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Epigenetic and Genetic Contributions to Adaptation in *Chlamydomonas*

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Abstract

Epigenetic modifications, such as DNA methylation or histone modifications, can be transmitted between cellular or organismal generations. However, there are no experiments measuring their role in adaptation, so here we use experimental evolution to investigate how epigenetic variation can contribute to adaptation. We manipulated DNA methylation and histone acetylation in the unicellular green alga *Chlamydomonas reinhardtii* both genetically and chemically to change the amount of epigenetic variation generated or transmitted in adapting populations in three different environments (salt stress, phosphate starvation, and high CO_2) for two hundred asexual generations. We find that reducing the amount of epigenetic variation available to populations can reduce adaptation in environments where it otherwise happens. From genomic and epigenomic sequences from a subset of the populations, we see changes in methylation patterns between the evolved populations over-represented in some functional categories of genes, which is consistent with some of these differences being adaptive. Based on whole genome sequencing of evolved clones, the majority of DNA methylation changes do not appear to be linked to *cis*-acting genetic mutations. Our results show that trangenerational epigenetic effects play a role in adaptive evolution, and suggest that the relationship between changes in methylation patterns and differences in evolutionary outcomes, at least for quantitative traits such as cell division rates, is complex.

Introduction

Evolutionary adaptation occurs when the population growth rate increases as a result of natural selection sorting heritable variation across individuals in fitness related traits, and the origin of this variation across individuals is usually characterised using genetic differences (Mayr, 1982; Hartl and Clark, 1997; Orr, 2005). However, it is now widely appreciated that heredity is not based on DNA sequence alone (Rassoulzadegan et al., 2006; Richards, 2006; Bonduriansky and Day, 2009; Jablonka and Raz, 2009; Crews et al., 2012; Daxinger and Whitelaw, 2012; Donelson et al., 2012; Salinas and Munch, 2012; Kelly, 2014; Taudt et al., 2016). Information not directly encoded in the DNA sequence can also be transmitted between generations. For example, non-genetic information can be transmitted when DNA or its associated proteins are modified, as is the case for DNA methylation and histone modifications (Cubas et al., 1999; Manning et al., 2006; Chinnusamy and Zhu, 2009; Johannes et al., 2009; Bossdorf et al., 2010; Verhoeven et al., 2010; Ou et al., 2012; Song et al., 2012; Verhoeven and van Gurp, 2012; Cortijo et al., 2014; Lauria et al., 2014). Collectively, these modifications are called epigenetic changes. It has now been established that epigenetic changes can be passed not only through mitotic cell division but also from parent to offspring (Johannes et al., 2009; Öst et al., 2014; Cortijo et al., 2014; Gaydos et al., 2014; Ragunathan et al., 2014; Audergon et al., 2015). Mutation accumulation experiments have shown that spontaneous epigenetic changes occur much like genetic mutations. However, one key difference is that epigenetic mutations occur at a faster rate, but may be less stable than genetic mutations. For example, rates of change in DNA methylation patterns have been estimated to be about five orders of magnitude higher than genetic mutations rates (Becker et al., 2011; Schmitz et al., 2011; van der Graaf et al., 2015).

The evidence for transmission of epigenetic variation opens up the question of how epigenetics affects evolutionary adaptation. Theoretical models and simulations predict that epigenetic variation has the potential to affect the rate and outcomes of adaptation (Day and Bonduriansky, 2011; Klironomos et al., 2013; Kronholm and Collins, 2016). Previous empirical research has either focused on adaptive plastic responses, and shown that plastic phenotypic changes have an epigenetic component (Bossdorf et al., 2010; Herrera et al., 2012; Verhoeven and van Gurp, 2012), or investigated adaptation occurring by independent epigenetic changes in wild populations indirectly by population genetic methods (Paun et al., 2010; Silveira et al., 2013).

Here, we investigate the effects of epigenetic variation directly using experimental evolution. This allows us to study how epigenetic variation affects adaptation over timescales that are long enough for novel adaptive genetic mutations to occur and increase in frequency in populations. Previously, adaptation on this timescale has been investigated and explained in purely genetic terms (Barrick et al., 2009; Blount et al., 2012; Dettman et al., 2012; Wong et al., 2012). We carried out laboratory experiments in four different environments using the unicellular green alga *Chlamydomonas reinhardtii*. We manipulated the production and transmission of epigenetic variation either genetically or chemically. Specifically, we genetically

manipulated epigenetic variation by generating a *sir2* mutant (see methods) to change the extent of histone
78 acetylation. *SIR2* is a NAD-dependent histone deacetylase that is conserved from archaea and bacteria to animals and plants (Frye, 2000). In all organisms studied, *SIR2* is involved in transcriptional silencing
80 (Tanny et al., 1999; Blander and Guarante, 2004; North and Verdin, 2004; Smith et al., 2008). The populations made up of *sir2* mutants were less able to produce epigenetic variation than wild-type
82 populations, but could transmit that variation. We chemically manipulated epigenetic variation by periodically subjecting evolving populations to chemical treatments that prevent either DNA-methylation, or
84 both methylation and histone deacetylation. The treatment "demet" contained demethylating agents 5-aza-deoxycytidine, L-Ethionine, and Zebularine and treatment "demet + acet" contained 5-aza-deoxycytidine, L-
86 Ethionine, and histone deacetylation inhibitor Trichostatin A. Published studies have shown that the concentrations used for 5-aza-2-deoxycytidine and L-Ethionine are effective for demethylation in *C.*
88 *reinhardtii* without decreasing growth (Feng and Chiang, 1984), and we confirmed the lack of effect on growth here. Thus the chemically-treated populations had reduced levels of epigenetic variation compared to
90 the untreated populations, with both reduced production and transmission of epigenetic variation. Since the 5-aza-deoxycytidine and Zebularine can be mutagenic, we included a UV-treated strain to account for an
92 increased supply of genetic mutations.

94 The selection experiment consisted of four strains (CC-2937, UV irradiated CC-2937, *sir2* mutant, complemented *SIR2* mutant), three chemical treatments (demet, demet + acet, control), and four selection
96 environments (high salt, high CO_2, low phosphate, control). Each selection environment imposes different selection on evolving populations. The experiment was run for approximately 200 asexual generations. Since
98 the UV-irradiated CC-2937 was used to account for changes (in this case an increase) in mutational supply, it is treated as a "strain" throughout the experiment. Throughout this study, we refer to strains as "strains",
100 chemical treatments as "treatments" and selection environments as "environments". See Figure 1A for a schematic of the selection experiment, and methods for details of strains and environments. We expect that
102 high salt and low phosphate environments are stressful environments and adaptation to these environments should increase growth rates, as in batch culture fitness should be proportional to maximum population
104 growth rate *r*. However, the high CO_2 environment is an enriched environment. While counter intuitive, previous work has revealed that evolution in high CO_2 environments either does not improve on the plastic
106 response, or reverses it to decrease growth (Collins and Bell, 2004; Schaum and Collins, 2014). This strategy evolves rapidly and repeatedly, and is associated with an increase in competitive ability and cell condition, so
108 that the best current interpretation is that the reduction in population growth rate is adaptive for chlorophytes in nutrient-rich, high CO_2 environments (Schaum and Collins, 2014; Collins, 2016). To understand the roles
110 of genetic and epigenetic differences in adaptation to a range of selection environments, we isolated clones from the CC-2937 control and demet chemical treatments and resequenced their genomes and methylomes
112 after the selection experiment. If it is the case that epigenetic changes are used in adaptation, we expect that manipulating the amount of epigenetic variation available for the populations we will reduce adaptation in
114 the different environments (Figure 1B). Furthermore, if we cluster the strains based on epigenetic changes

we should see similarities in the between strains that come from the same environment (Figure 1B).

116

We find that reducing the production or transmission of epigenetic variation available to the populations can

118 reduce growth rate evolution when populations adapt to novel environments. We also observed that when the methylation patterns of evolved populations were compared, populations evolved in the high salt clustered

120 together based on methylation differences, and among the genes containing methylation differences gene functions related to aminoglycan catabolism were enriched in all environments, membrane depolarization in

122 high CO_2, and transmembrane transport in high salt. Differences in methylation patterns were not associated with nearby genetic mutations, and have the potential to be adaptive.

124

Results

126

Environmental and chemical treatment effects on initial population growth rates

128

To investigate the effects of epigenetic transmission on adaptation as generally as possible, we used three

130 environments that exerted different selection pressures on the populations. Each one of these is a full evolution experiment. Here, the high NaCl environment exerted strong selection (indicated by a large initial

132 drop in population growth rates), while low phosphate was a more benign environment (indicated by a small initial drop in population growth rates) and high CO_2 was an enriched environment (indicated by an initial

134 increase in population growth rates). See Table 1 for list of initial (ancestral) growth rate responses to all environments for the different strains. This is consistent with previous experiments in *C. reinhardtii* showing

136 growth declines in high NaCl and low phosphate environments (Collins and de Meaux, 2009; Lachapelle and Bell, 2012; Lachapelle et al., 2015), and positive or no change in growth in high CO_2 environments (Collins

138 and Bell, 2004), and confirms that our environments exert different intensities of selection on evolving populations.

140

The initial effects of the different chemical treatments are listed in Table 2. The effects of the chemical

142 treatments were environment and strain dependent. While variability was high, averaged over all strains and environments, the demet treatment decreased growth by -6% and the demet + acet treatment had a stronger

144 effect as it decreased growth by -15% relative to the control treatment.

146 *Population extinctions during the selection experiment*

148 Populations were evolved for approximately 200 generations in their selection environments. Of the 432 populations in the selection experiment, 19 went extinct. Extinction events were not randomly distributed

150 among environments (Chi square goodness-of-fit test, $\chi^2 = 35.95$, df = 3, $p = 7.68*10^{-8}$). There were 2

extinctions in the control environment, 1 in the high CO_2 environment, 16 in the low phosphate environment,
152 and no extinctions in the high NaCl environment. In the low phosphate environment, strains had different extinction rates, with 13 populations of CC-2937, 2 populations of CC-2937 UV, 1 population of LM3 *sir2*,
154 and no populations of LM3 c*SIR2* going extinct (Chi square goodness-of-fit test, χ^2 = 27.5, df = 3, p = $4.63*10^{-6}$). CC-2937 may have had a higher extinction rate because its relatively fast growth rate led to rapid
156 phosphate depletion in the culture. Furthermore, phosphate depletion often caused CC-2937 cells to become sticky and clump together, which decreased their ability to be transferred in the experiment. UV irradiation
158 increases mutation rates in *C. reinhardtii* (see supplementary material), and although deleterious mutations will be more common with UV irradiation than without UV irradiation, so will beneficial ones, such that
160 selection is likely to be more effective in the large populations used here. Thus, a lower extinction rate in the UV-treated CC-2937 strain is consistent with evolutionary rescue made possible by an increased mutational
162 supply, and shows that in this experiment, the evolutionary potential of the UV-treated strain is different from the wild type within environments. Chemical treatment did not have a significant effect on extinctions in the
164 low phosphate environment (Chi square goodness-of-fit test, χ^2 = 3.88, df = 2, p = 0.1441).

166 *Decreasing epigenetic transmission affects growth rate evolution*

168

We have visualized the direct response to selection, which is calculated by dividing the growth rate of
170 populations evolved and measured in one of the three novel environments (high NaCl, low phosphate, high CO_2) by the growth rate of the populations evolved in the control environment but measured in the novel
172 selection environments. Populations were matched by chemical treatments (Figure 2). However, the statistical analysis has been performed on absolute growth rates (Figure S1), because this allows statistical
174 testing of differences between control and evolved populations. In this section we analyse the effects of epigenetic manipulations on adaptation using linear models. Since each selection environment was a
176 complete evolution experiment, we discuss the results for each environment separately. The statistical model includes terms for strain effect, effect of selection (whether population evolved in the one of the three novel
178 environments or the control environment), and the effect of chemicals and the interactions of these terms. We tested the full model first, and then dropped non-significant terms. The 3-way interaction of selection ×
180 chemical × strain tests the effect of epigenetic manipulation on adaptation varied across different strains. The interaction of selection × chemical tests the effect of epigenetic manipulation of adaptation and the selection
182 × strain tests the effect of different strains on adaptation. See Table 3 for a summary of evolutionary outcomes in terms of growth over all strains, chemical treatments, and selection environments. In all cases,
184 "growth rate" indicates population growth rate (increase in cell number over time) and not an increase in size off individual cells. We discuss the direct responses to selection here, and the indirect (correlated) responses
186 to selection, which are evolutionary changes that occurred but were not directly acted on by natural selection during the evolution experiment (Travisano et al., 1995), in the supplementary material.

188

High NaCl environment. In general, populations adapted to the high NaCl environment, and adaptation was

190 affected by the ability to produce and transmit epigenetic information. In the high NaCl environment, growth rates were initially low and an increase in growth rate is adaptive (Lachapelle et al., 2015). The 3-way

192 interaction of selection × chemical × strain was not significant. However, the selection × chemical interaction was significant ($F_{2,196}$ = 5.953, p = 0.0031), as populations subjected to control or only demet epigenetic

194 manipulation were able to adapt to high NaCl regardless of strain, while the more severe demet + acet epigenetic manipulation impeded adaptation to high salt (Figure 2A). The chemical × strain effect was not

196 significant, but the selection × strain effect was ($F_{3,196}$ = 3.628, p = 0.0140). The different strains responded to selection in a different manner with more adaptation in the UV-treated strain and in the complemented

198 mutant (Figure 2A).

200 In terms of the specific evolutionary responses for the wild-type strain CC-2937, populations evolved in high NaCl had an average of 21% (chemical control) and 15% (demet treatment) higher growth rates in the high

202 NaCl environment than CC-2937 populations evolved in the control environment. The UV-treated CC-2937 populations evolved in high NaCl had 72%, 58%, and 15% higher average growth rates in high NaCl than

204 populations selected in the control environment, for the chemical control, demet and demet + acet treatments respectively. The higher growth rates of the UV-treated strain reflects the increased genetic variation in the

206 UV-treated populations. For the *sir2* mutant, the chemical control, demet, and demet + acet populations had direct responses to selection of 24%, 37%, and 12% respectively. For the complemented *sir2* populations, the

208 control, demet, and demet + acet populations increased growth rate by 35%, 25%, and 19%. While there were slight differences between the *sir2* mutant and the complemented strain, the effect of the *sir2* mutation

210 was not significant (contrast: t = 1.26, df = 196, p = 0.2093). Overall, we see that decreasing epigenetic variation decreased or impeded adaptation to the high salt environment.

212

Low phosphate environment. While populations generally adapted to the low phosphate environment, but the

214 ability to produce and transmit epigenetic information did not statistically affect adaptation. In the low phosphate environment neither the 3-way interaction nor any of the 2-way interactions were significant.

216 Although previous work with *C. reinhardtii* detected substantial growth rate evolution in a low phosphate environment (Collins and de Meaux, 2009), the direct response to selection in this experiment was only 14%

218 on average over all other strains and treatments (Figure 2B, effect of selection: $F_{1,186}$ = 10.35, p = 0.0015). The effect of strain was significant ($F_{1,186}$ = 9.19, p = 1.06×10^{-5}), but the main effect of chemical was not.

220 The wild type CC-2937 strain populations evolved in low P had 28% (control), 10% (demet), and 15% (demet + acet) higher growth rates as corresponding populations evolved in the control environment. UV-

222 treated CC-2937 populations evolved in low P grew 12% (control), 18% (demet), and 0% (demet + acet) faster than their respective controls. The *sir2* mutant populations grew 7% (control), 9% (demet), and 40%

224 (demet + acet) faster than their control populations. For the complemented mutant populations, growth rates of the evolved populations were 25% (control), 8% (demet), and 8% (demet + acet) faster than control

226 populations, the effect of the sir2 mutation was not significant (contrast: t = −0.4, df = 186, p = 0.69). These

results suggest that epigenetic variation plays a smaller role in adaptation to low phosphate environment than
228 to high NaCl. Alternatively, given the increased variability and smaller responses to selection than in high NaCl environment (14% in low P vs 28% in high NaCl), we may lack the statistical power to detect a trend
230 associated with manipulating epigenetic variation chemically in the low phosphate environment.

232 *High CO_2 environment*. In the high CO_2 environment, the effects of decreasing the production and transmission of epigenetic information resulted in populations evolving higher growth rates than the control
234 chemical populations. Based on other studies on evolution in high CO_2 environments for unicellular chlorophytes, we suggest that the higher growth rates are maladaptive. High CO_2 is initially an improved
236 environment relative to the control environment for *C. reinhardtii*, and in previous studies wild type *C. reinhardtii* did not evolve to increase their growth rate beyond the initial plastic response in high CO_2
238 environments (Collins and Bell, 2004; Collins et al., 2006). Thus, we did not expect relative growth rate to increase in this environment in the wild-type populations. In addition, some unicellular chlorophytes
240 eventually decrease their growth rates over several hundred generations of growth in high CO_2 environments where growth initially increased (Schaum and Collins, 2014), suggesting that eventually slowing growth in
242 chronically enriched environments can be adaptive (Collins 2016).

244 In the high CO_2 environment 3-way interaction of selection × chemical × strain was significant ($F_{6,188} = 2.41$, $p = 0.0288$), indicating that adaptation depended on chemical treatment and strain. As expected, the high
246 CO_2-evolved wild type strain that is not chemically treated does not increase its growth rate relative to the control-selected population growing in the high CO_2 environment (Figure 2C) and their plastic response to
248 changes in CO_2 was lost or diminished (see absolute growth rates in supplementary material). The wild type CC-2937 populations evolved in high CO_2 had a change in growth of −18% (control), −9% (demet), and
250 19% (demet + acet) compared to populations evolved in control environment. The UV-treated CC-2937 populations had a change in growth of −13% (control), −13% (demet), and 19% (demet + acet) compared to
252 their respective controls. These results are consistent with studies suggesting that slower growth than predicted from the short-term (ancestral or control) response may be adaptive under chronically elevated CO_2
254 or other chronic environmental enrichment (Schaum and Collins, 2014; Collins, 2016). In contrast, when the most extreme epigenetic manipulation is used (demet + acet chemical treatment) in the CC-2937 genetic
256 background, populations evolved in high CO_2 evolve higher growth than the plastic response of control populations. This has never been reported for chlorophytes evolved under the moderate levels of CO_2
258 enrichment used here. It is also possible that the higher growth rate in the demet + acet treatments simply reflects a different, but adaptive, strategy than seen in the control treatments. The *sir2* mutant populations
260 had a change in growth of −13% (control), −21% (demet), and −9%. And finally, the complemented mutant had a change in growth of 10% (control), 13% (demet), and 5% (demet + acet) compared to populations
262 evolved in control environment. The effect of the *sir2* mutation was significant in control ($t = -4.24$, $df = 188$, $p = 3.48 \times 10^{-5}$), demet ($t = -4.96$, $df = 188$, $p = 1.54 \times 10^{-6}$), and suggestive in demet + acet ($t = -1.88$,

264 df = 188, p = 0.0619) treatment. The strains with the LM3 genetic background react differently, the *sir2* mutant does have lowered plastic response but the complemented mutant does not. In summary, populations
266 with the CC-2937 genetic background have likely adapted to high CO_2, while the demet + acet treatment may alter the evolutionary trajectory populations as they may have adapted using a different mechanism.

268

Manipulating epigenetic effects also affected the indirect responses to selection. Indirect responses were
270 smaller than direct responses, and are detailed in the supplementary material.

272 *Effects of UV-irradiation and changes in mutational supply*

274 Our UV-irradiation treatment increased mutational supply in UV-treated CC-2937 strain. Analysis of the possible effects of mutational supply on adaptation has to be done using absolute growth rates and this can
276 be found in supplementary material. However, we found little evidence that adaptation was limited by mutational supply in populations that did not go extinct. There was some evidence that conditionally neutral
278 mutations accumulated in the UV-treated strain but deleterious mutations did not prevent adaptation either.

280 *Effects of decreasing epigenetic transmission in the control treatment*

282 To measure how much transmitted epigenetic patterns affect growth rate of populations evolved in the control chemical treatment that had never undergone chemical treatment, we used an assay of phenotypic
284 stability in the face of chemical treatment. This was done by subjecting chemical control populations and ancestors to a single round of chemical treatment (demet + acet), followed by a growth cycle to allow
286 epigenetic marks to be re-established, and then measuring their growth rates. If epigenetic patterns have not contributed to the evolved phenotypes (and adaptation in purely genetic), then a single round of chemical
288 treatment should have the same magnitude of effect in the ancestor and the evolved populations. Conversely, if changes to epigenetic patterns have played a role in evolution, then we expect that the change in phenotype
290 in the evolved populations differ from the ancestor. Our reasoning is that chemical treatment will remove epigenetic marks; if adaptation is primarily genetic, then the phenotype should be stable except for any
292 effects of the drugs themselves, which will also be evident in the ancestor. On the other hand, if adaptation involved inherited epigenetic information, the adapted phenotype should be less (or more) stable in the face
294 of chemical treatment then the ancestral phenotype is. The toxic effects of drugs (if any) should remain constant or attenuate over time, so changes in response to chemical treatment over time indicates that there
296 have been changes in epigenetic marks that effect phenotype in the evolved populations. For this experiment we used CC-2937 populations evolved in the control treatment, and control, high CO_2, and high NaCl
298 environments, and their ancestor. Evolved low phosphate populations were excluded because many of them had gone extinct. Results discussed below are shown in Figure 3.

300

We find evidence that the changes to epigenetic patterns that are transmitted between generations affect
302 growth rate evolution in our experiment. The effect of the chemical treatment on growth rate is environment-specific in the CC-2937 ancestor (environment × chemical interaction, $F_{4,33}$ = 2.578, p = 0.0555). Chemical
304 treatment had negative effects on growth rate in all environments (Figure 3). For populations evolved in the control environment, there was a significant interaction between the chemical treatment and population ($F_{8,36}$
306 = 2.56, p = 0.0255), where the chemical treatment decreased growth in all but one of the evolved populations relative to the ancestor. If the one outlier population (Figure 3) is removed from the analysis, the interaction
308 is no longer significant. However, as the data come from replicate measurements made on independently grown and treated subcultures, it is likely that this reflects variation in evolutionary outcomes instead of
310 measurement error. This suggests that epigenetic configurations changed during the selection experiment in the control environment, and that the stability of the adapted phenotype requires direct transmission of these
312 epigenetic marks, such that the epigenetic configuration underlying the evolved phenotype cannot be re-established from genetic information alone. In contrast, in the high CO_2 environment, most populations did
314 not respond to chemical treatment differently than the ancestor as the interaction between chemical treatment and population was not significant ($F_{8,36}$ = 1.34, p = 0.27, Figure 3). This suggests that in these populations
316 adapted primarily through genetic changes (though these genetic changes could in turn direct epigenetic patterns). In the high NaCl environment, the responses to the chemical treatment did not differ between
318 evolved populations and the ancestor ($F_{7,28}$ = 0.63, p = 0.72, Figure 3). However, there is a non-significant trend for the chemical treatment to have less effect on the high NaCl-evolved populations than on the
320 ancestor, indicating that epigenetic configurations may have changed during adaptation. Together, the data from all three environments shows that while the phenotypic effect of epigenetic marks can evolve over
322 hundreds of generations, the frequency with which this occurs is environment-specific, and is likely less important than genetic variation during directional adaptation (in the selection environments) than under
324 stabilizing or reduced selection (in the control environment). Thus, while manipulating the production and transmission of epigenetic information affects evolutionary outcomes in environments where adaptation
326 occurs in this experiment, we also show that the role of directly-transmitted epigenetic changes, when they can occur, is low by the time populations have undergone a significant change in fitness. This is consistent
328 with modelling work showing that directly transmitted epigenetic marks can aid in the exploration of a fitness landscape, and contribute to the early stages of adaptation, but are often ultimately replaced by
330 genetic mutations later in adaptation (Klironomos et al., 2013; Kronholm and Collins, 2016).

332 *Phenotypes of evolved populations*

334 Manipulating sources of variation on which selection can act also has the potential to affect the evolution of traits other than growth rate. If this is the case, we expect that either the *sir2* mutant, the chemical treatments,
336 or both, affect the trait values of the populations at the end of the experiment relative to the rescued mutant and the control chemical treatment. Overall, we find that cell size, cell shape and chlorophyll content
338 changed during the experiment in most environments (Figure S3-S5). However, effects of epigenetic

manipulation on trait evolution was environment and phenotype dependent. The *sir2* mutation affected
340 responses to selection for cell size in high CO_2 and for chlorophyll content in high NaCl, and the chemical
treatments affected responses to selection for cell shape in high CO_2 and high NaCl, and chlorophyll content
342 in low phosphate. Many of the effects on trait evolution were small, at least on the timescale of this
experiment. Overall, effects of epigenetic manipulation on traits other than population growth were
344 environment and trait dependent. Detailed description of the results is given in Supplementary Material.

346 *Genome sequencing*

348 We used evolved populations of the strain CC-2937 from the control and demet chemical treatments to
examine the genetic and epigenetic basis of adaptation. We isolated single clones from our evolved
350 populations and sequenced the genomes and methylomes of clones from the control, high CO_2, and high
NaCl environments, from both the control and demet chemical treatments. The low phosphate environment
352 was excluded because of extinctions. We aimed to sequence the genomes and methylomes of 3 clones from
each of the three environments and chemical treatments. However, due to failures in library construction in
354 bisulfite sequencing, methylomes for some clones are missing. For control environment and control
treatment 2 methylomes were missing, leaving 1; and demet treatment 1 was missing leaving 2. For high
356 NaCl environment demet treatment 2 methylomes were missing leaving 1 (Table S2). We also sequenced the
methylomes of the ancestor in all three environments to originally assess the ancestral methylation states (but
358 see results below) and 3 methylomes of the ancestor in control and 3 methylomes of the ancestor in demet
chemical treatments to assess the effectiveness of the demethylation treatment.

360

Numbers of genetic mutations in control and demet chemical treatments

362

In the resequenced control chemical treatment clones we detected 77 mutations in total, with a mean number
364 of 9 mutations in the control environment clones, a mean of 5 mutations in the high CO_2 environment clones,
and a mean of 11 mutations in the high NaCl environment clones. Numbers of mutations for each clone are
366 presented in Table 4. The nine resequenced clones from the demet chemical treatment had 3594 genetic
mutations in total, with a mean of 428 mutations in the control environment clones, 571 mutations in the
368 high CO_2 environment clones, and 201 mutations in the high NaCl environment clones. We observed over 46
times more mutations in the demet treatment than in the control chemical treatment. This can be explained
370 by the mutagenicity of the cytosine analogs, 5'-aza-deoxycytidine and zebularine (Umen and Goodenough,
2001), where the modified cytosine tends to be replaced with guanine during DNA replication. This is
372 consistent with C↔G mutations in the two different treatments; there were 7 C↔G mutations out of 57
SNPs (12 %) in the control chemical treatment, but 3152 C↔G mutations out of 3544 SNPs (89 %) in the
374 demet chemical treatment. For the control chemical treatment the majority of the mutations were in UTRs
(untranslated region) and introns (49), with 26 mutations in coding regions, including 6 non-synonymous
376 mutations and 3 indels causing frameshifts. For the demet chemical treatment mutations in UTRs and introns

together contained most mutations (1745). In coding regions there were 1526 mutations, 1138 non-
378 synonymous, 379 synonymous, and 4 indels causing frame-shifts. A breakdown of mutations in different
functional regions is shown in Table 5.
380

The bioinformatics pipeline for calling mutations was the same for the control and demet treatments and
382 identical thresholds were applied. Both treatments were sequenced in the same run. We validated 20
mutations by Sanger sequencing (supplementary material) and in each case we confirmed the mutations.
384 Thus, it is not likely that differences in the quantity and identity of mutations are due to sequencing errors.

386 Across all environments the number of mutations in the demethylation treatment was correlated with the
number of generations those populations went through during the experiment (r = 0.89, n = 9, p = 0.0012),
388 but the control chemical treatment showed the opposite trend (r = -0.86, n = 9, p = 0.002) with more
mutations in populations that had gone through fewer generations. Here the effect of environment itself is
390 confounded with the number of generations as we do not have enough data to test the effect of generation
number within each environment, and there is little variation in generation number within environments.
392 However, given that the biological mechanism of more cell divisions in the presence of mutagen leads to
more mutations is reasonable, and we do not observe this pattern in the control treatment, suggest that it is
394 the number of generations that drive the relationship.

396 The number of mutations did not account for variation in growth rate (among all of the lines, effect of
number of mutations on growth rate: $F_{1,12}$ = 0.64, p = 0.438). This is consistent with genetic mutations in the
398 demet lines being neutral or nearly neutral, and with deleterious mutations being removed by natural
selection during the experiment. Alternatively, deleterious mutations may be offset by beneficial
400 (compensatory) mutations.

402 *Genetic changes in clones from the control chemical treatment*

404 Since control treatment populations adapted in the high salt and high CO_2 environments, some of the
mutations in these clones are probably beneficial. However, there are multiple mutations in each clone,
406 which makes pinpointing the mutations responsible for phenotypic changes impossible in an asexual
population. Many of the genes with mutations are of unknown function or annotated based on homology.
408 Mutations that occurred in sequenced chemical control clones are listed in supplementary table S3.

410 For mutations that occurred in annotated genes, there are some candidates for adaptive mutations. For
example, in the control environment, clone P3G11 has a deletion of one amino acid that preserves the
412 reading frame in Cre17.g723600, which is an intraflagellar transport protein 81, which may be involved in
cell motility. In the high NaCl environment clone P10C5, there is a deletion that causes a frameshift in
414 Cre03.g160050, which is annotated as flagellar associated protein. Another mutation of potential interest is a

SNP in 3' UTR of Cre14.g629650 (NIK1) which is a nickel transporter. In clone P12E4 there is an insertion
416 of 1 bp in the intron Cre17.g732150 which is a flagellar associated protein, as in clone P10C5. Another interesting mutation is a SNP in the intron of Cre02.g078400, a gene with a Bestrophin RFP-TM chloride
418 channel domain. In the high CO_2 environment there are no mutations in genes with functions obviously related to high CO_2.

420

Genetic changes in clones from the demet chemical treatment

422

The large number of mutations precludes discussing each one individually, so we concentrate on mutations
424 with multiple hits over different clones in genes with functions that are more obviously related to the selective environment. For clones evolved in the control environment we observed several mutations in
426 mitochondrial genes. In particular we observed five non-synonymous mutations in cytochrome b, two non-synonymous mutations in NADH dehydrogenase subunit 5, two non-synonymous mutations in subunit 4,
428 two in subunit 1, two mutations in RrnS4, which is gene producing an RNA of the S4 portion of small subunit rRNA and two non-synonymous mutations in rtl, a reverse transcriptase like protein.

430

For the clones evolved in the high CO_2 environment we also observed mutations in mitochondrial genes. We
432 observed four non-synonymous mutations in cytochrome b with one non-sense mutation causing an early stop codon. Another five non-synonymous mutations in NADH dehydrogenase subunit 5 with one non-sense
434 mutation. There were also five mutations in total in RrnS4, which is gene producing an RNA of the S4 portion of small subunit rRNA. Notable mutations in the nuclear genome were two non-synonymous
436 mutations in Cre01.g024400 which is a component of the TRAPP complex and is predicted to be involved in meiosis, three mutations in gene Cre03.g200250 which is an enzyme that catalyzes the reaction of isocitrate
438 to glyoxylate and succinate, one mutation was in 5' UTR region and two other were in clone P4C7, one of them intronic and one synonymous mutation.

440

For clones evolved in high NaCl we never observed genes with more than two mutations, in contrast to
442 clones evolved in high CO_2 and control environments. In mitochondrial genes, we only observed one non-synonymous mutation in cytochrome b, no mutations in NADH dehydrogenase subunit 5, and only one in
444 RrnS4. Other non-synonymous mitochondrial mutations were one mutation in rtl, and one mutation in NADH dehydrogenase subunit 2. For nuclear mutations, we observed one intronic and one non-synonymous
446 mutation in Cre09.g400850, which is a putative polycystin cation channel.

448 Since the demethylation treatment contained a large number of mutations we also looked at the function of genes that were overrepresented in terms of having multiple mutations across different environments. In the
450 control and high CO_2 environments we see an over representation of mutations in genes related to microtubule based movement (GO terms 0070018, 0070017, and 006928) (Table 6). These mutations could
452 reflect changes in swimming behaviour if swimming is costly for *C. reinhardtii*. In all environments, we also

observed mutations in genes related to cyclic nucleotide metabolism and biosynthesis (GO terms 0009187,
454 and 0009190). These changes may reflect adaptation to laboratory conditions and the growth media as these
terms are significant in all environments including the control (Table 6). In control and high NaCl
456 environments, terms for oxidative phosphorylation and electron transport were also significant. In high CO_2
and high NaCl environments GO terms for detecting external and abiotic stimulus, as well as those for
458 detection and response to mechanical stimulus were significant (Table 6). In the high NaCl environment, the
GO term for transmembrane transport was the most significant term, which may be related to osmoregulation
460 in the high salt environment.

462 *Bisulfite sequencing and general features of DMRs*

464 To examine the methylation changes that happened during adaptation, we compared the methylation patterns
of the evolved clones. Methylation levels were low in *C. reinhardtii* in this experiment, as expected (Feng et
466 al., 2010; Lopez et al., 2015). The mean value of CG methylation was 1.3 %, CHG methylation was 0.3 %,
and CHH methylation was 0.4 % for the ancestor in the control environment and treatment. It is unclear how
468 to interpret the biological effects of differences in methylation of single cytosines (Wibowo et al., 2016), so
we focused our analysis of differential methylation patterns on large contiguous stretches of methylation.
470 Differentially methylated regions (DMRs) were found by pairwise comparison of all the evolved samples
and the ancestor to each other, and regions that had differences in methylation were designated as DMRs.
472 See methods for detailed criteria required for a region to be defined as DMR.

474 We first considered DMRs that were detected when all clones were included in the analysis. We detected 924
such DMRs, with a median length of 61 bp (ranging from 9 bp to 1150 bp in length). Most DMRs (72 %)
476 were located within genes and 28 % were intergenic, which contrasts with results in *Arabidopsis* where 20 %
of DMRs were located within genes (Hagmann et al., 2015; Wibowo et al., 2016) but is consistent with *C.*
478 *reinhardtii* having mainly genic methylation (Feng et al., 2010; Lopez et al., 2015). Of the within- gene
DMRs, 54 % were in exons, 26 % in introns 1.8 % in 5' UTRs, and 19 % in 3' UTRs.

480

Clustering of clones based on methylation changes

482

When we clustered clones based on DMRs (Figure 4), the ancestor samples and the evolved clones separate
484 with high bootstrap support. The ancestor samples have higher methylation rate in the observed DMRs
(Figure 4). This may be attributable to storage effects on the ancestor, as the ancestor had to be stored on agar
486 slants. Based on hierarchical clustering, clones P11B4, P11B11, and P12E4 from the high NaCl environment
cluster together with bootstrap support of 100% (Figure 4), suggesting that the DMRs unique to these clones
488 are specifically involved in adaptation to high salt. The remaining clones cluster by chemical treatment,
indicating that the demethylation treatment affected methylation patterns in this experiment, although
490 bootstrap support for the branch separating the remaining evolved clones by chemical treatments is 89%.

Among the ancestor samples, the branch separating the chemical treatments has a bootstrap support of 100%.

Epigenetic changes among the control and demet treatment clones

Because the difference in ancestral and control evolved methylation patterns is so large (Figure 4), and may represent a storage effect rather than evolutionary differences relevant to this experiment, we considered the 542 DMRs that were polymorphic among the evolved clones and used parsimony to determine the ancestral state of the DMR. For each DMR, we assigned the DMR ancestral state to the most common state among the clones from the chemical control treatment of all environments. To resolve ties we included all the evolved clones. Among the evolved clones, most DMRs occurred only in one clone (Figure 5A), but some were present in multiple clones. Certain DMRs were also present across multiple clones that had evolved in different environments (Figure 5B). DMRs in the evolved clones had similar distribution of annotations as all DMRs (Figure 5C).

We observed 143 derived DMR changes in the clone from control chemical treatment and control environment. Among the control treatment clones from the high CO_2 environment there were on average 87 DMR changes per clone, and on average 149 changes per clone in the high NaCl treatment. Changes per clone are listed in Table 4. In the clones from the demet chemical treatment, we observed 70 DMRs on average in the control environment, 73 changes on average in the high CO_2 environment, and 123 changes in the high NaCl environment. While the total number of DMR changes was lower in the demet (483) than in the control treatment (852), this difference was only marginally significant (Wilcoxon-Mann-Whitney rank sum test, W = 34, p-value = 0.073). We also compared the mean methylation levels of DMRs in control and demet clones over all environments in different sequence contexts (Figure 5D). Methylation rates in CG context were 19.8 % for the control clones, and 10.2 % for clones strains (paired sample t-test, t = 24.8, df = 541, $p < 2.2 \times 10^{-16}$). For CHG context methylation rates were 6.1 % and 7.4 % (paired sample t-test, t = -0.96, df = 45, p-value = 0.347), for control and demet. samples respectively. For CHH context methylation rates were 4.9 %. and 5.5 % (paired sample t-test, t = -0.40, df = 48, p-value = 0.688). This shows that the demethylation treatment did reduce overall methylation levels in the CG context. The very low methylation rate in the first place limits our ability to detect differences in methylation in the other sequence contexts.

Next we investigated whether DMRs occurred evenly across the two chemical treatments. We observed that out of the 542 DMRs present in the evolved clones a change in 500 of them occurred in the control chemical treatment, and a change in 223 of them occurred in the demet chemical treatment. Of those changes, 319 were unique to the control treated lines, 181 occurred in both treatments, and 42 changes were unique to the demet chemical treatment. We tested whether we had expected amounts of unique changes by permuting (n = 1000) the clone labels, 95 % quantiles were: overlapping changes 174 – 253, 86 – 292 for changes unique to control clones, and 64 – 240 for changes unique to demet clones. Thus, there was an over representation of changes unique to control clones and under representation of changes unique to demet clones. Most of the

DMRs were gains in methylation. However, DMRs that changed in multiple clones were often losses of
530 methylation, especially in the demet treatment clones (Table 7). These results are consistent with the
demethylating effect of the chemical treatment and the demet clones being less likely to use changes in
532 methylation patterns to adapt as a result.

534 *No indication of genetic control of DMRs*

536 In clone P12E4, which evolved in high NaCl, there is a mutation in chromosome 16 at position 3227089 that
is close to three DMRs in that region. No other genetic mutations were within 1kb up or downstream of the
538 DMRs. There was only a single case of over overlap between genetic mutations and DMRs among the demet
clones. In clone P4C7, which evolved in high CO_2, a DMR occurred on chromosome 14, position 3546199
540 that was downstream and within 1 kb of a genetic mutation. Thus across all clones there were only two cases
where a genetic mutation was near a DMR. While we cannot exclude the possibility that some of the
542 mutations are *trans*-acting or *cis*-acting over very long distances the vast majority of the DMRs appear
independent of DNA sequence changes. Furthermore, clones from the demet treatment contained nearly the
544 same number of DMR changes as did clones from the control chemical treatment, despite having many more
genetic mutations. This supports the interpretation that many of the DMR differences were not directly
546 caused by genetic mutations.

548 *Enrichment of gene ontology annotations for DMRs*

550 To examine the potential effects of the DMRs on phenotype, we identified GO terms that were enriched in
DMRs that were polymorphic among the evolved clones regardless of chemical treatment in each of the
552 three environments. In the control environment, only a few GO terms were enriched ($p = 0.00032$), these are
involved in aminoglycan (GO:0006026) and chitin catabolic processes (GO:0006032). Enrichment of these
554 terms was due to DMRs on gene Cre10.g451600 and Cre10.g458350, which are both annotated as chitinases
(E.C 3.2.1.14). Based on computational predictions, *C. reinhardtii* has a chitin degradation pathway, but
556 there is no experimental support for this yet. In the high CO_2 environment, the GO term 0006026 was again
enriched ($p = 0.00020$), again due to polymorphic DMRs in genes Cre10.g451600 and Cre10.g458350. The
558 DMR in gene Cre10.g451600 was also polymorphic in the lines evolved in the high salt environment. While
the physiological role of the putative chitinase genes is unknown, these results indicate that the methylation
560 changes observed in these genes possibly contribute to adaptation to shared laboratory conditions since
changes were observed in all of the environments.

562

Specific changes to high CO_2 included enrichment of GO term for membrane depolarization during action
564 potential (GO:0086010, $p = 0.00036$). This was due to DMRs in genes Cre07.g333535 and Cre11.g467528,
which are both annotated as voltage gated Ca^{2+} channels. In the high salt environment no GO terms reached
566 the cut-off value of 0.01. The GO term 0055085, transmembrane transport, had the lowest lowest p-value (p

= 0.013). DMRs in genes that were responsible for enrichment of this GO term were Cre05.g234645 (sodium/hydrogen exchanger), Cre06.g260100 (adenosine 3'-phospho 5'-phosphosulfate transporter), Cre07.g327750 (ion transport protein), Cre07.g333535 as well, and Cre17.g725150 (xenobiotic-transporting ATPase).

For DMRs that were polymorphic among the ancestor samples, we did not find any significantly enriched GO terms that were represented by more than one gene.

Discussion

Based on theoretical models of adaptation with epigenetic variation (Klironomos et al., 2013; Kronholm and Collins, 2016), reducing the amount of epigenetic variation either genetically or chemically should decrease adaptation. Our study broadly supports these predictions; we see evidence for epigenetic contributions to adaptation in the high NaCl and high CO_2 environments, which are the two environments where a large direct response to selection occurred. Our major findings are summarized in Table 8.

Effects of epigenetic manipulation on adaptation in the selective environments

The results from the high NaCl environment most closely match the prediction that reducing the amount of epigenetic variation available with the chemical treatments decreased adaptation. This effect was not likely mediated by SIR2 dependent mechanisms as the *sir2* mutation had no consistent effects in high NaCl. We also observed that clones from the high NaCl environment clustered together based on their methylation changes. Taken together these data support the conclusion that epigenetic changes are important in adapting to this environment.

In the low phosphate environment, the evolutionary response in growth rate was small and no consistent effect of the chemical treatments or the *sir2* mutation was observed. In the CC-2937 strain many populations went extinct and this reduces our power to detect the effects of the chemical treatments for the CC-2937 strain. Overall, it may be that our power to detect an effect of epigenetic mechanisms is small due to higher variation in evolutionary responses, or there is little or no epigenetic regulation of phosphate metabolism in *Chlamydomonas*.

In contrast, the evolutionary response to high CO_2 was to decrease population growth rates. While this may seem counter-intuitive, previous studies of single-celled chlorophytes evolving in high CO_2 environments show that high growth rates result in low competitive fitness, and that this can associated with poor mitochondrial function (Schaum and Collins, 2014). Therefore the evolution or maintenance of low growth rates may be adaptive in high CO_2 environments, where rapid growth both compromises the ability of cells to withstand other challenges, and decreases competitive ability. Indeed, lineages with slower population

growth rates evolved in high CO_2 environments are generally better competitors than faster-growing lineages evolved in those same environments (Collins, 2010; Schaum and Collins, 2014). This evolutionary reversal of a plastic increase in cell division rates has been called “Prodigal Son dynamics”. Modelling studies suggest that Prodigal Son dynamics may occur when cells cannot evolve mechanisms to cope with the consequences of maintaining an overall increase in metabolism in enriched environments (Collins, 2016). Thus, if low growth rate is adaptive in high CO_2, we observed an adaptive evolutionary response in our experiment. We do not show in this study that higher growth rates are maladaptive, so it is also possible that increased growth in high CO_2 environments is adaptive for *Chlamydomonas*, but this interpretation goes against all of the empirical evidence to date, so we consider it to be unlikely here. The demethylation chemical treatment alone did not reduce adaptation but the demethylation + acetylation had a consistent adaptation reducing effect in the CC-2937 background. In terms of methylation patterns, strains from the high CO_2 environment cluster together with strains from the control treatment. This is consistent with our observation that demethylation alone had little effect, and suggests that changes in DNA methylation do not contribute to adaptation that is specific to high CO_2. However, other epigenetic modifications, such as histone acetylation, may do so. The plastic response to high CO_2 was diminished in the *sir2* mutant strain but not in the complemented strain, suggesting that SIR2 mediated silencing may be important for plastic response to high CO_2, but that the evolutionary response of the LM3 background is different from CC-2937 background.

Potential side effects of the chemical treatments

The chemicals 5-aza-deoxycytidine and Zebularine are mutagenic, as is evident by our sequencing results. This raises the possibility that an increased input of deleterious mutations, or mutational meltdown, could explain cases where little or no adaptation occurred. However, UV-treated populations, which also have extremely high mutation rates, adapted in a similar manner in all environments showing that increased mutation loads did not impede adaptation in this experiment. Thus, the decreased rates of adaptation in the chemical treatments are unlikely to be caused by an increased input of deleterious mutations, and are more likely to be caused by the effects of the drugs on the production and transmission of epigenetic information.

Epigenetic mechanisms (methylation, acetylation) have many cellular functions, some of which are related to normal functioning of the cell, so that chemically manipulating epigenetic marks could conceivably have general toxic effects on cells. However, general toxic effects are unlikely to be driving our results. First, the chemical treatments had no systematic effect on ancestral fitness in the control environment during pilot studies. Second, the effects of the chemical are strongly dependent on the environment. If the chemical treatments were acting through cytotoxic effects, we would expect them to have an effect in the same direction across the environments, which was not the case for the ancestors. This environmental dependence suggests that effects on growth are caused by changes in cellular function due to the modification of epigenetic marks rather than general cytotoxicity. Finally, the chemical treatments change growth rates, but do not systematically lower them, in the populations evolved in the control treatment. This strongly suggests

644 that the growth effects seen in the chemical treatments are due to the modification of epigenetic marks rather than cytotoxicity.

646

If cytotoxic effects exist, they could also have demographic effects, such as depressing population sizes or 648 slowing down cellular division rates, which would result in the chemically treated populations going through fewer generations over the experiment. We examined this possibility but found no evidence that the chemical 650 treatments caused demographic effects large enough within environments to explain variance in evolutionary outcomes (see Supplementary Information). Since we cannot find any reasonable indication that the effects 652 of the chemical treatments are only due to cytotoxicity or demographics effects, we conclude that the differences in growth associated with these treatments are likely attributable to their effects on the 654 transmission of epigenetic information between transfers.

656 *DNA methylation changes*

658 In both this and other studies, the role of changes to DMR patterns relative to genetic change appears both species and environment specific. Among the sequenced clones from chemical control treatment, we 660 observed the most genetic mutations and DMR changes in the high NaCl environment. Many more DMR changes occurred than genetic mutations, which is consistent with changes in DMRs being more common 662 than genetic mutations in *C. reinhardtii*. This is in line with mutation accumulation experiments that have shown rapid changes in methylated positions, with a rate for gain of methylation reported at 2.56×10^{-4}, and 664 loss 6.30×10^{-4} per CG site per generation in *Arabidopsis* (van der Graaf et al., 2015). However, there are also reports that DMRs can change at similar rates to genetic mutations (Becker et al., 2011). In *Arabidopsis*, 666 for example, natural variation in methylomes shows that DMR patterns tend to be stable and often under genetic control (Dubin et al., 2015; Hagmann et al., 2015). In contrast, our results show that for *C.* 668 *reinhardtii* the role of changes in DMR patterns relative to genetic variation is environment specific. Comparing our results with those from *Arabidopsis* indicates that the role of changes to DMR patterns is also 670 species specific.

672 Like genetic changes, we see evidence for limited “parallel evolution” (Bailey et al., 2015) of changes in DMR patterns between replicate populations evolving in the same environments. We observed some DMR 674 changes that occurred in parallel over many, but not all, lines within selection environments. These parallel changes suggest that changes to methylation patterns have the potential to be adaptive, but our study cannot 676 separate the possibilities of the changes either being environmentally induced from that of the changes being random and under positive selection (or some combination of these two). This would be an interesting 678 direction for future work, and would require a detailed comparison of the epigenetic changes associated with plastic and adaptive evolutionary responses in the same environments, as well as a reasonably accurate 680 epigenotype-phenotype map.

682 Previous studies on natural variation in DNA methylation have suggested that most methylation variants are

controlled by DNA sequence (Dubin et al., 2015; Hagmann et al., 2015). One example of this would be a
684 SNP or a transposable element insertion determining whether a downstream sequence gets methylated or not.
However, there is no evidence that genetic mutations caused the observed methylation changes in our study.
686 We observed only two cases across the 13 sequenced lines where a genetic mutation was within 1 kb of a
region that had a DMR change. In the chemical control lines we observed many more DMRs than genetic
688 mutations, so if genetic mutations were indeed responsible for a high proportion of DMR changes, they
would have to control multiple DMRs over long and variable distances in *trans*.

690

Effect of epigenetic variation on adaptation

692

Theoretical models predict that adaptation from epigenetic variation happens in two steps: first a population
694 adapts using epigenetic variation, and then epigenetic variation is replaced by genetic changes over a long
period of time (Klironomos et al., 2013; Kronholm and Collins, 2016). Our observations are in line with
696 some, but not all, of the model predictions. In our experiment, manipulating the epigenetic system slows
adaptation, which is consistent with the model prediction that epigenetic changes, which occur at a faster
698 rate, are available first to natural selection. Consistent with the prediction of epigenetic changes preceding
genetic ones, we observed more DMR changes than genetic mutations in the chemical control lines.
700 However, this study did not include a timecourse to monitor the rate at which genetic and epigenetic changes
were fixed in populations, nor the rate at which epigenetic changes disappeared. While our study did not test
702 the timescale on which epigenetic changes were replaced with genetic changes as predicted by models, the
outcome of test for phenotypic stability in the control chemical treatment populations is certainly consistent
704 with epigenetic changes being replaced with genetic changes during adaptation. Recently, Wang et al. (2015)
also demonstrated that a fission yeast mutant with uncontrolled heterochromatin spreading reverted back via
706 rapid epigenetic adaptation.

708 *Conclusion*

Epigenetic variation can contribute to adaptation, although the extent to which it does so depends on the
710 selection environment. These results highlight the need to consider epigenetic variation during
microevolution, even on timescales where genetic mutations can be used. While this study shows that
712 epigenetic variation can contribute to adaptation, it also points out the need to better characterize epigenetic
mutations in a way that will allow them to be included in standard theory. In particular, understanding the
714 link between genetic mutations and patterns of epigenetic change is required in order to advance our
mechanistic understanding of how phenotypes evolve.

716

Materials and methods

718

Chlamydomonas strains

We used four different *Chlamydomonas* strains in the selection experiment: the strain CC-2937, UV-treated CC-2937, a *sir2* mutant and a complemented mutant (the rationale for treating the UV-treated CC-2937 as a strain is detailed in the “selection experiment” section). The *sir2* mutant was generated in the genetic background hereafter called LM3 by insertional mutagenesis (see below). A complemented *SIR2* line was constructed by transforming the genomic region encompassing the *SIR2* gene into the mutant background. The LM3 strain has no cell wall, which allows for easier transformation. CC-2937 is a standard wild-type strain of *Chlamydomonas reinhardtii*, obtained from the *Chlamydomonas* Resource Center.

In order to manipulate the genetic mutational supply available, we treated the UV CC-2937 strain with UV-radiation every other transfer, using a UV-lamp placed 5 cm above the plate for 1 minute. This produced an irradiance of 33.75 W / m^2, giving a radiant exposure of 2025 J / m^2. This radiation dose was chosen based on preliminary experiments that showed an increased number of mutants appearing in a culture but did not substantially increase mortality (see supplementary material).

sir2 mutant and complementation

The *sir2* mutant was found using a screen for components involved in transgene silencing. Briefly, the screen was performed by using a reporter plasmid containing a 500 bp repetitive region from the 3' end of the L1 transposon, which was cloned upstream of a bleomycin/zeocin resistance cassette driven by the RBCS2 promoter (pMTBRBle-L1-3'-2F). The forward primer used to amplify the L1-region was: TTAGATCTATTGGAGACAACGCGCTGTACC and the reverse primer was: TTAGATCTGCCTTGCTCTTGTCCGGATGG. The plasmid also contained an *ARGININOSUCCINATE LYASE* gene for transformant selection. The plasmid was transformed into the *cw*15- 325 *arg*- strain, and a clone was selected that had silenced expression of the zeocin resistance gene, and was therefore sensitive to zeocin. The presence of silenced zeocin resistance cassette was checked via PCR and using the histone acetylation inhibitor Trichostatin A, which increased zeocin resistance. Insertional mutagenesis was performed by transforming the zeocin sensitive strain with a linearized pKanAPHVIII plasmid which contained a paromomycin resistance cassette driven by PSAD regulatory elements. Mutants were selected for both zeocin and paromomycin resistance. All transformations were performed using the glass-beads method (Kindle et al., 1991). The site of the insertion was determined by inverse PCR and sequencing which showed an insertion in intron 2 of the gene Cre10.g462200 (Figure S6), which belongs to the SIR2 family of class IV sirtuins. The site of insertion was confirmed by PCR amplification across the region of the insertion and Southern blotting. Complementation of the *sir2* mutant was performed with a 6172 bp genomic fragment encompassing the entire *sir2* gene and including 1.2 kb of the upstream promoter region. This was amplified by PCR using Pfusion polymerase (NEB) and oligonucleotides gSIR2F (attaatGAGCGATGTCGGTGGCCCC) and gSIR2R (attaatTTTGCGGTACCGGTCCCACG), and cloned into the *Ase* I site of the pMTH vector encoding a hygromycin resistance gene driven by a PSAD promoter

756 for transformant selection. Mutant strains were transformed, selected with hygromycin, and tested for complementation of the mutant phenotype by loss of expression of the zeocin resistance gene by qRT-PCR
758 (Figure S7) and by comparison of growth on media containing different zeocin concentrations (Figure S8).

760 *Chemical treatments*

Two different chemical treatments were used to decrease the transmission rates of epigenetic information
762 during our evolution experiment. To lower methylation rates, we used mixture of 5-aza-2-deoxycytidine at 0.2 mM and zebularine at 0.2 mM, both of which are cytosine analogs that cannot be methylated (Jones,
764 1985; Cheng et al., 2003), along with L-Ethionine at 0.2 mM, which blocks methylation by competing with methionine for the transfer of methyl groups to DNA (Moore and Smith, 1969). We refer to this treatment as
766 "demet". In the second chemical treatment we used 5-aza-2-deoxycytidine and L-Ethionine as before, but added a histone deacetylase inhibitor Trichostatin A (TSA) (Marks et al., 2000) at 0.1 μM. We refer to this
768 treatment as as "demet + acet". These concentrations of 5-aza-2-deoxycytidine and L-Ethionine have been shown to demethylate DNA in *C. reinhardtii* , after treating cells with 0.2 mM of the drugs, the same
770 concentration used in this study, no methylation was detected in chloroplast DNA in vegetative cells and approximately 45 % reduction in methylation occurred in gametes and zygotes, which are heavilty
772 methylated in Chlamydomonas (Feng and Chiang, 1984). Since the 5-aza-2-deoxycytidine stocks had to be dissolved in DMSO, a DMSO blank was included in the control chemical treatment.
774

Selection environments

776 Four different selection environments were used, each of which imposed a different selection pressure on evolving populations. The environments were: high CO_2, high salt, and low phosphate, and a control
778 environment that reflects standard laboratory conditions.

780 The experiment was done in customized incubators (Infors, Basel, Switzerland). For control environments, the CO_2-level was set at 420 ppm and temperature was set to +25 °C. In all environments the base growth
782 media was Sueoka high salt media (HSM) (Sueoka, 1960) with 20 mM Tris added (HSMT) buffered at pH 7.0. Populations were cultured in 200 μl of media under constant light. This reflects the usual culturing
784 conditions for these stains. We used AeraSeal breathable sealing films (Alpha Laboratories, Hampshire, UK) to cover the 96-well plates to allow even gas exchange across all wells.
786

The selection environments were modified as follows: in the high CO_2 environment we increased CO_2 levels
788 to 2000 ppm, in the high NaCl environment we added 43 mM of NaCl to the base media and in the low phosphate environment we decreased the phosphate available in the HSMT by 50-fold, from 13.56 mM to
790 0.2712 mM. Phosphate is added to HSMT as potassium salt, so we added KCl to the low phosphate media to achieve the level of 22 mM K^+ ions as in the control media.

792

Selection experiment

794 The selection experiment consisted of four strains (CC-2937, UV irradiated CC-2937, *sir2* mutant, complemented *SIR2* mutant), three chemical treatments (demet, demet + acet, control), and four selection
796 environments (high salt, high CO_2, low phosphate, control), for a total of 48 treatments. We used the control environment populations as an evolving control, and compared growth rates of the populations from the
798 other environments to these populations. We wanted to investigate how important epigenetic effects are for adaptation in general in different environments, so having an evolving control that adapts to the shared lab
800 environment allows us to investigate specific adaptation to the different environments. We treated the UV-irradiated CC-2937 as a strain throughout the experiment. UV-irradiation is used here to increase the genetic
802 variation produced. Each combination of strain, chemical treatment, and selection environment had 9 independent replicate populations. The selection experiment was carried out on 96 well plates (Corning, NY,
804 USA) using a split plot design, with the different chemical treatments randomized on the columns (plots) of each plate and genotypes randomized within plots, with blank wells present in a unique pattern within each
806 plate, both to monitor possible migration between wells, and to serve as a unique identifier for each plate. Edges of plates were filled with water to minimize edge effects. All populations were founded from single
808 cells, so that very little genetic variation was present within populations at the beginning of the experiment. All populations were acclimated to the selection environment for four days, after which a population of 5000
810 cells was used to start the selection experiment.

812 Populations were propagated by batch transfer. During each transfer we transferred 40 µl of control and low phosphate populations, 50 µl of the high NaCl, and 20 µl of the high CO_2 populations to a new plate with 200
814 µl of fresh medium. In the first four transfers 40 µl of the high NaCl populations and 20 µl of control populations were transferred but this amount was increased to 50 µl and 40 µl respectively in the subsequent
816 transfers. The populations were transferred twice a week, and population size measured at each transfer. During the experiment there was an interruption in the compressed air supply on transfers 7 and 8, during
818 this interruption the lights were turned off and the plates sat in the incubator for seven days. After this the experiment resumed normally. The selection experiment was run for 62 transfers (roughly 200 generations).
820 The selection experiment was not designed to give us detailed understanding of adaptation to each different environment, but to use different environments (three different selection experiments) to let us examine the
822 general role of epigenetic effects in adaptation.

824 *Standard curves for optical density and cell counts*

At the end of the selection experiment we constructed a standard curve for optical density and cell counts for
826 each of the populations both in the environment it evolved in and in the control environment and all other environments for populations evolved in the control environment. Dilution series were made on 96-well

828 plates and the cultures we measured spectrophotometrically. All spectrophotometric measurements were done using absorbance at 750 nm with an EL808 plate reader (BioTek, Potton, UK). Cell numbers were
830 measured in these cultures by flow cytometry (see supporting information for details). Standard curves were constructed by fitting a linear regression to the data (for all standard curves median $R^2 = 0.9902$). In all
832 subsequent experiments cell numbers were estimated by transforming absorbances into cell numbers using these curves.
834

Fitness measurements

836 Control and selection environments used for fitness assays were the same as during the selection experiment. Populations were acclimated to the environment in which fitness was measured for one transfer and then
838 transferred to a fresh medium. Growth curves in the environment of interest were made by measuring the cell density over 140 hours. Three replicate fitness measurements were performed for each population. The
840 fitness of evolved control populations was measured in all environments. Populations evolved in the high CO_2, high salt, and low phosphate environments were measured in their selection environment and in the
842 control environment. In all cases, chemical treatments used for the fitness assays are the same for each population as they experienced during the selection experiment unless otherwise noted.
844

Statistical analysis

846 To calculate fitness, we fitted growth curves to the cell number data. We used the baranyi growth curve model in the R package “nlstools” (Baty and Delignette-Muller, 2012) without lag phase. The curves were
848 fitted using the Levenberg-Marquardt algorithm implemented in the R package "minpack.lm" (Elzhov et al., 2013). Some populations experienced a lag phase, while others did not; in order to fit the same growth model
850 to all populations, we removed the data points within the lag phase when it were present. We extracted the maximum growth rate, *r*, from the growth model for each population and used this as a measure of fitness.
852 Relative fitness measures were calculated by standardizing the absolute fitness for each population by the mean fitness of control populations measured in that environment and chemical treatment. For example, the
854 relative fitness of populations of strain CC-2937 evolved in the high salt environment and demet chemical treatment was calculated by standardizing their absolute growth rate by the mean absolute growth rate of
856 CC-2937 populations evolved in the control environment and demet chemical treatment. By comparing the same chemical treatments to each other, any growth inhibiting effects that the chemicals themselves have are
858 controlled for. Fitness data was analyzed using linear models in R (R Core Team 2013), we fitted fitness as a response variable and assay environment, selection environment, chemical treatment, and strain and their
860 interactions as explanatory variables. First we fitted all terms and their interactions and then dropped non-significant interactions one by one to get to the final model. Contrasts were calculated using the R package
862 “contrast”. Throughout the study, responses are reported as mean ± SEM.

864 *Sample preparation for genome and bisulfite sequencing*

866 After the evolution experiment we chose three populations randomly from different experimental plates from control, high CO_2, and high salt environments, and the control chemical treatment and from the
868 demethylation (demet) treatment. We focused only on populations on the CC-2937 background, and low phosphate environment was excluded because of not enough populations were alive. We plated cultures of
870 these populations and picked single colonies from each at random that were stored for further experiments, hereafter called clones. This came to 18 evolved clones and the CC-2937 ancestor, 19 clones in total for
872 genome re-sequencing. In addition, we grew the ancestor in the three environments and in the control environment with and without demet treatment. For comparing the control and demet chemical treatments
874 we used three independent replicate cultures. In total for bisulfite sequencing we had 22 different clones, since five were lost due to failed library construction (Table S2). We grew the clones to high density in liquid
876 culture in the same environmental conditions that they had experienced during the evolution experiment and extracted DNA using phenol-chloroform extraction.

878

Genome re-sequencing and bisulfite sequencing was done at the Beijing Genomics Institute (BGI-Hong
880 Kong) with the Illumina HiSeq 2000 platform using paired-end sequencing. For re-sequencing Libraries were prepared by fragmenting DNA by sonication, ligating adapters, size selecting DNA, and PCR
882 amplified. Insert size in libraries was 500 bp. *Chlamydomonas* has a high GC content, so PCR conditions in library preparation were modified to accommodate high GC content following Aird et al. (2011). For
884 bisulfite sequencing, after fragmenting DNA methylated sequencing adaptors were ligated and DNA was bisulfite treated with the ZYMO EZ DNA Methylation-Gold kit, following manufacturer's instructions. Insert
886 size in libraries was 100-300 bp. Libraries were sequenced with the Illumina HiSeq 2000 platform using paired-end sequencing, read length was 90 bp. We obtained approximately 4 Gb of clean sequence for each
888 sample.

890 *Read mapping for DNA resequencing and genotyping*

892 In general for read mapping and genotyping we followed the approach of Ness et al. (2012). We mapped reads to the available *Chlamydomonas* reference genome using BWA (Li and Durbin, 2009) and realignment
894 of reads near indels was done using GATK 3.1-1 (McKenna et al., 2010; DePristo et al., 2011). Genotypes were called jointly for all samples with GATK Unified Genotyper with heterozygosity set to 0.01, minimum
896 base quality to 10, and sample ploidy set to diploid.

898 Mutations that had happened during the evolution experiment were identified as different genotype calls in the ancestor and a sample. We processed vcf files produced by GATK using Wormtable 0.1.5a2 (Kelleher et
900 al., 2013) and a custom Python script. We filtered low quality genotype calls by discarding all cases where read depth was less than five, genotype quality less than 30 for either ancestor or the sample. All

heterozygous positions were also discarded; we let Unified Genotyper run in diploid mode even though *Chlamydomonas* is haploid, as we observed that heterozygous genotype calls were indicative of read mapping errors. After a list of putative mutations had been produced by the filtering step, we checked mutations manually in IGV (Thorvaldsdóttir et al., 2013); visual inspection of read alignments in IGV revealed any potential sequencing or mapping errors. This allowed us to produce a manually curated list of mutations. For the control chemical treatment we manually checked all mutations and for the demethylation chemical treatment we checked 18 % of mutations prioritizing those with the lowest genotype qualities.

Read mapping for bisulfite sequencing and methylated base calling

For read mapping of bisulfite treated reads we used BWA-meth (Pedersen et al., 2014). BWA-meth uses *in silico* conversion of C's to T's in both reference genome and reads. Methylation status of C's is recovered by comparing mapped converted reads to the original reads. Calling of methylated cytosines was performed using BisSNP 0.82.2 (Liu et al., 2012). Based on a methylation bias plot, we trimmed 4 bp from both 5' and 3' ends of the reads. We let BisSNP call methylated cytosines in CG, CHG, and CHH contexts. Minimum base quality was set to 20 and minimum mapping quality to 60. All samples were called together making use of GNU parallel (Tange, 2011) to parallelize the task. We processed the vcf file using PyVCF 0.6.8 with a custom python script to extract methylated bases for downstream analysis.

Calling methylated regions (MRs) and differentially methylated regions (DMRs)

In order to identify differentially methylated regions in a statistically robust manner we followed the approach of Hagmann et al. (2015). First we identified contiguous methylated regions in each clone using a Hidden Markov Model (Molaro et al., 2011; Hagmann et al., 2015). The model considers each three sequence contexts, CG, CHG, and CHH separately with different methylation rate distributions, the model is trained on genomewide data to identify regions of high and low methylation. We set maximum distance between adjacent cytosines within an MR to 100 bp and trimmed those sites off the ends of the region that had methylation rate < 10 %. After training the model, the methylation rates within a region were summed to give a score for that region. Then whether the observed score was higher than expected by chance was tested by computing an empirical distribution of scores by permuting cytosines across the genome to obtain a p-value for that region. These p-values were corrected for false discovery rate (FDR) and those highly methylated regions that had FDR < 0.05 were kept as MRs.

Next we selected regions to be tested for differential methylation. For two clones every region could in principle be tested for differing methylation. However, we want to group clones based on differentially methylated regions, so identical regions need to be compared to each other. MRs are not necessarily identical in among clones, and for multiple clones this would result in a very large number of tests among all pairwise combinations of clones. Thus, we followed the rules defined by Hagmann et al. (2015) to select regions for

testing. The start and end coordinates of each MR across all clones formed a set of breakpoints and each combination of coordinates defined a segment to be tested for differential methylation. Then using the following rules segments were discarded to reduce the number of tests. Segment was kept if at least one clone was in high methylation state throughout this segment. To detect quantitative methylation differences entirely methylated segments in more than one clone were also compared to each other. Segments were discarded from pairwise comparison if less than two clones contained at least 8 cytosines covered by at least 3 reads each. Segments were discarded if they overlapped more than 70 % with a previously tested segment. Pairwise tests were not performed if both clones were in low methylation state in the region. To prevent testing regions with coverage imbalance clones were excluded if the number of positions covered by at least 3 reads was less than half of the maximum number of such positions of all clones in the same region.

Statistical analysis of DMRs

For data visualisation we used the “ggplot2” and “gplots” R packages. DMR clustering was performed with the “fastcluster” R package (Müllner, 2013). Bootsrap values for DMR clustering were calculated with the “pvclust” R package (Suzuki and Shimodaira, 2006).

Gene ontology term enrichment test

We extracted gene ontology (GO) terms from the *Chlamydomonas* genome annotation and supplemented these with annotations from the Uniprot database. We used reciprocal BLAST to identify matching genes between the *Chlamydomonas* genome annotation and the Uniprot database. All proteins which were annotated as from *Chlamydomonas* in the Uniprot database were blasted against proteins from the *Chlamydomonas* genome annotation and the best hit was identified. Then all proteins from the *Chlamydomonas* genome annotation were blasted against the Uniprot set and best hit was identified. If the both best hits were against the same proteins, the loci were designated as a pair and GO terms were propagated from one database to another. GO terms for proteins encoded by the organelle genomes were extracted from the Uniprot database. We used a GO term enrichment test, implemented in the R package “topGO” (Alexa et al., 2006), using the classic algorithm. P-values for the GO terms were calculated using Fisher's exact test.

For GO enrichment among the genes with genetic mutations, we included genes that had multiple mutations across the whole experiment among the demet treated strains. Parallel mutations in the experiment could potentially mean that those genes were involved in adaptation. We performed separate tests for the three different environments, while pooling all mutations that occurred in clones from the same environment. For GO enrichment among the DMRs, we included DMRs overlapping genes that were polymorphic at least in one environment. Separate tests were performed for all environments.

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References

988 Aird D., Ross M. G., Chen W.-S., Danielsson M., Fennell T., Russ C., Jaffe D. B., Nusbaum C. and Gnirke A. (2011) Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries. *Genome*
990 *Biology* 12: 1-14.

Alexa A., Rahnenführer J. and Lengauer T. (2006) Improved scoring of functional groups from gene
992 expression data by decorrelating GO graph structure. *Bioinformatics* 22: 1600-1607.

Audergon P. N. C. B., Catania S., Kagansky A., Tong P., Shukla M., Pidoux A. L. and Allshire R. C. (2015)
994 Epigenetics. Restricted epigenetic inheritance of H3K9 methylation. *Science* 348: 132-135.

Bailey S.F., Rodrigue N. and Kassen R. (2015) The effect of selection environment on the probability of
996 parallel evolution. *Molecular Biology and Evolution* 32: 1436-1448.

Barrick J. E., Yu D. S., Yoon S. H., Jeong H., Oh T. K., Schneider D., Lenski R. E. and Kim J. F. (2009)
998 Genome evolution and adaptation in a long-term experiment with *Escherichia coli*. *Nature* 461: 1243-1247.

1000 Baty F. and Delignette-Muller M.-L. (2012) nlstools: tools for non-linear regression diagnostics.

Becker C., Hagmann J., Müller J., Koenig D., Stegle O., Borgwardt K. and Weigel D. (2011) Spontaneous
1002 epigenetic variation in the *Arabidopsis thaliana* methylome. *Nature* 480: 245-249.

Blander G. and Guarante L. (2004) The SIR2 family of protein deacetylases. *Annual Review of Biochemistry*
1004 73: 417-435.

Blount Z. D., Barrick J. E., Davidson C. J. and Lenski R. E. (2012) Genomic analysis of a key innovation in
1006 an experimental *Escherichia coli* population. *Nature* 489: 513-518.

Bonduriansky R. and Day T. (2009) Nongenetic inheritance and its evolutionary implications. *Annual*
1008 *Review of Ecology, Evolution and Systematics* 40: 103-125.

Bossdorf O., Arcuri D., Richards C. L. and Pigliucci M. (2010) Experimental alteration of DNA methylation
1010 affects the phenotypic plasticity of ecologically relevant traits in *Arabidopsis thaliana*. *Evolutionary Ecology* 24: 541-553.

1012 Cheng J. C., Matsen C. B., Gonzales F. A., Ye W., Greer S., Marquez V. E., Jones P. A. and Selker E. U. (2003) Inhibition of DNA methylation and reactivation of silenced genes by Zebularine. *Journal of the*
1014 *National Cancer Institute* 95: 399-409.

Chinnusamy V. and Zhu J.-K. (2009) Epigenetic regulation of stress responses in plants. *Current Opinion in*
1016 *Plant Biology* 12: 133-139.
Collins S. (2010) Competition limits adaptation and productivity in a photosynthetic alga at elevated CO_2.
1018 *Proc Biol Sci* 278: 247-255
Collins S. (2016) Growth rate evolution in improved environments under Prodigal Son dynamics.
1020 *Evolutionary Applications* 9: 1179-1188.
Collins S. and Bell G. (2004) Phenotypic consequences of 1000 generations of selection at elevated CO_2 in a
1022 green alga. *Nature* 431: 566-569.
Collins S. and de Meaux J. (2009) Adaptation to different rates of environmental change in *Chlamydomonas*.
1024 *Evolution* 63: 2952-2965.
Collins S., Sültemeyer D. and Bell G. (2006) Changes in C uptake in populations in *Chlamydomonas*
1026 *reinhardtii* selected at high CO_2. *Plant, Cell and Environment* 29: 1812-1819.
Cortijo S., Wardenaar R., Colomé-Tatché M., Gilly A., Etcheverry M., Labadie K., Caillieux E., Hospital F.,
1028 Aury J.-M., Wincker P., Roudier F. c., Jansen R. C., Colot V. and Johannes F. (2014) Mapping the
Epigenetic Basis of Complex Traits. *Science* 343: 1145-1148.
1030 Crews D., Gillette R., Scarpino S. V., Manikkam M., Savenkova M. I. and Skinner M. K. (2012) Epigenetic
transgenerational inheritance of altered stress responses. *Proceedings of the National Academy of*
1032 *Sciences* 109: 9143-9148.
Cubas P., Vincent C. and Coen E. (1999) An epigenetic mutation responsible for natural variation in floral
1034 symmetry. *Nature* 401: 157-161.
Daxinger L. and Whitelaw E. (2012) Understanding transgenerational epigenetic inheritance via the gametes
1036 in mammals. *Nature Reviews Genetics* 13: 153-162.
Day T. and Bonduriansky R. (2011) A unified approach to evolutionary consequences of genetic and
1038 nongenetic inheritance. *American Naturalist* 178: E18-E36.
DePristo M. A., Banks E., Poplin R., Garimella K. V., Maguire J. R., Hartl C., Philippakis A. A., del Angel
1040 G., Rivas M. A., Hanna M., McKenna A., Fennell T. J., Kernytsky A. M., Sivachenko A. Y., Cibulskis
K., Gabriel S. B., Altshuler D. and Daly M. J. (2011) A framework for variation discovery and
1042 genotyping using next-generation DNA sequencing data.. *Nature Genetics* 43: 491-498.
Dettman J. R., Rodrigue N., Melnyk A. H., Wong A., Bailey S. F. and Kassen R. (2012) Evolutionary insight
1044 from whole-genome sequencing of experimentally evolved microbes. *Molecular Ecology* 21: 2058-2077.
Donelson J. M., Munday P. L., McCormick M. I. and Pitcher C. R. (2012) Rapid transgenerational
1046 acclimation of a tropical reef fish to climate change. *Nature Climate Change* 2: 30-32.
Dubin M. J., Zhang P., Meng D., Remigereau M.-S., Osborne E. J., Paolo Casale F., Drewe P., Kahles A.,
1048 Jean G., Vilhjálmsson B., Jagoda J., Irez S., Voronin V., Song Q., Long Q., Rätsch G., Stegle O., Clark R.
M. and Nordborg M. (2015) DNA methylation in Arabidopsis has a genetic basis and shows evidence of
1050 local adaptation. *eLife* 4: e05255
Durand S., Bouché N., Strand E. P., Loudet O. and Camilleri C. (2012) Rapid establishment of genetic
1052 incompatibility through natural epigenetic variation. *Current Biology* 22: 326-331.

Elzhov T. E., Mullen K. M., Spiess A.-N. and Bolker B. (2013) minpack.lm: R interface for to the
1054 Levenberg-Marquardt nonlinear least-squares algorithm found in MINPACK, plus support for bounds.
Feng S., Cokus S. J., Zhang X., Chen P.-Y., Bostick M., Goll M. G., Hetzel J., Jain J., Strauss S. H., Halpern
1056 M. E., Ukomadu C., Sadler K. C., Pradhan S., Pellegrini M. and Jacobsen S. E. (2010) Conservation and divergence of methylation patterning in plants and animals. *Proceedings of the National Academy of*
1058 *Sciences* 107: 8689-8694.
Feng T.-Y. and Chiang K.-S. (1984) The persistance of maternal inheritance in *Chlamydomonas* despite
1060 hypomethylation of chloroplast DNA induced by inhibitors. *Proc Natl Acad Sci U S A* 81: 3438-3442.
Frye R. A. (2000) Phylogenetic Classification of Prokaryotic and Eukaryotic Sir2-like Proteins . *Biochemical*
1062 *and Biophysical Research Communications* 273: 793-798.
Gaydos L. J., Wang W. and Strome S. (2014) H3K27me and PRC2 transmit a memory of repression across
1064 generations and during development. *Science* 345: 1515-1518.
van der Graaf A., Wardenaar R., Neumann D. A., Taudt A., Shaw R. G., Jansen R. C., Schmitz R. J., Colomé-
1066 Tatché M. and Johannes F. (2015) Rate, spectrum, and evolutionary dynamics of spontaneous epimutations. *Proceedings of the National Academy of Sciences* 112: 6676-6681.
1068 Hagmann J., Becker C., Müller J., Stegle O., Meyer R. C., Wang G., Schneeberger K., Fitz J., Altmann T., Bergelson J., Borgwardt K. and Weigel D. (2015) Century-scale Methylome Stability in a Recently
1070 Diverged *Arabidopsis* thaliana Lineage. *PLoS Genet* 11: e1004920.
Hartl D. L. and Clark A. G., (1997) *Principles of Population Genetics*. Sinauer Associates, Inc., Sunderland.
1072 Herrera C. M., Pozo M. I. and Bagaza P. (2012) Jack of all nectars, master of most: DNA methylation and the epigenetic basis of niche width in a flower living yeast. *Molecular Ecology* 21: 2602-2616
1074 Jablonka E. and Raz G. (2009) Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. *Quarterly Review of Biology* 84: 131-176.
1076 Johannes F., Porcher E., Teixeira F. K., Saliba-Colombani V., Simon M., Agier N., Bulski A., Albuisson J., Heredia F., Audigier P., Bouchez D., Dillmann C., Guerche P., Hospital F. and Colot V. (2009) Assessing
1078 the Impact of Transgenerational Epigenetic Variation on Complex Traits. *PLoS Genetics* **5**, e1000530.
Jones P. A. (1985) Altering gene expression with 5-Azacytidine. *Cell* 40: 485-486.
1080 Kelleher J., Ness R. W. and Halligan D. L. (2013) Processing genome scale tabular data with wormtable. *BMC Bioinformatics* 14: 1-5.
1082 Kelly W. (2014) Transgenerational epigenetics in the germline cycle of *Caenorhabditis elegans*. *Epigenetics & Chromatin* 7: 6.
1084 Kindle K., Richards K. L. and Stern D. B. (1991) Engineering the chloroplast genome: Techniques and capabilities for chloroplast transformation in Chlamydomonas reinhardtii. *Proceedings of the National*
1086 *Academy of Sciences* 88: 1721-1725.
Klironomos F., Berg J. and Collins S. (2013) How epigenetic mutations can affect genetic evolution: Model
1088 and mechanism. *BioEssays* 35: 571-578.
Kronholm I. and Collins S. (2016) Epigenetic mutations can both help and hinder adaptive evolution.
1090 *Molecular Ecology* 25: 1856-1868.

Lachapelle J. and Bell G. (2012) Evolutionary rescue of sexual and asexual populations populations in a
1092 deteriorating environment. *Evolution* 66: 3508-3518.
Lachapelle J., Bell G. and Colegrave N. (2015) Experimental adaptation to marine conditions by a freshwater
1094 alga. *Evolution* 69: 2662-2675.
Lauria M., Piccinini S., Pirona R., Lund G., Viotti A. and Motto M. (2014) Epigenetic Variation, Inheritance,
1096 and Parent-of-Origin Effects of Cytosine Methylation in Maize (Zea mays). *Genetics* 196: 653-666.
Li H. and Durbin R. (2009) Fast and accurate short read alignment with Burrows-Wheeler transform.
1098 *Bioinformatics* 25: 1754-1760.
Liu Y., Siegmund K., Laird P. and Berman B. (2012) Bis-SNP: Combined DNA methylation and SNP calling
1100 for Bisulfite-seq data. *Genome Biology* 13: R61.
Lopez D., Hamaji T., Kropat J., De Hoff P., Morselli M., Rubbi L., Fitz-Gibbon S., Gallaher S. D., Merchant
1102 S. S., Umen J. and Pellegrini M. (2015) Dynamic Changes in the Transcriptome and Methylome of
Chlamydomonas reinhardtii throughout Its Life Cycle. *Plant Physiology* 169: 2730-2743.
1104 Manning K., Mahmut T., Poole M., Hong Y., Thompson A. J., King G. J., Giovannoni J. J. and Seymour G.
B. (2006) A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor
1106 inhibits tomato fruit ripening. *Nature Genetics* 38: 948-952.
Marks P. A., Richon V. M. and Rifkind R. A. (2000) Histone Deacetylase Inhibitors: Inducers of
1108 Differentiation or Apoptosis of Transformed Cells. *Journal of the National Cancer Institute* 92: 1210-
1216.
1110 Mayr E., (1982) *The Growth of Biological Thought*. Belknap Press, Cambridge.
McKenna A., Hanna M., Banks E., Sivachenko A., Cibulskis K., Kernytsky A., Garimella K., Altshuler D.,
1112 Gabriel S., Daly M. and DePristo M. A. (2010) The Genome Analysis Toolkit: a MapReduce framework
for analyzing next-generation DNA sequencing data. *Genome Research* 20: 1297-1303.
1114 Molaro A., Hodges E., Fang F., Song Q., McCombie W. R., Hannon G. J. and Smith A. D. (2011) Sperm
methylation profiles reveal features of epigenetic inheritance and evolution in primates. *Cell* 146: 1029-
1116 1041.
Moore B. G. and Smith R. C. (1969) S-Adenosylethionine as an inhibitor of tRNA methylation. *Canadian
1118 Journal of Biochemistry* 47: 561-565.
Müllner D. (2013) fastcluster: Fast Hierarchical, Agglomerative Clustering Routines for R and Python.
1120 *Journal of Statistical Software* 53: 1-18.
Ness R. W., Morgan A. D., Colegrave N. and Keightley P. D. (2012) Estimate of the spontaneous mutation
1122 rate in Chlamydomonas reinhardtii. *Genetics* 192: 1447-1454.
North B. J. and Verdin E. (2004) Sirtuins: Sir2-related NAD-dependent protein acetylases. *Genome Biology*
1124 5: 224.
Orr H. A. (2005) The genetic theory of adaptation: a brief history. *Nature Reviews Genetics* 6: 119-127.
1126 Ou X., Zhang Y., Xu C., Lin X., Zang Q., Zhuang T., Jiang L., von Wettstein D. and Liu B. (2012)
Transgenerational Inheritance of Modified DNA Methylation Patterns and Enhanced Tolerance Induced
1128 by Heavy Metal Stress in Rice (Oryza sativa L.). *PLoS ONE* 7: e41143.

Paun O., Bateman R. M., Fay M. F., Hedren M., Civeyrel L. and Chase M. W. (2010) Stable epigenetic effects impact adaptation in allopolyploid orchids (Dactylorhiza: Orchidae). *Molecular Biology and Evolution* 27: 2465-2473

Pedersen B. S., Eyering K., De S., Yang I. V. and Schwartz D. A. (2014) Fast and accurate alignment of long bisulfite-seq reads. *arXiv:1401.1129 [q-bio.GN]*

R Core Team (2013) R: A language and environment for statistical computing.

Ragunathan K., Jih G. and Moazed D. (2014) Epigenetic inheritance uncoupled from sequence-specific recruitment. *Science* 348: 1256899.

Rassoulzadegan M., Grandjean V., Gounon P., Vincent S., Gillot I. and Cuzin F. (2006) RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse. *Nature* 441: 469-474.

Richards E. J. (2006) Inherited epigenetic variation - revisiting soft inheritance. *Nature Reviews Genetics* 7: 395-401.

Salinas S. and Munch S. B. (2012) Thermal legacies: transgenerational effects of temperature on growth in a vertebrate. *Ecology Letters* 15: 159-163.

Schaum C. E. and Collins S. (2014) Plasticity predicts evolution in a marine alga. *Proceedings of the Royal Society B: Biological Sciences* 281: 20141486.

Schmitz R. J., Schultz M. D., Lewsey M. G., O'Malley R. C., Urich M. A., Libiger O., Schork N. J. and Ecker J. R. (2011) Transgenerational epigenetic instability is a source of novel methylation variants. *Science* 334: 369-373.

Silveira A. B., Trontin C., Cortijo S., Barau J., Del Bem L. E. V., Loudet O., Colot V. and Vincentz M. (2013) Extensive natural epigenetic variation at a *de novo* originated gene. *PLoS Genetics* 9: e1003437.

Smith K., Kothe G., Matsen C., Khlafallah T., Adhvaryu K., Hemphill M., Freitag M., Motamedi M. and Selker E. (2008) The fungus Neurospora crassa displays telomeric silencing mediated by multiple sirtuins and by methylation of histone H3 lysine 9. *Epigenetics & Chromatin* 1: 5.

Song Y., Ji D., Li S., Wang P., Li Q. and Xiang F. (2012) The dynamic changes of DNA methylation and histone modifications of salt responsive transcription factor genes in soybean. *PLoS One* 7: e41274.

Sueoka N. (1960) Mitotic replication of deoxyribolucleic acid in *Chlamydomonas reinhardi*. *Proceedings of the National Academy of Sciences* 46: 83-91.

Suzuki R. and Shimodaira H. (2006) Pvclust: an R package for assessing the uncertainty in hierarchical clustering. *Bioinformatics* 22: 1540-1542.

Tange O. (2011) GNU Parallel - The Command-Line Power Tool. *;login: The USENIX Magazine* 36: 42-47.

Tanny J. C., Dowd G. J., Huang J., Hilz H. and Moazed D. (1999) An Enzymatic Activity in the Yeast Sir2 Protein that Is Essential for Gene Silencing. *Cell* 99: 735-745.

Taudt A., Colome-Tatche M. and Johannes F. (2016) Genetic sources of population epigenomic variation. *Nat Rev Genet* 17: 319-332.

Thorvaldsdóttir H., Robinson J. T. and Mesirov J. P. (2013) Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Briefings in Bioinformatics* 14: 178-192.

Travisano M., Vasi F. and Lenski R. E. (1995) Long-term experimental evolution in *Escherichia coli*. III.

Variation among replicate populations in correlated responses to novel environments. *Evolution* 49: 189-
1168 200.
Umen J. G. and Goodenough U. W. (2001) Chloroplast DNA methylation and inheritance in
1170 *Chlamydomonas*. *Genes and Development* 15: 2585-2597.
Verhoeven K. J. and van Gurp T. P. (2012) Transgenerational Effects of Stress Exposure on Offspring
1172 Phenotypes in Apomictic Dandelion. *PLoS ONE* 7: e38605.
Verhoeven K. J. F., Jansen J. J., van Dijk P. J. and Biere A. (2010) Stress-Induced DNA methylation changes
1174 and their heritability in asexual dandelions. *New Phytologist* 185: 1108-1118.
Wang J., Reddy B. D. and Jia S. (2015) Rapid epigenetic adaptation to uncontrolled heterochromatin
1176 spreading. *eLife* 4: e06179.
Wibowo A., Becker C., Marconi G., Durr J., Price J., Hagmann J., Papareddy R., Putra H., Kageyama J.,
1178 Becker J., Weigel D. and Gutierrez-Marcos J. (2016) Hyperosmotic stress memory in *Arabidopsis* is
mediated by distinct epigenetically labile sites in the genome and is restricted in the male germline by
1180 DNA glycosylase activity. e*Life* 5: e13546.
Wong A., Rodrigue N. and Kassen R. (2012) Genomics of adaptation during experimental evolution of the
1182 opportunistic pathogen *Pseudomonas aeruginosa*. *PLoS Genetics* 8: e1002928.
Öst A., Lempradl A., Casas E., Weigert M., Tiko T., Deniz M., Pantano L., Boenisch U., Itskov P., Stoeckius
1184 M., Ruf M., Rajewsky N., Reuter G., Iovino N., Ribeiro C., Alenius M., Heyne S., Vavouri T. and
Pospisilik J. (2014) Paternal Diet Defines Offspring Chromatin State and Intergenerational Obesity. *Cell*
1186 159: 1352-1364.

1188 Tables

1190 Table 1. Initial effects of the different environments on population growth rate of the ancestors. Comparisons are shown in percentages relative to ancestor in control environment.

Environment	Genotype	Difference to control (%)	±SE of difference (%)
High NaCl	CC-2937	-80	11
High NaCl	sir2	-45	11
High NaCl	cSIR2	-70	17
Low P	CC-2937	-27	12
Low P	sir2	-19	13
Low P	cSIR2	-35	12
High CO2	CC-2937	26	14
High CO2	sir2	60	13
High CO2	cSIR2	68	13

1192

Table 2. Initial effects of the chemical treatments to the population growth rate of the ancestors in the four
1194 environments assayed. Comparisons are shown in percentages relative to the control chemical treatment in

the appropriate environment and ancestor.

Environment	Genotype	Treatment	Difference to control (%)	±SE of difference (%)
Control	CC-2937	demet	9	10
Control	CC-2937	demet + acet	−40	14
Control	sir2	demet	−17	10
Control	sir2	demet + acet	−37	11
Control	cSIR2	demet	−32	17
Control	cSIR2	demet + acet	−36	13
High NaCl	CC-2937	demet	61	51
High NaCl	CC-2937	demet + acet	2	36
High NaCl	sir2	demet	−49	7
High NaCl	sir2	demet + acet	−8	10
High NaCl	cSIR2	demet	−41	49
High NaCl	cSIR2	demet + acet	15	51
Low P	CC-2937	demet	−7	10
Low P	CC-2937	demet + acet	0	9
Low P	sir2	demet	−7	14
Low P	sir2	demet + acet	−31	16
Low P	cSIR2	demet	27	20
Low P	cSIR2	demet + acet	−13	14
High CO2	CC-2937	demet	−5	9
High CO2	CC-2937	demet + acet	−25	9
High CO2	sir2	demet	−4	9
High CO2	sir2	demet + acet	0	7
High CO2	cSIR2	demet	−9	6
High CO2	cSIR2	demet + acet	−9	6

1196

1198 Table 3. Summary table of epigenetic effects.

The effect of reduced epigenetic transmission by chemical treatment on adaptation for strain CC-2937 was
1200 calculated as the ratio of growth rate of a population selected and assayed in the focal environment in a given chemical treatment relative to growth rate of a population selected and assayed in the same environment but
1202 in the control chemical treatment. Effect of the *sir2* mutation, which reduces the epigenetic mutation rate, was calculated as growth rate of the mutant relative to the growth rate of the complemented line.

Effect of reducing epigenetic contribution	Control environment	High NaCl	Low phosphate	High CO_2
Direct responses				
demet vs. control	0.83	1.04	0.97	0.99

demet + acet vs. control	0.83	0.76	0.92	0.98
sir2 mutation vs cSIR2	0.95	1.09	0.88	0.66

1204

Table 4. Genetic mutations and observed derived DMR changes in the evolved clones. All sequenced clones

1206 were of CC-2937 background.

Clone	Genetic mutations	DMRs	Environment	Chemical treatment
P1B3	8	143	Control	Control
P2B8	10	NA	Control	Control
P3G11	10	NA	Control	Control
P1D2	461	72	Control	Demet
P2D9	433	68	Control	Demet
P3B7	391	NA	Control	Demet
P4C5	8	77	High CO_2	Control
P5F6	3	88	High CO_2	Control
P6E8	5	97	High CO_2	Control
P4C7	642	61	High CO_2	Demet
P5B10	595	60	High CO_2	Demet
P6E2	475	99	High CO_2	Demet
P10C5	11	87	High NaCl	Control
P11B4	11	192	High NaCl	Control
P12E4	11	168	High NaCl	Control
P10C7	201	NA	High NaCl	Demet
P11B11	239	123	High NaCl	Demet
P12G10	162	NA	High NaCl	Demet

1208

Table 5. Observed mutations in evolved clones for control and demethylation chemical treatments by functional

1210 categories. All sequenced clones were of CC-2937 background.

	Control chemical treatment			Demethylation chemical treatment		
Category	All	SNP	Indel	All	SNP	Indel
UTR	14	13	1	729	719	10
5' UTR	6	6	0	143	143	0
3' UTR	8	7	1	586	576	10
Intron	35	24	11	1016	992	24
Coding region	26	19	7	1526	1517	9
Non-synonymous	6	6	NA	1138	1138	NA
Synonymous	13	13	NA	379	379	NA

Frameshift	3	NA	3	4	NA	4
Inframe	4	NA	4	5	NA	5
Intergenic	2	1	1	303	296	7
rRNA	0	0	0	27	27	0
tRNA	0	0	0	2	2	0
Total	77	57	20	3594	3544	50

1212

Table 6. Results of gene ontology (GO) enrichment test for genetic mutations for the demethylation treatment in the

1214 different environments. For each of the top ten most significant GO terms, shown are the number of genes that have been annotated this GO term, number of significantly enriched genes, expected number of genes and p-value for

1216 significant enrichment. All sequenced clones were of CC-2937 background.

	Control Environment				
GO ID	Term	Annotated	Significant	Expected	p-value
GO:0007018	Microtubule-based movement	75	11	1.19	1.8E-08
GO:0006928	Movement of cell or subcellular component	81	11	1.29	4.1E-08
GO:0009187	Cyclic nucleotide metabolic process	93	11	1.48	1.8E-07
GO:0009190	Cyclic nucleotide biosynthetic process	93	11	1.48	1.8E-07
GO:0007017	Microtubule-based process	127	11	2.02	4.1E-06
GO:0035556	Intracellular signal transduction	259	12	4.12	0.00071
GO:0042773	ATP synthesis coupled electron transport	12	3	0.19	0.00077
GO:0006119	Oxidative phosphorylation	13	3	0.21	0.00099
GO:0009165	Nucleotide biosynthetic process	232	11	3.69	0.00100
GO:1901293	Nucleoside phosphate biosynthetic process	233	11	3.71	0.00103
	High CO_2 environment				
GO:0009187	Cyclic nucleotide metabolic process	93	9	1.86	8.7E-05
GO:0009190	Cyclic nucleotide biosynthetic process	93	9	1.86	8.7E-05
GO:0006928	Movement of cell or subcellular component	81	8	1.62	0.00019
GO:0050982	Detection of mechanical stimulus	7	3	0.14	0.00026
GO:0009612	Response to mechanical stimulus	8	3	0.16	0.00041
GO:0070588	Calcium ion transmembrane transport	19	4	0.38	0.00047
GO:0035556	Intracellular signal transduction	259	14	5.19	0.00058
GO:0009581	Detection of external stimulus	9	3	0.18	0.0006
GO:0009582	Detection of abiotic stimulus	9	3	0.18	0.0006
GO:0007018	Microtubule-based movement	75	7	1.5	0.00068
	High NaCl environment				
GO:0055085	Transmembrane transport	411	11	3.87	0.0012

GO:0009187	Cyclic nucleotide metabolic process	93	5	0.88	0.0017
GO:0009190	Cyclic nucleotide biosynthetic process	93	5	0.88	0.0017
GO:0050982	Detection of mechanical stimulus	7	2	0.07	0.0018
GO:0009612	Response to mechanical stimulus	8	2	0.08	0.0024
GO:0009581	Detection of external stimulus	9	2	0.08	0.003
GO:0009582	Detection of abiotic stimulus	9	2	0.08	0.003
GO:0042773	ATP synthesis coupled electron transport	12	2	0.11	0.0054
GO:0006119	Oxidative phosphorylation	13	2	0.12	0.0064
GO:0022904	Respiratory electron transport chain	16	2	0.15	0.0096

1218 Table 7. Results on how often DMRs among the evolved lines gained or lost methylation in different DMR frequency classes in the control and demet chemical treatments. All sequenced clones were of CC-2937
1220 background.

DMRs in control treatment			
Frequency class	Gain	Loss	Frequency of loss
1	256	3	0.01
2	117	13	0.10
3	82	29	0.26
DMRs in demet treatment			
Frequency class	Gain	Loss	Frequency of loss
1	129	4	0.03
2	23	3	0.12
3	9	1	0.10
4	6	12	0.67
5	9	11	0.55
6	2	14	0.86

1222 Table 8. Summary of the major findings.

	Prediction	Observation
High NaCl	Evolutionary adaptation to a stressful environment by increasing growth rate. Reducing epigenetic variation lowers adaptation	Evolution of higher growth rate. Demet and demet + acet treatments reduced adaptation. Sequenced strains from high NaCl clustered together based on their methylation changes. SIR2 mutation had no consistent effect.
Low P	Evolutionary adaptation to a stressful environment by increasing growth rate. Reducing epigenetic variation lowers adaptation	Evolution of higher growth rates but no consistent effects of chemical treatments or the SIR2 mutation
High CO_2	Evolutionary adaptation by reducing growth rate as a result of losing the plastic	Plastic response to high CO_2 was diminished or lost in most strains, in

	response to high CO2. Reducing epigenetic variation lowers adaptation (increases growth rate)	the demet + acet treatment this response was retained. The complemented SIR2 mutant increased its growth rate in contrast to other strains. Sequenced strains from high CO_2 clustered together with control strains based on their DNA methylation changes.
Chemical treatments	Reducing epigenetic variation reduces adaptation	Chemical treatments reduced adaptation. Multiple lines of evidence suggest that cytotoxic effects unlikely to have caused the observed effects of the chemicals.
Methylation changes	Most methylation changes are under genetic control.	More methylation changes than genetic mutations in the sequenced control treatment clones. Both shared methylation changes that suggest environmental induction and but many unique changes that suggest random methylation changes. Genetic mutations unlikely to have caused methylation changes.

1224

1226 Figures

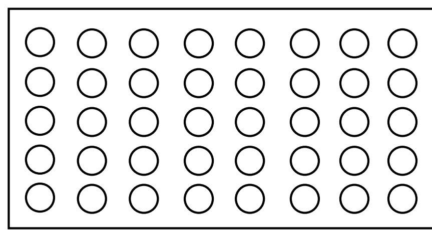
Figure 1. A) Schematic representation of the selection experiment. Four different environments were used. In
1228 each environment, there three different chemical treatments applied to each of four strains. Each population
was grown in nine replicates, resulting in a full factorial experiment with 4*3*4*9 = 432 populations in total.
1230 Populations were grown in 200 µl of media on 96-well plates and transferred every three and four days for
approximately 200 generations. The chemical treatments were randomized among the columns of the plate
1232 and strains were randomized within columns, such that each strain occurred once in each column. After batch
transfers for ~ 200 generations, fitness assays were performed for each population in the environment they
1234 evolved and in the control environment. Populations evolved in the control environment were measured in
all other environments. Fitness assays were performed in a fully randomized design. B) Expected results of
1236 the experiment. Top row shows the expected effect of manipulating epigenetic variation (either chemically or
genetically) during a adaptation. If epigenetic changes contribute to adaptation we expect treated populations
1238 in the high NaCl and low phosphate environments have lower relative growth rates than control treatment
after the selection experiment. In the high CO_2 environment, based on previous studies, we expect that initial
1240 plastic response to increase growth rate in high CO_2 will diminish in the control treated populations. If this
response is due to epigenetic changes, then in the treated populations this response may remain, resulting in
1242 increased relative growth rate. Bottom row shows expected results after clustering the evolved strains based
on epigenetic changes. If epigenetic changes contribute to adaptation strains should cluster by the
1244 environment but if not then clustering is expected to be random.

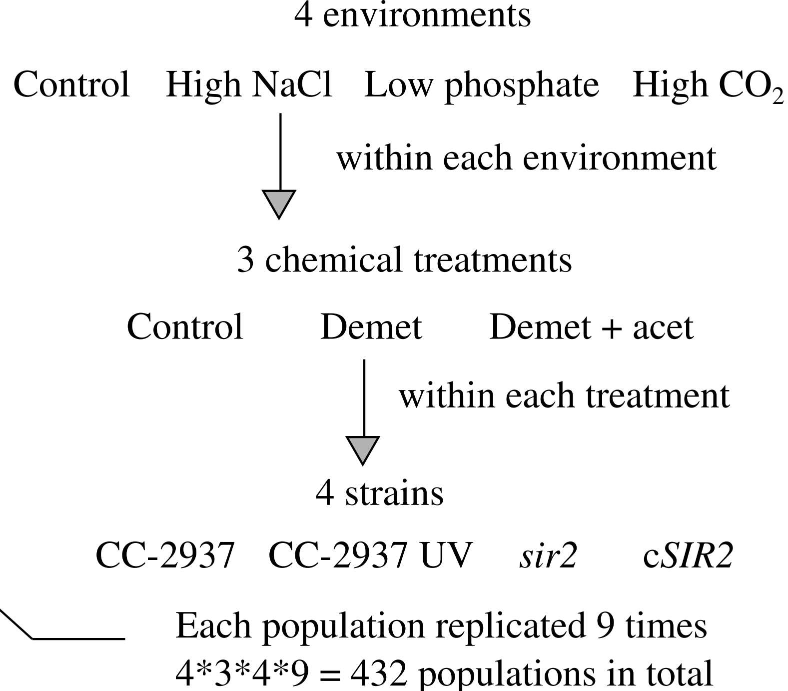
1246 Figure 2. Direct responses to selection in the different environments. Relative growth rates were calculated by taking the growth rate of populations evolved in one of the three environments (high NaCl, low P, and
1248 high CO_2) measured in the environment they evolved in, over growth rate of corresponding population evolved in the control environment but measured in the novel environment. Error bars indicate ± SEM.
1250 Dashed line indicates relative growth rate of one. (A) Populations evolved in the high NaCl environment. (B) Populations evolved in the low phospate environment. (C) Populations evolved in the high CO_2 environment.
1252

Figure 3. The effects of "knocking out" epigenetic transmission with the demet + acet chemical treatment in
1254 the ancestor and populations evolved in the control treatment. At the end of the experiment, we measured growth rates of populations that had the CC-2937 background and that had evolved in the control chemical
1256 treatment, both in the control and demet + acet chemical treatments. We calculated the effect of the demet + acet treatment as a contrast between these two growth measurements (growth in demet + acet treatment -
1258 growth in control treatment). The effect of the demet + acet chemical treatment is plotted on the horizontal axis. An effect of zero means that the demet + acet has no effect on growth, negative values indicate the the
1260 demet + acet treatment reduces growth relative to control and positive values indicate that growth is increased relative to control. If there have been no epigenetic changes during the experiment that contribute
1262 to phenotype, then the chemical treatment should have the same effect in the ancestor and the evolved populations. Populations are stacked on the vertical axis, point are estimates of the effects and error bars are
1264 one standard error. Facets show the different environments. The low phosphate environment was excluded from this experiment as most control treated populations had gone extinct. Vertical lines show the values for
1266 the ancestor.

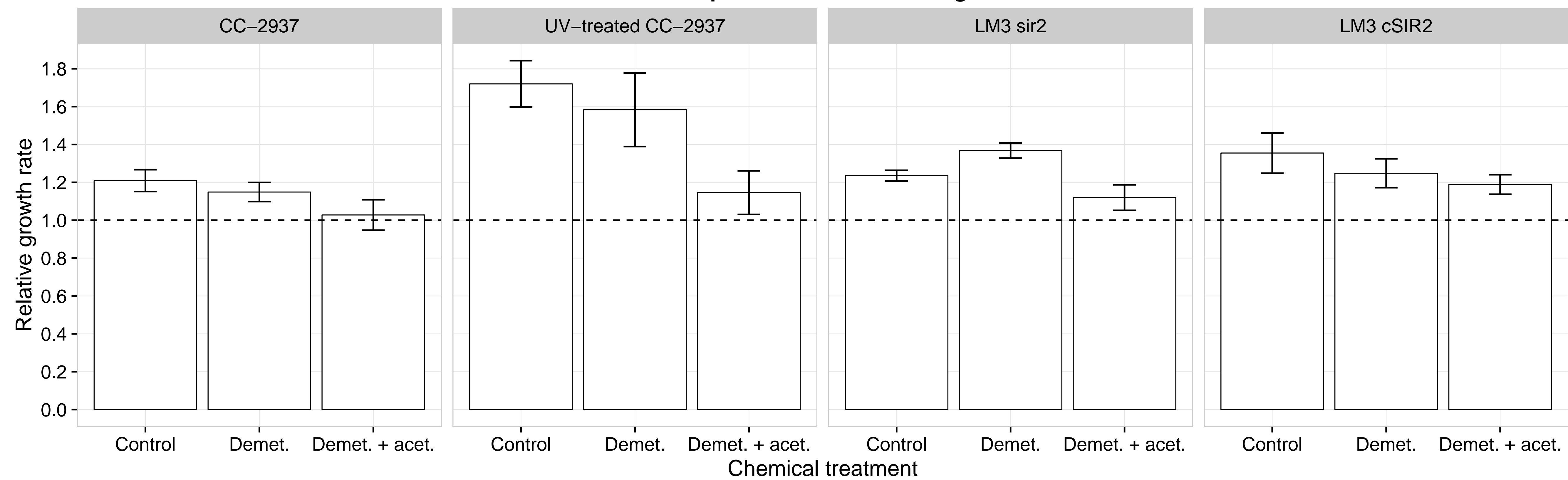
1268 Figure 4. Clustering of clones and ancestors based on DMRs in CG context. In the heatmap DMRs are on rows and samples in columns. Black lines in the heatmap represent missing data. Numbers on sample
1270 dendrogram are bootstrap values. Coloured boxes above the heatmap show clone treatments.

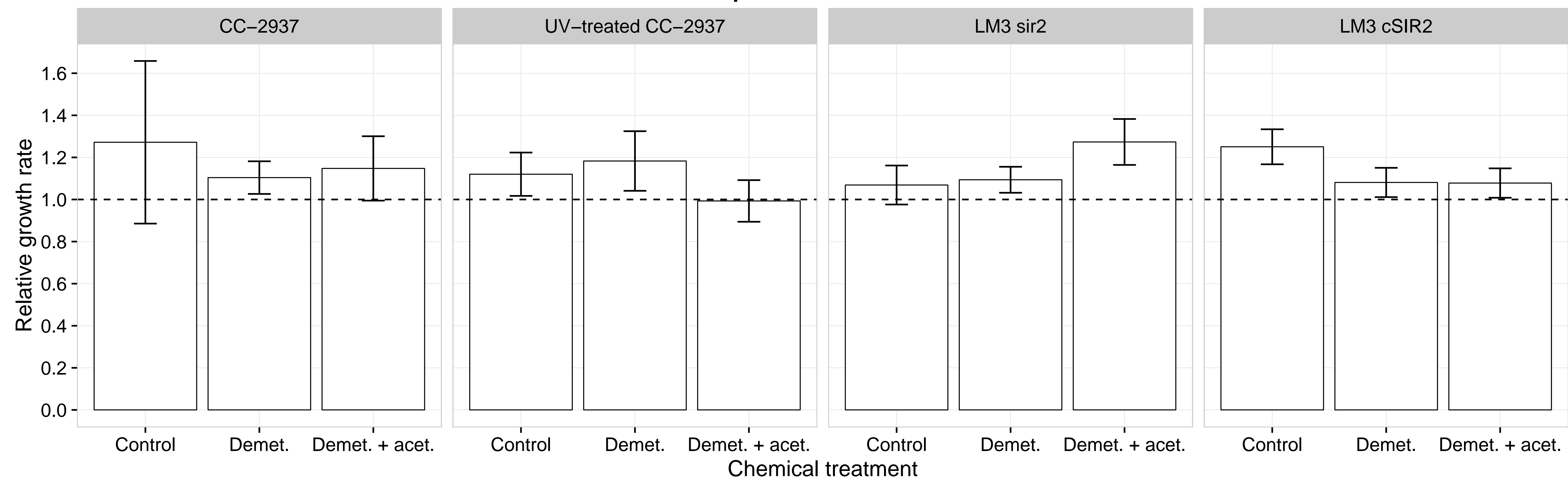
1272 Figure 5. Properties of DMRs among the evolved clones. (A) Frequency distribution of the derived DMRs among the evolved clones. Classes show the number of clones the DMRs are present out of 13 sequenced
1274 clones. (B) Venn diagram of DMR count overlaps among the evolved clones in different environments. (C) Distribution of annotations among the DMRs in the evolved clones and DMRs among the evolved clones
1276 and the ancestor. (D) Mean methylation frequencies of cytosines in within DMRs among the evolved clones that come from different chemical treatments and different sequence contexts.

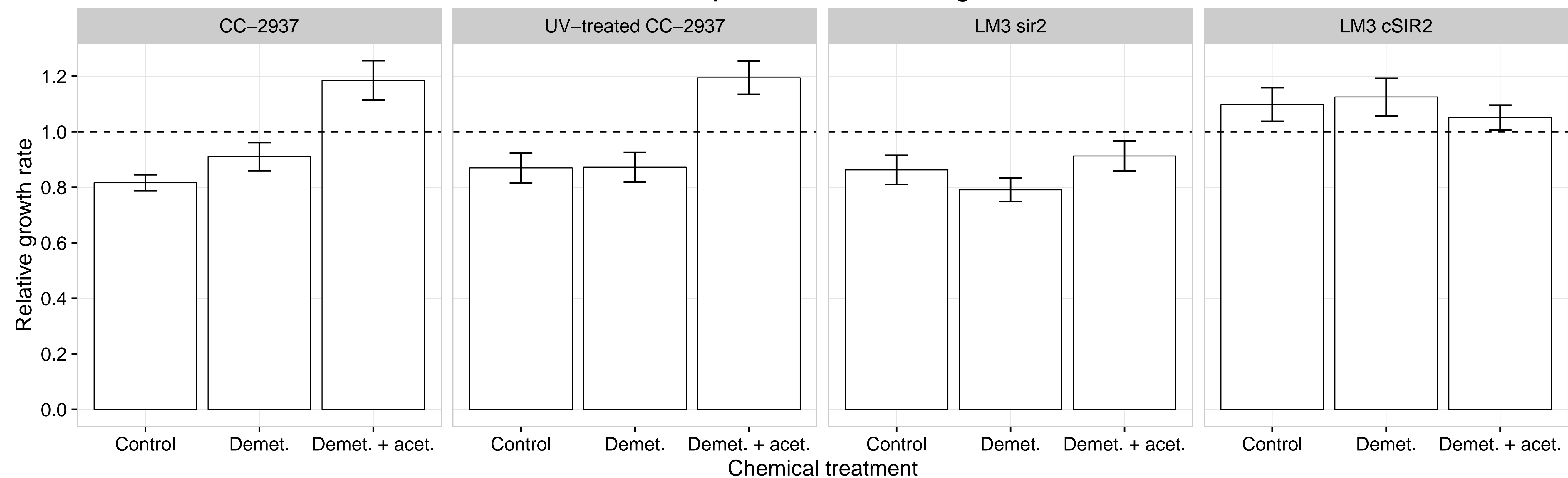

A
strains randomized within each column
chemical treatments randomized
among 96-well plate columns
Batch transfers for ~ 200 generations
Fitness assays in a fully randomized design

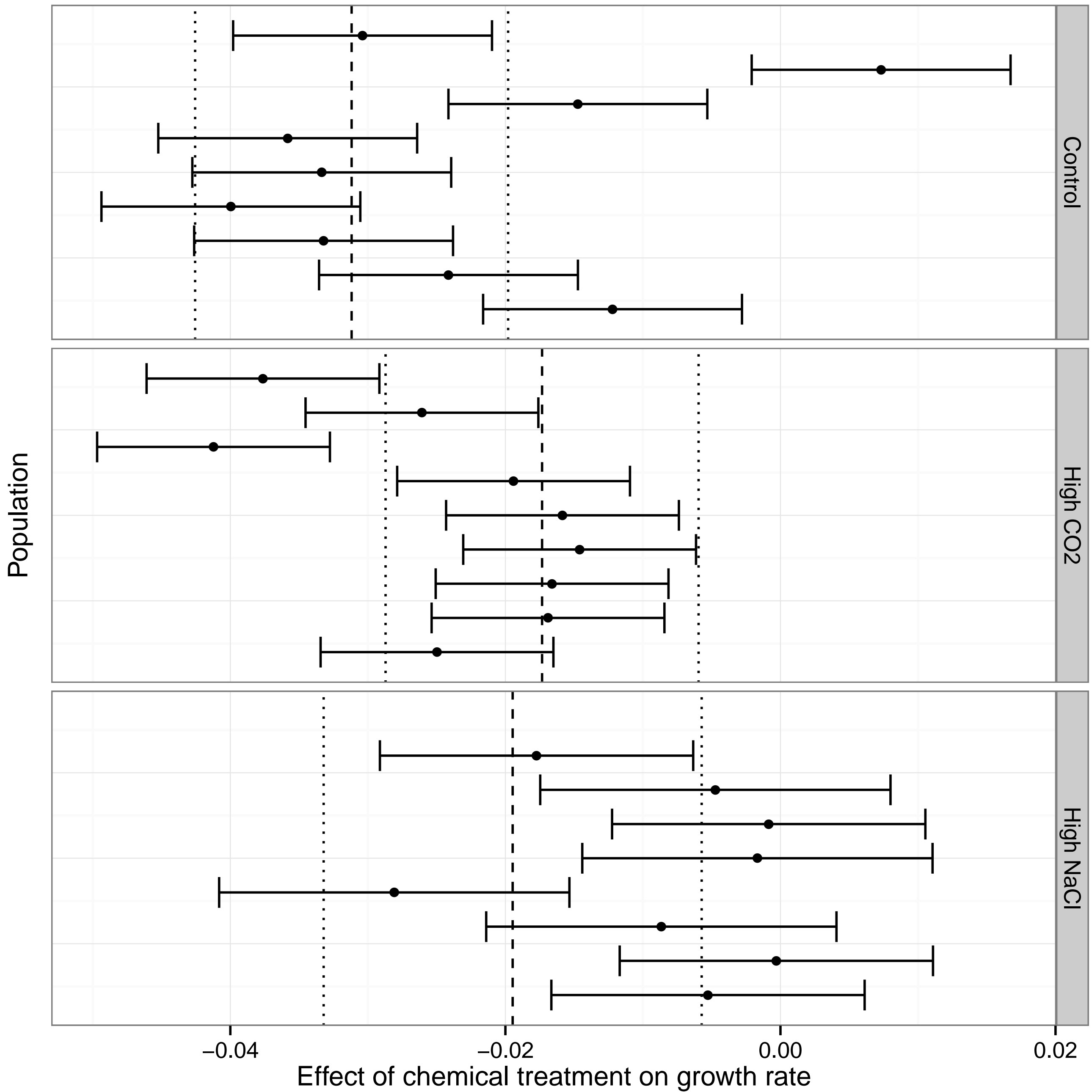

4 environments
Control
High NaCl
Low phosphate
High CO_2
within each environment
3 chemical treatments
Control
Demet
Demet + acet
within each treatment
4 strains
CC-2937
CC-2937 UV
sir2
c*SIR2*
Each population replicated 9 times
4*3*4*9 = 432 populations in total

B
Expected effect of manipulating epigenetic variation in the different environments
High NaCl
Low phosphate
High CO_2
Relative growth
Control
Treatment
Control
Treatment
Control
Treatment
Clustering strains based on epigenetic changes
Env.
1
1
1
2
2
2
3
3
3
Epigenetic changes
affect adaptation
Env.
1
2
3
1
1
2
3
2
3
Epigenetic changes
have no effect

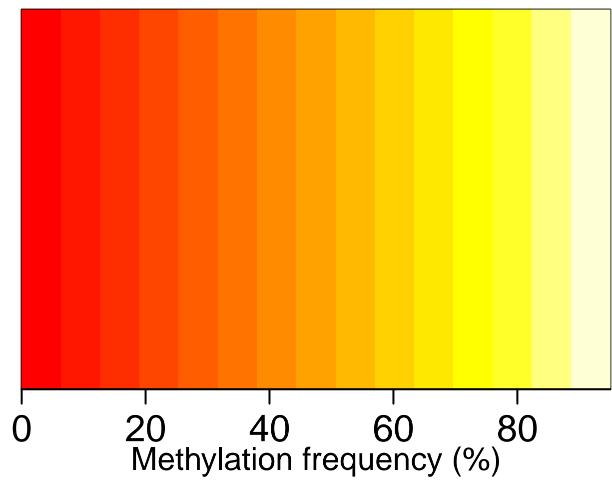

A
Direct response to selection in high NaCl
CC−2937
UV−treated CC−2937
LM3 sir2
LM3 cSIR2
Relative growth rate
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Control
Demet.
Demet. + acet.
Chemical treatment

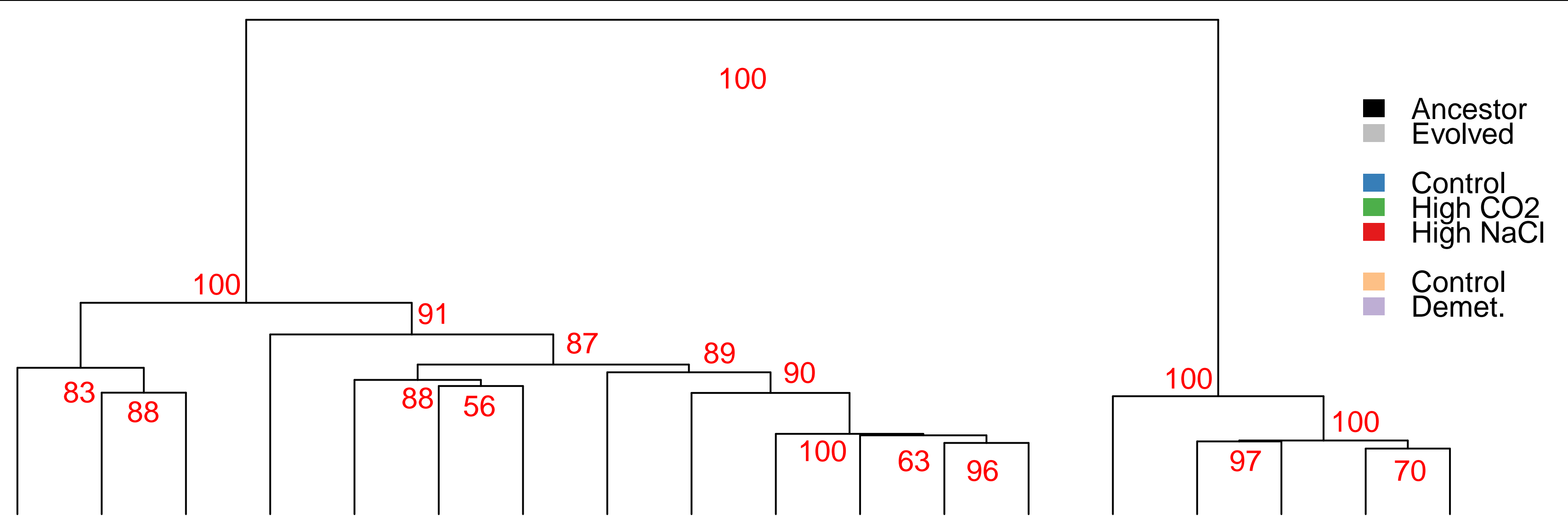

B
Direct response to selection in low P
CC−2937
UV−treated CC−2937
LM3 sir2
LM3 cSIR2
Relative growth rate
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Control
Demet.
Demet. + acet.
Chemical treatment


C
Direct response to selection in high CO2
CC−2937
UV−treated CC−2937
LM3 sir2
LM3 cSIR2
Relative growth rate
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Control
Demet.
Demet. + acet.
Chemical treatment

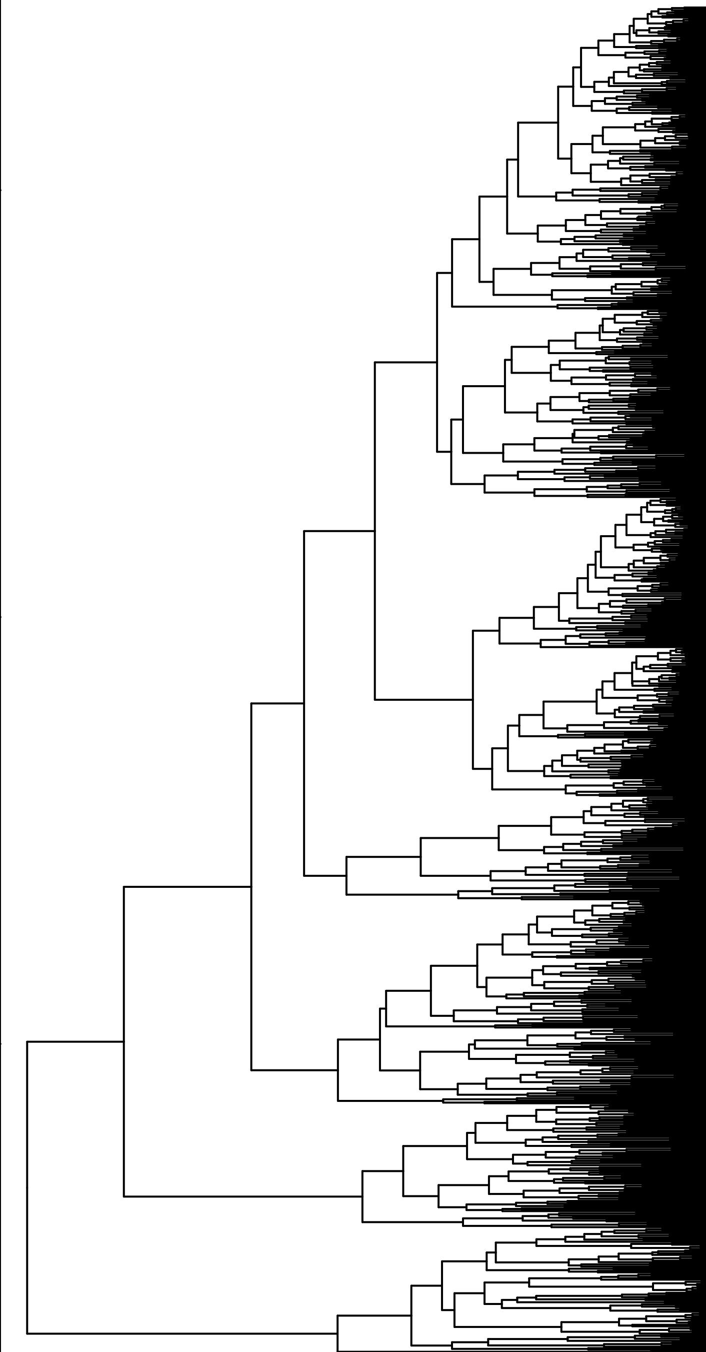


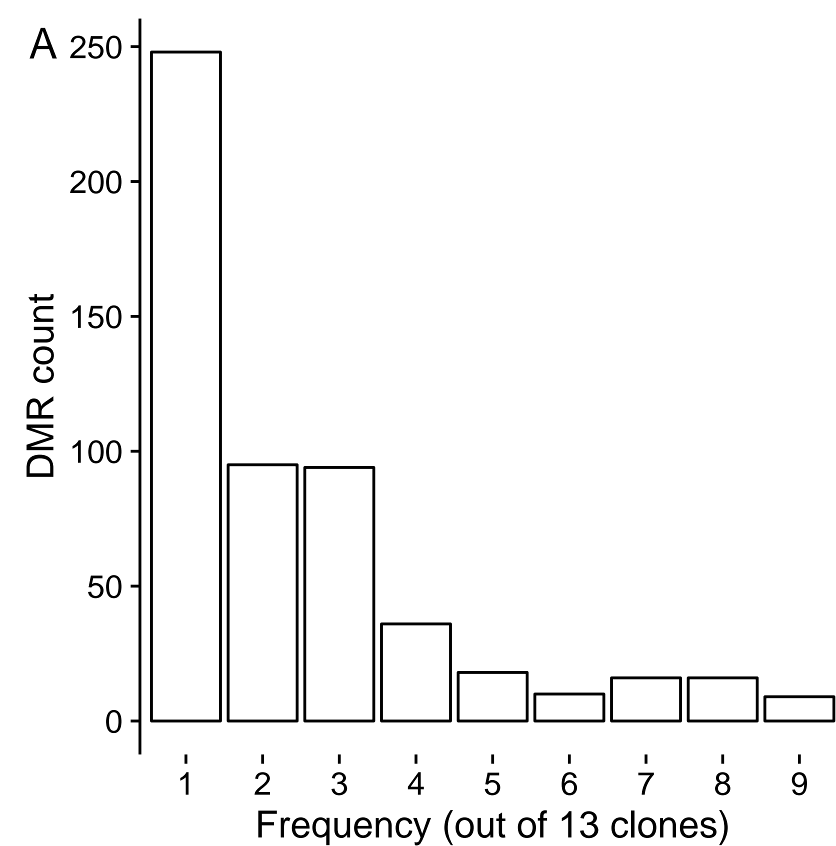
Control
High CO2
High NaCl
Population
−0.04
−0.02
0.00
0.02
Effect of chemical treatment on growth rate

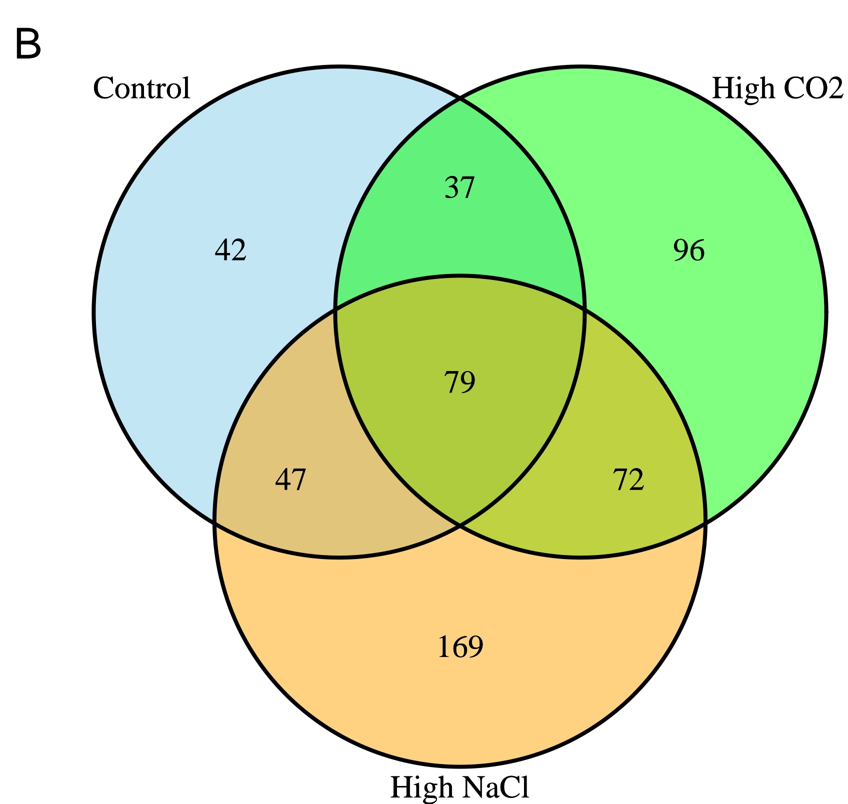

Color Key
0
20
40
60
80
Methylation frequency (%)

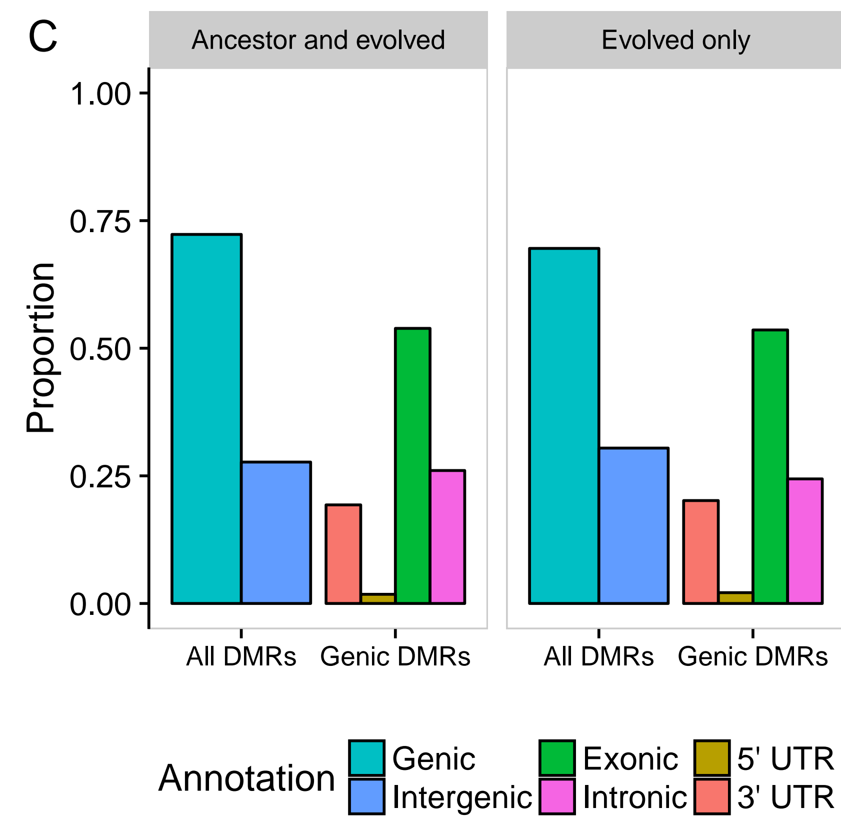

100
100
91
87
89
90
83
88
88
56
100
63
96
100
100
97
70
Ancestor
Evolved
Control
High CO2
High NaCl
Control
Demet.

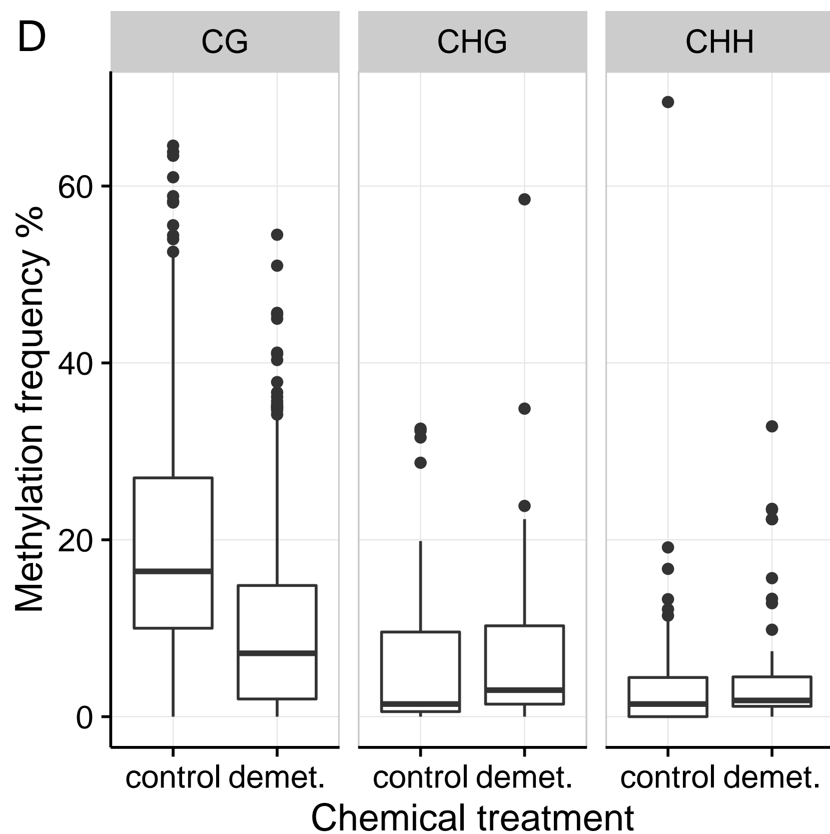
Treatment
Environment
History


P11B4
P11B11
P12E4
P1B3
P5F6
P6E8
P4C5
P10C5
P6E2
P5B10
P1D2
P4C7
P2D9
Demet
CC2937hco2
CC2937cont
CC2937hsalt
Control


A
DMR count
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
Frequency (out of 13 clones)


B
Control
High CO2
High NaCl
42
37
96
79
47
72
169


C
Ancestor and evolved
Evolved only
Proportion
1.00
0.75
0.50
0.25
0.00
All DMRs
Genic DMRs
All DMRs
Genic DMRs
Annotation
Genic
Intergenic
Exonic
Intronic
5' UTR
3' UTR


D
CG
CHG
CHH
Methylation frequency %
60
40
20
0
control
demet.
control
demet.
control
demet.
Chemical treatment

Supporting Information

Absolute growth rates

Absolute growth rates of the evolved populations in control and focal environments are shown in Figure S1. Evolutionary responses can be seen by comparing population that were selected in the control environment and populations that were selected in the focal environment, all assayed in the focal environment (dark blue vs. dark green bars). Indirect evolutionary responses can be seen by comparing populations selected in the control environment and populations that were selected in the focal environment, all assayed in the focal environment (light blue vs. light green bars). Plastic responses for the control populations can be seen by comparing the different assay environments for populations selected in the control environment (light blue vs. dark blue bars). Plastic responses for the populations selected in the focal environment can by seen by comparing the different assay environment (light green vs. dark green bars).

Indirect responses to selection

Indirect or correlated responses to selection happen when a population adapts to certain environment and certain genetic (or epigenetic) changes increase in frequency. These adaptive mutations may have correlated effects in environments that are not experienced by the population. For example, evolution in one environment may bring about phenotypic changes that cause the evolved populations to grow slower than the ancestor in the ancestral environment. This sort of indirect response would reveal a cost of adaptation that is manifested in some environment other than the selection environment. We investigated indirect responses to selection by comparing the growth of the populations evolved in high NaCl, low phosphate, or high CO_2 to growth of populations evolved in the control environment all assayed in the control environment, and calculated indirect responses as a ratio of populations from corresponding chemical treatments.

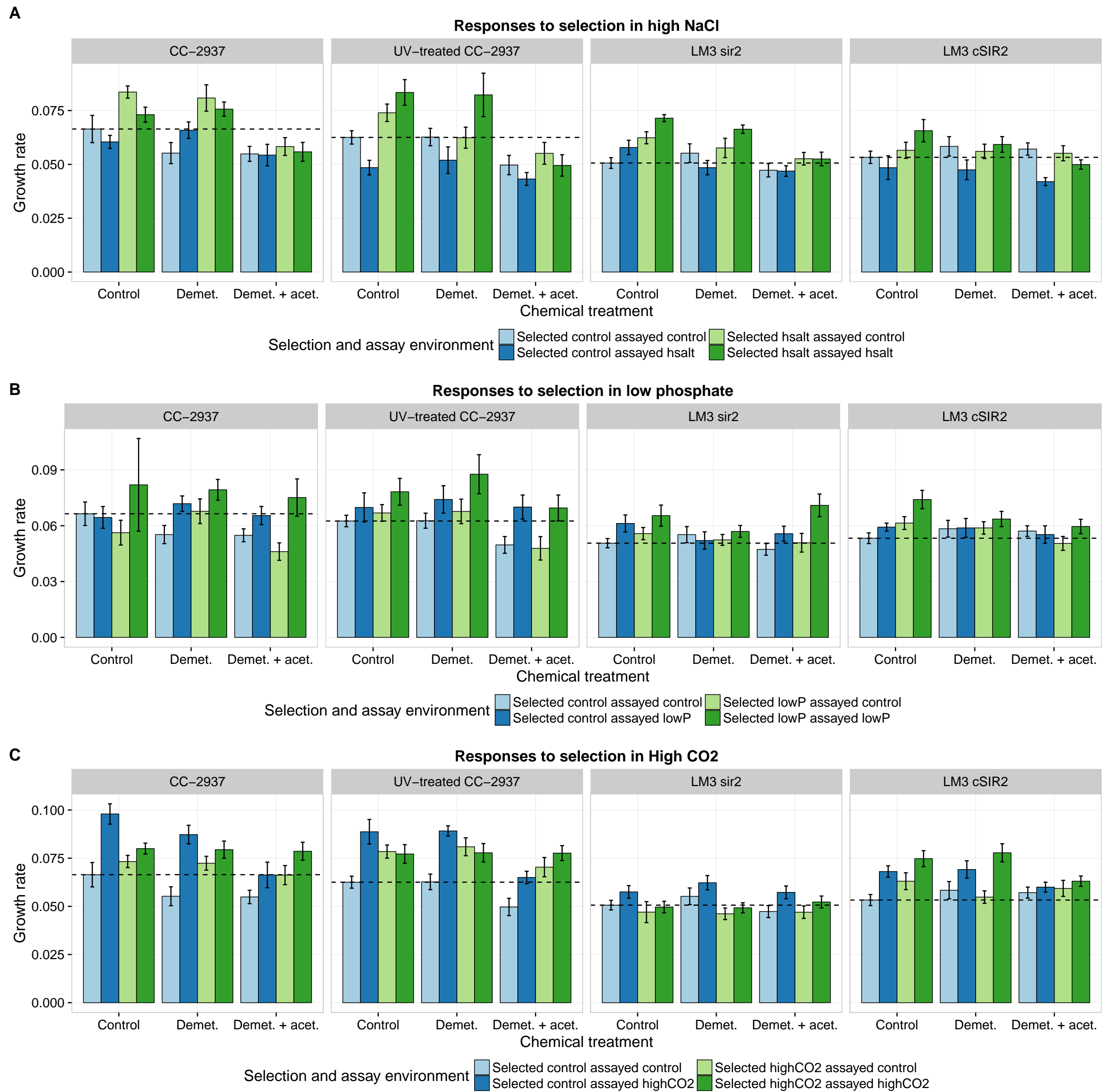


Figure S1: Absolute growth rates of the evolved populations. Different coloured bars show the different selective and assay environments. Vertical axis is the absolute growth rate, r_{max}. (A) Populations evolved in high NaCl. (B) Populations evolved in low phosphate. (C) Populations evolved in high CO_2.

26 In the following treatment we used the data for populations that were assayed in the control environment and had been selected in the control environment or one of the other en-
28 vironments. We analysed the three environments separately. The 3-way interaction selection × chemical × strain tests if the indirect effects of selection differ across chemical treatments
30 and strains. If this term was not significant it was dropped from the model and the other interactions were tested. Non-significant terms were subsequently dropped. The selection ×
32 chemical treatment tests if the indirect effects of selection differs across the different chemical treatments and the selection × strain interaction tests if the different strains have different
34 indirect effects.

High NaCl environment

36 For the high NaCl environment, the 3-way interaction was not significant. For the 2-way interactions selection × strain interaction was significant ($F_{1,194} = 3.832$, $p = 0.0107$) and
38 also strain × chemical interaction was significant ($F_{6,194} = 2.393$, $p = 0.0297$), while the selection × chemical interaction was not significant. The indirect responses of strain CC-
40 2937 were positive 26%, 46%, and 6% in the control, demet, and demet + acet chemical treatments respectively (Figure S2A). For the UV-treated CC-2937 strain indirect responses
42 were 18%, 0%, and 11% respectively. Smaller indirect response in the UV-treated strain may reflect the effects of conditionally deleterious mutations. For the *sir2* mutant the in-
44 direct responses were 23%, 4%, and 11% and for the complemented strain 6%, −4%, and −3% for the three chemical treatments respectively. The effect of the *sir2* mutation were
46 not significant for populations evolved in high NaCl but assayed in the control evironment. Strains of the CC-2937 background had positive indirect responses, while strains in the LM3
48 background had small responses. These results indicate that adaptation to high NaCl tended to increase growth also the control environment, possibly reflecting adaptation via general
50 stress tolerance mechanisms.

Low phosphate environment

52 For low phosphate environment, none of the interactions were significant, and only the effect of strain ($F_{3,192} = 3.447$, $p = 0.0277$) and chemical ($F_{2,192} = 10.578$, $p = 4.38 \times 10^{-5}$) were
54 significant. Suggesting that in general there were no consistent indirect effects of selection in this environment (Figure S2B). The average effects of the chemical treatments on indirect
56 responses were 4%, 7%, and -6% for control, demet, and demet + acet treatments respectively. For the *sir2* mutant the indirect responses were 10%, −5%, and 8% and for the complemented
58 strain 15%, 1%, and −12% for the three chemical treatments respectively. The effect of the *sir2* mutation is suggestive (contrast: $t = -1.92$, $df = 192$, $p = 0.0561$). Because the chemical
60 treatments did not affect growth in low phosphate for low phosphate-evolved populations in strain CC-2937 (Figure 2, Table 3, Figure S1), it is unlikely that the chemical effects on the
62 indirect response to selection are simply due to toxic effects of the chemicals, and are instead are more likely to indicate an environmentally dependent epigenetic response.

64 **High CO_2 environment**

The 3-way interaction was not significant in the high CO_2 environment, and of the 2-way
66 interactions only the selection × strain interaction was significant ($F_{3,202} = 9.000$, $p = 1.27 \times 10^{-5}$). Evolution in the high CO_2 environment increased the relative growth rate in the
68 control environment for the CC-2937 strain by 10%, 31%, and 21% in the three chemical treatments, and by 25%, 29%, and 42% for the UV-treated CC-2937 strain (Figure S2C).
70 The high indirect responses in the CC-2937 background may reflect adaptation to laboratory conditions that was facilitated by high population sizes these populations reached at the
72 high CO_2 environment. For the *sir2* mutant and the complemented strain indirect responses were different. The indirect responses of the *sir2* mutant were −7%, −17%, and −0.7% for
74 the three different chemical treatments and 18%, −7%, and 4% for the complemented strain (Figure S2C). The effect of the *sir2* mutation was significant (contrast: $t = -3.7$, $df = 202$,
76 $p = 0.0003$). The *sir2* mutant had trade-offs in adapting to high CO_2, while complemented

line increased its growth in the control chemical treatment.

Phenotypic measurements

Flow cytometry

We measured relative cell numbers, cell size, cell shape, and chlorophyll content by flow cytometry using a BD FACSCanto II (BD Biosciences, Oxford, UK) flow cytometer calibrated with CS&T beads. Dilution series were set up in 96-well plates and an HTS plate system was used for sample acquisition, with all events recorded for a volume of 30 µl, acquired at flow rate of 1 µl/s. The data were analyzed with the BD FACSDiva v6 software. An electronic acquisition gate was applied to the forward vs side scatter plot to exclude debris from intact material. Cells were excited at a wavelength of 488 nm and chlorophyll fluorescence emission was detected between 670-735 nm. We used forward scatter as a proxy for cell size and side scatter as a measure of cell shape and internal complexity. Chlorophyll content was calculated as detected fluorescence from chlorophyll divided by cell size.

Phenotypes of evolved populations

In the high NaCl environment all populations evolved similar cell sizes regardless of the chemical treatment, none of the interactions were significant, nor were the effects of chemical treatment or selection (effect of chemical: $F_{2,403} = 0.135$, $p = 0.8737$, effect of selection: $F_{1,403} = 2.424$, $p = 0.1203$). Only the difference in cell sizes between CC-2937 and LM3 backgrounds was significant (effect of strain: $F_{3,403} = 161.878$, $p < 2 \times 10^{-16}$). Figure S3A). For cell shape the three way interaction between selection × strain × chemical treatment was significant ($F_{6,394} = 2.977$, $p = 0.0074$). Cell shape responded differently to selection in the CC-2937 background and the LM3 background. In the LM3 background and the demet + acet treatment, different cell shape phenotype had evolved than in the other treatments, Figure S3B). Chlorophyll content increased as a response to selection in the CC-2937 background but decreased in the *sir2* mutant while the response was variable in the complemented line

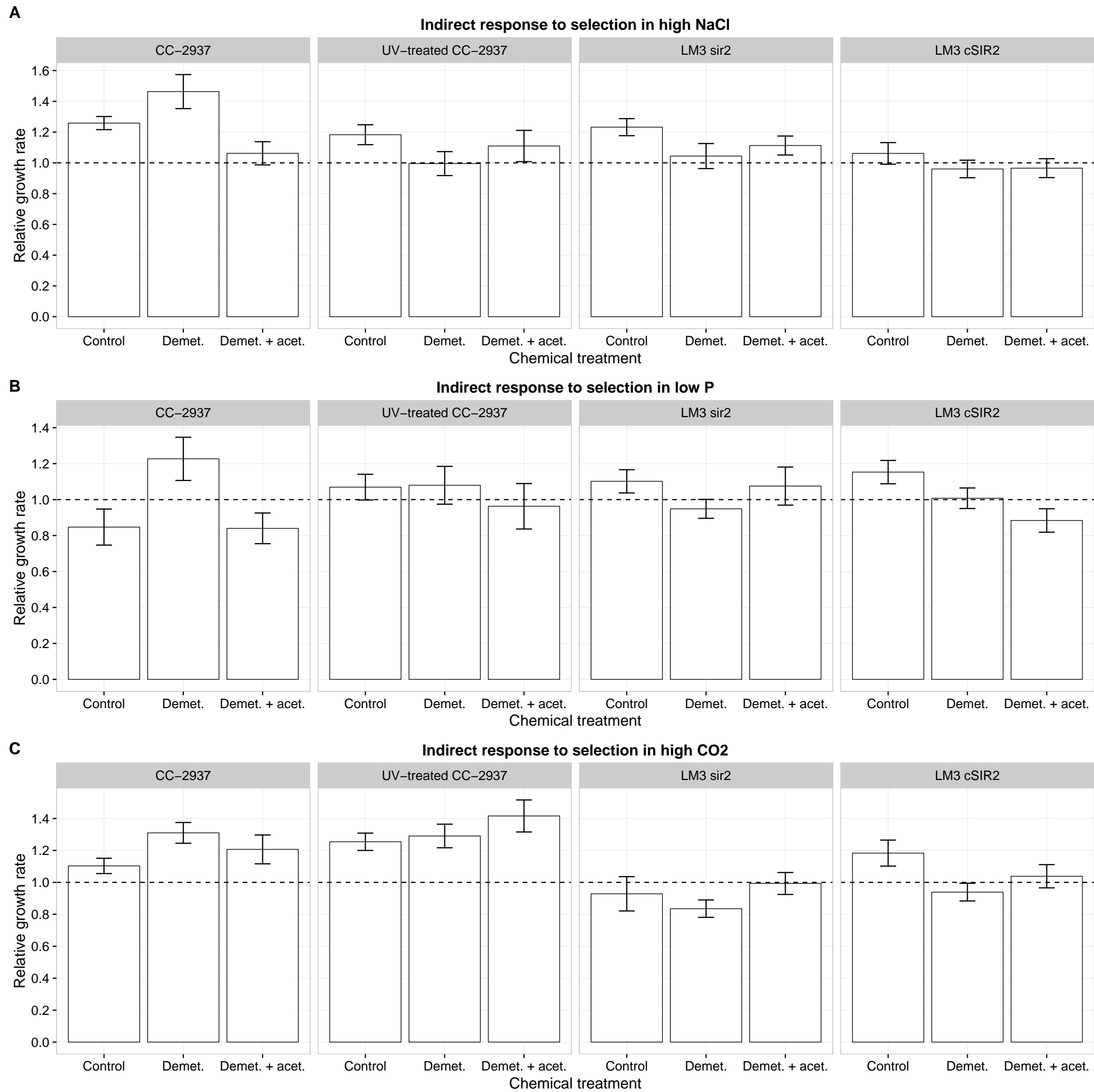


Figure S2: Indirect responses to selection in the different environments. Populations evolved in the different selection environments were assayed in the control environment. Their growth rates were standardised to populations evolved in the control environment of the same genotype and chemical treatment and assayed in the control environment. Error bars indicate ± SEM. Dashed lines indicates relative growth rate of 1. (A) Populations evolved in the high NaCl environment. (B) Populations evolved in the low phospate environment. (C) Populations evolved in the high CO_2 environment.

102 (selection × strain interaction, $F_{3,400} = 33.287$, $p < 2 \times 10^{-16}$). The chemical treatments had little effect on chlorophyll content in the CC-2937 background but increased the chlorophyll

104 content in the LM3 background (strain × chemical treatment interaction, $F_{6,400} = 4.434$, p = 0.0002, Figure S3C).

106 In the low phosphate environment the was a significant selection × strain interaction in cell size ($F_{3,371} = 3.182$, p = 0.0240), cell size responded differently to selection in the CC-

108 2937 and LM3 background (Figure S4A). Effects of chemical treatment were not significant. For cell shape, none of the interactions nor the effect of chemical treatment were signifi-

110 cant ($F_{2,371} = 0.633$, p = 0.5317, Figure S4B). However, cell shape responded to selection ($F_{1,371} = 7.941$, p = 0.0051). Chlorophyll content increased in response to selection and this

112 effect became smaller as more epigenetic mechanisms were removed (selection × chemical interaction: $F_{2,371} = 2.712$, p = 0.0677), although this effect was only marginally significant.

114 Moreover, the effect was stronger in the CC-2937 background (Figure S4).

In the high CO_2 environment there was a marginally significant chemical × strain in-

116 teraction ($F_{6,401} = 1.792$, p = 0.0994) for cell size, the chemical treatments decreased cell size for the complemented mutant (Figure S5A). Moreover, the selection × strain interaction

118 was significant ($F_{3,401} = 5.719$, p = 0.0008) indicating that cell size responded differently to selection in the different strains, this was due to indirect response to selection was stronger

120 in the complemented mutant. For cell shape the there was a significant interaction between chemical treatment and selection regime (selection × chemical interaction: $F_{2,401} = 3.904$,

122 p = 0.0209). The chemical treatments mainly affected the indirect response to selection; lines selected in high CO_2 had different cell shapes in the control environment when epige-

124 netic mechanisms were removed, relative to the control treatment (Figure S5B). There was no significant response to selection in chlorophyll content (effect of selection: $F_{1,401} = 0$, p

126 = 0.9911). Removing epigenetic mechanisms increased chlorophyll content in the CC-2937 background while in the LM3 background chlorophyll content tended to increase in response

128 to the chemical treatments only in populations selected in the control environment (chemical

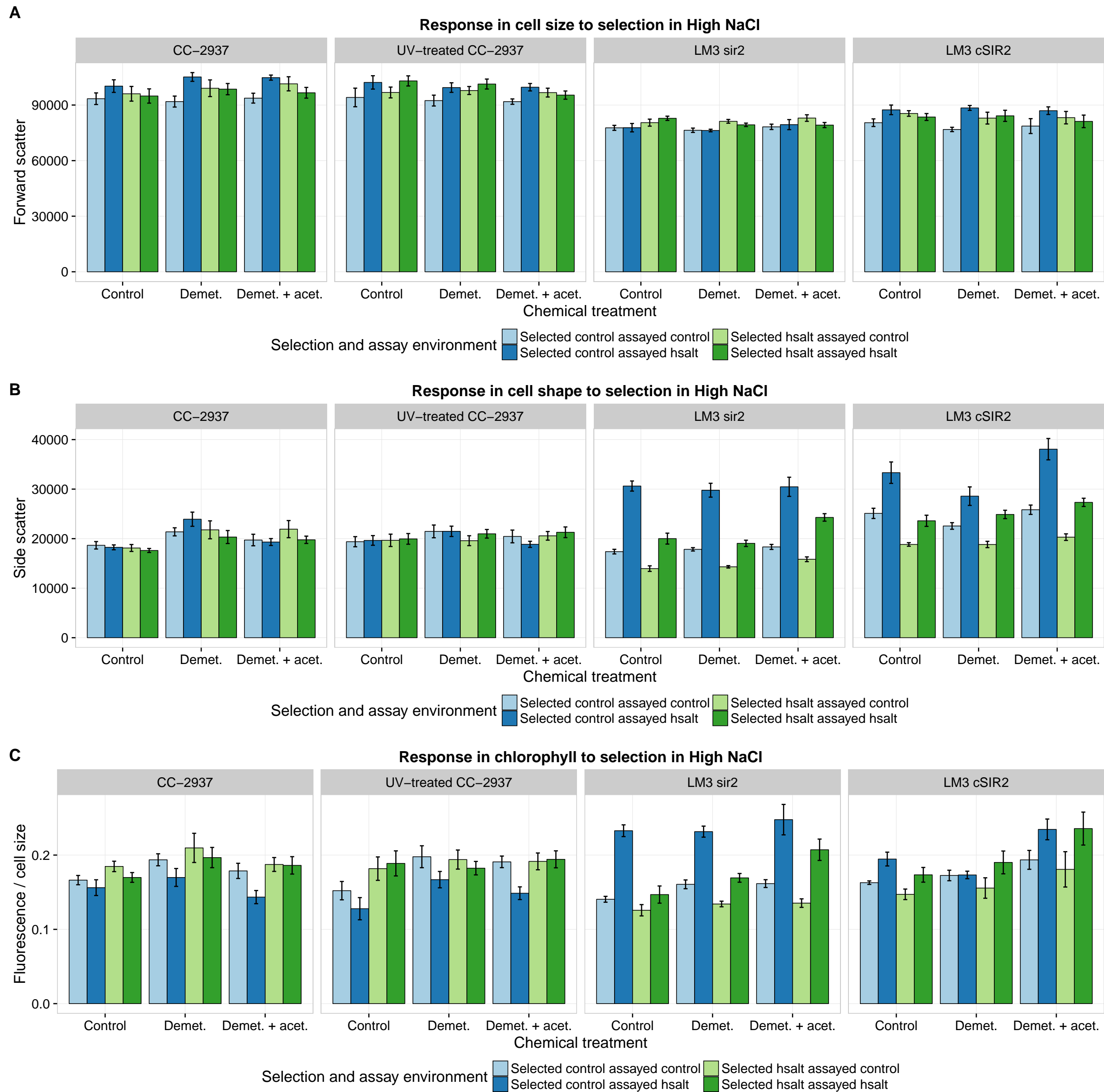


Figure S3: Phenotypic responses to selection in the high NaCl environment. Different colored bars show the selective and assay environments as indicated by the legend. Groups of bars on the horizontal axis are the different chemical treatments and facets show the different genotypes. (A) Response in cell size, vertical axis is forward scatter, a measure of relative cell size. (B) Response in cell shape, vertical axis is side scatter a measure of relative cell shape or internal complexity. (C) Response in chlorophyll content, vertical axis is fluorescence from chlorophyll divided by cell size, a measure of chlorophyll content.

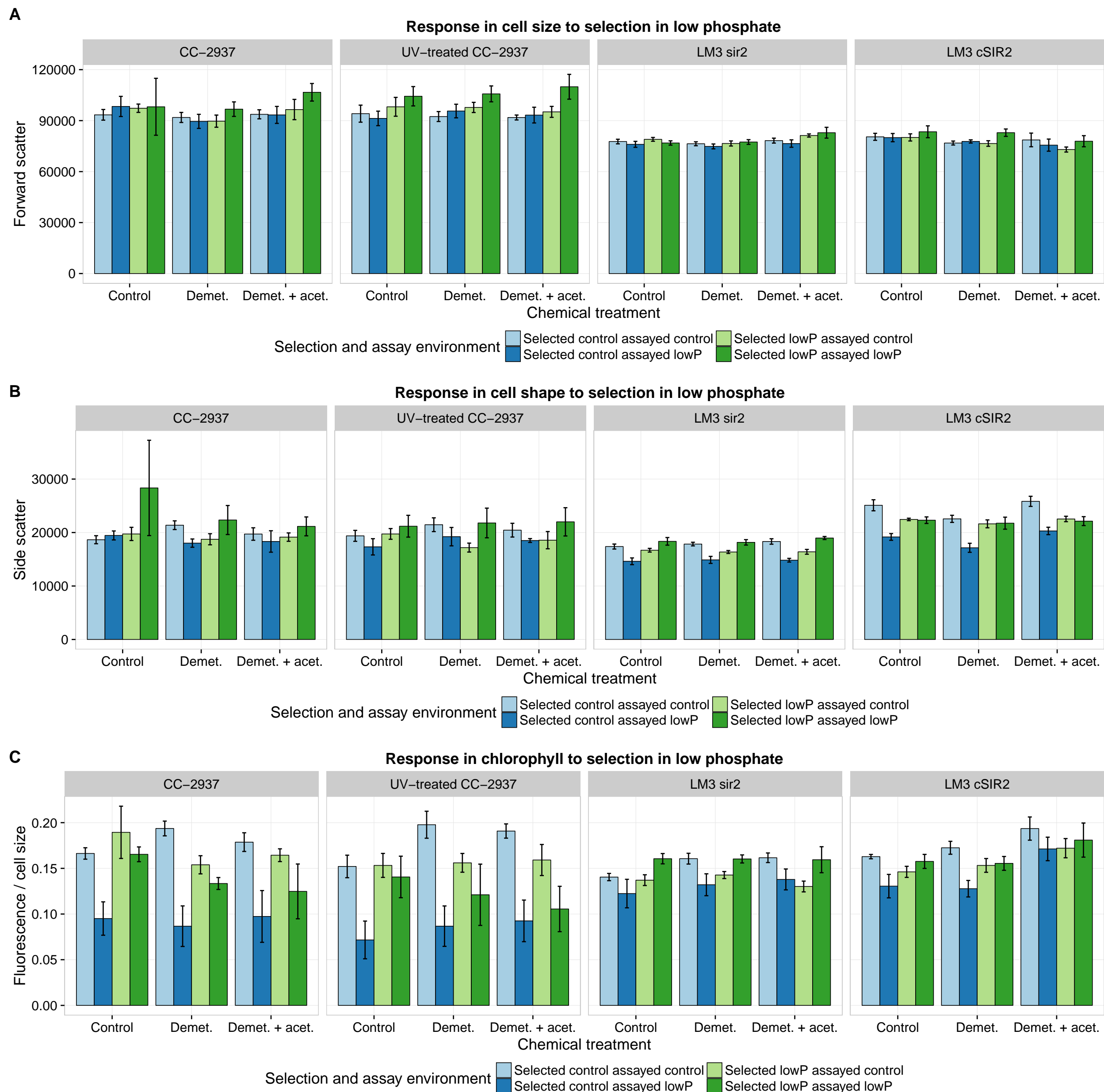


Figure S4: Phenotypic responses to selection in the low phosphate environment. Different colored bars show the selective and assay environments as indicated by the legend. Groups of bars on the horizontal axis are the different chemical treatments and facets show the different genotypes. (A) Response in cell size, vertical axis is forward scatter, a measure of relative cell size. (B) Response in cell shape, vertical axis is side scatter a measure of relative cell shape or internal complexity. (C) Response in chlorophyll content, vertical axis is fluorescence from chlorophyll divided by cell size, a measure of chlorophyll content.

$\times$ genotype interaction, $F_{6,401} = 2.833$, $p = 0.0103$, Figure S5C).

Effect of UV-radiation on mortality and mutation frequency in *Chlamydomonas*

To determine what dose of UV-radiation should be given to the CC-2937 UV populations in order to increase mutation rate but not increase mortality substantially we performed an experiment to determine how radiant exposure to UV-radiation affects mutation frequency and mortality.

The strain CC-2937 was used for the trial. We grew a single culture to high density and diluted it to a concentration of 10^6 cells/ml. We split this culture into 30 cultures of 200 µl on 96-well plate. We irradiated this plate with UV-radiation and removed cells from the plate after 0, 1, 3, 6, 9, and 12 minutes which corresponded to a radiant exposure of 0, 2025, 6075, 12150, 18225, and 24300 J/m^2 with our UV-lamp. Each time point had five replicates. For each time point we diluted the culture to 10^3 cells/ml and plated 200 µl of this dilution was plated on TAP-agar plates. After three minutes of UV-radiation corresponding to a radiant exposure of 6075 J/m^2 mortality increased drastically (Figure S6A). After six minutes mortality was nearly complete.

To determine how exposure to UV-radiation increases the frequency of mutants present in the cultures we scored the number of mutants that were resistant to the antibiotic paromomycin. We repeated the same experimental setup as when testing for mortality, except that only time points 0, 1, 3, and 6 were chosen with twelve replicates each. The whole 200 µL of the culture was plated TAP-agar plates containing 10 µg/ml paromomycin. Since all of the replicate populations were started from the same population the number of mutants naturally present at time zero should be the same and any increases in mutant frequency should be due to the mutagenic effect of UV-radiation. Only a few spontaneous mutants were observed in cultures that did not receive UV-radiation but even a radiant exposure of 2025 J/m^2 increased the number of mutants in the culture more than ten fold (Figure S6B).

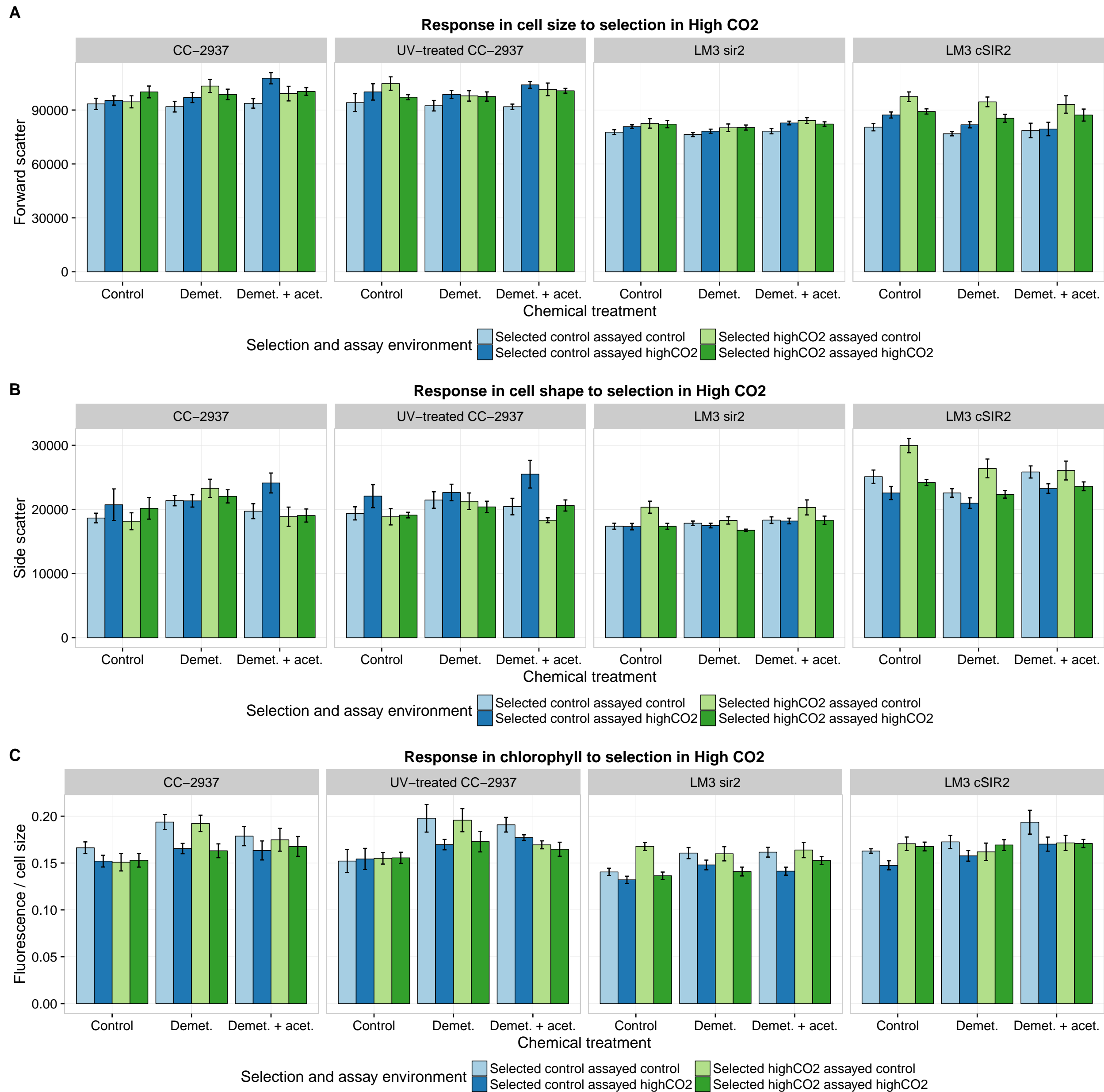


Figure S5: Phenotypic responses to selection in the high CO_2 environment. Different colored bars show the selective and assay environments as indicated by the legend. Groups of bars on the horizontal axis are the different chemical treatments and facets show the different genotypes. (A) Response in cell size, vertical axis is forward scatter, a measure of relative cell size. (B) Response in cell shape, vertical axis is side scatter a measure of relative cell shape or internal complexity. (C) Response in chlorophyll content, vertical axis is fluorescence from chlorophyll divided by cell size, a measure of chlorophyll content.

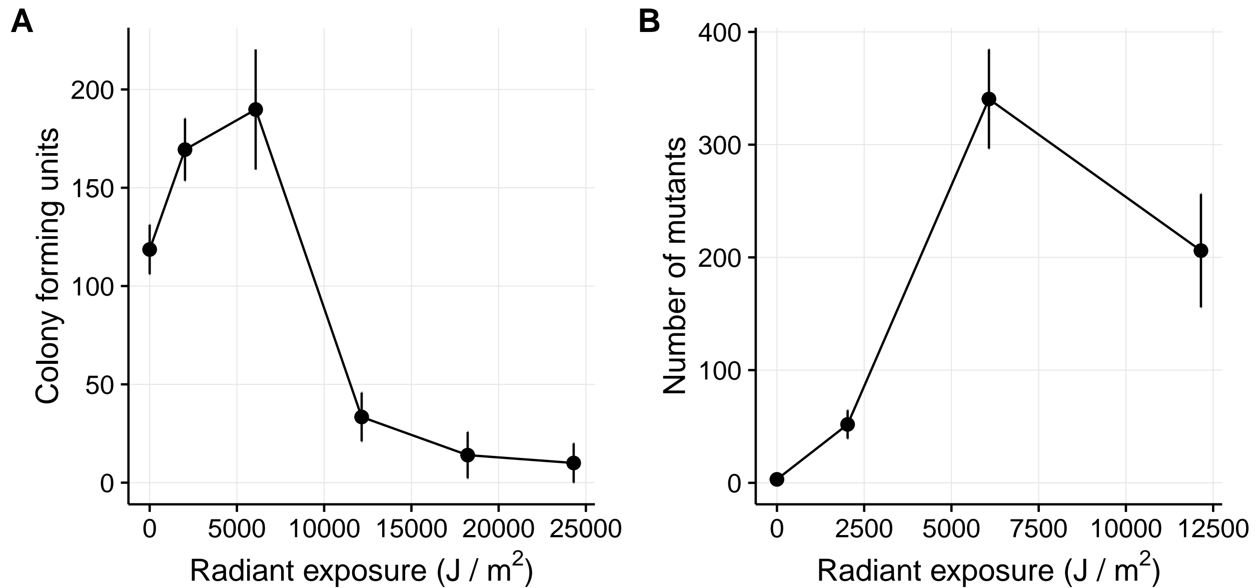


Figure S6: (A) Mortality induced by UV-radiation. Each culture was diluted to the same density and received increasing amounts of UV-radiation (as shown on the x-axis). The same amount of cells were plated on TAP-agar plates. Y-axis is the number of colonies that grew on the plates. Each point is the mean of 5 replicate cultures and error bars are ± 1 SE. (B) Mutagenicity of UV-radiation. Each culture was diluted to the same density and received increasing amounts of UV-radiation (as shown on the x-axis). The same amount of cells were plates on TAP-agar plates containing 10 µg/ml paromomycin. Y-axis shows the number of paromomycin resistant colonies that grew on the plates. Each point is the mean of 12 replicate cultures and error bars are ± 1 SE.

Further increases in radiation dose increased number of mutants observed. For increasing
156 the mutational supply in our selection experiment, we chose to use radiant exposure of 2025 J/m^2 as this dose already increased the number of observed mutants but did not cause an
158 observable increase in mortality (Figure S6).

Effects of UV-irradiation on mutational supply

160 If adaptation in any of the environments is mutation limited, we expect that the UV-irradiated strains show a greater response to selection than the wild type strains. The
162 effects of increased mutational supply must be examined using the absolute growth rates as increased mutational supply could lead to increased growth of the evolved lines in the new
164 environment if adaptation is mutation limited but also decreased growth in the new environment for the control lines if mutations have pleiotropic effects. Therefore comparing relative
166 growth rates could be misleading in this case. We used the final linear models for each environment and pre-planned contrasts, note that since final linear models were different for the
168 different environments, we could not test the same contrasts for each environment. See the results section for the models.

170 High NaCl environment

First we compared those populations of strain CC-2937 that had evolved in high NaCl and
172 were assayed in high NaCl to corresponding populations of the UV-treated CC-2937 strain to measure the effects of increased mutational supply. For populations evolved in the control
174 environment and assayed in high NaCl differences between CC-2937 and UV-treated CC-2937 were significant (pre-planned contrast: $t = 3.42$, $df = 196$, $p = 0.0008$), but for populations
176 evolved in high NaCl and assayed in high NaCl did not differ between the strains (contrast: $t = -1$, $df = 196$, $p = 0.3163$). This suggests that while adaptation to high salt environment
178 was not limited by mutational supply to a significant amount, populations for the UV-treated strain that evolved in the control environment accumulated mutations that were neutral in

180 the control environment but slightly deleterious in the high NaCl environment (Figure S1). This explains the elevated relative responses to selection (Figure 2A). For indirect effects 182 of selection we also observed that populations evolved in high NaCl but assayed in control environment differed between the strain in all chemical treatments (contrast: $t = 2.44$, df 184 $= 194$, $p = 0.0157$ for control chemical treatment, $t = 2.22$, $df = 194$, $p = 0.0277$ for demet treatment, and $t = 1.94$, $df = 194$, $p = 0.0543$ for the demet + acet treatment). See 186 also Figure S1. No significant differences were observed between populations selected and assayed in the control environment. These results further suggest that conditionally neutral 188 mutations accumulated in the UV-treated strain.

Low phosphate environment

190 We did not observe any significant differenes on absolute growth rates between strains CC-2937 and UV-treated CC-2937 in the low phosphate environment (contrast: $t = -0.63$, $df =$ 192 186, $p = 0.5287$). This maybe surprising, since we observed several extinctions of CC-2937 in the control chemical treatment at low phosphate, and populations of UV irradiated CC-2937 194 did not go extinct under the same conditions. This difference in extinction rates suggests that adaptation to low phosphate may be limited by mutation supply. We also did not detect 196 any differences between the strains for indirect effects of selection (contrast: $t = -0.4$, $df =$ 192, $p = 0.6921$).

198 **High CO_2 environment**

For the high CO_2 environment we did not observe significant differences between strains CC-200 2937 and UV-treated CC-2937 for populations assayed in the high CO_2 environment. For populations that were evolved in the high CO_2 and assayed in the control environment there 202 was a non-significant but suggestive difference between the strains (contrast: $t = -1.81$, $df =$ 202, $p = 0.0718$), with populations of the UV-treated strain growing faster (Figure S1), with 204 an average effect across all chemical treatments of 8%. This may reflect better adaptation of

UV-treated populations to the laboratory conditions.

Overall, we found little evidence that adaptation was limited by mutational supply in our experimental populations. Instead, we did find some evidence that increasing mutational supply increased the number of conditionally neutral mutations in the high NaCl environment. However, we also found no evidence that increased mutational supply prevented adaptation, indicating that our experimental populations were able to tolerate increased genetic loads.

Demographic effects

We verified that our chemical treatments were not driving the patterns we see through general toxic effects that may decrease growth rates, and thus the total number of generations during the selection experiment; or population sizes, and thus the effectiveness of natural selection. After each transfer we measured the optical density of the populations, and this measurement was transformed into a cell concentration using the prepared standard curves. Based on the known volumes used for the cultures and the transfer volume we could calculate the number of cells a population had reached at the point of transfer and the number of cells that were transferred to the next batch. The number of generations that happened in each transfer was calculated as

$$g = \log_2 \left(\frac{N_f}{N_i} \right) \quad (1)$$

where g is the number of generations, N_f is the final population size reached at the end of the transfer, and N_i is the initial population size.

The selection environment had a large effect on the number of generations that the populations went through during the experiment ($F_{3,414} = 1245.4$, $p < 2 \times 10^{-16}$). On average, the experiment lasted 247 generations in the high CO_2 environment, 180 generations in the control environment, 150 generations in the high salt environment, and 166 generations in the low phosphate environment. This is to be expected, since we deliberately chose three different selection environments that imposed different amounts of stress that resulted in different growth rates, from the extremely stressful low phosphate environment to the high CO_2

environment, which actually increases fitness. However, the effect of the chemical treatment on the number of generations over the course of the experiment was not significant ($F_{2,414}$ = 1.7, p = 0.182). There was an effect of chemical treatment on population size at transfer ($F_{2,412}$ = 9.9, p = 6.48×10^{-5}). The demet treatment increased population size by four percent, and the demet + acet decreased population size by seven percent. These differences in population sizes are not large enough to drive the differences in evolutionary outcomes in this study. To confirm this, we checked if the number of generations that each population went through or the mean population size was correlated with its final fitness. We used a linear model separately for each environment with, chemical treatment, genotype, number of generations, and mean populations size as predictors. In the control environment mean population size had a marginal effect ($F_{1,91}$ = 3.096, p = 0.0818), and the effect of generation number was not significant ($F_{1,91}$ = 0.362, p = 0.5489). For the other environments mean population size and generation number were not significant (High CO_2: $F_{1,93}$ = 1.193, p = 0.2770; $F_{1,93}$ = 0.001, p = 0.9720; High NaCl: $F_{1,89}$ = 0.128, p = 0.7213; $F_{1,89}$ = 2.52, p = 0.1159; Low P: $F_{1,79}$ = 1.345, p = 0.2497; $F_{1,79}$ = 0.123, p = 0.7270, for mean population size and number of generations respectively). Based on this, we conclude that demographic effects did not drive the differences in evolutionary outcomes in this study.

Site of insertion in the *sir2* mutant and transgene silencing

The site of insertion of the plasmid in the insertional mutant was identified by inverse PCR followed by Sanger sequencing and was found to be a sequence of 5'-GGAATGGTGGGG-3' in intron 2 of the gene Cre10.g462200 (Figure S7). This gene belongs to a SIR2 family of class IV sirtuins and is homologous to *Arabidopsis SRT1* and mouse *SIRT6*.

Transgene silencing in the *sir2* mutant and the complemented line was checked by RT-qPCR of the Zeocin resistance gene used as a reporter in the mutant screen. RNA was extracted using Trizol (Life Technologies), and reverse transcription carried out with 1 µg of total RNA using Superscript III in a 20 µl reaction volume. Quantitative PCR was per-

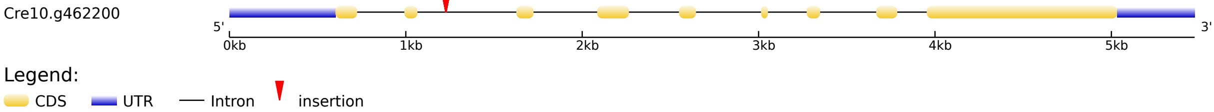


Figure S7: Gene structure of Cre10.g462200, a *Chlamydomonas* SIR2-like gene. Yellow blocks show the exons, blue blocks show the UTR's and red wedge indicates the position of the plasmid insertion in the mutant. The gene structure was drawn using GSDS 2.0 (http://gsds.cbi.pku.edu.cn/).

256 formed with primers amplifying the 5' end of the Zeocin resistance gene (Zeocin_F: ATGGCCAAGCTGACCAGCGC, Zeocin_R: ACTCGGCGTACAGCTCGTCCAG), and normalised
258 to the expression of RACK1 (RACK1_F: GCCACACCGAGTGGGTGTCGTGCG, RACK_R: CCTTGCCGCCCGAGGCGCACAGCG) using the $\Delta\Delta$Ct-method. Amplification was per-
260 formed with the SybrGreen Jumpstart Taq Ready Mix (Sigma #S4438) and detected on a Biorad Chromo4 thermal cycler. In the parental strain (Figure S8, "silenced") expression
262 of the Zeocin resistance gene is normally repressed by epigenetic mechanisms. In the *sir2* mutant this repression of transcription is relieved and the Zeocin resistance gene is expressed,
264 but silencing is restored again in the lines complemented with the genomic fragment of the *SIR2* gene (Figure S8). While the mutant was resistant to Zeocin the complemented lines
266 had become Zeocin sensitive (Figure S9).

Validation of mutations by Sanger sequencing

268 We confirmed that our genotyping criteria were reliable by confirming 20 mutations by Sanger sequencing. Mutations were chosen at random among the control treatment samples. We
270 amplified a region with a mutation by PCR, using the high-fidelity Phusion DNA polymerase (Thermo Scientific) following manufacturer's instructions: PCR reactions contained 1x Phu-
272 sion high GC buffer, 0.2 mmol/l each dNPT, 0.5 µmol/l of both primers, and 0.4 units of Phusion DNA polymerase. Total volume of each reaction was 20 µl. PCR program was: +98
274 °C for 30 s, then 35 cycles of +98 °C for 5 s, annealing temperature for 10 s, extension at +72 °C for 20 s, and a final extension at +72 °C for 1 min. All mutations were confirmed

Figure S8: Expression of silenced Zeocin resistance transgene measured by RT-qPCR. Results are means of three biological replicates and error bars show 95% confidence intervals. The samples are: silenced - strain transformed with a plasmid where a repetitive region had been cloned upstream of the Zeocin resistance gene, LM3: sir2 - insertional mutant in Cre10.g462200, LM3: gSIR2 a and b - two different lines where the mutant had been complemented with a genomic region of the *SIR2* gene.

Figure S9: Zeocin resistance in the insertional mutant (LM3: EV) and transformants complemented with the genomic fragment (LM3: gSIR2). Clones were grown on plates without Zeocin (left) and with Zeocin (right). Two clones indicated by the red boxes have become Zeocin sensitive again.

276 from Sanger reads. List of primers and corresponding annealing temperatures are in table S1.

Table S1: PCR primers used for Sanger sequencing to confirm mutations

Sample	Forward primer	Reverse primer	Annealing Temp. (°C)
P3G11	CTGCAGCCGTCCATGTCG	TCAACGAGCTGAAGGTGGAG	66
P12E4	ATGTGGAACCAAACAGACGC	CACTAGCGGCATCAGAAGCG	65
P12E4	CTGTCAAAGGCCCATCTGTG	CCATCAAGGGTGGGAGGAG	66
P10C5	CGTGTAGGTAAGCGGCTG	ATGACCCGGACGAGATACAC	62
P10C5	GTCCAGTCCTCTTCCTCCTG	GCTACTCCCGTCATTGCCTT	63
P4C5	GCTTACGCCCTGCTTCTTG	TAAGTCGATTGGGAGCTGCA	65
P11B4	GCTTCCTCTACCGTACCGTG	ACCGTAATACCGTGTGTCCC	63
P3G11	CCGTACTTGTACTAGGCTTTGC	ATGTCACCATGCGCTATTGC	62
P2B8	GAGTTTGATGTTGCCGCCAT	CATCAATGGGACCGTCATGC	67
P4C5	TCGGCGATGTAACTTGAAGC	CGGCTTCCACGGTATACTTTG	65
P4C5	AAGTGTTAGTGCGTCTGTGC	AATGACCGTCTGCAGCATG	65
P2B8	GCTTCCCTAGTGTGCATTGATA	GCATCCAGTACTCGAACACG	63
P2B8	TCGTCATGAGCAAAGTCAGC	GCAAGGTACACTGTGGCAAA	64
P4C5	GGACGTATGCCTTGAGTGGT	CCCTAATCCATTCTGCTGGA	64
P2B8	TCTCCGCGTCACTATCCTCT	GGTAAGCTGAAGCGTTTTCG	64
P10C5	GGGCATCTCCCTACAAACAG	CGTACATATGCTGCCTGGTG	63
P1B3	TATCAATTTTGCGTGCAACC	GCGCCATTTCGTGTTATCTC	63
P1B3	CAGCAGCAGGTGAGCATTAC	GGCCAACGTTTGACTCATGT	64
P11B4	CAACCCACCGTGCTACTACC	AGGTGAGTGCTGGCATTCTT	64
P3G11	ACCGCCACGTCAATACTCAT	ACACACCACGCATGATCCTA	64

278 Clones for genome and bisulfite sequencing

Table S2 shows the clones used for genome resequencing and bisulfite sequencing. Five
280 bisulfite libraries failed.

Genetic mutations in control treatment

282 List of all genetic mutations that occurred in the control chemical treatment in all of the different environment are shown in Table S3.

Table S2: Clones used for genome and bisulfite sequencing. The construction of some bisulfite libraries failed and those samples indicated by NA.

Clone	Genome re-sequencing	Bisulfite sequencing	Environment	Chemical treatment
P1B3	x	x	Control	Control
P1D2	x	x	Control	Demet
P2B8	x	NA	Control	Control
P2D9	x	x	Control	Demet
P3G11	x	NA	Control	Control
P3B7	x	NA	Control	Demet
P4C5	x	x	High CO2	Control
P4C7	x	x	High CO2	Demet
P5F6	x	x	High CO2	Control
P5B10	x	x	High CO2	Demet
P6E8	x	x	High CO2	Control
P6E2	x	x	High CO2	Demet
P10C5	x	x	High NaCl	Control
P10C7	x	NA	High NaCl	Demet
P11B4	x	x	High NaCl	Control
P11B11	x	x	High NaCl	Demet
P12E4	x	x	High NaCl	Control
P12G10	x	NA	High NaCl	Demet
CC2937	x	x	Control	Control
CC2937	NA	x	High CO2	Control
CC2937	NA	x	High NaCl	Control
CC2937	NA	x	Control	Control
CC2937	NA	x	Control	Control
CC2937	NA	x	Control	Control
CC2937	NA	x	Control	Demet
CC2937	NA	x	Control	Demet
CC2937	NA	x	Control	Demet

Table S3: List of genetic mutations that occurred in clones in the control treatment

Clone	Environment	Chr	Position	Mutation	Feature	Gene	Gene Annotation
P1B3	Control	1	2233644	Insertion TG	Intron	Cre01.g012200	Unknown function, SBP transcription factor domain and BTB/POZ domain
P1B3	Control	10	1258768	Insertion CGGGAAGTA	Intron	Cre10.g426850	Unknown function, Transferase activity
P1B3	Control	16	948791	A → T	Intron	Cre16.g648700	ABC-2 type transporter
P1B3	Control	12	1098394	G → T	Intron	Cre12.g490700	MIN1, the MIN1 protein is required for eyespot assembly and organization
P1B3	Control	16	3110680	A → G	5'UTR	Cre16.g665550	Unknown function
P1B3	Control	16	3444247	A → T	Intron	Cre16.g670261	Unknown function, Vacuolar protein 14 C-terminal Fig4p binding
P1B3	Control	7	6002449	G → T	Exon, AA substitution Ala to Ser	Cre07.g354650	Unknown function
P1B3	Control	16	1559986	C → A	Exon, AA substitution Asp to Glu	Cre16.g653550	Unknown function
P2B8	Control	9	5496506	Deletion TT	3'UTR	Cre09.g400367	RCL1, RNA terminal 3' phosphate cyclase
P2B8	Control	13	1930940	Deletion TGTCTG	Intergenic	NA	NA
P2B8	Control	1	7869398	C → A	Intron	Cre01.g060347	Unknown function
P2B8	Control	2	7108209	G → C	5'UTR	Cre02.g146851	Protein kinase, (Serine – Threonine)
P2B8	Control	7	2099905	G → T	Intron	Cre07.g326150	Unknown function
P2B8	Control	14	1354979	C → T	Intron	Cre14.g617050	WASP-interacting protein VRP1/WIP
P2B8	Control	16	3085884	C → G	Intron	Cre16.g665400	Small nuclear ribonucleoprotein SmD1
P2B8	Control	16	5677902	G → A	3'UTR	Cre16.g679150	Aminohydrolase family
P2B8	Control	9	1555172	C → T	Exon, AA substitution Ala to Val	Cre09.g397700	Dimethylaniline monooxygenase family

Continued on next page. . .

Table S3 – Continued

Clone	Environment	Chr	Position	Mutation	Feature	Gene	Gene Annotation
P2B8	Control	14	2202490	G → T	Exon, AA substituion Val to Leu	Cre14.g622800	Lipase (class 3)
P3G11	Control	1	2435976	Deletion of TGC	Exon, frame preserved	Cre01.g013600	Unknown function
P3G11	Control	2	5187774	Deletion of GAGGA	Exon, frameshift	Cre02.g105600	Unknown function, ABC1 family
P3G11	Control	17	3364293	Deletion of CTT	Exon, frame preserved	Cre17.g723600	Intraflagellar transport protein 81
P3G11	Control	6	7334211	C → T	3'UTR	Cre06.g298900	Unknown function
P3G11	Control	7	957897	G → A	Intron	Cre07.g319300	Alpha-glucan water dikinase
P3G11	Control	10	1624744	G → A	3'UTR	Cre10.g429702	Unknown function
P3G11	Control	14	1084222	G → A	Intron	Cre14.g614850	Flagellar Associated Protein with ankyrin repeats
P3G11	Control	17	5772836	T → G	Intron	Cre17.g739350	Unknown function
P3G11	Control	7	1815741	A → G	Exon, synonymous	Cre07.g325727	ABC1 family
P3G11	Control	12	3143160	G → A	Exon, AA substitution Ala to Val	Cre12.g499601	Unknown function
P4C5	high CO2	11	3619792	Insertion of GT	Intron	Cre11.g482100	Unknown function, Parkin co-regulated protein, ubiquitin / proteosome system
P4C5	high CO2	9	4682075	Insertion of TA	Exon, frameshift	Cre09.g396846	Unknown function, TBC1 domain family member GTPase-activating protein
P4C5	high CO2	2	4734753	A → G	Intron	Cre02.g102100	Unknown function
P4C5	high CO2	3	4261966	T → C	3'UTR	Cre03.g174350	Unknown function, PWR motif protein
P4C5	high CO2	7	2181491	G → A	5'UTR	Cre07.g326833	Unknown function
P4C5	high CO2	17	2441624	T → C	5'UTR	Cre17.g715700	Phosphoprotein phosphatase 2C

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Table S3 – Continued

Clone	Environment	Chr	Position	Mutation	Feature	Gene	Gene Annotation
P4C5	high CO2	6	1605533	T → G	Exon, AA substitution Asp to Ala	Cre06.g260650	Unknown function
P4C5	high CO2	9	1654348	C → G	Exon, AA substitution Asp to Glu	Cre09.g397050	Unknown function
P5F6	high CO2	11	1929988	Deletion of T	Intron	Cre11.g468350	Monooxegynase
P5F6	high CO2	13	858930	Insertion of GCC	Exon, frame preserved	Cre13.g567400	3',5'-cyclic-nucleotide phosphodiesterase
P5F6	high CO2	6	3470712	C → T	Exon, synonymous	Cre06.g278096	Selenoprotein U
P6E8	high CO2	7	4402271	Insertion CACA	Intron	Cre07.g346750	Unknown function, Nucleotide-diphospho-sugar transferase
P6E8	high CO2	14	1201445	G → T	Intron	Cre14.g616000	Rhodanese-related sulfurtransferase
P6E8	high CO2	17	959598	C → G	Intron	Cre17.g703050	Unknown function
P6E8	high CO2	17	7008963	G → A	Intergenic	NA	NA
P6E8	high CO2	13	1032153	G → A	Exon, early stop	Cre13.g568550	MEKK and related serine/threonine protein kinases
P10C5	high NaCl	2	7075920	Deletion of CGCCGC-CGCCGC	Exon, frame preserved	Cre02.g147150	Unknown function
P10C5	high NaCl	3	2531420	Deletion of A	Exon, frameshift	Cre03.g160050	Flagellar Associated Protein
P10C5	high NaCl	2	615887	G → T	Intron	Cre02.g077900	Unknown function
P10C5	high NaCl	2	4348202	C → A	Intron	Cre02.g099350	Scavenger receptor cysteine rich (SRCR) protein
P10C5	high NaCl	2	6069175	C → T	5'UTR	Cre02.g112750	transcription factor with SBP domain
P10C5	high NaCl	7	4536090	G → A	3'UTR	Cre07.g343650	Dihydrolipoamide acetyl/succinyl-transferase related
P10C5	high NaCl	10	1921268	C → A	Intron	Cre10.g432000	UV-damaged DNA binding complex subunit 1 protein
P10C5	high NaCl	11	3209786	T → G	Intron	Cre11.g479450	Unknown function

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Table S3 – Continued

Clone	Environment	Chr	Position	Mutation	Feature	Gene	Gene Annotation
P10C5	high NaCl	14	1844797	G → T	Intron	Cre14.g620300	Anthranilate synthase, beta subunit
P10C5	high NaCl	14	3279554	C → G	3'UTR	Cre14.g629650	NIK1, High-affinity nickel-transport protein
P10C5	high NaCl	14	445760	G → A	Exon, synonymous	Cre14.g610850	Unknown function
P11B4	high NaCl	1	4051301	Deletion of G	Intron	Cre01.g026400	EXS family protein
P11B4	high NaCl	3	3388341	Insertion of GT	Intron	Cre03.g166850	Unknown function, DNA helicase, RNA processing
P11B4	high NaCl	7	1412936	Insertion of TG	Intron	Cre07.g322900	Lipase, Carboxylic ester hydrolases
P11B4	high NaCl	7	4973492	Insertion of C	Intron	Cre07.g346750	RNA pseudouridylate synthase
P11B4	high NaCl	8	354594	C → T	Intron	Cre08.g358571	Adenylate and Guanylate cyclase catalytic domain
P11B4	high NaCl	12	1385935	A → G	Intron	Cre12.g488200	Unknown function
P11B4	high NaCl	14	2755207	A → C	Intron	Cre14.g627100	Unknown function, ankyrin repeat protein binding domain
P11B4	high NaCl	16	5410913	C → A	Intron	Cre16.g681351	Unknown function
P11B4	high NaCl	6	5903226	C → G	Exon, AA substitution Ala to Pro	Cre06.g288350	Unknown function
P11B4	high NaCl	13	4389340	T → C	Exon, AA substitution Ile to Val	Cre13.g603000	3'5'-cyclic nucleotide phosphodiesterase
P11B4	high NaCl	14	3564734	C → T	Exon, AA substitution Ala to Val	Cre14.g631050	Unknown function
P12E4	high NaCl	17	4448225	Insertion of C	Intron	Cre17.g732150	Flagellar Associated Protein
P12E4	high NaCl	1	304805	A → G	5'UTR	Cre01.g001700	Serine-Threonine protein kinase
P12E4	high NaCl	2	721333	G → T	Intron	Cre02.g078400	Has Bestrophin, RFP-TM, chloride channel domain
P12E4	high NaCl	5	707033	C → A	3'UTR	Cre05.g230650	Unknown function
P12E4	high NaCl	10	5899535	T → G	Intron	Cre10.g462000	Calcium-independent phospholipase A2 (IPLA2)-RELATED

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Table S3 – Continued

Clone	Environment	Chr	Position	Mutation	Feature	Gene	Gene Annotation
P12E4	high NaCl	10	6426518	G $\rightarrow$ A	Intron	Cre10.g465850	PAB-dependent poly(A)-specific ribonuclease subunit 3
P12E4	high NaCl	3	3951632	G $\rightarrow$ C	Exon, synonymous	Cre03.g171650	Helicase, involved in RNA metabolism
P12E4	high NaCl	6	2797986	G $\rightarrow$ T	Exon, synonymous	Cre06.g271188	Calcineurin-like phosphoesterase
P12E4	high NaCl	10	6069174	T $\rightarrow$ C	Exon, AA substitution Ser to Arg	Cre10.g463350	HRP3, Extracellular matrix protein (cell wall protein)
P12E4	high NaCl	13	2142230	G $\rightarrow$ T	Exon, AA substitution Ala to Ser	Cre13.g578250	Unknown function
P12E4	high NaCl	16	3227089	C $\rightarrow$ A	Exon, AA substitution Thr to Lys	Cre16.g666576	Unknown function