Strain-specific modifier genes of Cecr2-associated exencephaly in mice: genetic analysis and identification of differentially expressed candidate genes

Megan K. Kooistra, Renee Y. M. Leduc, Christine E. Dawe, Nicholas A. Fairbridge, Jay Rasmussen, Julie H. Y. Man, Mattea Bujold, Diana Juriloff, Kirst King-Jones, and Heather E. McDermid

1Department of Biological Sciences, University of Alberta, Edmonton, Alberta; 2Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada

Submitted 2 August 2011; accepted in final form 26 October 2011

Kooistra MK, Leduc RYM, Dawe CE, Fairbridge NA, Rasmussen J, Man JHY, Bujold M, Juriloff D, King-Jones K, McDermid HE. Strain-specific modifier genes of Cecr2-associated exencephaly in mice: genetic analysis and identification of differentially expressed candidate genes. *Physiol Genomics* 44: 35–46, 2012. First published November 1, 2011; doi:10.1152/physiolgenomics.00124.2011.—Although neural tube defects (NTDs) are common in humans, little is known about their multifactorial genetic causes. While most mouse models involve NTDs caused by a single mutated gene, we have previously described a nulligenic system involving susceptibility to NTDs. In mice with a mutation in *Cecr2*, the cranial NTD exencephaly shows strain-specific differences in penetrance, with 74% penetrance in BALB/cCr and 0% penetrance in FVB/N. Whole genome linkage analysis showed that a region of chromosome 19 was partially responsible for this difference in penetrance. We now reveal by genetic analysis of three subinterval congenic lines that the chromosome 19 region contains more than one modifier gene. Analysis of embryos showed that although a *Cecr2* mutation causes wider neural tubes in both strains, FVB/N embryos overcome this abnormality and close. A microarray analysis comparing neurulating female embryos from both strains identified differentially expressed genes within the chromosome 19 region, including *Arhgap19*, which is expressed at a lower level in BALB/cCr due to a stop codon specific to that strain. Modifier genes in this region are of particular interest because a large portion of this region is syntenic to human chromosome 10q25, the site of a human susceptibility locus.

neural tube defect; mouse; *Arhgap19*; microarray; subinterval congenics

NEURAL TUBE DEFECTS (NTDs) are among the most common human birth defects, affecting 1–2 infants per 1,000 live births (3). These severe birth defects are caused by the partial or complete failure to close the neural tube, the embryonic precursor to the brain and spinal cord (7). The type of NTD depends on the location of the closure failure. Anencephaly (and the murine equivalent exencephaly) results from a lack of cranial closure, craniorachischisis involves nearly the entire neural tube, and spina bifida occurs when the posterior neurocranium fails to close.

Both environmental factors, such as dietary folate levels, and genetic factors have been implicated in the development of human NTDs, and it is thought that the majority of cases are caused by the interaction of two or more risk factors (7). Because of this, the identification of susceptibility genes in model organisms could help to elucidate the genetic causes of human NTDs. Although human NTDs show a multifactorial inheritance, over 200 single-gene defects have been shown to result in NTDs in the mouse (reviewed in Ref. 9). These NTD-causing genes are involved in many different cellular processes, including apoptosis, noncanonical Wnt signaling, cell cycle, regulation of actin cytoskeleton, chromatin organization, and modifications such as methylation or acetylation (reviewed in Ref. 8).

The penetrance of NTDs is highly dependent on genetic background in many mouse mutants, including *Men1*, *Gpr161* (*vacuolated lens*), *Jarid2* (*jumonji*), *Nog*, *Ski*, and *Cecr2* (1, 2, 14, 15, 18, 25). For instance, a *Men1* mutation backcrossed into *C57BL/6* and 129S6/SvEv genetic backgrounds showed the NTDs exencephaly and dorsal oedema exclusively in 129S6/SvEv embryos (15). Variability in NTD penetrance between strains indicates the influence of genetic modifiers. In the *Gpr161* NTD mouse model, variability in spina bifida penetrance has been used to map two modifier loci, *Mod11* and 2 (17). The identification of modifier genes affecting NTD penetrance in mouse models may provide insight into the multifactorial etiology of human NTDs.

A gene-trap mutation in the chromatin remodeler gene *Cecr2* (*Cecr2<sup>Gt45Bic</sup>*/*Gt45Bic*) results in exencephaly and inner ear defects in homozygotes on a BALB/cCr genetic background (1, 5). The penetrance of exencephaly in homozygotes occurs in a strain-dependent manner. On a BALB/cCr background, *Cecr2<sup>Gt45Bic/Ko45Bic</sup>* embryos show 74.5% (35/47) penetrance for exencephaly compared with a 0% (0/42) penetrance on a FVB/N background (1). When congenic BALB/cCr *Cecr2<sup>Gt45Bic<sup>+/+</sup></sub>*) males were crossed to congenic FVB/N *Cecr2<sup>Gt45Bic/Gt45Bic</sup>* females, exencephaly was observed at only 2.9% (1/35) penetrance (4). Together, these experiments suggest that there is at least one semidominant or dominant modifier locus that produces a resistant phenotype in the FVB/N strain. Previously, collection of embryos from reciprocal crosses of FVB/BALB F1 *Cecr2<sup>Gt45Bic/Gt45Bic</sup>* to BALB/cCr *Cecr2<sup>Gt45Bic<sup>+/+</sup></sub>* mice revealed 28.1% (101/360) penetrance of exencephaly in the backcross embryos, indicating there are likely a small number of major modifiers that segregate independently (4). Whole genome linkage analysis revealed one significant (P < 0.001) candidate region on chromosome 19, which has a linkage peak with a logarithm of the odds (LOD) score of 4.35 (relative risk of 3.74). It was predicted that the presence of this FVB/N region reduces the incidence of exencephaly by 30–35% compared with a BALB/cCr allele. A suggestive linkage region on the X chromosome (LOD score of 3.04) was also found (4).

In the study reported here, we further characterized the influence of genetic background on *Cecr2* mutant phenotypes. We determined that, compared with wild-type FVB/N and BALB/cCr embryos, FVB/N *Cecr2<sup>Gt45Bic/Gt45Bic</sup>* mutant embryos have wide neural folds that close late, similar to the wide

Address for reprint requests and other correspondence: H. E. McDermid, Dept. of Biological Sciences, Univ. of Alberta, T6G 2E9, Canada (e-mail: hmcdermi@ualberta.ca).
neural folds of BALB/cCrl \textit{Cecr2^{Gt45Bic/Gt45Bic}} mutant embryos that do not close and result in exencephaly (5). Inner ear defects of the stereociliary bundles, a second abnormality found in BALB/cCrl \textit{Cecr2^{Gt45Bic/Gt45Bic}} mutants, were also absent in FVB/N \textit{Cecr2^{Gt45Bic/Gt45Bic}} mutants, showing a broader effect of FVB/N modifiers. A genetic analysis of subinterval congenic mouse lines revealed the presence of more than one neurulation modifier gene under the chromosome 19 linkage peak. To identify candidates for these neurulation modifiers, differential gene expression analysis of the candidate region on chromosome 19 compared neurulating embryos of the BALB/cCrl and FVB/N strains. \textit{Arhgap19} is significantly downregulated in BALB/cCrl and was found to have a nonsense mutation specific to the BALB/cCrl strain. \textit{Arhgap19} is widely expressed in the embryonic day (E) 9.0 embryo. Identification of \textit{Cecr2} modifier gene(s) may provide insight into the multigenic etiology of human NTDs, since the murine modifier region mapped for \textit{Cecr2}-related exencephaly is syntenic to a susceptibility locus mapped in 10q25 in a human NTD study (22).

\section*{MATERIALS AND METHODS}

\subsection*{Mice and Generation of Subinterval Congenic Mouse Lines}

All experiments were approved by the Animal Care and Use Committee of the University of Alberta. Mice were housed in a 14 h light/10 h dark cycle at 22 ± 2°C and were fed Laboratory Rodent Diet 5001 (LabDiet), with the exception of breeding animals who received Mouse Diet 9F 5020 (LabDiet). Wild-type mouse strains used for this study were BALB/cAnNCrl ("BALB/cCrl") (a University of Alberta colony originally from Charles River Laboratories), BALB/cJ (Jackson Laboratories), FVB/N (Jackson Laboratories), C57BL/6 (Charles River Laboratories), and 129S2/SvPasCrl ("129S2") (Charles River Laboratories). Two congenic \textit{Cecr2^{Gt45Bic}} mouse mutant lines were previously generated by backcrossing the allele onto a BALB/cCrl or FVB/N genetic background for over 10 generations (1). BALB/cCrl \textit{Cecr2^{Gt45Bic}} heterozygotes were crossed to generate embryos for microarray analysis, while FVB/N \textit{Cecr2^{Gt45Bic}} homozygotes were crossed for embryo collection.

Subinterval congenic mouse lines were produced to further characterize the modifier region on chromosome 19 (Fig. 1). Segments of the modifier region on chromosome 19 from FVB/N were transferred onto the BALB/cCrl background, as follows. Wild-type FVB/N and BALB/cCrl mice were crossed to produce F1 hybrids, which were

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{breeding_strategy.png}
\caption{Breeding strategy to make subinterval congenic mouse lines to further characterize the candidate region for the \textit{Cecr2} modifier region on chromosome 19. Black bars represent FVB/N chromosome 19 and white bars BALB/cCrl chromosome 19. \textit{Cecr2} is on chromosome 6, although the genotype appears beside the stylized chromosome 19 in the diagram [+, \textit{Cecr2} wild-type (WT) allele; m, \textit{Cecr2^{Gt45Bic}} mutant allele].}
\end{figure}
then backcrossed to BALB/cCtrl mice. DNA from the resulting mice was then genotyped at polymorphic loci across chromosome 19 to determine the FVB/N allelic portion of chromosome 19 in each mouse. Three mice were selected based on the size and location of the FVB/N fragment of chromosome 19. These three mice became the founders of the subinterval congenic lines MOD4, MOD5, MOD31 that together covered the entire candidate region and subdivided it into four subintervals. The founders were further backcrossed to BALB/cCtrl for three generations to create incipient (NS) congenic mouse lines. The Cecr2Gt45Bic allele congenic in the BALB/cCtrl background was then crossed into each line and Cecr2Gt45Bic heterozygotes that were homozygous for the FVB/N chromosome 19 fragment were mated for embryo collection. A final cross between MOD5 and MOD31 was done to generate mice that were at least heterozygous for FVB/N alleles across the entire candidate region, and homozygous FVB/N in an overlapping region. To ensure the FVB/N fragment remained intact within each MOD line prior to homozygosity, parents of each generation were typed based on polymorphic markers. A total of 12 microsatellite and single nucleotide polymorphism (SNP) (rs13483563, rs6259521, rs31272775, rs6381572, D19Mit88, rs3677115, rs2950750, rs13483644, rs13483654, rs13483669, rs37096715, and rs3712604) spaced ~3 Mb apart were used: MOD4 was typed from rs13483653 to D19Mit88, MOD5 from rs13483563 to rs13483654 and MOD31 from rs3677115 to rs3712604. An additional two SNPs were used to narrow down the breakdown in MOD5 (rs30659530 and rs31199765) and MOD31 (rs30714351 and rs30418423) to help localize candidate genes.

Embryo Collection

Various developmental ages of embryos were obtained through timed matings of wild-type, Cecr2Gt45Bic heterozygotes, or homozygote mice. Embryos were initially dissected into PBS (or DEPC-treated PBS) on ice, and a tail biopsy or extraembryonic membrane was collected for genotyping. Samples for RNA analysis were flash-frozen and stored at −80°C until analysis.

Neural Fold Analysis

Embryos from timed matings were dissected at E8.5–9.5 into ice-cold PBS and were immediately imaged for analysis. Each embryo was categorized by somite pairs count into age groups, ranging from 10 to 16 somite pairs, and then examined for the degree of neural tube closure. Only embryos with neural folds that were elevated but not yet fused were scored. To score the width of the neural tube, the distance between the widest portion of the cranial neural tube (from the outside of the neural epithelium across the dorsal lateral hinge points) was compared with the width at the branchial arches to create the neural fold-to-head width ratio (5).

DNA Extraction and Genotyping

Genomic DNA was isolated from tail biopsies or extraembryonic tissues. Multiplex PCR reactions were used to genotype the Cecr2Gt45Bic gene-trap insertion as previously described (1). SNPs were genotyped by a CEL assay, performing heteroduplex formation with known samples, then subsequent CELI endonuclease mismatch digest and separation on a ~1.5% agarose gel (done by The Centre for Applied Genomics, Hospital for Sick Children) or using the using the ABI PRISM SNaPshot Multiplex Kit (11, 21).

RNA Extraction and Microarray/qRT-PCR

Wild-type and Cecr2Gt45Bic heterozygote embryos were collected for RNA expression analysis by microarray and qRT-PCR as previously described (6). In brief, four female embryos with 11–14 somite pairs undergoing cranial neural tube closure (neural folds had elevated and hinge points formed, but fusion at closure site 2 had not yet started) were used for each genotype and strain (BALB/cCtrl and FVB/N wild-type and Cecr2Gt45Bic homozygotes). Total RNA was extracted from individual embryos using a QIAGEN Lipid RNA Extraction Kit (Invitrogen), and RNA quality and concentration were analyzed using Agilent 2100 Bioanalyzer RNA 6000 Nano chips (RNA integrity number >8).

Gene expression profiling of the Cecr2 chromosome 19 modifier region was performed by microarray, followed by confirmation of gene expression changes by quantitative real-time PCR (qRT-PCR) as previously described (6). An Affymetrix GeneChip Mouse Genome 430 2.0 chip was used according to manufacturer’s protocol by The Centre of Applied Genomics (Hospital for Sick Children, Toronto) and data (accession #GSE30957) was initially analyzed by R/gcRNA. Only genes with fold change >1.5 and P values <0.05 were further validated. A second method of microarray analysis was also done (see below). Validation of genes by qRT-PCR was done using TaqMan assays designed with the Roche Probefinder version 2.45 and probes from the Roche Universal ProbeLibrary, as previously described (6). Assays were done using a Fluidigm BioMark System for 48.48 dynamic arrays and NanoFlex IFC Controller. Four individual embryos for each genotype and strain were analyzed by qRT-PCR, with four technical replicates/sample, and the average fold change and 95% confidence intervals were determined. Four different embryos were analyzed by microarray and qRT-PCR, except for FVB/N wild-type embryos, where the same four mRNA samples were utilized for both analyses.

Sequenceing

A portion of the putative promoter region, the 12 exons and the 3′-untranslated region (UTR) of Arhgap19 were analyzed for sequence differences between various mouse strains. Each region was amplified by PCR of genomic DNA and analyzed by sequence chromatographs. Regions covered by the qRT-PCR probe sets were amplified by external primers and sequenced to verify that no SNPs that differed between the strains resulted in mismatches between either the primers or probes.

Inner Ear Analysis

Stereocilia rotation within the inner ear was analyzed in FVB/N wild-type and Cecr2Gt45Bic mutant fetuses as described (5, 20). In brief, cochleas from E18.5 fetuses were fixed in 4% paraformaldehyde; the sensory epithelium was microdissected and stained with anti-myosin VIIa antibody (1:500; Proteus Biosciences), Alexa Fluor 488-conjugated phalloidin (1:40, Invitrogen), and Cy3-conjugated goat anti-rabbit antibody (1:200, Jackson Immuno). At least 40 cells per region were analyzed, and the orientation of stereocilia was determined as previously described (5).

Generation, Genotyping, and Xgal Staining of the Arhgap19GsYH020248S Mouse Line

The mouse ES cell line YH0202, containing the β-gal splice-trap vector pGT01xRC within intron 2 of Arhgap19, was generated by BayGenomics. ES cells (strain 129P2) were injected into DBA/2J: C57BL/6 hybrid blastocysts by the Mutant Mouse Regional Resource Center at UC Davis. Germline transmission was achieved by mating chimeric male mice to wild-type BALB/cCtrl and FVB/N (Charles River Laboratories) females. F1 Arhgap19GsYH020248S heterozygous mice were then backcrossed to the appropriate wild-type mice. N2 Arhgap19GsYH020248S heterozygous females were crossed with F1 Arhgap19GsYH020248S heterozygous males to produce embryos for LacZ analysis.

Mice were genotyped by PCR from DNA extracted from tail clippings or extraembryonic membranes. Two separate PCs were utilized for each sample, one containing two Arhgap19 intron 2 specific primers to identify the wild-type allele (2i-F: 5′-GATGTT-GACTGGCTCGGTCT-3′ and 2i-R: 5′-CCCCATACTGTCAGCCT GGT-3′) and the other containing two pGT01xRC specific primers to

Physiol Genomics • doi:10.1152/physiolgenomics.00124.2011 • www.physiolgenomics.org
identify the mutant allele containing the gene trap (En2-1F: 5'-AACAAACTGGCCTCACCAG-3' and BGeo-R: 5'-AAATTCA-GACGGGCAA CGAC-3'). PCR amplicons were pooled and electrophoresed.

Xgal staining of E9.5 embryos was performed as described, with the following differences (5). Embryos were fixed in 4% paraformaldehyde for 1.5 h and then stained in 5 mg/ml Xgal for 24–48 h at 37°C.

Statistical Methods

A χ² test-of-independence was used to determine if the penetrance of exencephaly was statistically different between any of the MOD lines or parental strains. If a significant P value was obtained between all groups in a particular analysis, pair-wise comparisons were done to determine which groups differed significantly. A χ² goodness-of-fit test was used to indicate if a Mendelian inheritance of Cecr2<sup>Gt45Bic</sup> allele was obtained for each MOD line during embryo collection.

The microarray data (accession #GSE30957) and qRT-PCR data were analyzed as previously described (6). Differences in expression were expressed as fold change with 95% confidence intervals, and P values were calculated using a two-tailed t-test. Affymetrix microarrays were also analyzed with Arraystar 4.0 (DNASTAR), using robust multiarray averaging (RMA) for calculating signal strength and normalization. To identify candidate modifier genes, we filtered for genes that map to chromosome 19 and that showed significant changes in transcript levels based on the ANOVA Bonferroni algorithm using a cutoff P value of 0.05.

The stereociliary bundle orientation of inner ears was expressed as a ratio of misaligned to normal and analyzed using a χ² test-for-independence as previously described (5). Differences in the neural fold to head width ratios were expressed as means ± SE, and P values were determined using the Mann-Whitney test using InStat (GraphPad).

RESULTS

Cranial Neural Folds in FVB/N Cecr<sup>2Gt45Bic</sup> Mutant Embryos Are Abnormally Wide and Close Late

We have previously shown that in a BALB/c<sup>Crl</sup> background, the Cecr<sup>2Gt45Bic</sup> mutation results in both wider cranial neural folds in the homozygotes that do not undergo neural tube closure and a delay of closure in the heterozygotes (5). We therefore examined the process of cranial neural tube closure when the Cecr<sup>2Gt45Bic</sup> mutation is in the FVB<sup>N</sup> background, which is resistant to NTDs. The separation between the neural folds was determined as a ratio between the dorsolateral hinge points (the widest portion of the cranial neural tube) and the branchial arches (Fig. 2, A and B). The neural fold ratio was not significantly different between the wild-type BALB/c<sup>Crl</sup> and FVB<sup>N</sup> strains (means of 0.534 and 0.553, respectively; P > 0.05 by the Mann-Whitney test). The neural fold ratios of the Cecr<sup>2Gt45Bic</sup> homozygous mutants on both strains were also not significantly different (means of 0.690 and 0.684 for BALB/c<sup>Crl</sup> and FVB<sup>N</sup>, respectively; P > 0.05). All differences between wild-type and mutant were highly significant, regardless of strain (P < 0.0005), even though the penetrance of exencephaly is high in a BALB/c<sup>Crl</sup> background and extremely low in an FVB<sup>N</sup> background (Fig. 2E, data not shown). BALB/c<sup>Crl</sup> Cecr<sup>2Gt45Bic</sup> mutants showed 74% penetrance (35/47) at generation N6 or N7 (1) and 69% (36/52) at generation >N10 based on a recent analysis. Exencephaly penetrance for Cecr<sup>2Gt45Bic</sup> homozygous mutants in the FVB<sup>N</sup> background was 0% originally (0/42) (1) and 1.6% (1/61) in the current study (at generation >N10). Therefore, although the Cecr<sup>2Gt45Bic</sup> mutation results in the same increase in cranial neural tube width regardless of strain, FVB<sup>N</sup> embryos can overcome this defect and close their neural tubes, whereas the BALB/c<sup>Crl</sup> embryos usually cannot.

The BALB/c<sup>Crl</sup> Cecr<sup>2Gt45Bic</sup> heterozygotes have an average neural fold ratio of 0.566, which is not significantly different from either wild-type strain (P > 0.05). However, BALB/c<sup>Crl</sup> Cecr<sup>2Gt45Bic</sup> heterozygotes do show a delay in closure in the cranial region (5). At 14 somite pairs, the cranial neural tubes of most WT embryos are closed (C), while a significant number of the Cecr<sup>2Gt45Bic</sup> homozygous mutant neural tubes are still open (D). E: graphical representation of the neural fold-to-head width ratios of FVB<sup>N</sup> WT controls compared with FVB/N Cecr<sup>2Gt45Bic</sup> homozygotes. Ratios are represented as means ± SE. **P < 0.001. The number of embryos examined is labeled on each bar.

Fig. 2. Cranial neural folds of FVB/N Cecr<sup>2Gt45Bic</sup> homozygote embryos are wider and close later than FVB/N WT embryos. At 11–12 somite pairs, whole mount images of FVB/N WT (A) and Cecr<sup>2Gt45Bic</sup> homozygous mutants (B) show wider cranial neural folds in the latter. Black lines indicate the measurements used to produce neural fold-to-head width ratios (“width ratio”) shown in E. At 14–15 somite pairs, the cranial neural tubes of most WT embryos are closed (C), while a significant number of the Cecr<sup>2Gt45Bic</sup> homozygous mutant neural tubes are still open (D). E: graphical representation of the neural fold-to-head width ratios of FVB/N WT controls compared with FVB/N Cecr<sup>2Gt45Bic</sup> homozygotes. Ratios are represented as means ± SE. **P < 0.001. The number of embryos examined is labeled on each bar.

Physiol Genomics • doi:10.1152/physiolgenomics.00124.2011 • www.physiolgenomics.org
closure for the FVB/N Cecr2<sup>Gt45Bic</sup> mutants. By 14 somite pairs, most of FVB/N wild-type embryos showed neural tube closure (72.7%, 32/44) while few of the observed FVB/N Cecr2<sup>Gt45Bic</sup> homozygous mutants had closed (13.3%, 2/15) (Fig. 2, C and D). By 16 somite pairs all FVB/N wild-type neural tubes had closed (28/28), yet only approximately half of the FVB/N Cecr2<sup>Gt45Bic</sup> homozygous mutants had closed (45.5%, 5/11). By 19 somite pairs all FVB/N Cecr2<sup>Gt45Bic</sup> mutants had closed their neural tubes (13/13). Therefore the FVB/N Cecr2<sup>Gt45Bic</sup> mutants are also delayed in cranial neural tube closure, as well as having wider neural tube ratios, although this does not inhibit closure in this strain.

**FVB/N Cecr2<sup>Gt45Bic</sup> Mutant Embryos Do Not Show Stereociliary Bundle Misorientation**

The Cecr2<sup>Gt45Bic</sup> mutation causes similar widening of the neural folds in both BALB/cCrl and FVB/N backgrounds, although the consequences are exencephaly for the former and normal closure for the latter. The other major abnormality seen in BALB/cCrl Cecr2<sup>Gt45Bic</sup> homozygous mutants are inner ear defects: BALB/cCrl Cecr2<sup>Gt45Bic</sup> exencephalic mutants showed smaller cochlea with significant misorganization of stereociliary bundles within the sensory epithelium (5). Nonpenetrant mutants and heterozygotes showed lower levels of misorganization but were still significantly different from wild type. We therefore examined the effect of Cecr2<sup>Gt45Bic</sup> on inner ear morphology in an FVB/N background. We found no evidence of inner ear defects in FVB/N Cecr2<sup>Gt45Bic</sup> mutants. The cochlear size and length were equivalent to wild type, and analysis of the stereociliary bundle orientation within the inner ear revealed that there is no significant misorientation of the hair cells (Fig. 3). Xgal staining in both backgrounds showed similar expression of CECR2 along the longitudinal axis of the cochlear duct, specifically within the medial cochlear floor, indicating that the difference in phenotype between the strains does not result from a difference in expression (data not shown). These observations suggest that FVB/N-specific modifier genes act to counteract the effects of the Cecr2<sup>Gt45Bic</sup> mutation during formation of both the neural tube and inner ear, although it is not known whether the same modifier genes are responsible for the effects in both tissues.

**Subinterval Congenics Indicate the Presence of More Than One Neural Tube Modifier in the Chromosome 19 Region**

A Cecr2 modifier region associated with neural tube closure was previously mapped to a ∼30 MB region of chromosome 19, with a peak at ∼40.1 MB (corresponding to rs3677115) and an odds ratio of 3.74 (4). This chromosome 19 region contains ∼292 validated genes and ∼126 predicted genes, totaling ∼418 candidate modifier genes. To further characterize the modifier region, we produced three subinterval incipient (N5) congenic mouse lines, MOD4, MOD5, and MOD31, that contained overlapping fragments of chromosome 19 from the resistant FVB/N strain in the susceptible BALB/cCrl genetic background (∼96.9% BALB/cCrl) to minimize the remaining FVB/N genomic segments that may contain modifiers not on chromosome 19. The three FVB/N fragments, which divide the region into four subregions, and the markers used to define them are shown in Fig. 4.

The N5 cross for each MOD line introduced the Cecr2<sup>Gt45Bic</sup> allele in the BALB/cCrl background, followed by an intercross that produced Cecr2<sup>Gt45Bic</sup> heterozygotes that were homozygous for the FVB/N chromosome 19 fragment (Fig. 1). Crosses within each line then generated Cecr2<sup>Gt45Bic</sup> homozygotes that

---

**Fig. 3. Inner ear analysis of Cecr2 mutants on the FVB/N strain reveals no abnormalities.** A: macroanatomy of inner ear structures dissected from embryonic day (E) 18.5 embryos of WT and Cecr2<sup>Gt45Bic</sup> genotypes on various strains. The solid line indicates the cochlear portion of the inner ear; the remainder is vestibulum. In an FVB/N background, the Cecr2<sup>Gt45Bic</sup> mutant inner ear is the same size as the WT. Scale bar is 500 µm. B: sensory epithelium was stained with phalloidin-Alexa 488 antibody to visualize the actin-based stereociliary v-shaped bundles. The 5, 50, and 75% region relative to the basal end were analyzed (only a representative of the 50% region is shown). No difference was apparent. Layers of hair cells are identified as inner hair cells (IHC) and 3 rows of outer hair cells (OHCs). C: quantification of misaligned hair cells in the 50% region in WT and Cecr2<sup>Gt45Bic</sup> mutant embryos in both FVB/N and BALB/cCrl genetic backgrounds. Stereociliary v-shaped bundles are considered misaligned if they point between 30° and 330°. Levels of significance: ***P < 0.0005, **0.0005 ≤ P < 0.005, *0.005 ≤ P < 0.05, and no asterisk indicates a lack of significance (P > 0.05). Only BALB/cCrl Cecr2<sup>Gt45Bic</sup> mutant stereocilia show misalignment.

Physiol Genomics • doi:10.1152/physiolgenomics.00124.2011 • www.physiolgenomics.org
between the observed penetrance of exencephaly among any of the three MOD subcongenics lines or the MOD5/31 cross (P > 0.05). However, all four crosses do differ significantly from the parental BALB/cCrl penetrance of exencephaly of 69% (P < 0.05) based on a χ² test-of-independence. The parental BALB/cCrl penetrance of exencephaly of 69% (36/52) has been recently determined by a collection of BALB/cCrl embryos analyzed for inner ear morphology (5). Penetrance has not significantly changed (P > 0.05) from the original determination of 74.5% (35/47) (1), indicating that penetrance is stable in this line. Since there is no chromosome 19 FVB/N region common to all three MOD lines, yet all four crosses show a similar effect on penetrance, each producing a significant reduction from BALB/cCrl penetrance levels, this suggests the presence of more than one modifier gene within the chromosome 19 linkage region.

Assuming there are two modifier loci, one in region 1 and a second in region 4 (see DISCUSSION), we tested whether the MOD lines support a codominant, additive model to explain the similar penetrance decreases in the four MOD lines. Under this model, the modifier loci in the two regions would be equivalent, and any combination of two modifiers would give the same decrease in penetrance. We crossed MOD31 animals heterozygous for Cecr2Gt45Bic and containing two copies of FVB/N region 3 and 4, to BALB/cCrl Cecr2Gt45Bic heterozygotes. This produced MOD31/+ Cecr2Gt45Bic mutants with only one copy of FVB/N region 3 and 4. Under an additive model, we would expect a level of penetrance between the MOD31 animals (2 FVB/N modifiers) and BALB/cCrl wild-type and heterozygotes. This produced MOD31/+ Cecr2Gt45Bic mutants, which is not significantly different from the penetrance in the four MOD lines (4, 5, 31, and 5/31; Fig. 4) but is significantly different from the parental BALB/cCrl penetrance of exencephaly of 69%. This result does not support a codominant, additive model.

**Gene Expression Profiling by Microarray and qRT-PCR Identifies Candidate Genes on Chromosome 19**

Microarray analysis of gene expression differences between FVB/N and BALB/cCrl wild-type and Cecr2Gt45Bic mutant embryos was done with two different methods to identify candidate genes in the modifier region. The R/gcRMA analysis revealed 24 or 25 transcripts for each category that were differentially regulated between the strains (fold change > 1.5, P < 0.05) from the original determination of 74.5% (35/47) (1), P = 0.05 (Table 1). These transcripts represent 21 genes differing in their expression between wild-type embryos of the two strains and 20 genes differing between mutant embryos. Of these 20–21 genes, there are 14 genes that differ between the strains regardless of genotype. To test the robustness of our results we also analyzed the array data with the commonly used RMA algorithm followed by ANOVA analysis. This analysis identified 11 transcripts that were differentially regulated between the strains based on an ANOVA (Bonferroni corrected) P value of <0.05 (Table 2). Most genes were identified by both approaches. A subset of candidate genes from both analyses (Arhgap19, Dmrt2, Exosc1, Foxd4, Lipa, Lipo1, Scd2, Tmem180, and Vldlr) was selected for further validation based on known or predicted function. The expression of the nine genes was analyzed by qRT-PCR based on total RNA collected from four individual wild-type embryos at 11–14 somite pairs.
Chromosome 19 transcripts differentially regulated in BALB/cCRL and FVB/N embryos with 11–14 somite pairs using GC-RMA/Student's t-test analysis

Table 1. Chromosome 19 transcripts differentially regulated in BALB/cCRL and FVB/N embryos with 11–14 somite pairs using GC-RMA/Student's t-test analysis

<table>
<thead>
<tr>
<th>Affy Probe Set</th>
<th>Gene Symbol</th>
<th>MOD Region</th>
<th>FC</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1434911_s_at</td>
<td>Arhgap19</td>
<td>3</td>
<td>21.43</td>
<td>3.27E-08</td>
</tr>
<tr>
<td>1444218_at</td>
<td>D19Ertd737e</td>
<td>4</td>
<td>4.97</td>
<td>2.31E-04</td>
</tr>
<tr>
<td>1422318_at</td>
<td>Foxd4</td>
<td>1</td>
<td>2.65</td>
<td>3.08E-02</td>
</tr>
<tr>
<td>1423875_at</td>
<td>Fam160b1</td>
<td>4</td>
<td>2.27</td>
<td>4.89E-04</td>
</tr>
<tr>
<td>1438258_at</td>
<td>Fam160b1</td>
<td>4</td>
<td>2.24</td>
<td>3.09E-04</td>
</tr>
<tr>
<td>1426867_at</td>
<td>Dmr3</td>
<td>1</td>
<td>2.11</td>
<td>4.07E-02</td>
</tr>
<tr>
<td>1439561_at</td>
<td>2010012O05Rik</td>
<td>3</td>
<td>1.87</td>
<td>8.38E-03</td>
</tr>
<tr>
<td>1418473_at</td>
<td>Ctxt</td>
<td>3</td>
<td>1.73</td>
<td>6.06E-04</td>
</tr>
<tr>
<td>1429382_at</td>
<td>Vldlr</td>
<td>3</td>
<td>1.77</td>
<td>1.50E-02</td>
</tr>
<tr>
<td>1428139_at</td>
<td>Vldlr</td>
<td>4</td>
<td>1.61</td>
<td>1.27E-04</td>
</tr>
<tr>
<td>1415965_at</td>
<td>Scd1</td>
<td>1</td>
<td>1.64</td>
<td>3.45E-02</td>
</tr>
<tr>
<td>1434911_s_at</td>
<td>Tmem180</td>
<td>3</td>
<td>1.57</td>
<td>2.07E-02</td>
</tr>
<tr>
<td>1443854_at</td>
<td>Pten</td>
<td>4</td>
<td>1.56</td>
<td>3.02E-02</td>
</tr>
<tr>
<td>142983_at</td>
<td>2700078E11Rik</td>
<td>4</td>
<td>1.51</td>
<td>1.24E-04</td>
</tr>
<tr>
<td>1452845_at</td>
<td>Hif1an</td>
<td>3</td>
<td>1.50</td>
<td>2.42E-04</td>
</tr>
<tr>
<td>1435986_at</td>
<td>Sfn2</td>
<td>3</td>
<td>1.53</td>
<td>6.04E-04</td>
</tr>
<tr>
<td>1436075_at</td>
<td>Sprr5</td>
<td>3</td>
<td>1.52</td>
<td>5.86E-02</td>
</tr>
<tr>
<td>1415964_at</td>
<td>Sdn1</td>
<td>3</td>
<td>1.54</td>
<td>1.82E-04</td>
</tr>
<tr>
<td>1453180_at</td>
<td>Bubs</td>
<td>1</td>
<td>1.57</td>
<td>2.07E-02</td>
</tr>
<tr>
<td>1438561_x_at</td>
<td>Tmem180</td>
<td>3</td>
<td>1.73</td>
<td>3.08E-05</td>
</tr>
<tr>
<td>1415965_at</td>
<td>Sdn1</td>
<td>3</td>
<td>1.87</td>
<td>8.66E-05</td>
</tr>
<tr>
<td>1428139_at</td>
<td>Tmem180</td>
<td>3</td>
<td>2.04</td>
<td>2.80E-02</td>
</tr>
<tr>
<td>1423141_at</td>
<td>Lipa</td>
<td>1/2</td>
<td>2.41</td>
<td>7.30E-04</td>
</tr>
<tr>
<td>1415824_at</td>
<td>Scd2</td>
<td>3</td>
<td>2.67</td>
<td>4.03E-04</td>
</tr>
<tr>
<td>1429910_at</td>
<td>March5</td>
<td>2</td>
<td>2.78</td>
<td>2.24E-05</td>
</tr>
<tr>
<td>1432052_at</td>
<td>Exosc1</td>
<td>3</td>
<td>2.99</td>
<td>3.47E-07</td>
</tr>
<tr>
<td>1433914_at</td>
<td>Lipa</td>
<td>1</td>
<td>6.26</td>
<td>4.81E-05</td>
</tr>
</tbody>
</table>

Positive and negative fold changes indicate transcripts that are upregulated and downregulated, respectively, in FVB/N embryos relative to BALB/cCrl. FC, fold change.

Table 2. Chromosome 19 transcripts differentially regulated in BALB/cCRL and FVB/N embryos with 11–14 somite pairs using RMA/ANOVA analysis

<table>
<thead>
<tr>
<th>Affy Probe Set</th>
<th>Gene Symbol</th>
<th>MOD Region</th>
<th>FC</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1434911_s_at</td>
<td>Arhgap19</td>
<td>3</td>
<td>6.12</td>
<td>2.11E-08</td>
</tr>
<tr>
<td>1444218_at</td>
<td>D19Ertd737e</td>
<td>4</td>
<td>2.04</td>
<td>5.58E-06</td>
</tr>
<tr>
<td>1422318_at</td>
<td>Foxd4</td>
<td>1</td>
<td>1.45</td>
<td>2.15E-05</td>
</tr>
<tr>
<td>1423875_at</td>
<td>Fam160b1</td>
<td>4</td>
<td>1.29</td>
<td>1.52E-04</td>
</tr>
<tr>
<td>1438258_at</td>
<td>Fam160b1</td>
<td>4</td>
<td>1.29</td>
<td>1.03E-04</td>
</tr>
<tr>
<td>1438258_at</td>
<td>Vldlr</td>
<td>1</td>
<td>1.55</td>
<td>1.02E-03</td>
</tr>
<tr>
<td>1422443_at</td>
<td>Xpnpep1</td>
<td>4</td>
<td>1.29</td>
<td>1.61E-04</td>
</tr>
<tr>
<td>1442418_at</td>
<td>D19Ertd737e</td>
<td>4</td>
<td>3.25</td>
<td>4.72E-05</td>
</tr>
<tr>
<td>1438454_at</td>
<td>Exosc1</td>
<td>3</td>
<td>2.33</td>
<td>2.25E-05</td>
</tr>
<tr>
<td>1423875_at</td>
<td>Ctxt</td>
<td>3</td>
<td>1.34</td>
<td>7.76E-04</td>
</tr>
</tbody>
</table>

Positive and negative FC indicate transcripts that are upregulated and downregulated, respectively, in FVB/N embryos relative to BALB/cCrl. WT, wild type.
The 945T insertion was shown in boldface. BALB/cCrl and FVB/N embryos with 11–14 somite pairs were analyzed by qRT-PCR. Positive and negative FC indicate transcripts that are upregulated and downregulated, respectively, in FVB/N embryos relative to BALB/cCrl. CI, confidence interval.

affected. However, 4/11 of the Exosc1 oligomer probes contained known SNPs, which may account for the fact that the apparent expression difference was not validated by qRT-PCR. It is also possible that unidentified SNPs could account for the expression differences between qRT-PCR probe sets, and therefore all qRT-PCR probe sets used in this study were sequenced for both strains and found to have no sequence differences.

Based on the validated differential gene expression, Arhgap19, Foxd4, Lipo1, Scd2, and Tmem180 are good candidates for Ceca2 modifier genes. Arhgap19, Scd2, and Tmem180 are located in region 3 of the chromosome 19 candidate interval and are of FVB/N origin in MOD 5 and 31 (Fig. 4), whereas Foxd4 and Lipo1 are in region 1 and are of FVB/N origin in MOD 4 and MOD 5. Candidate genes identified by the microarray analysis in region 4 require validation and further characterization. Strong candidates include several genes of unknown function (D19Ertd737e, 2700078E11Rik, and Fam160b1).

We focused on further characterization of Arhgap19 as a candidate modifier gene. The expression level of Arhgap19 during cranial neurulation was determined by qRT-PCR in two additional strains. Similar expression levels of Arhgap19 during cranial NT closure were found in FVB/N, 129S2, and C57BL/6 strains compared with a decreased expression in the BALB/cCrl strain (Fig. 5A), indicating that BALB/cCrl is unusual. Preliminary evidence from crossing the Ceca2G045Bic mutation from BALB/cCrl into the 129S2 and C57BL/6 backgrounds suggests that these two lines are also susceptible to exencephaly; however, differences in modifiers outside of the chromosome 19 region could also be involved.

To identify a sequence variant responsible for the expression difference between strains, we sequenced from both BALB/cCrl and FVB/N DNA ~500 bp of the putative promoter region, the 12 exons flanked by splice acceptor and donor sequences and the 3′-UTR of Arhgap19. A number of polymorphisms were found in the 3′ UTR. No polymorphisms were found within the 5′-UTR or putative promoter region. Three synonymous variants were found within the coding region of Arhgap19. There were no variants found between BALB/cCrl and FVB/N within the splice acceptor or donor sites for all 12 exons.

One single nucleotide insertion (945T) was found in exon 6 in BALB/cCrl, resulting in a frame shift of the reading frame (Fig. 5B). This frame shift results in a truncated protein due to a premature stop codon at the end of exon 6 at position 309 (K309*). The mutation is located at the COOH terminus of the RhoGAP domain (Fig. 5C). The 945T insertion was unique to BALB/cCrl in all the strains tested (A/J, FVB/N, C3H/He, 129S2, 129P2, C57BL/6, and CD1). Furthermore, this insertion was absent from BALB/cJ (Jackson Laboratories) but present in BALB/cAnNcrl mice from Charles River Laboratories, the latter being the origin...
of our colony. BALB/cJ and BALB/cCrI strains diverged before 1940 (http://www.informatics.jax.org/external/ festing/mouse/docs/BALB.shtml) and are known to differ at a variety of loci; the 945T insertion constitutes a new addition to this list.

Expression of Arhgap19 Shows Low, Ubiquitous Expression During Neurulation

To study the role of Arhgap19 further, we obtained mice with a gene-trap mutation in the second intron of the gene. The gene trap produces an ARHGAP19-β-GAL fusion protein that can be stained for expression using Xgal (Fig. 6A). Shortly after neural tube closure at E9.5, Arhgap19 expression appears to be ubiquitous, based on whole-mount embryos (Fig. 6, B and C). Thus, Arhgap19 is expressed around the time of neurulation in the neural tube, as well as other tissues, consistent with Arhgap19 being involved in neurulation. Although not yet thoroughly characterized, embryos and adults with this Arhgap19 mutation appear to be superficially normal based on a visual examination (the inner ears were not examined). Analysis of the phenotype is ongoing. Using qRT-PCR analysis of three mutant embryos compared with their normal littermates we found that the Arhgap19 transcript is decreased by 15.3 ± 1.5-fold in the mutants, making this mutation a hypomorph with a very low expression.

Fig. 6. Generation of Arhgap19 gene-trap mutant mice and Arhgap19 expression patterns. A: structure of the Arhgap19 β-geo gene-trap allele. Position of the genotyping primers are noted. B, C: Xgal staining of an E9.5 Arhgap19Gt(YHD020)Byg/+ embryo compared with a WT control littermate. Embryos are on a mixed 129P2: BALB/cCrI background.

DISCUSSION

Most mouse NTDs result from targeted single gene mutations, whereas human NTDs are considered to involve multiple gene variants as well as environmental factors. The presence of modifier genes affecting exencephaly penetrance in Cecr2 mutants makes our mouse model a better approximation of the multigene situation in humans. The chromosome 19 modifier region, which shows the largest effect on the penetrance of Cecr2Gt45Bic-associated exencephaly, is of particular interest for two reasons. First, the FVB/N background also shows changes of NTD penetrance with both Splotch and Shroom mutations (4). This suggests at least some of the underlying modifier genes may play a broad role in resistance to NTDs, although that alone does not necessarily implicate the chromosome 19 region. Second, the region of conserved synteny for this murine chromosome 19 region is human 10q25.3, a region implicated in human NTD susceptibility (22). Therefore, identifying the mouse modifier gene(s) may lead to a better understanding of human NTDs.

Past studies have shown a major difference between Cecr2Gt45Bic mutants in BALB/cCrI and FVB/N backgrounds in the penetrance of exencephaly, which was 74 and 0%, respectively (1). We have now shown that the increase in cranial neural fold width typical of the BALB/cCrI mutants (5), which is presumably symptomatic of exencephaly in these
embryos, is also seen in the FVB/N mutants, which do complete neurulation. This implies that the FVB/N modifier genes act at or after this point, allowing the neural folds to close despite being widely placed. As a result, cranial neurulation is slightly delayed but with no apparent phenotypic consequences. In the BALB/cCrl background, the Cecc2\(^{Grd45bc}\) mutation also acts on the inner ear development. This is presumably also the case in the FVB/N background, but modifier genes, whether the same or different to those in the neural tube, rescue this phenotype as well. We recently developed a more severe targeted Cecc2 mutation, Cecc2\(^{2mol.1Hemc}\), that deletes the first exon and results in a lack of full-length protein (6). This mutation shows penetrance of exencephaly at \(\sim 96\%\) for BALB/cCrl and \(31\%\) for FVB/N. Therefore, with this severe Cecc2 mutation, the FVB/N modifiers can no longer rescue the exencephaly phenotype in all mutants. It would be interesting to see whether the FVB/N Cecc2\(^{2mol.1Hemc}\) mutants show some level of inner ear stereociliary orientation defects as well.

### Identifying Candidate Modifier Genes

Gene expression profiling has been used for other mouse mutants to identify candidate modifier genes. Microarray analysis of the Hrtfm2 locus involved in the transgenic CSQ heart phenotype revealed a candidate gene, Tnni3k, with a 12-fold higher expression in the susceptible C57BL/6 and AKR/J strains compared with the resistant DBA/2J strain (28). Congenic mice containing the AKR/J Hrtfm2 region in a DBA/2J genetic background had increased expression of Tnni3k and accelerated death of CSQ transgenic. The transgenic expression of Tnni3k in a DBA/2J background was then shown to accelerate heart failure in CSQ transgenics compared with nontransgenics. A caveat of the gene profiling strategy to identify candidate modifier genes is not all genes are represented on a microarray. A microarray will also not identify a gene variant that affects function but not mRNA expression level.

To identify candidates for the Cecc2-associated exencephaly modifiers genes, we examined microarray data comparing the BALB/cCrl and FVB/N strains at neurulation. In our case, although two inbred strains will likely show many expression differences, focusing on one small region (\(\sim 30\) Mb of chromosome 19) gave us a manageable number of candidates. We determined that 90% of known genes in this region are represented on the Affymetrix 430 2.0 microarray chip. Five genes were confirmed in both microarray and qRT-PCR to show at least a 1.5-fold expression difference between the strains: Arhgap19 (Rho GTPase-activating protein 19), Foxd4 (Forkhead box D4), Lipo1 (lipase, member O1), Scd2 (stearyl-Coenzyme A desaturase 2), and Tnmel180 (Transmembrane protein 180). Transcript levels of Arhgap19 and Foxd4 were elevated in FVB/N wild-type embryos compared with BALB/c, while Lipo1, Scd2, and Tnmel180 transcript levels were lower in FVB/N compared with BALB/cCrl.

### Candidate Modifier Genes

**Arhgap19 (region 3).** We focused on Arhgap19 as a candidate modifier gene. ARHGAP19 is a GTPase-activating protein (GAP) (16), and part of a family of proteins that stimulate the intrinsic GTP hydrolysis activity of Rh proteins. These proteins act as molecular switches involved in a variety of cellular events, including gene expression, cell cycle progression, and reorganization of the actin cytoskeleton (26). Our BALB/cAnNCrl colony showed a unique stop codon in Arhgap19. Although this mutation occurs directly after the conserved RhoGAP domain, the entire protein shows high amino acid sequence conservation between homologs, suggesting a critical function for the entire open reading frame. Wild-type BALB/cCrl mice therefore have significantly decreased Arhgap19 mRNA, probably due to nonsense-mediated decay, and the remaining transcript codes for a truncated protein, but with no apparent impact on development or survival or any pathological consequences. This suggests either that the truncated protein with an intact RhoGAP domain provides sufficient function for normal phenotype or that Arhgap19 is not essential and its loss is compensated for, perhaps by redundant GAP family members. The lack of obvious phenotypes in the new Arhgap19\(^{Gt(YHD020)Byg}\) gene-trap mutant animals would suggest the latter. However, it is also possible that homozygosity for this mutant Arhgap19 does result in susceptibility to defects when combined with the loss of Cecc2. Our expression analysis indicated that Arhgap19 is expressed generally throughout E9.5 embryos, including in the forming neural tube. Changes in the actin cytoskeleton are critical during neurulation, consistent with Arhgap19 as a candidate modifier. Further functional studies to examine the effect of the Arhgap19 gene-trap mutation on the penetrance of the Cecc2 mutant phenotype are currently underway.

**Foxd4 (region 1).** Foxd4 (previously Fkh2) is a member of the “winged helix” or “forkhead” transcription factors that are defined by a common 100 amino acid DNA binding domain (reviewed by Ref. 13). It is located proximally within MOD region one and falls outside the region syntenic to human 10q25. Human FOXD4 maps to 9p24.3. Foxd4 is one of the first genes expressed in the anterior neuroectoderm, the precursor of the forebrain, and is later seen in the developing midbrain (12). A knockout mouse has not been described for Foxd4; however, studies in Xenopus indicate that Foxd4 induces the expression of early neural genes and that overexpression can cause expansion of the neural plate and delay its differentiation (24). Foxd4 shows \(\sim 2.7\)-fold increased expression in FVB/N over BALB/c as determined by qRT-PCR.

**Lipo1 (region 1).** Lipo1 (previously AI747699) is a newly discovered acid lipase found only in rats (one copy) and mice (four copies) (10). Analysis of the GeneAltas GNF1M (gcRMA) microarray (http://biogps.gnf.org) indicates that Lipo1 is strongly expressed in the adult salivary gland, making it an unlikely candidate for a modifier gene affecting neural development. However, if the expression differential between FVB/N and BALB/cCrl is also present in the adult, this could affect the nutrition of the mother. The role of Lipo1 in embryos is unknown.

**Scd2 (region 3).** This gene is the embryonic stearyl-CoA desaturase, involved in the synthesis of monosaturated fatty acids such as oleate (19). Loss of Scd2 results in neonatal lethality, the penetrance dependent on the strain. Lethality is due to abnormalities of the skin permeability resulting in severe dehydration. Interestingly, surviving homozygous adults show a curly, twisted tail, a phenotype consistent with mild neural tube defects. Shaw et al. (23) used qRT-PCR to demonstrate moderate to high expression of Scd2 in E10.5–E12.5 brain and liver.
modifier loci in the candidate region. Two loci that are not far apart would not be resolved and give a single “false” peak between them.

The chromosome 19 FVB/N modifiers are likely dominant or codominant, since they represent 30–35% of the FVB/N resistance phenotype, and the original cross between FVB/N and BALB/cCrI gave a penetrance of 2.9% (1/35) (4). The existence of two additive codominant modifier loci in the chromosome 19 candidate region represents one possible explanation of the MOD congenic line data. One modifier locus would be located in region 1, which would be of FVB/N origin in both MOD4 and MOD5 (summarized in Fig. 7). A second modifier locus would be located in region 4, of FVB/N origin in MOD31 but not in MOD4 or 5. Under this hypothesis, each of the four congenic situations tested would have two copies of FVB/N modifier genes: MOD4, MOD5, and MOD31 would each have two copies of the same allele, whereas MOD5/ MOD31 would have one copy of the FVB/N allele from region 1 and one copy of the FVB/N allele from region 4. Under genetic additivity, these situations are equivalent, except for any differences in impact between the two loci. The homozygosity of region 3 would have no effect because there would be no modifier loci in region 3. Notably, under this hypothesis, Arhgap19, Scd2, and Tmem180, which are in region 3, would not be candidate genes. However, an additive model is not supported by the results of the MOD31/ + cross, which showed that the presence of one FVB/N modifier locus gave a penetrance value that was not significantly different from two FVB/N modifier loci but was significantly different from the parental BALB/cCrI Cecr2<sup>Gr45Bic</sup> congenic mice without FVB/N modifier loci.

An alternative, genetically more complex hypothesis to explain the congenic data involves nonadditive interactions between the modifier loci. One modifier would be located in region 1 (MOD4 region) and a second modifier would be in region 3 or 4 (MOD31 region). This would mean that MOD5 could contain both FVB/N modifiers, but the penetrance drop equivalent to MOD4 or 31 would reflect the fact that the effects are not additive if both FVB/N modifiers are dominant. This implies that the two modifiers are in the same or related pathways and that Arhgap19, Scd2, and Tmem180 remain candidate genes. It is also possible that more than two modifiers may be present in the chromosome 19 candidate region. A caveat to this analysis is that differences in decreased exencephaly penetrance among the MOD lines might be detected if larger numbers of embryos were analyzed, which could reveal more subtlety in the multiple modifier interactions. However, the decrease in penetrance of all three lines shows that more than one modifier gene must exist in the chromosome 19 candidate region.

Most of the murine chromosome 19 candidate region described here is syntenic to human chromosome 10q25, the location of one of the few genetic associations to NTDs found in humans (22). The resistance phenotype in FVB/N has been associated with not only Cecr2, but also Pax3 and Shroom NTDs, including spina bifida (4), suggesting that the FVB/N modifying effect may transcend genes and NTD type. Therefore, a chromosome 19 gene that causes this effect would be an excellent candidate to be involved in NTD susceptibility in humans.

**Characterizing the Candidate Region**

We approached the search for chromosome 19 modifier genes by trying to narrow the candidate region, since it contains over 418 genes. The subinterval congenic approach subdivided the region into four subregions, but all congenic lines showed statistically the same drop in penetrance, as did the MOD5/31 cross that covered the entire region with at least heterozygous FVB/N alleles. MOD5 and MOD31 subinterval congenic lines contain the FVB/N allele of Arhgap19, while MOD4 contains the BALB/cCrI allele of this gene. These four mouse lines were 96.9% BALB/cCrI background, except for the FVB/N subinterval on chromosome 19, and thus likely contain BALB/cCrI modifier variants throughout the rest of the genome. The Cecr2 mutation on a BALB/cCrI background originally showed 74% penetrance (35/47) (1), and a recent redetermination of that value shows the penetrance of exencephaly has not changed by a significant amount (69%, 36/52). The three subinterval congenics showed penetrance values of 36.2–50%. The chromosome 19 region was originally estimated to represent 30–35% of the overall difference in penetrance between the two strains (4), which approximates the decreases seen in each line. However, the MOD5/31 cross also showed a similar decrease. No one modifier could account for these differences. The single peak of LOD scores (Fig. 7) is, however, completely compatible with the existence of two modifier loci in the candidate region. Two loci that are not far apart would not be resolved and give a single “false” peak between them.

**Fig. 7.** Graphical representation of the chromosome 19 Cecr2 modifier region and the position of the candidate modifier genes. The region between the 2 vertical lines defines the Cecr2 modifier region, where the P value is >0.001. The locations of probes are indicated by filled circles on the LOD score peak, and the location of candidate modifier genes is indicated by diamonds. The FVB/N region contained in each MOD line/cross are indicated above the graph. Probe rs3090325 (26.806 Mb) was replaced by rs31272775 from the original linkage analysis, and rs3681148 (31.481 Mb) by rs6381572. Image adapted from Davidson et al. (4).

**Tmem180.** The function of this transmembrane protein is unknown. In situ hybridization of an E14.5 embryo demonstrated on the Eurexpress website showed expression in the orbitosphenoid viscerocranium and ribs (http://www.eurexpress.org/ee/databases/assayMontage.jsp?assayID=euxassay_010032); however, the expression during neurulation is unknown. In situ hybridization of an E14.5 embryo demonstrated on the Eurexpress website showed expression in the orbitosphenoid viscerocranium and ribs (http://www.eurexpress.org/ee/databases/assayMontage.jsp?assayID=euxassay_010032); however, the expression during neurulation is unknown.
ACKNOWLEDGMENTS
We thank Lisa-Rae Chisholm-Dumesnil, Michelle Miller, Peter Thompson, Edward Weiss, and Dr. Corey Davis for technical assistance and/or helpful discussions.

Current address of M. Kooistra: Ottawa Hospital Research Institute, 725 Parkdale Ave., Ottawa, Ontario, K1Y 4E9, Canada.

GRANTS
This work was supported by the Canadian Institutes of Health Research (CIHR) Grant MOP64361 and the WCHRI (Women and Children’s Health Research Institute) Internal Grant RES00002890. M. Kooistra, R. Leduc, C. Dawe, and N. Fairbridge were supported by scholarships from the Natural Sciences and Engineering Research Council of Canada (NSERC). N. Fairbridge was also supported by a scholarship from the Alberta Heritage Foundation for Medical Research.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES