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Loop-tail phenotype in heterozygous mice and neural tube defects in homozygous mice result from a nonsense mutation in the *Vangl2* gene

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ABSTRACT. *N*-ethyl-*N*-nitrosourea (ENU) is a powerful point mutagen that can generate random mutations. It has been used to generate mouse mutations to produce phenotypic models of human disease. Neural tube defects (NTD) are common birth defects in which the brain and/or spinal cord can be exposed; however, the mechanisms of these defects are poorly understood. Craniorachischisis is one type of NTD that bears a close resemblance to the phenotype of the *loop-tail* (*Lp*) mouse. Here we describe a C57BL/6J *Lp* mouse generated by ENU-induced mutagenesis. The mutation was mapped to the *Vangl2* gene on chromosome 1, near markers *D1Mit113* and *D1Mit149*. Sequence analysis of *Vangl2* heterozygotes (*Vangl2*^{m1Yzcm}/+) revealed a C/T transition mutation that resulted in substitution of a glutamine codon for a stop (nonsense) codon at position 449. The Vangl2 protein is involved in epithelium planar cell polarity. The predicted truncated protein would lack the PDZ-domain binding motif involved in protein-protein interaction; therefore, *Vangl2*^{m1Yzcm} may be a loss-of-function mutant. Morphological and histological examination of homozygous

mouse embryos revealed a neural tube closure defect that leads to craniorachischisis. This *Vangl2*^{m1Yzcm} mouse represents a valuable model for the study of NTDs in humans.

Key words: *N*-ethyl-*N*-nitrosourea; *Loop-tail*; Neural tube defect; *Vangl2*

INTRODUCTION

Planar cell polarity (PCP) is the process by which cells become polarized in the epithelial plane. This process has been studied in the epithelial tissues of adult *Drosophila*. Genetic studies have identified a group of so-called 'core' PCP molecules: stbm/vang; frizzled; the intracellular proteins Dishevelled and Prickle; and an atypical cadherin Flamingo, also known as Starry night; diego (Simons and Mlodzik, 2008; Vladar et al., 2009); and the cadherin-related proteins Fat and Dachous (Clark et al., 1995; Matakatsu and Blair, 2004). Vertebrate orthologs of the *Drosophila* core PCP genes have been identified and recent *in vivo* studies of mutations that inactivated some of these genes (*Vangl2*, *Celsr1*, *Scy*, *Crsh*, *Dvl1*, and *Dvl2*) indicate they regulate convergent extension, a process critical for proper gastrulation and formation of the neural tube (Torban et al., 2004).

N-ethyl-*N*-nitrosourea (ENU) is a powerful point mutagen that can generate random mutations in the mouse genome. An ENU-mutagenesis screen for dominant and recessive mutations recovered a large number of mouse mutants with a variety of phenotypes. These mutants provided a rich resource for identifying disease-related genes, deciphering pathogenic mechanisms, and developing new therapies and new drugs. Recently, this procedure established more than 20 lines of mutant mice by screening the ENU-induced mutants in a C57BL/6J background (Wu et al., 2003, 2009, 2010a,b,c; Chen et al., 2011). Interestingly, one of these lines displayed a phenotype similar to that of the *Vangl2* (*Lpp1*) mutant alleles.

In this paper, we report the molecular and phenotypic characterization of the *loop-tail* (*Lp*) mutant mice. We indentified a C/T transition mutation at position 1345 of *Vangl2*, which converted a glutamine codon to a stop (nonsense) codon at position 449 in the protein. The mutant mouse was named *Vangl2*^{m1Yzcm} (*Vangl2*; mutation 1, Yangzhou University Comparative Medicine Center). The preliminary phenotypic analysis of the *Vangl2*^{m1Yzcm} mouse is described.

MATERIAL AND METHODS

Ethics statement

C57BL/6J (B6) and C3H/HeJ (C3H) mice were obtained from the Shanghai Laboratory Animal Center (Shanghai, China). This study was conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council. The Animal Care and Use Committee of Yangzhou University approved all animal experiments and procedures (approval ID: SYXK (Su) 2007-0005).

ENU mutagenesis

Vangl2^{m1Yzcm} was isolated as an *Lp* phenotype inherited in a dominant fashion. Wild-

type B6 males were treated with a 3 x 100 mg/kg dose of ENU, allowed to recover fertility, and then mated to B6 females.

Alcian blue and alizarin red staining

Newborn mice were killed, eviscerated, placed in a 70°C water bath for 30 s, and skinned, then fixed in 100% ethanol for 3 days, followed by staining with alcian blue (15 mg in 80 mL ethanol/20 mL glacial acetic acid) for 8-12 h. Skeletons were rinsed in 100% ethanol overnight and cleared in 2% KOH for 6-8 h. Staining for bone was carried out using alizarin red (50 mg/L in 2% KOH) for 3-5 h. Skeletons were then cleared in 2% KOH and stored in 20% glycerol.

Linkage analysis

Vangl2^{m1Yzcm} heterozygotes of the B6 background were mated to C3H mice to generate F1 mice. Next, F1 mice with *loop-tail* were backcrossed to B6 mice to generate N2 mice. DNA samples of N2 mutant mice were prepared from tail samples by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. DNA samples were screened by polymerase chain reaction (PCR) for microsatellite markers; PCR products were separated on 4% agarose gels by electrophoresis and analysed.

Sequence analysis of the *Vangl2* gene

The exons and immediate flanking sequences of *Vangl2* were amplified from *Vangl2*^{m1Yzcm} heterozygotes and B6 genomic DNA. Primer sequences for *Vangl2* are available on request. PCR products were purified and sequences read using the Big Dye Terminator v3.1 kit on an ABI-PRISM 3730 instrument.

Reverse transcription (RT)-PCR

Total RNA was isolated from the heads of E12.5 embryos using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using a RevertAid First-Strand cDNA Synthesis Kit (Fermentas) with oligo(dT)18 primers. RT-PCR for *Vangl2* was performed with primers: forward: 5'-TACTACGAGGAAGCCGAGCATGA-3' and reverse: 5'-GCAGCCGCATGACGAACTTATGT-3'. PCR conditions consisted of one cycle of denaturation for 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 61°C, and 30 s at 72°C, ending with one cycle of elongation for 5 min at 72°C. PCR products were purified and sequenced.

Genotyping

The point mutation introduced an *Fsp*BI (*Bfa*I) restriction site in *Vangl2*^{m1Yzcm}. A 452-bp fragment that encompassed the point mutation was amplified from genomic DNA using forward primer: 5'-AAACACCCTAGCTATCTTAGAAAG-3' and reverse primer: 5'-GGAAGTAGGACTGGCAGAAATGTG-3'. Digestion of the PCR product with *Fsp*BI was predicted to yield DNA fragments of the following sizes: +/+ mice, 452 bp; +/- heterozygotes,

452, 133, and 319 bp; and -/- homozygous mice, 133 and 319 bp. Analysis after 2% gel electrophoresis revealed DNA bands of the expected sizes.

Histology

Heterozygous *Vangl2*^{m1Yzcm} mice were intercrossed and embryos at E12.5 and E18.5 were recovered, genotyped, and examined for the presence of neural tube defects (NTDs). Embryos at E12.5 were fixed in 4% paraformaldehyde in phosphate-buffered saline, dehydrated, wax-embedded and sectioned at 10 µm, and then stained with Harris hematoxylin and eosin-Y stain.

RESULTS

Phenotype of *Vangl2*^{m1Yzcm} heterozygous mice

The founder male mouse with an *Lp* appearance (Figure 1) was the progeny of an ENU-treated B6 male mouse. The mutant mouse was mated with B6 mice: 32/103 of the progeny produced had the *Lp* phenotype. The B6 heterozygotes were mated to C3H mice to generate F1 mice: 1/32 of the progeny had the *Lp* phenotype, indicating the presence of a background effect. Alcian blue and alizarin red staining showed that the *loop-tail* phenotype was caused by a contortion of the tail, and the skeletons of the heterozygotes were normal (Figure 2).

Figure 1. *Loop-tail* appearance of *Vangl2*^{m1Yzcm} heterozygous mouse.

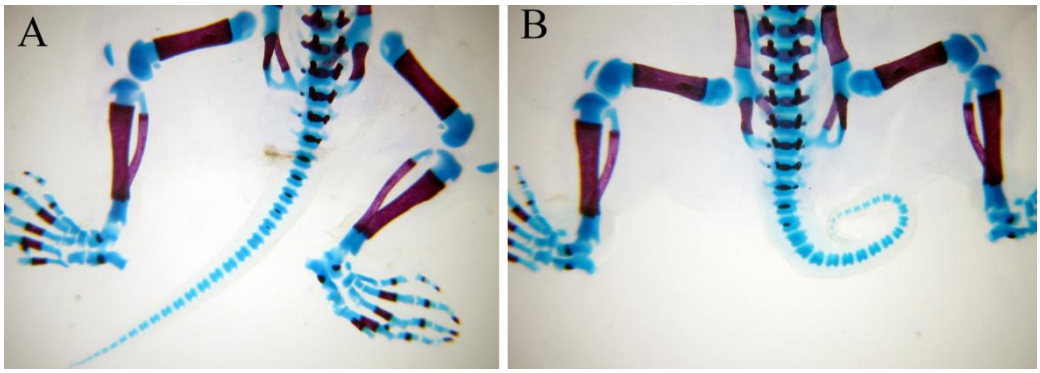

Figure 2. Alcian blue and alizarin red staining. **A.** Wild-type mouse. **B.** *Vangl2*^{m1Yzcm} heterozygous mouse.

Genetic mapping

We tested genomic DNA from 42 N2 samples by mapping microsatellite markers. Previous descriptions of the chromosomal location of the *Lp* mutant genes suggested that some microsatellite markers near these genes were used. The mutation was mapped to chromosome 1 and had no exchange with markers *D1Mit113* and *D1Mit149*, which were located at 79.54 and 80.13 cM, respectively. An *Lp*-associated gene, *Vangl2*, 79.54 cM from the centromere on chromosome 1, was chosen to be a particularly good candidate for mutation.

Lp phenotype is caused by a nonsense mutation in the *Vangl2* gene

On examination of the *Vangl2* gene, we found a single nucleotide change, a C/T substitution, in exon 8 at nucleotide position 1345. This substitution converted tyrosine to glutamine to a nonsense mutation at position 449 in the protein (Figure 3A, B, and C). The point mutation introduced an *Fsp*BI (*Bfa*I) restriction site in *Vangl2*^{m1Yzcm}.

Sequencing of *Vangl2* RT-PCR products from *Vangl2*^{m1Yzcm} heterozygous mutant mice showed that the mutant allele is transcribed, correctly spliced, and not subject to nonsense-mediated decay (Figure 3D). Translation of the mutant allele would produce a truncated protein.

Phenotype of the *Vangl2*^{m1Yzcm} homozygous mice

Embryos from *Vangl2*^{m1Yzcm} brother-sister matings were isolated at E12.5 and E18.5, genotyped (Figure 4), and studied to characterize the *Vangl2*^{m1Yzcm} phenotype. Several homozygous E18.5 embryos (4/16) were isolated and had an apparent NTD. Morphological examination of the homozygous mutant embryos revealed a malformed neural tube that was open from midbrain to tail and was clearly distinguishable from the closed neural tube of wild-type littermates; embryos also had failure of eyelid closure. The NTD in these embryos was also associated with smaller overall size, suggesting overall growth retardation (Figure 5). Histological examination of transverse sections through *Vangl2*^{m1Yzcm} homozygous mutant embryos at E12.5 illustrated defects that included an enlarged floor plate and exposed neuroepithelium (Figure 6).

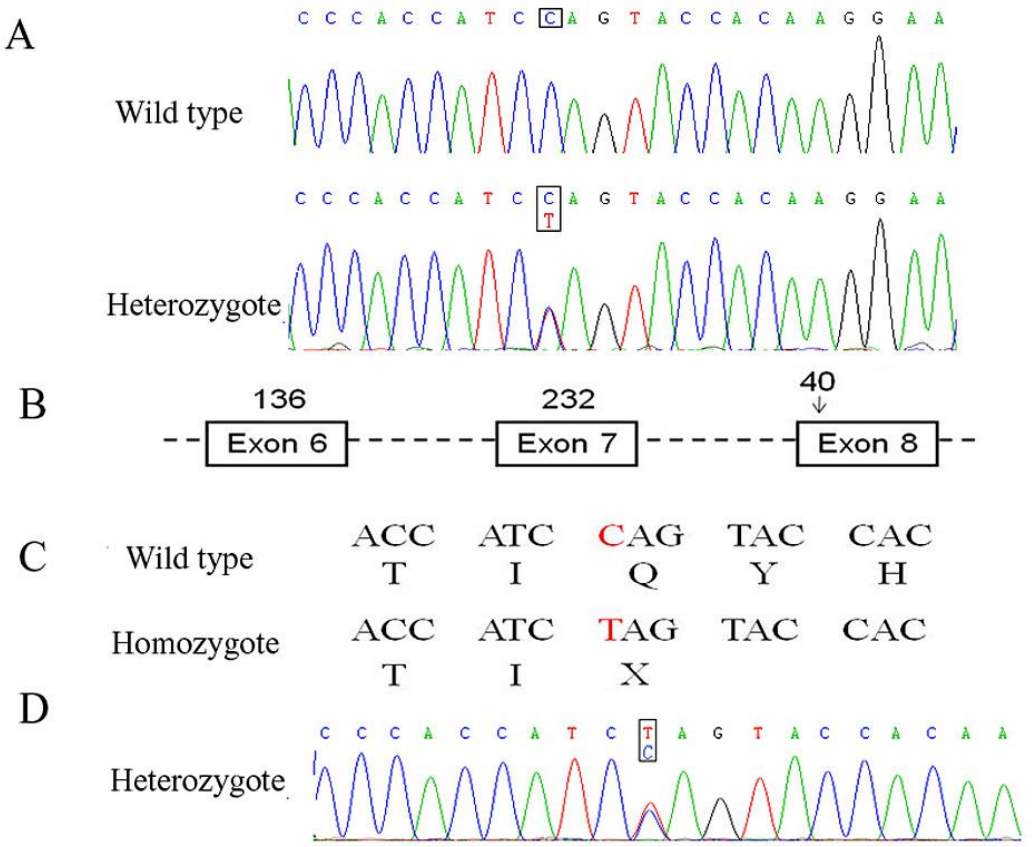

Figure 3. *Vangl2* carries a mutation in *Vangl2*^{m1Yzcm} mice. **A.** Sequence analysis of the *Vangl2* gene in *Vangl2*^{m1Yzcm} heterozygous mice by ABI-PRISM 3730. **B.** Partial exon and intron structure of the mouse *Vangl2* gene. The arrow shows the position of the *Vangl2*^{m1Yzcm} substitution. **C.** Translation of wild-type and homozygous *Vangl2*^{m1Yzcm} alleles flanking the mutation site. The nucleotide mutated in the *Vangl2*^{m1Yzcm} allele is highlighted in red. **D.** Sequence analysis of reverse-transcription polymerase chain reaction products of *Vangl2* in *Vangl2*^{m1Yzcm} heterozygous mice.

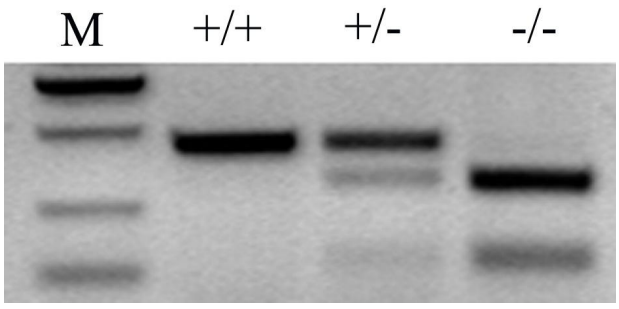

Figure 4. Genotyping of embryos from *Vangl2*^{m1Yzcm} brother-sister matings. *Lane M* = molecular weight markers; *lane* +/+ = wild-type mouse; *lane* +/- = heterozygous mouse; *lane* -/- = homozygous mouse.

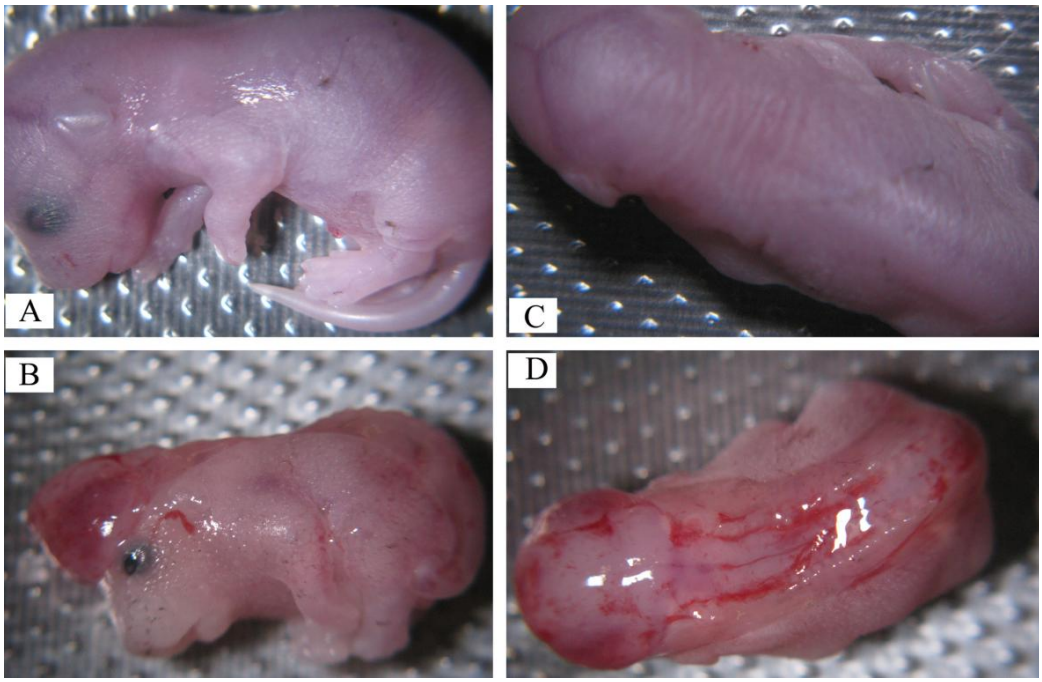

Figure 5. Morphological appearance of *Vangl2*^{m1Yzcm} homozygous embryos lateral (**A**, **B**) and dorsal (**C**, **D**) views of wild-type (**A**, **C**) and *Vangl2*^{m1Yzcm}/*Vangl2*^{m1Yzcm} (**B**, **D**) E18.5 embryos.

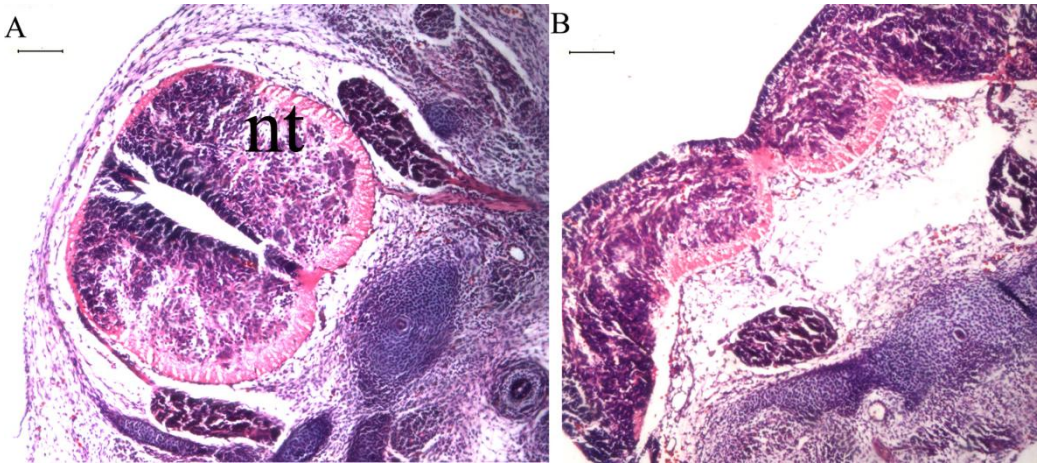

Figure 6. Histological examination of transverse sections through wild-type (**A**) and homozygous mutant *Vangl2*^{m1Yzcm}/*Vangl2*^{m1Yzcm} (**B**) embryos at E12.5. Sections were stained with hematoxylin and eosin. nt = neural tube. Scale bars = 100 µm.

DISCUSSION

Closure of the neural tube is essential for normal development of the brain and spinal cord. Failure of correct neural tube formation is one of the most common structural malformations of the central nervous system in humans and affects 1-2 infants per 1000 births (Copp et al., 1990; Harris and Juriloff, 1999; Kibar et al., 2001).

ENU-induced mutagenesis represents a powerful tool for the study of gene function and generation of human disease models. Three independent mutant alleles have been described for *Lp* mice: the naturally occurring *Lp* (Strong and Hollander, 1949) and the chemically induced Lp^{m1Jus} and Lp^{m2Jus} (ska^{17}). Three mutations, S464N, D255E, and R259L, were identified in *Lp*, Lp^{m1Jus} and Lp^{m2Jus}, respectively (Murdoch et al., 2001; Guyot et al., 2011).

We identified a novel ENU-induced *Lp* mutant mouse. The underlying genetic defect responsible for the *Lp* phenotype of $Vangl2^{m1Yzcm}$ mice is a nonsense mutation in exon 8 of the *Vangl2* gene, which, if translated, produces a truncated, 448-amino acid protein product. *Vangl2* (*Lpp1*) encodes a full-length protein of 521 amino acids, with 4 transmembrane domains that are related to the *Drosophila* protein Van Gogh (Vang) and a large intracellular domain with a PDZ-domain binding motif [post synaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)] at its carboxy terminus, which mediates protein-protein interaction (Murdoch et al., 2001; Torban et al., 2004). The predicted truncated protein product, therefore, lacks the PDZ-domain binding motif. We predict, therefore, that $Vangl2^{m1Yzcm}$ is a loss-of-function mutant.

$Vangl2^{m1Yzcm}$ heterozygous mice are characterized by an *Lp* appearance, while homozygous embryos show a severe form of NTD called craniorachischisis, in which the neural tube remains open throughout the hindbrain and spine. Thus, the allelic series of mutations in the *Vangl2* gene represent an extremely valuable genetic resource for understanding the function of this gene in PCP signaling. The $Vangl2^{m1Yzcm}$ mouse represents a valuable model for the study of NTDs in humans.

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