

14 **SUMMARY**

15 The induction of cellular reprogramming by forced expression of the transcription factors
16 OCT4, SOX2, KLF4, and C-MYC (OSKM) has been shown to allow the dedifferentiation
17 of somatic cells and ameliorate age-associated phenotypes in multiple tissues and
18 organs. Yet to date, the benefits of in vivo reprogramming are limited by the occurrence
19 of detrimental side-effects. Here, using complementary genetic approaches, we
20 demonstrated that continuous in vivo induction of the reprogramming factors leads to
21 hepatic and intestinal dysfunction resulting in decreased body weight and premature
22 death. By generating a novel transgenic reprogrammable mouse strain, which avoids
23 OSKM expression in both liver and intestine, we drastically reduced the early lethality
24 and adverse effects associated with in vivo reprogramming. This new reprogramming
25 mouse allows safe and long-term continuous induction of OSKM and might enable a
26 better understanding of in vivo reprogramming as well as maximize its potential effects
27 on rejuvenation and regeneration.

28

29 **KEYWORDS**

30 In vivo reprogramming, aging, regeneration, epigenetic, liver, intestine

31

32 **INTRODUCTION**

33 The simultaneous forced expression of four transcription factors (4F), OCT4, SOX2,
34 KLF4, and MYC (OSKM) has been shown to be sufficient to activate pluripotency
35 networks and dedifferentiate somatic cells to a pluripotent state (Takahashi and
36 Yamanaka, 2006). Interestingly, it has been demonstrated that transient induction of
37 reprogramming can reverse age-associated features in cells in vitro (Lapasset et al.,
38 2011; Olova et al., 2019; Roux et al., 2021; Sarkar et al., 2020), suggesting its capacity
39 to alter both cellular identity and age. Likewise, the conversion of differentiated cells into
40 pluripotency has been confirmed in vivo by multiple groups including ours (Abad et al.,
41 2013; Ocampo et al., 2016), opening the door for potential in vivo rejuvenation and

42 regeneration of tissues and organs. In this line, in vivo reprogramming can induce
43 beneficial effects on skin (Doeser et al., 2018), heart (Chen et al., 2021), skeletal muscle
44 (de Lazaro et al., 2019; Wang et al., 2021) and liver (Hishida et al., 2022) regeneration.
45 Furthermore, reprogramming of aged mice has the capacity to improve memory
46 (Rodriguez-Matellan et al., 2020), regenerate skeletal muscle and pancreas following
47 injury (Chiche et al., 2017; Ocampo et al., 2016), restore vision loss in injured retina (Lu
48 et al., 2020), and recently, rejuvenate multiple tissues and organs (Browder et al., 2022;
49 Chondronasiou et al., 2022). Finally, cyclic induction of reprogramming has been shown
50 to extend lifespan in a progeria mouse model (Alle et al., 2021; Ocampo et al., 2016). All
51 these observations represent a proof of concept and highlight the potential of in vivo
52 reprogramming for the improvement of human healthspan. However, despite many
53 benefits, previous studies have also indicated that continuous expression of OSKM in
54 vivo can lead to cancer development and teratoma formation (Abad et al., 2013; Mosteiro
55 et al., 2016; Ohnishi et al., 2014), and most importantly, early mortality (Abad et al., 2013;
56 Hishida et al., 2022; Ocampo et al., 2016; Rodriguez-Matellan et al., 2020).
57 Consequently, these strong adverse effects represent a barrier for the safe and long-
58 term continuous induction of reprogramming in vivo, and therefore limit its potential
59 benefits. Importantly, the etiology of the side-effects associated with in vivo
60 reprogramming remains unexplored, and the ultimate cause of death of reprogrammable
61 mice is still unclear.

62 In order to gain insight into the adverse effects of in vivo reprogramming, we performed
63 a comparative analysis of different reprogrammable mouse strains upon induction of
64 OSKM. As expected, continuous expression of reprogramming factors is highly toxic,
65 indicated by decreased body weight, temperature loss, decreased activity, and ultimately
66 mortality. Using genetic strategies to activate or inactivate the expression of the
67 reprogramming factors in hepatocytes and intestinal epithelial cells, we first
68 demonstrated that specific expression of OSKM in the liver and intestine compromises
69 the normal function of these organs and is lethal. Importantly, these severe adverse

70 phenotypes were drastically reduced in a novel reprogrammable mouse strain where
71 OSKM expression was avoided in the liver and intestine. Finally, we showed that safe
72 and long-term continuous induction of in vivo reprogramming can be achieved in the
73 absence of detrimental side effects by avoiding the expression of the Yamanaka factors
74 in these organs. We anticipate that these observations and this novel mouse strain might
75 open the door for reaching the maximum beneficial potential of in vivo reprogramming.

76

77

78 **RESULTS**

79 **In vivo reprogramming results in hepatic and intestinal dysfunction**

80 Despite the potential beneficial effects of in vivo reprogramming, the induction of OSKM
81 at the organismal level has been associated with body weight loss, tumor development,
82 and early mortality (Abad et al., 2013; Mosteiro et al., 2016; Ohnishi et al., 2014).
83 Importantly, although certain short-term induction protocols, which allow
84 reprogrammable mice to survive, lead to the development of tumor and teratomas, the
85 timing of these events is not compatible with the early mortality observed upon
86 continuous induction of in vivo reprogramming. For these reasons, in order to better
87 understand the deleterious consequences of this process, we first performed side-by-
88 side comparisons of two of the most studied reprogrammable mouse strains, which have
89 been previously used to investigate the effects of in vivo reprogramming at tissue and
90 organismal levels. These strains included the 4Fj developed by the laboratory of Rudolf
91 Jaenisch (Carey et al., 2010) and 4Fs-B generated by the group of Manuel Serrano
92 (Abad et al., 2013). Importantly, these strains are similar but not identical. Although each
93 of them carries the transcriptional activator (rtTA-M2) within the ubiquitously expressed
94 *Rosa26* locus, the doxycycline-inducible polycistronic cassette encoding the four murine
95 factors *Oct4* (*Pou5f1*), *Sox2*, *Klf4* and *c-Myc* (TetO 4F) is inserted in different genomic
96 locations, specifically in the *Col1a1* (4Fj) and *Pparg* (4Fs-B) loci (Figure 1A). First, to
97 induce the expression of the reprogramming factors in vivo, 4Fj and 4Fs-B mice were

98 treated with doxycycline in drinking water (1 mg/ml) continuously (Figure S1A). As
99 expected, both lines experienced body weight loss (Figure 1B), albeit to different extent,
100 lower activity (Figure 1C), and decreased body temperature (Figure S1B) after only a
101 few days of treatment, indicating early toxicity of in vivo reprogramming. Unsurprisingly,
102 mice of both strains began to die after 3 days of doxycycline treatment, with a significantly
103 different median survival of 5 days and 10 days for the 4Fj and 4Fs-B respectively (Figure
104 1D). Interestingly, gross postmortem examination of these animals showed neither
105 indication of tumor nor teratoma formation, suggesting that these events were not the
106 cause of premature death. For this reason, in order to investigate the early and
107 differential mortality associated with the expression of the Yamanaka factors in vivo, we
108 analyzed the expression of the reprogramming factors in multiple tissues and organs.
109 Although *Oct4* and *Sox2* transcripts were detected at similar levels in both strains in
110 organs such as small intestine and kidney, a higher expression of these factors was
111 observed in both liver and blood of 4Fj compared to 4Fs-B mice (Figure 1E and Figure
112 S1C). In the same line, OCT4 was detected in the small intestine of both strains at the
113 protein level, but only in the liver of 4Fj animals (Figures S1D). Notably, we observed a
114 significant increase of Ki67 positive cells in the liver of the 4Fj mice as well as the intestine
115 of both strains, indicating that reprogramming can induce cellular proliferation in these
116 organs (Figure S1F) in agreement with previous reports (Hishida et al., 2022; Ohnishi et
117 al., 2014).

118 Next, with the goal of investigating the potential organ dysfunction leading to the early
119 lethality, we performed chemistry analysis of plasma collected from 4Fj and 4Fs-B mice
120 after 4 days of continuous doxycycline treatment, the time point at which both strains
121 were found sick. Interestingly, a significant decrease in glucose levels was observed in
122 both strains (Figure 1F). Importantly, elevated levels of the circulating liver enzymes
123 aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), alkaline
124 phosphatase (ALP), and bilirubin, together with a decrease in albumin, was observed in
125 the 4Fj but not in 4Fs-B mice, suggesting hepatic failure only in this strain (Figure 1F). In

126 this line, analysis of 4Fj liver histology after doxycycline treatment showed a disorganized
127 lobule architecture, with many apoptotic bodies, scattered vacuolated hepatocytes and
128 anisonucleosis and numerous Kupffer cells. However, the liver parenchyma of treated
129 4Fs-B mice only showed some small droplets in the cytoplasm of the hepatocytes located
130 in zone 2 and 3 (Figure 1G). These observations correlate with an increase in cleaved
131 caspase-3 positive cells, a marker of apoptosis, in only 4Fj mice, confirming the hepatic
132 damage after reprogramming in this strain (Figure 1H). Histological analysis of the small
133 intestine showed alterations in both strains after doxycycline treatment. Specifically, the
134 intestine of 4Fs-B mice presented shortened villi, with less goblet cells, and
135 dedifferentiated enterocytes. In 4Fj mice, the intestine was completely atrophic, with very
136 short villi, apoptotic figures in the crypts and dedifferentiated smaller enterocytes with
137 complete loss of goblet cells and destruction of the brush border (Figure S1E). Lastly, no
138 changes in potassium, phosphate, or uric acid levels were detected in neither of the
139 strains suggesting normal kidney function at least after 4 days of in vivo reprogramming
140 (Figure S1F).

141 Strikingly, our findings indicate that the adverse phenotypes associated with in vivo
142 reprogramming can be related to organ failure prior to tumor or teratoma formation, which
143 may indeed represent the main cause of the early mortality observed in whole body
144 reprogrammable mouse strains.

145

146 **Liver and intestine-specific reprogramming leads to body weight loss and early** 147 **mortality**

148 Next, in order to determine whether hepatic and intestinal dysfunction resulting from in
149 vivo reprogramming is a direct effect of the expression of the Yamanaka factors in these
150 organs, and consequently the cause of premature death, we generated two novel tissue-
151 specific reprogrammable mouse strains for the specific expression of reprogramming
152 factors in these organs. Towards this goal, we used the 4Fj mice, which is the only strain
153 characterized by high OSKM expression in both organs. For this purpose, the reverse

154 tetracycline trans-activator (rtTA-M2) was substituted by a LoxP-STOP-LoxP (LSL)
155 cassette-blocked rtTA trans-activator and the resulting offspring were crossed with both
156 an Albumin-Cre mice (Postic et al., 1999) to generate the 4F Liver mice or with a Villin-
157 Cre mice (Madison et al., 2002) to generate 4F Intestine mice (Figure 2A). As expected,
158 the Cre lines showed expression of the Cre recombinase specifically in the liver or the
159 intestine (Figure S2A). Moreover, we confirmed the expression of the reprogramming
160 factors in the targeted organs upon doxycycline treatment (Figure 2B and Figure S2B).
161 Interestingly, significant body weight loss was observed after only 2 days of doxycycline
162 treatment in 4F Intestine mice, and 24 hours later in 4F Liver mice (Figure 2C), as well
163 as decreased body temperature (Figure S2C). Interestingly, plasma analysis revealed
164 liver failure associated with high levels of circulating hepatic enzymes (ASAT, ALAT and
165 ALP) and bilirubin, as well as low albumin levels in 4F Liver mice (Figure 2E), in
166 agreement with a recent study (Hishida et al., 2022). Conversely, we did not observe
167 changes in liver enzymes and metabolites in 4F Intestine mice, suggesting normal liver
168 function (Figure 2E). Instead, 4F Intestine mice displayed hallmarks of nutrient
169 malabsorption, including a significant decrease in glucose levels in plasma (Figure 2D)
170 and diarrhea. More importantly, all mice of both strains were found extremely sick after
171 several days of reprogramming and succumbed to illness with median survival of 3.5
172 days (4F Liver) or 5 days (4F Intestine mice) (Figure 2F).
173 Overall, these results indicate direct negative effects of the expression of the
174 reprogramming factors in the liver and intestine, which are sufficient to induce rapid body
175 weight loss and ultimately lead to death. Moreover, a comparison of the phenotypes
176 observed in these organ-specific strains and whole-body reprogrammable mice strongly
177 suggests that most of the early negative consequences associated with in vivo
178 reprogramming are due to hepatic and intestinal dysfunction.

179

180 **Bypassing the expression of OSKM in liver and intestine significantly reduces the**
181 **adverse effects of in vivo reprogramming**

182 To further validate that hepatic and intestinal reprogramming are major causes of early
183 mortality in whole-body 4F mice, we generated two novel mouse strains which express
184 OSKM in whole body with the exception of the liver or the intestine. To achieve this goal,
185 we substituted the 4F polycistronic cassette in the 4Fj rtTA-M2 mice, by a 4Fj cassette
186 flanked by loxP sites, which allows its Cre-mediated excision. Subsequently, we crossed
187 these mice to Albumin-Cre or Villin-Cre in order to remove the 4F cassette in the targeted
188 organs and generate 4F Non-Liver or 4F Non-Intestine mice respectively (Figure 3A).
189 Next, we confirmed the decrease expression of *Oct4* and *Sox2* transcript levels in the
190 liver or intestine of 4F Non-Liver and 4F Non-Intestine mice (Figure 3B and Figure S3A).
191 As expected, no reduction of OSKM expression was observed in other organs such as
192 the kidney upon doxycycline treatment when compared with whole-body 4F-Flox controls
193 (Figure 3B and Figure S3A).
194 Importantly, 4F Non-Intestine mice did not manifest abnormalities in body weight loss or
195 activity upon induction of in vivo reprogramming (Figure 3C-D). Moreover, none of the
196 mice of this line showed diarrhea or changes in the glucose level in plasma, suggesting
197 that intestinal function was not affected (Figure 3E). On the other hand, ASAT, ALAT,
198 ALP and bilirubin levels were highly elevated, indicating hepatic failure after 4 days due
199 to hepatic reprogramming (Figure 3E). More importantly, none of the animals survived
200 the continuous treatment with doxycycline treatment, showing a median survival
201 comparable to 4F-Flox controls of only 5 days (Figure 3F).
202 Conversely, 4F Non-Liver mice did not show any sign of liver failure, as observed by the
203 low levels of ASAT, ALAT ALP and bilirubin in the plasma (Figure 3E). However, despite
204 normal liver function, 4F Non-Liver mice displayed decreased activity and weight loss
205 starting after 3 days of treatment probably due to intestinal reprogramming (Figure 3C-
206 D). Remarkably, the median survival in this strain was significantly extended to 9 days
207 (Figure 3F).
208 Taken together, these data suggest that intestinal reprogramming leading to nutrient
209 malabsorption is one of the main causes of early adverse effects of reprogramming,

210 including body weight loss, diarrhea and low activity, whereas hepatic reprogramming
211 triggers liver failure and represents the primary cause of premature death in 4Fj
212 reprogrammable mouse strain. Moreover, avoiding the expression of OSKM in these
213 organs partially prevents the adverse effects of in vivo reprogramming including
214 decrease in body weight and premature death.

215

216 **Safe and long-term continuous reprogramming can be achieved in the absence of**
217 **hepatic and intestinal expression of OSKM**

218 Finally, with the goal of achieving the safe and long-term induction of in vivo
219 reprogramming, we generated a novel quadruple transgenic mouse strain in which the
220 4F cassette (4F-Flox) is simultaneously removed from both liver and intestine (4F Non-
221 Liver/Intestine mice) (Figure 4A). In order to validate this novel strain, we first confirmed
222 the absence of OSKM expression in the liver and intestine of these mice 4 days after
223 doxycycline treatment (Figure 4B and Figure S4A). Surprisingly, none of the adverse
224 effects associated with in vivo reprogramming including decrease in body temperature
225 (Figure 4SB), diarrhea, body weight loss, or abnormal activity (Figure 4C-D) were
226 detected in this strain following the induction by doxycycline treatment. Moreover, no
227 alterations in plasma parameters characteristic of liver failure, such as, albumin, ASAT,
228 ALAT, or ALP were detected (Figure 4E). Interestingly and compared to the whole-body
229 4F-Flox, the first 4F Non-Liver/Intestine mouse died after more than one week of
230 continuous induction of reprogramming, and 60% of these mice survived up to one month
231 of continuous doxycycline treatment, making this strain, the reprogrammable line known
232 to date that can survive the longest protocols of continuous induction of in vivo
233 reprogramming at high dose (Figure 4F). Subsequently, 4F Non-liver/intestine mice were
234 euthanized after a month of doxycycline treatment and multiples organs were collected.
235 Afterwards, we confirmed that the lack of expression of the reprogramming factors in the
236 liver and small intestine (Figure 4G and Figure S4C), and no alterations in plasma
237 parameters of 4F Non-Liver/Intestine mice after 4 weeks of treatment, when compared

238 with whole body reprogrammable mice after only 4 days (Figure S4D). On the other hand,
239 comparable or even higher level of *Oct4* and *Sox2* expression were detected in kidney
240 and blood of 4F Non-Liver/Intestine mice after continuous induction (Figure 4G and
241 Figure S4C).

242 Lastly and with the goal of confirming the safety of the long-term induction of in vivo
243 reprogramming in 4F Non-Liver/Intestine mice, we induce the expression of the
244 reprogramming factors for 4 days or 2 weeks and monitor these mice for 2 months after
245 doxycycline withdrawal. Importantly, none of the mice died after treatment withdrawal
246 (Figure 4SE), none the adverse effects described above typically associated with whole-
247 body reprogramming such as body weight loss were observed, and no visible tumors
248 were detected in these mice neither during the doxycycline treatment or up 2 months
249 following its withdrawal (Figure 4SF).

250 Taken together, these results demonstrate that avoiding OSKM expression in liver and
251 intestine allows the continuous induction of in vivo reprogramming for a longer period of
252 time without significant adverse effects. For these reasons, this novel reprogrammable
253 mouse strain might open the door for enhancing and extending the beneficial effects of
254 reprogramming to other tissues and organs.

255

256 **DISCUSSION**

257 The transient induction of cellular reprogramming has been shown to improve
258 regeneration in multiple organs and ameliorate age-associated features both in vitro and
259 in vivo, opening the door to a new era of regenerative medicine and possible reversion
260 of both molecular and physiological age-associated phenotypes. Despite its huge
261 potential, the field of in vivo reprogramming is still in its initial stages of development with
262 most studies being published in the past few years (Taguchi and Yamada, 2017). In
263 contrast to the studies of cellular reprogramming in vitro, there is a profound lack of
264 understanding of the effects of reprogramming in a living organism with many open
265 questions still unresolved. As an example, although tumor and teratoma formation after

266 continuous expression of OSKM has been well-reported (Abad et al., 2013; Mosteiro et
267 al., 2016; Ohnishi et al., 2014), remains unclear whether these events or alternative
268 mechanisms represent the major cause of premature death in reprogrammable mice.
269 Therefore, in order to avoid the adverse effects and early lethality associated with in vivo
270 reprogramming, most studies have typically focused on the short-term expression of the
271 reprogramming factors at organismal level, or more recently, the tissue- or organ-specific
272 expression. In this line, to induce whole body expression of OSKM in the absence of side
273 effects, researchers have commonly used short-term cyclic expression of the OSKM
274 factors, such as 2 days (Browder et al., 2022; Ocampo et al., 2016) or 3 days (Rodriguez-
275 Matellan et al., 2020) of doxycycline (1mg/ml) followed by 5 or 4 days withdrawal, or
276 lower concentration of doxycycline (0.2 mg/ul) for 7 days (Chondronasiou et al., 2022).
277 Alternatively, the generation of tissue- specific reprogrammable mouse models such as
278 skeletal muscle-specific (Wang et al., 2021), heart-specific (Chen et al., 2021; de Lázaro
279 et al., 2021) and liver-specific (Hishida et al., 2022), or the ectopic local expression of
280 Yamanaka factors by using adeno-associated virus (Lu et al., 2020; Senis et al., 2018)
281 have allowed to circumvent the negative consequences of organismal reprogramming.
282 Unfortunately, although these protocols have been shown to be effective on improving
283 the regeneration capacity of some specific organs and ameliorating some age-
284 associated phenotypes, it appears that the short-term or mild induction of in vivo
285 reprogramming is insufficient to induce significant systemic rejuvenation or extend the
286 lifespan of wild type mice. Thus, it seems that novel strategies based on better
287 understanding of the biology of in vivo reprogramming will be necessary to achieve its
288 induction in the absence of adverse side-effects and therefore maximize its potential.
289 Toward this goal, the comparative analysis of different reprogrammable strains
290 performed in this study indicates that despite their similarities, different reprogramming
291 strains behave differently and are characterized by differential expression of OSKM in
292 multiple tissues and organs. Moreover, our findings demonstrate that hepatic and
293 intestinal reprogramming directly leads to dysfunction and damage in these organs

294 causing strong adverse phenotypes, which are lethal within a few days. Importantly, the
295 cause of death of reprogrammable mice has been a fundamental and long-standing
296 question even a decade after these strains were generated. We hypothesize that the
297 sensitivity of liver and intestine to cellular reprogramming might be due to multiple factors
298 including their role in the digestive system and their proliferative and regenerative nature.
299 Consequently, organs implicated in the absorption and metabolism of nutrients, such as
300 the intestinal tract, liver, and kidney, are expected to be highly exposed to doxycycline
301 and therefore might express higher levels of Yamanaka factors. On the other hand, the
302 intestinal epithelium is one of the faster self-renewing tissues (Tetteh et al., 2015) and
303 adult hepatocytes exhibit spontaneous cellular reprogramming during liver regeneration
304 (Yanger et al., 2013) indicating high plasticity and susceptibility of these cell types to
305 reprogramming and dedifferentiation, and potentially leading to loss of function upon
306 OSKM expression.

307 Based on these observations and by generating a novel reprogrammable mouse strain
308 where expression of OSKM is avoided in the liver and intestine, we have reduced the
309 detrimental effects of continuous induction of OSKM including early mortality, allowing
310 safe long-term induction of in vivo reprogramming. Nevertheless, we cannot rule out the
311 possibility that removing the expression of the factors in these organs might cancel some
312 positive indirect effects of reprogramming at the organismal level. In this line, it is
313 tempting to speculate that the decrease in nutrient absorption and body weight observed
314 following the induction of intestinal reprogramming could be responsible for some of the
315 systemic benefits.

316 Importantly, although this new generation of reprogramming strains allows for long-term
317 continuous reprogramming in the whole body without early adverse effect and lethality,
318 the fact that some mice ultimately died after extended periods indicates that continuous
319 reprogramming was still partially toxic, maybe by affecting other tissues or organs that
320 remain unknown. For these reasons, it would be essential to continue investigating the

321 effects of in vivo reprogramming in additional organs and develop strategies to better
322 control the expression of the factors in these organs.

323 We believe that despite significant advances in this area of research and its enormous
324 translational potential, we have so far failed to extend to living animals many of the
325 beneficial effects of partial reprogramming observed in cells in vitro. Thus, it is possible
326 that recent data on the potential benefits of in vivo reprogramming, specially in wild type
327 mice, has been somehow not as significant as expected maybe due to the use of short-
328 term reprogramming protocols designed to avoid early adverse effects and premature
329 death.

330 Toward this ultimate goal, our findings represent initial advances in the knowledge of this
331 emergent field of research and open new avenues for the long-term and safer induction
332 of in vivo reprogramming in the absence of adverse effects, which we believe will be critical
333 for its clinical translation. Therefore, we hope that these findings can serve as the basis
334 for the study of organismal regeneration and rejuvenation, ultimately leading to the
335 amelioration of diseases and improvements in human health and lifespan.

336

337 **EXPERIMENTAL PROCEDURES**

338 **Animal housing**

339 All the experimental procedures were performed in accordance with Swiss legislation
340 after the approval from the local authorities (Cantonal veterinary office, Canton de Vaud,
341 Switzerland). Animals were housed in groups of five mice per cage with a 12hr light/dark
342 cycle between 06:00 and 18:00 in a temperature-controlled environment at around 25°C
343 and humidity between 40 and 70% (55% in average), with free access to water and food.
344 Wild type (WT) and transgenic mouse models used in this project were generated by
345 breeding and maintained at the Animal Facility of Epalinges and the Animal Facility of
346 the Department of Biomedical Science of the University of Lausanne.

347 **Mouse strains**

348 All WT and transgenic mice were used in C57BL/6J background. Whole-body
349 reprogrammable mouse strain 4Fj rtTA-M2, carrying the OSKM polycistronic cassette
350 inserted in the *Col1a1* locus and the rtTA-M2 trans-activator in *Rosa 26* locus (rtTA-M2),
351 was generated in the laboratory of professor Rudolf Jaenisch (Carey *et al.*, 2010) and
352 purchased from The Jackson Laboratory, Stock No: 011004. The reprogrammable
353 mouse strain 4Fs-B rtTA-M2, carrying the OSKM polycistronic cassette inserted in the
354 *Pparg* locus and the rtTA-M2 trans-activator in *Rosa 26* locus (rtTA-M2), were previously
355 generated by professor Manuel Serrano (Abad *et al.*, 2013) and kindly generously
356 donated by professor Andrea Ablasser. 4F-Liver and 4F-Intestine reprogrammable
357 mouse strains were generated by substituting the rtTA-M2 of the 4Fj with a lox-stop-lox
358 rtTA (LSLrtTA), (Belteki *et al.*, 2005), purchased in The Jackson Laboratory, Stock No:
359 005670. The resultant offspring was crossed with an Albumin-cre, Stock No 003574,
360 (Postic *et al.*, 1999) or Villin-cre, Stock No 021504, (Madison *et al.*, 2002) strains
361 expressing selectively Cre recombinase in the liver and intestine, respectively.
362 The 4F Non-liver and 4F Non-intestine mouse strains were generated by breeding the
363 4F-Flox strain, carrying loxP sites flanking the 4F cassette in the *Col1a1* locus, previously
364 generated by professor Jaenisch (Carey *et al.*, 2010), and purchased from The Jackson
365 Laboratory, Stock No: 011001, with Albumin-Cre (4F Non-liver), Villin-Cre (4F Non-
366 intestine) or Albumin-Cre and Villin-Cre (4F Non-Liver/Intestine) mice to specifically
367 remove the 4F cassette in these organs. All transgenic mice carry the mutant alleles in
368 heterozygosity.

369 **Doxycycline administration**

370 In vivo expression of OSKM in all reprogrammable mouse strains was induced by
371 continuous administration of doxycycline (Sigma, D9891) in drinking water (1 mg/ml) in
372 2-3-month-old mice.

373 **Mouse monitoring and euthanasia**

374 All mice were monitored at least three times per week. Upon induction of in vivo
375 reprogramming, mice were monitored daily to evaluate their activity, posture, alertness,

376 body weight, presence of tumors or wound, and surface temperature. Body temperature
377 was recorded using non-contact infrared thermometer (SBS-IR-360-B, Steinberg). Mice
378 were euthanized according to the criteria established in the scoresheet. We defined lack
379 of movement and alertness, presence of visible tumors larger than 1cm³ or opened
380 wounds, body weight loss of over 30% and surface temperature lower of 34°C as
381 imminent death points.

382 For survival and body weight experiments only males were used, and for tissue and
383 organ collection, mice of both genders were randomly assigned to control and
384 experimental groups. Animals were sacrificed by CO₂ inhalation (6 min, flow rate 20%
385 volume/min). Subsequently, before perfusing the mice with saline, blood was collected
386 from the heart. Finally, multiple organs and tissues were collected in liquid nitrogen and
387 used for DNA, RNA, and protein extraction, or placed in 4% formaline for histological
388 analysis.

389 **Mouse activity**

390 Locomotor activity was assessed by Open Field test in 2-month-old mice after 3.5 days
391 of continuous treatment of doxycycline. Briefly, mice were individually placed in the
392 center of a Plexiglas boxes (45 x 45 x 38 cm, Harvard Apparatus, 76-0439). Mice
393 movements were recorded for 15 minutes (Stoelting Europe, 60516) and then analyzed
394 using ANY-maze video tracking software (ANY-maze V7.11, Stoelting).

395 **RNA extraction**

396 Total RNA was extracted from mouse tissues and organs using TRIzol (Invitrogen,
397 15596018). Briefly, 500 µl of TRIzol was added to 20-30 µg of frozen tissue into a tube
398 (Fisherbrand 2 ml 1.4 Ceramic, Cat 15555799) and homogenized at 7000 g for 1 min
399 using a MagNA Lyser (Roche diagnostic) at 4°C. Subsequently, 200 µl of chloroform was
400 added to the samples and samples were vortexed for 10 sec and placed on ice for 15
401 min. Next, samples were centrifuged for 15 min at 12000 rpm at 4°C and supernatants
402 were transferred into a 1.5 ml vial with 200 µl of 100% ethanol. Finally, RNA extraction

403 was performed using the Monarch total RNA Miniprep Kit (NEB, T2010S) following the
404 manufacture recommendations and RNA samples were stored at -80°C until use.

405 **cDNA synthesis**

406 Total RNA concentration was determined using the Qubit RNA BR Assay Kit (Q10211,
407 Thermofisher), following the manufacture instructions and a Qubit Flex Fluorometer
408 (Thermofisher). Prior to cDNA synthesis, 2 µL of DNase (1:3 in DNase buffer) (Biorad,
409 10042051) was added to 700 ng of RNA sample, and then incubated for 5 min at room
410 temperature (RT) followed by an incubation for 5 min at 75°C to inactivate the enzyme.
411 For cDNA synthesis, 4 µL of iScript™ gDNA Clear cDNA Synthesis (Biorad,
412 1725035BUN) was added to each sample, and then placed in a thermocycler (Biorad,
413 1861086) following the following protocol: 5 min at 25°C for priming, 20 min at 46°C for
414 the reverse transcription, and 1 min at 95°C for enzyme inactivation. Finally, cDNA was
415 diluted using autoclaved water at a ratio of 1:5 and stored at -20°C until use.

416 **Semiquantitative RT-PCR**

417 The following specific primers were used to detect the expression of the Cre recombinase
418 in the cDNA of mice samples, Cre forward: 5'-GAACGAAAACGCTGGTTAGC-3', and
419 Cre: reverse 5'-CCCGGCAAAACAGGTAGTTA-3' at a final concentration of 0.4 µM.
420 DNA was amplified using DreamTaq Green PCR Master Mix 2X (Thermofisher, K1081)
421 following the amplification protocol: 3 min at 95°C + 33 cycles (30 s at 95°C + 30 s at 60
422 °C + 1 min at 72°C) + 5 min at 72°C. PCR product were loaded and run in an agarose
423 (1.6%) gel containing ethidium bromide (Carlroth, 2218.1). Images were scanned with a
424 gel imaging system (Genetic, FastGene FAS-DIGI PRO, GP-07LED).

425 **qRT-PCR**

426 qRT-PCR was performed using SsoAdvanced SYBR Green Supermix (Bio-Rad,
427 1725274) in a PCR plate 384-well (Thermofisher, AB1384) and using a Quantstudio 12K
428 Flex Real-time PCR System instrument (Thermofisher). Forward and reverse primers
429 were used at a ratio 1:1 and final concentration of 5 µM with 1ul of cDNA. *Oct4* and *Sox2*
430 mRNA levels were determined using the following primers: *Oct4* forward: 5'-

431 GGCTTCAGACTTCGCCTTCT-3' *Oct4* reverse: 5'-TGGAAGCTTAGCCAGGTTTCG-3',
432 *Sox2* forward: 5'-TTTGTCCGAGACCGAGAAGC-3', *Sox2* reverse: 5'-
433 CTCCGGGAAGCGTGTACTTA-3'. mRNA levels were normalized using the house
434 keeping gene *Gapdh* (forward: 5'-GGCAAATTCAACGGCACAGT-3', reverse: 5'-
435 GTCTCGCTCCTGGAAGATGG-3').

436 **Immunohistochemistry**

437 Mice were euthanized with CO₂ and multiple tissues and organs were collected, placed
438 in 4% formaline (Sigma, 252549) overnight, and then immersed in 30% sucrose in
439 phosphate buffered saline (PBS) for 72 h. Subsequently, samples were paraffin-
440 embedded with a Leica ASP300S tissue processor (Leica, Heerbrug, Switzerland),
441 sections prepared with a Microm HM 335 E microtome (Thermo Scientific, Walldorf,
442 Germany) and mounted on Superfrost Plus slides (Thermo Scientific). Next, slides were
443 deparaffinized and rehydrated with xylol and alcohol. Each section was routinely stained
444 with hematoxylin and eosin, mounted on glass slides, and examined. Sections were
445 immunostained with the primary antibody for 60 min and subsequently incubated with
446 Dako EnVision HRP secondary antibody (Dako) for 30 min. For Ki67 immunostaining,
447 the total number of immunopositive cells was quantified in the liver in three different
448 regions per animal and means of the three regions were calculated and normalized by
449 area. For cleaved Caspase-3, a complete liver slice per animal was quantified and
450 normalized by area using a Nikon microscope camera (Nikon, digital sight 1000).

451 Antibodies: rabbit Ki67 (1:100, Abcam, ab16667); rabbit cleaved CASP3, Asp175 (1:200,
452 Cell Signaling, 9661).

453 **Plasma analysis**

454 Mouse blood was collected in lithium heparin tubes (BD microtainer, 365966),
455 centrifugated at 6500 rpm for 5 min, and the supernatant (plasma) was transferred to a
456 new vial. Multiple parameters (glucose, ASAT, ALAT, ALP, bilirubin, albumin, potassium,
457 uric acid and phosphate) were analyzed and quantified by the Service de Chimie Clinique
458 du CHUV using a Cobas 8000 (Roche Diagnostics).

459 **Western blot**

460 Samples were quickly dissected on an ice-cold plate and stored at -80°C . Protein
461 extraction was prepared by homogenizing in ice-cold RIPA buffer (ThermoFisher, 89900).
462 Homogenates were centrifuged at 15,000 g for 15 min at 4°C . The resulting supernatants
463 were collected, and protein content determined by Quick Start Bradford kit assay (Bio-
464 Rad, 500-0203). 20-50 μg of total protein was electrophoresed on 10% SDS-
465 polyacrylamide gel, transferred to a nitrocellulose blotting membrane (Amersham Protran
466 0.45 μm , GE Healthcare Life Sciences, 10600002) and blocked in TBS-T (150 mM NaCl,
467 20 mM Tris-HCl, pH 7.5, 0.1% Tween 20) supplemented with 5% non-fat dry milk.
468 Membranes were incubated overnight at 4°C with the OCT4 primary antibody (Oct-3-4,
469 C-10, Santacruz, sc-5279) in TBS-T supplemented with 5% non-fat dry milk, washed
470 with TBS-T and next incubated with secondary HRP-conjugated anti-mouse IgG
471 (1:2,000, DAKO, P0447) for 1 hour at room temperature and developed using the ECL
472 detection kit (Perkin Elmer, NEL105001EA).

473 **Data analysis**

474 Statistical analysis was performed using SPSS 21.0 (SPSS® Statistic IBM®). The
475 normality of the data was studied by Shapiro-Wilk test and homogeneity of variance by
476 Levene test. For comparison of two independent groups, two-tail unpaired t-Student's
477 test (data with normal distribution), Mann-Whitney-Wilcoxon or Kolmogorov-Smirnov
478 tests (with non-normal distribution) was executed. For multiple comparisons, data with a
479 normal distribution were analyzed by one way-ANOVA test followed by a Tukey's (equal
480 variances) or a Games-Howell's (not assumed equal variances) post-hoc tests.
481 Statistical significance of non-parametric data for multiple comparisons was determined
482 by Kruskal-Wallis one-way ANOVA test.

483

484 **FIGURE LEGENDS**

485 **Figure 1: In vivo reprogramming results in hepatic and intestinal dysfunction in**
486 **different reprogrammable mouse strains.** (A) Schematic representation of

487 reprogrammable mouse strains used in this study carrying the rtTA (reverse tetracycline-
488 controlled transactivator) transgene at the *Rosa26* locus and a doxycycline-inducible
489 polycistronic cassette for the expression of the mouse 4F (*Oct4*, *Sox2*, *Klf4* and *c-Myc*)
490 in the *Col1a1* locus (4Fj) and *Pparg* locus (4Fs-B), (pA) polyA sequence, (TetO)
491 tetracycline operator minimal promoter. (B) Body weight changes in 4Fj and 4Fs-B mice
492 upon continuous administration of doxycycline, and untreated 4F control mice (Ctrl). (C)
493 Distance travelled in open field cage following 3.5 days of doxycycline treatment of
494 controls, 4Fj and 4Fs-B mice. (D) Survival of 4Fj and 4Fs-B mice upon continuous
495 administration of doxycycline and untreated 4F mice. (E) *Oct4* mRNA levels in liver, small
496 intestine, kidney and blood in untreated controls, 4Fj and 4Fs-B mice after 4 days of
497 doxycycline treatment. (F) Glucose, liver enzymes (ASAT, ALAT, ALP), bilirubin and
498 albumin levels in plasma of untreated animals (Ctrl) and 4Fj and 4Fs-B mice after 4 days
499 of induction of in vivo reprogramming. (G) Liver hematoxylin and eosin stain for an
500 untreated wildtype and 4Fj mice, and treated 4Fj and 4Fs-B mice during 4 days. (H)
501 Immunostaining and quantification of cleaved caspase-3 positive cells in liver of
502 untreated controls, 4Fj and 4Fs-B mice. Data are mean \pm SEM. Statistical significance
503 was assessed by (B-C, F-G) one-way ANOVA followed by Tukey's post hoc or Games
504 Howell tests and (D) log-rank (Mantel-Cox) test.

505 **Figure 2: Liver or intestine-specific reprogramming leads to body weight loss and**
506 **early mortality.**

507 (A) Schematic representation of liver- and intestine-specific reprogramming strains
508 carrying a polycistronic for the expression of the mouse reprogramming factors (*OSKM*)
509 in the *Col1a1* locus (4Fj), the LoxP-STOP-LoxP (LSL) cassette-blocked rtTA trans-
510 activator, and Cre recombinase transgenes under the control of the promoter of the
511 mouse *Albumin* and *Villin 1* genes. (B) *Oct4* transcript levels in the liver, small intestine,
512 and kidney of 4F-Liver, 4F-Intestine, and control mice (carrying only the 4Fj and LSLr-
513 tTA transgenes but no Cre) after 4 days of induction. (C) Changes in body weight of 4F-
514 Liver, 4F-Intestine and control mice upon continuous administration of doxycycline. (D-

515 E) Glucose, liver enzymes (ASAT, ALAT, ALP), bilirubin and albumin levels in plasma of
516 control, 4F-Liver, and 4F-Intestine mice after 4 days of induction of in vivo
517 reprogramming. (F) Survival of control, 4F-Liver and 4F-Intestine mice upon continuous
518 administration of doxycycline. Data are mean \pm SEM. Statistical significance was
519 assessed by (B-E) one-way ANOVA followed by Tukey's post hoc test and (F) log-rank
520 (Mantel-Cox) test.

521 **Figure 3: Reduction of adverse effects of in vivo reprogramming by abolishing the**
522 **expression of OSKM in the liver and intestine of reprogrammable mice.**

523 (A) Schematic representation of the 4F Non-Liver and 4F Non-Intestine mouse strains
524 carrying the polycistronic cassette for the expression of the reprogramming factors (*Oct4*,
525 *Sox2*, *Klf4* and *c-Myc*) under a doxycycline-inducible promoter flanked by loxP sites in
526 the *Col1a1* locus (4Fj-Flox), the reverse tetracycline-controlled transactivator (rtTA) in
527 the *Rosa26* locus, and Cre recombinase transgenes under the control of the promoter of
528 the mouse *Albumin* and *Villin 1* mouse genes. (B) mRNA levels of *Oct4* in the liver, small
529 intestine, and kidney, of the 4F-Flox (whole body reprogrammable mice), 4F Non-Liver,
530 4F Non- Intestine mice, after 4 days of induction of in vivo reprogramming. (C) Changes
531 in body weight of 4F-Flox (whole body reprogramming), 4F Non-Liver, 4F Non-Intestine
532 mice, upon continuous administration of doxycycline and (D) distance travelled in open
533 field cage following 3.5 days of treatment. (E) Glucose levels, liver enzymes (ASAT,
534 ALAT, ALP) and bilirubin levels in plasma of 4F-Flox, 4F Non-Liver, 4F Non-Intestine
535 after 4 days of reprogramming. (F) Survival of 4F-Flox (whole body), 4F Non-Liver and
536 4F Non-Intestine mice upon continuous administration of doxycycline (1mg/ml). Data are
537 mean \pm SEM. Statistical significance was assessed by (B-E) one-way ANOVA followed
538 by Tukey's post hoc test and (F) log-rank (Mantel-Cox) test.

539 **Figure 4: Generation of novel reprogrammable mouse strain for the safe and long-**
540 **term induction of in vivo reprogramming.**

541 (A) Schematic representation the 4F Non-Liver/Intestine quadruple transgenic mouse
542 strain carrying the polycistronic cassette for the expression of the mouse reprogramming

543 factors (OSKM) under a doxycycline-inducible promoter flanked by LoxP sites in the
544 *Col1a1* locus (4Fj-Flox), the reverse tetracycline-controlled transactivator (rtTA) in
545 *Rosa26* locus and Cre recombinase under the control of the promoter of the mouse
546 *Albumin* and *Villin 1* genes. (B) mRNA levels of *Oct4* in the liver and small intestine in
547 4F-Flox (whole body reprogrammable mice) and 4F Non-Liver/Intestine mice after 4 days
548 of reprogramming induction. (C) Body weight of control (no reprogrammable mice), 4F-
549 Flox (whole body reprogramming) and 4F Non-Liver/Intestine mice upon continuous
550 administration of doxycycline and (D) activity measured in distance travelled in open field
551 test following 3.5 days of treatment. (E) Glucose, liver enzymes (ASAT, ALAT, ALP) and
552 albumin levels in plasma of controls no reprogrammable mice, 4F-Flox (OSKM whole
553 body) and 4F Non-Liver/Intestine mice after 4 days of induction of in vivo reprogramming.
554 (F) Survival of control, 4F-Flox (whole body), and 4F Non-Liver/Intestine mice upon
555 continuous administration of doxycycline. (G) mRNA levels of *Oct4* in multiple organs of
556 4F whole body reprogrammable and 4F Non-Liver/Intestine mice after 4 days and 4
557 weeks respectively, of continuous doxycycline treatment. Data are mean \pm SEM.
558 Statistical significance was assessed by (B, G) two-sided unpaired t-test and Mann-
559 Whitney-Wilcoxon test, (C-E) one-way ANOVA followed by Tukey's post hoc test and (F)
560 log-rank (Mantel-Cox) test.

561

562 SUPPLEMENTAL FIGURE LEGENDS

563 **Figure S1: In vivo reprogramming induces liver and intestinal damage.**

564 (A) Experimental design indicating doxycycline administration protocol, activity measure
565 by open field (OF) test, and organs and plasma collection. (B) Superficial temperature of
566 4Fj and 4Fs-B mice upon continuous administration of doxycycline, as wells as untreated
567 4F control mice (Ctrl). (C) *Sox2* mRNA and (D) OCT4 protein levels in the indicated
568 organs of untreated 4F controls, and 4Fj and 4Fs-B mice after induction of 4 days. (E)
569 Small intestine hematoxylin and eosin staining for an untreated wildtype and 4Fj mice
570 and treated 4Fj and 4Fs-B mice during 4 days. (F) Immunostaining and quantification of

571 Ki67 positive cells in the liver and small intestine (n = 3 regions from four untreated
572 controls, five 4Fj, and four 4Fs-B mice). Scale bars, 100 μ m. (G) Potassium, phosphate,
573 and uric acid levels in plasma of untreated control mice (Ctrl), and 4Fj and 4Fs-B mice
574 after 4 days of induction of in vivo reprogramming. Data are mean \pm SEM. Statistical
575 significance was assessed by (B-D, F-G)) one-way ANOVA followed by Tukey's or
576 Games-Howells post hoc tests.

577 **Figure S2: Liver and intestine-specific reprogramming lead to decrease body**
578 **weight and early mortality.**

579 (A) Expression of Cre recombinase in the liver, small intestine, and kidney of control (4Fj
580 LSL-rtTA), 4F-Liver (4Fj LSLrtTA AlbCre), and 4F-Intestine (4Fj LSLrtTA Vil1Cre) mice.
581 (B) Sox2 mRNA levels in control, 4F-Liver, and 4F-Intestine mice after induction of
582 reprogramming for 4 days. (C) Superficial body temperature upon continuous
583 administration of doxycycline in controls and 4F tissue-specific reprogramming mice.
584 Data are mean \pm SEM. Statistical significance was assessed by (B-C) one-way ANOVA
585 followed by Tukey's post hoc test.

586 **Figure S3: Avoiding the expression of OSKM in liver and intestine diminishes**
587 **adverse phenotypes and premature death associated with in vivo reprogramming.**

588 (A) Sox2 mRNA levels in the liver, small intestine, and kidney of 4F-Flox (OSKM whole
589 body), 4F Non-Liver, and 4F Non-Intestine mice, after 4 days of induction of in vivo
590 reprogramming. (B) Superficial body temperature upon continuous administration of
591 doxycycline in whole body (4F-Flox), 4F Non-liver, and 4F Non-intestine mice. Data are
592 mean \pm SEM. Statistical significance was assessed by (A-B) one-way ANOVA followed
593 by Tukey's post hoc test.

594 **Figure S4: Safe and long-term continuous induction of in vivo reprogramming in**
595 **a novel reprogrammable strain.**

596 (A) Sox2 mRNA levels in control and 4F non-Liver/Intestine mice after induction of in vivo
597 reprogramming for 4 days. (B) Body temperature of control and 4F Non-Liver/Intestine
598 mice upon continuous administration of doxycycline. (C) mRNA levels of Sox2 in multiple

599 organs of 4F whole body reprogrammable and 4F Non-Liver/Intestine mice after 4 days
600 and 4 weeks of continuous doxycycline treatment. (D) Glucose, liver enzymes (ASAT,
601 ALAT, ALP) and bilirubin levels in plasma of untreated 4F controls, treated no
602 reprogrammable mice, 4F whole body after 4 days of treatment, and 4F Non-
603 Liver/Intestine mice after 4 weeks of induction of in vivo reprogramming. (E) Survival and
604 (F) body weight of 4F Non-Liver/Intestine mice non-treated or doxycycline-treated for 4
605 days or 2 weeks and monitored after doxycycline withdrawal. Data are mean \pm SEM. (A,
606 C) Two-sided unpaired t-test and Mann-Whitney-Wilcoxon test, (B, D, F) one-way
607 ANOVA followed by Tukey's post hoc test and (E) log-rank (Mantel-Cox) test.

608

609 **AUTHOR CONTRIBUTIONS**

610 A.P. was involved in the design of the study, in all experiments, data collection, and
611 statistical analysis. A.V. performed mouse doxycycline treatment, immunostainings, and
612 qRT-PCR experiments. G.D. and C. M. contributed to RNA and protein extraction, qRT-
613 PCR, and western blot analysis. K.P. made intellectual contributions. C.R. and F.B. was
614 implicated in RNA extraction and qRT-PCR experiments. C.B. was involved in mouse
615 genotyping and sample collection. A.O. directed and supervised the study and designed
616 the experiments. A.P. and A.O wrote the manuscript with input from all authors.

617

618 **DECLARATION OF INTEREST**

619 The authors declare no competing interests.

620

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636

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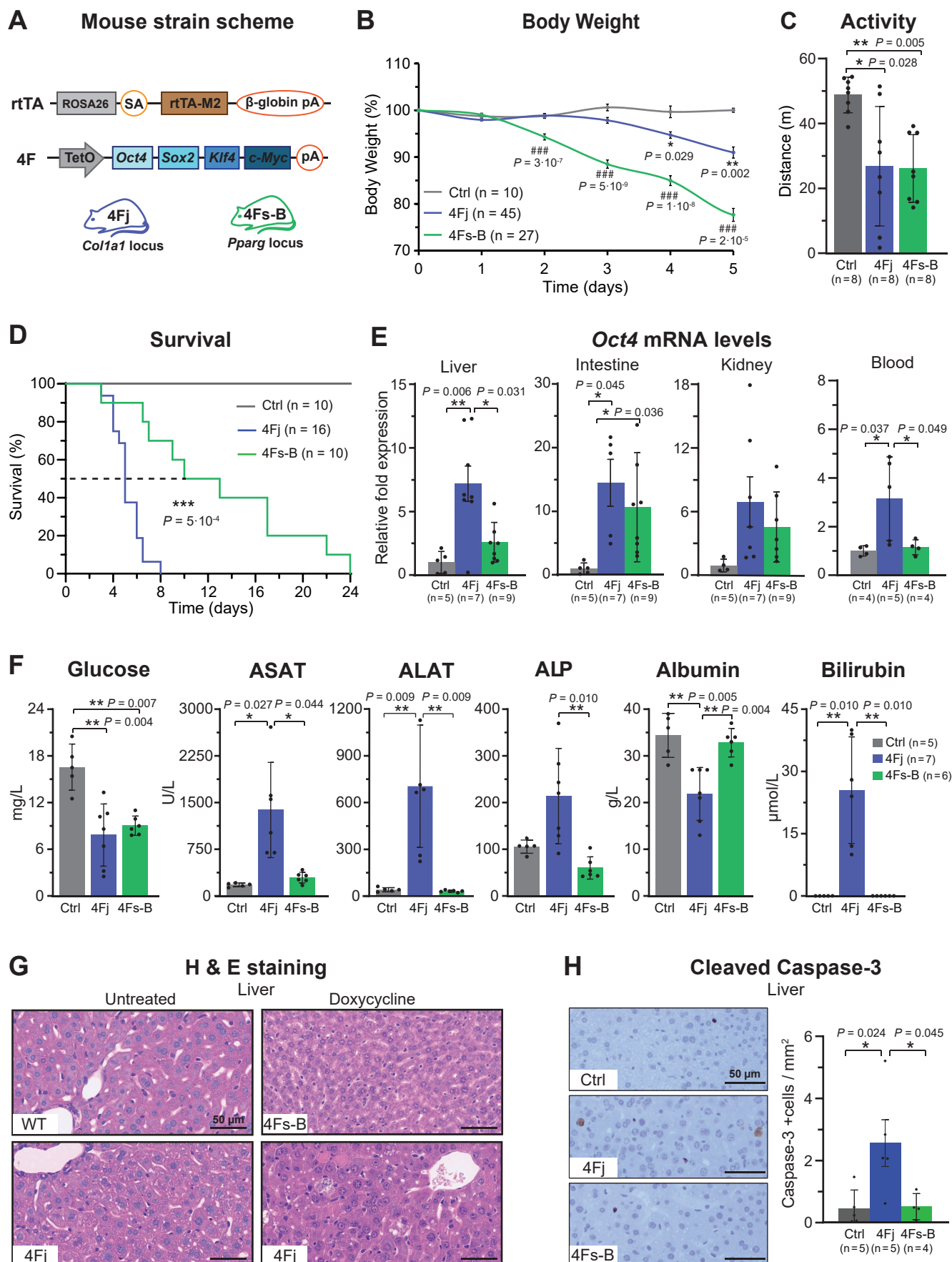


Figure 1

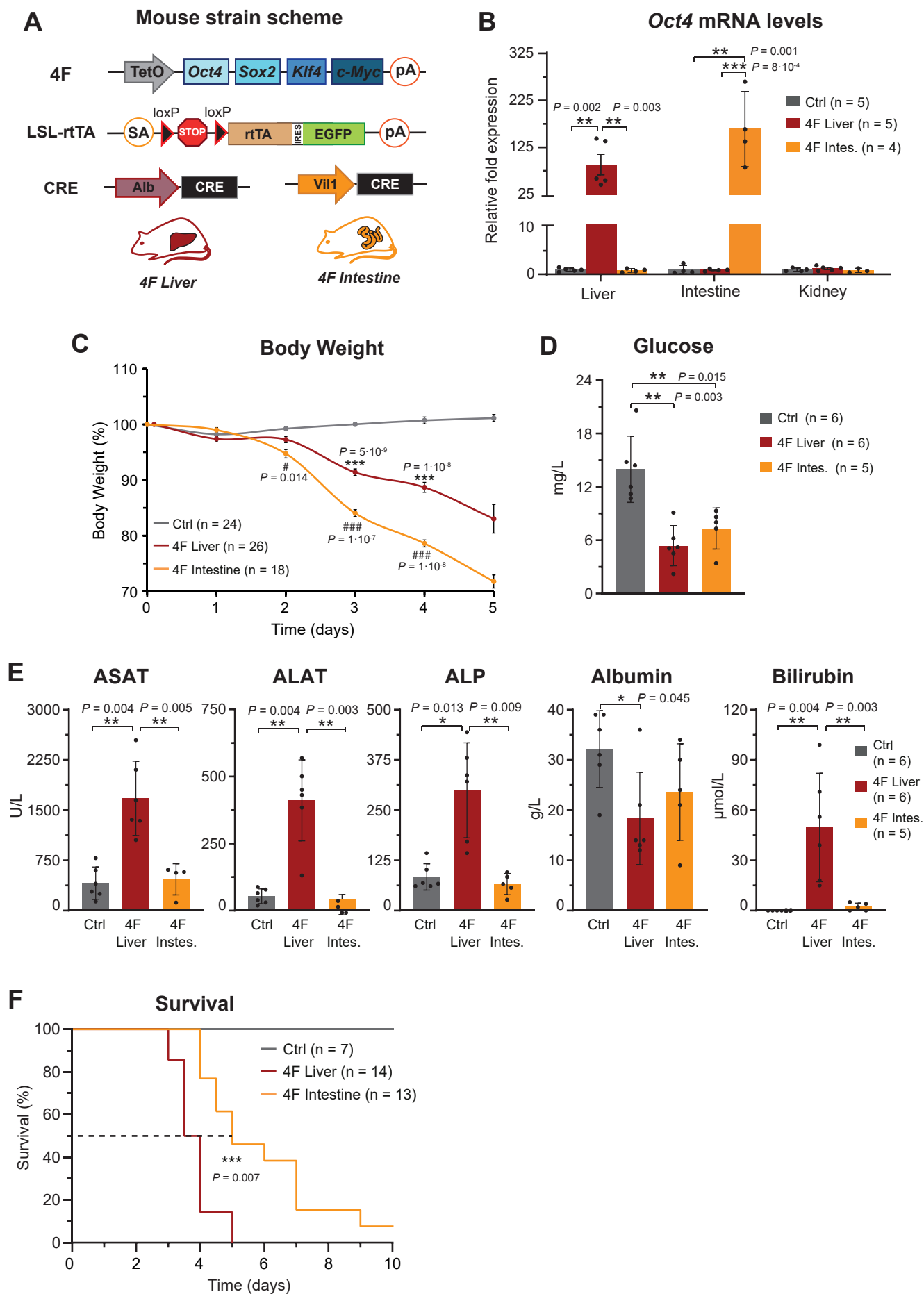


Figure 2

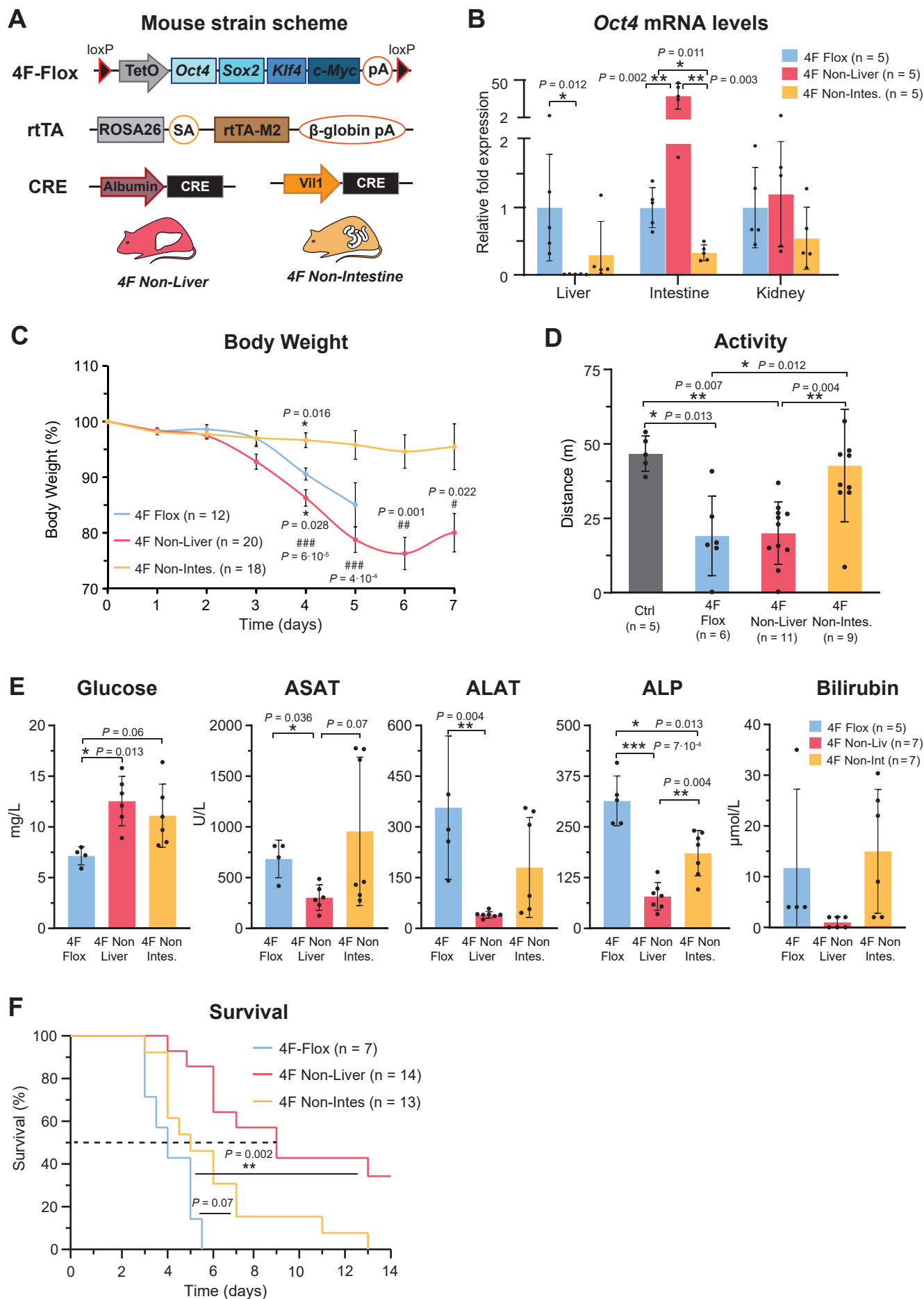


Figure 3

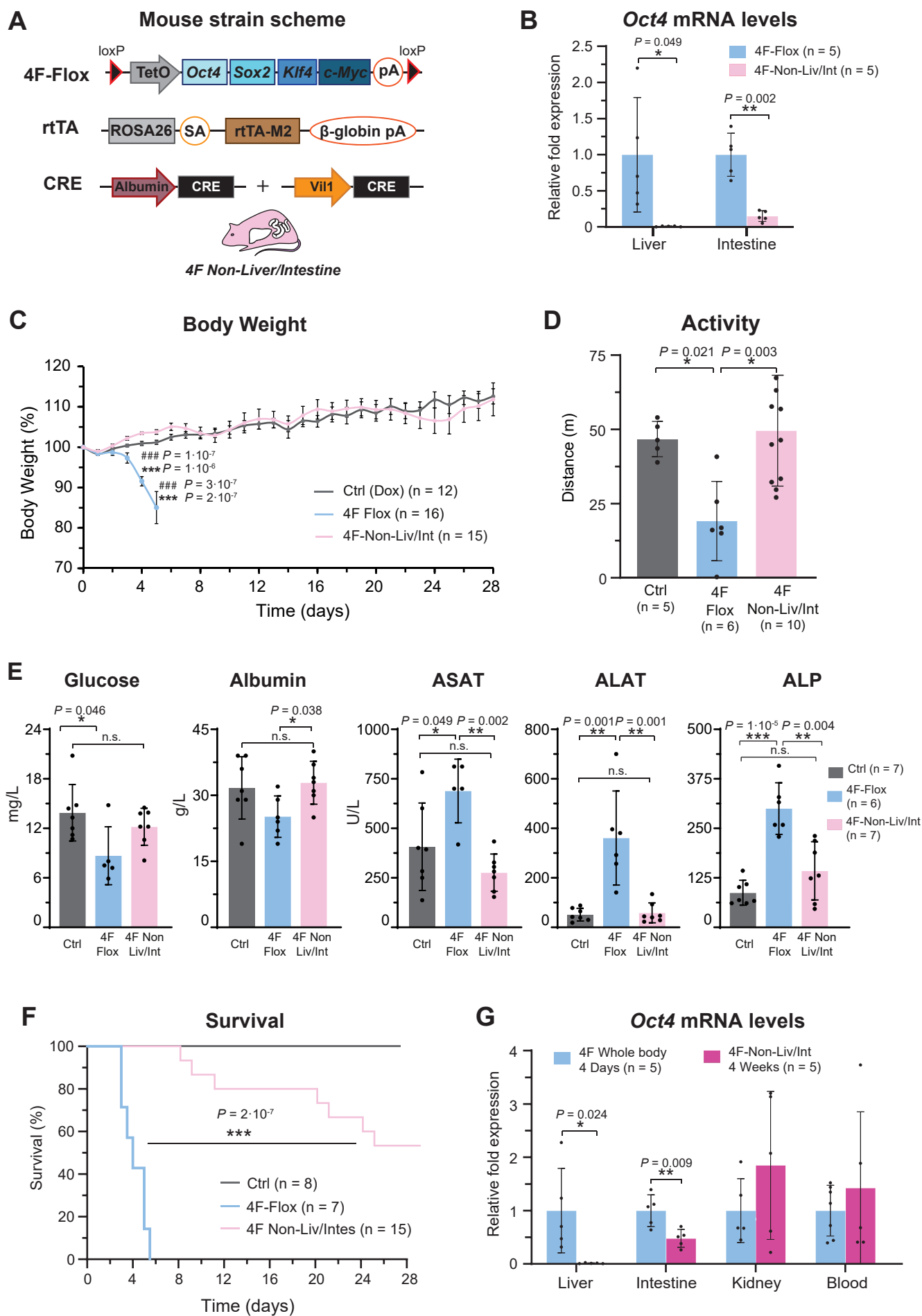


Figure 4

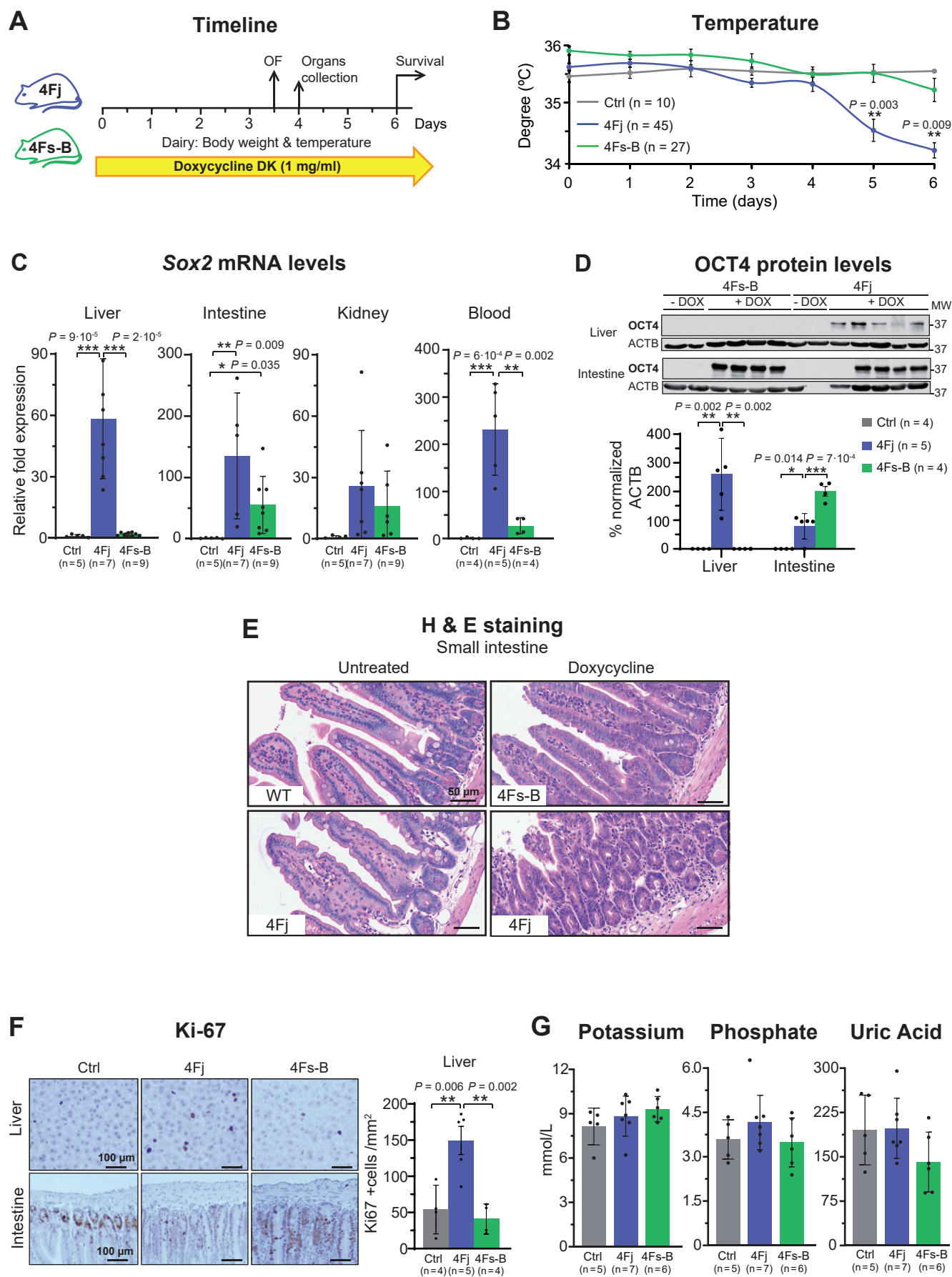


Figure S1

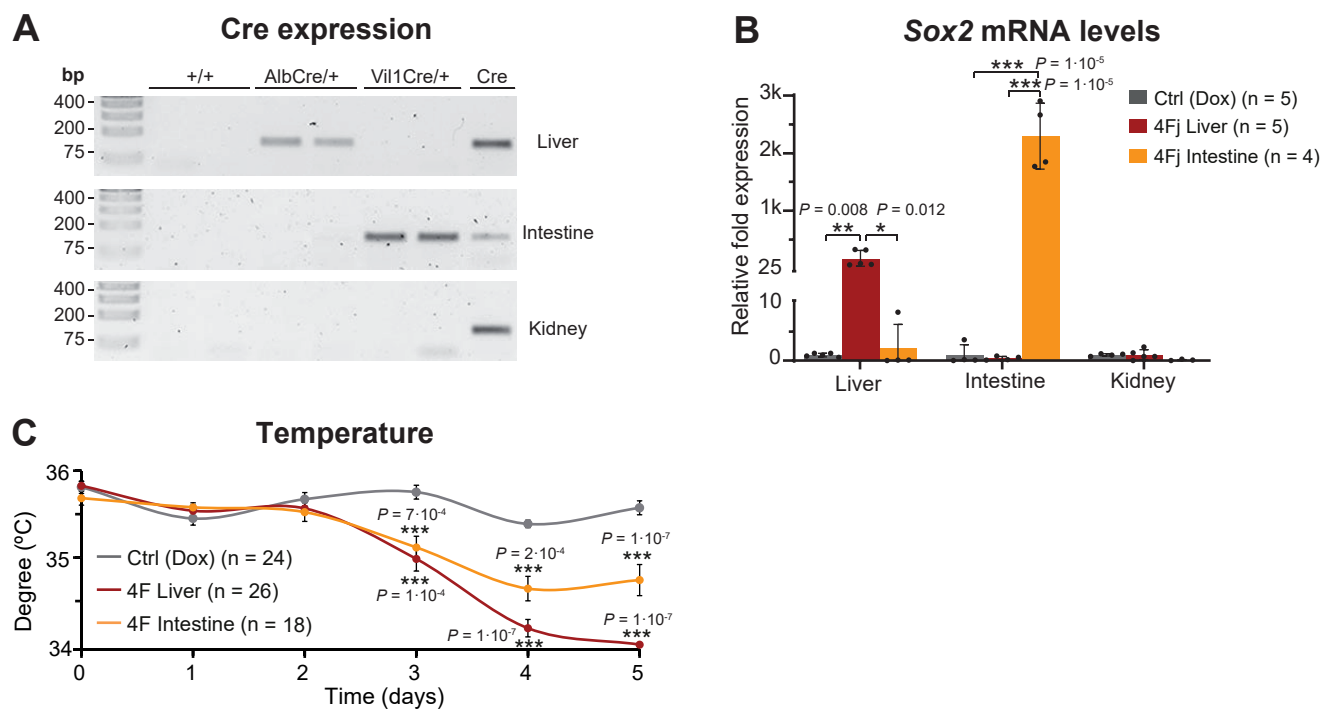


Figure S2

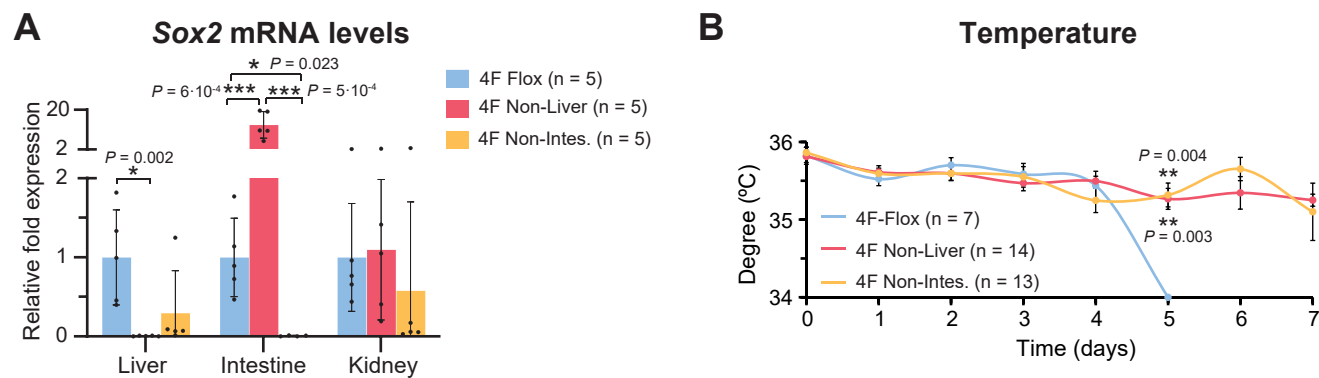


Figure S3

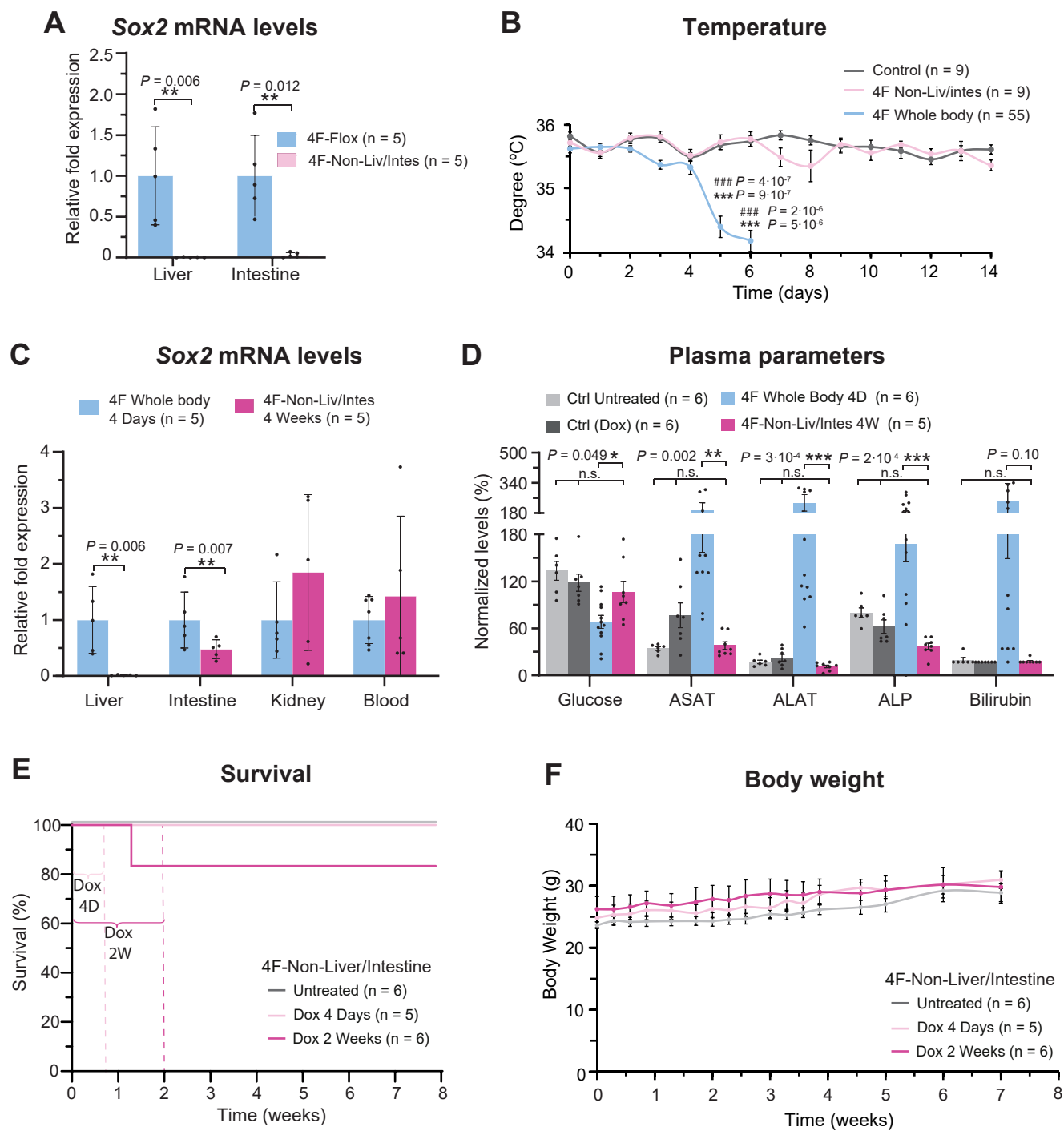


Figure S4