A piggyBac insertion disrupts Foxl2 expression that mimics BPES syndrome in mice

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Blepharophimosis, ptosis, epicanthus inversus syndrome (BPES) is an autosomal dominant genetic disorder characterized by small palpebral fissures and other craniofacial malformations, often with (type I) but could also without (type II) premature ovarian failure. While mutations of the forkhead transcription factor FOXL2 are associated with and likely be responsible for many BPES cases, how FOXL2 affects craniofacial development remain to be understood. Through a large-scale piggyBac (PB) insertion mutagenesis, we have identified a mouse mutant carrying a PB insertion ∼160 kb upstream of the transcription start site (TSS) of Foxl2. The insertion reduces, but not eliminates, the expression of Foxl2. This mutant, but not its revertant, displays BPES-like conditions such as midface hypoplasia, eyelid abnormalities and female subfertility. Further analysis indicates that the mutation does not affect mandible, but causes premature fusion of the premaxilla–maxilla suture, smaller premaxilla and malformed maxilla during midface development. We further identified an evolutionarily conserved fragment near the insertion site and observed enhancer activity of this element in tissue culture cells. Analyses using DNase I hypersensitivity assay and chromosome conformation capture assay in developing maxillary and periocular tissues suggest that the DNA region near the insertion site likely interacts with Foxl2 TSS. Therefore, this mutant presents an excellent animal model for mechanistic study of BPES and regulation of Foxl2.

INTRODUCTION

Blepharophimosis, ptosis, epicanthus inversus syndrome (BPES) is an autosomal dominant genetic disorder characterized by eyelid malformations, and in some cases also with premature ovarian failure (1). BPES patients not only develop small palpebral fissures, epicanthus inversus and telecanthus, but also manifest non-ocular facial features, such as a broad nasal bridge and a short philtrum. Human genetic studies demonstrated that the forkhead transcription factor FOXL2 on chromosome 3q23 was mutated in BPES patients (2,3). Intragenic mutations and genomic rearrangements affecting FOXL2 are associated with 72 and 16% of all BPES patients, respectively (4,5).

Foxl2 expresses in the ovarian follicles, the periciliary mesenchyme (PM) and the brachial arches (BAs) during mouse development (2,6). In the ovarian follicles, Foxl2 is essential for the differentiation of granulosa cells (7,8). Induced ablation of Foxl2 in adult ovary resulted in transdifferentiation of granulosa cells into Sertoli cells (9). Consistently, deletion of the Foxl2 suppressor Dmrt1 in adult Sertoli cells led to feminized testes (10). In the PM, Foxl2 transcription is negatively regulated by the Notch signaling. A gain-of-function Notch transgene led to agenesis of the eyelid levator muscle, while disruption of Foxl2 resulted in absent eyelids (8,11). In the embryonic mandibular arch, Foxl2 was repressed by mandible-specific transcription factors Dlx5 and Dlx6 (6,12). However, the roles that Foxl2 plays in the maxillary development are still unknown.

Deleterious mutations of long-range regulatory elements are reported to be responsible for several genetic diseases (13,14). For example, loss of the downstream regulatory region of PAX6 results in aniridia (15), and disruption of a distant SHH enhancer causes preaxial polydactyly (16,17). The goat polled intersex syndrome (PIS) has been shown to be caused by an 11.7 kb upstream deletion that affects FoxL2 expression.

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Six percent of the BPES cases are caused by mutations outside of the FOXL2 transcription unit, also suggests the existence of long-range regulatory elements of the disease gene (4,5). In fact, genomic studies have identified three upstream fragments required for the correct expression of FOXL2 in cultured human cells (21).

piggyBac (PB) insertion has been shown to be an effective way to generate genetic mutations for functional study (22,23). PB insertion often reduces but not eliminates gene expression, facilitating the identification of specific functions of genes with highly pleiotropic knockout phenotypes. We have performed a large-scale PB insertion mutagenesis to identify novel disease-related functions of the genome. In this study, we show that a PB insertion (~160 kb upstream of the transcription start site (TSS)) of Foxl2 partially disrupted the expression of Foxl2, resulted in BPES-like conditions such as midface hypoplasia, eyelid abnormalities and female subfertility in the homozygotes. We report our analysis of the effects of this mutation on midface development and Foxl2 transcription.

RESULTS
Growth retardation caused by midface defects in the mutant mouse

We have identified a growth retardation mutant from a large-scale insertional mutagenesis project with the PB transposon in mice (22,23). At birth, the body weight of both heterozygotes (PB/+ ) and homozygotes (PB/PB) mice is similar to their wild-type (+/+ ) littermates. However, PB/PB animals started to lose weight on postnatal Day 17 (P17) and became 38.4% lighter at the age of weaning (P21) (Fig. 1A). Most of the homozygotes (19/23) did not survive by the end of the first month of their lives, only 8.7% (2/23) grew to adulthood (Fig. 1B).

Since malocclusion is a known cause of lower body weight at weaning, we tested this possibility by following postnatal craniofacial development of PB/PB mice. A shortened and curved midface profile could be recognized at birth and became more prominent at the age of 3 weeks (Fig. 1D). Mutant incisors could not occlude properly after eruption and grew unrestrictedly in the oral cavity. At the age of weaning, 75% (24/32) of the mutants occupied overgrown mandibular incisors, while others had broken incisors probably due to daily gnawing. Overgrowth of the mandibular incisors became more severe in older survivors, resulted in a tusk-like appearance (Fig. 1F). In addition to malocclusion, PB/PB mice often developed palpebral anomalies. Among 32 homozygous animals examined, we found 27 with bilateral small palpebral fissures and one case of unilateral small palpebral fissures. Among these 27 mice, six also showed unilateral periocular hair loss, while another one lost its periocular hair on both sides (Fig. 1D). No obvious difference in malocclusion or the palpebral defects were observed between males and females (data not shown).

To quantify changes of the craniofacial structure, we measured the skulls of 2-week-old mice by microcomputerized tomography (CT). We found normal mandibles (md), but malformed premaxillae (pmx) and maxillae (mx) in PB/PB mice (Fig. 1H and J). Compared with that of a heterozygous individual, the premaxilla length on the basal aspect was reduced by 48.4%, while the premaxillary angle on the lateral aspect (rpm) was increased by 24.7% in the homozygote (Fig. 1O and P). Compared with the heterozygous maxilla, homozygous maxilla has a similar total length. However, the distance from the infraorbital fissure to the first molar (mxa) was reduced by 21.0% on the basal aspect. In contrast, the distance from the first molar to the most posterior maxilla-palatine suture (mxp) was increased by 7.8% (Fig. 1O).

Midface defects were caused by the PB insertion

The PB insertion was mapped to a TTAA site in the intron of AK087804, an EST without long-coding sequences (Fig. 2A). To verify that mutant phenotypes were caused by the PB insertion, but not by unknown mutations potentially generated during the mutagenesis, we generated revertants by crossing PB/PB mice with a transposase line Act-PBase. Ubiquitously expressed PBase can induce precise excision of the PB insertion both in somatic cells and in gametes, resulted in a true revertant carrying restored wild-type alleles (+/+ ) in the following generation (22) (Fig. 2B and C). We did not detect craniofacial defects in all 11 revertant progeny, while all three non-revertant littermates still possess the anomalies (Fig. 2D and E; Supplementary Material, Fig. S1A). These results suggested that the PB insertion is the causative factor of mutant defects.

PB insertion partially disrupts the expression of Foxl2

We then carried out experiments to identify the gene of which the expression is affected by the PB insertion and responsible for the craniofacial phenotype. We first excluded the non-protein-coding gene AK087804 because it does not express in the head region of the embryo, nor in the testis, the brain, the liver, or the muscle in mice of 28 days old (Supplementary Material, Fig. S2). We next evaluated the expression of protein-coding genes within ~250 kb from the PB insertion in embryonic facial tissues (Fig. 2A; Supplementary Material, Table S1). Compared with those of the wild type, expression of most of the genes was unaffected in PB/PB embryos. However, the expression of Foxl2 was modestly but significantly reduced (Supplementary Material, Fig. S3 and Table S1).

Because the expression of Foxl2 in specific cell types could be more prominently affected by the insertion mutation, we further analyzed the expression changes of this gene by RNA in situ hybridization. Foxl2 expression could be detected in embryonic BAs and PM. In the BA1, Foxl2 signals are prominent at the

Comparisons...
anterior and dorsal sites of the maxillary arch (mxBA1), and weaker in the maxillary–mandibular junction at embryonic Day 10.5 (E10.5). Moderate expression of Foxl2 could also be detected in the second BA and the third BA (BA2 and BA3, respectively) at the same stage (Fig. 3A and B). In E10.5 PB/PB embryos, expression of Foxl2 became more restricted in mxBA1 (Fig. 3C), where quantification analysis revealed a 66% reduction of the in situ hybridization signals (Fig. 3J). Consistently, the expression of Osr2, a potential target of FOXL2 (26,27), was also significantly reduced in the maxillary–mandibular junction of these embryos (Fig. 3K and L). In the PM, Foxl2 expression is dominant at E11.5 and E12.5 (Fig. 3D, E, G and H), but significantly reduced in the PB/PB embryos (Fig. 3F and I). Upon the precise excision of PB insertion in revertant animals, Foxl2 expression was restored to a comparable level to that of the wild-type allele (Supplementary Material, Fig. S1B and C). Taken together, these data indicated that Foxl2 expression and function are significantly downregulated by the PB insertion. The mutation was therefore named as Foxl2PB.

Figure 1. Growth retardation and midface defects in homozygous PB-insertion mutants. Growth curves showing that homozygous mutants start to lose weight on P17 (A) and gradually die after P23 (B). Representative images of the mutants at P21 (C and D) and P35 (E and F). Homozygous mice display characteristic midface profiles, small palpebral fissures (arrow, D) and overgrown lower incisors (F). (G–J) Micro-CT images of the skulls of P14 pups reveal normal mandibles (md), but malformed premaxillae (pmx) and maxillae (mx) in the homozygotes. Small infraorbital fissures (iof) (arrow, H) and short palatine foramina (pf) (circle, J) are observed in the homozygotes. (K–N) Alizarin red and Alcian blue staining of the skulls of P14 mutants. The interdigitated pattern observed in lateral (arrow, K) and ventral (arrowhead, M) views suggests a well-developed premaxillary–maxillary suture in the heterozygotes, while a smooth suture indicates premature fusion in the homozygotes (L and N). (O and P) Craniofacial analysis of the P14 skulls reveals reduced anterior–posterior ratio of the maxilla (O) and larger premaxilla angle (P) in the homozygotes. The craniofacial landmarks are: n, the nasion; p, the most inferior and anterior point on alveolar process of premaxilla; m, the intersection point between the maxillary bone and the mesial surface of the upper first molar; ua, the most inferior point of the infraorbital fissure; up, the most posterior point of the maxilla-palatine suture; la, the most inferior and anterior point on alveolar process of mandible; lp, the most posterior point of angular process of mandible. Scale bars: 2 mm in (G and H), (I and J), (K and L) and (M and N); 0.5 mm in (K and L) and (M and N) insets. Data in (A), (O) and (P) represent the means ± SEM. ∗P < 0.05, ∗∗P < 0.01 and ∗∗∗P < 0.001; ns, not significant.
A long-range regulatory element of \textit{Foxl2} near the \textit{PB} insertion

Partial disruption of \textit{Foxl2} expression by a distant \textit{PB} insertion suggested the existence of long-range regulatory elements. Sequence analysis identified four evolutionarily conserved fragments (ECFs) within a 25 kb region flanking the \textit{PB} insertion (Fig. 4A). To test whether they have transcription regulation capabilities, we generated luciferase reporters driven by a \textit{CMV} minimal promoter in combination with individual ECF (Fig. 4B). We found ECF1, but not other three elements, could significantly enhance the expression of luciferase in 293 T cells. The luciferase activity was 25-fold more active at the presence of ECF1 (Fig. 4C). This result suggested that ECF1 may act as a transcription enhancer.

Long-range regulatory elements can recruit transcription factors and interact with their target promoters \textit{in vivo} (29,30). To test whether ECF1 was accessible to regulatory proteins during craniofacial development, we mapped the DNase I hypersensitive sites in E10.5 maxillary cells. We observed a hypersensitive subband with a probe targeting the ECF1-containing fragment, indicating that ECF1 was localized in an open chromatin region (Fig. 5A and C). This result suggested that ECF1 may act as a transcription enhancer.

Ovarian follicle retardation and subfertility in female mutants

The craniofacial anomalies of \textit{Foxl2} PB/PB mice are reminiscent of the conditions of BPES patients. To further explore if \textit{Foxl2} PB/PB have the ovarian defects commonly observed in type 1 BPES patients, we examined the expression of \textit{Foxl2} and the related changes in the mutant ovaries. We detected reduced ovarian expression of FOXL2 and smaller uteri and ovaries in P14 \textit{Foxl2} PB/PB mice (Fig. 6A and C). Histological analysis showed less secondary follicles in the homozygous ovaries at this stage (Fig. 6D and E). Ovarian abnormalities persisted when \textit{Foxl2} PB/PB females grow older. At the age of P35, while heterozygous ovaries developed antral follicles, no such follicles were observed in the homozygotes. In addition, some of the \textit{Foxl2} PB/PB follicles occupied fewer layers of granulosa cells at this stage (Fig. 6F and G). These phenotypes may suggest retardation rather than deficiency of the ovarian follicles, since \textit{Foxl2} PB/PB female could still produce a small number of progeny at adulthood (Fig. 6H).

DISCUSSION

We described a new allele of \textit{Foxl2} in mice. The allele, \textit{Foxl2} PB, was generated by a \textit{PB} insertion \~160 kb upstream from the TSS of \textit{Foxl2}. It partially disrupted the expression of \textit{Foxl2}, resulted in midface hypoplasia, eyelid malformation and female subfertility in homozygous animals. We also reported an evolutionary conserved element ECF1 close to the \textit{PB} insertion. ECF1 may function as a long-range enhancer of \textit{Foxl2} during craniofacial development. It not only showed transcription stimulation...
activity in tissue culture cells but also interacted with Foxl2 promoter in the developing maxillary and periocular cells.

**Foxl2 controls maxillary development in mice**

The forkhead transcription factor Foxl2 is involved in the specification of mouse gonad cells (9,10). Recently, Foxl2 was also reported to be regulated by Notch signaling during the formation of eyelid levator smooth muscles (11). The craniofacial defects observed in Foxl2PB/PB mice revealed an essential role of Foxl2 during midface skeletal development. Downregulation of Foxl2 led to premature fusion of the premaxillary–maxillary suture, shortened premaxilla and malformed maxilla. Thus, Foxl2 may not only contribute to the growth but also be involved in the pattern formation of the midface skeleton. The downstream targets of Foxl2 during midface development are still unknown. Although transcriptomic analysis has suggested that the zinc finger transcription factor OSR2 and several other factors are potential targets of FOXL2 in human granulosa cells (26), in situ hybridization or real-time PCR failed to detect differential expression of these genes in the maxillary arch of homoygous animals (data not shown). Further analysis of global gene expression in specific tissues may be needed to identify targets of FOXL2 involved in specific physiological functions.

It is interesting to note that Foxl2 expression may be under complex spatial and temporal regulations. In Foxl2PB/PB embryos, Foxl2 transcription was reduced by two-thirds in the midface progenitor tissue mxB1 at E10.5 (Fig. 3C and J), while milder alterations were observed in other BA tissues and in the PM at different stages (Fig. 3F and I; Supplementary Material, Fig. S3). This may explain the phenotypic discrepancy between Foxl2PB/PB and heterozygous Foxl2 knockout animals. In the latter case, loss of one copy Foxl2-coding sequence, which presumably leads to 50% reduction of the transcripts, resulted in no dramatic morphological changes in the midface (8). Alternatively or additionally, this discrepancy may reflect a difference in sensitivity to Foxl2 expression level between these two strains with different genetic backgrounds.

Foxl2PB/PB mice do not display dramatic reduction in gene expression even in specific head regions, suggesting that normal development of midface is highly sensitive to the expression level of Foxl2. Our results may provide an explanation for that many known BPES cases are associated with mutations in the regulatory region of FOXL2 (4,5,21). In addition, the fact that many other BPES-associated FOXL2 mutations are likely reduction-of-function point mutations is also consistent with the idea that midface development demands full strength of Foxl2 function (4). Therefore, our results demonstrate the value of genetic analysis using reduction-of-function mutations and PB-mediated mutagenesis in functional study of the genome.

**ECF1 as a long-range regulatory element of Foxl2**

Regulatory sequence mutations of Foxl2 orthologs in different species have been previously reported. The PIS deletion reduces Foxl2 expression from 280 kb away upstream in goat (18–20). Deletions affecting upstream genomic sequences of FOXL2 also exist in 4% of the BPES patients (4,5). We identified an evolutionary conserved fragment ECF1 close to the PB insertion in mice. Cross-species alignment of the regulatory sequences of Foxl2 orthologs showed that ECF1 is highly conserved among goat, mouse and human (Supplementary Material, Fig. S4A). A 3C study has detected three upstream genomic fragments interacting with FOXL2 promoter in human KGN cells, centralizing at −177, −283 and −360 kb, respectively (21). Goat orthologs of ECF1 and PIS deletion are close to the sequences that are conserved with the −177 and −283 kb fragments, respectively (Supplementary Material, Fig. S4B). Meanwhile, the human ortholog of ECF1 is contained in the −177 kb fragment. Chromosome breakpoints 130, 160 or 171 kb upstream of FOXL2 have been detected in BPES patients with balanced translocations (31–35). These translocations may isolate the transcription regulatory elements, which likely include the hECF1, result in misregulation of FOXL2 in patients. We found that ECF1 could not only stimulate transcription but also physically interact with Foxl2 promoter in developing...
maxillary and pericocular cells. These results were consistent with the expectation that ECF1 plays an evolutionarily conserved role in regulating Foxl2 expression in mice, though we could not exclude the possibility that some mouse-specific genomic fragments nearby also participate in the regulation. In fact, sequence analysis does reveal that ECF1 contains several homeodomain-binding sites (28,36) (Supplementary Material, Fig. S4A). The PB insertion may interfere with the interaction between ECF1-containing regulatory sequences and Foxl2 promoter, or alter the high-order chromatin structure.

**Foxl2PB/PB** mice as a model of BPES

BPES patients develop eyelid abnormalities including blepharophimosis, ptosis, epicantus inversus and telecanthus. They also manifest non-ocular features such as a broad nasal bridge.
and a short philtrum. Foxl2<sup>PB/PB</sup> animals exhibited comparable craniofacial defects: small palpebral fissures, shortened premaxilla and malformed maxilla. Premature ovarian failure is a typical symptom of type I BPES patients. Similarly, we observed follicle retardation and subfertility in female mutants. The fact that Foxl2<sup>PB/PB</sup> mutant recapitulated the craniofacial and ovarian conditions of type I BPES patients makes it an animal model of the disease. Quantitative description of the craniofacial defects provided here may help future in-depth characterization of human patients. This mutant may also benefit further studies of the function and transcriptional regulation of Foxl2.

**MATERIALS AND METHODS**

**Mouse strains**

All animal procedures were approved by the Animal Care and Use Committee of the Institute of Developmental Biology and Molecular Medicine. Both Foxl2<sup>PB</sup> and Act-PBase strains were kept on the FVB/N background.

**Histology and micro-CT**

Ovaries were fixed in 4% paraformaldehyde and dehydrated in 30% sucrose before embedded in Frozen Section Medium Neg-50 (Richard-Allan Scientific). After frozen in liquid nitrogen-cooled isopentane, sections of 8 μm were prepared and stained with hematoxylin and eosin. Alizarin red and Alcian blue staining of the skeleton were performed as previously described (24).

Micro-CT data were collected by an Inveon MMCT system (SIEMENS). Cranio metric analysis was performed in 3D visualization with the illustrated landmarks (Fig. 1K and L).

**Reverse transcription and real-time RT-PCR**

Total RNAs were extracted with Trizol (Invitrogen) and reverse transcribed (Takara), real-time PCR was performed using Brilliant QPCR Master Mix (Stratagene) following the manufacturer’s instruction.

**Whole-mount in situ hybridization**

The Foxl2 and Osr2 cDNAs were amplified by PCR and cloned into the T-vector. RNA probes were synthesized using a DIG RNA labeling kit (Roche). Embryo whole-mount in situ hybridization was performed as described (37,38) and photographed with a Leica MZFLIII microscope. Hybridization signals are quantified by ImageJ (39).

**Western blot**

Ovary proteins were extracted in RIPA with freshly added 1 mM PMSF and 1× protease inhibitor (Roche). Proteins were separated on 10% SDS–PAGE. FOXL2 and GAPDH were detected by a rabbit anti-FOXL2 (1 : 1000, F0805, Sigma) and...
a mouse anti-GAPDH (1 : 2000, KC-5G4, Kangcheng) antibody, respectively.

Comparative genomic analysis
A 25 kb mouse genomic sequence centralizing at the PB insertion (chr 9: 98 682 701–98 707 700, NCB137/mm9) was aligned with its human ortholog (chr 3: 140 145 248–140 558 578, NCB136/hg18) by zPicture (40). Evolutionarily conserved elements were identified under a stringent requirement of at least 80% in similarity and 200 bp in length. Cross-species alignment of the Foxl2 upstream sequences from human (chr3: 140 145 248–140 558 578, NCB136/hg18), mouse (chr9: 98 489 179–98 858 248, NCB137/mm9) and goat (chr1: 127 322 877–127 806 801, CHIR1.0) were performed by mVISTA with the same stringency requirement (41).

Luciferase enhancer assay
A minimal CMV promoter (pNBio, DQ520290) was synthesized to replace the EcoRv–BglII fragment of pGL4.20 to generate CMV-Luc2. ECF1–4 fragments were amplified by PCR and cloned into the XhoI site of CMV-Luc2 in the same direction of Foxl2 transcription to generate individual reporter plasmid. 293T cells were cultured in DMEM (GIBCO-BRL) with 10% fetal bovine serum at 37 °C and 5% CO2. Transfections were carried out with Lipofectamine 2000 (Invitrogen) in 24-well plates. One microgram of a reporter plasmid and 0.1 μg of the control plasmid (pCX-nLacZ) were applied for each well. Cells were harvested 48 h later and assayed for the luciferase and β-galactosidase activities according to standard protocols (42).

Mapping the DNase I hypersensitive sites
The maxillae from 50 E10.5 embryos were dissected and homogenized to prepare the nuclear extracts (43). Nuclear extracts equivalent to 10 embryos were digested with 0, 0.25, 0.5, 1 or 2 U DNase I (D4263, Sigma), respectively, in a total volume of 500 μl at 37 °C for 5 min. The reaction was attenuated by addition of 125 μl 0.05 μM EDTA; 2.5% SDS. Genomic DNA was then purified and analyzed by Southern blot according to the standard protocol (42).

3C assay
The 3C assay were performed as described (44,45). Cross-linked chromatin DNA from 1 × 10^7 maxillary or periocular cells was treated with 500 U BglII (NEB) and ligated by 400 U T4 ligase (NEB). After purification, 200 ng DNA was PCR amplified for 40 cycles to detect the ligation product. The BAC (RP23-9M20) containing Foxl2 TSS and ECF1 was used to generate randomly ligated BAC DNA for positive control template as described (45).

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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