

1 **Decreased Activity of the *Ghrhr* and *Gh* Promoters Causes**
2 **Dominantly Inherited GH Deficiency**

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23 Running title: ER-localized mutant GH decreases GH1 mRNA

24

25 **Abstract**

26

27 Isolated growth hormone deficiency type II (IGHD2) is mainly caused by
28 heterozygous splice-site mutations in intron 3 of the *GH1* gene. A dominant negative
29 effect of the mutant growth hormone (GH) lacking exon 3 on wild-type GH secretion has
30 been proposed; however, the molecular mechanisms involved are elusive. To uncover
31 the molecular systems underlying GH deficiency in IGHD2, we established IGHD2
32 model mice, which carry both wild-type and mutant copies of the human *GH1* gene,
33 replacing each of the endogenous mouse *Gh* loci. Our IGHD2 model mice exhibited
34 growth retardation associated with intact cellular architecture and mildly activated ER
35 stress in the pituitary gland, caused by decreases in the growth hormone releasing
36 hormone receptor (*Ghrhr*) and *Gh* gene promoter activities. Decreases in *Ghrhr* and *Gh*
37 promoter activities were likely caused by reduced levels of nuclear CREB3L2, which
38 was demonstrated to stimulate the activity of the *Ghrhr* and *Gh* promoters. This is the
39 first *in vivo* study revealing a novel molecular mechanism of GH deficiency in IGHD2,
40 representing a new paradigm, differing from widely accepted models.

41

42 Key Words: dominant negative effect/ endoplasmic reticulum stress/ growth hormone/
43 growth hormone releasing hormone receptor/ isolated growth hormone deficiency type
44 II

45

46 **Introduction**

47

48 Isolated growth hormone deficiency type II (IGHD2) is a dominantly inherited
49 growth hormone (GH) deficiency, first described in 1994, and mainly caused by
50 heterozygous splice-site mutations in intron 3 of the *GH1* gene (Binder & Ranke, 1995;
51 Cogan et al, 1994). The wild-type *GH1* allele transcript includes 5 exons and produces a
52 22-kDa wild-type GH protein; however, the mutant *GH1* allele transcript generates a
53 17.5-kDa exon 3 deletion-mutant GH (Δ 3 GH), as a result of in-frame skipping of exon 3.
54 The fact that patients harboring a deletion in one *GH1* allele exhibit normal stature
55 indicates that a single wild-type *GH1* allele is sufficient to produce normal levels of
56 wild-type GH secretion (Akinci et al, 1992); however, patients with IGHD2 have low
57 serum concentrations of wild-type GH, despite having a wild-type *GH1* allele. Thus, it
58 has been suggested that Δ 3 GH exerts a dominant negative effect on wild-type GH
59 secretion; however, the precise molecular mechanisms involved have remained elusive
60 for more than 20 years.

61 Several *in vitro* studies have demonstrated that Δ3 GH is not secreted
62 extracellularly (Graves et al, 2001; Iliev et al, 2005; Kannenberg et al, 2007; Mullis et al,
63 2002; Salemi et al, 2006), suggesting that the dominant negative effect of Δ3 GH is
64 exerted within somatotropic cells of the pituitary, where GH is generated. Generally,
65 mutant proteins exert dominant negative effects on secretory pathways of wild-type
66 factors at the protein level (Deladoey et al, 2001; Ito et al, 1999; Jacobson et al, 1997),
67 which has led many researchers to focus on wild-type and Δ3 GH protein interactions,
68 such as heterodimer formation; however, no study has yet demonstrated definitive
69 evidence of heterodimers comprising wild-type and Δ3 GH proteins. At present, it is
70 widely accepted that Δ3 GH itself is not harmful to somatotroph (Graves et al, 2001),
71 and that wild-type GH contributes to the degradation of Δ3 GH via protein interactions,
72 leading to impairment of the wild-type GH secretory pathway (Kannenberg et al, 2007;
73 McGuinness et al, 2003).

74 In contrast, previous *in vitro* studies revealed that Δ3 GH localizes to the
75 endoplasmic reticulum (ER), due to its aberrant protein structure (Graves et al, 2001;
76 Salemi et al, 2006), and is degraded by the proteasome (Ariyasu et al, 2013;
77 Kannenberg et al, 2007), indicating that Δ3 GH potentially causes ER stress in the
78 somatotroph. We previously demonstrated the involvement of ER stress and apoptosis
79 in IGHD2, using rat GH4C1 cells stably expressing wild-type GH and Δ3 GH (Ariyasu et
80 al, 2013), indicating that Δ3 GH itself impairs ER functions *in vitro*, without the
81 involvement of wild-type GH, inconsistent with the widely accepted hypothesis
82 described above.

83 Since GH secretion is regulated by growth hormone releasing hormone (GHRH)
84 signaling, it is important to establish a usable model that includes the
85 hypothalamus-pituitary axis. One *in vitro* study has authentically mimicked the
86 hypothalamus-pituitary axis (Petkovic et al, 2010); however, *in vivo* animal models are
87 imperative to clarify the molecular mechanisms involved in the GH deficiency of IGHD2.
88 McGuinness et al. established Δ3 GH transgenic mice and reported that they exhibited
89 widespread pituitary damage and severe macrophage invasion (McGuinness et al,
90 2003). This mouse model had been the sole *in vivo* model used to represent the human
91 IGHD2 phenotype. *In vivo* studies have been performed using this model, including Δ3
92 GH knockdown by shRNA (Lochmatter et al, 2010), and altering splicing efficiency using
93 butyrate (Miletta et al, 2016), which ameliorated impaired GH secretion; however, the
94 mechanisms underlying IGHD2 have not been determined *in vivo* (Miletta et al, 2017).

95 To clarify the molecular processes causing impaired GH secretion in IGHD2, we
96 established IGHD2 model mice by exchanging endogenous mouse *Gh* genes for the

97 human wild-type *GH1* (*wtGH1*) and mutant *GH1* ($\Delta 3GH1$; generates $\Delta 3$ GH) genes,
98 using the 'gene exchange system' previously reported by our laboratory (Araki et al,
99 2002). Our IGHD2 model mice demonstrated significant growth failure, associated with
100 a marked decrease in *wtGH1* mRNA, and no apoptosis was detected in the pituitary
101 glands, despite the clear growth retardation phenotype.

102 Here, we show that $\Delta 3$ GH decreases transcription from the growth hormone
103 releasing hormone receptor (*Ghrhr*) gene promoter, which has a fundamental role in
104 somatotroph proliferation, as well as *GH1* gene transcription, leading to impaired GH
105 production before birth in IGHD2 model mice. These decreases in promoter activity
106 were mediated by a reduction in nuclear CREB3L2, one of the Creb3 family of bZip
107 transcription factors, which was found to stimulate transcription from the *Ghrhr* and the
108 *Gh* promoters coordinately with POU class 1 homeobox 1 (POU1F1). This is the first *in*
109 *vivo* study to reveal a novel molecular mechanism underlying GH deficiency in IGHD2,
110 and provides a new paradigm, different from the widely accepted model.

111

112 **Results**

113

114 **IGHD2 model mice exhibited mild growth retardation**

115 To establish a mouse model that authentically demonstrates the $\Delta 3$ GH-mediated
116 dominant negative effect observed in IGHD2, one each of the mouse endogenous *Gh*
117 gene alleles was exchanged for the human *wtGH1* and $\Delta 3GH1$ genes, using the gene
118 exchange system previously reported by our laboratory (Araki et al, 2002). Briefly, we
119 inserted a *neoR* gene cassette, flanked by a left-element mutated *loxJT15* and *loxP* site,
120 at the *Gh* gene locus by homologous recombination, producing a mouse *Gh* knock-out
121 (KO) allele (*Gh*⁻) (Fig EV1A and C). Then, we constructed gene exchange vectors,
122 containing right-element mutated *loxKR3* and the *wtGH1* or $\Delta 3GH1$ genes, followed by
123 a puromycin resistance gene and the *loxP* site (Fig EV1A and C). The *neoR* gene
124 cassette was exchanged for the *wtGH1* or $\Delta 3GH1$ genes using Cre-mediated
125 recombination (Fig EV1A and B). Since recombination between *loxJT15* and *loxKR3*
126 produced a *lox* site with both sides mutated, which is resistant to Cre-mediated excision,
127 we were able to efficiently generate recombined embryonic stem (ES) cell clones (Fig
128 EV1A) (Araki et al, 2002).

129 Schematic representations of the original mouse endogenous *Gh* allele (*Gh*⁺), KO
130 allele (*Gh*⁻), and exchanged human *GH1* alleles (*Gh*^{*wtGH1*} or *Gh* ^{$\Delta 3GH1$}) are presented in
131 Fig 1A. By crossing *Gh*⁺⁻, *Gh*^{*wtGH1*}, and *Gh* ^{$\Delta 3GH1$} mice, we successfully established
132 *Gh*^{*wtGH1/wtGH1*} mice (human healthy control model), *Gh*^{*wtGH1/-*} mice (GH1 heterozygous

133 deletion model), $Gh^{wtGH1/\Delta3GH1}$ mice (IGHD2 model), and $Gh^{-/-}$ mice ($GH1$ homozygous
134 deletion model). The body weights and body lengths of these model mice are shown in
135 Fig 1B and C. $Gh^{-/-}$ mice demonstrated severe postnatal growth retardation, associated
136 with serum insulin-like growth factor 1 (IGF-1) levels below the detection range,
137 indicating that, as in humans, postnatal growth in mice is dependent on GH activity (Fig
138 1B–E). $Gh^{wtGH1/wtGH1}$ and $Gh^{wtGH1/-}$ mice showed longitudinal growth and serum IGF-1
139 levels comparable with those of $Gh^{+/+}$ mice, suggesting that the human wild-type GH
140 molecule is capable of binding the mouse GH receptor and producing IGF-1, and that
141 one exchanged human wild-type $GH1$ allele is sufficient for IGF-1-mediated longitudinal
142 growth in mice (Fig 1B–E). $Gh^{wtGH1/\Delta3GH1}$ mice exhibited mild growth retardation,
143 associated with significantly reduced serum IGF-1 values, which were intermediate
144 between those of $Gh^{wtGH1/wtGH1}$ and $Gh^{-/-}$ mice (Fig 1B–E). These data indicate that
145 $Gh^{wtGH1/\Delta3GH1}$ mice successfully demonstrate the dominant negative effect of $\Delta3$ GH, and
146 that the growth retardation of $Gh^{wtGH1/\Delta3GH1}$ mice is caused by impaired GH activity.

147 We also established $Gh^{mGh/\Delta3GH1}$ mice, in which the mouse Gh gene was inserted
148 at the endogenous mouse Gh locus, using the gene exchange system in a similar way
149 to that used to obtain $Gh^{wtGH1/\Delta3GH1}$ mice (Fig EV1A). $Gh^{mGh/\Delta3GH1}$ mice also
150 demonstrated growth failure, as for $Gh^{wtGH1/\Delta3GH1}$ mice, although $Gh^{+/+}$ mice did not
151 (Fig EV1E). The phenotypic discrepancy between $Gh^{+/+}$ and $Gh^{mGh/\Delta3GH1}$ mice was
152 attributable to a significant difference in the abundance of mRNA transcribed from the
153 endogenous and exchanged Gh alleles, as demonstrated by qRT-PCR (Fig EV1F). In
154 this study, we used mouse lines in which both alleles were exchanged for human $GH1$
155 genes, because the transcriptional efficiencies of the $wtGH1$ and $\Delta3GH1$ alleles were
156 basically equivalent in human IGHD2 patients. These decreases in transcriptional
157 efficiency of the exchanged human $GH1$ genes did not cause the growth failure in
158 $Gh^{wtGH1/wtGH1}$ mice (Fig 1B and C), and IGF-1 levels in $Gh^{wtGH1/wtGH1}$ mice were
159 comparable with those in $Gh^{+/+}$ mice (Fig 1D), indicating that $wtGH1$ mRNA expression
160 levels were sufficient for production of human wild-type GH protein, required for the
161 IGF-1-mediated longitudinal growth of mice.

162

163 **The growth retardation of $Gh^{wtGH1/\Delta3GH1}$ mice is caused by decreased $wtGH1$ mRNA
164 expression**

165 We evaluated the expression levels of wild-type and $\Delta3$ GH proteins in pituitary
166 glands at 4 weeks of age, the period in which $Gh^{wtGH1/\Delta3GH1}$ mice demonstrated
167 significant growth retardation, based on their growth curves (Fig 1B and C).
168 Immunoblotting showed significant decreases in the content of the 22 kDa wild-type GH

169 in whole $Gh^{wtGH1/\Delta3GH1}$ pituitary, compared with $Gh^{wtGH1/wtGH1}$ and $Gh^{wtGH1/-}$ pituitaries (Fig
170 2A left and B), indicating that the growth retardation of $Gh^{wtGH1/\Delta3GH1}$ mice was caused by
171 impaired GH production in the somatotroph. $\Delta3$ GH expression was barely detected by
172 long exposure (Fig 2A right), despite comparable affinities of the anti-GH antibody for
173 wild-type and $\Delta3$ GH proteins (Fig EV2A). Immunostaining revealed that both the
174 wild-type GH content in each somatotroph and the number of somatotrophs were
175 reduced in $Gh^{wtGH1/\Delta3GH1}$, compared with $Gh^{wtGH1/wtGH1}$ pituitaries (Fig 2C).

176 Since we had demonstrated impaired production of wild-type GH in $Gh^{wtGH1/\Delta3GH1}$
177 pituitary, we next evaluated $GH1$ transcript levels. Using whole pituitary glands from
178 4-week-old animals, RT-PCR detecting both the $wtGH1$ and $\Delta3GH1$ transcripts, with a
179 sense primer in exon 1 and an antisense primer in exon 5, revealed markedly
180 decreased $wtGH1$ transcript levels in the $Gh^{wtGH1/\Delta3GH1}$ pituitary, compared with those in
181 $Gh^{wtGH1/wtGH1}$ and $Gh^{wtGH1/-}$ pituitaries (Fig 2D). qRT-PCR analysis, detecting the $wtGH1$
182 mRNA alone, using a sense primer in exon 3 and an antisense primer spanning exons 3
183 and 4, also demonstrated that the abundance of the $wtGH1$ mRNA in $Gh^{wtGH1/\Delta3GH1}$
184 pituitary was approximately one sixth of that in $Gh^{wtGH1/wtGH1}$ pituitary in 4-week-old
185 animals (Fig 2E). Decreases in $wtGH1$ mRNA levels in $Gh^{wtGH1/\Delta3GH1}$ pituitaries were
186 demonstrated from embryonic day E19.5 to 4 weeks of age (Fig 2F), suggesting that
187 GH production in $Gh^{wtGH1/\Delta3GH1}$ pituitaries was already impaired before birth. *In situ*
188 hybridization analysis to detect $wtGH1$ mRNA using an RNA probe for $GH1$ exon 3, in
189 E19.5 and 4-week-old pituitaries, revealed that both the abundance of $wtGH1$ mRNA in
190 each somatotroph and the number of somatotrophs were decreased in $Gh^{wtGH1/\Delta3GH1}$
191 pituitaries, consistent with the results of immunostaining (Fig 2C and G). These data
192 indicate that the impaired production of wild-type GH in $Gh^{wtGH1/\Delta3GH1}$ pituitary is caused
193 by a decrease in $wtGH1$ mRNA.

194 RT-PCR showed that levels of the $wtGH1$ and $\Delta3GH1$ transcripts were
195 comparable (Fig 2D); however, immunoblotting revealed that expression of the $\Delta3$ GH
196 protein was drastically reduced compared with that of wild-type GH protein in
197 $Gh^{wtGH1/\Delta3GH1}$ pituitaries (Fig 2A). These data indicate that $\Delta3$ GH is degraded in the
198 somatotroph, and that wild-type GH is not involved in the degradation, inconsistent with
199 the currently accepted hypothesis. Thus, to evaluate whether wild-type GH
200 preferentially interacts with $\Delta3$ GH, 3-D protein structures of wild-type and $\Delta3$ GH were
201 analyzed under pH conditions in the ER and docking simulation analysis was conducted
202 using ZDOCK (Chen et al, 2003; Nakamura et al, 2017). The average binding affinity
203 score for heterodimers of wild-type and $\Delta3$ GH was significantly lower than that for the
204 wild-type GH homodimer (Table 1), indicating that wild-type and $\Delta3$ GH heterodimer

205 formation is unlikely to be involved in impaired GH secretion in IGHD2.

206

207 **Electron microscopy reveals markedly decreased numbers of secretory vesicles**
208 **and enlarged ER in $Gh^{wtGH1/\Delta3GH1}$ pituitaries**

209 Considering the possibility that somatotroph loss due to apoptosis, necrosis, and
210 inflammation, contribute to the decrease in $wtGH1$ mRNA described above, we
211 conducted histological evaluation of pituitary glands (Fig 3). Stereomicroscopic analysis
212 of four-week-old $Gh^{wtGH1/\Delta3GH1}$ pituitaries revealed a slightly atrophic and
213 semi-translucent appearance, which was intermediate between those of $Gh^{wtGH1/-}$ and
214 $Gh^{-/-}$ mice, consistent with the decreased somatotroph number in $Gh^{wtGH1/\Delta3GH1}$ mice
215 described above (Fig 3A); however, the decrease in somatotroph number was not
216 caused by somatotroph loss, since hematoxylin-eosin staining demonstrated that the
217 cellular architecture was intact, with no signs of necrosis or inflammation. Further,
218 analysis by TdT-mediated dUTP nick end labeling (TUNEL) assay revealed that the
219 decrease in the $wtGH1$ mRNA in $Gh^{wtGH1/\Delta3GH1}$ mice was not associated with apoptosis
220 (Fig 3B).

221 The absence of somatotroph loss in $Gh^{wtGH1/\Delta3GH1}$ mice led us to evaluate the
222 characteristics of the cellular organelles in pituitary glands using transmission electron
223 microscopy (TEM). TEM images of 4-week-old $Gh^{wtGH1/wtGH1}$ pituitary glands showed
224 intact cell organelles, including appropriately developed rough ER, and many secretory
225 vesicles containing mature wild-type GH protein (Fig 3C). In contrast, images of
226 $Gh^{wtGH1/\Delta3GH1}$ pituitary glands revealed a clear decrease in the number of secretory
227 vesicles, abnormal enlargement of the rough ER, and protein aggregates in the cytosol
228 (Fig 3C). To evaluate the impact of $\Delta3$ GH itself on cellular morphology, we obtained
229 $Gh^{\Delta3GH1/-}$ mice by crossing $Gh^{wtGH1/-}$ and $Gh^{wtGH1/\Delta3GH1}$ animals. In contrast to the current
230 understanding that $\Delta3$ GH itself is not harmful to somatotroph, TEM images of $Gh^{\Delta3GH1/-}$
231 pituitaries revealed extreme enlargement of the rough ER and protein aggregates in the
232 cytosol, whereas such abnormalities were not visible in $Gh^{-/-}$ pituitaries (Fig 3D). The
233 observed protein aggregates were connected to the ER (Fig 3E), suggesting that the
234 proteins accumulated in the ER were retro-translocated to the cytosol, leading to
235 aggregate formation. These data led us to confirm the cellular localization of $\Delta3$ GH by
236 immunofluorescence analysis. Using $Gh^{wtGH1/\Delta3GH1-myc}$ mice, expressing $\Delta3$ GH with a
237 C-terminal myc tag (Fig EV1A), $\Delta3$ GH was also demonstrated to localize within the ER
238 *in vivo* (Fig 3F).

239 To evaluate the contents of the protein aggregates in the cytosol, we dissociated
240 $Gh^{wtGH1/\Delta3GH1}$ anterior pituitary cells and separated them into soluble and insoluble

241 fractions, in the presence or absence of treatment with the proteasome inhibitor, MG132,
242 and evaluated the distributions of wild-type and Δ3 GH by immunoblotting, because
243 several studies have demonstrated that cytosolic protein aggregates have low solubility
244 (Ariyasu et al, 2013; Imai et al, 2001; Kannenberg et al, 2007; Ward et al, 1995). A
245 significant proportion of Δ3 GH was detected in the insoluble fraction following MG132
246 treatment, although the majority of wild-type GH was sorted into the soluble fraction (Fig
247 EV2B). These data suggest that ER-localized Δ3 GH is degraded by the proteasome,
248 leading to Δ3 GH aggregation, which overwhelms the degradative capacity in the
249 cytosol, and that most wild-type GH is not involved in the aggregates.

250

251 **Δ3 GH-mediated ER stress is not a direct cause of the growth failure of**
252 ***Gh*^{wtGH1/Δ3GH1} mice**

253 The enlargement of the rough ER and Δ3 GH aggregates in the cytosol suggest
254 that the *Gh*^{wtGH1/Δ3GH1} somatotrophs are under ER stress, and we have previously shown
255 that Δ3 GH causes ER stress to the somatotroph *in vitro* (Ariyasu et al, 2013). These
256 data led us to investigate Δ3 GH-mediated ER stress *in vivo*. PKR-like endoplasmic
257 reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol
258 requirement 1 (IRE1), are well-characterized ER membrane-located proteins which
259 sense ER stress. In the presence of ER stress, PERK is activated by
260 trans-autophosphorylation, ATF6 activates expression of the ER chaperone
261 immunoglobulin heavy-chain binding protein (BiP), and IRE1 activates splicing of X-box
262 binding protein 1 (*Xbp1*) mRNA via the PERK, ATF6, and IRE1 pathways, respectively
263 (Ariyasu et al, 2017; Yoshida, 2007). PERK phosphorylation can be detected by
264 immunoblotting, as phosphorylated PERK is associated with a mobility shift during
265 SDS-PAGE (Harding et al, 1999). Immunoblotting revealed that the PERK was
266 phosphorylated in 2 and 4-week-old *Gh*^{wtGH1/Δ3GH1} pituitary glands (Fig 4A). Further,
267 qRT-PCR demonstrated a significant increase in *BiP* mRNA abundance in 4-week-old
268 *Gh*^{wtGH1/Δ3GH1} pituitary (Fig 4B). *Xbp1* mRNA splicing was evaluated by competitive
269 RT-PCR, using primers flanking the 26 bp sequence spliced out by IRE1α (Yoshida et al,
270 2001). *Xbp1* mRNA splicing was significantly increased at 1, 2, and 4 weeks of age in
271 *Gh*^{wtGH1/Δ3GH1} pituitaries (Fig 4C and D).

272 These data suggest that Δ3 GH can activate the three major ER stress pathways
273 *in vivo*; however, activation of these ER stress pathways is not sufficiently strong to
274 cause somatotroph apoptosis, since no apoptotic cells were detected by TUNEL assay,
275 as described above (Fig 3B), and no caspase-3 activation was detected in *Gh*^{wtGH1/Δ3GH1}
276 pituitary glands by immunoblotting (Fig 4A). In other established ER stress-related

277 endocrine diseases, apoptosis is required to cause organ dysfunction (Ariyasu et al,
278 2017; Fonseca et al, 2010; Hayashi et al, 2009; Oyadomari et al, 2002; Yoshida, 2007).
279 These data suggest that $\Delta 3$ GH-mediated ER stress is not likely to be causally related
280 to the growth retardation in $Gh^{wtGH1/\Delta 3GH1}$ mice.

281

282 **Decreased *Ghrhr* gene promoter activity contributes to decreased *wtGH1* gene
283 expression in $Gh^{wtGH1/\Delta 3GH1}$ mice**

284 Our data (described above) indicate that $\Delta 3$ GH itself can decrease *wtGH1* mRNA
285 levels, without interacting with wild-type GH protein, ER stress, or apoptosis. RT-PCR
286 revealed that both *wtGH1* and $\Delta 3GH1$ mRNA were equally decreased in $Gh^{wtGH1/\Delta 3GH1}$
287 pituitary (Fig 2D), leading us to evaluate the expression levels of genes contributing to
288 upstream regulation of *GH1* gene transcription.

289 As *wtGH1* mRNA was primarily decreased in $Gh^{wtGH1/\Delta 3GH1}$ somatotroph, we would
290 expect the *Ghrhr* gene to be overexpressed, because of negative feedback
291 mechanisms reflecting the GH deficiency in this tissue. Consistent with this hypothesis,
292 4-week-old $Gh^{-/-}$ mice had significantly increased abundance of *Ghrhr* mRNA compared
293 with $Gh^{wtGH1/wtGH1}$ mice, because of a negative feedback mechanism, reflecting their
294 complete GH deficiency (Fig 5A lane 1 and 3, and B). However, $Gh^{wtGH1/\Delta 3GH1}$ mice
295 demonstrated significantly decreased *Ghrhr* mRNA compared with $Gh^{wtGH1/wtGH1}$ mice,
296 despite their marked GH deficiency (Fig 5A lane 1 and 2, and B). These data suggest
297 that the decrease in *wtGH1* mRNA in $Gh^{wtGH1/\Delta 3GH1}$ pituitary is mediated, at least in part,
298 by that of *Ghrhr* mRNA. In agreement with this hypothesis, $Gh^{\Delta 3GH1/-}$ mice demonstrated
299 significantly decreased abundance of *Ghrhr* mRNA, compared with $Gh^{-/-}$ mice, despite
300 the fact that both strains lack the ability to secrete wild-type GH (Fig 5A lane 3 and 4,
301 and B). Note that $Gh^{\Delta 3GH1/-}$ mice demonstrate exactly the same degree of growth
302 retardation as $Gh^{-/-}$ mice, because $\Delta 3$ GH is not secreted (Fig 5C and EV2C). Further,
303 the decrease in both the abundance of *wtGH1* mRNA in each somatotroph and the
304 number of somatotrophs in $Gh^{wtGH1/\Delta 3GH1}$ pituitaries (Fig 2G), can be explained by this
305 decrease in *Ghrhr* mRNA, because GHRH signaling is essential for *GH1* gene
306 transcription and somatotroph proliferation (Lin et al, 1993). Taken together, $\Delta 3$ GH
307 contributes to decreased *Ghrhr* mRNA levels, without the assistance of wild-type GH,
308 leading to the impaired GHRH signaling and reduced *wtGH1* transcription in
309 $Gh^{wtGH1/\Delta 3GH1}$ somatotroph.

310 To evaluate the mechanisms underlying decreases in *Ghrhr* mRNA, a mouse
311 model with the *LacZ* gene knocked in to the *Ghrhr* gene locus ($Ghrhr^{+/-LacZ}$) was
312 established using the CRISPR/Cas9 gene editing system (Fig EV3A and B). E19.5 and

313 4-week-old $Ghrhr^{+/LacZ};Gh^{wtGH1/\Delta3GH1}$ pituitary showed significantly decreased X-gal
314 staining compared with $Ghrhr^{+/LacZ};Gh^{wtGH1/wtGH1}$ pituitary, indicating that the decreased
315 $Ghrhr$ mRNA levels are caused by a reduction in $Ghrhr$ promoter activity (Fig 5D).

316

317 **Nuclear expressions of CREB3L2 is decreased in $Gh^{wtGH1/\Delta3GH1}$ pituitary glands**

318 The decreased $Ghrhr$ promoter activity detected in $Gh^{wtGH1/\Delta3GH1}$ mice suggests
319 that the expression of nuclear transcription factors crucial for the $Ghrhr$ expression is
320 disturbed by ER-localized $\Delta 3$ GH (Fig 5D). Furthermore, the abnormal cellular
321 organelles and mildly activated ER stress pathway caused by $\Delta 3$ GH, indicate that $\Delta 3$
322 GH causes a deterioration in ER function by inducing ER stress (Fig 3C–E, Fig 4A–D).
323 Immunoblotting revealed that abundance of the nuclear POU1F1 protein, a well-known
324 transcription factor involved in regulation of $Ghrhr$ and Gh promoter activities, was
325 comparable in 4-week-old $Gh^{wtGH1/wtGH1}$ and $Gh^{wtGH1/\Delta3GH1}$ pituitaries (Fig EV3C),
326 suggesting that other, unknown, pituitary transcription factors were involved in the
327 decreased $Ghrhr$ promoter activity in $Gh^{wtGH1/\Delta3GH1}$ mice. These data led us to focus on
328 the Creb3 family of bZip transcription factors, a recently described family of ER stress
329 transducers, all of which are ER-bound factors that undergo proteolysis in the Golgi
330 apparatus, leading to production of active N-terminal fragments, which translocate to
331 the nucleus and activate transcription of target genes (Kondo et al, 2011). Five Creb3
332 family members, CREB3/LUMAN, CREB3L1/OASIS, CREB3L2/BBF2H7,
333 CREB3L3/CREBH, and CREB3L4/TISP40, have been identified to date (DenBoer et al,
334 2005; Kondo et al, 2005; Kondo et al, 2007; Nagamori et al, 2005; Omori et al, 2001).

335 Of these, CREB3L1 and CREB3L2 are essential for the differentiation and
336 proliferation of osteoblasts and chondrocytes, respectively. Active N-terminal fragments
337 of CREB3L1 and CREB3L2 bind to cAMP response element (CRE)-like sequences in
338 the promoter regions of target genes, and enhance their expression in osteoblasts and
339 chondrocytes, respectively (Murakami et al, 2009; Saito et al, 2009). Interestingly,
340 *Creb3l1* KO mice display mild growth failure, which is not rescued by osteoblast-specific
341 *Creb3l1* overexpression, but can be ameliorated by exogenous GH treatment,
342 suggesting that they have impaired GH secretion (Murakami et al, 2011). Furthermore,
343 TEM images of *Creb3l1*-deficient osteoblasts and *Creb3l2*-deficient chondrocytes
344 showed abnormal enlarged ER (Murakami et al, 2009; Saito et al, 2009), similar to
345 those detected in $Gh^{wtGH1/\Delta3GH1}$ somatotroph. These similarities between $Gh^{wtGH1/\Delta3GH1}$
346 and Creb3 family KO mice led us to hypothesize that one or more Creb3 family
347 members are crucial for $Ghrhr$ gene expression, and that the growth failure of
348 $Gh^{wtGH1/\Delta3GH1}$ mice is mediated by decreased levels of nuclear active N-terminal Creb3

349 protein fragments.

350 RT-PCR analysis of pituitary glands from 4-week-old C57BL/6 mice revealed that
351 the *Creb3l1* and *Creb3l2* genes were strongly expressed in this tissue (Fig 6A). Next,
352 we evaluated expression of these two genes in 4-week-old *Gh*^{wtGH1/wtGH1} and
353 *Gh*^{wtGH1/Δ3GH1} pituitaries by qRT-PCR and immunoblotting. In *Gh*^{wtGH1/Δ3GH1} pituitary, the
354 abundance of *Creb3l1* mRNA was increased, while that of the N-terminal CREB3L1
355 protein was comparable with levels in the *Gh*^{wtGH1/wtGH1} pituitary (Fig 6B and C).
356 Furthermore, in *Gh*^{wtGH1/Δ3GH1} pituitary, *Creb3l2* mRNA levels were comparable, while
357 those of N-terminal CREB3L2 protein were reduced, compared with *Gh*^{wtGH1/wtGH1}
358 pituitary (Fig 6B and C). Both CREB3L1 and CREB3L2 were demonstrated to be
359 expressed in somatotroph by immunofluorescence (Fig EV3D). These data indicate that
360 expression levels of N-terminal CREB3L1 and CREB3L2 proteins are disturbed in
361 *Gh*^{wtGH1/Δ3GH1} mice.

362 Since involvement of N-terminal CREB3L1 and CREB3L2 in GH production has
363 not been described previously, we evaluated the impact of N-terminal CREB3L1 and
364 CREB3L2 on *Ghrhr* and the *Gh* promoter activities *in vitro*. Luciferase assays revealed
365 that N-terminal CREB3L2 strongly stimulated transcription from both the *Ghrhr* and *Gh*
366 promoters, in combination with POU1F1 (Fig 6D). Decreased nuclear CREB3L2 levels
367 were also demonstrated at embryonic stages in *Gh*^{wtGH1/Δ3GH1} pituitary glands by
368 immunoblotting. Ratios of N-terminal CREB3L2 to full-length CREB3L2 decreased in
369 *Gh*^{wtGH1/Δ3GH1} pituitary glands at embryonic day E19.5 (Fig 6E and F), indicating that
370 translocation of CREB3L2 to the nucleus was disturbed by ER-localized Δ3 GH. Sec23a
371 mRNA, an established target gene of nuclear CREB3L2, was also decreased in
372 *Gh*^{wtGH1/Δ3GH1} pituitary at E19.5 (Fig 6G). These data suggest that CREB3L2 stimulates
373 *Ghrhr* and the *Gh* gene expression levels, and that impaired GH production in
374 *Gh*^{wtGH1/Δ3GH1} somatotroph is mediated by a decrease in nuclear CREB3L2 levels.
375

376 Discussion

377
378 The gene exchange system used in this study is a useful method for establishing
379 mouse models of human diseases, because once researchers obtain a gene of interest
380 flanked by mutated *lox* sites, they can easily exchange the endogenous genes with
381 various human genes, via Cre-mediated integration. Using this system, we established
382 a mouse model that expresses the *wtGH1* and *Δ3GH1* genes, instead of endogenous
383 mouse *Gh*. *Gh*^{wtGH1/Δ3GH1} mice have two advantages: 1) endogenous mouse GH is not
384 produced and 2) one copy each of human wild-type GH and Δ3 GH are expressed

385 under the control of the *Gh* promoter. Thus, $Gh^{wtGH1/\Delta3GH1}$ mice are a genetically ideal
386 model that mimics the growth patterns of human patients with IGHD2.

387 For functional analysis of secretory molecules regulated by feedback mechanisms
388 involving in multiple tissues, such as GH, it is important to establish an applicable model
389 that replicates the human disease as precisely as possible. Since patients with IGHD2
390 were first described in 1994, many researchers have focused on interference of
391 wild-type GH protein trafficking by $\Delta 3$ GH, and several *in vitro* studies have been
392 conducted to investigate the dominant negative effect of $\Delta 3$ GH (Ariyasu et al, 2013;
393 Graves et al, 2001; Hayashi et al, 1999; Iliev et al, 2005; Kannenberg et al, 2007; Lee et
394 al, 2000; McGuinness et al, 2003; Salemi et al, 2006; Salemi et al, 2007); however, in
395 these studies, wild-type and $\Delta 3$ GH expression were driven by homeostatic promoters,
396 such as the CMV promoter, and were thus independent of the feedback mechanisms
397 that regulate GH expression *in vivo*. Furthermore, pituitary-derived cell lines, such as
398 GC, GH3, GH4C1, and AtT-20, used in these studies do not express GHRHR. Thus,
399 previous *in vitro* studies have been limited by the absence of feedback mechanisms
400 mimicking those present *in vivo*.

401 The $\Delta 3$ GH transgenic mice reported by McGuinness demonstrated massive
402 pituitary damage (McGuinness et al, 2003); however, our $Gh^{wtGH1/\Delta3GH1}$ mice did not
403 show similar somatotroph loss. The pituitary damage in the $\Delta 3$ GH transgenic mice is
404 likely caused by overexpression of the transgene, compared with endogenous mouse
405 *Gh*, since our data indicate that $\Delta 3$ GH caused GH deficiency, under conditions of
406 comparable transcriptional efficiency between the mouse *Gh* and $\Delta 3GH1$ alleles (Fig
407 EV1E and F). Further, a previous study using cultured lymphocytes from patients with
408 IGHD2 revealed that ratios of mutant to wild-type *GH1* transcripts were correlated with
409 the severity of GH deficiency (Hamid et al, 2009).

410 In this study, we reveal two important findings: 1) Impaired GH secretion in
411 $Gh^{wtGH1/\Delta3GH1}$ mice is caused by decreased activity of the *Ghrhr* and *Gh* promoters; 2)
412 these decreases in promoter activity are mediated by reduced levels of nuclear
413 CREB3L2. A schematic representation of the molecular mechanisms underlying GH
414 deficiency in IGHD2 is presented in Fig 7. Our data, including the abnormal TEM
415 images and activated ER stress responses in $Gh^{wtGH1/\Delta3GH1}$ pituitary, indicate that
416 ER-localized $\Delta 3$ GH diminishes ER function by invoking ER stress. $\Delta 3$ GH-mediated ER
417 stress leads to a decrease in COPII vesicles, which are essential for ER-Golgi transport,
418 because $\Delta 3$ GH disturbs ER-Golgi transport (Graves et al, 2001) and COPII vesicles
419 can be reduced under ER stress conditions (Shaheen, 2018). Full-length CREB3L2 is
420 transported to the Golgi, where it is cleaved to produce the active N-terminal

421 transcription factor (Kondo et al, 2011); thus a decrease in ER-Golgi transport is
422 expected to reduce nuclear N-terminal CREB3L2 levels, leading to downregulation of
423 both *Ghrhr* and *GH1* gene transcription. The decreased levels of N-terminal CREB3L2
424 disturb ER-Golgi transport in vicious cycle, since *Sec23a*, an established target gene of
425 N-terminal CREB3L2, encodes SEC23, which is a COPII vesicle coat protein (Saito et al,
426 2009). Decreased ER-Golgi transport will also influence intracellular protein trafficking
427 of wild-type GH and GHRHR, exacerbating the impaired secretion of wild-type GH.
428 Furthermore, decreased GHRH signaling contributes to GH deficiency through inhibition
429 of somatotroph proliferation. In contrast, decreased $\Delta 3GH1$ gene transcription limits the
430 accumulation of $\Delta 3$ GH in the ER, which likely protects the somatotroph from massive
431 ER stress and apoptosis (Fig 7). To our knowledge, this is the first *in vivo* study that has
432 come close to determining the molecular mechanisms underlying GH deficiency in
433 IGHD2.

434 The involvement of other transcription factors, such as CREB3L1 and/or
435 unknown molecules, in the reduction of *Ghrhr* and *Gh* promoter activities is possible.
436 Creb3 members can interact with other bZip transcription factors as heterodimers, to
437 coordinately stimulate transcription of target genes (Saito et al, 2012; Vecchi et al, 2009;
438 Zhang et al, 2006). Hence, identifying the partner molecule of CREB3L2 will be
439 necessary for complete elucidation of the molecular mechanisms involved in IGHD2 GH
440 deficiency.

441 We presume that CREB3L2 has an important role in the differentiation and
442 proliferation of somatotroph at the late embryonic stage, by stimulating *GHRHR* and
443 *GH1* gene transcription and increasing their secretion, similar to its role in chondrocytes.
444 Postnatal growth has not been described in *Creb3l2*-deficient mice because systemic
445 *Creb3l2* KO mice are lethal soon after birth (Saito et al, 2009). Thus, establishment of
446 somatotroph-specific *Creb3l2* conditional KO mice, and/or *Gh*^{WTGH1/Δ3GH1} mice
447 overexpressing *Creb3l2* specifically in the somatotroph, would be warranted in the
448 future.

449 Interestingly, exon 3 of the *GH1* gene has weak splice sites, and a small amount
450 of $\Delta 3$ GH is produced in the pituitary glands, even in healthy individuals (Lecomte et al,
451 1987; Ryther et al, 2004). $\Delta 3$ GH may have a physiological role in preventing
452 hyperproliferation of somatotroph and overexpression of the *GH1* gene, through the
453 CREB3L2-mediated inhibition of *GHRHR* and *GH1* transcription.

454 This study has some limitations: 1) it remains unclear whether decreased activity
455 of the *Ghrhr* and *Gh* promoters contributes to the GH deficiency in human IGHD2
456 patients, because patient pituitary samples are not available. Establishment of

457 somatotrophs using induced pluripotent stem (iPS) cells derived from IGHD2 patients
458 will help address this issue. 2) The observed decreases in transcriptional activity could
459 be attributed to the influence of the gene exchange process (described in Fig EV1F) on
460 amount of Δ3 GH protein produced, and the cellular characteristics of somatotrophs.

461 In this study, CREB3L1 and CREB3L2 were demonstrated to be involved in GH
462 production in somatotrophs. These findings suggest that patients with GH deficiency
463 may have mutations in the *Creb3l1* or *Creb3l2* genes, or their binding sites in the *Ghrhr*
464 and *Gh* promoters.

465 In conclusion, IGHD2 model mice, created using our gene exchange system,
466 reveal a novel molecular mechanism underlying GH deficiency, where ER-localized Δ3
467 GH leads to decreased levels of nuclear CREB3L2, and a consequent reduction in the
468 activities of the *Ghrhr* and *Gh* promoters.

469

470 **Materials and Methods**

471

472 **Isolation of the *wtGH1* and *Δ3GH1* genes**

473 The *wtGH1* genomic sequence was amplified from healthy human control
474 lymphocyte DNA using standard PCR methods with a sense primer (GH1-gf1) in the
475 5'-untranslated region (UTR) and an antisense primer (GH1-gr1) including the
476 termination codon. The *Δ3GH1* genomic sequence, which contains a c.291+1 g>a
477 mutation, was created by mutagenesis, using sense (GH1IVS3-F) and antisense
478 (GH1IVS3dsmut-R) primers containing the single nucleotide substitution. None of the
479 nucleotide polymorphisms, reported to be significantly associated with adult height
480 (Hasegawa et al, 2000) were included in the sequences. All primers used in this study
481 are listed in Supplementary Table 1.

482

483 **Plasmids**

484 For KO of the *Gh* gene, the 5' (7 kb) and 3' (3 kb) arms were isolated by standard
485 PCR methods, using a mouse bacterial artificial chromosome containing the *Gh* gene
486 locus as a template, with sense (Gh-5arm-F and Gh-3arm-F) and antisense (Gh-5arm-R
487 and Gh-3arm-R) primers. These 5' and 3' arms were inserted into the gene KO vector
488 (Fig EV1A).

489 The *wtGH1*, *Δ3GH1*, *Δ3GH1-myc*, or *Gh* gene genomic sequences were inserted
490 to the cassette exchange vector using appropriate restriction enzymes.

491 To establish the mouse model with *LacZ* knocked-in at the *Ghrhr* gene locus, 5'
492 (500 bp) and 3' (450 bp) arms were isolated by standard PCR methods, using sense

493 (Ghrhr-5arm-f5 and Ghrhr-3arm-f6) and antisense (Ghrhr-5arm-r5 and Ghrhr-3arm-r6)
494 primers, and inserted into a plasmid containing the *LacZ* gene (Fig EV3A).

495 For CRISPR/Cas9 system plasmids, two pairs of oligonucleotides
496 (Ghrhr-CRI-f1/r1, Ghrhr-CRI-f2/r2, Ghrhr-CRI-f4/r4, Ghrhr-CRI-f5/r5) were annealed
497 and inserted into the pX335-U6-Chimeric_BB-CBh-hSpCas9n(D10A) plasmid (Addgene
498 #42335) (pX335-Ghrhr-1, 2, 4, 5, and pX335-Rosa-3, 4).

499 For POU1F1, CREB3L1, and CREB3L2 expression plasmids their open reading
500 frames (ORF) were isolated by standard PCR from C57BL/6 pituitary gland template
501 cDNA using sense (Pou1f1-vf1, Creb3l1-vf1, and Creb3l2-vf1) and antisense
502 (Pou1f1-vr1, Creb3l1-vr1, and Creb3l2-vr1) primers, and inserted into the pcDNA4
503 vector (Invitrogen).

504 For luciferase assay plasmids, the promoter regions of the *Ghrhr* (500 bp) and *Gh*
505 (400 bp) genes were isolated from C57BL/6 mouse genomic DNA by standard PCR and
506 inserted into the pGL4.10 vector (Promega).

507 For RNA probes to detect *GH1* and *Ghrhr* mRNAs by *in situ* hybridization, exon 3
508 of the *GH1* gene (120 bp) and the *Ghrhr* ORF (1272 bp) were amplified by PCR using
509 sense (GH1ex3-f1 and Ghrhr-vf1) and antisense (GH1ex3-r1 and Ghrhr-vr1) primers,
510 with C57BL/6 pituitary gland cDNA as a template, and inserted into the pSP73 vector
511 (Promega).

512

513 **Embryonic stem cell culture and electroporation**

514 KTPU8 feeder free ES cells, derived from F1 mice obtained by crossing C57BL/6
515 and CBA strains, were seeded into dishes pretreated with 0.15% gelatin solution, and
516 cultured in Glasgow minimum essential medium, 14% knockout serum replacement,
517 and 100 IU/ml leukemia inhibitory factor. To establish *Gh*^{+/−} ES cells, KTPU8 cells
518 cultured in a 10 cm culture dish were electroporated (0.8 kV/3 µF) with 20 µg linearized
519 KO vector containing a neomycin resistance (*neoR*) gene flanked by the 5' and 3' arms,
520 and seeded into four 10 cm culture dishes. Culture medium containing 180 µg/mL
521 neomycin was exchanged daily. Nine days after electroporation, the surviving clones
522 were picked and DNA from each clone was evaluated by Southern blotting (Fig EV1A
523 and B).

524 To exchange the *neoR* gene for human *GH1* genes, the *Gh*^{+/−} ES clones cultured
525 in one 10 cm culture dish were electroporated (0.4 kV/125 µF) with 20 µg exchangeable
526 vector and 10 µg Cre expression vector. Culture medium containing 0.8 µg/mL
527 puromycin was exchanged daily, and the DNA samples of surviving clones were
528 evaluated by Southern blotting (Fig EV1A and B).

529 To establish a mouse model in which the *LacZ* gene was knocked-in at the *Ghrhr*
530 gene locus, ES cells derived from C57BL/6 mice were electroporated (0.4 kV/125 μ F)
531 with 25 μ g plasmid containing the *LacZ* gene flanked by *Ghrhr* homology arms,
532 pX335-Ghrhr-1 and pX335-Ghrhr-2 (15 μ g each) (Fig EV3A). For this process, we took
533 advantage of D10A mutant Cas9, to avoid making double-strand breaks (Cong et al,
534 2013; Jinek et al, 2012).

535

536 **Southern Blotting**

537 Southern blotting was conducted using DIG, according to the manufacturer's
538 protocol. For *Gh* gene knockout, homologous recombination of the *neoR* gene was
539 confirmed by Southern blotting using probes for sequences flanking the 5' and 3' arms,
540 and the *neoR* gene (Fig EV1A and B). Substitution of the *GH1* gene for *neoR* was also
541 evaluated by Southern blotting, using probes for the puromycin resistance gene (*PuroR*)
542 (Fig EV1B). To evaluate knock-in of the *LacZ* gene at the *Ghrhr* gene locus, Southern
543 blotting was performed, using probes for sequences flanking the 5' and 3' arms (Fig
544 EV3A and B).

545

546 **RT-PCR and quantitative RT-PCR (qRT-PCR)**

547 Total RNA was extracted from mouse pituitaries using an RNeasy mini kit
548 (Qiagen) and 250 ng converted to cDNA using Revertra Ace (TOYOBO, Osaka, Japan,
549 FSQ-101). cDNA aliquots were used for RT-PCR and qRT-PCR.

550 For qRT-PCR, THUNDERBIRD SYBR qPCR Mix (TOYOBO, QPS-201) was used
551 as Taq polymerase and reactions were performed using the SYBR method on an
552 Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City,
553 CA). In experiments using relative quantification, the relative concentrations of target
554 mRNAs were calculated using a standard curve and normalized to β -actin expression.

555

556 **SDS-PAGE and immunoblotting**

557 SDS-PAGE and immunoblotting were carried out using polyacrylamide gels,
558 polyvinylidene difluoride membranes, and ECL select detection reagent (Amersham,
559 Buckinghamshire, England, RPN2235) according to standard procedures (Sambrook et
560 al).

561

562 **Antibodies**

563 For immunoblotting assays, the following primary antibodies were used: 1)
564 anti-GH rabbit polyclonal antibody (Dako, A0570) (dilution 1:3000); 2) anti-PERK rabbit

565 monoclonal antibody C33E10 (Cell Signaling Technology, 3192) (dilution 1:1000); 3)
566 anti-caspase-3 rabbit monoclonal antibody 8G10 (Cell Signaling Technology, 9665)
567 (dilution 1:1000); 4) anti-GRP78 rabbit monoclonal antibody EPR4041(2) (Abcam,
568 ab108615) (dilution 1:500); 5) anti-GAPDH mouse monoclonal antibody 6C5 (Santa
569 Cruz, sc-32233) (dilution 1:5000); 6) anti-LAMIN A/C rabbit polyclonal antibody (Cell
570 Signaling Technology, 2032) (dilution 1:1000); 7) anti-POU1F1 mouse monoclonal
571 antibody 2C11 (Abcam, ab10623) (dilution 1:1000); 8) anti-CREB3L1 mouse
572 monoclonal antibody 44c7 (Merck Millipore, MABE1017) (dilution 1:1000); 9)
573 anti-CREB3L2 rabbit polyclonal antibody (kindly provided by Prof. Imaizumi, Hiroshima
574 University, Japan) (dilution 1:1000). Anti-rabbit immunoglobulin (IgG) (Dako, P0399)
575 and anti-mouse immunoglobulin (Abcam, ab205719) (dilution 1:5000) secondary
576 antibodies were used.

577 For immunohistochemistry, the following primary antibodies were used at a 1:100
578 dilution: 1) anti-GH rabbit polyclonal antibody (Dako, A0570); 2) anti-GH mouse
579 monoclonal antibody (Abcam, ab15317); 3) anti-CREB3L1 rabbit polyclonal antibody
580 (Abcam, ab33051); and 4) anti-CREB3L2 rabbit polyclonal antibody (kindly provided by
581 Prof. Imaizumi). Goat anti-rabbit IgG (Abcam, ab150079, and Dako, P0448) (dilution
582 1:100) and M.O.M. biotinylated anti-mouse IgG reagent (VECTOR, MKB-2225)
583 secondary antibodies were used. Fluorescein avidin DCS (VECTOR, A-2011) was used
584 with the anti-mouse IgG reagent (MKB-2225).

585

586 **Luciferase assay**

587 BMT10 cells were seeded into 96-well plates at a density of 5000 cells/well. Next
588 day, plasmids expressing POU1F1, CREB3L1-N, and CREB3L2-N or empty vector
589 were mixed in various combinations and transfected using Lipofectamine 2000
590 (Invitrogen). Forty-eight hours later, luciferase assay was performed using
591 Dual-Luciferase Reporter Assay System (Promega).

592

593 **3-D structural analyses of GH proteins**

594 The 3-D structure of wild-type GH was obtained from the Protein Data Bank (PDB
595 ID: 1AXI) and that of Δ3 GH was analyzed using the homology model function of MOE
596 software (Chemical Computing Group Inc.), as described previously (Nakamura et al,
597 2017; Ogasawara et al, 2016). Hydrogen atoms were then added to each protein, using
598 the protonate 3D function of MOE under ER pH conditions (Kim et al, 1998). These
599 structures were then subjected to molecular mechanics (MM) calculations using MOE,
600 with the AMBER99 force field, until the root mean square gradient was 0.01 kcal/mol/Å.

601 After heating for 250 ps to attain 310 K as the starting temperature, a 5000 ps
602 production run of the molecular dynamic (MD) simulation was performed at 310 K, with
603 NPT ensemble using NAMD software (Phillips et al, 2005).

604

605 **Docking simulation analysis of dimeric GH molecules**

606 Docking simulations were performed using ZDOCK (Chen et al, 2003) with
607 residues within 10 Å of cysteine residues (numbers 79, 191, 208, and 215) defined as
608 docking sites in each protein (Ogasawara et al, 2016; Yasui et al, 2013). Two thousand
609 docking runs were performed for each pair of GH molecules and docking poses were
610 classified according to the distance between cysteine residues in the two GHs. The
611 number and binding affinities of docking poses which could form dimers via
612 intermolecular disulfide bonds (Grigorian et al, 2005) were analyzed.

613

614 **Animals**

615 ICR and C57BL/6 mice were purchased from CLEA Japan, Inc. Egg zona
616 pellucida from E2.5 ICR mouse embryos were removed and aggregated with KTPU8
617 ES clones. The resulting blastocysts were transferred to the uteruses of ICR female
618 mice mated with vasoligated male mice. F0 mice, with 100% chimerism, were crossed
619 with C57BL/6 mice, to obtain N1 offspring. $Gh^{wtGH1/wtGH1}$ and $Gh^{wtGH1/\Delta3GH1}$ mice were
620 backcrossed to C57BL/6 mice for at least ten generations.

621

622 **Genotyping**

623 To determine mouse genotypes, standard PCR reactions were performed using
624 template DNA samples extracted from toe clips from 7-day-old mice (Fig EV1A and D).
625 A reverse primer with the 3' end converted to thymine from cytosine at the first base of
626 the intron 3 in the *GH1* gene (primer No. 4, completely matching the $\Delta3GH1$ allele) was
627 used. PCR reactions to detect both *wtGH1* and $\Delta3GH1$ alleles used an annealing
628 temperature of 60°C, with those to detect only the $\Delta3GH1$ allele using an annealing
629 temperature of 66°C (Fig EV1D). The sequences of all primers used in this study are
630 listed in Table 1.

631

632 **Measurement of mouse body weight and length**

633 Mouse body weights were measured every week from 1 to 16 weeks of age. Body
634 lengths (distances from nose to anus) were measured under anesthesia, induced using
635 isoflurane, every 4 weeks from 4 to 16 weeks of age, using a ruler.

636

637 **Measurement of mouse serum IGF-1 concentration**

638 Blood samples were obtained from mouse orbital veins at 4 weeks of age, using a
639 heparinized tube. Samples were centrifuged at 3000 rpm for 5 min, to collect serum
640 samples collected. IGF-1 concentrations were evaluated using a Mouse/Rat IGF-1
641 ELISA Kit (ALPCO, 22-IG1MS-E01).

642

643 **Hematoxylin and eosin (HE) staining, TUNEL assay, and Immunohistochemistry**

644 Pituitary glands were fixed in 4% paraformaldehyde (PFA) for 24 h at 15-25
645 °C, dehydrated through increasing concentrations of ethanol, equilibrated with xylene,
646 embedded in paraffin wax, and sectioned at 4 µm. Pituitary sections were stained with
647 hematoxylin and eosin and examined by light microscopy. TUNEL assays were
648 performed using an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore,
649 S7100). Thymus sections were used as positive controls. For immunostaining of GH,
650 pituitary sections were deparaffinized, rehydrated, and treated with 20 µg/ml proteinase
651 K as an antigen retrieval step. Antibodies are described in the 'antibodies' section above.
652 Staining was performed using diaminobenzidine (DAB) and hematoxylin.

653

654 **Immunofluorescence assay**

655 Pituitary glands were fixed in 4% PFA for 2 h at room temperature, dehydrated
656 through increasing concentrations of sucrose, embedded in Optimal Cutting
657 Temperature Compound, and sectioned at 6 µm. Frozen pituitary sections were
658 subjected to immunofluorescence analysis.

659

660 ***In situ* hybridization**

661 Pituitary glands were fixed in 4% PFA for 48 h at room temperature.
662 Deparaffinization, cell conditioning, prehybridization, and stringency washing were
663 automated using a staining workstation (Ventana Discovery XT). RNA probes were
664 synthesized by *in vitro* transcription, and labelled with digoxigenin (DIG), using a DIG
665 RNA labeling kit (Roche, 11175025910). Probes for *wtGH1* and *Ghrhr* mRNAs were
666 manually hybridized at 0.5 ng/ml, 65°C for 6 h, and 10 ng/ml, 68°C for 6 h, respectively.

667

668 **Transmission electron microscopy**

669 Pituitary glands from four-week-old mice were used for TEM observation, as
670 described previously (Shibata et al, 2015). Briefly, tissues were dissected out and fixed
671 in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4°C. After 2 h
672 post-fixation with 1% OsO₄ and dehydration through ethanol, then acetone with n-butyl

673 glycidyl ether (QY1) including a graded concentration of Epon with QY-1, they were
674 embedded into 100% Epon. Following 72 h of polymerization in pure Epon, 70 nm
675 ultrathin coronal pituitary gland sections were prepared on copper grids and stained with
676 uranyl acetate and lead citrate for 10 min. Sections were observed under a TEM (JEOL
677 JEM-1400 plus).

678

679 **X-gal staining of pituitary glands**

680 Pituitary glands were removed from four-week-old mice and fixed in 4% PFA for 1
681 h on ice. Then, samples were permeabilized using rinse buffer (phosphate buffered
682 saline (PBS) containing 2 mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% Nonidet
683 P-40) for 2 h on ice, washed three times for 30 min in PBS, and stained in rinse buffer
684 containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml
685 5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside (X-gal) overnight at 37°C. For E19.5
686 embryo samples, heads were removed and fixed in 4% PFA for 30 min on ice. Then,
687 pituitary glands were exposed by removing the skull bone and brain before
688 permeabilization using rinse buffer.

689

690 **Dissociation of pituitary cells**

691 Anterior pituitary cells were dissociated, according to published methods, using
692 0.5 % trypsin-EDTA (Oomizu et al, 1998).

693

694 **Soluble/insoluble fraction assay, cytoplasmic/nuclear fraction assay**

695 For soluble/insoluble fraction assays, anterior pituitary cells were dissociated as
696 described above, and 0.5 x 10⁶ cells seeded in DMEM with 10% FBS in a 24-well plate
697 with 10 μM MG132 (Peptide Institute, Osaka, Japan), or DMSO, for 4 h. Detailed
698 procedures have been described elsewhere (Ariyasu et al, 2013). To separate
699 cytoplasmic and nuclear fractions, a NE-PER Nuclear and Cytoplasmic Reagent Kit
700 (Thermo Fischer Scientific, 78833) was used.

701

702 **Statistics**

703 Data were analyzed by 2-tailed unpaired Student's *t* test for comparisons of two
704 groups. For all bar graphs, means ± SD are plotted.

705

706 **Study approval**

707 The Animal Care Committee and Institutional Biosafety Committee of the
708 Kumamoto University approved all mouse protocols. All experiments were performed in

709 accordance with the Declaration of Helsinki and were approved by the Kumamoto
710 University Ethics Committee for Animal Experiments (authorization number in
711 Kumamoto University: C23-262, C24-278).

712

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721

722 **Author contributions**

723 DA and KA conceived the study. DA and KA designed the experiments. DA, EK,
724 DH, YT, and SS performed the experiments and analyzed the data. DA, SS, and YT
725 wrote the initial draft of the paper. TH, MS, KI, and KA critically revised the manuscript.
726 All authors reviewed and edited the manuscript.

727

728 **Conflict of Interest**

729 The authors have declared that no conflict of interest exists.

730

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991 **Figure Legends**
992

993 Figure 1
994 $Gh^{wtGH1/\Delta3GH1}$ mice exhibit a $\Delta3$ GH-mediated dominant negative phenotype.
995 (A) Schematic representations of wild-type and genetically modified alleles. Vertical
996 long open and closed rectangles represent the 5 exons of the endogenous *Gh* gene and

997 the exchanged human *GH1* gene, respectively. Orange and purple rectangles,
998 untranslated regions of the *Gh* gene. Red closed circle, c.291+1 g>a mutation. hACp,
999 human actin promoter; neoR, neomycin resistance gene; pPGK, phosphoglycerate
1000 kinase promoter; PuroR, puromycin resistance gene; pA, polyadenylation signal.
1001 (B, C) Growth curves of model mice. (B) Body weight (BW) and (C) body length (BL)
1002 were measured every week and every 4 weeks, respectively, up to 16 weeks of age.
1003 Asterisks indicate that data from *Gh*^{wtGH1/Δ3GH1} mice are significantly different to those
1004 from *Gh*^{wtGH1/wtGH1} mice. Means calculated from the indicated numbers of model mice
1005 (bottom right in (B)) are shown. Error bars represent the standard deviation (SD) of
1006 values in *Gh*^{wtGH1/wtGH1} and *Gh*^{wtGH1/Δ3GH1} mice. *p<0.01, **p<0.005.
1007 (D) Serum IGF-1 values in 4-week-old model mice. Mean and SD values from six mice
1008 with each genotype are shown. **p<0.005.
1009 (E) Photograph of male *Gh*^{wtGH1/wtGH1}, *Gh*^{wtGH1/-}, *Gh*^{wtGH1/Δ3GH1}, and *Gh*^{-/-} mice at 8 weeks
1010 old.
1011

1012 Figure 2

1013 Evaluation of wild-type and Δ3 GH expression in *Gh*^{wtGH1/wtGH1} and *Gh*^{wtGH1/Δ3GH1} pituitary
1014 samples.
1015 (A) Immunoblotting using anti-GH antibody. This antibody recognizes Δ3 GH; however,
1016 the 17.5 kDa signals were too faint to be detected in *Gh*^{wtGH1/Δ3GH1} pituitary glands. An
1017 image obtained by long exposure is shown on the right.
1018 (B) Evaluation of wild-type GH expression in (A) by densitometry. *p<0.05.
1019 (C) Results of immunohistochemistry using the same antibody used in (A). Scale bars,
1020 100 μM.
1021 (D) Abundance of *wtGH1* and *Δ3GH1* mRNAs, evaluated by RT-PCR using a sense
1022 primer in exon 1 and an antisense primer in exon 5.
1023 (E) Evaluation of *wtGH1* mRNA abundance by qRT-PCR, using a sense primer in exon
1024 3 and an antisense primer spanning exons 3 and 4. Data presented are mean and SD
1025 values from three independent samples, with relative abundance calculated by
1026 normalization to *β-actin* expression. ***p<0.005.
1027 (F) Results of qRT-PCR to evaluate the abundance of *wtGH1* mRNA using pituitary
1028 samples from *Gh*^{wtGH1/wtGH1} and *Gh*^{wtGH1/Δ3GH1} mice at E19.5, and 1, 2, 3, and 4 weeks of
1029 age. **p<0.01, ***p<0.005.
1030 (G) *In situ* hybridization of pituitary gland samples from E19.5 and 4-week-old mice,
1031 using an antisense probe complementary to *GH1* exon 3 mRNA. Scale bars, 100 μM.
1032

1033 Figure 3

1034 Histological evaluation of pituitary glands from $Gh^{wtGH1/wtGH1}$ and $Gh^{wtGH1/\Delta3GH1}$ mice.

1035 (A) Stereomicroscope images of pituitary glands from 4-week-old $Gh^{+/+}$, $Gh^{wtGH1/wtGH1}$,
1036 $Gh^{wtGH1/-}$, $Gh^{wtGH1/\Delta3GH1}$, and $Gh^{-/-}$ mice. Scale bars, 2 mm.

1037 (B) HE (scale bars, 50 μ M) and TUNEL staining (scale bars, 100 μ M) of pituitary glands
1038 from 4-week-old $Gh^{wtGH1/wtGH1}$ and $Gh^{wtGH1/\Delta3GH1}$ mice.

1039 (C, D) TEM images of pituitary glands from 4-week-old (C) $Gh^{wtGH1/wtGH1}$ and
1040 $Gh^{wtGH1/\Delta3GH1}$, and (D) $Gh^{-/-}$ and $Gh^{\Delta3GH1/-}$, mice. Marked enlargement of the rough ER
1041 (arrow) and cytosolic protein aggregates (arrowhead) were observed in $Gh^{wtGH1/\Delta3GH1}$
1042 and $Gh^{\Delta3GH1/-}$ somatotroph. Scale bars, 10 μ M (low power field) and 2 μ M (high power
1043 field).

1044 (E) Magnified view of the open square in (C). The rough ER membrane is connected to
1045 the protein aggregates (arrowhead). Scale bar, 1 μ M.

1046 (F) Evaluation of cellular localization of $\Delta3GH$ -myc by immunofluorescence, using
1047 antibodies against the myc-tag ($\Delta3GH$ -myc), KDEL (ER marker), and GM130 (Golgi
1048 marker).

1049

1050 Figure 4

1051 $\Delta3$ GH activates three major ER stress response pathways.

1052 (A) Evaluation of phosphorylation of PERK, expression of BiP, and activation of
1053 caspase-3 by immunoblotting using pituitary glands from 2 and 4-week-old $Gh^{wtGH1/wtGH1}$
1054 and $Gh^{wtGH1/\Delta3GH1}$ mice.

1055 (B) qRT-PCR evaluation of the abundance of *BiP* mRNA in pituitary glands from 2 and
1056 4-week-old $Gh^{wtGH1/wtGH1}$ and $Gh^{wtGH1/\Delta3GH1}$ mice. **p<0.01.

1057 (C) RT-PCR evaluation of *Xbp1* mRNA splicing. Primers surrounding the region spliced
1058 out by IRE1 were used. PCR products of 140 and 114 bp, represent unspliced (*Xbp1(u)*)
1059 and spliced (*Xbp1(s)*) *Xbp1*, respectively. M, PCR molecular weight marker.

1060 (D) Evaluation of the ratio of *Xbp1(s)* to *Xbp1(u)* by densitometry. *p<0.05, **p<0.01,
1061 ***p<0.005.

1062

1063 Figure 5

1064 $\Delta3$ GH decreases *Ghrhr* gene promoter activity.

1065 (A) Evaluation of *Ghrhr* mRNA abundance by qRT-PCR using samples from
1066 $Gh^{wtGH1/wtGH1}$, $Gh^{wtGH1/\Delta3GH1}$, $Gh^{-/-}$, and $Gh^{\Delta3GH1/-}$ mice at 4 weeks old. Data presented are
1067 mean and SD values from three independent samples, with relative abundance
1068 calculated by normalization to β -actin expression. ***p<0.005.

1069 (B) *In situ* hybridization evaluation of *Ghrhr* mRNA expression in pituitary glands from
1070 E19.5 and 4-week-old *Gh*^{wtGH1/wtGH1}, *Gh*^{wtGH1/Δ3GH1}, *Gh*^{-/-}, and *Gh*^{Δ3GH1/-} mice. Scale bars,
1071 100 μM.

1072 (C) Growth curves of male *Gh*^{-/-} and *Gh*^{Δ3GH1/-} mice.

1073 (D) Results of X-gal staining of pituitary glands from 4-week-old and E19.5 *Ghrhr*^{+/LacZ};
1074 *Gh*^{wtGH1/wtGH1} and *Ghrhr*^{+/LacZ}; *Gh*^{wtGH1/Δ3GH1} mice. Arrows indicate pituitary glands. Scale
1075 bars, 2 mm.

1076

1077 Figure 6

1078 Levels of nuclear CREB3L2 are decreased in pituitary glands from *Gh*^{wtGH1/Δ3GH1} mice.

1079 (A) Evaluation of Creb3 family member expression in 4-week-old whole pituitary glands
1080 by RT-PCR.

1081 (B) Evaluation of *Creb3l1* and *Creb3l2* gene expression in pituitary glands from
1082 4-week-old *Gh*^{wtGH1/wtGH1} and *Gh*^{wtGH1/Δ3GH1} mice by qRT-PCR. *p<0.05.

1083 (C) Evaluation of cytoplasmic (Cy) and nuclear (N) expression levels of CREB3L1 and
1084 CREB3L2 by immunoblotting. Anterior pituitary cells from four-week-old *Gh*^{wtGH1/wtGH1}
1085 and *Gh*^{wtGH1/Δ3GH1} mice were dissociated and separated into Cy and N fractions. GAPDH
1086 and LAMIN A/C were used as positive controls for Cy and N fractions, respectively.

1087 (D) Reporter assay to evaluate expression driven by the *Ghrhr* (500 bp) and *Gh* (400
1088 bp) promoters using vectors expressing POU1F1, CREB3L1-N, and CREB3L2-N.

1089 (E, F) Evaluation of CREB3L2 protein levels in pituitary glands from E19.5 mouse
1090 embryos by immunoblotting. **p<0.01.

1091 (G) Evaluation of the abundance of Sec23a mRNA in pituitary glands from E19.5 mouse
1092 embryos by qRT-PCR. *p<0.05.

1093

1094 Figure 7

1095 A schematic representation of Δ3 GH-mediated dominant negative effects in IGHD2
1096 model mice.

1097

1098 Tables

1099

1100 Table 1

| | wild type GH - wild type GH | wild type GH-Δ3 GH |
|----------------|-----------------------------|-----------------------|
| | | |
| ZDOCK score | 150.6±16.58 (n=234) | 146.21±19.18 (n=938)* |

1101 Results of docking simulation of wild-type and $\Delta 3$ GH. Higher ZDOCK score indicates
1102 more stable binding affinity. Numbers indicate times of the docking runs. ***p<0.001.

1103

1104 **Expanded View Figure Legends**

1105

1106 Expanded View Figure 1

1107 Establishment of IGHD2 model mice using the gene exchange system.

1108 (A) Schematic representation of the gene exchange system. Vertical long open and
1109 grey rectangles represent the open reading frames of the endogenous *Gh* and
1110 exchanged human *GH1* genes, respectively. Orange and purple rectangles represent
1111 untranslated regions of the *Gh* gene. Black bold lines represent homology arms. Red
1112 and blue bold lines represent probes used for Southern blotting to detect homologous
1113 recombination of the *neoR* gene and exchange of the *GH1* gene. Red and blue vertical
1114 lines represent EcoRI and KpnI recognition sites, respectively. Green arrows numbered
1115 from 1 to 4, genotyping primers. DT-A, diphtheria toxin A.

1116 (B) Results of Southern blotting using probes indicated in (A).

1117 (C) Intact and mutant loxP sequences used in this study; red letters indicate mutant
1118 sequences.

1119 (D) Typical results of PCR genotyping using primers 1 to 4. Signals detecting *Gh*⁺, *Gh*⁻,
1120 and *Gh*^{*wtGH1*} or *Gh*^{*Δ3GH1*} are shown. M: PCR molecular weight marker.

1121 (E) Growth curves of male *Gh*^{*+Δ3GH1*} and *Gh*^{*mGh/Δ3GH1*} mice.

1122 (F) qRT-PCR evaluating the abundance of mRNA transcribed from the endogenous and
1123 exchanged *Gh* alleles in pituitary glands from 4-week-old mice.

1124

1125 Expanded View Figure 2

1126 (A) GH4C1 cells were transfected with *wtGH1* and/or $\Delta 3GH1$ cDNA constructs in the
1127 presence or absence of treatment with the proteasome inhibitor, MG132. Cell lysate
1128 samples were subjected to immunoblotting analysis using anti-GH antibody. Upper
1129 numbers indicate the quantities of transfected plasmid DNA, encoding either *wtGH1* or
1130 $\Delta 3GH1$, cDNA. Note that $\Delta 3$ GH is degraded by the proteasome and the anti-GH
1131 antibody used in this study has comparable affinities for both wild-type and $\Delta 3$ GH
1132 proteins.

1133 (B) Evaluation of the distribution of wild-type and $\Delta 3$ GH by soluble/insoluble fraction
1134 assay. Four-week-old anterior pituitary cells from *Gh*^{*wtGH1/Δ3GH1*} mice were dissociated,
1135 separated into soluble (S) and insoluble (I) fractions, and subjected to immunoblotting in
1136 the presence or absence of MG132 treatment (10 μ M, 4 h). GAPDH was used as a

1137 positive control for the soluble fractions.

1138 (C) Photograph of male $Gh^{/-}$, and $Gh^{\Delta 3GH1/-}$ mice at 8 weeks old.

1139

1140 Expanded View Figure 3

1141 (A) A strategy for establishing *LacZ* knocked-in model mice, using the CRISPR/Cas9
1142 system. The *LacZ* gene was inserted into exon 1 of the *Ghrhr* gene by homologous
1143 recombination. Blue and orange bars, 5' and 3' homology arm CRISPR oligonucleotides,
1144 respectively. Purple bar, probe used for Southern blotting. NLS, nuclear-localization
1145 signal; B, BgIII recognition site.

1146 (B) Results of Southern blotting using the probe indicated in (A).

1147 (C) Evaluation of cytoplasmic and nuclear POU1F1 expression levels by
1148 immunoblotting. Anterior pituitary cells from four-week-old $Gh^{wtGH1/wtGH1}$ and
1149 $Gh^{wtGH1/\Delta 3GH1}$ mice were dissociated, separated into cytoplasmic (Cy) and nuclear (N)
1150 fractions, and subjected to immunoblotting analysis. GAPDH and LAMIN A/C were used
1151 as positive controls for Cy and N fractions, respectively.

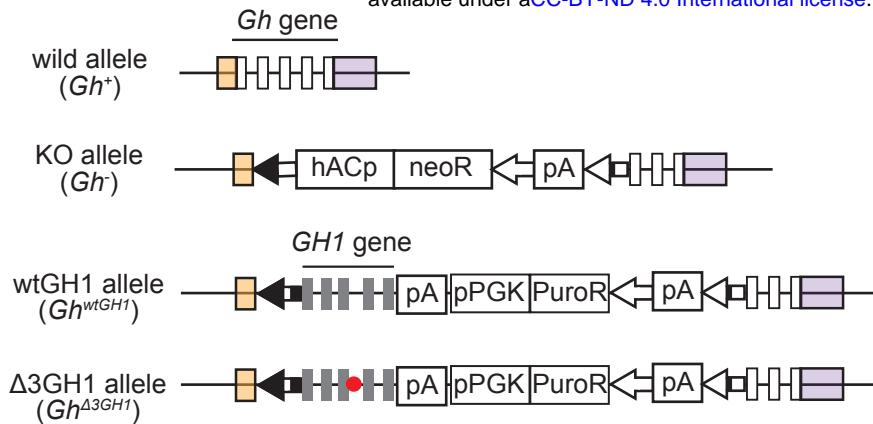
1152 (D) Evaluation of wild-type GH, CREB3L1, and CREB3L2 expression in pituitary glands
1153 from 4-week-old $Gh^{wtGH1/wtGH1}$ and $Gh^{wtGH1/\Delta 3GH1}$ mice by immunofluorescence analysis.

1154

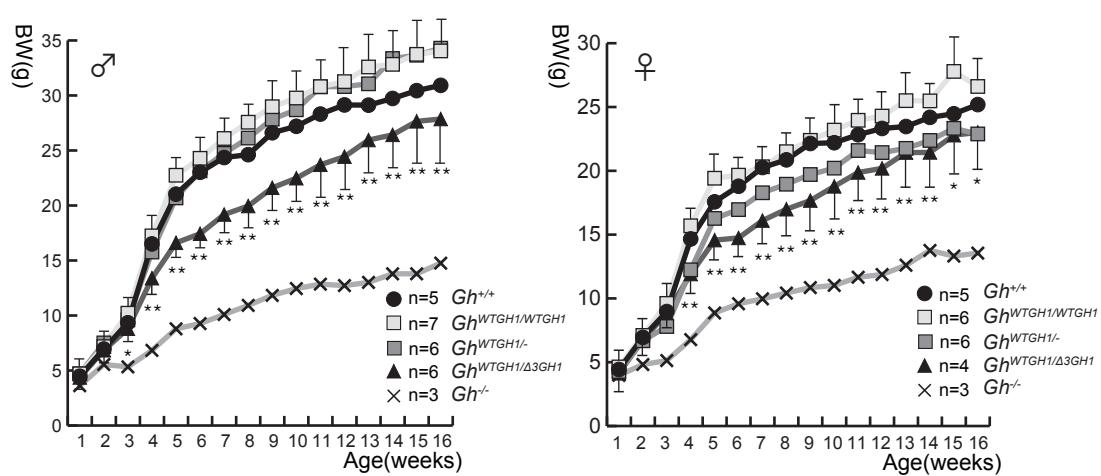
Figure 1

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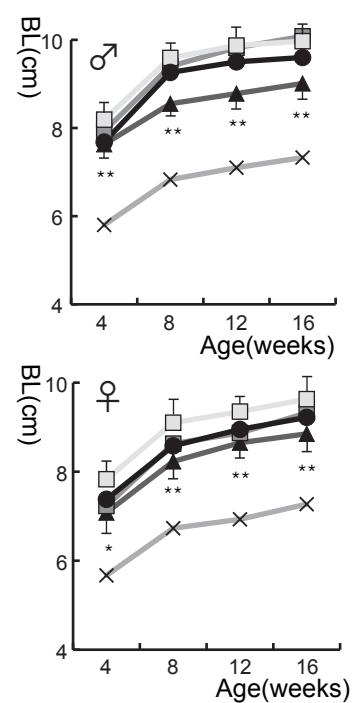
A



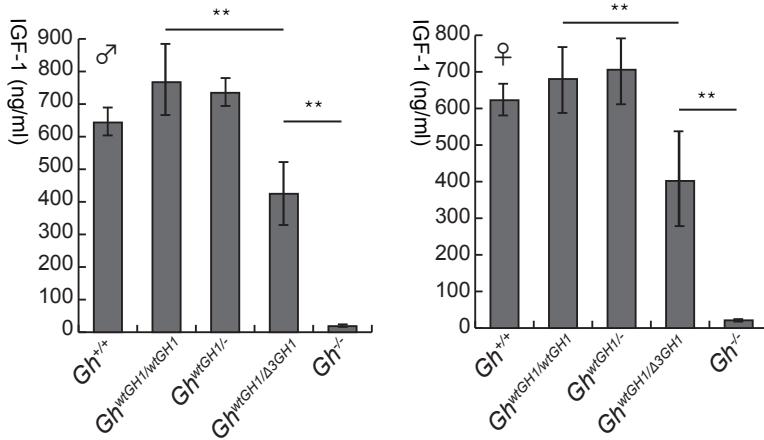
B



C



D



E

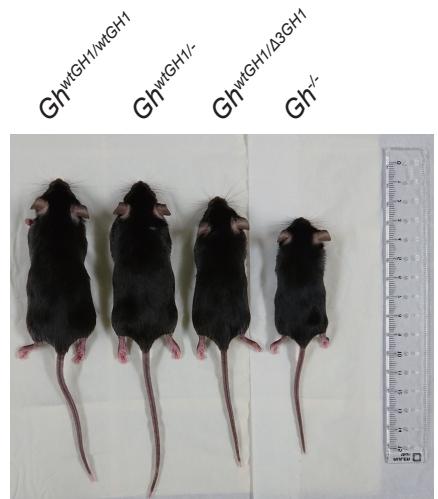


Figure 2 | bioRxiv preprint doi: <https://doi.org/10.1101/545384>; this version posted February 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

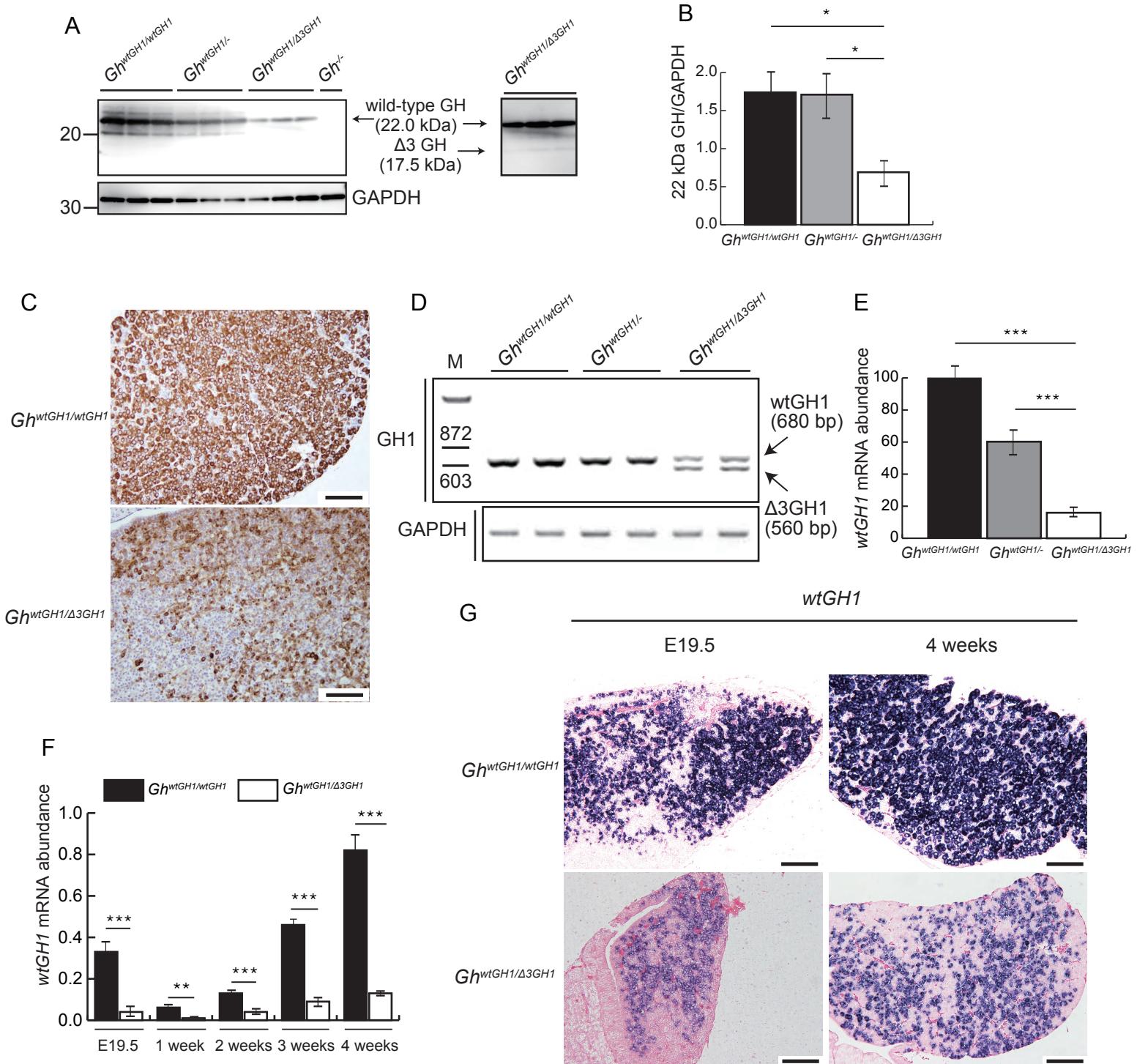


Figure 3

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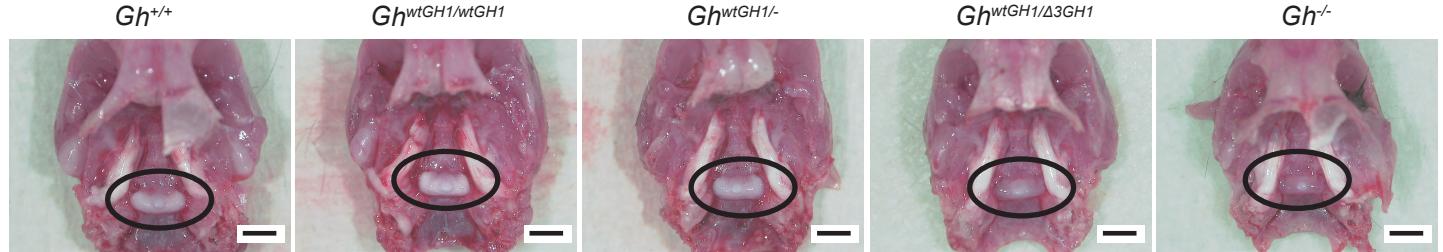
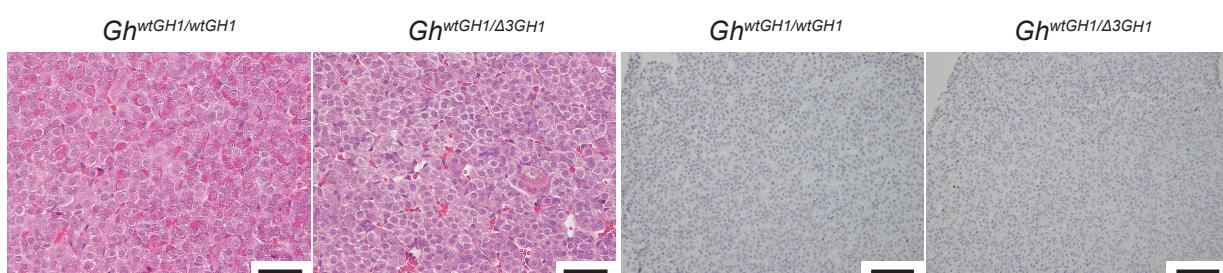
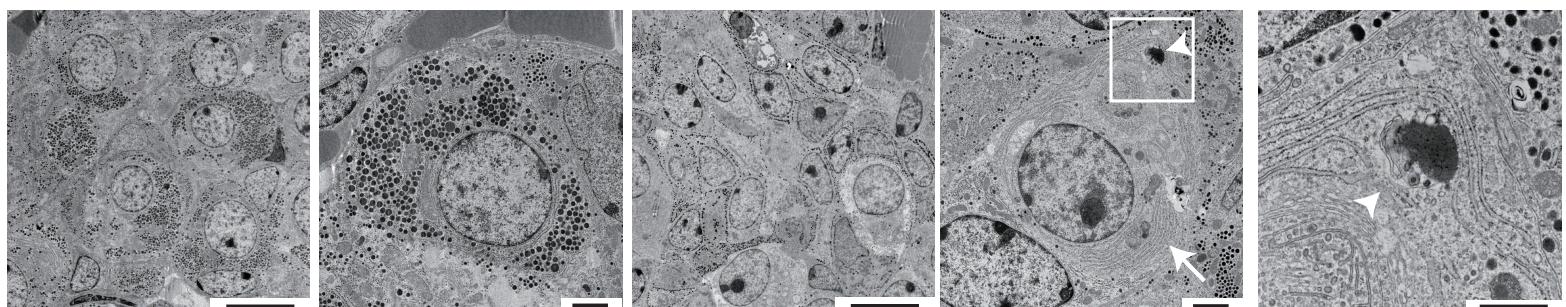
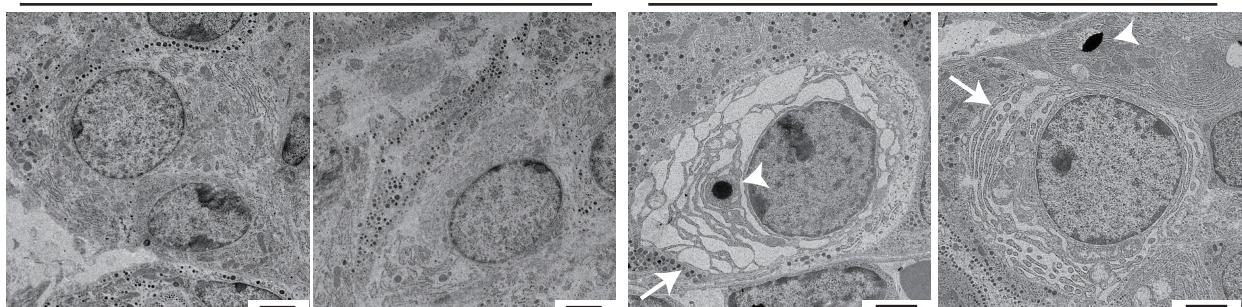
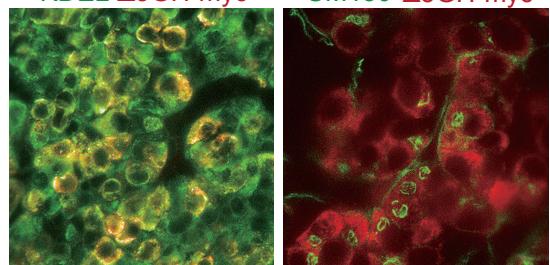
A**B****HE****TUNEL****C****GhwtGH1/wtGH1****GhwtGH1/Δ3GH1****E****D****Gh-/-****GhΔ3GH1/-****F****GhwtGH1/Δ3GH1-myc****KDEL Δ3GH-myc****GM130 Δ3GH-myc**

Figure 4 bioRxiv preprint doi: <https://doi.org/10.1101/545384>; this version posted February 8, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

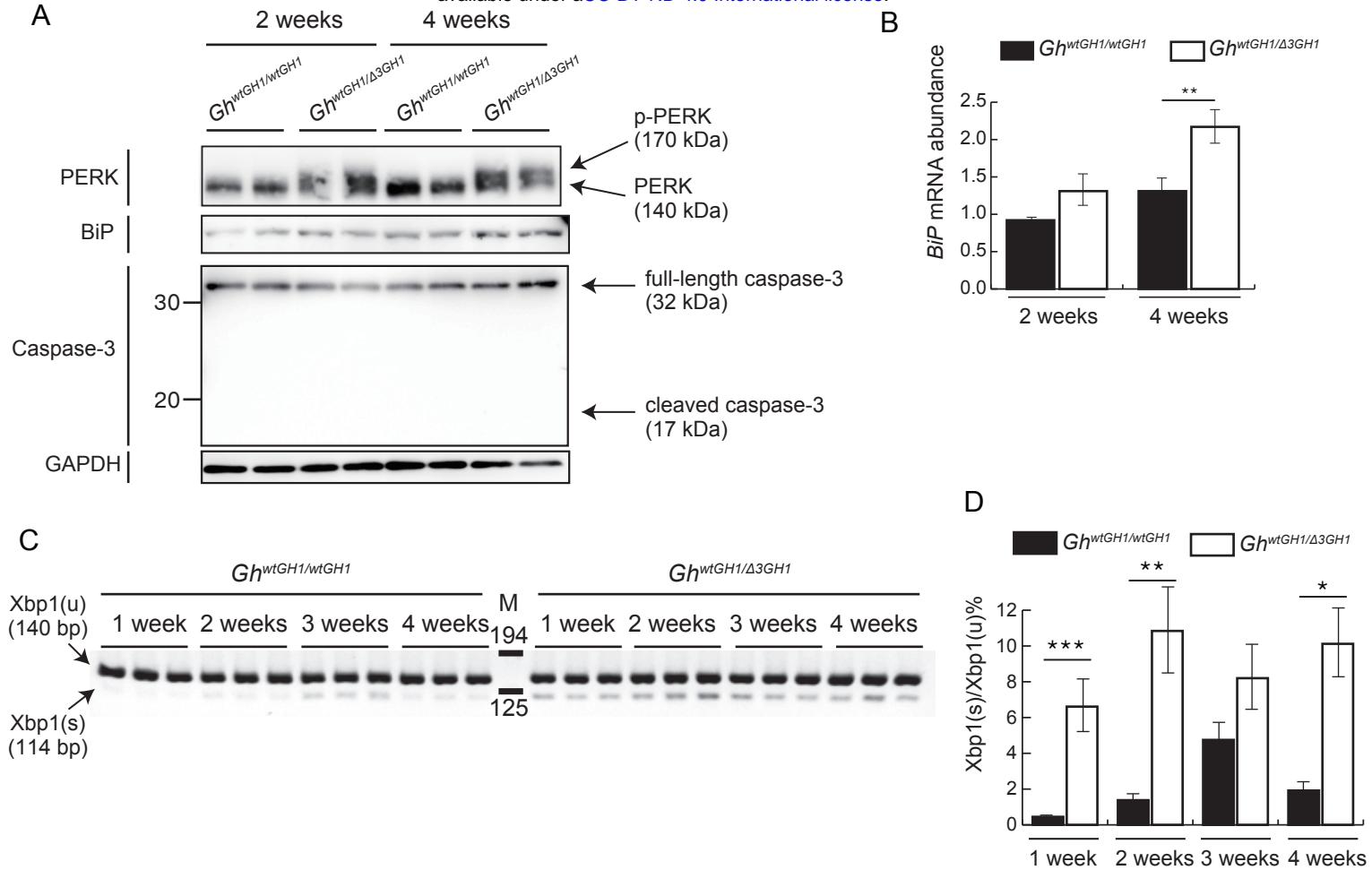
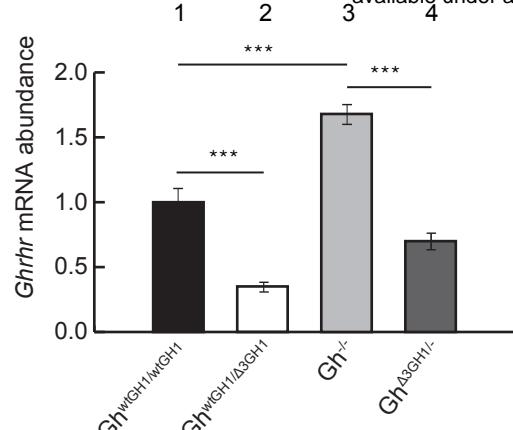


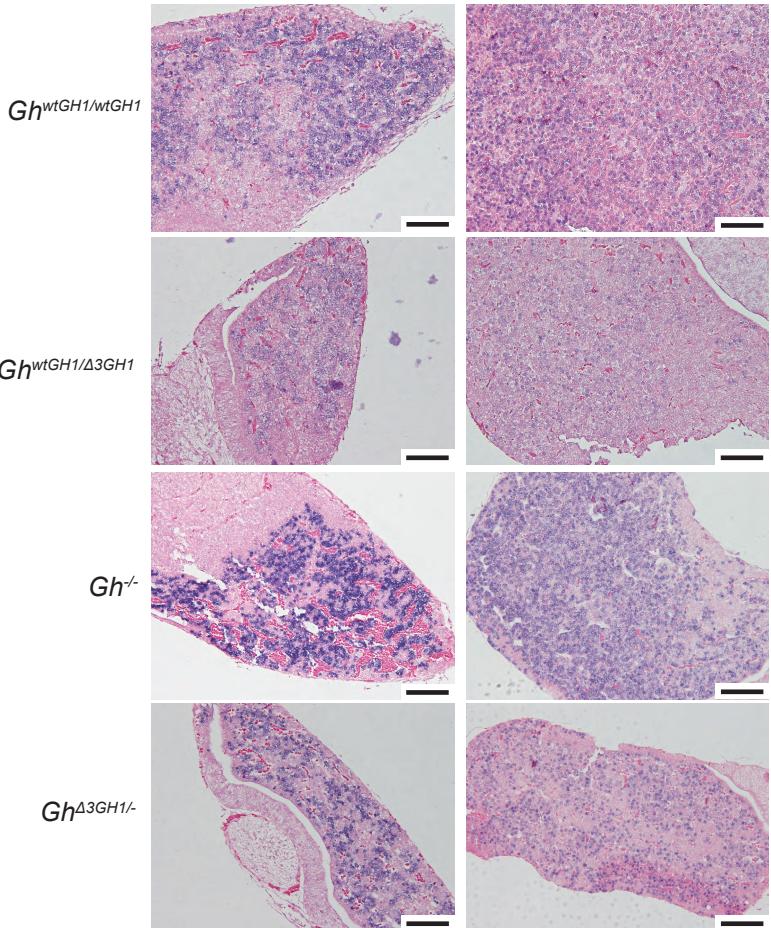
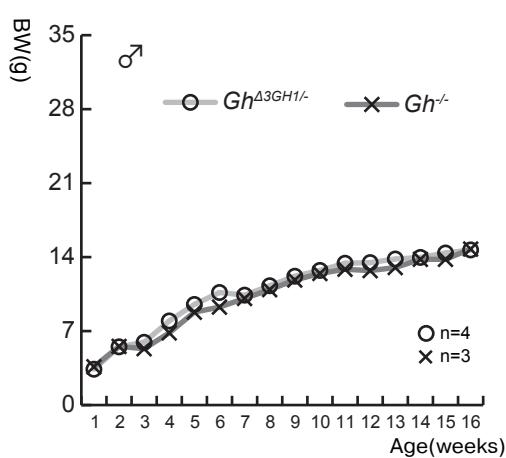
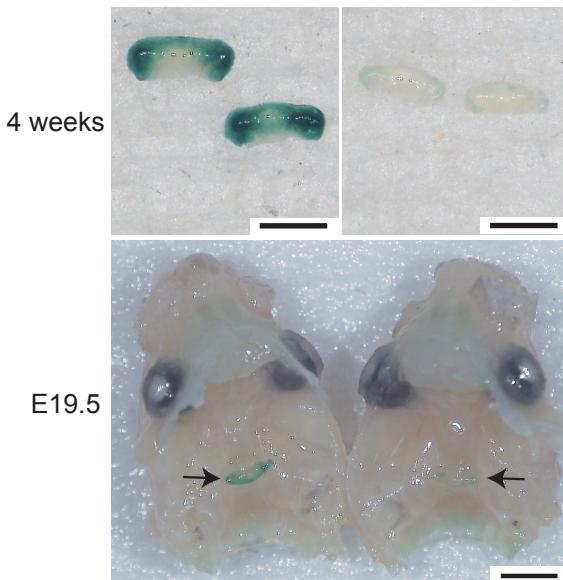
Figure 5

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A

E19.5

4 weeks

**C****D** $Ghrhr^{+/LacZ}, Gh^{WTGH1/WTGH1}$ $Ghrhr^{+/LacZ}, Gh^{WTGH1/\Delta 3GH1}$ 

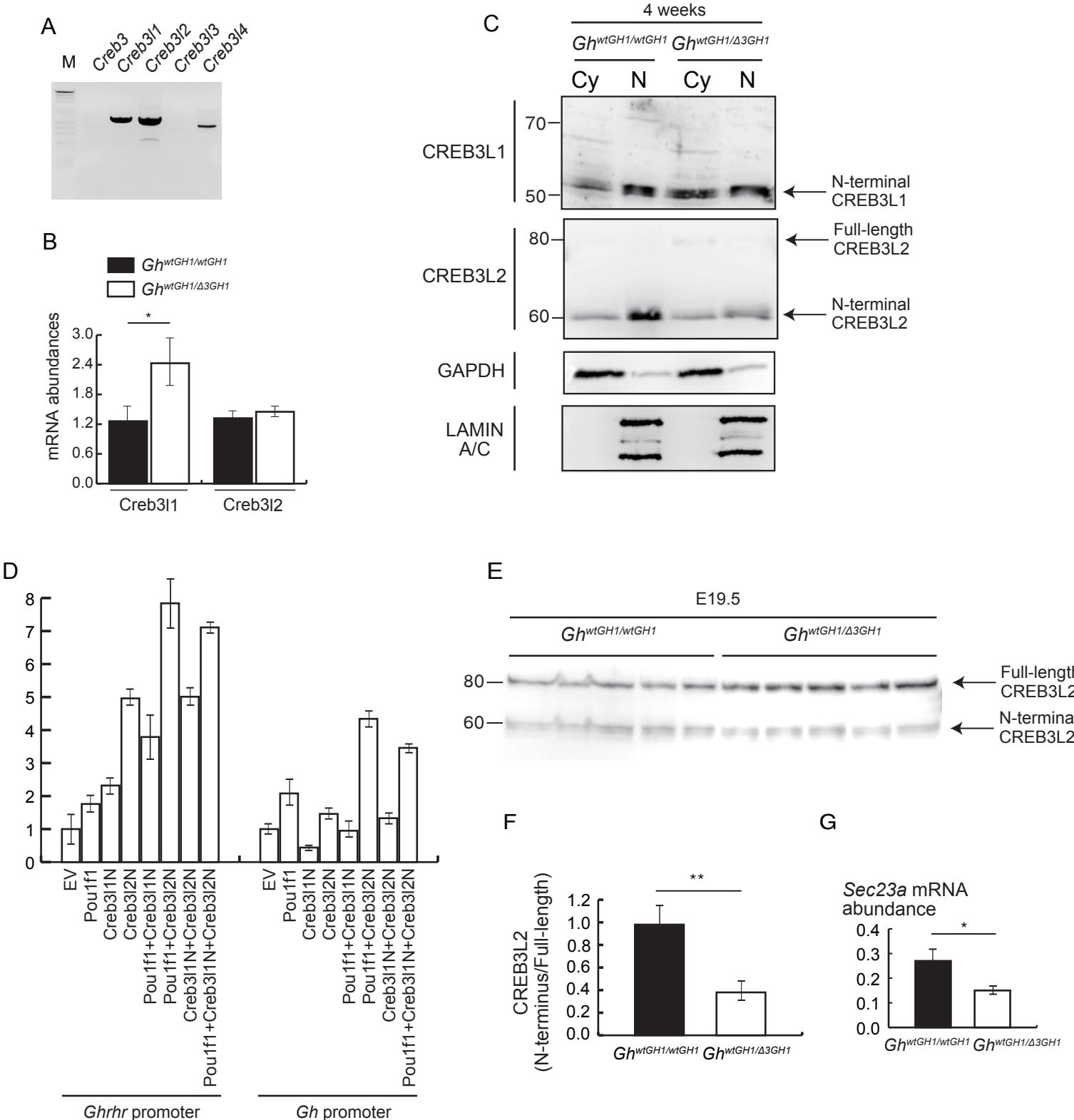
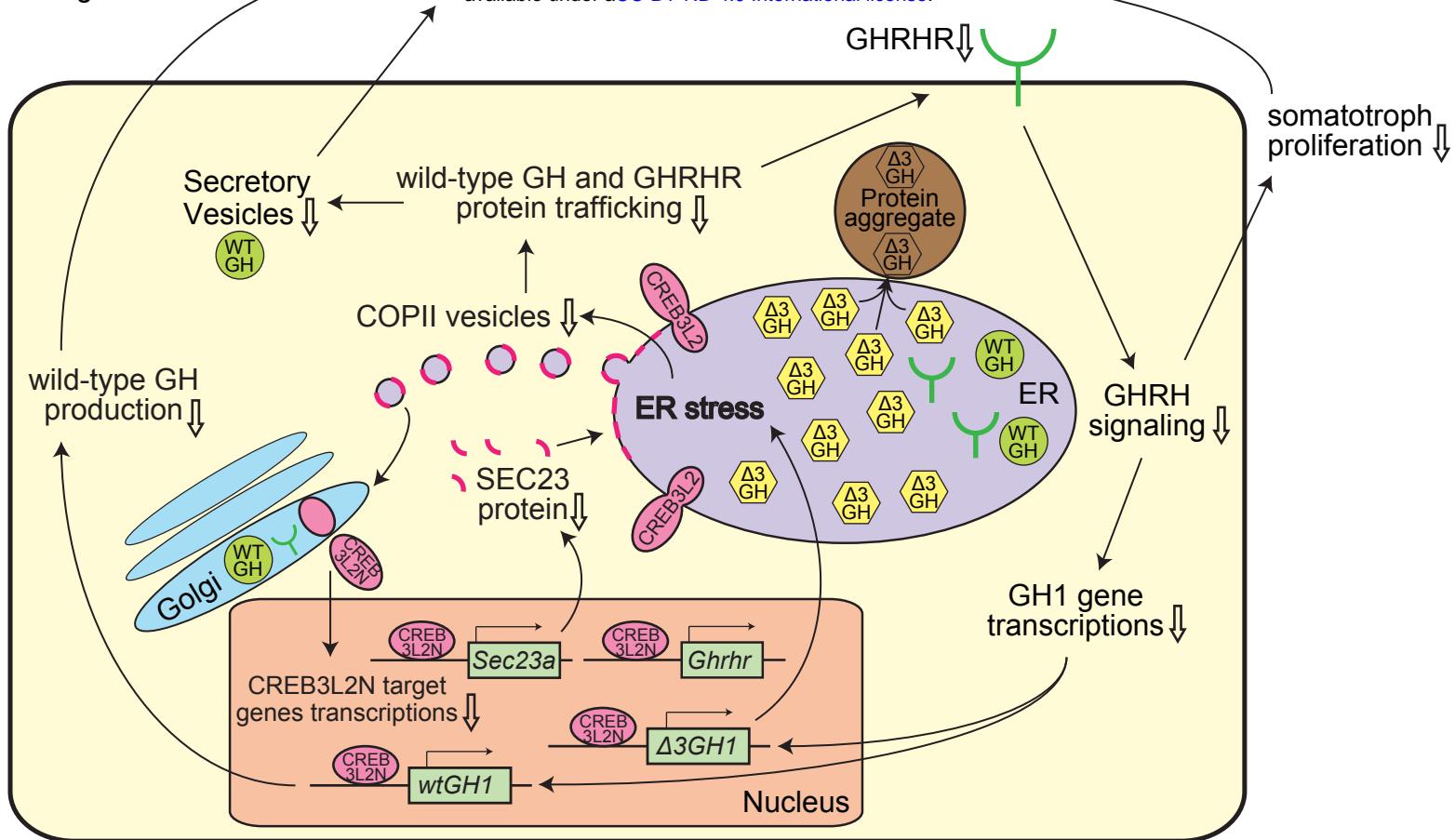


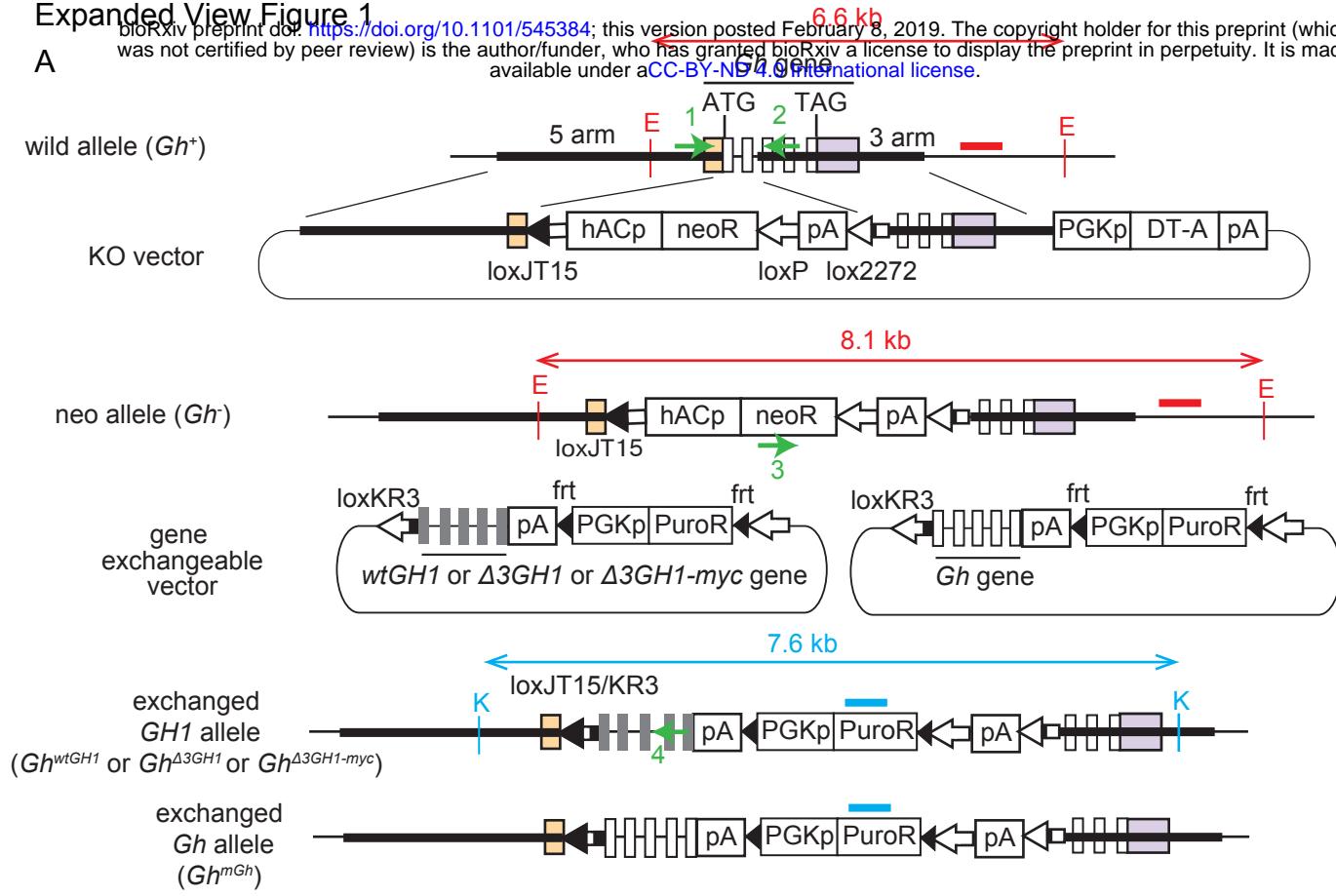
Figure 7



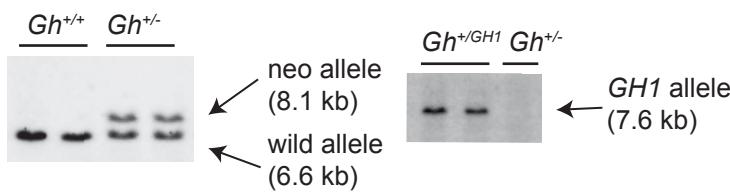
Expanded View Figure 1

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A



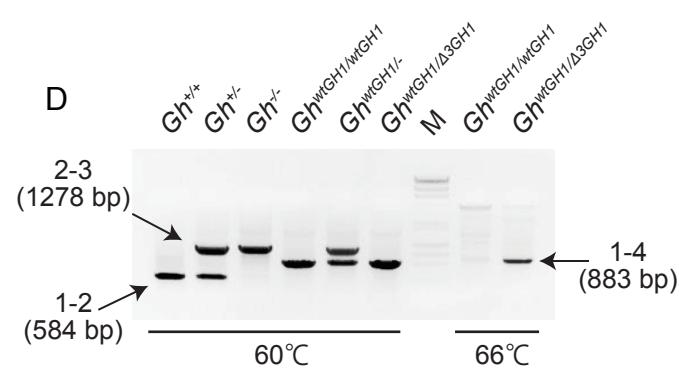
B



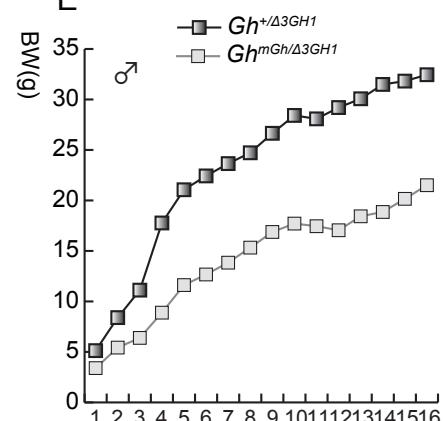
C

loxP ATAACTTCGTATAGCATACATTATACGAAGTTAT
 loxJT15 A~~TTT~~ATTCGTATAGCATACATTATACGAAGTTAT
 loxKR3 ATAACTTCGTATAGCATACATTATAC~~CTT~~GTTAT

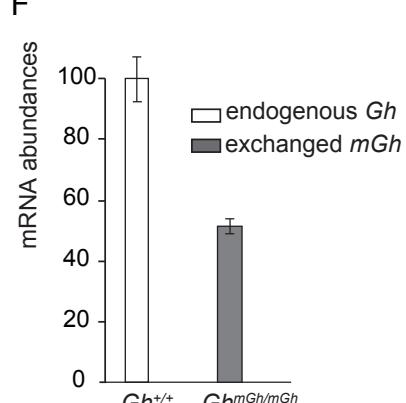
D



E

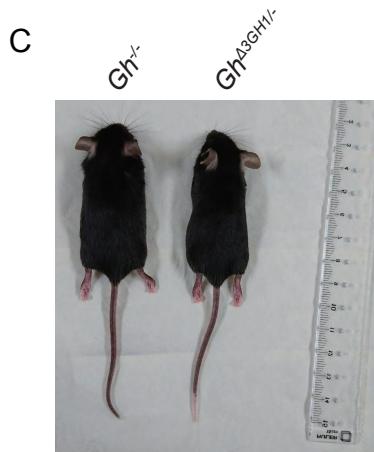
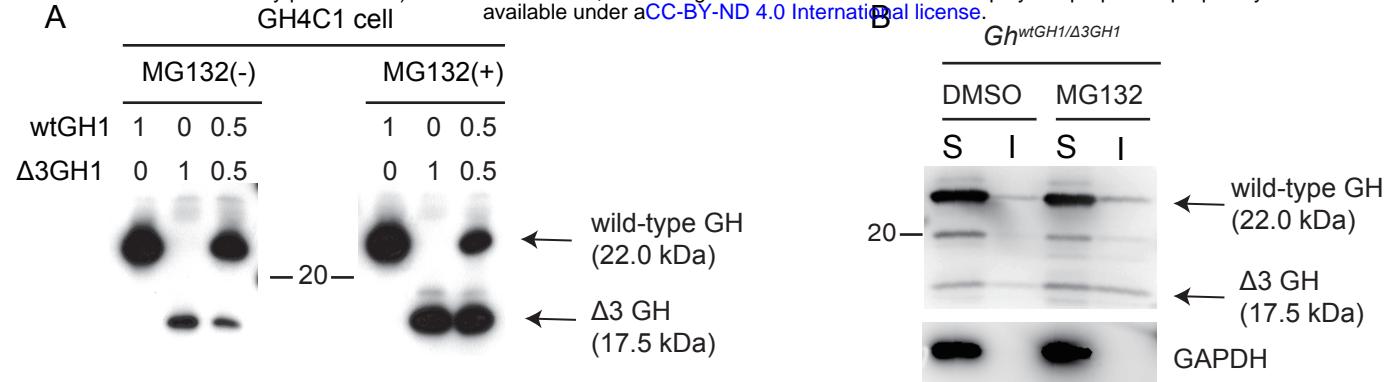


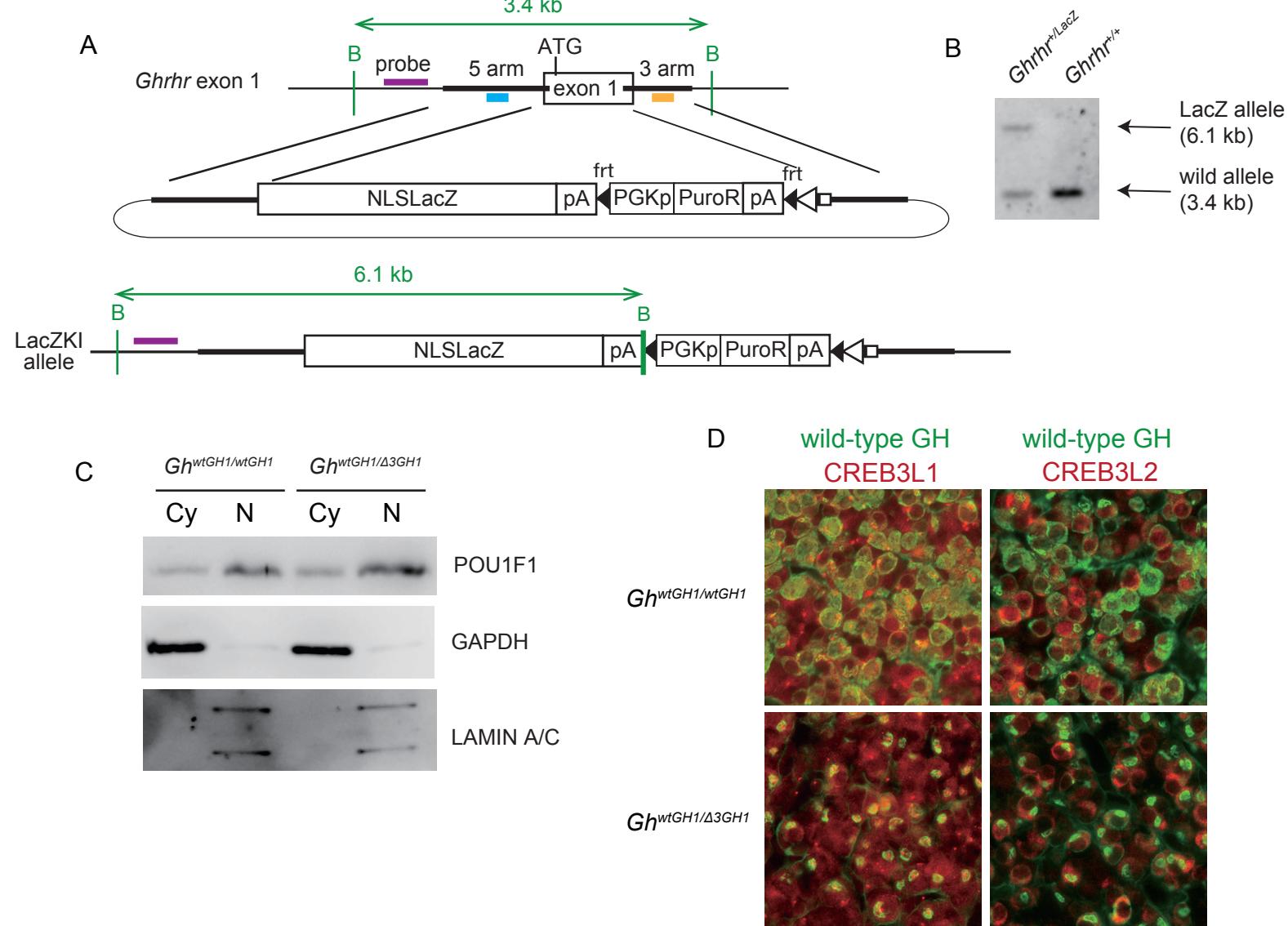
F



Expanded View Figure 2

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Supplementary table 1

Sequences of primers used in this study.

| | |
|----------------|--|
| Primer 1 | AGTCCAGATTCCAAACTGCT |
| Primer 2 | AAGAAGGGTGGTCAGTGAGG |
| Primer 3 | AGAGGCTATTGGCTATGAC |
| Primer 4 | CCCTCTTCCGTAGGTGAGTA |
| Gh-5arm-F | AGCAGGGAGCTCTCATCGCGAGTCAGTGGAACATTG |
| Gh-5arm-R | CATTGCCGAGCTCTGATCTGTCACAGGACTCTG |
| Gh-3arm-F | AGTAATGATGTCGACGAGACACAAGCTG |
| Gh-3arm-R | ATATATGCAGGTCGACATAGAGACCAGAAG |
| GH1-gf1 | GCTGTCGACAGCTCACCTAGC |
| GH1-gr1 | TAGGAATTCCCTAGAACGCCACAGCTGCCCTCACAGAG |
| GH1IVS3-F | CCCAGGCGGGATGGGGA |
| GH1IVS3dsmut-R | GAGAAGGCATCCACTCACGGATT |
| GH1-cf1 | TCCTGTGGACAGCTCACCTAG |
| GH1-cr1 | CTAGAACCCACAGCTGCCCT |
| GH1ex3-f1 | TTCAGATCTGAAGAACGCTATATCCCAAAG |
| GH1ex3-r1 | TCCGATATCGGATTCTGTTGTGTTCTC |
| Ghrhr-5arm-f5 | TTCTCGTTGGAATTCTCAGCTCACCCCTC |
| Ghrhr-5arm-r5 | AATGGTGTCTGAATTCAAGCCTTC |
| Ghrhr-3arm-f6 | CAGACCTTGTCTCGAGGGTGGTGGCAG |
| Ghrhr-3arm-r6 | CAGAGGCAAACGAAAGCTCGAGTCACAG |
| Ghrhr-vf1 | CATAGATCTATGGATGGCCTGATGTGGGC |
| Ghrhr-vr1 | TAGCTCGAGCTAGCACTCAGAGGTAGCA |
| Ghrhr-CRI-f1 | CACCGAGTAAGGCTATTGGTGAAC |
| Ghrhr-CRI-r1 | AAACGTTCACCAATAGCCTTACTC |
| Ghrhr-CRI-f2 | CACCAGAAACAGCTACTGCTCCC |
| Ghrhr-CRI-r2 | AAACGGGAGCAGTAGCTGGTTCT |
| Ghrhr-CRI-f4 | CACCGGATCAAAGTCACTCAGAGA |
| Ghrhr-CRI-r4 | AAACTCTCTGAGTGACTTTGATCC |
| Ghrhr-CRI-f5 | CACCCCTCTCTGGGACTCACACC |
| Ghrhr-CRI-r5 | AAACGGTGTGAGTCCCAGAGAGAG |
| Xbp1-f1 | AGGCCAAGGGAGTGGAGTA |
| Xbp1-r1 | AGGCAACAGTGTCAAGAGTCC |
| wtGH1-qf1 | AGCCTATATCCCAAAGGAAC |
| wtGH1-qr1 | AGCAGCTCTAGGTTGGATT |
| Ghrhr-qf1 | TCTCTCACTTGGCTCAGACAC |
| Ghrhr-qr1 | TCTTCACCGTGGAGAACGTACGA |
| Actb-qf1 | TCTGTGTGGATCGGTGGCTCCA |
| Actb-qr1 | CCTGCTTGCTGATCCACATCTG |
| WZ-14 | GCTCTCTACAGGTGGATCAAG |
| WZ-17 | GCAGTTCAATCAGCTGCTTTC |
| Hspa5-qf2 | AACTGTAACAATCAAGGTCT |
| Hspa5-qr2 | CAAAAGTGAATTCAATCTGG |
| Pou1f1-vf1 | CATGGTACCATGAGTTGCCAATCTTCACCTC |
| Pou1f1-vr1 | TAAGGTACCTTATCTGCACTCTAGATGTTCC |
| Creb3l1-vf1 | CATGGTACCATGGACGCCGTTGGAACCC |
| Creb3l1-vr1 | TAGGGTACCCCTAGGAGAGTTGATGGTGG |
| Creb3l1 ex2-1 | UUUCUUUGAUGACCCUGUGCGUUUUAGAGCUAUGCUGUUUUG |
| Creb3l1 ex2-2 | GAGCACAGCUACUCCCUGAGGUUUUAGAGCUAUGCUGUUUUG |
| Creb3l2-vf1 | CATGAATTCATGGAGGTGCTGGAGAGCGG |
| Creb3l2-qr1 | TGAGAATTCTCAGAACGGTGGCGTTCACTCT |
| Luman-cf1 | CATGAATTCATGGATCCTGGTGGTCAGGAT |
| Luman-cr1 | TAGGAATTCTAACCTGAATACCTGCCCTG |
| CrebH-cf1 | CATAGATCTATGGATGGGACATAGCGGCT |
| CrebH-cr1 | TGAAGATCTCACAGCACCCCCAATGCATC |
| loxP-CRIf1 | AGGGGTATGCTATACGAAGTTATT |
| loxP-CRIr1 | AAACAATAACTCGTATAGCATAAC |