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A novel ENU-induced mutation, *peewee*, causes dwarfism in the mouse

Lee Bon-Ryon¹, Kiyoshi Kano^{1,*}, Jay Young², Simon John², Patsy M Nishina², Jurgen K Naggert², and Kunihiko Naito¹

¹ Laboratory of Applied Genetics, Graduate School of Agricultural and Life Science, University of Tokyo, Tokyo 113-8657, Japan

² The Jackson Laboratory, Bar Harbor, ME 04609, USA

Abstract

We identified a novel fertile, autosomal recessive mutation, called *peewee* and that results in dwarfing, in a region-specific ENU-induced mutagenesis. These mice at litter size were smaller than those of other strains. Histological analysis revealed that the major organs appear normal, but abnormalities in cellular proliferation were observed in bone, liver and testis. Haplotype analysis localized the *peewee* gene to a 3.3-Mb region between *D5Mit83* and *D5Mit356.3*. There are 18 genes in this linkage area, and we also performed *in silico* mapping using the PosMedSM program, which searches for connections among keywords and genes in an interval, but no similar phenotype descriptions were found for these genes. In the *peewee* mutant compared to the normal, C57BL/6J mouse, only *Slc10a4* expression was lower. Our preliminary mutation analysis examining the nucleotide sequence of three exons, two introns and an untranslated region of *Slc10a4* did not find any sequence difference between the *peewee* mouse and the C57BL/6J mouse. Detailed analysis of *peewee* mice might provide novel molecular insights into the complex mechanisms regulating body growth.

Introduction

Growth-retarded mice provide a valuable model system with which to elucidate the molecular mechanisms that interplay among growth, body size and genetic influences. Some spontaneous genetic mutations in mice cause dwarfism by altering endocrine systems, such as Snell dwarf (*dw*), Ames dwarf (*df*), and little (*lit*) mice (Bartke 1964; Eicher and Beamer 1976; Schaiber and Gowen 1961; Snell 1929). These mutant models were important for elucidating the mechanisms of growth hormone regulation and the role of transcription factors such as *Prop1* (Sornson et al. 1996) and *Pit1* (Li et al. 1990) as regulators underlying neuroendocrine axis activation and organization. Genetic mutations in these two factors cause defects in anterior pituitary cell types, resulting in poor or no production of GH, prolactin, and TSH, but normal basal levels of FSH and LH (Bartke 1964, 1965; Cheng et al. 1983; Sinha et al. 1975; van Buul-Offers 1983). However, dwarfism is not limited to disorders of the pituitary gland and hypothalamus. Besides central endocrine mechanisms, peripheral defects retard growth, as seen in hypothyroid (*hyt*), congenital goiter (*cog*), growth-retarded (*grt*), thyroid peroxidase (*tpo*), *Pax8*, and discoidin domain receptor 2 (*slie*) homozygous mutant mice (Beamer et al. 1987; Beamer et al. 1981; Flamant et al. 2002; Kano et al. 2008; Takabayashi et al. 2006; Yoshida et al. 1994).

* Address all correspondence and requests for reprints to: Kiyoshi Kano Laboratory of Applied Genetics, Graduate School of Agricultural and Life Science, University of Tokyo, Tokyo 113 8657, Japan Phone: +81 3 5841 5919 Fax: +81 3 5841 7568 akano@mail.ecc.u-tokyo.ac.jp.

Mice homozygous for dwarf mutations often manifest infertility (Bartke 2000). The infertility in Snell dwarf mice, for example, appears to be due to gonadal dysfunction arising from a lack of neuro-endocrine axis activation in either sex (Bartke 2000; Bartke and Lloyd 1970; Smith and MacDowell 1931).

In mice, forward genetic screens are powerful tools with which to identify biological pathways and the genetic components of complex phenotypes. A program was conducted to mutagenize the mouse genome with ENU and to specifically recover mutations in the region spanned by the rump-white (*Rw*) inversion on Chr 5 (Wilson et al. 2005). *Rw* is a radiation-induced mutation causing depigmentation of the posterior and ventral abdomen in heterozygotes and embryonic lethality in homozygotes (Stephenson et al. 1994). We discovered a novel ENU-induced mutant mouse with fertility defects and dwarfing, *peewee*.

In this study, we characterize and map the *peewee* mutant. Detailed analysis of *peewee* mice might provide novel molecular insights into the complex mechanism of body growth.

Materials and methods

Animals and phenotyping

The *peewee* mutation was generated through the ENU mouse mutagenesis project by Dr. Simon John (The Jackson Laboratory). The *peewee* mice were maintained on a mixed genetic background of C57BL/6J (including the Rump white (*Rw*) inversion) and C3H/HeJ (Schimenti and Bucan 1998; Wilson et al. 2005). Homozygous mice were obtained by intercrossing homozygous *peewee* mice. The *peewee* mice will be available at the Bio Resource Center of RIKEN, Japan.

All mice used in this study were bred and maintained in vivariums at either the University of Tokyo or The Jackson Laboratory. Their respective institutional Animal Care and Use Committees approved all procedures in these studies. A minimum of three mice were analyzed at each time point.

Blood chemical analysis was conducted in Rodents Multi-Analyte Profiles by Rules-Based Medicine, Inc., (Austin, TX).

Microsatellite marker analysis

As previously reported, *peewee* was never observed to recombine in the *Rw* inversion, which spans the ~27- to 74-Mb interval on chromosome 5. To reduce the critical region, a mating cross of Balb/cCrSlc $\times$ *peewee* was established to generate F1 offspring. The heterozygous F1 mice, which do not exhibit dwarfism, were intercrossed for the initial linkage mapping study. The resultant F2 progeny mice were weighed at 10 weeks of age to distinguish affected animals. Genomic DNA from F2 offspring was analyzed using microsatellite markers to construct a genetic map. Microsatellite marker primer pairs for the genome scan were purchased from Research Genetics; Invitrogen Life Technologies, Inc. (Carlsbad, CA). For genomic PCR amplification, 25 ng of tail DNA was used in a 10 μ l volume containing 50 mM KCl, 10 mM Tris-Cl pH 8.3, 2.5 mM MgCl₂, 0.2 mM oligonucleotides, 200 μ M dNTP, and 0.02 U BIOTAQ DNA polymerase (Bioline Inc., Randolph, MA). The cycling conditions were 95°C for 2 min, followed by 49 cycles at 94°C, 20 sec; 50°C, 20 sec; 72°C, 30 sec; and a 7 min extension at 72°C. PCR products were separated by electrophoresis on a 4% MetaPhor agarose gel (FMC, Rockland, ME) and visualized by ethidium bromide staining.

Mutation analysis

Mutation analysis was performed by direct sequencing of genomic DNA and cDNA using primers that were designed flanking the entire genomic region of *Slc10a4* and the cDNA of *Tec*. Genomic PCR and RT-PCR were performed using primers spanning the coding regions of each gene within the *peewee* critical interval (Supplemental Table 1). For genomic PCR and RT-PCR, 25 ng of genomic DNA or cDNA was used in a 20 µl volume containing 0.5 µM oligonucleotides, 200 µM dNTP, and 0.4 U Phusion DNA polymerase (Finnzymes OY, Espoo, Finland). The cycling conditions were 98°C for 30 sec, followed by 35 cycles at 98°C, 10 sec; 58°C, 30 sec; 72°C, 30 sec; and a 7 min extension at 72°C. The appropriate bands were excised. DNA was recovered using the NucleoSpin Extract II (Macherey-Nagel GmbH & Co., KG, Düren, Germany) per the manufacturer's instructions. Purified genomic PCR and RT-PCR products were sequenced using the 3130 Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan).

Histology and immunohistochemical analysis

Bone samples were collected from wild-type and *peewee* mice at 2 and 10 weeks of age (n>3 per sample and age). Bone was decalcified and fixed in Bouin's reagent overnight. Samples were dehydrated, embedded in paraffin, cut into 5 µm sections, and processed for hematoxylin and eosin, TUNEL, or immunohistochemical staining.

To localize PCNA in *peewee* and wild-type homozygotes, polyclonal antibodies to PCNA were used (sc-56; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Following deparaffinization, the sections were boiled in sodium citrate buffer (pH 6.0) for 5 min in a microwave oven for antigen retrieval. The sections were pre-incubated with 5% normal goat serum for 30 min, followed by 2 hr incubation with the primary antibody (1:500), then with biotinylated secondary antibody (1:400; Vector Laboratories, Burlingame, CA) for 1 hr, and next with streptavidin-biotin-HRP complex (Vectastain Elite ABC, Vector Laboratories) for 30 min. As a negative control, sections without the primary antibody were processed (data not shown). All the reactions were carried out at room temperature. Terminal deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining was performed according to the manufacturer's protocol (DeadEnd Colorimetric TUNEL System; Promega, Madison, WI). PCNA- and TUNEL-positive cells were counted across each tissue on sections in three wild-type and three *peewee* mice. PCNA staining was quantitated by counting 500 cells each in random sections.

Expression analysis

For semi-quantitative RT-PCR, total RNAs were isolated from brain, thymus, lung, heart, spleen, intestine, ovary and eye of wild-type and *peewee* mutant mice using TRIzol reagent (Invitrogen). cDNA was synthesized from total RNA with the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). Table 1 lists the primers used to detect *Gabrg1*, *Gabra2*, *Cox7b2*, *Gabra4*, *Gabrb1*, *Commd8*, *Atp10d*, *Corin*, *Nfx11*, *Zar1*, *EG545758*, *Cnga1*, *Npal1*, *Txk*, *Tec*, *Slain2*, *Slc10a4*, *Fryl* and housekeeping gene *Gapdh* (also called *G3PDH*). PCR was performed using AccuPrime Pfx DNA Polymerase (Invitrogen). The amplification conditions used were 98°C, 30 s followed by 15-25 cycles of 98°C, 10 s, 58°C, 30 s; 72°C, 30 s. The number of cycles used ensured that the reaction could be quantified within the log phase of the amplification reaction.

In silico positional cloning

In silico positional cloning was carried out using PosMedSM, developed by RIKEN (<http://omicspace.riken.jp/PosMed/>). It provides a bioinformatics approach to find the most promising candidate genes in a candidate interval restricted by genetic mapping analysis.

Statistical analysis

Descriptive statistics (mean and SEM) were calculated for the body weight of *peewee* and C57BL/6J mice, the number of PCNA- and TUNEL- positive cells, and the expression of *Slc10a4* in the tissues of wild-type and *peewee* mutant mice. Mann-Whitney tests were used to detect significant statistical differences among the experimental groups. Differences were considered significant at $P < 0.05$ (StatView, SAS Institute, Cary, NC).

Results

Dwarfism phenotype of *peewee* mutant mice

Peewee is an autosomal recessive mutation, and is induced by ENU mutagenesis directed at the *Rw* inversion region of proximal mouse chromosome 5. At birth, *peewee* mutants are indistinguishable from wild-type mice, and *peewee* mutant mice have no abnormalities other than their stature. Their weight gain is slower and they lack the juvenile growth spurt observed in wild-type littermates after weaning (Fig. 1). The litter size was significantly smaller compared with other strains (Supplementary Fig. 1).

Cellular proliferation and apoptosis in *peewee* mutant

To investigate the physiological and morphological consequences of the *peewee* mutant, the major organs of homozygous wild-type and *peewee* mutant mice were compared at 10 weeks of age. No gross morphological abnormalities were observed in the organs and tissues examined (Supplemental Fig. 2). Plasma analysis showed that EGF, INF- δ , IL- α , Apo-A1, leptin, IL-6 and OSM levels were significantly decreased, and fibrinogen, myoglobin, TNF- α , VEGF, MIP-1 α , MDC and IL-10 levels were significantly increased in *peewee* mutant mice (Supplementary Table 2).

As indicated by the plasma analysis, some cell growth factors were altered in the *peewee* mutant, which could promote cellular proliferation or cell death. In order to determine whether a greater incidence of cellular proliferation or apoptosis occurs in *peewee* mutants, we performed immunohistochemical analysis labeling with PCNA and TUNEL assays in juvenile (2-week-old) wild-type and *peewee* mutant mice in proliferative tissue. Similar levels of PCNA positive staining were present in thymus (Fig. 2A and B), lung (Fig. 2 C and D) and small intestine (Fig. 2 G and H) of wild-type and *peewee* mutants. However, more significant numbers of positive cells were observed in chondrocytes of bone (Fig. 2 K and L) and fewer positive cells in hepatocyte of liver (Fig. 2 E and F) and spermatogonia of testis (Fig. 2 I and J) of *peewee* mutant compared to wild-type. The altered ratio of PCNA-positive cells between wild-type and *peewee* mutant mice at 2 weeks of age indicates a tissue-specific imbalance of proliferation (Fig. 2M). At 2 weeks of age, similar levels of TUNEL-positive cell staining were present in liver, testis, and bone of wild-type and *peewee* mutants (Fig. 3).

Genetics and chromosomal mapping of *peewee* allele

We initially scanned the *Rw* inversion region using four microsatellite markers at approximately 50 cM intervals (Fig. 4A). Initial genome scanning indicated significant linkage between *D5Mit232* and *D5Mit356.3* (Fig. 4A). A high-resolution genetic map of the region was constructed using the flanking markers *D5Mit83* and *D5Mit356.3* on 492 F2 progeny from a *peewee* homozygote $\times$ Balb/cCrSlc F1 intercross and their associated phenotype (Fig. 4B). The fine structure genetic map encompassed an estimated 3.3 Mb physical region on Chromosome 5 containing the *peewee* mutation. According to annotations in the *M. musculus* genome assembly version 37 (<http://www.ensembl.org>), the *peewee* critical region contained 18 gene loci.

Candidate genes

Eighteen candidate genes were identified within the *peewee* critical region (Table 2). We performed *in silico* positional cloning using PosMedSM program, which searches for connections among keywords and genes in an interval. We entered a keyword, dwarf, in the PosMedSM and identified five candidate genes--*Gabra2*, *Gabra4*, *Corin*, *Txk* and *Tec*—that are related to the *peewee* phenotype in the critical region (Table 2).

Six of the candidate genes (*Gabra2*, *Gabra4*, *Corin*, *Zar1*, *Txk* and *Tec*) in the *peewee* critical region have already been knocked out in mice, and have not exhibited the dwarfism phenotype observed in *peewee* mice. To analyze the expression levels of these candidate genes, we performed semi-quantitative RT-PCR on cDNA of tissue highly expressed for individual genes from both wild-type and *peewee* homozygotes (Fig. 5A). Among the 15 candidate genes, the levels of *Slc10a4* gene expression were relatively low in *peewee* mutants, and no significant expression differences were observed in any of the other genes, including the genes picked out by PosMedSM (Fig. 5A). The expression level of *Slc10a4* mRNA significantly differed between *peewee* and wild-type mice, with *G3PDH* used as an internal control (Fig. 5B).

We examined the genomic sequence of *Slc10a4* in *peewee* and wild-type mice. No nucleotide sequence differences were observed in the genomic DNA of the *peewee* mice compared to C57BL/6J in 7 kb of *Slc10a4* genomic DNA, which included 162 nucleotides in the 5' and 1096 nucleotides in the 3' - untranslated region.

Discussion

In this study, we determined the morphogenic and genetic defects associated with a new fertile dwarf mutant mouse, *peewee*. Mutant mice harboring the *peewee* mutation showed normal appearance and histology, but we observed altered cellular proliferation in bone, liver and testis. We identified a possible candidate gene for *peewee* mutation in the *Rw* region, but did not observe a nucleotide alteration in the gene.

Most mice homozygous for dwarf mutations are a result of reproductive dysfunction (Bartke 2000). For example, infertility in the Snell dwarf mice appears to be due to gonadal dysfunction arising from a lack of neuro-endocrine axis activation (Bartke 2000; Bartke and Lloyd 1970; Smith and MacDowell 1931). It has been proposed that decreased GH levels lead directly to diminished circulating insulin and IGF-1, both of which are necessary for normal body size and for aging in Snell, as well as in Ames dwarf mice (Bartke and Brown-Borg 2004; Bartke et al. 1998; Brown-Borg et al. 1996; Flurkey et al. 2002; Flurkey et al. 2001; Kemp 1938; Sornson et al. 1996). Deficiency of pituitary gland function is a direct consequence of a deficiency of growth hormone in many dwarf mice. In Smallie mice, the absence of discoidin domain receptor 2 leads to growth retardation and gonadal dysfunction due to peripheral defects in hormonal-responsive pathways (Kano et al. 2008).

The *peewee* mutant was generated by a region-specific ENU-induced mutagenesis, which is a powerful and efficient phenotype-driven approach to define gene function relevant to phenotype and disease. Unlike other dwarf mice, the *peewee* mutants are fertile, although they have smaller litters compared to most inbred and F1 mice. Histological examination of various tissues from *peewee* mice did not reveal any gross abnormalities. These results confirm that the peripheral reproductive organs and central endocrine mechanisms were normal. The normality of these tissues reveals that the smallness of the litters might not be due to deficiencies in the reproductive system of *peewee* mice. The small litters could, however, be attributed to their small body size; it is well known that relatively small inbred

mice have fewer pups (Mouse Phenome Database (MPD); <http://phenome.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home>).

Plasma analysis to detect alteration in blood chemistry identified apoptotic and cell proliferative factors that could affect growth. To further examine these findings, we performed immunohistochemical analyses using PCNA (to examine proliferation), and TUNEL assay (to examine apoptosis) in the proliferative tissue of juvenile wild-type and *peewee* mutant mice. In chondrocytes, more proliferative cells were observed in *peewee* mutant mice than in wild-type mice. Endochondral bone formation has some important processes, including proliferation of chondrocytes (Kronenberg 2003). Further analysis is necessary, but the increased proliferation of chondrocytes could be caused by abnormalities of other processes, such as hypertrophicity of endochondral bone formation, in *peewee* juvenile mutant mice. Alternatively, repressive factors might be activated in the *peewee* chondrocytes and suppress the proliferation of bone growth. Additionally, low proliferative activity in the cells of liver and testis and equal levels of apoptosis in various tissues of *peewee* mutant mice might reflect that the growth retardation of the *peewee* mutant results from the complete elimination of total decreasing of cellular proliferative activity.

Due to the region-specific targeted ENU mutagenesis, we easily refined the critical region of *peewee* to distal to the *Rw* region on chromosome 5. We mapped the *peewee* locus to a region between *D5Mit232* and *D5Mit356.3* on the *Rw* region of chromosome 5 by standard linkage analysis and refined the *peewee* mutation to a 3.3 Mb interval between markers *D5Mit83* and *D5Mit356.3*. Bioinformatic methodologies were employed by entering the keyword “dwarf” to PosMed™ and by performing a mutant search of the MGI (<http://www.informatics.jax.org/>) database. We finally identified five candidate genes--*Gabra2*, *Gabra4*, *Corin*, *Txk* and *Tec*--by PosMed™. *Gabra2* and *Gabra4* are members of the GABA-A receptor family, and both of these knockout mice mainly show phenotypes of behavior (Knabl et al. 2008; Chandra et al. 2006). *Corin* is highly expressed in the heart and is the pro-ANP-converting enzyme. Corin-mediated pro-ANP activation may play a role in regulating blood pressure (Yan et al. 2000). Mouse *Tec* is a nonreceptor-type protein-tyrosine kinase that is highly expressed in many hematopoietic cell lines (Sato et al. 1994). *Tec* is involved in immune system and bone homeostasis in signaling molecules shared by B cells and osteoclasts (Shinohara et al. 2008). *Txk* is a member of the *Tec* subfamily of SRC-type (nonreceptor) tyrosine kinases. *Txk* expression is mainly detected in T cells and some myeloid cell lines. In this study, none of the candidate genes identified by PosMed™ could be verified, because no similar phenotypes and descriptions were found for these candidate genes. However, if functions and descriptions of candidate genes are well analyzed in the future, PosMed™ should be useful for analyzing relationships between phenotypes and genes, enabling the easy identification of potent candidate genes.

Gene expression of the candidate genes in the *peewee* critical region was analyzed. The expression of one gene, *Slc10a4*, a solute carrier, was lower in *peewee* mice than in wild-type mice both in both brain and pituitary glands. Solute carrier (SLC) families comprise passive transporters, ion-coupled symporters, and antiporters in the plasma membrane and other cellular membrane compartments, and each SLC family is thought to have relationships to physiological, pathological, pathophysiological and pharmacological functions (Hediger et al. 2004). SLC10A4, is an orphan transporter with ubiquitous expression, with high expression in the nervous tissue and pituitary gland (GNF SymAtlas v1.2.4). While SLC10A4 is a solute carrier or transporter of other physiological molecules, it does not appear to transport bile acids (Geyer et al. 2006). Thus far, there is no evidence or reports that associates SLC10A4 function with cell proliferation or other defects in mice, but mutations in another solute carrier, SLC7A7, a member of the SLC family, causes fetal growth retardation by downregulating *Igf-1* in the mouse (Sperandeo et al. 2007). In our

preliminary experiment, Northern blot analysis indicated that liver *Igf-1* mRNA levels did not differ between *peewee* and wild-type mice (data not shown). This indicates that dwarfism in *peewee* mice might not be due to hormonal control such as growth hormone or *Igf-1* regulation through an SLC10A4 transporter like SLC7A7.

Using recombination mapping, we narrowed the *peewee* genetic interval to less than 3.3 Mb, but did not find any mutations in a candidate gene, *Slc10a4*. It is possible that the mutation is in a regulatory element that affects the expression of *Slc10a4* outside the genetically defined critical region. The mutation may be in nontranslated transcript elements such as a microRNA. However, there are no such known elements in this region. Thus, DNA sequencing of the entire critical region may be required to identify the *peewee* mutation. Although *Slc10a4* appears to be a good candidate gene with different expression between wild-type and *peewee* mice, we should consider another possibility that a point mutation in *peewee* mice causes functional loss of the protein. Transgenic rescue to each candidate gene might provide new evidence about the relationships between the phenotype and the responsible gene in *peewee* mice. Detailed analysis of the *peewee* phenotypes and identification of the mutation may provide molecular insights into the complex mechanism of body size and growth regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Growth curves of male and female wild-type and *peewee* mutant mice from 4 to 9 weeks of age (n=5/group). ** $P < 0.01$.

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Figure 2.

Immunohistochemistry for PCNA in juvenile (2-week-old) wild-type (A, C, E, G, I, K) and *peewee* mutant mice (B, D, F, H, J, L) in proliferative tissue. Similar levels of PCNA-positive staining were present in the thymus (A, B), lung (C, D) and small intestine (G, H) of wild-type and *peewee* mutants. However, greater numbers of positive cells were observed in the chondrocyte of bone (K, L, M) and fewer positive cells in hepatocyte of liver (E, F, M) and spermatogonia of testis (I, J, M) of *peewee* mutant compared to wild-type. Comparison of the ratio of PCNA-positive cells ratio between wild-type and *peewee* mutant mice at 2 weeks of age (n=4/group). Scale bar, 50 μ m (A, B, G, H), 100 μ m (C, D, I, J), 200 μ m (E, F, K, L). * P <0.05.

Figure 3. TUNEL analyses of thymus (A, B), testis (C, D) and bone (E, F) from wild-type (A, C, E) and *peewee* (B, D, F) mutants at juvenile (2 weeks of age). Similar levels of TUNEL-positive cell staining were present in these tissues of wild-type and *peewee* mutants. Arrowheads indicate PCNA-positive cells. Scale bar, 50 μ m.

Figure 4. Map of the *peewee* region. (A) Map of the *Rw* inversion region of mouse chromosome 5. The map is based upon the linkage analysis of phenotype-positive F₂ progeny obtained by intercrossing F₁ animals. The *peewee*-critical region is indicated. (B) Haplotype analysis of F₂ mice. A black box indicates homozygosity. A white box indicates a heterozygous allele or a homozygous Balb/c allele. Number of F₂ mice is shown.

Figure 5. Semi-quantitative RT-PCR analysis of *peewee* candidate genes in the *peewee* critical region in wild-type and *peewee* mutant mice (A). Differences were detected for *Slc10a4* between wild-type and *peewee* mutant mice, with *G3PDH* used as an internal control. No differences were detected for any other candidate genes between wild-type and *peewee* mutant mice. Significant differences were detected for *Slc10a4* between wild-type and *peewee* mice, with *G3PDH* used as an internal control (B; n=3/group). * $P < 0.05$.

Table 1

Primer Sequences

	Forward Primer	Reverse Primer
<i>Gabrg1</i>	CTTGTTACTAACCTGCATCTGG	ATAGAGAACCCTTCCATCACTCC
<i>Gabra2</i>	CTTCTGACTCCGTTTCAGGTTG	TGTATCATGACGGAGCCTTTC
<i>Gabra4</i>	CCTGTGCTGAAGGAGAAACAC	CTGCAGATGAAAGACCTCTGG
<i>Gabrb1</i>	ACCTACATGCCTTCCCACTG	GCACTCTGGTCTTGTGCTC
<i>Commd8</i>	TCACAGACACTCCAGCAACTG	GGCTGTGACTTCAGCTCTTC
<i>Atp10d</i>	CTTCAGTAGTCCCATGGAAACAG	CATAGGAGGTGACAGTTTGGTC
<i>Corin</i>	CACTGGCCAGCTCAATCTAAG	GAGACGGAATGAGCTGACTG
<i>Nfxl1</i>	TAAGCGAAAAGAACCTCCTCAG	TGGCTTCTGCTTCTTAACTC
<i>EG545758</i>	TTATGGCTTAGCTACCTCTTCC	CTTTAGCTGATCCAGGTGTAAGC
<i>Npa11</i>	TAAGCGTATTGGGGTCAACTG	TGTAGGAGGAAGATGCCATTG
<i>Txk</i>	CAAGAGCTCGAAGGCATACAC	CCTTGACAAAAGCCAACCTG
<i>Tec</i>	GATTAACCCATCAGAGCTGACC	CGCCAGAAGAACTTGTGTACTG
<i>Slain2</i>	AGTCATCCGACTCTACTTTGCTG	ACTTGACCTTACAGAACACCTC
<i>Slc10a4</i>	CAGACAGGTAAAAGGGACCAC	GAAGTGATGTTTCAGCTCCCG
<i>Fryl</i>	CACTGGCCAGCTCAATCTAAG	GAGACGGAATGAGCTGACTG

Table 2

Candidate genes in the *peewee* critical region

Gene	Description	Exons	PosMed	highly expressed	KO/mt
<i>Gabrg1</i>	GABA-A receptor, subunit gamma 1	11		brain	
<i>Gabra2</i>	GABA-A receptor, subunit alpha 2	10	yes	brain	yes
<i>Cox7b2</i>	cytochrome c oxidase subunit VIIb2	3		NA	
<i>Gabra4</i>	GABA-A receptor, subunit alpha 4	9	yes	brain	yes
<i>Gabbr1</i>	GABA-A receptor, subunit beta 1	9		brain	
<i>Commf8</i>	COMM domain containing 8	5		thymus	
<i>Atp10d</i>	ATPase, class V, type 10D	24		lung	
<i>Corin</i>	corin	22	yes	heart	yes
<i>Nfxl1</i>	nuclear transcription factor, X-box binding-like 1	23		spleen	
<i>Zar1</i>	zygote arrest 1	4		NA	yes
<i>EG545758</i>	predicted gene, EG545758	3		brain	
<i>Cnga1</i>	cyclic nucleotide gated channel alpha 1	9		retina	
<i>Npal1</i>	NIPA-like domain containing 1	6		large intestine	
<i>Txk</i>	TXK tyrosine kinase	15	yes	thymus	yes
<i>Tec</i>	cytoplasmic tyrosine kinase, Dscr28C related	19	yes	liver	yes
<i>Slain2</i>	SLAIN motif family, member 2	9		ovary	
<i>Slc10a4</i>	solute carrier family 10, member 4	3		brain	
<i>Fryl</i>	furry homolog-like	63		eye	