic incorporation of D into C-D bonds does not appear to strongly affect the growth of *S.*
 381 *pombe*. The resulting changes in stability and/or dynamics of proteins and other biomolecules,
 382 carrying C-D bonds, therefore appears negligible next to the observed solvent effects of exchanging

H₂O for D₂O. However, upon substituting H₂O with D₂O, loosely-bound hydrogen atoms such as those bound to amide/amino nitrogen or hydroxy oxygen will, depending on solvent exposure, exchange with deuterium atoms in the D₂O solvent. Here, the properties of hydrogen bonds compared to the stronger and slightly longer deuterium bonds likely become highly relevant. Hence, deuterium transfer reactions are expected to be less efficient than the corresponding proton transfer reactions. Also, the more ordered nature of D₂O compared to H₂O suggests that proteins and other biomolecules are less dynamic and more tightly wrapped in D₂O than in H₂O [49]. Accordingly, D₂O is known to both directly (as a solvent) and indirectly (through deuteration) affect the structure and stability of proteins and other macromolecules *in vitro* [25, 50-54].

One of the direct effects of the solvent exchange that we observed was on the central metabolism, where we directly and on a very short time-scale (2 minutes) noted a clear accumulation of α -G6P, suggesting that G6P isomerase is inhibited. The solvent exchange also led to an activation of a general stress response pathway. Typically, this pathway is provoked by protein misfolding events, suggesting that solvent exchange may destabilize proteins *in vivo*. However, inducing molecular chaperones through a heat shock prior to incubation with D₂O did not protect cells from the D₂O-induced growth retardation. This indicates that activation of the stress response pathway is not tuned to counter the adverse effects of the solvent exchange, although it has been reported that overexpression of Hsp70 may protect budding yeast cells from D₂O [55]. In line with chaperones being unable to protect fission yeast cells against D₂O, we were unable to adapt *S. pombe* to growth at higher D₂O concentrations and we did not identify any chaperone mutants as hypersensitive or resistant to D₂O. Conversely, it was not surprising to find that pre-treating cells with D₂O led to an increased tolerance towards heat and oxidizing conditions, which is most likely caused by the D₂O-induced activation of the stress response pathway. The activated stress response and slow growth phenotypes are probably also connected with our observation that exposure to D₂O caused a strong

407 decrease in the expression of several tRNAs. In budding yeast and in bacteria it has been shown that
 408 tRNAs are degraded in response to stress conditions [56, 57], and we speculate that perhaps a similar
 409 mechanism operates in *S. pombe*.

410 The multiple effects, we observed for cells in the presence of D₂O, not surprisingly, suggest that the
 411 effects of the solvent exchange are pleiotropic. Accordingly, it is easy to imagine a number of genetic
 412 defects that may cause cells to become hypersensitive to D₂O. However, it is harder to reconcile
 413 D₂O resistant mutants with the observed pleiotropic phenotypes, and although heavy metals,
 414 oxidizing agents and increased temperatures also induce multiple cellular effects [58-61], these
 415 conditions occur naturally and cells have therefore evolved specific transcriptional programs driving
 416 the expression of proteins that fend against such insults. Since the natural abundance of D₂O is
 417 miniscule (<0.02% in water), any D₂O resistant mutant must therefore be a consequence of some
 418 cellular pathway, which indirectly improves fitness in response to D₂O. We identified a null mutant
 419 in *pmk1* (orthologue of human *MAPK7*) which displays increased tolerance to D₂O. This is in line
 420 with Pmk1 acting as a MAP kinase that regulates cell wall biogenesis [44, 62, 63] and the observed
 421 D₂O-induced cell wall and cell septation defects. Accordingly, the cell integrity pathway is activated
 422 in response to D₂O, and when we selected for spontaneous D₂O-tolerant strains, we recovered
 423 mutants in other components of the pathway. Moreover, we found that deletion of the Rho2 GAP,
 424 Rga7, which normally down-regulates the cell integrity pathway [43, 44], leads to a D₂O
 425 hypersensitive phenotype. However, besides *rga7Δ*, other mutants that we scored as D₂O sensitive
 426 have also been reported in BioGRID to display genetic interactions with components of the cell
 427 integrity pathway (supporting material file 3). Intriguingly, the D₂O-dependent cell wall effects, we
 428 report here, resemble those observed upon deletion of the kinase Kin1, which regulates cell polarity
 429 [64, 65]. Similar to D₂O treated cells, the *kin1Δ* strain displays positive genetic interactions with
 430 *pck2Δ* and *pmk1Δ* mutants [64].

431 Activation of the cell integrity pathway is in agreement with its role in mediating an appropriate
 432 response to allow survival during sudden changes in water activity [66] and earlier studies suggesting
 433 that D₂O causes osmotic stress [67, 68]. It does, however, pose the question of how the solvent
 434 exchange, or what property of D₂O, causes activation of the pathway? Several upstream membrane-
 435 spanning sensors of the cell integrity pathway, including Wsc1 and Mid2, have been identified in
 436 *Saccharomyces cerevisiae*. Recent studies suggest that these sensors physically couple the cell wall
 437 with the plasma membrane, and in a spring-like manner function to sense mechanical perturbations
 438 or elasticity changes in the cell wall and/or plasma membrane [69]. Accordingly, D₂O is known to
 439 affect phospholipid membranes [70] and ion channels [68], and we speculate that this or related
 440 effects on the cell wall glycans may cause a direct activation of the cell integrity pathway.

441 Identifying D₂O-resistant strains has important practical applications, since the phenotypes imposed
 442 by D₂O severely hinder the production of deuterated proteins for structural studies [71], but also
 443 potentially for biological production of deuterated pharmaceuticals, which in some cases are superior
 444 to their protonated counterparts [72], and are therefore increasingly being considered as drug
 445 candidates [73-75]. Although bacteria can be adapted to grow in 100% D₂O [17], growth is often
 446 poor and the bacteria quickly lose the D₂O tolerance when returned to H₂O media. The results
 447 presented here provide an important first step towards an understanding of how D₂O affects cell
 448 biology and for generating D₂O tolerant strains for production of deuterated biomolecules.

449 **Materials and Methods**

450

451 *Heavy water and deuterated glucose*

452 The used D₂O (deuterium oxide) was purchased as 99.8% D₂O/0.2% H₂O (Sigma). Deuterated
453 glucose (D-glucose-1,2,3,4,5,6,6-d₇) was purchased as 97 atom % D (Sigma).

454

455 *Yeast strains and techniques*

456 The fission yeast strains that were used in this study are all derivatives of the wild type *972h⁻* and
457 *975h⁺* heterothallic strains that either carry no auxotrophic markers (no marker wild type), or the three
458 auxotrophic markers *ura4-D18*, *leu1-32*, and *ade6-216* (3 marker wild type). The gene deletion
459 strains were from the fission yeast haploid deletion library purchased from Bioneer [76]. The *LifeAct-*
460 *GFP* and *atb2-GFP* strains have been described before [77, 78]. The *pck2-8* strain was kindly
461 provided by Dr. Jeremy Hyams. The *pmk1-HA* strain was kindly provided by Dr. Marisa Madrid [47].
462 Growth assays in liquid YES (yeast extract with supplements) media (5 g/L yeast extract, 30 g/L
463 glucose, 225 mg/L adenine, 225 mg/L uracil, 225 mg/L leucine) were performed at 30 °C with
464 vigorous shaking by following the optical density (OD) at 600 nm. Growth assays on solid media,
465 either YES media (YES medium with 20 g/L agar) or EMM2 (Edinburgh minimal media) [79, 79]
466 were performed at 25 or 30 °C essentially as described [80]. Sporulation assays were performed using
467 malt extract agar plates (30 g/L malt extract, 20 g/L agar). For iodine staining, solid iodine was heated
468 in a closed beaker and the plates briefly exposed to the iodine vapor. The wild type diploid strain was
469 constructed by mating *ade6-210 h⁺* cells with *ade6-216 h⁻* cells on malt extract and selecting for cells
470 that could grow in the absence of adenine.

471 β-glucanase (Sigma) resistance was determined (in water) for cells grown for 25 hours in 100% D₂O-
472 based YES medium as described [81].

Cell viability was determined by staining cells using VitaBright-48 (ChemoMetec) and a NucleoCounter NC-3000 (ChemoMetec) as described by the manufacturer.

To monitor cell cycle progression, cultures treated or untreated with D₂O, were stained with SYTOX Green and the cellular DNA content was quantified using a NucleoCounter NC-3000 (ChemoMetec) as described by the manufacturer.

Cell imaging and microscopy

Calcofluor white (Sigma) staining was used to monitor cell septation as described previously [82]. Hoechst (Sigma) staining was used to mark the nucleus. Cells were observed on 2 % agarose pads made in EMM with H₂O or D₂O. Samples were examined using a Zeiss Z1 AxioObserver inverted fluorescence microscope, equipped with a motorized stage, in a temperature-controlled incubation chamber. A 100x, 1.4 NA oil immersion lens and a Cool-Snap HQ2 CCD camera (Photometrics) controlled by Axiovision software (Zeiss) was used for image capture. All image analysis was performed using ImageJ [83]. Electron microscopy was performed essentially as described [84].

Hyperpolarized extract ¹³C-NMR

A previously published protocol [35] was followed for substrate incubation, metabolite extraction, and extract hyperpolarization. Briefly, 100 µL 120 mM U-¹³C, d₇ glucose dissolved in either D₂O- or H₂O-based morpholinoethanesulfonate (MES) buffer (50 mM MES, 100 mM NaCl, pH 5.5) was added to the yeast cells and the respective samples were incubated for 2 minutes or 10 minutes at 30 °C after which the metabolism was stopped by addition of 400 µL perchloric acid and the soluble metabolites were extracted according to the protocol. The freeze-dried extracts were re-dissolved in a hyperpolarization matrix and polarized at 3.35 T and 1.4 °K. Hyperpolarized extracts were hereafter dissolved in 4 mL D₂O or H₂O based MES-buffer pH 5.5 and injected into a 5 mm NMR tube and a

single spectrum ^{13}C -NMR was acquired on a 9.4 T Varian Inova spectrometer immediately with a 90 degree pulse.

NMR spectra were analyzed with the MNova software. Metabolites were identified by reference to the human metabolome database [85].

501

RNA sequencing

Total RNA was isolated using the hot phenol method [86] from cells that were either untreated or treated with 100% D₂O for 5 or 24 hours. Conversion to cDNA and sequencing was performed by the Beijing Genome Institute using the Illumina sequencing as single-end with final read lengths of 50 bp after demultiplexing. This produced 23.9-24.1 million reads per sample. The raw reads were aligned to the *S. pombe* genome using bwa mem (v0.7.12) with default settings giving an alignment rate of 98.4-98.7% for all 12 samples. Mapped reads were assigned to genes using GFOLD ('gfold count') followed by GFOLD statistical analysis ('gfold diff') to detect differentially expressed (DE) genes [87].

The significance cut-off for DE genes was set at $\text{FDR} \leq 5\%$ and absolute $\log_2$ fold change ≥ 2 . For figures the DESeq2 (R package) [88] normalized gene counts ('rld') were used. The *S. pombe* reference genome and gene annotation were downloaded from GenBank (date stamped 27-FEB-2015, Rel. 123, Last updated, Version 14). Supplementary data tables from Chen *et al.* (Chen et al., 2003) were downloaded from <http://128.40.79.33/projects/stress/>. The data from Vjestica *et al.* are primarily from the supplementary material (Vjestica et al., 2013).

Our annotation also includes rRNA, tRNA, ncRNA, snoRNA, and mitochondrial genes, that in many cases are not present in the older annotations used by Chen *et al.* (Chen et al., 2003) and Vjestica *et al.* (Vjestica et al., 2013). Thus, it was not possible to compare the expression levels of many, in

particular non-coding, genes across the studies. The DESeq2 Bioconductor package in R (Anders et al., 2015) was used for statistical analyses of differentially expressed genes.

Screening the S. pombe deletion library

The *S. pombe* haploid gene deletion library was first pinned to YES (0% D₂O) agar plates in 384 format. Once large colonies had formed, cells were transferred to YES with 0% D₂O and YES with 100% D₂O agar plates in 384 format. After 8 days of incubation at 30 °C, the plates were photographed and cell growth was scored by manual inspection. All cell handling was performed using a ROTOR HDA pinning robot (Singer Instruments).

Screening for spontaneous D₂O-tolerant mutants and whole-genome sequencing

Wild type cells were inoculated in 1 mL 100% D₂O YES medium in separate tubes and incubated at 30 °C. After about 1 week, when slight growth was apparent in some of the tubes, cells from each culture were spread on solid 100% D₂O YES media plates and only one single large colony from each plate was selected for further analyses. Genomic DNA was purified by phenol-chloroform extraction as described [89]. The purified DNA from the three selected *hwr* mutants and the wild type parent was sequenced by the Beijing Genome Institute.

Illumina sequencing was applied using paired-end with final read lengths of 150 bp after demultiplexing and produced 78.4-87.5 million read pairs per sample, which leads to an average coverage >1000 reads per reference base.

The raw reads were trimmed using Trimmomatic (v0.32; default setting except: HEADCROP=10, SLIDINGWINDOW=4:30, MINLEN=30) [90]. The trimmed reads were aligned to the *S. pombe* genome (version ASM294v2.38) with 'bwa mem' (v0.7.15; default settings except: -M) [91] and the resulting alignments locally refined using Stampy (v1.0.32; default settings except: --sensitive--

544 bamkeepgoodreads -M) [92] for higher mapping sensitivity. With Picard tools (v4.0.1.1)
 545 (<http://broadinstitute.github.io/picard>) the alignments were coordinate sorted (sortSam) and read
 546 duplicates annotated (MarkDuplicates). The alignment rate was >99% for all four samples.
 547 To call variations (SNPs, insertions and deletions) between wild type cells and the three independent
 548 *hwr* mutants we used GATK LeftAlignIndels followed by GATK Mutect2 (v4.0.1.1; default settings
 549 except: -ploidy 1 -tumor <Mutant> -normal <Wild-type>) [93, 94]. The Mutect2 generated list of
 550 putative mutations were quality filtered with 'bcftools filter' (v1.3.1; settings: 'AF[0]>0.75').
 551 Mutations were also called on the alignment files using Freebayes (v1.2.0; default settings except: --
 552 ploidy 1 --min-alternate-count 5 --min-alternate-fraction 0.4) followed by quality filtering by 'bcftools
 553 filter' (settings: 'AO>200 && ODDS>100 && GL[*]<-100 && AC=1') [95]. Mutect2 and Freebayes
 554 agree on the main findings. The mutations were annotated to genes (ENSEMBL, *S. pombe* version
 555 ASM294v2.38) using ANNOVAR (version: July 17 2017) [96]. Comparison to the reference genome
 556 revealed that our strains contained two mutations on chromosome II (A153954T and T153964A) in
 557 the *mal1* gene encoding maltase alpha-glucosidase resulting in a variant of Mal1 containing an R131R
 558 silent and F135I missense substitution.
 559 Three mutations were selected for PCR validation, and multiple alignments in ClustalW comparing
 560 the annotated full length gene, the wild type PCR sequences and the *hwr* PCR sequences all verified
 561 the predicted mutations.
 562 PCR sequencing was performed by Eurofins. The used primers were: *mkh1* forward:
 563 GATAATGTGTATGACAATGACGC, *mkh1* reverse: GCATGTCGTAAAGATTCGTG, *pek1*
 564 forward: TGACGTCAAAAGGTCAAGTG, *pek1* reverse: CTAATCAGACCAGACTTGACG, *pck2*
 565 forward: AAGCCCAGATGGTCATG, *pck2* reverse: GGATGAGTCATGACATCTTCTG.

566

567 *Electrophoresis and blotting*

568 Whole cell lysates were prepared with TCA and glass beads as described [84]. Proteins were resolved
569 by SDS-PAGE on 12% acrylamide gels and subsequently transferred to nitrocellulose membranes
570 (0.2- μ m pore size, Advantec, Toyo Roshi Kaisha Ltd.). The blots were blocked in PBS (133 mM
571 NaCl, 2.7 mM KCl, 6.5 mM Na₂HPO₄, and 1.5 mM KH₂PO₄ (pH 7.4)) with 5% fat-free milk powder
572 and 0.01% Tween 20. The antisera, diluted 1:1000, were as follows: anti-HA (clone 3F10, Roche),
573 anti-P-p44/42 (Phospho-p44/42 MAPK #9101, Cell Signaling Technology), and anti- α -tubulin (clone
574 TAT1, Abcam). Secondary HRP-conjugated antibodies were from Dako Cytomation. Blots were
575 developed using the Pierce ECL Plus detection kit (Thermo Scientific).
576

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584 **Competing interests**

585 No competing interests declared.

587 **Author contributions**

588 C.K., C.H., M.K., S.K.G., M.H.L., I.J. conducted the experiments. C.K., C.H., I.J., M.K., M.H.L.,
589 B.B.K., J.V.J., and R.H.P. analyzed the data. C.K., J.V.J., H.N.B., M.H.L., J.R.W. and R.H.P.
590 designed the experiments. J.R.W. and R.H.P. conceived the study. C.K., J.R.W. and R.H.P. wrote the
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Table 1

<i>Gene deletions scored as D₂O hypersensitive in high-throughput screen</i>		
Systematic ID	Gene name	Function
<i>SPAC13G7.03</i>	<i>upf3</i>	up-frameshift suppressor 3 family protein
<i>SPBC1539.08</i>	<i>arf6</i>	ADP-ribosylation factor, Arf family Arf6
<i>SPAC2F3.02</i>	-	ER protein translocation subcomplex subunit
<i>SPAC328.01c</i>	<i>msn5</i>	karyopherin/importin beta family nuclear import/export signal receptor
<i>SPAPB2B4.02</i>	<i>grx5</i>	mitochondrial [2Fe-2S] cluster assembly & transfer glutaredoxin Grx5
<i>SPAC4D7.03</i>	<i>pop2</i>	F-box/WD repeat protein Pop2
<i>SPCC970.10c</i>	<i>brl2</i>	ubiquitin-protein ligase E3 Brl2
<i>SPBC18H10.06c</i>	<i>swd2</i>	Set1C complex subunit
<i>SPBC19C7.02</i>	<i>ubr1</i>	N-end-recognizing protein E3 Ubr1
<i>SPAC17H9.10c</i>	<i>ddb1</i>	Cul4-RING E3 adaptor Ddb1
<i>SPAC26H5.05</i>	<i>mga2</i>	IPT/TIG ankyrin repeat transcription regulator of fatty acid synthesis
<i>SPBC106.10</i>	<i>pka1</i>	cAMP-dependent protein kinase catalytic subunit Pka1
<i>SPAC6F6.01</i>	<i>cch1</i>	plasma membrane calcium ion import channel Cch1
<i>SPAC3H8.02</i>	<i>csr102</i>	Sec14 family, phospholipid-intermembrane transfer protein Csr102
<i>SPBC13G1.08c</i>	<i>ash2</i>	Ash2-trithorax family protein
<i>SPCP1E11.06</i>	<i>apl4</i>	AP-1 adaptor complex gamma subunit Apl4
<i>SPAC10F6.13c</i>	<i>caa1</i>	cytoplasmic aspartate aminotransferase Caa1
<i>SPBC23G7.08c</i>	<i>rga7</i>	RhoGAP, GTPase activating protein Rga7
<i>SPBC27B12.08</i>	<i>sip1</i>	Pof6 interacting protein Sip1, AP-1 accessory protein
<i>SPBC21C3.20c</i>	<i>git1</i>	C2 domain protein Git1
<i>SPAC806.07</i>	<i>ndk1</i>	nucleoside diphosphate kinase Ndk1
<i>SPAC644.06c</i>	<i>cdr1</i>	NIM1 family serine/threonine protein kinase Cdr1/Nim1
<i>SPBC16E9.13</i>	<i>ksp1</i>	serine/threonine protein kinase Ksp1
<i>SPCC1739.14</i>	<i>npp106</i>	nucleoporin Npp106
<i>SPAC7D4.06c</i>	<i>alg3</i>	dolichol-P-Man-dependent alpha(1-3) mannosyltransferase Alg3
<i>SPBC3B9.11c</i>	<i>ctf1</i>	mRNA cleavage and polyadenylation specificity factor subunit Ctf1
<i>SPAC2F7.07c</i>	<i>cph2</i>	Clr6 histone deacetylase associated PHD protein Cph2
<i>SPBC16E9.08</i>	<i>mcp4</i>	prospore membrane protein Mcp4/Mug101
<i>SPAC926.03</i>	<i>rlc1</i>	myosin II regulatory light chain Rlc1
<i>SPBC13G1.03c</i>	<i>pex14</i>	peroxisomal docking protein Pex14
<i>SPBC776.15c</i>	<i>kdg2</i>	dihydrolipoamide S-succinyltransferase Kdg2
<i>SPAC513.03</i>	<i>mfm2</i>	M-factor precursor Mfm2
<i>SPAC26F1.04c</i>	<i>etr1</i> *	enoyl-[acyl-carrier protein] reductase
<i>SPBP8B7.11</i>	<i>nxt3</i>	ubiquitin protease cofactor Nxt3
<i>SPAC12B10.12c</i>	<i>rhp41</i>	DNA repair protein Rhp41
<i>SPCC777.10c</i>	<i>ubc12</i>	NEDD8-conjugating enzyme Ubc12
<i>SPAC4H3.13</i>	<i>pcc1</i>	EKC/KEOPS complex subunit Pcc1
<i>SPAC5D6.05</i>	<i>med18</i>	mediator complex subunit Med18
<i>SPCC970.06</i>	<i>erv29</i>	COP II adaptor Erv29

829 *: Upregulated in RNA seq. at 24 hours in D₂O.

830

Table 2

Mutect2 predicted genome sequence differences in the hwr strains

Mutant	Chr.*	Nucleotide change*	Location	Gene	Type
<i>hwr1-1</i>	II	A4313777del	exon	<i>pek1</i>	frameshift
<i>hwr1-1</i>	III	G2337547A	exon	<i>trm72</i>	missense (G253E)
<i>hwr2-1</i>	I	613724CTATGTTCAAGins	exon	<i>mkh1</i>	frameshift
<i>hwr2-1</i>	I	1670566Tins	splicing	<i>gta2</i>	-
<i>hwr2-1</i>	I	T5448804del	5' UTR	<i>SPAC1039.02#</i>	-
<i>hwr3-1</i>	I	G3273325C	ncRNA	<i>SPNCRNA.899</i>	-
<i>hwr3-1</i>	II	C2310230T	exon	<i>pck2</i>	missense (G870S)

*: Chr.: chromosome, del: deletion, ins: insertion; #: Downregulated in RNA seq. after 5 hours in D₂O.

834 Figure legends

835

836 Figure 1 – *D₂O* inhibits cell growth.

837 (A) Cell growth of wild type (no marker) *S. pombe* cells in rich medium was followed by measuring
838 the turbidity of the culture at the indicated concentrations of *D₂O*. The error bars indicate the standard
839 deviation (n=3). (B) From growth experiments as shown in (A), the doubling time of wild type *S.*
840 *pombe* cells in exponential phase was determined at the indicated concentrations of *D₂O*. The error
841 bars indicate the standard deviation (n=3). (C) A close up of the growth curves shown in (A) during
842 the first three hours of culturing at the indicated concentrations of *D₂O*. Note that the growth
843 retardation caused by *D₂O* occurs rapidly. The error bars indicate the standard deviation (n=3). (D)
844 The viability of wild type cells grown at 0% and 100% *D₂O* was determined by VitaBright staining
845 and quantified by automated fluorescence microscopy. The error bars indicate the standard deviation
846 (n=3). (E) The growth of wild type cells, pre-incubated for 5 hours at 0, 50, 75 or 100% *D₂O* in liquid
847 YES media, was compared by serial diluting and spotting onto solid rich media agar plates with the
848 indicated concentrations of *D₂O* at 30 °C. (F) The growth of wild type cells was compared on media
849 prepared with *D₂O* or *H₂O* with either hydrogenated glucose (Glu (H)) or deuterated glucose (Glu
850 (D)) by serial diluting and spotting onto solid rich media or synthetic minimal media agar plates at
851 30 °C.

852

853 Figure 2 – *D₂O* affects cell morphology and causes cell septation defects.

854 (A) Wild type cells grown at 0% and 100% *D₂O* for the indicated times were stained with calcofluor
855 (to mark the septa) and Hoechst (to mark the nucleus) and analyzed by microscopy. Note that the
856 thickness of the septa is increased in the presence of *D₂O*. Bar = 5 µm. (B) The percentages of septated
857 cells (septation index) and of septated cells with multiple septa were determined after 5 and 24 hours
858 at the indicated concentrations of *D₂O*. The error bars indicate the standard deviation (n=4). (C)
859 Transmission electron microscopy images of wild type cells after 24 hours in 0% or 100% *D₂O* YES
860 medium. Note the irregular septa and thickening of the cell wall (black arrow head). Bar = 5 µm. (D)
861 Transmission electron microscopy images of wild type cells after 24 hours in 0% or 100% *D₂O* YES
862 medium as in panel A. Bar = 5 µm. (E) Cell lysis of wild type (no marker) cells grown at the indicated
863 *D₂O* concentrations for 24 hours was recorded over time by measuring turbidity of cultures treated
864 with β-glucanase (in water). The error bars indicate the standard deviation (n=4).

865

866 Figure 3 – *D₂O* inhibits glucose metabolism.

867 (A) Hyperpolarized ¹³C NMR spectrum of cellular metabolite extract following incubation with the
868 isotope labelled glucose tracer for 10 minutes in *D₂O* buffer (red spectrum) and *H₂O* buffer (black
869 spectrum). Red insert shows a zoom-in on the carbonyl region showing gluconate (GA) and 6-
870 phosphogluconate (6PGA). The asterisk marks an unidentified metabolite. Blue insert shows a zoom-
871 in on the aliphatic sugar region, revealing a multiplet centred at 61.2 ppm originating from 6-¹³C-
872 glucose. The multiplet at 62.8 ppm originates from the α-form of glucose-6-phosphate (α-G6P) in the
873 spectrum from the *D₂O* experiments (red) and from the β-form of glucose-6-phosphate (β-G6P) in

the spectrum from the H₂O experiments (black). (B) Quantitative difference between accumulated glucose-6-phosphate (G6P) signal in D₂O and H₂O exposed cells following incubation with ¹³C₆-d₇-labelled glucose for 10 minutes. The error bars indicate the standard deviation (n=4). (C) Glucose metabolic pathways when *S. pombe* cells are exposed to D₂O and H₂O. Metabolites identified in panel A are highlighted in red. Glucose in its β-form is in both D₂O and H₂O buffers converted into gluconate (GA) and further into 6-phosphogluconate (6PGA) in the pentose phosphate pathway (PPP). In D₂O, the α-form of glucose-6-phosphate (α-G6P) is accumulating whereas the β-form is metabolized more rapidly. Contrary, in H₂O, no accumulation of α-G6P can be detected. An explanation for this difference is an altered activity of glucose-6-phosphate isomerase (GPI, EC 5.3.1.9) when the cells are exposed to D₂O. This enzyme catalyzes the isomerization reactions between α-G6P and β-G6P, α-G6P and β-F6P, and β-G6P and β-F6P (blue reactions).

Figure 4 – RNA sequencing of cells treated with D₂O.

(A) Total RNA was sequenced from wild type (no marker) cells that were either untreated (0% D₂O) or treated with 100% D₂O for 5 or 24 hours in quadruplicates. Heatmap showing hierarchical clustering on the Euclidian distances between full set of gene counts for all samples (after DESeq2's own log transformation and variance stabilization). (B) Plot showing the first two principal components from a principal component analysis (PCA) on the full set of gene counts. (C) Heatmap of CESR genes as defined by Chen *et al.* for D₂O regulated genes, *mas5Δ*, *ssa2Δ*, heat shock [97], *sty1Δ*, *atf1Δ* [60], and *bag101* overexpression (OE) [98]. The combined datasets were quantile normalized to make them comparable. Color key including value frequency is shown next to the heatmap. Note that the D₂O response resembles the heat stress, *mas5Δ*, *ssa2Δ* and *bag101* OE response, but not that in the other strains. (D) Plot showing the first two principal components from a principal component analysis (PCA) on the same data shown in (C). (E) The growth at 30 °C of wild type (no marker) cells that were either untreated (control) or subjected to a 30 minute heat shock at 43 °C was compared in the presence of the indicated concentrations of D₂O by serial diluting and spotting onto solid rich media. (F) The growth at 30 °C of wild type cells that were either untreated (control) or exposed to 0.07 mM H₂O₂ for 1 hour, was compared in the presence of the indicated concentrations of D₂O by serial diluting and spotting onto solid rich media. (G) Wild type cells that were either untreated (control) or subjected to 5 hours incubation in rich medium containing 100% D₂O were challenged with the indicated heat shock conditions prior to spotting onto solid rich media at 30 °C. (H) The growth of wild type cells that were either untreated (control) or subjected to 5 hours incubation in rich medium containing 100% D₂O was compared in the presence of the indicated concentrations of H₂O₂ by serial diluting and spotting onto solid rich media at 30 °C.

Figure 5 – D₂O sensitive and D₂O resistant *S. pombe* mutants.

(A) The growth of a 3 marker wild type strain and the otherwise isogenic *caa1Δ* were compared in the presence of the indicated concentrations of D₂O by serial diluting and spotting onto solid rich media at 30 °C. (B) The growth of a 3 marker wild type strain and the otherwise isogenic deletion mutants were compared in the presence of the indicated concentrations of D₂O by serial diluting and spotting onto solid rich media at 30 °C. (C) Schematic representation of pairwise genetic or physical

interactions listed in the BioGRID database (version 3.4.164) between the deleted genes/proteins in the isolated D₂O hypersensitive strains (grey) and D₂O tolerant strain (Pmk1, black).

Figure 6 – D₂O activates the cell integrity pathway.

(A) The growth of 3 marker wild type cells and the isolated *hwr* mutants was compared by serial diluting and spotting onto solid rich media agar plates. (B) The cell integrity pathway in *S. pombe* cells as described by Madrid *et al.* [47]. The pathway is known to be activated by salt stress or cell wall damaging agents (red arrows), and consists of small GTPases that activate Pck1 and Pck2. In turn, this leads to activation of Mkh1, Pek1 and finally of the MAPK Pmk1 which ultimately triggers increased cell wall synthesis. Rga7 functions as a Rho2 GAP that limits signaling [43]. The signaling components identified with mutations in the *hwr* strains are shown in green. The D₂O hypersensitive phenotype observed for the *rga7Δ* strain is shown in red. (C) The growth of 3 marker wild type cells and the indicated mutants in the cell integrity pathway was compared by serial diluting and spotting onto solid rich media agar plates at 25 °C. (D) Wild type cells (3 marker) at 100% D₂O for 24 hours were stained with calcofluor and analyzed by microscopy. Bar = 5 μm. (E) Whole cell lysates of wild type cells expressing HA-tagged Pmk1 were analyzed by SDS-PAGE and Western blotting using antibodies to phosphorylated Pmk1 (P-p44/42), the HA-tag on Pmk1 and, as a loading control, to α-tubulin. Note that the level of phosphorylated Pmk1 is increased in response to D₂O.

Figure 1

Figure 3

A

B

C

Figure 3

A

B

C

Figure 6