

# Genetic Background Modifies Inner Ear and Eye Phenotypes of *Jag1* Heterozygous Mice

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## ABSTRACT

Mice heterozygous for missense mutations of the Notch ligand Jagged1 (*Jag1*) exhibit head-shaking behavior indicative of an inner ear vestibular defect. In contrast, mice heterozygous for a targeted deletion of the *Jag1* gene (*Jag1<sup>del1</sup>*) do not demonstrate obvious head-shaking behavior. To determine whether the differences in inner ear phenotypes were due to the types of *Jag1* mutations or to differences in genetic background, we crossed *Jag1<sup>del1</sup>* heterozygous mice onto the same genetic background as the missense mutants. This analysis revealed that variation of the *Jag1* mutant inner ear phenotype is caused by genetic background differences and is not due to the type of *Jag1* mutation. Genome scans of N2 backcross mice identified a significant modifier locus on chromosome 7, as well as a suggestive locus on chromosome 14. We also analyzed modifiers of an eye defect in *Jag1<sup>del1</sup>* heterozygous mice from this same cross.

THE Notch signaling pathway is an evolutionarily conserved, intercellular signaling mechanism (BRAY 2006; EHEBAUER *et al.* 2006). Notch family receptors are large single-pass transmembrane proteins. Four Notch family receptors (Notch1–Notch4) have been described in mammals. Notch receptors interact with ligands that are also single-pass transmembrane proteins. In mammals, the Notch ligands are encoded by the Jagged (*Jag1* and *Jag2*) and Delta-like (*Dll1*, *Dll3*, and *Dll4*) gene families.

Notch signaling plays a critical role in the differentiation of hair cells and supporting cells in the inner ear by mediating lateral inhibition via the Dll1 and Jag2 ligands (LANFORD *et al.* 1999; DAUDET and LEWIS 2005; KIERNAN *et al.* 2005). The Notch ligand Jag1 is required at an earlier stage in inner ear development, during specification of the sensory regions of the ear (KIERNAN *et al.* 2001, 2006; BROOKER *et al.* 2006). Supporting a role for the *Jag1* gene in sensory organ development in the ear, mice heterozygous for ethylnitrosourea (ENU)-induced point mutations in the *Jag1* gene exhibit head-tossing behavior characteristic of mice with defects of the vestibular system (KIERNAN *et al.* 2001; TSAI *et al.* 2001; VRIJENS *et al.* 2006). Analysis of these mice revealed missing posterior and anterior semicircular canals and ampullae, the structures that house the sensory epithelia at the base of the semicircular canals. However, mice heterozygous for a targeted null mutation of the *Jag1* gene (*Jag1<sup>del1</sup>/+*) (XUE

*et al.* 1999) did not exhibit any head-tossing behavior. Two possible explanations for the differences of these *Jag1*-heterozygous mutant phenotypes are the differences in the type of *Jag1* mutant allele (missense *vs.* null mutation) and differences in genetic background of the *Jag1<sup>mutant</sup>/+* mice. We demonstrate here that the absence of an inner ear phenotype in *Jag1<sup>del1</sup>/+* mice is due to differences in genetic background. In addition, we show that anterior chamber eye defects in *Jag1<sup>del1</sup>/+* mice are also modified by genetic background and describe efforts to map these genetic modifiers.

## MATERIALS AND METHODS

**Mice:** Our targeted *Jag1* null allele, referred to as *Jag1<sup>dnsl</sup>* (XUE *et al.* 1999) or *Jag1<sup>del1</sup>* (KIERNAN *et al.* 2006) (Mouse Genome Informatics nomenclature: *Jag1<sup>tm1Grid</sup>*), was described previously. *Jag1<sup>del1</sup>/+* mice normally were maintained on a C57BL/6J (B6) background (N9–N10 backcross generation). For these studies, B6-*Jag1<sup>del1</sup>/+* mice were crossed to C3HeB/FeJ (C3H) mice.

**Ear and eye analyses:** Paint filling of dissected inner ears from late embryonic and neonatal mice was performed as described (KIERNAN 2006). Scanning electron microscopy was performed as described (KIERNAN *et al.* 2005, 2006). An alternative method was used to score the ear phenotype of adult *Jag1<sup>del1</sup>/+* mice for the genome scan. Since paint filling inner ears of adult mice is very difficult, inner ears from the N2 backcross generation instead were dissected out, fixed, and decalcified to view the ampullae. The inner ears were then scored for the presence or absence of the ampullae, the structures that lie at the base of each semicircular canal and house the sensory cristae. *Jag1<sup>del1</sup>* mice that were missing both anterior and posterior ampullae in at least one ear were categorized as “affected” and given a value of 1. Mice in which the

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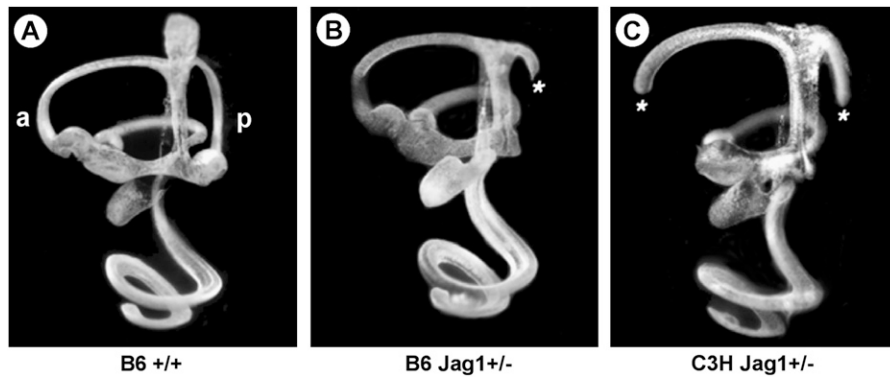


FIGURE 1.—Vestibular defects in *Jag1<sup>del1</sup>/+* inner ears. (A) An inner ear from a wild-type littermate on the B6 background that has been painted filled to display its overall morphology (a, anterior semicircular canal; p, posterior semicircular canal). (B) This B6-*Jag1<sup>del1</sup>/+* inner ear exhibits a truncation (asterisk) of the posterior semicircular canal and deletion of the ampulla at the base of this canal. (C) This C3H-*Jag1<sup>del1</sup>/+* inner ear exhibits truncations (asterisks) of both the anterior and posterior semicircular canals and deletions of their ampullae.

anterior ampullae were present in both ears were categorized as “unaffected” and given a value of 0.

Anterior eye chambers were examined and photographed by slit-lamp microscopy. For the genome scan for eye phenotypes, *Jag1<sup>del1</sup>* mice were categorized as affected if at least one eye exhibited an obvious dysmorphology (most commonly, a pupil dysmorphology).

**Statistical analysis:** To identify modifiers of the *Jag1<sup>del1</sup>/+* phenotype, *Jag1<sup>del1</sup>/+* (B6 × C3H)<sub>F1</sub> mice were crossed to C3H mice to generate N2 backcross mice, and *Jag1<sup>del1</sup>/+* [(B6 × C3H) × C3H]<sub>N2</sub> mice were assessed for ear and eye phenotypes as described above. For the genomewide scans, only two phenotypic categories, unaffected (value = 0) or affected (value = 1), were used for both the ear and eye phenotypes.

Mice were genotyped by polymerase chain reaction with simple sequence length polymorphic markers with an average spacing across the genome of 20 cM. Genome scan analysis was carried out in the R/qtl software package (BROMAN *et al.* 2003). Significance thresholds were determined on the basis of 1000 permutations of the original data (CHURCHILL and DOERGE 1994). Confidence intervals were established using the posterior density method (SEN and CHURCHILL 2001).

## RESULTS

**Effect of genetic background on head-shaking behavior of *Jag1<sup>del1</sup>/+* mice:** *Jag1<sup>del1</sup>/+* mice (XUE *et al.* 1999) were generated by gene targeting in strain 129S1/SvImJ embryonic stem cells and were then subsequently backcrossed to C57BL/6J mice for 9–10 generations. The ENU-induced *Jag1* alleles *slalom* and *headturner* (KIERNAN *et al.* 2001; TSAI *et al.* 2001; VRIJENS *et al.* 2006) were generated using two different C3H substrains (C3HeJ and C3HeB/FeJ). We investigated the effects of genetic background on the *Jag1<sup>del1</sup>/+* mutant phenotype by crossing *Jag1<sup>del1</sup>/+* mice on the C57BL/6J (B6) background (B6-*Jag1<sup>del1</sup>/+*) to C3HeB/FeJ (hereafter designated C3H) mice. *Jag1<sup>del1</sup>/+* (B6 × C3H)<sub>F1</sub> offspring did not show any head-shaking behavior. However, when the *Jag1<sup>del1</sup>/+* (B6 × C3H)<sub>F1</sub> offspring were crossed again to C3H mice (N2 backcross generation), 32% of the *Jag1<sup>del1</sup>/+* offspring (55/171) exhibited head-shaking behavior similar to that described for the ENU-induced alleles (KIERNAN *et al.* 2001; TSAI *et al.* 2001; VRIJENS *et al.* 2006).

To further investigate inner ear defects in these mice, timed matings were set up between C3H-*Jag1<sup>del1</sup>/+* males (N4 backcross generation) and C3H females. Litters (N5 generation) were taken at embryonic day (E) 15.5, and inner ears were painted filled (KIERNAN 2006) to assess their overall morphology (Figure 1). Four of eight C3H-*Jag1<sup>del1</sup>/+* inner ears demonstrated a truncation of the anterior and posterior canals, and the respective ampullae were absent (Figure 1C). Two of the C3H-*Jag1<sup>del1</sup>/+* inner ears showed a truncation in the posterior canal and missing posterior ampullae whereas the anterior canal was complete but had a smaller ampulla. The final two C3H-*Jag1<sup>del1</sup>/+* inner ears showed only a truncated posterior canal with a normal anterior canal. These types of defects were identical to those observed in mice heterozygous for ENU-induced *Jag1* missense mutations (KIERNAN *et al.* 2001; TSAI *et al.* 2001; VRIJENS *et al.* 2006). Inner ears from wild-type littermates ( $n = 9$ ) exhibited a completely normal morphology (Figure 1A).

We performed a similar study on B6-*Jag1<sup>del1</sup>/+* inner ears. Since these mice exhibited no obvious head-tossing phenotype, we were surprised to find that B6-*Jag1<sup>del1</sup>/+* inner ears did not develop normally. Eight of 12 B6-*Jag1<sup>del1</sup>/+* inner ears exhibited a truncated posterior canal and a missing ampulla (Figure 1B). The anterior canal appeared normal. Of the remaining four B6-*Jag1<sup>del1</sup>/+* inner ears, three demonstrated a complete posterior canal but an absent ampulla, while in one ear the ampulla was present but small. These results show that, in the mouse, inner ear defects can be present in the vestibular system without producing obvious behavioral defects. These results also suggest that the C3H modifiers are exacerbating a defect already present in *Jag1<sup>del1</sup>/+* mice, rather than causing a novel defect.

**Cochlear defects on C3H and B6 backgrounds:** Since the ENU-induced *Jag1* alleles also exhibited defects in cochlear patterning (KIERNAN *et al.* 2001; TSAI *et al.* 2001; VRIJENS *et al.* 2006), we examined the effect of the C3H background on development of the cochlea, which contains the sensory region responsible for auditory function. Cochleae from B6 wild-type (+/+), C3H-*Jag1<sup>del1</sup>/+* (N5 generation), and B6-*Jag1<sup>del1</sup>/+* (N9–10 generation) mice were examined by scanning electron

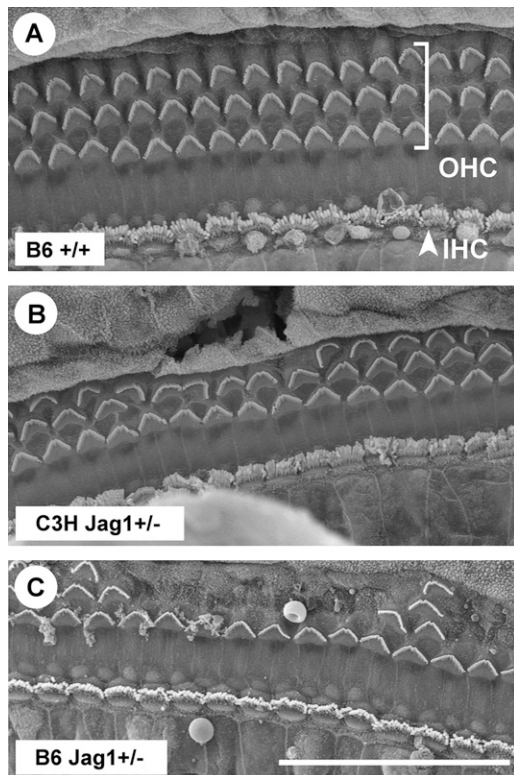


FIGURE 2.—Cochlear defects in *Jag1*<sup>del1</sup>/<sup>+</sup> inner ears: scanning electron micrographs of the sensory epithelium of the cochlea. (A) Wild-type (B6 +/+) inner ear. The wild-type cochlea has a single row of inner hair cells (IHC) and three rows of outer hair cells (OHC). (B) C3H-*Jag1*<sup>del1</sup>/<sup>+</sup> inner ear. (C) B6-*Jag1*<sup>del1</sup>/<sup>+</sup> inner ear. *Jag1*<sup>del1</sup>/<sup>+</sup> mice on both genetic backgrounds exhibited similar cochlear defects, with reduced numbers of outer hair cells and some additional inner hair cells. Bar, 50  $\mu$ m.

microscopy. The cochleae of wild-type mice contain a single row of inner hair cells and three rows of outer hair cells (Figure 2A). Previous studies from the ENU-induced *Jag1* alleles showed that the numbers of outer hair cells were reduced in the mutants and were often arranged in one or two rows rather than the normal three rows. In addition, defects were present in the inner hair cell row, with occasional extra inner hair cells and atypical cells that exhibited outer hair cell stereocilia but were present in the inner hair cell row. In the C3H-*Jag1*<sup>del1</sup>/<sup>+</sup> cochleae, a similar phenotype was observed, with reduced numbers of outer hair cells and occasional extra inner hair cells and atypical cells (Fig-

ure 2B). However, B6-*Jag1*<sup>del1</sup>/<sup>+</sup> cochleae appeared very similar (Figure 2C), indicating that the cochlear phenotype was not significantly modified on the C3H background.

**The *Jag1*<sup>del1</sup>/<sup>+</sup> eye phenotype is also modified on the C3H background:** We had shown previously that *Jag1*<sup>del1</sup>/<sup>+</sup> mice displayed eye defects and that genetic background could affect the penetrance of these defects (XUE *et al.* 1999). The most consistently observed eye defect in B6-*Jag1*<sup>del1</sup>/<sup>+</sup> mice is abnormalities in the shape and location of the pupil (Figure 3, A and B). These pupil dysmorphologies affect at least one eye and often occur in both eyes. Less frequently, the B6-*Jag1*<sup>del1</sup>/<sup>+</sup> mice develop corneal opacity, and rarely the mice show small or underdeveloped eyes (microphthalmia). When B6-*Jag1*<sup>del1</sup>/<sup>+</sup> mice were crossed to the C3H background, the penetrance of observed eye defects decreased (Figure 3C and Table 1). This effect became stronger as the relative percentage of the C3H genome increased. For example, in the F<sub>1</sub> generation the frequency of pupil dysmorphologies (for at least one eye) dropped from 100 to  $\sim$ 50%. In the N<sub>2</sub> backcross generation, the frequency of this eye defect dropped to  $\sim$ 30% (Table 1). These data show that although the C3H background enhances inner ear defects of *Jag1*<sup>del1</sup>/<sup>+</sup> mice, it suppresses eye defects of these mice. However, in the N<sub>2</sub> generation there was no correlation between the *Jag1*<sup>del1</sup>/<sup>+</sup> mice whose inner ears were affected and those that exhibited suppression of the eye phenotype, suggesting that the genetic modifiers for the ear and eye phenotypes were likely to be independent.

**Backcross mapping of modifier loci:** To localize modifier loci underlying the variations of the inner ear defects in N<sub>2</sub> *Jag1*<sup>del1</sup>/<sup>+</sup> mice, a phenotypic assessment of their inner ears was performed. In addition to a behavioral assessment (which consisted of a yes/no assessment of head-shaking behavior), we assessed truncated semicircular canals and missing ampullae. We found a strong correlation between the number of missing cristae and whether the mouse exhibited head-shaking behavior, indicating that the semicircular canal defects led to the head-shaking phenotype.

For the genome scan of the ear phenotype, mice that were missing both anterior and posterior ampullae in at least one ear were categorized as affected. Mice in which the anterior ampullae were present in both ears were categorized as unaffected. Ninety mice (49 affected and 41 unaffected) from the N<sub>2</sub> backcross generation were

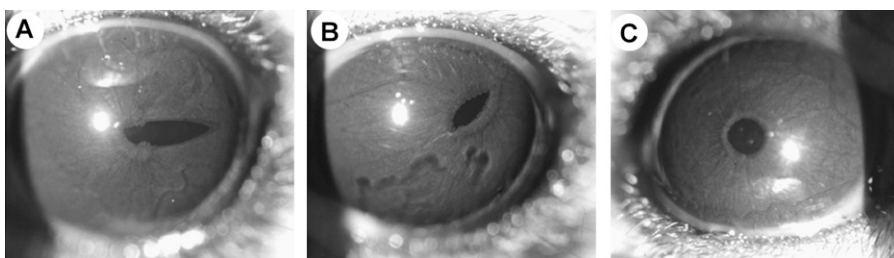


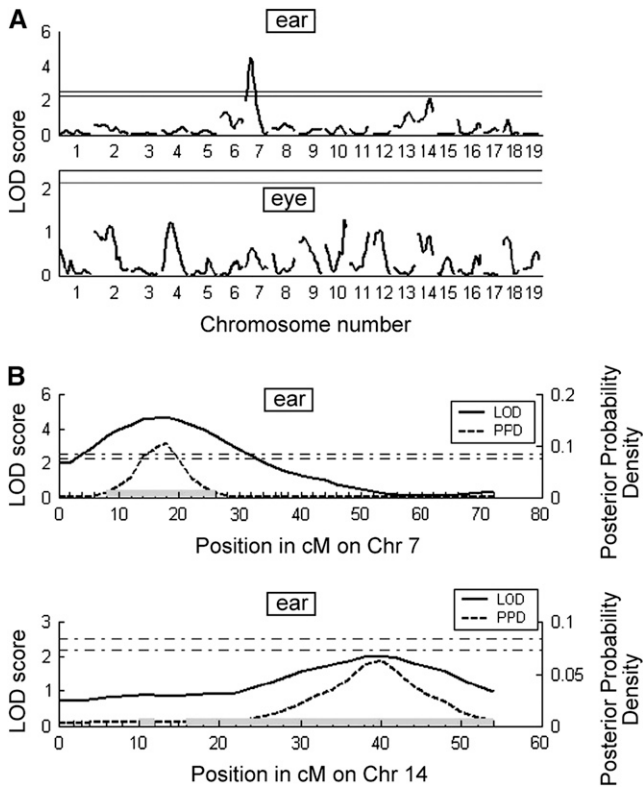
FIGURE 3.—Eye dysmorphologies of *Jag1*<sup>del1</sup>/<sup>+</sup> backcross mice. All mice shown are *Jag1*<sup>del1</sup>/<sup>+</sup> [(B6  $\times$  C3H)  $\times$  C3H]N<sub>2</sub> mice. (A and B) These eyes exhibit the dysmorphology of the pupil typical of B6-*Jag1*<sup>del1</sup>/<sup>+</sup> mice. (C) This eye exhibits normal morphology, with a centrally located, round pupil.

**TABLE 1**  
**Eye dysmorphologies of *Jag1<sup>del1</sup>* mice**

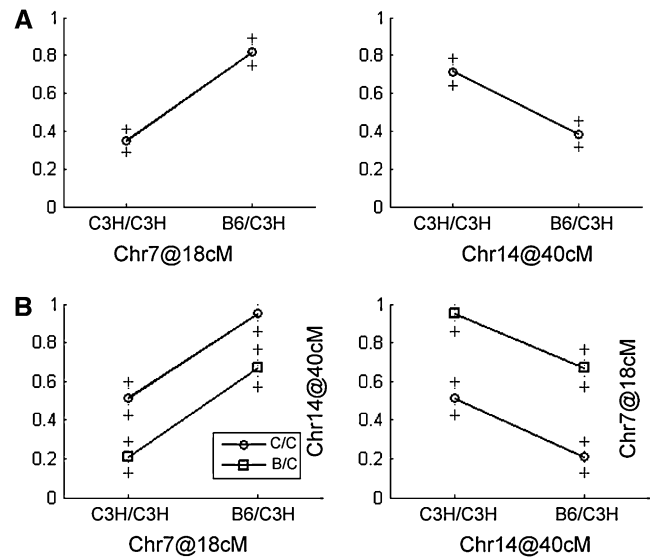
Background	<i>n</i>	Both eyes affected (%)	One eye affected (%)	Neither eye affected (%)
B6	15	5 (33)	10 (67)	0 (0)
(B6 × C3H)F <sub>1</sub>	8	1 (13)	3 (38)	4 (50)
[(B6 × C3H) × C3H]N2	100	5 (5)	28 (28)	67 (68)

typed using microsatellite markers with an average spacing of ~20 cM. The eye phenotype was also assessed in these animals and included 34 affected (in which at least one eye exhibited a pupil defect) and 56 unaffected (no eye defects). These analyses identified a significant modifier for the ear dysmorphology on chromosome (Chr) 7, as well as a suggestive modifier on Chr 14 (Figure 4A). These loci were considered modifiers, rather than quan-

titative trait loci, because the phenotypic effects were assessed as a binary (0 or 1) trait. No significant modifiers for the eye dysmorphology were identified. The maximum LOD score for the Chr 7 ear dysmorphology modifier was 4.6. This modifier is centered at 16 cM on Chr 7, with 95% confidence limits spanning from 8 to 26 cM (Figure 4B). Surprisingly, the Chr 7 modifier was positively correlated with B6/C3H heterozygosity in this region (Figure 5A). However, this is likely counteracted by the Chr 14 modifier (and potentially other modifiers) that is positively correlated with C3H/C3H homozygosity (Figure 5A). The Chr 7 and Chr 14 modifiers do not interact with each other, but have a joint additive effect on the ear phenotype (Figure 5B).



**FIGURE 4.**—Modifier analysis of *Jag1<sup>del1</sup>/+* backcross mice. (A) Genomewide scan of modifiers of the ear and eye phenotypes in *Jag1<sup>del1</sup>/+* [(B6 × C3H) × C3H]N2 mice. A significant ear modifier was detected on Chr 7, and a suggestive ear modifier was detected on Chr 14. No significant or suggestive individual modifiers were identified for the eye phenotype. (B) Interval map of the Chr 7 and Chr 14 ear modifiers. For each scan, the posterior probability density shown is a likelihood statistic that gives the 95% confidence interval (indicated by the shaded bar). The top and bottom horizontal lines in the scans represent significant ( $P < 0.05$ ) and suggestive ( $P < 0.01$ ) LOD thresholds (2.5 and 2.2), respectively, as determined by permutation testing.



**FIGURE 5.**—Allele effect and interaction plots for ear modifiers. (A) Allele effect plots for the Chr 7 and Chr 14 ear modifiers. Plotted on the y-axis are the mean values for the ear phenotype measured as a binary trait (unaffected = 0; affected = 1) for mice with the indicated genotype at the Chr 7 and Chr 14 modifier loci. Crosses denote the standard error. The Chr 7 modifier is positively correlated with B6/C3H heterozygosity, while the Chr 14 modifier is positively correlated with C3H/C3H homozygosity. (B) Interaction plots for the Chr 7 and Chr 14 ear modifiers. Mean ear phenotype values are plotted on the y-axis. The parallel lines indicate that the Chr 7 and Chr 14 modifiers do not interact, but have a joint additive effect on the ear phenotype. Inset symbols: C/C, C3H/C3H; B/C, B6/C3H.

## DISCUSSION

In this work, we have demonstrated that the behavioral differences (*i.e.*, the head-tossing phenotype) between the *Jag1* missense mutants and the *Jag1* targeted null mutant are due to more extensive semicircular canal defects in the vestibular system. Moreover, the more severe vestibular defects are due to differences in genetic background and not to differences in the nature of the *Jag1* mutations. *Jag1<sup>mutant</sup>/+* mice, whether bearing missense or null *Jag1* mutant alleles, exhibit more severe semicircular canal defects on a C3H genetic background than on a B6 background. We and others have shown that, during inner ear development, the *Jag1* gene is required for specification of the prosensory regions, the regions competent to differentiate into both sensory hair cells and nonsensory support cells. Conditional homozygous deletion of *Jag1* gene function using the *Foxg1-Cre* line results in more severe truncations of the semicircular canals and their ampullae than the truncations present in *Jag1<sup>mutant</sup>/+* heterozygotes (BROOKER *et al.* 2006; KIERNAN *et al.* 2006). It is interesting that, while the vestibular phenotype of *Jag1<sup>mutant</sup>/+* heterozygotes was modified by genetic background, the cochlear phenotype was not. This may suggest either that there are different cofactors involved in sensory specification in the cochlea and vestibule or that there may be different threshold requirements for *Jag1* function during specification and differentiation of these sensory epithelia.

The eye dysmorphologies exhibited by *Jag1* mutants are also modified by genetic background, and these modifiers are independent of the ear phenotype modifiers. Interestingly, the eye modifiers act in an opposite manner to the ear modifiers, in that the C3H background suppresses the eye dysmorphologies exhibited by *Jag1<sup>mutant</sup>/+* heterozygotes on a B6 background. Only one modifier with a statistically significant effect on the ear phenotype was identified in this analysis. No statistically significant modifier was identified for the eye phenotype. This indicates that, for both of these traits, multiple modifiers with small effects act in a combinatorial fashion to influence the phenotypic outcome.

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