

1 **Detailed metabolic phenotyping of four tissue specific**
2 **Cas9 transgenic mouse lines**

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25 **Abstract**

26 CRISPR/Cas9 technology has revolutionized gene editing and fast tracked our capacity to
27 manipulate genes of interest for the benefit of both research and therapeutic applications.
28 Whilst many advances have, and continue to be made in this area, perhaps the most utilized
29 technology to date has been the generation of knockout cells, tissues and animals by taking
30 advantage of Cas9 function to promote indels in precise locations in the genome. Whilst the
31 advantages of this technology are many fold, some questions still remain regarding the effects
32 that long term expression of foreign proteins such as Cas9, have on mammalian cell function.
33 Several studies have proposed that chronic overexpression of Cas9, with or without its
34 accompanying guide RNAs, may have deleterious effects on cell function and health. This is
35 of particular concern when applying this technology *in vivo*, where chronic expression of
36 Cas9 in tissues of interest may promote disease-like phenotypes and thus confound the
37 investigation of the effects of the gene of interest. Although these concerns remain valid, no
38 study to our knowledge has yet to demonstrate this directly. Thus, in this study we used the
39 lox-stop-lox (LSL) spCas9 ROSA26 transgenic (Tg) mouse line to generate four tissue-
40 specific Cas9-Tg models with expression in the heart, liver, skeletal muscle and adipose
41 tissue. We performed comprehensive phenotyping of these mice up to 20-weeks of age and
42 subsequently performed molecular analysis of their organs. We demonstrated that Cas9
43 expression in these tissues had no detrimental effect on whole body health of the animals, nor
44 did it induce any tissue-specific effects on energy metabolism, liver health, inflammation,
45 fibrosis, heart function or muscle mass. Thus, our data suggests that these models are suitable
46 for studying the tissue specific effects of gene deletion using the LSL-Cas9-Tg model, and
47 that phenotypes observed utilizing these models can be confidently interpreted as being gene
48 specific, and not confounded by the chronic overexpression of Cas9.

49

50 **Introduction**

51 Since the discovery and proven utility of CRISPR/Cas9 based gene editing technologies,
52 there has been a proliferation of applications that take advantage of this ground-breaking
53 technology. Whilst the potential for this relatively simple but precise, genetic manipulation
54 tool is obvious, the speed at which the field is developing often means that subtle off-target
55 and deleterious effects of such an approach can be overlooked. Studies over the past 5 years
56 have demonstrated that each system requires important optimization to ensure accurate gene
57 editing, whilst minimizing off-target editing and potential toxicity induced by the
58 introduction of foreign genetic machinery (Broeders et al., 2020; Molla and Yang, 2019).

59

60 A major advantage of CRISPR based editing in the pre-clinical biomedical arena is the rapid
61 development of animal models that harbor global gene deletions or conditional targeting of
62 alleles. These models historically took 2-3 years to generate, where now a global deletion
63 model using CRISPR can take 3 months or less to generate (Singh et al., 2015). Moreover,
64 CRISPR overcomes the need to generate one mouse model per gene of interest, as is the case
65 with floxed alleles. Indeed, by over-expressing Cas9 globally in mice, or in a tissue specific
66 manner, one can generate a model where almost any gene can be manipulated simply by
67 introducing a guide RNA that targets your gene of interest. This flexibility of manipulation
68 has made it feasible to use one mouse model, or even an existing disease model, to study the
69 effect of manipulating one or many genes in combination.

70

71 One such model developed is the lox-STOP-lox spCas9-transgenic (LSL-spCas9Tg) mouse
72 (Platt et al., 2014). This model harbors the spCas9 gene at the ROSA26 locus, but is silenced
73 in the basal state by commonly applied repressor elements. By flanking the repressor or

74 “STOP” element with loxP sites, the construct becomes inducible in the presence of Cre-
75 recombinase. This model has been used by a number of groups to demonstrate robust Cas9
76 expression induced by Cre-recombinase, and subsequent CRISPR mediated gene editing
77 upon the administration of a guide RNA (Laidlaw et al., 2020; Shamsi et al., 2020; Zhu et al.,
78 2020).

79

80 One concern with such an approach is whether chronic expression of the foreign CRISPR
81 machinery mediates phenotypic effects *per se* (Broeders et al., 2020). To this end, we have
82 successfully developed four tissue-specific Cas9 transgenic mouse models that include key
83 tissues of interest in the metabolism field (skeletal muscle, liver, adipose tissue and heart).
84 We phenotyped these animals for readouts of whole body metabolism, adiposity, glucose
85 tolerance, toxicity and cardiac function, and demonstrated that none of the models were
86 impacted by the chronic (~12 weeks) presence of Cas9. These findings provide important and
87 much needed confidence for researchers who wish to use these mouse models in the future,
88 who can now confidently ascribe any observed metabolic phenotype to their gene of interest,
89 and not to underlying unwanted side effects of the model itself.

90 **Results**

91 **Tissue Specific and Inducible Expression of Cas9 in four mouse models.**

92 To generate tissue specific Cas9 transgenic mice, we crossed the LSL-spCas9 Tg mouse
93 (Platt et al., 2014) with four different tissue specific Cre-recombinase transgenic mouse lines.
94 Three of these models (ACTA1-Cre, AdipoQ-Cre and MHCalpha-Cre) were tamoxifen
95 inducible via the use of Cre-recombinase that was fused to the modified estrogen receptor
96 (mER), often referred to as ERT2. The other model (albumin-Cre) was constitutively active
97 and expressed from the albumin promoter (Alb-Cre). These breeding strategies resulted in
98 four separate mouse models with an expected tissue specific expression of Cas9 in liver
99 (Albumin), heart (MHC-alpha), skeletal muscle (ACTA1), and adipose tissue (AdipoQ),

100 **(Figure 1A).**

101

102 Because three of the Cre-models were inducible (with tamoxifen), we administered tamoxifen
103 or vehicle (oil) using specific regimens for each model (see methods) between 6-8 weeks of
104 age, then allowed 2 weeks for maximal gene expression before any phenotyping was
105 performed. For the inducible models, we studied four cohorts of mice per model, which
106 included: Cre+OIL (Cre-inactive), Cas9+Cre+OIL (Cas9+Cre-inactive), Cre+Tamoxifen
107 (Cre-active) and Cas9+Cre+Tamoxifen (Cas9+Cre-active) mice. For the constitutively active
108 Albumin-Cre model, there was no oil/tamoxifen treatment so there were only two cohorts;
109 Cre-active and Cas9+Cre-active.

110

111 To characterize the different models, we performed phenotyping that included assessment of
112 body weight, fat mass and lean mass by EchoMRI, and glucose tolerance tests (GTTs) which
113 were performed at various times for different models over the subsequent 12 weeks. We also

114 performed some model specific phenotyping, including echocardiography of the MHC-alpha
115 model to analyze heart function.

116

117 At the completion of each study (mice up to approximately 20-22 weeks of age), tissues were
118 collected, processed and analyzed to first confirm that each model displayed the expected
119 expression profiles. Using qPCR analysis, we demonstrated that each model exhibited tissue
120 specific Cre-recombinase expression, with robust expression in the expected tissue, but no
121 detected expression in other tissues (**Figure 1B**). Importantly, we also demonstrate using
122 qPCR that Cas9 expression was tissue specific, and that this expression was dependent on
123 both Cre-expression and the administration of tamoxifen in the inducible models. As a
124 secondary confirmation we also determined the abundance of GFP by qPCR, which is co-
125 expressed from the same transgene cassette as Cas9, but is independently processed by the
126 ribosome (i.e. not tagged to Cas9). We demonstrated that both Cas9 and GFP exhibited the
127 expected tissue specific expression profiles including liver (**Figure 1C**), heart (**Figure 1D**),
128 muscle (**Figure 1E**) and white adipose tissue (WAT, **Figure 1F**). The level of Cas9 induction
129 varied slightly across the lines, which is likely a reflection of local transcriptional machinery,
130 number of cells per unit of tissue, and the differential activity of Cre-recombinase in each
131 tissue.

132

133 **Tissue Specific Expression of Cre-Recombinase, Cas9 and GFP does not Impact Animal**
134 **Body Weight or Tissue Weights.**

135 Upon demonstrating that each model expressed Cas9 in a tissue specific manner, we next
136 sought to test previously raised concerns that chronic over-expression of “foreign” enzymes
137 such as Cre and Cas9 in metabolic tissues, might lead to phenotypic differences in animal
138 growth and development. A simple way of testing for toxicity or growth inhibition is to

139 compare body weight and individual tissue weights from each model at study end. We
140 demonstrate that body weights for each model were comparable between cohorts at the time
141 of cull, with no significant differences in body weight, whether they were expressing Cre,
142 Cas9, or if they had been treated with tamoxifen/oil (**Figure 2A**). Moreover, assessment of
143 liver, WAT, Muscle (*Tibialis anterior*; TA) and heart weights at the time of cull,
144 demonstrated no difference in the weight of any of these tissue between the various groups
145 within each model (**Figures 2B-2E**).

146

147 **Tissue Specific Expression of Cre-Recombinase, Cas9 and GFP does not Alter
148 Molecular or Physiological Readouts of Tissue Function.**

149 Whilst it was important to demonstrate that there was no effect of Cas9 expression on gross
150 tissue weights or animal growth, we also sought to investigate whether tissue specific
151 pathways were being impacted by chronic over expression of Cas9 in each tissue. Therefore,
152 we performed a series of analyzes on each tissue to investigate these parameters, utilizing
153 qPCR, histology and functional assessment.

154

155 In the liver specific Cas9 model (Alb-Cre), we used qPCR to analyze the expression of genes
156 that were representative of pathways that provided insight into the health and activity of the
157 liver. These included *Colla2* and *Vim* as markers of fibrosis, *Chop* for ER stress, *Plin2* for
158 lipid storage and *Tnfa* and *Il1b* for inflammation (**Figure 3A**). We demonstrated that none of
159 these genes were differentially expressed in mice with livers expressing Cas9 compared to
160 control livers, indicating that the expression of Cas9 in the liver was not impacting on liver
161 inflammation, fibrosis or lipid handling. Moreover, representative histological sections
162 stained with hematoxylin and eosin (H&E) demonstrated no gross changes in liver
163 morphology (**Figure 3B**).

164

165 In the heart specific Cas9 model (MHCa-Cre), we used qPCR to measure pathways in the
166 heart that are indicative of cardiac health and function. These included classic molecular
167 markers of heart failure including Atrial Natriuretic Peptide (ANP) (*Nppa*) and B-type
168 Natriuretic Peptide (BNP) (*Nppb*) as well as α MHC (*Myh6*) and β MHC (*Myh7*). We also
169 measured a marker of fibrosis (*Col1a1*) and a marker of cardiac contractile function via the
170 sarcoplasmic reticulum /endoplasmic reticulum Ca²⁺ ATPase 2a (SERCA; *tp2a2*) (**Figure**
171 **3C**). We demonstrate that were no differences in the expression of any of these markers in
172 the left ventricle (LV) across the four groups of mice, implying that these pathways were not
173 altered by the expression of Cas9 (or Cre-recombinase). This was supported by data
174 demonstrating that the weight of the whole heart and the different regions of the heart from
175 these mice including; atria, LV and right ventricle (RV), were also not different between
176 groups (**Figure 3D**). Consistently, the lung weights, spleen weight and kidney weight (which
177 are useful readouts of health in cardiac models) were all comparable across groups
178 demonstrating no peripheral effects of cardiac specific overexpression of Cas9. Lastly, we
179 assessed heart function in these mice using echocardiography. We demonstrated that at
180 comparable heart rates (HR), under anesthesia there were no differences in fractional
181 shortening (FS%) – a measure of systolic function, between the four groups (**Figure 3E**).
182 Thus, collectively these data demonstrate that overexpression of Cas9 in cardiomyocytes has
183 no impact on heart health and function.

184

185 Specific phenotyping of the muscle specific Cas9 model (ACTA1-Cre) was also performed
186 using qPCR. We measured the expression of muscle specific genes that are known readouts
187 of muscle development and growth in the TA muscle. These included myogenic transcription
188 factors *Myod*, *Myog* and *Mef2c*, as well as the pro-fusion protein myomaker (*Tmem8c*) and

189 mature muscle marker *Mck* (**Figure 3F**). As with our previous models, we demonstrated no
190 difference in the expression of these genes between the four groups of mice, indicating that
191 there were no major differences in the growth and function of adult skeletal muscle in the
192 presence of Cas9 expression. In support of this data, using EchoMRI we demonstrated that
193 there was no difference in lean muscle mass across the four groups, at any time point
194 throughout the study period (**Figure 3G**). Finally, histological analyzes of TA muscle
195 sections using H&E staining, indicated that there were no major morphological differences in
196 the muscle structure between Cas9 positive and Cas9 negative mice (**Figure 3H**).
197 Collectively, these data indicate that Cas9 expression in skeletal muscle has no impact on
198 muscle health and maturation.

199

200 The final model we characterized was the adipose-specific Cas9 mouse (AdipoQ-Cre). As
201 with previous models, using qPCR we demonstrated that there was no major difference in the
202 expression of genes related to adipocyte differentiation and health in WAT, including the
203 adipogenic transcription factors PPARgamma (*Pparg*) and C/EBPalpha (*Cebpa*), the lipid
204 transporter *Cd36*, inflammatory markers *Tnfa* and *Il1b*, and the browning marker *Ucp1*
205 (**Figure 3I**). Moreover, using EchoMRI we demonstrated that there was no difference in fat
206 mass between the four groups in this AdipoQ-Cre model at any time point throughout the
207 study period (**Figure 3J**). Finally, histological assessment of WAT sections using H&E
208 staining, indicated that there were no major morphological differences in adipocyte size or
209 structure between Cas9 positive and Cas9 negative mice (**Figure 3K**). Collectively, these
210 data indicate that Cas9 expression in adipose tissue has no impact on WAT health and
211 development.

212

213 **Tissue Specific Expression of Cre-Recombinase, Cas9 and GFP does not Alter Whole
214 Body Glucose Homeostasis.**

215 Given that many groups which study metabolism have an interest in glucose homeostasis and
216 how it relates to tissues such as liver, adipose, skeletal muscle and the heart, we sought to
217 determine if the expression of Cas9 in these tissue led to any changes in whole body glucose
218 handling. To investigate this, we performed fasting blood glucose measurements and oral
219 glucose tolerance tests on all groups and models within the final two weeks of the study
220 period. We demonstrated that there was no difference in fasting blood glucose levels between
221 Cas9 positive and Cas9 negative mice in each of the four tissue specific mouse models
222 (**Figures 4A, 4D, 4G and 4J**). In order to test the glucose tolerance of these models, we
223 challenged each cohort with a standardized oral dose of glucose (2mg/kg lean mass), and
224 subsequently measured their blood glucose concentration over two hours in an oral glucose
225 tolerance test (oGTT). We demonstrated that all groups and models showed a peak glucose
226 concentration of approximately 18-20mmol/L at 15 minutes post glucose delivery, which
227 mostly returned to baseline by 60 minutes after delivery of the glucose bolus (**Figures 4B,**
228 **4E, 4H and 4K**). We also demonstrated that there was no difference in the clearance of
229 glucose across any of the groups and in each of the models, indicating that there was no
230 difference in the glucose tolerance of these animals. This is further demonstrated
231 quantitatively by assessing the 90 minute cumulative area under the curve (AUC) for the
232 tolerance test (**Figures 4C, 4F, 4I and 4L**), confirming that there was no difference in
233 glucose tolerance between the groups in each tissue specific Cas9 model.

234
235 Collectively, the data presented above demonstrates that long term overexpression of Cas9 in
236 four different tissue specific models, does not lead to any effects on body weight, tissue
237 weight, the expression of markers of pathological pathways, or readouts of whole body

238 glucose metabolism. These findings provide an important foundation for future studies that
239 wish to use the LSL-Cas9 mouse model to study their gene of interest, and affords confidence
240 to researchers that metabolic phenotypes they measure are unlikely to be impacted by the
241 chronic over expression of Cas9 or Cre-recombinase in these models.

242 **Discussion**

243 The discovery and implementation of CRISPR Cas9 as a gene editing tool has far reaching
244 implications for furthering knowledge gain in biomedical science. The flexibility and
245 comparatively simple execution of this technology means it can be utilized by most research
246 laboratories around the world, accelerating the opportunity for discovery by several fold over
247 existing technologies. Whilst CRISPR Cas9 has indeed been adopted quickly and efficiently
248 by the scientific community, this has often been accomplished without due consideration for
249 the potential negative effects on metabolic readouts that might arise from such
250 methodologies, particularly if appropriate optimisation has not been performed. Many studies
251 have shown that spurious gene editing can occur in the setting of chronic, high level
252 expression of Cas9 (Hendel et al., 2015) and their accompanying single guide RNAs
253 (sgRNAs) (Fu et al., 2013; Link et al., 2018), whilst others have expressed concern over the
254 impacts of long term exogenous expression of CRISPR machinery (Cas9/sgRNAs), causing
255 unwanted effects on target cells (Charlesworth et al., 2019; Enache et al., 2020). Such
256 unwanted effects on targets cells would be particularly concerning in the in vivo setting,
257 where even minor disruptions to tissue function over many months has the potential to
258 substantially impact on animal health and disease risk. Unfortunately, the impact of these
259 unwanted effects is mostly unknown at this point, and is difficult to predict without
260 performing the experiments directly. This is likely time consuming and laborious, and thus
261 these important “control group comparisons” are often the first experiments to be overlooked
262 when designing new CRISPR editing experiments.

263

264 With regard to in vivo CRISPR editing, the generation of the inducible spCas9 transgenic
265 mouse (LSL-spCas9Tg) by Zhang and colleagues has been an important tool to enable tissue
266 and temporal specific Cas9 expression in mice (Platt et al., 2014). This model has been used

267 in several labs around the world to successfully delete genes of interest, most commonly in
268 myeloid or neuronal cell lineages (Laidlaw et al., 2020; Shamsi et al., 2020; Zhu et al., 2020).
269 Whilst there are obvious advantages to using this LSL-spCas9Tg mouse model, it is less
270 obvious what the potential disadvantages are - if any do exist. This is particularly true when
271 generating tissue specific Cas9 models for the first time, as there would be no data available
272 as to whether chronic Cas9 overexpression will impact the tissue of interest and the whole
273 body phenotypes of interest.

274

275 Given our group has a major interest in metabolism and the organs that regulate whole body
276 energy status, we are constantly performing studies in pertinent metabolic tissues such as the
277 liver, muscle, adipose and heart. Unfortunately, to date, few studies have performed *in vivo*
278 CRISPR editing in these tissues using the LSL-spCas9Tg mouse, and thus it is unclear as to
279 whether this model would be suitable for investigating CRISPR-mediated gene deletion in the
280 aforementioned tissues, without the risk of unwanted side effects due to chronic Cas9
281 overexpression.

282

283 We thus generated liver-, muscle-, adipose- and heart-specific Cas9 expressing mice using
284 the LSL-Cas9Tg model, and demonstrated that these four models appear to be unaffected by
285 the chronic expression of Cas9 and/or Cre-recombinase. We performed comprehensive
286 metabolic phenotyping of all four lines including body composition, glucose tolerance,
287 molecular and biochemical measurements, and functional readouts on various tissues. These
288 analyses demonstrated clear tissue specificity of Cas9 expression, driven by temporal
289 activation of Cre-recombinase using tamoxifen in all three of the inducible lines (muscle,
290 adipose and heart), as well as the constitutive albumin (liver) line. Importantly, we were

291 unable to detect any deleterious effects on metabolic pathways, morphology of tissues, body
292 composition or glucose tolerance in any lines over-expressing Cas9, supporting the notion
293 that there is no negative impact of chronic Cas9 expression in these tissues. Moreover,
294 echocardiography also demonstrated no impact on systolic heart function in cardiac-specific
295 Cas9 expressing mice after 20 weeks of induction, providing evidence that heart function in
296 these mice was not affected by chronic Cas9 expression.

297

298 In summary, we provide critical evidence that the metabolism and general health of four
299 different metabolic tissue specific mouse lines are unaffected by the chronic expression of
300 Cas9. These findings provide confidence for researchers moving forward, who wish to use
301 these Cas9 mouse models to manipulate the expression of genes in these particular tissues.
302 The minimal impact of Cas9 in these studies will likely reduce the need for future studies to
303 perform specific controls groups, reducing animal numbers and sparing expensive resources.
304 Our data will also provide confidence that observed phenotypes related to gene deletions in
305 these models in future studies, are likely to be specific to the gene of interest rather than
306 being related to the chronic over-expression of Cas9. Thus these findings provide an
307 important resource for the research community.

308

309

310

311 **Methods**

312 **Generation of Tissue Specific Cas9 Animal Models**

313 All animal experiments were approved by the Alfred Research Alliance (ARA) Animal
314 Ethics committee (E/1756/2017/B) and performed in accordance with the research guidelines
315 of the National Health and Medical Research Council of Australia. Tissue specific
316 expression of Cas9 was achieved using the Cre-Lox system, where Cre-recombinase was
317 used to remove the “STOP” sequence from the lox-stop-lox (LSL) cassette separating the
318 promoter and Cas9 genes in the mouse described by Zhang et al (Platt et al., 2014). The four
319 different mouse lines were generated by crossing the LSL-spCas9-Tg mouse with either the
320 Albumin-Cre, ACTA1-Cre-ERT2, AdipoQ-Cre-ERT2 or MHCalpha-Cre-ERT2 mice. All
321 mice were on a C57BL/6J background and are available from Jackson Laboratories. We
322 generated two cohorts of n=8-10 male mice of Alb-Cre-Cas9Tg mice (Cre & Cre+Cas9), and
323 2 cohorts of n=16-20 male mice of the inducible Cre-Cas9Tg mouse lines (Cre & Cre+Cas9),
324 the latter of which were split further into two groups each and treated with either vehicle
325 (sunflower oil) or Tamoxifen in sunflower oil, generating the following four groups; Cre
326 inactive (OIL), Cre+Cas9 inactive (OIL), Cre active (tamoxifen) and Cre+Cas9 active
327 (tamoxifen). Only the final group (Cre+Cas9 active) was expected to express Cas9, with the
328 others serving as either Cre or tamoxifen control groups.

329

330 **Animal Treatments and Husbandry**

331 All mice were bred and sourced through the ARA Precinct Animal Centre and randomly
332 allocated into their respective groups. For tamoxifen inducible models, they were treated as
333 follows. For ACTA1-Cre-ERT2 and AdipoQ-Cre-ERT2 models, mice were aged to 6-8
334 weeks old before being gavaged with either Tamoxifen (80mg/kg) in sunflower oil, or
335 sunflower oil alone, for 3 consecutive days. For the MHC-alpha-Cre-ERT2 model, mice were

336 IP injected once with 40mg/kg of Tamoxifen in sunflower oil, or sunflower oil alone.
337 Following tamoxifen treatment, mice were left to recover for 2 weeks, after which they were
338 maintained on a normal chow diet (Normal rodent chow, Specialty feeds, Australia) and
339 housed at 22°C on a 12hr light/dark cycle with access to food and water *ad libitum* with cages
340 changed weekly for 12 weeks. Cohorts of mice were subjected to EchoMRI and body weight
341 analysis throughout the study period. In the last two weeks of the study period, all animals
342 underwent oral glucose tolerance tests, whilst the MHC-alpha mice were also subjected to
343 cardiac function assessment via echocardiography. At the end of the study, mice were fasted
344 for 4-6 hours and then anesthetized with a lethal dose of ketamine/xylazine before blood and
345 tissues were collected, weighed and snap frozen for subsequent analysis.

346

347 **Glucose Tolerance Tests**

348 Oral glucose tolerance tests (oGTT) were performed as previously described (Bond et al.,
349 2019a; Bond et al., 2021). In the final two weeks of the study period mice were fasted for 4-6
350 hours and gavaged at a glucose dose of 2g/kg of lean mass as determined by EchoMRI.
351 Blood glucose was determined using a glucometer at the following times points; 0, 15, 30,
352 45, 60, 90 and 120 minutes.

353

354 **EchoMRI**

355 Body composition was analyzed using the 4 in 1 NMR Body Composition Analyzer for Live
356 Small Animals, according to the recommendations of the manufacturer (EchoMRI LLC,
357 Houston, TX, USA). This provides measurements of lean mass and fat mass in living animals
358 as previously described (Bond et al., 2019a; Bond et al., 2021).

359

360 **Histology**

361 Liver and muscle were embedded cut side down in OCT before being frozen in a bath of
362 isopentane submerged in liquid nitrogen. After freezing, blocks were brought to -20°C and
363 5µm sections were cut using a Leica Cryostat. Sections were mounted and dried overnight at
364 room temperature before being fixed in Methanol. WAT samples were fixed in formalin and
365 mounted in Paraffin, before 5µm sections were cut on a Leica microtome. All sections were
366 stained with hematoxylin and eosin and slide images were captured using Olympus Slide
367 scanner VS120 (Olympus, Japan) and viewed in the supplied program (OlyVIA Build 13771,
368 Olympus, Japan).

369

370 **Quantitative PCR (qPCR)**

371 RNA was isolated from tissues using RNAzol reagent and isopropanol precipitation as
372 previously described (Bond et al., 2021; Bond et al., 2019b). Briefly, cDNA was generated
373 from RNA using MMLV reverse transcriptase (Invitrogen) according to the manufacturer's
374 instructions. qPCR was performed on 10ng of cDNA using the SYBR-green method on an
375 ABI 7500, using primer sets outlined in Table 1. Primers were designed to span exon-exon
376 junctions where possible, and were tested for specificity using BLAST (Basic Local
377 Alignment Search Tool; National Centre for Biotechnology Information). Amplification of a
378 single amplicon was estimated from melt curve analysis, ensuring only a single peak and an
379 expected temperature dissociation profile were observed. Quantification of a given gene was
380 determined by the relative mRNA level compared with control using the delta-CT method,
381 which was calculated after normalisation to the housekeeping gene *Ppia* or *Rplp0*.

382

383 **Echocardiography**

384 Echocardiography was performed on mice anaesthetised with isoflurane (1.5-2%) at the end
385 of the 12-week period following tamoxifen induction, using a 15-MHz linear transducer L15-

386 7io with a Philips iE33 Ultrasound Machine (North Ryde, NSW, Australia). Data were
387 analyzed and verified by two independent researchers according to QC procedures and
388 validation measures as outlined previously (Donner et al., 2018).

389

390 **Data Inclusion and Exclusion Criteria**

391 For animal experiments, phenotyping data points were excluded using the following pre-
392 determined criteria: if the animal was unwell at the time of analysis, there were identified
393 technical issues (such as unclear signal from echocardiography) or data points were identified
394 as outliers using Tukey's Outlier Detection Method (Q1 minus 1.5 IQR or Q3 plus 1.5 IQR).
395 If repeated data points from the same mouse failed QC based on pre-determined criteria, or
396 several data points were outliers as per Tukey's rule, the entire animal was excluded from
397 that given analysis (i.e. during glucose tolerance tests, indicating inappropriate gavage). For
398 in vivo and in vitro tissue and molecular analyzes, data points were only excluded if there
399 was a technical failure (i.e. poor RNA quality, failed amplification in qPCR), or the value
400 was biologically improbable. This was performed in a blinded fashion (i.e. on grouped
401 datasets before genotypes were known).

402

403

404 **Tables**

| Gene | Forward primer (5' – 3') | Reverse primer (5' – 3') |
|---------------|-----------------------------|----------------------------|
| <i>Cre</i> | AGGGCGCGAGTTGATAGCT | GAGCGATGGATTCCGTCTCT |
| <i>spCas9</i> | CCAAGAGGAACAGCGATAAG | CACCACCAGCACAGAATAG |
| <i>GFP</i> | CAGGAGCGCACCATCTTCTT | CTTGTGCCAGGGATGTTG |
| <i>Colla2</i> | GGGAATGGAGCAAGACAGTCTT | TGCGATATCTATGATGGGTAGTCTCA |
| <i>Chop</i> | AGGAGCCAGGGCCAACA | TCTGGAGAGCGAGGGCTTT |
| <i>Vim</i> | GAAATTGCAGGAGGAGATGC | GGATTCCACTTCCGTTCAA |
| <i>Plin2</i> | CCCGTATTGAGATCCGTGT | TAGGTATTGGCAACCGCAAT |
| <i>Tnfa</i> | CCAGACCCCTCACACTCAGATC | CACTTGGTGGTTGCTACGAC |
| <i>Il1b</i> | GACGGCACACCCACCC | AAACCGTTTTCCATCTTCTTT |
| <i>Nppa</i> | GGGGGTAGGATTGACAGGAT | AGGGCTTAGGATCTTTGCG |
| <i>Nppb</i> | ACAAGATAGACCGGATCGGA | AAGAGACCCAGGCAGAGTCA |
| <i>Atp2a2</i> | AATATGAGCCTGAAATGGGC | TCAGCAGGAACCTTGTCACC |
| <i>Myh6</i> | AAGATAGTGGAACCGCAGGGA | CTCTTCAGCAGCGGTTTGAT |
| <i>Myh7</i> | AGCATTCTCCTGCTGTTCC | GAGCCTTGGATTCTCAAACG |
| <i>Colla1</i> | GGTTTCCACGTCTCACCATT | ACATGTTAGCAGTTGTGGACC |
| <i>Mck</i> | TGAGGTCTGGTACTCCTCC | CCTCCACAGCACAGACAGAC |
| <i>Tmem8c</i> | GGAGGCCATGGTCTACCTCT | GGGCTGTTCCATAGATGCTG |
| <i>Mef2c</i> | GCCGGACAAACTCAGACATTG | GGGTTTCCCAGTGTGCTGAC |
| <i>Myog</i> | CAACCAGGAGGAGCGCGATCTCCG | AGGCGCTGTGGGAGTTGCATTCACT |
| <i>Myod</i> | AGGCCGTGGCAGCGA | GCTGTAATCCATCATGCCATCA |
| <i>Cd36</i> | TTGTACCTATACTGTGGCTAAATGAGA | CTTGTGTTTGAACATTTCTGCTT |
| <i>Cebpa</i> | TGGACAAGAACAGCAACGAG | GTCACTGGTCAACTCCAGCA |
| <i>Pparg</i> | GTTTTATGCTGTTATGGGTG | GTAATTCTTGTGAAGTGCTCATAG |
| <i>Ucp1</i> | ACTGCCACACCTCCAGTCATT | CTTGCCTCACTCAGGATTGG |

405

406

407 **Table 1**

408 Forward and reverse primer sets for detection of the designated mouse genes using qPCR.

409

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419

420 **Author contributions**

421 BGD and ACC designed and conceived the study. BGD wrote the manuscript and all other
422 authors read and/or edited the manuscript. BGD, STB, DCH, AZ, CY, EAMG, YL, HK,
423 KIW and GIL performed animal experiments and phenotyping. BGD, STB, AZ, TS, YL, YF
424 and YT analyzed data, processed tissue samples and performed molecular and biochemical
425 experiments. PG, JRM, PJM and ACC provided reagents, experimental advice and access to
426 infrastructure and resources.

427

428 **Conflicts of interest**

429 The authors declare that they have no conflicts of interest.

430

431

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480

481 **Figure Legends**

482 **Figure 1: Tissue Specific and Inducible Expression of Cas9 in four mouse models.**

483 **A.** Schematic outlining the breeding strategy and generation of the four tissue specific Cas9
484 transgenic mouse lines. The LSL-spCas9Tg mouse was bred with four different Cre-lines
485 (Albumin-Cre = Liver, AdipoQ-Cre-ERT2 = adipose, MHC-alpha-Cre-ERT2 = cardiac,
486 ACTA1-Cre/-ERT2 = skeletal muscle) to generate lines that were independently maintained
487 and studied. **B.** Relative Cre-recombinase expression as determined by qPCR for each line
488 (annotated across the bottom) in the various tissues (annotated across the top) for each mouse
489 line. Data are normalized to a housekeeping gene (*Rplp0*) and presented as arbitrary unit
490 (AU) for “Cas9+Cre Active” compared to “Cre active” groups. Adjusted expression of Cas9
491 and GFP as determined by qPCR and presented as fold change to control in the heart, liver
492 and muscle of **C.** spCas9Tg+Alb-Cre constitutively active Cre line, showing just the two
493 groups per tissue = Cre-Cas9 and Cre+Cas9 as indicated by the (+) and (-) signs at the bottom
494 of the graph, Cas9 and GFP in the **D.** spCas9Tg+MHC-alpha-Cre/-ERT2 mice, **E.**
495 spCas9Tg+ACTA1-Cre/-ERT2 mice and **F.** spCas9Tg+AdipoQ-Cre/-ERT2 mice. Because
496 the MHC-alpha-, ACTA1- and AdipoQ-Cre are inducible (tamoxifen, TAM) lines, there are
497 four groups per tissue = Cre-Cas9 (no TAM), Cre-Cas9 (plus TAM), Cre+Cas9 (no TAM)
498 and Cre+Cas9 (plus TAM) as indicated by the (+) and (-) signs at the bottom of the graphs.
499 All data are presented as mean±SEM, n=4-12/group. LSL = lox-STOP-lox, spCas9 = Cas9
500 from *S. Pyogenes*, Tg = transgenic, eGFP = enhanced green fluorescent protein, ERT2 = 2 x
501 tamoxifen sensitive mutant estrogen receptor

502

503 **Figure 2: Tissue Specific Expression of Cre-Recombinase, Cas9 and GFP does not**
504 **Impact Animal Body Weight or Tissue Weights.** All four tissue specific Cas9 mouse lines
505 were aged to approximately 18-20 weeks of age and analyzed for **A.** Body weight, **B.** Liver

506 weight at cull, **C.** Fat mass by EchoMRI (final two weeks), **D.** Lean mass by EchoMRI (final
507 two weeks) and **E.** heart weight at cull. The four groups per line are as follows: Cre (inactive
508 - light grey), Cre+Cas9 (inactive – dark grey), Cre (active – light blue) and Cre+Cas9 (active
509 – dark blue). All data are presented as mean±SEM, n=6-11/group. Albumin Cre-line is
510 constitutively active so the inactive groups were designated as “not inducible”. ND = not
511 determined in this line.

512

513 **Figure 3: Tissue Specific Expression of Cre-Recombinase, Cas9 and GFP does not Alter**
514 **Molecular or Physiological Readouts of Tissue Function.** Molecular and functional read
515 outs of tissue function specific were performed. Alb-Cre line (liver specific) was investigated
516 for changes in **A.** hepatic mRNA expression for pathways indicative of fibrosis (*Colla2*,
517 *Vim*), ER stress (*Chop*), lipid metabolism (*Plin2*) and inflammation (*Tnfa*, *Il1b*) as analyzed
518 by qPCR (n=12/group), and **B.** representative images of liver tissue morphology as assessed
519 by histology with H&E staining. The MHC-alpha-Cre-ERT2 line (cardiac specific) was
520 analyzed for changes in **C.** cardiac mRNA expression for pathways indicative of cardiac
521 pathology (*Nppa*, *Nppb*, *Atp2a2*, *Myh6* and *Myh7*) and fibrosis (*Colla1*), **D.** Mass of tissues
522 pertinent to cardiac pathology (heart, atria, left ventricle (LV), right ventricle (RV), and lung)
523 and whole body animal health (spleen, kidney and liver) and **E.** measurement of heart
524 function including heart rate and fractional shortening (FS%) as analyzed by
525 echocardiography, n=6-10/group. The ACTA1-Cre-ERT2 line (muscle specific) was
526 investigated for changes in **F.** *Tibialis anterior* (TA) mRNA expression for pathways
527 indicative of muscle maturation (*Mck*), regeneration (*Mef2c*, *Myod* and *Myog*) and fusion
528 (*Tmem8c*) as analyzed by qPCR, **G.** Temporal changes in lean (muscle) mass during the
529 study (at timepoints indicated on graph) as analyzed by EchoMRI and **H.** muscle (TA)
530 morphology as assessed by histology with H&E staining n=5-8/group. The AdipoQ-

531 Cre/ERT2 line (adipose specific) was investigated for changes in **F.** white adipose tissue
532 (WAT) gene expression for pathways indicative of lipid uptake (*Cd36*), adipocyte
533 differentiation (*Cebpa*, *Pparg*) inflammation (*Tnfa*, *Il1b*) and WAT browning (*Ucp1*) as
534 analyzed by qPCR, **G.** Temporal changes in fat (adipose) mass during the study (at
535 timepoints indicated on graph) as analyzed by EchoMRI and **H.** WAT morphology as
536 analyzed by histology with H&E staining, n=4-9/group. All data are presented as
537 mean \pm SEM, scale bar represents 100 μ m.

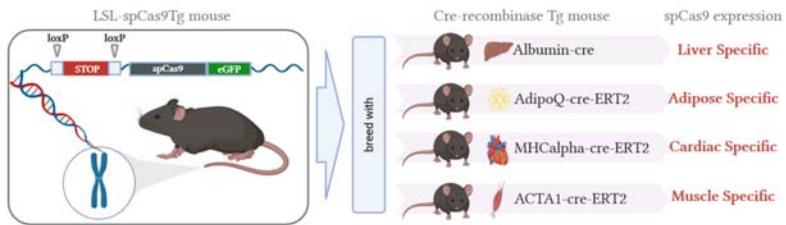
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539 **Figure 4: Tissue Specific Expression of Cre-Recombinase, Cas9 and GFP does not Alter**
540 **Whole Body Glucose Homeostasis.** All four Cas9 lines were phenotyped for parameters of
541 whole body glucose homeostasis at the end of the study. This included assessment of fasting
542 blood glucose for **A.** Alb-Cre (n=11-12/group), **D.** MHC-alpha-Cre-ERT2 (n=7-10/group),
543 **G.** ACTA1-Cre-ERT2 (n=8-9/group) and **J.** AdipoQ-Cre-ERT2 (n=6-8/group) and two-hour
544 glucose tolerance as performed by oral glucose tolerance tests (oGTT) on **B.** Alb-Cre **E.**
545 MHC-alpha-Cre-ERT2 **H.** ACTA1-Cre-ERT2 and **K.** AdipoQ-Cre-ERT2 mice as quantified
546 by 90 minute cumulative area under the curve (AUC) analysis for **C.** Alb-Cre **F.** MHC-alpha-
547 Cre-ERT2 **I.** ACTA1-Cre-ERT2 and **L.** AdipoQ-Cre-ERT2. All data are presented as
548 mean \pm SEM.

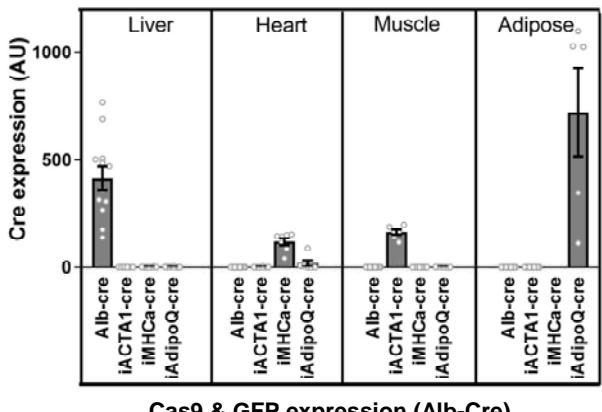
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Figure 1

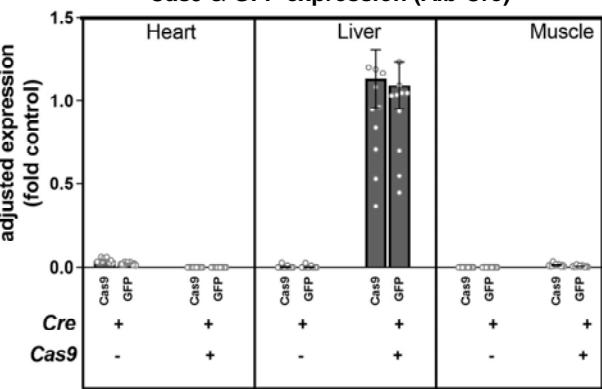
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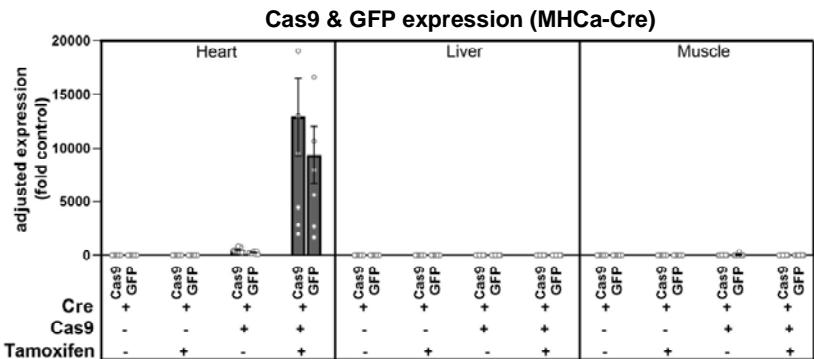
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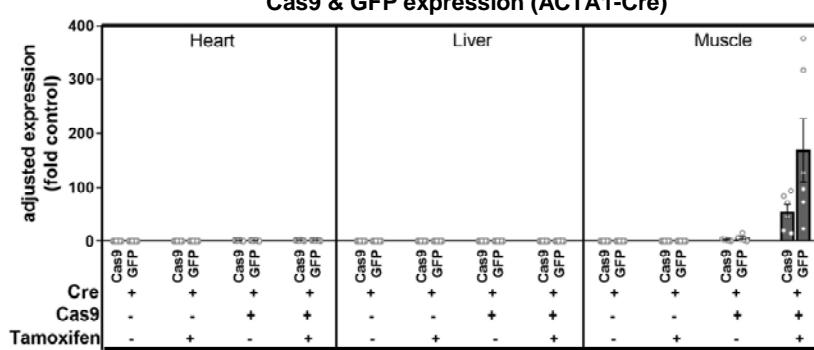
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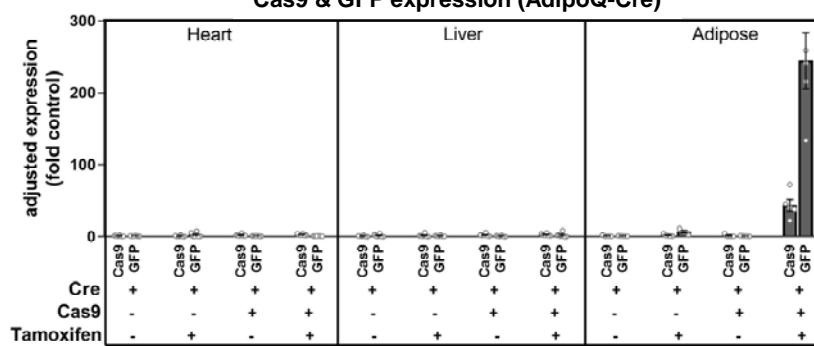


Figure 2

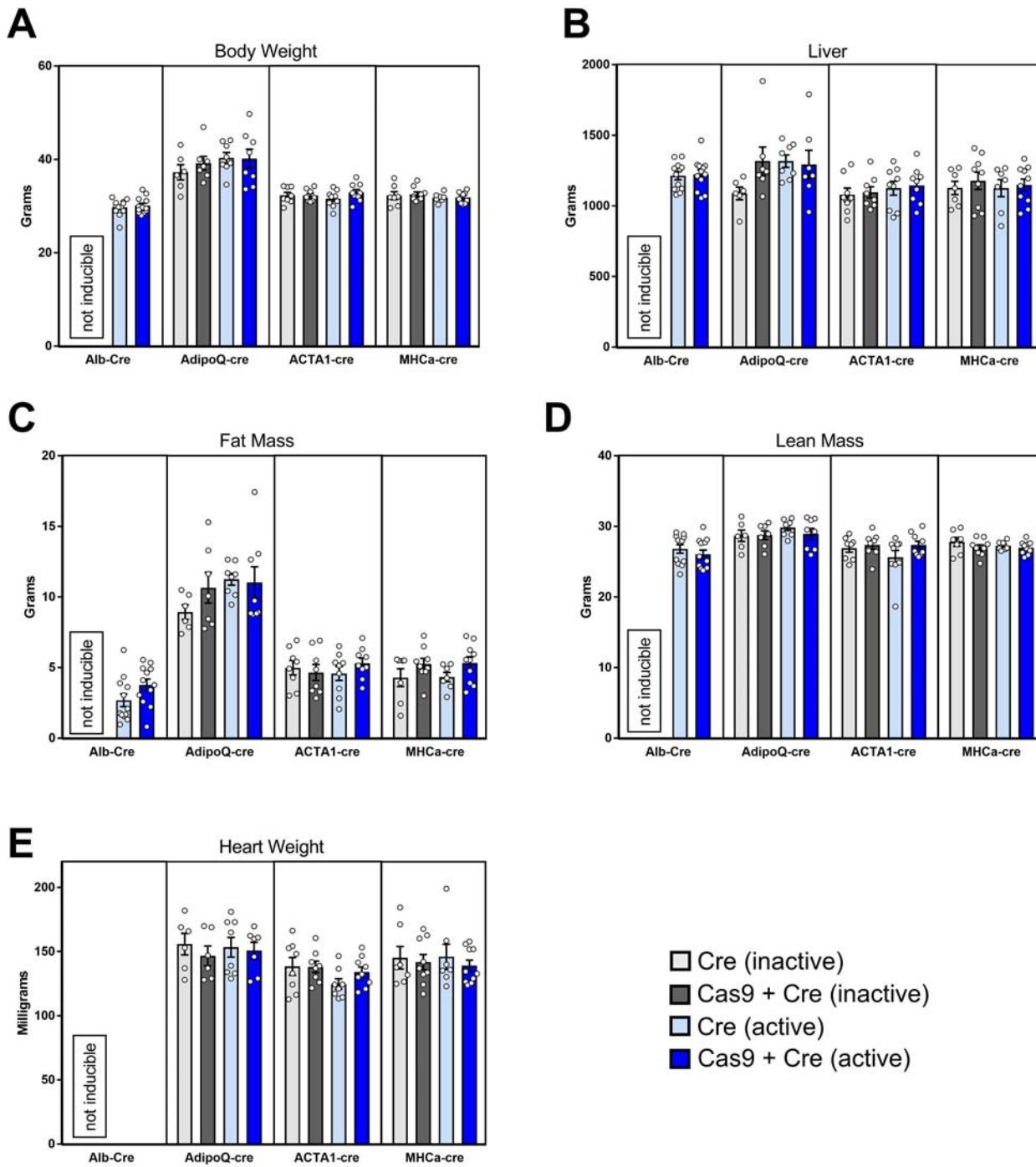


Figure 3

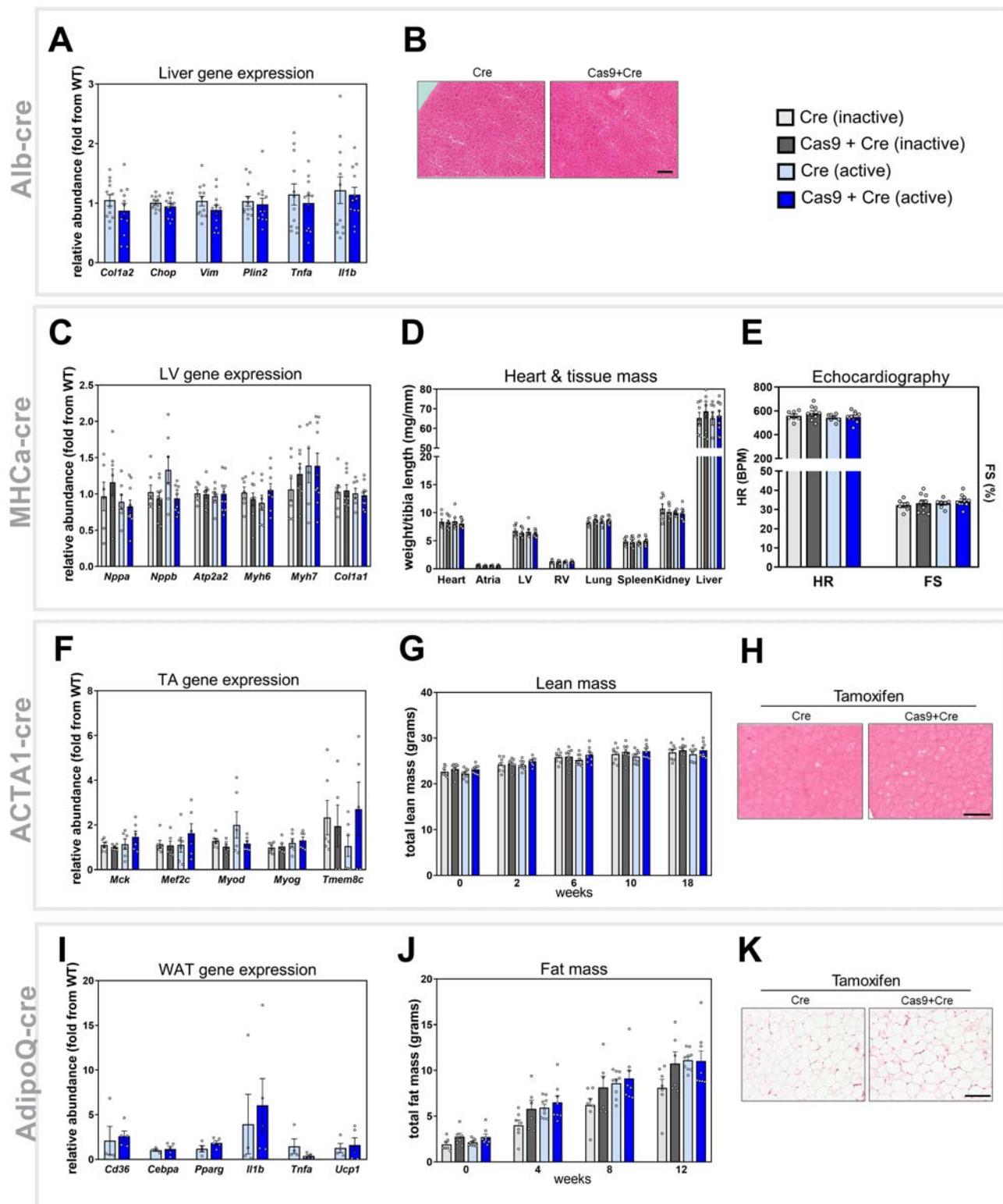


Figure 4

