

## BRIEF REPORT

Mutations in *VANGL1* Associated with Neural-Tube Defects

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## SUMMARY

Neural-tube defects such as anencephaly and spina bifida constitute a group of common congenital malformations caused by complex genetic and environmental factors. We have identified three mutations in the *VANGL1* gene in patients with familial types (V239I and R274Q) and a sporadic type (M328T) of the disease, including a spontaneous mutation (V239I) appearing in a familial setting. In a protein–protein interaction assay V239I abolished interaction of *VANGL1* protein with its binding partners, disheveled-1, -2, and -3. These findings implicate *VANGL1* as a risk factor in human neural-tube defects.

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NEURAL-TUBE DEFECTS AFFECT 1 TO 2 INFANTS PER 1000 BIRTHS<sup>1</sup> AND are caused by a partial or complete failure of the neural tube to close during embryogenesis.<sup>1</sup> The most common forms of neural-tube defects are described as open defects (including anencephaly and myelomeningocele, or spina bifida), which result from the failure of fusion in the cranial and spinal region of the neural tube, respectively.<sup>1</sup> Other open dysraphisms (including myeloschisis, hemimyelomeningocele, and hemimyelocoele) are sometimes associated with a Chiari II malformation. A number of skin-covered (closed) neural-tube defects are categorized clinically depending on the presence of a subcutaneous mass (lipomyeloschisis, lipomyelomeningocele, meningocele, and myelocystocele) or the absence of such a mass (complex dysraphic states, including split cord malformations, dermal sinus, caudal regression, and segmental spinal dysgenesis).<sup>2</sup> All infants with anencephaly are still-born or die shortly after birth, whereas many infants with spina bifida now survive but with severe disabilities.<sup>1</sup>

Maternal periconceptional supplementation of folic acid reduces the incidence of neural-tube defects by 50 to 70%.<sup>3</sup> Polymorphic variants in genes of the folate and homocysteine pathways have been associated with an increased risk of neural-tube defects, including a common variant (C677T) in the *MTHFR* gene (5,10-methylene tetrahydrofolate reductase).<sup>3</sup> However, population- and family-based studies indicate a complex multigenic cause of neural-tube defects.<sup>1</sup> Although no causative gene has been identified in humans, mutated *Vangl2* is present in the *Loop-tail* (*Lp*) mouse mutant<sup>4,5</sup> with a severe defect known as craniorachischisis.<sup>6</sup> *Vangl2* is the mammalian homologue of the drosophila (fly) gene *Stbm/Vang*, which is required for establishing planar cell polarity in the developing eye, wing, and leg tissues.<sup>7,8</sup>

In the fly, planar cell polarity is controlled by a membrane-associated signaling complex composed of several proteins, including Stbm/Vang, frizzled (Fz), disheveled (Dvl), flamingo (Fmi), and prickle (Pk). Activation of planar cell polarity involves an asymmetric redistribution of these core proteins in which cytoplasmic Dvl and Pk are recruited to the plasma membrane, forming complexes with Fz and Stbm/Vang, respectively.<sup>9</sup> In vertebrates, a second *VANGL* gene (*VANGL1*) has been described. *VANGL1* and *VANGL2* proteins are highly similar. Each has four predicted transmembrane domains and a cytoplasmic domain that includes a PDZ-binding motif that mediates protein–protein interaction.<sup>10</sup> In mice, *Vangl1* and *Vangl2* messenger RNAs (mRNAs) are expressed in the ventral and dorsal portions of the developing neural tube, respectively.<sup>11</sup>

We tested the hypothesis that mutations in the *VANGL1* and *VANGL2* genes cause neural-tube defects and abrogate the physical interaction of the *VANGL1* protein and disheveled proteins.

## METHODS

### PATIENTS

This study included 144 patients with neural-tube defects, including 137 Italian patients with non-syndromic spinal dysraphisms who were recruited at the Spina Bifida Center of the Gaslini Hospital in Genoa and 7 fetuses with craniorachischisis that were obtained from Groupe Hôpitalier Necker-Enfants Malades in Paris. The Spina Bifida Center of Gaslini Hospital has recruited children with neural-tube defects since 1976, mainly from the north and south of Italy. All mothers of Italian patients lacked periconceptional supplementation of folic acid. Thirteen of the Italian patients had a positive family history documented by clinical records — including findings on magnetic resonance imaging (MRI) and radiography, which were obtained from parents of patients — whereas all other patients had sporadic disease (Table 1, and Table 1 of the Supplementary Appendix, which is available with the full text of this article at [www.nejm.org](http://www.nejm.org)).

The control group consisted of 106 unrelated, randomly selected children who had been admitted to the Gaslini Children's Hospital for miscellaneous illnesses and healthy young adults who had contributed samples to the blood bank of the Gaslini Institute. None of the patients had a

**Table 1. Characteristics of the Patients.**

Group and Variable	Finding
<b>Italian</b>	
No. of patients	137
Male sex (%)	51
Mean age (yr)	15.3
Familial type (%)	9
Neural-tube defect (no. of patients)	
Myelomeningocele with Chiari II	33
Myelomeningocele without Chiari II	47
Lipomyelomeningocele	7
Lipomyeloschisis	15
Caudal regression	35
<b>French</b>	
No. of fetuses	7
Neural-tube defect (no. of fetuses)	
Craniorachischisis	7

first-degree relative with a neural-tube defect. The samples from all control subjects were anonymous. Information associated with these samples included sex, region of birth, and age.

All patients and control subjects were Italian with ancestors from all parts of the country, as determined during genetic-counseling sessions. The experimental protocol was approved by the local ethics committee, and written informed consent was obtained from all patients or their parents. We analyzed additional samples from 65 subjects in the control group obtained from the Centre d'Etude du Polymorphisme Humain (CEPH) repository, which contains samples from French, Venezuelan, and Amish persons and also persons from Utah with northwestern European ancestry.

### SCREENING FOR MUTATIONS

The coding exons of *VANGL1* (National Center for Biotechnology Information accession number, NM\_138959) were amplified by polymerase-chain-reaction (PCR) assay from genomic DNA with the use of primers flanking the exon–intron junctions, as described previously.<sup>4</sup> Primer sequences are available from the authors on request. The PCR products were purified with the use of a Qiaquick PCR purification kit (Qiagen) and were sequenced with the use of the BigDye Terminator V2.0 cycle sequencing kit and an automated ABI 3700 instrument (Applied Biosystems). All mutations were

verified by the dideoxynucleotide termination method and <sup>33</sup>P sequencing with the use of the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech).

#### INTERACTIONS BETWEEN PROTEINS

Physical interactions between human VANGL1 and disheveled proteins were monitored as described previously.<sup>10</sup> For details, see the Methods section of the Supplementary Appendix.

### RESULTS

We identified three Italian patients who were heterozygotic for missense mutations in VANGL1 (V239I, R274Q, and M328T). These mutations were predicted to affect amino acid residues that are conserved across members of the VANGL family. We did not detect these variants in ancestrally matched controls or in CEPH controls. We observed V239I and R274Q in two patients with familial types of disease (Patient 103 and Patient 48, respectively) and M328T in a patient with a sporadic type (Patient 151). The remaining 11 patients with familial types of a neural-tube defect did not carry any disease-specific mutation in the open reading frame of VANGL1. We identified eight other sequence variants in the open reading frame of VANGL1 that were silent (that is, they did not change the predicted amino acid), that were present in both patients with neural-tube defects and control subjects, or that did not affect highly conserved residues (Table 2 of the Supplementary Appendix). On the other hand, we sequenced VANGL2 in both series of patients and identified several variants but none that were predicted to affect protein sequence (data not shown and in agreement with previous findings).<sup>11</sup>

We detected a missense mutation in VANGL1 that is predicted to result in the substitution of valine with isoleucine at position 239 (V239I) in Patient 103, a 10-year-old Italian girl who had a severe form of caudal regression (type IV of sacral agenesis, according to Pang's classification<sup>12</sup>) with lipomyeloschisis, anorectal malformation, hydromelia, and tethered spinal cord (Table 1 of the Supplementary Appendix). The girl's mother showed no clinical signs of neural-tube defect, and her brother had a milder form of the disease, dermal sinus. V239I was present in the brother and mother of Patient 103 and was

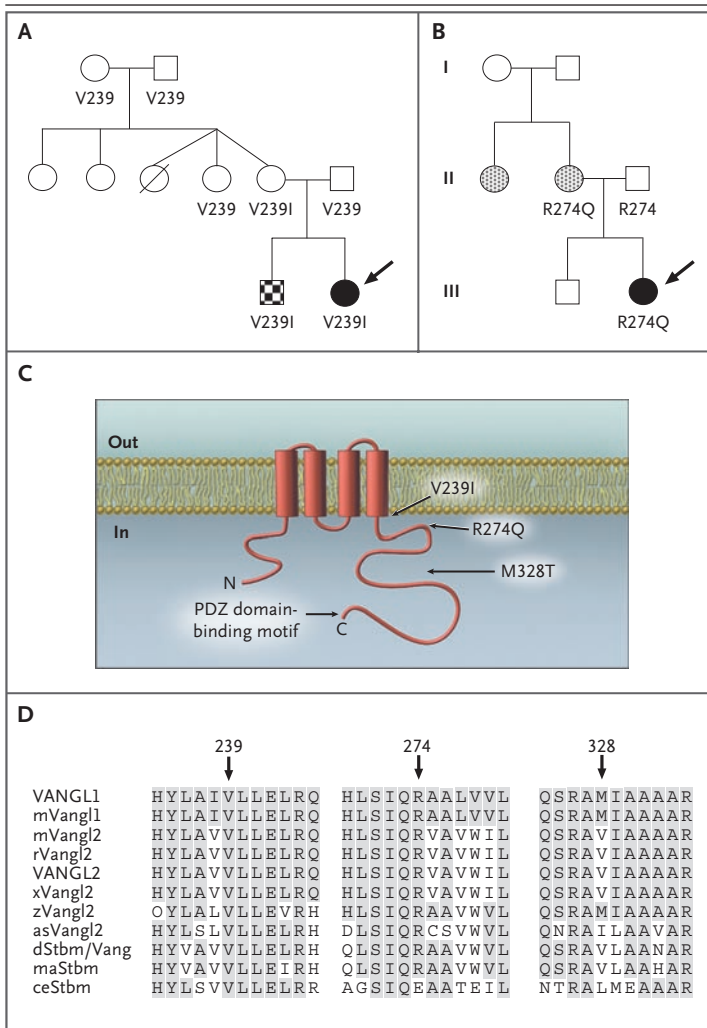
absent in the father and maternal aunt. However, the maternal grandparents did not carry the mutation, indicating that it had arisen de novo in the germ line of one of the maternal grandparents or somatically in the mother and was subsequently transmitted through the mother's germ line (Fig. 1A). Additional genotyping confirmed paternity, maternity, and DNA authenticity in all family members (including the paternity and maternity of the mother of the proband) from whom DNA was available (not shown). Valine at position 239 is in the fourth predicted transmembrane domain of the VANGL1 protein (Fig. 1C). It is invariant and part of a "VLLE" motif, which is conserved across all known Vangl proteins (Fig. 1D). Although a substitution with isoleucine preserves the hydrophobic character of valine, it introduces a bulky side chain, which may have structural or functional consequences.

We identified a missense mutation that is predicted to result in the substitution of glutamine for arginine at position 274 (R274Q) in Patient 48, a 19-year-old Italian girl who had myelomeningocele (level L5–S1), hydrocephalus, and congenital club feet (Table 1 of the Supplementary Appendix). Her mother and maternal aunt had vertebral schisis, a minimal sign of a neural-tube defect. R274Q was present in the mother but not in the father (DNA samples from the patient's maternal aunt and brother were not available for analysis) (Fig. 1B). R274Q is in the cytoplasmic domain of VANGL1 (Fig. 1C). It is invariant in all known orthologues (genes in two or more species that have evolved from a common ancestor) except that of *Caenorhabditis elegans*, in which it is replaced by glutamate (Fig. 1D). Although a substitution of glutamate in the *C. elegans* orthologue is nonconservative, it preserves the charged character of arginine, a physical property that is not preserved by substitution with glutamine.

We detected a missense mutation that is predicted to result in the substitution of methionine with threonine at position 328 (M328T) in a 21-year-old woman with sporadic disease; she had myelomeningocele (level L3–S1), hydrocephalus, Chiari II malformation, tethered spinal cord, club feet, lumbosacral scoliosis, and sacrococcygeal kyphosis. M328T is also in the predicted cytoplasmic domain of VANGL1 (Fig. 1C). The hydrophobic character of methionine is conserved at this position across evolution (Fig. 1D). M328T is not

### Figure 1. VANGL1 Mutations Associated with Neural-Tube Defects.

The familial pedigrees are shown for Patient 103 (Panel A) and Patient 48 (Panel B), who carry the V239I (715G→A) and R274Q (821G→A) mutations, respectively. Patient 103 (arrow) was affected with lipomyeloschisis, hydromyelia, and caudal regression. Her brother was affected with dermal sinus (patterned box). V239I was present in the brother and mother and absent in the father and the maternal aunt and grandparents. Patient 48 (arrow) was affected with lumbosacral myelomeningocele (L5–S1) and hydrocephalus. Her mother and maternal aunt were affected with vertebral schisis (dotted circles). R274Q was present in the mother and absent in the father. Panel C shows a topologic model of VANGL1 with the approximate positions of V239I, R274Q, and M328T indicated. Panel D shows a partial alignment of VANGL1 with 10 other vertebrate and invertebrate *Stbm/Vangl* sequences. Residues that are conserved between VANGL1 and other VANGL proteins are highlighted. VANGL1 substitutions of V239I (left), R274Q (center), and M328T (right) that are found in patients with neural-tube defects affect conserved residues (indicated by arrows). National Center for Biotechnology Information accession numbers are NP\_620409 for VANGL1, XP\_049695 for VANGL2, NP\_277044 for mouse (m) *Vangl2*, XP\_222896 for rat (r) *Vangl2*, AAK70879 for xenopus (x) *Vangl2*, AAL30891 for zebrafish (z) *Vangl2*, AK113083 for ascidian (as) *Vangl2*, NP\_477177 for drosophila (d) *Stbm/Vang*, EAA00250 for African malaria mosquito (ma) *Stbm*, and NP\_508500 for *Caenorhabditis elegans* (ce) *Stbm*. The protein sequence of mouse *Vangl1* was initially reported by Wolff and Rubin in 1998.<sup>8</sup>



a conservative substitution because threonine contains a hydroxyl side chain that substantially increases the hydrophilicity at this position.

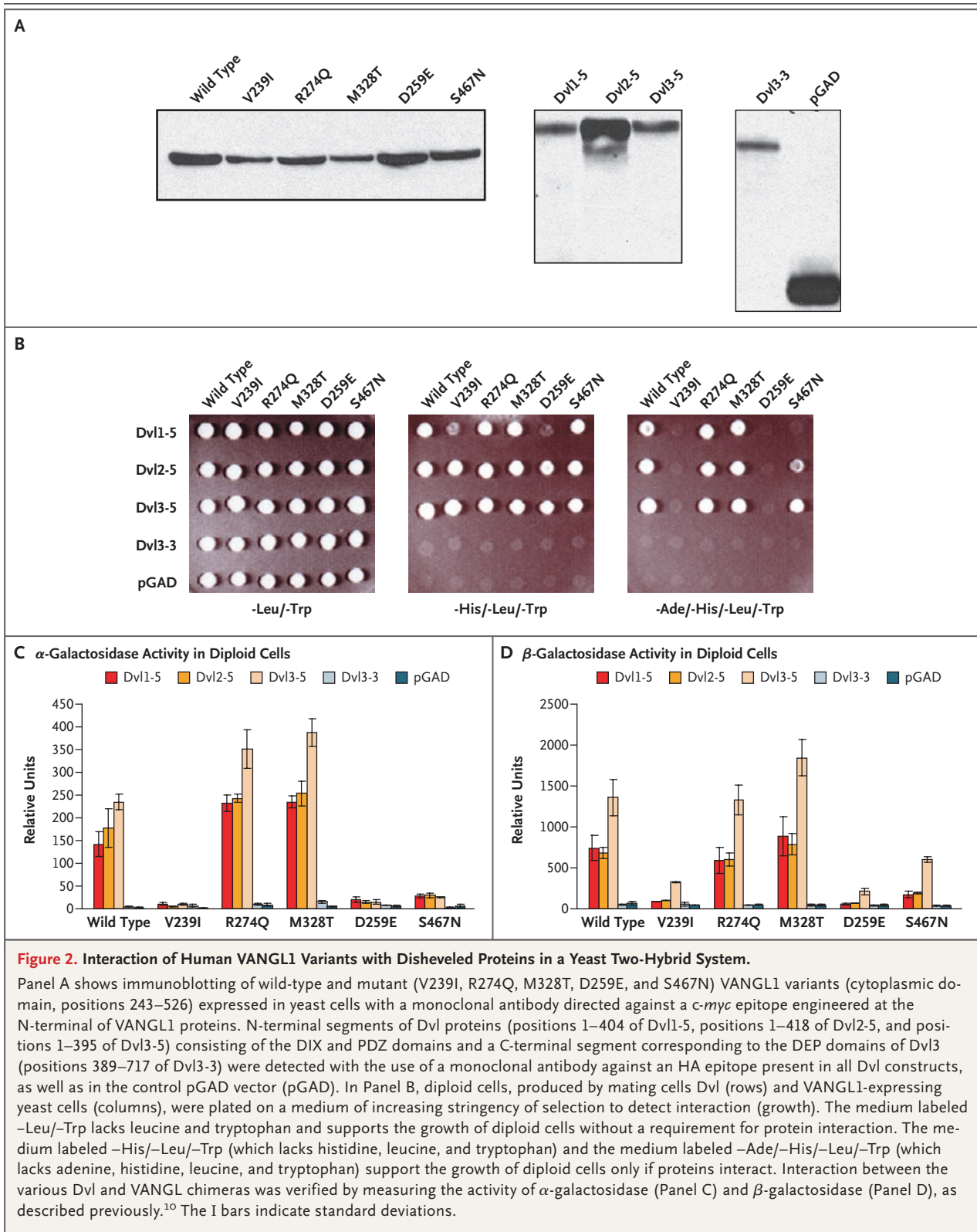
Mouse *Vangl1* and *Vangl2* proteins physically interact with cytoplasmic Dvl1, Dvl2, and Dvl3; in mice, *Lp*-associated mutations of *Vangl2* disrupt this interaction.<sup>10</sup> Therefore, we tested the ability of V239I, R274Q, and M328T variants of VANGL1 to interact with the Dvl proteins with use of the yeast two-hybrid system. All VANGL1 and Dvl constructs were stably expressed at similar levels in *Saccharomyces cerevisiae* (Fig. 2A). Our results indicate that VANGL1 interacts with the N-terminal half of Dvl proteins and that this interaction is disrupted either completely (D259E) or partially (S467N) by *Lp*-associated mutations (Fig. 2B). The V239I mutation in VANGL1 abrogated interaction between VANGL1 and all three Dvl proteins, whereas R274Q and M328T had no apparent effect on this interaction in this assay (Fig. 2B). We ob-

tained similar results regardless of the “reporter” of this interaction (the secretion of  $\alpha$ -galactosidase or the production of  $\beta$ -galactosidase) in diploid yeast cells (Fig. 2C and 2D).

## DISCUSSION

We have identified three VANGL1 mutations (V239I, R274Q, and M328T) in patients with sporadic and familial neural-tube defects. These three mutations affect residues conserved in *Vangl* proteins across species. Our findings contrast with those of a previous study in which no disease-specific mutation was identified in VANGL1 in a group of 66 patients with neural-tube defects.<sup>11</sup>

The phenotype associated with V239I varied among patients. This finding is consistent with the



proposed multifactorial model for neural-tube defects; V239I probably has a partial or complete loss-of-function effect and interacts with other genetic loci or unknown environmental factors to modulate the incidence and severity of the defect. Notably, the mother of the proband with the V239I de novo mutation also had the mutation (de novo) but no phenotype for a neural-tube defect, possibly reflecting mosaicism (with respect to cells carrying the mutation) or variable penetrance or expressivity. At the biochemical level, V239I abolishes the interaction of VANGL1 with Dvl proteins, which may compromise the recruitment of Dvl to the plasma membrane and thus perturb molecular signaling during gastrulation and neural-tube closure.

Noncoding regulatory polymorphisms in VANGL1, particularly regulatory variants modulating the level of gene transcription, could also confer susceptibility to neural-tube defects. A thorough analysis of the promoter region of VANGL1 in our series of patients may uncover additional mutations predisposing to the condition. However, the identification of such polymorphisms is challenging in the absence of detailed information on *cis*-acting sequence elements that regulate temporal and tissue-specific expression of VANGL1 during embryogenesis.

Our results are consistent with studies that underscore the critical role of core planar-cell-polarity genes (*Vangl2*, *Celsr1*, *Dvl*, and *Fz*) in controlling convergent extension, a process necessary for neural-tube closure.<sup>4,5,13-16</sup> Moreover, it was shown that mutations in *Ptk7* interact genetically with *Vangl2* in mice that are heterozygous for both mutations and consequently develop neural-tube defects.<sup>17</sup> Studying the effect of periconceptual supplementation with folic acid, inositol, and methionine on the emergence of neural-tube defects in these mutants may shed further light on the preventive mechanism associated with these nutrients. Additional tests of association between mutated VANGL1 and neural-tube defects in humans is warranted, as is the systematic genetic and biochemical testing of the other members of the planar-cell-polarity signaling pathway for a role in the cause of this disease.

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No potential conflict of interest relevant to this article was reported.

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