Localization of modifier loci for the *vacuolated lens* mutant, a mouse model of spina bifida and congenital cataracts

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Running head: *Vacuolated lens* modifiers

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ASTRACT

Vacuolated lens (*vl*) is a recessive mouse mutation that arose spontaneously on the C3H/HeSnJ background. *Vl* mutant embryos display neural tube defects (NTDs) while mutant adults exhibit congenital cataract and occasionally a white belly spot. We recently determined these phenotypes are due to a mutation in an orphan G protein coupled receptor (GPCR) called *Gpr161*. When the *vl* mutation was crossed onto different genetic backgrounds (C57BL/6J, CAST/Ei and MOLF/Ei) to map the locus, a strain dependent modifying effect on the penetrance of the *vl* phenotypes was observed. We now report the incidence of the different vl phenotypes on the isogenic C3H strain and the effect of the three genetic backgrounds on the penetrance of these phenotypes. Previous analysis using spina bifida and cataract as co-variants mapped QTL (Modifiers of *vacuolated lens*, *Modvl1-3*). We now map three additional QTL using the belly spot phenotype as a co-variant. Two to novel chromosomal positions (Modvl4 and 5) and one to same location as Modvl1. Haplotype analysis was used to fine map the 95% confidence intervals for all QTL, which successfully delimited the regions for *Modvl4* and *Modvl5*. Bioinformatics identified biologically relevant candidate genes (X and X) for each locus while re-sequencing and modeling identified candidate SNPs that may be responsible for the modifying effect of these genes. Our mapping of distinct non-overlapping QTL underscore the complex inheritance of NTDs and congenital cataracts in the *vl* mutant and establishes it as a model to study the multi-genic inheritance of these human disorders in mice. Words? 250 max

Keywords: QTL analysis, neural tube defects, *Gpr161*, *Fzd6*, *Mc5r*.

INTRODUCTION

The *vacuolated lens* mutation arose spontaneously on the C3H/HeSnJ background and was identified by the presence of cataract in post-natal mice. Adult mutants with cataract also occasionally display a white belly spot (**ref**). Previous research has documented two embryonic spinal cord phenotypes: lumbar-sacral spina bifida and if the neural tube closes, an attenuated dorsal midline and a dilated dorsal ventricle. Interestingly, these later phenotypes are remarkably similar to a closed human NTD called embryonic hydromyelia (**refs**).

Neurulation is the process where the neural plate undergoes a series of morphogenetic movements to form the neural tube. This requires bending of the neural plate medially and dorso-laterally, elevation of the neural folds and then finally apposition and fusion of the neural folds on the dorsal midline. Previous histological, ultra-structural EM and *in vitro* culture studies indicate that the *vl* mutation likely affects the last step of neurulation, apposition and fusion of the neural folds (13, 26, 61-63). These studies indicate that spina bifida is likely due to a complete failure of neural fold fusion while the *vl* closed NTD/dorsal midline phenotype results from abnormal and/or incomplete apposition and fusion. These previous conclusions are consistent with mutant embryos displaying both phenotypes on the isogenic C3H background.

We have recently determined that these *vl* phenotypes are due to a mutation in an orphan G protein coupled receptor (GPCR) called *Gpr161* (**ref**). Orphan GPCRs share nucleotide and amino acid homology with other GPCRs but their ligands are unknown. *Gpr161* is expressed in both the neural plate and lens, consistent with the *vl* mutant phenotypes. Most GPCRs have a similar secondary protein structure with an extracellular

N terminus, seven trans-membrane domains and a cytoplasmic C terminal tail. The *vl* mutation is an eight base pair deletion that causes a frameshift and premature stop codon, truncating the C terminal tail by 143 amino acids (**ref**). The C terminal tail of GPCRs is known to function as an important regulator of receptor activity (10, 21, 22, 28, 32, 45, 46, 65). We have demonstrated that the mutation has multiple effects on Gpr161 protein, including decreased receptor-mediated endocytosis, which is a common mechanism to attenuate GPCR signaling. These studies have identified a new receptor mediated pathway necessary for neural tube closure and lens development. The identification of the ligand and other components of the pathway will be important for understanding how Gpr161 activation regulates neurulation and lens development.

To map the *vl* locus and clone *Gpr161*, the mutation was crossed onto three different genetic backgrounds (C57BL/6J, CAST/Ei and MOLF/Ei). In all three crosses, the penetrance of the *vl* phenotypes was altered suggesting the presence of modifier loci. QTL analysis was then employed to map the position of these modifiers and we have reported previously the mapping of three QTL (Modifiers of *vacuolated lens*, *Modvl1-3*). These QTL affect the penetrance of the *vl* spina bifida and cataract phenotypes, suggesting that these modifiers likely represent other genes that function in the novel Gpr161 pathway. These studies have also determined that the *vl* spina bifida and cataract phenotypes can be suppressed by unlinked loci, establishing *vl* as a mouse model to study the multi-genic inheritance of human NTDs and cataracts.

We now extend these observations by detailing the penetrance of the different *vl* phenotypes on the C3H and three mixed genetic backgrounds as well as mapping three additional QTL. In addition, we have employed haplotype mapping, sequencing and

bioinformatic tools to narrow the QTL regions. This analysis has identified biologically relevant genes and candidate functional SNPs for two of the loci.

MATERIALS AND METHODS

Animals and crosses

Cryopreserved *vacuolated lens* mice were rederived from The Jackson Laboratory, Bar Harbor, ME. To determine the penetrance of the different adult and embryonic phenotypes, +/*vl* x +/*vl* matings were performed and when necessary pregnant females were sacrificed and E10.5-E12.5 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 (C57BL6/J, CAST/Ei and MOLF/Ei), which were obtained from The Jackson Laboratory. For the C57BL/6J and CAST/Ei crosses, wild type females were mated to C3H/HeSnJ-*vl/vl* males and for the MOLF/Ei cross wild type males were mated to C3H/HeSnJ-*vl/vl* females to produce F_1 progeny. The F_1 mice were then intercrossed to produce F_2 progeny. Because the *vl* mutation arose on the C3H background, *vl/vl* were identified by the genotype of markers that flanked the previously mapped chromosomal position of the *vl* locus (43). For the B6 cross, 86 F_2 progeny were identified as C3H/C3H between *D1Mit143* and *D1Mit15* and used for the QTL analysis. For the CAST cross 86 F_2 progeny were identified as C3H/C3H between *D1Mit506* and *D1Mit15*; for the MOLF cross 127 F_2 progeny were identified as C3H/C3H between *D1Mit506* and *D1Mit15* and used for the analysis.

Mice were housed in a climate-controlled facility with a 14-hour: 10-hour light-dark cycle with 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

DNA was isolated from a centimeter of tail digested overnight in 500 μl of 1X digestion buffer (50 mM Tris-Cl pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl, 1% SDS, 1mg/ml proteinase K) in a 55^{O}C water bath. Digests were mixed with 1 volume of 25:24:1 phenol:chloroform:isoamyl alcohol and centrifuged for 5 minutes at 14,000 rpm at room temperature. DNA was precipitated by adding 2 volumes of 100% ethanol to the isolated aqueous phase. The dried DNA pellets were resuspended in 1 ml TE (10mM Tris-HCl, 1mM EDTA, pH 7.5-8.0). Genotyping by PCR using mouse MIT MapPairs primers (Research Genetics, Huntsville, AL) was carried out under standard conditions at an annealing temperature of 55°C. Polymorphisms were detected either by capillary electrophoresis (ABI), standard electrophoresis using 4% agarose (Nusieve) or 7.5% acrylamide (19:1 acrylamide:bis-acrylamide) gels. The B6, CAST and MOLF crosses used 75, 60, and 80 markers distributed across the genome, respectively. For the MOLF cross, QTL analysis was first performed on 92 F_2 *vl/vl* mice. To confirm and refine the identified QTL intervals, 35 F_2 mice were added and flanking SSLP markers to selected QTL regions were genotyped. To genotype for the *vl* mutation, standard PCR conditions at 55°C annealing temperature were used with primers that flank the 8bp deletion (F 5'CCGTTCTACCAATGCCAACTTTG3'; R 5'GTGAGGGGTTTTCAGGGTT TTTAC3'; 168 bp amplicon +/+, 160 bp amplicon *vl/vl*)

Phenotyping

The belly spot was recorded in F_2 progeny from the B6 and MOLF intercrosses. The effect of genetic background on *vl* associated lethality was determined by comparing the percentage of F_2 *vl/vl* adults generated in the three different intercrosses to the ~50% lethality observed on the C3H background.

Statistical analysis

We performed genome-wide scans for QTL by using the method of Sen and Churchill (51), which is similar to the interval mapping procedure of Lander and Botstein (33) 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 (11). Significant and suggestive QTL meet or exceed the 95% and 90% genome-wide thresholds, respectively. Simultaneous genome scans for all pairs of markers were then implemented to detect epistatic interactions. The search strategy has been described by Sen and Churchill and Sugiyama et al (51, 54). 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 genome-wide 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 genome-wide screening. A stringent nominal significance level (0.001) is used for interaction test and only those locus pairs passing

both tests are deemed to be interacting. For the B6 and MOLF crosses, the presence of a belly spot was given a numerical value of 1 while an absence of a belly spot was scored as 0. The software package used in this study, R/QTL version 0.97-21, is available at http://www.biostat.jhsph.edu/~kbroman/qtl/ (7).

To test whether loci segregated with the lethality in F_2 progeny, we analyzed all markers by chi-square test (single-marker search) and contingency table testing (marker-pair search). A chi-square analysis of the data was performed to determine whether the segregation of alleles differed from the 1:2:1 ratio for autosomes and 2:1:1 ratio for sex chromosomes, which is expected for normal Mendelian segregation. Chi-square 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 if 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.

Haplotype analysis

Recent evidence demonstrates that most genetic variation in inbred mouse strains predates the derivation of the common laboratory strains (Wade, 2002). Co-localized QTL that are mapped in multiple crosses using different strains are then likely to represent shared alleles. Haplotype comparisons using the different parental strains can then be used to fine map QTL (Park, 2003) (Wade, 2002) (DiPetrillo, 2005). Single

nucleotide polymorphisms (SNPs) used in haplotype analysis were obtained from the Mouse Phenome Database (www.jax.org/phenome).

Sequencing of Candidate Genes

Genomic DNA from C57BL/6J, C3H/HeSnJ, and MOLF/EiJ was obtained from mouse DNA resource at The Jackson Laboratory (http://www.jax.org/dnares/index.html). Coding regions and splicing sites of the candidate genes were amplified using custom designed primers (program?). Direct sequencing was performed on the PCR products using Big Dye Terminator Cycle Sequencing Chemistry and the ABI 3700 Sequence Detection System (Applied Biosystems). Results were analyzed using the Sequencher software (version 4.8).

Protein modeling

RESULTS

Penetrance of *vl* phenotypes

Prior to the positional cloning of the *vl* locus, published experiments have been limited to a small number of matings using adult mice with cataracts, making it difficult to determine the penetrance of the different mutant phenotypes in *vl/vl* and +/*vl* mice and embryos. In addition, it was unknown whether the *vl* mutation results in lethality in some percentage of homozygotes, which is commonly observed in other mouse NTD mutants (**refs**). To investigate this further, +/*vl* x +/*vl* matings were performed and the progeny were genotyped for the *vl* mutation. In crosses of adult heterozygotes, *vl* homozygotes mice were observed ~50% less than their +/+ littermates (+/+: 26/109, +/*vl*: 69/109, *vl/vl*: 14/109), indicating that this percentage of *vl/vl* die before weaning. 100% of viable adult vl/vl^{C3H} mice display congenital cataracts with less than 5% also exhibiting a small white belly spot. When E9.5-E12.5 litters were sacrificed, 100% of the *vl/vl* progeny display neural tube phenotypes. 41% of *vl/vl* embryos display spina bifida (23/56), while all the remaining embryos display a posterior spinal cord phenotype similar to human embryonic hydromyelia. Because all reported NTD mutants never survive past P0 (**refs**), it is likely that *vl/vl* spina bifida affected embryos die before birth contributing to the post-natal lethality phenotype. *Vl/vl* embryos with a closed NTD phenotype likely survive but display cataracts as neonates.

In our crosses to B6, CAST/Ei and MOLF/Ei, we have reported previously modifying effects on the spina bifida and cataract phenotypes (**ref**). We also noticed additional effects on the incidence of *vl*-associated lethality and the belly spot phenotype. When the mutation was crossed onto the MOLF/Ei background, the lethality was

significantly reduced. 192/854 or 22.5% of the F_2 progeny were genotyped as *vl/vl*, which was not statistically significant different from the expected 25% (Chi-test; *P*=.56)(Table 1a). In addition, nearly half (49.2%) of the surviving adult $vl/vl^{C3H/MOLF}$ F_2 progeny were indistinguishable from their +/+ littermates (Table 1b), indicating that the MOLF background is able to completely rescue all obvious *vl* post-natal phenotypes.

The remaining $vl/vl^{C3H/MOLF}$ F_2 progeny exhibit either congenital cataracts and/or a white belly spot. We have reported previously a significant reduction in the penetrance of the cataracts (**ref**) but also noticed an enhancement of the belly spot phenotype (C3H <5%; C3H/MOLF 41.3%)(Table 1b; Fig 1C). These results indicate that ~40% of the vl/vl embryos$^{C3H/MOLF}$ rescued for the embryonic lethality go on to display a belly spot phenotype as adults while the remaining mutants did not display any obvious phenotype.

The B6 and CAST/Ei backgrounds had the most significant effects on the *vl* spina bifida phenotype. Adult *vl/vl* mice on a mixed C3H/B6 and C3H/CAST background now displayed an obvious lumbar-sacral lesion and hind-limb paralysis (**ref**), phenocopying important aspects of human spina bifida that has not been reported for other mouse spina bifida mutants. We have only very rarely observed this phenotype on the C3H or C3H/MOLF backgrounds. Two additional effects of the B6 and CAST/Ei backgrounds for other *vl* phenotypes were noted. One, both backgrounds increased *vl* associated lethality since the number of F_2 *vl/vl* mice generated from the crosses was lower than expected (Table 1b) and two, the incidence of the belly spot phenotype was increased by the B6 (29% of F_2 progeny) but not the CAST/Ei background (Table 1b).

QTL analysis

In order to map additional modifier loci that segregated with the belly spot or lethality phenotypes, QTL analyses were employed on F_2 progeny from the three different intercrosses. Only F_2 progeny homozygous for the *vl* mutation were used in the study. All F_2 *vl* homozygotes were individually genotyped for 60-80 SSLP markers spaced evenly throughout the genome, enabling us to detect both additive and interactive QTL in our three crosses (Table 2). No interacting QTL were identified so only single QTL are presented. Permutation testing was used to determine significance thresholds.

When the belly spot was used as a covariant, three additional QTL were identified, one for the B6 and two for the MOLF cross. The B6 belly spot QTL was mapped to chromosome 5 with a peak at 44cM linked to *D5Mit309* (LOD 3.7). The B6 alleles segregated with the presence of a belly spot in a recessive fashion (**Fig X**). Interestingly, the same QTL was mapped previously when the spina bifida phenotype was used as a covariant (*Modvl1*, *D5Mit309*, 44cM, LOD 3.7). allele effect This suggests the underlying locus influences both spina bifida and belly spot phenotypes. *Modvl1* accounts for 18.7% of the F_2 belly spot phenotypic variance.

Two additional QTL (*Modvl4* and *Modvl5*) were identified when the belly spot phenotype was used as a covariant in the MOLF cross. *Modvl4* mapped to chromosome 15 with a peak at 15 cM linked to *D15Mit252* (LOD 4.4) and *Modvl5* mapped to chromosome 18 with a peak at 41 cM linked to *D18Mit50* (LOD 5.0)(Fig X). *Modvl4* and *Modvl5* account for 12.7% and 16.8% of the F_2 phenotypic variance. For both loci the C3H allele segregates with the belly spot phenotype in an additive fashion, indicating that the MOLF alleles of these QTL were inherited in phenotypically normal F_2 *vl/vl* progeny (**Figs X**).

We also investigated whether any QTL segregated with the increase of *vl*-associated lethality on the C3H/B6 and C3H/CAST backgrounds. Chi-square and contingency table testing were used to determine whether any marker alleles were under-represented in F_2 *vl/vl* mice but no significant QTL were identified (data not shown).

Haplotyping of QTL regions

Because most mouse inbred strains share ancestors, large parts of their genomes are identical by descent (IBD). We can use this to narrow the QTL and the number of genes within the QTL by excluding the regions where the strains share a haplotype. We took the SNP data (www.jax.org/phenome) and gene lists (Ensembl build 36) from the 95% confidence intervals (Table 2) and performed haplotype analysis MM. The *Modvl1* region between B6 and C3H and the *Modvl2* region between CAST and C3H have mostly different haplotypes and we were unable to significantly reduce the intervals.

Although not significant, we also observed the *Modvl4* and *Modvl5* QTL in the B6 x C3H cross. data Therefore, we made the assumption that the underlying genes should be in a region that is IBD between MOLF and B6, but different from C3H. With these assumptions we narrowed the gene list for *Modvl4* from 137 to 25 genes and for *Modvl5* from 111 to 29 genes (Supplemental Table 1). Explain better From these lists, *Fzd6* and *Mc5r* spell out both encode GPCRs, are expressed at the same embryonic stage as *Gpr161*, and have been associated with neural tube and hair phenotypes (refs). We therefore focused on these genes as strong candidates underlying the *Modvl4* and *Modvl5* QTL.

Sequencing and protein modeling of *Fzd6* and *Mc5r*

Re-sequenced. We found several SNPs in the coding regions of *Fzd6* of which two were non-synonymous leading to an amino acid change. In addition we found a three base pair deletion leading to the deletion of one amino acid in the protein. However, all these polymorphisms were found in MOLF and where not shared with B6. When using the assumption that the polymorphism we are looking for is shared between B6 and MOLF these are not the causative polymorphism. However, there are several SNPs in the region upstream of the gene that might lead to expression differences.

We found multiple SNPs in the coding regions of *Mc5r* between the three different strains. However, only one SNP was shared by B6 and MOLF and different from C3H. This SNP (rs8256628) results in an amino acid difference (hydrophobic Phenylalanine in B6 and MOLF, polar Tyrosine in C3H). Using the structure of cow rhodopsin (which is very similar to MC5R) the amino acid is predicted to be in alpha helix 3. This helix, in rhodopsin, is involved in a large conformational change with other helices in the structure when the protein is activated (Choi et al 2002 Biochemistry 41:7318-7324).

DISCUSSION

We have previously mapped *Modvl1-3* that have specific modifying effects on the spina bifida and cataract phenotypes. We have extended these observations by mapping additional belly spot QTL and using haplotype mapping, sequencing and bioinformatics to identify candidate genes for all the QTL.

Vl/vl mice on the C3H isogenic background very occasionally display a white belly spot phenotype while on the MOLF/Ei and B6 mixed backgrounds this phenotype is increased in incidence. A white belly spot is indicative of a melanocyte defect. Melanocytes are derived from neural crest cells, which are generated from extreme lateral neural fold cells during neural tube closure (8, 49). Other mouse mutants that affect neurulation or spinal cord development can also display a white belly spot (18, 47, 48, 57). Because the *vl* mutation affects the last step of neurulation, apposition and fusion of the neural folds (13, 26, 61-63), a possible explanation for this observation is that the spina bifida phenotype has been rescued but subsequent spinal cord development is abnormal, resulting in the neural crest/melanocyte defect. This possibility is supported by our B6 QTL analysis. When the belly spot was used as a co-variant, a QTL at the same genetic position as the spina bifida QTL was identified but with the opposite allele effects (B6 recessive for belly spot, C3H dominant for spina bifida).

In the MOLF/Ei cross adult *vl/vl* mice do not exhibit an obvious lumbar-sacral lesion or hind-limb paralysis but rescue of the lethality and an increase in the belly spot phenotype was observed. Approximately 50% of the adult *vl/vl* mice were phenotypically indistinguishable form +/+ littermates, suggesting that this background is able to completely rescue *vl* associated phenotypes. When the belly spot was used as a co-

variant, two QTL (*Modvl4* and *Modvl5*) were identified. Because the MOLF/Ei alleles of *Modvl4* and *5* segregate with mice that are phenotypically normal, these modifiers may be sufficient to suppress the neurulation and subsequent neural crest defects. Congenic analysis for *Modvl5* supports this possibility (Lazar et al, unpublished observations).

On the B6/C3H and CAST/C3H backgrounds, an increase in the *vl* associated lethality was observed. Although further histological analysis is required to determine the cause of the lethality, a likely contributor is the spina bifida for two reasons. One, adult lethality and embryonic spina bifida are both observed in approximately the same percentages of vl/vl^{C3H} progeny. Two, the surviving adult vl/vl^{C3H} mice never exhibit an obvious lumbar-sacral lesion, indicating that the spina bifida-affected embryos either die or the defect repairs itself later in development. Embryonic death would be consistent with other mouse NTD mutants not surviving past P0 and embryonic resorptions being routinely observed in +/*vl* x +/*vl* matings post-neurulation (Desai et al., unpublished observations). If our hypothesis is correct that the *vl* lethality is due to the spina bifida, this suggests that the B6 and CAST backgrounds have modifiers that can bypass the *vl* associated lethality but also have QTL that can enhance this phenotype. We attempted to map the position of these modifiers by identifying B6 and CAST alleles that were under-represented in the F_2 mice from these crosses. No significant findings were observed, suggesting that there may be multiple modifiers that are responsible for the increased lethality.

The *vl* mutation affects the orphan GPCR, *Gpr161*, which is expressed in the neural plate, where neural crest cells are generated, and in the anterior epithelium of the developing lens (Desai et al. submitted). This expression pattern is consistent with the *vl*

spina bifida, belly spot and cataracts phenotypes. The mutation deletes a significant portion of the C terminal tail. We have determined that the mutation does not affect the transcriptional regulation or targeting of the mutant receptor to the plasma membrane. Instead the mutation decreases steady state protein levels but also results in increased cell surface expression due to reduced receptor mediated endocytosis (Desai et al. submitted). This result is consistent with numerous published reports demonstrating a role of the C terminal tail in receptor mediated endocytosis (10, 21, 32, 45, 46, 65). In addition, the C terminal tail often functions as a scaffold for other proteins (GPCR Interacting Proteins-GIPs), which then either positively or negatively regulate receptor activity (3-5, 17). 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 cataracts, belly spot and spina bifida.

How might the mapped QTL modify the different *vl* phenotypes? It is well established that GPCRs form oligomeric complexes with other GPCRs (6, 42). One possible way that the mutant effects of the C terminal tail deletion could be bypassed by crossing onto different genetic backgrounds is if these modifying backgrounds express higher levels of GPCRs that interact with Gpr161. These oligomeric complexes would contain a greater percentage of functional C terminal tails presumably allowing them to be internalized into endosomes. Interestingly, GPCRs map to the chromosomal locations of all five QTL (www.gpcr.org). Other mechanisms are also possible such as modifiers functioning upstream or downstream of Gpr161 and counteracting the signaling effects of

the mutation. The cloning of these modifiers is likely to identify other genes in the Gpr161 pathway and provide insight into how this pathway regulates neurulation and lens development. Speculation on how Fzd6 and Mc5r could be involved?

Mc5r is expressed in the eye and plays a role in ocular immunity (Taylor 2006)

Fzd6 controls neural tube closure (Wang 2006)

ACKNOWLEDGEMENTS

We thank Theolyn Gilley for technical support. This work was supported by research grants from The New Jersey Commission on Spinal Cord Research (02-3016-SCR-S-0, 04-2901-SCR-E-0) to J.H.M.

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Table 1a. Modifying effect of genetic background on *vl/vl* associated lethality

cross	expected[a]	observed[b]	effect[c]
B6	131	105	+
CAST	168	94	+
MOLF	107	192	-

[a]- Number of F_2 *vl/vl* expected to survive based upon a 50% lethality rate observed on the C3H isogenic background

[b]- Number of F_2 progeny scored as *vl/vl* by genotype

[c]- Effect on *vl* lethality: (+) increased lethality, (-) decreased lethality

Table 1b. Modifying effect of genetic background on *vl* spina bifida associated phenotypes.

cross	phenotype	expected[a]	observed[b]
B6	non-phenotypic	0	18/105
	belly spot	1.3/105	30/105
	adult spina bifida	0	57/105
CAST	non-phenotypic	0	52/94
	belly spot	4.7/94	0/94
	adult spina bifida	0	42/94
MOLF	non-phenotypic	0/126	62/126
	belly spot	3.4/126	52/126
	adult spina bifida	0/126	0/126

[a]- Number of F_2 *vl/vl* progeny displaying phenotype based upon frequency observed on isogenic C3H background. Belly spot phenotype was estimated to be 5% of the total number of adult *vl/vl.*

[b]- Number of F_2 *vl/vl* progeny displaying phenotype on different mixed backgrounds

Table 2. Summary of modifier loci for the *vl* mutation.

Cross	QTL	Peak (cM)	Nearest Marker	95% CI (Mb)	LOD	Phenotype	high allele	mode of inheritance
A	*Modvl1*	5 (44)	*D5Mit309*		3.7	spina bifida	C3H	dominant
					4.3	belly spot	B6	recessive
B	*Modvl2*	1 (26)	*D1Mit236*		3.3	spina bifida	C3H	additive
C	*Modvl3*	4 (51)	*D4Mit168*		4.2	cataract	MOLF	additive
	Modvl4	15 (15)	*D15Mit252*		4.4	belly spot	C3H	additive
	Modvl5	18 (41)	*D18Mit50*		5.0	belly spot	C3H	additive

Supplemental table 1. Genes in the narrowed *Modvl4* and *Modvl5* regions according to the Ensembl database (build 36).

Modvl4		*Modvl5*	
start (Mb)	**gene**	**start (Mb)**	**gene**
21.06	*Cdh12*	60.34	*NP_00102893*
23.12	*B230220E17R*	60.39	*NP_00103003*
23.42	*Cdh18*	62.63	*Fbxo38*
23.43	*XR_004732.1*	62.67	*A230091H23R*
25.08	*9230109A22R*	62.72	*Isk7*
25.31	*Basp*	62.73	*Q3uts8*
25.36	*NP_00101381*	63.83	*Wdr7*
28.15	*Dnahc5*	64.38	*St8sia3*
28.45	*XM_890135.1*	64.47	*Onecut2*
31.54	*Cct5*	64.57	*Amd1*
31.55	*A930016P21R*	64.58	*Fech*
32.12	*Tas2r119*	64.65	*Atp8b1*
32.19	*Sema5a*	65.05	*Nedd4l*
32.87	*Sdc2*	65.39	*Alpk2*
33.03	*Pgcp*	65.56	*Malt1*
33.35	*1700084J12R*	65.71	*Zfp532*
38.43	*Azin1*	65.93	*Sec11l3*
38.76	*Baalc*	66.00	*Grp*
38.84	*Fzd6*	66.18	*Ccbe1*
41.28	*Oxr1*	66.64	*Q8c5c2*
42.25	*Angpt1*	67.21	*Gnal*
43.26	*Q3ufn4*	67.52	*Tubb6*
43.31	*4921531G14R*	67.53	*Afg3l2*
43.69	*Tmem74*	67.59	*BC019561*
44.03	*Trhr*	67.61	*Spire1*
		67.90	*Seh1l*
		68.39	*4933403F05*
		68.43	*Rnmt*
		68.46	*Mc5R*

Figure 1. **(A)** A (C3H/HeSn-*vl*/*vl* x C57BL/6J) F_2 intercross animal with caudal spina bifida and a *vl*/*vl* littermate without spina bifida. Both animals have cataracts. **(B)** The same animal from a different angle. **(C, D)** (C3H/HeSn-*vl*/*vl* x MOLF/Ei) F_2 intercross mice are shown with congenital cataracts (**C**) or a belly spot phenotype (**D**)(left and right-belly spot, middle-no belly spot).

Figure 2. **(A)** Genome-wide scan of the (C3H/HeSn-*vl*/*vl* x C57BL/6J) F_2 intercross for spina bifida. The genome-wide scan for belly spot (not shown) looked similar. Suggestive (P =0.10), and significant (P =0.05) thresholds are indicated. **(B)** LOD score plot of chromosome 5 for spina bifida with *Modvl1*. **(C, D)** Allele effect plots of the peak marker (*D5Mit309*) for spina bifida and belly spot.

Figure 3. **(A)** Genome wide scan of the (C3H/HeSn-*vl*/*vl* x CAST/Ei) F_2 intercross for spina bifida. Suggestive (P =0.10), and significant (P =0.05) thresholds are indicated. **(B)** LOD score plot of chromosome 1 with *Modvl2*. **(C)** Allele effect plot of the peak marker (*D1Mit236*).

Figure 4. **(A)** Genome wide scan of the (C3H/HeSn-*vl*/*vl* x MOLF/Ei) F_2 intercross for cataract. Suggestive (P =0.10), and significant (P =0.05) thresholds are indicated. **(B)** LOD score plot of chromosome 4 with *Modvl3*. **(C)** Allele effect plot of the peak marker (*D4Mit168*).

of the peak marker (*D15Mit252*). **(D)** LOD score plot of chromosome 18 with *Modvl5*, and **(E)** allele effect plot of the peak marker (*D18Mit50*).