Fgf10-CRISPR mosaic mutants demonstrate the gene dose-related loss of the accessory lobe and decrease in the number of alveolar type 2 epithelial cells in mouse lung

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Abstract

CRISPR/Cas9-mediated gene editing often generates founder generation (F0) mice that exhibit somatic mosaicism in the targeted gene(s). It has been known that *Fibroblast growth factor 10* (*Fgf10*)-null mice exhibit limbless and lungless phenotypes, while intermediate limb phenotypes (variable defective limbs) are observed in the *Fgf10*-CRISPR F0 mice. However, how the lung phenotype in the *Fgf10*-mosaic mutants is related to the limb phenotype and genotype has not been investigated. In this study, we examined variable lung phenotypes in the *Fgf10*-targeted F0 mice to determine if the lung phenotype was correlated with percentage of functional *Fgf10* genotypes. Firstly, according to a previous report, *Fgf10*-CRISPR F0 embryos on embryonic day 16.5 (E16.5) were classified into three types: type I, no limb; type II, limb defect; and type III, normal limbs. Cartilage and bone staining showed that limb truncations were observed in the girdle, (type I), stylopodial, or zeugopodial region (type II). Deep sequencing of the *Fgf10*-mutant genomes revealed that the mean proportion of codons that encode putative functional FGF10 was 8.3 ± 6.2% in type I, 25.3 ± 2.7% in type II, and 54.3 ± 9.5% in type III (mean ± standard error of the mean) mutants at E16.5. Histological studies

showed that almost all lung lobes were absent in type I embryos. The accessory lung lobe was often absent in type II embryos with other lobes dysplastic. All lung lobes formed in type III embryos. The number of terminal tubules was significantly lower in type I and II embryos, but unchanged in type III embryos. To identify alveolar type 2 epithelial (AECII) cells, known to be reduced in the *Fgf10*-heterozygous mutant, immunostaining using anti-surfactant protein C (SPC) antibody was performed: In the E18.5 lungs, the number of AECII was correlated to the percentage of functional *Fgf10* genotypes. These data suggest the *Fgf10* gene dose-related loss of the accessory lobe and decrease in the number of alveolar type 2 epithelial cells in mouse lung. Since dysfunction of AECII cells has been implicated in the pathogenesis of parenchymal lung diseases, the *Fgf10*-CRISPR F0 mouse would present an ideal experimental system to explore it.

KEYWORDS: Genome editing, Fibroblast growth factor 10, Mouse, Lung, Somatic mosaicism

Introduction

The recently developed CRISPR/Cas9 system provides a highly efficient means for editing the genomes of model and non-model organisms. This powerful tool can help elucidate pathophysiological mechanisms underlying various genetic diseases (reviewed in [1,2]). CRISPR/Cas9 promises the possibility of an ultimate cure for genetic diseases by enabling replacement of mutated genes with normal alleles (reviewed in [3]); however, genome-edited founder mice often exhibit somatic mosaicism in the targeted gene, meaning more than two mutated alleles for that gene are mixed in the same mouse [4-6]. Such mosaicism may be undesirable when it complicates phenotypic analysis [7]. However, mosaic mice may help the study of genes whose constitutive mutations are lethal, or the study of interactions between mutant and normal cells in the same individual [8]. Furthermore, recent DNA sequencing advances have enabled us to identify genetic mosaicism even in phenotypically normal individuals, the pathological significance of which is still unclear (reviewed in [9,10]).

Previously, we reported that somatic mosaicism of a homeobox gene *Pax6* mutation causes variable eye phenotypes correlated with its gene dosage [11]. In this study, we

75 focused on the variable phenotypes of genome-edited *Fgf10* founder generation (F0) mice.
76 The *Fgf10* gene is required for limb and lung formation; *Fgf10* null mutants suffer
77 embryonic lethality at birth due to lung agenesis [12,13]. Recent studies have shown that
78 a fraction of *Fgf10* genome-edited F0 mice exhibits typical limbless and lungless
79 phenotypes, and that the severity of the limbless phenotype depends on the *Fgf10*
80 mutation rate [7,14,15]. However, the lung phenotype has not been described. In this
81 study, we sought to determine the relationship between lung and limb phenotypes, and
82 correlation of these phenotypes to putative functional FGF10 dosage.
83
84

Materials and methods

Reagents and equipment used in this study are listed in S1 Table.

Mice and ethical statement

The animal experimental design was approved by the Committee of Animal Experiments of Tokushima University, Tokushima, Japan, and by the Animal Care and Use Committee, Okayama University, Okayama, Japan (Permit numbers: T27-16, T30-8, OKU-2017404, OKU2018605). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. *In vitro* fertilized (IVF) eggs were prepared from male and female BDF1 (C57BL/6 x DBA2 F1) mice as previously reported [14]. CRISPR/Cas9 genome editing was performed on one-cell stage zygotes by microinjection (analysis on E16.5 embryos) with *Cas9* mRNA and sgRNA targeted at exon 3 of the *Fgf10* gene according to Yasue et al. (2014) [14] (S1 Fig). After culturing genome-edited zygotes until the two-cell stage, embryos were transferred to the uterine tube of foster mice (Jcl: MCH (ICR) strain developed by CLEA Japan, Inc. (Tokyo, Japan); Jcl for Japan clea, MCH for Multi-Cross Hybrid, and ICR for Institute of Cancer

Research) and allowed to develop until E16.5. To obtain E18.5 F0 embryos, *Cas9* mRNA (100 ng/μL) and synthesized guide RNA (50 ng/μL) were electroporated into one-cell stage IVF eggs and further manipulation of embryos was performed according to Hashimoto et al. (2016) [7]. CRISPR-control embryos were obtained by electroporation of medium only into IVF eggs before processing them for sequencing and histological examination. To know developmental variations, the weight of E18.5 embryos was measured with a standard electronic scale.

Enzyme mismatch cleavage assay

Approximately 25 μg genomic DNA were extracted from neck skin tissue (epidermis and dermis) of collected embryos using a Qiagen DNeasy Blood & Tissue Kit. The genomic region encompassing the CRISPR/Cas9 target site was amplified by polymerase chain reaction (PCR) using a primer set specific for the *Fgf10* gene (S3A Fig, S1 Table) [14]. The amplicons were processed for a mismatch cleavage assay using the Guide-it Mutation

Detection Kit and the products were analyzed by 2% agarose gel electrophoresis (S3B Fig).

DNA sequencing

To verify the presence of the target site mutation, single bands of PCR amplicons of approximately 500 bp derived from the collected embryos at E16.5 (neck skin tissues) were subjected to Sanger sequencing. The resulting DNA sequence waves were dissociated using a web tool for TIDER data analysis available at https://tide.nki.nl [16].

Deep sequencing analysis using DNA extracted from neck skin, lung, and/or limb dermal tissues (Table 1 for E18.5; neck skin only for E16.5 embryos in Table 3; neck skin only for additional E18.5 embryos in S3 Table) for the *Fgf10* target site was performed according to the previous report on Illumina MiSeq system [17]. Briefly, the *Fgf10* on-target site from each individual embryo was amplified using custom barcode primers shown in S1 Table. Library construction and sequencing were performed at the National Institute for Basic Biology (NIBB, Japan) and Bioengineering Lab (Kanagawa, Japan). The pooled sequence data was demultiplexed into each sample and analyzed subsequently

131 using a web-based tool, CLiCKAR [17].

132

133 Correction and calculation of putative somatic mutation rate
134 in the case of large insertions/deletions (indels)

135 PCR amplicons for deep sequencing were analyzed using TAPEStation 4200 system.
136 When larger or smaller DNA bands were detected above or below the main bands, their
137 approximate molecular size was recorded. “Peak Molarity” value for each DNA band was
138 documented and the percentage of large indel was estimated by calculating the total value.
139 The putative somatic mutation rate for large indels was calculated proportionally. In
140 embryos #3 (type II, E16.5), #23 (type III, E18.5), #25 (type III, E18.5), and #11_2 (type
141 II, E18.5), and #22_2 (type III, E18.5), large indels were detected and corrected
142 accordingly.

143

144 Cartilage and bone staining

After Cesarean dissection, E18.5 embryos, removed their skin and internal organs, were fixed in 95% ethanol and processed for whole mount skeletal staining with Alcian blue 8GX and Alizarin red S according to standard procedures [18].

Histology and analysis of terminal tubules

Hematoxylin-eosin (HE) staining was performed on mouse sections according to standard procedures. Briefly, sections of 5 μm-thickness were made of the pectoral region of paraffin-embedded embryos and mounted on microscope slides. After deparaffinization and rehydration, sections were stained with Mayer's hematoxylin solution and 0.125% eosin. Following staining, sections were washed, dehydrated, and mounted with DPX. After HE staining, photos were taken with a 10x objective lens for each embryonic lung (n = 3 for each type, at E16.5) (Fig 5E-H) and tiled using ImageJ (https://imagej.nih.gov/ij/index.html) (RRID:SCR_003070). Terminal tubules were scored in the tiled full-field image (n = 1 for each embryo). The area for the full-field was calculated by ImageJ and the number of terminal tubules per mm^2 were obtained and further analyzed statistically.

Immunohistochemistry and analysis of SPC-positive cells

Immunohistochemistry was performed on the 5 μm-thick deparaffinized sections using ImmPRESS Polymer Detection Kit for IHC. A rabbit polyclonal antibody against a synthetic peptide fragment of human prosurfactant protein C (SPC) (within residues 1-100) was used at 1:5,000 dilution. The specificity of the antibody for AECIIs in the mouse lung has been validated [19]. The public identifier from the Antibody Registry is RRID: AB_10674024. After immunohistochemistry, SPC-positive cells were scored in random portions of a section in eight photomicrographs using a 40x objective lens. The total number of 6627 cells on average was counted per sample. The results were presented as a ratio of the number of positive cells per total number of cells [20]. This method has a limitation, in which as *Fgf10* mutations have been known to affect epithelial tube formation and branching [21] and therefore the number of distal airway epithelial cells in total may be impacted, the accuracy of the data would be ensured by normalizing AECII cell number to total epithelial cell number.

Microscopy and image processing

Images for sections were collected with a Nikon DS-Fi1 camera on a Leica DM5000B microscope. Embryos, skeletons, and intestines were observed and imaged using a Leica DFC310FX camera on a Leica M165FC stereomicroscope. Image manipulation such as levels and color balance adjustments, made to some images, and assembly of figures were performed with Adobe Photoshop CS6 Extended (RRID:SCR_014199).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Dissected embryonic lungs (E18.5) were immersed in RNAlater at 4 °C overnight and stored at -80 °C until further use. Total RNA was extracted using NucleoZOL. Five micrograms of RNA were reverse-transcribed to cDNA with FastGene cDNA Synthesis 5x ReadyMix. RT-qPCR was performed in duplicate wells with BrightGreen 2X qPCR MasterMix-No Dye and LightCycler Nano System using primers as shown in S4 Table. The PCR conditions employed were according to the manufacturer's protocol: 10 min at 95°C for enzyme activation, 40 cycles of 15 sec at 95°C (denaturation) and 60 sec at 60°C

193 (annealing and extension). We performed no template negative control experiments, as
194 well as melting curve analysis according to manufacturer's instructions. Gene expression
195 was normalized to a housekeeping gene, *Glyceraldehyde-3-phosophate dehydrogenase*
196 (*Gapdh*). Relative mRNA levels were determined by the comparative Ct method (Winer
197 et al. 1999). Error bars in S6 Fig show standard deviation and p values were calculated
198 by Microsoft Excel (RRID:SCR_016137) and confirmed by WaveMetrics Igor Pro
199 (RRID:SCR_000325).
200

201 Morphometric analysis of the cecum

202 Wild type (n = 3), type I (n = 4), and type II (n = 2) dissected intestines containing the
203 cecum, colons, and part of small intestines at E18.5 were photographed at the same
204 magnification (S7 Fig). The length of each cecum was measured using ImageJ. Briefly,
205 multi-points were set from the most proximal point abutting the small intestine and colon
206 to the most distal cecum, approximately 16 to 18 points in the case of wild type cecum.
207 The sum of distance between each point was regarded as the length of the cecum.
208

Statistical analysis

Significance was determined by one-way analysis of variance (ANOVA) (Fig 4B), Dunnett's test (Fig 5I), or two-tailed unpaired Student's t-test (S5L Fig, S7J Fig). Correlation analysis was done by calculating correlation coefficient (Fig 6J-L, S5M Fig). Chi-square test was used for the comparison of deep sequencing data on DNA from two or three different tissues (Table 4). Data were presented as mean ± standard error of the mean (SEM). Values of $p<0.05$ were considered significant otherwise stated. Data analysis was essentially performed using Microsoft Excel except for Dunnett's test, which was done using R (https://www.r-project.org) (RRID:SCR_001905).

Results

Generation of *Fgf10*-CRISPR founder embryos and their limb skeletal structures

To generate *Fgf10*-CRISPR mice, we used a single guide RNA (sgRNA) to target exon 3, as previously described (S1 Fig) [14]. According to Hashimoto and Takemoto (2015) [15], the resultant *Fgf10*-CRISPR F0 embryos were classified by the limb phenotype into three types: type I, no limb; type II, limb defect; and type III, normal limbs. Since skeletal pattern of these three limb phenotypes has not been revealed, cartilage and bone staining was performed on E18.5 *Fgf10*-crispants (n = 4 for wild type; n = 11 for mutant embryos) (Table 2, Fig 1, S2 Fig). Normally, the appendicular skeleton consists of girdle ("pectoral" for forelimb and "pelvic" for hindlimb) and limb elements. The skeletons of the fore- and hindlimbs consist of stylopod for humerus/femur, zeugopod for the radius/ulna and tibia/fibula, and autopod for hand/foot (S2I-K Fig). In type I embryos (n = 6), all limb bones were lost and remaining girdle bones were affected to varying degrees (Fig 1A-D; S2A-D Fig). The pectoral girdle consists of the scapula and clavicle, while the pelvic girdle consists of the ilium, pubis, and ischium (S2I-K Fig) (for review [22]). In four out

234 of six type I embryos, the size of scapula was reduced and its spine was not formed,
235 indicating that the posterior blade was lost (Fig 1A). As for the pelvic girdle, the ischial
236 and pubic bones were lost, while iliac bones reduced bilaterally (Table 2 and Fig 1B). In
237 the remaining two embryos, the scapula was intact with a spine, while the pubis and
238 ischium were lost or reduced with the ilium intact bilaterally (Table 2 and Fig 1C, D; S2C,
239 D Fig). Thus, we regarded the latter phenotype as a type between I and II (type I/II). In
240 type II embryos (n = 2), all limb elements were lost in one or two appendages and in the
241 remaining appendages, limb truncations were observed at the stylopod or zeugopod level
242 (Table 2 and Fig 1E-O; S2E, F Fig). In type III embryos examined (n = 3), all limb and
243 girdle bone structures were normal (Fig 1P-R). These limb skeletal patterns indicated that
244 limb truncations were observed in the stylopodial, zeugopodial (type II), or pelvic region
245 (type I). Formation of the clavicle, and the superior part of the scapula and pelvic girdle
246 were not affected by lack of *Fgf10* gene products.
247

248 ## Genomic analysis of *Fgf10*-CRISPR founder embryos

To prescreen mutations at the *Fgf10* locus, we performed an enzyme mismatch cleavage assay (S3A, B Fig) and Sanger sequencing with the use of DNA from neck skin tissues of the E16.5 embryos. We estimated mutation frequencies based on band intensities [23], and found an embryo (type II, #4) with obvious limb defects but showed faint band intensities for mutated DNA after endonuclease treatment (S3B Fig). Sanger sequencing indicated a mixed genotype of wild type and a single nucleotide insertion in this embryo (S3C Fig).

To get more accurate mutation frequencies, we performed deep sequencing of PCR amplicons containing the *Fgf10* target site by use of embryonic (E16.5) neck DNA. By multiple amplicon analysis, we could distinguish between wild type, in-frame and frameshift mutations (Fig 2). We also deduced amino acid sequences encoded by the targeted *Fgf10* locus (Fig 3). Among in-frame mutations, we found that codons that encode Lys196 and His201 were preserved or those that encode His201 alone were preserved as in #3, #5, and #9 embryos; both residues are known to be indispensable for FGF receptor dimerization [24]. We calculated the percentage of the gene sequence that still encodes putative functional FGF10 proteins with both residues in each type of mutant

265 at E16.5, finding 8.3 ± 6.2% for type I, 25.3 ± 2.7% for type II, and 54.3 ± 9.5% for type
266 III, n = 3 for each type, mean ± SEM) (Table 3) (Fig 4A, B). In the case of E18.5 neck
267 DNA, it was 3.4 ± 2.9% for type I (n = 3), 33% for type II (n = 1), and 66.5 ± 8.8% for
268 type III, respectively (n = 8) (Table 1).

269 To know whether genotyping results using DNA from different tissues were similar,
270 we performed deep sequencing on DNA extracted from lung and limb dermal tissues as
271 well as neck skin tissues of the E18.5 embryos (Table 1; S2 Table). We evaluated deep
272 sequencing results on type III embryos (n = 10) with Chi-square test (Table 4). We found
273 that in 6 embryos there was a significant difference in the frequencies of the functional
274 *Fgf10* genotypes from the different tissues and in 4 embryos there was no significant
275 difference in the frequencies of the functional *Fgf10* genotypes from the different tissues
276 (Table 4).

277

278 Analysis of lung phenotypes

279 We next examined the lung histology of *Fgf10*-mosaic F0 embryos on E16.5 (Table 3,
280 Fig 5, S4 Fig). In the normal mouse lung, there are five lung lobes, four on the right

281 (cranial, middle, caudal, and accessory) and one on the left (Fig 5A). The lung histology
282 of type I embryos demonstrated either the absence of all lobes (two of three embryos) or
283 only the presence of a residual lobe (one of three) (Fig 5B). We found that the accessory
284 lobe was absent in two out of three type II embryos, and that either middle or caudal lobe
285 was additionally absent or hypoplastic (Fig 5C). In type III embryos, all five lung lobes
286 were present (Fig 5D). In E18.5 type II embryos, the accessory lung lobe was absent from
287 all three embryos examined (embryo #11_2, 24_2, 25_2; S5I-K Fig).

288 Mouse lung development is divided into five stages, namely embryonic,
289 pseudoglandular (E9.5 to 16.5), canalicular (E16.5 to 17.5), saccular (E17.5 to P5), and
290 alveolar (P5 to P28) [25]. At E16.5, after branching morphogenesis the lung is in the
291 transition of pseudoglandular to canalicular stage. We found that the number of terminal
292 tubules was significantly decreased in type I and II residual lungs (Fig 5F, G, I), but not
293 significantly altered in type III lungs (Fig 5H, I), compared with wild type (Fig 5E, I). By
294 E18.5, the lung enters the saccular stage and cuboidal cells that produce surfactant protein
295 C (SPC), i.e. alveolar type 2 epithelial (AECII) cells, differentiate on the wall of the
296 developing airway space. Since AECII cell differentiation is perturbed in the *Fgf10*-

297 heterozygous lung [26,27], we performed immunohistochemistry on E18.5 lung sections
298 to detect AECII. We counted the number of SPC-positive cells in lungs of type III
299 embryos and examined whether it was correlated to the percentage of functional *Fgf10*
300 genotype. Representative data of immunostaining for type III lungs are shown (Fig 6B,
301 E) along with those for type II lungs (Fig 6A, D) and positive/negative controls (Fig. 6C,
302 F, G-I). We found that the number of SPC-positive cells in lungs of type III embryos was
303 correlated to the percentage of functional *Fgf10* genotype (Fig 6J, correlation coefficient
304 is 0.909 for limb DNA; Fig. 6K, 0.924 for neck DNA). Since the delay in development
305 can be caused by variation between embryos even from a same litter due to crowding in
306 the uterus, we also examined whether the number of SPC-positive cells was correlated to
307 the weight of each embryo, as an index for developmental stage (Table 1). It seemed that
308 there was no correlation between them (Fig 6L; correlation coefficient is 0.301).

309 On the other hand, since it was reported that the mesenchymal FGF10 is also important
310 for proper mesenchymal lineage formation during lung development as revealed by
311 *Fgf10*-hypomorphic lungs [20,28], we examined expression of mesenchymal and its
312 related marker genes [29] in type III lungs at E18.5 by quantitative PCR (Table 1, S4

313 Table). However, we could not detect a significant decrease in expression of these genes
314 (S6 Fig), possibly because the percentage for functional *Fgf10* genotypes was too high to
315 mimic a *Fgf10*-hypomorphic phenotype in lung mesenchymal lineage formation (61.19%
316 in #11, 65.98% in #12, and 70.11% in #17 embryos). This expression analysis should be
317 further clarified on earlier stage lungs, and in those which have more loss-of-function
318 mutations in the *Fgf10* gene.
319

Discussion

Fgf10 genome edited F0 mice are classified into three limb phenotypes: no limb (type I), limb defect (type II), and normal limbs (type III). In this study, we first examined appendicular skeletal patterns of the *Fgf10* mosaic mutant mice by cartilage and bone staining. We found that varying degrees of limb and girdle bone truncations were observed in type I and type II embryos. We next estimated the rate of putative functional *Fgf10* genotypes in each mutant type and the mean percentage for type II embryos is 25.3 ± 2.7 by deep sequencing of neck DNA. Comparison of deep sequencing results on DNA from different tissues (neck, lung, and limb dermis) revealed that in half of the embryos the rate of putative functional *Fgf10* genotypes were significantly varied between the three DNA. Lung phenotypes were examined on two embryonic stages, E16.5 and E18.5. In type II embryos, the accessory lobe was lost and the number of terminal tubules was significantly reduced. In type II and "normal limb (type III)" embryos, the number of alveolar type 2 cells, immunostained by anti-surfactant C protein, was correlated with the rate of putative functional *Fgf10* genotypes.

Truncation patterns of girdle and limb bones in *Fgf10*-mosaic mutants

Skeletal staining in this study revealed that in type II embryos, limb bone structures are truncated in more distal structures rather than forming a miniature of whole limb and girdle structures. Also, in the mutants, the zeugopodial element consists of one ulna- or tibia-like bone: This pattern is different from those after extirpation of apical ectodermal ridge in chicken limb buds, where both zeugopod bones are truncated [30]. Although the number of type II embryos examined in this study is only two and therefore further studies are needed, a reduced number of *Fgf10*-expressing cells in the mosaic mutants does not seem to know the morphological pattern to be formed. Looking the expression of zeugopod patterning genes could help to elucidate the underlying mechanisms.

Regarding girdle bones, it has been known that the posterior (inferior) half of the scapula is absent and most of the pelvic structures is lacking in *Fgf10*-null embryos [12,13]. This study showed that in type I/II *Fgf10*-mosaic mutants the posterior scapula with the spine retained. In the mouse, while the pelvic girdle and most of the scapular blade derives solely from the lateral plate mesoderm (LPM), the medial edge of the blade

arises from dermomyotome cells and the neural crest cells contribute to the scapular spine [31-33]. Thus, it is likely that a reduced number of *Fgf10*-expressing cells in the LPM and neural crest cells [34] contributed to the formation of the posterior (inferior) scapula in type I/II embryos. In the case of pelvic girdle, a reduced number of *Fgf10*-expressing cells in the LPM contributed to the formation of the inferior pelvic structures in type I/II embryos.

Fgf10 mutation rate may not be identical in different tissues

Here we showed that there is a correlation between limb phenotypes, lung phenotypes, and overall defective mutation rate in *Fgf10*-CRISPR knockout F0 mice. It has been shown that even if CRISPR-genome editing materials are introduced into fertilized eggs at the one-cell stage, mosaicism in the target genotype is caused under certain experimental conditions until the target sequences are mutated [6,7]. During the early embryonic stage, mutated cells and non-mutated cells are intermingled and distributed to lung and limb primordia proportionally [35], which we believe contributes to the correlation between limb and lung phenotypes. However, statistical analysis on deep

368 sequencing data of the genotypes shows in more than half cases there is a significant
369 difference of the defective mutation rate in DNA from different tissues. In this study, we
370 could not identify the reason why there were differences in the rate of the functional *Fgf10*
371 genotypes. In some developmental settings, *Fgf10*-expressing normal cells might have a
372 growth advantage than *Fgf10*-deficient cells, which should be further explored in the next
373 studies [10].

374 *Fgf10*-CRISPR F0 mice possess degrees of lung dysgenesis that
375 correlate with putative functional FGF10 dosage

376 Accessory lobe formation was firstly impaired due to reduction in putative functional
377 FGF10 dosage. Ramasamy et al. (2007) reported that *Fgf10*-hypomorphic mice, in which
378 *Fgf10* expression is reduced by 27%, compared with *Fgf10*-heterozygous mutants, lack
379 accessory lobe formation [20]. Since *Fgf10* expression is highest in the mesenchyme of
380 the accessory lobe on E11.5 and persists as such until at least E18.5 [21,29], high *Fgf10*
381 dosage was believed to be required for accessory lobe formation. Our result shows that
382 more than 25.3 ± 2.7% (from the analysis of E16.5 embryos) of functional *Fgf10* gene
383 product is required for accessory lobe formation. Since reduction or loss of accessory lobe

384 formation has been observed in loss-of-function mutants of *Gli3*, *Fog2,* and *Gata4* [36-
385 38], FGF10 signaling may be related to the expression of these transcription factors
386 during accessory lobe formation.

387 Lineage-tracing analysis of *Fgf10*-expressing cells during mouse lung development has
388 revealed that there are two waves of *Fgf10* expression; the first begins from E11.5 and
389 the second from E15.5 [29]. We think that a reduction in the latter cells would cause the
390 decrease in the number of terminal tubules in type I and type II *Fgf10*-CRISPR F0
391 embryos. A recent study has shown that AECII as well as AECI independent progenitors
392 are present at E13.5 as revealed by single cell RNA sequencing analysis [39]. By the
393 saccular stage, the lung mesenchyme surrounding the epithelium becomes thinner and
394 cuboidal AECII cells, characterized by the production of surfactant proteins, begin to
395 differentiate (summarized in [40]). The number of AECII cells is found to be lower in the
396 *Fgf10*-heterozygous lung [26,27], which is similar to our result in type III *Fgf10*-mosaic
397 mice. Taken together, *Fgf10* expression levels regulate the number of alveolar type 2
398 epithelial cells in mouse lung in somewhat dose-dependent manner.

399 Regarding FGF10 dosage, the *Fgf10*-hypomorphic mouse aforementioned [20,28] has
400 also clarified that higher FGF10 dosage is required for full development of colonic crypts
401 [41]. Our preliminary observation (S7 Fig) showed that in type I embryos the length of
402 the cecum was significantly shorter than the wild type and there was an atresia of the
403 colon as reported in the *Fgf10*-null mutants [41,42]. The length of the blind colon varied
404 depending upon the embryos (S7D-G Fig), suggesting a correlation with their FGF10
405 dosage.

406

407 *Fgf10*-CRISPR F0 mice can serve as a series of lung disease
408 models

409 Volckaert et al. (2013) reported that lung branching morphogenesis does not require
410 localized *Fgf10* expression in the distal mesenchyme, because ubiquitous *Fgf10*
411 overexpression can induce lung formation in *Fgf10* knockout mice [43]. Furthermore,
412 *Fgf10* expression after lung initiation is required for branching and proximal-distal
413 differentiation by regulating *Sox2/9* expression in the epithelium [43]. Our result also

414 supports this property of *Fgf10* as the number of terminal tubules decreased in the *Fgf10*-
415 mosaic mutants where functional FGF10 dosage is reduced.

416 Recent papers have reported that mutations and single nucleotide polymorphisms
417 (SNPs) in the *Fgf10* gene are correlated with human lung disease (reviewed in [44]). For
418 example, the absence of the right medial-basal airway is associated with a type of chronic
419 obstructive pulmonary diseases and two types of SNPs are found within the same intron
420 of *Fgf10* in those cohorts [45]. Although some molecular and cellular differences have
421 been identified between mouse and human lungs (reviewed in [46]), the F0 mice
422 generated by CRISPR/Cas9-mediated *Fgf10* gene editing can become a model animal to
423 study the pathophysiology of human pulmonary hypoplasia and related chronic lung
424 diseases that may be rooted in the developmental stage as recently postulated [47]).

425

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430

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571

572 Tables

573 **Table 1. Summary of *Fgf10*-CRISPR F0 embryos at E18.5. These embryos were**
574 **genotyped by deep sequencing (NGS).**

575 *SPC (+) cells/total cells (mean ± SEM %) is shown in parenthesis of IHC for Fig 6J and
576 K. -, no limb; c-b staining, cartilage and bone staining; N.A., not applicable; N.A.**, not
577 applicable due to low expected frequency (<5); N.D., not done; qPCR, quantitative PCR.
578 ***contains wild type genotype and in-frame genotype retaining nucleotides for Lys196
579 and His201.

580

581 **Table 2. Summary of *Fgf10*-CRISPR F0 embryos (E18.5) processed for cartilage**
582 **and bone staining.**

583 -, no bone; ±, bone hypoplasia; +, normal bones; N.A., not applicable; N.D., not
584 determined due to separation of proximal parts of the limb from the trunk for lung analysis.

585

586 **Table 3. Summary of limb and lung phenotypes of *Fgf10*-CRISPR F0 embryos at**
587 **E16.5.**

588 -, no limb or no lung lobe; ±, limb defect or small lung lobes; +, normal limbs or normal
589 appearance of lung lobes. *a residual lung lobe. Genotypes of crispants (types I-III) were
590 prescreened by an enzyme mismatch cleavage assay (S3B Fig) and determined by deep
591 sequencing (NGS).
592
593 **Table 4. Chi-square test for genotyping results of DNA derived from different tissues.**
594 The number of sequence reads in deep sequencing is shown in contingency tables for
595 observed frequencies. Wt, wild type *Fgf10* genotype; Out, frameshift mutations in the
596 *Fgf10* gene; W/o & Out, in-frame mutation without nucleotides encoding Lys196 and/or
597 His201 plus frameshift mutations in the *Fgf10* gene; In (w), in-frame mutations with
598 nucleotides encoding Lys196 and His201; In (w/o), in-frame mutations without
599 nucleotides encoding Lys196 and/or His201. When p-values <0.05, there is statistically
600 significant difference in the *Fgf10* genotypes of the different tissues.
601

Table 1. Summary of *Fgf10*-CRISPR F0 embryos at E18.5. These embryos were genotyped by deep sequencing (NGS).

Type	Embryo No.	Forelimb		Hindlimb		Weight (gram)	Analysis*	Tissues for NGS	Chi-square test of NGS results (*p*-value)	Functional *Fgf10* genotype (%) in neck DNA	Functional *Fgf10* genotype (mean±SEM %) in neck DNA	Functional *Fgf10* genotype (%) in lung DNA	Functional *Fgf10* genotype (%) in limb DNA	Genotype identity between different DNA	Figure
		Left	Right	Left	Right										
I	#18	—	—	—	—	0.6303	collected for c-b staining	Neck	N.A.	9.27	3.4 ± 2.9	N.D.	N.D.		
	#19	—	—	—	—	0.6899	collected for c-b stainig	Neck	N.A.	0.50		N.D.	N.D.		
	#26	—	—	—	—	0.4682	C-b staining	Neck	N.A.	0.45		N.D.	N.D.		
II	#14	—	Humerus only	—	Short femur	0.5784	C-b staining	Neck	N.A.	32.96	N.A.	N.D.	N.D.		Fig 1J-O
III	#11	Normal	Normal	Normal	Normal	0.7799	Lung qPCR	Limb, lung, neck	3.34357E-29	67.75	65.8 ± 9.0	61.19	64.23	Not identical	
	#12					0.9111	Lung qPCR	Limb, lung	0.751528903	N.D.		65.98	66.45	Identical	Fig 1P-R
	#13					0.9699	Lung collected for qPCR	Limb, lung	4.01545E-05	N.D.		82.59	84.77	Not identical	
	#15					0.9572	Lung collected for qPCR	Limb, lung, neck	2.89945E-11	59.78		56.84	56.22	Not identical	
	#16					0.9254	Lung collected for qPCR	Limb, lung	N.A.**	N.D.		100.00	100.00	Identical	
	#17					0.7524	Lung qPCR	Limb, lung	3.37691E-50	N.D.		70.11	64.00	Not identical	
	#20					1.0555	IHC (19.9 ± 0.7)	Limb, neck	1.892E-09	100.00***		N.D.	100.00***	Not identical***	Fig 6J-M
	#21					0.7742	IHC (16.9 ± 0.8)	Limb, neck	0.176029751	56.92		N.D.	57.52	Identical	Fig 6J-M
	#22					0.8757	IHC (9.8 ± 0.5)	Limb, neck	0.271069452	28.92		N.D.	29.94	Identical	Fig 6B,E,H;Fig 6J-M
	#23					0.9232	IHC (24.2 ± 0.8)	Limb, neck	N.A.**	100.00		N.D.	97.20	Identical	Fig 6J-M
	#24					0.9976	IHC (18.8 ± 0.7)	Limb, neck	1.18483E-26	73.06		N.D.	82.26	Not identical	Fig 6J-M
	#25					0.9832	IHC (15.2 ± 1.4)	Limb, neck	0.139527497	40.75		N.D.	40.16	Identical	Fig 6J-M
Wild type	#2	Normal	Normal	Normal	Normal	0.7472	Lung qPCR	Limb, lung, neck	N.A.**	99.63	N.A.	100.00	100.00	Identical	

*SPC (+) cells/total cells (mean ± SEM %) is shown in parenthesis of IHC for Fig 6J and K. -, no limb; c-b staining, cartilage and bone staining; N.A., not applicable; N.A.**, not applicable due to low expected frequency (<5); N.D., not done; qPCR, quantitative PCR. ***contains wild type genotype and in-frame genotype retaining nucleotides for Lys196 and His201.

Table 2. Summary of *Fgf10*-CRISPR F0 embryos (E18.5) processed for cartilage and bone staining.

Type	Subtype	Embryo No.	Forelimb		Pectoral girdle				Hindlimb		Pelvic girdle						Functional *Fgf10* genotypes (%) in neck DNA	Functional *Fgf10* genotypes (%) in lung DNA	Functional *Fgf10* genotypes (%) in limb DNA	Figure
			Left	Right	Left		Right		Left	Right	Left			Right						
					Scaplua	Clavicle	Scaplua	Clavicle			Ilium	Ischium	Pubis	Ilium	Ischium	Pubis				
I	I	#4_18	-	-	±	+	±	+	-	-	±	-	-	±	-	-	N.A.	N.A.	N.A.	Fig 1,Fig S2
		#26	-	-	±	+	±	+	-	-	±	-	-	±	-	-	0.45	N.A.	N.A.	
		#34	-	-	±	+	±	+	-	-	±	-	-	±	-	-	N.A.	N.A.	N.A.	
		#35	-	-	±	+	±	+	-	-	±	-	-	±	-	-	N.A.	N.A.	N.A.	
	I/II	#40	-	-	+	+	+	+	-	-	+	±	-	+	±	-	N.A.	N.A.	N.A.	
		#46	-	-	+	+	+	+	-	-	+	±	-	+	-	-	N.A.	N.A.	N.A.	Fig 1,Fig S2
II		#14	N.D.	Humerus only	N.D.	N.D.	+	+	-	Truncated at distal femur	+	±	-	+	-	±	32.96	N.A.	N.A.	Fig 1
		#33	+	Humerus, radius only,no autopod	+	+	+	+	-	Truncated at distal tibia, no fibula	+	+	-	+	+	+	N.A.	N.A.	N.A.	Fig 1,Fig S2
III		#11	+	+	+	N.D.	+	N.D.	+	+	+	+	+	+	+	+	67.75	61.19	64.23	
		#12	+	+	+	N.D.	+	N.D.	+	+	+	+	+	+	+	+	N.A.	65.98	66.45	Fig 1
		#17	+	+	+	N.D.	+	N.D.	+	+	+	+	+	+	+	+	N.A.	70.11	64.00	
Wild type		#1	+	+	+	N.D.	+	N.D.	+	+	+	+	+	+	+	+	N.A.	N.A.	N.A.	
		#4	+	+	+	N.D.	+	N.D.	+	+	+	+	+	+	+	+	N.A.	N.A.	N.A.	
		#5	+	+	+	N.D.	+	N.D.	+	+	N.D.	N.D.	N.D.	+	+	+	N.A.	N.A.	N.A.	
		#7_18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N.A.	N.A.	N.A.	Fig 1,Fig S2

-, no bone; ±, bone hypoplasia; +, normal bones; N.A., not applicable; N.D., not determined due to separation of proximal parts of the limb from the trunk for lung analysis.

Table 3. Table 3. Summary of limb and lung phenotypes of *Fgf10*-CRISPR F0 embryos at E16.5.

Type	Embryo No.	Forelimb		Hindlimb		Lung					# of terminal tubules /mm2	# of terminal tubules/mm2 (mean ± SEM)	Functional *Fgf10* genotype (%) (neck DNA)	Functional *Fgf10* genotype (mean ± SEM %)	Figure
		Left	Right	Left	Right	Cranial lobe	Middle lobe	Caudal lobe	Accessory lobe	Left lobe					
I	#9	-	-	-	-	-	-	-	-	-	0	6.2 ± 6.2	0.00	8.3 ± 6.2	Fig S4
	#5	-	-	-	-	-	-	-	-	-	0		4.63		Fig S4
	#7	-	-	-	-	±*				-	18.7		20.35		Fig 5,Fig S4
II	#11	±	±	±	±	+	+	±	-	+	87.4	84.4 ± 13.9	20.60	25.3 ± 2.7	Fig S4
	#3	±	±	±	±	+	-	+	-	+	59.0		25.39		Fig 5,Fig S4
	#4	±	+	+	±	+	+	+	+	+	106.8		29.96		Fig S4
III	#1	+	+	+	+	+	+	+	+	+	113.4	120.9 ± 4.0	42.83	54.3 ± 9.5	Fig 5,Fig S4
	#2	+	+	+	+	+	+	+	+	+	127.1		46.97		Fig S4
	#8	+	+	+	+	+	+	+	+	+	122.2		73.06		Fig S4
WT	#1	+	+	+	+	+	+	+	+	+	131.2	135.6 ± 11.6	Not determined		Fig 5,Fig S4
	#2	+	+	+	+	+	+	+	+	+	117.9				Not shown
	#3	+	+	+	+	+	+	+	+	+	157.6				Not shown

-, no limb or no lung lobe; ±, limb defect or small lung lobes; +, normal limbs or normal appearance of lung lobes. *a residual lung lobe. Genotypes of crispants (types I-III) were prescreened by an enzyme mismatch cleavage assay (Fig S3B) and determined by deep sequencing (NGS).

Table 4. Chi-square test for genotyping results of DNA derived from different tissues.

Embryo No.	Type	Observed frequencies					Expected frequencies (EF)					*p*-value
#2	Wild type		Neck	Lung	Limb	Total		Neck	Lung	Limb	Total	N.A.
		Wt	4877	8760	58889	72526	Wt	4894	8758	58874	72526	
		Out	18	0	0	18	Out	0.067	2.174	15	18	
		Total	4895	8760	58889	72544	Total	4895	8760	58889	72544	
								EF: less than 5				
#11	III		Neck	Lung	Limb	Total		Neck	Lung	Limb	Total	3E-29
		Wt	6686	12893	18549	38128	Wt	6290	13430	18408	38128	
		Out	3182	8178	10331	21691	Out	3578	7641	10472	21691	
		Total	9868	21071	28880	59819	Total	9868	21071	28880	59819	
#12	III		Lung	Limb	Total			Lung	Limb	Total		0.752
		Wt	706	19627	20333		Wt	710.8	19622	20333		
		Out	364	9911	10275		Out	359.2	9916	10275		
		Total	1070	29538	30608		Total	1070	29538	30608		
#13	III		Lung	Limb	Total			Lung	Limb	Total		4E-05
		Wt	4203	45331	49534		Wt	4304	45230	49534		
		Out	886	8147	9033		Out	784.9	8248	9033		
		Total	5089	53478	58567		Total	5089	53478	58567		
#15	III		Neck	Lung	Limb	Total		Neck	Lung	Limb	Total	3E-11
		Wt	7295	20266	22243	49804	Wt	6952	20313	22539	49804	
		W/o & Out	4908	15388	17318	37614	Out	5251	15341	17022	37614	
		Total	12203	35654	39561	87418	Total	12203	35654	39561	87418	
#16	III		Lung	Limb	Total			Lung	Limb	Total		N.A.
		Wt	15698	38223	53921		Wt	15698	38223	53921		
		Out	0	0	0		Out	0	0	0		
		Total	15698	38223	53921		Total	15698	38223	53921		
								EF: less than 5				
#17	III		Lung	Limb	Total			Lung	Limb	Total		3E-50
		Wt	3441	21706	25147		Wt	3505	21642	25147		
		In (w)	2869	13857	16726		In (w)	2331	14395	16726		
		Out	2690	20007	22697		Out	3164	19533	22697		
		Total	9000	55570	64570		Total	9000	55570	64570		
#20	III		Neck	Limb	Total			Neck	Limb	Total		2E-10
		Wt	29282	5549	34831		Wt	29043	5788	34831		
		In (w)	11875	2653	14528		In (w)	12114	2414	14528		
		Total	41157	8202	49359		Total	41157	8202	49359		
#21	III		Neck	Limb	Total			Neck	Limb	Total		0.176
		Wt	23077	10726	33803		Wt	23153	10650	33803		
		Out	17464	7923	25387		Out	17388	7999	25387		
		Total	40541	18649	59190		Total	40541	18649	59190		
#22	III		Neck	Limb	Total			Neck	Limb	Total		0.271
		Wt	2653	963	3616		Wt	2677	938.6	3616		
		Out	6521	2253	8774		Out	6497	2277	8774		
		Total	9174	3216	12390		Total	9174	3216	12390		
#23	III		Neck	Limb	Total			Neck	Limb	Total		N.A.
*including large deletion		Wt	82324	1146	83470		Wt	82291	1179	83470		
		Out*	0	33	33		Out*	32.53	0.466	33		
		Total	82324	1179	83503		Total	82324	1179	83503		
									EF: less than 5			
#24	III		Neck	Limb	Total			Neck	Limb	Total		1E-26
		Wt	8862	2633	11495		Wt	9095	2400	11495		
		Out	3268	568	3836		Out	3035	800.9	3836		
		Total	12130	3201	15331		Total	12130	3201	15331		
#25	III		Neck	Limb	Total			Neck	Limb	Total		0.155
**including large insertion		ln(w)	12665	10417	23082		ln(w)	12582	10500	23082		
		Out**	18415	15520	33935		Out**	18498	15437	33935		
		Total	31080	25937	57017		Total	31080	25937	57017		

The number of sequence reads in deep sequencing is shown in contingency tables for observed frequencies. Wt, wild type *Fgf10* genotype; Out, frameshift mutations in the *Fgf10* gene; W/o & Out, in-frame mutation without nucleotides encoding Lys196 and/or His201 plus frameshift mutations in the *Fgf10* gene; In (w), in-frame mutations with nucleotides encoding Lys196 and His201; In (w/o), in-frame mutations without nucleotides encoding Lys196 and/or His201. When p-values <0.05, there is statistically significant difference in the *Fgf10* genotypes of the different tissues.

Figure legends

Fig 1. Cartilage and bone staining to reveal limb skeletal structures.

A-I, L-R, left or right lateral views of limb and girdle regions (at E18.5) are shown. Cartilage is stained with Alcian blue and bone is stained with Alizarin red. **A, B,** type I #4_18 embryo. **C, D,** type I/II #46 embryo. **E-I,** type II #33 embryo. **L-O,** type II #14 embryo. **P-R,** type III #12 embryo. **J, K,** Lateral views of type II #14 embryo are shown. Arrows in (**K**) show truncated limbs. au, autopod; f, femur; fi, fibula; h, humerus; il, ilium; is, ischium; p, pubis; r, radius; sc, scapula; sp, spine; st, stylopod t, tibia; u, ulna; ze, zeugopod. Scale bars: 1 mm.

Fig 2. Genomic analysis of the *Fgf10*-CRISPR F0 embryos at E16.5 as revealed by deep sequencing.

The target nucleotide sequence is highlighted in yellow. Proto-spacer adjacent motif sequence is shown in blue. Insertion and deletion sequences are shown in red.

Fig 3. Deduced amino acids for in-frame mutations after deep sequencing.

Wild type (WT) sequences are shown on the top. The embryo (E16.5) number (#), nucleotide number of small insertion (+) or deletion (-) (Indels) are shown on the left. Classified "types" are shown on the right. Amino acids corresponding to the guide RNA sequence are underlined. Deleted and altered amino acids are indicated in red.

Fig 4. Schematic representation of genomic analysis of *Fgf10*-CRISPR F0 embryos at E16.5 by deep sequencing.

A, percentage of total reads for the *Fgf10* crispants. wt, wild type *Fgf10* target nucleotide sequence; in, in-frame mutations by small insertion or deletion; out, frameshift mutations. Among in-frame mutations, the percentage of those that preserve the codons for Lys196 and His201 is shown in green, while the percentage that eliminate either of them is shown in yellow. **B**, percentage of wild type and in-frame mutations in which nucleotides for both Lys196 and His201 are retained: 8.3 ± 6.2% for type I, 25.3 ± 2.7% for type II, and 54.3 ± 9.5% for type III. Data are presented as means ± SEM. *p<0.01 (p=0.008).

Fig 5. Limb phenotypes and lung histology of *Fgf10*-CRISPR F0 embryos at E16.5.

Representative embryos are shown for wild type (WT), type I (embryo #7), type II (#3), and type III (#1). **A**–**D**, transverse section of the embryonic chest region. **E**–**H**, close-up of the embryonic lung (boxed area) shown in (**A–D**), respectively. Asterisks show putative terminal tubules in the lung. **I**, the number of lung terminal tubules per unit area. In type I and type II embryos, there is a significant decrease in the number compared with that of wild type. Data are presented as means ± SEM. Source data are available in Table 3. Ac, accessory lobe; Ca, caudal lobe; Cr, cranial lobe; L, left lobe; Mi, middle lobe. Scale bars: 500 μm in (**A–D**), and 100 μm in (**E–H**).

Fig 6. Immunohistochemistry of the lung in type II and type III *Fgf10*-crispants and wild type embryos at E18.5.

Representative data are shown. Nuclei are stained with Hematoxylin. **A-C**, Localization of Surfactant protein C (SPC) (alveolar type 2 epithelial cells) is indicated by brown staining. **D-F**, close-up of the boxed area shown in (**A–C**), respectively. **G-I**, negative control, using normal rabbit IgG instead of anti-SPC antibody. Scale bars: 100 μm in (**A-C**); 50 μm in (**D-I**). **J**, **K**, the number of SPC-positive cells in type III embryos is

83 correlated to the percentage of functional *Fgf10* genotypes in limb (**J**) or neck DNA (**K**).
84 **L**, the number of SPC-positive cells in type III embryos is not to correlated to their weight
85 (**K**). Source data for (**J-L**) are available in Table 1. **M**, Schematic drawings to show
86 percentage of total reads for the type III *Fgf10* crispants. wt, wild type *Fgf10* target
87 nucleotide sequence; in, in-frame mutations by small insertion or deletion; out, out-of-
88 frame mutations. Among in-frame mutations, the percentage of those that preserve the
89 codons for Lys196 and His201 is shown in green, while the percentage that eliminate
90 either of them is shown in yellow.
91

Supporting information

Supplementary Tables

S1 Table. List of reagents and equipment used in this study.

S2 Table. Summary of deep sequencing data on DNA from different tissues of E18.5 embryos.

The percentage of sequence reads for each genotype category is shown in contingency tables. Wt, wild type *Fgf10* genotype; In, in-frame mutations in the *Fgf10* gene; Out, frameshift mutations in the *Fgf10* gene; N.D., not done; N.A., not applicable. *p*-values of Chi-square test (see Table 4) are shown for reference.

S3 Table. Summary of type II and type III embryos examined for E18.5 lungs by immunohistochemistry, shown in Fig 6 and Fig S5.

*Approximately 295- (#11_2) and 79- (#22_2) bp insertion were detected by microchip electrophoresis and the number of sequence reads in deep sequencing was corrected accordingly (see Materials and methods). N.D., not determined.

S4 Table. Primers used for quantitative PCR (qPCR) analysis.

Supplementary Figures

S1 Fig. Structure of the mouse *Fgf10* gene indicating the target sites for CRISPR/Cas9 system.

S2 Fig. Cartilage and bone staining to reveal skeletal structures.

A-H, whole mount staining. Left (**A, C, E, G**) and right (**B, D, F, H**) lateral views are shown. Arrows show truncated limb and girdle bones. **I-K,** wild type skeletal structures, showing scapula and forelimb (**I**), hindlimb (**J**), and pelvic girdle (**K**). au, autopod; f, femur; fi, fibula; h, humerus; il, ilium; is, ischium; p, pubis; r, radius; sc, scapula; sp, spine; st, stylopod; t, tibia; u, ulna; ze, zeugopod. Scale bars: 1 mm.

S3 Fig. Mismatch cleavage assay.

123 **A**, PCR primers were designed in the upstream region of the exon 3 and in the exon 3,
124 giving rise to a PCR amplicon size of 501 bp. DNA fragments of 309 bp and 192 bp are
125 generated by the Resolvase when the *Fgf10* genome has been cleaved by Cas9 and non-
126 homologous end joining has been achieved. **B**, Electrophoresis of the enzyme-treated
127 mouse genomic DNA from the *Fgf10*-CRISPR F0 embryonic necks. The DNA ladder for
128 DNA size reference and a result of DNA from a wild type (WT) mouse are shown on the
129 left. Three DNA fragments of approximately 500 bp (▼), 300 bp (▽ in gray), and 200
130 bp (▽) are seen in all the lanes except for the wild type and #4 lanes. In embryo #3, an
131 extra band for large insertion (328 base) is shown (see Fig 2). **C**, Genomic analysis of the
132 #4 embryo as revealed by Sanger sequencing. Deduced amino acid sequences are also
133 shown. Lys-196 and His-201 are highlighted in yellow and green, respectively. Altered
134 amino acids are indicated in red. Asterisks indicate stop codons.
135
136 **S4 Fig. Limb phenotypes and lung histology of all embryos examined at E16.5 as**
137 **summarized in Table 3.**
138 Scale bars: 500 μm.

S5 Fig. Limb phenotypes, lung histology, and the number of SPC-positive cells per total cell number of type II and type III embryos at E18.5.

Source data for (**L, M**) are available in S3 Table. Lateral views of type II (**A-F**), and type III (**G, H**) embryos at E18.5. Arrowheads show limb defects. (**I-K**) In all three type II embryos, the accessory lobe was lost. In embryo #24_2, (**J**), the middle lobe (Mi) was also undetectable. Data in (**L**) are presented as means ± SEM. (**M**) In these embryos, the number of SPC-positive cells was more correlated to the percentage of wild type *Fgf10* genotype (correlation coefficient [R] was 0.728 for neck DNA) than that including in-frame mutations with Lys196 and His201 retained (R=0.334). Scale bar: 2 mm (**A-H**).

S6 Fig. Quantitative PCR analysis for mRNA expression of mesenchymal and its related marker genes in wild type and type III lungs at E18.5.

S7 Fig. Cecum and colons of the wild type and *Fgf10*-CRISPR F0 (type I and type II) embryos at E18.5.

155 **A-C**, wild type (Wt) cecum (c), colon, and small intestine (si) are shown from three
156 embryos examined. Ileum and colon were cut at dissection. **D-G**, type I cecum is reduced
157 compared with the wild type. Whether the cecum epithelium is absent or not cannot be
158 identified from these photos. Type I embryos show an atresia of the colon, but the length
159 varies depending upon the embryos. **H-I**, type II embryos examined (n = 2) do not exhibit
160 a reduced cecum or an atresia of the colon. The colons presented here were cut as distally
161 as possible. **J**, the approximate length of the cecum. The length of type I cecum is
162 significantly decreased compared with the wild type. The length of two type II embryos
163 examined is also shown for reference. Scale bar: 1 mm (in A for all to scale).