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**Over-expression of *Grhl2* causes spina bifida in the *Axial defects* mutant mouse**

Brouns, Madeleine R., Sandra C.P De Castro, Els A. Terwindt-Rouwenhorst, Valentina Massa, Johan W. Hekking, Caroline S. Hirst, Dawn Savery, Chantal Munts, Darren Partridge, Wout Lamers, Eleonore Köhler, Henny W. Van Straaten, Andrew J. Copp, and Nicholas D.E Greene. "Over-expression of *Grhl2* causes spina bifida in the *Axial Defects* mutant mouse." *Human Molecular Genetics* 20.8 (2011): 1536-546.

**Objectives:**

Failure of neural tube closure during development can result in an opening in the spinal cord or brain called a neural tube defect (NTD). NTDs are among the most common human birth defects. Spina bifida, an isolated spinal NTD, occurs in a high frequency in the *Axial defects* (*Axd*) mutant mouse model. The causative gene of the *Axd* mutation is unknown. In this study, researchers mapped the mutation to a region of chromosome 15. They were unable to identify a mutation within the critical region, but were able to link the phenotype to an over-expression of *Grhl2*.

**Experimental Approach and Results:**

The experimenters first wanted to link phenotype to genotype. Heterozygous *Axd* mice (*Axd*/+) were intercrossed and offspring were used to map the *Axd* mutation by linkage disequilibrium to a region of chromosome 15. Further mapping and crossing refined the critical region to a 1.1 Mb interval. This region is syntenic to humans at chromosome 8q22. A marker with a 100% association between homozygous alleles and *Axd* phenotype that could be used for genotyping was also identified. The offspring of these crosses were then genotyped and the phenotypes were analyzed.

Homozygous *Axd* mutants displayed an enlarged posterior neuropore (PNP), elevated neural folds, delayed eyelid closure, increased ventral curvature, and a shortened tail compared to wild type mice and spina bifida. Heterozygous embryo PNPs eventually closed, but the closure time was lengthened and this manifested as tail flexion defects in ~40-50% of the mice.

The 1.1 Mb critical region contained 6 *Axd* candidate genes that were then sequenced. No mutations were found in the coding regions of these genes or the intron-exon boundaries. To determine if a regulatory mutation was responsible for the disease phenotype, the expression levels of the 6 candidate genes were measured. Only the expression of *Grhl2* was significantly altered in the *Axd*/*Axd* embryos; there was up to a 5-fold over-expression of *Grhl2*. *Axd*/+ mutants displayed an intermediate level of increased expression. Expression levels of *Grhl2* plotted against PNP length reveal a linear relationship indicating the up-regulation of Grhl2 correlates with a failure of PNP closure. In *situ* hybridization for *Grhl2* revealed more intense staining in *Axd*/*Axd* embryos than wild-type controls. *Grhl2* was expressed in otic vesicles, the pharyngeal region, forebrain, and hindgut endoderm. Further sequencing of *Grhl2* was done on 3’ and 5’ UTRs, including 1.6 kb upstream from the start codon and a 5’ conserved sequence; no mutations were found.

Data mining revealed *Grhl2* shares close homology and consensus-binding sequences with *Grhl3*. The down-regulation of *Grhl3* in *curly tail* (*ct*) mutants leads to spina bifida. Furthermore, *Grhl3* null mutants exhibited delayed eyelid closure (a feature of the *Axd* phenotype).

Excess expression of *Grhl2* appears responsible for NTDs in the *Axd* model based on linkage analysis, up-regulation of expression, and phenotypic similarities to *Grhl3* mutants. This could be further supported if the reduction of expression reduced the disease phenotype. A loss of function allele of *Grhl2* (*Grhl2GT*) was generated that also contained a reporter gene (β-geo). Heterozygous loss of function mutants (*Grhl2GT*/+) were stained and the location of expression was found to be comparable to expression seen from *in situ* hybridization and added branchial arches, nasal pits, Rathke’s pouch, and the ectoderm lining the limb buds as sites of expression . A small amount of the heterozygous embryos (15%) displayed cranial NTDs displayed as exencephaly (‘split face’). Interestingly, homozygous loss of function embryos (*Grhl2GT*/*GT*) all developed cranial NTDs and 88% developed spina bifida. The loss of *Grhl2* expression causing such a high frequency of NTDs indicates *Grhl2* as a requirement for neural tube closure.

Compound heterozygotes (Axd/*Grhl2GT*) were generated by crossing Axd/+ and *Grhl2GT*/+ and the expression of *Grhl2* was analyzed in the offspring. An expected up-regulation and down-regulation of *Grhl2* was seen in Axd/+ and *Grhl2GT*/+ embryos, respectively. PNP length was analyzed as well. *Grhl2GT*/+ and +/+ embryos completed spinal neurulation at similar stages of development, Axd/+ displayed an expected delay of PNP closure, and the compound heterozygotes Axd/*Grhl2GT* revealed an overall significant reduction in the length of PNP. Thus, a reduction of *Grhl2* expression levels acted to normalize PNP closure.

It was noted in *ct* mice, that a down-regulation of *Grhl3* caused a decrease in cellular proliferation in the hindgut. This led to increased ventral curvature that mechanically opposed PNP closure. In the *Axd* mice, *Grhl2* is up-regulated in the hindgut and increased ventral curvature was seen in *Axd*/*Axd* mice. In order to test if a similar mechanism causes the delay in or lack of PNP closure, researchers took a measure of the amount of cells undergoing mitosis in *Axd*/*Axd* embryos (mitotic index). There was a significantly lower mitotic index in the hindgut of *Axd*/*Axd* embryos compared to wild-type implicating that a decrease in cellular proliferation might be responsible for mechanical inhibition of neural tube closure.

Finally, due to the sequence similarities and their association of altered expression and NTDs, *Grhl2* and *Grhl3* were tested to see if they could genetically interact. *Axd*/+ and *ct*/*ct* were intercrossed and their offspring were analyzed for tail flexion defects (similar phenotype of both heterozygotes). Compound heterozygote (*Axd*/+;*ct*/+) embryos displayed a 2.5-fold increase of the curly tail phenotype than *ct*/+;+/+ embryos. This suggests that *Axd* and *ct* mutations can genetically interact to prolong spinal neural tube closure.

**Conclusion:**

In this study, the data determined a primary role for *Grhl2* in the *Axd* phenotype. *Grhl2* is located within the 1.1 Mb critical region, up-regulation is associated with increase in PNP closure time in *Axd*/*Axd* mice, over-expression of *Grhl2* is visible in the surface ectoderm and hindgut tissues (tissues implicated in NTDs) in *Axd*/*Axd* mice, loss of function of *Grhl2* causes NTDs, sequence similarity to *Grhl3* and shared phenotypes (loss of function of *Grhl3* causes NTDs and delayed eyelid fusion), and PNP closure is normalized in compound heterozygotes creating one loss of function *Grhl2* allele. The researches were well supported in titling the article, “Over-expression of *Grhl2* CAUSES spina bifida in the *Axial defects* mutant mouse”.

Furthermore, the *ct* and *Axd* mutations were shown to genetically interact to prolong PNP closure. It was also shown that a supported mechanism for PNP closure failure was mechanical inhibition caused by decreased proliferation in hindgut cells producing increased ventral curvature, the same mechanism seen in *ct* mice. These results and the similarities between *Grhl2* and *Grhl3* implicate this gene family in neural tube closure.

Together, the researchers have provided significant data implicating *Grhl2* as a candidate gene for human NTDs.

**Future Research and Critique:**

The first step of future work should be to attempt to characterize the *Axd* mutation. The researchers were unable to find the mutation, although they narrowed the range to a 1.1 Mb critical region. Also, *Grhl2* is a transcription factor, it appears that the *Axd* phenotype could be due to the misregulation of key downstream genes. It would be beneficial to see expression levels of targets in the *Axd* and *ct* models and the loss of function *Grhl2* mutants. This would reveal if the same downstream targets are responsible in each model and help elucidate why down-regulation of *Grhl2* causes cranial NTDs, but not up-regulation. All this future research would also be beneficial towards human diagnoses and treatments of NTDs.

I feel the researchers should have further attempted to discover the regulatory mutation of the *Axd* model. Only 6 genes in the 1.1 Mb critical region were sequenced. Furthermore, the article never explained why those 6 genes were chosen to be sequenced. Also, the discussion of the article had extensive new information that was not conferred prior and seemed to be more speculation about *Grhl2* and NTDs than their work. Finally, the dilemma of gaining knowledge, after the research there are now more questions than before.