Edinburgh Research Explorer

Epigenetic gene silencing by heterochromatin primes fungal resistance

Epigenetic gene silencing by heterochromatin primes fungal resistance

Sito Torres-Garcia, Imtiyaz Yaseen, Manu Shukla, Pauline N. C. B. Audergon[†],
Sharon A. White, Alison L. Pidoux, Robin C. Allshire[*]

Wellcome Centre *for* Cell Biology and Institute of Cell Biology,

School of Biological Sciences,

The University of Edinburgh,

Mayfield Road,

Edinburgh EH9 3BF, UK.

† Present address: Centre for Genomic Regulation (CRG),

The Barcelona Institute of Science and Technology,

Barcelona 08003, Spain

* Corresponding author: E-mail: robin.allshire@ed.ac.uk

1   **Summary:**

2   **Genes embedded in H3 lysine 9 methylation (H3K9me)–dependent**

3   **heterochromatin are transcriptionally silenced[1-3]. In fission yeast,**

4   ***Schizosaccharomyces pombe*, H3K9me-mediated heterochromatin can be**

5   **transmitted through cell division provided the counteracting demethylase Epe1**

6   **is absent[4,5]. Under certain conditions wild-type cells might utilize**

7   **heterochromatin heritability to form epimutations, phenotypes mediated by**

8   **unstable silencing rather than DNA changes[6,7]. Here we show that resistant**

9   **heterochromatin-dependent epimutants arise in threshold levels of caffeine.**

10   **Unstable resistant isolates exhibit distinct heterochromatin islands, which**

11   **reduce expression of underlying genes, some of which confer resistance when**

12   **mutated. Targeting synthetic heterochromatin to implicated loci confirms that**

13   **resistance results from heterochromatin-mediated silencing. Our analyses**

14   **reveal that epigenetic processes promote phenotypic plasticity, allowing wild-**

15   **type cells to adapt to non-favorable environments without altering their**

16   **genotype. In some isolates, subsequent or co-occurring gene amplification**

17   **events augment resistance. Caffeine impacts two anti-silencing factors: Epe1**

18   **levels are downregulated, reducing its chromatin association; and Mst2 histone**

19   **acetyltransferase expression switches to a shortened isoform. Thus,**

20   **heterochromatin-dependent epimutant formation provides a bet-hedging**

21   **strategy that allows cells to remain genetically wild-type but adapt transiently**

22   **to external insults. Unstable caffeine-resistant isolates show cross-resistance**

23   **to antifungal agents, suggesting that related heterochromatin-dependent**

24   **processes may contribute to antifungal resistance in plant and human**

25   **pathogenic fungi.**

26

27

28

29

30

31

Main Text:

H3K9me-heterochromatin can be copied by a read-write mechanism[4,5,8] and has been observed to arise stochastically at various loci, albeit only in the absence of key anti-silencing factors[9-13] or specific growth conditions[14]. We reasoned that if heterochromatin can redistribute in wild-type *S. pombe* cells, epimutations could be generated, allowing adaption to external insults. Unlike genetic mutants we predicted that such epimutants would be unstable, resulting in gradual loss of resistance following growth without the insult. We chose to use caffeine because deletion of genes with a variety of cellular roles confers caffeine resistance[15], thereby increasing the chance of obtaining epimutations. We also reasoned that unstable epimutants would occur more frequently at moderate caffeine concentrations that prevent most cells from growing (16 mM) rather than the higher stringency (20 mM) used in screens for genetic caffeine-resistant mutants[15].

As secondary events might occur upon prolonged growth on caffeine, we froze an aliquot of each isolate upon resistant colony formation and also froze consecutive aliquots of each isolate after continued growth on caffeine (Fig. 1a). This 'time series' permitted detection and separation of potential initiating and subsequent events. Colonies that grew after plating wild-type fission yeast (972 *h*⁻) cells in 16 mM caffeine (+CAF) were picked. Following freezing, isolates were then successively propagated without caffeine (-CAF). Re-challenging isolates with caffeine revealed that 23% lost caffeine resistance after 14 days of non-selective growth ('unstable resistant', UR) whereas 13% remained caffeine resistant ('stable resistant', SR). 64% of isolates did not display a clear phenotype ('unclear') (Fig. 1b and Extended Data Fig. 1a-c). Deletion of *clr4⁺* (the sole *S. pombe* H3K9 methyltransferase[16,17]), but not a control

3

56   locus, from resistant isolates resulted in loss of caffeine resistance in unstable, but

57   not stable isolates (Fig. 1c and Extended Data Fig. 1d). Thus, caffeine resistance in

58   unstable isolates requires heterochromatin.

59   Whole genome sequencing (WGS) of stable isolate SR-1 uncovered a mutation in

60   *pap1+* responsible for the caffeine-resistant phenotype (Extended Data Fig. 1e)[18].

61   ChIP-seq for H3K9me2 on SR-1 revealed no changes in heterochromatin distribution.

62   WGS of unstable isolates revealed no genetic changes in any sequence involved in

63   either caffeine resistance or H3K9me2-mediated silencing, and 8 of 30 analyzed

64   unstable isolates had no detectable genetic change compared to wild-type (Extended

65   Data Fig. 2a-e and Supplementary Table 1).

66   H3K9me2 ChIP-seq on unstable isolates revealed altered heterochromatin

67   distributions. UR-1 exhibited a new H3K9me2 island over the *hba1* locus, whereas

68   UR-2-to-UR-6 exhibited H3K9me2 islands over the *ncRNA.394*, *ppr4*, *grt1, fio1* and

69   *mbx2* loci, respectively (Fig. 2 and Supplementary Table 1). Deletion of *hba1+* confers

70   caffeine resistance[19], suggesting that caffeine-induced heterochromatin islands may

71   drive resistance by silencing underlying genes. Accordingly, RT-qPCR analysis

72   revealed reduced expression of genes underlying the observed *hba1*

73   heterochromatin island (Extended Data Fig. 2f).

74   The *ncRNA.394*, *ppr4*, *grt1, fio1* and *mbx2* loci have not previously been implicated

75   in caffeine resistance. Interestingly, 24/30 unstable isolates exhibited a

76   heterochromatin island over the *ncRNA.394* locus (Extended Data Fig. 3a, b and

77   Supplementary Table 1), and reduced underlying transcript levels (Extended Data Fig.

78 2f and 3c), suggesting that transcriptional silencing within these loci mediates

79 caffeine resistance.

80 *ncRNA.394* was previously identified as a heterochromatin island that gains

81 H3K9me2 in the absence of counteracting Epe1 demethylase[9,20]. We detected no

82 H3K9me2 over *ncRNA.394* in untreated wild-type cells (Fig. 2b and Extended Data

83 Fig. 3a, b). Deletion of *ncRNA.394* did not result in caffeine resistance (Extended Data

84 Fig. 3d). Prolonged growth without caffeine of cells exhibiting the *ncRNA.394*

85 heterochromatin island resulted in H3K9me2 loss over this region, whereas growth

86 with caffeine extended the H3K9me2 domain over the *SPBC17G9.13c$^+$* and

87 *SPBC17G9.12c$^+$* genes (Extended Data Fig. 3e). Deletion of *SPBC17G9.12c$^+$* or

88 *eno101$^+$* did not result in caffeine resistance (Extended Data Fig. 3d). *SPBC17G9.13c$^+$*

89 is essential for viability, precluding testing its deletion for resistance.

90 To test if heterochromatin formation at these specific loci alone results in caffeine

91 resistance, *tetO* binding sites were inserted at *hba1*, *ncRNA.394* and *mbx2* loci to

92 force synthetic heterochromatin assembly upon recruitment of TetR-Clr4* fusion

93 protein[4,5]. Combining *tetO* with TetR-Clr4* without anhydrotetracycline (-AHT)

94 resulted in novel H3K9me2 domains and growth on caffeine (Fig. 3 and Extended

95 Data Fig. 4a-d). Thus, heterochromatin-mediated silencing at *hba1*, *ncRNA.394* or

96 *mbx2* loci results in caffeine resistance.

97 Remarkably, strains with forced synthetic heterochromatin at either *hba1* or

98 *ncRNA.394* loci displayed resistance to the widely-used antifungals clotrimazole,

99 tebuconazole and fluconazole (Fig. 3 and Extended Data Fig. 4e). Unstable caffeine-

100 resistant isolates with heterochromatin islands at *hba1* (UR-1) or *ncRNA.394* (UR-2)

101   loci also displayed resistance to antifungals and produced small interfering RNAs

102   (siRNAs) homologous to surrounding genes (Extended Data Fig. 5a-c). Consistent

103   with RNAi pathway involvement, caffeine resistance was abolished upon removal of

104   RNAi components (*dcr1∆, ago1∆*; Extended Data Fig. 5d).

105   TetR-Clr4* tethering close to *SPBC17G9.13c*[+], upstream of *ncRNA.394*, resulted in

106   caffeine resistance (Fig. 3c), suggesting that reduced *SPBC17G9.13c*[+] expression

107   may mediate resistance. We therefore reduced expression of *SPBC17G9.13c*[+]

108   (named *cup1*[+]*, <u>c</u>affeine <u>u</u>nstable <u>p</u>henotype 1*) by increasing degradation of its mRNA

109   (*LocusPX:cup1-3xDSR*) or attenuating its transcription (*cup1-TT*; see Methods). Both

110   approaches resulted in reduced *cup1*[+] transcript levels and caffeine resistance

111   (Extended Data Fig. 6a, b). Cup1 contains a LYR domain often found in mitochondrial

112   proteins[21] and Cup1-GFP exhibited mitochondrial localisation (Extended Data Fig.

113   6c). LYR-domain mutation led to caffeine resistance (Extended Data Fig. 6d). Thus,

114   reduced expression or mutation of mitochondrial protein Cup1 (SPBC17G9.13c)

115   renders cells caffeine resistant. We conclude that *cup1*[+] silencing by heterochromatin

116   island formation mediates caffeine resistance.

117   In addition to the *ncRNA.394/cup1* heterochromatin island, analysis of ChIP-seq

118   input DNA indicated that many independent unstable caffeine-resistant isolates also

119   contained increased copy number of a chromosome III region (Extended Data Fig.

120   7a). The minimal region of overlap in 11/12 isolates contained *cds1*[+], whose

121   overexpression confers caffeine resistance[22]. To determine if *cds1*[+] amplification

122   occurred before or after *ncRNA.394/cup1* heterochromatin island formation, we

123   analyzed UR-2 samples frozen at earlier and later time points. The *ncRNA.394/cup1*

124   H3K9me2 island was detected in the initial caffeine-resistant isolate (4day/+CAF),

125   whereas *cds1* locus amplification arose later (7day/+CAF) (Extended Data Fig. 7b).

126   Thus, development of resistance appears to be a multistep process where

127   combinatorial events facilitate adaption to the insult.

128   In agreement with this hypothesis, deletion of *clr4⁺* from the initial UR-2 isolate

129   (4day/+CAF) resulted in caffeine resistance loss in all transformants (6/6). However,

130   only half of the transformants (3/6; transformants 1, 4 and 5) lost caffeine resistance

131   upon *clr4⁺* deletion from the later UR-2 isolate with *cds1* locus amplification

132   (7day/+CAF). Transformants that retained resistance after *clr4⁺* removal (3/6;

133   transformants 2, 3 and 6) exhibited higher *cds1⁺* copy numbers compared to *clr4Δ*

134   transformants that lost resistance or to wild-type cells (Extended Data Fig. 7c). We

135   conclude that once *cds1* locus amplification occurs heterochromatin is no longer

136   required for caffeine resistance. In UR-2 the new *ncRNA.394/cup1* heterochromatin

137   island arose before *cds1⁺* amplification, but it is likely that these events are stochastic

138   and occur in no fixed order. Interestingly, both adaptations – island formation and

139   locus amplification – are unstable and lost following growth without caffeine

140   (Extended Data Fig. 7d).

141   Instability of the amplified region suggested it resulted from excision and

142   extrachromosomal circular DNA (eccDNA) formation which can be rapidly

143   accumulated and lost[23-26]. CNV plots revealed repetitive elements at junctions of

144   putative eccDNA (*5S rRNA.24/26* for UR-2 (7day/+CAF) and *LTR3/27* for UR-4). PCR

145   specific for putative circle junctions and Southern analysis confirmed the presence of

146   chromosome-III-derived eccDNA (Extended Data Fig. 8). Therefore, repeat-mediated

147   eccDNA generation provides an alternative, or supplementary, mechanism for the

148   evolution of caffeine, and perhaps other, resistances in fission yeast. Accumulation

149   of additional changes may allow further adaption to insults through other pathways

150   or by bolstering silencing at particular loci[27].

151   To investigate the dynamics of heterochromatin island formation in response to

152   caffeine we exposed wild-type cells to low (7 mM) or medium (14 mM) doses of

153   caffeine. Cells in low or medium caffeine doubled ~8 or ~3 times, respectively, in 18

154   hours (Extended Data Fig. 9a). Several H3K9me2 heterochromatin islands were

155   detected following exposure to low caffeine (Fig. 4a top and Extended Data Fig. 9b,

156   c). These low-caffeine-induced islands represent a subgroup of those that

157   accumulate H3K9me2 in the absence of Epe1[9,10,12], including *ncRNA.394/cup1*, but

158   did not overlap with H3K9me2-heterochromatin domains that accumulate without

159   nuclear exosome function[13] or at 18°C[14]. Remarkably, ectopic heterochromatin was

160   restricted to *ncRNA.394/cup1* following medium caffeine treatment and H3K9me2

161   levels at this locus were ~4-fold greater after medium compared to low caffeine

162   exposure (Fig. 4a and Extended Data Fig. 9d). These data indicate that exposure to

163   near-lethal doses of caffeine (14 mM) allows wild-type cells to develop resistance

164   rapidly by forming heterochromatin over a locus (*ncRNA.394/cup1*) that confers

165   resistance when silenced.

166   To determine if other insults also induce heterochromatin islands, we exposed wild-

167   type cells to oxidative stress (1 mM hydrogen peroxide). Heterochromatin islands

168   were detected at similar locations to those observed in low caffeine, albeit H3K9me2

169   levels were lower (Extended Data Fig. 9b, c and e).

170 The heterochromatin profile of wild-type cells treated with low caffeine resembles that

171 of untreated *epe1Δ* cells (Extended Data Fig. 9c). We hypothesized that caffeine might

172 negatively regulate Epe1, thereby allowing adaptive ectopic heterochromatin islands

173 to form in wild-type cells. TetR-Clr4*-mediated synthetic heterochromatin can be

174 transmitted through cell division upon release of TetR-Clr4* from *tetO* sites only in

175 cells lacking Epe1[4,5]. To further test if caffeine imparts an *epe1Δ*-like phenotype, we

176 treated wild-type cells with low caffeine and released TetR-Clr4* from *4xtetO* sites

177 inserted at *ura4+* (Fig. 4b). Caffeine treatment, like *epe1Δ*, allowed heterochromatin

178 retention at the tethering site for longer compared to untreated cells. *epe1+* RNA

179 levels were not significantly altered by caffeine, suggesting post-transcriptional

180 regulation (Extended Data Fig. 9f). 3xFLAG-Epe1 levels decreased by 33% and Epe1

181 association with various heterochromatic locations was reduced following exposure

182 to caffeine (Fig. 4c, d). These data suggest that down-regulation of Epe1 putative

183 H3K9 demethylase levels plays a critical role in the response to external insults by

184 allowing formation of adaptive ectopic H3K9me-heterochromatin islands that, in turn,

185 reduce expression of underlying genes to confer resistance. Consistent with this

186 scenario, *epe1Δ* cells form more, and *clr4Δ* cells fewer, caffeine resistant colonies

187 than wild-type cells (Extended Data Fig. 9g).

188 Although caffeine down-regulates Epe1 protein levels, higher levels of H3K9me2

189 accumulate at heterochromatin islands following caffeine exposure than in untreated

190 *epe1Δ* cells (Extended Data Fig. 9c). Therefore, reduced Epe1 levels alone cannot

191 account for the high levels of H3K9me2 observed at islands upon caffeine treatment.

192 Mst2 histone acetyltransferase acts synergistically with Epe1 to prevent

193 heterochromatin island formation[10]. Interestingly, caffeine exposure results in

194　production of a shorter Mst2 protein by wild-type cells (52 kDa versus 62 kDa;

195　Extended Data Fig. 10a). RNA-seq suggests this shorter isoform arises through use

196　of an alternative transcriptional start site in caffeine, such as that detected in other

197　stresses[28] (Extended Data Fig. 10b). We suggest that this caffeine-induced shortened

198　Mst2 isoform, lacking the MYST-Zinc finger domain[29], may be inactive and unable to

199　prevent heterochromatin island formation. Thus, caffeine, by both lowering Epe1

200　levels and likely disabling Mst2, allows greater accumulation of H3K9me2 at islands

201　than in *epe1Δ* cells. These findings reveal an adaptive epigenetic response to external

202　insults that stimulates phenotypic plasticity, and suggest that stress-response

203　pathways may regulate heterochromatin modulation activities, thereby ensuring cell

204　survival in fluctuating environmental conditions (Fig. 4e).

205　DNA methylation-dependent epimutations frequently arise in plants and are

206　propagated by maintenance methyltransferases[30,31]. RNAi-mediated epimutations

207　occur in the fungus *Mucor circinelloides*[32], but their DNA methylation or

208　heterochromatin dependence is unknown. As fission yeast lacks DNA methylation[33,34]

209　this epigenetic mark cannot be responsible for the epimutations described here.

210　Instead our analyses indicate that these adaptive epimutations are transmitted in

211　wild-type cells by the Clr4/H3K9me read-write mechanism[4,5,8].

212　Why have epimutants not been detected previously in mutant screens? Stringent

213　phenotypic screens mean strong mutants are investigated further and eccentric

214　mutants discarded. Here we selected for weak mutants by applying sublethal doses

215　of drug at the threshold of growth prevention. Selection was time-limited to maximize

216　identification of isolates exhibiting unstable phenotypes prior to development of

217　genetic alterations.

218 Fungal infections are on the rise, especially in immunocompromised humans. Few

219 effective antifungal agents exist and resistance is rendering them increasingly

220 ineffective[35]. Widespread use of related azole compounds to control fungus-mediated

221 crop deterioration may leave residual antifungals in the soil, possibly allowing

222 unwitting selection of resistant epimutants in fungi, ultimately driving increasing cases

223 of azole-resistant Aspergillosis and Cryptococcosis in the clinic. Monitoring

224 resistance in clinical isolates involves mutation identification by genome sequencing,

225 but resistance due to epimutations – similar to those described here – would be

226 missed, leading to inaccurate diagnoses. Re-engineering existing so-called

227 'epigenetic drugs' – compounds that inhibit histone-modifying enzymes – or

228 development of novel agents, may identify molecules that specifically block fungal,

229 not host, heterochromatin formation, hence reducing the emergence of antifungal

230 resistance in clinical and agricultural settings.

231

232

233

234

235

236

237

238

References

239

240   1.   Bannister, A. J. *et al.* Selective recognition of methylated lysine 9 on histone H3

241   by the HP1 chromo domain. *Nature* **410,** 120–124 (2001).

242   2.   Lachner, M., O'Carroll, D., Rea, S., Mechtler, K. & Jenuwein, T. Methylation of

243   histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410,** 116–

244   120 (2001).

245   3.   Allshire, R. C. & Madhani, H. D. Ten principles of heterochromatin formation

246   and function. *Nat. Rev. Mol. Cell Biol.* **45,** 153 (2017).

247   4.   Audergon, P. N. C. B. *et al.* Epigenetics. Restricted epigenetic inheritance of

248   H3K9 methylation. *Science* **348,** 132–135 (2015).

249   5.   Ragunathan, K., Jih, G. & Moazed, D. Epigenetics. Epigenetic inheritance

250   uncoupled from sequence-specific recruitment. *Science* **348,** 1258699–

251   1258699 (2015).

252   6.   Jeggo, P. A. & Holliday, R. Azacytidine-induced reactivation of a DNA repair

253   gene in Chinese hamster ovary cells. *Mol. Cell. Biol.* **6,** 2944–2949 (1986).

254   7.   Oey, H. & Whitelaw, E. On the meaning of the word 'epimutation'. *Trends*

255   *Genet.* **30,** 519–520 (2014).

256   8.   Zhang, K., Mosch, K., Fischle, W. & Grewal, S. I. S. Roles of the Clr4

257   methyltransferase complex in nucleation, spreading and maintenance of

258   heterochromatin. *Nat. Struct. Mol. Biol.* **15,** 381–388 (2008).

259   9.   Zofall, M. *et al.* RNA elimination machinery targeting meiotic mRNAs promotes

260   facultative heterochromatin formation. *Science* **335,** 96–100 (2012).

261   10.   Wang, J., Reddy, B. D. & Jia, S. Rapid epigenetic adaptation to uncontrolled

262   heterochromatin spreading. *eLife* **4,** 80 (2015).

263   11.   Parsa, J.-Y., Boudoukha, S., Burke, J., Homer, C. & Madhani, H. D. Polymerase

264   pausing induced by sequence-specific RNA-binding protein drives

265   heterochromatin assembly. *Genes Dev.* **32,** 953–964 (2018).

266   12.   Sorida, M. *et al.* Regulation of ectopic heterochromatin-mediated epigenetic

267   diversification by the JmjC family protein Epe1. *PLoS Genet.* **15,** e1008129

268   (2019).

269   13.   Yamanaka, S. *et al.* RNAi triggered by specialized machinery silences

270   developmental genes and retrotransposons. *Nature* **493,** 557–560 (2013).

271   14.   Gallagher, P. S. *et al.* Iron homeostasis regulates facultative heterochromatin

272   assembly in adaptive genome control. *Nat. Struct. Mol. Biol.* **25,** 372–383

273   (2018).

274   15.   Calvo, I. A. *et al.* Genome-wide screen of genes required for caffeine tolerance

275   in fission yeast. *PLoS ONE* **4,** e6619 (2009).

276   16.   Ivanova, A. V., Bonaduce, M. J., Ivanov, S. V. & Klar, A. J. The chromo and SET

277   domains of the Clr4 protein are essential for silencing in fission yeast. *Nat.*

278   *Genet.* **19,** 192–195 (1998).

279   17.   Nakayama, J., Rice, J. C., Strahl, B. D., Allis, C. D. & Grewal, S. I. Role of histone

280   H3 lysine 9 methylation in epigenetic control of heterochromatin assembly.

281   *Science* **292,** 110–113 (2001).

282   18.   Kudo, N., Taoka, H., Toda, T., Yoshida, M. & Horinouchi, S. A novel nuclear

283   export signal sensitive to oxidative stress in the fission yeast transcription factor

284   Pap1. *J. Biol. Chem.* **274,** 15151–15158 (1999).

285   19.   Castillo, E. A., Vivancos, A. P., Jones, N., Ayté, J. & Hidalgo, E.

286   *Schizosaccharomyces pombe* cells lacking the Ran-binding protein Hba1 show

287   a multidrug resistance phenotype due to constitutive nuclear accumulation of

288   Pap1. *J. Biol. Chem.* **278,** 40565–40572 (2003).

289   20. Zofall, M., Smith, D. R., Mizuguchi, T., Dhakshnamoorthy, J. & Grewal, S. I. S.

290   Taz1-Shelterin Promotes Facultative Heterochromatin Assembly at

291   Chromosome-Internal Sites Containing Late Replication Origins. *Mol. Cell* **62,**

292   862–874 (2016).

293   21. Angerer, H. Eukaryotic LYR Proteins Interact with Mitochondrial Protein

294   Complexes. *Biology* **4,** 133–150 (2015).

295   22. Wang, S. W., Norbury, C., Harris, A. L. & Toda, T. Caffeine can override the S-

296   M checkpoint in fission yeast. *J. Cell. Sci.* **112**, 927–937 (1999).

297   23. Libuda, D. E. & Winston, F. Amplification of histone genes by circular

298   chromosome formation in *Saccharomyces cerevisiae*. *Nature* **443,** 1003–1007

299   (2006).

300   24. Møller, H. D., Parsons, L., Jørgensen, T. S., Botstein, D. & Regenberg, B.

301   Extrachromosomal circular DNA is common in yeast. *Proc. Natl. Acad. Sci.*

302   *U.S.A.* **112,** E3114–22 (2015).

303   25. Hull, R. M. *et al.* Transcription-induced formation of extrachromosomal DNA

304   during yeast ageing. *PLoS Biol.* **17,** e3000471 (2019).

305   26. Wu, S. *et al.* Circular ecDNA promotes accessible chromatin and high

306   oncogene expression. *Nature* **575,** 699–703 (2019).

307   27. Stajic, D., Perfeito, L. & Jansen, L. E. T. Epigenetic gene silencing alters the

308   mechanisms and rate of evolutionary adaptation. *Nat. Ecol. Evol.* **3,** 491–498

309   (2019).

310   28.   Thodberg, M. *et al.* Comprehensive profiling of the fission yeast transcription

311   start site activity during stress and media response. *Nucleic Acids Res.* **47,**

312   1671–1691 (2019).

313   29.   Yan, Y., Barlev, N. A., Haley, R. H., Berger, S. L. & Marmorstein, R. Crystal

314   structure of yeast Esa1 suggests a unified mechanism for catalysis and

315   substrate binding by histone acetyltransferases. *Mol. Cell* **6,** 1195–1205 (2000).

316   30.   Cubas, P., Vincent, C. & Coen, E. An epigenetic mutation responsible for natural

317   variation in floral symmetry. *Nature* **401,** 157–161 (1999).

318   31.   Heard, E. & Martienssen, R. A. Transgenerational epigenetic inheritance: myths

319   and mechanisms. *Cell* **157,** 95–109 (2014).

320   32.   Calo, S. *et al.* Antifungal drug resistance evoked via RNAi-dependent

321   epimutations. *Nature* **513,** 555–558 (2014).

322   33.   Antequera, F., Tamame, M., Villanueva, J. R. & Santos, T. DNA methylation in

323   the fungi. *J. Biol. Chem.* **259,** 8033–8036 (1984).

324   34.   Wilkinson, C. R., Bartlett, R., Nurse, P. & Bird, A. P. The fission yeast gene

325   *pmt1$^+$* encodes a DNA methyltransferase homologue. *Nucleic Acids Res.* **23,**

326   203–210 (1995).

327   35.   Fisher, M. C., Hawkins, N. J., Sanglard, D. & Gurr, S. J. Worldwide emergence

328   of resistance to antifungal drugs challenges human health and food security.

329   *Science* **360,** 739–742 (2018).

330

331

332

333

Figure 1. Identification of heterochromatin-dependent epimutants resistant to caffeine

a, Screening strategy. *S. pombe* wild-type (wt) cells were plated on caffeine-containing (+CAF) media. Caffeine-resistant isolates were picked and grown on +CAF for 4 days. Isolates were then grown on +CAF for a total of 7 or 20 days or on non-selective (-CAF) media for 2 and 14 days.

b, Unstable (UR) and stable (SR) caffeine-resistant isolates were identified. After non-selective growth for 2 and 14 days, caffeine-resistant isolates were serially diluted and spotted on -CAF and +CAF plates to assess resistance to caffeine**.**

c, Caffeine resistance in UR isolates depends on the Clr4 H3K9 methyltransferase. *clr4$^+$* (*clr4Δ*) or an unlinked intergenic region (*controlΔ*) were deleted in unstable (UR-1) and stable (SR-1) caffeine-resistant isolates.

Experiments in (**b**) and (**c**) were independently repeated at least twice with similar results.

Figure 2. Ectopic islands of heterochromatin are detected in unstable (UR) caffeine-resistant isolates

a-b, Genome-wide (**a**) and locus-specific (**b**) H3K9me2 ChIP-seq enrichment in wild-type (wt) cells and UR isolates. Data are represented as relative fold enrichment over input. Sequencing was performed once, and results were confirmed by qChIP. Red arrows in (**b**) indicate essential genes.

Figure 3. Forced synthetic heterochromatin targeting to the identified loci is

366 **sufficient to drive caffeine resistance in wild-type cells**

367 **a,** TetR-Clr4* mediates H3K9me deposition at *4xtetO* binding sites. Addition of

368 anhydrotetracycline (+AHT) releases TetR-Clr4* from *4xtetO* sites, resulting in

369 removal of H3K9me.

370 **b-d,** Wild-type (wt) cells harbouring *4xtetO* binding sites at the *hba1* or *ncRNA.394*

371 loci (or *ura4* as control) and expressing TetR-Clr4* were assessed for caffeine (+CAF)

372 or clotrimazole (+CLZ) resistance in the absence or presence of AHT. qChIP of

373 H3K9me2 levels on *hba1* (**b**), *SPBC17G9.13c* (**c**) and *ura4* (**d**) loci. Data are mean ±

374 s.d. from three biological replicates. Dumbbells indicate primer pairs used. Red

375 arrows indicate essential genes. Note *hba1* is not present in *hba1Δ*.

376

377

378

379

380

381

382

383

384

385

386

387

388

389

390

391

392

393

394

395

Figure 4. Dynamic heterochromatin redistribution following short exposure to caffeine in wild-type cells

a, H3K9me2 ChIP-seq enrichment at *ncRNA.394/cup1* and *mcp7* loci (or at pericentromeric *dgl/dhl* repeats of chromosome I as control) in wild-type (wt) cells following 18 hr exposure to low (7 mM, *top*) or medium (14 mM, *bottom*) concentrations of caffeine. Data are represented as relative fold enrichment over input. Red arrows indicate essential genes.

b, Effect of caffeine treatment on retention of synthetic heterochromatin upon release of tethered Clr4 methyltransferase. qChIP of H3K9me2 levels on *4xtetO-ura4$^+$* before and after TetR-Clr4* release in wt cells untreated or treated with low caffeine. *epe1Δ* cells were used as positive control. Dumbbells indicate primer pairs used. H3K9me2 levels were normalized to spike-in control. Data are mean ± s.d. from three biological replicates.

c, *Top:* Western analysis of 3xFLAG-Epe1 (endogenous gene tagged) levels before and after low caffeine treatment. Loading control: α-tubulin. For gel source data, see Supplementary Figure 1a.

Bottom: Quantification of 3xFLAG-Epe1 protein levels normalized to α-tubulin. Data are mean ± s.d. from four biological replicates. *P* value: two-tailed Student's *t*-test.

d, Effect of caffeine treatment on association of Epe1 with chromatin. qChIP analysis of Epe1-GFP levels at sub-telomeric *tlh2* locus and centromere 1 (*dg* repeats: *cen-dg*; outer boundary: *cen-IRC*) in wt cells treated with no, low or medium caffeine. Epe1-GFP levels were normalized to spike-in control. Data are mean ± s.d. from three biological replicates**.**

e, Model. Resistant isolates arise following exposure to a lethal insult. Resistance could be mediated by permanent, DNA-based changes (resistant mutants) or reversible, heterochromatin-based epimutations (resistant epimutants). Upon insult removal, resistant epimutants can revert to wild-type (sensitive phenotype) by disassembling ectopic heterochromatin islands, whereas resistant mutants continue displaying the mutant phenotype due to the genetic nature of DNA mutations.

Methods

Yeast strains and manipulations

Standard methods were used for fission yeast growth, genetics and manipulation[36]. *S. pombe* strains used in this study are described in Supplementary Table 2. Oligonucleotide sequences are listed in Supplementary Table 3. For pDUAL-adh21-TetR-2xFLAG-Clr4-CDΔ (abbreviated as TetR-Clr4*), the *nmt81* promoter of pDUAL-nmt81-TetR-2xFLAG-Clr4-CDΔ[4], was replaced by the *adh21* promoter (pRAD21, gift from Y. Watanabe). *Not*I-digested plasmid was integrated at *leu1*+.

To reduce expression of *SPBC17G9.13c*+/*cup1*+ we used two independent strategies. First, we expressed an additional copy of *cup1*+ with three nuclear exosome RNA degradation motifs (DSR; Determinant of Selective Removal[37,38]) fused to its 3' untranslated region from an intergenic locus (*LocusPX:cup1-3xDSR*). Following insertion of *cup1-3xDSR* at *LocusPX*, endogenous *cup1*+ was deleted and cells expressing only *cup1-3xDSR* were analysed. Second, the 144-bp transcriptional terminator site from *ura4*+ was inserted in place of part of the putative *cup1*+ promoter (*cup1-TT*) and cells were analysed.

pap1-N424STOP, *clr5-Q264STOP meu27-S100Y*, *LocusPX:cup1-3xDSR*, *cup1-TT*, *cup1-L73G*, *cup1-F99G*, *cup1-GFP, 3xFLAG-epe1* and strains carrying *4xtetO* insertions were constructed by CRISPR/Cas9-mediated genome editing using the *SpEDIT* system (Allshire Lab; available on request) with oligonucleotides listed in Supplementary Table 3. The mitochondrial protein Arg11[39], Epe1 and Mst2 were C-terminally tagged with mCherry (Arg11), GFP (Epe1) or 13xMyc (Mst2) using the Bähler tagging method[40].

449   Yeast extract plus supplements (YES) was used to grow all cultures. 16 mM caffeine

450   (Sigma, C0750) was added to media for caffeine resistance screens and serial dilution

451   assays. To screen for unstable caffeine-resistant isolates, caffeine-resistant colonies

452   that formed seven days after plating wild-type cells on 16 mM caffeine YES (+CAF)

453   plates were picked and patched to +CAF plates. After four days of growth, isolates

454   were frozen (4day/+CAF). 4day/+CAF isolates were re-patched and grown for three

455   days on +CAF plates and then frozen (7day/+CAF). Subsequently, 7day/+CAF

456   isolates were re-patched every three days on +CAF plates up to twenty days of total

457   growth on +CAF plates and then frozen (20day/+CAF).

458   0.29 μM clotrimazole (Sigma, C6019) was added to media for clotrimazole resistance

459   serial dilution assays. 1.6 μM tebuconazole (Sigma, 32013) was added to media for

460   tebuconazole resistance serial dilution assays. 0.6 mM fluconazole (Sigma, PHR1160)

461   was added to media for fluconazole resistance serial dilution assays.

462   7 or 14 mM caffeine (Sigma, C0750), or 1 mM hydrogen peroxide (Sigma, H1009)

463   were added to media for 18 hours for drug treatment experiments. To release *TetR-*

464   *Clr4**, 10 μM anhydrotetracycline (AHT) was added to the media.

465   **Serial dilution assays**

466   Equal amounts of starting cells were serially diluted five-fold and then spotted onto

467   appropriate media. Cells were grown at 30-32°C for 3-5 days and then photographed.

468

469

Chromatin immunoprecipitation (ChIP)

471 ChIP experiments were performed as previously described[41] using anti-H3K9me2

472 (5.1.1, gift from Takeshi Urano) or anti-GFP (Invitrogen, A11122).

473 Immunoprecipitated DNA was recovered with Chelex-100 resin (BioRad) for ChIP-

474 qPCR (qChIP) experiments or with QIAquick PCR Purification Kit (Qiagen) for ChIP-

475 seq experiments.

Quantitative ChIP-qPCR (qChIP)

477 qChIPs were analysed by real-time PCR using Lightcycler 480 SYBR Green (Roche)

478 with oligonucleotides listed in Supplementary Table 3. All ChIP enrichments were

479 calculated as % DNA immunoprecipitated at the locus of interest relative to the

480 corresponding input samples and normalized to % DNA immunoprecipitated at the

481 $act1^+$ locus. For spike-in qChIPs, an equal number (~20%) of *Schizosaccharomyces*

482 *octosporus* cells (H3K9me2 spike-in qChIP)[41] or Sgo1-GFP *Saccharomyces*

483 *cerevisiae* cells (GFP spike-in qChIP)[42] (gift from Adele Marston) were added to initial

484 *S. pombe* pellets. Histograms represent data averaged over three biological

485 replicates. Error bars represent standard deviations.

ChIP-seq library preparation and analysis

487 Illumina-compatible libraries were prepared as previously described[41] using

488 NEXTflex-96 barcode adapters (Bioo Scientific) and Ampure XP beads (Beckman

489 Coulter). Libraries were then pooled to allow multiplexing and sequenced on an

490 Illumina HiSeq2000, NextSeq or MiniSeq system (150-cycle high output kit) by 75 bp

491 paired-end sequencing.

492 Approximately 6-10 million 75 bp paired-end reads were produced for each sample.

493 Raw reads were then de-multiplexed and trimmed using Trimmomatic (v0.35)[43] to

494 remove adapter contamination and regions of poor sequencing quality. Trimmed

495 reads were aligned to the *S. pombe* reference genome (*972h⁻*, ASM294v2.20) using

496 Bowtie2 (v2.3.3)[44]. Resulting bam files were processed using Samtools (v1.3.1)[45] and

497 picard-tools (v2.1.0) (http://broadinstitute.github.io/picard) for sorting, removing

498 duplicates and indexing. Coverage bigwig files were generated by BamCoverage

499 (deepTools v2.0) and ratios IP/input were calculated using BamCompare (deepTools

500 v2.0)[46] in SES mode for normalisation[47]. Peaks were called using MACS2[48] in PE mode

501 and broad peak calling (broad-cutoff = 0.05). Region-specific H3K9me2 enrichment

502 plots were generated using the Sushi R package (v1.22)[49]. Heatmaps were generated

503 using computeMatrix and plotHeatmap (deepTools v2.0)[46] with genomic coordinates

504 indicated in Supplementary Table 4.

505 **SNP and indel calling**

506 SNPs and indels were called as previously described[50]. Trimmed reads were mapped

507 to the *S. pombe* reference genome (*972h⁻*, ASM294v2.20) using Bowtie2 (v2.3.3)[44].

508 GATK[51,52] was used for base quality score recalibration. SNPs and indels were called

509 with GATK HaplotypeCaller[51,52] and filtered using custom parameters. Functional

510 effect of variants was determined using Variant Effect Predictor[53].

511 **Copy number variation analysis**

512 Copy number variation was determined using CNVkit[54] in Whole-Genome

513 Sequencing (-wgs) mode. Wild-type ChIP-seq input bam files were used as reference.

Extrachromosomal circular DNA diagnostic PCRs and Southern analysis

ChIP-input DNA samples were used as template for PCR with Taq polymerase (Roche, 4728858001) according to manufacturer's instructions. Two types of PCR were performed: control PCR for loci present on endogenous chromosome III (expected to be present in wild-type, UR-2 (7day/+CAF) and UR-4) and circle-specific PCRs specific for putative extrachromosomal circles predicted to be present in UR-2 (7day/+CAF) or UR-4. For wild-type and UR-2 (7day/+CAF): control primers were located on either on side of *5S rRNA.24* (primers A (forward), B (reverse); see Supplementary Table 3) and *5S rRNA.26* (primers C, D); circle-specific primers were located on either side of a predicted junction between *5S rRNA.24* and *5S rRNA.26* (primers C and B). For wild-type and UR-4: control primers were located on either on side of *LTR3* (primers E, F) and or *LTR27* (primers G, H); circle-specific primers were located on either side of a predicted junction between *LTR3* and *LTR27* (primers G and F). For some locations, more than one forward and/or reverse primer was used, for instance: forward primers C1, C2 with reverse primers D1, D2. PCR products were electrophoresed on 2% agarose gels containing Ethidium Bromide.

For Southern analysis, genomic DNA was prepared from wild-type, UR-2 (7day/+CAF) and UR-4 cultures grown in YES. Briefly, cells were incubated with Zymolyase 100T (AMS Biotechnology) to digest the cell wall, pelleted, resuspended in TE and lysed with SDS, followed by addition of potassium acetate and precipitation with isopropanol. After treatment with RNase A and proteinase K, phenol chloroform and chloroform extractions were performed. DNA was precipitated in the presence of sodium acetate and ethanol, followed by centrifugation and washing of the pellet

537 with 70% ethanol. After air drying the pellet was resuspended in TE. Approximately 8

538 μg of DNA was digested with the following restriction enzymes: wild-type and UR-2

539 (7day/+CAF): *Bsm*BI, *Eco*RV, *Nde*I; wild-type and UR-4: *Eco*RI, *Bam*HI + *Xba*I.

540 Digested DNA was subjected to electrophoresis in a 0.9 % agarose gel containing

541 ethidium bromide. Southern blotting was achieved by the alkali transfer method.

542 Briefly, the gel was depurinated with 0.3 M HCl for 10 minutes, washed with distilled

543 water, followed by two 15 min incubations in Denaturing Solution (0.5 M NaOH, 1.5

544 M NaCl). Overnight capillary transfer was used for transfer to Hybond XL membrane

545 (Amersham), which was then washed with 50 mM Na_2HPO_4 pH7.2, followed by air

546 drying. After drying at 80°C for 2 hours and UV-crosslinking, membranes were

547 prehybridized in Church Buffer (0.5 M Na_2HPO_4 pH 7.2, 7% SDS, 1 mM EDTA, 1%

548 BSA (Sigma, A0281) for 1 hour at 65°C. Probes were made using High Prime kit

549 (Roche, 11585592001) and α-^{32}P-dCTP (NEN), according to the manufacturer's

550 instructions. Heat denatured probes in Church Buffer were hybridized with relevant

551 membranes at 65°C overnight with rotation. Following washes with Wash Buffer (40

552 mM Na_2HPO_4 pH 7.2, 1 mM EDTA, 1% SDS) blots were exposed to XAR-5 film

553 (Kodak) at -80°C with an intensifying screen for several hours.

554 **Cytology**

555 *Schizosaccharomyces pombe* cultures were fixed before processing for

556 immunofluorescence as described[41]. Briefly, cells in YES culture were fixed with 3.7%

557 formaldehyde (Sigma, F8775) for 30 min, followed by cell wall digestion with

558 Zymolyase-100T (AMS Biotechnology) in PEMS buffer (100 mM PIPES pH 7, 1 mM

559 EDTA, 1 mM $MgCl_2$, 1.2 M Sorbitol). After permeabilization with Triton-X100, cells

560 were washed, blocked in PEMBAL (PEM containing 1% BSA, 0.1% sodium azide,

561 100 mM lysine hydrochloride). Rabbit anti-GFP (Invitrogen, A11122) was used in

562 PEMBAL at 1:500 dilution, and Alexa 488-coupled chicken-anti-rabbit secondary

563 antibody (Invitrogen, A21441) at 1:1000 dilution. Arg11-mCherry fluorescence

564 survived fixation and no antibodies were used for localisation. Cells were stained with

565 DAPI and mounted in Vectashield. Microscopy was performed with a Zeiss Imaging

566 2 microscope (Zeiss) using a 100x 1.4NA Plan-Apochromat objective, Prior filter

567 wheel, illumination by HBO100 mercury bulb. Image acquisition with a Photometrics

568 Prime sCMOS camera (Photometrics, https://www.photometrics.com) was

569 controlled using Metamorph software (Version 7; Universal Imaging Corporation).

570 Exposures were 3000 ms for FITC/Alexa-488 channel (Cup1-GFP/Alexa 488), 500 ms

571 for TRITC channel (Arg11-mCherry) and 100 ms for DAPI. For display of images,

572 maximum intensity was determined for e.g. Cup1-GFP staining in Cup1-GFP Arg11-

573 mCherry strain (B4909) and this maximum was applied for scaling of all B4909 and

574 B4912 (expresses only Arg11-mCherry) images. FITC and TRITC channels were

575 scaled in this way; DAPI images were autoscaled.

576 **qRT–PCR analysis**

577 Total RNA was extracted using the Monarch Total RNA Miniprep Kit (New England

578 Biolabs) according to the manufacturer's instructions. Contaminating DNA was

579 removed by treating with Turbo DNase (Invitrogen) and reverse transcription was

580 performed using LunaScript RT Supermix Kit (New England Biolabs).

581 Oligonucleotides used for qRT-PCR are listed in Supplementary Table 3. qRT-PCR

582 histograms represent three biological replicates; error bars correspond to the

583 standard deviation.

RNA-seq library preparation and analysis

Total RNA was extracted using the Monarch Total RNA Miniprep Kit (New England Biolabs) according to the manufacturer's instructions. Contaminating DNA was removed by treating with Turbo DNase (Invitrogen). rRNA was removed using the Ribo-Zero Gold rRNA removal kit (Yeast) (Illumina) before library construction using NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs). Libraries were pooled and sequenced on an Illumina NextSeq platform by 75 bp paired-end sequencing. Adapter-trimmed reads were aligned to the *S. pombe* reference genome (*972h⁻*, ASM294v2.20) using STAR (v2.2.1)[55] and processed using Samtools (v1.3.1)[45]. Coverage bigwig files were generated by BamCoverage (deepTools v2.0)[46].

Differential expression was analysed using the Bioconductor Rsamtools (v2.0.3), GenomicFeatures (v1.36.4)[56] and DESeq2 (v.1.24)[57] R libraries. Log2 fold changes were shrunk using the apeglm method[58] and a MA-plot was generated using R. Genes with an adjusted *p* value below 0.01 are shown in red.

Small RNA-seq

50 mL of log-phase cells were collected and processed using the mirVana miRNA Isolation kit (Invitrogen). Resulting sRNA was treated with TURBO DNase (Invitrogen) and used for library construction using NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs) according to manufacturer's instructions. Libraries were pooled and sequenced on an Illumina NextSeq platform by 50 bp single-end sequencing. Raw reads were then de-multiplexed and processed using Cutadapt (v1.17) to remove adapter contamination and discard

607   reads shorter than 19 nucleotides or longer than 25 nucleotides. Coverage plots were

608   generated using SCRAM[59].

609   **Protein extraction and western analysis**

610   Protein samples were prepared as previously detailed[60]. Western blotting detection

611   was performed using anti-FLAG-HRP (Sigma, A8591), anti-Myc (Cell Signalling,

612   9B11), anti-α-tubulin (gift from Keith Gull)[61], goat anti-mouse (Sigma, A4416), anti-

613   Bip1[62], goat anti-rabbit (Sigma, A6154), anti-Cdc11 (gift from Ken Sawin) and donkey

614   anti-sheep (Abcam, ab6900). Gels were visualised using the ChemiDoc imaging

615   system (BioRad) and analysed with ImageJ.

616

617

618

619

620

621

622

623

624

625

Additional references for Methods section

36. Moreno, S., Klar, A. & Nurse, P. Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Meth. Enzymol.* **194,** 795–823 (1991).

37. Harigaya, Y. *et al.* Selective elimination of messenger RNA prevents an incidence of untimely meiosis. *Nature* **442,** 45–50 (2006).

38. Watson, A. T. *et al.* Optimisation of the *Schizosaccharomyces pombe urg1* expression system. *PLoS ONE* **8,** e83800 (2013).

39. Delerue, T. *et al.* Loss of Msp1p in *Schizosaccharomyces pombe* induces a ROS-dependent nuclear mutator phenotype that affects mitochondrial fission genes. *FEBS Lett.* **590,** 3544–3558 (2016).

40. Bähler, J. *et al.* Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast* **14,** 943–951 (1998).

41. Tong, P. *et al.* Interspecies conservation of organisation and function between nonhomologous regional centromeres. *Nat. Commun.* **10,** 2343 (2019).

42. Nerusheva, O. O., Galander, S., Fernius, J., Kelly, D. & Marston, A. L. Tension-dependent removal of pericentromeric shugoshin is an indicator of sister chromosome biorientation. *Genes Dev.* **28,** 1291–1309 (2014).

43. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30,** 2114–2120 (2014).

44. Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **9,** 357–359 (2012).

45. Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25,** 2078–2079 (2009).

649   46.   Ramírez, F. *et al.* deepTools2: a next generation web server for deep-
650   sequencing data analysis. *Nucleic Acids Res.* **44,** W160–5 (2016).

651   47.   Diaz, A., Park, K., Lim, D. A. & Song, J. S. Normalization, bias correction, and
652   peak calling for ChIP-seq. *Stat Appl Genet Mol Biol* **11,** Article 9 (2012).

653   48.   Zhang, Y. *et al.* Model-based analysis of ChIP-Seq (MACS). *Genome Biol.* **9,**
654   R137 (2008).

655   49.   Phanstiel, D. H., Boyle, A. P., Araya, C. L. & Snyder, M. P. Sushi.R: flexible,
656   quantitative and integrative genomic visualizations for publication-quality multi-
657   panel figures. *Bioinformatics* **30,** 2808–2810 (2014).

658   50.   Jeffares, D. C. *et al.* The genomic and phenotypic diversity of
659   *Schizosaccharomyces pombe*. *Nat. Genet.* **47,** 235–241 (2015).

660   51.   McKenna, A. *et al.* The Genome Analysis Toolkit: a MapReduce framework for
661   analyzing next-generation DNA sequencing data. *Genome Res.* **20,** 1297–1303
662   (2010).

663   52.   Van der Auwera, G. A. *et al.* From FastQ data to high confidence variant calls:
664   the Genome Analysis Toolkit best practices pipeline. *Curr. Protoc.*
665   *Bioinformatics.* **43,** 11.10.1–33 (2013).

666   53.   McLaren, W. *et al.* The Ensembl Variant Effect Predictor. *Genome Biol.* **17,** 122
667   (2016).

668   54.   Talevich, E., Shain, A. H., Botton, T. & Bastian, B. C. CNVkit: Genome-Wide
669   Copy Number Detection and Visualization from Targeted DNA Sequencing.
670   *PLoS Comput. Biol.* **12,** e1004873 (2016).

671   55.   Dobin, A. *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29,**
672   15–21 (2013).

673   56.   Lawrence, M. *et al.* Software for computing and annotating genomic ranges.

674   *PLoS Comput. Biol.* **9,** e1003118 (2013).

675   57.   Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and

676   dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15,** 550 (2014).

677   58.   Zhu, A., Ibrahim, J. G. & Love, M. I. Heavy-tailed prior distributions for sequence

678   count data: removing the noise and preserving large differences. *Bioinformatics*

679   **35,** 2084–2092 (2019).

680   59.   Fletcher, S. J., Boden, M., Mitter, N. & Carroll, B. J. SCRAM: a pipeline for fast

681   index-free small RNA read alignment and visualization. *Bioinformatics* **34,**

682   2670–2672 (2018).

683   60.   Braun, S. *et al.* The Cul4-Ddb1(Cdt)^2 ubiquitin ligase inhibits invasion of a

684   boundary-associated antisilencing factor into heterochromatin. *Cell* **144,** 41–54

685   (2011).

686   61.   Woods, A. *et al.* Definition of individual components within the cytoskeleton of

687   *Trypanosoma brucei* by a library of monoclonal antibodies. *J. Cell. Sci.* **93 (Pt**

688   **3),** 491–500 (1989).

689   62.   Pidoux, A. L. & Armstrong, J. The BiP protein and the endoplasmic reticulum

690   of *Schizosaccharomyces pombe*: fate of the nuclear envelope during cell

691   division. *J. Cell. Sci.* **105 (Pt 4),** 1115–1120 (1993).

692

693

694

695

696 **End notes**

697 **Acknowledgments**

713 **Author contributions**

714 S.T-G., P.N.C.B.A. and R.C.A. conceived the project. S.T-G. and P.N.C.B.A.
715 performed preliminary studies. S.T-G. performed experiments and bioinformatics.
716 M.S. designed *cup1-3xDSR* experiments and contributed to ChIP-seq and qChIP
717 experiments. A.L.P. performed cytology, *cup1-TT* and eccDNA experiments. I.Y.
718 constructed Epe1 and Mst2 strains and performed western analysis. S.A.W.

719   generated Cup1 point mutants, Cup1-GFP strain and contributed to Epe1 and Mst2

720   experiments. S.T-G., A.L.P. and R.C.A. wrote the manuscript.

721   **Competing interests**

722   The authors declare no competing interests.

723   **Additional information**

724   Supplementary Information is available for this paper.

725   Correspondence and requests for materials should be addressed to Robin Allshire.

726   Reprints and permissions information is available at www.nature.com/reprints

727   **Data availability**

728   Sequence data generated in this study have been submitted to GEO under accession

729   number: GSE138436.

730   **Code availability**

731   The complete Workflow Description Language (WDL) pipeline script used for ChIP-

732   seq and variation analyses is available at:

733   https://github.com/SitoTorres/Torres-Garcia-et-al.-2019.

734

735

736

Extended Data Figure 1. Identification of heterochromatin-dependent epimutants resistant to caffeine

a, Frequency of unstable (UR) and stable (SR) caffeine-resistant isolates obtained from 3 independent screens. 64% of isolates did not display a clear phenotype (unclear).

b, Unstable (UR) and stable (SR) caffeine-resistant isolates were identified using this screening strategy. After growth on non-selective media for 14 days caffeine resistance is lost in UR isolates but not in SR isolates.

c, Caffeine resistance is lost progressively in unstable (UR) isolates but maintained in stable (SR) isolates.

d, Caffeine resistance in UR isolates depends on the Clr4 H3K9 methyltransferase. *clr4*+ (*clr4Δ*) or an unlinked intergenic region (*controlΔ*) were deleted in unstable (UR-2) and stable (SR-2) caffeine-resistant isolates.

e, A mutation in *pap1*+ confers caffeine resistance in the stable isolate SR-1. *Left:* Whole genome sequencing of the stable isolate SR-1 revealed a 7-nucleotide insertion in *pap1*+. The insertion results in a truncated Pap1 protein (Pap1-N424STOP) that lacks the Nuclear Export Signal (NES). CRD: Cysteine-rich domain. *Right:* Pap1-N424STOP is resistant to caffeine. The 7-nucleotide insertion identified in SR-1 was introduced into the *pap1*+ gene of wild-type cells (Pap1-N424STOP) and caffeine resistance assessed. *hba1Δ* and SR-1 cells were used as positive controls.

Experiments in (**b-d**) and (**e,** *right*) were independently repeated at least twice with similar results.

Extended Data Figure 2. Unstable (UR) caffeine-resistant isolates are *bona fide* epimutants.

a-e, Genetic changes (*clr5-Q264STOP meu27-S100Y*) found in 4 of 30 unstable isolates do not contribute to the caffeine-resistant phenotype nor cause the formation of ectopic heterochromatin.

a, Whole genome sequencing of unstable isolates UR-1/3/5/7 revealed a Single Nucleotide Polymorphism (SNP) in *clr5+* (*clr5-Q264STOP*) and in *meu27+* (*meu27-S100Y*).

b, *Left:* Schematic of experiment to determine whether *clr5-Q264STOP meu27-S100Y* cells form more caffeine-resistant colonies than wild-type cells. Wild-type (wt) and *clr5-Q264STOP meu27-S100Y* cells were plated on +CAF media (10^5 cells per plate, 20 plates per strain). Caffeine-resistant colonies were counted after 7 days. *Right: clr5-Q264STOP meu27-S100Y* form a similar number of caffeine-resistant colonies to wt cells. Data are mean from twenty technical replicates. *P* value from a two-tailed Student's *t*-test is indicated.

c, *clr5-Q264STOP meu27-S100Y* cells are not resistant to caffeine. *clr5-Q264STOP meu27-S100Y* cells were serially diluted and spotted on -CAF and +CAF plates to assess caffeine resistance. *hba1Δ* cells served as a positive control. Experiment was independently repeated at least twice with similar results.

d, Genome-wide H3K9me2 ChIP-seq enrichment in wt and *clr5-Q264STOP meu27-S100Y* cells. Data are represented as relative fold enrichment over input.

e, H3K9me2 ChIP-seq enrichment at known heterochromatin islands detected in *epe1Δ* cells[9] in wt and *clr5-Q264STOP meu27-S100Y* cells. Data are represented as relative fold enrichment over input.

f, Gene transcript levels within and flanking ectopic heterochromatin islands in individual isolates. See Figure 2b. Data are mean ± s.d. from three biological replicates. *P* values < 0.05 from a two-tailed Student's *t*-test are indicated.

Extended Data Figure 3. 24 of 30 unstable (UR) caffeine-resistant isolates

display an ectopic heterochromatin island over the *ncRNA.394* locus

a, H3K9me2 ChIP-seq enrichment at the *ncRNA.394* locus in individual isolates (*left:* coverage tracks; *right:* heatmaps). Data are represented as relative fold enrichment over input. Relevant genes within and flanking ectopic heterochromatin islands are indicated. Red arrows indicate essential genes. Dumbbells indicate primer pairs used in **b, c** and **e**.

b, Quantitative chromatin immunoprecipitation (qChIP) of H3K9me2 levels on *ncRNA.394* in individual isolates*.* Data are mean ± s.d. from three biological replicates. Primer pairs used are indicated in **a** (*ncRNA.394*, primer pair 5).

c, *SPBC17G9.13c*[+] gene transcript levels in individual isolates. Data are mean ± s.d. from three biological replicates. *P* values from a two-tailed Student's *t*-test are indicated. Primer pairs used are indicated in **a** (*SPBC17G9.13c*[+], primer pair 3).

d, Deletion of *ncRNA.394* or non-essential adjacent genes does not result in caffeine resistance. Experiment was independently repeated at least twice with similar results.

e, qChIP of H3K9me2 levels at the *ncRNA.394* locus in UR-2 cells. UR-2 cells were grown in the absence (-CAF) or presence (+CAF) of caffeine overnight or in the absence of caffeine for 14 days (+14day/-CAF). Data are mean ± s.d. from three biological replicates. Primer pairs used are indicated in **a**.

Extended Data Figure 4. Forced synthetic heterochromatin targeting to the

830 **identified loci is sufficient to drive caffeine resistance in wild-type cells**

831 **a-c,** Quantitative chromatin immunoprecipitation (qChIP) of H3K9me2 levels in wild-

832 type (wt) cells harbouring *4xtetO* binding sites at the identified ectopic

833 heterochromatin loci (or *ura4* as control) and expressing TetR-Clr4* in the absence or

834 presence of AHT. **a,** *hba1* locus. **b,** *ncRNA.394* locus. **c,** *ura4* locus. Data are mean ±

835 s.d. from three biological replicates. Dumbbells indicate primer pairs used. Red

836 arrows indicate essential genes.

837 **d**, Forced synthetic heterochromatin targeting to the *mbx2* locus is sufficient to drive

838 caffeine resistance in wt cells. qChIP of H3K9me2 levels in wt cells harbouring *4xtetO*

839 binding sites at the *mbx2* ectopic heterochromatin locus and expressing TetR-Clr4*

840 in the absence or presence of AHT. Data are mean ± s.d. from three biological

841 replicates. Dumbbells indicate primer pairs used.

842 **e,** Strains from **a-c** were assessed for resistance to the antifungal agents

843 tebuconazole (+TEZ) and fluconazole (+FLZ). Experiments were independently

844 repeated at least twice with similar results.

845

846

847

848

849

850

851

852

853

854

855

856

857

858

Extended Data Figure 5. Unstable (UR) caffeine-resistant isolates show cross-resistance to antifungals and siRNA generation at ectopic heterochromatin islands

a, Unstable caffeine-resistant isolates UR-1 and UR-2 were serially diluted and spotted on non-selective (N/S), caffeine (+CAF), clotrimazole (+CLZ), tebuconazole (+TEZ) and fluconazole (+FLZ) media to assess resistance. Experiment was independently repeated at least twice with similar results.

b-c, *Left:* small RNA sequencing detects siRNAs (21-24 nucleotides) homologous to ectopic heterochromatin islands in UR-1 (**b**, *hba1 locus*) and UR-2 (**c**, *ncRNA.394* locus) compared to wild-type (wt) cells. *Right:* siRNAs mapping to pericentromeric *dgl/dhl* repeats of chromosome I shown as control. Sequencing was performed once. *Transcripts mapping to the highly-expressed gene *eno101*$^+$ in euchromatic wild-type conditions (note these are unidirectional RNAs and not siRNAs).

d, Caffeine resistance depends on RNAi. *dcr1*$^+$ (*dcr1Δ*), *ago1*$^+$ (*ago1Δ*) or an unlinked intergenic region (*controlΔ*) were deleted in UR-2 cells. Experiment was independently repeated at least twice with similar results.

Extended Data Figure 6. Decreased *cup1⁺* transcript levels or Cup1 LYR-domain mutation results in caffeine resistance

a, An additional copy of *cup1⁺* with 3x Determinant of Selective Removal (DSR) motifs fused to its 3' untranslated region was inserted at an intergenic region (*LocusPX:cup1-3xDSR*). *Bottom left:* After deletion of endogenous *cup1⁺,* cells expressing only *cup1-3xDSR* were assessed for caffeine resistance. *Bottom right:* Transcript levels of *cup1⁺* and *SPBC17G9.12c⁺* (as control) in *cup1Δ locusPX:cup1-3xDSR* cells compared to wild-type. Data are mean ± s.d. from three biological replicates. *P* value from a two-tailed Student's *t*-test is indicated. Dumbbells indicate primer pairs used.

b, The 144-bp transcriptional terminator site from *ura4⁺* was inserted in place of part of the putative *cup1⁺* promoter (*cup1-TT*). *Bottom left:* Cells were assessed for caffeine resistance. *Bottom right:* Transcript levels of *cup1⁺* and *SPBC17G9.12c⁺* (as control) in *cup1-TT* cells compared to wild type. Data are mean ± s.d. from three biological replicates. *P* value from a two-tailed Student's *t*-test is indicated. Dumbbells indicate primer pairs used.

c, Cup1 localises to mitochondria. Cells expressing either untagged Cup1 (top row) or Cup1-GFP (bottom three rows) were fixed and processed for immunofluorescence with anti-GFP antibody and Alexa-488 secondary antibody and DNA was stained with DAPI. The mitochondrial protein Arg11-mCherry served as a positive control for mitochondrial localisation. All images in the green channel (Cup1-GFP) are scaled relative to each other, as are those in the red channel (Arg11-mCherry); DAPI images are autoscaled. Bar, 5 μm.

d, Point mutations (L73G and F99G) were introduced in the LYR domain of Cup1 and cells were assessed for caffeine resistance. Mutations were designed based on *Phyre2* tool analysis. *hba1Δ* cells were used as positive control.

Experiments in (**c**) and (**d**) were independently repeated at least twice with similar results.

921 **Extended Data Figure 7. Copy Number Variation (CNV) analysis reveals a partial**
922 **duplication of chromosome III in 12 of 30 unstable (UR) caffeine-resistant**
923 **isolates**

924 **a,** Chromosome III coverage plots with overlaid segments in UR isolates showing
925 partial duplication of chromosome III. Location of *cds1⁺* is highlighted. Wild-type
926 ChIP-seq input data were used as the reference.

927 **b-d,** Epigenetic changes preceded genetic changes (CNV) in unstable caffeine-
928 resistant isolate UR-2.

929 **b,** H3K9me2 ChIP-seq enrichment at the *ncRNA.394/cup1* locus (*left*) and
930 chromosome III coverage plots with overlaid segments (*right*) in UR-2 (4day/+CAF)
931 cells and following their prolonged growth on +CAF for an additional 3 days
932 (7day/+CAF). Wild-type ChIP-seq input data were used as the reference for CNV
933 analysis.

934 **c,** *clr4⁺* (*clr4Δ*) or an unlinked intergenic region (*controlΔ*) were deleted in UR-2 cells
935 (4day/+CAF) and UR-2 (7day/+CAF). All (6/6) UR-2 (4day/+CAF) *clr4Δ* transformants
936 lost resistance to caffeine whereas only 50% (3/6, transformants 1, 4 and 5) UR-2
937 (7day/+CAF) lost resistance to caffeine. Experiments were independently repeated at
938 least twice with similar results. *cds1⁺* DNA levels in extracted genomic DNA were
939 assessed by qPCR. Data are mean ± s.d. from three biological replicates.

940 **d,** H3K9me2 ChIP-seq enrichment at the *ncRNA.394/cup1* locus (*left*) and
941 chromosome III coverage plots with overlaid segments (*right*) in UR-2 (7day/+CAF)
942 cells and following their prolonged growth on non-selective media for 14 days
943 (7day/+CAF→14day/-CAF). Wild-type ChIP-seq input data were used as the
944 reference for CNV analysis.

945
946
947
948
949
950
951
952

Extended Data Figure 8. Copy Number Variation (CNV) of chromosome III corresponds to extrachromosomal circular DNA (eccDNA)

Junctions of putative extrachromosomal circles were identified at repetitive sequences by inspection of CNV plots for UR-2 (7day/+CAF) (**a**) and UR-4 (**b**). *Maps and lower panels:* Positions of *5S rRNA.24* and *5S rRNA.26* (pink arrows), *LTR3* and *LTR27* (green arrows) and flanking genes are indicated. PCR primers (half arrows) flanking *5S rRNA.24* (A (forward); B1,2 (reverse)) and *5S rRNA.26* (C1,2; D1,2) were used to amplify products from wild-type (wt) and UR-2 (7day/+CAF) ChIP input samples, along with primer combinations (C1,2; B1,2) specific for the putative circle junctions (vertical black lines). Primers flanking *LTR3* (E; F1,2) and *LTR27* (G1,2; H) were used to amplify products from wild-type and UR-4 ChIP input samples, along with primer combinations (G1,2; F1,2) specific for the putative circle junction. Shaded boxes indicate primer locations and predicted circle junctions (pink: *5S rRNA.24/26*, green: *LTR3/27*). *Right:* Restriction enzyme-digested genomic DNA isolated from wild-type (wt), UR-2 (7day/+CAF) and UR-4 was separated on an Ethidium Bromide (EtBr)-containing gel followed by Southern analysis using the indicated probes (925: blue; 520: purple; 44: red). Relevant restriction enzyme sites are indicated. Predicted sizes of hybridising fragments and DNA size markers are indicated (kb). PCR experiments were independently repeated at least twice with similar results. For gel source data, see Supplementary Figure 1b.

Extended Data Figure 9. The heterochromatin profile of low caffeine-treated wild-type cells resembles that of untreated *epe1Δ* cells

a, Growth of cells in caffeine. Wild-type (wt) cells were grown in the presence of low (7 mM) or medium (14 mM) caffeine for 18 hours. Cell number was counted every 6 hours. Note: a larger inoculum was used for 14 mM caffeine culture to obtain an equivalent final number of cells. Data are mean ± s.d. from three biological replicates. Cells from the 18-hr time point were used for **d**.

b-c, H3K9me2 ChIP-seq enrichment at previously-detected facultative heterochromatin loci (described in Zofall *et al.,* 2012[9] (**b** and **c**), Yamanaka *et al.*, 2013[13] (**b**), Wang *et al.,* 2015[10] (**b**), Sorida *et al.,* 2019[12] (**b**) and Gallagher *et al.,* 2019[14] (**b**)), in wt cells treated with low or medium dose of caffeine or low dose (1 mM) of H_2O_2, compared to untreated *epe1Δ* and wt cells. Data are represented as relative fold enrichment over input. A subset of facultative heterochromatin loci detected in untreated *epe1Δ* cells (Zofall *et al.,* 2012[9], Wang *et al.,* 2015[10] and Sorida *et al.,* 2019[12]) was detected in low caffeine-treated wt cells. Asterisks in **c** indicate loci with similar H3K9me2 patterns in low caffeine-treated wt cells and untreated *epe1Δ* cells, but not untreated wt cells. Facultative heterochromatin loci formed in the absence of the exosome (Yamanaka *et al.,* 2013[13]) or in wt cells grown at 18°C (Gallagher *et al.,* 2019[14]) were not detected in wt cells treated with low or medium caffeine or low H_2O_2.

d, Quantitative ChIP (qChIP) of H3K9me2 levels on *ncRNA.394/cup1* in wt cells following 18 hr exposure to low or medium caffeine. H3K9me2 levels were normalized to *S. octosporus* spike-in control. Data are mean ± s.d. from three biological replicates.

e, H3K9me2 ChIP-seq enrichment at *ncRNA.394/cup1* and *mcp7* loci (or at pericentromeric *dgl/dhl* repeats of chromosome I as control) in wt cells following 18 hr exposure to low H_2O_2. Data are represented as relative fold enrichment over input. Red arrows indicate essential genes. Lower levels of H3K9me2 at pericentromeric repeats upon H_2O_2 treatment may be due to H_2O_2-specific regulation of limiting heterochromatin factors at this locus.

f, *epe1+* RNA levels do not change upon caffeine treatment. Total RNA-seq of wt cells treated with low caffeine. Components of the Clr4 H3K9 methyltransferase CLRC complex (*clr4+, rik1+, raf1+, raf2+, pcu4+ and rbx1+*) and the antisilencing factors *epe1+*

1017 *and mst2⁺* are highlighted. Experiment was independently repeated twice with similar

1018 results.

1019 **g,** *epe1Δ* cells display increased resistance to caffeine. *Left:* Schematic of

1020 experiment. Wild-type, *epe1Δ* and *clr4Δ* cells were plated on +CAF media (10^5

1021 cells/plate, 40 plates/strain). Caffeine-resistant colonies were counted after 7 days.

1022 *Right:* Compared to wt cells, *epe1Δ* forms more, whereas *clr4Δ* forms fewer, caffeine-

1023 resistant colonies. Note that the total number of resistant colonies also includes

1024 genetic mutants. Data are mean from forty technical replicates. *P* values from a two-

1025 tailed Student's *t*-test are indicated.

1026

1027

1028

1029

1030

1031

1032

1033

1034

1035

Extended Data Figure 10. A shortened version of the anti-silencing factor Mst2 is produced upon exposure to caffeine

a, Western analysis of Mst2-13xMyc (*left*) and Gcn5-13xMyc (as HAT control, *right*) before and after caffeine treatment (medium concentration, 14 mM). Tagged proteins are expressed from their endogenous loci. Loading controls: *left:* Bip1; *right:* Cdc11. Experiments were independently repeated at least twice with similar results. For gel source data, see Supplementary Figure 1c.

b, Total RNA-seq for *mst2 (left)* and *gcn5* (as HAT control, *right*) of untreated wild-type cells (*top*) or wild-type cells treated with medium caffeine concentration (*bottom*). Diagrams illustrate *mst2* and *gcn5* transcripts and predicted protein domains. Reads are normalized to RPKM. Red dashed lines indicate the region of full length *mst2* transcript absent from the short isoform. The MYST zinc finger (ZnF) domain, required for *S. cerevisiae* Esa1 acetyltransferase activity[29], is truncated in the short isoform of Mst2. The alternative *mst2* TSS utilised in caffeine conditions was previously annotated[28]. Experiment was independently repeated twice with similar results.

Figure 1   **Torres-Garcia** *et al.*

Figure 2

Torres-Garcia *et al.*

a ChIP-seq: H3K9me2

b ChIP-seq: H3K9me2

Main Fig2

Figure 3   **Torres-Garcia** *et al.*

Figure 4   **Torres-Garcia *et al.***
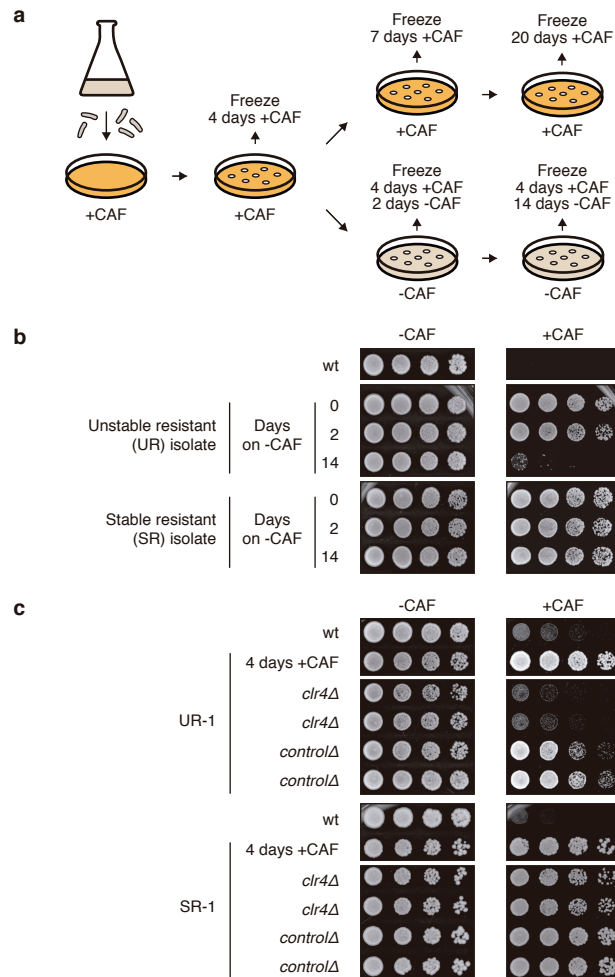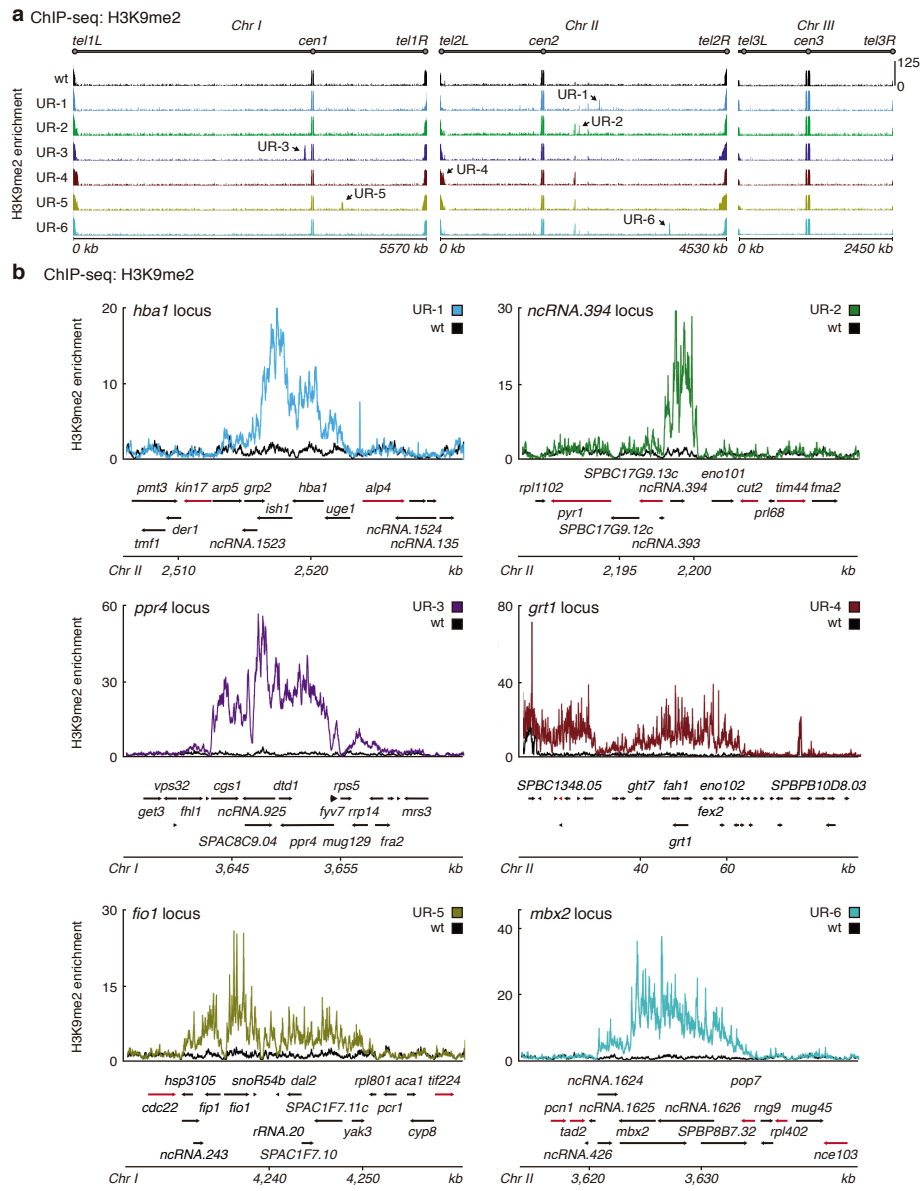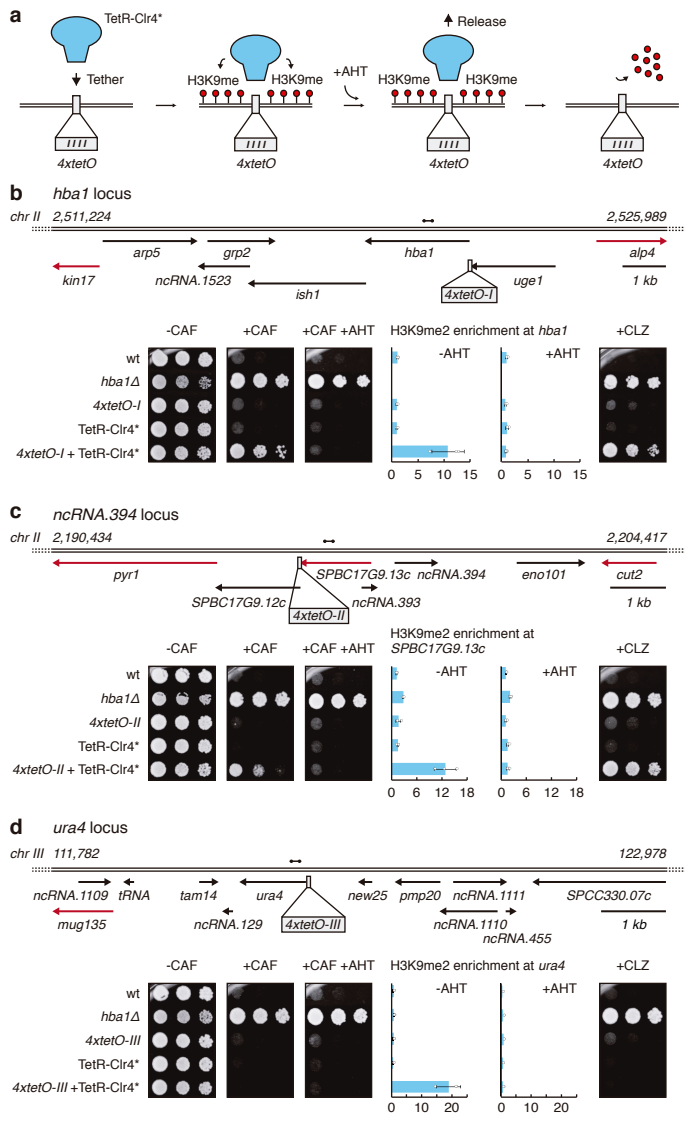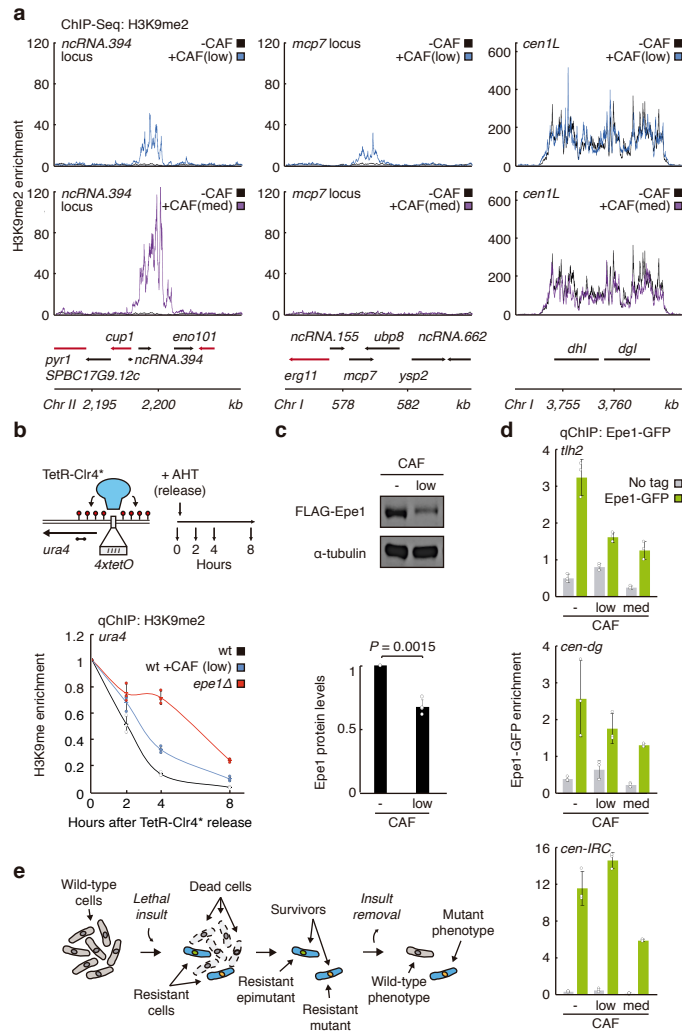
Main Fig4

a Total analyzed resistant isolates (n=176)
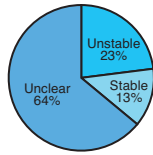
Screen	Plated cells	Resistant isolates	Analyzed isolates	Unstable (UR)	Stable (SR)	Unclear
1	1.2×10^5	87	48	19%	8%	73%
2	6.4×10^5	367	47	21%	29%	49%
3	8.5×10^5	371	81	26%	6%	68%

b

c

d

e

Pap1

CRD
NES

N— bZIP —C
1 552

wt
TTT CAT AAT TAT TTC CAT AAT TCT AAC GAA AAT GGA GAC TTG
414 F H N Y F H N S N E N G D L 427

SR-1
TTT CAT AAT TAT TTC CAT AAT TCT AAC Gtc taa cgA AAA TGG
F H N Y F H N S N V *

insertion

wt
hba1Δ
SR-1
Pap1-N424STOP

Extended Data Fig1

a

Clr5
1 ⎯⎯⎯⎯⎯⎯⎯⎯ 689

wt
CAT GCC GTT AAC GGT CAG CCA CAA GCT
259 H A V N G Q P Q A 267

clr5-Q264STOP
CAT GCC GTT AAC GGT TAG CCA CAA GCT
H A V N G *

Meu27
1 ⎯⎯⎯⎯⎯⎯⎯⎯ 736

wt
CAG GAA AAT AAG TCT TCA AAC CAA AAT
96 Q E N K S S N Q N 104

meu27-S100Y
CAG GAA AAT AAG TAT TCA AAC CAA AAT
Q E N K Y S N Q N

b

wt / clr5-Q264STOP meu27-S100Y

+CAF / +CAF

+CAF / +CAF

Count colonies

Resistant colonies per 10⁵ plated cells

n.s. (*P* = 0.11)

wt   clr5-Q264STOP meu27-S100Y

c

-CAF   +CAF

wt
hba1Δ
clr5-Q264STOP
meu27-S100Y

d

ChIP-seq: H3K9me2

wt   clr5-Q264STOP meu27-S100Y   H3K9me enrichment

Chr I cen1
Chr II cen2
Chr III cen3

-1.5 0 +1.5 -1.5 0 +1.5 *kb*
Centre of gene

e

ChIP-seq: H3K9me2

wt   clr5-Q264STOP meu27-S100Y   H3K9me enrichment

isl 1
isl 2
isl 3
isl 4
isl 5
isl 6
isl 7
isl 8
isl 9
isl 10
isl 11
isl 12
isl 13
ncRNA.394 locus - isl 14
isl 15
isl 16
isl 17
isl 18
isl 19
isl 20
isl 21

-3 0 +3 -3 0 +3 *kb*
Centre of peak

f RT-qPCR

hba1 locus UR-1 wt
P = 2.1x10⁻³
slu7 ish1 hba1 alp4

ncRNA.394 locus UR-2 wt
P = 1.8x10⁻² *P* = 1.1x10⁻⁴ *P* = 1.5x10⁻² *P* = 1.5x10⁻⁴ *P* = 1.1x10⁻⁴
pyr1 SPBC17G9.13c eno101 cut2
SPBC17G9.12c ncRNA.394

ppr4 locus UR-3 wt
P = 3.2x10⁻⁴ *P* = 1.8x10⁻³
vps32 cgs1 ppr4

grt1 locus UR-4 wt
P = 1.8x10⁻²
grt1 eno102 aes1

fio1 locus UR-5 wt
P = 1.4x10⁻³
cdc22 fip1 fio1 cyp8

mbx2 locus UR-6 wt
P = 3.8x10⁻⁶
pcn1 mbx2 nce103

Ratio to *act1⁺*

Extended Data Fig2

a ChIP-seq: H3K9me2

b qChIP: H3K9me2
ncRNA.394

c RT-qPCR
SPBC17G9.13c

d

e qChIP: H3K9me2

Extended Data Fig3

a *hba1 locus*
chr II 2,511,224 — 2,525,989

qChIP: H3K9me2

- AHT

+AHT

- wt
- TetR-Clr4*
- 4xtetO-I
- 4xtetO-I + TetR-Clr4*

b *ncRNA.394 locus*
chr II 2,190,434 — 2,204,417

qChIP: H3K9me2

-AHT

+AHT

- wt
- TetR-Clr4*
- 4xtetO-II
- 4xtetO-II + TetR-Clr4*

c *ura4 locus*
chr III 111,782 — 122,978

qChIP: H3K9me2

-AHT

+AHT

- wt
- TetR-Clr4*
- 4xtetO-III
- 4xtetO-III + TetR-Clr4*

d *mbx2 locus*
chr II 3,620,488 — 3,628,813

qChIP: H3K9me2

- AHT   + AHT

H3K9me2 enrichment at *mbx2*

e *hba1 locus*   *ncRNA.394 locus*   *ura4 locus*

Extended Data Fig4

a

	N/S	+CAF	+CLZ	+TEZ	+FLZ
wt					
hba1Δ					
UR-1					
UR-2					

b Small RNA-seq

hba1 locus

wt — Reads per million (rpm) — 21 nt, 22 nt, 24 nt

UR-1 — Reads per million (rpm) — 21 nt, 22 nt, 24 nt

kin17 arp5 grp2 ish1 hba1 uge1 alp4

ncRNA.1523

Chr II   2,510 — 2,526   kb

cen1L

wt — 21 nt, 22 nt, 24 nt

UR-1 — 21 nt, 22 nt, 24 nt

dhl   dgl

Chr I   3,753 — 3,766   kb

c Small RNA-seq

ncRNA.394 locus

wt — Reads per million (rpm) — 21 nt, 22 nt, 24 nt

UR-2 — Reads per million (rpm) — 21 nt, 22 nt, 24 nt

rpl1102 pyr1 SPBC17G9.13c ncRNA.394 eno101 cut2

SPBC17G9.12c   ncRNA.393

Chr II   2,190 — 2,204   kb

cen1L

wt — 21 nt, 22 nt, 24 nt

UR-2 — 21 nt, 22 nt, 24 nt

dhl   dgl

Chr I   3,753 — 3,766   kb

d

	-CAF	+CAF
wt		
4 days +CAF		
dcr1Δ-1		
dcr1Δ-2		
ago1Δ-1		
ago1Δ-2		
controlΔ-1		
controlΔ-2		

UR-2

Extended Data Fig5

a

ncRNA.394 locus

chr II 2,194,561 — 2,200,000

SPBC17G9.12c   ncRNA.393

cup1   ncRNA.394   0.5 kb

PX locus

chr II 2,700,000 — 2,705,000

mak5   mrpl39   cup1-3xDSR   str1

0.5 Kb

cup1 strain		-CAF	+CAF	RT-qPCR Ratio to *act1*+	

wt cup1+

3xDSR
cup1Δ locusPX:cup1-3xDSR

P = 6.5x10⁻³

b

ncRNA.394 locus

chr II 2,194,561 — 2,200,000

SPBC17G9.12c   ncRNA.393

cup1   ncRNA.394   0.5 kb

TT-ura4

cup1 strain		-CAF	+CAF	RT-qPCR Ratio to *act1*+	

wt cup1+

TT-ura4
cup1-TT   TT

P = 1.1x10⁻²

c

Arg11-mCherry

Arg11-mCherry
Cup1-GFP

GFP   mCherry   DAPI   merge

d

Cup1

LYR domain

0 — 317

L73G   F99G

48 — 108

CTA → GGA

TTT → GGT

-CAF   +CAF

wt
hba1Δ
Cup1-L73G
Cup1-F99G

Extended Data Fig6

a Whole Genome Sequencing - CNV analysis

b ChIP-seq: H3K9me2

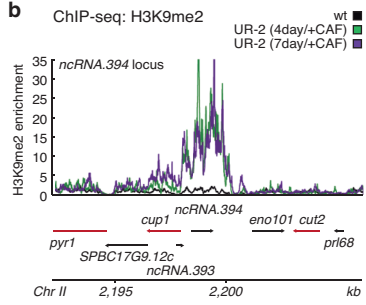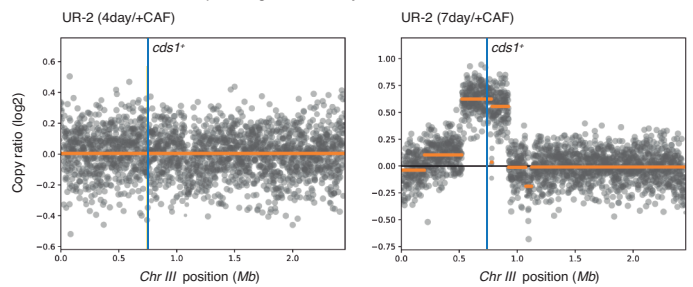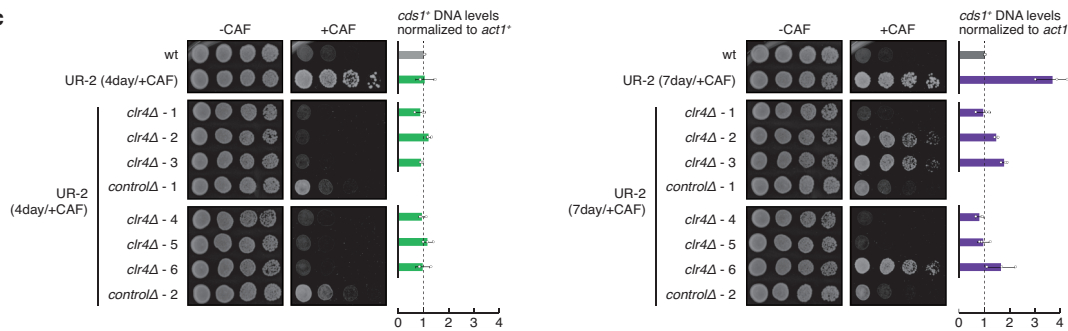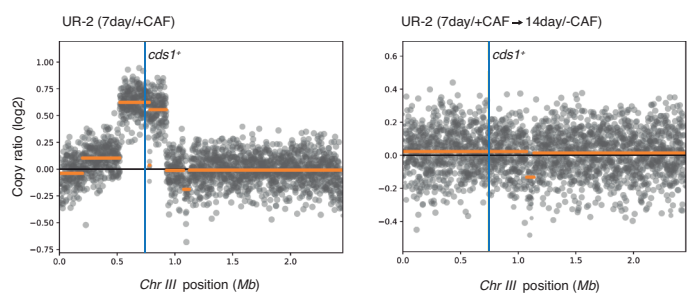Whole Genome Sequencing - CNV analysis

c

d ChIP-seq: H3K9me2
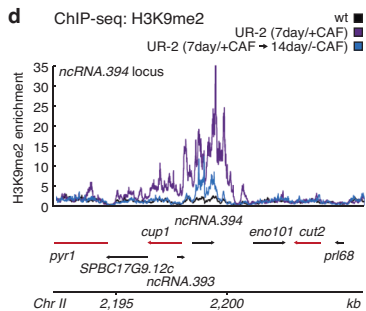
Whole Genome Sequencing - CNV analysis

Extended Data Fig7

a UR-2 (7day/+CAF)

b UR-4

Extended Data Fig8

a Growth curve: wt cells

b ChIP-seq: H3K9me2

c ChIP-seq: H3K9me2

d qChIP: H3K9me2 ncRNA.394

e ChIP-seq: H3K9me2

f Total RNA-seq: +CAF (low) vs -CAF

g wt epe1Δ clr4Δ

Extended Data Fig9

a

	CAF	
	−	+

Mst2-Myc — Mst2 (full length) / Mst2 (short isoform)

α-Bip1

	CAF	
	−	+

Gcn5-Myc

α-Cdc11

b

Total RNA-seq: *mst2*

−CAF

RPKM
1200
0

mst2 transcript (full length)

e1   e2   e3

main TSS

Mst2 protein (full length)

103   154   372

MYST ZnF domain   MYST HAT domain

+CAF

RPKM
5300
0

mst2 transcript (short isoform)

e1   e2   e3

annotated alternative TSS

Mst2 protein (short isoform)

141   154   372

Truncated MYST ZnF domain   MYST HAT domain

Total RNA-seq: *gcn5*

−CAF

RPKM
2200
0

gcn5 transcript (full length)

TSS

Gcn5 protein (full length)

118   278   354   434

GNAT domain   Bromodomain

+CAF

RPKM
1600
0

gcn5 transcript (full length)

TSS

Gcn5 protein (full length)

118   278   354   434

GNAT domain   Bromodomain

Extended Data Fig10

Supplementary Figure 1. a, Source gel data for Fig. 4c. **b,** Source gel data for Extended Data Fig. 8. **c,** Source gel data for Extended Data Fig. 10a. Yellow and dashed boxes indicate data used in figures.

Supplementary Table 1. Summary of epigenetic (H3K9me2 domains) and genetic (SNPs, indels and copy number variation) changes found in unstable (UR) caffeine-resistant isolates.

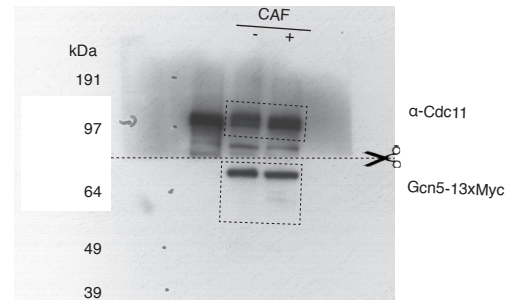| Isolate	Ectopic heterochromatin location		SNPs or indels in coding sequences?	Partial duplication of *Chr III*?
	ncRNA.394	other loci		
UR-1		✓ (*hba1*)	Clr5-Q264STOP / Meu27-S100Y	
UR-2	✓		Sdo1-R11C	
UR-3		✓ (*ppr4*)	Clr5-Q264STOP / Meu27-S100Y	
UR-4		✓ (*grt1*)	-	✓
UR-5		✓ (*fio1*)	Clr5-Q264STOP / Meu27-S100Y	✓
UR-6		✓ (*mbx2*)	-	✓
UR-7		✓ (*ppr4*)	Clr5-Q264STOP / Meu27-S100Y	
UR-8	✓		-	
UR-9	✓		-	
UR-10	✓		Cob1-F318L	
UR-11	✓		-	
UR-12	✓		-	
UR-13	✓		-	✓
UR-14	✓		Npp-W300STOP / SPBC16H5.13-S1011L	✓
UR-15	✓		-	✓
UR-16	✓		-	
UR-17	✓		SPCC777.02-R120R	✓
UR-18	✓		SPCC777.02-R120R	✓
UR-19	✓		Sdo1-R11C	✓
UR-20	✓		-	
UR-21	✓		-	✓
UR-22	✓		-	✓
UR-23	✓		Pch1-Q234STOP	
UR-24	✓		-	
UR-25	✓		-	✓
UR-26	✓		SPBC1271.08c-A133A	
UR-27	✓		SPCC4B3.13-A229V	
UR-28	✓		Mug72-N116S	
UR-29	✓		Mug72-N116S	
UR-30	✓		-	

Supplementary Table 2. *Schizosaccharomyces pombe* strains used in this study.

Strain number	Name	Description
143	wt	*h*- ED972 wild-type
B4411	SR-1	Stable 16 mM Caffeine Resistant Isolate – From wt – 1
B4412	SR-2	Stable 16 mM Caffeine Resistant Isolate – From wt – 2
B4413	UR-1	Unstable 16 mM Caffeine Resistant Isolate – From wt – 1
B4414	UR-2	Unstable 16 mM Caffeine Resistant Isolate – From wt – 2
B4415	UR-3	Unstable 16 mM Caffeine Resistant Isolate – From wt – 3
B4416	UR-4	Unstable 16 mM Caffeine Resistant Isolate – From wt – 4
B4417	UR-5	Unstable 16 mM Caffeine Resistant Isolate – From wt – 5
B4418	UR-6	Unstable 16 mM Caffeine Resistant Isolate – From wt – 6
B4419	UR-7	Unstable 16 mM Caffeine Resistant Isolate – From wt – 7
B4420	UR-8	Unstable 16 mM Caffeine Resistant Isolate – From wt – 8
B4421	UR-9	Unstable 16 mM Caffeine Resistant Isolate – From wt – 9
B4422	UR-10	Unstable 16 mM Caffeine Resistant Isolate – From wt – 10
B4423	UR-11	Unstable 16 mM Caffeine Resistant Isolate – From wt – 11
B4424	UR-12	Unstable 16 mM Caffeine Resistant Isolate – From wt – 12
B4425	UR-13	Unstable 16 mM Caffeine Resistant Isolate – From wt – 13
B4426	UR-14	Unstable 16 mM Caffeine Resistant Isolate – From wt – 14
B4427	UR-15	Unstable 16 mM Caffeine Resistant Isolate – From wt – 15
B4428	UR-16	Unstable 16 mM Caffeine Resistant Isolate – From wt – 16
B4429	UR-17	Unstable 16 mM Caffeine Resistant Isolate – From wt – 17
B4430	UR-18	Unstable 16 mM Caffeine Resistant Isolate – From wt – 18
B4431	UR-19	Unstable 16 mM Caffeine Resistant Isolate – From wt – 19
B4432	UR-20	Unstable 16 mM Caffeine Resistant Isolate – From wt – 20
B4433	UR-21	Unstable 16 mM Caffeine Resistant Isolate – From wt – 21
B4434	UR-22	Unstable 16 mM Caffeine Resistant Isolate – From wt – 22
B4435	UR-23	Unstable 16 mM Caffeine Resistant Isolate – From wt – 23
B4436	UR-24	Unstable 16 mM Caffeine Resistant Isolate – From wt – 24
B4437	UR-25	Unstable 16 mM Caffeine Resistant Isolate – From wt – 25
B4438	UR-26	Unstable 16 mM Caffeine Resistant Isolate – From wt – 26
B4439	UR-27	Unstable 16 mM Caffeine Resistant Isolate – From wt – 27
B4440	UR-28	Unstable 16 mM Caffeine Resistant Isolate – From wt – 28
B4441	UR-29	Unstable 16 mM Caffeine Resistant Isolate – From wt – 29
B4442	UR-30	Unstable 16 mM Caffeine Resistant Isolate – From wt – 30
B4443	SR-1 *clr4Δ* - 1	SR-1 *clr4Δ::NAT* - transformant 1
B4444	SR-1 *clr4Δ* - 2	SR-1 *clr4Δ::NAT* - transformant 2
B4445	SR-1 NAT control - 1	SR-1 *NAT:3' of ura4* - transformant 1
B4446	SR-1 NAT control - 2	SR-1 *NAT:3' of ura4* - transformant 2
B4447	SR-2 *clr4Δ* - 1	SR-2 *clr4Δ::NAT* - transformant 1
B4448	SR-2 *clr4Δ* - 2	SR-2 *clr4Δ::NAT* - transformant 2
B4449	SR-2 NAT control - 1	SR-2 *NAT:3' of ura4* - transformant 1
B4450	SR-2 NAT control - 2	SR-2 *NAT:3' of ura4* - transformant 2
B4451	UR-1 *clr4Δ* - 1	UR-1 *clr4Δ::NAT* - transformant 1
B4452	UR-1 *clr4Δ* - 2	UR-1 *clr4Δ::NAT* - transformant 2
B4453	UR-1 NAT control-1	UR-1 *NAT:3' of ura4* - transformant 1
B4454	UR-1 NAT control-2	UR-1 *NAT:3' of ura4* - transformant 2
B4455	UR-2 *clr4Δ* - 1	UR-2 *clr4Δ::NAT* - transformant 1
B4456	UR-2 *clr4Δ* - 2	UR-2 *clr4Δ::NAT* - transformant 2
B4457	UR-2 NAT control - 1	UR-2 *NAT:3' of ura4* - transformant 1
B4458	UR-2 NAT control - 2	UR-2 *NAT:3' of ura4* - transformant 2
B5022	UR-2 *dcr1Δ* - 1	UR-2 *dcr1Δ::NAT* - transformant 1
B5023	UR-2 *dcr1Δ* - 2	UR-2 *dcr1Δ::NAT* - transformant 2
B5024	UR-2 *ago1Δ* - 1	UR-2 *ago1Δ::NAT* - transformant 1
B5025	UR-2 *ago1Δ* - 2	UR-2 *ago1Δ::NAT* - transformant 2
B4352	Pap1-N424STOP	*h- pap1-N424STOP*
B4752	Clr5-Q264STOP Meu27-S100Y	*h- clr5-Q264STOP meu27-S100Y*
B4459	UR-2 (+14day/-CAF)	UR-2 after growth on -CAF media for 14 days

B4460	*hba1Δ*	*h- hba1Δ::NAT*
B4461	*SPBC17G9.12cΔ*	*h- SPBC17G9.12cΔ::NAT*
B4462	*ncRNA.393Δ*	*h- ncRNA.393Δ::NAT*
B4463	*ncRNA.394Δ*	*h- ncRNA.394Δ::NAT*
B4464	*eno101Δ*	*h- eno101Δ::NAT*
B3797	*TetR-Clr4**	*h+ leu1+adh21-TetROFF-2xFLAG-Clr4-cdd*
B3808	*4xtetO-II*	*h- 4xtetO 3' of SPBC17G9.13c leu1-32*
B3813	*4xtetO-I*	*h- 4xtetO 5' of hba1 leu1-32*
B3820	*4xtetO-III*	*h- 4xtetO 5' of ura4 leu1-32*
B4707	*4xtetO-IV*	*h- 4xtetO 5' of mbx2*
B4465	*TetR-Clr4* + 4xtetO-II*	*h+ leu1+adh21-TetROFF-2xFLAG-Clr4-cdd 4xtetO 3' of SPBC17G9.13c*
B4466	*TetR-Clr4* + 4xtetO-I*	*h+ leu1+adh21-TetROFF-2xFLAG-Clr4-cdd 4xtetO 5' of hba1*
B4467	*TetR-Clr4* + 4xtetO-III*	*h+ leu1+adh21-TetROFF-2xFLAG-Clr4-cdd 4xtetO 5' of ura4*
B4807	*TetR-Clr4* + 4xtetO-IV*	*h+ leu1+adh21-TetROFF-2xFLAG-Clr4-cdd 4xtetO 5' of mbx2*
B4885	*cup1-3xDSR*	*h- cup1Δ LocusPX:cup1-3xDSR (cup1=SPBC17G9.13c)*
B5005	*cup1-TT*	*h- cup1-TT (cup1=SPBC17G9.13c)*
B4688	Cup1-L73G	*h- cup1-L73G (cup1=SPBC17G9.13c)*
B4690	Cup1-F99G	*h- cup1-F99G (cup1=SPBC17G9.13c)*
B4567	Cup1-GFP	*h- cup1-GFP (cup1=SPBC17G9.13c)*
B4909	Cup1-GFP Arg11-mCh	*h- cup1-GFP arg11-mCherry-NAT (cup1=SPBC17G9.13c)*
B4912	Arg11-mCherry	*h- arg11-mCherry-NAT*
B4468	UR-2 (7day/+CAF)	UR-2 after growth on +CAF media for 3 days
B4469	UR-2 (7day/+CAF →14day/-CAF)	UR-2 after growth on +CAF media for 3 days and then on -CAF media for 14 days
B4621	*epe1Δ*	*h- epe1Δ*
B2835	Epe1-GFP	*h- epe1-GFP-KAN*
B4958	3xFLAG-Epe1	*h- 3xFLAG-epe1*
B4767	*TetR-Clr4* + 4xtetO-III epe1Δ*	*h+ epe1Δ::NAT leu1+adh21-TetROFF-2xFLAG-Clr4-cdd 4xtetO 5' of ura4*
B1008	*clr4Δ*	*h- clr4Δ::NAT*
B3250	*S. cerevisiae* Sgo1-GFP	*S. cerevisiae SGO1-yeGFP::KanMX6 MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11, 15, can1-100, GAL, psi+*
B3111	*S. octosporus* wt	*h90 S. octosporus wild-type*
B4108	Mst2-13xMyc	*h- mst2-13xMyc-NAT*
B0505	Gcn5-13xMyc	*h? gcn5-13xMyc-NAT cc2Δ6Kb:cc1 ade6-704-HYG*

Supplementary Table 3. Oligonucleotides used in this study.

Name	Sequence	Description
qAct1-F	GGTTTCGCTGGAGATGATG	qPCR *act1+* - F
qAct1-R	ATACCACGCTTGCTTTGAG	qPCR *act1+* - R
qDg-F	AATTGTGGTGGTGTGGTAATAC	qPCR *dg* repeats - F
qDg-R	GGGTTCATCGTTTCCATTCAG	qPCR *dg* repeats - R
ST-52	GAATTGTGGAGCCATGTCCC	qPCR *slu7+* - F
ST-53	TCTTCTCCTGTCCAACGAGC	qPCR *slu7+* - R
ST-872	GAAACCCAGAAATTCGCAGGT	qPCR *kin17+* - F - primer pair 1 *hba1* locus
ST-873	ATGAGTTGCTTGGGCATCCA	qPCR *kin17+* - R - primer pair 1 *hba1* locus
ST-62	CAGCAAATTGGGGACTGTGT	qPCR *ish1+* - F - primer pair 2 *hba1* locus
ST-63	CTCAAGAAGCCTGGGAGTCA	qPCR *ish1+* - R - primer pair 2 *hba1* locus
ST-64	CGATGATCTGGTTGTATGGTGG	qPCR *hba1+* - F - primer pair 3 *hba1* locus
ST-65	TGCTCAGTACGCCATCTTGA	qPCR *hba1+* - R - primer pair 3 *hba1* locus
ST-66	GGGCTATCCTTAACGCTCTTC	qPCR *hba1+cds* - F - primer pair 4 *hba1* locus
ST-67	CGCCTCCTCTGAACCAAAAG	qPCR *hba1+cds* - R - primer pair 4 *hba1* locus
ST-58	CTTCCCACATCGCGTTCATT	qPCR *alp4+* - F - primer pair 5 *hba1* locus
ST-59	ACCTAAATCATCGCTGCTGG	qPCR *alp4+* - R - primer pair 5 *hba1* locus
ST-393	GGGCATGACAATCTCCGACT	qPCR *pyr1+* - F - primer pair 1 *ncRNA.394* locus
ST-394	GGCCTACCTCGGTGATCTTG	qPCR *pyr1+* - R - primer pair 1 *ncRNA.394* locus
ST-401	CCGTATGGTGAAGCAGGGTT	qPCR *SPBC17G9.12c+* - F - primer pair 2 *ncRNA.394* locus
ST-402	CCCGATCTCCGTGTAAGCAA	qPCR *SPBC17G9.12c+* - R - primer pair 2 *ncRNA.394* locus
ST-184	TTCGTCGTATGCCCTCTTGC	qPCR *SPBC17G9.13c+* - F - primer pair 3 *ncRNA.394* locus
ST-185	AAAATCCGCCATTTGCCCAG	qPCR *SPBC17G9.13c+* - R - primer pair 3 *ncRNA.394* locus
ST-251	TGCTGTAGTGATGCAGAGGAG	qPCR *ncRNA.393+* - F - primer pair 4 *ncRNA.394* locus
ST-252	GCGGCCATTTTGTTTACATTCC	qPCR *ncRNA.393+* - R - primer pair 4 *ncRNA.394* locus
ST-190	GAAAATTAGCGCGGCCGTTA	qPCR *ncRNA.394+* - F - primer pair 5 *ncRNA.394* locus
ST-191	TCAATCTGCTTGTCCCACCC	qPCR *ncRNA.394+* - R - primer pair 5 *ncRNA.394* locus
ST-263	GTGCTGCCCAAAAGAAGCTC	qPCR *eno101+* - F - primer pair 6 *ncRNA.394* locus
ST-264	TGGGAACCACCGTTCAAGAC	qPCR *eno101+* - R - primer pair 6 *ncRNA.394* locus
ST-249	AGCTTTCAAGGTAGCGGGTG	qPCR *cut2+* - F
ST-250	TTCCTCTGCTCAGCGTAGAC	qPCR *cut2+* - R
PA-354	CAGTTAGTTTCAGGTTTCCC	qPCR +2.5 kb *ura4+* - F - primer pair 1 *ura4* locus
PA-355	GCAGAGTAATGGTGATTGG	qPCR +2.5 kb *ura4+* - R - primer pair 1 *ura4* locus
ST-874	CACACAGTTTCAGAAGAAC	qPCR *tam14+* - F - primer pair 2 *ura4* locus
ST-875	GTTACGAGGAATCTTGGTAG	qPCR *tam14+* - R - primer pair 2 *ura4* locus
ST-796	CGCGACTGACAAGTTGCTTT	qPCR *ura4+* - F - primer pair 3 *ura4* locus
ST-797	AGCTAGAGCTGAGGGGATGA	qPCR *ura4+* - R - primer pair 3 *ura4* locus
ST-800	TGGTTTAAATCAAATCTTCCATGCG	qPCR 5' of *ura4+* - F - primer pair 4 *ura4* locus
ST-801	TGAGCAAACTGCTTTTGTGGT	qPCR 5' of *ura4+* - R - primer pair 4 *ura4* locus
ST-788	GGATGAAGCTGTCTCCCTGG	qPCR *new25+* - F - primer pair 5 *ura4* locus
ST-789	TATTGCTGCTTCTTCCCTGGC	qPCR *new25+* - R - primer pair 5 *ura4* locus
ST-876	GGAATCTATGTCGTTGCCG	qPCR *pmp20+* - F - primer pair 6 *ura4* locus
ST-877	GTAAACTCTCCGTTCCAGTC	qPCR *pmp20+* - R - primer pair 6 *ura4* locus
Clr4-KO-F	ATTTTTTAAATTCGTTCAGGCATCATTTGGAGGGTTTGCTAAAAATCATCTCACCAAACAAGAGGTTATTAGTTTTGCGACGGATCCCCGGGTTAATTAA	KO of *clr4+* with Bahler construct - F
Clr4-KO-R	AAATGAATGACCTTTTTCAGTTTAACAGTAATGGAGAAAAACAAATTGTAATTATTGGAGTCAACCAGTAATAAATTAGCGAATTCGAGCTCGTTTAAAC	KO of *clr4+* with Bahler construct - R
ST-155	ATAGCTTAGGATTCATTATTTTTTAAGAGACAAATTTCTCGTCAATTGAATGAAACCTTCCGCCTTTATTTTCTTTTTGACGGATCCCCGGGTTAATTAA	KO of *dcr1+* with Bahler construct - F
ST-156	GACTTGAAATATACAGTATTTCATTTTTTATGACCGCGGCCCTTGTAACTTTTCAAACTTTCAATTTGGGTCTCCAAAGCGAATTCGAGCTCGTTTAAAC	KO of *dcr1+* with Bahler construct - R

ST-157	GGTTTGGTATATATAAGCTTCCAACCGCCAAAGCGAATTGTCTTCAGCCAACTCGTCCTTTATGATTCAGAGTGAGTAGGCGGATCCCCGGGTTAATTAA	KO of *ago1*[+] with Bahler construct - F
ST-158	TAAGGAAGTAAAAGTTGTGGGCAATCCAGTAGTCAATCGTATATCTATTTCATTACTTATTGCATGCAATCCATCAAACAGAATTCGAGCTCGTTTAAAC	KO of *ago1*[+] with Bahler construct - R
ST-3	GTCCAACACCCAGTTGTTAACTGCTTATAATGACGCGTATGATTGCGATATTTTAAGACTCTGGCCATCCACCGCTTTATCCGACGGATCCCCGGGTTAATTAA	Inserting natMX6 marker 3' of *ura4*[+] (*controlΔ*) - F
ST-12	GCAGGTTCTAGTAATGCGCATTCAATTTGTAGTATTCTTAAATAATCATTAAACGACAAGGGCCTTCCGTGCTATAGTGTGAATTCGAGCTCGTTTAAAC	Inserting natMX6 marker 3' of *ura4*[+] (*controlΔ*) - R
ST-866	CtagaGGTCTCgGACTCTCCATTTTCGTTAGAATTAGTTTcGAGACCcttCC	Golden Gate cloning pap1-sgRNA-1-F
ST-867	GGaagGGTCTCgAAACTAATTCTAACGAAAATGGAGAGTCcGAGACCtctaG	Golden Gate cloning pap1-sgRNA-1-R
ST-868	AGCATGGCGCGAACCCGCTGAATCATTGGACAAAGAATTCTTTAACGACGAGGGTGAAATAGATGATGTTTTTCATAATTATTTTCATAATTCTAACGTC	Pap1-N424STOP - HR template - F
ST-869	GCTCAGGGAATGATTCGTTGGCATTCTCCAGAAAATCAAGACCATGCAATGAATTAGTGATCAAGTCTCCATTTTCGTTAGACGTTAGAATTATGAAAAT	Pap1-N424STOP - HR template - R
ST-284	CAGCTGTGTGTTTGATTGAATCCACATTCGTCCTCATGTACTCATAGCTAGGTGAAATATATTAGGCTTTCAGTGATTCGCGGATCCCCGGGTTAATTAA	KO of *hba1*[+] with Bahler construct - F
ST-285	GAATGAATAAGAACCATAGTGAAGAGCTAAAAAAGAATCGAAAAGTACTTACTATTTTACGAGTGGATCTTCTATCTCGCGAATTCGAGCTCGTTTAAAC	KO of *hba1*[+] with Bahler construct - R
ST-391	TCTTCTGCCTAACCATACTACTTCTTCTAGCCTTCAGACTTAAAAGCTTCGCCTTTAGAAAACATCTCTATTCCTTCAAACGGATCCCCGGGTTAATTAA	KO of *SPBC17G9.12c*[+] with Bahler construct - F
ST-392	CAAGAGAGATGGAAAACAGAGGAATTGTGAACGTTCTCCTTATTCATATTTCCATAAAGCTTCTCCAATGACCTTTATTGGAATTCGAGCTCGTTTAAAC	KO of *SPBC17G9.12c*[+] with Bahler construct - F
ST-307	GATAAAATCTTAGAGATTGTTGCTAAATAAGCAAACAGTGTCTTTGCTGTAACTGGTGAGATATGTTTAAAATTAAATCACGGATCCCCGGGTTAATTAA	KO of *ncRNA.393*[+] with Bahler construct - F
ST-308	TGATATAATATATTTTCCTTCTTTACTATTACATTTCCTATTTTTTCACCATTTACGATATGTGTAACACTATCTAACCCGAATTCGAGCTCGTTTAAAC	KO of *ncRNA.393*[+] with Bahler construct - R
ST-95	TAATGAAAAAGGTTGCTAATTGGTTTGTTATATAAGAGTATGTCGCATTTGTTTACGATAGGAGAGAGCGATTTTCCACACGGATCCCCGGGTTAATTAA	KO of *ncRNA.394*[+] with Bahler construct - F

ST-96	TATTACTATGACTCTGGTTCTAGCT CGACTCTGACCCTTGCCTGACATA CAAATACTTTGCTCTTTTCAAAATG TACCGTGAATTCGAGCTCGTTTAA AC	KO of *ncRNA.394+* with Bahler construct - R
ST-305	ATATATAGAGTGGAAGGGCCGTCC GTTAGGACTTGTTTCAGTAAGAAT CAATTAGTATTCTACAGTAAACATC GTTAATCCGGATCCCCGGGTTAAT TAA	KO of *eno101+* with Bahler construct - F
ST-306	CTACTTCTACTACAACAACAGTTTA CTTTAATACTAATAATAAATAAACA CGCAACCTGGCAAATTAATCCAAA ACGCAAGAATTCGAGCTCGTTTAA AC	KO of *eno101+* with Bahler construct - R
ST-756	CtagaGGTCTCgGACTGGTGCTTGA CTTCTAATCTTGTTTcGAGACCcttC C	Golden Gate cloning *4xtetO-I*-sgRNA-F
ST-757	GGaagGGTCTCgAAACAAGATTAG AAGTCAAGCACCAGTCcGAGACCt ctaG	Golden Gate cloning *4xtetO-I*-sgRNA-R
ST-732	AAACGCTAATCTAGCATGTCATGA AGG	Making *4tetO-I*-HR-template - 1F
ST-733	actagtaggccttgCCGTATTGAAATCA AAATTATTAATAATGAGTAAGTGAA TATATACCA	Making *4tetO-I*-HR-template - 1R
ST-734	TGATTTCAATACGGcaaggcctactagt gcatgca	Making *4tetO-I*-HR-template - 2F
ST-735	TCTATAACTTTTACGTTAGctggatttc gtttacctcaccac	Making *4tetO-I*-HR-template - 2R
ST-736	gaggtaaacgaaatccagCTAACGTAAA AGTTATAGACAGTATTATAACAAGT ATTATTGTAAAA	Making *4tetO-I*-HR-template - 3F
ST-737	TTTAATTGTATTTTTTTATTCAAAGG TTCTACTTTGTCAATCATTTTCAA	Making *4tetO-I*-HR-template - 3R
ST-752	CtagaGGTCTCgGACTATTTCTTTTG CTTTACGGTCGTTTcGAGACCcttC C	Golden Gate cloning *4xtetO-II*-sgRNA-F
ST-753	GGaagGGTCTCgAAACGACCGTAA AGCAAAAGAAATAGTCcGAGACCtc taG	Golden Gate cloning *4xtetO-II*-sgRNA-R
ST-720	TTGAATTAATTCATAGAGTATGATA AAAATTGATAGTAAATTCATTGG	Making *4tetO-II*-HR-template - 1F
ST-721	cactagtaggccttgATGCATGCTAATA AATCATCGTAACTCAAGTAG	Making *4tetO-II*-HR-template – 1R
ST-722	TTTATTAGCATGCATcaaggcctactag tgcatgca	Making *4tetO-II*-HR-template – 2F
ST-723	TTTTTTTTTTCATAAATATTTActgga tttcgtttacctcaccacc	Making *4tetO-II*-HR-template – 2R
ST-724	tggtgaggtaaacgaaatccagTAAATATT TATGAAAAAAAAAATAAATGATTCA TAACAAGCAGATGAAAA	Making *4tetO-II*-HR-template - 3F
ST-725	TTTGTAATGTATAATCTTCATTTATT TTGAAGAGTCCTAATTCGT	Making *4tetO-II*-HR-template – 3R
ST-760	CtagaGGTCTCgGACTATATTTTAGA TAGTTCTGTGGTTTcGAGACCcttC C	Golden Gate cloning *4xtetO-III*-sgRNA-F
ST-761	GGaagGGTCTCgAAACCACAGAAC TATCTAAAATATAGTCcGAGACCtct aG	Golden Gate cloning *4xtetO-III*-sgRNA-R
ST-744	CGGTAAGAAAACACGACATGTGCA G	Making *4tetO-III*-HR-template – 1F
ST-745	catgcactagtaggccttgTATAATTAAGA TGTTTTAGAGACTTATACAATTTTG TCTTTATAAATTCT	Making *4tetO-III*-HR-template – 1R
ST-746	CTAAAACATCTTAATTATAcaaggcct actagtgcatgca	Making *4tetO-III*-HR-template – 2F
ST-747	TTTGCACTTTGTGAATctggatttcgttt acctcaccacca	Making *4tetO-III*-HR-template – 2R
ST-748	gtaaacgaaatccagATTCACAAAGTGC AAACATTATCATGAAAAAGAAC	Making *4tetO-III*-HR-template – 3F

ST-749	TGAAAAAGATAATCAGCCTTATAAT CTTTACAAAAGTAAGAAATTCT	Making *4tetO-III*-HR-template – 3R
ST-858	CtagaGGTCTCgGACTGATTTGCCG TTCTACGACGGGTTTcGAGACCctt CC	Golden Gate cloning *4xtetO-IV*-sgRNA-F
ST-859	GGaagGGTCTCgAAACCCGTCGTA GAACGGCAAATCAGTCcGAGACCt ctaG	Golden Gate cloning *4xtetO-IV*-sgRNA-R
ST-836	CTCAACAAACCACTGGTTACATGG C	Making *4tetO-IV*-HR-template – 1F
ST-837	actagtaggccttgGATACTTCGCAAAA TTCTAAGCATGGTGC	Making *4tetO-IV*-HR-template – 1R
ST-838	TTTGCGAAGTATCcaaggcctactagtg catgca	Making *4tetO-IV*-HR-template – 2F
ST-839	TCGATACCACCTTTTActggatttcgttt acctcaccacc	Making *4tetO-IV*-HR-template – 2R
ST-840	gtaaacgaaatccagTAAAAGGTGGTAT CGAGGAATGGCA	Making *4tetO-IV*-HR-template – 3F
ST-841	ATCCAATAGTTAATAAATCGATGCT TAATTTGGTGG	Making *4tetO-IV*-HR-template – 3R
ST-952	CtagaGGTCTCgGACTAGCTTGTGG CTGACCGTTAAGTTTcGAGACCctt CC	Golden Gate cloning clr5-sgRNA-F
ST-953	GGaagGGTCTCgAAACTTAACGGT CAGCCACAAGCTAGTCcGAGACCt ctaG	Golden Gate cloning clr5-sgRNA-R
ST-954	CTTATTTGCAGCAGCCTTTCCAAA TACCCTCTCAACGTTTCTCTCGAC AGCAACAATCTCATCCATTCCCTG CTGCTCAACATGCAGTTAACGGTT AGCC	Making clr5-Q264STOP-HR-template – F
ST-955	CAGATTGGTTTGAAGAAGCAAACA TGGTGGAGCCCATTGGGACATTTC TAGATTGGTAGATGAAAGGATACA AAGCTTGTGGCTAACCGTTAACTG CATG	Making clr5-Q264STOP-HR-template – R
ST-956	CtagaGGTCTCgGACTTATTAGCCT TTGAAGGATTTGTTTcGAGACCcttC C	Golden Gate cloning meu27-sgRNA-F
ST-957	GGaagGGTCTCgAAACAAATCCTTC AAAGGCTAATAAGTCcGAGACCtct aG	Golden Gate cloning meu27-sgRNA-R
ST-958	GCCAAAATCAATAGAGAACAATTA TACTTTAAAAAAAAAAAATGAAGAA GGCTTCTTAAGTCAACAGGAAAAT AAGTATTCAAATCAAAATCCTTCAA AG	Making meu27-S100Y-HR-template – F
ST-959	GAGGTGCCGCCCAATTGCAGTAT ACAAGCTATGAATGTTATTGGCTT GCTTACGCCGAGCTTGTCGAAAA GGTTATTAGCCTTTGAAGGATTTTG ATTTG	Making meu27-S100Y-HR-template – F
ST-1044	CCTTGGTTTTTATGTATTTACTAGT AAATCTATAATAATCATTAACT	Making cup1-3xDSR construct – 1F
ST-1045	TATATTTGAATAAgagaatggaaggaag gttcgttagcAAAAGGTAGGAGGAAG CAAGAAATGG	Making cup1-3xDSR construct – 1R
ST-1046	gctaacgaaccttccttccattctc	Making cup1-3xDSR construct – 2F
ST-1047	cAAGGGAGccAaGGatAttGgaaagtg gatgaacaagatcatttggagc	Making cup1-3xDSR construct – 2R
ST-1048	tcCaaTatCCtTggCTCCCTTgtaacttat gactctcgtttacac	Making cup1-3xDSR construct – 3F
ST-1049	ttaatacggagtttaaagcacacgatttgctacc	Making cup1-3xDSR construct – 3R
ST-1050	caaatcgtgtgctttaaactccgtattaaaccca ttcttaaactaatcttcatttattttgaagagtccta attcgtcatcattttcatctgcttgttat	Making cup1-3xDSR construct – 4F
ST-1051	gttacgatgatttattagcatgcatccggaccgt aaagcaaaagaaattaaatatttatgaaaaaaa aaataaatgattcataacaagcagatgaaaatg	Making cup1-3xDSR construct – 4R

ST-1052	tccggatgcatgctaataaatcatcgtaacGTCATCTTTTGGCATATAGGGTAAAGGGG	Making cup1-3xDSR construct – 5F
ST-1053	AAATTTACTCTAACAGACGATATTGTCTCACTATCC	Making cup1-3xDSR construct – 5R
ST-1054	CtagaGGTCTCgGACTAGGCCTTAATATTAACCCCCGTTTcGAGACCcttCC	Golden Gate cloning LocusPX-sgRNA-F
ST-1055	GGaagGGTCTCgAAACGGGGGTTAATATTAAGGCCTAGTCcGAGACCtctaG	Golden Gate cloning LocusPX-sgRNA-R
ST-1056	CtagaGGTCTCgGACTttCGAACATTTTAGGTAGCCGTTTcGAGACCcttCC	Golden Gate cloning endogenous cup1-sgRNA-F
ST-1057	GGaagGGTCTCgAAACGGCTACCTAAAATGTTCGaaAGTCcGAGACCtctaG	Golden Gate cloning endogenous cup1-sgRNA-R
ST-989	GATGAAAATGATGACGAATTAGGACTCTTCAAAATAAATGAAGATTATACATTACAAACTTTGGTCTGACTTTTTAAAGCACACGATTTGTGAAGTATTT	Making endogenous *cup1Δ*-HR-template – F
ST-990	TGATTTAATTTTAAACATATCTCACCAGTTACAGCAAAGACACTGTTTGCTTATTTAGCAACAATCTCTAAGATTTTATCAAATACTTCACAAATCGTGT	Making endogenous *cup1Δ*-HR-template – R
TagJ-sgRNA-F	CTAGAGGTCTCGGACTGCTCAGGCTAAACGTCGGAAGTTTCGAGACCCTTCC	Golden Gate cloning of cup1-tag sgRNA-F
TagJ-sgRNA-R	GGAAGGGTCTCGAAACTTCCGACGTTTAGCCTGAGCAGTCCGAGACCTCTAG	Golden Gate cloning of cup1-tag sgRNA-R
JTHR-Sito-F	ATGACGAATTAGGACTCTTCAAAATAAATGAAGATTATACATTACAAACTTTGGTCTGACTTTTTAAAGCACACGATTTGCTATTTGTATAGTTCATCCA	Making HR template for C-terminal tagging of cup1 with GFP
JTHR-Sito-R	TTGTATCGTGGGACTCTTTGTCAGACATTCAGCTCAGGCTAAACGTCGGAAAAGTTCTTAAAAAGTCAGTCAAAAAAAGGAGTAAAGGAGAAGAACTTTT	Making HR template for C-terminal tagging of cup1 with GFP
lyrkosgRNA-F	CTAGAGGTCTCGGACTTCTTAATGTTGAGCCTGTTTGTTTCGAGACCCTTCC	Golden Gate cloning of cup1-LYR sgRNA-F
lyrkosgRNA-R	GGAAGGGTCTCGAAACAAACAGGCTCAACATTAAGAAGTCCGAGACCTCTAG	Golden Gate cloning of cup1-LYR sgRNA-R
HR-L73F	ACTCGAAGATTCTGGGATCCAATGGCATCCCAAATCGGAATCTCACATTTTCAAGCTTACGCCCGAAAAGCTCGCAAGTCAAATCCTAATCCTCACAAGCGAC	Making cup1-L73G-HR-template – F
HR-L73R	AGCTTGATGGTCGCCATCATTGGCGCGCTCAATACGACATGCAAAAGTTTTTAGGAGATTGCGAAACCGTTTTATGCGTCGCTTGTGAGGATTAGGATTT	Making cup1-L73G-HR-template – R
HR-F99F	CAATGGCATCCCAAATCCTAATCTCACATTTTCAAGCTTACGCCCGAAAAGCTCGCAAGTCAAATCCTAATCCTCACAAGCGACGCATAAAACGGGGTCG	Making cup1-F99G-HR-template – F
HR-F99R	TCGTAAAGTCTGCCATATAGCTTGATGGTCGCCATCATTGGCGCGCTCAATACGACATGCAAAAGTTTTTAGGAGATTGCGACCCCGTTTTATGCGTCGC	Making cup1-F99G-HR-template – R
WC258-sg-393-F	CtagaGGTCTCgGACTcaaatgctgtagtgatgcagGTTTcGAGACCcttCC	Golden Gate cloning of cup1/ncRNA393 region sgRNA-F

WC259-sg-393-R	GGaagGGTCTCgAAACctgcatcactacagcatttgAGTCcGAGACCtctaG	Golden Gate cloning of cup1/ncRNA393 region sgRNA-R
WC279-cup1DP-uraTT-F	GATCATTTGGAGCTTGGAATGGTCTCCTTTTGGTAACTGTAGAAATAAATCTCATGAGTAAGGAATTTTGTATGAATGAAGCTTGTGATATTGACGAAAC	To amplify 144-bp ura4 transcriptional terminator to replace 697 bp of cup1 upstream/promoter region (to make cup1-TT)- F
WC280-cup1DP-uraTT-R	ATTACCGTCTAAAGCGCGCAATTTCACAGATGCCGCAAATTTGACATCTGGGTCTTTCAAGTCTTGTTAGAACATTGAATAACTATGTACAAAGCCAATG	To amplify 144-bp ura4 transcriptional terminator to replace 697 bp of cup1 upstream/promoter region (to make cup1-TT) - R
WC267-arg11-Ctag-F	TAAAGGTGCTGCTACTCAAGCTCTCCAGAATCTCAATCTGTCGTGTGGTTACGATGAATATGCCGGTATCCATTTGGATcggatccccgggttaattaa	C-terminal tagging of arg11[+] with mCherry using Bahler construct - F
WC268-arg11-Ctag-R	AATATTTGTAACAAAAAATATCCAAATGGTACACAGAAAGAATAAAAATAACAAAAGAATGGGCTACAAAAAATATAAgaattcgagctcgtttaaac	C-terminal tagging of arg11[+] with mCherry using Bahler construct - R
WC301-UR2-C1-F	GGGAACCACATACAATGAATG	C1 primer upstream of 5S rRNA.26 – F ChrIII control PCR and putative circle junction in UR-2
WC305-UR2-C2-F	TAGTCAGTATATACTGAGCGG	C2 primer upstream of 5S rRNA.26 – F ChrIII control PCR and putative circle junction in UR-2
WC306-UR2-B1-R	TCGCTGCTTATTGACTTTGAG	B1 primer downstream of 5S rRNA.24 – R ChrIII control PCR and putative circle junction in UR-2
WC308-UR2-B2-R	AACTGCTCTACTACTATAACG	B2 primer downstream of 5S rRNA.24 – R ChrIII control PCR and putative circle junction in UR-2
WC310-CHR3-2-D1-R	ATCATCTCGATAAGTGCTTTC	D1 primer downstream of 5S rRNA.26 – R ChrIII control PCR
WC311-CHR3-2-A-F	TCCGACTATTTGCATAAGACC	A primer upstream of 5S rRNA.24 – F ChrIII control PCR
WC312-CHR3-2-D2-R	AGAACTTTGTTGTAGCCTGAG	D2 primer downstream of 5S rRNA.26 – R ChrIII control PCR
WC321-UR4-UR17-G1-F	TTCTCCTTTGAACCCAGAAGG	G1 primer upstream of LTR27 – F ChrIII control PCR and putative circle junction in UR-4
WC325-UR4-UR17-G2-F	AATTCCATCCAAATTCTCTGG	G2 primer upstream of LTR27 – F ChrIII control PCR and putative circle junction in UR-4
WC326-UR4-F1-R	TATCACAACAGTTCTGCAACG	F1 primer downstream of LTR3 – R ChrIII control PCR and putative circle junction in UR-4
WC328-UR4-F2-R	TGGAAGCTTTGATAGAAAGGG	F2 primer downstream of LTR3 – R ChrIII control PCR and putative circle junction in UR-4
WC329-CHR3-4-E-F	ACGAATACGGTGTTGTATGAC	E primer upstream of LTR3 – F ChrIII control PCR
WC330-CHR3-4-17-H-R	CGCATCGTTAATGAGTTCATC	H primer downstream of LTR27 – R ChrIII control PCR
WC313-PROBE-925-F	TAAGCCAAAGGGTCCAATTCC	To amplify probe fragment at ~925 kb on chrIII – F
WC314-PROBE-925-R	CATTCATTGTATGTGGTTCCC	To amplify probe fragment at ~925 kb on chrIII – R
WC315-PROBE-520-F	TATGCGGCAAAGTGCAATGTC	To amplify probe fragment at ~520 kb on chrIII – F
WC316-PROBE-520-R	TGTTGCTAAGAGTGATAGCAG	To amplify probe fragment at ~520 kb on chrIII – R
WC317-PROBE-44-F	ACTACAGCAGTATCCTTCATG	To amplify probe fragment at ~44 kb on chrIII – F
WC318-PROBE44-R	TTCGTCCTCTCTGTCATTTCC	To amplify probe fragment at ~44 kb on chrIII – R
ST-9	CCATAGAATCTCCTTAGTTTGCATCGCAATTTTATAGTTACCTTTTTGCTAGTAAGCAATTAATTTTTGGGACTTTTAAGCGGATCCCCGGGTTAATTAA	KO of epe1[+] with Bahler construct - F
ST-10	TGTGAACTACTCAAGAATCATAAGCACGTGGGGATAAATATTCAATGGTAGCCGAAGGAAATAAAAAGTGCCGAGGTACTGAATTCGAGCTCGTTTAAAC	KO of epe1[+] with Bahler construct - R

ST-1064	AAAATAACATTTATGATTTTGAAGA TCACTCTCCTGTTAGGGAAAAATG GGGGCACAGGCTTCGGTCCAGAG GTGCTAGTCGGATCCCCGGGTTA ATTAA	C-terminal tagging of *epe1+* with GFP using Bahler construct - F
ST-1065	CTTAATTATTTGATGAAACCTTCAT GATATACTCATAAAATGTGAACTAC TCAAGAATCATAAGCACGTGGGGA TAAATAGAATTCGAGCTCGTTTAAA C	C-terminal tagging of *epe1+* with GFP using Bahler construct - R
ST-1058	CtagaGGTCTCgGACTGGACTTTTA AGATGGATTCCGTTTcGAGACCctt CC	Golden Gate cloning epe1-sgRNA-F
ST-1059	GGaagGGTCTCgAAACGGAATCCA TCTTAAAAGTCCAGTCcGAGACCtc taG	Golden Gate cloning epe1-sgRNA-R
ST-1062	GTGAACTACTCAAGAATCATAAGC ACGTGGGGATAAATATTCAATGGT AGCCGAAGGAAATAAAAGTGCC GAGGTACTTCTTAAAAGTCCCAAA AATTA	Making *epe1Δ*-HR-template – F
ST-1063	CCATAGAATCTCCTTAGTTTGCAT CGCAATTTTATAGTTACCTTTTTGC TAGTAAGCAATTAATTTTTGGGACT TTTAAGAAGTACCTCGGCACTTTTT A	Making *epe1Δ*-HR-template – R
ST-1060	TTTATAGTTACCTTTTTGCTAGTAA GCAATTAATTTTTGGGACTTTTAAG ATGGACTACAAAGACCATGACGGT GATTATAAAGATCATGACATCGAC TA	Making 3xFLAG-Epe1-HR-template – F
ST-1061	GAATATCAATGTCTTGATTTATAAT GTCATCGTATTCAAGCCAGGAATC GCTGCCTCCTCCCTTGTCATCGTC ATCCTTGTAGTCGATGTCATGATC TTT	Making 3xFLAG-Epe1-HR-template – R
ST-36	CAGGAGTGTGTACAGGAGGT	qPCR *vps32+* - F
ST-37	AGATGAATTGGCCAACGAGTT	qPCR *vps32+* - R
ST-44	CTCGCCTGAAACTTGCTACA	qPCR *cgs1+* - F
ST-45	GCACGAGGTTGATTACGCAT	qPCR *cgs1+* - R
ST-48	GTCACGGGCGATTTTAGGAC	qPCR *ppr4+* - F
ST-49	TCCCTTGTCCGGCAGAATAA	qPCR *ppr4+* - R
ST-1012	TCTGCGTGACACTTGTTCGT	qPCR *grt1+* - F
ST-1013	TAGAGACTCCAGCGCATCCT	qPCR *grt1+* - R
ST-1010	GTCAGGTGCTCCTTGCAGAT	qPCR *eno102+* - F
ST-1011	GCGTTCCTGCATAGATTGCG	qPCR *eno102+* - R
ST-1006	ATTGGATAGGCGTCACCGTC	qPCR *aes1+* - F
ST-1007	AGCCGGATCCTCATTGACAT	qPCR *aes1+* - R
ST-1018	GATTGGGCCGAGTTGAAGGA	qPCR *cdc22+* - F
ST-1019	AAGCAGTAGGCATTGGTGCT	qPCR *cdc22+* - R
ST-1028	AAGCAGTAGGCATTGGTGCT	qPCR *fip1+* - F
ST-1029	CCCTTTTTCACCGTTCTGCG	qPCR *fip1+* - R
ST-1024	GCACCGGAGATGATACCCAG	qPCR *fio1+* - F
ST-1025	GCACCATTTCCGATCGTTGG	qPCR *fio1+* - R
ST-1022	TCACACATCGTGGCTATCCG	qPCR *cyp8+* - F
ST-1023	TCGTTCACGAGATCCCTCCA	qPCR *cyp8+* - R
ST-1040	CCTGCTGCCGAATTTCAACG	qPCR *pcn1+* - F
ST-1041	TGCAGCTAAAACGAACACCC	qPCR *pcn1+* - R
ST-1032	GTCTCCGGGTGCTACAGTTC	qPCR *mbx2+* - F
ST-1033	GTGCGTTTGCCTACGATGAC	qPCR *mbx2+* - R
ST-1038	CTCGTGTTCCTGAGACCACC	qPCR *nce103+* - F
ST-1039	AACGAGGAACGACATTGGCA	qPCR *nce103+* - R
ST-277	CATTTTGCGGGACAATGGGT	qPCR *cds1+* - F
ST-278	CCTCCGTCCAAGTTGACGTT	qPCR *cds1+* - R
ST-1068	tgctgaatgtaaccaacatca	qPCR *cen-IRC-L1*
ST-1069	gcctcaattgcctattagtgct	qPCR *cen-IRC-R1*
ST-1066	GGATAAGCCAATCATCGTTGAG	qPCR *tlh2* - F
ST-1067	GTAGTTGACGCTCCTTGGAAG	qPCR *tlh2* - R
ST-1070	CCGAGGCTTTCATAGCTTA	qPCR *S. cerevisiae CEN4* – F (for GFP spike-in qChIP)
ST-1071	ACCGGAAGGAAGAATAAGAA	qPCR *S. cerevisiae CEN4* – R (for GFP spike-in qChIP)

ST-161	CCCAATTGTTGTGATTGCTG	qPCR *S. octosporus* CEN3 heterochromatin – F (for H3K9me2 spike-in qChIP)
ST-162	GCGGATGCAGTATTTCGTTT	qPCR *S. octosporus* CEN3 heterochromatin – R (for H3K9me2 spike-in qChIP)
Mst2-C-Tag-F	ACCTTTTACTTAAAGAAAATATACT TATTCCTCTACCTCAAAAGCGTCT ATTAGATAACTCTCATCATCTGGAT TCCGTTCGGATCCCCGGGGTTAATT AA	C-terminal tagging of *mst2*$^+$ with 13xMyc using Bahler construct - F
Mst2-C-Tag-R	TATAGAGCAACAACCAAGCCGTAG ATGATACAAATGCTTCACGACAAA TATCGAAAGATTAAAATACTTATTT ATTTGAAGAATTCGAGCTCGTTTAA AC	C-terminal tagging of *mst2*$^+$ with 13xMyc using Bahler construct - R

Supplementary Table 4. Genomic coordinates used to generate heatmaps for heterochromatin islands.

Chromosome	Start	End	Name	Reference
I	578000	582000	*Island 1*	Zofall et al., Science, 2012
I	2447000	2449000	*Island 2*	Zofall et al., Science, 2012
I	2521000	2525000	*Island 3*	Zofall et al., Science, 2012
I	3647000	3651000	*Island 4*	Zofall et al., Science, 2012
I	3727000	3730000	*Island 5*	Zofall et al., Science, 2012
I	4534000	4540000	*Island 6*	Zofall et al., Science, 2012
I	4653000	4656000	*Island 7*	Zofall et al., Science, 2012
II	898000	903000	*Island 8*	Zofall et al., Science, 2012
II	1472000	1479000	*Island 9*	Zofall et al., Science, 2012
II	1551000	1554000	*Island 10*	Zofall et al., Science, 2012
II	1670000	1680000	*Island 11*	Zofall et al., Science, 2012
II	1692000	1698000	*Island 12*	Zofall et al., Science, 2012
II	1869000	1873000	*Island 13*	Zofall et al., Science, 2012
II	2199000	2202000	*Island 14*	Zofall et al., Science, 2012
II	2338000	2342000	*Island 15*	Zofall et al., Science, 2012
II	3628000	3631000	*Island 16*	Zofall et al., Science, 2012
II	3640000	3642000	*Island 17*	Zofall et al., Science, 2012
III	958000	968000	*Island 18*	Zofall et al., Science, 2012
III	1036000	1040000	*Island 19*	Zofall et al., Science, 2012
III	2369000	2371000	*Island 20*	Zofall et al., Science, 2012
III	2422000	2424000	*Island 21*	Zofall et al., Science, 2012
I	1465847	1469848	*HOOD-1*	Yamanaka et al., Nature, 2013
I	1564163	1568414	*HOOD-2*	Yamanaka et al., Nature, 2013
I	2544835	2561773	*HOOD-3*	Yamanaka et al., Nature, 2013
I	2927156	2941954	*HOOD-4*	Yamanaka et al., Nature, 2013
I	2977013	2988899	*HOOD-5*	Yamanaka et al., Nature, 2013
I	2994807	3009469	*HOOD-6*	Yamanaka et al., Nature, 2013
I	3361499	3365727	*HOOD-7*	Yamanaka et al., Nature, 2013
I	3736902	3743575	*HOOD-8*	Yamanaka et al., Nature, 2013
I	3791825	3796114	*HOOD-9*	Yamanaka et al., Nature, 2013
I	3996353	4000615	*HOOD-10*	Yamanaka et al., Nature, 2013
I	4022326	4026545	*HOOD-11*	Yamanaka et al., Nature, 2013
I	5069824	5083205	*HOOD-12*	Yamanaka et al., Nature, 2013
I	5191103	5195325	*HOOD-13*	Yamanaka et al., Nature, 2013
I	5195656	5199909	*HOOD-14*	Yamanaka et al., Nature, 2013
I	5234000	5250176	*HOOD-15*	Yamanaka et al., Nature, 2013
II	91600	101684	*HOOD-16*	Yamanaka et al., Nature, 2013
II	347505	354250	*HOOD-17*	Yamanaka et al., Nature, 2013
II	898107	902233	*HOOD-18*	Yamanaka et al., Nature, 2013
II	1812684	1816937	*HOOD-19*	Yamanaka et al., Nature, 2013
II	1965175	1969519	*HOOD-20*	Yamanaka et al., Nature, 2013
II	2126590	2128479	*HOOD-21*	Yamanaka et al., Nature, 2013
II	4414469	4418768	*HOOD-22*	Yamanaka et al., Nature, 2013
II	4442538	4449562	*HOOD-23*	Yamanaka et al., Nature, 2013
III	173841	176400	*HOOD-24*	Yamanaka et al., Nature, 2013
III	254411	256353	*HOOD-25*	Yamanaka et al., Nature, 2013
III	778123	782331	*HOOD-26*	Yamanaka et al., Nature, 2013

III	1047657	1056145	*HOOD-27*	Yamanaka et al., Nature, 2013
III	1168500	1176000	*HOOD-28*	Yamanaka et al., Nature, 2013
III	1179500	1182650	*HOOD-29*	Yamanaka et al., Nature, 2013
III	1196050	1196500	*HOOD-30*	Yamanaka et al., Nature, 2013
III	1763512	1775613	*HOOD-31*	Yamanaka et al., Nature, 2013
III	2320230	2324503	*HOOD-32*	Yamanaka et al., Nature, 2013
I	239913	249656	*SPAC806.04c & SPAC806.05*	Wang et al., eLife, 2015
I	4527389	4533031	*LTR & SPAC27D7.11c*	Wang et al., eLife, 2015
III	273261	279340	*mae2*	Wang et al., eLife, 2015
II	2301681	2308661	pfk1/sad1	Sorida et al., PLoS Genet, 2019
I	94000	97000	SPAC1F8.05	Gallagher et al., NSMB, 2019
I	125000	129000	SPAC11D3.11c	Gallagher et al., NSMB, 2019
I	125000	129000	SPAC11D3.10	Gallagher et al., NSMB, 2019
I	1036000	1042000	SPAC23C4.05c	Gallagher et al., NSMB, 2019
I	1036000	1042000	SPAC23C4.06c	Gallagher et al., NSMB, 2019
I	1513000	1517000	SPAC57A7.13	Gallagher et al., NSMB, 2019
I	1513000	1517000	SPAC57A7.12	Gallagher et al., NSMB, 2019
I	2147000	2150000	SPAC23C11.09	Gallagher et al., NSMB, 2019
I	2174000	2179000	SPAC13F5.03c	Gallagher et al., NSMB, 2019
I	2395000	2398500	SPAC15F9.01c	Gallagher et al., NSMB, 2019
I	2977500	2985500	SPAPB1A11.02	Gallagher et al., NSMB, 2019
I	3476500	3481000	SPAC328.03	Gallagher et al., NSMB, 2019
I	5309000	5311500	SPNCRNA.1068	Gallagher et al., NSMB, 2019
I	5309000	5311500	SPNCRNA.1069	Gallagher et al., NSMB, 2019
I	5390000	5396000	SPAC3G6.07	Gallagher et al., NSMB, 2019
II	102000	105000	SPBC359.01	Gallagher et al., NSMB, 2019
II	167500	170500	SPBC1683.12	Gallagher et al., NSMB, 2019
II	200500	203000	SPBC660.05	Gallagher et al., NSMB, 2019
II	221500	224000	SPBC660.14	Gallagher et al., NSMB, 2019
II	337500	340500	SPNCRNA.103	Gallagher et al., NSMB, 2019
II	459500	464000	SPBC428.10	Gallagher et al., NSMB, 2019
II	538000	541000	SPBC649.04	Gallagher et al., NSMB, 2019
II	919500	922500	SPBC216.07c	Gallagher et al., NSMB, 2019
II	948000	951000	SPNCRNA.334	Gallagher et al., NSMB, 2019
II	948000	951000	SPNCRNA.335	Gallagher et al., NSMB, 2019
II	1223500	1226000	SPBC725.10	Gallagher et al., NSMB, 2019
II	1309000	1312000	SPBC30B4.04c	Gallagher et al., NSMB, 2019
II	1495000	1499000	SPBC11B10.07c	Gallagher et al., NSMB, 2019
II	2184000	2186500	SPBC17G9.08c	Gallagher et al., NSMB, 2019
II	3344629	3348817	SPBC17D1.17	Gallagher et al., NSMB, 2019
II	3530000	3532500	SPBC1105.12	Gallagher et al., NSMB, 2019
II	3530000	3532500	SPBC1105.13c	Gallagher et al., NSMB, 2019
II	4103000	4108500	SPBC56F2.06	Gallagher et al., NSMB, 2019
II	4409500	4414500	SPBC1289.14	Gallagher et al., NSMB, 2019
III	31500	34500	SPCP20C8.03	Gallagher et al., NSMB, 2019
III	34500	39000	SPCC1884.01	Gallagher et al., NSMB, 2019
III	739000	742000	SPCC18B5.11c	Gallagher et al., NSMB, 2019
III	1265500	1268000	SPCC23B6.01c	Gallagher et al., NSMB, 2019
III	2072000	2075000	SPCPB1C11.02	Gallagher et al., NSMB, 2019
III	2286000	2289500	SPCC965.04c	Gallagher et al., NSMB, 2019
III	2410325	2415970	SPCC569.08c	Gallagher et al., NSMB, 2019

| III | 2429000 | 2433000 | SPCC569.03 | Gallagher et al., NSMB, 2019 |