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GATA2 functions in adrenal chromaffin cells

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36

37   **Abstract**

38   Catecholamine synthesized in the sympathoadrenal system, including sympathetic

39   neurons and adrenal chromaffin cells, is vital for cardiovascular homeostasis. It has been

40   reported that GATA2, a zinc finger transcription factor, is expressed in murine

41   sympathoadrenal progenitor cells. However, a physiological role for GATA2 in adrenal

42   chromaffin cells has not been established. In this study, we demonstrate that GATA2 is

43   specifically expressed in adrenal chromaffin cells. We examined the consequences of

44   *Gata2* loss-of-function mutations, exploiting a *Gata2* conditional knockout allele crossed

45   to neural crest-specific Wnt1-Cre transgenic mice (*Gata2* NC-CKO). The vast majority

46   of *Gata2* NC-CKO embryos died by embryonic day 14.5 (e14.5) and exhibited a decrease

47   of catecholamine-producing adrenal chromaffin cells, implying that a potential

48   catecholamine defect might lead to the observed embryonic lethality. When intercrossed

49   pregnant dams were fed with synthetic adrenaline analogs, the lethality of the *Gata2* NC-

50   CKO embryos was partially rescued, indicating that placental transfer of the adrenaline

51   analogs complements the lethal catecholamine-deficiency in the *Gata2* NC-CKO

52   embryos. These results demonstrate that GATA2 participates in the development of

53   neuroendocrine adrenaline biosynthesis, which is essential for fetal survival.

54   **Introduction**

55   Catecholamines, including dopamine, noradrenaline and adrenaline, play a crucial role in

56   the development and maintenance of the cardiovascular system. In sympathoadrenal cells,

57   tyrosine hydroxylase (TH) converts tyrosine to L-DOPA, which is converted to dopamine

58   by L-aromatic amino acid decarboxylase (AADC) (Zhou et al., 1995; Rahman et al.,

59   1995). Subsequently, the enzymatic activity of dopamine β-hydroxylase (DBH) produces

60   noradrenaline (Thomas et al., 1995). In adrenal chromaffin cells, phenylethanolamine N-

61   methyltransferase (PNMT) converts noradrenaline to adrenalines (Martin et al., 2001).

62   Mice deficient in either TH or DBH have diminished catecholamine levels and exhibit

63   midgestational lethality due to cardiovascular failure (Thomas et al., 1995; Zhou et al.,

64   1995; Kobayashi et al., 1995).

65   Early embryonic neural crest cells differentiate into sympathoadrenal progenitor cells

66   in response to instructive signaling, including bone morphogenetic protein secreted from

67   the dorsal aorta (Goridis & Rohrer, 2002). Sympathoadrenal progenitors subsequently

68   migrate to their final destinations, including the sympathetic ganglia and the adrenal

69   medulla, which in turn give rise to sympathetic neurons and adrenal chromaffin cells,

70   respectively (Goridis & Rohrer, 2002). Over the years, a series of transcriptional

71   regulators have been identified that play a role in the development of adrenal chromaffin

72   cells (Unsicker et al., 2005). Among these transcriptional regulators is GATA3, which

73   belongs to the GATA family of transcription factors containing two C_4 zinc fingers that

74   serve as its DNA binding domain and recognize the cognate consensus motif

75   (A/T)GATA(A/G) (Yamamoto et al., 1990; Ko and Engel, 1993; George et al., 1994;

76   Lakshmanan et al., 1999; Lim et al., 2000). *Gata3*-deficient embryos die from

77   noradrenaline deficiency around embryonic day 10.5 (e10.5) (Pandolfi et al., 1995; Lim

78   et al., 2000; Moriguchi et al., 2006). Homozygous mutant embryonic lethality can be

79   rescued to the perinatal stage by feeding heterozygous intercrossed pregnant dams with

80   catecholamine intermediates, underscoring that GATA3 plays a critical role in

81   catecholamine biosynthesis, which itself is essential for midgestational survival (Lim et

82   al., 2000). The drug-rescued *Gata3* null-deficient perinatal embryos had far fewer adrenal

83   chromaffin cells, indicating the essential requirement of GATA3 for chromaffin cell

84   development (Lim et al., 2000).

85   A separate study has shown that GATA2 could be required for early sympathetic

86   neuronal development in chick embryos (Tsarovina et al., 2004). However, the expression

87   pattern of GATA2 and the roles played by GATA2 in adrenal chromaffin cell

88   development in mammals has not been determined. In this study, we demonstrate that

89   GATA2 is specifically expressed in murine adrenal chromaffin cells and that conditional

90   deletion of GATA2 in adrenal medullary cells by Wnt1-Cre reduces the number of

91   chromaffin cells. Consequently, the GATA2 conditionally mutant mice expired at around

92   e14.5. Notably, this embryonic lethality was partially rescued by feeding pregnant dams

93   synthetic adrenaline analogs. These results demonstrate that GATA2 participates in

94   sympathetic catecholamine biosynthesis.

95   **Results**

96   ***GATA2 is specifically expressed in adrenal medullary cells***

97   We have previously demonstrated that GATA3 is specifically expressed in TH-positive

98   adrenal chromaffin cells and plays a crucial role in the development and maintenance of

99   chromaffin cells (Lim et al., 2000; Moriguchi et al., 2006). Therefore, we examined

100   whether GATA2 and GATA3 are co-expressed in adrenal chromaffin cells. For this

101   purpose, we employed *Gata2* GFP knock-in ($Gata2^{G/+}$) and *Gata3* LacZ knock-in

102   ($Gata3^{Z/+}$) mice, in which GFP and LacZ reporter genes were inserted at the translational

103   initiation site of the *Gata2* and *Gata3* genes, respectively (van Doorninck et al., 1999;

104   Suzuki et al., 2006). The $Gata2^{G/+}$ and $Gata3^{Z/+}$ mice were intercrossed, and the recovered

105   $Gata2^{G/+}::Gata3^{Z/+}$ compound heterozygous mutant mice were examined by anti-GFP

106   and anti-β-gal co-immunohistochemical analysis. We found that GFP (GATA2) and β-

107   galactosidase (GATA3) expression were clearly colocalized in adrenal chromaffin cells

108   (Fig. 1). This result indicates that GATA2 and GATA3 are co-expressed in adrenal

109   chromaffin cells, implying that GATA2 could play a functional role in adrenal chromaffin

110   cells.

111

112   ***Neural crest-specific GATA2 deletion results in embryonic lethality***

113   *Gata2* null mice die at around e9.5 due to severe hematopoietic failure (Tsai et al. 1994;

114   Lim et al., 2012), which hampers the analysis of adrenal chromaffin cells that only

115   develop at later stages of embryogenesis. To circumvent this limitation and to address

116   possible physiological functions of GATA2 in adrenal chromaffin cells, we employed

117   Wnt1-Cre transgenic mice that direct Cre recombinase activity specifically in neural

118   crest-derived cells (Wilson et al., 2004). When the Wnt1-Cre mice were crossed with the

119   ROSA26 *LacZ* reporter strain, β-gal activity was specifically detected in chromaffin cells

120   of the adrenal medulla, confirming chromaffin cell-specific Wnt1-Cre activity (Fig. 2B).

121   We then crossed the Wnt1-Cre transgene into $Gata2^{GFP/+}$ heterozygous deficient mice

122   (Fig. 2A). Thereafter, $Gata2^{GFP/+}$::Wnt1-Cre mice were crossed to $Gata2^{f/+}$ heterozygous

123   floxed mutant mice to recover *Gata2*GFP/f::Wnt1-Cre mutants (hereafter referred to as

124   *Gata2* NC-CKO, for <u>Gata2</u> <u>n</u>eural <u>c</u>rest-specific <u>c</u>onditional <u>k</u>nock<u>o</u>ut).

125   No *Gata2* NC-CKO mice were recovered at weaning (175 two-week-old mice were

126   genotyped from 32 litters, Table 2), suggesting that GATA2 deficiency in neural crest-

127   derived tissue leads to peri- or prenatal lethality. Therefore, we conducted timed-breeding

128   experiments and embryos were collected at e11.5, e12.5, e14.5, e16.5 and e18.5. *Gata2*

129   NC-CKO embryos were recovered at approximately Mendelian expectation until e14.5,

130   while no further live *Gata2* NC-CKO mutant embryos were detected from e16.5 onward

131   (Table 2). These results indicate that most of the *Gata2* NC-CKO embryos expired

132   between e14.5~16.5. Gross observation of the e12.5 *Gata2* NC-CKO embryos showed

133   an almost normal gross physiological appearance, indicating that fetal hematopoiesis was

134   largely unaffected in the *Gata2* NC-CKO embryos and that another defect likely

135   accounted for the observed mid-embryonic lethality from defective neural crest-derived

136   tissue (Fig. 2C and 2D).

137

138   ***Reduced size of adrenal gland in Gata2 NC-CKO***

139   *Gata3*-null mutant embryos succumb to noradrenaline deficiency during the mid-

140   gestational period (Lim et al., 2000). Given the colocalization of GATA2 and GATA3 in

141   chromaffin cells (Fig. 1), we speculated that *Gata2* NC-CKO mice might also die from

142   similar catecholamine deficiency. To address this hypothesis, we histologically assessed

143   the adrenal glands of *Gata2* NC-CKO embryos at e14.5. In the HE-stained adrenal gland

144   sections, *Gata2* NC-CKO had a reduced number of basophilic medullary cells (Fig. 3B

145   and 3D), while littermate controls had more abundant medullary cells (Fig. 3A and 3C).

146   Chromogranin A, a neuroendocrine marker for adrenal chromaffin cells, was decreased

147   in the *Gata2* NC-CKO embryos in comparison with the controls (Fig. 3E and 3F).

148   Consequently, the size of the adrenal gland was reduced in the *Gata2* NC-CKO embryos

149   compared with the control embryos (compare Fig. 3A and 3B). These results suggest that

150   GATA2 plays a role in the development and/or maintenance of adrenal chromaffin cells.

151

152   ***Diminished adrenal chromaffin cells in Gata2 NC-CKO***

153   TH serves as the rate-limiting enzyme in the catecholamine biosynthetic pathway and is

154   specifically expressed in adrenal chromaffin cells (Unsicker et al., 2005). We found that

155 TH-positive chromaffin cells were significantly decreased in the adrenal gland of *Gata2*
156 NC-CKO embryos, while the control littermates had more abundant TH-positive
157 chromaffin cells (Fig. 4A-D). Quantification of the TH-immunoreactive surface area and
158 the immune-fluorescence intensity in serial sections of these embryonic adrenal glands
159 showed a significant reduction in the *Gata2* NC-CKO embryos in comparison with the
160 control littermates (Fig. 4E). These results suggest that GATA2 is important for the
161 development of chromaffin cells and maintenance of the TH expression in the mouse
162 adrenal gland.
163
164 ***Adrenaline feeding partially rescues the lethality of the Gata2 NC-CKO embryos***
165 We next asked whether the embryonic lethality of the *Gata2* NC-CKO embryos was
166 due to a presumptive adrenaline deficiency. To this end, we fed L-phenylephline (α-
167 adrenoreceptor agonist) and isoproterenol (β-adrenoreceptor agonist) in the drinking
168 water of pregnant intercrossed dams from approximately e6.5 onwards. Subsequently, we
169 analyzed the numbers of recovered mutant embryos at embryonic stages. We found that
170 feeding these adrenaline analogs to the pregnant dams efficiently extended the lifespan
171 of the *Gata2*-NCKO embryos beyond the e14.5 stage up to the perinatal period (Table 3).
172 This pharmacological adrenaline complementation rescue allowed us to examine gene
173 expression profile in the dissected adrenal glands of *Gata2* NC-CKO embryos at later
174 stages. As anticipated, GATA2 mRNA expression level was significantly diminished in
175 the adrenal gland of the e16.5 *Gata2* NC-CKO embryos, confirming efficient deletion of
176 *Gata2* in the adrenal glands (Fig. 4F). TH mRNA expression level was diminished less
177 than 37% in the *Gata2* NC-CKO adrenal gland consistent with the reduced TH-
178 immunoreactivity (Fig. 4E and 4F). We found the GATA3 mRNA expression was
179 relatively maintained in the adrenal glands of the *Gata2* NC-CKO embryos, suggesting
180 that GATA3 was not under intense regulatory influence of GATA2 in the adrenal
181 chromaffin cells (Fig 4F). These results indicate that GATA2 plays a role for maintenance
182 of the TH expression in the adrenal gland and that the embryonic lethality of *Gata2* NC-
183 CKO mice can be attributed, at least partially, to adrenaline deficiency in mid-gestational
184 embryos.
185
186 **Discussion**

187   In the present study, we discovered that GATA2 is specifically expressed in adrenal

188   chromaffin cells. The vast majority of *Gata2* NC-CKO embryos died *in utero* and showed

189   a decrease in TH expression in adrenal chromaffin cells, which possibly led to a decrease

190   in catecholamine in the embryos. GATA2 is known as an important regulator in the

191   hematopoietic system; thus, *Gata2* null- or hematopoietic cell-specific deficiency leads

192   to early (before e9.5) embryonic lethality primarily due to hematopoietic defects (Tsai et

193   al. 1994; Lim et al., 2012). In contrast, *Gata2* NC-CKO embryos did not exhibit anemia

194   (this study). Given these findings, we concluded that the fetal lethality observed in the

195   *Gata2* NC-CKO embryos is not due to hematopoietic deficiency.

196   Catecholamine plays a crucial role in cardiovascular development in mouse embryos.

197   Catecholamine deficiencies in both TH- or DBH-deficient mice leads to embryonic

198   lethality due to cardiovascular failure, which can be pharmacologically rescued by

199   feeding catecholamine analogs (Zhou et al., 1995; Thomas et al., 1995; Kobayashi et al.,

200   1995). Since the midgestational lethality of the *Gata2* NC-CKO embryos was partially

201   rescued by the catecholamine feeding, we hypothesized that the lethality of the *Gata2*

202   NC-CKO embryos could be attributable at least in part to catecholamine deficiency. We

203   did not detect apparent cardiovascular defects in the *Gata2*-NC CKO mice by histological

204   examination (data not shown). We therefore speculate that subtle changes in

205   cardiovascular function, such as inability to maintain sufficient heart rate or contractility,

206   may affect the viability of the *Gata2*-NC CKO embryos.

207   We have previously reported that *Gata3* homozygous deficient embryos showed early

208   embryonic lethality (e10.5) with a significant decrease in sympathetic neurons and

209   adrenal chromaffin cells (Pandolfi et al., 1995; Lim et al., 2000; Moriguchi et al., 2006).

210   In contrast, the *Gata2* NC-CKO embryos exhibited somewhat later embryonic lethality

211   (e14.5) with a modest decrease in chromaffin cells. These results suggest that GATA3

212   plays a dominant role to GATA2 in the maintenance or function of adrenal chromaffin

213   cells. It has been previously shown that GATA2 mRNA expression was decreased in

214   *Gata3*-deficient sympathetic progenitor cells, indicating that GATA2 expression might

215   be directly or indirectly regulated by GATA3 (Tsarovina et al., 2004). In contrast, in the

216   present study, GATA3 expression was not significantly affected in GATA2-deficient

217   chromaffin cells. These results suggest that GATA2 may function hierarchically

218   downstream of GATA3. The sustained GATA3 might account for the remaining TH

219 expression in the *Gata2*-NC CKO adrenal gland. Further elucidation of the regulatory

220 relationships between GATA2 and GATA3 and their distinct and cooperative functions

221 in adrenal chromaffin cells would be of particular value.

222   The results of this study suggest that GATA2 is involved in the development of adrenal

223 chromaffin cells. Recent studies have demonstrated that GATA2 and GATA3 are highly

224 expressed in noradrenaline-producing human primary neuroblastoma and neuroblastoma

225 cell lines and likely play a role in the proliferation of neuroblastoma cells (Boeva et al.,

226 2017). Thus, GATA2 could be a promising therapeutic target for neuroblastoma. Precise

227 elucidation of the physiological roles played by GATA2 during the development and

228 maintenance of the sympathoadrenal system may help to elucidate how GATA2

229 participates in the tumorigenesis of chromaffin cells.

230 **Experimental procedures**

231 **Mutant mice.** *Gata2* eGFP knockin (*Gata2*$^{GFP/+}$) and *Gata3 LacZ* knockin (*Gata2*$^{LacZ/+}$)

232 mice carry eGFP and *LacZ* reporter genes, which are inserted into the translational

233 initiation site of the *Gata2* and *Gata3* alleles, respectively (Fig. 2A) (Suzuki et al., 2006;

234 van Doorninck et al., 1999). The generation and characterization of the *Gata2*^{flox}

235 conditional allele was described previously (Lim et al., 2012). Wnt1-Cre transgenic mice

236 and the ROSA26 *LacZ* reporter strain of mice were supplied by Jackson Laboratory

237 (Wilson et al., 2014; Soriano et al., 1999). *Gata2* NC-CKO were generated by mating

238 *Gata2*$^{GFP/+}$::Wnt1-Cre mice and *Gata2*$^{f/+}$ or *Gata2*$^{f/G}$ mice. Offsprings genotyped as

239 *Gata2*$^{+/+}$::Wnt1-Cre or *Gata2*$^{f/+}$::Wnt1-Cre were used as controls. Genotyping primers

240 for *Gata2*^{GFP}, *Gata2*^{f}, Wnt1-Cre and ROSA26 *LacZ* are listed in Table 1. These mice

241 were maintained in the C57BL/6J genetic background. To pharmacologically

242 complement catecholamine deficiency, pregnant dams were given freshwater daily

243 containing 100 μg/ml L-phenylephrine (α-adrenoreceptor agonist) (Sigma P6126), 100

244 μg/ml isoproterenol (β-adrenoreceptor agonist) (Sigma I5627) and 2 mg/ml ascorbic acid

245 (Sigma A0278) at e 6.5 onward (Lim et al., 2000). All mice were handled according to

246 the regulations of the Standards for Human Care and Use of Laboratory Animals of

247 Tohoku Medical and Pharmaceutical University and the Guidelines for the Proper

248 Conduct of Animal Experiments from the Ministry of Education, Culture, Sports, Science

249 and Technology (MEXT) of Japan. All animal experiments were approved by the Tohoku

250   Medical Pharmaceutical University Animal Experiment Committee (registration number:

251   18034-cn).

252

253

254   **Histological analysis.** Embryonic tissues were fixed overnight in 4% paraformaldehyde

255   (PFA) at 4°C and then processed for immunostaining as frozen or paraffin-embedded

256   sections. For hematoxylin and eosin (HE) staining, serial paraffin sections (4 μm) were

257   subjected to a regular protocol. For immunofluorescence analysis, mouse anti-tyrosine

258   hydroxylase (TH) (SIGMA T1299) antibody and M. O. M. Fluorescein Kit (VECTOR

259   FMK-2201) were used. For detection of adrenal chromaffin cells, rabbit anti-

260   chromogranin A antibody (Abcam ab15160) was used. The colocalization of β-

261   galactosidase and GFP was performed using a Cy3-conjugated rabbit anti-β-galactosidase

262   antibody (Moriguchi et al., 2006) and rabbit anti-GFP antibody (Molecular Probes).

263   Fluorescence was visualized using a Leica DM inverted microscope. Separate images

264   were taken and merged using OpenLab software. Section X-gal staining of embryos was

265   performed as previously described (Lakshmanan et al., 1999).

266

267   **Quantitative real time RT-PCR (RT-qPCR).** Total RNA was extracted from the fetal

268   adrenal gland using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan). cDNA was

269   synthesized using ReverTra Ace (TOYOBO, Osaka, Japan). Quantitative real-time

270   PCR(RT-qPCR) was performed with THUNDERBIRD SYBR qPCR Mix (TOYOBO,

271   Osaka, Japan) on CFX96 TouchTM Detection System (Bio-Rad Laboratories, Hercules,

272   CA). The primers used in the RT-qPCR are listed in Table 1.

273

274   **Morphometric analysis**

275   Tyrosine   hydroxylase   immunoreactivity-positive   surface   area   and   the

276   immunofluorescence intensity of the equally thresholded area was morphometrically

277   quantified using ImageJ 1.53a software (https://imagej.nih.gov/ij/download.html). Four

278   sections of bilateral adrenal glands from five embryos from each genotype were subjected

279   to analysis. Measurements from wild-type littermate control mice were arbitrarily set at

280   100%.

281

287

288   **References**

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369

370   **Figure legends**

371   **Figure 1. GATA2 and GATA3 expression in the adrenal medulla.** The GFP (i.e.,

372   GATA2) and β-galactosidase (i.e., GATA3) expression were colocalized in the adrenal

373   medulla cells of *Gata2*^GFP/+::*Gata3*^Z/+ compound heterozygous mutant mice (2-week-old).

374   Scale bar: 100 μm.

375

376   **Figure 2.** (A) Schematic diagram of *Gata2*^{GFP}, *Gata2*^{f} and recombined *Gata3*$^-$ null alleles.

377   *Gata2*^{GFP} functions as a null allele. Primers used to detect the *Gata2*^{f} allele are depicted.

378   (B) Activity of Wnt1-Cre indicated by LacZ expression (blue signal) is detected in the

379   adrenal chromaffin cells of Wnt1-Cre::ROSA26 *LacZ* e12.5 embryos. The dotted line

380   indicates the outer margin of the primordial adrenal gland. VC; vertebral cartilage; K,

381   kidney. (C and D) Gross observation of *Gata2*$^{GFP/f}$::Wnt1-Cre (*Gata2* NC-CKO) and

382   control littermate embryos at e12.5. *Gata2* NC-CKO embryos show normal outer

383   appearance. Asterisks indicate fetal liver. Representative images from analysis of three

384   e12.5 embryos in each group are depicted. Scale bar: 100 μm.

385

386   **Figure 3.** (A and B) The size of the adrenal medullary region (encircled with red dotted

387   lines) was smaller in the *Gata2* NC-CKO than in the littermate control embryos at e14.5

388   stage. *Gata2* NC-CKO showed a reduced population of basophilic medullary cells, while

389   the control embryos showed more abundant medullary cells (arrowheads in C and D;

390   large magnification of the rectangles in A and B). (E and F) Chromogranin A-positive

391   adrenal chromaffin cells were decreased in the *Gata2* NC-CKO in comparison with the

392   control embryos. Representative images from three e14.5 embryos in each group are

393   depicted. Scale bars are 200 μm (A and B) and 100 μm (C, D, E and F).

394

395   **Figure. 4.** Tyrosine hydroxylase (TH)-positive chromaffin cells were significantly

396   reduced in the adrenal gland of *Gata2* NC-CKO embryos at e14.5 (B and D). The control

397   littermates showed more abundant TH-positive cells (A and C). Representative images

398   from analysis of three embryos in each group are depicted. Scale bars are 100 μm (A and

399   B) and 50 μm (C and D). (E) Quantification of relative TH-positive surface area and

400   intensity of TH-immunofluorescence (TH-if). Data are presented as mean±SD (n=3 in

401   each group). (F) mRNA levels in the adrenal gland of individual e16.5 embryos

402   (normalized to GAPDH mRNA) were assayed by RT-qPCR. Data are presented as

403   mean±SD (n=5 in each group). The statistical significance of the differences between

404   *Gata2* NC-CKO and the control is indicated (*P<0.05, ** P<0.01; Student's t-test). N.S.;

405   not significant.

406   **Table 1.** Sequence of primers used for genotyping

Gene	Sense primer	Antisense primer	Assay
Gata2 flox	TCCGTGGGACCTGTTTCCTTAC	GCCTGCGTCCTCCAACACCTCTAA	genotyping
GFP	CTGAAGTTCATCTGCACCACC	GAAGTTGTACTCCAGCTTGTGC	genotyping
Wnt1-Cre	CAG CGC CGC AAC TAT AAG AG	CAT CGA CCG GTA ATG CAG	genotyping
ROSA26 *LacZ*	AAAGTCGCTCTGAGTTGTTAT	GCGAAGAGTTTGTCCTCAACC	genotyping
Th	AGTTCTCCCAGGACATTGGACTT	ACACAGCCCAAACTCCACAGT	RT-qPCR
Gata2	ACCTGTGCAATGCCTGTGGG	TTGCACAACAGGTGCCCGCT	RT-qPCR
Gata3	GGTGGACGTACTTTTTAACATCGA	CCCTGACGGAGTTTCCGTAG	RT-qPCR
Gapdh	CCTGCACCACCAACTGCTTA	TCATGAGCCCTTCCACAATG	RT-qPCR

407

408 **Table 2.** Genotyping frequency of *Gata2* NC-CKO progenies from *Gata2*[G/+]::Wnt1-Cre

409 and *Gata2*[f/+] intercrosses. The observed and expected number of *Gata2* NC-CKO

410 embryos and the total number of littermates are denoted. The expected number of *Gata2*

411 NC-CKO embryos was estimated as one-eighth of the total number of littermates.

Stage	Number of *Gata2* NC-CKO observed (expected)	Number of littermates
E11.5	2 (2)	16
E12.5	3 (2.5)	20
E14.5	3 (4)	32
E16.5	0 (1.9)	15
E18.5	0 (2.25)	18
P14	0 (21.9)	175

412

413

414   **Table 3.** Genotyping frequency of *Gata2* NC-CKO progenies from breeding

415   *Gata2*$^{G/+}$::Wnt1-Cre and *Gata2*$^{f/G}$ intercrosses with catecholamine feeding. **Drug; L-

416   phenylephrine and isoproterenol feeding. The expected number of *Gata2* NC-CKO

417   embryos was estimated as one-sixth of the total number of littermates.

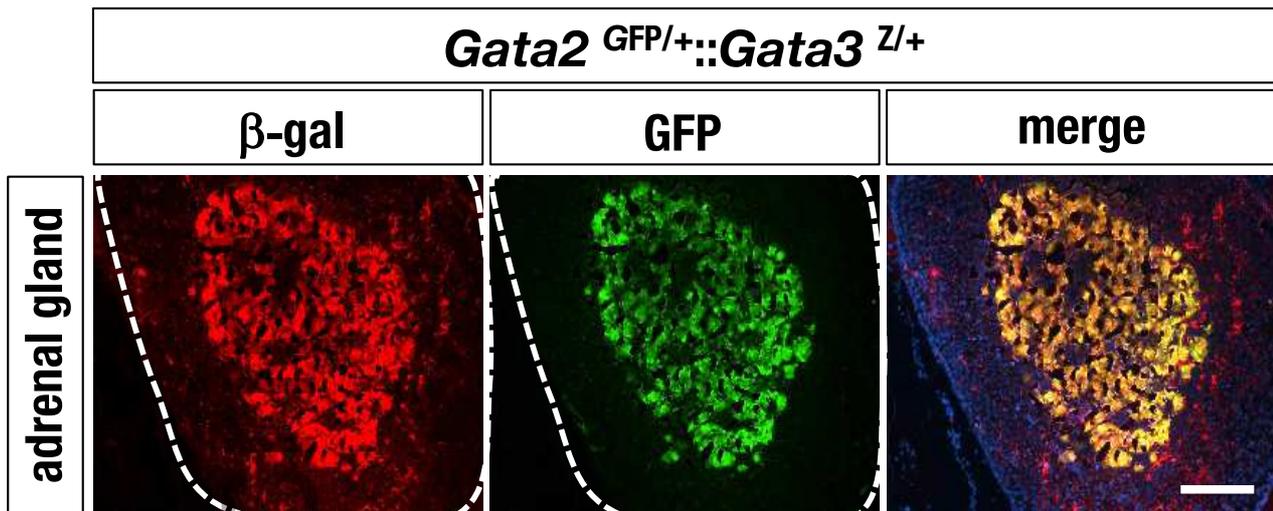| Stage	*Drug	Number of *Gata2* NC-CKO observed (expected)	Number of littermates
E14.5	(+)	4 (3)	18
E15.5	(+)	7 (5.8)	35
E16.5	(+)	5 (6)	36
E18.5	(+)	5 (5.4)	32

418

Figure 1. GATA2 and GATA3 expression in adrenal medulla. The GFP (i.e., GATA2) and β-galactosidase (i.e., GATA3) expression were co-localized in the adrenal medulla cells of the *Gata2*GFP/+::*Gata3*Z/+ compound heterozygous mutant mice (2-week-old). Scale bar: 100μm.
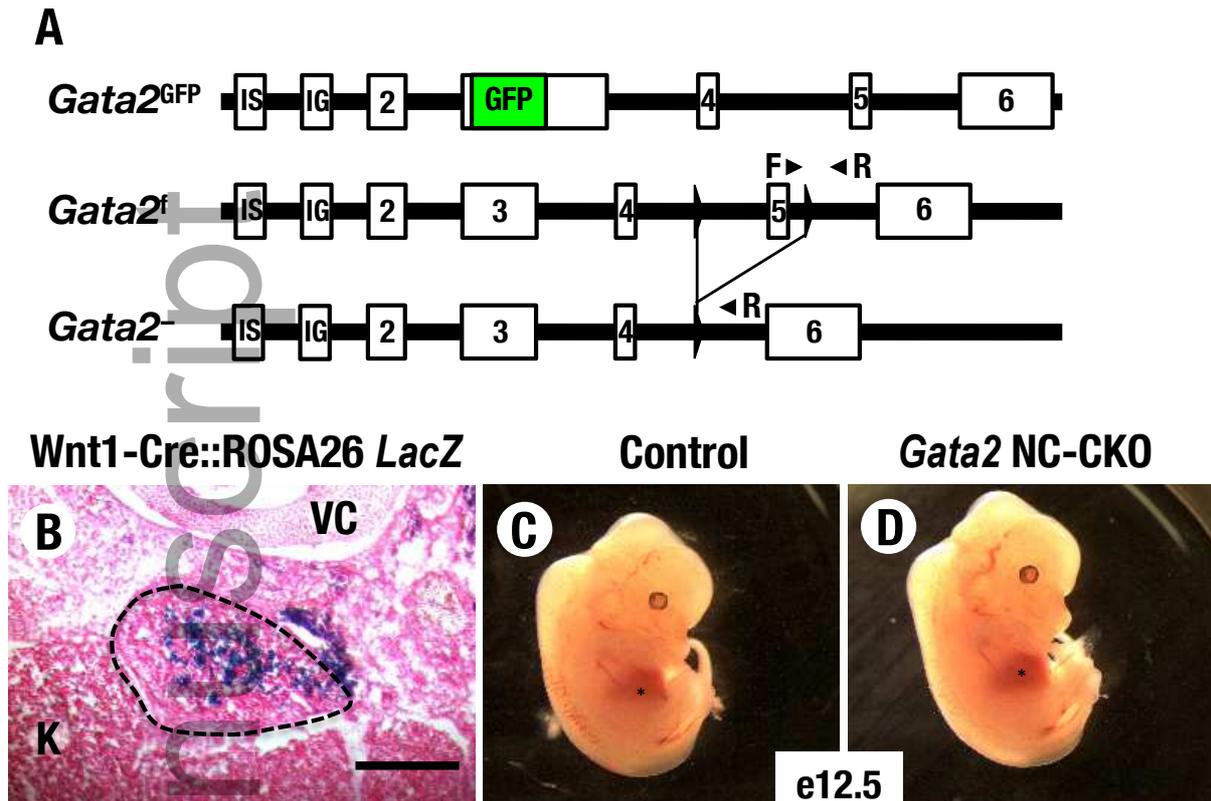
A

$Gata2^{GFP}$ | IS | IG | 2 | GFP | | 4 | 5 | 6 |

$Gata2^f$ | IS | IG | 2 | 3 | 4 | F▶ ◀R 5 | 6 |

$Gata2^-$ | IS | IG | 2 | 3 | 4 | ◀R 6 |

Wnt1-Cre::ROSA26 *LacZ* **Control** ***Gata2* NC-CKO**

B
VC
K

C

D

e12.5

Figure 2. (A) Schematic diagram of *Gata2*^{GFP}, *Gata2*^{f} and recombined *Gata3*$^-$ null alleles. *Gata2*^{GFP} functions as a null allele. Primers used to detect the *Gata2*^{f} allele are depicted. (B) Activity of Wnt1-Cre indicated by LacZ expression (blue signal) is detected in the adrenal chromaffin cells of Wnt1-Cre::ROSA26 *LacZ* e12.5 embryos. The dotted line indicates the outer margin of the primordial adrenal gland. VC; vertebral cartilage; K, kidney. (C and D) Gross observation of *Gata2*$^{GFP/f}$::Wnt1-Cre (*Gata2* NC-CKO) and control littermate embryos at e12.5. *Gata2* NC-CKO embryos show normal outer appearance. Asterisks indicate fetal liver. Representative images from analysis of three e12.5 embryos in each group are depicted. Scale bar: 100μm.
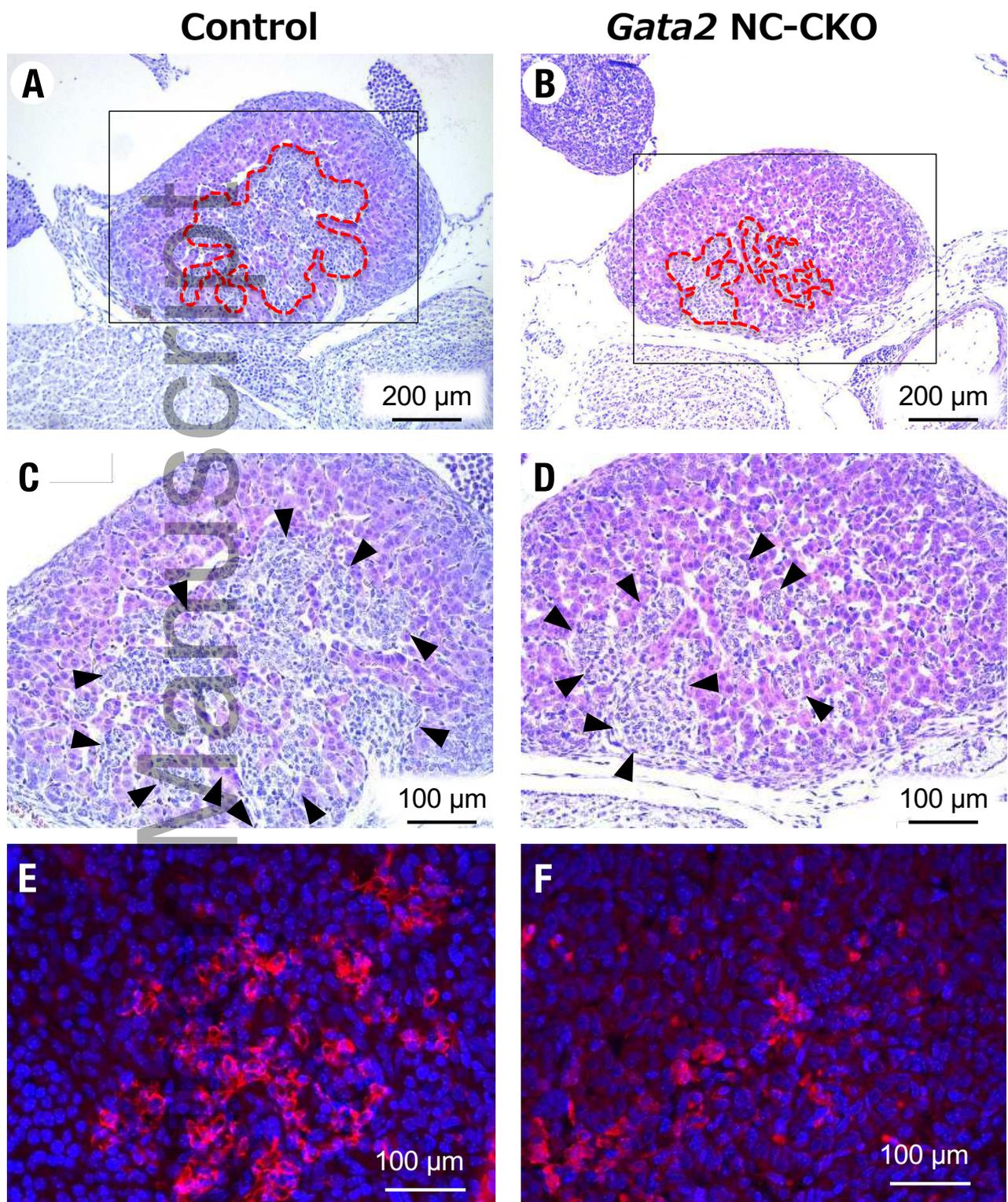
Figure 3. (A and B) The size of adrenal medullary region (encircled with red dotted lines) was smaller in the *Gata2* NC-CKO than in the littermate control embryos at e14.5 stage. *Gata2* NC-CKO showed reduced population of basophilic medullary cells, while the control embryos showed more abundant medullary cells (arrow heads in C and D; large magnification of the rectangles in A and B). (E and F) Chromogranin A-positive adrenal chromaffin cells were decreased in the *Gata2* NC-CKO in comparison with the control embryos. Representative images from analysis of three e14.5 embryos in each group are depicted. Scale bars are 200 µm (A and B) and 100 µm (C, D, E and F).
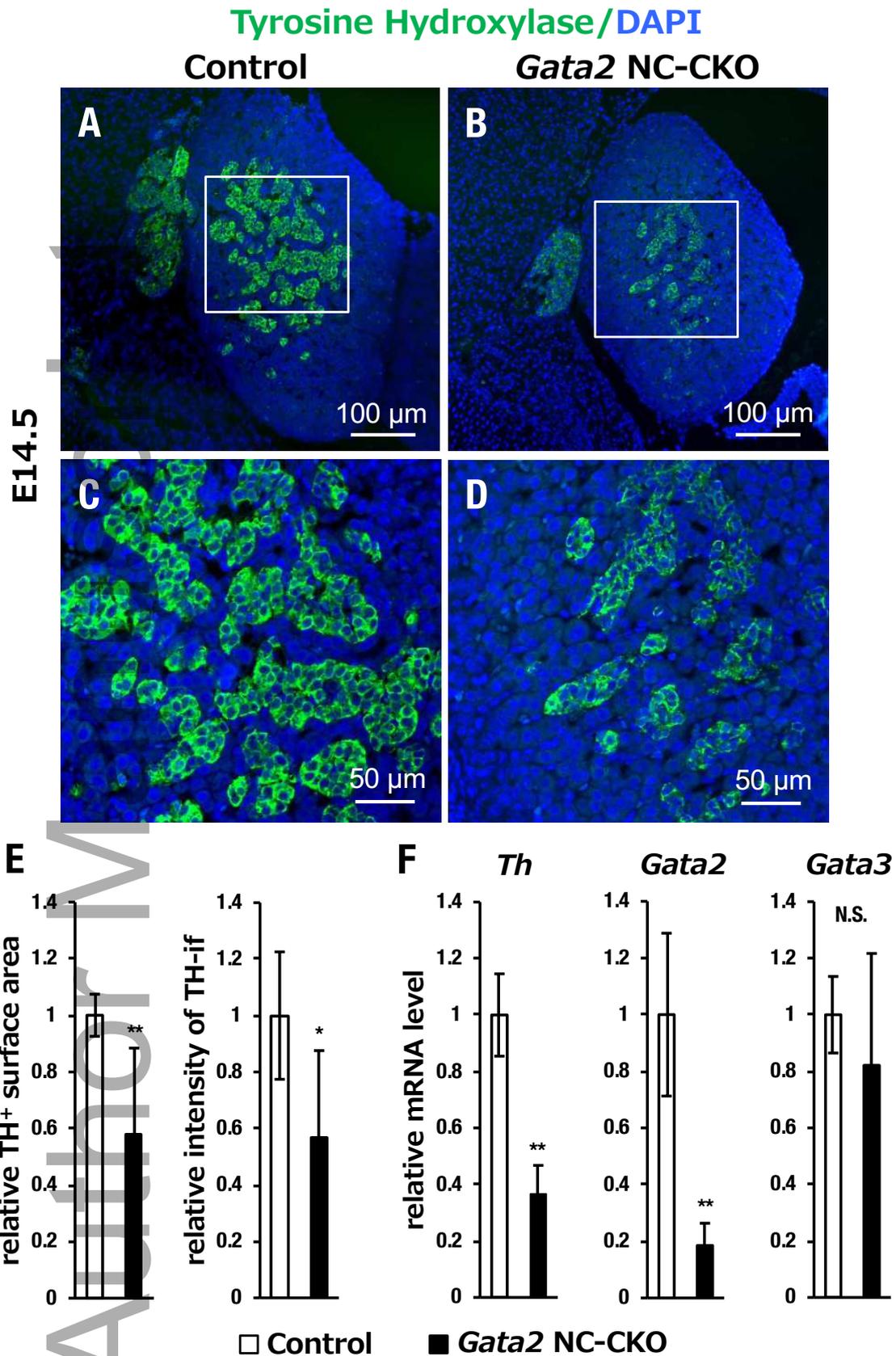
Figure 4. Tyrosine hydroxylase (TH)-positive chromaffin cells were significantly reduced in the adrenal gland of *Gata2* NC-CKO embryos at e14.5 (B and D). The control littermates showed more abundant TH-positive cells (A and C). Representative images from analysis of three embryos in each group are depicted. Scale bars are 100 μm (A and B) and 50 μm (C and D). (E) Quantification of relative TH-positive surface area and intensity of TH-immunofluorescence (TH-if). Data are presented as mean±SD (n=3 in each group). (F) mRNA levels in the adrenal gland of individual e16.5 embryos (normalized to GAPDH mRNA) were assayed by RT-qPCR. Data are presented as mean±SD (n=5 in each group). The statistical significant differences between *Gata2* NC-CKO and the control is indicated (*$P<0.05$, ** $P<0.01$; Student's t-test). N.S.; not significant.