Quantitative trait loci affecting phenotypic variation in the vacuolated lens mouse mutant, a multigenic mouse model of neural tube defects

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Quantitative trait loci affecting phenotypic variation in the vacuolated lens mouse mutant, a multigenic mouse model of neural tube defects. Physiol Genomics 35: 296–304, 2008. First published September 16, 2008; doi:10.1152/physiolgenomics.90260.2008.—The vacuolated lens (vl) mutation was identified initially by the congenital cataract phenotype observed in postnatal mice. A small percentage of these mutants also display a white belly spot (14). Two vl-associated NTD phenotypes have been reported. One group of embryos displayed lumbar-sacral spina bifida, while in the other subset the neural tube closed but an attenuated dorsal midline and a dilated dorsal ventricle were observed (44–47). These later phenotypes are remarkably similar to a closed human NTD called embryonic hydromyelia (22).

Neurulation requires bending of the neural plate medially and dorso-laterally, elevation of the neural folds, and then finally apoposition and fusion of the neural folds on the dorsal midline (11). Previous histological, ultrastructural electron microscopy (EM), and in vitro culture studies indicate that the vl mutation affects the last step of neurulation, apoposition, and fusion of the neural folds (11, 20, 44, 46–48). These studies led to the conclusion that the vl spina bifida phenotype is likely due to a complete failure of neural fold fusion, while the vl closed NTD phenotype results from abnormal or incomplete apoposition and fusion.

We previously reported (28) that these vl phenotypes are due to a mutation in an orphan G protein-coupled receptor (GPCR), Gpr161. Approximately 360 human nonsensory GPCRs exist, with the ligand identified for ~200 receptors and the remaining 160 receptors called orphan GPCRs because their endogenous ligands are not known (16). Our previous in situ analysis demonstrated that Gpr161 is expressed in the lateral neural folds during neural tube closure and at all stages of lens development, consistent with the vl NTD and cataract phenotypes. The vl mutation is an 8-bp deletion, which causes a frameshift and premature stop codon truncating ~70% of the cytoplasmic COOH-terminal tail (143/203 amino acids) (28). We have demonstrated that mutant protein is expressed on the plasma membrane but the COOH-terminal tail truncation results in reduced receptor-mediated endocytosis. It is well established that attenuation of GPCR signaling is accomplished through receptor-mediated endocytosis, which is commonly initiated through COOH-terminal tail phosphorylation (21, 25, 34, 35, 42). These previous studies identified Gpr161 as a new receptor-ligand mediated pathway necessary for neural tube closure and lens development.

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NEURAL TUBE DEFECTS (NTDs) are the second-most common human congenital disorder, with an incidence of ~1/1,000 in American Caucasian live births. Increased risk for NTDs is due to both genetic susceptibility and nongenetic factors such as the environment (13). Although >250 mouse NTD mutants have been identified, few of them model the multigenic basis of the human disorder (12, 23).

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To map the vl locus and clone Grprl6l, the mutation was crossed onto three different genetic backgrounds (C57BL/6J, CAST/Ei, and MOLF/Ei). We previously reported (28) that the penetrance of the vl NTD and cataract phenotypes was significantly affected in these crosses, enabling us to map three quantitative trait loci (QTL) (modifiers of vacuolated lens, Modvl1-3). In these B6 and CAST crosses, ~50% of adult F2 vl/vl mice display an obvious lumbar-sacral lesion and hindlimb paralysis (28), phenocopying important aspects of human spina bifida that have not been replicated in other mouse mutants (11, 23, 41). The modifying effects of these backgrounds on the vl NTD phenotype were mapped to chromosomes 5 (Modvl1/C57BL/6J) and 1 (Modvl2/CAST/Ei), Modvl3 affects the incidence of the cataract phenotype in the MOLF/Ei cross. Previous phenotype-based bioinformatics analysis identified Foxe3, a winged helix forkhead transcription factor, as a candidate for Modvl3. Foxe3 is an important regulator of lens development, and mutations in the gene result in cataract in both mice and humans (1, 29, 33, 38). Our resequencing of Foxe3 identified a nonsynonymous coding variant between C3H and MOLF. Additional bioinformatic, genetic, and molecular data demonstrated that this coding variant is functional and likely contributes to the Modvl3 cataract phenotype (28, 29).

We now extend these previous results by 1) identifying additional modifying effects for vl NTD-associated phenotypes (embryonic lethality, adult belly spot) on the three mixed backgrounds (B6/C3H, CAST/C3H, and MOLF/C3H), 2) performing further QTL analysis and mapping two novel modifiers (Modvl4 and 5), and 3) employing bioinformatics to identify candidate genes responsible for these modifying effects. These studies further establish vl as a mouse model for studying the multigenic basis of NTDs.

**Materials and Methods**

**Animals and Crosses**

Cryopreserved vacuolated lens (vl) mice were rederived from the Jackson Laboratory. To determine the penetrance of the different adult and embryonic phenotypes, +/vl × +/vl matings were performed and when necessary pregnant females were killed and litters were genotyped and phenotyped. To map the vl locus and to generate F2 vl/vl mice for QTL analysis, vl/vl C3H mice were crossed to three different inbred strains (C57BL/6J, CAST/EiJ, and MOLF/EiJ), which were obtained from the Jackson Laboratory. For the C57BL/6J and CAST/EiJ crosses wild-type females were mated to C3H/HeSnJ-vl/vl males and for the MOLF/EiJ cross wild-type males were mated to C3H/HeSnJ-vl/vl females to produce F1 progeny. The F1 mice were then intercrossed to produce F2 progeny. Because the vl mutation arose on the C3H background, vl/vl were identified by the C3H/C3H genotype of simple sequence-length polymorphism (SSLP) markers flanking the vl locus (B6: D1Mit143, D1Mit15; CAST and MOLF: D1Mit506, D1Mit15) (31). For the B6 and CAST crosses, 86 and 94 F2 vl/vl mice were typed for genome scan markers, respectively, and then analyzed for QTL. For the MOLF cross, 92 F2 vl/vl mice were typed for genome scan markers and analyzed for QTL. To refine the identified QTL intervals, 35 F2 C3H/MOLF mice were added and flanking SSLP markers to selected QTL regions were genotyped.

Mice were housed in a climate-controlled facility with a 14:10-h light-dark cycle and free access to food and water throughout the experiment. After weaning, mice were maintained on a Chow diet (Old Guilford 234A, Guilford, CT). All experiments were approved by the Animal Care and Use Committees of the Jackson Laboratory and UMDNJ-Robert Wood Johnson Medical School.

**DNA Isolation and Genotyping**

Tail DNA was isolated and genotyped as described previously (28). The B6, CAST, and MOLF crosses used 75, 60, and 80 SSLP markers distributed across the genome, respectively. To genotype for the vl mutation, standard PCR conditions at 55°C annealing temperature were used with primers that flank the 8-bp deletion (F: 5′CCGTTTACCACT-GCAACITITG3′; R: 5′TGAGGGTGTTICAGGGTTTACC3′; 168-bp amplicon +/+; 160-bp amplicon vl/vl).

**Statistical Analysis**

We performed genomewide scans for QTL by using the method of Sen and Churchill (39), which is similar to the interval mapping procedure of Lander and Botstein (27) but uses a different imputation algorithm. First, we carried out one-dimensional genome scans on a single-QTL basis to detect QTL with main effects. Logarithm of odds ratio (LOD) scores were computed at 2-cM intervals across the genome, and significance was determined by permutation testing (9). Significant QTL meet or exceed the 95% genomewide thresholds, respectively. Simultaneous genome scans for all pairs of markers were then implemented to detect epistatic interactions. The search strategy has been described previously (39, 40). Briefly, the genome scan searches through all pairs of loci by fitting a two-way ANOVA model with an interaction item. A LOD score contrasting the full model to a null model (with no genomic effects) is computed, and genomewide significance is established by permutation analysis. A secondary test for the significance of the interaction term is computed only for those pairs that pass the genomewide screening. A stringent nominal significance level (0.001) is used for the interaction test, and only those locus pairs passing both tests are deemed to be interacting. The software package used in this study, R/QTL version 0.97-21, is available at http://www.biostat.jhsph.edu/~kbroman/qtl/(4).

To test whether loci segregated with the lethality in F2 progeny, we analyzed all markers by $\chi^2$-test (single-marker search) and contingency table testing (marker pair search). A $\chi^2$-analysis of the data was performed to determine whether the segregation of alleles differed from the expected for normal Mendelian segregation. $\chi^2$-Tests were performed on each marker separately except for markers on distal chromosome 1 flanking the vl locus. The obtained $P$ values determined whether segregation distortion was observed, which would suggest the existence of protective loci. To investigate whether possible interacting marker pairs demonstrated distorted segregation, contingency table testing was applied to the genotyping data. Each possible pair of markers was tested, except for neighboring linked marker pairs.

**Bioinformatic and Resequencing Analysis**

To identify candidate genes that may contribute to the modifying effects of the mapped QTL, we searched www.informatics.jax.org/phenotypes.shtml for genes within the 95% confidence intervals (CI) that caused similar phenotypes. Validated single nucleotide polymorphisms (SNPs) between C3H and MOLF for candidate genes were then identified by mining dbSNP (www.ncbi.nlm.nih.gov/geneguide/mouse/). Noncoding SNPs were mapped relative to potential cis-regulatory sequences (ESPERR Regulatory Potential; genome.ucsc.edu), and potential effects on transcription factor binding were investigated by using Transcription Element Search Software (TESS; www.cbl.upenn.edu/tes). To identify and validate additional coding polymorphisms in Fzd6 and Mc5r, C57BL/6J, C3H/HeSnJ, and MOLF/EiJ genomic DNA was obtained from the Jackson Laboratories (http://www.jax.org/dnares/index.html), and the coding regions plus splicing sites were amplified with custom-designed primers (Primer3; frodo.wi.mit.edu/).
sequencing was performed on the PCR products with Big Dye Terminator Cycle Sequencing Chemistry and the ABI 3700 Sequence Detection System (Applied Biosystems). Results were analyzed with Sequencher software (version 4.8). The potential functional effects of nonsynonymous coding SNPs were evaluated with PolyPhen (genetics.bwh.harvard.edu/pph), which also generated protein alignments between orthologs and paralogs. NetPhos (www.cbs.dtu.dk/services/NetPhos/) at www.expasy.org/determined the potential effect of coding SNPs on protein phosphorylation. Protein secondary structure analysis was performed with McVector v9.0 (Robson-Garnier algorithm).

**Protein Modeling**

Protein sequences for Mc5r in mouse, rat, human, cow, chicken, frog, zebrafish, and pufferfish were obtained from Ensembl release 48. These protein sequences were aligned with CLUSTALW2 (http://www.ebi.ac.uk/Tools/clustalw2/), and the alignment was viewed in JalView version 2.3 (http://www.jalview.org) (10). A molecular model of three-dimensional protein structure for mouse MC5R (SWISS-PROT protein sequence P41149) was obtained from the ModBase resource (http://modbase.compbio.ucsf.edu/modbase/) that used the X-ray crystallographic structure of bovine rhodopsin (PDB code 1L9H) as a template (32). RasMol version 2.7.2.1 (http://www.rasmol.org) was used to visualize the structure (36). The potential functional effects of Mc5r rs8256628 were evaluated by PAN-THER (http://www.pantherdb.org/tools/csmsScoreForm.jsp) and SIFT (http://blocks.fhcrc.org/sift/SIFT.html). The effect of the Tyr121 substitution on Mc5r ModBase structure was visualized by SwissPDBViewer (version 3.7) (http://www.expasy.org/spdbv) (18).

**RESULTS**

**Penetrance of vl Phenotypes**

**C3H isogenic background.** Before the positional cloning of the vl locus, published experiments were limited to a small number of matings in which adult mice with cataracts were either mated to each other or mated to unaffected littermates. Mutant adults were identified previously by their cataract phenotype, which also occasionally displayed a belly spot. For the published vl embryonic analysis, pregnant females were killed and embryos with an obvious NTD defect were examined experimentally (44, 46, 47). Because vl mutants were identified by their phenotype and not by the causative mutation, the penetrance of the cataract, belly spot, and NTD phenotypes has never been determined precisely. In addition, since open NTD phenotypes in mice typically result in embryonic lethality (11, 20, 23), some percentage of mutant embryos may die during gestation, but this has never been investigated.

To determine the penetrance of the vl NTD phenotypes and whether the vl mutant displays embryonic lethality on an isogenic C3H background, +vl intercrosses were performed. Over 100 adult progeny were generated and genotyped for the vl mutation. Vl homozygotes were observed ~50% less than their +/+ littermates (+/+: 26/109, +vl/vl: 69/109, vl/vl: 14/109), indicating that half of the vl homozygotes die before weaning (Table 1). When embryonic day (E)9.5–E12.5 litters were killed and genotyped, all of the vl/vl progeny displayed neural tube phenotypes: 41% of vl/vl embryos displayed lumbar-sacral spina bifida (23/56), while all of the remaining vl/vl embryos displayed a posterior spinal cord phenotype similar to human embryonic hydromelia (Table 1). For litters killed between E14.5 and E18.5 (n = 64 embryos), no spina bifida-affected vl/vl embryos were observed and an increase in resorptions was detected. These data indicate that the vl mutation results in embryonic lethality on the C3H background, which has not been previously recognized. This lethality is likely due to the embryonic spina bifida phenotype, since other open mouse NTD mutants typically die during embryogenesis or at birth (20, 23).

The penetrance of the adult cataract and belly spot phenotype was also determined. Through our intercrosses to maintain the vl colony over the last 6 years, >400 vl/vl adult mice have been generated and 100% display congenital cataracts, with <5% also exhibiting a small white belly spot (Table 1). The percentages of these vl phenotypes on the isogenic C3H background were then used to determine the effect of the three modifying backgrounds (MOLF/Ei, CAST/Ei, B6) in our previous crosses.

**MOLF/Ei cross.** We previously reported (28) that the MOLF background significantly reduced the penetrance of the cataract phenotype (Table 2). Our results now indicate that the MOLF background has additional effects on the penetrance of the vl-associated embryonic lethality and adult belly spot phenotype (Tables 2–4).

If the MOLF background completely rescues the vl-associated lethality, we would expect 25% of the F2 C3H/MOLF progeny to genotype as vl/vl. In our cross 22.5% of the F2 progeny were vl homozygotes, which is not statistically different from the expected 25% (χ²: P = 0.09) (Table 3). These data indicate that the MOLF background can rescue the vl embryonic lethality phenotype (Table 2).

The surviving F2 C3H/MOLF vl/vl mice were of two phenotypic classes: 1) 49.6% of the F2 vl/vl mice used for our QTL analysis (63/127) displayed no obvious postnatal phenotype and were indistinguishable from their +/- littermates; these data demonstrate that the MOLF background is able to rescue all obvious vl postnatal phenotypes in a subset of vl/vl, 2) The remaining C3H/MOLF vl/vl F2 progeny (50.4%, 64/127) were phenotypically abnormal, exhibiting congenital cataract and/or a white belly spot. Although we previously noted (28) that the penetrance of the cataracts was reduced considerably (Table 2), we could now determine that the MOLF background significantly exacerbated the incidence of the belly spot phenotype (40.9%, 52/127) (Tables 2 and 4).

Together these results indicate that ~50% of the C3H/MOLF vl/vl embryos are rescued for the embryonic lethality, which is likely due to the spina bifida. As adults these rescued vl/vl F2 C3H/MOLF mice are either phenotypically normal or display a white belly spot. Interestingly, a belly spot phenotype is often due to defects in early central nervous system (CNS) development, suggesting that the MOLF modifiers may rescue the spina bifida defect but subsequent spinal cord development is abnormal.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Phenotype</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>Lethality</td>
<td>49%</td>
</tr>
<tr>
<td></td>
<td>Congenital cataract</td>
<td>100%*</td>
</tr>
<tr>
<td></td>
<td>Belly spot</td>
<td>&lt;5%*</td>
</tr>
<tr>
<td>Embryonic</td>
<td>Spina bifida (open NTD)</td>
<td>41%</td>
</tr>
<tr>
<td></td>
<td>Embryonic hydromelia (closed NTD)</td>
<td>59%</td>
</tr>
</tbody>
</table>

A vacuolated lens modifier identified in the C3H/B6 background.

**Table 2. Summary of vl-modifying effects**

<table>
<thead>
<tr>
<th>Background</th>
<th>Postnatal Spina Bifida</th>
<th>Congenital Cataract</th>
<th>Embryonic Lethality</th>
<th>Postnatal Belly Spot</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOLF</td>
<td>–</td>
<td>+ Rescue</td>
<td>+ Rescue</td>
<td>+ Exacerbate</td>
</tr>
<tr>
<td>B6</td>
<td>+ New</td>
<td>ND</td>
<td>+ Exacerbate</td>
<td>+ Exacerbate</td>
</tr>
<tr>
<td>CAST</td>
<td>+ New</td>
<td>ND</td>
<td>+ Exacerbate</td>
<td>–</td>
</tr>
</tbody>
</table>

*New, background results in a new phenotype not observed on the isogenic C3H background; + Exacerbate, background increases incidence of phenotype; – background has no modifying effect; ND, not determined. *Previously reported in Matte-son et al. (28).

**Table 4. Modifying effect of genetic background on vl belly spot phenotype**

<table>
<thead>
<tr>
<th>Cross</th>
<th>Expected</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOLF</td>
<td>6.3/127</td>
<td>52/127</td>
</tr>
<tr>
<td>B6</td>
<td>5.2/105</td>
<td>30/105</td>
</tr>
<tr>
<td>CAST</td>
<td>4.7/94</td>
<td>0/94</td>
</tr>
</tbody>
</table>

Expected, number of F2 vlvl progeny displaying phenotype based on frequency observed on isogenic C3H background (belly spot phenotype was estimated to be 5% of the total number of adult vlvl); observed, number of F2 vlvl progeny displaying phenotype on different mixed backgrounds.

Both the MOLF and B6 backgrounds increased the penetrance of the belly spot phenotype. To map possible modifier loci, F2 B6/C3H and MOLF/C3H vlvl progeny with a belly spot were given a numerical value of 1, while absence of a belly spot was scored as 0. QTL analysis was then performed for these two crosses, and three QTL were identified. For the B6 cross, a QTL was mapped to chromosome 5 with a peak at 44 cM linked to D5Mit309 (LOD 3.7). The B6 alleles segregated with the presence of a belly spot in a recessive fashion (Table 5). Interestingly, the same QTL was mapped previously when the adult spina bifida phenotype was used as a trait (Modvl1, D5Mit309, 44 cM, LOD 3.7) but with the B6 allele segregating in a dominant fashion (28). These data suggest that the same locus influences both phenotypes. Modvl1 accounts for 18.7% of the F2 belly spot phenotypic variance. When the belly spot phenotype was used as a trait in the MOLF cross, two additional QTL (Modvl4 and Modvl5) were identified. Modvl4 maps to chromosome 15, with a peak at 15 cM linked to D15Mit252 (LOD 4.4). Modvl5 maps to chromosome 18, with a peak at 41 cM linked to D18Mit50 (LOD 5.0). Modvl4 and Modvl5 account for 12.7% and 16.8% of the F2 phenotypic variance. For both Modvl4 and 5, the C3H allele segregates with the belly spot phenotype in an additive fashion, while the MOLF alleles for these QTL are inherited with phenotypically normal F2 vlvl progeny (Table 5, Fig. 1). We also investigated whether any QTL could be identified for the increase in vl-associated lethality on the C3H/B6 and C3H/CAST backgrounds (Table 3); \( \chi^2 \) and contingency table testing were used to determine whether any marker alleles were underrepresented in F2 vlvl mice, but no significant QTL were identified (data not shown).

**Bioinformatic Analysis**

We were previously successful (28) in using bioinformatics and resequencing to identify a functional SNP in *Foxe3* that contributes to the cataract-modifying effects of Modvl3. A similar approach was then employed for Modvl1, 2, 4, and 5. The 95% CIs for these QTL were searched (http://informatics.jax.org) for genes that result in NTDs or skin/pigmentation defects when mutated. Fourteen genes were identified: three for Modvl1, four for Modvl2, three for Modvl4, and four for Modvl5 (Table 6).

To prioritize candidates for further analysis, GPCRs were examined. It is well established that GPCRs form oligomeric complexes with each other (3, 30), so we hypothesized that functional polymorphisms affecting the activity or expression of different GPCRs could potentially bypass the mutant effects of the Gpr161 COOH-terminal tail truncation. The above
phenotype-based bioinformatic analysis identified two GPCRs that cause NTD and skin/pigmentation defects: Frizzled homolog 6 (Fzd6) for Modvl4 and Melanocortin 5 receptor (Mc5r) for Modvl5. Our analysis identified no GPCRs for Modvl1 or 2. Both Fzd6 and Mc5r are expressed during early CNS development (genome.ucsc.edu). The Fzd6 knockout results in craniorachischisis, a open neural tube along the entire anterior-posterior axis, while both the Fzd6 and Mc5r mutants exhibit a hair or skin phenotype (7, 19, 43). These observations focused our resequencing and bioinformatic analysis on Fzd6 and Mc5r.

Fzd6. To identify possible functional polymorphisms, available dbSNP data were first mined for validated polymorphisms between C3H and MOLF. None of the validated SNPs between these strains is in protein coding sequence, but four of them mapped in or near (<100 bp) predicted cis-regulatory sequences (ESPERR Regulatory Potential; genome.ucsc.edu) (24, 26). All of these SNPs are situated in binding sites for transcription factors expressed during neural tube closure or in the skin and may affect binding (Table 7).

We then resequenced the Fzd6 protein-coding region from C3H and MOLF to either identify novel polymorphisms or determine whether previously annotated SNPs were polymorphic between these two strains. This analysis validated polymorphic differences for five SNPs and identified one new polymorphism (Table 8).

Two of these polymorphisms (rs50388052, rs48579948) result in a nonsynonymous coding changes, and one 3-bp deletion causes a deletion of an amino acid. All of these amino acids are situated in the COOH-terminal tail of Fzd6. We then investigated whether any of these polymorphisms are predicted to be functional. The bioinformatic analysis did not identify rs48579948 or the deleted amino acid as potentially functional, but significant results were obtained for rs50388052. This SNP replaces a polar SerC3H residue at position 618 with a nonpolar ProMOLF residue (S618P). Interestingly, the ProMOLF allele is conserved among multiple vertebrate species in Fzd6 and a related protein, Fzd3, while the Ser allele has only been reported for C3H and B6 mouse strains (Fig. 2; data not shown). The S618P change is predicted to be damaging by PolyPhen [position-specific independent counts (PSIC) score difference = 1.748] and to affect the secondary structure of the Fzd6 protein-coding region (Fig. 2). The S618P also removes a potential Ser phosphorylation site in the COOH-terminal tail (data not shown), which is often used to initiate receptor-mediated endocytosis (16). These data are consistent with the S618P alteration possibly affecting Fzd6 activity.

Table 6. Candidate genes for vl modifiers

<table>
<thead>
<tr>
<th>Cross</th>
<th>QTL</th>
<th>LOD</th>
<th>Chr (cM)</th>
<th>Peak Marker</th>
<th>95% CI, mb</th>
<th>Phenotype</th>
<th>High Allele</th>
<th>Inheritance</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAST</td>
<td>Modvl2*</td>
<td>3.3</td>
<td>1 (26)</td>
<td>D1Mit236</td>
<td>0–66</td>
<td>Spina bifida</td>
<td>C3H</td>
<td>Additive</td>
</tr>
<tr>
<td>MOLF</td>
<td>Modvl3*</td>
<td>4.2</td>
<td>4 (51)</td>
<td>D4Mit50</td>
<td>90–121</td>
<td>Cataract</td>
<td>MOLF</td>
<td>Additive</td>
</tr>
<tr>
<td></td>
<td>Modvl4</td>
<td>4.4</td>
<td>15 (15)</td>
<td>D15Mit252</td>
<td>12–68</td>
<td>Belly spot</td>
<td>B6</td>
<td>Recessive</td>
</tr>
<tr>
<td></td>
<td>Modvl5</td>
<td>5.0</td>
<td>18 (41)</td>
<td>D18Mit50</td>
<td>55–72</td>
<td>Belly spot</td>
<td>C3H</td>
<td>Additive</td>
</tr>
</tbody>
</table>

QTL, quantitative trait locus; LOD, logarithm of odds ratio; Chr, chromosome; CI, confidence interval. *Previously reported in Matteson et al. (28).
Table 7. Bioinformatic analysis for Fzd6 and Mc5r noncoding SNPs

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Allele</th>
<th>Factor</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fzd6</td>
<td>rs31547795</td>
<td>A (C3H)</td>
<td>CREB</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>API1</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>YY1</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>rs31770137</td>
<td>A (MOLF)</td>
<td>Hormone receptor</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>rs32052549</td>
<td>A (C3H)</td>
<td>YY1</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>rs33858730</td>
<td>C (MOLF)</td>
<td>POU</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>POU</td>
<td>2.0</td>
</tr>
<tr>
<td>Mc5r</td>
<td>rs37038905</td>
<td>C (C3H)</td>
<td>RXR</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>rs37170486</td>
<td>A (MOLF)</td>
<td>c-Myb</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>rs37252221</td>
<td>C (C3H)</td>
<td>C/EBP</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NFI</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C/EBP</td>
<td>1.2</td>
</tr>
</tbody>
</table>

SNP, single nucleotide polymorphism; factor, TESS-predicted transcription factor binding site; score, TESS La/score (0–2), log-likelihood score/base pair length of site with 2.0 being the maximum score.

**Mc5r.** Data mining and resequencing identified or validated five noncoding polymorphisms in potential cis-regulatory sequences and six protein-coding SNPs. Three of the noncoding SNPs are predicted to affect transcription factor binding (Table 7). Four of the coding SNPs are nonsynonymous (Table 8), but only rs8256628 is predicted to be potentially deleterious (PSIC score difference = 1.876). Rs8256628 is situated at the start of the second transmembrane domain and substitutes a polar Tyr residue at position 121 for a hydrophobic Phe residue (Y121F). Interestingly, CLUSTALw2 protein sequence alignments indicate that the PheMOLF allele is conserved among a number of vertebrate Mc5r orthologs (pufferfish, zebrafish, frog, chicken, rat, cow, human) and all McR paralogs (Mc1r-4r) (Fig. 3; data not shown). The Tyr121 allele is only observed in a subset of Mus musculus strains (C3H/HeSn, A/J, DBA/2J, BALB/cJ, NOD/LtJ) (www.ncbi.nlm.nih.gov/SNP), suggesting that this SNP may be functional.

Protein modeling was then performed to investigate the potential effects of this substitution. Mc5r is part of the class A rhodopsin-like GPCR gene family as classified by the GPCR database (http://www.gpcr.org). A molecular model of three-dimensional protein structure for mouse Mc5r was obtained from the ModBase resource, which used the X-ray crystallographic structure of bovine rhodopsin as a template (32). Phe121 is predicted to be in close apposition with three residues (S179, L180, and I183) in the third transmembrane α-helix (Fig. 3). Previous reports have established that the third α-helix of bovine rhodopsin undergoes a large conformational change on ligand binding and activation (8). This possible functional effect of the Y121F substitution was examined with PANTHER cSNP and SIFT tools. PANTHER cSNP reports a functional effect of the Y121F substitution was examined with PANTHER cSNP and SIFT tools. PANTHER cSNP reports a subPSEC (substitution position-specific evolutionary conservation) score of −6.18, with a score of 0 being neutral and greater negative values predictive of more deleterious substitutions. The SIFT analysis also indicates that the Y121F substitution may be functional. The effect of the Tyr substitution was then evaluated with protein modeling by mutating F121 to a tyrosine. Each of the nine rotamers, which are frequently observed side chain conformations compiled from crystallographic structures, was tested for favorable interactions with neighboring amino acids. Three of the rotamers had neutral interactions, while the other six were unfavorable, consistent with our bioinformatic results that the Y121F substitution may affect Mc5r function. These analyses have identified possible functional SNPs in both Fzd6 for Modvl4 and Mc5r for Modvl5 that may contribute to the modifying effects of these QTL and will be the basis of future analysis.

Table 8. Resequencing analysis for Fzd6 and Mc5r protein-coding polymorphisms

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Annotation</th>
<th>C3H</th>
<th>MOLF</th>
<th>C3H</th>
<th>MOLF</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fzd6</td>
<td>rs31767403</td>
<td>S G A</td>
<td>T T</td>
<td>258</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs46408062</td>
<td>S C T</td>
<td>S S</td>
<td>609</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs50388052</td>
<td>N T C</td>
<td>S P</td>
<td>618</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs48579948</td>
<td>N A G</td>
<td>N D</td>
<td>628</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-bp deletion</td>
<td>GGC</td>
<td>G</td>
<td>638</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs48368216</td>
<td>S T C</td>
<td>N N</td>
<td>648</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mc5r</td>
<td>rs8256631</td>
<td>N T C</td>
<td>L S</td>
<td>57</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs37267055</td>
<td>S T C</td>
<td>D D</td>
<td>66</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs8256630</td>
<td>N T C</td>
<td>L S</td>
<td>78</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs8256628</td>
<td>N A T</td>
<td>Y F</td>
<td>121</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs8256625</td>
<td>S C G</td>
<td>T T</td>
<td>202</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs36935918</td>
<td>N G A</td>
<td>V I</td>
<td>357</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Annotation: N, nonsynonymous amino acid changes; S, synonymous amino acid changes.
but go on to display congenital cataract with 100% penetrance postnatally.

Adult C3H<sup>vl/vl</sup> mice can also display a white belly spot at a low frequency. This phenotype is indicative of a melanocyte defect. Melanocytes are derived from neural crest cells, which are specified from the extreme lateral neural plate cells during neurulation (5, 37). These lateral neural plate cells are also important for neural fold apposition and fusion, which is affected by the <i>vl</i> mutation. EM studies have determined that cellular protrusions normally extend from these neural plate cells, which then interdigitate on contact during neural fold fusion (17). Interestingly, <i>Gpr161</i> is specifically expressed in lateral neural plate cells during neurulation, and in <i>vl</i> the cellular protrusions have an abnormal ultrastructural morphology (48). Thus in <i>vl/vl</i> embryos with a closed NTD phenotype, aberrant neural fold apposition and fusion may also cause a neural crest/melanocyte defect that then results in the postnatal belly spot phenotype.

Our MOLF cross increases the frequency of the belly spot phenotype but also rescues the <i>vl</i>-associated lethality. This result suggests that MOLF modifiers can bypass the spina bifida phenotype but on some occasions subsequent spinal cord development is abnormal, resulting in the belly spot/neural crest phenotype. Our B6 QTL analysis also supports this conclusion. The B6 background increases both the frequency of the belly spot phenotype and the number of postnatal <i>vl/vl</i> mice exhibiting lumbar-sacral spina bifida and hindlimb paralysis. When the belly spot and spina bifida were used as traits, the same QTL was mapped to chromosome 5 (<i>Modvl1</i>), but interestingly the opposite allele effects were observed (B6 recessive for belly spot, C3H dominant for spina bifida). These results suggest that the underlying locus influences both phenotypes and further support the hypothesis that the <i>vl</i> closed NTD and belly spot phenotypes are causally related.

When the belly spot was used as a trait in our MOLF cross, <i>Modvl4</i> and <i>Modvl5</i> were identified. Approximately 50% of adult <i>vl/vl</i>C3H/MOLF mice are also phenotypically indistinguishable from <i>+/+</i> littermates, indicating that the MOLF background is able to completely rescue all obvious postnatal <i>vl</i>-associated phenotypes. For both <i>Modvl4</i> and <i>Modvl5</i>, the MOLF/Ei alleles segregate with phenotypically normal mice, suggesting that these modifiers may be sufficient to suppress the lethality, neurulation, and neural crest defects. Our initial characterization of <i>Modvl5</i> congenic mice supports this possibility (Lazar G, Desai J, Matteson PG, unpublished observations).

Unlike the MOLF cross, the B6/C3H and CAST/C3H backgrounds increased the incidence of the <i>vl</i>-associated lethality. These results suggest that the B6 and CAST backgrounds have modifiers that exacerbate the incidence or severity of the embryonic spina bifida phenotype. However, it is also possible that these modifiers cause lethality through a different mechanism. We attempted to map the position of these modifiers by identifying B6 and CAST alleles that were underrepresented in the <i>F<sub>2</sub> vl/vl</i> mice. No significant findings were observed, suggesting that these loci could not be mapped because of their heterogeneity or low penetrance.

The B6 and CAST backgrounds both modify the adult spina bifida and embryonic lethality phenotypes in a similar manner, while these traits are affected differently by the MOLF cross. Both the B6 and CAST crosses resulted in adult <i>F<sub>2</sub> vl/vl</i> mice with spina

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**DISCUSSION**

Our C3H phenotypic analysis indicates that the <i>vl</i> NTD and cataract phenotypes are fully penetrant but <5% of the <i>vl/vl</i> exhibit a belly spot as adults. These phenotypes are inherited in a recessive manner, with no <i>vl</i> heterozygotes exhibiting any of the phenotypes. The <i>vl</i> mutation also results in lethality in ~50% of <i>vl/vl</i>. Interestingly, a nearly equal percentage of E9–E12 <i>vl/vl</i> embryos display spina bifida, consistent with this NTD phenotype causing lethality. This possibility is supported by three other observations: 1) Spina bifida-affected C3H<sup>vl/vl</sup> embryos are not observed at later developmental ages, which is also comcomitant with increased resorptions. 2) Other mouse NTD mutants universally do not survive past P0 (20, 23), and three adult C3H<sup>vl/vl</sup> mice do not exhibit an obvious lumbar-sacral lesion, indicating that either the spina bifida-affected embryos die or the defect repairs itself later in development. All remaining C3H<sup>vl/vl</sup> embryos display a closed NTD phenotype. These <i>vl/vl</i> mutants are likely to survive embryogenesis
bifida, which was never observed on the C3H/MOLF background. The B6 and CAST backgrounds also increased the embryonic lethality, while the MOLF background rescued this phenotype. One factor that may play a role in these results is the directionality of the crosses (+/+B6 or CAST × v/lvlC3H vs. v/lvlC3H × +/+MOLF), suggesting that unrecognized sex differences may contribute to some of the vl-modifying effects.

We have shown that the vl mutation is due to a frameshift mutation in the orphan GPCR Gpr161, which truncates a significant portion of the COOH-terminal tail. The mutation does not affect the developmental expression of the Gpr161 gene or targeting of the mutant receptor to the plasma membrane. Instead, the truncation decreases steady-state protein levels but also results in increased cell surface expression due to reduced receptor-mediated endocytosis (28). Our data are in agreement with numerous published reports demonstrating a role of the COOH-terminal tail in receptor-mediated endocytosis (2, 6, 16, 25, 34, 35, 42). In addition, the COOH-terminal tail functions as a scaffold for other proteins [GPCR interacting proteins (GIPs)], which then positively or negatively regulate receptor activity (2, 15). These findings are consistent with the vl mutation causing a complex Gpr161 signaling phenotype, which could vary between cell types depending on the expression of GIPs or other proteins in the Gpr161 pathway. This could explain why all three backgrounds can modify the various vl phenotypes and why different QTL have been mapped for the cataract, spina bifida, and belly spot phenotypes.

Our previous analysis (28) indicated that Foxe3, a forkhead transcription factor important for lens development, is a contributor to the Modvl3 cataract-modifying effect. We have now used a similar approach to identify biologically relevant candidate genes for the other four QTL. Resequencing and bioinformatic analysis were focused on two GPCRs, Fzd6 and Mc5r, that may bypass the mutant effects of the vl mutation. One nonsynonymous coding SNP in each gene (Fzd6-rs30388052; Mc5r-rs8256628) is of particular interest. For both SNPs the MOLF allele is evolutionarily conserved among different paralogs and orthologs, and the substitution is predicted to alter protein activity and structure. The Fzd6 Ser^{C3H} allele for rs30388052 is expected to affect the secondary structure of the COOH-terminal tail as well as provide an additional site for phosphorylation, both of which may affect the binding of GIPs and GPCR activity. Mc5r protein modeling supports the idea that rs8256628 could be functional with a majority of the polar Tyr^{C3H} rotamers being unfavorable. Molecular and biochemical methods will be used to investigate the potential functionality of these SNPs. Other candidate genes identified by our bioinformatics will be analyzed similarly. Together with ongoing congeneric analysis these experiments will hopefully identify the genes and polymorphisms that contribute to the modifying effect of these QTL.

In summary, our QTL analysis has demonstrated that numerous loci from three different backgrounds affect the penetrance and expressivity of the vl NTD phenotypes. The identification of these modifier genes will aid in understanding how the Gpr161 pathway regulates neural fold apposition and fusion. The multigenic inheritance of the vl NTD phenotypes on these mixed backgrounds will also serve as a mouse model to study the genetic and developmental basis of this common human disorder.

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