

*Preprint*

## **Hyper-variability in Circulating Insulin and Physiological Outcomes in Male High Fat-fed *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* Mice in a Conventional Facility**

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### **Author Contributions**

A.E.M. performed experiments and co-wrote the manuscript.

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## 1 Abstract

2 Insulin is an ancient, multi-functional hormone with essential roles in glucose homeostasis and  
3 energy storage. Recently, our group has taken advantage of the ability to limit insulin secretion *in vivo*  
4 by reducing insulin gene dosage to demonstrate that insulin hypersecretion is a requirement for diet-  
5 induced obesity. Our previous studies employed male *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* mice that exhibit a complete  
6 inhibition of diet-induced hyperinsulinemia relative to *Ins1<sup>+/+</sup>:Ins2<sup>-/-</sup>* littermate controls, as well as  
7 female *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice with transient, partial reduction in circulating insulin relative to *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>*  
8 littermates. In the present study, we sought to extend these studies to male *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice on the  
9 same chow and high fat diets. Surprisingly, while reduced *Ins2* gene dosage appeared capable of  
10 reducing *Ins2* mRNA, insulin protein levels in these mice were not significantly reduced. Moreover,  
11 there was a marked hyper-variability in circulating insulin levels within and between two independent  
12 cohorts of mice that persisted over at least the first year of life. In Cohort 1, we observed a paradoxical  
13 increase in body weight in some high fat-fed male *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice relative to *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* littermate  
14 controls. This phenomenon is consistent with the known satiety effects of insulin and our previous  
15 observations with *Ins2* can be expressed in the brain. Collectively, our data reveal unexpected  
16 complexity associated with the *Ins2* gene in male mice, and establish the *Ins2* gene as a candidate for  
17 studying the effects of modifier genes and/or environmental influences on gene-to-phenotype  
18 variability. Further studies are required to define the molecular mechanisms of this phenotypic hyper-  
19 variability and to define the role of reduced *Ins2* gene dosage in the brain.

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## 25 **Introduction**

26 Insulin genes are highly conserved, playing critical roles in glucose homeostasis in all species  
27 studied to date [1, 2]. Unlike humans, mice have two insulin genes, *Ins1* and *Ins2* [3]. Most studies  
28 have shown that *Ins1* is restricted to pancreatic  $\beta$ -cells, where it contributes to approximately 1/3 of the  
29 expressed and secreted insulin [3, 4]. The peptide product of the *Ins1* gene differs from that of the *Ins2*  
30 gene by two amino acids in the  $\beta$ -chain, at the B9 and B29 location, and is missing two amino acids in  
31 the connecting C-peptide [5]. *Ins1* also lacks an intron present in *Ins2* [5]. *Ins2* is the ancestral gene,  
32 with gene structure, parental imprinting, and a broad tissue distribution similar to human *INSULIN* [3,  
33 4, 6]. Notably, there is evidence that both mouse *Ins2* and human *INSULIN* are expressed at low levels  
34 in within sub-populations of cells in the brain [4, 7]. The two murine insulin genes are partially  
35 redundant and capable of compensating for the loss of one another [8]. However, some studies, such as  
36 those comparing the effect of the expression of the *Ins1* versus *Ins2* in the thymus in the context of type  
37 1 diabetes, have shown that the two genes are not entirely redundant [9]. Outside of type 1 diabetes (i.e.  
38 in conditions of relative normoglycemia), the effects of changed *Ins* gene dosage, and ultimately insulin  
39 levels, remain to be fully elucidated.

40 Studies of human populations and animal models of obesity have demonstrated that elevated levels  
41 of fasting insulin, known as hyperinsulinemia, precede weight gain [10-23]. Moreover, some studies  
42 have suggested that humans with class I allele VNTR in the *INSULIN* gene produce and release more  
43 insulin from the pancreatic islets and are also more susceptible to obesity [24-26], although this  
44 observation remains controversial [27]. On the other hand, studies of invertebrates with reduced insulin  
45 or insulin signalling have reported leaner, smaller bodies, along with increased lifespan [28, 29].  
46 Similarly, studies in mammalian models, such as the Zucker fatty rats, have shown that treatment with  
47 diazoxide, a compound that reduces insulin secretion, results in reduced weight and improved glucose  
48 intolerance [30, 31]. Treatment of obese patients with diazoxide is also associated with weight loss in

49 some small clinical trials [32, 33]. Lustig and his group found similar results using Octreotide, a  
50 somatostatin agonist that binds the sst5 somatostatin receptor, found on  $\beta$ -cells, which inhibits insulin  
51 release [34-36]. Therefore, such observations have raised the question of whether hyperinsulinemia  
52 itself is a primary defect in obesity. Recently, our group has extended the observations that a full  
53 complement of insulin genes appears to be required for substantial high fat diet-induced obesity in  
54 mammals [4, 37].

55 Here, we report on the phenotype of male *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> mice fed two different diets in a  
56 conventional facility. Surprisingly, the effect of *Ins2* gene dosage on circulating insulin peptide was  
57 highly variable in these mice, displaying strong cohort dependence. This precluded definitive  
58 conclusions about the effects of this gene on weight gain, but provide insight into the regulation and  
59 effects of insulin production from this locus.

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## 62 **Materials and Methods**

### 63 **Experimental Animals**

64 The *Ins1*<sup>-/-</sup> and *Ins2*<sup>-/-</sup> mice were previously generated by Jacques Jami (INSERM) and are described  
65 elsewhere. [8] A neo cassette was used to disrupt the *Ins1* gene and replace most of its sequence. A  $\beta$ -  
66 geo (Neo/LacZ) cassette was used to disrupt most of the *Ins2* gene sequence. [8] We used DNeasy  
67 Blood and Tissue Kit (Qiagen, Valencia, CA) to isolate DNA from tail samples. The genotyping  
68 primers are detailed in Table 1.

69

70 **Table 1.** Primers used for genotyping the presence or absence of *Ins1* or *Ins2* alleles.

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Gene	Sequence 5'-3'	Annealing	PCR Product
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		Temperature °C	(bp)
<i>Ins1</i>	CCAGATACTTGAATTATTCCTGGTGTTTTATCAC		
&	GCT GCA CCA GCA TCT GCT CCC TCT ACC	60	273 <i>Ins1</i> & 550 Neo
Neo	TTC TCG GCA GGA GCA AGG TGA GAT GAC		
<i>Ins2</i>	TGC TCA GCT ACT CCT GAC TG	54	193
	GTG CAG CAC TGA TCT ACA AT		
LacZ	ACG GCA CGC TGA TTG AAG CA	59	420
	CCA GCG ACC AGA TGA TCA CA		

71

72 We divided the mice into two diet groups at weaning (3 weeks); one group was kept on the chow  
73 diet (CD; total calories = 3.81 kcal/g; 25.3% calories from fat, 19.8% calories from protein, 54.9%  
74 calories from carbohydrate; Catalog #5LJ5; PMI Nutrition International, St. Louis, MO) and we put the  
75 second group on a high fat diet (HFD; total calories = 5.56 kcal/g; 58.0% calories from fat, 16.4%  
76 calories from protein, 25.5% calories from carbohydrate; Catalog # D12330 Open Source  
77 Diets/Research Diets, New Brunswick, NJ). Diets are detailed in Table 2. One-year-old mice were  
78 scanned for whole body fat to lean mass ratio using NMR Spectroscopy at the 7T MRI Research Center  
79 at the University of British Columbia (Vancouver, BC).

80

81 **Table 2.** Comparison of the control medium fat and the high fat diets.

Diet	5015 (CD)	D12330 (HFD)
Protein	19.805 kCal%	16.4 kCal%
Carbohydrate	54.858 kCal%	25.5 kCal%
Fat	25.337 kCal%	58.0 kCal%

Total Calories	3.81 kCal/g	5.55 kCal/g
Type of Fat	Lard	Hydrogenated Coconut Oil

82

### 83 **Glucose Tolerance, Insulin Tolerance, and Hormone Secretion**

84 We measured body weight and fasting glucose (OneTouch glucometer, LifeScan Canada, Burnaby,  
85 BC) weekly in four-hour fasted mice. The fasting was initiated at approximately 8 am (start of light  
86 cycle was at 7 am). For glucose tolerance tests, mice were injected intraperitoneally with 11.1  $\mu$ L per  
87 gram of body weight of 18% glucose in 0.9% NaCl saline. For insulin tolerance tests, mice received  
88 0.75 U of insulin (Lispro Humalog VL-7510 in 0.9% NaCl solution) per gram of body weight.  
89 Ultrasensitive mouse insulin ELISA kits (80-INSMSU-E01; ALPCO Diagnostics, Salem, NH) were  
90 used to measure serum insulin levels and leptin ELISA kits (90030; CrystalChem Inc., Downers Grove,  
91 IL) were used to measure serum leptin levels. Blood was collected from the tail vein.

92

### 93 **Metabolic Cage Analyses**

94 We placed 8-week old mice (N = 3-5) from each group in PhenoMaster indirect calorimetry cages  
95 (TSE Systems Inc., Chesterfield MO) for three complete days. The cages also measured food, drink  
96 and body weight as well as activity using infrared beam grid in the x, y and z axes. All cages were  
97 contained in an environmental chamber to ensure constant temperature (21°C). The room's light cycles  
98 were from 7 am - 7 pm. Data collected from the first 4 hours were not included in the study. The  
99 average of data collected from each of the 3 days were presented as a prototypical day for each  
100 genotype, as in our previous publications [4, 37].

101

### 102 **Tissue Collection and Analyses**

103 At one year of age, mice were euthanized for the purpose of tissue collection. The following tissues  
104 were collected: pancreas, epididymal fat pads, soleus muscle, liver, brain, kidney, spleen, heart, thymus  
105 and tibia. Some samples were snap-frozen in liquid nitrogen and stored in -80°C freezer. The rest of the  
106 samples were fixed in 4% paraformaldehyde (PFA) for tissue sectioning. For removal of non-bone  
107 tissue the tibias were incubated in 2% KOH for for physical measurements. Sections were made  
108 serially at 5 µm thickness paraffin sections. The Child and Family Research Institute Histology Core  
109 Facility (Vancouver, BC) were responsible for making the sections. Pancreatic islets at 200 µm apart  
110 were stained with guinea pig anti-insulin and rabbit anti glucagon (Linco/Millipore). Using the insulin  
111 positive area morphology and hormone expression were approximated. The secondary antibodies of  
112 choice were Alexa Fluor 488 and 594 raised in goat (Life Technologies, Abtenau, Austria). The  
113 antibody dilutions were 1:100 for the primary antibodies and 1:400 for the secondary antibodies.  
114 Samples were incubated with primary antibodies overnight at 4 °C and one hour at room temperature  
115 with the secondary antibody. Vectashield solution with DAPI (Reactolab SA, Switzerland) was used as  
116 the mounting media. Imaging was done with a Zeiss 200M inverted microscope equipped with a 10x  
117 (1.45 numerical aperture) objective, individual filter cubes for each color, and a CoolSnap HQ2  
118 Camera (Roper Scientific). Image analysis was done using the Slidebook software (Intelligent Imaging  
119 Innovations) as previously described [38].

120

## 121 **Statistical Analyses**

122 Most results are expressed as means ± SEM. The area under the curve (AUC) was used to measure  
123 statistical significance in different groups for the glucose tolerance and insulin secretion tests and is  
124 described elsewhere [4]. The area over the curve (AOC) was used for insulin tolerance studies and are  
125 also described in detail elsewhere [4]. SPSS 15.0 software or Prism 5 (Graphpad) software was used to  
126 perform the statistical analyses. Two-way ANOVAs were used to compare factors of genotype and

127 diet, or alternatively in the case of significant interactions, one-way ANOVAs with Bonferroni  
128 corrections were used. We used Levene's test to validate homogeneity of variance. In all statistical  
129 analyses, if  $p < 0.05$  the differences were considered to be significant.

130

131

## 132 **Results**

### 133 **Effects of reducing *Ins2* gene dosage on *Ins2* mRNA, insulin production, and** 134 **insulin secretion**

135 We varied the *Ins2* gene dosage in mice lacking both alleles of *Ins1*, placing mice on a chow diet  
136 with moderate fat, or a diet high in fat (Fig. 1A). Deleting one of the two *Ins2* alleles reduced the *Ins2*  
137 mRNA levels in the *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> mice regardless of diet (Fig. 1B). Insulin content in isolated islets  
138 was not statistically different between genotypes, but showed a tendency to be decreased in *Ins1*<sup>-/-</sup>  
139 :*Ins2*<sup>+/-</sup> mice compared to littermate control *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/+</sup> mice ( $p = 0.095$ ; Fig. 1C).  
140 Immunofluorescent staining of islets from year-old mice (Fig. 1D) mirrored the measurements of  
141 insulin protein in isolated islets at 8 weeks (Fig. 1C). We did not detect significant differences in  
142 pancreatic  $\beta$ -cell area between groups (Fig. 1E), in contrast to *Ins1*<sup>+/-</sup>:*Ins2*<sup>-/-</sup> male mice in our previous  
143 report [4].

144

145 **Figure 1. Reduced *Ins2* gene dosage and mRNA expression does not equate to consistently**  
146 **reduced insulin secretion in male *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> mice. (A)** Experimental design for mice with varying  
147 *Ins2* gene dosage on an *Ins1* null background. **(B)** Proportionally reduced *Ins2* mRNA in *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup>  
148 mice regardless of diet in islets isolated from 8 week old mice (n = 3-4 per group). As expected, *Ins1*  
149 mRNA was not found in these samples (not shown). **(C)** Insulin protein content in 30 size-matched



150 islets isolated from 8 week old mice (n = 3-4 per group). The samples in Panels A-C, which required  
151 euthanasia for collection, were from neither Cohort 1 nor Cohort 2, which were followed for 1 year.  
152 **(D,E)** Insulin immunoreactivity in pancreas secretions from cohort 1 mice collected at 1 year of age  
153 and used to assess beta-cell area. **(F)** Circulating insulin was measured at 4 time points in both Cohort 1  
154 (n = 3) and Cohort 2 (n = 2-3).  $p \leq 0.05$  denoted by \* for CD vs HFD, # for *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/+</sup> vs *Ins1*<sup>-/-</sup>  
155 :*Ins2*<sup>+/-</sup>, and § for cohort 1 vs cohort 2.

156

157 We next measured fasting insulin, which is the product of the number of  $\beta$ -cells and their basal  
158 insulin exocytosis. Interestingly, fasting insulin was hyper-variable in these mice, with only cohort 1  
159 exhibiting significant HFD-induced elevated insulin at 8 weeks of age, and lowered fasting insulin in  
160 year-old *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> mice compared to *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/+</sup> mice (Fig. 1F). Fasting insulin was also  
161 significantly different between cohorts at 8 and 52 weeks of age (Fig. 1F). Collectively, these data  
162 demonstrate that while reducing *Ins2* gene dosage has the expected effect of reducing *Ins2* mRNA,  
163 compensatory post-transcriptional mechanisms appear to have resulted in wide variation in circulating  
164 insulin levels in these mice.

165

## 166 **Glucose Homeostasis in *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> Mice**

167 Glucose homeostasis was tracked over 1 year in *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/-</sup> mice and *Ins1*<sup>-/-</sup>:*Ins2*<sup>+/+</sup> littermate  
168 controls. No significant differences in fasting blood glucose levels between groups were observed  
169 throughout the experiment (Fig. 2A; both cohorts pooled). Consistent with the observed fasting insulin  
170 results (Fig. 1F), there were significant differences between cohorts for glucose-stimulated insulin  
171 secretion values at 7 and 52 weeks of age. However, in spite of differences in value magnitude, there  
172 tended to be similar patterns of the effects of diet and genotype across both cohorts for stimulated  
173 insulin secretion and blood glucose response to intraperitoneal glucose or insulin, and so the cohorts

174 were pooled for these data. Glucose-stimulated insulin secretion was higher in high fat-fed mice  
175 compared to chow-fed mice at 7 weeks of age, but there were no statistically significant differences in  
176 stimulated insulin secretion detected between  $Ins1^{-/-}:Ins2^{+/-}$  mice and their  $Ins1^{-/-}:Ins2^{+/+}$  littermate  
177 controls at any time point (Fig. 2B). This indicates that a single allele of the *Ins2* gene is sufficient to  
178 generate enough insulin to mount an appropriate response to glucose in these male mice, unlike the  
179 previously described male  $Ins1^{+/-}:Ins2^{-/-}$  mice [4] or female  $Ins1^{-/-}:Ins2^{+/-}$  mice [37]. Effects of genotype  
180 on glucose tolerance were very modest, with  $Ins1^{-/-}:Ins2^{+/-}$  mice exhibiting slightly worsened glucose  
181 tolerance than their  $Ins1^{-/-}:Ins2^{+/+}$  littermates only at 25 weeks (Fig. 2C). In addition, a minimal degree  
182 of HFD-induced glucose intolerance was observed by 52 weeks of age (Fig. 2C). Interestingly, high  
183 fat-fed  $Ins1^{-/-}$  mice (regardless of *Ins2* gene dose) were paradoxically insulin hypersensitive at all of the  
184 time points from 12 weeks onward (Fig. 2D). On chow diet, 6 week-old mice with reduced *Ins2* gene  
185 dosage tended to be hypersensitive to exogenous insulin, but this was not observed in older mice (Fig.  
186 2D).

187

188 **Figure 2. Insulin secretion and glucose homeostasis in high fat fed  $Ins1^{-/-}:Ins2^{+/-}$  mice.**

189 (A) Weekly blood glucose after a 4-hour fast. (B) Glucose-stimulated insulin release. Insets show area  
190 under the curve (AUC). (C) Intraperitoneal glucose tolerance. Insets show area under the curve (AUC).  
191 (D) Insulin tolerance (0.75 U/g) after four hours of fasting. Insets show area over the curve (AOC). n =  
192 6-14 per group. Cohort data are pooled.  $p \leq 0.05$  denoted by \* for CD vs HFD and # for  $Ins1^{-/-}:Ins2^{+/-}$   
193 vs  $Ins1^{-/-}:Ins2^{+/-}$ .

194

195 **Cohort-dependent Effects of *Ins2* Gene Dosage on Diet-induced Obesity**

196 Comparing the effects of a chow diet and high fat feeding on  $Ins1^{-/-}:Ins2^{+/-}$  mice and control  $Ins1^{-/-}$   
197  $:Ins2^{+/+}$  mice revealed a complex, cohort-dependent gene-environment interaction. In the first cohort,

198 *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* mice did not gain weight on a high fat diet (Fig. 3A), leading us to speculate that the  
199 hypersecretion of the *Ins1* gene product could have been required for the proper storage of lipids in  
200 adipose tissue. Remarkably, however, the majority of *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice in the first cohort exhibited  
201 striking weight gain on the high fat diet (Fig. 3A). Surprisingly, these somewhat paradoxical  
202 differences were not observed in a second cohort (Fig. 3B). Together, these data illustrate a hyper-  
203 variability of the physiological response to reduced *Ins2* gene dosage in these male mice.

204

205 **Figure 3. Cohort-dependent and diet-dependent effects on body weight in *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice on**  
206 **high fat diet.** Pooled body weight tracked weekly for 1 year in Cohort 1 (A) and Cohort 2 (B).  
207 Individual body weight are also shown across 1 year in Cohort 1 (A) and Cohort 2 (B). In cohort 1, n =  
208 6-8, and in cohort 2, n = 3. \*<sup>+/-</sup> denotes  $p < 0.05$  for CD- vs HFD-fed *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice.

209

### 210 **Characterization of Weight Gain in the First Cohort of *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* Mice**

211 Weight gain in the first cohort of high fat-fed *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice, relative to the other groups,  
212 appears to have been due to a combination of increased adiposity and increased somatic growth of  
213 various organs (Figs. 4A-F). An unusual heterogeneity in adipocyte size was observed in all groups of  
214 *Ins1<sup>-/-</sup>* mice (Fig. 4A), reminiscent of mice lacking adipocyte insulin receptors [39, 40]. Circulating  
215 leptin tended to be proportional to epididymal fat pad weight and fat-to-lean ratio (Figs. 4 B,C,F). We  
216 did not detect differences in the circulating free fatty acids between groups in this cohort (Fig. 4D).  
217 Together, these data suggest that, in some currently un-defined conditions, reducing *Ins2* expression  
218 can be associated with facilitating greater weight gain, via a mechanism that is active specifically in the  
219 context of a high fat diet.

220

221 **Figure 4. High Fat Diet-dependent Adiposity in  $Ins1^{-/-};Ins2^{+/-}$  Mice From Cohort 1. (A)**  
222 Hematoxylin and eosin-stained epididymal fat revealed heterogeneity in adipocyte size (representative  
223 image from 1 of 3 mice assessed per group). **(B)** Whole body fat to lean ratio measure with NMR (n =  
224 3). **(C)** Circulating leptin levels (n = 3). **(D)** Serum free fatty acids (n = 3). **(E,F)** Tibial length and  
225 tissues weights from 1 year old mice (n = 6-8).  $*^{+/+}$  denotes  $p < 0.05$  for CD- vs HFD-fed  $Ins1^{-/-};Ins2^{+/+}$   
226 mice. Data are collected from Cohort 1.

227

228 Given the statistical similarity between genotypes for the average circulating insulin levels prior to  
229 one year of age, these observations hint at the possibility of altered local effects of *Ins2* in the brain. It  
230 is well established that insulin can act in the brain as a satiety factor [41, 42]. We have confirmed that  
231 *Ins2* is expressed in multiple regions of the brain that can potentially control and project to feeding,  
232 reward and memory centers, raising the possibility that central *Ins2* gene expression may regulate food  
233 intake [4]. In this first cohort, where reduced *Ins2* gene dosage was associated with increased weight  
234 gain on high fat diet, there was a tendency for  $Ins1^{-/-};Ins2^{+/-}$  mice to show elevated food intake on HFD  
235 versus CD, whereas lean  $Ins1^{-/-};Ins2^{+/+}$  littermates tended to to reduce caloric intake in response to high  
236 fat feeding (Fig. 5A). Other parameters such as activity and energy expenditure were not statistically  
237 different between any of the groups (Figs. 5B-E). Taken together, these experiments indicate that,  
238 within this cohort, reduced *Ins2* gene dosage led greater weight gain on high fat diet, which may have  
239 been associated with increased relative food intake.

240

241 **Figure 5. Food intake and metabolic parameters in Cohort 1. (A-D)** Food intake, activity, and  
242 oxygen consumption (normalized to body weight, or total oxygen consumption) were measured over 3  
243 days in indirect calorimetry cages at 8 weeks of age in male mice from Cohort 1 (n = 5-14).

244

245

## 246 **Discussion**

247 The present study was initiated to investigate the effects of reduced *Ins2* gene dosage, in the absence  
248 of *Ins1*, on glucose homeostasis and weight gain in male mice in the context of chow and high fat diets.  
249 To our surprise, reduced *Ins2* gene dosage did not translate into consistent differences in circulating  
250 insulin. We observed a large degree of variability within and between two cohorts of mice. These  
251 observations are in contrast to our experience with female mice of the same genotypes on the same  
252 diets, and in contrast with our experience reducing *Ins1* gene dosage in male mice [4, 37].

253 Insulin is the most studied hormone in biology, yet the present study provides new insight into the  
254 range of physiological functions that can be modulated by insulin. Specifically, our data point to  
255 somewhat unique roles for, and post-transcriptional regulation of, the *Ins2* gene, relative to the *Ins1*  
256 gene. Elegant studies in flies and worms have demonstrated that deleting specific insulin genes  
257 increases lifespan and prevents diseases associated with adiposity, and clearly indicate that individual  
258 insulin-like peptide genes have distinct physiological functions despite signalling through a single  
259 receptor [28, 29]. It has been previously shown that the mouse *Ins1* and *Ins2* genes have opposing  
260 effects on type 1 diabetes incidence in the NOD mouse due to the induction of thymic tolerance by  
261 *Ins2*, [43, 44]. Specifically, *Ins2* expression was associated with reduced incidences of type 1 diabetes  
262 and the reverse was true for the expression of the *Ins1* gene [43, 44]. Our previous observation that the  
263 pancreatic-specific *Ins1* is dose-dependently required for diet-induced obesity in male mice [4], defines  
264 the first specific role for *Ins1* outside the context of type 1 diabetes. Similarly, a follow-up study  
265 revealed that a modest and transient reduction in circulating insulin in female *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice  
266 relative to *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* littermate controls was sufficient to provide long-term protection from diet-  
267 induced obesity [37]. In the present study, the high fat diet was unable to consistently increase fasting  
268 insulin or beta-cell mass in a statistically significant manner. This meant that we were unable to

269 formally test the hypothesis that reducing hyperinsulinemia by reducing *Ins2* gene dosage might protect  
270 these mice from high-fat diet-induced obesity, as we could in our previously published studies [4, 37].  
271 Clearly, additional studies with greater statistical power and more diet groups would be required to  
272 formally rule in or out a role for hyperinsulinemia stemming from the *Ins2* gene in diet-induced  
273 obesity.

274 One interesting observation from the present study was that male *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* from the first cohort  
275 were heavier than their *Ins1<sup>+/-</sup>:Ins2<sup>-/-</sup>* littermates fed the same high-fat diet. Considerable mouse-to-  
276 mouse heterogeneity was observed in these measurements, but they were relatively consistent within  
277 each mouse over time. Taken at face value, these observations suggest the possibility that *Ins2*  
278 expression in the brain made have played a role in this weight gain, because only the *Ins2* gene is  
279 robustly expressed in the brain [4], and because we only detected modest and late-onset differences  
280 between genotypes in circulating insulin levels. The central nervous system is known to play important  
281 roles in peripheral energy homeostasis and body weight regulation [45-47]. For example, it has been  
282 reported that insulin receptor knockout in the brain leads to increased high fat food intake and obesity  
283 [48], suggesting the possible presence of a local signalling network. The presence of small amounts of  
284 insulin protein and mRNA in the mammalian brain has long been reported [49](reviewed in [4]). The  
285 production of *Ins2* mRNA and protein in specific brain regions was confirmed by our group using *Ins1<sup>-/-</sup>*  
286 *Ins2<sup>-/-</sup>* as negative controls, and *Ins2<sup>βGal/+</sup>* mice as positive staining controls [4].

287 Since insulin has been proposed to be a satiety factor [50], downregulation of insulin expression  
288 and/or action may be expected to increase food intake. Consistent with this notion, *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice  
289 in our first cohort tended to show increased high fat food intake and were obese when compared to the  
290 high fat-fed *Ins1<sup>-/-</sup>:Ins2<sup>+/+</sup>* littermate controls. Although our experimental treatment was expected to  
291 reduce *Ins2* gene dosage in multiple tissues, including the pancreas, thymus and brain, several lines of  
292 evidence could potentially hint that a partial reduction of brain *Ins2* was associated with the diet-

293 dependent hyperphagia and obesity, at least in some specific conditions. Other investigators have  
294 shown that *Ins2* knockout in the thymus had no effect on body weight [51], further suggesting that the  
295 brain could have play a role in this phenomenon. Our work should open new avenues for investigating  
296 the biology of insulin in neurons and their connections. However, it is imperative that these data are  
297 interpreted with caution, as the second cohort of *Ins1<sup>-/-</sup>:Ins2<sup>+/-</sup>* mice did not gain additional weight with  
298 high fat feeding. A complete understanding of the role of *Ins2* in the central nervous system will  
299 require conditional *Ins2* knockout mice.

300 The phenotypes we observed in the present study were hyper-variable, sex-specific, and diet-  
301 dependent with respect to reduction of the *Ins2* gene. The animal facility where this work was done no  
302 longer exists, so it is impossible to formally repeat these studies in the same environment. Similar  
303 studies were also conducted in a modern specific-pathogen free facility, and cohort-dependent  
304 variability is described in a companion paper. Remarkably, in this distinct animal facility we observed  
305 opposite outcomes for the effect of *Ins2* gene dosage on weight gain than those which were shown in  
306 the current experiments. Collectively, the experience of our laboratory is that modulation of the *Ins2*  
307 gene, in the absence of *Ins1*, leads to striking variability in body weight. The source of this variability  
308 will be investigated by our collaborators specializing in epigenetics and translational control.  
309 Phenotypic hyper-variability is a poorly understood phenomenon in biology, but our studies illustrate  
310 that varying *Ins2* gene dosage could be a useful model with clear differences in physiological  
311 outcomes.

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313

## 314 **Acknowledgements**

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316





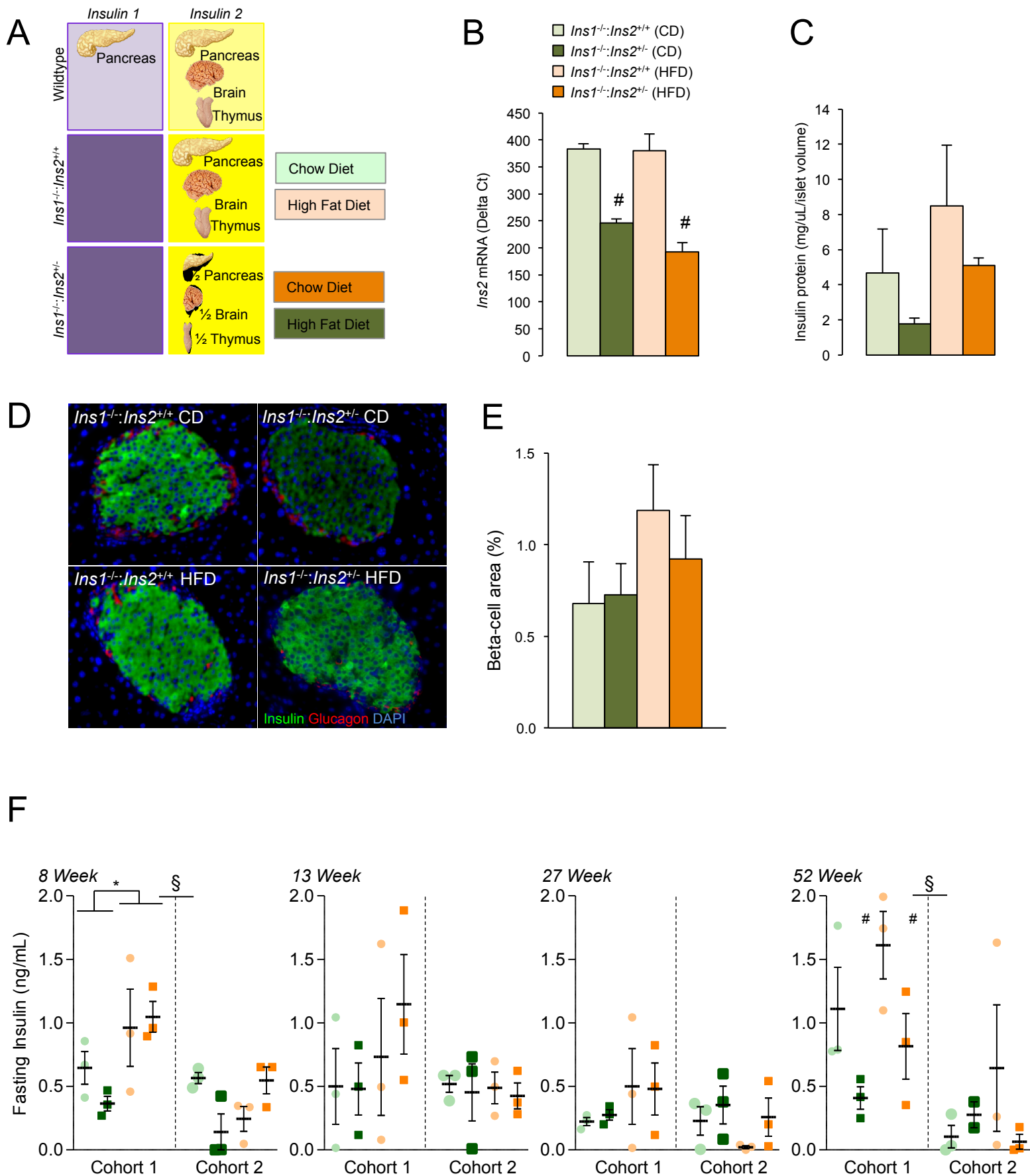
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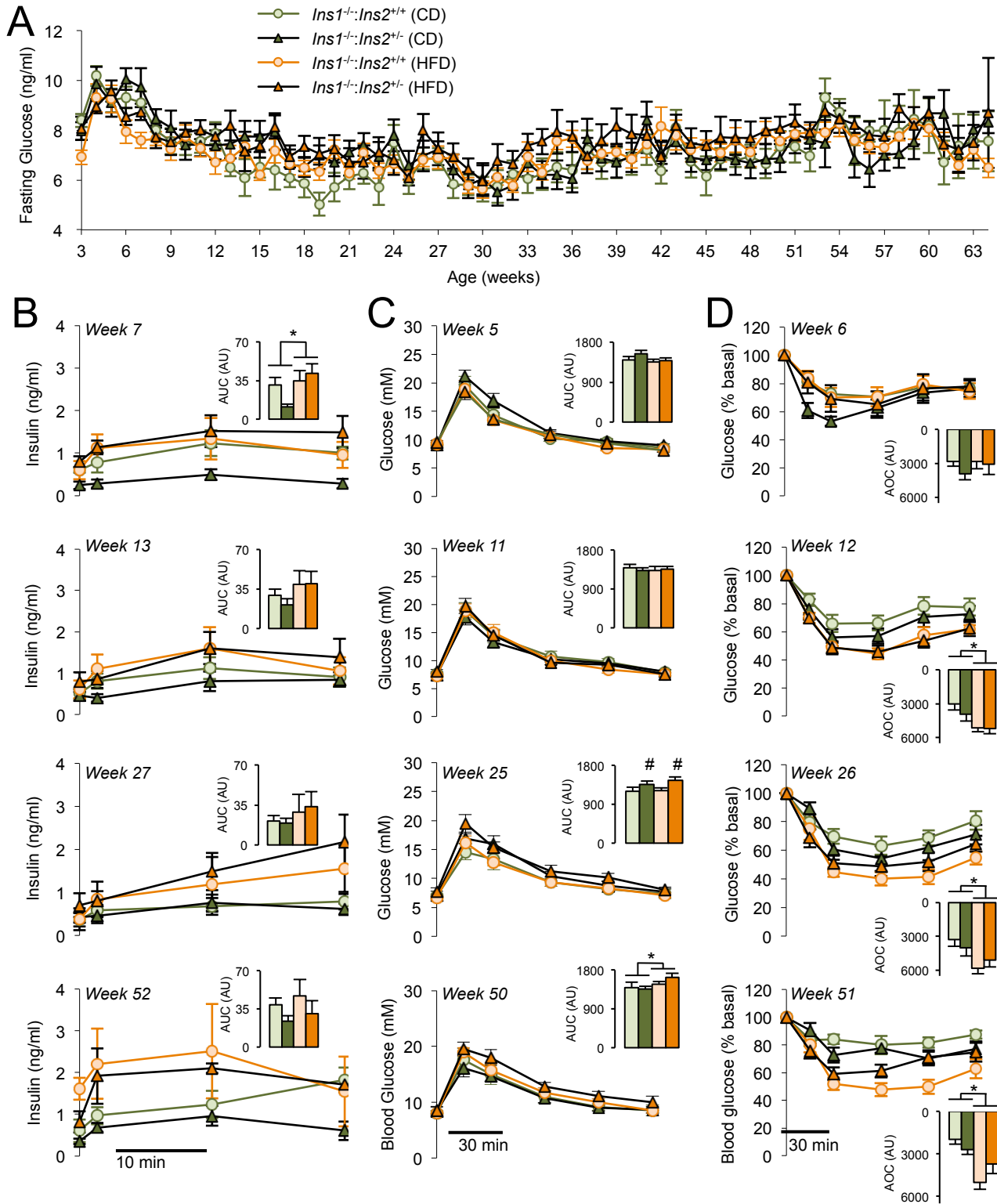
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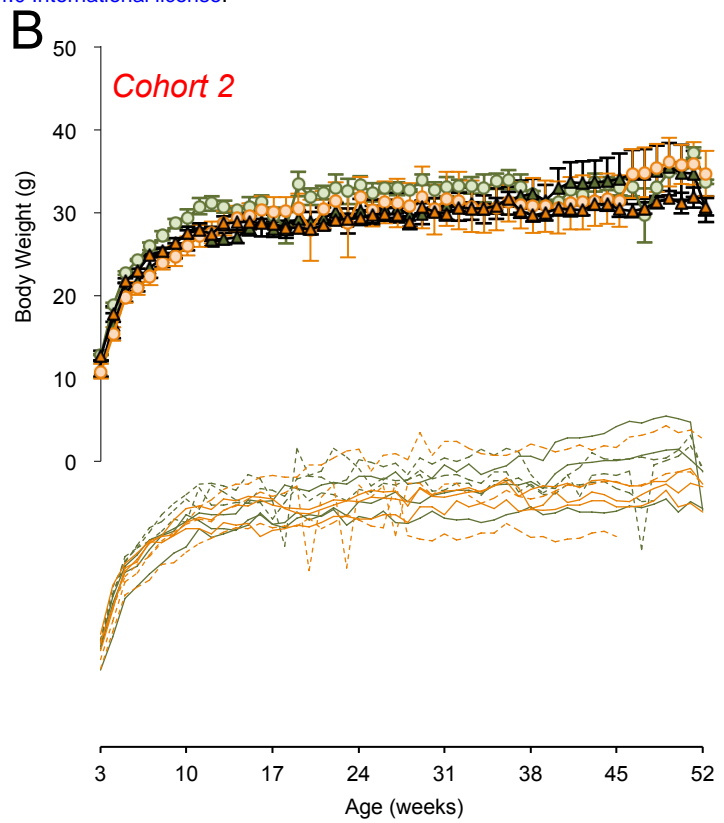
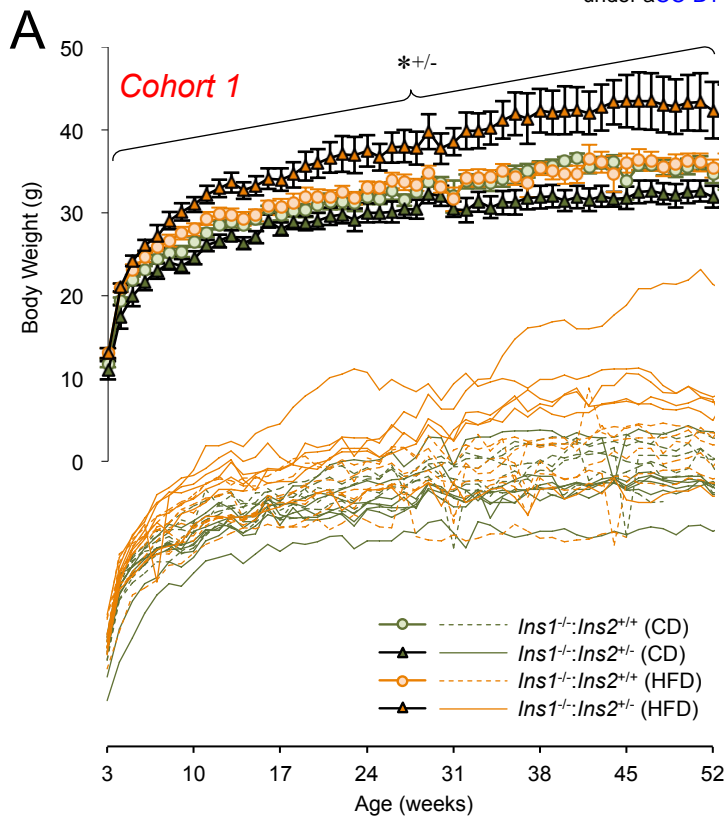
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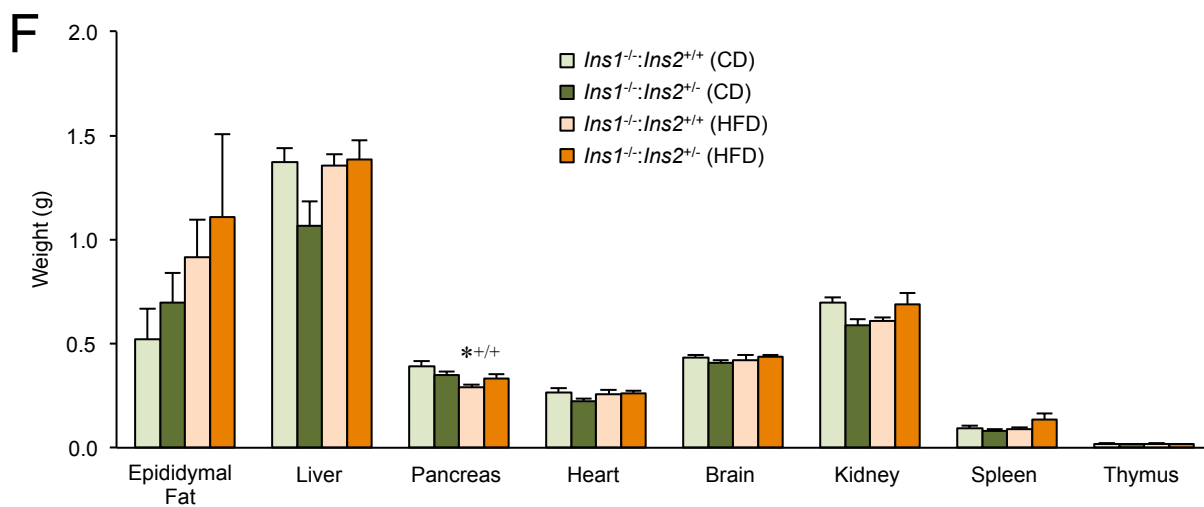
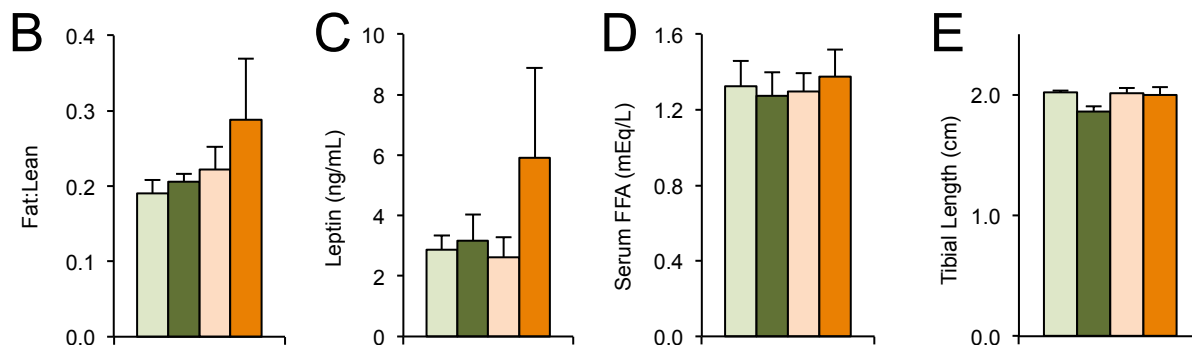
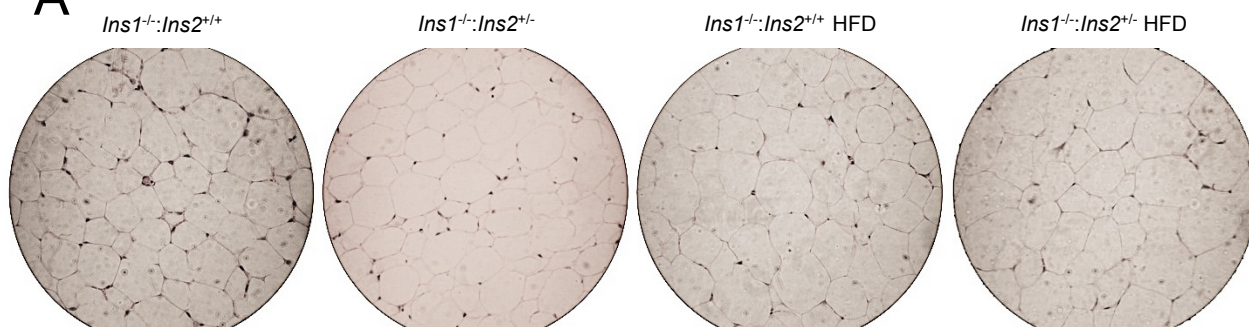








## A Cohort 1





# Figure 5

## Cohort 1

