Mouse genetics reveals Barttin as a genetic modifier of Joubert syndrome

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Genetic and phenotypic heterogeneity and the lack of sufficiently large patient cohorts pose a significant challenge to understanding genetic associations in rare disease. Here we identify Bsnd (alias Barttin) as a genetic modifier of cystic kidney disease in Joubert syndrome, using a Cep290-deficient mouse model to recapitulate the phenotypic variability observed in patients by mixing genetic backgrounds in a controlled manner and performing genome-wide analysis of these mice. Experimental down-regulation of Bsnd in the parental mouse strain phenocopied the severe cystic kidney phenotype. A common polymorphism within human BSND significantly associates with kidney disease severity in a patient cohort with CEP290 mutations. The striking phenotypic modifications we describe are a timely reminder of the value of mouse models and highlight the significant contribution of genetic background. Furthermore, if appropriate managed, this can be exploited as a powerful tool to elucidate mechanisms underlying human disease heterogeneity.

Significance

Our current understanding of genetic disease is often inadequate, largely due to genetic background effects that modify disease presentation. This is particularly challenging for rare diseases that lack sufficient numbers of patients for genome-wide association studies. We show in a series of experiments using a murine model of Joubert syndrome, a multisystem ciliopathy, that a single locus is a modifier of cystic kidney disease. We go on to show that the human homolog plays a similar role in disease using a cohort of patients. These findings make a significant contribution to the underplayed (and often ignored) role of genetic background in murine models and how this can be exploited to understand further rare inherited disease.

Rar e disease represents a healthcare burden estimated to affect ~350 million people worldwide (1). Phenotypic variability is a confounding factor in understanding genetic disease and often results from the underlying background genetics. The concept of modifying genes that have little or no effect on a phenotype in isolation but can alter the phenotype of a particular mutation was recognized a century ago in Drosophila (2) and subsequently shown to be an important factor in human disease (3). While the identification of modifying genes has been simplified by developments in genome-wide analyses and the availability of increasingly large patient cohorts (4, 5), genetic modifiers in rare disease remain inherently intractable due to the scarcity of patients.

Defects of the primary cilium result in a number of syndromes, collectively known as ciliopathies, that exemplify the challenges faced when attempting to understand genotype–phenotype heterogeneity in rare disease (6–8). Joubert syndrome is regarded as the archetypal ciliopathy, with one of the most common causes being biallelic mutations in the CEP290 gene (9, 10). However, mutations in CEP290 can result in a phenotypic spectrum (11–17) ranging from retinal degeneration alone (Leber congenital amaurosis) to embryonic lethality (Meckel–Gruber syndrome). In many cases, phenotypic variability is evident among patients harboring identical mutations, with no clear genotype–phenotype correlations.

Recently, the mouse has been shown to provide a good model for numerous ciliopathies and these models have begun to elucidate the underlying mechanisms of these diseases (18–21). Furthermore, there is evidence to suggest that murine ciliopathy phenotypes are influenced by genetic modifiers. The Cep290Gt(CC0582)Wtsi mouse, for example, which presents with retinal degeneration, slowly progressing cystic kidney disease, and hydrocephalus on a 129/Ola genetic background, is embryonic lethal on a C57BL/6 background and shows a variable phenotype ranging from embryonic lethality to severe cystic kidney disease on a 129/Sv genetic background (19). This heterogeneity is indicative of the presence of strain-specific genetic modifiers of the phenotype and is consistent with the heterogeneity seen in CEP290 ciliopathy patients.

Results and Discussion

In order to identify potential loci modifying murine Joubert syndrome and establish whether mouse genetics could provide a convenient way of identifying modifier genes in rare human diseases more generally, we intercrossed Cep290Gt(CC0582)Wtsi mice...
(a hypomorphic allele, MGI:3870362; hereafter referred to as Cep290<sup>Gt/Gt</sup> mice) to mix their genetic background in a controlled manner (Fig. 1A). Heterozygous Cep290<sup>Gt/Gt</sup> first generation (F1) (129/Ola x C57BL/6) animals were bred to generate homozygous Cep290<sup>Gt/Gt</sup> F2 mice that were analyzed at 3 wk of age. The number of homozygous mutant mice recovered from these crosses deviated significantly from the expected Mendelian ratio ($\chi^2$, $P < 0.005$), suggesting that $\sim$40% of homozygous animals died in utero, consistent with previous reports (22, 23). Viable homozygous animals tended to be smaller than littermates (6.6 vs. 11.6 g at postnatal day 21; SI Appendix, Fig. S1B), displaying varying degrees of cranial doming, a feature associated with hydrocephalus (SI Appendix, Fig. S1A).

Retinal abnormalities were observed in homozygous Cep290<sup>Gt/Gt</sup> F2 mice, similar to the phenotype previously described for the inbred (129/Ola) strain (19), with complete loss of the outer segment of the photoreceptor layer in all homozygous mutant animals (SI Appendix, Fig. S1C). This suggests that the photoreceptor phenotype is not affected by genetic modifiers, given that it is severe in both the genetic backgrounds we have studied. Variability in the extent of cell loss within the outer and inner nuclear and plexiform layers was observed but showed no consistent trends across the cohort, and association analyses revealed no genetic associations (SI Appendix, Fig. S2 E and F). It should be noted that at the time point of phenotyping (P21), retinal development is ongoing and therefore the observed variability may be in part due to differences in rates of development.

Homozygous F2 Cep290<sup>Gt/Gt</sup> mice displayed hydrocephalus, as previously reported for the inbred (129/Ola) strain (19). However, high-resolution ex vivo MRI (in a subset of animals) revealed structural defects within the cerebellum (Fig. 1B and C and SI Appendix, Fig. S1D) that are not found in mutant mice on a 129/Ola genetic background, indicating that the phenotype of the Cep290<sup>Gt/Gt</sup> Joubert syndrome mouse can be modified to include cerebellar aplasia phenotypes typical of patients by alteration of the genetic background. Cerebellar lobule-specific defects observed include hypoplasia of the folium–tuber vermis (lobule VII) and pyramus (VIII), with some mice also displaying additional degeneration within the uvula (IX) and nodulus (X). It is noteworthy that mutant mice with structural abnormalities of the nodulus (X), which forms part of the vestibulocerebellum and is crucial for maintaining balance, displayed overt symptoms of ataxia, a common feature of Joubert syndrome patients (10, 24, 25). It should be noted that the presence of foliation defects did not correlate with the degree of hydrocephalus, suggesting that additional, tissue-specific, phenotypic modifiers exist.

Fig. 1. Phenotypic spectrum of an F2 Cep290<sup>Gt/Gt</sup> mouse model of Joubert syndrome. (A) Schematic showing mouse breeding strategy. Cep290<sup>Gt/Gt</sup> heterozygous mice on a 129/Ola background were bred with wild-type C57BL/6 mice to generate F1 hybrids. F1 Cep290<sup>Gt/Gt</sup> heterozygous mice were then interbred to give F2 Cep290<sup>Gt/Gt</sup> homozygous animals with a randomized set of alleles from both parent strains. Representative alleles from 129/Ola and C57BL/6 mice are shown in yellow and blue, respectively. Variable coat colors in F2 mice from this cross are shown including black, chinchilla, albino, and agouti (Left to Right). (B) High-resolution ex vivo MRI showing morphologically aberrant cerebellar structures in P21 F2 Cep290<sup>Gt/Gt</sup> mice. A subset of F2 Cep290<sup>Gt/Gt</sup> mice shows localized aplasia within the cerebellum, specifically within the folium–tuber vermis, pyramus, uvula, and nodulus (lobules VII to X, respectively; blue overlay). Lobules I to VI appear largely unaffected (red overlay); flattening of the cerebellum against the skull can be observed in all Cep290<sup>Gt/Gt</sup> animals as a result of hydrocephalus. Representative images of wild-type control, mild, and severe animals are shown. (Scale bar, 1 mm.) (C) Quantification of the sagittal cross-sectional area of lobules I to VI compared with VII to X of the cerebellum in mild and severe F2 Cep290<sup>Gt/Gt</sup> animals (Student’s $t$ test, *$P < 0.01$; ns, not significant). (D) Hematoxylin and eosin (H&E) and Masson’s Trichrome images of kidneys from F2 Cep290<sup>Gt/Gt</sup> mice showing a large degree of phenotypic heterogeneity in terms of the number and size of cysts, as well as the amount of interstitial fibrosis as indicated by increased collagen deposition (blue staining). (Scale bars, 1 mm [main images] and 100 μm [expanded views].) (E) Immunofluorescence staining of P21 mouse kidney from F2 Cep290<sup>Gt/Gt</sup> mice showing the expression of the ciliary GTPase Arl13b (green) and the water channel Aquaporin 2 (Aqp2; magenta). The 2 Lower in each column are magnified regions from the Upper identified by dotted boxes. (Scale bars, 10 μm.) (F and G) Quantification of cilia length (F) and tortuosity (G) in F2 Cep290<sup>+/+</sup> and Cep290<sup>Gt/Gt</sup> animals (1-way ANOVA, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).
While the majority of homozygous mutant kidneys appeared grossly normal, several were pale and enlarged, resembling the kidney previously reported from a Cep290<sup>−/−</sup> mouse bred on a mixed C57BL/6/J-129SvJ background (23). Renal histopathology revealed a striking variability in cystic burden ranging in severity from a few small cysts to multiple large cysts with early stages of fibrosis and collagen deposition typical of nephronophthisis (Fig. 1D and SI Appendix, Fig. S1E). In order to avoid bias, cystic burden was quantified in an automated way to give a “cystic index” value that enabled the severity of kidney disease to be ranked from 0 to 52.5%, with the majority of animals (n = 66) falling in the 0 to 5% range (SI Appendix, Figs. S1F and S4). There was no correlation between cystic index of the kidneys and renal layer loss (SI Appendix, Fig. S1G), while the most severe cerebellar disease was associated with severe cystic kidney disease (SI Appendix, Fig. S1H).

Cep290, a transition-zone protein, has been shown to be required for normal cilia morphology in both human and mouse (19, 26–28). Immunofluorescence staining for Arl13b, a GTPase which is spatially restricted to the ciliary membrane, revealed elongated primary cilia in all F2 homozygous mutant kidneys compared with wild-type littermates (Fig. 1E and F). Costaining with Aquaporin 2 (Aqp2), a water channel found at the apical surface of principal cells of the collecting duct, revealed that cysts containing elongated cilia were found predominantly in the distal part of the nephron (Fig. 1E). Kidneys with the highest cystic index, however, presented with significantly longer cilia than those with a low cystic index, and a strong association was observed between cilia length and average cyst size (R<sup>2</sup> = 0.82, P < 0.0001; SI Appendix, Fig. S1I). Cilia from kidneys with a high cystic index also displayed increased tortuosity (Fig. 1E and G and SI Appendix, Fig. S1J), consistent with the elongated, tortuous cilia seen in Joubert syndrome patient renal biopsies (28). Furthermore, epithelial cells of the collecting duct showed an almost complete loss of Aqp2 expression from the apical surface in kidneys from the most severely affected animals (Fig. 1E).

Given that our cohort of F2 mutant mice presented with a range of phenotypes consistent with the presence of strain-specific modifier loci, we designed a panel of 932 informative single-nucleotide polymorphisms (SNPs; of which 789 subsequently passed quality control) across the genome (SI Appendix, Fig. S2A) to identify the most severe (Gt/Gt) mice from the population of homozygous Cep290<sup>−/−</sup> mice was 39, which was invariant across the cystic kidney phenotypic range (SI Appendix, Fig. S2C and D). The approach was first validated using coat color as a variable trait. As predicted, no correlation between cystic index of the kidneys and retinal layer loss (SI Appendix, Figs. S1F and S4). There was no correlation between cystic index of the kidneys and renal layer loss (SI Appendix, Fig. S1G), while the most severe cerebellar disease was associated with severe cystic kidney disease (SI Appendix, Fig. S1H).

Having identified a locus modifying the severity of kidney disease in murine Joubert syndrome, we went on to investigate loci associated with the severity of renal disease in Joubert syndrome patients. Initially, a cohort of 6 patients was assembled, 3 with early-onset end-stage renal disease (ESRD) and 3 with mild/no renal involvement, selected on the basis of having identical causative mutations in CEP290 [homozygous c.5668G>T; p.(Gly1890*)]. A whole exome-wide search of variants segregating with disease severity in these patients revealed over 300 potential modifier loci associated with kidney disease. However, selecting only loci within the region syntenic to the mouse modifier locus (human chromosome 1p32) resulted in SNPs associated with severe kidney disease in Joubert syndrome patients linked to just 2 adjacent genes—BSND (rs2500341) and TMEM61 (rs2253466) (Table 1).

Of note, Barttin expression is found in small cysts of homozygous F2 mutant mice but appears to be lost in large cysts (SI Appendix, Fig. S3A). Of note, Barttin expression is found in small cysts of homozygous F2 mutant mice but appears to be lost in large cysts (SI Appendix, Fig. S3A). Bartter syndrome patients with BSND mutations have reduced urine-concentrating capacity, resulting in increased urine production (polyuria) and chronic kidney disease which may progress to ESRD (31, 32), as in Joubert syndrome patients with renal involvement (nephronophthisis) (33, 34). It is noteworthy that the mutations in CFTR encoding an apical chloride channel may also modify cystic kidney disease phenotypes in patients with autosomal dominant polycystic kidney disease, highlighting the importance of chloride transport in cyst expansion (35). rs2500341 is located within the 5′ UTR of BSND and the variant associated with severity of kidney disease (G) has a frequency of 0.595 within 1000 Genomes (global) (https://www.internationalgenome.org) but is markedly more prevalent in European (G = 0.759) and American (G = 0.70) populations. In addition, rs2500341 is annotated as an eQTL, with the G allele linked with lower levels of BSND expression within the brain (https://gtexporta-lorg/home). Coupled with the reduced expression observed in the most severely affected mouse kidneys, we hypothesized that lower expression of Bsnd from the C57BL/6 locus could be responsible for increased cystic disease. To test this hypothesis, we sought to determine the consequences of reducing Bsnd expression in pure, inbred 129/Ola homozygous mutant Cep290<sup>−/−</sup> mice (Fig. 3A). Systemic administration, via tail vein injection, of a Bsnd antisense oligonucleotide (ASO) targeting the transcriptional start site over 12 d resulted in a significant reduction in Bsnd protein levels within the kidney (Fig. 3B and SI Appendix, Figs. S3C and S5A). Immunohistochemical analysis revealed loss of Aquaporin 2 in principal cells of the cortical collecting duct and primary cilia with increased tortuosity, compared with control cilia.
129/Ola Cep290Gt kidneys (Fig. 3 C and D). Injection of Bsnd ASO in wild-type 129/Ola mice did not produce any ciliary phenotype (SI Appendix, Fig. S5), consistent with the concept of a genetic modification that has little or no phenotypic effect in isolation. These data indicate that reduction of Bsnd within kidneys of pure 129/Ola Cep290Gt/Gt mutant mice phenocopies the severe F2 mice that are homozygous for C57BL/6–derived Bsnd. Furthermore, immunohistochemical analysis of a Joubert syndrome patient (NPH621: G/G at rs2500341; SI Appendix, Table S1) kidney biopsy at ESRD reveals a remarkably similar phenotype to the mouse models (both Bsnd knockdown in 129/Ola and severe F2 mutant Cep290GtGt), including loss of Aquaporin 2 expression and elongated, tortuous primary cilia (Fig. 3E). A reduction of Barttin expression in epithelial cells lining the cysts in kidney tissue from patient NPH621 can also be seen (SI Appendix, Fig. S3B), consistent with the findings observed in F2 Cep290GtGt mice.

Having identified Bsnd as a modifier gene for the renal manifestation of murine Joubert syndrome and observed confirmatory evidence in a patient biopsy, we extended our analysis in humans to assess the relevance of this finding in a wider cohort of CEP290 ciliopathy patients with variable phenotypes (SI Appendix, Table S1). Patients were defined as having a high severity...
A high disease score indicates limited or no residual kidney function and/or the presence of multiple cysts within the kidney. CKD, chronic kidney disease; ESRD, end-stage renal disease.

![Image](53x196 to 107x249)

**Table 1.** Common SNPs in **BSND** and **TMEM61** are associated with increased kidney involvement in Joubert syndrome patients with **CEP290** mutations

| Sample | CEP290 mutation 1 | CEP290 mutation 2 | Kidney phenotype | Kidney disease score† | MAF (C) | MAF (G) |
|--------|-------------------|-------------------|------------------|-----------------------|---------|---------|
| F394   | c.5668G>T; p.(Gly1890*) | c.5668G>T; p.(Gly1890*) | Enlarged cystic kidneys | High | GG | TT |
| F700   | c.5668G>T; p.(Gly1890*) | c.5668G>T; p.(Gly1890*) | Bilateral small hyperechogenic kidneys | High | GG | TT |
| F944   | c.5668G>T; p.(Gly1890*) | c.5668G>T; p.(Gly1890*) | Bilateral small hyperechogenic kidneys | High | GG | TT |
| B1106  | c.5668G>T; p.(Gly1890*) | c.5668G>T; p.(Gly1890*) | Hyperechogenic kidneys | Low | CG | CT |
| F02    | c.5668G>T; p.(Gly1890*) | c.5668G>T; p.(Gly1890*) | Hyperechogenic kidneys | Low | CG | CT |
| A1188  | c.5668G>T; p.(Gly1890*) | c.5668G>T; p.(Gly1890*) | Hyperechogenic kidneys | Low | CG | CT |

†Patients were scored based on their level of kidney function and the presence of cysts in the kidneys as shown by renal ultrasound scanning. A high disease score indicates limited or no residual kidney function and/or the presence of multiple cysts within the kidney. CKD, chronic kidney disease; ESRD, end-stage renal disease; MAF, minor allele frequency. Genotype is denoted as GG, TT, CG, or CT; where G is guanine, T is thymine, and C is cytosine.

Common SNPs in **BSND** and **TMEM61** are associated with increased kidney involvement in Joubert syndrome patients with **CEP290** mutations.

**Fig. 3.** Down-regulation of **Bnsd** in mouse recapitulates the human kidney phenotype. (A) Schematic showing the injection schedule of **Cep290** knockout (KO) animals from a 129/Ola background. Mice were i.v. injected with an antisense oligonucleotide (ASO) targeted against the **Bnsd** gene, which codes for the CLC-type chloride channel accessory protein Barttin. (B) Western blot of murine kidney showing the reduction in Barttin protein level following knockdown with **Bnsd** ASO. (C) Immunofluorescence images of P21 mouse kidney from F2 **Cep290** KO animals on a 129/Ola background showing the expression of Arl13b (green) and Aqp2 (magenta) following injection of an ASO targeted against **Bnsd**. (Scale bars, 5 μm.) (D) Quantification of cilia tortuosity in **Cep290** KO and **Cep290** KO animals (Student’s t test, *P < 0.05). (E) Immunofluorescence images showing the expression of Arl13b (green) and Aqp2 (magenta) in a section of a kidney biopsy from a Joubert syndrome patient (NPH621: G/G at rs2500341; **SI Appendix, Table S1**) with end-stage renal disease secondary to mutations in **CEP290**. (Scale bars, 10 μm.)

Table 1. Common SNPs in **BSND** and **TMEM61** are associated with increased kidney involvement in Joubert syndrome patients with **CEP290** mutations

| Sample | CEP290 mutation 1 | CEP290 mutation 2 | Kidney phenotype | Kidney disease score† | MAF (C) | MAF (G) |
|--------|-------------------|-------------------|------------------|-----------------------|---------|---------|
| F394   | c.5668G>T; p.(Gly1890*) | c.5668G>T; p.(Gly1890*) | Enlarged cystic kidneys | High | GG | TT |
| F700   | c.5668G>T; p.(Gly1890*) | c.5668G>T; p.(Gly1890*) | Bilateral small hyperechogenic kidneys | High | GG | TT |
| F944   | c.5668G>T; p.(Gly1890*) | c.5668G>T; p.(Gly1890*) | Bilateral small hyperechogenic kidneys | High | GG | TT |
| B1106  | c.5668G>T; p.(Gly1890*) | c.5668G>T; p.(Gly1890*) | Hyperechogenic kidneys | Low | CG | CT |
| F02    | c.5668G>T; p.(Gly1890*) | c.5668G>T; p.(Gly1890*) | Hyperechogenic kidneys | Low | CG | CT |
| A1188  | c.5668G>T; p.(Gly1890*) | c.5668G>T; p.(Gly1890*) | Hyperechogenic kidneys | Low | CG | CT |

†Patients were scored based on their level of kidney function and the presence of cysts in the kidneys as shown by renal ultrasound scanning. A high disease score indicates limited or no residual kidney function and/or the presence of multiple cysts within the kidney. CKD, chronic kidney disease; ESRD, end-stage renal disease; MAF, minor allele frequency. Genotype is denoted as GG, TT, CG, or CT; where G is guanine, T is thymine, and C is cytosine.

**Materials and Methods**

Further information can be found in **SI Appendix, Figs. S1–S6, and Table S1**.

**Statistics.** All individual tests of numerical data were performed using an unpaired Student’s t test or a 1-way ANOVA followed by a Bonferroni-corrected post hoc test when comparing 2 or more groups. A P value of less than 0.05 was considered statistically significant.

**Study Approval.** Ethical approval was obtained from the National Research Ethics Service Committee North East (14/NE/1076), United Kingdom. All animal experiments were performed under licenses granted from the Home Office (United Kingdom) in accordance with the guidelines and regulations for the care and use of laboratory animals outlined by the Animals (Scientific Procedures) Act 1986, and conducted according to protocols approved by the Animal Ethics Committee of Newcastle University and the Home Office, United Kingdom.

**Subjects.** We obtained DNA samples (blood/saliva) after obtaining informed consent from individuals with Joubert syndrome (with and without renal involvement) and Leber congenital amaurosis. Criteria for Joubert syndrome were based on the presence of cerebellar vermis aplasia/hypoplasia and/or molar tooth sign on brain MRI. Leber congenital amaurosis/retinal degeneration was diagnosed by an ophthalmologist. The diagnosis of renal disease was based on clinical course and renal ultrasound scan results.

**Data Availability.** We declare that all data supporting the findings of this study are available within the article, **SI Appendix**.
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