

1 **Endogenous formaldehyde scavenges cellular glutathione resulting in cytotoxic redox disruption**

2 Carla Umansky^{1,6}, Agustín Morellato^{1,6}, Marco Scheidegger^{1,6}, Matthias Rieckher^{2,6}, Manuela R. Martinefski³,
3 Gabriela A. Fernandez³, Ksenia Kolesnikova² Anna J. Vesting⁴, Ismene Karakasilioti⁴, Hernán Reingruber¹, Yan
4 Wei⁵, Rongqiao He⁵, Mariela Bollini³, María Eugenia Monge³, Björn Schumacher² and Lucas B. Pontel^{1,*}

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6 1. Instituto de Investigación en Biomedicina de Buenos Aires (IBioBA), CONICET - Partner Institute of the
7 Max Planck Society, C1425FQD, Buenos Aires, Argentina.

8 2. Institute for Genome Stability in Ageing and Disease, Medical Faculty, University of Cologne, and
9 Cologne Excellence Cluster for Cellular Stress Responses in Aging-Associated Diseases (CECAD), and
10 Center for Molecular Medicine Cologne (CMMC), 50931 Cologne, Germany.

11 3. Centro de Investigaciones en Bionanociencias (CIBION), Consejo Nacional de Investigaciones
12 Científicas y Técnicas (CONICET), C1425FQD, Buenos Aires, Argentina.

13 4. Max Planck Institute for Metabolism Research, Cologne Excellence Cluster on Cellular Stress
14 Responses in Aging-associated Diseases (CECAD), Center for Endocrinology, Diabetes and Preventive
15 Medicine (CEDP), 50931 Cologne, Germany.

16 5. State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, Chinese Academy of
17 Sciences, Beijing 100101, China; CAS Key Laboratory of Mental Health, Institute of Psychology, Chinese
18 Academy of Sciences, Beijing 100101, China.

19 6. These authors contributed equally

20 *Correspondence: lpontel@ibioba-mpsp-conicet.gov.ar

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24

25 **Abstract**

26 Formaldehyde (FA) is a ubiquitous endogenous and environmental metabolite that is thought to exert
27 cytotoxicity through DNA and DNA-protein crosslinking. We show here that FA can cause cellular damage
28 beyond genotoxicity by triggering oxidative stress, which is prevented by the enzyme alcohol dehydrogenase
29 5 (ADH5/GSNOR). Mechanistically, we determine that endogenous FA reacts with the redox-active thiol group
30 of glutathione (GSH) forming S-hydroxymethyl-GSH, which is metabolized by ADH5 yielding reduced GSH thus
31 preventing redox disruption. We identify the *ADH5*-ortholog gene in *Caenorhabditis elegans* and show that
32 oxidative stress also underlies FA toxicity in nematodes. Moreover, we show that endogenous GSH can protect
33 cells lacking the Fanconi Anemia DNA repair pathway from FA, which might have broad implications for
34 Fanconi Anemia patients and for healthy *BRCA2*-mutation carriers. We thus establish a highly conserved
35 mechanism through which endogenous FA disrupts the GSH-regulated cellular redox homeostasis that is
36 critical during development and aging.

37 **Keywords:** Glutathione, Formaldehyde, Oxidative Stress, Fanconi Anemia, ADH5, GCLM, p53, S-
38 hydroxymethylglutathione, Cancer, Genotoxicity.

39

40 **Introduction**

41 FA is a potent genotoxin classified by the World Health Organization (WHO) as a human carcinogen¹.
42 In the body, FA can originate from cellular metabolism, i.e. histone and DNA demethylation reactions or the
43 one-carbon cycle; it can also arise from the diet and it is ubiquitously found in the environment²⁻⁵. Indeed, this
44 aldehyde is more abundant in the body than previously thought; different works have reported FA
45 quantification in healthy human blood samples with levels in the 10-50 μ M range^{6,7}. Endogenous FA has been
46 suggested as a causative agent for several human diseases such as Fanconi Anemia and the Ruij-Aalfs
47 syndrome, and it might drive cancer in *BRCA2*-mutation carriers^{5,8}. Indeed, Fanconi Anemia patients carrying
48 a mutation in the acetaldehyde/FA metabolizing gene *ALDH2* present accelerated progression of bone marrow
49 failure (BMF)⁹⁻¹¹. Moreover, mice lacking *ADH5* and the Fanconi Anemia DNA repair pathway show severe

50 BMF, liver and kidney dysfunction and early cancer onset⁹, indicating that endogenous FA can drive cancer
51 initiation and Fanconi Anemia phenotypes.

52 Genotoxicity has been widely indicated as the main consequence of FA reactivity in cells⁴. However,
53 the strong reactivity of the FA carbonyl group might also affect other molecules than DNA. *In vitro*, the
54 spontaneous electrophilic attack of the FA carbonyl group to the thiol-group of GSH leads to the formation of
55 the covalent product S-hydroxymethyl-GSH (HSMGSH)¹². This reaction might be strongly favored inside cells,
56 where GSH levels are in the millimolar range¹³. Accordingly, ADH5 metabolizes HSMGSH yielding formate,
57 which is directed to the one-carbon cycle for nucleotide synthesis³ (Fig. 1A).

58 Considering the electrophilicity of FA and the abundance of GSH we hypothesize that the reaction
59 between FA and GSH might affect the GSH pool having detrimental biological consequences. Indeed,
60 alterations in GSH homeostasis have been reported in multiple pathologies such as hemolytic anemia,
61 diabetes, liver diseases, cystic fibrosis, neurodegeneration and cancer¹⁴⁻¹⁷. GSH not only neutralizes reactive
62 oxygen species (ROS), but can also promote chemoresistance by forming GSH-xenobiotic conjugates that are
63 pumped out of the cell *via* multiple resistance-associated protein transporters (MRP)¹⁸. To replenish
64 intracellular GSH, cells synthesize GSH in a two-step metabolic pathway centered on the rate-limiting enzyme
65 glutamate cysteine ligase (GCL), which is composed of a catalytic (GCLC) and a regulatory (GCLM) subunit, and
66 the GSH synthetase (GS) (**Fig. 1a**)¹⁸. Cells might thus also need to maintain the balance between GSH and the
67 oxidized GSH disulfide form (GSSG) -GSH:GSSG- to limit free FA and to prevent redox disruption.

68 We report here that FA toxicity is inflicted by the reaction between FA and the redox-active thiol group
69 present in GSH, which disables the antioxidant property of GSH. Our data also support a previously
70 unrecognized function of GSH in the protection against FA toxicity, and an evolutionary conserved mechanism
71 that maintains GSH:GSSG balance by salvaging reduced GSH from FA-GSH covalent adducts. These data might
72 have wide implications not only for Fanconi Anemia patients and *BRCA2*-mutation carriers but also for cancer
73 cells that would have to overcome blood FA level for a successful disease progression^{8,9}.

74

75 Results

76 ADH5 prevents FA toxicity in human cancer cells

77 FA levels in blood from different species have been reported in the 10-50 μM range (Reingruber and
78 Pontel, 2018 and references therein). With the aim to determine the amount of FA in blood, we set out to
79 measure this aldehyde in serum samples from 6-month old mice. FA was detected and quantified in mouse
80 blood with a mean concentration of $9.95 \pm 1 \mu\text{M}$ ($n=14$), which is in the same order as values reported for
81 healthy human blood^{6,7}(**Fig. 1b**). In mice, ADH5 limits the toxicity of FA by converting it into the less toxic
82 formate. To address whether cancer cells also rely on ADH5 activity to prevent FA toxicity, we inactivated the
83 *ADH5* gene in HCT116 human colorectal carcinoma cells by CRISPR/Cas9 (**Extended Data Fig. 1a,b**). *ADH5*-
84 deficient cells were not able to form tumor-spheroids in presence of FA, and they became sensitive to levels
85 of FA near to those present in human blood (**Extended Data Fig. 1c,d**). Moreover, ADH5 prevented the early
86 apoptosis markers Annexin V, a blockage of the cell cycle at G2/M phase and sub-G1 DNA accumulation (**Fig.**
87 **1e,f and Extended Data Fig. 1c**), indicating that ADH5 limits FA-triggered cell death. Consistently,
88 lymphoblastic leukemia Nalm6 cells lacking *ADH5* also presented strong sensitivity to blood FA levels,
89 indicating that ADH5 protects unrelated human cancer cells from FA toxicity (**Extended Data Fig. 1d**).

90 p53 orchestrates a FA response

91 Cell death can be a consequence of extensive damage to cellular components such as DNA, which
92 might be detected by cell-fate regulators like p53¹⁹. Indeed, p53 has been shown to trigger a cellular response
93 leading to acetaldehyde-mediated cell death in hematopoietic cells deficient in the Fanconi Anemia DNA
94 crosslink repair pathway¹⁰. We set out to determine whether p53 could also orchestrate a cellular response
95 to FA in HCT116 cells proficient for DNA repair leading to cell death. Surprisingly, the simultaneous inactivation
96 of *P53* and *ADH5* only slightly suppressed the cytotoxicity of FA observed in HCT116 $\Delta ADH5$ cells (**Fig. 2a and**
97 **Extended Data Fig. 1e,f**). In contrast, we found that inactivating *P53* significantly suppressed the severe
98 colony-formation phenotype detected in $\Delta ADH5$ cells at FA concentrations as low as 12.5 μM but only mildly
99 restored the formation of colonies at 25 μM FA (**Fig. 2b**), suggesting that FA can trigger cell death by both p53-

100 dependent and independent pathways. HCT116 cells are proficient for the Fanconi Anemia DNA crosslink
101 repair pathway, which might limit lethal FA genotoxicity. We therefore interrogated whether DNA damage
102 was leading to a p53 response and to the accumulation of the double-strand break marker γ -H2AX. We
103 detected p53 phosphorylation, indicative of the activation of p53, in $\Delta ADH5$ but not in Wild type (WT) cells
104 (**Fig. 2c,d and Extended Data Fig. 1e**), which correlated with cell cycle blockage at G2/M phase (**Fig. 1f**).
105 However, we could not detect a significant induction of γ -H2AX by blood-FA levels neither in WT nor in $\Delta ADH5$
106 cells (**Fig. 2c,d**). In contrast to FA treatment, exposure to the DNA-damaging drugs cisplatin, hydroxyurea (HU)
107 or mitomycin C (MMC) resulted in a profound induction of those DNA-damage markers (**Fig. 2c,d**). To confirm
108 that a 48-h exposure to micromolar levels of FA is not lethally genotoxic for cells proficient in DNA repair, we
109 addressed genome instability by direct visualization of single chromosome damage (**Fig. 2e,f**). Indeed, we
110 found that most of the metaphases in WT as well as in $\Delta ADH5$ cells were normal and only few of them
111 presented chromosome damage. In stark contrast, severe chromosome damage was evident upon treatment
112 with the DNA crosslinking agent mitomycin C, thus suggesting that when DNA repair is functional, FA might be
113 causing cell death by damaging other cellular components than DNA.

114 **Oxidative stress underlies FA cytotoxicity**

115 With the aim of discovering physiologically relevant cellular targets of FA, we reasoned that the strong
116 avidity of the FA-carbonyl group toward electron-rich thiol groups might affect the antioxidant GSH. Indeed,
117 the reaction between FA and the thiol group in GSH would block the redox capability of GSH, impairing its
118 redox function. Moreover, the abundance of GSH (1-10 mM) might favor the spontaneous reaction between
119 GSH and FA, which, if not limited, could diminish cellular GSH levels leading to oxidative stress. We therefore
120 measured the cellular oxidative status by quantifying the oxidation of the probe 2',7'-
121 dichlorodihydrofluorescein diacetate (H2DCFDA). Interestingly, FA induced a significant oxidation of H2DCFDA
122 in $\Delta ADH5$ cells (**Fig. 3a,b**). This oxidation level was comparable to that observed when exposing cells to the
123 GSH-synthesis inhibitor L-buthionine-sulfoximine (L-BSO), and could be reverted by expressing ADH5 *in trans*.
124 In order to test this more thoroughly, we incorporated the genetically-encoded cytosolic ROS sensor roGFP²⁰.

125 Exposure to FA induced a population of cells in which the sensor is oxidized in the absence of *ADH5*. These
126 results indicate that FA detoxification is necessary to prevent oxidative stress (**Fig. 3c,d**).

127 To address the causal contribution of FA-induced oxidative stress to cell death, we set out to test
128 whether cell toxicity could be rescued by the antioxidants N-acetylcysteine (NAC), glutathione monoethyl
129 ester (GSH-MEE) or Trolox (water-soluble vitamin E). The death phenotype and the 3D-sphere formation
130 defect could be almost fully reverted by incubating with GSH-MEE or NAC, indicating that an increase in free-
131 thiols can prevent FA cytotoxicity (**Fig. 3e,f**). Remarkably, GSH-MEE and NAC led to an overgrowth of WT 3D-
132 spheres (**Fig. 3f,g**). In contrast, Trolox, a non-thiol antioxidant, was unable to limit FA toxicity, suggesting that
133 oxidative stress *per se* is not sufficient to poison $\Delta ADH5$ cells (**Fig. 3e,f**). NAC can work by directly scavenging
134 free FA or by boosting endogenous GSH²¹. To further interrogate the suppressive effect observed with this
135 thiol-rich antioxidant, we combined L-BSO and NAC. Remarkably, NAC could still rescue the toxicity caused by
136 FA even when GSH synthesis was inhibited by L-BSO (**Fig. 3e**). However, blocking GSH synthesis limited the
137 overgrowth phenotype observed in 3D-spheres exposed to NAC (**Fig. 3e,g**). Moreover, GSH synthesis inhibition
138 reduced the NAC-rescue of 3D-sphere formation in $\Delta ADH5$ cells exposed to FA from 96.5 % to 75 % (**Fig. 3h**).
139 Altogether, these observations indicate that ADH5 limits oxidative stress induction by FA and that supplying
140 GSH can prevent FA toxicity.

141 **GSH biosynthesis limits FA toxicity**

142 Exogenous GSH precursors can prevent FA toxicity; we therefore predicted that limiting endogenous
143 GSH should increase FA toxicity even in presence of ADH5. First, we selected concentrations of the GSH
144 synthesis inhibitor L-BSO that were not cytotoxic to the human cancer cells HCT116 and Nalm6 (**Extended**
145 **Data Fig. 2a**). The viability of WT HCT116 and Nalm6 cells in presence of FA was significantly reduced in
146 presence of L-BSO, indicating that GSH synthesis contributes to cellular FA tolerance (**Fig. 3e,4a**). Surprisingly,
147 a non-cytotoxic L-BSO concentration affected the formation of 3D-spheres in both WT and $\Delta ADH5$ HCT116
148 cells even in absence of exogenous FA (**Fig. 4b**). In Nalm6 cells, which grow in suspension, the treatment with
149 L-BSO increased the sensitivity of $\Delta ADH5$ cells to FA (**Fig. 4a**), suggesting that GSH biosynthesis and ADH5

150 independently contribute to prevent FA toxicity in this lymphoblastic human cancer cell. Although L-BSO is
151 neither cytotoxic to HCT116 nor Nalm6 cells at the concentrations used in our experiments (**Extended Data**
152 **Fig. 2a**), it is still a pharmacological avenue that might have off-target effects. We therefore set out to
153 genetically inactivate GSH biosynthesis (*GCLM*) by CRISPR/Cas9 in HCT116 cells (**Extended Data Fig. 2b,c,d**).
154 Concordantly with the pharmacological experiments, *GCLM* deficiency reduced cellular tolerance to FA (**Fig.**
155 **4c**). The simultaneous inactivation of *ADH5* and *GCLM* did not further affect viability (**Fig. 4c**). This result
156 indicates that for cell viability, *ADH5* is the dominant factor in protecting HCT116 cells against FA. The 3D-
157 sphere formation phenotype was affected by the sole inactivation of *GCLM* (**Fig. 4d**), concordantly with the
158 results observed using the GSH-synthesis inhibitor L-BSO (**Fig. 4b**). In contrast, the formation of colonies was
159 further impaired in $\Delta GCLM \Delta ADH5$ cells compared to single knockout counterparts, thus revealing an
160 independent contribution of GSH biosynthesis and *ADH5* to this phenotype (**Fig. 4e,f**). The disparity observed
161 in viability and colony survival assays might indicate that in absence of both *ADH5* and *GCLM* some phenotypes
162 such as cell-cell interaction might be affected without necessarily impairing cell viability.

163 **Endogenous FA reacts with GSH yielding HSMGSH**

164 GSH and FA metabolisms are linked as FA spontaneously reacts with GSH yielding HSMGSH (**Fig. 5A**),
165 a substrate of *ADH5*. We hypothesized that this reaction might occur *in vivo* affecting the endogenous level of
166 GSH as well as limiting the reactivity of free FA. By *in house* synthesis and reaction monitoring using
167 ultraperformance liquid chromatography coupled to high resolution mass spectrometry (UPLC-HRMS), we first
168 confirmed that GSH and FA react *in vitro* yielding HSMGSH, which was subsequently used as chemical standard
169 (**Extended Data Fig. 3**). Should cellular metabolism generate endogenous FA, we might be able to detect the
170 formation of HSMGSH. By UPLC-HRMS, we were able to detect this compound together with GSH and GSSG
171 in cell extracts (**Fig. 5b,c and Extended Data Fig. 4a-d**). The continuous generation of FA from cellular
172 metabolism might need a constant recovery of reduced GSH from HSMGSH formation to sustain endogenous
173 GSH. Indeed, cells lacking *ADH5* presented significantly lower levels of GSH compared to WT cells (**Fig. 5d**).
174 This decrease is in line with the accumulation of HSMGSH relative to GSH (**Fig. 5e**). However, the net amount
175 of total GSH and HSMGSH was lower in $\Delta ADH5$ cells, thus we cannot rule out the participation of efflux

176 mechanism(s) pumping out HSMGSH when this product accumulates (**Fig. 5e,f**). To confirm that $\Delta ADH5$ cells
177 present lower levels of GSH, we interrogated GSH by using an indirect fluorescent reagent. According to this
178 assay, cells lacking *ADH5* contained 17.9 % less reduced GSH than the WT counterparts, corroborating that *in*
179 *vivo* *ADH5* significantly contributes to cellular GSH (**Fig. 5g**). The genetic inactivation of the regulatory
180 component in the rate-limiting step of GSH biosynthesis (*GCLM*) or the treatment with L-BSO further depleted
181 endogenous GSH in both $\Delta ADH5$ and WT cells, denoting that the mechanism by which *ADH5* contributes to
182 GSH homeostasis is downstream GSH synthesis (**Fig. 5g**).

183 **HSMGSH metabolism prevents GSH:GSSG imbalance**

184 In the cytosol, GSH and GSSG levels have been reported to be around 10 mM and 200 nM, respectively,
185 determining a cytosolic GSH redox potential (E_{GSH}) of -320 mV¹³. Despite the high level of reduced GSH, a small
186 change in the ratio between the reduced and the oxidized GSH form (GSH:GSSG) can substantially affect E_{GSH} ,
187 thus impairing cellular redox balance²². We reasoned that blocking the GSH supply through *ADH5* would affect
188 the GSH:GSSG ratio, which might consequently lead to oxidative stress (**Fig. 3a,d**). We therefore measured
189 relative levels of GSSG in both WT and $\Delta ADH5$ cells (**Fig. 5h**) and calculated the GSH:GSSG ratio from UPLC-
190 HMRS data (**Fig. 5i**), observing a 5.9-fold reduction in $\Delta ADH5$ compared to WT cells (**Fig. 5i**). To interrogate the
191 role of *ADH5* in maintaining the GSH:GSSG ratio upon FA stress, we incorporated the cytoplasmic version of
192 the reporter Grx1-roGFP2²³ in HCT116 WT and $\Delta ADH5$ cells. This ratiometric reporter ($\lambda_{em} = 510$ nm) contains
193 two cysteines that can form a reversible disulfide bond that is in equilibrium with the endogenous GSH:GSSG
194 couple. In a more oxidant environment, the ratio between GSH and GSSG will decrease leading to a more
195 oxidized Grx1-roGFP2 sensor. The fraction of the oxidized sensor (OxD Grx1-roGFP2) can be calculated from
196 the ratio between the Grx1-roGFP2 emission at $\lambda = 510$ nm when it is excited at $\lambda = 405$ and $\lambda = 488$ nm
197 ($R_{405/488}$)²³. We found that *ADH5* prevented the FA-dependent oxidation of Grx1-roGFP2 in the cytosol (**Fig.**
198 **5j**), concordantly with the detection of H2CDFDA and roGFP oxidation (**Fig. 3a-d**). In summary, these results
199 show that HSMGSH metabolization by *ADH5* can prevent cytoplasmic GSH:GSSG imbalance by supplying
200 cellular GSH.

201 **The role of ADH5 is conserved**

202 In order to interrogate the relevance of GSH metabolism and ADH5 beyond human cancer cells, we
203 explored the presence of genes coding for ADH5-like proteins in the metazoan model *Caenorhabditis elegans*
204 (**Extended Data Fig. 5a**). In the nematode, the uncharacterized gene H24K24.3 codes for the ortholog of the
205 human ADH5 enzyme. Transgenic expression of ADH-5 fused with GFP under the control of the endogenous
206 *adh-5* promoter (Ex[*p_{adh-5}*ADH-5::GFP; *p_{myo-2}*tdTomato]) presented a ubiquitous cytoplasmic expression in
207 larvae and in the adult nematode (**Fig. 6a**). To assess whether H24K24.3 participates in the prevention of FA
208 toxicity in worms, we generated a null mutant via CRISPR/Cas9 by introducing multiple stop codons in all three
209 reading frames²⁴. Animals lacking H24K24.3 showed an extreme hypersensitivity to FA (**Fig. 6b,c**), affecting
210 the survival throughout development (**Extended Data Fig. 5b**), overall indicating that H24K24.3 is the ortholog
211 of *ADH5* in *C. elegans*. We thus refer to H24K24.3 from now on as *adh-5*. While *adh-5(sbj21)* mutant *C. elegans*
212 larvae did not survive FA exposure, a pre-treatment with only 10 μ M NAC significantly restored survival of
213 *adh-5* mutants and also allowed animals to develop into adulthood, assessed 72 h post FA treatment (**Fig. 6b-**
214 **d and Extended Data Fig. 5b,c**). Conversely, treatment of L1 larvae with a sublethal FA concentration and
215 simultaneous exposure to the prooxidant paraquat (PQ), which generates ROS in *C. elegans*²⁵, severely
216 affected the development of L1 *adh-5* larvae (**Fig. 6e,f**). These results indicate that providing an antioxidant
217 can reduce FA toxicity, while additional oxidative damage increases FA stress in nematodes, strongly
218 supporting our model of oxidative GSH imbalance as a FA-cytotoxic effect.

219 Finally, we reasoned that the reaction between FA and GSH forming HSMGSH molecules and their
220 metabolization by ADH5 might limit free FA. It has been shown that cells lacking the interstrand-crosslinking
221 (ICL)-DNA repair pathway Fanconi Anemia are very sensitive to FA⁹⁻¹¹. We therefore predict that GSH will be
222 required to prevent FA toxicity in Fanconi Anemia. To assess our hypothesis, we exposed Nalm6 cells deficient
223 in *FANCB*, a Fanconi Anemia DNA crosslink repair gene, to FA in presence of L-BSO. As predicted, Δ *FANCB* cells
224 were sensitive to FA and this phenotype was largely exacerbated by blocking GSH synthesis (**Fig. 6g**).
225 Interestingly, cells lacking *FANCB* were significantly sensitive to GSH inhibition even in absence of FA, which

226 might be a consequence of an increase in endogenous free FA, overall suggesting that GSH supply might be
227 fundamental for Fanconi Anemia patients (**Fig. 6h**).

228 Discussion

229 In this work we reveal that FA can cause cytotoxicity by triggering oxidative stress (**Fig. 3a-d**),
230 explaining earlier observations of oxidative damage in tissues and cells exposed to FA ^{26,27}. We determined
231 that FA reacts with GSH, affecting the GSH:GSSG ratio and the cellular redox balance. We describe a conserved
232 mechanism to salvage GSH from FA-GSH covalent products (HSMGSH) limiting FA cytotoxicity not only in
233 cancer cells but also in *C. elegans*. This pathway is centered on the enzyme ADH5 and it is downstream of the
234 *de novo* GSH synthesis pathway (**Fig. 6i**).

235 At physiological FA concentrations as they occur in human blood, ADH5 is essential for cellular growth
236 and viability (**Fig. 1c,d, 2c,d**). In $\Delta ADH5$ cells FA treatment triggers p53 activation that accounts for some
237 aspects of the FA response such as proliferation arrest at non-cytotoxic FA concentrations (**Fig. 2b**), while p53
238 is dispensable for the decline in viability (**Fig. 2c**) suggesting other cell-fate regulators may trigger FA-induced
239 cell death. It is likely that FA-induced DNA damage upregulates a p53 response that blocks cell cycle at G2/M
240 phase, thus impairing the formation of colonies. However, in presence of functional DNA repair mechanisms,
241 DNA damage would be alleviated before it reaches the threshold required for triggering p53-dependent
242 apoptosis. On the other hand, the FA-induced metabolic disruption might lead to p53-independent cell death.
243 Further research should reveal the identity of the p53-independent mechanisms that respond to FA.

244 The detection of HSMGSH in cells not exposed to exogenous FA indicates that cellular metabolism
245 produces sufficient FA to react with GSH yielding HSMGSH (**Fig. 5b,c**). ADH5 restores GSH by metabolizing
246 HSMGSH and thus maintaining the cellular GSH balance to limit oxidative stress. Several factors -in addition
247 to GSH- have been implicated in the cellular protection against oxidative stress, most of them being under the
248 control of the master regulator NRF2²⁸. Upon detecting oxidative stress, the NRF2 partner KEAP1 no longer
249 ubiquitin-labels NRF2 for degradation, resulting in NRF2 stabilization and activation of NRF2-response genes.
250 Indeed, inactivating *Keap1* has been shown to rescue a phenotype of diet-induced steatohepatitis reported in

251 *Adh5*^{-/-} mice²⁹. NRF2 is a tumor suppressor gene that also controls stem cell fate and the crosstalk between
252 NRF2 and HSMGSH metabolism might have significant consequences beyond cancer.

253 ADH5 can also metabolize S-nitrosoglutathione (GSNO) producing ammonia and GSH¹². This enzymatic
254 activity gave origin to the alternative name GSNOR and it has prompted the development of pharmacological
255 inhibitors that might be used for modulating nitric oxide homeostasis in inflammatory diseases³⁰. Remarkably,
256 GSH is the common product of the enzymatic activity of ADH5 using either HSMGSH or GSNO as substrates.
257 Thus, blocking ADH5 might trigger adverse effects such as GSH redox imbalance and increased toxic
258 endogenous FA. On the other hand, GSH biosynthesis has been explored as a therapeutic target to overcome
259 resistance to cancer combinatorial therapies. However, cancer cells can compensate GSH depletion by
260 inducing the thioredoxin (TXN) pathway, which helps to maintain cellular antioxidant capacity³¹, and by
261 maintaining protein homeostasis through deubiquitinating enzymes (DUB)³². Since FA was shown to induce a
262 proteotoxic stress response³³, thus ADH5 inhibition might improve the efficacy of DUB inhibitors and GSH
263 depletors in cancer therapy.

264 Our findings may have wide implications for the human disease Fanconi Anemia. Metabolic ROS were
265 shown to induce DNA damage in hematopoietic stem cells (HSCs) when they start cycling to exit quiescence,
266 which impairs blood production in *Fanca*^{-/-} mice³⁴. It is known that oxygen can exacerbate chromosome
267 aberrations in lymphocytes from Fanconi Anemia patients³⁵. Moreover, the GSH precursor NAC has been
268 shown to improve genome stability in these lymphocytes³⁶. It is likely that an increase in ROS as consequence
269 of oxygen exposure would affect GSH pool, thus indirectly leading to accumulation of FA and genome
270 instability. A combined therapy using a FA sponge such as metformin³⁷ and GSH-precursors might succeed in
271 benefiting Fanconi Anemia patients. Furthermore, a diet rich in GSH-precursors might delay cancer onset in
272 healthy *BRCA2*-mutation carriers by limiting FA toxicity, overall highlighting the broad reach of the findings
273 reported here.

274 **Methods**

275 **Experimental Model and Subject Details**

276 **Cells and animals**

277 HCT116 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) high glucose (Thermo
278 Scientific, #12100061), supplemented with 1 % Penicillin/Streptomycin and 10 % FBS (Natocor)³⁸. Nalm6 cells
279 were maintained in Roswell Park Memorial Institute 1640 medium (RPMI) (Thermo Scientific, #31800105)³
280 containing 10 % FBS, 1 % Penicillin/Streptomycin and 50 μ M β -mercaptoethanol. All the cell lines were
281 regularly tested for mycoplasma infection.

282 Housing and handling of mice were performed in agreement with animal protection guidelines of the
283 district president of Cologne. All procedures were approved and authorized by the LANUV with identification
284 number 84-02.04.2015.A484. 'Role of ageing-associated DNA damage in energy homeostasis-regulating
285 neurons. Mice were maintained in individually ventilated cages (IVCs) on autoclaved bedding and food and
286 sterile-filtered water in a barrier facility at the University of Cologne and the MPI for Metabolism Research.
287 Mice were subjected to a constant 12-h day–night cycle and a constant room temperature of 22°C. Starting
288 from 2 months of age, mice were fed a control diet (CD) consisting of 67 kJ % carbohydrates, 20 kJ % proteins
289 and 13 kJ % fat (Sniff). Mice had ad libitum excess to food and water. The NPY-GFP mice were backcrossed for
290 at least two generations to the C57BL/6N background³⁹.

291 *Caenorhabditis elegans* was maintained using standard methods⁴⁰. N2, Bristol *C. elegans* wild isolate
292 was obtained from *Caenorhabditis* Genetics Center (CGC), Minneapolis, MN, USA.

293 **CRISPR/Cas9 generation of $\Delta ADH5$ and $\Delta GCLM$ cell lines**

294 HCT116 $\Delta ADH5$, $\Delta GCLM$, $\Delta P53 \Delta ADH5$ and $\Delta GCLM \Delta ADH5$ cell lines were generated by targeting exon
295 3 of *ADH5* (sgRNA: TGCTGGAATTGTGAAAGTGTT) and exon 1 of *GCLM* (sgRNA: ACGGGGAACCTGCTGAACTG) in
296 the corresponding parental cell lines. Briefly, sgRNAs were cloned into the pX458 vector (Addgene, #48138)
297 and transfected using lipofectamine 3000 (Thermo Scientific, L3000015). GFP-expressing cells were sorted and
298 clonally diluted in 96-well plates. After 20 days, cells were expanded, and inactivation of the desire gene
299 confirmed by western blot using GCLM (Atlas antibodies, #HPA023696) or ADH5 antibodies⁹. The mutations
300 generated by Cas9 at the target exons were obtained by preparing genomic DNA from the selected clones and
301 amplifying the exons with the primers: Fwd (hA5-ck1): 5'- TCTTGTATCTGTACCTCTGA-3'; Rv (hA5-ck1rv): 5'-

302 CCTTCAGCTTAGTAACTC -3' for *ADH5*, and Fwd (hGCLM-ck_834Fw): 5'-GAAGCACTTTCTCGGCTACG -3' ; Rv
303 (hGCLM-834_Rv): 5'-TCCTTTACTGGACAGGGTG-3' for *GCLM*. PCR results were analyzed by gel
304 electrophoresis, cloned and sequenced using universal M13 primers.

305 **Generation of cells stably expressing ADH5**

306 Cells carrying the ADH5-expressing plasmid pLox-ADH5-FLAG-CT-BSR³ were selected using 4 µg/ml
307 Blasticidin (BSR). BSR-resistant cells were clonally diluted and ADH5-expression verified by western blot
308 against FLAG epitope. Cells carrying the ADH5-expressing plasmid pLox-ADH5-FLAG-CT-BSR³ were selected
309 using 4 µg/ml Blasticidin (BSR). BSR-resistant cells were clonally diluted and ADH5-expression verified by
310 western blot against FLAG epitope (Abcam, #ab49763).

311 **Generation of *C. elegans* lines**

312 The ADH-5::GFP reporter line *Ex*[*p_{adh-5}*ADH-5::GFP; *p_{myo-2}*tdTomato] was produced via co-injecting the
313 clone (5736523864883943 G06, tagged gene: H24K24.3) of the TransgeneOme fosmid library⁴¹ together with
314 the selection marker for tdTomato expression in the pharynx, using standard *C. elegans* microinjection⁴². For
315 imaging, various stages of the transgenic animals were mounted on 5 % agar pads with polystyrene
316 nanoparticles (Polysciences, 2.5 % by volume) as previously described⁴³ and imaged at an AxioImager M.2
317 fluorescence microscope (Zeiss, Jena, Germany).

318 The *C. elegans* orthologue of human *adh-5* gene (H24K24.3) was knocked out using the CRISPR/Cas9
319 system as previously reported: The preassembled CRISPR/Cas9 ribonucleoprotein complexes and linear single
320 stranded DNAs as repair templates were directly injected into the gonad of young adult hermaphrodites⁴⁴. To
321 generate *adh-5* null mutant, we utilized a universal STOP-IN cassette that contained an exogenous Cas9 target
322 site, multiple stop codons in all three reading frames and the recognition site of the *NheI* restriction enzyme
323 ²⁴. The *C. elegans adh-5* sgRNA with GGG protospacer adjacent motif was designed using Benchling
324 (<https://benchling.com/>) and targeted exon 3 of the *adh-5* gene (5'-CTTCATGTCCCAAGACGACA-3'). The *C.*
325 *elegans* DNA repair oligo included a STOP-IN cassette and two short homology arms identical to the sequences
326 flanking the Cas9 cleavage site (5'-
327 GCCACACGGACGCCTACACCCTCGACGGACACGATCCGGAAGGTCTCTCCCTGTGGGAAGTTTGTCCA

328 GAGCAGAGGTGACTAAGTGATAAGCTAGCCGTCTTGGGACATGAAGGGTCTGGAATTGTTCGAGA-3'). To
329 facilitate screening, a co-conversion strategy with dominant phenotypic roller marker was used⁴⁵.
330 Microinjection was performed as previously described⁴² using the following injection mix: KCl (25 mM), Hepes
331 pH 7.4 (7.5 mM), tracrRNA (200 ng/μl), *dpy-10* crRNA (150 ng/μl), *dpy-10* ssODN (13.75 ng/μl), *adh-5* sgRNA
332 (300 ng/μl), *adh-5* ssODN (100 ng/μl), Cas9 (416 ng/μl, NEB, USA). F1 worms carrying roller phenotype were
333 preselected and cloned 4-6 days after the injection. The F2 progeny was subsequently screened for the desired
334 edit by PCR amplification using the *adh-5* forward primer (5'- CGATCCAAGTGGCTCCACCGAA-3') and the
335 *adh-5* reverse primer (5'- TTCCACATCCCAAAGCGAAACC -3'). The presence of the STOP-IN cassette was
336 verified via Sanger sequencing (Eurofins Genomics, Germany) with the *adh-5* sequencing primer (5'-
337 CGATTAACCGACACCCTTGCTC-3').

338 **Survival and development assays in *C. elegans***

339 For the combined N-acetylcysteine (NAC, Sigma-Aldrich, #A7250) and formaldehyde (FA, Pierce,
340 #28908) treatment worm stages were first synchronized via bleach-synchronization. Gravid adult animals and
341 eggs were harvested from NGM plates with 5 ml M9 buffer (3 g KH₂PO₄, 6 g Na₂HPO₄, 5 g NaCl, in 1 l H₂O;
342 autoclaved and added 1 ml 1 M MgSO₄) using a cell scraper and transferred to 15 ml tubes, before adding 1
343 ml bleach solution (5M NaOH and sodium hypochlorite in a 1:1 ratio). The tubes were then constantly vortexed
344 for 5 min and centrifuged (Centrifuge 5810R, Eppendorf) at 2800 rpm for 1 min. After removing the
345 supernatant, the worms were washed three times with 5 ml M9 medium by shaking the tubes and then
346 centrifuging at 2800 rpm for 1 min. Finally, they were kept in 10 ml M9 medium overnight (16 h) under rotation
347 at 35 rpm (Multiple-Axle-Rotating-Mixer RM10W-80V, CAT) to allow animals to hatch. Prior to FA treatment,
348 L1-staged worms were centrifuged at 1300 rpm for 1 minute, and the volume was reduced to 1 ml M9 medium.
349 The number of worms was determined under a stereoscope in a representative volume of 3 μl and a final
350 concentration of approx. 50-100 worms per μl was adjusted. A solution was prepared by pelleting a saturated
351 OP50 *E. coli* bacterial culture, which was first heat-inactivated (60 °C O/N), at 4000 rpm for 10 minutes, and
352 then concentrated two-fold in M9 plus cholesterol (5 μg/ml). 5 ml aliquots were prepared and 1000 worms
353 were added in a volume of 10-20 μl. NAC (500 mM stock solution in H₂O) was added to a final concentration

354 of 10 mM to half of the aliquots and incubated under rotation for 2 h. Thereafter, various concentrations of
355 FA (10 mM and 12 mM) were added to the tubes and incubated under rotation for another 4 h. To this end,
356 methanol-free 16 % FA (w/v; Thermo Scientific) was first adjusted to a 1 M stock solution in H₂O, which was
357 prepared fresh for each experiment. After the NAC/FA treatment, the solutions were centrifuged at 1300 rpm
358 for 1 minute, followed by two washing steps with 5 ml M9 medium. Finally, worms were pelleted again and
359 the volume was reduced to 500 µl. A volume of 25-50 µl (approx. 50-100 worms) was transferred to OP50-
360 seeded NGM plates, on which the survival rate was scored under a stereoscope. Worms were qualified as
361 dead when no locomotion could be detected and when stimulation with a wormpick did not cause a response.
362 The survival count was repeated after 24 h, 48 h and 72 h. In parallel, developmental stages of worms were
363 determined under the stereoscope at 48 h and 72 h post-treatment and qualified in the categories L1-L3, L4
364 and adult.

365 The combined PQ and FA treatment was performed in the same way as the NAC/FA treatment, with
366 the exception that PQ was added at the same time as FA (2 mM) and incubated together for a total of 5 h. PQ
367 (Methyl viologen dichloride hydrate, Sigma-Aldrich, #856177) was always freshly prepared and first adjusted
368 to a 1 M stock in H₂O, which was further diluted for the treatment.

369 **Viability and survival assays**

370 For determining cell viability, cells were seeded into 96-well plates at a density of 3000 cells per well
371 and allowed to attach for 24 h at 37°C, 5 % CO₂. Then, FA and/or L-buthionine-sulfoximine (L-BSO, Sigma-
372 Aldrich, #B2515) and/or antioxidants were added to a final volume of 200 µl per well. 3 days later, resazurin
373 (Sigma-Aldrich, #R7017) was added to a final concentration of 30 µM in the growing medium. Fluorescence
374 ($\lambda_{\text{ex}} = 525 \text{ nm}$; $\lambda_{\text{em}} = 590 \text{ nm}$) was measured 3 h later in an Enspire Plate Reader (Perkin Elmer). For Nalm6,
375 5000 cells per well were seeded into 96-well plates and the drugs to be tested added immediately afterward.
376 Viability was determined 5 days later. In all cases, the experiments were done by triplicate and data
377 represented as percentage of the fluorescence obtained with the untreated samples of the corresponding cell
378 line.

379 The colony survival assay was done by seeding 600 cells per well in 6-well plates. Immediately
380 afterward, FA was added at the concentrations described in the text in a final volume of 2 ml (DMEM). Plates
381 were maintained during 7-10 days at 37°C, 5 % CO₂. Staining was done using a fixative/staining solution (0.5
382 % crystal violet, 6 % glutaraldehyde) for 30 minutes, following of extensive rinse with tap water. Visible
383 colonies were counted, and the results expressed as percentage of the untreated wells. Experiments were
384 done by duplicated and repeated the number of times indicated in each corresponding figure.

385 **ROS measurement by H2DCFDA**

386 ROS measurement was performed using 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA, Sigma-
387 Aldrich #D6883). Briefly, 4x10⁴ HCT116 cells were seeded per well in 24-well plates and allowed to adhere
388 overnight. Cells were then treated with 0, 60 and 150 µM of FA and 0 or 100 µM of L-BSO, for 48 h. H₂O₂ 500
389 µM was used as a positive control and added 15 minutes prior H2DCFDA staining. After treatment, H2DCFDA
390 was added to each well at a final concentration of 10 µM, kept 30 min at 37 °C. Then, cells were lifted and
391 transferred to flow cytometry tubes, which were kept at 4°C until measuring was performed. Fluorescence
392 ($\lambda_{\text{ex}} = 488\text{nm}$; $\lambda_{\text{em}} = 530\text{nm}$) was measured by flow cytometry using a Becton Dickinson's FACS Canto II Flow
393 cytometer.

394 **ROS and GSH redox status determination by genetic sensors**

395 The cytosolic roGFP2 sensor was cloned from Addgene 49435 into a retroviral backbone pLPCX by
396 Gibson Assembly. Plasmid sequence was confirmed by sequencing. Retroviral infection was carried out
397 transfecting HEK293T cells with pBS-CMV-gagpol (Addgene, #35614) and pCAG-VSVG (Addgene, #35616)
398 vectors in addition to pLPCX cyto Grx1-roGFP2 (Addgene, #64975) or pLPCX cyto roGFP (Addgene, #49435).
399 Conditioned medium was collected and recipient HCT116 cells infected adding Polybrene (Merk, #TR-1003-G
400) (1 µg/µL). Infection was confirmed by GFP-expression (90 % efficiency). Cells expressing the desired reporter
401 were selected with 0.5 µg/mL puromycin. No clonal selection was carried out to prevent single clone artefacts.
402 For ROS measurement, cells were seeded into a 24-well plate at 3.5x10⁴ cells per well and allowed to adhere
403 overnight. Cells were then treated with 0, 60 and 150 µM of FA, and 0 or 100 µM of L-BSO, for 48 h. H₂O₂ 500
404 µM, which was used as a positive control, was added 15 minutes prior cell analysis. After treatment, culture

405 media was removed, and cells were washed with PBS. Cells were then trypsinized and transferred into clear
406 flow cytometry tubes containing phosphate buffer saline (PBS) supplemented with 2 % fetal bovine serum
407 (FBS). Tubes were kept at 4°C until measuring was performed. Fluorescence ($\lambda_{ex} = 405\text{nm}$ and 488nm , $\lambda_{em} =$
408 510nm) was measured by flow cytometry using a Becton Dickinson's FACS Aria II flow cytometer.
409 For determination of the glutathione (GSH) redox potential, cells expressing pLPCX cyto Grx1-roGFP2 were
410 exposed to FA and/or L-BSO for 48 h. Then, cells were collected and fluorescence ($\lambda_{ex} = 405\text{nm}$ and 488nm ,
411 $\lambda_{em} = 510\text{nm}$) determined by flow cytometry as described above ²³. The fraction of oxidized Grx1-roGFP2
412 sensor was calculated using the formula:

413

$$OxD_{roGFP2} = \frac{R - R_{red}}{\left(\frac{I_{488\ min}}{I_{488\ max}}\right) \times (R_{ox} - R) + (R - R_{red})}$$

414 **Metaphases analysis**

415 To assess single-chromosome damage, HCT116 Wild type (WT) and $\Delta ADH5$ cells were plated in P60
416 dishes allowed to adhere and then treated with mitomycin C (MMC, Santa Cruz, #sc-3514) 0.5 $\mu\text{g/ml}$ during
417 24 h or with FA 150 μM during 48 h. 16 h before harvesting the cells Colcemid (Gibco, #15212-012) was added
418 at the concentration of 0.08 $\mu\text{g/ml}$ without replacing the culture medium. Cells were washed with PBS and
419 trypsin added to a final concentration of 0.125 %. Complete medium was added to stop trypsin reaction and
420 clumps of cells disrupted by pipetting. Then, cells were centrifuged and resuspended into 2 ml of prewarmed
421 hypotonic solution (KCl 0.075 mM) and incubated in 14 ml of this solution for 15 minutes at 37 °C. Then, 1 ml
422 of fixative solution was added (3:1 methanol:glacial acetic acid) dropwise. Cells were washed twice with
423 fixative solution and then dropped onto chilled humid slides, where cells were left to dry overnight. The day
424 after, slides were stained in 2 % Giemsa solution (Thermo Scientific, #10092013) prepared in Gur buffer (Gibco,
425 #10582-013), left to dry and mounted using BC solution (Cicarelli, #891). Pictures were taken using a Zeiss
426 Axiobserver Z1 microscope with a 40x oil-immersion objective and analyzed using ImageJ⁴⁶. To guarantee
427 unbiased quantitation, pictures were taken by a microscopy technician, who labeled the images with numbers.
428 After scoring of chromosome damage, the identities of the images were revealed.

429

430 **3D-Spheroid assay**

431 96-well plates were coated with 50 μ L of 1.5 % sterile agarose 2 h before cell plating. 100 μ L of HCT116 cells
432 (WT, Δ ADH5, Δ ADH5 complemented, Δ GCLM or Δ ADH5 Δ GCLM) were seeded at a concentration of 2×10^3 or
433 4×10^3 cells/well. Immediately after seeding, cells were treated with 100 μ L of DMEM 10 % FBS containing 2x
434 concentrations of the drugs used. The final concentrations of the drugs were 0, 50, 100 and 150 μ M FA; 0 and
435 100 μ M L-BSO; 0 and 500 μ M NAC; 0, 1 mM Glutathione monoethyl ester (GSH-MEE, Santa Cruz, # sc-203974);
436 0 and 1 mM Trolox (Sigma-Aldrich, ##238813). Plates were kept at 37 °C. Spheroid formation was assayed by
437 microscopy (Zeiss Axio A1 inverted microscope) 5-7 days after seeding and registered using a CANON Rebel
438 T3i camera attached to the microscope with an appropriate adaptor, at 40x magnification. For sphere-size
439 quantification Fiji was used to measure the area of the formed sphere.

440 **Phylogenetic analysis**

441 Eukaryote orthologs of ADH5 were obtained from NCBI, CLUSTAL at phylogeny.fr was used to align
442 the sequences and TreeDyn at phylogeny.fr for tree generation⁴⁷.

443 **Western blot analysis**

444 Cells were washed with PBS containing 1 mM N-Ethylmaleimide (NEM, Santa Cruz, #sc-202719), then
445 lysed with Laemmli Sample buffer containing 2 % SDS, 4 % glycerol, 40mM Tris-Cl (pH 6.8), 5 % 2-
446 Mercaptoethanol, 0.01 % bromophenol blue, 1 mM NEM, 1 mM Phenylmethylsulfonyl fluoride (PMSF),
447 protease inhibitor mixture (Roche, #COEDTAF-RO), and phosphatase inhibitor mixture (Roche,
448 #04906837001). Samples were bath-sonicated (3 pulses 30'' ON 30'' OFF) and boiled for 10 minutes. Sample
449 concentration was relativized by Coomassie Brilliant Blue staining. For separation, samples were loaded onto
450 12 % polyacrylamide gels and subjected to electrophoresis. Protein was transferred to nitrocellulose
451 membranes, which were blocked with 2 % BSA in Tris-buffered saline (TBS) or 5 % non-fat milk in TBS.
452 Membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with secondary
453 antibodies conjugated with either horseradish peroxidase or fluorescent dye. DNA damaging agents were
454 MMC, cisplatin (Santa Cruz, #sc-200896) and Hydroxyurea (Santa Cruz, #sc-29061) Proteins were visualized
455 using ECL prime chemiluminescence reagent or fluorescence emission, respectively. Primary antibodies used

456 were p53 (CST, #9282), phospho-P53 (CST, #9284), p21 (CST, #2947), phospho-histone H2A.X (CST, #9718),
457 Vinculin (Santa Cruz, #sc-73614), alpha-tubulin (CST, #2144), and beta-actin (Santa Cruz, #sc-47778).
458 Secondary antibodies used were horseradish peroxidase-linked anti-rabbit (CST, ##7074), horseradish
459 peroxidase-linked anti-mouse (CST, #7076), DyLight-800 4x PEG-linked anti-rabbit (CST, #5151), and DyLight
460 680-linked anti-mouse (CST, #5470).

461 **Cell cycle assay and apoptosis determination**

462 Cells were plated at a final concentration of 3×10^5 cells per well in DMEM supplemented with 10 % FBS.
463 After 24 h, cells were treated with 0, 60, and 150 μ M FA for 24 h. After this period, cells were harvested by
464 trypsinization and pelleted by centrifugation (5 min, 1000 x g). Cells were washed with cold PBS and then fixed
465 with 70 % cold ethanol for 15 min on ice. Cells were washed twice with PBS and treated with 30 μ g
466 ribonuclease A and 15 μ g of propidium iodide. Cells were run on a BD FACS Canto II flow cytometer and the
467 data was analyzed with FlowJo 10.0.7 (Tree Star). For apoptosis determination, the BD PE Annexin V Apoptosis
468 Detection Kit (BD Pharmigen, #579563). Briefly, cells were plated and 24 h later exposed to the indicated
469 concentrations of FA. 24 h later, cells were lifted, washed with cold PBS and stained with PE-Annexin V
470 antibody and 7-AAD. Samples were run on a BD FACSAria II flow cytometer and data analyzed with FlowJo
471 10.0.7 (Tree Star).

472 **GSH measurement**

473 GSH was determined using the GSH-Glo™ Glutathione Assay (Promega, #V6911). Briefly, 10000 cells per
474 well were seeded in a 96-well plate. A duplicated plate was prepared to determine viability. 48 h later GSH
475 was determined following the instructions provided in the kit. In parallel, the viability was scored using
476 resazurin and the results adjusted for the percentage of viable cells relative to the GSH content of WT cells.

477 **Formaldehyde determination in blood**

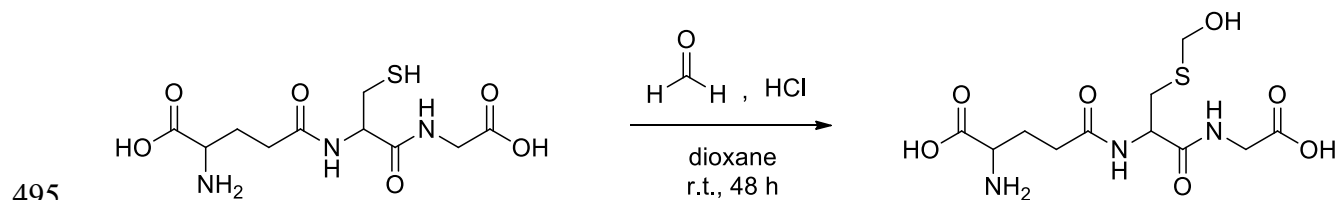
478 Mice were sacrificed at 6 months of age by decapitation, full blood was collected, and serum was
479 separated from red blood cells by centrifugation (15,000 x g, 30 min, 4 °C). Serum was transferred to a new
480 Eppendorf tube and sera of three mice of the same age, sex and diet were pooled and subsequently subjected
481 to trichloroacetic acid (Guoyao, #80132618) (20 % w/v, in ultrapure water) precipitation. Therefore,

482 trichloroacetic acid was added in a 1:1 ratio to the serum, vortexed for 30 sec. and centrifuged (15,000 x g, 30
483 min, 4°C). The supernatant was transferred to a fresh Eppendorf tube and stored at -80 °C until further
484 processing.

485 The concentration of FA was detected by high-performance liquid chromatography (HPLC) as previously
486 described⁴⁸. Serum samples (0.08 ml each) were mixed with 0.02 ml 10 % trichloroacetic acid, 0.08 ml
487 acetonitrile (Thermo Scientific, #A998-4), and 0.02 ml 2,4-dinitrophenylhydrazine (Beijingshiji, #550626).
488 Samples were centrifuged (15,000 x g, 4 °C, 10 min) and then reacted in a 60°C water bath for 30 min; this
489 step was followed by a centrifugation (15,000 x g, 4 °C, 10 min) and filtered (0.22 µm). 20 µl of the solution
490 was then subjected to HPLC (LC-20A, Shimadzu, Japan). FA-DNPH derivatives were detected with an ultraviolet
491 detector (Cas: 228-34016, Shimadzu, Japan) and a C18 reversed-phase column (Sigma-Aldrich, #50208-U),
492 using 65 % acetonitrile as the mobile phase.

493 **Synthesis of S-hydroxymethyl-glutathione**

494 Reaction:



496

497 Procedure:

498 In a 50 ml-bottom flask, FA solution (12 µL, 37 wt. % in water, Sigma-Aldrich, #F8775) and HCl (0.5 ml, 36.5-
499 38.0%) were dissolved in 2 ml of dioxane (Sintorgan, #SIN-083003-63). The mixture was stirred at room
500 temperature over 5 min and glutathione (Santa Cruz, #sc-29094) (50 mg, 0.16 mmol) was added in small
501 portions. After stirring at room temperature over 48 h, the mixture was neutralized with saturated aqueous
502 NaHCO₃ solution and partitioned between ethyl acetate and water. The aqueous phase was lyophilized (0.03
503 mBar, -80 °C, 72 h) to obtain a white solid using a Telstar LYOQuest-85 freeze dryer (Telstar, Madrid, Spain). A
504 portion of 10 mg of the solid was resuspended in 1 ml of a CH₃OH:CH₃CN (1:1) mixture, centrifuged and the
505 supernatant was diluted to be analyzed by UPLC-HRMS. Estimated reaction yield 95.8 %⁴⁹.

506 **Sample preparation for UPLC-HRMS analysis**

507 HCT116 WT and $\Delta ADH5$ cells were counted and cultured in 100 cm plates at 1×10^6 cells/plate. Two
508 independent rounds of sample preparation were carried out in consecutive weeks. 6 plates in the first week
509 and 5 plates in the second week for each cell line were set up and allowed to grow for 72 h. One plate in each
510 round was used for protein and cell count. Once 80 % confluence was reached, cells were gently washed with
511 5 ml of a 0.9% NaCl aqueous solution at 0 °C. Subsequently, enzymatic activity was quenched by adding liquid
512 N₂. Cells were scrapped immediately after with 1.4 ml of a cold (0 °C) CH₃OH:CH₃CN (50:50 v/v) solution and
513 subsequently frozen using liquid N₂. After one freeze-thaw cycle, samples were vortex-mixed during 30 s and
514 centrifuged at $5000 \times g$ for 5 min at 4 °C. Supernatants were collected and stored at -20 °C for 2 h and
515 subsequently centrifuged at $15000 \times g$ for 10 min at 4 °C. Afterwards, 1.4 ml of ultrapure water was added to
516 supernatants and these solution were immediately frozen and stored at -80 °C until lyophilization.

517 Process blanks consisting of incubating culture media in plates without cells were generated in parallel with
518 samples, and followed the same protocol described above. For protein and cell count, cells were lifted and
519 counted using trypan blue as viability marker. Afterwards, cells were lysed in a solution containing 1 μ M EDTA;
520 10 μ M Tris pH 8; 200 μ M NaCl and 0.2 % Triton, and total protein was determined by the Bradford assay using
521 BSA as standard. Samples were lyophilized at -80 °C and 50 mTorr for 48 h using a Telstar LYOQuest-85 freeze
522 dryer (Telstar, Madrid, Spain) and stored at -80 °C until analysis by UPLC-HMRS. All sample residues from each
523 batch were reconstituted the same day in a water: methanol (90/10 v/v) solution. Reconstitution factors were
524 selected to reach the same protein content for all samples. After reconstitution, samples were vortex-mixed
525 for 30 s and centrifuged at $21382 \times g$ for 20 min and 4 °C. Supernatants were stored until use at -80 °C. Quality
526 control (QC) samples were prepared by pooling an aliquot of 15 μ L from each sample, vortex-mixed for 30 s,
527 split into 4 micro tubes, and stored at -80 °C until use for analysis.

528 A pooled QC sample spiked with GSH (14.3 μ M), GSH disulfide (15.5 μ M) and S-hydroxymethylglutathione (20
529 μ M) was used to verify the stability of retention times, peak shapes and areas during the analysis.

530 **UPLC-HRMS analysis**

531 UPLC-HMRS analyses were performed using a Waters ACQUITY UPLC I Class system fitted with a Waters
532 ACQUITY UPLC BEH C₁₈ column (2.1×100 mm, 1.7 μm particle size, Waters Corporation, Milford, MA, USA,
533 catalog #186002352), and coupled to a Xevo G2S QTOF mass spectrometer (Waters Corporation, Manchester,
534 UK, SN: YDA 375) with an electrospray ionization (ESI) source operated in ESI positive ionization mode. The
535 typical resolving power and mass accuracy of the Xevo G2S QTOF mass spectrometer were 32,000 FWHM and
536 0.3 ppm at *m/z* 556.2771, respectively. The mobile phase consisted of water with 0.1 % formic acid (Fisher
537 Chemical, #F/1900/PB15) (mobile phase A) and methanol (Fisher Chemical A454-4, (UN 1230-CL3)) (mobile
538 phase B). The flow rate was constant at 0.3 ml min⁻¹, the elution gradient was set as follows: 0-1.6 min 0-0 %
539 B; 1.6-2 min 0-20 % B; 2-6 min 20-70% B; 6-7 min 70-70 % B; 7-14 min 70-90 % B; 14-17.5 min 90-90 % B; 17.5-
540 18 min 90-95 % B; 18-21 min 95-95 % B. After each sample injection, the gradient was returned to its initial
541 conditions in 9 min (total run time was 30 min). The eluates from the analytical column were diverted by
542 automatically switching the valve to waste, except for the elution window from 0 to 8 min. The column and
543 autosampler tray temperatures were set at 35 and 5°C, respectively. The injection volume was 2 μL.
544 A solvent blank, which consisted of a water : methanol (90:10 v/v) solution, and a process blank were analyzed
545 at the beginning and end of each batch. Samples were randomly analyzed within a defined template of spiked
546 QC samples, and the analysis order was balanced based on sample classes. QC samples were used to condition
547 the LC-MS system before sample analysis. A total of 20 randomized samples (WT cells n=10 and $\Delta ADH5$ cells
548 n=10) were analyzed along 3 consecutive days. UPLC–MS sample lists were set up as follows (sample type
549 (technical replicates)): zero consisting of mobile phase analysis without injection (1); solvent blank (2); process
550 blank (2); QC samples (5); spiked QC sample (1); randomized, and balanced samples (12) with 1 spiked QC
551 sample analyzed every 4 samples; spiked QC sample (1); process blank (2); solvent blank (1).
552 The mass spectrometer was operated in positive ion mode with a probe capillary voltage of 2.5 kV and a
553 sampling cone voltage of 30.0 V. The source and desolvation gas temperatures were set to 120 and 300 °C,
554 respectively. The nitrogen gas desolvation flow rate was 600 L h⁻¹, and the cone desolvation flow rate was 10
555 L h⁻¹. The mass spectrometer was daily calibrated across the range of *m/z* 50-1200 using a 0.5 mM sodium
556 formate solution prepared in 2-propanol/water (90:10 v/v). Data were drift corrected during acquisition using

557 a leucine enkephalin (m/z 556.2771) reference spray (Waters cop, #700008842) infused at $5 \mu\text{l min}^{-1}$, every 45
558 seconds. Data were acquired in MS continuum mode in the range of m/z 50-1200, and the scan time was set
559 to 0.5 seconds.

560 Principal component analysis (PCA) was conducted using MATLAB R2015a (The MathWorks, Natick, MA, USA)
561 with the PLS Toolbox version 8.1 (Eigenvector Research, Inc., Manson, WA, USA). PCA was used to track data
562 quality and to identify and remove outliers in the dataset. Two samples were identified as outliers by PCA, one
563 from WT and one from $\Delta ADH5$ cells, and were not further considered for data analysis.

564 For UPLC-MS/MS experiments, the product ion mass spectra were acquired with collision cell voltages
565 between 10 and 30 V, depending on the analyte. Ultra-high-purity argon ($\geq 99.999\%$) was used as the collision
566 gas. Data acquisition and processing were carried out using MassLynx version 4.1 (Waters Corp., Milford, MA,
567 USA).

568 Chemical standards were prepared in ultrapure water and were analyzed under identical conditions as
569 samples to validate metabolite identities by chromatographic retention time and MS/MS fragmentation
570 pattern matching. Spiking experiments were also conducted with the authentic chemical standards on samples
571 to address retention time differences caused by matrix effects.

572 Two different normalization strategies were independently used for sample analysis: data were normalized by
573 number of viable cells or by protein content.

574 **Quantification and Statistical Analysis**

575 Prism software package (GraphPad Software 7) was used for statistical analysis with the level of significance
576 of 0.05 (95 % confidence) and one-way ANOVA using the Tukey's algorithm for multiple comparisons. For mass
577 spectrometry data whisker plots and Mann-Whitney test was used to assess significance. The box and whiskers
578 plots are represented by a line in the box corresponding to the median; the edges are the 25th and 75th
579 percentiles and the whiskers extend to the most extreme values in data. Additional information about
580 statistical tests, sample number and P-values are described in figure legends. Unless otherwise stated,
581 experiments were done using technical replicates (2 or 3 wells per condition) and repeated the n times

582 described in the figure legends with each symbol in a bar plot representing the average of the technical
583 replicates for a given biological sample.

584 **Data availability**

585 All data generated during this study are included in the published paper including source data for figure 2,
586 Extended data Fig. 1 and Extended data Fig. 5.

587 **Acknowledgements**

588 This work was supported by CONICET (PUE 2016 22920160100010CO), FOCEM MERCOSUR (COF 03/11),
589 ANPyCT (PICT-PRH 2017-4668) and (PRH-PICT-2015-0022). LBP is a collaborative Group Leader between
590 IBioBA, MPI for Metabolism Research (Cologne, Germany) and MPI for Biophysical Chemistry (Göttingen,
591 Germany), from whom he receives support. B.S. acknowledges funding from the Deutsche
592 Forschungsgemeinschaft (SCHU 2494/3-1, SCHU 2494/7-1, SCHU 2494/10-1, SCHU 2494/11-1, SFB 829, KFO
593 286, KFO 329, GRK 2407) and the Deutsche Krebshilfe (70112899). IK acknowledges EMBO (ALTF 1029-2014).
594 LBP would like to acknowledge Prof. Jens Brüning and Prof. Thomas Wunderlich for providing support for
595 mouse breeding at MPI-MR. Also, to IBioBA staff for general support, to Gerry Crossan, Ricardo Biondi,
596 Alejandro Leroux and Carolina Perez Castro for the critical reading of the manuscript. CU, AM, HR, MM and AF
597 are CONICET fellows. MEM, MRM, MB and LBP are Research Staff Members from CONICET.

598 **Author contributions**

599 CU, AM, MS and HR carried out cellular experiments assisted by LBP. MR, KK and BS contributed with *C.*
600 *elegans* data generation and analysis. MRM and MEM designed the sample preparation protocol for
601 metabolite extraction, developed the UPLC-QTOF-MS-based method, and performed data analysis. MRM
602 conducted UPLC-MS/MS experiments. AF and MB synthesized S-hydroxymethyl-GSH. AV and IK processed
603 mouse blood samples. YW and RH measured blood FA. LBP conceived the work and wrote the paper. All
604 authors revised the manuscript.

605

606 **Ethics declarations**

607 The authors declare no competing interests.

608 **References**

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715

716 **Fig. titles and legends.**

717 **Fig. 1. ADH5 prevents formaldehyde toxicity in human cancer cells**

718 **a.** Scheme showing formaldehyde (FA) metabolism, glutathione (GSH) *de novo* synthesis and the central role
719 of ADH5 (alcohol dehydrogenase 5). GCL: glutamate cysteine ligase; GS: glutathione synthetase; ROS: Reactive
720 Oxygen Species.

721 **b.** FA in blood of 6-month old mice determined by high-performance liquid chromatography with ultraviolet
722 detection (UV-HPLC) (mean \pm s.e.m., n = 7 (males), n = 7 (females), n = 14 (both sexes)).

723 **c.** Representative images of 3D-sphere formation for HCT116 Wild type (WT), Δ ADH5 and Δ ADH5/pADH5 cells
724 (scale bar 0.5 mm).

- 725 **d.** Resazurin-based viability assay expressed as % of fluorescence relative to untreated cells. Each point
726 represents the mean \pm s.e.m. of 6 independent experiments done by triplicate. Asterisks represent the
727 statistical significance according to one-way ANOVA for multiple comparison using a Tukey-corrected test
728 between Wild type and $\Delta ADH5$ cells.
- 729 **e.** Apoptosis determined by Annexin V detection in cells exposed to FA over 24 h at the concentrations
730 indicated in the figure (mean \pm s.e.m., n=5, unpaired t-test).
- 731 **f.** Cell cycle analysis of Wild type and $\Delta ADH5$ HCT116 cells exposed to 60 and 150 μ M FA over 24 h (mean \pm
732 s.e.m., n = 4).

733 **Fig. 2. Formaldehyde triggers a p53 response in absence of ADH5**

- 734 **a.** Resazurin-based viability assay expressed as % of fluorescence relative to untreated cells in Wild Type (WT),
735 $\Delta ADH5$, $\Delta P53$ and $\Delta P53 \Delta ADH5$ cells exposed to increasing concentrations of formaldehyde (FA) (mean \pm
736 s.e.m., n=8).
- 737 **b.** Colony survival assay prepared seeding 600 cells in 6-well plates in presence of the indicated concentration
738 of formaldehyde (FA). Colonies were stained and quantified after 10 days (mean \pm s.e.m., n=4, one-way
739 ANOVA using Tukey multiple comparison test).
- 740 **c.** Western blot showing the induction of p-p53 and γ -H2AX after 48 h of exposure to the indicated
741 concentrations of FA or 24 h of exposure to the genotoxic compounds cisplatin (4 μ M), hydroxyurea (HU, 1
742 mM) and mitomycin C (MMC, 1.5 μ M).
- 743 **d.** Quantitation of p-p53 and γ -H2AX western blots using ImageJ (p-p53: FA 0, 60, 150 and cisplatin n=5; HU
744 n=4, MMC n=3. γ -H2AX: FA 0, 60, 150, cisplatin and HU n=4; MMC n=3; mean \pm s.e.m., unpaired t-test
745 comparing against the same cell line untreated)
- 746 **e.** Quantitation of metaphase scoring (mean \pm s.e.m., n=49, one-way ANOVA, Tuckey's multiple comparison
747 test) denoting the induction of chromosome damage by MMC (1.5 μ M) but not by FA (150 μ M).
- 748 **f.** Representative images of Wild Type and $\Delta ADH5$ cells exposed to 150 μ M FA and to 1.5 μ M MMC (scale bar
749 1 μ m).

750 **Fig. 3. Oxidative stress underlies formaldehyde cytotoxicity**

751 **a.** Oxidative stress determination by 2',7'-dichlorodihydrofluorescein diacetate (HFDCDA) in Wild type,
752 $\Delta ADH5$, and complemented $\Delta ADH5$ ($\Delta ADH5/pADH5$) HCT116 cells upon 48 h exposure to formaldehyde (FA).

753 Data is represented as the % of fluorescence detected in the untreated samples from the same cell line (mean
754 \pm s.e.m., n=3, one-way ANOVA, Tuckey's multiple comparison test).

755 **b.** Representative plots of oxidative stress determination as described in a.

756 **c.** Flow cytometry representative plots obtained from Wild type and $\Delta ADH5$ cells harboring the cytoplasmic-
757 roGFP reporter. Cells were excited at $\lambda = 405$ or $\lambda = 488$ nm and emission recorded at $\lambda = 510$ nm.

758 **d.** Quantitation of experiments shown in d (mean \pm s.e.m., n=3, one-way ANOVA, Tuckey's multiple
759 comparison test).

760 **e.** Resazurin-based viability at 0, 60 and 150 μ M FA denoting the rescue of cell viability by N-acetylcysteine
761 (NAC, 500 μ M), Trolox (1 mM) and glutathione monoethyl ester (GSH-MEE, 1 mM). L-buthionine-sulfoximine
762 (L-BSO) was used at 100 μ M. The data represent the mean \pm s.e.m. of 4 experiments done in triplicate (one-
763 way ANOVA, Tuckey's multiple comparison test).

764 **f.** Representative images of the 3D-tumour spheroid formation phenotype in presence of the indicated FA
765 concentration and the combination of the antioxidants described in e (scale bar 0.5 mm).

766 **g.** Quantitation of 3D-sphere area from 10 formed spheres at day 5 after seeding (mean \pm s.e.m., one-way
767 ANOVA, Tuckey's multiple comparison test).

768 **f.** Quantitation of sphere formation phenotype at day 5 after seeding 2000 cell/well of WT or $\Delta ADH5$ cells in
769 presence of FA (150 μ M); FA and NAC (500 μ M); or FA, NAC and L-BSO (100 μ M). The plots correspond to a
770 part of the whole representation (WT + FA, n=30; $\Delta ADH5$ + FA, n=28; WT + FA + NAC, n=29; $\Delta ADH5$ + FA +
771 NAC, n=29; WT + FA + NAC + L-BSO, n=27; $\Delta ADH5$ + FA + NAC + L-BSO, n=28).

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776 **Fig. 4. GSH biosynthesis limits formaldehyde toxicity**

777 **a.** Resazurin-based viability assay in Wild type (WT) and $\Delta ADH5$ Nalm6 cells in presence of different
778 concentrations of formaldehyde (FA) with or without 50 μ M L-buthionine-sulfoximine (L-BSO) (mean \pm s.e.m.,
779 n=6).

780 **b.** HCT116 3D-spheroid formation in presence of 100 μ M L-BSO and the indicated concentrations of FA.
781 Pictures were taken 5 days after seeding cells on agarose-coated plates (scale bar 0.5 mm).

782 **c.** Resazurin-based viability assay performed with Wild type, $\Delta ADH5$, $\Delta GCLM$ and $\Delta ADH5 \Delta GCLM$ cells in
783 response to increasing concentrations of FA (mean \pm s.e.m., n=5, asterisks represent the statistical significance
784 according to one-way ANOVA for multiple comparison using a Tukey-corrected test between Wild type and
785 $\Delta GCLM$).

786 **d.** Representative images of HCT116 3D-spheroid formation for the same cell lines described in (C). Pictures
787 were taken at day 5 after seeding (scale bar 0.5 mm).

788 **e.** Colony survival assay prepared seeding 600 of WT, $\Delta ADH5$, $\Delta GCLM$ and $\Delta GCLM \Delta ADH5$ cells in 6-well plates
789 in presence of the indicated concentration of FA (mean \pm s.e.m., n=5, one-way ANOVA using a Tukey's multiple
790 comparison test).

791 **f.** Representative images of the colony survival assay quantified in e.

792 **Fig. 5. Endogenous formaldehyde reacts with GSH altering the GSH:GSSG ratio**

793 **a.** Scheme showing the spontaneous reaction between formaldehyde (FA) and glutathione (GSH) yielding S-
794 hydroxymethylglutathione (HSMGSH).

795 **b.** Extracted ion chromatograms for $[HSMGSH + H]^+$ ion at m/z 338.1022 ± 0.0500 generated from a non-
796 spiked Wild type (WT) sample (green, left axis), a 20 μ M spiked WT sample (black dotted line, right axis), and
797 a 20 μ M HSMGSH standard solution (blue, left axis).

798 **c.** Product ion mass spectra of $[HSMGSH + H]^+$ precursor ion for a WT sample (green), and for a 20 μ M HSMGSH
799 standard solution (blue), using a collision cell voltage of 10 V.

- 800 **d.** Box and whiskers plot for GSH content in WT and $\Delta ADH5$ cells calculated as normalized chromatographic
801 peak areas relative to the number of viable cells (n=9, Mann-Whitney test, *** P<0.0001). The box and
802 whiskers plots are represented by a line in the box corresponding to the median; the edges are the 25th and
803 75th percentiles and the whiskers extend to the most extreme values in data.
- 804 **e.** Left: Box and whiskers plot for HSMGSH content relative to GSH in WT and $\Delta ADH5$ cells (n=9, Mann-Whitney
805 test). Right: Net HSMGSH content in WT and $\Delta ADH5$ cells calculated as normalized peak areas relative to the
806 number of viable cells (n=9, Mann-Whitney test).
- 807 **f.** Box and whiskers plot for total GSH (GSH disulfide (GSSG) plus GSH) content in WT and $\Delta ADH5$ cells
808 calculated as normalized peak areas relative to the number of viable cells (n=9, Mann-Whitney test).
- 809 **g.** Bar plots for GSH content in WT (n=17), $\Delta ADH5$ (n=15), $\Delta ADH5/pADH5$ (n=8), $\Delta GCLM$ (n=4) and $\Delta ADH5$
810 $\Delta GCLM$ (n=4) cells, and in WT (n=7) and $\Delta ADH5$ (n=7) cells exposed to 100 μ M L-BSO for 48 h. Every dot is the
811 percentage of fluorescence intensity in a single well relative to the average fluorescence of WT samples run
812 the same day and corrected for viability determined using resazurin (mean \pm s.e.m., unpaired t-test).
- 813 **h.** Box and whiskers plot for GSSG content in WT and $\Delta ADH5$ cells calculated relative to the number of viable
814 cells (n=9, Mann-Whitney test).
- 815 **i.** Box and whiskers plot for GSH:GSSG ratio in WT and $\Delta ADH5$ cells (n=9, Mann-Whitney test).
- 816 **j.** Flow cytometry representative plots from WT and $\Delta ADH5$ cells harboring the cytosolic Grx1-roGFP2
817 reporter. Data was recorded 48 h post-FA exposure.
- 818 **k.** Quantitation of oxidized Grx1-GFP2 (OxD (Grx1-roGFP2)) sensor from plots depicted in (J) (mean \pm s.e.m.,
819 n=4, unpaired t-test).

820 **Fig. 6. The *ADH5* ortholog in *C. elegans* and GSH synthesis in Fanconi Anemia-deficient cells**

- 821 **a.** Various developmental stages of a *C. elegans* transgenic line expressing *Ex[p_{adh-5}ADH-5::GFP; p_{myo-2}tdTomato]*. White arrows point to nuclei. Scale bars represent 50 μ m. Gray inlays show the corresponding
822 DIC image. (i) Adult stage boxes refer to ii and iii. (ii) Head area with focus on the intestine of the adult depicted
823

824 in i, recorded with 40x magnification. (iii) Tail area with focus on the cuticle of the animal shown in i, recorded
825 with 40x magnification. (iv) L4 stage. (v) L3 stage. (vi) Late embryo.

826 **b.** Scheme depicting the protocol used to treat *C. elegans* with formaldehyde (FA) and N-acetylcysteine (NAC).

827 **c.** Survival of L1-staged Wild type (N2) and *adh-5* mutant upon exposure to the indicated FA concentrations
828 and 10 μ M NAC measured directly after treatment (mean \pm S.D., n=3).

829 **d.** Development profile of surviving animals 72 h after FA exposure (mean \pm S.D., n=3).

830 **e.** Scheme depicting the protocol used to treat *C. elegans* with FA and paraquat (PQ).

831 **f.** Development profile of surviving animals 72 h upon FA and PQ exposure (mean \pm S.D., n=3).

832 **g.** Resazurin-based viability assay for Nalm6 cells exposed to increasing concentrations of FA and 50 μ M L-
833 buthionine-sulfoximine (L-BSO), denoting the protection against FA by glutathione (GSH) biosynthesis (mean
834 \pm s.e.m., n=6, asterisks represent one-way ANOVA for multiple comparison using a Tukey-corrected test
835 between Δ FANCB and Δ FANCB + L-BSO 50 μ M).

836 **h.** Resazurin-based viability assay for Nalm6 cells exposed to increasing concentrations of L-BSO (mean \pm
837 s.e.m., n=6, asterisks represent the statistical significance according to one-way ANOVA for multiple
838 comparison using a Tukey-corrected test between Wild type (WT) and Δ FANCB cells).

839 **i.** Model for FA metabolism and the crosstalk with GSH metabolism highlighting the formation of HSMGSH
840 adducts and the metabolization through ADH5. GSH supply by ADH5 limits oxidative stress and sustains the
841 balance between GSH and GSH disulfide (GSSG).

842 **Supplemental Information titles and legends**

843 **Extended Data Fig. 1, Δ ADH5 cell generation and P53 response to formaldehyde**

844 a. Western blot analysis of ADH5 expression in clones edited by CRISPR/Cas9. **b.** *ADH5* gene showing the exon
845 targeted by CRISPR/Cas9 and the genetic modifications of the Δ ADH5 clone used in this work. **c.** Left:
846 Representative flow cytometry plot for Annexin V determination in Δ ADH5 cells untreated or exposed to 150
847 μ M formaldehyde (FA). Right: Gating strategy for determining apoptotic cells. **d.** Resazurin-based viability
848 assay for Nalm6 cells exposed to increasing concentrations of FA (mean \pm s.e.m., n=6, asterisks represent the
849 statistical significance according to one-way ANOVA for multiple comparison using a Tukey-corrected test

850 between WT and $\Delta ADH5$). **e.** Western blot analysis of ADH5 expression in $\Delta P53$ clones edited by CRISPR/Cas9.
851 **f.** *ADH5* gene showing the exon targeted and the genetic modifications of the $\Delta P53 \Delta ADH5$ clone used in this
852 work. **g.** Western blot against p21 and p53. Loading control β -actin. **h.** Quantitation of western blots against
853 p21 (left) and p53 (right) (s.e.m., n=3).

854

855 **Extended Data Fig. 2, GSH biosynthesis inactivation**

856 **a.** Resazurin-based viability assay for HCT116 (left) and Nalm6 (right) Wild type (WT) and $\Delta ADH5$ cells at 100
857 and 50 μM L-buthionine-sulfoximine (L-BSO), respectively (s.e.m., n=5). **b.** Western blot analysis of *GCLM*
858 expression in clones edited by CRISPR/Cas9. **c.** *ADH5* gene showing the exon targeted by CRISPR/Cas9 and the
859 genetic modifications of the $\Delta ADH5$ clone on which *GCLM* was inactivated. **d.** *GCLM* gene showing the exon
860 targeted by CRISPR/Cas9 and the genetic modifications of the $\Delta GCLM$ clones used in this work.

861

862 **Extended Data Fig. 3, S-hydroxymethylglutathione synthesis**

863 **a.** Extracted ion chromatograms for $[\text{GSH} + \text{H}]^+$ ion at m/z 308.0916 generated from a 10.4 μM glutathione
864 (GSH) standard solution before reaction (t_0 : green dash line) and after 48h of reaction (t_{48} : green solid line);
865 and for $[\text{S-hydroxymethylglutathione (HSMGSH)} + \text{H}]^+$ ion at m/z 338.1022 generated from a 10.4 μM GSH
866 standard solution before reaction (t_0 : blue dash line) and after 48h reaction (t_{48} : blue solid line). **b.** Mass
867 spectrum for the solvent at t_0 , with no signals detected at m/z 308.0916 or m/z 338.1022. **c.** Mass spectrum
868 for a GSH standard solution at t_0 , with no signal detected at m/z 338.1022. **d.** Mass spectrum for the reaction
869 mixture at t_{48} .

870

871 **Extended Data Fig. 4, *in vivo* detection of S-hydroxymethylglutathione**

872 **a.** Mass spectrum for $[\text{S-hydroxymethylglutathione (HSMGSH)} + \text{H}]^+$ ion at m/z 338.1022 in a Wild type (WT)
873 sample (green), and its simulated isotopic pattern (blue). **b.** Extracted ion chromatograms for $[\text{Glutathione}$
874 $(\text{GSH}) + \text{H}]^+$ ion at m/z 308.0916 \pm 0.0500 generated from a non-spiked QC sample (green), a 43 μM spiked QC
875 sample (red), and a 14.3 μM GSH standard solution (blue). **c.** Mass spectrum for $[\text{GSH} + \text{H}]^+$ ion at m/z 308.0916

876 obtained from a QC sample (green), and its simulated isotopic pattern (blue). **d.** Product ion mass spectrum
877 for [GSH + H]⁺ precursor ion obtained from a QC sample (green), and a 14.3 μM GSH standard solution (blue),
878 using a collision cell voltage of 10 V. **e.** Extracted ion chromatograms for [GSH disulfide(GSSG) + H]⁺ ion at *m/z*
879 613.1598 ± 0.0500 generated from a non-spiked QC sample (green), a 15.5 μM spiked QC sample (red), and a
880 15.5 μM GSSG standard solution (blue). **f.** Mass spectrum for [GSSG + H]⁺ ion at *m/z* 613.1598 obtained from
881 a QC sample (green), and its simulated isotopic pattern (blue). **g.** Product ion mass spectrum for [GSSG + H]⁺
882 precursor ion obtained from a QC sample (green), and a 15.5 μM GSSG standard solution (blue) using a
883 collision cell voltage of 20 V. **h.** Extracted ion chromatograms for [GSSG + 2H]²⁺ ion at *m/z* 307.0838 ± 0.0500
884 generated from a non-spiked QC sample (green), a 15.5 μM spiked QC sample (red), and a 15.5 μM GSSG
885 standard solution (blue). **i.** Mass spectrum for [GSSG + 2H]²⁺ ion at *m/z* 307.0838 obtained from a QC sample
886 (green), and its simulated isotopic pattern (blue). **j.** Mass spectrum for [GSSG + H]⁺ precursor ion obtained
887 from a 15.5 μM GSSG standard solution (blue).

888

889 **Extended Data Fig. 5, ADH5 is conserved and prevents FA toxicity in *C. elegans*, related to Figure 6**

890 **a.** Phylogenetic analysis of *ADH5*-homolog genes in eukaryote highlighting the ortholog gene
891 (*gi_71997431/H24K24.3*) found in *C. elegans*.

892 **b.** Survival of L1-staged Wild type (N2) and *adh-5* mutant upon exposure to the indicated FA concentrations
893 and 10 μM NAC measured 0, 24, 48 and 72 h after treatment (mean ± S.D., n=3).

894 **c.** Development profile of surviving animals 48 h after FA exposure (mean ± S.D., n=3).

895

896 **Source data:**

897 **Source Data Fig. 2:** Uncropped images for western blots from Fig. 2

898 **Source Data Extended Data Fig. 1:** Uncropped images for western blots from Extended Data Fig. 1

899 **Source Data Extended Data Fig. 5:** List of sequences used for phylogenetic analysis.

900

Figure 1

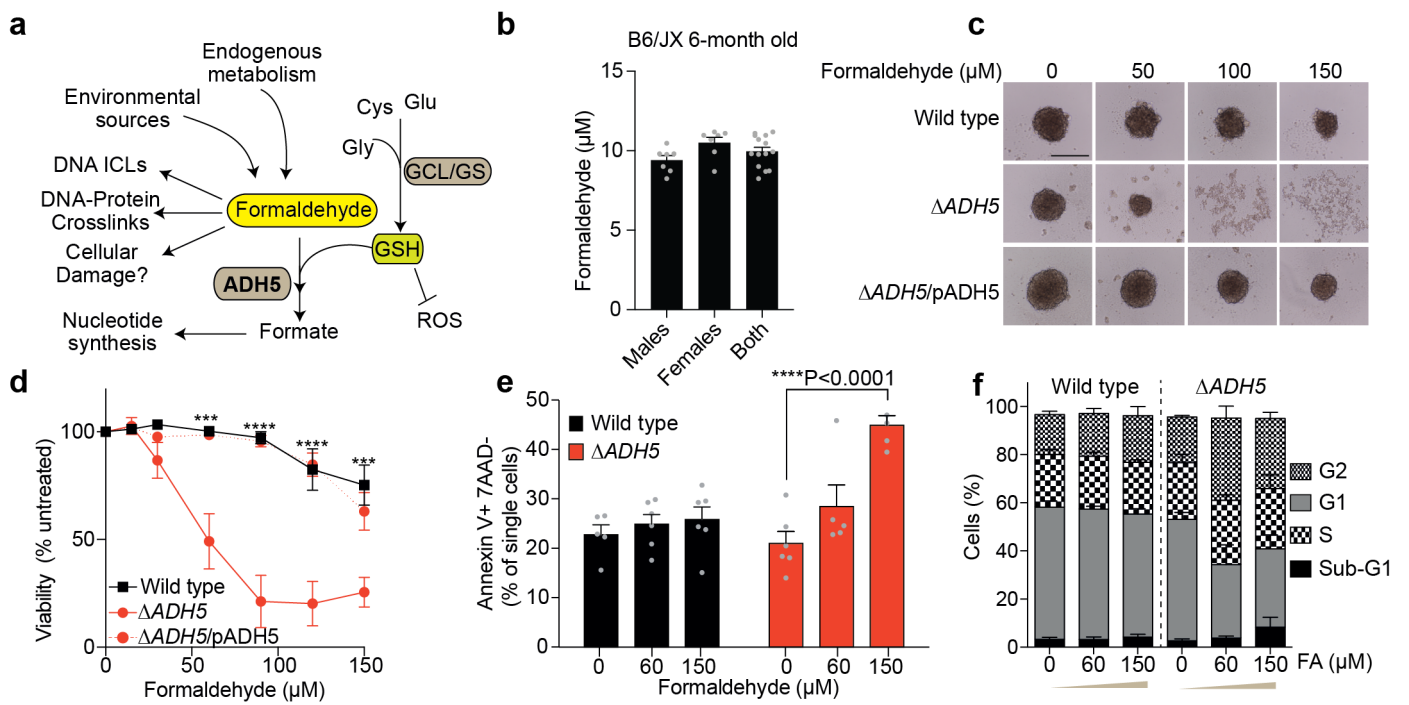


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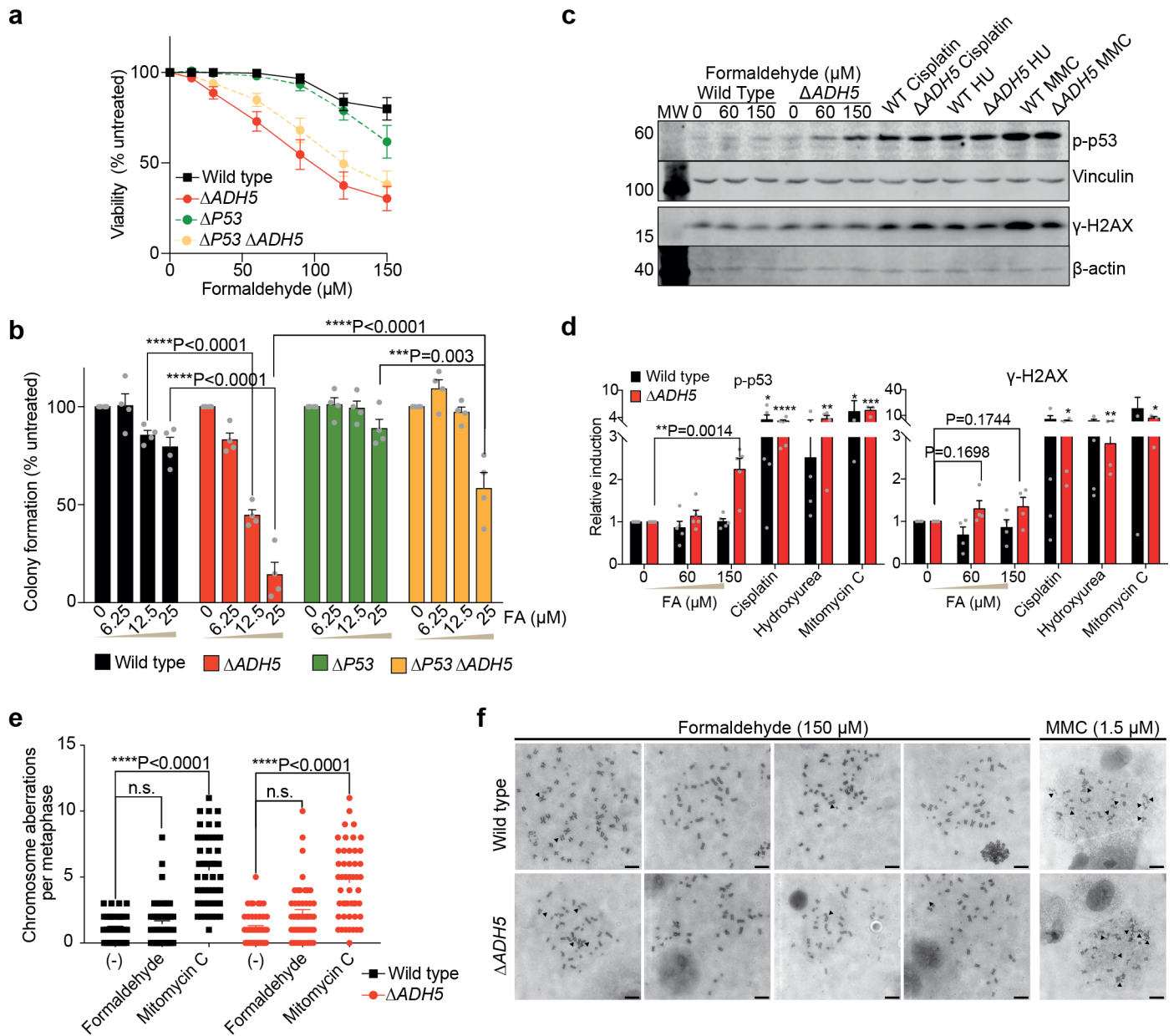


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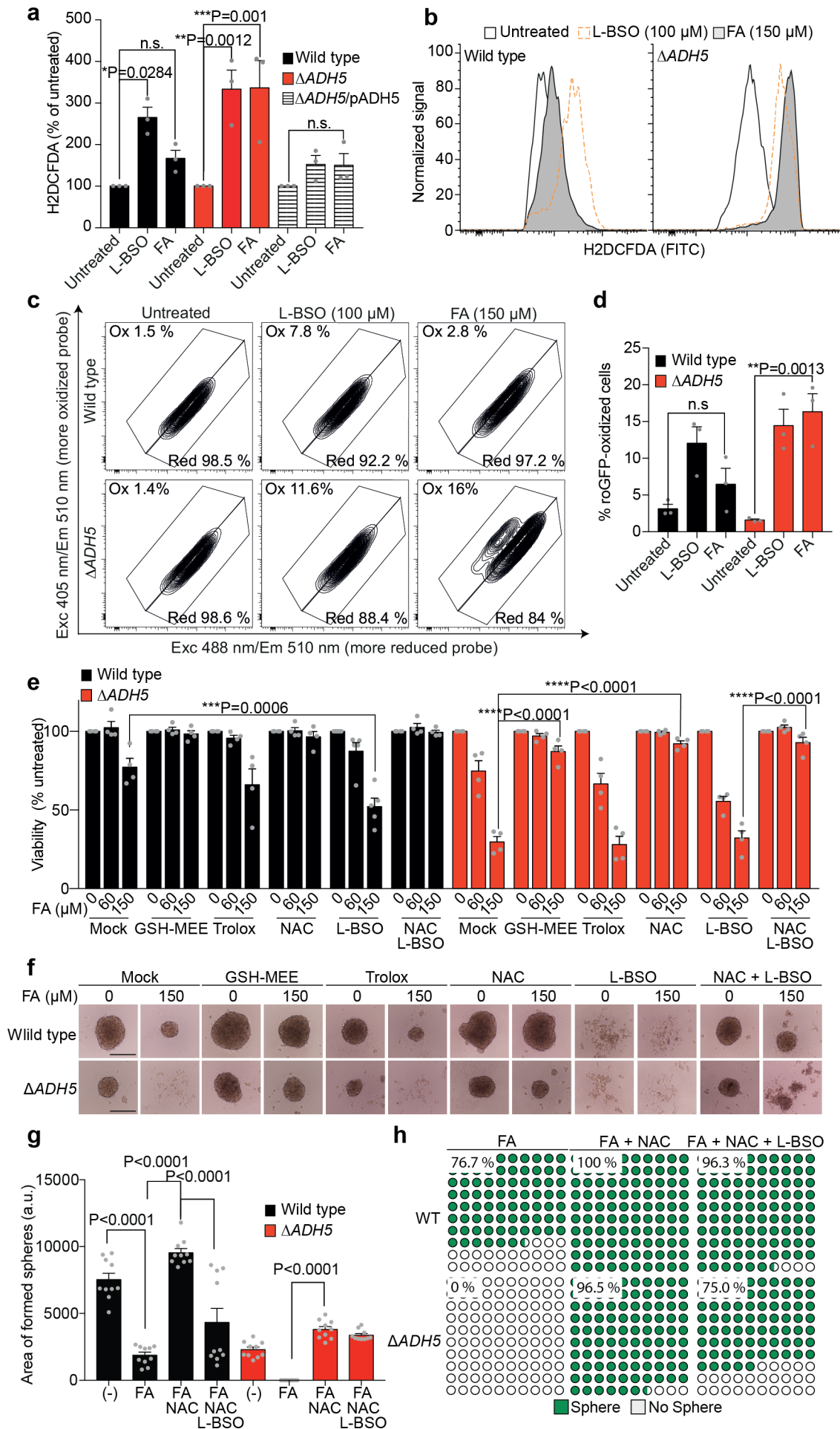


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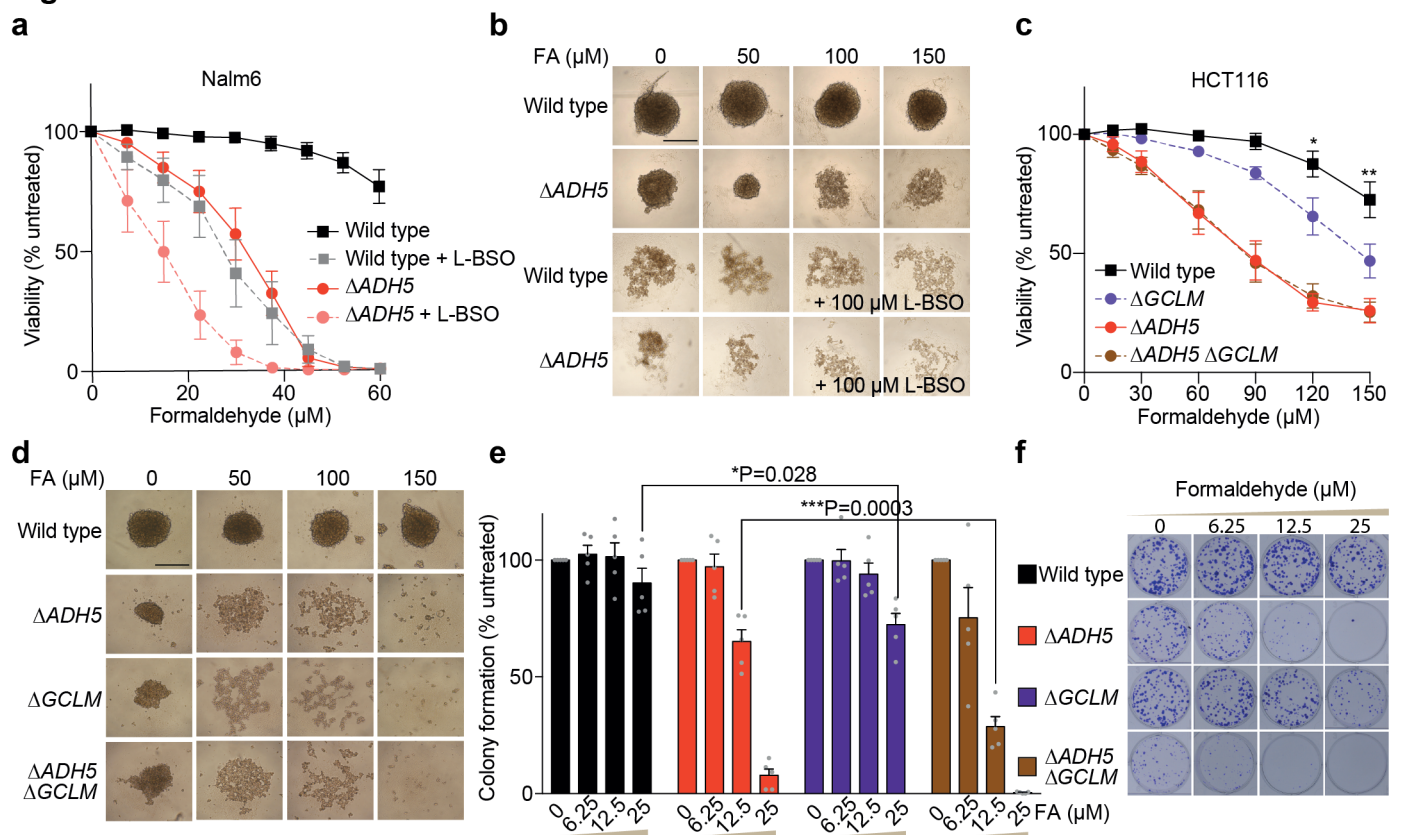


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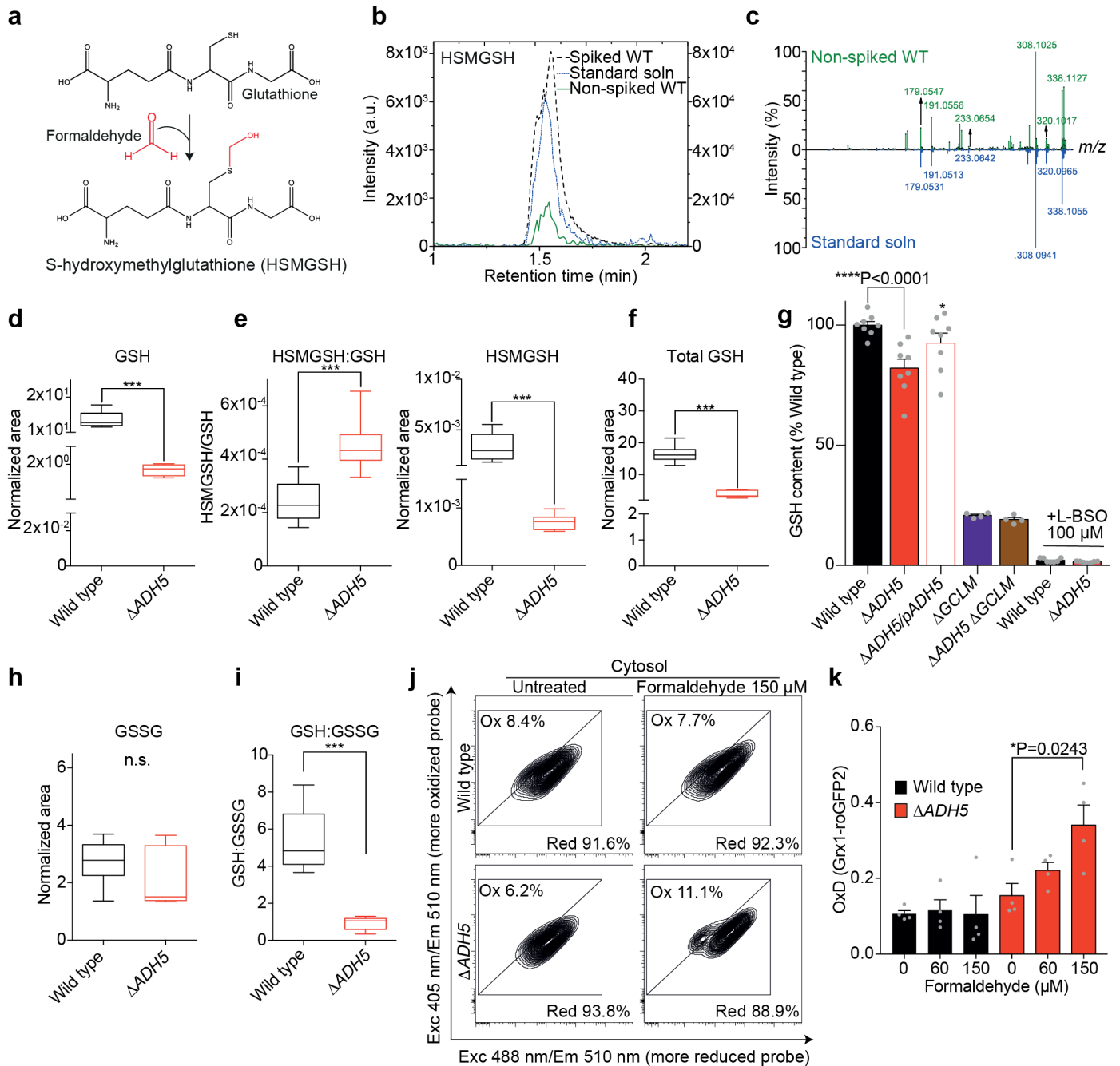
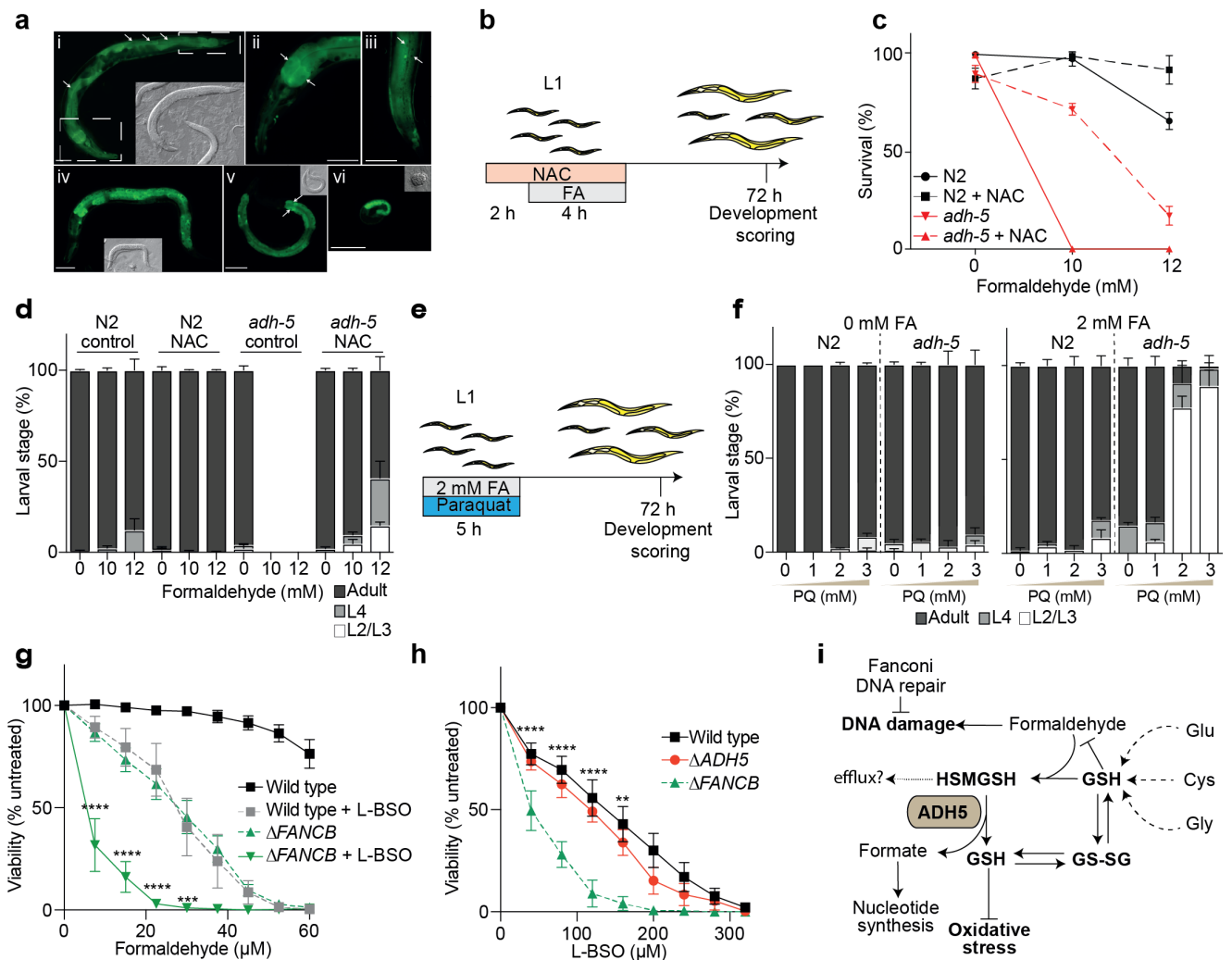
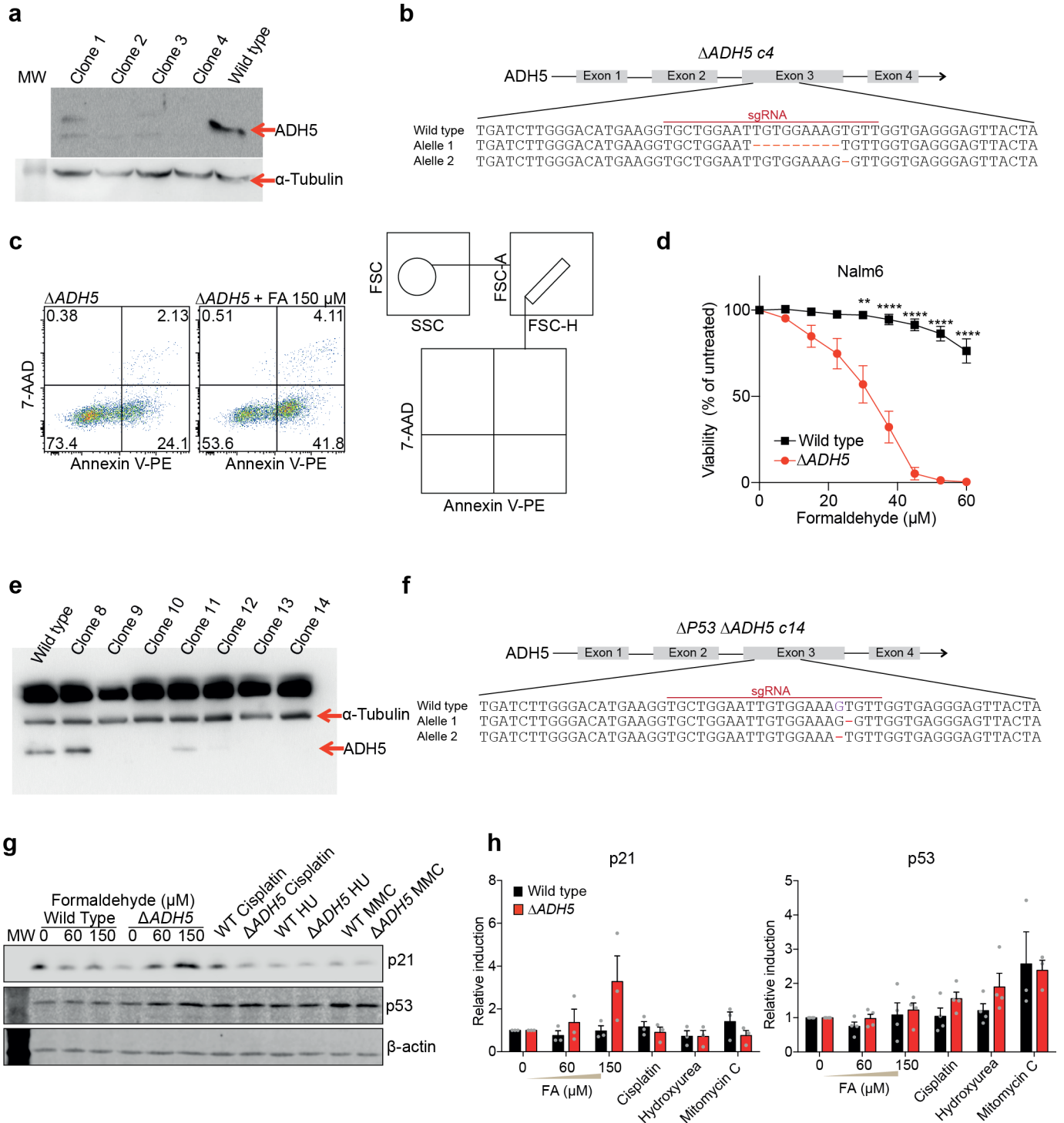


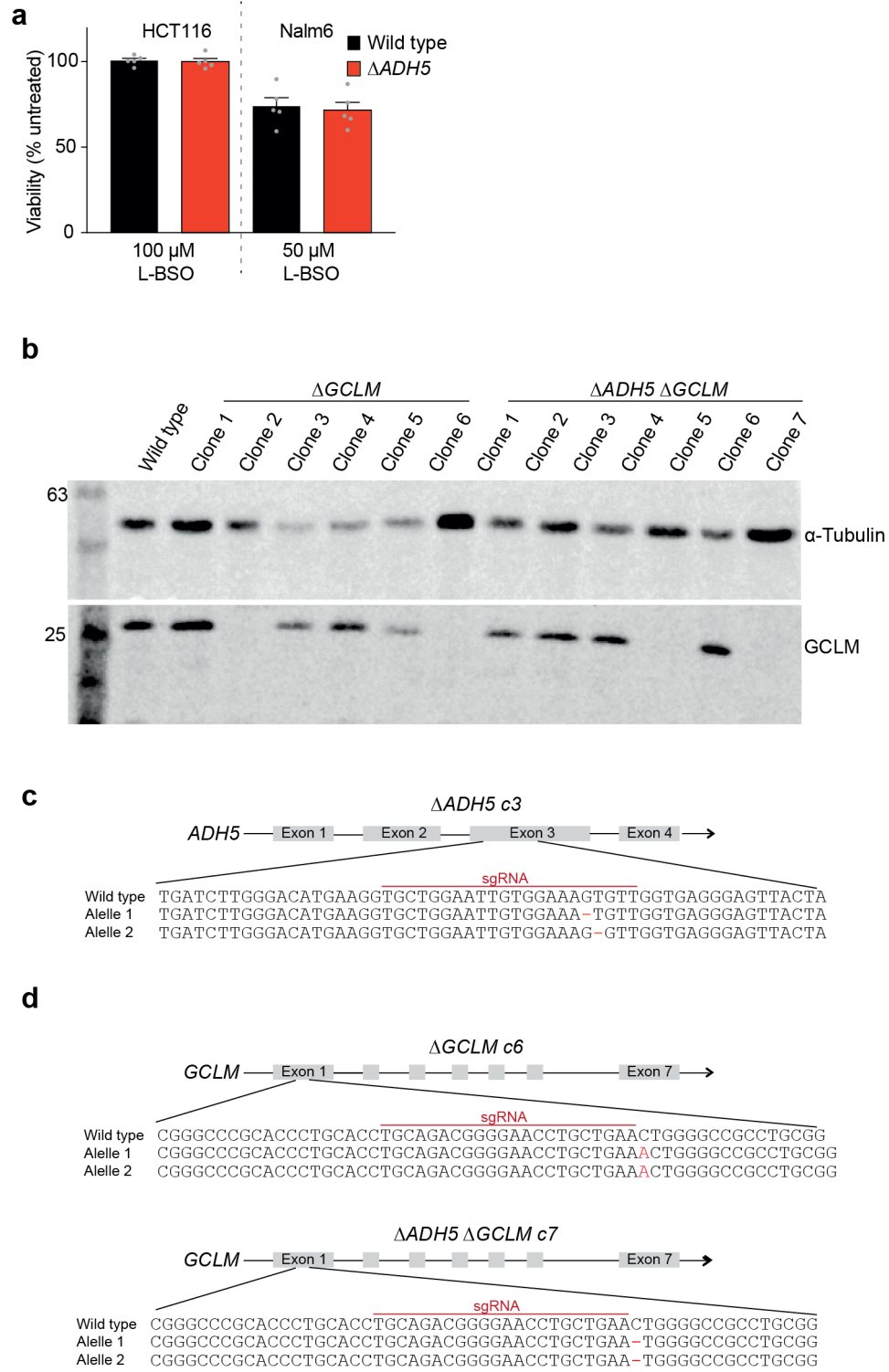
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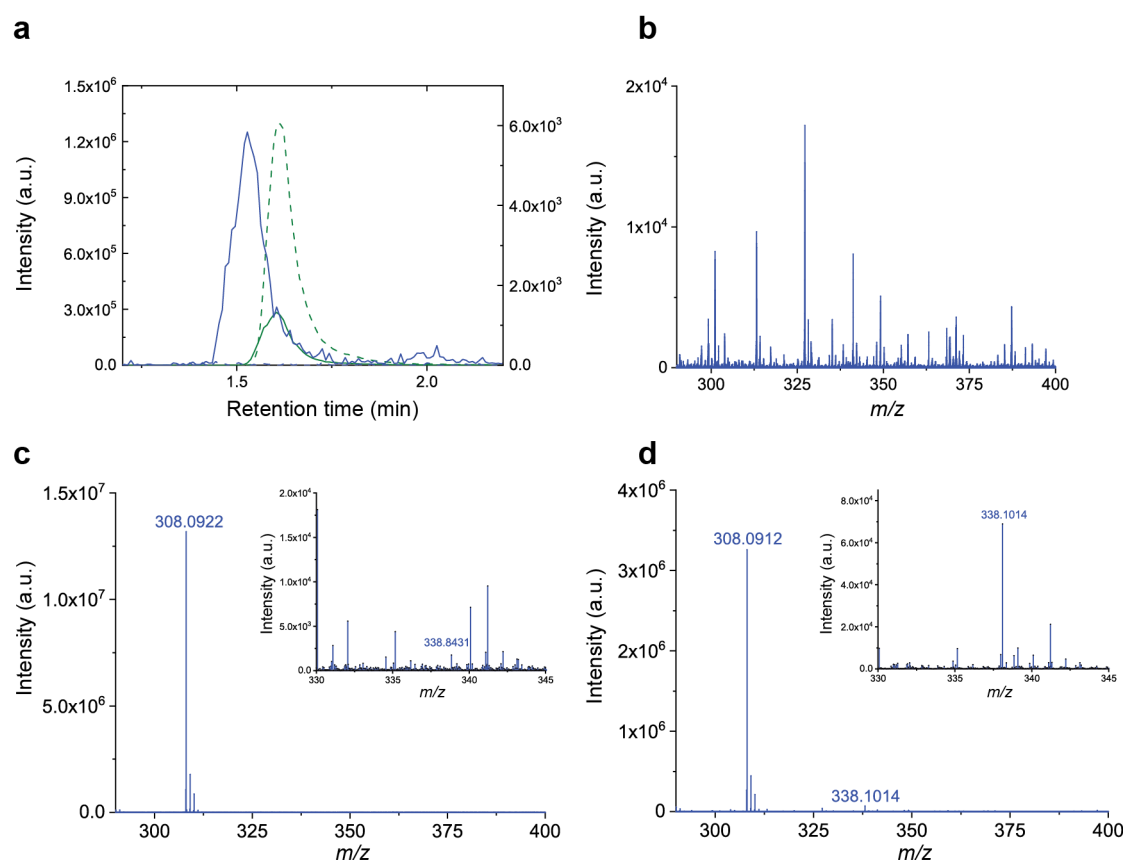
Extended Data Fig. 1



Extended Data Fig. 2

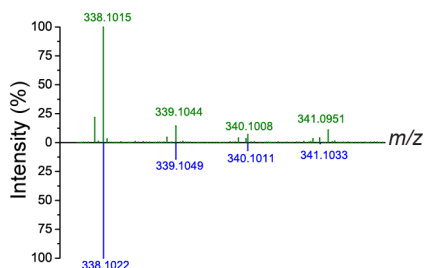


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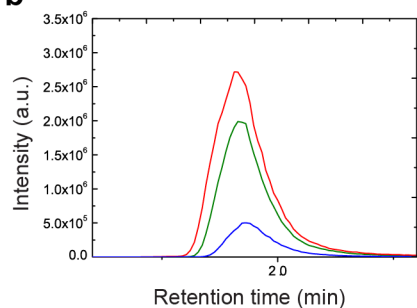


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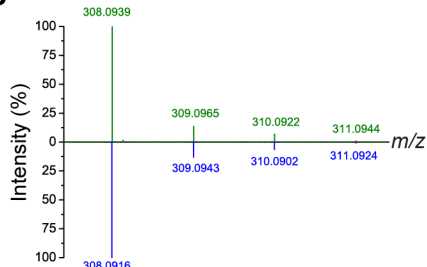
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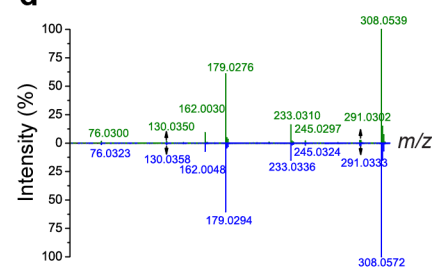
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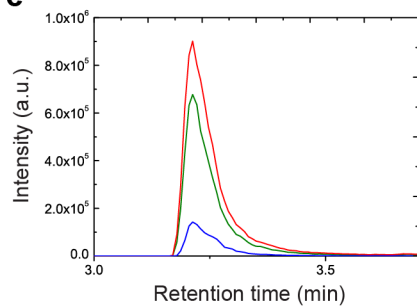
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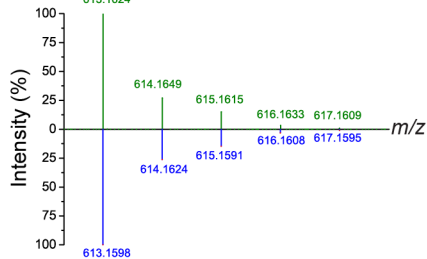
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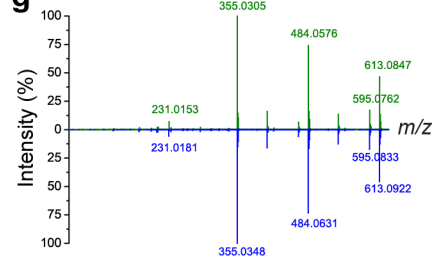
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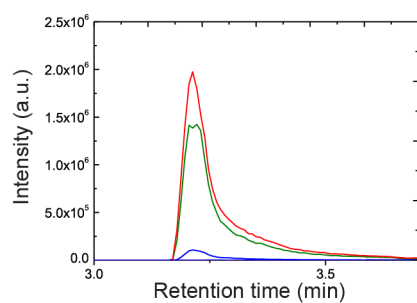
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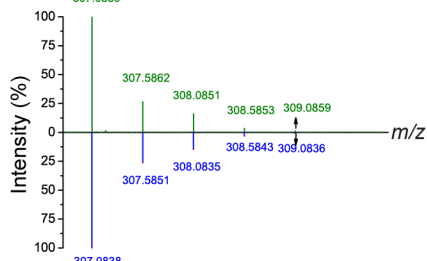
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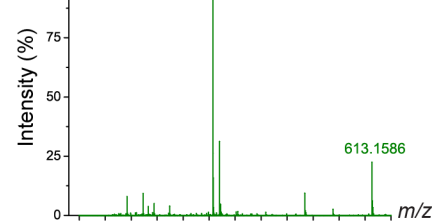
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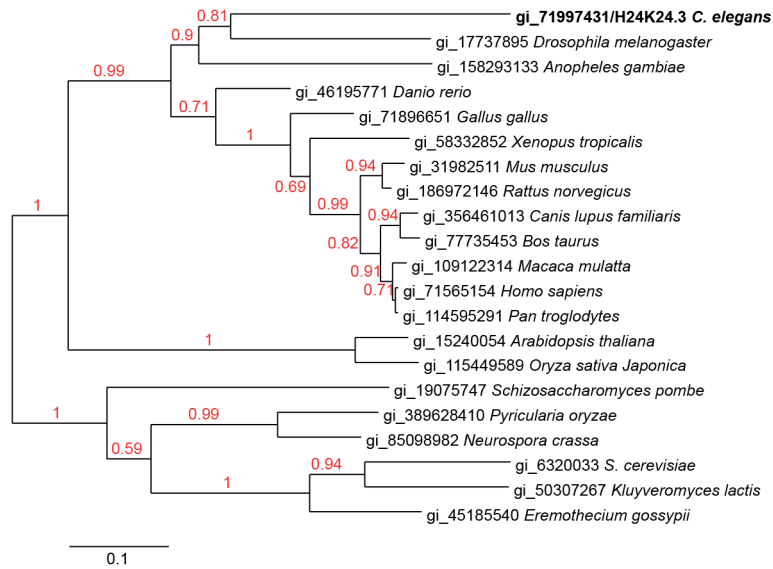


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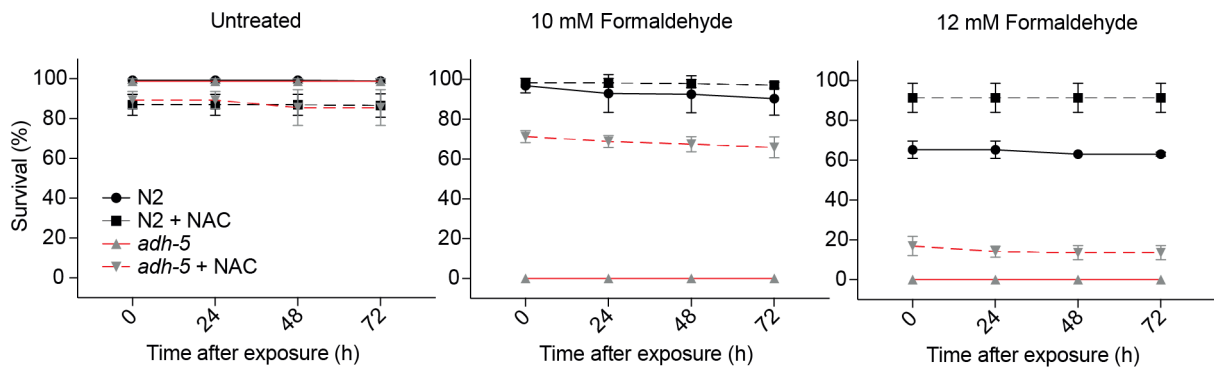


Extended Data Fig. 5

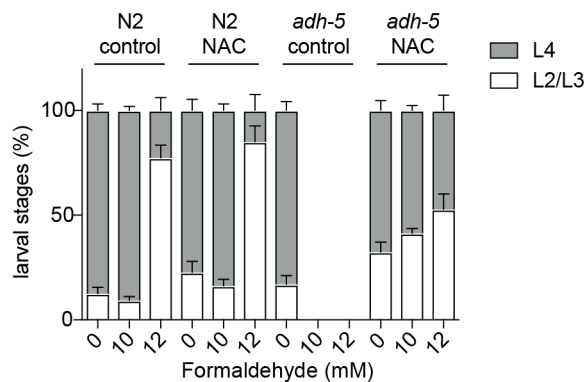
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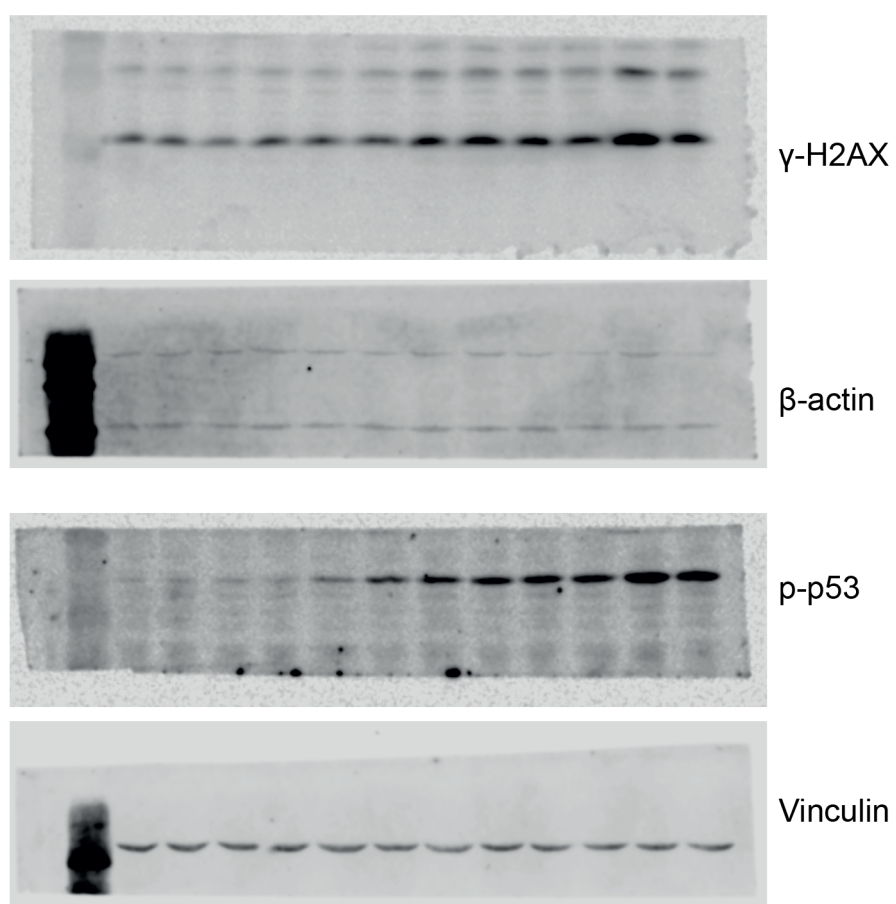
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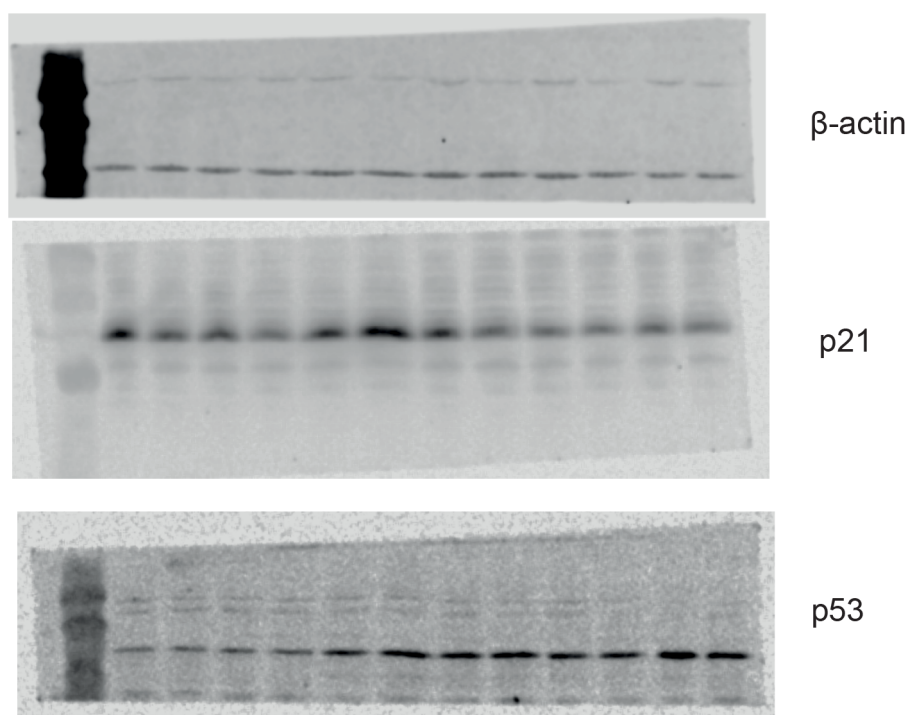
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Source Data Fig. 2: Uncropped images Fig. 2



Source Data Extended Data Fig. 1: Uncropped images Extended Data Fig.1



Source Data Extended Data Fig. 5

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