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Permalink
https://escholarship.org/uc/item/68g8m815

Journal
Human molecular genetics, 27(24)

ISSN
0964-6906

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Publication Date
2018-12-01

DOI
10.1093/hmg/ddy313

Peer reviewed
Overexpression of Grainyhead-like 3 causes spina bifida and interacts genetically with mutant alleles of Grhl2 and Vangl2 in mice

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Abstract

The genetic basis of human neural tube defects (NTDs), such as anencephaly and spina bifida (SB), is complex and heterogeneous. Grainyhead-like genes represent candidates for involvement in NTDs based on the presence of SB and exencephaly in mice carrying loss-of-function alleles of Grhl2 or Grhl3. We found that reinstatement of Grhl3 expression, by bacterial artificial chromosome (BAC)-mediated transgenesis, prevents SB in Grhl3-null embryos, as in the Grhl3 hypomorphic curly tail strain. Notably, however, further increase in expression of Grhl3 causes highly penetrant SB. Grhl3 overexpression recapitulates the spinal NTD phenotype of loss-of-function embryos, although the underlying mechanism differs. However, it does not phenocopy other defects of Grhl3-null embryos such as abnormal axial curvature, cranial NTDs (exencephaly) or skin barrier defects, the latter being rescued by the Grhl3-transgene. Grhl2 and Grhl3 can form homodimers and heterodimers, suggesting a possible model in which defects arising from overexpression of Grhl3 result from sequestration of Grhl2 in heterodimers, mimicking Grhl2 loss of function. This hypothesis predicts that increased abundance of Grhl2 would have an ameliorating effect in Grhl3 overexpressing embryo. Instead, we observed a striking additive genetic interaction between Grhl2 and Grhl3 gain-of-function alleles. Severe SB arose in embryos in which both genes were expressed at moderately elevated levels that individually do not cause NTDs. Furthermore, moderate Grhl3 overexpression also interacted with the Vangl2Lp allele to cause SB, demonstrating genetic interaction with the planar cell polarity signalling pathway that is implicated in mouse and human NTDs.
Introduction

Although neural tube defects (NTDs) are among the most common birth defects worldwide and have a strong genetic component, the specific contributors to genetic risk are not well understood (1,4). Identifying the molecular determinants of human NTDs is hindered by their complex, multifactorial nature and likely heterogeneity between cases. Moreover, most cases are sporadic rather than familial and de novo mutations may also play a significant role in spina bifida (SB) causation (5,6). Patterns of recurrence risk support oligogenic or polygenic models in which most NTDs result from a combination of one or more genetic factors with contribution from environmental risk factors, both positive and negative (7). It is anticipated that large-scale whole-exome and whole-genome sequencing efforts will provide a greater understanding of the genetic basis of NTDs but analysis of these large data sets and assignment of causation to coding variants and/or potential regulatory mutations will not be trivial.

Candidate genes for human NTDs may be indicated by knowledge of environmental risk factors, such as folate status and maternal diabetes, and causative genes in genetic models of which several hundred have been identified in mice (2,8). Mouse models have demonstrated a crucial role for members of the grainyhead-like family of transcription factors in neural tube closure. Grhl3-null embryos develop fully penetrant SB (9,10) and a hypomorph allele of Grhl3 is the main genetic cause of SB in the curly tail strain (11). Each of these strains also exhibit a low frequency of exencephaly, which results from incomplete closure of the cranial neural tube and leads to anencephaly in late gestation. Analysis of Grhl3-null and tissue-specific knockout embryos indicates that the initial defect leading to failure of spinal neuralization is localized to the surface ectoderm component of the closing neural folds, corresponding with its prominent early expression in this cell layer (12). Grhl3 is also expressed in the node-streak border/caudo-lateral epiblast and, later and transiently, in the neuroepithelium as well as in the gut endoderm (9,11,12). Among these tissues, knockout of Grhl3 in the gut endoderm causes spinal NTDs, but with later onset than in null embryos. These defects result from excess ventral curvature of the body axis, as in curly tail (Grhl3<sup>−/−</sup>) mutant embryos (12,13). Hence, Grhl3 deficit leads to tissue-specific abnormalities that inhibit closure at two successive stages of spinal neuralization.

The extent to which GRHL3 mutations may contribute to human NTDs is not yet clear. Both de novo and rare inherited missense mutations of GRHL3 have been reported in SB cases at a frequency that suggests a role in determining NTD predisposition (6,14). GRHL3 mutations have also been reported in individuals with cleft lip and palate and individuals with syndromic (Van der Woude syndrome) and non-syndromic isolated cleft palate (15,17), consistent with GRHL3 expression in the oral ectoderm. Sharing of a missense mutation in independent SB and cleft palate cases suggests the concept that GRHL3 may contribute to both defects (6,15).

The potential for regulatory mutations in GRHL3 to contribute to causation of human NTDs has been largely unexplored to date. In mice, a regulatory mutation likely underlies the diminished expression of Grhl3, which causes SB in the curly tail mouse (11). Tissues from human fetuses with NTDs have revealed hypomethylation of CpG islands within the 5′UTR and introns of GRHL3 (18), perhaps suggesting that GRHL3 misexpression may lead to NTDs. In the current study we examined this possibility in mouse models. Notably, we found that overexpression of Grhl3 causes a high frequency of severe SB. Moreover, even moderately elevated abundance of Grhl3 was found to cause SB when in combination with mutant alleles of other NTD genes: Grhl2 or Vangl2. Hence, insufficient or excess levels of Grhl3 can both cause SB.

Results

Overexpression of Grhl3 causes NTDs

Reinstatement of Grhl3 expression, mediated by bacterial artificial chromosome (BAC) transgenesis, prevents spinal NTDs in curly tail mice that have partial loss of function of Grhl3 (11). We investigated the potential effect of increasing levels of Grhl3 expression by intercross of hemizygous Grhl3 BAC-transgenic mice (Grhl3<sup>c<sub>0</sub>c<sub>0</sub>TgGrhl3<sup>+/−</sup></sup>) to generate litters that include embryos carrying the BAC in homozygosity (Grhl3<sup>c<sub>0</sub>c<sub>0</sub>TgGrhl3<sup>+/+</sup></sup>). Litters were genotyped by BAC-specific polymerase chain reaction (PCR) and quantitative genomic PCR (GqPCR). As in our previous study (11), Grhl3<sup>c<sub>0</sub>c<sub>0</sub>TgGrhl3<sup>+/+</sup></sup> embryos (i.e. c/c embryos also hemizygous for the Grhl3-BAC) did not display spinal NTDs, whereas a proportion of Grhl3<sup>c<sub>0</sub>c<sub>0</sub>TgGrhl3<sup>+/−</sup></sup> embryos developed SB and/or tail flexion defects (TFDs) (Fig.1). Remarkably, we observed SB in 67% of Grhl3<sup>c<sub>0</sub>c<sub>0</sub>TgGrhl3<sup>+/−</sup></sup> embryos, suggesting that overexpression of Grhl3 prevents neural tube closure (Fig.1).

The NTD phenotype of Grhl3 overexpressing embryos could theoretically result from homoygous insertion of the transgene into an essential endogenous locus. To investigate this, the genomic location of the BAC was determined using inverse PCR (Fig. 2A and B). Sequence analysis indicated that the transgene insertion site is at position 3,005,382 on chromosome 18, in a low-complexity region. This was confirmed by PCR amplification of genomic DNA, using a series of primer pairs located in the transgene and putative chromosome 18 location (Fig. 2C and Supplementary Material, Fig. S1A). This site is more than 100 kb from the nearest recognized gene (vmn1r238) and 266 kb from the nearest gene (Creml) that has detectable embryonic expression at E10.5 (Supplementary Material, Fig. S1B). Fluorescence in situ hybridization (FISH) analysis on whole blood cultures confirmed that the BAC is present in only one location (Supplementary Material, Fig. S1D and E). We conclude that insertional mutagenesis is very unlikely to explain the NTDs observed in Grhl3 overexpressing embryos.

Further evidence that Grhl3 overexpression causes NTDs was provided by backcross of the Grhl3 transgene onto a wild-type BALB/c genetic background. NTDs did not occur among wild-type embryos (n = 20), but among hemizygous (+/<sup>TgGrhl3<sup>+/−</sup></sup>) embryos we observed a low frequency of SB or TFDs (3/20; 15%). These defects are predicted to arise because the level of endogenous Grhl3 expression from the wild-type allele is higher than in the hypomorph ct strain such that overexpression mediated by a single copy of the BAC is sufficient to exceed the level that is compatible with neural tube closure. Consistent with this, all homozygous (+/<sup>TgGrhl3<sup>+/−</sup></sup>) embryos developed SB on this genetic background (18/18; 100%).

SB in Grhl3 overexpressing embryos results from early failure of posterior neuropore closure

In addition to a higher frequency of SB than in Grhl3<sup>c<sub>0</sub>c<sub>0</sub>TgGrhl3<sup>+/−</sup></sup> embryos, the size of the open lesion was also greater in Grhl3 overexpressing embryos at E11.5–13.5 (Fig. 1 and Supplementary Material, Fig. S2A–D). Previous studies showed that the posterior neuropore (PNP) length of Grhl3<sup>c<sub>0</sub>c<sub>0</sub>TgGrhl3<sup>+/−</sup></sup> embryos becomes larger than genetically matched wild-type embryos from the 25–27
Figure 1. Spinal NTDs in Grhl3-transgenic mice. (A) Occurrence of SB among offspring of matings between Grhl3ct/ct; TgGrhl3/0 (BAC-hemizygous) and Grhl3ct/ct mice analysed at E11.5–18.5 (*, significant difference from other genotypes; P < 0.001, Chi-square). (B–E) Embryos of the three genotypes at E11.5. All Grhl3ct/ct; TgGrhl3/0 embryos appeared normal with a straight caudal region (C), whereas a proportion of Grhl3ct/ct (B) and Grhl3ct/ct; TgGrhl3/TgGrhl3 (D, E) exhibited TFDs and/or SB (extent of open region indicated by arrowheads). Scale bar represents 1 mm.

Figure 2. Localization of BAC-transgene in Grhl3ct/ct; TgGrhl3/0 embryos. (A) Inverse PCR was used to isolate genomic fragments adjacent to the insertion site of the Grhl3-containing BAC. (B) Sequence tags most closely aligned to the reference genomic sequence in a region on chromosome 18 with insertion of the BAC at 18:3,005,382 in a low complexity repeat. Inverse PCR fragments also show homology to regions on chromosome 16 but with lower identity.

somite stage (E10.5) (11). In order to assess the stage at which spinal neurulation fails in Grhl3 overexpressing embryos, we collected litters generated by intercross of Grhl3ct/ct; TgGrhl3/0 mice. Among embryos analysed at E9–10.5, the PNP length was already larger among Grhl3ct/ct; TgGrhl3/0 embryos than Grhl3+/+ littermates from the 12–15 somite stage (E9) onwards (Fig. 3A and Supplementary Material, Fig. S2E). This earlier failure of closure explains why the extent of the open SB lesion is greater among Grhl3 overexpressing fetuses than in their hypomorphic littermates.

Among embryos at E9.5 and E10.0, Grhl3 mRNA was upregulated by 2–3-folds in the caudal region of Grhl3ct/ct; TgGrhl3/0 embryos and 4–6-folds in Grhl3ct/ct; TgGrhl3/TgGrhl3, compared with Grhl3+/+ (Fig. 3B). This equates to ~1.3–1.5- and 2.5–3-fold higher expression than in wild-type embryos with a similar genetic background (based on our finding that Grhl3 abundance in ct/ct embryos is ~50% of that in partially congenic (+/ct) wild types (11)). Hence, Grhl3 mRNA abundance (by qRT-PCR) correlated with the number of copies of the Grhl3 gene, determined by GqPCR (Fig. 3C). We conclude that during spinal neurulation the PNP becomes enlarged in Grhl3ct/ct hypomorphic embryos compared with Grhl3ct/ct; TgGrhl3/0 embryos that have mildly elevated Grhl3 expression, but a further increase in Grhl3 expression results in an even larger PNP in Grhl3ct/ct; TgGrhl3/TgGrhl3 embryos (Fig. 3D), resulting in SB. Scanning electron microscopy showed that the enlarged PNP of Grhl3 overexpressing embryos was characteristically very narrow (compare Fig. 3E–F with Fig. 3G–H), suggesting that NTDs do not result from a defect in elevation or bending of the neural folds.

Balance between excess and insufficient expression of Grhl3 in spinal neurulation

Homozygous embryos for Grhl3-null (Grhl3−/−) or gain-of-function (Grhl33+/−; TgGrhl3/TgGrhl3) alleles develop severe spinal NTDs. In order to further investigate the correlation between Grhl3 expression level and neural tube closure we asked whether transgenic expression was sufficient to rescue Grhl3-null NTDs and/or vice versa. Grhl3−/− and Grhl33+/−; TgGrhl3/TgGrhl3 mice were crossed and offspring with genotype Grhl3−/−; TgGrhl3/TgGrhl3 were intercrossed to generate experimental litters carrying combinations of the null allele and the Grhl3-BAC (Table 1). Grhl3−/− and Grhl33+/−; TgGrhl3/TgGrhl3 fetuses developed SB as expected. The majority of embryos with Grhl33+/− genotype exhibited TFDs, with SB also present in
Figure 3. Excess expression of Grhl3 results in spinal NTDs owing to failure of PNP closure. (A) Among litters from Grhl3ct/ct;TgGrhl3/0 matings analysed at E8.5–10.5, the PNP was significantly enlarged among Grhl3ct/ct;TgGrhl3/TgGrhl3 embryos (n = 64) from the 12–15 somite stage onwards (#, significant difference from both other genotypes, P < 0.001). PNP closure was normalized in Grhl3ct/ct;TgGrhl3/0 embryos (n = 123) compared with Grhl3ct/ct littermates (n = 176) at 28–31 somites (*, significantly different from Grhl3ct/ct, P < 0.001). Mean ± SEM values; n = 4–77 embryos/genotype/stage (see Supplementary, Fig. S2 for plot of individual data). (B) Abundance of Grhl3 mRNA varies significantly with genotype at E9.5 and E10.5 (*P < 0.0001 ANOVA; Holm-Sidak pairwise analysis). For context, we previously found that Grhl3 expression in ct/ct embryos at E10.5 was ∼50% of that in partially congenic wild-type (+ct) embryos (11). (C) Analysis of individual embryos at E10.5 (28–29 somites) shows that increased abundance of Grhl3 genomic DNA (in transgenic embryos) results in gene dosage-dependent increase in Grhl3 mRNA expression. qG-PCR signal corresponds to 2, 3 and 4 copies of Grhl3 in ct/ct, hemizygous and homozygous transgenics, respectively. (D) Moderate overexpression of Grhl3 normalizes PNP closure in individual Grhl3ct/ct;TgGrhl3/0 embryos, compared with Grhl3ct/ct, whereas excess expression prevents PNP closure, in Grhl3ct/ct;TgGrhl3/TgGrhl3. (E–H) Scanning electron micrographs at E9 (13 somite stage) show typical appearance of the closing PNP. Neural folds are elevated and apposed in all three genotypes (E–G) but failure of closure progression is already apparent in Grhl3ct/ct;TgGrhl3/TgGrhl3 embryos (G,H), leaving a narrow unclosed region (compare region adjacent to white arrow E–H). Scale bars represent 0.1 mm.

Abnormal function of the surface ectoderm is implicated in causation of spinal NTDs in Grhl3 overexpressing embryos

Partial rescue of NTDs resulting from excess Grhl3 by deletion of the endogenous allele suggested that the deleterious effect of Grhl3 overexpression on neural tube closure was localized to one or more of the endogenous sites of expression, as opposed to ectopic activity. At E8.5, Grhl3 is expressed in the surface ectoderm and in the posterior part of the embryo corresponding to the node-streak border and caudo-lateral epiblast (11). At E9.5, Grhl3 mRNA is also detected in the neural plate of the PNP and then, at E10–10.5, also in the hindgut endoderm. Whole mount in situ hybridization (WMISH) of Grhl3ct/ct;TgGrhl3/TgGrhl3 embryos confirmed Grhl3 expression to be more intense but localized in the same tissues as in wild-type and hemizygous Grhl3-transgenic embryos (Fig. 4A and B) (11,12). Analysis of Grhl3 expression in the context of lack of the endogenous allele further confirmed expression of the transgene in the normal expression domain (Fig. 4C).

In Grhl3ct/ct embryos, the timing of closure failure and the phenotype of conditional knockouts suggest that the earliest abnormality of spinal neural tube closure results from a defect in the surface ectoderm (12). The surface ectoderm is the precursor of the epidermis, in which Grhl3 regulates terminal differentiation, barrier formation and repair (10,19–21). We asked whether expression of epithelial/epidermal genes was altered at
Table 1. Phenotypes of embryos carrying combinations of Grhl3 loss of function and overexpressing alleles

| Genotype                  | No. embryos | Straight tail | Spinal phenotype % (n) | SB & TFDs | P     |
|---------------------------|-------------|---------------|------------------------|-----------|-------|
| Grhl3ct/ct                | 13          | 77 (10)       | 15 (2)                 | 8 (1)     |       |
| Grhl3 ct/ct; TgGrhl3/0    | 8           | 100 (8)       | 0 (0)                  | 0 (0)     |       |
| Grhl3 ct/ct; TgGrhl3/TgGrhl3 | 9       | 11 (1)        | 11 (1)                 | 78 (7)    | <0.001|
| Grhl3 ct/ct               | 10          | 20 (2)        | 30 (3)                 | 50 (5)    |       |
| Grhl3 ct/-                | 11          | 30 (3)        | 0 (0)                  | 70 (7)    | <0.001|
| Grhl3 -/-                 | 12          | 100 (12)      | 0 (0)                  | 100 (11)  |       |
| Grhl3 ct/-; TgGrhl3/0     | 10          | 62 (5)        | 0 (0)                  | 38 (3)    | <0.001|
| Grhl3 ct/-; TgGrhl3/TgGrhl3 | 8       | 62 (5)        | 0 (0)                  | 38 (3)    | <0.001|

Offspring of Grhl3ct/-; TgGrhl3/0 intercrosses were assessed at E11.0–18.5 for the presence of NTDs and/or TFDs. NTDs did not arise among embryos heterozygous for the Grhl3 transgene irrespective of the endogenous genotype. Differences in the number (0, 1 or 2) of Grhl3 transgene copies was associated with significant variation in the distribution of spinal phenotypes among Grhl3ct/ct, Grhl3ct/-, and Grhl3-/- embryos (P < 0.001; Chi-square).

Figure 4. Overexpression of Grhl3 and Cdh1 in Grhl3-transgenic embryos. (A and B) Grhl3 expression in hemizygous (A) and homozygous (B) Grhl3-BAC transgenics detected by WMISH. Expression is detected in the expected domains in the surface ectoderm (se), neural plate (np) and hindgut (arrows in whole mounts; dotted lines in sections). Transverse sections are at the level of white dotted line on corresponding whole mount images; sagittal sections are shown at E9.5. (C) On a Grhl3-null background, the expression pattern of Grhl3 (entirely from the transgene), resembles the previously reported endogenous expression pattern. (D and E) Cdh1 shows the expected expression in surface ectoderm (se) and gut endoderm (g) in Grhl3ct/ct embryos (D). In Grhl3-transgenic embryos (E), Cdh1 expression appears more intense in the surface ectoderm (se) and occasional Cdh1-positive cells (arrows in E”–E”) are present in the recently closed neural tube (nt; 1) and the neuroepithelial component (ne) of the open neural folds (F”, inset). Figure shows representative embryos at E9.5 (19–20 somite stage; D,E) with site of neural fold closure shown by white arrow in whole mounts. Scale bar = 0.5 mm in whole mounts; 0.1 mm in sections.

neurulation stages. Using qRT-PCR, we found that known Grhl3 targets in late-fetal epidermis, Lor and Tgm1, were already upregulated in the caudal region of Grhl3 overexpressing embryos at E10.5 (Supplementary Material, Table S1).

At E9, the stage at which neurulation begins to fail in Grhl3 mutant embryos, Trp63 (encoding TAp63) (22), a key transcriptional regulator of epidermal specification, was also upregulated in Grhl3ct/ct; TgGrhl3/TgGrhl3 (Supplementary Material,
compared with Fig. S3). We hypothesize that earlier overexpression of may lead to persistent expression of (neuromesodermal progenitors) (24), and in the neural plate contains precursors of the neuroepithelium and mesoderm in the node-streak border and caudo-lateral epiblast, which

The premature upregulation of markers such as Trp63, Lor and Tgm1, and the abnormal expression of Cdhh1, suggest that NTDs caused by Grhl3 gain of function could result primarily from dysregulation of gene expression in the surface ectoderm. A contribution from other Grhl3 expressing tissues appears unlikely as follows: overexpression of Grhl3 in the neural plate occurs prior to closure failure but there is no obvious defect in neural fold elevation (Fig. 3). Grhl3 is expressed in the node-streak border and caudo-lateral epiblast (Fig. 4A) but we found no evidence for altered patterning of the caudal region in Grhl3 overexpressing embryos (Supplementary Material, Fig. S4). Loss of function of Grhl3 in the hindgut alone is sufficient to prevent closure at all stages of spinal neuralisation (12). However, in Grhl3 overexpressing embryos, closure fails prior to onset of hindgut expression (Figs 3 and 4) and there is no increase in ventral curvature (Supplementary Material, Fig. S5), the mechanism by which insufficient Grhl3 expression in the hindgut inhibits closure (13).

Overexpression of Grhl3 does not exacerbate cranial NTDs or cause skin barrier defects

In addition to SB, loss of function of Grhl3 has been shown to cause cranial NTDs (encephaly) and epidermal defects (9,10). We therefore asked whether overexpression of Grhl3 recapitulates these phenotypes.

In Grhl3+/- models, the frequency of exencephaly was reported as 2% and 14% (9,10). Unlike SB this is not markedly higher than in the curly tail strain, in which exencephaly typically affects 6–8% of embryos (25–27). Among Grhl3+/-;Tgm1-Lmnb1 Lmnb1 -null embryos (as observed in Grhl3-null embryos) (27). These findings support the hypothesis that diminished Grhl3 expression is not the main cause of cranial NTDs in the ct strain and that the underlying mechanism therefore differs from Grhl3-null embryos. Instead, the principal contribution may be from other deleterious variants in the ct genetic background, Lmnb1 and Mish611 having been identified as potential modifiers of NTDs in the ct strain (25,27).

Grhl3 is required for differentiation and repair of the epidermal barrier at late fetal stages (10) and for post-natal repair of the epidermal barrier after injury (21). Skin histology of Grhl3-null fetuses becomes abnormal by E16.5, while the epidermal permeability barrier, which normally begins to form at E16–17, fails to develop by E18.5 (10). In the Grhl3 null/transgenic crosses in the current study, we confirmed that the epidermal barrier fails to form in Grhl3-/- fetuses, using a dye penetration assay (Fig. 5A and B). The skin barrier was complete by E18.5 in 6/6 wild-type and 16/16 Grhl3 +/- fetuses but not in Grhl3-/- littermates (0/5). Transgenic expression of Grhl3 was sufficient to rescue the epidermal barrier defect phenotype in Grhl3-/- fetuses, both in hemizygous (Grhl3+/-;TgGrhl3/0) and homozygous (Grhl3+/-;TgGrhl3/0) transgenics (3/3 of each genotype tested) (Fig. 5). Known Grhl3 targets in the epidermis were upregulated in skin at E8.5 (Fig. 5D). However, in contrast to the NTDs produced by overexpression of Grhl3, we did not observe epidermal abnormalities in Grhl3+/-;TgGrhl3/0 fetuses (barrier complete in 3/3; Fig. 5). The histological appearance of skin in Grhl3-overexpressing fetuses was also comparable to controls at E18.5, with normal staining for the basal marker p63 (Supplementary Material, Fig. S6).

Additional phenotypes reported in Grhl3-null fetuses include the presence of open eyelids at E18.5 and an abnormal limb phenotype (28). During normal development, the digits become separated by E15 and then undergo a temporary epithelial fusion, with displacement of intervening periderm cells (29). Digit fusion appears to occur normally in Grhl3-null fetuses but the distal limb appears swollen (28) (Fig. 5C). Consistent with normal barrier formation in Grhl3 overexpressing embryos, limb development was also apparently normal (Fig. 5B). In contrast, the open-eye phenotype that accompanies SB in Grhl3-null fetuses was also present in Grhl3 transgenics with SB at E18.5, whether wild type or mutant at the endogenous Grhl3 locus.

Genetic interaction of Grhl3 and Grhl2 overexpression alleles

Grhl2 and Grhl3 both form homodimers but also exhibit protein–protein interactions to form heterodimers (30,31), although the functional role of these interactions is unknown. We speculated that excessive abundance of Grhl3 could disturb the relative abundance of Grhl2 and Grhl3 proteins and favour formation of heterodimers, thereby inhibiting function of Grhl3 homodimers and causing spinal NTDs (as observed in Grhl2-null embryos) (32). Similarly, it could
Figure 5. Establishment of the epidermal barrier is not compromised by Grhl3 overexpression. (A–C) Integrity of the permeability barrier was determined by dye penetration assay. At E16.5 (A), all genotypes show dye penetration, whereas at E18.5 (B) Grhl3\textsuperscript{-/-} is the only genotype with incomplete epidermal barrier. Note that barrier formation is rescued by transgenic expression of Grhl3 in Grhl3\textsuperscript{-/-}; TgGrhl3/TgGrhl3 fetuses (in parallel with rescue of NTDs). Although Grhl3 overexpression causes SB, the epidermal barrier is established by E18.5 in hemizygous and homozygous transgenics. Note that limbs appear normal in transgenic fetuses, unlike in Grhl3\textsuperscript{-/-} (\*; abnormal forelimbs in B,C). Moreover, partially or completely open eyelids are visible in the transgenic fetuses (B). Scale bar represents 1 mm; CT: curly tail; ST: straight tail. (D) Analysis of gene expression in E18.5 skin samples in litters from compound mutant/transgenic or Grhl3\textsuperscript{ct/ct}; TgGrhl3/0 intercrosses shows that Grhl3 overexpression is associated with increased expression of Lor, Tgm1 and Cdh1 (significant differences from Grhl3\textsuperscript{ct/ct}; \* P < 0.05, \*\* P < 0.001; ANOVA).

be predicted that spinal NTDs caused by overexpression of Grhl2 in Axd mutants could result from suppression of Grhl3 function. These models predict that overexpressing Grhl2 in Grhl3-transgenic embryos would normalize spinal neural tube closure by compensating for excess of Grhl3. To test this hypothesis we generated embryos that overexpress both Grhl2 and Grhl3, by intercross of Axd\textsuperscript{-/} and +/- TgGrhl3 mice. Spinal NTDs occurred only at very low frequency in single heterozygotes as expected (Fig. 6A). Remarkably, however, a large SB was observed in 100% (n = 19) of doubly heterozygous Axd\textsuperscript{-/+} and +/- TgGrhl3 embryos (Fig. 6A–C). Analysis of a further series of embryos collected at E9.0–10.5 showed that doubly heterozygous embryos exhibit failure of closure, with a significantly enlarged PNP from E9.5 (15–19 somites stage) onwards (Fig. 6D). We confirmed that expression of Grhl2 and Grhl3 was elevated in the embryos carrying the Grhl2\textsuperscript{Axd} allele and Grhl3 transgene, respectively (Fig. 6E), without evidence of reciprocal regulation. Hence, occurrence of SB results from an additive genetic interaction of the Grhl2 and Grhl3 alleles, and we can rule out a mechanism of NTDs based on mutual repression of function by gene overexpression.

The Vangl2\textsuperscript{Lp} allele genetically interacts with Grhl3 overexpression to cause SB

We next asked whether moderate overexpression of Grhl3 interacts with another genetic risk factor for NTDs, the loop-tail (Lp) mutation in Vangl2, encoding a core component of the PCP signalling pathway. Homozygosity for Vangl2\textsuperscript{Lp} causes the severe NTD craniarachischisis, (33,34), while heterozygosity for Vangl2 can also cause craniarachischisis or SB when in combination with mutant alleles of other genes including Fzd2, Ptk7, Scrib and Sdc4 (35–37). Previous studies have also demonstrated genetic interaction of Vangl2\textsuperscript{Lp} with the loss of function Grhl3\textsuperscript{-/-} and Grhl3-null alleles.
and regulation (Vangl2Lp/frequency of SB occurred in genetics (Table 2b). Among litters analysed at E11.5 a high Grhl3-/ Grhl3 with analyses (10,21,27) or in qRT-PCR analysis of region of E9.5 embryos, mRNA abundance of (Vangl2 sites were not identified in the 10 kb region upstream of Vangl2 forelimb bud at E13.5 (C) (in Grhl3ct/ct Grhl3 prevent any effect of hemizygosity for the confirming previous findings (38). However, in contrast to the Grhl3ct/ct heterozygotes were intercrossed with of this defect in heterozygotes, correlating with the almost complete penetrance transgene (Table 2a).

Similarly, Vangl2 expression did not involve direct regulation of Vangl2 expression was not associated with Grhl3 loss of function in our previous transcriptomic (39). We intercrossed Vangl2Lp/+ mice with Grhl3ct/+;TgGrhl3LO transgenics and analysed litters of embryos at E11.5 (Table 2b). Among litters analysed at E11.5 a high frequency of SB occurred in Vangl2Lp/+;Grhl3ct/+ embryos, confirming previous findings (38). However, in contrast to the preventive effect of hemizygosity for the Grhl3-BAC on NTDs in Grhl3ct/ct and Grhl3+/- embryos (Table 1), the presence of the Grhl3-transgene did not prevent the NTDs in Vangl2Lp/+;Grhl3ct/+ embryos (Table 2b). These findings provide further evidence for an exacerbating combinatorial effect of Vangl2 mutation and Grhl3 overexpression on spinal neural tube closure. This does not appear to involve direct regulation of Vangl2 expression by Grhl3 as altered Vangl2 expression was not associated with Grhl3 loss of function in our previous transcriptomic analyses (10,21,27) or in qRT-PCR analysis of Grhl3+/+ and Grhl33 embryos at E9.5 (P > 0.05; three or more per genotype). Similarly, Vangl2 expression did not differ between Grhl3ct/ct;Grhl3ct/+;TgGrhl3L0 or Grhl3ct/ct;Grhl3ct/+;TgGrhl3 LO embryos at E9.5 (P > 0.05; three or more per genotype). Moreover, Grhl3 binding sites were not identified in the 10 kb region upstream of Vangl2 (39).

**Discussion**

A requirement for sufficient Grhl3 expression to enable spinal neural tube closure is shown by the presence of SB in Grhl3-null embryos (10), hypomorphic Grhl3ct/ct embryos (11) and in tissue-specific knockouts (12). In the current study we found that overexpression of Grhl3 also causes SB, emphasizing the exquisite sensitivity of the closure process to the abundance of Grhl3 (Table 3). The induction of SB by excess Grhl3 appears to involve a defect in the surface ectoderm: PNP closure fails soon after initiation of closure when Grhl3 is strongly expressed in the surface ectoderm bordering the PNP and is not associated with a defect of neural fold elevation or altered molecular patterning of the caudal region. Moreover, Grhl3 overexpressing embryos do not exhibit the excess body curvature that results from Grhl3 loss of function in the hindgut and prevents the later stages of PNP closure (12).

The Grhl3 transgene is expressed in the endogenous domain during neural tube closure and rescue of SB by the presence of the transgene in Grhl3ct/ct and Grhl3+/- embryos shows that the target genes necessary for neurulation are appropriately regulated. Similarly, rescue of skin barrier defects shows that key epidermal genes are activated in the correct spatiotemporal manner. Grhl3 is known to regulate numerous genes in the epidermis (derived from the surface ectoderm) at late-fetal and post-natal stages, including cell adhesion molecules, lipid metabolizing enzymes and structural proteins of the stratum corneum (10,19–21).

The genome-wide binding pattern of Grhl3 appears dynamic with regulation of distinct sets of genes depending on the functional state of the epidermis. For example, comparison
alleles of which cause cranial and spinal NTDs (32,41,42), while events differed (21). The potential for context-dependent of the epidermal barrier following post-natal injury shows of epidermis during differentiation and during re-establishment of the epidermal barrier following post-natal injury shows overlap of Grhl3 targets, but more than half of the binding events differed (21). The potential for context-dependent and diverse transcriptional regulatory activity of Grhl3 suggests that the requirement during neural tube closure could involve overlapping and/or distinct functions compared with later epidermal differentiation and repair. Notwithstanding the possible difference in Grhl3 targets at different stages, we found a subset of epidermal and epithelial markers to be upregulated in Grhl3 overexpressing embryos at neural tube closure stages, suggesting a potential dysregulation of surface ectoderm properties. Such an effect could be incompatible with closure, consistent with the site of initial contact of the neural folds being mediated by surface ectoderm cells at the border with the neural plate (40).

The finding that both insufficient and excess abundance of Grhl3 cause SB is similar to observations with Grhl2, null alleles of which cause cranial and spinal NTDs (32,41,42), while upregulation causes SB (32). Interestingly, while Grhl2- and Grhl3-null embryos exhibit multiple defects alongside NTDs (e.g. skin barrier, urothelial differentiation, kidney and placental defects) (10,41,43,44), we found that their overexpression counterparts display isolated SB that more closely resembles the corresponding human condition. Overall, findings in mouse models suggest that GRHL2 and GRHL3 represent strong candidates for potential involvement in human NTDs, with the consideration that not only loss-of-function variants but also regulatory and gain-of-function mutations could plausibly play a role. In addition to the development of NTDs in homozygous transgenic embryos it is notable that even moderate overexpression of Grhl3 was sufficient to cause SB when in combination with heterozygous mutations in Grhl2 or Vangl2. Such gene–gene interactions appear likely to more closely resemble the multigenic etiology of human NTDs than single gene mutants.

### Materials and Methods

**Mice**

Curly tail mice were maintained as a homozygous, closed random-bred colony. The transgenic curly tail line Grhl3<sup>+/Grhl3<sup>+/</sup></sup>,...
transgenics were generated by intercross of Grhl3<sup>ct/ct</sup> background, crossed to generate experimental litters. To generate embryos carrying combinations of Grhl3 alleles with the Grhl3-BAC we crossed Grhl3<sup>ct/+</sup> and Grhl3<sup>-/ct</sup> mice. The offspring with genotype Grhl3<sup>-/ct</sup> were intercrossed to generate experimental litters.

To transfer the Grhl3-BAC onto a partial BALB/c genetic background, Grhl3<sup>ct/ct</sup> were backcrossed with wild-type BALB/c mice for three generations. These mice were on a CBA/Ca genetic background and the C57BL/6 reference genetic background and the C57BL/6 reference chromosomal location owing to variation between the unique Grhl3 locus such that relative signal was 1 (2 endogenous copies), 2 (2 endogenous + 1 transgene) and 3 (2 endogenous + 2 transgene) (Fig. 3C).

**BAC localization by inverse PCR**

DNA was extracted from transgenic embryos using the QiAamp DNA Mini kit (Qiagen), digested with RsaI (Invitrogen) or Haell (Fermentas), circularized and re-linearized with BamHI (Promega). The linearized product was then amplified by PCR with BAC inverse primers (327D13-R1 inverse 5′-CCCTAATGATG ACCACGTTA-3′ and pTARG-13-R1 inverse 5′-TAGTGTACCCATGTTGCTGAC-3′). PCR products were separated on a 1% agarose gel and all obvious bands were excised from the gel and purified using QIAquick Gel Extraction kit (Qiagen). DNA was eluted in water for subsequent sequencing with each of the inverse primers (BigDye Terminator Cycle Sequencing kit, Applied Biosystems).

Sequence tags did not show 100% identity with a specific chromosomal location owing to variation between the unique curly tail genetic background and the C57BL/6 reference sequence. However, sequences generated from inverse PCR were aligned to the reference genomic sequence, with closest homology to a region on chromosome 18 that indicates insertion of the BAC at 18:3005382 in a low complexity repeat. A series of primers (R1–R5; Supplementary Material, Table S5) complementary to chromosome 18 were used to amplify genomic DNA with the BAC-specific primer (pTARG-13-R1v) (Supplementary Material, Fig. S1). To confirm localization genomic PCR was performed using a BAC specific primer (pTARG-13-R1v) with a series of primers located in chromosome 18 (Supplementary Material, Table S4).

**Preparation of blood cultures and FISH**

Interphase nuclei were prepared on slides using peripheral blood from curly tail and hemizygous Grhl3 transgenic male mice. FISH analysis was performed on DAPI-stained interphase nuclei spreads on slides according to standard procedures using the BAC probe RP24-327D13 (BACPAC Resources Center at Childrens Hospital Oakland Research Institute).

Whole mount in situ hybridization was performed as previously reported (25). For sectioning, embryos were embedded in albumin-gelatine and 40 μm sections obtained on a vibratome (Leica VT 1000s, Leica Microsystems). Photographs of whole embryos were taken using a stereo microscope (Leica MZFL III) using a Leica DC500 camera. Bright-field image acquisition of sections was performed with an Axiohot 2 microscope (Zeiss) with Leica DC500 camera software (AxioVision). Images were processed using Photoshop (Version 6.0) for cropping and figures were prepared using Adobe Illustrator software.

Cdhl1-positive cells were counted on serial 40 μm sections after WMSH for Cdhl1. For each embryo the total number of ectopic cells was divided by the total number of sections. The mean and the standard error of the mean (mean ± SEM) were plotted for different genotypes. To analyse the axial distribution of Cdhl1 positive cells in the neuroepithelium, four regions were defined and for each region the total number of positive cells per section was calculated as a percentage of the total number of sections (all embryos combined at each stage).
Whole-mount antibody staining
4% PFA-fixed, methanol-dehydrated whole E8.5–E9.0 embryos were post-fixed (methanol/DMSO) overnight at 4°C. After bleaching (methanol/DMSO/30% H2O2) at room temperature for 4 h, embryos were blocked (phosphate buffered saline (PBS) containing 10% heat-inactivated Sheep serum/2% Bovine Serum Albumin/0.5% Triton X-100) for 4 h. Primary purified mouse anti-E-cadherin (BD Transduction Lab, 1:150 dilution) and relevant secondary (Alexa Fluor 488 goat anti-mouse, Life Technologies, 1/500) antibodies were applied in the same blocking solution overnight at 4°C. All the washes were performed with PBS 0.5% Triton X-100. Embryos were counterstained with DAPI for 2 h at room temperature. Prior to imaging, embryos were incubated in Scale A2 for clearing the tissue. Embryos were positioned in ‘wells’ cut into 4% agarose gels such that the PNP faced upwards. All images were captured on a Zeiss Examiner LSM880 confocal microscope using a 20×/NA1.0 Plan Apochromat dipping objective immersed in PBS. Low-magnification images were captured at 0.7× zoom with a pixel size of 0.6 μm and a Z step of 2 μm. High-magnification images were taken at 2× zoom with a pixel size of 0.1 μm and a Z step of 2 μm. Salt and pepper noise was subtracted, brightness and contrast were adjusted evenly across each image and maximum intensity projections were obtained in Fiji (Image, NIH, PMID 22743772). Digital reslicing of confocal Z-stacks was also performed in Fiji, as previously (46).

Quantitative real-time PCR
RNA was isolated from the caudal region of E8.5 (10–14ss, cut at somite 10), E9.5 (15–16ss, cut at somite 12) and E10.5 (26–31ss, cut at somite 14) embryos, and the cranial region, respectively from the level of the otocyst (excluding branchial arches). Total RNA was isolated using TRIzol Reagent (Gibco) followed by DNase treatment (DNA-free, Ambion). cDNA was generated using the SuperScript VILO kit (Invitrogen) or SuperScript II Reverse Transcriptase (RT) kit (Invitrogen). Normalization was performed using glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as a reference gene (11). Quantitative RT-PCR was performed using iTAQ Universal SYBR Green Supermix assay (Bio-Rad) on a CFX96 system (Bio-Rad) with analysis using Bio-Rad CFX Manager software (see Supplementary Material, Table S5 for primer sequences). For each experiment a calibrator sample was chosen to normalize levels of cDNA expression. Individual experiments were combined and analysed using SigmaStat v 3.5 software (ANOVA or t-test).

Supplementary Material
Supplementary Material is available at HMG online.

Acknowledgements
The mouse Cdh1 plasmid was provided by Marc Stemmler (Institute of Experimental Medicine I, Nikolaus-Fiebiger Center for Molecular Medicine).

Conflict of Interest statement. None declared.

Funding
Medical Research Council (G0802163, J003794 to N.G. and A.C.); Child Health Research CIO (to N.G.); Wellcome Trust (087525 to A.C. and N.G., 107474 to G.L.G.); National Institutes of Health Grant (R01AR44882 to B.A.); N.G., P.S. and A.C. are supported by Great Ormond Street Hospital Children’s Charity and the National Institute for Health Research Biomedical Research Centre at Great Ormond Street Hospital for Children NHS Foundation Trust and University College London.

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