Over-expression of \textit{Grhl2} causes spina bifida in the \textit{Axial defects} mutant mouse

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Received December 14, 2010; Revised and Accepted January 19, 2011

Crani al neural tube defects (NTDs) occur in mice carrying mutant alleles of many different genes, whereas isolated spinal NTDs (spina bifida) occur in fewer models, despite being common human birth defects. Spina bifida occurs at high frequency in the \textit{Axial defects} (\textit{Axd}) mouse mutant but the causative gene is not known. In the current study, the \textit{Axd} mutation was mapped by linkage analysis. Within the critical genomic region, sequencing did not reveal a coding mutation whereas expression analysis demonstrated significant up-regulation of \textit{grainyhead-like 2} (\textit{Grhl2}) in \textit{Axd} mutant embryos. Expression of other candidate genes did not differ between genotypes. In order to test the hypothesis that over-expression of \textit{Grhl2} causes \textit{Axd} NTDs, we performed a genetic cross to reduce \textit{Grhl2} function in \textit{Axd} heterozygotes. \textit{Grhl2} loss of function mutant mice were generated and displayed both cranial and spinal NTDs. Compound heterozygotes carrying both loss (\textit{Grhl2} null) and putative gain of function (\textit{Axd}) alleles exhibited normalization of spinal neural tube closure compared with \textit{Axd}/+ littermates, which exhibit delayed closure. \textit{Grhl2} is expressed in the surface ectoderm and hindgut endoderm in the spinal region, overlapping with \textit{grainyhead-like 3} (\textit{Grhl3}). \textit{Axd} mutants display delayed eyelid closure, as reported in \textit{Grhl3} null embryos. Moreover, \textit{Axd} mutant embryos exhibited increased ventral curvature of the spinal region and reduced proliferation in the hindgut, reminiscent of \textit{curly tail} embryos, which carry a hypomorphic allele of \textit{Grhl3}. Overall, our data suggest that defects in \textit{Axd} mutant embryos result from over-expression of \textit{Grhl2}.

INTRODUCTION

Formation of the neural tube in higher vertebrates depends on elevation, folding, apposition and fusion of the neural folds, a process known as primary neurulation (1,2). Failure of this process results in open neural tube defects (NTDs), which are among the most common congenital malformations in humans (3). Although there is strong evidence for a genetic component in NTDs, identification of the causative genes is complicated by the fact that most cases appear to involve a combination of genetic and environmental factors (4,5). The large number of mouse genetic models in which NTDs arise as part of the phenotype emphasize the possible genetic complexity of these defects and provide insight into the multiple molecular requirements for neural tube closure (3,6,7).

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In humans, cranial NTDs (anencephaly) and caudal NTDs (spina bifida) occur at approximately equal frequency (8,9). By comparison, a survey of the various NTD mutants in mice reveals a striking predominance of models in which cranial NTDs occur. Thus, exencephaly occurs in isolation (~75% of mutants) or in combination with spina bifida (~20% of mutants) in most models (7). In contrast, isolated spina bifida is observed in only ~5% of the roughly 250 mouse NTD mutants. Among NTDs induced by various teratogens, exencephaly is also induced more frequently than spina bifida (10). Thus, cranial neural tube closure appears exquisitely sensitive to disruption in mice. It may be that cranial neural tube closure in the mouse embryo is more complex than in humans, requiring coordinated function of many gene products. Hence, there is a relative paucity of mouse models for isolated spinal NTDs, despite the importance of these malformations in humans.

The Axial defects (Axd) mutation arose spontaneously in the 1980s and was recognized on the basis of a curled tail phenotype inherited as a semi-dominant Mendelian trait (11). The penetrance of tail defects among Axd/+ mice varies with genetic background, being highest on the BALB/c background with ~50–60% of mice affected (12). Intercrosses of heterozygotes produced litters containing neonates with spina bifida, presumed to be homozygous mutants (11). At embryonic day (E) 12.5, ~25% of embryos showed spina bifida, suggesting that spinal NTDs are fully penetrant among homozygotes at this stage (12). Cranial NTDs were not observed. Among neonates, there was a lower incidence of curled tails and spina bifida than expected. This was suggested to indicate possible correction of tail defects and/or soft tissue healing over open lesions during late gestation (11,12). However, inability to genotype litters precluded further analysis of this possibility.

In the current study, we mapped the Axd mutation to a region of chromosome 15 containing the grainyhead-like 2 (Grhl2) gene, which encodes a transcription factor. We found that loss of function of Grhl2 causes cranial and spinal NTDs, as previously observed for Grhl3 mutants (13–15). Expression analysis in Axd mutant embryos showed over-expression of Grhl2, whereas reduction of Grhl2 expression levels resulted in normalization of spinal neural tube closure. We conclude that both insufficient and excessive expression of Grhl2 can cause NTDs.

RESULTS

Axial defects mutant embryos display severe spina bifida

The phenotype of embryos among litters from Axd/+ intercrosses was examined by scanning electron microscopy (70 embryos) and light microscopy (>300 embryos), at developmental stages during and after the stage of spinal neural tube closure (E9.5–10.5 and later). Initial studies were performed prior to linkage analysis, with the prediction that embryos exhibiting failure of neural tube closure would correspond to homozygous mutant embryos. These phenotype–genotype correlations were confirmed following mapping of the Axd mutation (see what follows). Abnormalities in spinal neural tube closure could be detected from around the 24 somite stage (E9.5), when homozygous Axd/Axd embryos exhibited an enlarged posterior neuropore (PNP) compared with wild-type littermates (Fig. 1A and B). The PNP (the final region of open spinal neural folds) is very small or closed by the 31–32 somite stage (E10.5) in wild-type embryos (Figs 1C and E, and 2C), whereas homozygous mutants display very large open PNP s, of up to 3 mm in length (Figs 1D and F, and 2C). The rostral limit of the open PNP was at the level of somites 22–26, indicating that closure had failed to progress beyond this axial level.

Examination of embryos at more advanced somite stages, at E10.5 and E11.5, showed that the PNP of Axd mutants did not reduce in size but remained persistently open. This defect corresponds to spina bifida, which can be readily visualized in affected fetuses as a typical protrusion of tissue from the open lesion at E16.5 (Fig. 1G). Transverse sections at the level of the lesion reveal the degeneration of spinal cord tissue (Supplementary Material, Fig. S1C and D). Damage appears most severe at the sacral level where none of the typical architecture of the neural tube can be discerned, whereas motor neurons are still detectable in the lumbar region (Supplementary Material, Fig. S1A and B). By birth, the exposed nervous tissue has degenerated and the open vertebral arches are exposed at the dorsal surface (Supplementary Material, Fig. S1E). Some fetuses with spina bifida at E16.5 and 17.5 exhibit a ‘remnant’ of the tail which we hypothesize to result from apparent degeneration of the tail (Supplementary Material, Fig. S2D).

In the majority of heterozygous Axd/+ embryos, closure of the PNP was complete by the 35 somite stage or soon after (Fig. 2C). This reflects a delay in closure compared with wild-type embryos, apparent as a higher mean PNP length in heterozygotes than wild-type littermates at the 26–28, 29–31 and 32–34 somite stages (P < 0.001). Delayed PNP closure manifests at later stages as a tail flexion defect or ‘curly tail’ in ~40–50% of Axd/+ mice on the BALB/c background.

The Axd mutation maps to proximal chromosome 15

In order to map the mutation underlying the Axd phenotype, a congenic strain was first generated on the BALB/c genetic background, selected for high penetrance of the dominant (heterozygous) phenotype (11). In the recombinant line, 97% of alleles tested were homozygous BALB/c.

In order to map the Axd mutation, a cross was performed between Axd males (on BALB/c background) and C57BL/6 females. AFFECTED HETEROZYGOSITY ON THE BALB/c GENETIC BACKGROUND: RECOMBINANT INBREEDING. The rationale of this approach was that the Axd mutation would remain associated with the BALB/c genetic background, and not be replaced by C57BL/6 genomic DNA in affected individuals. Details of the linkage strategy are provided in the Supplementary Material. In summary, genomic DNA from 105 microsatellite markers that were polymorphic between BALB/c and C57BL/6 and evenly spread over the autosomes (~30 cM intervals) were used. The involvement of the sex chromosomes had previously been excluded (11). In the absence of linkage to a genomic locus, a 1:1 ratio of BALB/c and C57BL/6 alleles would be expected in embryos with spina bifida. Only chromosome 15 showed a divergence from the 1:1
This apparent association between an Axdl locus on chromosome 15 and the spina bifida phenotype was further investigated by individual analysis of affected embryos. The Axdl critical region was refined using 40 additional informative markers (of 100 tested), which revealed a peak of BALB/c and Axdl alleles (non-C57BL/6) between D15Mit252 and D15Mit171 (22.6–79.4 Mb). Within this region, a peak of Axdl alleles (non-BALB/c or C57BL/6) was present at 38.5–43.6 Mb. Using additional novel polymorphic markers, a critical region of 1.1 Mb was defined between markers CA15–16 and CA15–05 (36.4–37.5 Mb). This region contains 10 known genes (black boxes), as well as 3 RNA genes and various putative processed transcripts (as of Ensembl release 60). The marker D15Mit250 showed 100% association of homozygosity for Axdl alleles with spina bifida. In embryos that were genotyped using D15Mit250, the PNP length was compared at stages during and after spinal neural tube closure. The PNP fails to shorten and close in homozygous Axdl/Axdl embryos (black circles), leading to spina bifida. In contrast, most wild-type embryos exhibit closed PNPs by the 29–31 somite stage (white circles) and Axdl/+ embryos complete closure by the 35–37 somite stage in most cases (grey circles).

Figure 1. Failure of PNP closure in Axdl mutant embryos. (A, C and E) Scanning electron microscopy of wild-type embryos reveals that PNP closure has progressed beyond the last formed somite and is at the level of the hindlimb bud at the 24 somite stage (arrow in A); by the 31 somite stage (C and E), the PNP has reduced in size to a small slit (arrow in E). (B, D and F) In contrast, Axdl mutant embryos exhibit dramatically enlarged PNPs at both 26 and 31 somite stages (B and D, respectively), in which closure does not progress beyond the level of somite 24 (arrow in B). At the 26 somite stage (B), the neural folds of the mutant embryo appear elevated, although closure has not progressed. By the 31 somite stage, the neural folds are splayed wide apart (D and F). (G and H) In an Axdl mutant fetus at E16.5, open spina bifida is evident (arrow in G) affecting the low thoracic, lumbar and sacral regions. Transverse sections at the levels indicated by dashed lines in (G) are included in Supplementary Material, Fig. S1. Scale bars: 500 μm (A–D), 100 μm (E), 200 μm (F), 2 mm (G) and 1 mm (H).

Figure 2. The Axdl mutation maps to a critical region on mouse chromosome 15. (A) In DNA from embryos with spina bifida from an intercross of Axdl and C57BL/6 mice, typing of polymorphic markers showed a peak of BALB/c and Axdl alleles (non-C57BL/6) between D15Mit252 and D15Mit171 (22.6–79.4 Mb). Within this region, a peak of Axdl alleles (non-BALB/c or C57BL/6) was present at 38.5–43.6 Mb. (B) Using additional novel polymorphic markers, a critical region of 1.1 Mb was defined between markers CA15–16 and CA15–05 (36.4–37.5 Mb). This region contains 10 known genes (black boxes), as well as 3 RNA genes and various putative processed transcripts (as of Ensembl release 60). The marker D15Mit250 showed 100% association of homozygosity for Axdl alleles with spina bifida. (C) In embryos that were genotyped using D15Mit250, the PNP length was compared at stages during and after spinal neural tube closure. The PNP fails to shorten and close in homozygous Axdl/Axdl embryos (black circles), leading to spina bifida. In contrast, most wild-type embryos exhibit closed PNPs by the 29–31 somite stage (white circles) and Axdl/+ embryos complete closure by the 35–37 somite stage in most cases (grey circles).
c and Axd alleles (i.e. absence of C57BL/6 alleles) between D15Mit252 and D15Mit71 (22.6–79.4 Mb). Within a narrower interval (38.5–43.6 Mb), 11 informative markers could discriminate the Axd alleles from BALB/c and C57BL/6, confirming association (Fig. 2A). High-resolution mapping, using a combination of additional D15Mit markers, novel polymorphic DNA markers based on nucleotide repeat sequences and an additional cross to C57BL/6 (generating a second recombinant line), further narrowed the critical region to an interval of 1.1 Mb (36.4–37.5 Mb) flanked by heterozygous sequences (Fig. 2B). A 100% association was observed between homozygous Axd alleles for D15Mit250 (at 36.7 cM) and the presence of a large PNP (n > 150 embryos) in embryos at E10.5 (Fig. 2C). Therefore, in subsequent experiments, this marker was used to genotype embryos from heterozygous Axd intercrosses on the BALB/c background.

Grhl2 is over-expressed in Axd mutant embryos

The Axd critical region harbours 10 protein-coding genes (Fig. 2B) and three RNA genes (AC137871.1, AC114008.1 and AC121814.1) encoding micro-RNA or non-coding RNA, as well as three loci encoding putative processed transcripts (as at Ensembl release 60). The corresponding human syntenic region is at chromosome 8q22. The coding regions and intron–exon boundaries of Ankrd46, Pabpc1, Ywhaz, Zfp706, Grhl2 and Ncald were sequenced and no mutations were detected (data not shown). In order to investigate the possibility that a regulatory mutation might result in abnormal expression of one of these Axd candidate genes, expression levels were analysed by quantitative real-time RT–PCR (qRT–PCR). A series of embryos at stages during and immediately after spinal neurulation were selected that either displayed a large PNP or spina bifida and genotyped as Axd/Axd (n = 14) or had a normal or closed PNP and typed as wild-type (n = 9). The expression of Grhl2 showed marked up-regulation in Axd/Axd embryos, with an overall expression elevation of ~2.5-fold (P < 0.001, Mann–Whitney rank sum test) and a peak at the 30–35 somite stage, when up to 5-fold increase in expression was observed (Fig. 3A). None of the other five candidate genes analysed showed a significant difference in...
expression between mutant and wild-type embryos (Supplementary Material, Fig. S3).

We further analysed mRNA levels in the isolated caudal regions of a separate series of 30–31 somite stage embryos to focus on the embryonic region undergoing spinal neurulation (Fig. 3B). This analysis showed that Grhl2 expression is significantly elevated, by ≏4-fold, in homozygous mutants, while heterozygotes exhibit a level intermediate between +/− and Axd/Axd (Fig. 3B). In contrast, expression of the other genes in the critical region did not vary with genotype. Together, these studies demonstrate that up-regulation of Grhl2 correlates with failure of PNP closure in Axd/Axd embryos. Indeed, this can be visualized by plotting the PNP length against relative expression of Grhl2 which demonstrates an approximately linear relationship over at least a 5-fold range of Grhl2 levels (Supplementary Material, Fig. S4). Whole-mount in situ hybridization for Grhl2 showed markedly more intense staining in Axd/Axd embryos compared with wild-type or heterozygous littermates (Fig. 3C–F), including in the pharyngeal region, otic vesicles, forebrain and hindgut. Therefore, Axd/Axd embryos appear to exhibit an up-regulation of Grhl2 at all sites of expression.

Grhl2 has close homology and shares a consensus-binding sequence with Grhl3 (16,17), whose loss of function following gene targeting or down-regulation in the curly tail mutant results in spina bifida (13–15). Delayed eyelid closure has been noted in Grhl3 mutant mice, with closure not completed in any Grhl3 null fetuses by E16.5 compared with 100% closure in wild-type (18). Similarly, we noted lack of eyelid closure at E16.5 in Axd/Axd mutants (seven out of seven; Fig. 1G and Supplementary Material Fig. S2), whereas closure was complete in all wild-type and heterozygous fetuses examined (0 out of 11 with open eyelids).

Generation of a loss of function allele of Grhl2

We hypothesized that Axd represents a hypermorphic (overexpressing) allele of Grhl2 based on linkage analysis, up-regulation of expression at the stage of spinal neurulation and phenotypic similarities to Grhl3 mutants. We reasoned that if excess expression of Grhl2 is responsible for NTDs in the Axd model, then reduction of expression should normalize spinal neurulation. In order to test this idea genetically, we generated a loss of function allele, using a gene-trap of Grhl2 (Sanger Institute Gene Trap Resource, cell line AC0205). This allele carries the pGT0Ixr trap vector in intron 14 (Fig. 4A). The Grhl2GT allele, therefore, generates a fusion tran-

Figure 4. A gene-trap allele of Grhl2 results in NTDs. (A) The gene-trap vector is inserted in intron 14 of the Grhl2 gene. Schematics of wild-type allele (top) and β-geo-containing gene-trap allele (bottom). F1, F2, R1, R2: PCR primers. Grey bars: exons. (B) Efficacy of gene-trapping was confirmed by RT–PCR using cDNA generated from Grhl2GT/+ (lanes 1–4) and +/+ (lanes 6–9) embryos (lanes 5 and 10 are no RT controls). Primer pairs that span exons 13–16 (F1 and R2; lanes 4 and 9) or exons 14–16 (F2 and R2; lanes 2 and 7) amplify the wild-type transcript from both +/+ and Grhl2GT/+ embryos, whereas the trapped allele (amplified by primer F1 or F2 with R1) was detected only in Grhl2GT/+ samples (lanes 1 and 3).

(C–F) X-Gal staining of Grhl2GT/+ embryos on the C57BL background, shown in side view (C and D), ventral view (E) and sectioned at the level of the dashed line in (C) (F). Efficacy of gene-trapping is confirmed, as is the Grhl2 expression pattern which closely resembles that observed by whole-mount in situ hybridization (Fig. 3). Note expression in hindgut (arrows in C and D), branchial arches (ba), nasal pit (np) and Rathke’s pouch (white arrowhead in E), otic vesicles (ov), foregut (arrows) and ectoderm lining the limb buds (arrowheads in F). In some heterozygous embryos on the C57BL background, we observed exencephaly with ‘split face’ (EX in D). (G and H) On the BALB/c background, exencephaly with split face was observed together with spina bifida (SB: neural folds open to the level of arrowhead in H) in homozygous Grhl2GT/GT embryos (H), whereas heterozygotes were usually normal (G). Scale bars: 1 mm in all panels, except (F) (0.5 mm).
script lacking exons 15 and 16, which encode the 59 C-terminal amino acids of the Grhl2 protein (full-length protein is 625 amino acids). The location of the gene-trap vector was determined by PCR using combinations of intronic and vector primers. Sequencing of PCR products showed that the insertion site was at 6611 bp of the 10 515 bp intron 14. Efficacy of gene-trapping was confirmed by RT–PCR and X-Gal staining of embryonic stem cell colonies (not shown) and heterozygous embryos (Fig. 4), exploiting the expression of β-gal from the trapped allele. Staining was evident in several ectodermal locations, mimicking the Grhl2 expression pattern obtained by in situ hybridization. Sites included the surface ectoderm lining the branchial arches and limb buds, oral ectoderm, nasal pits, otic vesicles and Rathke’s pouch (Fig. 4C–F; Supplementary Material, Fig. S5).

Among heterozygous Grhl2GT/+ embryos analysed at E9.5–10.5, we observed a low frequency of cranial NTDs (15%; 11 out of 75 embryos) that was not observed among wild-type littermates. The cranial NTD phenotype involves failure of closure of the neural folds in the forebrain (‘split face’) and throughout the midbrain (Fig. 4D). In initial experiments, we did not recover homozygous embryos at stages later than E9.5 from intercrosses of heterozygotes, on a mixed 129/Sv and C57Bl/6 background. However, once the Grhl2GT allele was backcrossed onto a BALB/c background for two generations (prior to intercross to Axd, see below), homozygous mutant (Grhl2GT/Grhl2GT) embryos could be recovered at E10.5 and these embryos exhibited not only severe exencephaly with ‘split face’ but also spina bifida (Fig. 4H). Cranial NTDs occurred with 100% penetrance (17 out of 17 homozygotes at E10.5–11.5), and spina bifida occurred in 88% of homozygotes. These findings suggest a key requirement for Grhl2 in neural tube closure at both rostral and caudal levels of the body axis. The NTD phenotypes that we observed in Grhl2GT mutant embryos resemble those recently reported in Grhl2 knock-out mice (17), suggesting that the Grhl2GT allele corresponds to a loss of function.

Normalization of PNP closure in Axd embryos by loss of Grhl2 function

In order to test the hypothesis that over-expression of Grhl2 causes spinal NTDs in Axd mutant mice, we crossed Axd/+ and Grhl2GT/+ mice to generate Axd/Grhl2GT compound heterozygotes. To provide a quantitative measure of the degree to which spinal neurulation is affected by genotype, the length of the PNP was analysed with respect to developmental stage (Fig. 5A). Spinal neurulation was complete, signified by complete closure of the PNP, in the majority of +/- and Grhl2GT/+ embryos by the 31 somite stage. In contrast, the mean PNP length in Axd/+ embryos was significantly larger than in wild-type, with closure not occurring until after the 34 somite stage. The delayed closure of the PNP is likely responsible for the tail flexion defects observed in heterozygous animals. Among Axd/Grhl2GT embryos, there was an overall significant reduction in the mean PNP length compared with Axd/+ embryos at the 30–34 somite stages (Fig. 5A). Thus, although the presence of the Grhl2GT allele did not rescue the Axd heterozygous phenotype in every embryo, there was a striking shift towards smaller PNP lengths compared with stage-matched Axd/+ embryos. Moreover, 6 out of 35 Axd/Grhl2GT embryos had...
Over-expression of Grhl2 in the hindgut may contribute to increased curvature and altered proliferation in the caudal region

The Grhl2 expression pattern was determined both by whole-mount in situ hybridization (Fig. 3) and by X-Gal staining (Fig. 4). In the caudal region, the site of spinal neurulation, expression was not detected in the neuroepithelium but intense staining was evident ventral to the neural tube. Transverse sections through the caudal region confirmed that this tissue corresponds to the hindgut (Figs 3E and F, and 6A–C). Detection of Grhl2 in the hindgut at E10.5, but apparently not in the neural folds, is reminiscent of Grhl3 expression (15) and suggests that failure of neural tube closure in Axd mutant embryos results from a defect in a non-neural tissue. In curly tail (ct/ct) mice, reduced expression of Grhl3 in the hindgut results in diminished cellular proliferation and consequently increased ventral curvature that mechanically opposes PNP closure (19,20). The possibility that a similar mechanism might contribute to spinal NTDs in Axd mice was raised by the fact that embryos with a large open PNP at E10.5, subsequently genotyped as Axd/Axd, were frequently noted to exhibit abnormal ventral curvature of the caudal region (Fig. 6D–E). Measurements of the caudal region in a series of embryos at E10.5 showed that the angle of curvature of the caudal region was indeed significantly greater in Axd homozygous mutant embryos than in wild-type or heterozygous littersmates (Fig. 6F).

We next investigated whether the increased ventral curvature of Axd/Axd embryos could result from a proliferation imbalance in the caudal region, as in curly tail mutants. Immunostaining for phospho-histone H3 on transverse sections through the caudal region revealed a significantly lower mitotic index in the hindgut endoderm of Axd/Axd compared with wild-type embryos at the 23–28 and 31–33 somite stages (Fig. 6G and H). A small but significant difference in the mitotic index was also noted in the neural folds (Fig. 6G) at the earlier stage but not at the 31–33 somite stage, the stage at which spinal neurulation should be approaching completion (Fig. 6H).

Since spinal NTDs occur in association with altered expression of Grhl genes in both curly tail and Axd mutants and have phenotypic similarities, we tested whether the ct and Axd mutations interact genetically. Homozygous ct/ct mice were crossed with Axd/+ heterozygotes and the
offspring examined for tail flexion defects. We observed a 2.5-fold increased frequency of curled tails among $ct^+/Axd^+\times ct^+/Axd^+$ mice compared with $ct^++/Axd^+$ (two-tailed Fisher’s exact probability test), suggesting that the $Axd$ and $ct$ mutations can genetically interact, leading to delay of spinal neural tube closure.

**DISCUSSION**

The relative paucity of mouse models for isolated spina bifida presents a challenge in identifying candidate genes for the corresponding defects in humans. A parallel hypothesis could be that the genetic aetiology of human spina bifida will prove less complex than anencephaly, in which many more genes are potentially implicated from mouse models.

In the current study, we provide evidence that over-expression of $Grhl2$ is the cause of spina bifida in $Axd$ mutant mice. Although the $Axd$ mutation has not yet been characterized, several lines of evidence support a primary role for $Grhl2$ in the $Axd$ phenotype. First, $Grhl2$ is located in the $Axd$ critical region and exhibits misregulated expression in association with failure of spinal neurulation. Second, $Grhl2$ is over-expressed in the surface ectoderm and hindgut of $Axd^+/Axd^+$ embryos, both tissues that have been functionally implicated in NTDs in other models (3). Third, we find that loss of function of $Grhl2$, as well as $Grhl3$, causes NTDs, implicating this gene family in neural tube closure (17,21,22). Fourth, $Axd$ mutants show eyelid fusion defects reminiscent of those observed in $Grhl3$ mutants (18). Finally, delayed closure of the PNP in embryos that are heterozygous for the $Axd$ allele can be ameliorated by reduction of $Grhl2$ function, following replacement of the wild-type allele with a $Grhl2^{ct}$ allele.

As we could not identify a coding region mutation in $Grhl2$ in $Axd$ mutants, we carried out additional sequencing of the 5′ and 3′ UTRs including 1.6 kb upstream of the start codon (at chromosome 15: 37 163 142) or a conserved 5′ region (37 111 871–37 112 536). This did not reveal any difference to the mouse genomic sequence database (UCSC Genome Browser). Nevertheless, we hypothesize that the $Axd$ phenotype results from a regulatory mutation which leads to increased expression of $Grhl2$. Knock-in of $Grhl2$ into the $Grhl3$ locus has recently been found to generate embryos with spina bifida which resemble $Grhl3$ null mutants (17). This was interpreted to indicate that $Grhl2$ is unable to compensate for the function of $Grhl3$ in spinal neural tube closure (17). An alternative explanation, however, is that over-expression of $Grhl2$ might have contributed to development of spinal NTDs in these knock-in embryos, as in $Axd$.

$Grhl2$ functions as a transcription factor (23), and failure of neural tube closure presumably results from misregulation of key downstream genes. The finding of a similar spina bifida phenotype following over-expression or knock-out of $Grhl2$ raises questions about the underlying molecular mechanisms and whether the same downstream targets are implicated in each model. In contrast to the spinal neural tube, cranial neurulation appears unaffected by the $Axd$ mutation, whereas $Grhl2$ null embryos display a severe phenotype with complete absence of closure in fore- and midbrain. This discrepancy could suggest that differing mechanisms underlie NTDs, including spina bifida, in $Axd$ and $Grhl2$ null embryos. Alternatively, the key $Grhl2$ target genes might differ between cranial and spinal regions and those that are essential in the cranial region may be resistant to excessive $Grhl2$ levels.

Certain features of $Axd$ resemble the known pathogenesis in curly tail mutant embryos, in which reduced expression of $Grhl3$ in the hindgut leads to diminished proliferation and enhanced ventral curvature of the caudal region of the embryo (21). $Grhl2$ is also expressed in the hindgut and we find diminished proliferation in this tissue along with increased ventral curvature of the caudal region. We hypothesize that this curvature may contribute to development of spinal NTDs through mechanical inhibition of closure as in curly tail. $Grhl2$ and $Grhl3$ can form heterodimers as well as homodimers (24), and we speculate that the presence of increased levels of $Grhl2$ could favour formation of heterodimers in $Axd$ mutants at the expense of $Grhl3$ homodimers. Such an effect could lead to suppression of $Grhl3$ function, thereby mimicking the $Grhl3$ hypomorph, curly tail. Indeed, we do observe genetic interaction in double heterozygotes for the $Axd$ and $ct$ mutations.

Partial penetrance among mouse models of NTDs offers the opportunity to investigate gene–environment interactions and possible prevention of defects by exogenous agents. Spinal NTDs in curly tail mice can be partially ameliorated by exposure to retinoic acid, but in vivo treatment of $Axd^+/Axd^+$ dams did not alter the frequency of NTDs among litters (25). In contrast, methionine supplementation at neurulation stages, which is ineffective in curly tail (26), was found to reduce the frequency of spina bifida in $Axd$ litters although a low incidence of exencephaly was also observed (12). A possible association between elevated methionine and cranial NTDs is consistent with findings from embryo culture studies in wild-type mice (27). It will be of interest to determine whether effects of methionine treatment on neural tube closure in $Axd$ is associated with alteration in expression of $Grhl2$. For example, reduced expression of $Grhl2$ could be associated with normalization of spinal neurulation in $Axd/Axd$ embryos but with induction of cranial NTDs in wild-type embryos.

In humans, $GRHL2$ has been implicated in age-related hearing impairment (28), with the identification of a $GRHL2$ mutation in autosomal dominant non-syndromic hearing loss (29). These conditions are thought to relate to expression of $Grhl2$ in the otic vesicle (as we also observe in mice) and the cochlea later in development (29). In the current study, our findings suggest that either loss or gain of function of $Grhl2$ can cause spina bifida in the mouse, suggesting a key requirement for regulation of $Grhl2$ during spinal neurulation. Moreover, loss of function models demonstrate a critical requirement for $Grhl2$ in cranial neural tube closure, including in the forebrain (current study and reference 17). These studies implicate $Grhl2$ as a candidate gene for NTDs in humans. It is striking that presumed regulatory mutations affecting expression of both $Grhl2$ ($Axd$; current study) and $Grhl3$ (curly tail; reference 15) have now been found to result in spinal NTDs, perhaps emphasizing the need to consider regulatory as well as missense mutations in searching for the genes contributing to human NTDs.
MATERIALS AND METHODS

Mouse strains

Ax{d} mice were obtained upon rederivation of cryopreserved embryos from Rutgers University, NJ, USA. These mice had a mixed genetic background including the original Ax{d} strain (of unknown origin), BALB/c, C57BL/6 and C3H/HeN. Affected heterozygous Ax{d} mice were backcrossed multiple times to BALB/c (BALB/cAnNCrl; Charles River) to obtain a congenic strain while monitoring the genetic background using polymorphic microsatellite markers. For linkage analysis, congenic mice were crossed with C57BL/6 (C57BL/6NCrl; Charles River) and then intercrossed to obtain litters which were analysed at E13.5 for the presence of open spina bifida (see Supplementary Materials for details of linkage analysis). Initially, the Ax{d} strain (AxBR3) was maintained by a breeding scheme in which heterozygous mice (determined by a curled tail phenotype) were mated with wild-type mice (determined by a lack of phenotype in three subsequent crosses). Following linkage analysis, mice and embryos were genotyped using the D15Mit250 polymorphic marker, which lies between Pahpc1 and Ywhaz.

For generation of mice carrying a gene-trap allele of Grhl2 (denoted Grhl2{gt}), the AC0205 embryonic stem cell line, carrying the pGT01xr vector inserted in intron 14 of Grhl2, was obtained from the International Gene Trap Consortium. Chimeric mice carrying the Grhl2{gt} allele were generated by blastocyst injection of ES cells (UCL Institute of Child Health ES Cell Facility), and chimeras were mated to C57BL/6 to test for germline transmission and establish a colony of heterozygous mice. Efficacy of the gene-trap vector and targeting of Grhl2 were confirmed by X-Gal staining of ES cells and embryos and sequencing of RT–PCR products, generated using a forward primer in exon 14 of Grhl2, with a reverse primer in the trap vector (see what follows for RT–PCR details; sequence of primers available on request). The gene-trap vector insertion site was localized in intron 14 using a series of 20 primer pairs designed against the intron 14 sequence, and targeted embryos were matched for axial level using the hindlimb bud as morphological landmark (14). Mitotic index was calculated for neuroepithelium and hindgut in each section based on the number of stained cells expressed as a percentage of the total number of cells (as determined by number of DAPI-stained nuclei), as described previously (15,31).

Whole-mount in situ hybridization and immunohistochemistry

Whole-mount in situ hybridization was performed as described previously (15), using a digoxygenin-labelled cRNA probe which was complementary to nucleotides 366–1217 of the Grhl2 transcript (NM_026496). Embryos were embedded in gelatine-albumin and sectioned at 50 μm thickness on a vibratome, as described previously (30). Embryos were embedded in paraffin wax and 7 μm transverse sections were immunostained for phospho-histone H3 (Upstate Biotechnology). Sections through the caudal region were matched for axial level using the hindlimb bud as morphological landmark (14). Mitotic index was calculated for neuroepithelium and hindgut in each section based on the number of stained cells expressed as a percentage of the total number of cells (as determined by number of DAPI-stained nuclei), as described previously (15,31).

Scanning electron microscopy

Embryos were fixed in Bodian’s fluid [90 ml of ethanol (80%), 5 ml of acetic acid (99%) and 5 ml of formaldehyde (37%)] and prepared for scanning electron microscopy. After ethanol dehydration, they were critical-point-dried using liquid CO2, attached to aluminium stubs with silver paint, coated with gold/palladium and viewed at 10 kV with a Philips-XL30 scanning electron microscope (SEM).

RNA isolation, cDNA synthesis and real-time qRT–PCR

For analysis of the six candidate genes in the Ax{d} critical region, total RNA was isolated from affected (AxBR4CR1F2), wild-type (AxBR3F2) and BALB/c embryos at 25–43 somite stage (E10.5), using TRI Reagent (Invitrogen) and subsequently purified (RNaseasy, Qiagen). First-strand cDNA synthesis was performed according to the manufacturer’s instructions (iScript cDNA Synthesis Kit, BioRad). Relative expression of candidate genes was analysed by qRT–PCR using an iCyclerTM Real-Time PCR Detection System (Bio-Rad) with iQ SYBR Green Supermix (Bio-Rad). Primers, which spanned exons, were designed using Primer Express 1.5 software (ABI) based on the sequence entries in GenBank (Supplementary Material, Table S2).
Cyclophilin, glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and ribosomal RNA 18S (18S) were used as reference genes. Multiple wild-type and affected samples were tested simultaneously. Sample duplicates differing by more than 0.5 in Ct value were excluded. Values were determined using LinRegPCR, analysis of real-time data, version 9.30 beta. Between-session variation was accounted for using the Factor Correction program (32).

For further analysis of Grhl2 expression in Axd and Axd/Grhl2GT embryos, RNA was purified from isolated caudal regions (cut at the level of somite 21), genic DNA removed by DNase I digestion (DNA-free, Ambion) and first-strand cDNA synthesis performed (SuperScript VIIo cDNA Synthesis Kit, Invitrogen). qRT–PCR was performed (MESA Blue Mastermix for SYBR Assay, Eurogentec) on a 7500 Fast Real-Time PCR System (Applied Biosystems), with each sample analysed in triplicate. Primers were designed to amplify exons 10–14 and 14–16 of the mouse Grhl2 cDNA (Supplementary Material, Table S2), and results were normalized to Gapdh as previously (15).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

The authors are grateful to Dawn Lau (UCL) for technical assistance and to Peter Gustavsson (Karolinska Institute), Diana Juriloff and Muriel Harris (University of British Columbia) for helpful discussions. Henk Blom, Cees Oudejans, Margot van Eck van der Sluijs-van de Bor and Matthijs Verhage (VU University and VU University Medical Center, Amsterdam) supported and facilitated this work.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by funding from the Medical Research Council (G0802163 to N.D.E.G., A.J.C.); the Wellcome Trust (087525 to A.J.C., N.D.E.G.); and the Netherlands Organisation for Scientific Research (NWO; 916.36.142). Funding to pay the Open Access publication charges for this article was provided by Wellcome Trust.

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