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Multiple chemo-genetic interactions between a toxic metabolite and the ubiquitin pathway in yeast

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34 14 Running title: AICAR and the ubiquitin pathway
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3 19 **Abstract**

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7 21 AICAR is the precursor of ZMP, a metabolite with anti-proliferative properties in yeast and
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9 22 human. We aim at understanding how AICAR (and its active form ZMP) affects essential
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11 23 cellular processes. In this work, we found that ZMP accumulation is synthetic lethal with a
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13 24 hypomorphic allele of the ubiquitin activating enzyme Uba1. A search for gene dosage
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15 25 suppressors revealed that ubiquitin overexpression was sufficient to restore growth of the
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17 26 *uba1* mutant upon AICAR treatment, suggesting that the ubiquitin pool is critical for cells to
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19 27 cope with AICAR. Accordingly, two mutants with constitutive low ubiquitin, *ubp6* and *doa1*,
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21 28 were highly sensitive to AICAR, a phenotype that could be suppressed by ubiquitin
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23 29 overexpression. We established, by genetic means, that these new AICAR-sensitive mutants
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25 30 act in a different pathway from the *rad6/bre1* mutants which were previously reported as
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27 31 sensitive to AICAR (Albrecht et al., 2016). Two ubiquitin-conjugating enzymes (Ubc4 and
28
29 32 Cdc34) and a ubiquitin ligase (Cdc4) were found to contribute to the ability of cells to cope
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31 33 with ZMP. This study illustrates the complexity of chemo-genetic interactions and shows how
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33 34 genetic analyses allow deciphering the implicated pathways, the individual gene effects and
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35 35 their combined phenotypic contribution. Based on additivity and suppression patterns, we
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37 36 conclude that AICAR treatment shows synthetic interactions with distinct branches of the
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39 37 yeast ubiquitin pathway.

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42 39 Key words:

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45 40 Ubiquitin, metabolic intermediate, yeast, suppression, additivity

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43 INTRODUCTION

44 ZMP, the monophosphate derivative of Amino-Imidazole CarboxAmide Ribonucleoside
45 (AICAR), is a precursor of AMP in the purine *de novo* biosynthesis pathway (Fig. 1a) and as
46 such is naturally present in cells at low concentrations (micro molar range, (Daignan-Fornier
47 and Pinson, 2012; Hurlimann et al., 2011)). At higher concentrations, ZMP is a potent low-
48 energy mimetic which stimulates AMP-activated protein kinase (AMPK) by mimicking
49 activation by AMP (Sullivan et al., 1994a; Sullivan et al., 1994b). *In vivo*, this AMPK
50 activating effect was shown to increase endurance of sedentary mice (Narkar et al., 2008). At
51 high concentrations, AICAR induces cell cycle arrest and/or apoptosis (Rattan et al., 2005). *In*
52 *vivo* antitumor effects of AICAR have been reported in non-orthotopic mouse models (Guo et
53 al., 2009; Liu et al., 2014; Rattan et al., 2005; Robert et al., 2009; Tang et al., 2011) and
54 AICAR, also pharmacologically referred to as Acadesine, is considered as a potential
55 antitumor agent. A Phase I/II study has been successfully conducted to determine the safety
56 and tolerability of AICAR to treat patients with chronic lymphocytic leukemia (CLL) (Van
57 Den Neste et al., 2013). We and others have shown that AICAR cytotoxicity was not affected
58 in the absence of AMPK (Ceschin et al., 2014; Liu et al., 2014) indicating that AICAR has
59 other important targets that could strongly contribute to its potential anti-tumor effects but
60 could also cause unwanted side effects. In line with this remark, ZMP, the monophosphate
61 derivative of AICAR is accumulated in several purine genetic diseases and could contribute to
62 the poorly understood etiology of these diseases (Ceballos-Picot et al., 2015; Daignan-Fornier
63 and Pinson, 2012). For all these reasons, understanding, how AICAR (and its active form
64 ZMP) affects cellular processes and results in beneficial or an adverse effects, is crucial.

65 To identify cellular functions affected by AICAR, we took advantage of yeast genetics to
66 isolate and characterize several yeast mutants showing higher sensitivity to AICAR (Albrecht
67 et al., 2016; Ceschin et al., 2015; Ceschin et al., 2014). Of note, search for AICAR-sensitive
68 mutants cannot easily be done at a genome wide scale since a specific and complex genetic
69 set-up (*ade16 ade17 ade8 his1*) is required to reveal AICAR-sensitivity (Fig. 1a) (Ceschin et
70 al., 2015; Hurlimann et al., 2011). Recently, we found that sensitivity to AICAR is connected
71 with the ubiquitin pathway in yeast and mammals. In particular, AICAR treatment is synthetic
72 lethal with loss of function of several histone-modifying enzymes in yeast and human cells
73 (Albrecht et al., 2016). Indeed, in yeast cells, the deletion of *BRE1*, that catalyzes the
74 ubiquitination of H2B (H2Bub), results in a severe inhibition of growth under AICAR

75 treatment. Accordingly, the non-ubiquitinable form of H2B, H2BK123R, was also found to be
76 highly sensitive to AICAR. It is well known that H2Bub is a signal of transactivation for two
77 methyltransferases, Dot1 and Set1/COMPASS. We showed that a *set1* mutant was exquisitely
78 sensitive to AICAR, as was the non-methylable form of its targets H3K4. Remarkably, the
79 knock-down of the corresponding orthologs, Rnf40 for Bre1, Ash2l and Mll4/KMT2D for
80 COMPASS, were found to exacerbate AICAR sensitivity of human HCT116 cells (Albrecht
81 et al., 2016).

82 Here, we show that a mutation in the *UBA1* gene encoding the yeast ubiquitin activating
83 enzyme (E1) is synthetic lethal with AICAR treatment. This result prompted us to examine
84 more systematically the connections between AICAR and the ubiquitin-pathway (Fig. 1b).
85 We identified the ubiquitin pool as having a central role in AICAR sensitivity and established
86 that mutants with reduced ubiquitin pools, such as *ubp6* or *doa1*, are highly sensitive to the
87 drug. Based on suppression patterns and additivity, these mutants were found to belong to a
88 distinct branch of the ubiquitin-pathway which is required for yeast cells to cope with AICAR
89 and acts in parallel to the Rad6/Bre1 branch identified previously (Albrecht et al., 2016). The
90 complex chemo-genetic interactions between a metabolite and the ubiquitin pathway reported
91 here, illustrate how poly-pharmacological effects can result in phenotypic synergy.

93 **Material and methods**

95 **Yeast media**

96 SD is a synthetic minimal medium containing 0.5% ammonium sulfate, 0.17% yeast nitrogen
97 base without amino acids and ammonium sulfate (BD-Difco), 2% glucose. SDcasaW is SD
98 medium supplemented with 0.2% casamino acids (BD-Difco) and tryptophan (0.2 mM).
99 When indicated, adenine (0.3 mM) and/or uracil (0.3 mM) were added in SDcasaW medium
100 resulting in media named SDcasaWA (+ adenine), SDcasaWU (+ uracil) and SDcasaWAU (+
101 adenine + uracil). SC medium was prepared as described (Sherman, 1986). SC complete
102 medium is SC medium supplemented with adenine (0.3 mM), uracil (0.3 mM), histidine (0.06
103 mM), leucine (0.4 mM), lysine (0.06 mM) and tryptophan (0.2 mM).

105 **Strains and plasmids**

106 All yeast strains are listed in Table 1 and belong to, or are derived from, a set of disrupted

107 strains isogenic to BY4741 or BY4742 purchased from Euroscarf. Multi-mutant strains were
108 obtained by crossing, sporulation and micromanipulation of meiosis progeny. For each cross,
109 systematic tetrad analysis was done to verify correct segregation of the markers (mating type,
110 auxotrophy, temperature sensitivity, PCR detection of KanMX4 knock-out...). The presence
111 of the *uba1-ol* allele in the Y8033 strain used for gene dosage screening was verified by
112 sequencing of the *UBA1* locus. All plasmids used in this study are listed in Table 2 and were
113 obtained by PCR amplification on genomic DNA and cloning in indicated vectors. Cloning
114 details are available upon request.

115

116 **Growth test**

117 Overnight pre-cultured yeast cells were re-suspended in sterile water at $3 \cdot 10^7$ cells/ml and
118 submitted to 1/10 serial dilutions. Drops (5 μ l) of each dilution were spotted on freshly
119 prepared medium plates and were incubated at indicated temperature for 48-72 h before
120 imaging.

121

122 **Isolation of multicopy suppressors of the *uba1-ol* AICAR-sensitive phenotype**

123 Multicopy suppressors of the *ade16 ade17 ade8 his1 uba1-ol* AICAR-sensitive phenotype
124 were obtained by transforming the Y8033 strain with a multicopy-plasmid library (PFL44L
125 backbone, 2 μ *URA3*; generous gift from F. Lacroute). Transformants selected on SD_{cas}WA
126 medium at 25 °C were then replica-plated on the same medium containing or not AICAR (5
127 mM) and plates were incubated for 1-3 days at 33 °C. As described in the Results section, we
128 focused our attention on 5 clones containing ubiquitin-encoded genes: *UBI4*, *RPL40A* and
129 *RPL40B*. Among those, the plasmid p4814 corresponded to a chromosome IX fragment
130 (coordinates from 67814 bp to 71209 bp) containing *RPL40A* as the only entire gene. Three
131 other plasmids contained fragments of chromosome XI with *RPL40B* as the only entire
132 common gene. Finally, the fifth plasmid presented a fragment of chromosome XII
133 (coordinates from 63188 bp to 67952 bp) containing 3 entire open reading frames (ORF):
134 *UBI4*, *ENT4* and *YLL037w*. Subcloning of the *UBI4* ORF alone (P5504) in a YEplac195
135 plasmid (Gietz and Sugino, 1988) allowed us to show that this ORF was sufficient for
136 suppression of the AICAR-sensitive phenotype.

137

138 **Western blotting**

139 Total protein extraction was performed by disruption of exponential growing cells with glass-
140 beads in trichloro-acetic acid 5 %, as described in (Escusa et al., 2006). Protein extracts were

141 separated by SDS-PAGE, transferred onto 0.45 μ PVDF membrane and protein of interest
142 were detected by western blotting using anti Act1 (generous gift from I. Sagot (Bordeaux,
143 France), 1/100,000), anti-H2B (Active motif ; #39237, 11/2,000) or anti Ubi (Abcam,
144 #ab19247 1/10,000). Blots Quantification was done with Image J (NIH).

146 Results

148 *Synthetic lethality of a uba1 mutation with ZMP accumulation can be rescued by ubiquitin* 149 *overexpression*

150 Our previous report establishing a connection between sensitivity to AICAR and the ubiquitin
151 pathway (Albrecht et al., 2016) prompted us to examine whether sensitivity to AICAR could
152 be affected under conditions where the ubiquitin-pathway is dysfunctional. We started with
153 the *UBA1* gene encoding the ubiquitin activating enzyme (E1) at the apex of the ubiquitin
154 pathway, hence governing all the ubiquitin-dependent processes (McGrath et al., 1991). We
155 asked whether accumulation of ZMP could lead to a synthetic phenotype when combined with
156 a temperature sensitive allele (*uba1-ol*) of the *UBA1* gene (Shimada et al., 2002). The *uba1-*
157 *ol* mutant was mated to an *ade16 ade17* double mutant which constitutively accumulates
158 ZMP (Pinson et al., 2009) and meiosis of the resulting diploid was induced. Strikingly, in the
159 meiotic progeny, no *ade16 ade17 uba1-ol* triple mutant could be obtained at permissive
160 temperature unless rescued by a plasmid expressing the wild-type *UBA1* gene (Fig. 2a) thus
161 revealing synthetic lethality between *ade16 ade17* and *uba1-ol*. Importantly, this synthetic
162 lethality could be totally abolished by *ade8* and *his1* mutations (Fig. 2b) blocking ZMP
163 synthesis upstream of Ade16 and Ade17 (Fig. 1a) and thus eliminating ZMP accumulation
164 (Hurlimann et al., 2011). Finally, in the *uba1-ol ade16 ade17 ade8 his1* mutant, the synthetic
165 growth defect was recapitulated by addition of the ZMP-precursor AICAR at semi-permissive
166 temperature (33°C), a condition that did not affect growth of the *uba1-ol ade16 ade17 ade8*
167 *his1* mutant in the absence of AICAR (Fig. 2b). We conclude that the *uba1-ol* mutant is
168 synthetic lethal with ZMP accumulation supplied by two different means, either endogenously
169 from the purine pathway (Fig. 2a) or exogenously from the growth medium (Fig. 2b).
170 Together these results reveal that partial loss of function of the ubiquitin-conjugating enzyme
171 Uba1 is synthetic lethal with ZMP accumulation. Hence, the ubiquitin pathway appears
172 critical for yeast cells to cope with ZMP.

173 As a first step toward understanding how AICAR affects the ubiquitin pathway, we searched
174 for gene-dosage suppressors that would restore growth of the *uba1-ol ade16 ade17 ade8 his1*
175 mutant in the presence of AICAR. A genomic multicopy plasmid library was transformed in
176 the *uba1-ol ade16 ade17 ade8 his1* mutant and a set of 37 clones showing improved capacity
177 to grow in the presence of AICAR at 33°C were studied. Among them no plasmid carrying
178 the *UBA1* gene was found suggesting that it was not present in the genomic library possibly
179 because of the large size of the coding region >3kb. Indeed, in a reconstitution experiment,
180 the growth defect was fully suppressed by a plasmid expressing *UBA1* (not shown). Five
181 plasmids contained either the *ADE16* (3 clones) or the *ADE17* (2 clones) gene, thus restoring
182 ATIC enzymatic activity and preventing ZMP accumulation. These plasmids were not further
183 studied. Among the remaining clones, we only studied those carrying a single chromosome
184 fragment and presenting a robust suppression phenotype after retransformation. Eleven such
185 plasmids revealed *DDP1* (1 clone), *ECM21* (1 clone), *RPL40A* (1 clone), *RPL40B* (3 clones),
186 *UBI4* (1 clone) and *URA6* (4 clones) as gene-dosage suppressors after individual subcloning
187 (Fig. 2c). *DDP1* encodes an inositol polyphosphate phosphatase, *ECM21* encodes an arrestin
188 and *URA6* a uridylylate kinase. The three remaining genes, *UBI4*, *RPL40A* and *RPL40B*, encode
189 precursors of free ubiquitin (Finley et al., 1989; Finley et al., 1987) and were therefore
190 directly related to *UBA1* function as the ubiquitin-activating enzyme. We focused on those in
191 the rest of the work. We conclude that overexpression of ubiquitin allows bypassing of
192 AICAR sensitivity of the *uba1-ol ade16 ade17 ade8 his1* mutant. This result suggested that
193 the ubiquitin pool could play an important role in AICAR sensitivity in yeast. Importantly, the
194 free-ubiquitin pool was significantly higher in the *uba1-ol* mutant than in the control strain
195 (Fig. 2d, arrow) indicating that the exacerbated sensitivity of the *uba1-ol* mutant to AICAR is
196 not due a lower pool of free-ubiquitin. In both wild-type and *uba1-ol* strains overexpression
197 of *UBI4* resulted in increased free-ubiquitin (Fig. 2d), though the amount of free ubiquitin was
198 higher in the *uba1-ol* mutant possibly reflecting its defective utilization by the E1 mutant
199 enzyme. In both strains, *UBI4* overexpression strongly increased the signal of ubiquitylated
200 proteins indicating that under physiological conditions ubiquitin synthesis limits the
201 ubiquitylation process. Finally, we found no effect of AICAR treatment on the ubiquitin pool
202 (Fig. 2d). From these results, we conclude that AICAR-sensitivity of the *uba1-ol ade16*
203 *ade17 ade8 his1* mutant is not associated to a lower free-ubiquitin pool due to the mutation or
204 to AICAR treatment, but rather to a defect in the ubiquitylation process that can be efficiently
205 suppressed by overexpressing ubiquitin. We interpret these results as an indication that, in the

206 *uba1-ol ade16 ade17 ade8 his1* mutant, a function aggravated by AICAR treatment can be
207 rescued by ubiquitin overexpression.

208
209 ***Mutants with reduced ubiquitin pools are highly sensitive to AICAR but do not recapitulate***
210 ***the rad6/bre1 phenotypes***

211 We next asked whether AICAR treatment itself could impose an enhanced demand for
212 ubiquitin that would be exacerbated by the *uba1* mutation to a point leading to the observed
213 AICAR-hypersensitivity of this mutant. Several yeast mutants such as, *doa1* and *ubp6*, have
214 constitutively reduced free-ubiquitin pools, due to defective ubiquitin recycling (Amerik et
215 al., 2000; Johnson et al., 1995). The *doa1* and *ubp6* knock-out mutations were introduced in
216 the *ade16 ade17 ade8 his1* background allowing controlled accumulation of ZMP as a result
217 of feeding with AICAR (Hurlimann et al., 2011). Both mutations were found to lower the
218 ubiquitin pool (Fig. 3a) as previously reported (Amerik et al., 2000; Johnson et al., 1995) and
219 to severely impair growth of yeast cells in the presence of AICAR (Fig. 3b). Importantly this
220 growth defect could be robustly suppressed by overexpression of *UBI4* (Fig. 3b), indicating
221 that the low pool of ubiquitin was implicated in the exacerbated AICAR sensitivity of these
222 mutants.

223 We then examined whether the chemo-genetic interaction between AICAR and the *doa1* or
224 *ubp6* mutants was phenocopying the previously described synthetic lethality between ZMP
225 and the *rad6* or *bre1* mutants (Albrecht et al., 2016). Indeed, in a previous work, we found
226 that the ubiquitin conjugating enzyme Rad6 together with the ubiquitin ligase Bre1 were
227 critical for yeast cells to grow in the presence of AICAR (Albrecht et al., 2016). This growth
228 defect resulted from the lack of ubiquitylation of histone H2B in the absence of Rad6/Bre1
229 together with a cell cycle default associated to defective entry of the cyclin Cln3 in the
230 nucleus due to AICAR treatment (Albrecht et al., 2016). Since free ubiquitin is present in
231 limiting amounts in cells, changes in the ubiquitin pool directly affect ubiquitin modifications
232 (Groothuis et al., 2006) (Fig. 2d) and accordingly the free ubiquitin pool was found to impact
233 on histone ubiquitination in mammalian cells (Dantuma et al., 2006). Hence, we suspected
234 that *doa1* and *ubp6* mutants could directly affect H2B ubiquitination. Indeed, *doa1* and *ubp6*
235 mutants showed a decreased ubiquitination of H2B that could be restored by *UBI4*
236 overexpression (Fig. 3c). These results suggested that low-ubiquitin mutants could be
237 sensitive to AICAR in part because of the reduced ubiquitylation of H2B that could mimic the

238 *bre1* or *rad6* phenotype. This assumption was directly assessed by genetic means, i.e. by
239 comparing their suppression pattern. The rationale here was that if *doa1* and *ubp6* mutants are
240 sensitive to AICAR because they mimic *rad6* or *bre1*, they should be suppressed by the same
241 suppressors. As previously reported (Albrecht et al., 2016), *CLN3* overexpression could
242 efficiently suppress the AICAR growth-defect of the *bre1* mutant (Fig. 3b), however it had
243 little or no effect on *ubp6* or *doa1* mutations (Fig. 3b). We conclude that part of the effects of
244 *ubp6* or *doa1* mutations are likely due to their lower ability to ubiquitylate H2B, but that a
245 remaining part of their effect is independent of Rad6/Bre1 and can be suppressed by
246 increasing cellular ubiquitin (Fig. 3B). These results suggested that another effector in the
247 ubiquitin pathway was involved. We then aimed at identifying this effector.

249 ***Genetic analysis reveals new E2 and E3 AICAR-sensitive mutants***

250 Ubiquitin once activated by Uba1 is conveyed to ubiquitin-conjugating enzymes (E2s), which
251 in turn, with or without the help of specific ubiquitin ligases (E3s), transfer the ubiquitin
252 moiety to the target proteins (Fig. 1b). In yeast there are 11 E2s and dozens of E3s (Finley et
253 al., 2012). To further explore how AICAR affects the ubiquitylation pathway downstream of
254 Uba1 (E1), we started with a systematic genetic analysis of the ubiquitin conjugating enzymes
255 (E2s). Among the eleven yeast E2s, nine are encoded by non-essential genes (Finley et al.,
256 2012). The corresponding knock-out mutations were combined with the *ade16 ade17 ade8*
257 *his1* mutations allowing ZMP accumulation in yeast when yeast cells are fed with AICAR
258 (Hurlimann et al., 2011). While most E2 mutations did not affect the sensitivity to AICAR
259 accumulation (Fig. 4a), the *rad6 (ubc2)* knock-out strongly altered growth in the presence of
260 AICAR (Fig. 4a), thus confirming our previous report (Albrecht et al., 2016). In addition,
261 *ubc4* was also sensitive to AICAR accumulation (Fig. 4a) although to a lesser extent. Ubc4 is
262 known to be involved in protein degradation. In particular, Ubc4 coupled with Not4 poly-
263 ubiquitinates the H3K4 demethylase, Jhd2, leading to its degradation (Mersman et al., 2009).
264 Thus, Jhd2 stabilization in the *ubc4* mutant would lead to lower H3K4 methylation (Mersman
265 et al., 2009) hence phenocopying a defect in H3K4 methylation by Set1. Since Set1
266 methylation of H3K4 is dependent on H2B ubiquitylation by Rad6/Bre1 (Nakanishi et al.,
267 2009) a defective Ubc4 should mimic the *rad6/bre1* defect and should show the same
268 suppression pattern. Indeed, the *ubc4* mutant growth defect in the presence of AICAR was
269 robustly suppressed by *CLN3* overexpression but not by *UBI4* (Fig. 4b), hence phenocopying

270 a *bre1* mutant (Fig. 3d). This result thus phenotypically positioned the *ubc4* mutant in the
271 “*rad6/bre1/set1* pathway”.

272 We then examined the effect of a defect in the two remaining essential E2s, Ubc1 and
273 Ubc3/Cdc34, on AICAR sensitivity. We found that, in the S288c background, *UBC1* is
274 essential for germination but not for vegetative growth and we could thus construct a viable
275 *ade16 ade17 ade8 his1 ubc1* quintuple mutant. Clearly, *ubc1* knock-out did not affect growth
276 in the presence of AICAR (Fig. 4c) (compared to *set1* shown here as an AICAR-sensitive
277 control). For *cdc34*, AICAR-sensitivity was assayed on a temperature sensitive allele *cdc34-2*
278 (Liu et al., 1995) at semi-permissive temperature. The *cdc34-2* mutant was highly sensitive to
279 endogenous ZMP accumulation (Fig. 4d) or AICAR as an exogenous source of ZMP (Fig.
280 4e). Cdc34 catalyzes substrate ubiquitination via the Skp1-Cullin-F-box (SCF) ubiquitin
281 protein ligase complex in which the F-box proteins function as substrate-specific adaptors
282 (Feldman et al., 1997; Goebel et al., 1988; Skowrya et al., 1997). Cdc4 is one of these F-box
283 proteins and we asked whether it would be required for growth under conditions leading to
284 ZMP accumulation. The *cdc4-3* mutation was highly sensitive to ZMP accumulation whether
285 it was from an internal source (*ade16 ade17* background; Fig. 4f) or upon AICAR feeding
286 (*ade16 ade17 ade8 his1* background; Fig. 4g). We conclude that Cdc34 and Cdc4 define a
287 new ubiquitin sub-pathway required for cells to cope with AICAR.

289 *Suppression and additivity patterns unveil complex interactions between ZMP and the* 290 *ubiquitin pathway*

291 Phenotypic suppression of the newly identified *cdc34* and *cdc4* mutants by increased gene
292 dosage of *CLN3* or *UBI4* was evaluated. The AICAR sensitivity of the *cdc34-2* mutant was
293 suppressed by overexpression of *UBI4* but not by *CLN3* (Fig. 5a). By contrast, *cdc4-3* was
294 slightly suppressed by *CLN3* but not by *UBI4* increased gene dosage (Fig. 5b-c). Finally,
295 *CLB5* a known dosage suppressor of *cdc4* (Cui et al., 2002) indeed suppressed the high
296 sensitivity to AICAR of the corresponding *cdc4-3* temperature sensitive mutants (Fig. 5c) but
297 also that of a *cdc34-2* mutant (Fig. 5d). Still, *CLB5* overexpression had no effect on AICAR-
298 sensitivity of the *bre1*, *set1*, *doa1*, *ubp6* and *uba1-ol* mutants (Fig. 5e-i). Thus based on gene-
299 dosage suppression patterns, we can distinguish the various mutants of the ubiquitin pathway
300 leading to AICAR sensitivity (Fig. 6). A simple assumption derived from this model is that
301 mutations affecting the distinct “branches” should have additive effects. This was assayed by

302 constructing a *bre1 ubp6* strain capable of accumulating ZMP upon AICAR feeding (*ade16*
303 *ade17 ade8 his1* background). We found that the *bre1 ubp6* mutant was much more sensitive
304 to AICAR than a *bre1* or an *ubp6* strain alone at low AICAR concentration (2 mM, Fig. 7a).
305 This additivity indicates that the chemo-genetic interaction of these two mutants with AICAR
306 involves different functions. Accordingly, *UBI4* overexpression could still suppress a part, but
307 not all, of the sensitivity to AICAR of the *bre1 ubp6* strain, resulting in a level of AICAR-
308 sensitivity equivalent to that of a single *bre1* mutant (Fig. 7a). Additivity was also found for
309 the *set1* and *doa1* mutations (Fig. 7b) confirming the idea that these mutations result in
310 AICAR sensitivity through different means as deduced from their suppression pattern (Fig. 6).
311 Finally, co-overexpression of *CLN3* and *UBI4* in the *uba1-ol* mutant showed an additive
312 suppression effect (Fig. 7c) indicating that the *uba1* mutation affects both downstream
313 branches, as expected for a mutation affecting the enzyme which is at the apex of the
314 ubiquitin pathway. Interestingly, the *uba1-ol* mutant exhibited a strong defect in H2B
315 ubiquitination at semi-permissive temperature and this could be restored by *UBI4*
316 overexpression (Fig. 7d). Hence, while *UBI4* overexpression restored H2B ubiquitylation, it
317 only partially suppressed the *uba1-ol* growth phenotype (Fig. 7c) suggesting that the
318 Rad6/Bre1 branch might affect one or several other targets than H2B that would be
319 suppressed by *CLN3* overexpression but not or only partially by *UBI4* overexpression. We
320 conclude that ZMP accumulation results in multiple chemo-genetic interactions with the
321 ubiquitin pathway in yeast, thus illustrating the complexity of drug action and the helpfulness
322 of genetics to address it.

324 DISCUSSION

325 In a previous work we identified histone ubiquitylation mutants as synthetic lethal with
326 AICAR treatment (Albrecht et al., 2016). Here we further uncovered phenotypic interactions
327 between AICAR/ZMP and the ubiquitin pathway and identified several new mutants (*uba1*,
328 *ubc4*, *cdc34*, *cdc4*, *ubp6* and *doa1*) showing exacerbated sensitivity to AICAR. Differential
329 patterns of gene dosage suppression as well as phenotypic additivity revealed that these
330 mutants affect distinct branches, hence suggesting that AICAR interferes with one or several
331 functions particularly required when the ubiquitin pathway is impaired. Among the mutants
332 studied here and in our previous study, *uba-ol* was the only one resulting in synthetic lethality
333 with the *ade16 ade17* mutations leading to constitutive ZMP accumulation as revealed by our

334 inability to recover viable spores. This pronounced sensitivity could reflect the fact that Uba1
335 (E1) is at the apex of the ubiquitin pathway and therefore conditions all downstream ubiquitin
336 reactions. Among the 11 ubiquitin conjugating enzyme (E2) mutants, three (*rad6*, *ubc4* and
337 *cdc34*) were affected by the drug hence revealing the major pathways downstream of Uba1
338 that are required for yeast cells to resist AICAR. Following-up on Cdc34, we could identify
339 the Cdc4 ubiquitin ligase (E3) as required for AICAR tolerance.

340 Why is AICAR toxic for ubiquitin pathway mutants? We have shown in this work that the
341 abundance of ubiquitin itself is an important factor in the drug toxicity though we
342 demonstrated that AICAR does not directly affect it. Clearly in the case of *doa1* and *ubp6* it is
343 the low ubiquitin pool that is responsible for the exacerbated toxicity of AICAR. It is likely
344 that more than one function is involved in the phenotype and indeed these mutants were not
345 efficiently suppressed by overexpression of neither *CLN3* nor *CLB5*. For *cdc34* and *cdc4*,
346 both mutants were suppressed by *CLB5* overexpression, while this was not the case for the
347 other AICAR-sensitive mutants, suggesting that a negative effector stabilized in these mutants
348 could be displaced by *CLB5* overexpression. It remains to be established whether the multiple
349 connections, between AICAR and the ubiquitin pathway, revealed here, are more than
350 serendipitous i.e. whether there is a common target that when affected by ZMP results in
351 synthetic toxicity with the various ubiquitin mutants. The fact, that up to now, we identified
352 no common gene dosage suppressor to the various mutants, does not support this hypothesis.
353 However, Uba1 was found among ZMP-binders by affinity chromatography (our unpublished
354 results) and could therefore be a direct and central target. In any case, the strong connections
355 between AICAR and the ubiquitin pathway could be highly relevant to understand why
356 AICAR selectively inhibits proliferation of aneuploid cells (Tang et al., 2011). Indeed,
357 aneuploidy is known to result in proteotoxicity (Donnelly and Storchova, 2015; Santaguida
358 and Amon, 2015) and as such could impose a strong demand on the ubiquitin pathway. This
359 work thus provides a lead for further work on AICAR selective effects on aneuploid cells.

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488 **Table 1** Yeast strains used in this study

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Strain	Genotype	References
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Euroscarf
BY4742	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	Euroscarf
Y1093	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 ade16::KanMX4 ade17::KanMX4</i>	(Hurlimann et al., 2011)
Y1162	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 ade16::KanMX4 ade17::KanMX4</i>	(Hurlimann et al., 2011)
Y2950	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4</i>	(Pinson et al., 2009)
Y3273	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cdc4-3</i>	(Li et al., 2011)
Y3275	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cdc34-2</i>	(Li et al., 2011)
Y6853	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 uba1-o1</i>	(Li et al., 2011)
Y6987	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 uba1-o1</i>	This study
Y7070	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 ade17::KanMX4 uba1-o1</i>	This study
Y7071	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 ade16::KanMX4 uba1-o1</i>	This study
Y7117	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 ade16::KanMX4 ade17::KanMX4 uba1-o1 [ptet-UBA1]</i>	This study
Y8033	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 uba1-o1 [ptet-empty, CEN LEU2]</i>	This study
Y8385	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 ade16::KanMX4 ade17::KanMX4 cdc4-3</i>	This study
Y8406	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 ade16::KanMX4 ade17::KanMX4 cdc34-2</i>	This study
Y8675	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 can1::KanMX4 cdc4-3</i>	This study
Y8679	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 cdc34-2</i>	This study
Y9168	<i>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 set1::KanMX4</i>	(Albrecht et al., 2016)
Y9217	<i>MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 rad6(ubc2)::KanMX4</i>	This study
Y9516	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4</i>	This study

	<i>ade17::KanMX4 his1::KanMX4 bre1::kanMX4</i>	
Y9915	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 ubc11::URA3</i>	This study
Y9923	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 ubc5::KanMX4</i>	This study
Y9925	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 ubc10::URA3</i>	This study
Y9929	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 ubc13::URA3</i>	This study
Y9931	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 ubc8::URA3</i>	This study
Y9964	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 ubc7::KanMX4</i>	This study
Y10034	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 ubc6::LEU2</i>	This study
Y10072	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 ubc4::KanMX4</i>	This study
Y10296	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 ubp6::KanMX4</i>	This study
Y10524	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 trp1::LEU2 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 set1::URA3</i>	This study
Y10696	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 doa1::KanMX4</i>	This study
Y10757	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 bre1::KanMX4 ubp6::KanMX4</i>	This study
Y10817	<i>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ubc1::KanMX4</i>	This study
Y10840	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 ubc1::KanMX4</i>	This study
Y11352	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 uba1-ol</i>	This study
Y11389	<i>MATα his3Δ1 leu2Δ0 trp1::LEU2 ura3Δ0 ade8::KanMX4 ade16::KanMX4 ade17::KanMX4 his1::KanMX4 doa1::KanMX4 set1::URA3</i>	This study

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492 **Table 2** Plasmid used in this study

Plasmid	Backbone	characteristics	Sources
pCM189	pCM189	<i>CEN ARS URA3 tet-OFF promoter</i>	(Gari et al., 1997)
pFL44L	pFL44L	2 micron <i>URA3</i>	(Bonneaud et al., 1991)
YCpLac111	YCplac111	<i>CEN ARS LEU2</i>	(Gietz and Sugino, 1988)
YEplac 181	YEplac 181	2 micron <i>LEU2</i>	(Gietz and Sugino, 1988)
YEplac 195	YEplac 195	2 micron <i>URA3</i>	(Gietz and Sugino, 1988)
p4515	pCM189	<i>CEN ARS URA3 tet-OFF promoter-UBA1</i>	This study
p4742	YEplac 195	<i>CLN3</i> 2 micron <i>URA3</i>	(Albrecht et al., 2016)
p4807	pFL44L	2 micron <i>URA3 RPL40B PTR2</i>	This study
p4814	pFL44L	2 micron <i>URA3 RPL40A</i>	This study
p4859	YEplac 195	2 micron <i>URA3 DDPI</i>	This study
p4919	YEplac 195	2 micron <i>URA3 URA6</i>	This study
p4943	YCplac111	<i>UBA1 CEN ARS LEU2</i>	This study
p5183	pFL44L	2 micron <i>URA3 ECM21</i>	This study
p5504	YEplac 195	<i>UBI4</i> 2 micron <i>URA3</i>	This study
p5516	YEplac 181	<i>UBI4</i> 2micron <i>LEU2</i>	This study
p5791	YEplac 195	<i>CLB5</i> 2 micron <i>URA3</i>	This study

495 **Legend of figures**

1 496

2 497 **Fig. 1** Schematic representation of the *de novo* purine and histidine pathways (**a**) and the
3 498 ubiquitin cascade (**b**) in yeast. **a** Only the enzymes mentioned in the text are shown (in green).
4 499 AICAR: 5-Amino-Imidazole 4-CarboxAmide Ribonucleoside; AMP: adenosine 5'-
5 500 monophosphate; GMP: Guanosine 5'-monophosphate; IMP: Inosine 5'-monophosphate;
6 501 PRPP: α -D-ribofuranose 5-phosphate 1-diphosphate; ZMP: AICAR monophosphate. **b** E1, E2
7 502 and E3 stand for ubiquitin-activating enzyme, ubiquitin-conjugating enzymes and ubiquitin-
8 503 protein ligases, respectively. Target: protein target. Ub: ubiquitin.

9 504

10 505 **Fig. 2** AICAR toxicity in the hypomorphic *uba1-ol* mutant is alleviated by ubiquitin
11 506 overexpression. **a** The *ade16 ade17 uba1-ol* is viable only if *UBA1* is ectopically expressed.
12 507 Wild-type (BY4742) and mutant (*ade16 ade17*: Y1093; *uba1-ol*: Y6853; *ade17 uba1-ol*:
13 508 Y7070; *ade16 uba1-ol*: Y7071 and *ade16 ade17 uba1-ol*: Y7117) strains containing a
14 509 plasmid allowing expression of *UBA1* gene under the control of a doxycycline-repressible
15 510 promoter (p4515) were grown on SDcasaWA medium containing (+) or not (-) doxycycline
16 511 (Dox;100 mg/l). **b** Growth of the *uba1-ol* mutant is impaired at semi-permissive temperature
17 512 when ZMP is accumulated by exogenous addition of its riboside precursor AICAR in the
18 513 *ade8 ade16 ade17 his1* (Quad) genetic background. Strains (Y6987, Y2950 and Y8033) were
19 514 grown for 48 hours at indicated temperatures on SDcasaWAU medium containing or not
20 515 AICAR (10 mM). **c** Overexpression of gene-dosage suppressors restored growth of the *uba1-*
21 516 *ol* mutant in the presence of AICAR. The *ade8 ade16 ade17 his1 uba1-ol* quintuple mutant
22 517 was transformed with plasmids allowing overexpression of indicated genes (*ECM21*: p5183;
23 518 *URA6*: p4919; *DDP1*: p4859; *RPL40B*: p4807; *RPL40A*: p4814 and *UBI4*: p5504) or with an
24 519 empty vector (YepLac195). Growth of transformants was scored for 72 h at 32°C on
25 520 SDcasaWA medium in the presence or not of AICAR (6 mM). **d** Free ubiquitin and ubiquitin-
26 521 conjugated signals were strongly increased when *UBI4* gene is overexpressed (OE). Strains
27 522 (Y2950 and Y8033) were transformed with a plasmid allowing *UBI4* overexpression (p5504)
28 523 or the cognate empty vector (YEplac195). Total protein extracts were prepared from
29 524 transformants, overexpressing (+) or not (-) *UBI4*, grown in SDcasaWA medium and treated
30 525 (+) or not (-) for 2 hours with AICAR (1 mM). Proteins were revealed by western blotting
31 526 with anti-Ubi and anti-Act1 antibodies. Arrow points to free ubiquitin. Expo: Overexposure of
32 527 the dashed box region revealed with anti-Ubi antibody.

33 528

529 **Fig. 3** Mutants with low ubiquitin are highly sensitive to AICAR. **a** Conjugated ubiquitin is
1 530 drastically decreased in *ubp6* and *doa1* mutants. Total protein extracts were obtained from
2 531 strains (Y2950 (Control), Y10296 and Y10696) grown in SDcasaWAU medium, separated by
3 532 SDS-page and ubiquitin-conjugated proteins were revealed by western blotting with an anti-
4 533 ubiquitin antibody. Act1 was used as a loading control. **b** Overexpression of *UBI4* is
5 534 sufficient to restore growth of the *ubp6* and *doa1* mutants in the presence of AICAR. Yeast
6 535 strains (Y2950 (Control), Y10696, Y10296 and Y9168) were transformed with plasmids
7 536 allowing overexpression (OE) of either *UBI4* (p5504) or *CLN3* (p4742), or with the empty
8 537 vector (none, YEplac195). Transformants were grown at 37 °C for 48 h on SDcasaWA
9 538 medium containing or not AICAR (2 mM). **c** Ubiquitylation of H2B is increased when *UBI4*
10 539 is overexpressed (OE). Total protein extracts were obtained on transformants from Fig. 3b.
11 540 H2B pools were revealed by western blotting using an anti-H2B antibody. Act1 was used as a
12 541 loading control. Quantifications were set at 1 for the control strain transformed with the empty
13 542 vector (-). ND: not detectable.
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16 544 **Fig. 4** Different “ubiquitin sub-pathways” are required for yeast to cope with AICAR toxicity.
17 545 **a** Systematic analysis of AICAR-sensitivity of ubiquitin-conjugating enzyme (E2) mutants.
18 546 Control (Y2950) and mutant (Y9217, Y10072, Y9923, Y10034, Y9964, Y9931, Y9925,
19 547 Y9915 and Y9929) strains were grown in SDcasaWAU medium for 48 h at 30°C in the
20 548 presence or absence of AICAR. **b** The AICAR-growth defect of *ubc4* mutant is suppressed by
21 549 *CLN3* but not *UBI4* overexpression. Strains (Y2950, Y10072) were transformed with
22 550 plasmids overexpressing either *CLN3* or *UBI4*, or by the empty vector (none). Transformants
23 551 were grown in SDcasaWAU medium for 48 h at 30°C. **c** AICAR-sensitivity is not affected in
24 552 an *ubc1* deletion mutant. Strains (Y10817, Y2950: control, Y10840, Y9168) were grown for
25 553 48 h at 30°C on SDcasaWAU medium containing or not AICAR. **d,f** Growth of the *cdc34-2*
26 554 (**d**) and the *cdc4-3* (**f**) mutants is severely impaired by endogenous ZMP accumulation.
27 555 Strains (Y1162, Y3273, Y3275, Y8385 and Y8406) were grown for 48 h on SDcasaWAU
28 556 medium. **e,g** The *cdc34-2* (**e**) and *cdc4-3* (**g**) mutants are highly sensitive to AICAR treatment.
29 557 Strains were grown for 48 h at 32 °C on SDcasaWAU medium containing or not AICAR.
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32 559 **Fig. 5** Gene-dosage suppression of AICAR-sensitivity of the *cdc4-3* and *cdc34-2* mutants.
33 560 Yeast strains (Y8679 (**a, d**), Y8675 (**b, c**), Y9516 (**e**), Y9168 (**f**), Y10696 (**g**), Y10296 (**h**) and
34 561 Y8033 (**i**)) were transformed with plasmids allowing overexpression (OE) of either *CLB5*
35 562 (p5791), *CLN3* (p4742) or *UBI4* (p5504) genes, or with an empty plasmid (YEplac195,
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563 none). In all panels, transformants were grown on SDcasaWA medium containing or not
1 564 AICAR at 30°C (**b**) 32°C (**c, i**), 33°C (**a, d**) or 37°C (**e-h**).

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7 567 **Fig. 6** Schematic representation of the “ubiquitin sub-pathways” revealed by gene-dosage
8 suppression of the AICAR-sensitivity. Mutant names in white-bold letters correspond to the
9 568 AICAR-sensitive or -insensitive mutants characterized in this study.

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14 571 **Fig. 7** Combination of AICAR-sensitive mutants and gene dosage suppressors revealed
15 complex genetic relationships between AICAR-sensitivity and ubiquitin pathways. **a** The
16 572 double *ubp6 bre1* mutant is hypersensitive to AICAR treatment. Yeast strains (Y10757,
17 Y2950, Y9516 and Y10296) were transformed with the *UBI4* overexpressing plasmid or the
18 573 empty vector (none). Transformants were grown on SDcasaWA medium at 37°C for 48 h. **b**
19 Additive AICAR sensitivity was observed by combining *set1* and *doal* mutations. Strains
20 574 (Y2950, Y10696, Y10524 and Y11389) were grown in SDcasaWAU medium for 72 h at
21 37°C. **(c)** Double *CLN3* and *UBI4* overexpression is required to restore growth of the *ade8*
22 575 *ade16 ade17 his1 uba1-o1* in the presence of AICAR. Yeast strain (Y11352) was co-
23 transformed with plasmids overexpressing or not (empty vectors) *CLN3* and *UBI4* genes.
24 576 Transformants were grown for 72 h on SC medium lacking leucine and uracil. **(d)** The
25 577 ubiquitylation-defect of H2B in the *ade8 ade16 ade17 his1 uba1-o1* is restored by *UBI4*
26 overexpression. Yeast strains (Y2950, Y8033 and Y9516) were transformed with a plasmid
27 578 overexpressing *UBI4* (+) or the cognate empty vector (-). Total protein extracts were obtained
28 on transformants grown in SDcasaWA medium at 32 °C and treated (+) or not (-) with
29 579 AICAR (1 mM; 2 h). H2B and Act1 (loading control) were revealed by western blotting.
30 Quantification was set at 1 for the control strain transformed with the empty vector (-). ND:
31 580 not detectable.

47 589

Fig. 1

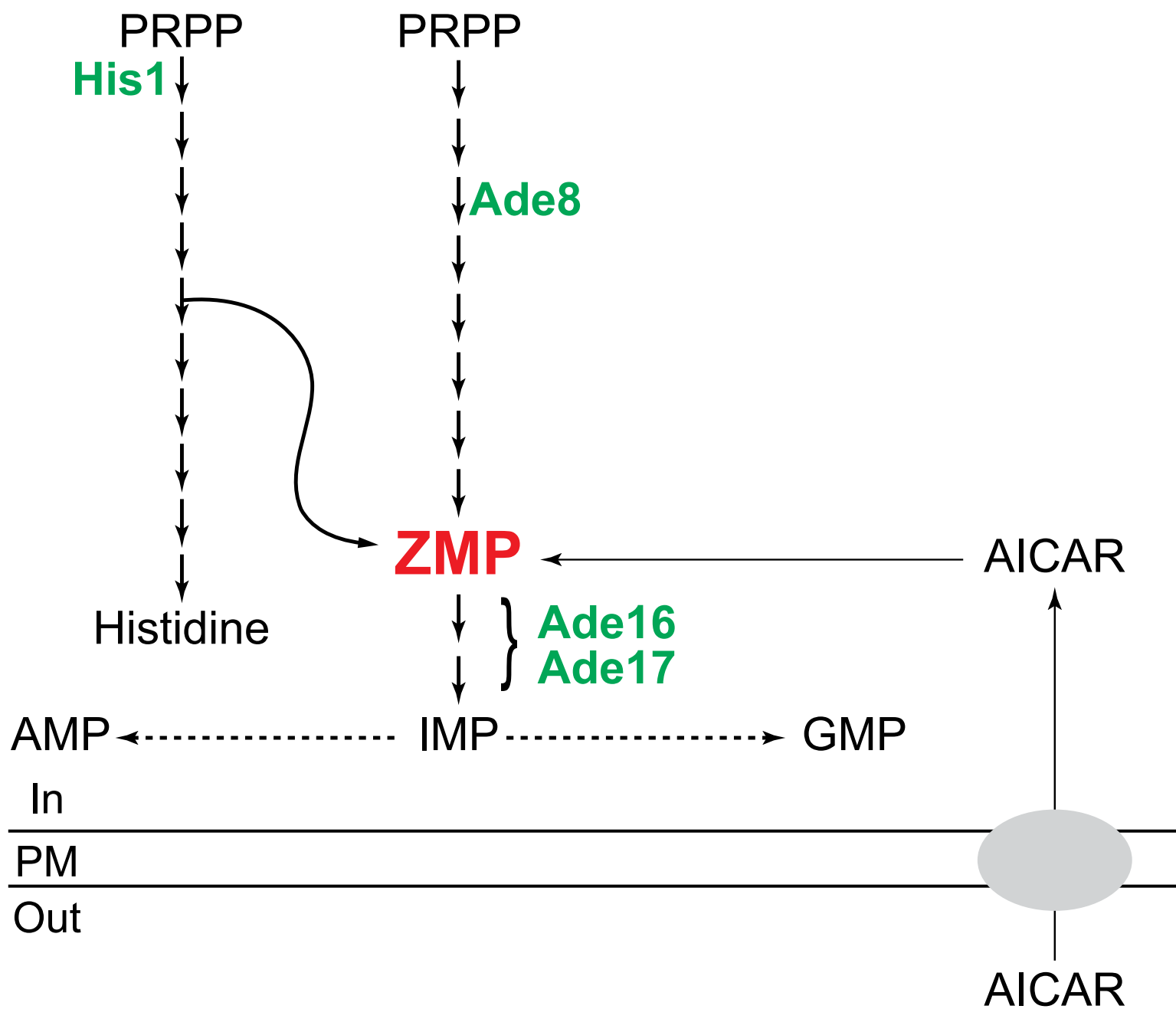
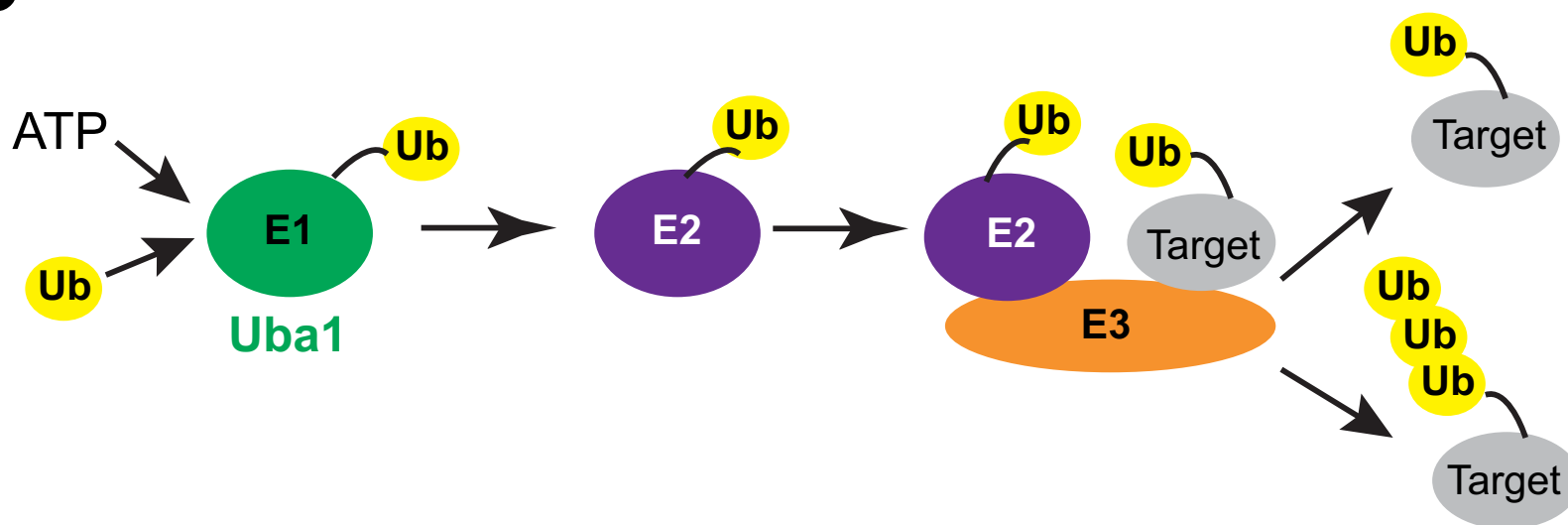
a**b**

Fig. 3

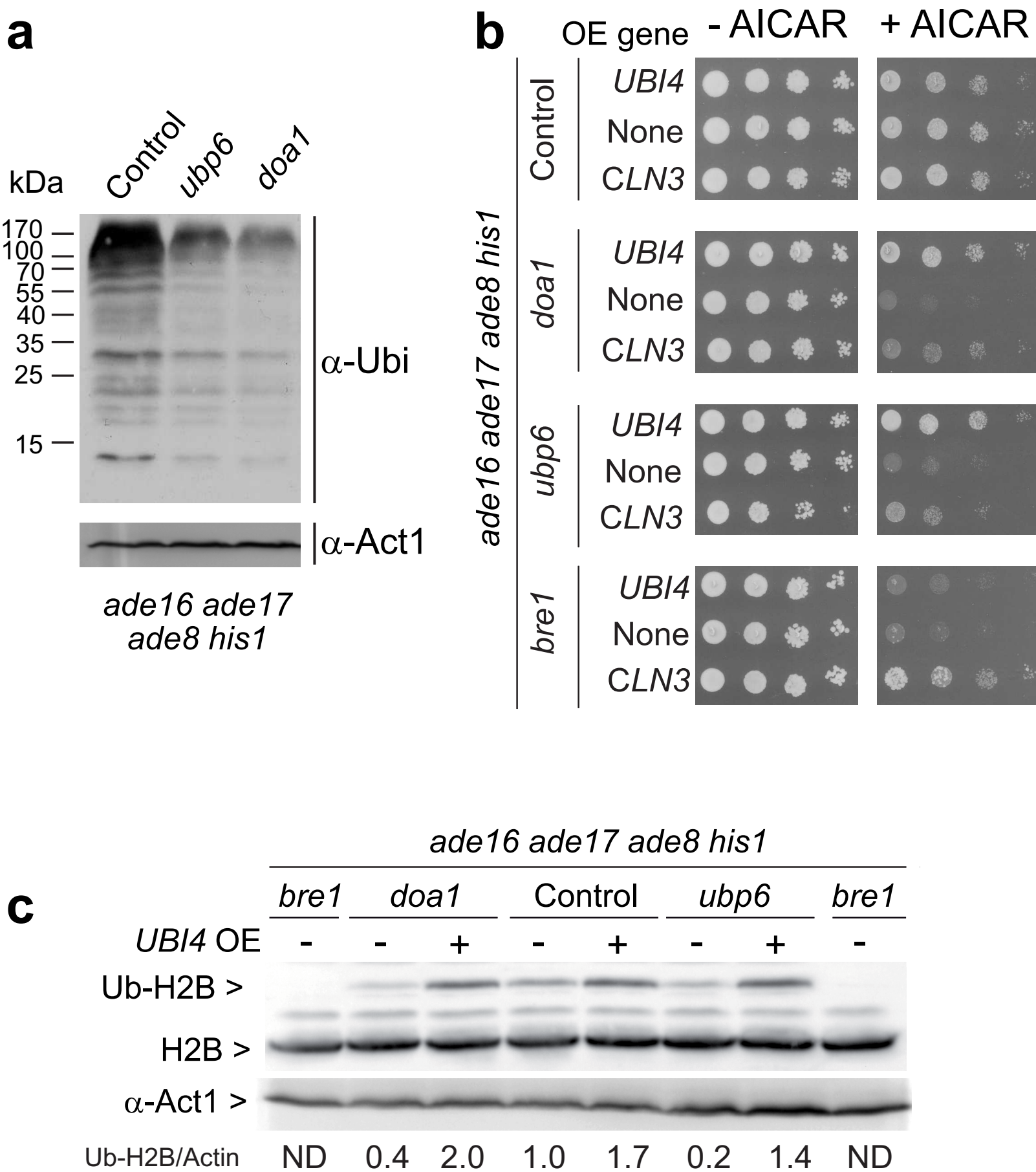


Fig. 4

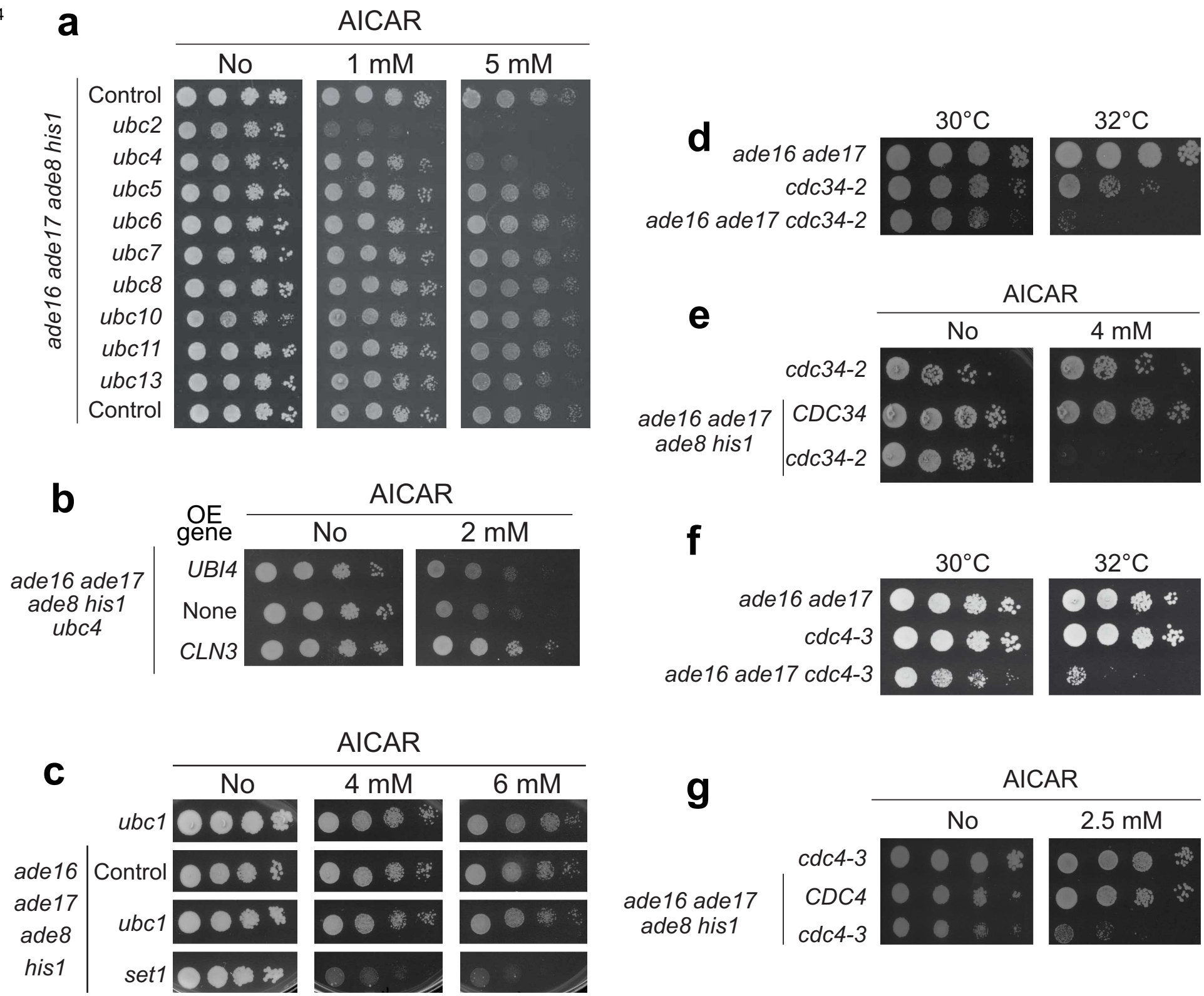
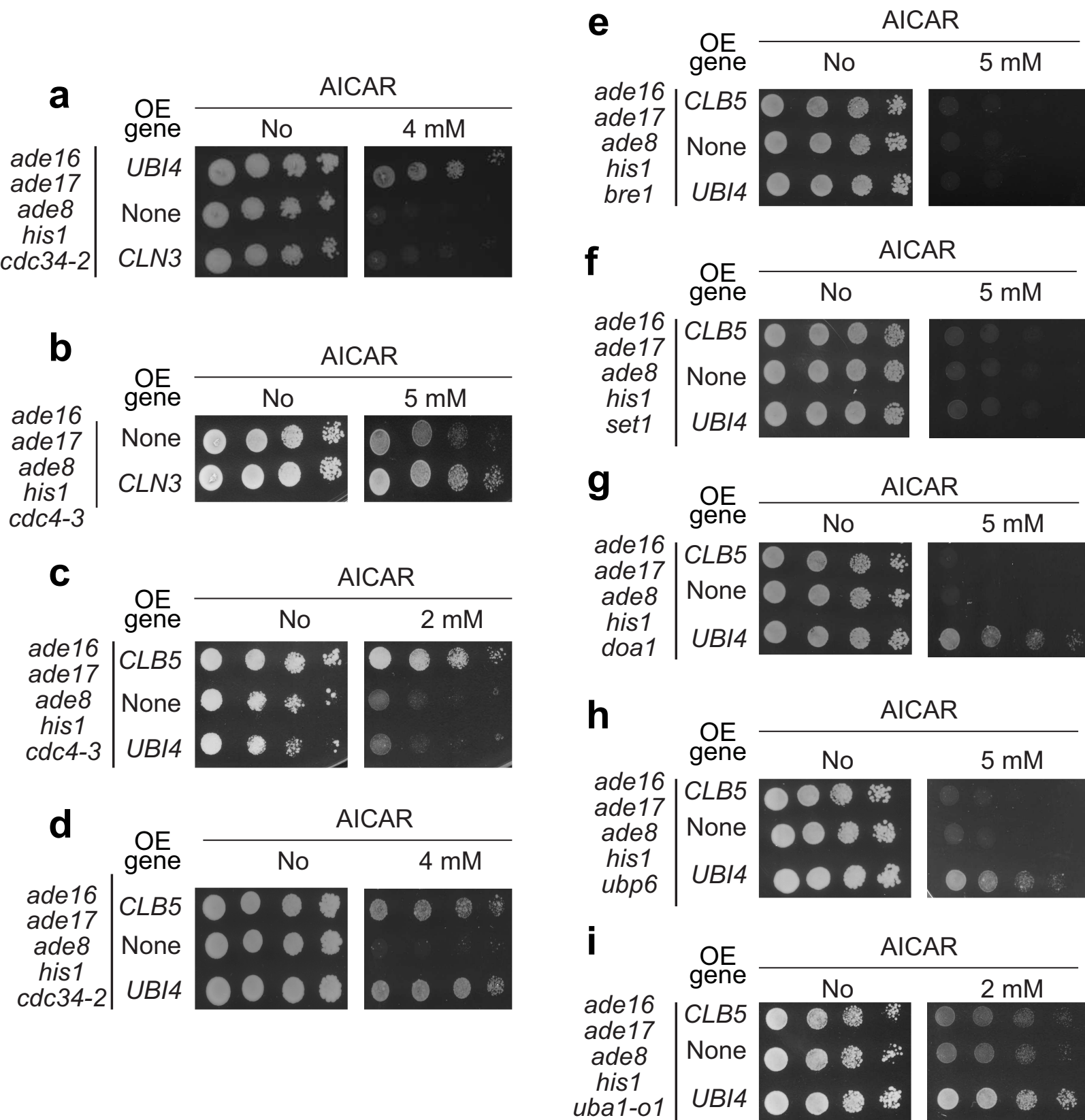


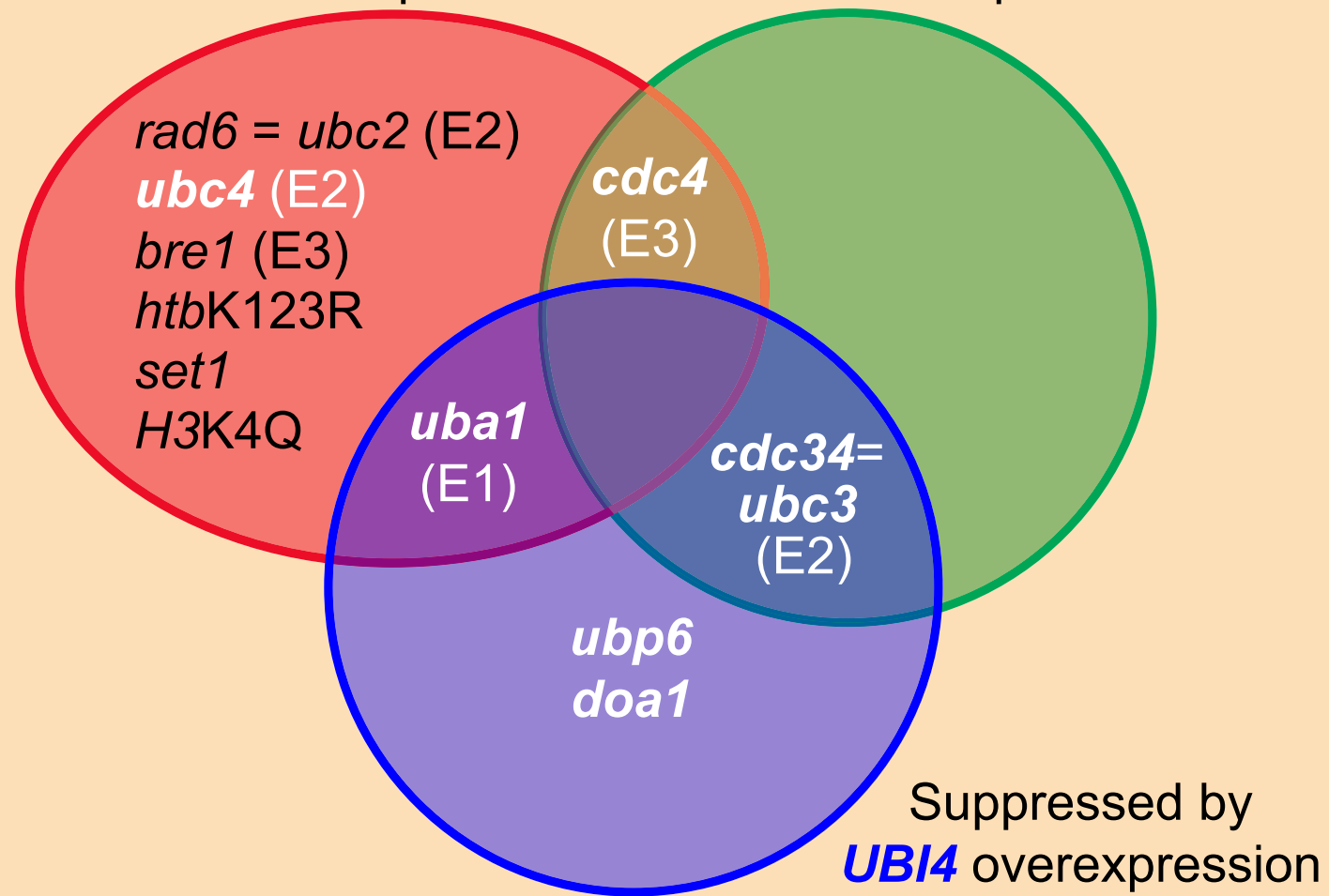
Fig. 5



AICAR-sensitive mutants

Suppressed by **CLN3** overexpression

Suppressed by **CLB5** overexpression



AICAR-insensitive mutants

- ubc1* (E2)
- ubc5* (E2)
- ubc6* (E2)
- ubc7* (E2)
- ubc8* (E2)
- ubc10* (E2)
- ubc11* (E2)
- ubc13* (E2)

