

1 **Chemical reprogramming ameliorates cellular hallmarks of**
2 **aging and extends lifespan**

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11 **ABSTRACT**

12 The dedifferentiation of somatic cells into a pluripotent state by cellular
13 reprogramming coincides with a reversal of age-associated molecular hallmarks.
14 Although transcription factor induced cellular reprogramming has been shown to
15 ameliorate these aging phenotypes in human cells and extend health and lifespan
16 in mice, translational applications of this approach are still limited. More recently,
17 chemical reprogramming via small molecule cocktails have demonstrated a similar
18 ability to induce pluripotency in vitro, however, its potential impact on aging is
19 unknown. Here, we demonstrated that partial chemical reprogramming is able to
20 improve key drivers of aging including genomic instability and epigenetic alterations
21 in aged human cells. Moreover, we identified an optimized combination of two
22 reprogramming molecules sufficient to induce the amelioration of additional aging
23 phenotypes including cellular senescence and oxidative stress. Importantly, in vivo
24 application of this two-chemical combination significantly extended *C. elegans*
25 lifespan. Together, these data demonstrate that improvement of key drivers of aging
26 and lifespan extension is possible via chemical induced partial reprogramming,
27 opening a path towards future translational applications.

28

29 **KEYWORDS**

30 Aging, cellular reprogramming, chemical reprogramming, epigenetics, lifespan

31

32 **INTRODUCTION**

33 Biological aging is a global process associated with a loss of homeostasis and
34 functional decline across cellular and physiological systems leading to the
35 development of age-associated chronic diseases and finally death (Rando and
36 Chang, 2012; Kennedy *et al.*, 2014). For this reason, a significant increase in

37 human life expectancy over the last decades has resulted in an extended period of
38 life spent in morbidity (Garmany, Yamada and Terzic, 2021). As aging is a key risk
39 factor for chronic diseases such as cardiovascular disease or neurodegenerative
40 disorders, new therapeutic strategies that target aging are now under intense
41 investigation (Niccoli and Partridge, 2012; Mahmoudi, Xu and Brunet, 2019).
42 Several hallmarks of aging including epigenetic dysregulation, genomic instability,
43 cellular senescence, and stem cell exhaustion have been identified as potential
44 targets for such an optimized therapeutic strategy (López-Otín *et al.*, 2013). Current
45 interventions that target aging include cellular reprogramming, dietary restriction
46 and related mimetics, systemic blood factors, and senolytics. Among these, cellular
47 reprogramming offers a unique prospective for its ability to reset the epigenome and
48 restore multiple aging hallmarks (Conboy *et al.*, 2005; Baker *et al.*, 2011; Lapasset
49 *et al.*, 2011; Longo *et al.*, 2015; Ocampo *et al.*, 2016; Olova *et al.*, 2019).
50 During development, cellular reprogramming induces zygotic and primordial germ
51 cell formation following a dramatic chromatin reorganization to create totipotent and
52 pluripotent cells free of aged molecular defects demonstrating that both cell identity
53 and age are reversible (Seisenberger *et al.*, 2013; Kerepesi *et al.*, 2021).
54 Importantly, this manipulation of cell identity has been recapitulated *in vitro* by
55 several methods including somatic cell nuclear transfer, forced expression of
56 transcription factors, and most recently treatment with small molecules (Gurdon,
57 1962; Takahashi and Yamanaka, 2006; Hou *et al.*, 2013).
58 Although restoration of aged phenotypes such as telomere length, mitochondrial
59 function, proliferation, and transcriptomic signature *in vitro* was demonstrated over
60 a decade ago, application of cellular reprogramming *in vivo* was initially proven
61 unsafe due to the loss of cellular identity leading to tumor and teratoma formation
62 (Lapasset *et al.*, 2011; Abad *et al.*, 2013). To overcome this issue, *in vivo* partial

63 reprogramming by short-term cyclic induction of Oct4, Sox2, Klf4, and c-Myc
64 (OSKM) was a critical advance as it avoided the detrimental loss of cellular identity.
65 Importantly, this limited cyclic expression of OSKM was sufficient to ameliorate
66 multiple aging hallmarks and extend the lifespan of a progeroid mouse strain
67 (Ocampo *et al.*, 2016). Improved regenerative capacity and function has also been
68 demonstrated following therapeutic application of cellular reprogramming in
69 multiple tissues and organs including the intervertebral disc, heart, skin, skeletal
70 muscle, liver, optic nerve, and dentate gyrus (Ocampo *et al.*, 2016; Kurita *et al.*,
71 2018; de Lázaro *et al.*, 2019; Lu *et al.*, 2020; Rodríguez-Matellán *et al.*, 2020; Chen
72 *et al.*, 2021; Cheng *et al.*, 2022; Hishida *et al.*, 2022). Furthermore, several groups
73 have demonstrated the ability to restore multiple aging phenotypes and reset the
74 epigenetic clock utilizing translational non-integrative methods such as modified
75 mRNAs encoding for six transcription factors (OSKM + Lin28 and Nanog) or adeno-
76 associated virus for expression of three factors (OSK) (Sarkar *et al.*, 2020; Lu *et al.*,
77 2020). While promising, methods that require transcription factor expression face
78 significant barriers for their clinical translation such as risk of tumorigenicity and low
79 delivery efficiency (Abad *et al.*, 2013; Ohnishi *et al.*, 2014). In this line, c-Myc and
80 Klf4 have been identified as proto-oncogenes while Oct4 and Sox2 are highly
81 expressed in a variety of human cancers (Klimczak, 2015). For this reason, the
82 clinical application of in vivo reprogramming may require further development.
83 Most recently, small molecule cocktails have been shown to produce chemical
84 induced pluripotent stem cells (ciPSCs) from mouse and human somatic cells (Hou
85 *et al.*, 2013; Guan *et al.*, 2022). These reprogramming compounds fall broadly into
86 three categories including epigenetic, cell signaling, and metabolic modulators
87 (Knyazer *et al.*, 2021). Importantly, small molecule reprogramming and OSKM
88 expression share the ability to overcome multiple reprogramming barriers while

89 retaining a distinct cell fate trajectory (Zhao *et al.*, 2015; Haridhasapavalan *et al.*,
90 2020). To date, the effects of chemical reprogramming on aging hallmarks and
91 lifespan have not been investigated. Considering both the rejuvenating effects of
92 partial reprogramming by short-term expression of OSKM and the ability of small
93 molecule cocktails to induce pluripotency, we proposed the use of chemical induced
94 partial reprogramming for the amelioration of aging phenotypes.
95 Here, we report that short-term treatment of human cells with seven small
96 molecules (7c), previously identified for their capacity to induce pluripotent stem
97 cells, leads to the improvement of molecular hallmarks of aging. In addition, we
98 show that an optimized cocktail, containing only two of these small molecules (2c),
99 is sufficient to restore multiple aging phenotypes including genomic instability,
100 epigenetic dysregulation, cellular senescence, and elevated reactive oxygen
101 species. Finally, *in vivo* application of this 2c reprogramming cocktail extends
102 lifespan in *C. elegans*.

103

104 **RESULTS**

105 **Chemical induced partial reprogramming significantly improves aging** 106 **hallmarks in aged human fibroblasts**

107 Multiple hallmarks of aging can be ameliorated following partial cellular
108 reprogramming by expression of the Yamanaka factors (OSKM) *in vitro* and *in vivo*
109 (Ocampo, Reddy and Belmonte, 2016). On the other hand, although chemical
110 reprogramming with seven small molecules (7c) has been shown to generate
111 chemically induced pluripotent stem cells (Hou *et al.*, 2013), whether chemical
112 induced partial reprogramming is also able to restore aged phenotypes is unknown.
113 Therefore, we sought to determine the effect of short-term 7c treatment on aging
114 phenotypes in primary aged human fibroblasts (Fig. 1a). Specifically, we asked

115 whether chemical induced partial reprogramming could improve multiple hallmarks
116 of aging, including DNA damage, heterochromatin loss, cellular senescence, and
117 reactive oxygen species (ROS) *in vitro*. Towards this goal, primary human
118 fibroblasts isolated from aged dermal tissue samples were treated for 6 days with a
119 7c cocktail including: CHIR99021, DZNep, Forskolin, TTNPB, Valproic acid (VPA),
120 Repsox, and Tranylcypromine (TCP). Notably, the levels of the DNA damage
121 marker γ H2AX were significantly decreased in aged cells after treatment (Fig. 1b).
122 Interestingly, a decrease in γ H2AX levels was also observed when 7c was added
123 for 6 days to aged cells that were pretreated with the DNA damage inducing agent
124 doxorubicin for 2 days, indicating a significantly improved DNA damage response
125 upon 7c treatment (Fig. 1c). Thus, we determined that short-term 7c treatment
126 improves DNA damage in primary aged human fibroblasts.

127 Epigenetic dysregulation and heterochromatin loss are key molecular markers of
128 aging (Haithcock *et al.*, 2005; Scaffidi and Misteli, 2006; Ni *et al.*, 2012; Brunet and
129 Berger, 2014; Djeghloul *et al.*, 2016; Kane and Sinclair, 2019). For this reason, we
130 next examined the effect of 7c treatment on the constitutive and facultative
131 heterochromatin marks H3K9me3 and H3K27me3. Our results show that 6 days of
132 7c treatment significantly increased the constitutive heterochromatin mark
133 H3K9me3 in aged human fibroblasts (Fig. 1d). In aged cells, previous work has
134 shown that the facultative heterochromatin marker H3K27me3 is decreased at the
135 senescence-associated *p16* gene locus (*CDKN2A*) leading to increased
136 expression, cell cycle arrest, and senescence (Dhawan, Tschen and Bhushan,
137 2009). Interestingly, we observed that H3K27me3 was significantly increased after
138 6 days of 7c treatment (Fig. 1e). Next, as cellular senescence has been shown to
139 be a key driver of aged tissue dysfunction and ablation of senescent cells has been
140 demonstrated to extend health and lifespan in mice (Baker *et al.*, 2011), we

141 determined the impact of chemical induced partial reprogramming on senescence-
142 associated gene expression. For this purpose, we serially passaged aged human
143 fibroblasts for 28 days to promote replicative induced senescence (RIS) in the
144 presence of continuous 7c treatment. Importantly, the 7c treated group showed a
145 downregulation of the senescence-associated cell cycle genes *p21* and *p53* relative
146 to untreated controls (Fig. 1f). On the other hand, we noted that the senescence-
147 associated secretory cytokine *IL6* was significantly upregulated upon long-term
148 treatment with 7c and therefore could not conclude a positive effect of 7c treatment
149 on senescence (Fig. 1f and S1a).

150 Next, further characterization of the transcriptomic effects of 7c treatment for 6 days
151 was performed by bulk RNA sequencing on aged fibroblasts treated with either 7c
152 or vehicle control. Principle component analysis (PCA) showed that 7c treated cells
153 clustered separately from the control group indicating that a distinct transcriptomic
154 profile emerges following chemical induced partial reprogramming (Fig. 1g). Gene
155 ontology (GO) enrichment analysis revealed that developmental processes were
156 significantly upregulated following 7c treatment relative to control, whereas mitosis
157 and cell proliferation programs were significantly downregulated (Fig. 1h).
158 Interestingly, numerous cellular reprogramming, stem cell, and self-renewal genes
159 within the GO term developmental pathways were significantly upregulated
160 following 7c treatment compared to control including *WNT5a*, *NOTCH1A*, *SOX4*,
161 *SALL1*, *NOG*, and *BMP4* indicating that 7c induced a shift towards a developmental
162 associated transcriptomic profile (Fig. 1i and S1b).

163 Since our RNA seq analysis indicated a downregulation in mitosis and proliferation
164 programs, we next evaluated the effect of 7c treatment on cellular proliferation. In
165 agreement with these results, we observed that 7c significantly decreased cell
166 density based on MTS assay (Fig. 2a). This observation was confirmed by a strong

167 decrease in the proliferation associated marker Ki67 in cells treated with 7c (Fig.
168 2b). Subsequently, to determine whether the effect of 7c on proliferation was dose-
169 dependent, a serial dilution assay of 7c treatment was performed. Notably, different
170 concentrations of 7c continued to impair proliferation (Fig. S2a). Overall, these
171 results validate our transcriptomic findings indicating that mitosis and proliferation
172 related programs are downregulated upon 7c treatment.
173 Next, in order to investigate whether the decrease in DNA damage upon 7c
174 treatment was independent of cell cycle impairment, we tested the effect of 7c
175 treatment under non-proliferative conditions in the presence of low-serum culture
176 conditions (i.e. 1% FBS culture media). Remarkably, regardless of growth
177 conditions and proliferation, 7c treatment still induced a reduction in γ H2AX levels
178 (Fig. 2c), suggesting that the impact of 7c on DNA damage is independent from its
179 effects on proliferation. In addition, to gain further insight into the metabolic changes
180 induced upon 7c treatment, we investigated the levels of reactive oxygen species
181 (ROS), which are associated with mitochondrial function, cellular stress and DNA
182 damage (Shields, Traa and Van Raamsdonk, 2021). Notably, a significant increase
183 in reactive oxygen species (ROS) in aged fibroblasts was observed upon 7c
184 treatment (Fig. 2d). Thus, although 7c treatment can decrease γ H2AX levels, we
185 find that it also leads to impaired proliferative capacity, even at low concentrations,
186 and an upregulation of ROS. Taken together, chemical induced partial
187 reprogramming via 7c treatment in aged human fibroblasts lacks the multiparameter
188 rejuvenation associated with OSKM-induced reprogramming. In particular, 7c
189 treatment results in the improvement of several key aging phenotypes such as DNA
190 damage, epigenetic dysregulation, and senescence markers while at the same time
191 leading to an impairment of proliferation, increased ROS, and upregulation of *IL6*.

192 These results suggest that further optimization of chemical reprogramming may be
193 required.

194

195 **A reduced reprogramming cocktail efficiently improves multiple molecular
196 hallmarks of aging**

197 In order to create an optimized cocktail for the amelioration of age-associated
198 phenotypes by chemical reprogramming, we first sought to remove compounds with
199 deleterious effects, while still retaining the three key functional categories of
200 chemical reprogramming compounds: epigenetic modifiers, cell signaling modifiers,
201 and metabolic switchers (Knyazer *et al.*, 2021). First, using an MTS assay, we
202 observed that while cell survival was unaffected or enhanced by Repsox or TCP
203 treatment respectively, it was significantly impaired by CHIR99021, DZNep,
204 Forskolin, TTNPB, and VPA, in agreement with previous publications and
205 suggesting their removal (Fig. S2b) (Wu *et al.*, 2006; Jung *et al.*, 2008; Rodriguez
206 *et al.*, 2013; Fujiwara *et al.*, 2014). In addition, DZNep has a known S-
207 adenosylhomocysteine (SAH) hydrolase mediated inhibitory effect on the H3K27
208 methyltransferase EZH2, further supporting its exclusion (Girard *et al.*, 2014). The
209 remaining compounds, TCP and Repsox, met the selection criteria for chemical
210 reprogramming functional categories and were thus selected. Therefore, we next
211 treated aged human fibroblasts with this reduced two-chemical cocktail (2c) for 6
212 days to determine its effect on aging hallmarks. Strikingly, similar to our previous
213 results with 7c, γ H2AX levels were significantly decreased upon 2c treatment (Fig.
214 3a). Furthermore, improvement on γ H2AX levels was observed when cells were
215 treated with 2c following addition of the DNA damaging agent doxorubicin (Fig.
216 S3b). Moreover, 2c significantly increased both H3K9me3 and H3K27me3 levels

217 (Fig. 3b-c). Taken together, these data indicate that 2c treatment improves DNA
218 damage and heterochromatin marks similar to 7c treatment.

219 Next, we sought to determine the impact of 2c treatment on cellular senescence in
220 both a genotoxic stress induced senescence model using doxorubicin application
221 and RIS after multiple passages. First, doxorubicin induced senescent cells
222 pretreated with 2c showed a significant decrease in senescence-associated beta-
223 galactosidase (SA-beta-gal) levels and *p21* gene expression compared to
224 untreated controls (Fig. 3d-e). Interestingly, 2c significantly decreased SA-beta-gal
225 levels only when added prior to induction, indicating a protective rather than
226 senolytic effect upon genotoxic treatment (Fig. S3c). Moreover, in our RIS model,
227 SA-beta-gal levels were significantly decreased in aged fibroblasts with continuous
228 2c treatment (Fig. 3f). In addition, senescence-associated and age-related stress
229 response genes *p21*, *p53*, and *IL6*, were also downregulated upon 2c treatment
230 after 6 days or 29 days of treatment (Fig. 3g). Taken together, these data show that
231 2c treatment reduces cellular senescence and significantly decreases *IL6* levels in
232 contrast to 7c treatment.

233 Next, as 7c treatment previously led to impaired proliferation and increased ROS
234 levels, we sought to determine the impact of 2c on these cellular phenotypes.
235 Importantly, 2c treatment had only a mild effect on cell proliferation compared to the
236 50% decrease induced by 7c treatment (Fig. 3h). In addition, in clear contrast to the
237 impact of 7c, 2c treatment significantly decreased ROS levels in aged fibroblasts,
238 indicating that 2c can markedly improve cellular homeostasis (Fig. 3i). Similarly, we
239 observed an improvement in ROS levels with 2c treatment when cells were co-
240 treated with the pro-oxidant Antimycin A (Fig. S3a).

241 Overall, these results show that the reduced 2c cocktail is able to improve multiple
242 age-related hallmarks including genomic instability, epigenetic dysregulation, and

243 cellular senescence. Importantly, treatment with 2c had a minor effect on
244 proliferation and led to a decrease in ROS levels. Taken together, these data
245 indicate that 2c is an optimized cocktail capable of improving multiple age-related
246 markers in vitro.

247

248 **2c treatment increases *C. elegans* lifespan**

249 Finally, in order to determine whether our 2c cocktail could also impact biological
250 aging in vivo, we tested the effect of 2c treatment on the lifespan of a commonly
251 used aging model organism, the nematode *Caenorhabditis elegans*. Towards this
252 goal, we monitored survival in *C. elegans* treated with either 2c, Repsox, or TCP at
253 three different concentrations (50, 100, or 200 μ M) alongside a vehicle control.
254 Strikingly, we observed that 2c treatment at 50 μ M was sufficient to extend *C.*
255 *elegans* median lifespan from 19 to 27 days, corresponding to a 42.1% increase
256 relative to vehicle control (Fig. 4a, e). To a lesser extent, Repsox or TCP alone at
257 50 μ M also increased *C. elegans* median lifespan to 25 days, a 31.6% increase
258 over vehicle control (Fig. 4a, e). These results indicate that Repsox and TCP are
259 each able to extend median lifespan in *C. elegans*, and when combined as part of
260 the 2c cocktail, can lead to an even greater increase in median lifespan.

261 In addition, we observed dose-dependent effects across all treatments (Fig. 4b-e).
262 Interestingly, the 2c cocktail or Repsox alone did not increase *C. elegans* lifespan
263 at 200 μ M (Fig. 4b-c, e), suggesting that this higher dose may impact off target
264 mechanisms and be slightly toxic. On the other hand, TCP still increased median
265 lifespan by 15.8% at 100 or 200 μ M relative to vehicle controls even though it was
266 most effective at 50 μ M (Fig. 4d-e). Taken together, these data demonstrate that
267 the optimized 2c cocktail can both ameliorate multiple aging hallmarks in aged
268 human fibroblasts in vitro and extend *C. elegans* lifespan in vivo.

269

270 **DISCUSSION**

271 The molecular identity and age of somatic cells have proven to be plastic states
272 that can be reset by cellular reprogramming (Gurdon, 1962; Campbell *et al.*, 1996;
273 Takahashi and Yamanaka, 2006; Lapasset *et al.*, 2011). As aging and age-
274 associated diseases are a major societal burden, the need for aging interventions
275 such as cellular reprogramming has grown (Mahmoudi, Xu and Brunet, 2019;
276 Garmany, Yamada and Terzic, 2021). Although multiple groups have now
277 demonstrated that *in vivo* partial reprogramming via transient application of OSKM
278 can rejuvenate molecular hallmarks of aging, restore tissue function, and extend
279 lifespan in mouse models, the risks of oncogenesis and inefficient gene delivery
280 hinders clinical development (Ocampo *et al.*, 2016; Kurita *et al.*, 2018; de Lázaro *et*
281 *al.*, 2019; Lu *et al.*, 2020; Rodríguez-Matellán *et al.*, 2020; Sarkar *et al.*, 2020; Chen
282 *et al.*, 2021; Cheng *et al.*, 2022; Hishida *et al.*, 2022). Interestingly, a more
283 translational approach for the induction of cellular reprogramming based on the use
284 of small molecules has been recently developed (Hou *et al.*, 2013; Zhao *et al.*, 2015;
285 Cao *et al.*, 2018; Guan *et al.*, 2022). Still, the effects of small molecule induced
286 cellular reprogramming on aging hallmarks and lifespan were, until now, unknown.
287 Here, we demonstrated for the first time that partial chemical reprogramming
288 induces multiparameter rejuvenation of key aging hallmarks including genomic
289 instability, epigenetic dysregulation, and cellular senescence *in vitro* while
290 simultaneously extending the lifespan of *C. elegans* *in vivo*. In particular, we
291 demonstrated that the seven chemical reprogramming cocktail, defined by Hou *et*
292 *al.*, was able to improve age-associated DNA damage, epigenetic alterations, and
293 induce a unique transcriptomic profile enriched for developmental processes in
294 aged human fibroblasts *in vitro*. Our observations that 7c significantly impaired

295 proliferation and increased ROS levels might contribute to the previously observed
296 low efficiency of mouse iPSC induction (Hou *et al.*, 2013). We further revealed that
297 an optimized two-compound cocktail (2c) is sufficient to decrease the levels of the
298 DNA damage marker γH2AX, increase H3K9me3 and H3K27me3, prevent both
299 replicative and genotoxic induced senescence, and decrease oxidative stress. Most
300 importantly, we found that our 2c cocktail applied *in vivo* was able to extend the
301 median lifespan of *C. elegans* by 42.1%. Interestingly, the highest doses of 2c or
302 single compounds were less effective to extend lifespan indicating potential off-
303 target effects that may require further optimization.

304 Previous reports have demonstrated that *in vivo* treatment with a modified small
305 molecule reprogramming cocktail similar to Hou *et al.* could enhance regeneration
306 in the liver and heart, thus providing proof of principle that treatment with these
307 reprogramming-associated chemicals could benefit tissue repair (Tang and Cheng,
308 2017; Huang *et al.*, 2018). On the other hand, we have now shown that chemical
309 reprogramming can improve multiple molecular hallmarks of aging similarly to
310 OSKM-induced reprogramming, and extend the lifespan of *C. elegans*.
311 Multiparameter rejuvenation across aging hallmarks is a defining trait of cellular
312 reprogramming albeit future work is required to properly identify the mechanisms
313 responsible for these benefits (Chondronasiou *et al.*, 2022; Gill *et al.*, 2022). In this
314 line, attempts to induce multiparameter amelioration are now emerging as a
315 strategy for rejuvenation even outside the field of cellular reprogramming. In this
316 regard, Shaposhnikov *et al.* recently demonstrated a synergistic effect by targeting
317 multiple aging hallmarks simultaneously, producing a significant increase in
318 *Drosophila melanogaster* lifespan compared to single interventions (Shaposhnikov
319 *et al.*, 2022).

320 Importantly, several translational advantages highlight the potential use of chemical
321 induced partial reprogramming for the amelioration of age-associated phenotypes,
322 including the fact that small molecules are cell permeable and therefore easy to
323 deliver. Furthermore, their effects can be modulated via dosage, and are transient
324 and reversible, thus avoiding oncogenic pitfalls associated with transcription factor
325 induction (Zhao, 2019). In this proof of principle study, our observations indicate
326 that chemical reprogramming represents both a valuable opportunity for the
327 development of future anti-aging interventions, along with the mechanistic
328 understanding of the complex inter-relationships of aging hallmarks and their
329 respective amelioration.

330

331 **METHODS**

332 **Cell culture and maintenance.** Human dermal fibroblasts were freshly extracted
333 using Collagenase I (Sigma, C0130) and Dispase II (Sigma, D4693) and cultured
334 in DMEM (Gibco, 11960085) containing non-essential amino acids (Gibco,
335 11140035), GlutaMax (Gibco, 35050061), Sodium Pyruvate (Gibco, 11360039) and
336 10% fetal bovine serum (FBS, Hyclone, SH30088.03) at 37°C in hypoxic conditions
337 (3% O₂). Subsequently, fibroblasts were passaged and cultured according to
338 standard protocols. Aged donor samples were of 56 and 83 years of age.

339

340 **Immunofluorescence staining.** Cells were washed with fresh PBS and then fixed
341 with 4% paraformaldehyde (Roth, 0964.1) in PBS at room temperature (RT) for 15
342 minutes. After fixation, cells were washed 3 times, followed by a blocking and
343 permeabilization step in 1% bovine serum albumin (Sigma, A9647-50G) in PBST
344 (0.2% Triton X-100 in PBS) for 60 min (Roth, 3051.3). Cells were then incubated at
345 4°C overnight with appropriate primary antibody, washed in PBS, followed by

346 secondary antibody incubation with DAPI staining at RT for 60 min. Coverslips were
347 mounted using Fluoromount-G (Thermofisher, 00-4958-02), dried at RT in the dark
348 for several hours, stored at 4°C until ready to image and -20°C for long-term.

349

350 **Immunofluorescence imaging.** Confocal image acquisition was performed using
351 the Ti2 Yokogawa CSU-W1 Spinning Disk (Nikon), using the 100X objective and
352 with 15 z-sections of 0.3 µm intervals. Appropriate lasers were used (405 nm and
353 488 nm) with a typical laser intensity set to 5-10% transmission of the maximum
354 intensity for methylated histones, Ki67 and ROS, and 30-40% for phosphorylated
355 histones. Exposure time and binning were established separately to assure
356 avoidance of signal saturation.

357

358 **Antibodies and compounds.** Antibodies were provided from the following
359 companies. Abcam: anti-H3K27me3 (ab192985); Cell Signaling: anti-H3K9me3
360 (13969), anti-γH2AX (9718), anti-Ki67 (15580); Bioconcept: anti-H3 (13969);
361 Sigma: anti-β-Actin (A2228); Thermofisher: anti-Rabbit (A32790); Agilent: anti-
362 Rabbit Immunoglobulins/HRP (P0448), anti-Mouse Immunoglobulins/HRP
363 (P0447); Roth: DAPI (6843.1)

364 Compounds were purchased from the following companies. Thermo Fisher: DHE
365 (D11347); Cayman: Valproic Acid (13033), CHIR99021 (13122), Repsox (14794),
366 Forskolin (11018), Doxorubicin (15007); Acros Organics: TCP (130472500);
367 APExBIO: DZNep (A8182); Seleckchem: TTNPB (S4627); Roth: X-beta-Gal
368 (2315.3)

369

370 **RNA analysis.** Total RNA was extracted using Monarch Total RNA Miniprep Kit
371 (New England Biolabs, T2010S) according to manufacturer's instructions with

372 DNase treatment (Qiagen, 79254) for 15 minutes (1:8 in DNase buffer). Total RNA
373 concentrations were determined using the Qubit RNA BR Assay Kit (Thermofisher,
374 Q10211). cDNA synthesis was performed by adding 4 μ L of iScriptTM gDNA Clear
375 cDNA Synthesis (Biorad, 1725035BUN) to 500ng of RNA sample and run in a
376 Thermocycler (Biorad, 1861086) with the following protocol: 5 min at 25°C for
377 priming, 20 min at 46°C for reverse transcription, and 1 min at 95°C for enzyme
378 inactivation. Final cDNA was diluted 1:5 using autoclaved water and stored at -
379 20°C. qRT-PCR was performed using SsoAdvanced SYBR Green Supermix (Bio-
380 Rad, 1725272) in 384-well PCR plates (Thermofisher, AB1384) using the
381 QuantStudioTM 12K Flex Real-time PCR System instrument (Thermofisher).
382 Forward and reverse primers (1:1) were used at a final concentration of 5 μ M with
383 1 μ L of cDNA sample. Primer sequences are listed in Table S1.

384

385 **RNA sequencing, processing, analysis.** RNA-Seq library preparation and
386 sequencing was performed by Novogene (UK) Company Limited on an Illumina
387 NovaSeq 6000 in 150 bp paired-end mode. Raw FASTQ files were assessed for
388 quality, adapter content and duplication rates with FastQC. Reads were aligned to
389 the Human genome (GRCh38) using the STAR aligner (v2.7.9a) (Dobin *et al.*, 2013)
390 with '--sjdbOverhang 100'. Number of reads per gene was quantified using the
391 featureCounts function in the subread package (Liao, Smyth and Shi, 2013).
392 Ensembl transcripts were mapped to gene symbols using the mapIds function in
393 the AnnotationDbi package (Pagès *et al.*, 2022) with the org.Hs.eg.db reference
394 package (Carlson, 2019). Raw counts were normalized by library size and
395 converted to counts per million (CPM) for downstream analysis. Dimensionality
396 reduction was performed via Principal Component Analysis (PCA) using the R
397 software. Differentially expressed genes (DEG) were computed by the limma R

398 package (Ritchie *et al.*, 2015), by fitting a linear model on each gene, with an
399 adjusted p-value of 0.05. Gene set enrichment analysis (GSEA) for gene ontology
400 (GO) (Ashburner *et al.*, 2000) was performed using the clusterProfiler package (Wu
401 *et al.*, 2021); (Ashburner *et al.*, 2000), from the list of DEG (with a valid Entrez ID)
402 ranked by logFoldChange. We used org.HS.eg.db as a reference, selected
403 Biological Processes (BP) only, and an adjusted p-value of 0.05 (Bonferroni).
404 Pathways were ranked by Normalized Enrichment Score (NES). Z-Score were
405 calculated for each gene to plot as a heatmap.

406

407 **MTS cell proliferation assay.** Cell viability and proliferation assays were
408 performed by Tetrazolium MTS assay. Control and treated cells were cultured for 1
409 day in 96-well plates then treated with small molecules for 3 consecutive days
410 before incubation with 120 μ L fresh media containing 20 μ L of CellTiter 96®
411 AQueous One Solution (Promega, G3580) for 1 to 4 hours at 37°C in a humidified,
412 5% CO₂ atmosphere. The amount of product formed was measured by recording
413 the absorbance at 490nm using a BioTek Epoch 2 microplate reader. Relative
414 proportion of viable cells was determined as a relative reduction of the optical
415 density (OD) compared to control OD.

416

417 **Crystal violet staining.** Cells were cultured as previously described for the MTS
418 cell proliferation assay. Culture media was then carefully removed from wells and
419 cells were washed three consecutive times with room temperature PBS (Gibco,
420 21600069) followed by 45 minutes incubation with crystal violet (Roth, T123.2)
421 solution (Crystal Violet 0.05%, Formaldehyde 0.4%, Methanol 1% in PBS 1X).
422 Plates were then washed by careful immersion in tap water 2 times, drained upside
423 down, and air dried. Finally, solubilization in 1% SDS was performed on an orbital

424 shaker until no dense areas of coloration persisted and absorbance was measured
425 at 570nm using a BioTek Epoch 2 microplate reader. Relative proportion of viable
426 cells was determined as a relative reduction of the optical density (OD) compared
427 to control OD.

428

429 **Senescence-associated β -galactosidase assay.** Senescence-associated beta-
430 galactosidase (SA- β gal) assay was performed as described in the literature
431 (Debacq-Chainiaux *et al.*, 2009). Briefly, light fixation was performed on cells plated
432 on glass coverslips using a solution of 3% paraformaldehyde and 0.2%
433 glutaraldehyde in PBS buffer for 5 minutes. Fixation solution was then removed,
434 wells were washed several times and stained overnight at 37°C in a CO2-free
435 incubator in a solution of 40 mM citric acid/Na phosphate buffer, 5 mM
436 K4[Fe(CN)6]3H2O, 5 mM K3[Fe(CN)6], 150 mM sodium chloride, 2 mM
437 magnesium chloride and 1 mg/mL X-gal (Roth, 2315.1) with a pH of 5.9-6.0. Finally,
438 coverslips were stained with DAPI, followed by standard immunofluorescence
439 protocol, images were taken using bright-field microscopy, and proportion of β -Gal-
440 positive cells was then quantified.

441

442 **Reactive Oxygen Species assay.** Mitochondrial reactive oxygen species (ROS)
443 were measured using the superoxide indicator dihydroethidium (DHE). Briefly, first,
444 cells were incubated in fresh FBS-free media containing 5 μ M DHE and incubated
445 at 37°C in a humidified, 5% CO2 atmosphere for 30 minutes. Following incubation,
446 wells were washed with room temperature PBS, fixed with 4% paraformaldehyde
447 for 15 min and then stained with DAPI. Immediately, images were taken at 554 nm
448 and standard immunofluorescence imaging protocol was followed.

449

450 **Quantification and statistical analysis.** Analysis of immunofluorescence
451 microscopy images was performed using ImageJ. A minimum of 50-100 cells were
452 imaged per condition. Maximal projections of z-stacks were analyzed and total
453 fluorescence intensity per cell were determined.

454 All statistical parameters such as statistical analysis, statistical significance and n
455 values are reported in the figure legends. Statistical analyses were performed using
456 GraphPad Prism 9.0.0. Outliers were systematically removed using the ROUT
457 method (Q=1%). For in vivo experiments, n corresponds to the numbers of animals
458 and statistical analysis was performed using SPSS 27.0.1.0 (IBM® SPSS®
459 Statistics).

460

461 **C. elegans strains and maintenance.** Wildtype *C. elegans* (N2) were obtained
462 from the Caenorhabditis Genetics Center (CGC), University of Minnesota, USA. N2
463 wildtype worms were maintained at 20°C and were grown on standard Nematode
464 Growth Media (NGM) plates.

465

466 **Lifespan analysis.** Animals were synchronized and lifespan analyses were
467 conducted at 20°C as previously described (Porta-de-la-Riva *et al.*, 2012) and were
468 transferred onto NGM plates containing treatment or vehicle at stage L4. TCP was
469 dissolved in water at 100 mM and Repsox was dissolved in DMSO at 200 mM. TCP
470 and Repsox were added directly into the molten agar to a final concentration of 50,
471 100 or 200 µM each before pouring. After proper drying of plates, UV killed OP50
472 bacteria were seeded (150 µl of 120 mg/ml UV killed OP50 per P60 plate) and
473 FUDR (150 µM) added as reproductive suppressant. Treated and control plates
474 contained an equivalent DMSO concentration. Animals that crawled off the plate or

475 displayed extruded internal organs were censored. Lifespan analyses were
476 assessed manually by counting live and dead animals based on movement.

477

478 **AUTHOR CONTRIBUTIONS**

479 A.O., P.T.P. and L.S. designed the study. A.O. and L.S. were involved in all
480 experiments, data collection, analysis and interpretation. A.O., P.T.P. and L.S.
481 prepared the figures and wrote the manuscript with input from all authors. L.S.,
482 G.B.P. and N.H.K. performed and analyzed *in vivo* experiments. C.M. performed
483 skin fibroblast extractions. L.S. and K.P. prepared and analyzed RNA-seq data.
484 A.O. and P.T.P. provided assistance, supervision, and guidance.

485

486 **COMPETING INTERESTS**

487 The authors declare no competing interests.

488

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497

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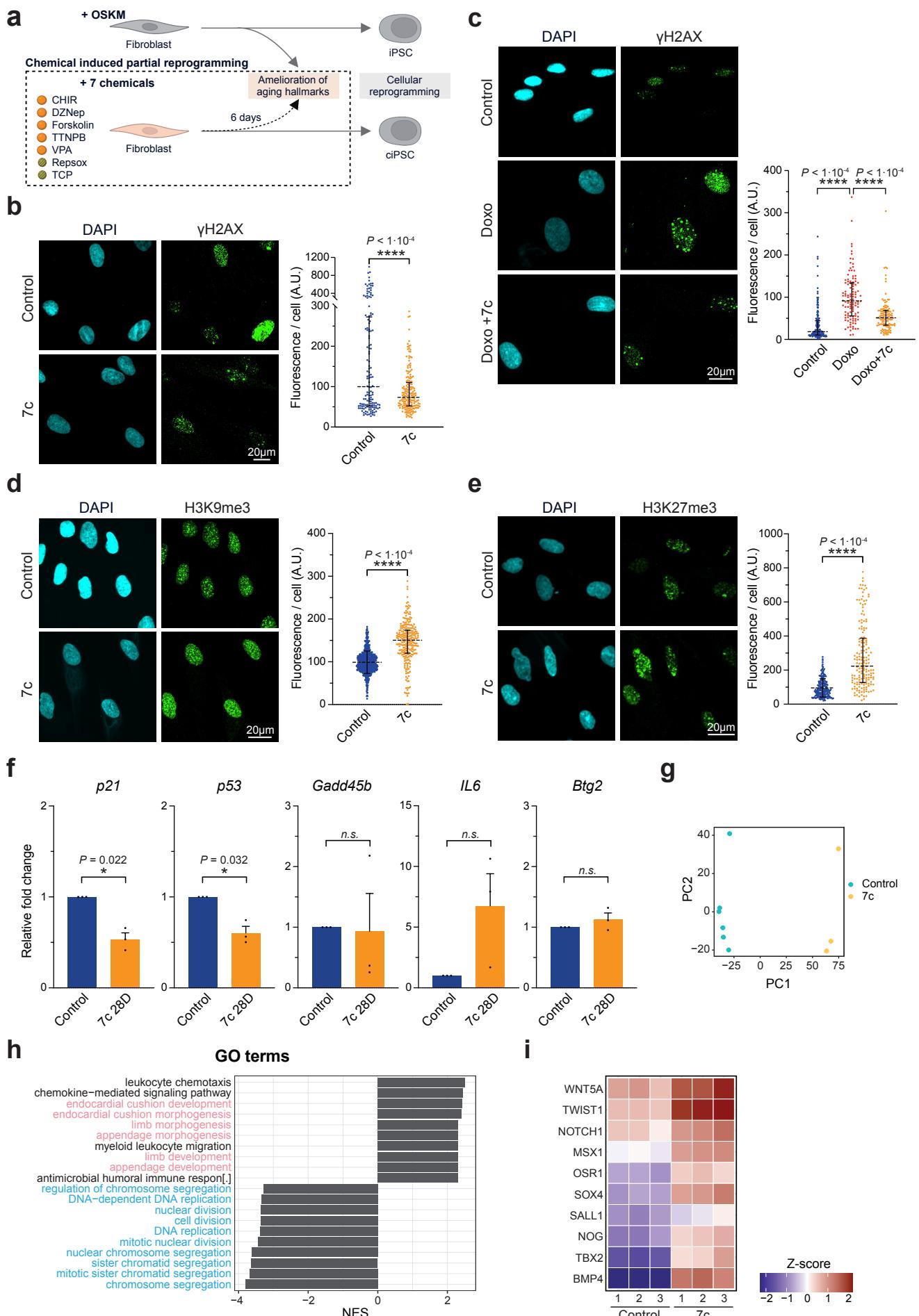


Figure 1 | Chemical induced partial reprogramming significantly improves aged hallmarks in aged human fibroblasts. **a**, Schematic representation of chemical induced partial reprogramming via 6 days treatment with the 7 chemicals previously shown to induce mouse chemical iPSC (Hou *et al.*, 2013). **b-c**, Immunofluorescence and quantification of γH2AX following 7c treatment (**b**) or Doxorubicin (100 nM) and 7c treatment (**c**). **d-e**, Immunofluorescence and quantification of H3K9me3 (**d**) and H3K27me3 (**e**) following 7c treatment. **f**, mRNA levels of senescence-associated and age-related stress response genes in the *p53* tumor suppressor pathway following 7c treatment during replicative induced senescence (RIS; 28 days). **g**, Principal component analysis (PCA) of control (blue) and 7c treated (orange) fibroblasts. **h**, Gene ontology (GO) enrichment analysis following 7c treatment with developmental (in pink) and cell cycle (in blue) pathways highlighted. **i**, List of differentially expressed genes associated with developmental pathways following 7c treatment. Data are median ± IQR (**b-c**), mean ± SEM (**d-f**). (**b-c**) n=2, (**d-i**) n=3. Statistical significance was assessed by comparison to untreated control using paired two-tailed *t*-test (**b-f**). NES, Normalized enrichment scores.

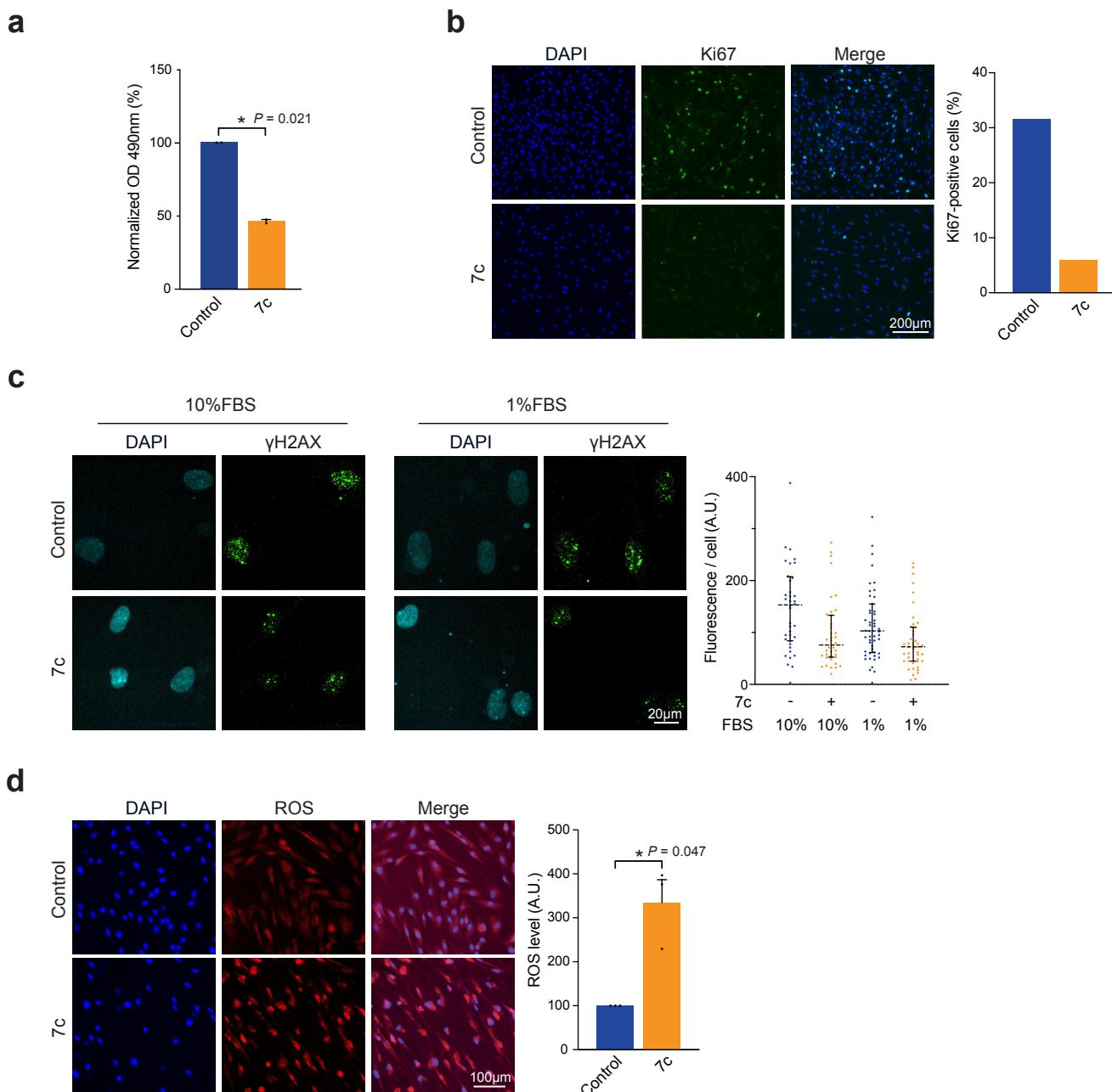


Figure 2 | ciPR via 7 chemicals does not fully induce multiparameter rejuvenation. **a**, MTS quantification of cell density following 7c treatment until confluence. **b**, Immunofluorescence and quantification of Ki67 following 7c treatment (6 days, “6D”). **c**, Immunofluorescence and quantification of γH2AX following 7c treatment (6D) in proliferative (10%FBS) and non-proliferative (1%FBS) conditions (6D). **d**, Fluorescence detection and quantification of reactive oxygen species (ROS) following 7c treatment (6D). Data are mean \pm SEM (**a**, **d**), median \pm IQR (**c**). (**a-b**) n=2, (**c**) n=1, (**d**) n=3. Statistical significance was assessed by comparison to untreated control using paired two-tailed *t*-test (**a**, **d**). OD, Optical Density.

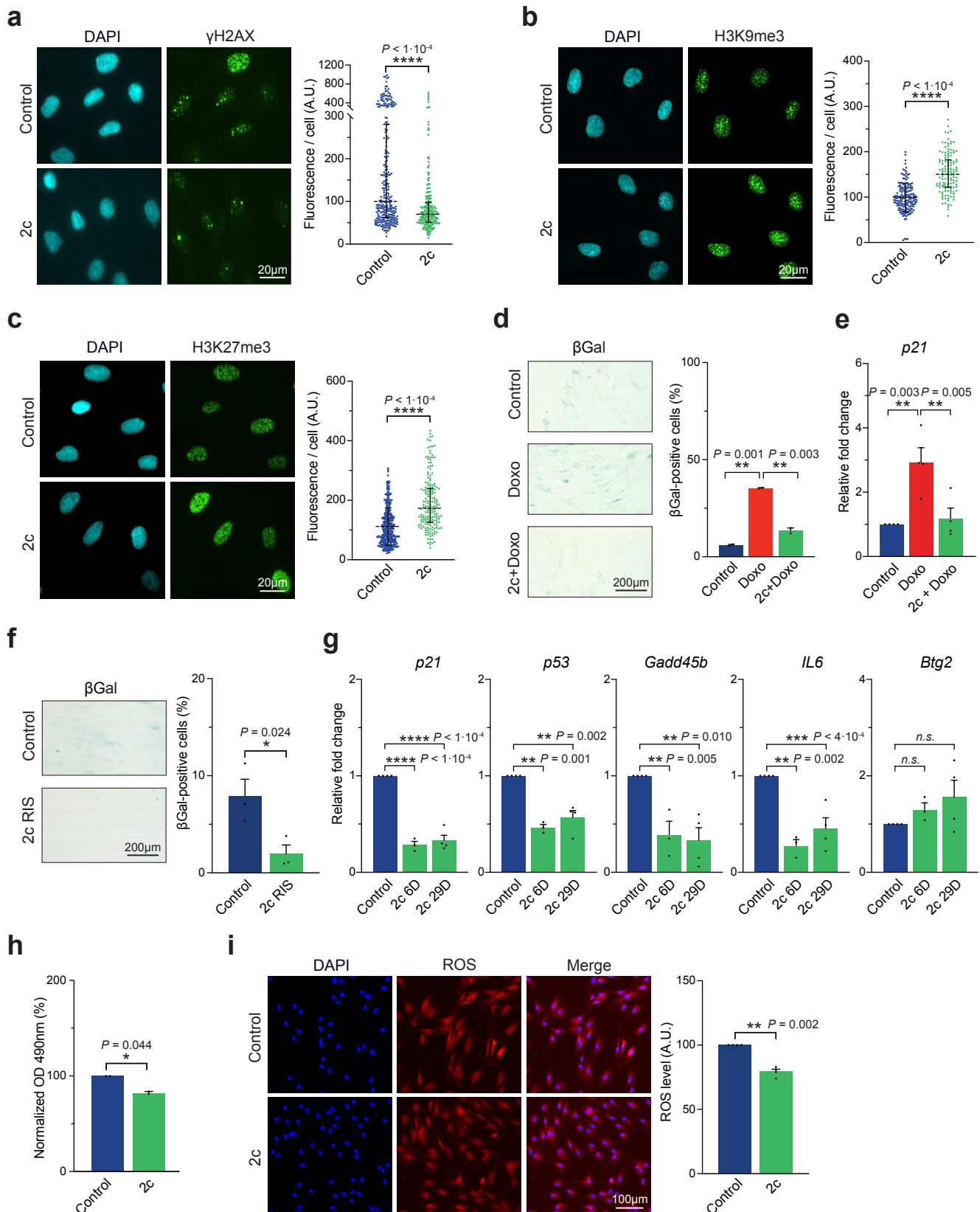


Figure 3 | Optimized cocktail (2c) efficiently improves multiple molecular hallmarks of aging.

a, Immunofluorescence and quantification of γ H2AX following TCP + Repsox (2c, 5 μ M each) treatment (6 days, “6D”). **b-c**, Immunofluorescence and quantification of H3K9me3 (**b**) and H3K27me3 (**c**) following 2c treatment (6D). **d**, Senescence-associated beta-galactosidase (SA-beta-gal) staining and quantification following Doxorubicin (100 nM) in 2c pre-treated fibroblasts (6D). **e**, mRNA levels of senescence-associated *p21* expression following Doxorubicin (100 nM) in 2c pretreated fibroblasts (29D). **f**, SA-beta-gal staining and quantification of replicative induced senescence (RIS) following long-term (29D) 2c treatment. **g**, mRNA levels of senescence-associated and age-related stress response genes in the *p53* tumor suppressor pathway following 2c treatment. **h**, MTS quantification of cell density following 2c treatment until confluence. **i**, Fluorescence detection and quantification of ROS following 2c treatment (6D). Data are median \pm IQR (**a**), mean \pm SEM (**b-i**). (**a-c, e-g, i**) $n \geq 3$, (**d, h**) $n=2$. Statistical significance was assessed by comparison to untreated control using paired two-tailed *t*-test (**f, h-i**), one-way ANOVA and Dunnett correction (**d-e, g**). OD, Optical Density.

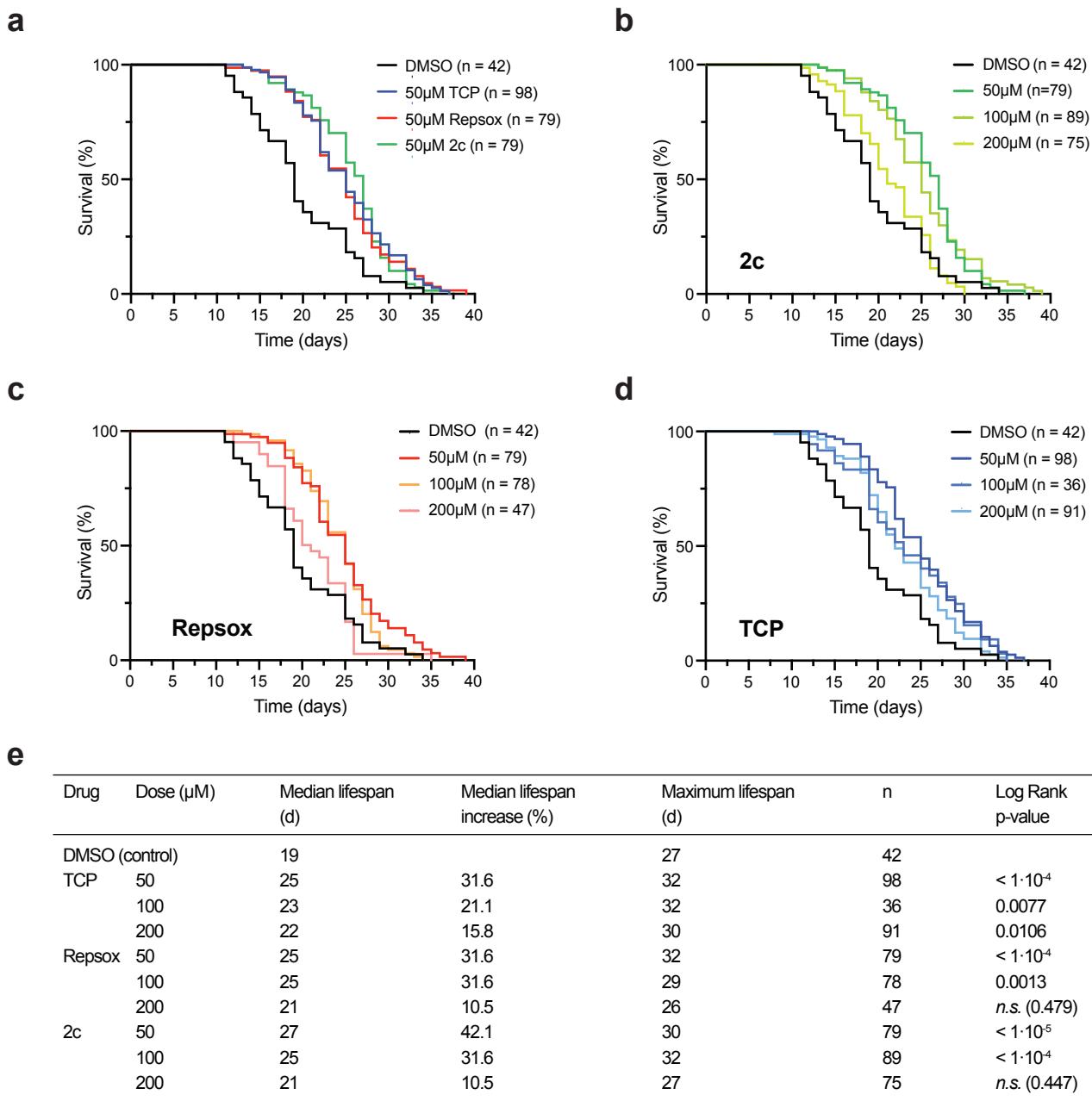


Figure 4 | Treatment with 2c increases *C. elegans* lifespan. **a**, Survival of N2 *C. elegans* upon treatment of TCP (50 µM), Repsox (50 µM), and 2c (TCP + Repsox, 50 µM each). **b-d**, Survival of N2 *C. elegans* upon treatment with 2c (**b**), Repsox (**c**), and TCP (**d**) at 50, 100, or 200 µM. **e**, Summary of survival assay results including median lifespan, maximal (90%) lifespan, and statistical analyses. Median lifespan increase relative to vehicle control. Statistical significance was assessed by comparison to untreated control using Log-Rank (Mantel-Cox) test.

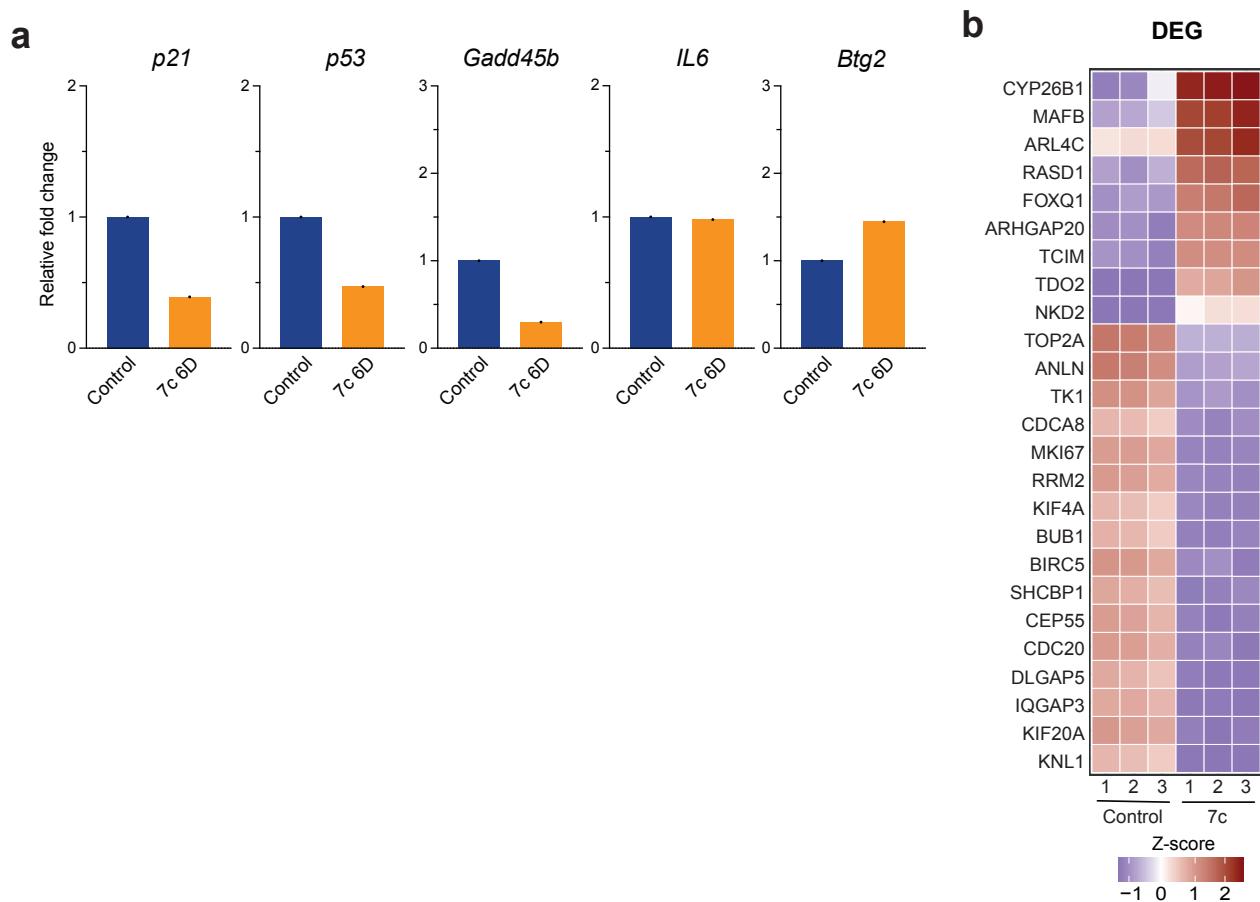


Figure S1 | Gene expression analysis of chemical induced partial reprogramming with 7c treatment. a, mRNA levels of senescence-associated and age-related stress response genes in the *p53* tumor suppressor pathway following 7c treatment (6 days). b, Top differentially expressed genes following 7c treatment relative to untreated controls in human fibroblasts. (a) n=1, (b) n=3. DEG, differentially expressed genes.

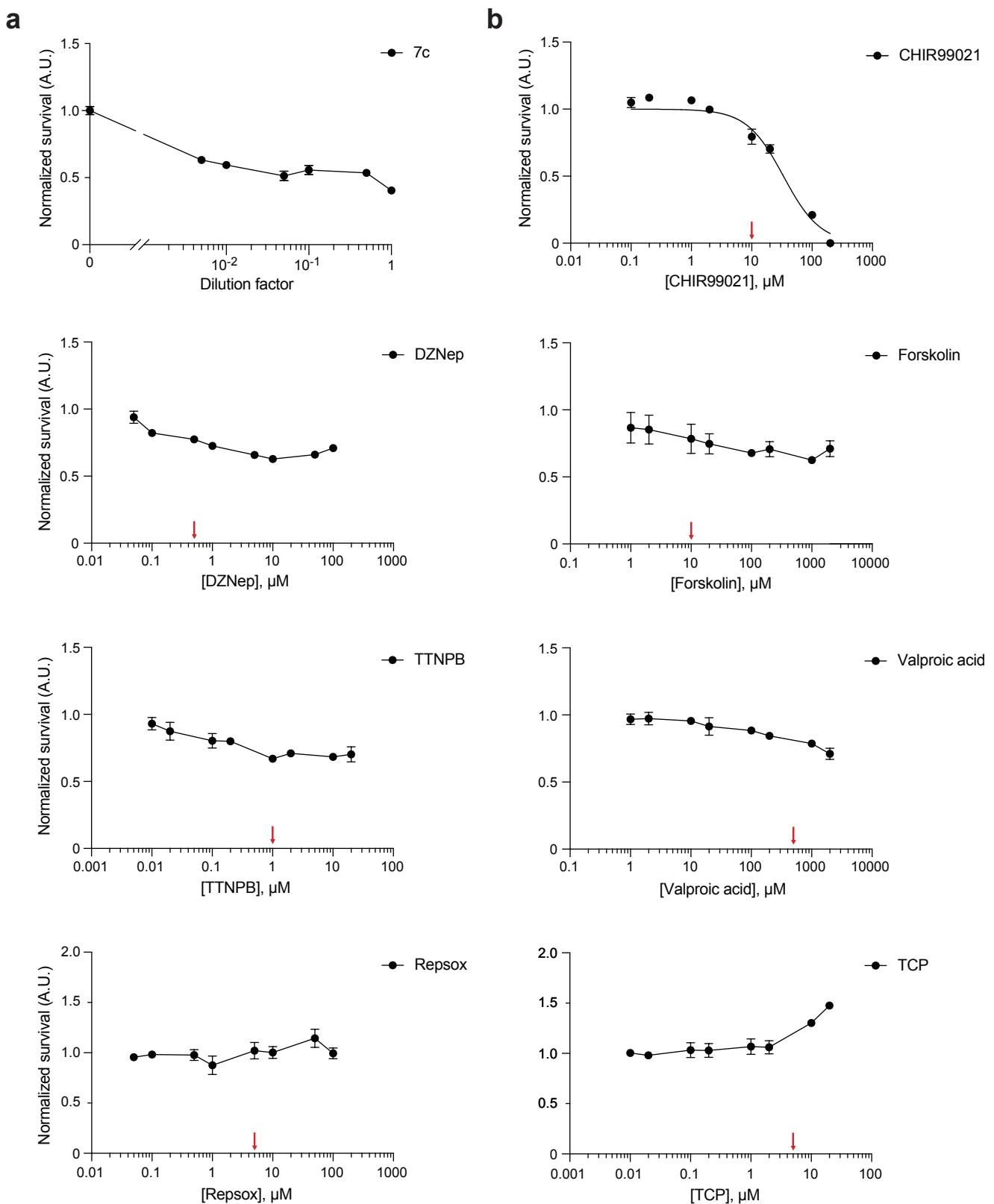


Figure S2 | Serial dilution of the reprogramming chemicals. **a**, Crystal violet quantification of cell density following treatment until confluence with serial dilutions of the 7c reprogramming cocktail. **b**, MTS quantification of cell density following treatment until confluence with different concentration of the reprogramming chemicals. Red arrows indicate experimental concentrations. Initial concentrations noted in Table S2. Nonlinear regression displayed when possible. Data are mean \pm SEM.

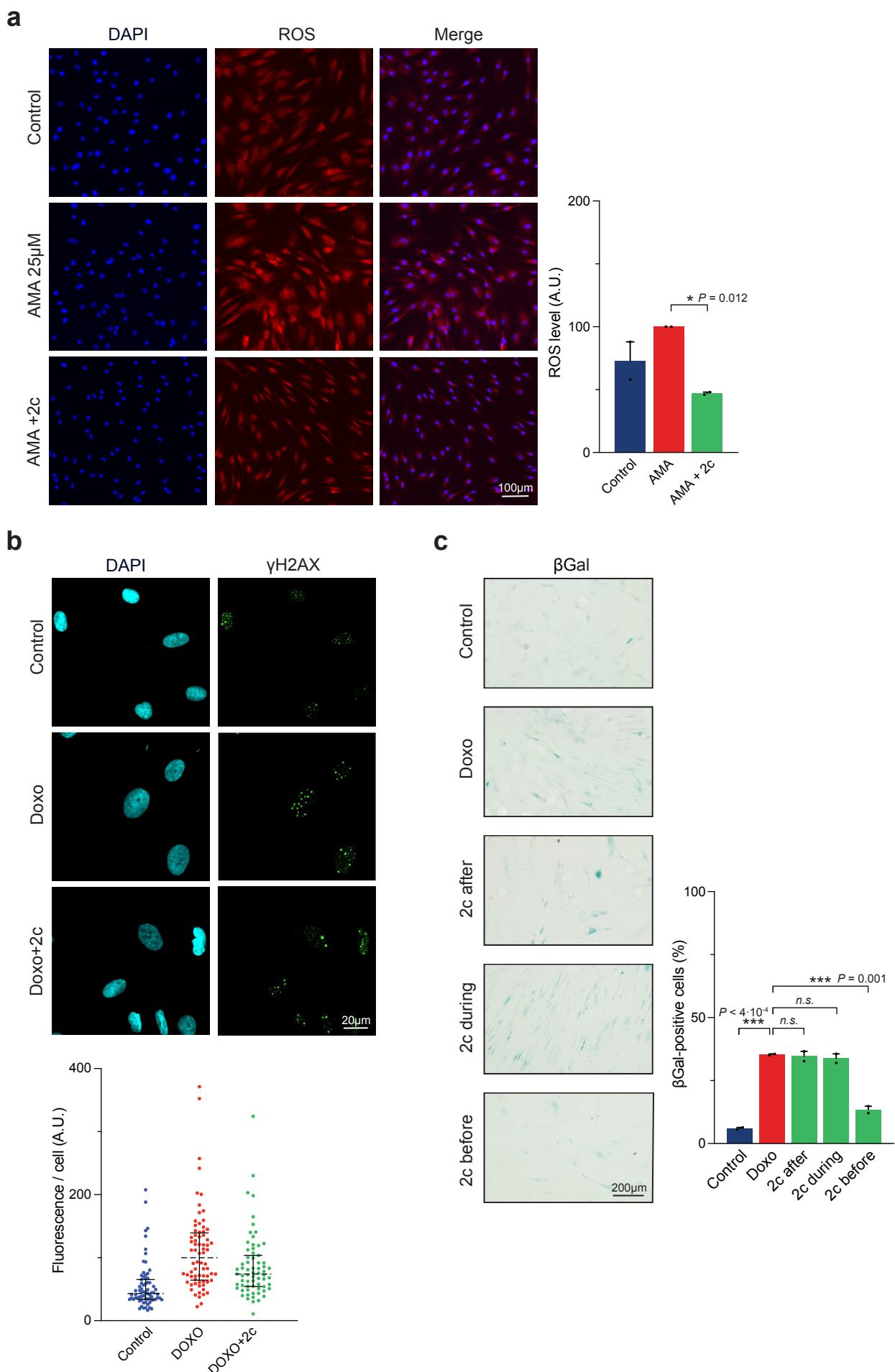


Figure S3 | Reduced 2c cocktail efficiently ameliorates multiple hallmarks of aging.

a, Fluorescence detection and quantification of ROS following AMA (25 μ M) and 2c treatment (6 days, “6D”). **b**, Immunofluorescence and quantification of γ H2AX following 2c treatment. **c**, Senescence-associated beta-galactosidase (SA-beta-gal) staining and quantification in 2c treated fibroblasts before, during, and after Doxorubicin (100 nM) treatment (6D). Data are mean \pm SEM (**a**, **c**), median \pm IQR (**b**). (**a-c**) n=2, (**b**) n=1. Statistical significance was assessed by comparison to untreated control using paired two-tailed *t*-test (**a**), one-way ANOVA and Dunnett correction (**c**).

Human gene		Sequence (5' → 3')
18S	Forward	GGCGCCCCCTCGATGCTCTAG
	Reverse	GCTCGGGCCTGCTTGAAACACTCT
<i>p16</i>	Forward	GGGTCGGGTGAGAGTGG
	Reverse	CGAATAGTTACGGTCGGAGG
<i>p21</i>	Forward	CATGGGTTCTGACGGACATC
	Reverse	TGCCGAAGTCAGTCCTTGT
<i>p53</i>	Forward	GCTTCACGACGGTGAC
	Reverse	GCTCGACGCTAGGATCTGAC
<i>Il6</i>	Forward	AGTGAGGAACAAGGCCAGAGC
	Reverse	GTCAGGGTGGTTATTGCAT
<i>Gadd45b</i>	Forward	ACAGTGGGGTGTACGAGTC
	Reverse	GATGTCATCCTCCTCCTCCTC
<i>Btg2</i>	Forward	CTCCAGGAGGCACTCACAG
	Reverse	ATGATGGGTCCATCTTGTC

Table S1 | Primers set for qRT-PCR.

Drug	Mol. Weight (kDa)	Concentration used (μM)
Valproic acid	166.2	500
CHIR99021	465.3	10
Repsox	287.3	5
Tranylcipromine	169.7	5
Forskolin	410.5	10
DZNep	298.7	0.5
TTNPB	348.5	1

Table S2 | Table of reprogramming chemicals and respective concentrations used.