Programmed bet hedging in yeast responses to osmotic stress

Yoshikazu Hirate[1], Samuel Bottani[2] and Suzannah Rutherford[1]

1 Fred Hutchinson Cancer Research Center, Seattle, WA, 2 Laboratoire Matière et Systèmes Complex, Paris Diderot, Paris, France

Abstract

Rapid reproduction contributes to evolutionary fitness but can be lethal under stress. Microbes are thought to limit risk in clonal populations by bet hedging; the stochastic expression of a low frequency of slow growing cells constitutively resistant to unpredictable environmental stresses including antibiotics. However fitness depends on rapid recovery and resumption of growth in potentially lethal environments whose severity and duration are also unpredictable. Here we describe trade-offs between osmotic stress-responsive signaling, survival and proliferation in 50 ecologically distinct strains of budding yeast. By contrast with prior examples, programmed bet hedging responses were heritable, stress-specific and varied continuously in our population. During rapid growth strong osmotic stress signaling promoted survival. Weak signaling predicted lower viability, intense rebound signaling, and robust recovery. Older cultures survived and adapted to unprecedented stress with fitness depending on reproducible, strain-specific proportions of cells with divergent strategies. The most 'cautious' cells survive extreme stress without dividing; the most 'reckless' cells attempt to divide too soon and fail, killing both mother and daughter. Heritable proportions of cautious and reckless cells generate a tunable, rapidly diversifying template for microbial bet hedging that resembles natural variation and would evolve in different patterns of environmental stress.

In order to understand the evolutionary trajectories of populations and species we need to understand the effects of natural genetic variation on mechanisms of development and expression of phenotypic variation. The mapping between genetic variation and the spectrum of attributes and behaviors upon which selection acts defines population-level properties such as the capacity to evolve (evolvability), the capacity to withstand genetic and environmental perturbation (robustness or canalization), and the optimization, within a given genotype, of phenotypic responses across different environments (reaction norms)[1–3]. Here we describe strain-specific differences in hyperosmotic stress responsive signaling and associated behaviors in a synthetic population of budding yeast.

The high osmolarity glycerol (HOG) signaling pathway is central to an elaborate stress response that reduces cellular damage and death in unpredictably changing osmotic environments where the balance between external solutes and free water pressure in the cell can change suddenly[4]. A main function of the HOG pathway is the production and accumulation of intracellular glycerol, which restores water balance and, as demonstrated by a large body of work from many labs, is essential for survival, adaptation and proliferation in hyperosmotic stress[4–9]. In the wild, yeast and other microorganisms must balance immediate survival against evolutionary fitness. Multiplicative fitness favors clonal populations that respond as rapidly as possible to improved conditions with earlier cell cycle reentry and proliferation[10]. On the other hand, individual survival requires that cells carefully sense the amplitude and direction of environmental change to more safely reenter the cell cycle after stress[7]. The HOG pathway consists of at least two highly-conserved, multi-component osmotic stress sensors linked to a parallel series of at least 15 kinases and accessory proteins that ultimately alter the activity of nearly 10% of the yeast genome[4,6]. The sheer numbers of genes involved in HOG signaling, their conservation, and their elaborate circuitry suggest that a nuanced response to osmotic stress has been crucial and strongly selected throughout evolutionary history. As the hyperosmotic stress response of budding yeast has well-characterized and accessible signaling and phenotypic traits that can be measured in the lab and are almost certainly under strong selection in nature, this system is ideal for characterizing the mapping between signaling and behavior in a diverse population[4,6,7].

We measured osmotic stress signaling, survival and adaptation in both exponentially growing and nearly quiescent cultures of diploid yeast. As glycerol-3-phosphate dehydrogenase (*GPD1*) is rate-limiting for glycerol production[11], the synthesis and accumulation of green fluorescent protein (GFP) integrated into the gene for *GPD1* was a proxy for HOG pathway activity. A synthetic population of diverse yeast genotypes was made by crossing *GPD1*::GFP in the genetic background of a standard laboratory strain (BY4742 Mat alpha) to a panel of wild and industrial genetic backgrounds –e.g. fifty diverse haploids of the opposite mating type extracted from globally diverse, sequence-validated *Saccharomyces cerivisiae* diploid strains deposited to the collection of the Royal Netherlands Academy of Arts and Sciences over the past 100 years (CBS; Table 1 and Table S1).

77 **HOG signaling and behavior in young cultures**

78 The behavior of single cells before and after their exposure to osmotic stress was followed by
79 time-lapse video microscopy of monolayer cultures in custom microfluidics devices[12]. When cells
80 in exponential growth were exposed to sudden hyperosmotic stress, cell volume decreased, cell
81 division and budding immediately stopped, and daughter cells retracted[13]. After a lag period
82 proportional to the severity of the stress GFP fluorescence driven by the *GPD1* promoter began
83 to accumulate in the cytoplasm of surviving cells. Cells that did not accumulate *GPD1*::GFP to
84 high levels did not survive or adapt, developed large vacuoles, and began to die, remaining in
85 view as shrunken cell ghosts. As GFP accumulated to saturation levels in the surviving cells,
86 they adapted to the higher osmotic pressure, resumed cell division, budded and began to divide
87 with a longer half time, producing daughter cells with similarly high fluorescence[13].

88 Viability per culture and *GPD1*::GFP accumulation per cell were measured using flow
89 cytometry of statistically large numbers of cells from all 50 strains (~10,000 cells / sample; Figure
90 S1). The rate and extent of mean *GPD1*::GFP accumulation in exponentially growing cultures
91 exposed to hyperosmotic media depended on the severity of the stress and the genetic
92 background of each strain (Figure 1A). Prior to the osmotic stress mean *GPD1*::GFP
93 fluorescence and viability were uncorrelated. After 2 hours in moderate 0.75 M KCl viability
94 decreased and became steeply correlated with accumulated *GPD1*::GFP (Figure1B and C). As
95 expected, natural variation in the strength of HOG signaling was directly responsible for variation
96 among the strains in osmotic stress survival.

97

98 **Negative feedback drives a robust recovery**

99 The initially strong positive correlation between variation in *GPD1*::GFP accumulation and
100 variation in viability reversed as cells adapted and began to divide (Figure 1C; 4 hours). This
101 distinguished two phases of the response, an early phase (0 – 2 hours) when viability decreased
102 markedly and acute HOG signaling promoted osmotic stress survival and a later phase (2 – 4
103 hours) when viability recovered but became negatively correlated with HOG signaling and
104 *GPD1*::GFP accumulation. The switch from positive to negative correlations might have indicated
105 that stronger HOG signaling, initially beneficial, suddenly caused lower viability. However we
106 think it likely that negative feedback increased signaling in the surviving cells of the less viable
107 strains. Negative feedback controls, occurring at many levels and timescales, are present in
108 essentially all of the varied mechanisms that act in concert to increase intracellular glycerol and
109 restore water balance. For example (1) unequal water pressures activate osmotic stress sensors,
110 glycerol channels and other pressure-sensitive components whose activities control and depend
111 on water balance (e.g. see Figure 5 in Hohman 2002[4,6]), (2) GPD1p indirectly controls and is
112 controlled by osmotic stress-sensitive kinases that respond to upward and downward changes in
113 water balance[14], and (3) nuclear Hog-1 MAP kinase increases the transcription of phosphatases
114 that restore its own cytoplasmic localization and basal activity[15–17].

115 Consistent with acting negative regulation, we found a strong and highly significant
116 correlation between early mortality (0 – 2 hour decreases in viability) and later accumulations of
117 *GPD1*::GFP (2 – 4 hours; Table 2). We reasoned that cells and strains that adapt quickly
118 experience lower and less sustained effects of osmotic stress (e.g. water loss) with more rapidly
119 attenuated HOG pathway activity and lower *GPD1*::GFP accumulation. Conversely, surviving
120 cells of strains that were slower to adapt and less viable would experience higher and more
121 sustained osmotic stress (and likely other stresses). Prolonged osmotic stress would sustain
122 HOG signaling and maintain *GPD1* transcription – also activated by general stress responses[18] –
123 further promoting *GPD1*::GFP accumulation (e.g. negative feedback regulation of viability at the
124 level of general stress responses). Indeed, even as *GPD1*::GFP and viability became negatively
125 correlated, their *rates of change* remained positively correlated (2 – 4 hours; Table 2) prompting
126 a parsimonious interpretation that osmotic stress signaling promotes adaptation and viability
127 during both the initial and recovery phases of the response (Figure 1C, insets).
128 After 4 hours all strains had adapted to a new steady state in 0.75M KCl and later
129 viability remained largely unchanged (Figure 1C inset, lower right). Interestingly, initial decreases
130 in steady-state viability (0 – 2 hour mortality) were almost perfectly restored by 4 hours (Figure
131 1D). Notably, by 6 hours early mortality and recovery were 98% correlated (R^2 = 0.9852,
132 P<0.0001; not shown). The biological robustness of adaptation and complete recovery of
133 steady state viability further support the idea that negative feedback restores viability through
134 continued activation of stress responses. Indeed, continued accumulation of *GPD1* and glycerol
135 – directly responsible for restoration of water balance and reduction of osmotic stress –suggests
136 that the accumulation of glycerol integrates the cumulative activities of many facets of the
137 osmotic stress response (e.g. a plausible biological mechanism for "integral feedback"[19]). The
138 presence of integral feedback virtually assures perfect adaptation[17,19]. However, despite their
139 resilience, strains that were relatively slower to adapt would be ultimately less fit than rapidly
140 adapting strains due to their higher death rate, slower recovery, and lower steady-state viabilities
141 before and after hyperosmotic shifts.
142
143 **Extreme stress resistance of older cultures**
144 By contrast with exponential cultures, when the aging yeast cultures (post-diauxic) were exposed
145 to hyperosmotic media they survived and adapted after long periods in unprecedented conditions
146 (Movies 1 and 2). As aging cultures deplete available glucose in their media they undergo a
147 metabolic change called the diauxic shift[20]. During post-diauxic growth stress response proteins
148 accumulate, cell division slows and then stops, and cells enter quiescence[21]. Post-diauxic
149 cultures survived up to 5 weeks in 3 M KCl (41/50 strains). They could not adapt and did not
150 grow in 3 M KCl, but recovered rapidly and grew when plated on fresh isotonic media ('static
151 viability'; Figure S2). When we tested their limits of adaptation in increasing concentrations of KCl
152 all but one strain could grow on 2.6 M KCl media and, remarkably, three strains grew on media

153 containing 2.9 M KCl (Table 3). We are unaware of previous reports of such extreme osmotic
154 stress survival or adaption limits for budding yeast of any growth stage or genotype.
155
156 **Heterogeneity of older cultures**
157 By contrast with cultures in exponential growth, in post diauxic growth the genetically identical
158 cells within each strain and culture were surprisingly heterogeneous in size, shape and signaling
159 behaviors (compare Figures S1 and S3). Neither total *GPD1*::GFP fluorescence nor rates of
160 change in fluorescence was strongly correlated with viability. After several hours in 2.5 M KCl
161 *GPD1*::GFP increased sharply in one group of cells as they began to divide. More surprising,
162 other cells also induced *GPD1*::GFP to high levels, started to divide and then popped, killing both
163 the mother and daughter (Movie 3). Another group of cells had slower signaling and cell division
164 while the most 'cautious' cells failed to signal or divide but remained in a cellular state of static
165 viability without dividing.
166 We used a Gaussian mixture model to assign the cells in each sample to four
167 Gaussian distributions described by eight parameters – means and covariances – numbered
168 according to increasing levels of fluorescence (G_0-G_3 distributions; Figure S3). Only
169 *GPD1*::GFP pre-accumulated into cells of the G_3 distribution during post-diauxic growth *prior* to
170 the osmotic challenge and therefore unrelated to osmotic stress signaling, predicted survival at
171 any time. The expression of a low frequency of cells in each strain expressing stress resistance
172 in rich media and normal growth conditions could be analogous to previously-defined stochastic
173 bet hedging in yeast and bacteria[22,23]. The amount of *GPD1*::GFP accumulated in G_{3_0} cells
174 predicted early but not later viability, and this relationship was better fit by 2nd order quadratic
175 rather than linear functions of *GPD1*::GFP, suggesting early survival was higher in strains with
176 intermediate G_3 level signaling (more variation explained and lower mean square errors; Table
177 S3). Despite the fine-scaled characterization of osmotic stress signaling behaviors of different
178 groups of cells in each strain, none of the distributions learned by the Gaussian mixture model –
179 neither pre-accumulated G_{3_0}, total *GPD1*::GFP fluorescence, nor stress-induced *GPD1*::GFP in
180 any distribution – embodied features of the osmotic stress signaling that were important for later
181 survival.
182
183 **Continuous variation in signaling behavior**
184 In order to map osmotic stress-responsive signaling onto survival more directly we next
185 quantified osmotic stress signaling behaviors of cells in each strain directly, as they unfolded
186 over time, unbiased by Gaussian assumptions or approximations. First, normalized levels of
187 induced *GPD1*::GFP were binned (as in a histogram), creating a 'sample vector' of cell numbers
188 in each of 100 successive intervals of fluorescence intensity. Next, sample vectors for increasing
189 time points in each strain were linked to create time line vectors (700-mers). For comparison of
190 averaged strain behaviors the time line vectors were clustered using a mean distance matrix,
191 constructed from 17,000 randomly chosen permutations of the data over replicate samples and

192 times (mean clusters; see methods). Finally, the fraction of permutations in which each strain
193 grouped with more than half of the other strains in its mean cluster was used to rank that strain's
194 behavior relative to the other strains in its group (clustering statistics; Table 4).
195 The unique signaling trajectories of most strains were highly reproducible (Figure S3).
196 Based on their shared and strain-specific (heritable) signaling behaviors the 50 strains rapidly
197 converged onto two large groups made up of six mean clusters (Figure 2). Each strain could be
198 further ordered within and between mean clusters and clades based on their clustering statistics,
199 with their rank order describing increasingly rapid accumulations of *GPD1*::GFP and 'reckless'
200 signaling (Figure S4). Tellingly, both mean cluster and rank now predicted viability over time
201 (Figure S5 and Table 4; respectively) thereby confirming the biological relevance of 'cautious'
202 versus 'reckless' osmotic stress signaling, validating the clustering method and supporting the
203 role of natural osmotic stress signaling differences between strains in shaping variation in fitness
204 during osmotic stress.
205
206 **Evidence for bet hedging**
207 As cautious and reckless behaviors were found both within and between strains, we wondered
208 whether bet hedging, the expression of alternate, conditionally-adaptive phenotypes within a
209 clone of genetically identical organisms[10,24–27], could explain the observed variation in osmotic
210 stress signaling and survival. Indeed, rank predicted survival in several different osmotic
211 environments. Higher-ranked strains with more aggressive osmotic stress signaling strategies
212 were favored in milder conditions, but with increasing time in extreme osmotic stress more
213 cautious strains and behaviors became more fit (Table 4). For example, W178 at rank 50 was
214 most viable in moderate 0.75 M KCl, but the optimum rank shifted to 25 after 20 hours in 2.5 M
215 KCl, 20 after 72 hours, and 9.6 after 168 hours (1 week). Viability after 168 hours decreased
216 most markedly among the most reckless strains (Figure S5).
217 Demonstration of differential relative fitness of strains with more cautious behavior
218 under increasing osmotic stress constitutes strong empirical evidence for bet hedging[25]. To
219 confirm that the relative fitness of 'cautiousness' was really correlated to the amount of stress, we
220 tested the idea that the yeast experienced increasing stress over time in severe osmotic
221 conditions. We again incubated cultures in 2.5 M KCl for 168 hours, but they were first exposed
222 to a mild pre-stress (2 hours in 0.5 M KCl) to pre-induce osmotic stress proteins and make them
223 more resistant to subsequent stress. If the optimum rank depended solely on time independent of
224 the degree of stress experienced by different strains, then it should be unaffected by the short
225 pre-stress. However optimum rank was shifted toward more reckless behaviors (rank 9.6 to rank
226 18; $P<0.0001$) after the pre-stress and viability increased by ~10% as expected if the pre-treated
227 cells experienced lower osmotic stress. After 168 hours in 2.5 M KCl the most reckless cells in
228 the highest-ranking strains began to selectively disappear. For example, replicate cultures of
229 strain W242 (rank 49) that had lower viability also had fewer cells with high accumulations of
230 *GPD1*::GFP, smaller G3 distributions, and correspondingly larger distributions with lower mean

231 *GPD1*::GFP (Figure 3A). The loss of cells with the highest accumulations of *GPD1*::GFP could
232 indicate that *GPD1*::GFP levels simply decrease over time. However, G3 distributions were
233 stable over most time points and in most strains. More likely, after 168 hours in 2.5 M KCl the
234 most aggressive cells in the highest-ranking strains attempt to divide and fail (e.g. Movie 3). This
235 suggests that rapid signaling and adaptation, a fitness advantage in milder conditions, becomes
236 a liability in severe or prolonged osmotic stress. On the other hand static viability – the survival of
237 non-dividing cultures in 3 M KCl (Figure 3B) – would usually have dramatically reduced
238 evolutionary fitness, but allows more cautious cells and strains to survive severe stress.
239

240 **Evolution of bet hedging**

241 Heritable osmotic stress signaling behavior predicted survival in increasing severity and duration
242 of osmotic stress. Since cautious and reckless strains reliably expressed a range of cells with
243 different behaviors and fitness depending on the environment we wondered if a simple, 2-state
244 bet hedging model including a heritable control of the relative proportions of cautious and
245 reckless cell types could account for the observed variation in osmotic stress signaling and
246 explain the complex relationship between rank and viability. If more aggressive osmotic stress
247 signaling with rapid recovery and resumption of growth in potentially lethal environments were
248 the default, ancestral behaviors, then cautious signaling and behavior could have arisen as a
249 later, adaptive response to the unpredictable severity and duration of potentially lethal osmotic
250 environments. We therefore assumed that the heritable probability of daughters with cautious
251 signaling and behavior (P) was the derived, bet-hedging trait and asked whether it could evolve.
252 We considered changes between three very general osmotic stress environments that
253 discriminate cautious versus reckless behaviors: (E0) a permissive environment in which both
254 cautious and reckless cells grow equally well, (E1) a restrictive environment approximating
255 moderate osmotic stress reckless cells divide and cautious cells survive without dividing, and
256 (E2) a killing osmotic stress where reckless cells die and cautious cells survive without cell
257 division. After several generations including each of 9 possible environmental shifts between
258 the three environments, we calculated the relative fitness (cell numbers) of each strategy
259 (probability (P) of cautious cells where $0 \leq P \leq 1$). Most environmental shifts favor optimum
260 strategies of either all cautious ($P = 1$) or all reckless cell types ($P = 0$; Figure 3D). Strictly
261 intermediate strategies ($0 < P < 1$) and bet hedging prevail only when the osmotic environment
262 shifted from moderate to more severe, with the optimum P depending on the number of
263 generations in the first environment (E1 -> E2). Shorter lag periods – corresponding to less
264 severe osmotic conditions – and more cell divisions in E1 initially favor lower P and a higher
265 proportion of reckless cells. Longer lag periods – corresponding to more severe conditions and
266 fewer cell divisions – favor higher P and a higher proportion of cautious cells. Indeed,
267 worsening osmotic environments are common in nature (for example, during fermentation or
268 drying). A worsening environment was experienced with increasing time by the cells and strains
269 in our experiments; lower-ranked strains with more cautious signaling behaviors, longer lag

270 periods and fewer attempted cell divisions were increasingly fit over time and with increasing
271 severity of osmotic stress (Table 4).
272 While the 2-state model of bet hedging behaviors may be overly simplistic relative to up
273 to four learned distributions of cell types that best fit the data (Figure S3), it provides a conceptual
274 framework for understanding the observed variation in osmotic stress signaling strategies and
275 generates testable hypotheses for further studies. Bet hedging in microorganisms has been
276 previously thought to arise almost exclusively through stochastic switching with a low probability
277 of alternate phenotypes that are independent of the environmental challenge[10,22,23,28]. By
278 contrast, the variation in osmotic stress signaling and behavior we observe is heritable, occurs in
279 a programmed response of cells to the osmotic stress and, since strains have a low to high
280 frequency of cells displaying cautious behavior, is one of few known examples in a
281 microorganism similar to the rapidly diversifying, within-clutch bet hedging strategies of plants
282 and animals[10,28]. If the heritable probabilities of cautious cells and behavior we observed were
283 sculpted by selection, we reasoned that milder and/or slowly worsening osmotic environments
284 would generate more reckless strains (e.g. W242, rank 49 isolated from rotting banana; W282,
285 rank 48 isolated from fermenting fruit) and more severe or rapidly deteriorating osmotic
286 environments would generate more cautious strains and behavior (e.g. W455, rank 1 from
287 molasses; W217, rank 4 from sugar cane syrup). Indeed, even though our synthetic population
288 is represented by lab/wild heterozygotes rather than inbred strains, we found that the general
289 environment from which each strain was isolated weakly predicted its aggressiveness (but not
290 rank; mean percent of cells above threshold, $P = 0.030$ by ANOVA), plausibly suggesting
291 adaptation to growth in rotting fruit or wine fermentation favors more aggressive osmotic stress
292 behavior than growth in more hyperosmotic sugar cane syrups, molasses or olive wastes.
293 Classical evolutionary models assign fitness directly to genotypes, mutations, and
294 mean trait values without consideration of the genotype-to-phenotype map; molecular models
295 provide detailed mechanistic understanding of development but rarely consider the effects of
296 natural genetic variation. The osmotic stress response is extensively characterized in a few
297 strains and genotypes but until now osmotic stress signaling and behavior in a population had
298 not been documented. Labyrinthine developmental mechanisms – themselves controlled by
299 genetic variation – translate genotypes into phenotypes with a variable fidelity that allows for the
300 possibility of phenotypic heterogeneity and the evolution of bi-stable states[29]. Our view of
301 osmotic stress signaling and response on a backdrop of natural variation enabled identification of
302 negative feedback controlling a robust recovery of steady-state viability in exponential growth.
303 In post diauxic cultures continuous, heritable variation in the distribution of cautious to reckless
304 osmotic stress signaling is a risk spreading strategy. Microorganisms in nature including yeast
305 spend a high fraction of their time in post-diauxic or quiescent phases[21]. Our simple, 2-state
306 model demonstrates how cells and strains can balance constraints between survival and
307 evolutionary fitness through programmed bet hedging responses that are adapted to different
308 patterns of environmental stress.

309 **Materials and methods**

310 **Strain acquisition and deposition**

311 Wild and industrial diploid strains of *Saccharomyces cerevisiae* were obtained from the fungal
312 diversity collection of Centraalbureau voor Schimmelcultures (CBS), an institute of the Royal
313 Netherlands Academy of Arts and Sciences in Utrecht, Netherlands
314 (http://www.cbs.knaw.nl/index.php/collection). Strains modified for this report are listed in
315 Tables 1 and S1. They have been deposited to the Yeast Genetic Resources Lab of the
316 National BioResource Project in Osaka, Japan (http://yeast.lab.nig.ac.jp/nig/index_en.html).
317

318 **Creation of haploid *MATa* library of wild and industrial genotypes**

319 For unique identification of each strain and prevention of homothalism, the *HO* locus of each wild
320 or industrial diploid strain was deleted and replaced with the KanMX4 marker gene and unique
321 barcode sequences[30,31]. The KanMX4 gene was PCR-amplified for transformation of each strain
322 with primers containing unique sets of barcode sequences[32] (Table S1). Kanamycin-resistant
323 transformants were sporulated in 1% potassium acetate sporulation medium and *MAT**a*** haploids
324 were selected by testing for schmoo formation in 96-well plates containing alpha factor. Mating
325 type was confirmed by test mating with a dominantly marked *MATa* tester strain on replica plates.
326 The *URA3* gene of the sporulated wild haploids (*MAT**a***) was deleted using standard gene
327 deletion method. In brief, pJL164 plasmid DNA was transformed and cells were plated onto
328 YPD plate grown for overnight at 30°C. Cells were replica plated on 5-FOA and grown for 2
329 days at 30°C. The *ho* and *ura3* deletions and barcode sequences were verified by PCR and
330 resulting wild haploids were in many cases validated by their colony morphologies. Forty-nine
331 wild strains and a laboratory strain met these criteria (see Tables 1 and S1 for strain details).
332

333 **Construction of synthetic population of *GPD1*::GFP wild/lab diploids**

334 The common laboratory strain BY stably integrated *GFP* reporter strain was created by replacing
335 the *GPD1* gene with *GFP* gene using a deletion cassette containing a *URA3* marker for selection
336 of transformants on SC-URA plates[32,33]. Wild *MATa* haploids were mated with *MATalpha* GFP
337 reporter strains. Briefly, *MAT**a*** and *MATalpha* cells were mixed on SC-URA plates for 2 hours
338 and streaked onto selective SC-URA+G418 plates. To test the BY4742 background for low
339 function alleles that could critically compromise osmotic stress function, a homozygous
340 laboratory strain was made by crossing BY4742a to cells of the opposite mating type to create a
341 50th strain in our population (BY4741alpha; Table 2).
342

343 **Ploidy analysis**

344 Polyploids had higher GFP and tolerance to osmotic stress. To screen for triploids or
345 tetraploids obtained after mating, wild/lab yeast were inoculated into 2 ml YPD with appropriate
346 antibiotics and cultured for 24 h at 21°C on rotator after which 40 ul of the cultured cells were
347 then added to fresh 2 ml YPD and cultured for 4 h at 21°C on rotator. 800 ul of cells were fixed

348 with 1.7 ml cold ethanol and stored at -20°C at least 20 minutes, centrifuged at 2300 rpm for 5
349 min, washed with 100 mM Tris-HCl (pH 7.8) twice, and suspended in 500 ul of 100 mM Tris-HCl
350 (pH 7.8). To digest ribonucleic acids 10 ul of 10 mg/ml RNase A was added to cell suspension
351 and they were incubated for at least 60 min at 37°C. Digested cells were then centrifuged at
352 2300 rpm for 5 min and suspended in 500 ul of FACS buffer (0.5 M Tris-HCl, pH 7.5, MgCl,
353 NaCl.). To dissociate cell clumps cells, they were sonicated for 10 sec at setting 3 (Sonifier Cell
354 Disrupter, Model W185). Cells were stained with 30 ug/ml Propidium Iodide (PI). After leaving
355 them for 20 min in the dark, cells were examined for their ploidy with FACS Calibur (Becton
356 Dickinson).
357
358 **Plating assays**
359 Cells cultured in YPD with G418 for 4 d at 21 ^{o}C on rotator were diluted to OD_{600} 0.1 with
360 exhausted YPD and sonicated for 5 sec at setting 2.5. 5 ul of cells were plated on YPD with or
361 without KCl and incubated at 21 ^{o}C for 2 months. To determine adaptation limits at which strains
362 could resume growth in concentrations up to 3 M KCl, we inoculated PD cells in a 96-well plate,
363 agar media containing increasing concentrations of KCl ranging from 2 to 3 M. After 2-months
364 incubation at 21°C, we documented adaptation limit, the highest concentration of KCl in which
365 colonies could grow.
366
367 **Microfluidics**
368 We employed custom made microfluidics devices with two fluid inputs as described previously[12].
369 Experiments were run at ambient room temperature. Yeast cells were observed using Nikon
370 TS100 and recorded using a digital camera (Photometrics CoolSnap HQ2) operated by Metavue
371 (Molecular Dynamics). Analysis of acquired images was performed using Image J software.
372
373 **Flow cytometry**
374 After osmotic stress challenge 4 ml of PBS was added to each tube. Cells were isolated by
375 centrifugation and resuspended in 1 ml PBS, transferred to FACS tubes, sonicated, and stained
376 with 3 ug/ml propidium iodide (PI) to monitor viability. After 20 min GFP fluorescence and
377 viability were quantified in a FACS Calibur flow cytometer (Becton Dickinson). Data were
378 initially analyzed using magnetic windows in FlowJo software to eliminate cell fragments and
379 dead (PI-positive) cells.
380
381 **Flow cytometry data analysis**
382 Raw cell data were initially processed and trimmed to identify cells and the fraction of viable cells
383 using magnetic windows in FloJO data analysis software. Cell data for each sample including
384 forward scatter and GFP fluorescence data were then extracted into an executable SQL
385 database for analysis of single cell data. We performed multi-normal fits to extract quantitative
386 values of GPD1::GFP for the different cellular conditions of a strain at a given time point. The

387 exact number of distributions to be fit is a parameter that is not determined by the model and was
388 determined from the data. We found that fitting both *GPD1*::GFP and forward scatter worked
389 slightly better than *GPD1*::GFP in fitting the data, and that 4 distributions provided more stable
390 fits than 3 distributions.< Figure S3- fit distributions G0-G4). Multi-normal fits were performed with
391 the sklearn.mixture.GMM Gaussian Mixture Model algorithm of Python scikit package
392 (http://scikit-learn.org/stable/index.html). The exact number of distributions to be fit is a
393 parameter that is not determined by the model, not determined from the data. We found that
394 fitting both *GPD1*::GFP and forward scatter worked slightly better than *GPD1*::GFP in fitting the
395 data, and that 4 distributions was better (smaller ??) than 3 distributions.< Figure S3- fit
396 distributions G0-G4).
397
398 **Clustering**
399 We used a clustering algorithm of *GDP1*::GFP accumulations to characterize the signaling
400 behaviors of the strains over time. In brief, the *GPD1*::GFP distribution of each strain at every
401 time point was converted into a vector of cell density (numbers of cells) in a small section (100
402 sections) of a normalized density plot of cells at each time point. Two-dimensional clustering on
403 the Kullbak-Leibler distance function was used to follow the behavior of each strain over time.
404 Strains and timepoints were replicated between 4 and 15 times. To control for variation in
405 sampling and clustering outcomes samples were randomly drawn for each strain and time point
406 with equal probability for 17000 permutations.
407 Strains were ranked from most static signaling to most aggressive, providing a
408 continuous relationship between signaling, survival and adaptation with the number of
409 permutations in which strains clustered with a majority of other strains in their clade was used to
410 rank strains from the most static to the most aggressive. Computational sorting of time-series
411 distributions resolved 6 clades differentiated for rates of GFP accumulation, adaptation and
412 survival. We used a clustering algorithm of *GDP1*::GFP and forward scatter accumulations to
413 characterize the signaling behaviors of the strains over time. We found that clustering based on
414 both the combined *GPD1*::GFP and forward scatter data worked slightly better than only
415 *GPD1*::GFP. In brief, for an experiment we consider for each cell the *GPD1*::GFP and forward
416 scatter data. This 2D distribution of each strain at every time point was converted first into a 2D
417 100x100 grid with binned values, then into a normalized linear 1000 elements state vector
418 characteristic for the *GPD1*::GFP and forward scatter data of cells at each time point. The
419 vectors for all the *n* time points were then successively appended to form a nx1000 time line
420 vector representing the time course of the combined GPD1::GFP and forward scatter data. The
421 vectors were then used to compute a distance matrix between strains at each time point using a
422 Kullback-Leibler divergence based distance function. We used hierarchical clustering with
423 Ward"s method using the fastcluster implementation in Python[34](http://www.jstatsoft.org/v53/i09/).
424 We found stronger cluster when performing the same analysis using the combined GPD1::GFP
425 and forward scattering for each cell in the flow cytometry, and unless stated otherwise the

426 reported results are in this case. Data from experimental replicates were representatively
427 sampled for each time point to generate a random average sampling of time line vectors for each
428 strain.
429
430 **Bet hedging model**
431 The bet hedging 'strategy' P was defined as the heritable probability of cautious cells for $0 \leq P \leq$
432 1. Relative fitness was measured for representative strategies (0, 0.1,0.2,…1.0) after 10
433 generations in each environment. Nine possible 2-state environmental shifts between three
434 general osmotic stress environments were considered: permissive (E0; all cells grow equally
435 well), restrictive (E1; reckless cells divide, cautious cells survive without dividing), and killing (E2;
436 reckless cells die, cautious cells survive without cell division). For simplicity, the natural attrition
437 of older cells and rates of cell division were assumed to be equal for all cell types.
438 Results were independent of the number of generations in the first environment except as shown
439 when E1 was the first environment.
440

441 **References**
442

443 1. Kirschner, M. & Gerhart, J. Evolvability. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 8420–8427
444 (1998).
445 2. West-Eberhard, M. J. *Developmental plasticity and evolution*. *Nature* **424,** (2003).
446 3. Rutherford, S. L. From genotype to phenotype: buffering mechanisms and the storage of
447 genetic information. *Bioessays* **22,** 1095–105 (2000).
448 4. Hohmann, S. Osmotic Stress Signaling and Osmoadaptation in Yeasts. *Microbiol. Mol.*
449 *Biol. Rev.* **66,** 300–372 (2002).
450 5. Hohmann, S., Krantz, M. & Nordlander, B. *Yeast Osmoregulation*. *Methods Enzymol.* **428,**
451 (Elsevier Masson SAS, 2007).
452 6. Saito, H. & Posas, F. Response to hyperosmotic stress. *Genetics* **192,** 289–318 (2012).
453 7. Clotet, J. & Posas, F. *Control of Cell Cycle in Response to Osmostress: Lessons from*
454 *Yeast*. *Methods Enzymol.* **428,** (Elsevier Masson SAS, 2007).
455 8. Nadal, E. de, Alepuz, P. & Posas, F. Dealing with osmostress through MAP kinase
456 activation. *EMBO Rep.* **3,** 735–40 (2002).
457 9. Babazadeh, R., Furukawa, T., Hohmann, S. & Furukawa, K. Rewiring yeast osmostress
458 signalling through the MAPK network reveals essential and non-essential roles of Hog1 in
459 osmoadaptation. *Sci. Rep.* **4,** 4697 (2014).
460 10. Ratcliff, W. C., Hawthorne, P. & Libby, E. Courting disaster: How diversification rate affects
461 fitness under risk. *Evolution* 1–10 (2014). doi:10.1111/evo.12568
462 11. Remize, F., Barnavon, L. & Dequin, S. Glycerol export and glycerol-3-phosphate
463 dehydrogenase, but not glycerol phosphatase, are rate limiting for glycerol production in
464 Saccharomyces cerevisiae. *Metab. Eng.* **3,** 301–312 (2001).
465 12. Cookson, S., Ostroff, N., Pang, W. L., Volfson, D. & Hasty, J. Monitoring dynamics of
466 single-cell gene expression over multiple cell cycles. *Mol. Syst. Biol.* **1,** 2005.0024 (2005).
467 13. Miermont, A. *et al.* Severe osmotic compression triggers a slowdown of intracellular
468 signaling, which can be explained by molecular crowding. *Proc. Natl. Acad. Sci. U. S. A.*
469 **110,** 5725–30 (2013).
470 14. Lee, Y. J., Jeschke, G. R., Roelants, F. M., Thorner, J. & Turk, B. E. Reciprocal
471 Phosphorylation of Yeast Glycerol-3-Phosphate Dehydrogenases in Adaptation to Distinct
472 Types of Stress. *Mol. Cell. Biol.* **32,** 4705–4717 (2012).
473 15. Jacoby, T. *et al.* Two Protein-tyrosine Phosphatases Inactivate the Osmotic Stress
474 Response Pathway in Yeast by Targeting the Mitogen-activated Protein Kinase, Hog1. *J.*
475 *Biol. Chem.* **272,** 17749–17755 (1997).
476 16. Wurgler-Murphy, S. M., Maeda, T., Witten, E. a & Saito, H. Regulation of the
477 Saccharomyces cerevisiae HOG1 mitogen-activated protein kinase by the PTP2 and
478 PTP3 protein tyrosine phosphatases. *Mol. Cell. Biol.* **17,** 1289–1297 (1997).
479 17. Muzzey, D., Gómez-Uribe, C. a., Mettetal, J. T. & van Oudenaarden, A. A Systems-Level
480 Analysis of Perfect Adaptation in Yeast Osmoregulation. *Cell* **138,** 160–171 (2009).
481 18. Boy-Marcotte, E., Perrot, M., Bussereau, F., Boucherie, H. & Jacquet, M. Msn2p and
482 Msn4p control a large number of genes induced at the diauxic transition which are
483 repressed by cyclic AMP in Saccharomyces cerevisiae. *J. Bacteriol.* **180,** 1044–52 (1998).
484 19. Yi, T. M., Huang, Y., Simon, M. I. & Doyle, J. Robust perfect adaptation in bacterial
485 chemotaxis through integral feedback control. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 4649–
486 4653 (2000).
487 20. Galdieri, L., Mehrotra, S., Yu, S. & Vancura, A. Transcriptional Regulation in Yeast during
488 Diauxic Shift and Stationary Phase. *Omi. A J. Integr. Biol.* **14,** 629–638 (2010).
489 21. Gray, J. V *et al.* 'Sleeping beauty': quiescence in Saccharomyces cerevisiae. *Microbiol.*
490 *Mol. Biol. Rev.* **68,** 187–206 (2004).
491 22. Levy, S. F., Ziv, N. & Siegal, M. L. Bet hedging in yeast by heterogeneous, age-correlated

expression of a stress protectant. *PLoS Biol.* **10,** (2012).

23. De Jong, I. G., Haccou, P. & Kuipers, O. P. Bet hedging or not? A guide to proper classification of microbial survival strategies. *BioEssays* **33,** 215–223 (2011).
24. Philippi, T. & Seger, J. Hedging one's evolutionary bets, revisited. *Trends Ecol. Evol. (Personal Ed.* **4,** 41–44 (1989).
25. Simons, A. M. Modes of response to environmental change and the elusive empirical evidence for bet hedging. *Proc. Biol. Sci.* **278,** 1601–1609 (2011).
26. King, O. D. & Masel, J. The evolution of bet-hedging adaptations to rare scenarios. *Theor. Popul. Biol.* **72,** 560–575 (2007).
27. Meyers, L. A. & Bull, J. J. Fighting change with change: Adaptive variation in an uncertain world. *Trends Ecol. Evol.* **17,** 551–557 (2002).
28. Ratcliff, W. C. & Denison, R. F. Individual-Level Bet Hedging in the Bacterium Sinorhizobium meliloti. *Curr. Biol.* **20,** 1740–1744 (2010).
29. Rutherford, S. L. Between genotype and phenotype: protein chaperones and evolvability. *Nat. Rev. Genet.* **4,** 263–74 (2003).
30. Giaever, G. *et al.* Functional profiling of the Saccharomyces cerevisiae genome. *Nature* **418,** 387–391 (2002).
31. Shoemaker, D. D., Lashkari, D. a, Morris, D., Mittmann, M. & Davis, R. W. Quantitative phenotypic analysis of yeast deletion mutants using a highly parallel molecular bar-coding strategy. *Nat. Genet.* **14,** 450–456 (1996).
32. Wach, A., Brachat, A., Pöhlmann, R. & Philippsen, P. New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. *Yeast* **10,** 1793–1808 (1994).
33. Gietz, R. D. & Woods, R. a. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol.* **350,** 87–96 (2002).
34. Müllner, D. fastcluster : Fast Hierarchical , Agglomerative Clustering Routines for R and Python. *J. Stat. Softw.* **53,** 1–18 (2013).
35. Mortimer, R. K. & Johnston, J. R. Genealogy of principal strains of the yeast genetic stock center. *Genetics* **113,** 35–43 (1986).
36. Brachmann, C. B. *et al.* Designer deletion strains derived from Saccharomyces cerevisiae S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14,** 115–132 (1998).

Supplementary information

Python script for bet hedging model

Flow cytometry database

3 movies

Acknowledgements

The wild strain collection was made in the Rutherford lab thanks to the help of numerous teams of summer undergraduates. We thank Wyming Lee Pang and Jeff Hasty for help with microfluidics and Stéphane Douady for advice on scaling single cell data. This research was supported by a Scholar Award from the Damon Runyon Cancer Research Foundation (SR), NIH R01 GM068873 (SR) and the Lady Tata Foundation (YH).

Author information

The current address for YH is Center for Experimental Animals, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan. None of the authors have competing interests.

544 **Tables**

545 **Table 1. Strains and aliases used in this study.** See Table S1 for details about each of the 49
546 wild haploid strain derivatives (WHXXX). For brevity, figures are labeled with the wild parent
547 strain number (WXXX; see Table S1 for details).

548

Strain	Genotype	Source
WHXXX	*MATa ura3D0 hoD::barcode::KanMX4*	This study; haploid Mata isolates of wild strains. See Table S1 for details.
BY41	*MATa his3D1 leu2D0 met15D0 ura3D0 flo8-1 hoD::barcode::KanMX4*	Mata derivative of BY4742.
BY4742	*MATalpha his3D1 leu2D0 lys2D0 ura3D0 flo8-1*	This study; BY4741 and BY4742 backgrounds derive from a wild diploid isolated in Nerced, California in 1938 on figs (EM93[35]; S228C[36]). They are distinguished primarily by the many generations it has been under laboratory selection.
G01	*MATalpha his3D1 leu2D0 lys2D0 ura3D0 flo8-1 gpd1D::GFP::URA3*	This study; BY4742 background. For monitoring GPD1.
WXXX.BY16	*MATa/MATalpha LYS2/lys2D0 ura3D0/URA3 FLO8/flo8-1 hoD::barcode::KanMX4/ho*	This study; 49 wild/lab plus 1 BY41 control. See Table S1 for details.
WXXX.G01	*MATa/MATalpha HIS3/his3D1 LEU2/leu2D0 LYS2/lys2D0 ura3D0/ura3D0 FLO8/flo8-1 hoD::barcode::KanMX4/ho GPD1/gpd1D::GFP::URA3*	This study. 49 wild/lab plus 1 lab/lab diploids for GPD1 quantification. See Table S1 for details.

549

550 **Table 2. Negative feedback between rates of change in *GPD1*::GFP accumulation and**
551 **viability.** To control for potential deviations from normality, non-parametric pairwise
552 correlations between rates of change in *GPD1*::GFP accumulation and viability within (upper 3
553 rows) and between 2 hours time intervals (below). Changes occurring in earlier intervals, which
554 were potentially causal for later changes, are listed first. Results were qualitatively similar for
555 Pearson's correlations with all significant P-values remaining <0.0001. Significant comparisons
556 are indicated in bold.
557

Interval	Variable	Interval	Variable	Spearman's r	Prob>|r|
0 – 2 hours	**Δ*GPD1*::GFP**	**0 - 2 hours**	**Δviability**	**0.7725**	**<.0001**
2 – 4 hours	**Δ*GPD1*::GFP**	**2 - 4 hours**	**Δviability**	**0.7217**	**<.0001**
4 - 6 hours	Δ*GPD1*::GFP	4 - 6 hours	Δviability	-0.1992	0.4282
0 – 2 hours	**Δviability**	**2 - 4 hours**	**Δ*GPD1*::GFP**	**-0.7411**	**<.0001**
0 – 2 hours	**Δviability**	**2 - 4 hours**	**Δviability**	**-0.9503**	**<.0001**
0 – 2 hours	**Δ*GPD1*::GFP**	**2 - 4 hours**	**Δ*GPD*1::GFP**	**-0.7697**	**<.0001**
0 – 2 hours	**Δ*GPD1*::GFP**	**2 - 4 hours**	**Δviability**	**-0.7696**	**<.0001**
1 - 2 hours	Δviability	4 - 6 hours	Δviability	-0.2178	0.3854
1 - 2 hours	Δviability	4 - 6 hours	Δ*GPD1*::GFP	-0.2549	0.3073
1 - 2 hours	Δ*GPD1*::GFP	4 - 6 hours	Δviability	-0.0464	0.8548
1 - 2 hours	Δ*GPD1*::GFP	4 - 6 hours	Δ*GPD1*::GFP	-0.4572	0.0565
2 - 4 hours	Δviability	4 - 6 hours	Δviability	0.0733	0.7726
2 - 4 hours	Δviability	4 - 6 hours	Δ*GPD1*::GFP	0.3333	0.1765
2 - 4 hours	Δ*GPD1*::GFP	4 - 6 hours	Δviability	0.1207	0.6332
2 - 4 hours	Δ*GPD1*::GFP	4 - 6 hours	Δ*GPD1*::GFP	0.3602	0.1421

558
559

560 **Table 3. Growth of post diauxic cells at unprecedented limits of adaptation.** Shown are
561 concentrations of agar media on which post diauxic strains could grow and form colonies.
562

[KCl] M	Wild/lab (G*PD1*) diploids*
2.0	W455
2.6	W027, W035, W167, W202, W203, W242, W285, W454
2.7	W033, W041, W042, W134, W136, W150, W166, W178, W195, W215, W217, W219, W235, W248, W282, W291, W292, W294, BC41
2.8	W037, W044, W050, W153, W157, W163, W164, W179, W189, W206, W238, W244, W245, W249, W255, W276, W301, W340
2.9	W173, W211, W343

563
564

565 **Table 4. Osmotic stress signaling behavior (rank) predicted early and late viability of**
566 **post diauxic cultures in osmotic stress.** Least squares predictions of early and late viability by
567 linear and 2nd order quadratic fits of fluorescence pre-accumulated into the G3 Gaussian at time
568 0 (G3_0) and ranked signaling behavior. The Bonferroni cutoff at the 0.05 level, based on 4
569 tests per data set, was 0.0125. Significant fits with lowest root mean squared errors and
570 highest fraction of variation explained (R^2) shown in bold, predicted values for optimum (x) and
571 value at optimum (y) for non-significant fits are marked NS.
572

	G3_0 fluorescence (AU)		Signaling (rank)	
	quadratic	**linear**	**quadratic**	**linear**
0 hours, 0M KCl				
probability > F	**0.0006**	0.0016	0.9629	0.8454
R_square	**0.2715**	0.1891	0.0016	0.0008
root_mean_square_error	**1.3935**	1.4548	1.6313	1.6149
max_viability at optimum (%)	**98.8**		NS 98.2	
optimum	**2626.6**		NS 31.9	
20 hours, 2.5M KCl				
probability > F	**< 0.0001**	< 0.0001	**0.0055**	0.6023
R_square	**0.4286**	0.3138	**0.1987**	0.0057
root_mean_square_error	**7.0949**	7.6940	**8.4022**	9.2615
max_viability at optimum (%)	**86.7**		**86.3**	
optimum	**2662.5**		**24.4**	
48 hours, 2.5M KCl				
probability > F	**< 0.0001**	0.0003	**0.0013**	0.9159
R_square	**0.3148**	0.2371	**0.2464**	0.0002
root_mean_square_error	**7.6281**	8.1323	**8.1679**	9.3096
max_viability at optimum (%)	**82.2**		**82.9**	
optimum	**2622.3**		**25.7**	
72 hours, 2.5M KCl				
probability > F	**0.0010**	0.0033	**0.0062**	0.8464
R_square	**0.2556**	0.1660	**0.1943**	0.0008
root_mean_square_error	**9.9877**	10.4608	**10.3903**	11.4501
max_viability at optimum (%)	**73.5**		**74.3**	
optimum	**2586.8**		**25.1**	
96 hours, 2.5M KCl				
probability > F	**0.0060**	0.0018	**0.0065**	0.0954
R_square	**0.1956**	0.1862	**0.1927**	0.0569
root_mean_square_error	**8.4186**	8.3789	**8.4338**	9.0201
max_viability at optimum (%)	**71.7**		**71.0**	
optimum	**3389.8**		**21.3**	
120 hours, 2.5M KCl				
probability > F	0.0791	0.0413	**0.0004**	0.0037
R_square	0.1023	0.0839	**0.2859**	0.1625
root_mean_square_error	8.5666	8.5636	**7.6405**	8.1881
max_viability at optimum (%)	NS 67.7		**74.7**	
optimum	NS 1679		**18.1**	

144 hours, 2.5M KCl				
probability > F	0.3473	0.1675	**0.0047**	0.0501
R_square	0.0440	0.0393	**0.2038**	0.0776
root_mean_square_error	9.6837	9.6059	**8.8377**	9.4123
max_viability at optimum (%)	NS 61.9		**68.3**	
optimum	NS 1481.3		**20.4**	
168 hours, 2.5M KCl				
probability > F	0.1785	0.3433	**<0.0001**	< 0.0001
R_square	0.0707	0.0187	**0.5733**	0.4911
root_mean_square_error	12.3795	12.5879	**8.3885**	9.0652
max_viability at optimum (%)	NS 56.2		**68.4**	
optimum	NS 2077		**9.7**	
24 hours, 3M KCl				
probability > F	0.0107	0.0292	**< 0.0001**	< 0.0001
R_square	0.1757	0.0952	**0.5254**	0.2980
root_mean_square_error	11.8963	12.3332	**9.0267**	10.8635
max_viability at optimum (%)	71.7		**65.0**	
optimum rank	2029.2		**18.1**	
48 hours, 3M KCl				
probability > F	0.0294	0.0140	**< 0.0001**	0.0003
R_square	0.1394	0.1193	**0.5382**	0.2402
root_mean_square_error	12.8903	12.9029	**9.4420**	11.9849
max_viability at optimum (%)	NS 57.1		**62.3**	
optimum	NS 2867.3		**19.7**	
72 hours, 3M KCl				
probability > F	0.0519	0.0395	**< 0.0001**	0.0002
R_square	0.1183	0.0853	**0.5459**	0.2460
root_mean_square_error	12.4241	12.5218	**8.9158**	11.3687
max_viability at optimum (%)	NS 51.4		**56.9**	
optimum	NS 2651		**19.7**	
Adaptation limit				
probability > F	0.5435	0.2699	**0.0002**	0.0086
R_square	0.0262	0.0258	**0.3099**	0.1379
root_mean_square_error	0.1344	0.1330	**0.1132**	0.1251
max_concentration optimum (M)	NS 2.8		**2.8**	
optimum	NS -95.6		**31.2**	

575 **Table S1. Haploid derivatives of wild strains.** The source for all wild strains in this study
576 was the strain collection of the Royal Netherlands Academy of Arts and Sciences over the past
577 100 years (Table 1 and Table S1). This resource has been deposited at the Yeast Genetic
578 Resources Lab of the National BioResource Project in Osaka, Japan.
579

Alias	MAT	Genotype	Comments
WH027	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 459; isolated in 1938 in Castellina, Italy from grape must; barcode #29(Uptag sequence: GGCCCGCACACAATTAGGAA, Downtag sequence: GCGCCGCATTAACTAAACTA)
WH030	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1508; isolated in 1927 from starter for sorghum brandy; YH note: mating defective, hard to make wild/lab diploids; barcode #16(Uptag sequence: GTCCGAACTATCAACACGTA, Downtag sequence: GCGCACGAGAAACCTCTTAA)
WH033	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 405 ; isolated in 1925 in West Africa from catabo for billi wine, from Osbeckia grandiflora; barcode #187(Uptag sequence: CCGTGTACTGAATTACGATC, Downtag sequence: CCATCTTTGGTAATGTGAGG)
WH035	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 5822; isolated in 1967 from malt wine; barcode #30(Uptag sequence: GGTCTATGCAAACACCCGAA, Downtag sequence: GCCGTCTTGACAACCTTATA)
WH037	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1395; isolated in 1922 from an unknown source ; barcode #235(Uptag sequence: GGCTAAGGGACAACACCTCA, Downtag sequence: GCCCGGCACATAGAAGTAAC)
WH041	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 5635; isolated in 1958 in South Africa from grape must; barcode #2(Uptag sequence: CCATGATGTAAACGATCCGA, Downtag sequence: TATATGGCAGCAGATCGCCG)
WH042	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 3081; isolated in 1958 in Spain from alpechin; barcode #12(Uptag sequence: GTGCGAACCAACGTACTACA, Downtag sequence: GCAGGAACACCACAGGGTTA)
WH044	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 422; isolated in 1926 in Odessa, Ukraine from beer; barcode #135(Uptag sequence: CCCGCGATTGTAATGAATAG, Downtag sequence: CATACTACGTGGGACAGTTG)
WH050	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 5112; isolated in 1962 in Spain from grape must; barcode #49(Uptag sequence: CTTACTGATAGCGTAGAGGT, Downtag sequence: GTGGTCTGCAAACCCAACAA)
WH134	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 2964; isolated in 1947 in Copenhagen, Denmark from distiller's rum yeast; barcode #18(Uptag sequence: GCCCTGATAACAAGGTGTAA, Downtag sequence: GCGCCTATTACACAAACGTA)

WH136	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 2811; isolated in 1957 from wine; wine yeast; barcode #20(Uptag sequence: GTGAGCGAAACACCGCGTAA, Downtag sequence: GGTAATACGCAACTCCTCTA)
WH150	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 2962; isolated in 1947 in Copenhagen, Denmark from ; distiller's rum yeast; barcode #15(Uptag sequence: GCCGTAGCCACAAGAGTTAA, Downtag sequence: GCGGCCACTTACACAAATTA)
WH153	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 6458; isolated in 1972 location not reported; barcode #37(Uptag sequence: GGGACCGCCAAAGCTATCAA, Downtag sequence: GTGAACAATAACGGCCTTGA)
WH157	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 6506; isolated in 1973 in UK brewery; killer yeast; barcode #53(Uptag sequence: CTGAGCGTAGGATATTCCGT, Downtag sequence: GCCGGTCGCAAACTCATAAA)
WH163	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 6914; isolated in 1977 in Spain from white wine; barcode #51(Uptag sequence: CTACGTCGGCTCATAGTCGT, Downtag sequence: GCTCTCGGCCAAGGAAACAA)
WH164	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 6978; isolated in 1984 from wine; wine yeast; barcode #59(Uptag sequence: CACTCGGATTCAGTTCTAGT, Downtag sequence: GGCCTTGCCAAACAGTCAAA)
WH166	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 7072; isolated in 1980 from distillate; barcode #62(Uptag sequence: CCTAGTTCGAGATTGCGAGT, Downtag sequence: GTGGTCGCCCAAGCAACAAA)
WH167	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 7173; isolated in 1985 from catabo for billi wine; wine yeast; barcode #43(Uptag sequence: CAGTATGCTAGATTCCGGGT, Downtag sequence: GTCCTCGCAAGAAAGGCCAA)
WH173	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 8615; isolated in 1998 in Italy from grape must; dry yeast for wine making; barcode #61(Uptag sequence: CCTGTAGTACGAGTATGAGT, Downtag sequence: GGTCTGCCCAAAGTCACAAA)
WH178	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1192; isolated in 1928 from wine; wine yeast; barcode #155(Uptag sequence: CGCACACGATTAAGGTCCAG, Downtag sequence: CACTGTTGGTAAGGTCTATG)
WH179	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1193; isolated in 1928 from wine; wine yeast; barcode #70(Uptag sequence: CAATAGGGTGTGACAGTTCT, Downtag sequence: CTACTTCGCGTGAGCTGGTT)
WH189	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1241; isolated in 1930 from an unknown source ; barcode #212(Uptag sequence: CCACTTAGTTCAATAGGCGC, Downtag sequence: CCGAGTATTACATTCTCACG)

WH195	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1256; isolated in 1937 from port wine; barcode #123(Uptag sequence: CGTGGAGCAGTTCGTATAAT, Downtag sequence: CTCGACGCTGGACGTTATGT)
WH202	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 7372; isolated in 1988; killer yeast, K2Rd (Young & Yagiu), K2R2 (Wickner); barcode #119(Uptag sequence: CAACGTAGAGTGAGGTACAT, Downtag sequence: CACTTAGCTTAGACTCGTGT)
WH203	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 7438; isolated in 1989 from wine; wine yeast; barcode #65(Uptag sequence: CTTTCGGACGTATGTGCAGT, Downtag sequence: CCTTGATGATAGAGGGCTTT)
WH206	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 7833; isolated in 1994 in Missouri, USA from lung of a man with immune deficiency syndrome; virulent strain; barcode #82(Uptag sequence: CATACAAAGAGAGGTGTCCT, Downtag sequence: CCCTTGCGATTGGTGCAGTT)
WH211	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 7838; isolated in 1994 in USA from patient; barcode #144(Uptag sequence: CGATACAAGTAAGTTGCGAG, Downtag sequence: CCTCTTACGAGATAGCGGTG)
WH215	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 7962; isolated in 1984 in Sao Paulo, Brazil from fermenting concentrated syrup from sugar cane; barcode #94(Uptag sequence: CCCGATTGAGGCATGGTTAT, Downtag sequence: CGCTTCGAGTATGGGATATT)
WH217	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 7964; isolated in 1995 in Sao Paulo, Brazil from fermenting concentrated syrup from sugar cane; barcode #92(Uptag sequence: CGCGGAGTATAGAGCTTTAT, Downtag sequence: CAATCGCTCGGAGGCGTATT)
WH219	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 439 ; isolated in 1933 in Lager Schartel, Germany from Silvaner grapes; barcode #93(Uptag sequence: CGACCCTGATGATCCTTTAT, Downtag sequence: CTACGGGCTCGATGCCTATT)
WH235	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 5952; isolated in 1968; barcode #102(Uptag sequence: GGCTACGATACATCTTCATC, Downtag sequence: CATTTGTAACCAGTTCGCTC)
WH238	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 6223; isolated in 1969 in Chile from grape juice; radiation resistant; barcode #104(Uptag sequence: CTATGTGCGGTAAGACGTAT, Downtag sequence: CGGCGTAGATTGTTAGCATT)
WH242	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 6333; isolated in 1942 in Costa Rica from rotting banana; Strain name NRRL Y-1350 (synonymous designation; NRRL YB-210, NRRL-210, NRRL-B210; Mortimer and Johnston (1986), Genetics 113: 35); barcode #56(Uptag sequence: CCTGTAGATTGACGTGTAGT, Downtag sequence: GCCCTCGTGACAAATCGAAA)

WH244	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 8266; isolated in 1996 in from rotting fig; Strain name X2180, derived from S288C by self-diploidization (Mortimer and Johnston (1986), Genetics 113: 35). Did not survive freeze-drying.; barcode #122(Uptag sequence: CAGAGGGCACTGTTCTTAAT, Downtag sequence: CCCTGCTGTAGAGGTTATGT)
WH245	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 3000; isolated in 1956 in Pakistan from palm wine; wine yeast; barcode #138(Uptag sequence: CACATCGTTTAACACTGGAG, Downtag sequence: CTAGGAGGTTACAGTCATTG)
WH248	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 8049; isolated in 1981 from fish food; feed for fish and crustaceans; barcode #108(Uptag sequence: CGACCCGATGTAGTAGATAT, Downtag sequence: CCGCCGGATGTGATATAATT)
WH249	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 6069; isolated in 1981; hybrid strain (Y55-2 x JJ101); barcode #87(Uptag sequence: CACTGTGACCGAGGGATACT, Downtag sequence: CGCGCTATTATACTCGACTT)
WH255	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 3090; isolated in 1958 from white grape must; barcode #72(Uptag sequence: CACTGTGGACGATACGGTCT, Downtag sequence: CTGTACGTGCGATACTCGTT)
WH276	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1394; isolated in 1924 from pressed yeast; distillery yeast; barcode #176(Uptag sequence: CCACCGATGTAATTTGAGTC, Downtag sequence: CACTCTGCGTTAATGTTGGG)
WH282	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1460; isolated in 1927 in Indonesia from fermenting fruit; barcode #115(Uptag sequence: CATACTTAGGGATCAGGGAT, Downtag sequence: CCTTGTCTGAGAGCCGTTGT)
WH285	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1479; isolated in 1928 from wine; wine yeast; barcode #240(Uptag sequence: GCGGCCAATAGTAAACTTCA, Downtag sequence: GCCGCCGTGATAAGAAACAC)
WH291	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1576; isolated in 1931 in Sulawesi, Indonesia from sap of Arenga palm; barcode #117(Uptag sequence: CCTGAGGACTTATTCACGAT, Downtag sequence: CATTGGATTAGACCGTGTGT)
WH292	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1582; isolated in 1948 in Portugal from wine; barcode #118(Uptag sequence: CCGATTAGAGGTTGACAGAT, Downtag sequence: CACTGACTTCGAGGTCGTGT)
WH294	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1585; isolated in 1934 from sake-moto; sake yeast; barcode #180(Uptag sequence: CATTAAGGCGCACGTTTATC, Downtag sequence: CTATCCTAGAGATTTGAGGG)
WH301	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1594; isolated in 1936 from juice of aren palm; barcode #182(Uptag sequence: CACGTTTGCGAATAGGTATC, Downtag sequence: CAGATACTATTAAGTGCCGG)

WH340	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 2805; isolated in 1954 from wine; wine yeast, particularly suitable for fruit wines; barcode #233(Uptag sequence: GCCGGGCTTAAATTGAATCA, Downtag sequence: GCTCCGACTGAAGAACTAAC)
WH343	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 2808; isolated in 1954 from grapes (Blauer Portugieser); wine yeast, suitable for fruit wines, yields more than 18% of alcohol; barcode #224(Uptag sequence: CCCGTGAATATAAGTGAAGC, Downtag sequence: CCTGGATTTGAAGCGTATAG)
WH454	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 6412; isolated in 1952 from sake; sake yeast; barcode #192(Uptag sequence: CCTTAGGGATAATGAGTTGC, Downtag sequence: CCAGTGTTCTAACGTGCAGG)
WH455	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 440 ; isolated in 1934 in Taiwan from molasses; barcode #249(Uptag sequence: GCCCAGGCTAAATGTTAAGA, Downtag sequence: GAAGTACGCTCAAGACCGAC)
BC4741 (BY41)	a	MATa his3D1 leu2D0 met15D0 ura3D0 flo8-1 hoD::barcode::KanMX4	Original CBS #: Lab strain, BY4741; isolated in 1938 in Merced, CA, USA from rotting fig; derived from S288C, of which strain 88% of the gene pool is contributed by strain EM93 (Mortimer and Johnston (1986), Genetics 113: 35). Barcode #266(Uptag sequence: GGCCTAACTCAACAGACGGA, Downtag sequence: GCGCTCGACTAAGAGAAACC)

580

581

Table S2. Clustering statistics used to rank signaling behavior. Statistics showing the fraction of 17,000 permutations in which strains ranked with 50% of other strains in each mean cluster. These data were used to rank total signaling behaviors from most cautious (1) to most reckless (50) based on the fraction of time each strain was associated with its mean cluster (characteristic of that cluster). See Figure 2.

Rank	Cluster	Strain	MC0	MC1	MC3	MC2	MC5	MC4
1	MC0	W455	0.8484	0.1516	0	0	0	0
2	MC0	W167	0.8365	0.1635	0	0	0	0
3	MC1	W219	0.6375	0.3625	0	0	0	0
4	MC1	W217	0.5536	0.4460	0.0004	0	0	0
5	MC1	W027	0.5214	0.4755	0.0028	0.0002	0	0
6	MC1	W042	0.4850	0.4892	0.0191	0.0044	0.0023	0.0001
7	MC1	W235	0.1976	0.5532	0.2266	0.0226	0	0.0002
8	MC1	W340	0.1700	0.5803	0.2354	0.0142	0	0
9	MC1	W454	0.3355	0.6610	0.0034	0	0	0
10	MC1	W134	0.1686	0.7376	0.0821	0.0117	0	0
11	MC1	W276	0.1617	0.7643	0.0658	0.0083	0	0
12	MC1	W294	0.1692	0.7834	0.0440	0.0034	0	0
13	MC1	W157	0.1195	0.7851	0.0866	0.0088	0	0
14	MC1	W202	0.1671	0.7855	0.0446	0.0027	0	0
15	MC1	W238	0.1337	0.8048	0.0580	0.0036	0	0
16	MC1	W035	0.1293	0.8086	0.0572	0.0050	0	0
17	MC1	W248	0.1494	0.8331	0.0172	0.0003	0	0
18	MC3	W130	0.0003	0.0671	0.7020	0.2307	0	0
19	MC3	W136	0	0.0092	0.6572	0.3334	0.0002	0
20	MC3	W203	0.0025	0.1348	0.6410	0.2214	0.0002	0
21	MC3	W285	0.0003	0.0806	0.6245	0.2924	0.0022	0
22	MC3	W163	0.0011	0.1474	0.6228	0.2285	0.0002	0
23	MC3	W206	0	0.0036	0.6203	0.3747	0.0014	0
24	MC3	BY41	0	0.0123	0.6102	0.3768	0.0008	0
25	MC3	W041	0.0002	0.0233	0.5625	0.4090	0.0050	0
26	MC3	W343	0.0012	0.0432	0.5522	0.3910	0.0123	0.0002
27	MC3	W292	0.0201	0.1962	0.4883	0.2893	0.0062	0
28	MC3	W189	0.0001	0.0205	0.4757	0.4715	0.0319	0.0003
29	MC3	W211	0	0	0.3698	0.5572	0.0696	0.0033
30	MC2	W245	0	0	0.2579	0.6898	0.0523	0
31	MC2	W291	0	0	0.2211	0.6786	0.0994	0.0009
32	MC2	W164	0	0	0.1517	0.6601	0.1852	0.0031

33	MC2	W249	0	0	0.3349	0.6417	0.0233	0.0001
34	MC2	W166	0	0	0.3372	0.6407	0.0220	0.0001
35	MC2	W179	0	0.0002	0.3569	0.6212	0.0217	0
36	MC2	W244	0	0	0.1478	0.6160	0.2225	0.0137
37	MC2	W173	0	0.0003	0.3801	0.6005	0.0188	0.0002
38	MC2	W215	0	0.0012	0.3162	0.6000	0.0815	0.0011
39	MC2	W037	0	0	0.0714	0.5308	0.3863	0.0115
40	MC2	W255	0	0.0011	0.4771	0.5141	0.0077	0
41	MC2	W050	0	0	0.0567	0.4672	0.4417	0.0343
42	MC5	W044	0	0	0.0021	0.0728	0.8501	0.0750
43	MC5	W195	0	0	0.0019	0.0744	0.8501	0.0736
44	MC5	W301	0	0	0.0029	0.0882	0.8203	0.0887
45	MC5	W150	0.0001	0.0004	0.0006	0.0414	0.7978	0.1598
46	MC5	W033	0	0	0	0.0279	0.7196	0.2525
47	MC5	W153	0	0	0	0.0181	0.6545	0.3274
48	MC4	W282	0	0	0	0	0.0596	0.9404
49	MC4	W242	0	0	0	0.0002	0.1477	0.8521
50	MC4	W178	0	0	0	0.0017	0.1697	0.8286

Table S3

Plate key

Left

	1	2	3	4	5	6
A	W027.BY01	W044.BY01	W153.BY01	W173.BY01	W203.BY01	W235.BY01
B	W033.BY01	W050.BY01	W157.BY01	W178.BY01	W206.BY01	W238.BY01
C	W035.BY01	W130.BY01	W163.BY01	W179.BY01	W211.BY01	W242.BY01
D	W037.BY01	W134.BY01	W164.BY01	W189.BY01	W215.BY01	W244.BY01
E	W041.BY01	W136.BY01	W166.BY01	W195.BY01	W217.BY01	W245.BY01
F	W042.BY01	W150.BY01	W167.BY01	W202.BY01	W219.BY01	W248.BY01

Right

	1	2	3
A	W249.BY01	W292.BY01	W455.BY01
B	W255.BY01	W294.BY01	
C	W276.BY01	W301.BY01	
D	W282.BY01	W340.BY01	
E	W285.BY01	W343.BY01	
F	W291.BY01	W454.BY01	

597 **Figure legends**

598 **Figure 1. Rate of osmotic stress signaling in exponential cultures with negative feedback**
599 **predicts early survival and robust recovery in moderate osmotic stress.**

600 A. Time course of mean accumulated *GPD1*::GFP fluorescence in exponential cultures
601 exposed to 0.75 and 1.5 M KCl. Each point represents an independent replicate
602 measurement; curves connect strain means at each time. In the absence of stress, all
603 strains had high steady-state viability (propidium iodide dye exclusion; range 96.3 –
604 98.7%; mean 97.6%) and relatively low mean GFP fluorescence indicating low
605 background activity of HOG pathway signaling through the GPD1 promoter and low
606 *GPD1*::GFP accumulation (range 12.7 – 34.8 AU; mean 18.8 AU).

607 B. Pie charts show relative changes after 2 hours in 0.75M KCl of mean viability (shaded
608 area), mortality (white area) and *GPD1*::GFP accumulation (percent opacity). Strains
609 ordered by viability at 2 hours, which was proportional to viability of non-disrupted
610 controls having two intact copies of the GPD1 gene (R^2 = 0.7085; P<0.0001;).

611 C. Relationship between mean *GPD1*::GFP accumulation and viability in mid-exponential
612 cultures exposed to 0.75 M KCl for 0, 2, 4, and 6 hours (h). Each data point represents
613 the average of at least three replicates per strain (~10,000 cells/ sample). The ellipses
614 indicate correlations between viability and fluorescence at alpha = 0.95. The inserts show
615 relationships between changes in *GPD1*::GFP and viability over each time interval.

616 D. Robust recovery of steady-state viability after 4 hours in 0.75 M KCl driven by negative
617 feedback (see text; recovery = (0.7670) early mortality + 3.4936; R^2 = 0.9351; P<
618 0.0001).

619 E. Plausible integrating negative control via multiple feedback mechanisms resulting in
620 accumulation of intracellular glycerol. Integrating feedback control would assure perfect
621 adaptation of stress responses, water balance and steady state viability. Note that the
622 persistence of dead cells over the course of the experiment precludes 100% recovery.

623

624 **Figure 2. Signaling behaviors predict the survival and fitness of older cultures in severe**
625 **osmotic stress.**

626 A. Strains classified by mean cluster (MC0 – MC5) and ranked top (1) to bottom (50)
627 according to changes in GPD1::GFP accumulation over time (see Table S2 and methods).
628 Each time point shows representative distributions of GPD1::GFP accumulation (green)
629 and relative survival red (99.7% viability) to blue (11.7% viability). Cells above the 89th
630 percentile (top 11%) are shown in black. Prior to osmotic challenge steady-state viabilities
631 were uniformly high (range 93.0 – 99.6%; mean 98.2%). Rank-ordered mean clusters are
632 topographically equivalent to a sequential ordering.

633 B. B. Relative viability of post-diauxic cultures (WXXX.BY01 controls) incubated in 3 M KCl
634 before plating on iso-osmolar media. Cultures were re-ordered according to the ranked
635 signaling behavior given in Figure 2A. The same experiment as originally plated is shown

in Figure S1. Strains color coded as in Figure 1C for comparison of exponential and post diauxic cultures.

Figure 3. Extreme cautious and reckless cell types observed in post diauxic cultures produce observed variation in cautious to reckless signaling strategies in a simple bet hedging model.

a. Cells with the most aggressive signaling began to die after long periods in severe stress leaving increasing fraction of cells with lower *GPD1*::GPF accumulations. Shown are distributions of accumulated *GPD1*::GFP and viability in replicate cultures of W242 (rank 49) after 168 hours in 2.5 M KCl. Mean (x), standard deviation (std), and weight (w; the fraction of cells in each distribution) are given. Sum (red) shows the cumulative fit of the 4 learned Gaussians.
b. Static viability of post diauxic cells of strain W027 exposed to 3 M in microfluidic chambers. Individual cell behaviors mirror population behaviors measured by flow cytometry – e.g longer lag periods and increased accumulations of GPD1::GFP with increasing osmotic stress. Colored traces indicate accumulated fluorescence in representative cells in 1.5 (green), 2.0 (blue), 2.5 (red) and 3.0 M KCl (yellow). Arrows indicate average time to the first cell division +/- standard deviations.
c. Generation of intermediate optima in simple 2-state bet hedging model with heritable production of cautious (static) and reckless cell types. Bet hedging strategy P was defined as the probability of cautious cells for $0 \leq P \leq 1$. Relative fitness was measured for all strategies after 10 generations in each environment. All nine possible environmental shifts are shown. Nine possible 2-state environmental shifts between three general osmotic stress environments were considered: permissive (E0; all cells grow equally well), restrictive (E1; reckless cells divide, cautious cells survive without dividing), and killing (E2; reckless cells die, cautious cells survive without cell division). Only in shifts from moderate to more severe (E1 -> E2) do bet hedging, intermediate strategies ($0 < P < 1$) prevail. The optimum strategy P depends on the number of generations in the first moderate stress environment. Results were independent of the number of generations in the first environment except as shown when E1 was the first environment.

Figure S1. Monophasic signaling behavior of exponential cultures described well by mean *GPD1*::GFP accumulations at each time point.

Mean (x), standard deviation (std), and weight (w; the fraction of cells in each distribution) are given. Sum (red) shows the cumulative fit of the 4 learned Gaussians. The representative strains shown are color-coded as in Figure 1B.

Figure S2. Static viability and survival of post diauxic cultures in extreme osmotic stress.

675 Strains were incubated for up to 5 weeks in 3 M KCl plated on iso-osmolar media. A plate key
676 is given in Table S3. The same data are shown sorted by rank in Figure 2B.
677
678 **Figure S3a–c. Reproducibility of cell distributions in strains with cautious to reckless**
679 **signaling.** Learned distributions of GPD1::GFP accumulation for post diauxic strains exposed
680 to 2.5 M KCl for the times shown. Mean (x), standard deviation (std), and weight (w; the
681 fraction of cells in each distribution) are given. Sum (red) shows the cumulative fit of the 4
682 learned Gaussians. The representative strains shown are color-coded as in Figure 1B.
683
684 **Figure S4. Rank predicts increasingly aggressive osmotic stress signaling.** Rank
685 predicts the relative proportion of cells above a threshold set at the top 11% of accumulation of
686 *GPD1*::GFP in post diauxic cultures exposed to 2.5 M KCl for the times shown. Mean clusters
687 are indicated with increasingly lighter grey scale in order of signaling (MC0, MC1, MC3, MC2,
688 MC5, MC4).
689
690 **Figure S5. Mean clusters were differentiated for predicts viability of post diauxic cultures.**
691 Mean cluster predicted viability. Shown are average viabilities each mean cluster were
692 analyzed by ANOVA or where appropriate by Welch's ANOVA at each time. Asterisks indicate
693 significance at the ≤0.05 level. Decreasing mean viabilities at each time are indicated
694 (horizontal lines).
695