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Author(s)	Kurosaka, Hiroshi; Mushiake, Jin; Mithun, Saha et al.
Citation	
Version Type	AO
URL	https://hdl.handle.net/11094/79001
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1	Synergistic role of retinoic acid signaling and Gata3 during primitive choanae formation
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#### 28 Abstract

29Developmental defects of primitive choanae, an anatomical path to connect the embryonic nasal and 30 oral cavity, result in disorders called choanal atresia, which are associated with many congenital 31diseases and require immediate clinical intervention after birth. Previous studies revealed that 32reduced retinoid signaling underlies the etiology of choanal atresia. In the present study, by using 33 multiple mouse models which conditionally deleted *Rdh10* and *Gata3* during embryogenesis, we 34showed that Gata3 expression is regulated by retinoid signaling during embryonic craniofacial 35development and plays crucial roles for development of the primitive choanae. Interestingly, Gata3 36 loss of function is known to cause hypoparathyroidism, sensorineural deafness and renal disease 37(HDR) syndrome, which exhibits choanal atresia as one of the phenotypes in humans. Our model 38partially phenocopies HDR syndrome with choanal atresia, and is thus a useful tool for investigating 39 the molecular and cellular mechanisms of HDR syndrome. We further uncovered critical synergy of 40Gata3 and retinoid signaling during embryonic development, which will shed light on novel 41molecular and cellular etiology of congenital defects in primitive choanae formation.

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# 43 Introduction

44	Craniofacial defects account for approximately 30% of all congenital anomalies, a rate largely due to
45	the complexity of craniofacial development (1). Although the etiology and pathogenesis of cleft lip
46	and/or palate are relatively well investigated (2), the developmental origins of many craniofacial
47	anomalies are not well understood at either the molecular or cellular level. Choanal atresia (CA) is a
48	craniofacial malformation characterized by a blocked nasal airway (3, 4). The incidence of CA is 1
49	in 5000 live births, and in cases of bilateral CA can be lethal. Therefore CA typically requires
50	immediate intervention (5). In spite of this clinical significance, the basic etiology and pathogenesis
51	of CA remains elusive. Although theoretical mechanisms have been proposed to explain the basis of
52	choanal atresia, very little basic research using animal models has been performed, and conclusive
53	answers have not been provided (6). It is well known that CA can occur in concert with various
54	genetic disorders such as CHARGE syndrome and Crouzon syndrome (3). This indicates that
55	multiple genetic pathways may underlie the development of primitive choanae formation and
56	furthermore that disruption of these signaling pathways could result in CA. However, our knowledge
57	of the interplay among molecular pathways during primitive choanae development is still
58	rudimentary. In past studies, reduced retinoic acid signaling was proven to result in defects in
59	primitive choanae formation and choanal atresia (4, 7). We previously identified multiple genes
60	which exhibit significantly altered spatiotemporal patterns of expression in embryos with reduced

61	retinoic acid signaling (4). For example, the transcription factor Gata3, was significantly
62	downregulated. Gata3 is normally expressed at high levels in the developing facial processes during
63	primitive choanae development in the normal situation, and interestingly, GATA3 mutation in
64	humans are associated with hypoparathyroidism, sensorineural deafness and renal disease (HDR)
65	syndrome, which can also include craniofacial anomalies, such as CA (8-10). Multiple studies have
66	investigated the role of Gata3 in the parathyroid (11), cochlea (12, 13) and nephron duct (14) in
67	order to determine the cellular and molecular mechanism underlying the development of each
68	phenotype in HDR syndrome. In contrast, the pathogenesis of CA in HDR syndrome remains
69	unknown. Here we generated tamoxifen-inducible <i>Gata3</i> knockout mice $(Ert2Cre:Gata3^{fx/fx})$ to
70	elucidate the role of <i>Gata3</i> in craniofacial and primitive choanae development. We discovered that
71	temporal excision of Gata3 during embryonic frontonasal development resulted in reduced cell
72	division of both epithelial and mesenchymal cells, which led to failure of the development of
73	primitive choanae. We also uncovered a critical interaction between retinoid and Gata3 function
74	during craniofacial development, the disruption of which underlies the etiology of choanal atresia.
75	
76	Results
77	Reduced <i>Gata3</i> expression is associated with choanal atresia (CA) in <i>Ert2Cre:Gata3<sup>fx/fx</sup></i>

78 embryos

79	To discover signaling pathway(s) potentially involved in regulating primitive choanae formation, we
80	analysed RNA-seq datasets we generated from the maxillary complex of E11.5 <i>Ert2Cre:Rdh10<sup>fx/fx</sup></i>
81	and control littermate embryos, which exhibit CA (4). Putative protein interactome analyses were
82	performed using the genes whose expression was either significantly reduced (blue) or elevated
83	(red), with known associated proteins (green) (Figure 1A and B). From this analysis we detected
84	several networks or clusters which included Gata3 and whose expression was significantly reduced
85	in <i>Ert2Cre:Rdh10<sup>fx/fx</sup></i> mice (Figure 1B). In parallel we also assessed the expression of <i>Gata3</i> in the
86	frontonasal process of E11.5 embryos via in situ hybridization. While strong Gata3 expression could
87	be observed in the lambdoidal region in control E11.5 where the lateral nasal prominence (LNP),
88	medial nasal prominence (MNP) and maxillary portion of the first pharyngeal arch (MXP), embryos,
89	a substantial reduction was evident in E11.5 Ert2Cre:Rdh10 <sup>fx/fx</sup> embryos (Figure1C and D). These
90	results identified retinoid signaling as a candidate regulator of Gata3 expression in the developing
91	lambdoidal region, which anatomically presages the developing primitive choanae.
92	
93	Expression pattern of <i>Gata3</i> mRNA and <i>RARE-LacZ</i> during primitive choanae formation
94	To further assess the correlation between Gata3 expression and retinoid signaling during primitive
95	choanae development, we performed <i>in situ</i> hybridization for <i>Gata3</i> in parallel with $\beta$ galactosidase
96	staining of RARE-LacZ embryos, which report retinoid signaling activity throughout the early stages

97	during frontonasal development. Strong Gata3 expression was detected in the lambdoidal region in
98	E11.0 embryos (Figure 2A). Frontal sections of stained embryos revealed that Gata3 was expressed
99	in both the epithelium and mesenchyme of the developing medial nasal and lateral nasal process at
100	the position where the medial and lateral nasal processes were fusing (Figure 2B and D, red
101	arrowhead). RARE-LacZ activity was detected around the developing lambdoidal region at E11.0
102	(Figure 2C), and frontal sections revealed an overlap of RARE-LacZ expression with Gata3
103	expression at the junction where the medial and lateral nasal processes fuse (Figure 2B and D, red
104	arrowhead). As development proceeded, the expression domain of Gata3 became prominent
105	specifically in the primitive choanae (Figure 2E, red arrowhead) and became restricted
106	predominantly in the mesenchyme (Figure 2F). The expression of RARE-LacZ likewise became
107	prominent around the primitive choanae at the E12.0 stage (Figure 2G) with intense retinoid
108	signaling activity present in both the epithelium and mesenchyme (Figure 2H). The overlap in
109	expression of Gata3 and retinoid signaling around the primitive choanae (Figure 2F and H, red
110	arrowhead), implying a synergistic role for these factors in regulating primitive choanae formation
111	and development.
112	

# 113 Elimination of *Gata3* disrupts formation of primitive choanae

114 To functionally test the global role of *Gata3* during primitive choanae formation, tamoxifen-

115	inducible Gata3 knockout mice were produced by intercrossing Gata $3^{fx/fx}$ mice (15) with Ert2Cre
116	mice to generate $Ert2Cre:Gata3^{fx/fx}$ mice (16). E9.5 tamoxifen treatment results in reduction but not
117	elimination of intact Gata3 in the developing choanae at E12.5 in Ert2Cre:Gata3 <sup>fx/fx</sup> embryos, as
118	shown by <i>in situ</i> hybridization using RNA oligo probe against exon 4 of Gata3 (Figure 3A and B).
119	Tamoxifen administration at E9.5 results in 31% of Ert2Cre:Gata3 <sup>fx/fx</sup> embryos exhibiting either
120	choanal atresia or choanal stenosis at E13.5 (Table 1). A further 34% were lethal at the same stage
121	(Table 1). Some of the <i>Ert2Cre:Gata3</i> <sup>fx/fx</sup> embryos presented with agenesis of the primitive choanae,
122	while control <i>Gata3<sup>fx/fx</sup></i> littermates exhibited normal choanae development (Figure 3C-F). Frontal
123	histological sections of <i>Ert2Cre:Gata3<sup>fx/fx</sup></i> embryos also confirmed the nasal cavity was blocked
124	(Figure 3G and H). These results strongly indicate that Gata3 expression during embryonic
125	craniofacial development is critical for primitive choanae development, and that Gata3 loss-of-
126	function results in defects in primitive choanae formation.
127	
128	Nasal cavity morphogenesis and shape are malformed in <i>Ert2Cre:Gata3<sup>fx/fx</sup></i> embryos
129	To further characterize malformation of the choanae and investigate the role of Gata3 in nasal cavity
130	formation, we generated three dimensional reconstructions of the volumetric shape of the nasal
131	cavity in red and oral cavity in yellow. Micro CT scanning was therefore performed on both E13.5
132	control $Gata3^{fx/fx}$ and $Ert2Cre:Gata3^{fx/fx}$ embryos to which tamoxifen was administered at E9.5. The

133	shape of the nasal cavity was continuous from the nostril through the end of the nasal cavity and
134	showed clear connection to the oral cavity in the control Gata3 <sup>fx/fx</sup> embryos (Figure 4A,B and C),
135	whereas in mutant embryos, the nasal cavity was discontinuous and abnormally shaped and lacked
136	the connection with the oral cavity (Figure 4D E and F). These results indicate that Gata3 plays a
137	critical role in primitive choanae formation as well as continuous nasal cavity development.
138	
139	Reduced cell proliferation and increased cell death underly the etiology of CA, CS and nasal
140	cavity deformation in <i>Ert2Cre:Gata3<sup>fx/fx</sup></i> mice
141	We previously revealed that reduced cell proliferation is a primary cause of CA during craniofacial
142	development (4). We therefore assessed the pattern of cell proliferation during primitive choanae
143	development via phosphorylated histone H3 (PHH3) immunostaining of sections through the
144	frontonasal process. In control E11.5 embryos, both the nasal epithelium and craniofacial
145	mesenchyme contained PHH3-positive cells (Figure 5A and B). The number of PHH3-positive cells
146	was significantly reduced in Ert2Cre: Gata3 <sup>fx/fx</sup> embryos both in the epithelium and mesenchyme
147	cells during nasal cavity development (Figure 5C-F). Moreover, the nasal epithelial cells were
148	irregularly aligned at the position of invagination in Ert2Cre:Gata3 <sup>fx/fx</sup> embryos (Figure 5D). In
149	parallel with analyses of alterations in proliferation we also examined the frontonasal processes for
150	the induction of cell death. TUNEL staining revealed an endogenously low level of cell death in the

151	frontonasal process of control embryos (Figure 5G and H). In contrast, a significant elevation in the
152	level of cell death was detected in both the frontonasal epithelium and mesenchyme in
153	Ert2Cre:Gata3 <sup>fx/fx</sup> embryos (Figure 5I,J,K and L). These results indicate that Gata3 regulates
154	proliferation and cell survival which are crucial for normal primitive choanae and nasal cavity
155	development. Interestingly, the overall spatiotemporal patterns of cell death and proliferation in
156	<i>Ert2Cre:Gata3<sup>fx/fx</sup></i> embryos closely resembled that in <i>Ert2Cre:Rdh10<sup>fx/fx</sup></i> embryos (4). This further
157	substantiates the notion that retinoid signaling and Gata3 play central and perhaps synergistic roles
158	in the development of the primitive choanae and nasal cavity.
159	
160	Vitamin A deficient diet in dams results in early lethality of <i>Ert2Cre:Gata3<sup>fx/fx</sup></i> embryos
160 161	<b>Vitamin A deficient diet in dams results in early lethality of</b> <i>Ert2Cre:Gata3<sup>fx/fx</sup></i> <b>embryos</b> To test for synergy between retinoid signaling and <i>Gata3</i> function during embryogenesis, the
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160 161 162 163	Vitamin A deficient diet in dams results in early lethality of <i>Ert2Cre:Gata3<sup>fx/fx</sup></i> embryos         To test for synergy between retinoid signaling and <i>Gata3</i> function during embryogenesis, the         pregnant dams of <i>Ert2Cre:Rdh10<sup>fx/fx</sup></i> embryos were placed on a vitamin A deficient diet from before         mating and subsequently administered tamoxifen at E9.5 and the embryos were collected at E13.0.
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<ol> <li>160</li> <li>161</li> <li>162</li> <li>163</li> <li>164</li> <li>165</li> <li>166</li> <li>167</li> </ol>	Vitamin A deficient diet in dams results in early lethality of <i>Ert2Cre:Gata3<sup>fc/fx</sup></i> embryos         To test for synergy between retinoid signaling and <i>Gata3</i> function during embryogenesis, the         pregnant dams of <i>Ert2Cre:Rdh10<sup>fc/fx</sup></i> embryos were placed on a vitamin A deficient diet from before         mating and subsequently administered tamoxifen at E9.5 and the embryos were collected at E13.0.         We observed a significant elevation in the incidence of developmental malformations, including         lethality, in the <i>Ert2Cre:Gata3<sup>fc/fx</sup></i> embryos on a vitamin A deficient diet (Table 1). Most of the <i>Ert2Cre:Gata3<sup>fc/fx</sup></i> embryos whose mothers consumed a vitamin A deficient diet for more than 20         days showed signs of early lethality (no blood or heartbeat and opaque tissue, or resorption at E13.0)

169 act synergistically during embryogenesis to ensure embryo survival.

171	Discussion
172	Investigation of critical factors associated with retinoid signaling during primitive choanae
173	formation
174	Multiple signaling pathways have been identified as key regulators of embryonic craniofacial
175	development and retinoid signaling is one of the best studied molecular pathways (17, 18).
176	Eliminating <i>Rdh10</i> , a rate-limiting enzyme in the synthesis of retinoic acid causes severe craniofacial
177	defects, including CA (4, 19, 20). Using a temporal conditional model of retinoid deficiency we
178	previously identified a critical role for <i>Rdh10</i> and retinoid signaling in formation of the primitive
179	choanae. (4). However, the molecular etiology of CA has not been fully elucidated and thus requires
180	further investigation. From protein interactome analyses using the results obtained from RNAseq of
181	Rdh10 mutant and control embryo maxillary complexes (4), we discovered one network or cluster
182	which pivoted on Gata3 and exhibited significantly reduced expression (Figure 1A and B). Previous
183	reports have shown that Gata3 loss-of-function in mice results in early lethality around E11 with
184	severe defects including craniofacial anomalies (21, 22). In addition, GATA3 mutations in human
185	result in hypoparathyroidism, sensorineural deafness and renal disease (HDR) syndrome, a
186	constellation of anomalies that also includes craniofacial defects such as CA (8, 23). The fact that

187	Gata3 is strongly expressed around the developing frontonasal process in combination with its
188	connection to human disease motivated us to investigate the role of Gata3 in CA and its association
189	with retinoid signaling (24). First, we confirmed a substantial reduction of Gata3 expression around
190	the developing primitive choana in E11.5 <i>Rdh10</i> mutant mouse embryos (Figure 1C and D). These
191	results strongly suggest that retinoid signaling maybe responsible for activating Gata3 in specific
192	tissues during craniofacial development, especially around the primitive choana. Additionally, the
193	spatiotemporal patterns of Gata3 expression and Rare-LacZ reporter activity overlap during
194	craniofacial development, especially around the frontonasal process. Notably, the ventral part of the
195	developing nasal epithelium in the frontonasal process, a critical tissue for epithelial invagination
196	and for forming oronasal membrane (3), co-expresses both Gata3 and Rare-LacZ reporter (Figure
197	2B and D, red arrowhead). The overlapping expression of <i>Gata3</i> and <i>Rare-LacZ</i> reporter activity in
198	the craniofacial epithelium implies a synergistic role for these molecular pathways in the process of
199	primitive choanae development.
200	
201	Disturbed expression of <i>Gata3</i> results in congenital defects including CA
202	To evaluate the functional role of Gata3, we utilized tamoxifen-inducible knock out mice
203	( <i>Ert2Cre;Gata3</i> <sup>fx/fx</sup> ) in this study (16). Importantly, when tamoxifen was administered at E9.5, 31%
204	(11/35) of the E13.5 <i>Ert2Cre; Gata3<sup>fx/fx</sup></i> embryos exhibited CA or choanal stenosis (CS) which

205	exhibit narrower primitive choanae (Table 1, Figure 3C-H). These results clearly demonstrate an
206	essential role of Gata3 in primitive choanae development. Interestingly, the expression of mRNA
207	containing Gata3 exon 4, which is located between the loxP sites, was not completely eliminated
208	around the developing choanae (Figure 3A and B). Additionally, 34% (12/35) of E13.5
209	Ert2Cre;Gata3 <sup>fx/fx</sup> embryos exhibited early lethality. Gata3 null mice are embryonic lethal around
210	E11.5 (21). These results indicate that <i>Ert2Cre;Gata3<sup>fx/fx</sup></i> embryos retain some limited expression of
211	Gata3 which enables them to survive long enough to present with CA and CS at E13.5. The fact that
212	the remaining 34% of <i>Ert2Cre; Gata3<sup>fx/fx</sup></i> embryos did not show either CA or CS suggests that there is
213	a threshold level of Gata3 expression required for normal primitive choanae development, below the
214	defects occur.
215	
216	The cytological role of <i>Gata3</i> in primitive choanae and nasal cavity development
217	Nasal cavity development requires continuous epithelial invagination and branching morphogenesis
218	(25). Multiple genes and their pathways, such as $Fgf$ and retinoid signaling, are known to be
219	involved in this process (4, 26). In the present study, we discovered severe nasal cavity deformation
220	in <i>Ert2Cre;Gata3</i> <sup>fx/fx</sup> embryos (Figure 4). We also found a significant reduction of cell proliferation
221	in both the epithelium and mesenchyme surrounding the developing nasal cavity (Figure 5A-F).
222	Epithelial proliferation is one mechanism known to be critical for epithelial folding and branching

223	morphogenesis in various organs, such as the salivary glands and lungs (27). Interestingly, Gata3 has
224	been reported to play critical roles in ductal invasion during mammary gland development (28). In
225	addition to alterations in proliferation significant elevation in cell death was also observed in
226	Ert2Cre;Gata3 <sup>fx/fx</sup> embryos, especially in the ventral portion of the nasal cavity in the frontonasal
227	processes (Figure 5G-L). Interestingly, a lack of retinoid signaling also causes similar cellular and
228	developmental defects resulting in CA (4). These findings strongly suggest that Gata3 regulates cell
229	proliferation and cell survival in the developing nasal cavity, and this dysregulation of balance leads
230	to nasal cavity defects, including CA and CS.
231	
232	Synergistic effect of retinoid and <i>Gata3</i> signaling in embryonic development
232 233	<b>Synergistic effect of retinoid and</b> <i>Gata3</i> <b>signaling in embryonic development</b> To evaluate the synergistic effects of <i>Gata3</i> and retinoid signaling during frontonasal and choanae
232 233 234	Synergistic effect of retinoid and <i>Gata3</i> signaling in embryonic development         To evaluate the synergistic effects of <i>Gata3</i> and retinoid signaling during frontonasal and choanae         development, we reduced the level of retinoid signaling in <i>Ert2Cre;Gata3<sup>fx/fx</sup></i> embryos by
<ul><li>232</li><li>233</li><li>234</li><li>235</li></ul>	Synergistic effect of retinoid and <i>Gata3</i> signaling in embryonic development         To evaluate the synergistic effects of <i>Gata3</i> and retinoid signaling during frontonasal and choanae         development, we reduced the level of retinoid signaling in <i>Ert2Cre; Gata3<sup>fx/fx</sup></i> embryos by         administering vitamin A deficient food to pregnant dams. Interestingly, this experimental model
<ul> <li>232</li> <li>233</li> <li>234</li> <li>235</li> <li>236</li> </ul>	Synergistic effect of retinoid and <i>Gata3</i> signaling in embryonic development         To evaluate the synergistic effects of <i>Gata3</i> and retinoid signaling during frontonasal and choanae         development, we reduced the level of retinoid signaling in <i>Ert2Cre;Gata3<sup>fx/fx</sup></i> embryos by         administering vitamin A deficient food to pregnant dams. Interestingly, this experimental model         resulted in an exaggerated malformation phenotype together with a significant increase in lethality
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<ol> <li>232</li> <li>233</li> <li>234</li> <li>235</li> <li>236</li> <li>237</li> <li>238</li> <li>239</li> </ol>	Synergistic effect of retinoid and Gata3 signaling in embryonic developmentTo evaluate the synergistic effects of Gata3 and retinoid signaling during frontonasal and choanaedevelopment, we reduced the level of retinoid signaling in Ert2Cre; Gata3 <sup>fc/fc</sup> embryos byadministering vitamin A deficient food to pregnant dams. Interestingly, this experimental modelresulted in an exaggerated malformation phenotype together with a significant increase in lethality(Table 1 and Figure 6). Interestingly, critical interactions between Gata3 and retinoid signaling hasbeen proposed for nephric duct insertion during kidney development (29). Our study however is thefirst report to show a critical interaction between Gata3 and retinoid signaling during embryonic

241 provide new knowledge about the contribution of Gata3 to craniofacial development and advance

242	our understanding	of the molecular	and cellular me	echanisms unde	rpinning	CA and CS.
	0					

243

244 Material and Methods

245 Animals

- 246 Previously reported *Ert2Cre; Gata3<sup>fx/fx</sup>* male mice were mated with *Gata3<sup>fx/fx</sup>* female mice in
- order to get *Ert2Cre; Gata3<sup>fx/fx</sup>* embryos (15, 16).  $Rdh10^{fx/fx}$  mice were derived from ES cells
- 248 generated through KOMP and maintained as previously described (4, 20). These mice are
- equivalent to C57BL/6N-Rdh10<sup>tm1a(KOMP)Wtsi</sup>. Cre-ER<sup>T2</sup> (B6.129
- 250 Gt(ROSA)26Sortm1(cre/ERT2)Tyj/J, Jax stock #008463) from the Jackson Laboratory. For
- embryonic staging, the morning of identification of the vaginal plug was defined as E0.5.
- 252 Ert2Cre and Rare-LacZ reporter mice were obtained from RIKEN BRC (STOCK Tg(RARE-
- 253 Hspa1b/lacZ)12Jrt). Vitamin A deficient diet was purchased from CLEA Japan.

254

### 255 **Protein interactome analysis**

- 256 Mouse protein interactome data was obtained from the iRefIndex database (30). After
- 257 interactions between the same genes (self-interactions) were removed, 46,512 interactions were

258	extracted. Among th	ose interactions, 316	associations that	t interacted w	ith the differentially
	8	) = -			5

- 259 expressed genes were visualized using Cytoscape software (31).
- 260

## 261 Administration of tamoxifen

- 262 In order to excise *Gata3* from the developing embryos, 40 ug/g-body of tamoxifen which was
- dissolved in 90% corn oil and 10% ethanol solution was administered by intraperitoneal
- 264 injection of pregnant  $Gata3^{fx/fx}$  dams at E9.5.
- 265

### 266 Whole mount *in situ* hybridization

- 267 Whole-mount *in situ* hybridization of mouse embryos was performed as previously described (32).
- 268 A minimum of three embryos of each genotype were examined per probe.
- 269

### 270 β-galactosidase staining

271 Staining for $\beta$ -galactosidase expression in <i>RARE-lacZ</i> reporter mice w	as performed b	y fixing	the
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- tissue in 2% formaldehyde and 0.8% glutaraldehyde solution for 30 min at 4°C followed by
- treatment with 1mg/ml X-gal (Promega, # V3941) solution for 2 hours at room temperature.

### 275 Whole-mount nuclear fluorescent imaging

276	For analyzing choanal structure, the maxilla of the embryos were fixed in 4% PFA overnight at
277	4°C. Fixed tissue were washed several times in PBS and stained directly with DAPI (Dojindo)
278	(1:1,000) in PBS overnight at 4°C and visualized with a Olympus SZX16 stereomicroscope (33).

279

# 280 Micro-CT analysis for evaluating the shape of nasal cavity

- 281 The head of E12.5 embryos were stained with 0.3% phosphotungstic acid / 70% ethanol solution
- overnight and scanned with an R\_mCT2 (Rigaku) with 10 um slice pitch. The shape of the nasal
- 283 cavity was traced and the images were processed using ITK-SNAP(General Public License) for
- 284 3D reconstruction.

285

# 286 Immunohistochemistry

Antibodies against phospho-Histone-H3 (PHH3) (#05806, 1:200, Millipore) and E-Cadherin
(#3195, 1:200, Cell Signaling Technology) were used with appropriate secondary antibodies. Cell

289	death analysis was performed using the In Situ Cell Death Detection kit (Roche, #11684795910)
290	following the manufacturer's instructions. The differences among the mean numbers of PHH3
291	and TUNEL positive cells in the epithelium and mesenchyme between control and
292	<i>Ert2Cre;Gata3<sup>fx/fx</sup></i> embryos were evaluated by Student's two-tailed unpaired t-test. P < 0.05
293	indicated statistical significance.

# 295 Statistical analysis

296 Two-proportion Z test was performed to evaluate the incidence of phenotypes (Table 1). P < 0.05

297 indicated statistical significance.

298

# 299 Acknowledgements

300 The authors thank the members of the Department of Orthodontics and Orthopedics, Graduate School

301 of Dentistry, Osaka University, for their insights and comments throughout the project. The authors

302 also deeply appreciate Dr. Sachiko Iseki at Tokyo Medical Dental University, Dr. Kazuyo Moro

303 at RIKEN IMS and Dr. Jinfang Zhu for providing the animal models used in this research. The

304 work was supported by grants-in-aid for scientific research from the Japan Society for the Promotion

305 of Science (#16K15836, 15H05687 and 19H03858 to HK). Research in the Trainor lab is supported

306 by the Stowers Institute for Medical Research.

### 308 Figures



# 318 Figure.1. Reduction of RA signaling results in reduced Gata3 expression during craniofacial

319 development. (A) The result of protein interactome analysis using the dataset of genes whose

320 expression had been previously shown by RNAseq to be altered by reduced retinoid signaling (4).

321 (B) A cluster pivoting Gata3. (C) RNA in situ hybridization of Gata3 in the developing maxillary

- 322 complex. Strong *Gata3* expression could be detected in the lambdoidal region of the developing
- 323 maxillary complex (red arrowhead). (D) Substantial reduction of Gata3 mRNA expression could be

324 observed in the retinoid deficient  $ErtCre:Rdh10^{fx/fx}$  mutant to which tamoxifen was administered at

325 E7.5.



327 Figure 2. *Gata3* and retinoid signaling during frontonasal development. (A-F) *In situ* 

328 hybridization of *Gata3* and (C-H) β galactosidase staining of the retinoid signaling reporter *Rare* 

329 LacZ in developing head. Whole mount in situ hybridization for Gata3 using embryonic head of

330 E11.0 in frontal view (A) and E12.0 in ventral view (E). Frontal section of E11.0 (B and D) and

- 331 E12.0 (F and H). Red arrowheads indicate the ventral epithelium of nasal cavity, which is important
- 332 for primitive choanae formation. MNP, medial nasal process. LNP, lateral nasal process. MXP,
- 333 maxillary process. NC, nasal cavity. MN, mandible.



## **Figure 3. Temporal reduction of Gata3 expression result in defects in primitive choanae**

development. (A and B) In situ hybridization of exon 4 of Gata3 at E12.5 in control (G) and

337 Ert2Cre; Gata3<sup>fx/fx</sup> (H) embryos. (C-F) Ventral view of developing choanae in E13.5 maxilla in

- control (C and E) and *Ert2Cre; Gata3*<sup>fx/fx</sup> (D and F) embryos. (G and H) Hematoxylin and eosin
- staining of frontal section of E13.5 heads in control (E) and *Ert2Cre; Gata3<sup>fx/fx</sup>* (F) embryos. Red
- 340 arrowheads indicate the position of developing choanae. TMX, tamoxifen treatment.





342 Figure 4. Three-dimensional reconstruction of the shape of developing nasal and oral cavity. (A

343 and D) Overlaid image of reconstructed head surface (light green), and nasal (red) and oral (yellow)

344 cavity seen from lateral view in E13.5  $Gata3^{fx/fx}$  control (A) and  $Ert2Cre; Gata3^{fx/fx}$  (D) mouse to

- 345 which tamoxifen was administered at E9.5 (B, C, E and F). 3-dimensional reconstruction of the
- shape of developing nasal (red) and oral (yellow) cavity in *Gata3*<sup>fx/fx</sup> control (B and C) and
- 347 *Ert2Cre; Gata3<sup>fx/fx</sup>* (E and F) embryo. E, eye. MX, maxilla. MN, mandible.
- 348





362 Figure 5. Cell proliferation and death in the process of development of primitive choana. (A-D)

363 Immunohistochemistry of PHH3 (magenta) and E-CADHERIN (green) of frontal section of

- developing nasal cavity in control (A and B) and *Ert2Cre;Gata3*<sup>fx/fx</sup> embryos (C and D). (G-J)
- 365 TUNEL staining to detect cell death (magenta) in developing nasal cavity in control (G and H) and
- 366 Ert2Cre; Gata3<sup>fx/fx</sup> (I and J) embryos. White dashed line indicates the boundary of nasal epithelium

367	and mesenchyme. B,D,H and J show magnified images of the ventral nasal cavity of A,C,G and
368	I, respectively. Statistical analysis was performed using the number of PHH3- (E and F) and
369	TUNEL-positive cells (K and L) in nasal epithelium and in the mesenchyme of nasal processes both
370	in control (white bar) and <i>Ert2Cre; Gata3<sup>fx/fx</sup></i> embryos (black bar). MNP, medial nasal process.
371	LNP, lateral nasal process. NC, nasal cavity. C, control. M, mutant ( <i>Ert2Cre; Gata3<sup>fx/fx</sup></i> ). Student T-
372	test ** $P < 0.01$ .



375 Figure 6. Retinoid-deficient diet in *Ert2Cre; Gata3<sup>fx/fx</sup>* embryos resulted in increased early

- **lethality.** (A) E13.0 control embryo with retinoid-deficient food for 38 days. (B-E)
- *Ert2Cre;Gata3<sup>fx/fx</sup>* embryos whose mother was fed retinoid-deficient food before fertilization. The
- 378 days indicated in each figure show the duration of retinoid-deficient diet.

Table.1 Varia	ation of the phe	enotype in pres	ent study	
	Choanal Deformation ( CA + CS )	Lethal	CD + Lethal	
Gata3 <sup>fx/fx</sup>	0% ( 0/50 )	4% ( 2/50 )	4%(2/50) -	*
Ert2Cre;Gata3 <sup>fx/fx</sup>	31%(11/35)	34%(12/35)	66% ( 23/35 ) -	J
Vitamin A deficient <i>Gata3<sup>fx/fx</sup></i>	0%(0/14)	7%(1/14)	7%(1/14) _	*
Vitamin A deficient <i>Ert2Cre;Gata3<sup>fx/fx</sup></i>	6%(1/17)	94%(16/17)	100%(17/17) <sup>-</sup>	
CA, Choanal Atrasia; CS,	Choanal Stenosis	; CD, Choanal Def	ormation * : p<0	).05
			Table	<b>).1</b> .

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