NFIA haploinsufficiency is associated with a CNS malformation syndrome and urinary tract defects

LU, Weining, et al.

Abstract

Complex central nervous system (CNS) malformations frequently coexist with other developmental abnormalities, but whether the associated defects share a common genetic basis is often unclear. We describe five individuals who share phenotypically related CNS malformations and in some cases urinary tract defects, and also haploinsufficiency for the NFIA transcription factor gene due to chromosomal translocation or deletion. Two individuals have balanced translocations that disrupt NFIA. A third individual and two half-siblings in an unrelated family have interstitial microdeletions that include NFIA. All five individuals exhibit similar CNS malformations consisting of a thin, hypoplastic, or absent corpus callosum, and hydrocephalus or ventriculomegaly. The majority of these individuals also exhibit Chiari type I malformation, tethered spinal cord, and urinary tract defects that include vesicoureteral reflux. Other genes are also broken or deleted in all five individuals, and may contribute to the phenotype. However, the only common genetic defect is NFIA haploinsufficiency. In addition, previous analyses of Nfia(-/-) [...]
NFIA Haploinsufficiency Is Associated with a CNS Malformation Syndrome and Urinary Tract Defects

Weining Lu1,2,a, Fabiola Quintero-Rivera3,4,a, Yanli Fan1,a, Fowzam S. Alkurya1, Diana J. Donovan4, Qiongchao Xi1, Annick Turbe-Doan5, Qing-Gang Li2, Craig G. Campbell5, Alan L. Shanske6, Elliott H. Sherr7, Ayesha Ahmad8, Roxana Peters1, Benedict Rilliet9, Paloma Parvex10, Alexander G. Bassuk11, David J. Harris12, Heather Ferguson13, Chantal Kelly13, Christopher A. Walsh12,14,15, Richard M. Gronostajski16, Koenraad Devriendt17, Anne Higgins4, Azra H. Ligon4, Bradley J. Quade4, Cynthia C. Morton4,6,13, James F. Gusella3, Richard L. Maas1*

1 Genetics Division, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts, United States of America, 2 Renal Section, Boston University Medical Center, Boston, Massachusetts, United States of America, 3 Center for Human Genetic Research, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, United States of America, 4 Department of Pathology, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts, United States of America, 5 Division of Neurology, Children’s Hospital of Western Ontario, London, Ontario, Canada, 6 Children’s Hospital at Montefiore, Albert Einstein College of Medicine, Bronx, New York, United States of America, 7 Department of Neurology, University of California San Francisco, San Francisco, California, United States of America, 8 Division of Genetic and Metabolic Disorders, Department of Pediatrics, Wayne State University, Detroit, Michigan, United States of America, 9 Department of Neurosurgery, University Hospital, Geneva, Switzerland, 10 Department of Nephrology, University Hospital, Geneva, Switzerland, 11 Departments of Pediatrics and Neurology, Northwestern University Feinberg School of Medicine, Chicago, Illinois, United States of America, 12 Genetics Division, Children’s Hospital Boston and Harvard Medical School, Boston, Massachusetts, United States of America, 13 Department of Obstetrics, Gynecology and Reproductive Biology, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts, United States of America, 14 Department of Neurology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts, United States of America, 15 Howard Hughes Medical Institute, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts, United States of America, 16 Department of Biochemistry, State University of New York at Buffalo, Buffalo, New York, United States of America, 17 Centre for Human Genetics, University of Leuven, Leuven, Belgium

Complex central nervous system (CNS) malformations frequently coexist with other developmental abnormalities, but whether the associated defects share a common genetic basis is often unclear. We describe five individuals who share phenotypically related CNS malformations and in some cases urinary tract defects, and also haploinsufficiency for the NFIA transcription factor gene due to chromosomal translocation or deletion. Two individuals have balanced translocations that disrupt NFIA. A third individual and two half-siblings in an unrelated family have interstitial microdeletions that include NFIA. All five individuals exhibit similar CNS malformations consisting of a thin, hypoplastic, or absent corpus callosum, and hydrocephalus or ventriculomegaly. The majority of these individuals also exhibit Chiari type I malformation, tethered spinal cord, and urinary tract defects that include vesicoureteral reflux. Other genes are also broken or deleted in all five individuals, and may contribute to the phenotype. However, the only common genetic defect is NFIA haploinsufficiency. In addition, previous analyses of NFIA knockout mice indicate that Nfia deficiency also results in hydrocephalus and agenesis of the corpus callosum. Further investigation of the mouse Nfia+/− and Nfia−/− phenotypes now reveals that, at reduced penetrance, Nfia is also required in a dosage-sensitive manner for ureteral and renal development. Nfia is expressed in the developing ureter and metanephric mesenchyme, and Nfia+/− and Nfia−/− mice exhibit abnormalities of the ureteropelvic and ureterovesical junctions, as well as bifid and megaureter. Collectively, the mouse Nfia mutant phenotype and the common features among these five human cases indicate that NFIA haploinsufficiency contributes to a novel human CNS malformation syndrome that can also include ureteral and renal defects.

Introduction

Complex human developmental phenotypes represent an especially difficult problem in human genetics. In many cases, congenital birth defects are believed to result from the combined effect of many genes, often with an environmental contribution, and frequently culminate in perinatal demise. Thus, for many cases, extended families do not exist, and approaches to disease gene identification based on linkage analysis are not possible. In addition, many developmental disorders are genetically heterogeneous, making the ascertainment of single contributory genes difficult.

The analysis of human balanced chromosome rearrangements offers a potential approach to this problem. Although
unforeseen rearrangements and position effects may super- vene [1,2], and a background rate of birth defects exists, human translocations provide powerful tools to identify genes that are essential to human development. Translocations may result in haploinsufficiency, the generation of fusion transcripts, or position effects, or act in combination with a second loss-of-function allele. The Developmental Genome Anatomy Project, DGAP (http://dgap.harvard.edu), has as its specific goal the ascertainment, recruitment, and analysis of individuals with chromosomal rearrangements and developmental disorders. A conspicuous class of such disorders is that involving the formation of the CNS and visceral organs.

Within the CNS, the corpus callosum is the largest interconnecting white matter tract in the brain, and it connects the association fibers of both hemispheres. Agenesis of the corpus callosum (ACC) is among the most common brain malformations in humans, with an incidence of 1 per 4,000 live births [3–5] and a prevalence as high as 3%-5% in individuals with neurodevelopmental disabilities [6,7]. In human embryos, the corpus callosum begins to develop at 11–12 wk gestation when the first fibers cross the midline to form the genu in the region of the comissural plate, and subsequent development proceeds from anterior to posterior, with formation of the anterior body, posterior body, and splenium, followed by a progressive enlargement that reflects the rapid expansion of the cerebral hemispheres [8]. Abnormalities of the corpus callosum can occur through a number of mechanisms, including defects in the genesis or survival of neuronal cells whose axons form the corpus callosum, and defects in axonal outgrowth, pathfinding, and midline crossing [9]. The etiology of ACC is thus heterogeneous and multifactorial, and both autosomal recessive and X-linked recessive mechanisms have been described [9] (see also Online Mendelian Inheritance in Man [OMIM, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM]). ACC is associated with certain chromosomal rearrangements [9] and occurs as a component of other genetic syndromes [10] and metabolic conditions [11], but its genetic heterogeneity and phenotypic pleiotropy have limited identification of the responsible genes to only a few of the more than 20 distinct loci that are associated with ACC, including one on the short arm of Chromosome 1 [12].

In addition to other CNS defects with which ACC is frequently associated, ACC may occur in conjunction with visceral organ malformations. For example, ACC and its associated brain and spinal cord lesions have been linked to vesicoureteral reflux (VUR), cystic kidney disease, renal agenesis and insufficiency, and neurogenic lower urinary tract dysfunction, a condition that includes neurogenic bladder and VUR [13–16]. Alternatively, single gene mutations may produce developmental defects in both the CNS and urinary tract. We show here that a novel human syndrome involving both CNS and urinary tract defects is associated with disruption or deletion of the NFIA gene at 1p31.3, which encodes a member of the Nuclear Factor I (NFI) family of transcription factors [17]. Disruption of Nfia in mice results in perinatal lethality, hydrocephalus, and ACC [18], and a recent study shows that NFIA controls the transition from neurogenesis to gliogenesis in the developing spinal cord [19]. Similar studies indicate that two other Nfi family members, Nfia and Nfib, are essential for lung, brain and tooth development [20,21]. However, the role that NFI transcription factors play in human disease has been unknown. Our results establish that NFIA haploinsufficiency is a likely contributor to a range of CNS defects, including ACC, hydrocephalus, ventriculomegaly, Chiari type I malformation, and tethered spinal cord, and that renal defects can also result from a disturbance in ureteral development.

Results

CNS and Urinary Tract Defects in Individuals with 1p31 Rearrangements

We investigated five individuals enrolled in DGAP (i.e., DGAP104, 089, 174, 205–1, and 205–1s), each with a similar spectrum of CNS defects, and in three cases, of urinary tract defects. All five individuals had chromosomal rearrangements that variously involved 1p31. DGAP104 is a 6-y-old female diagnosed at birth with congenital hydrocephalus, a thin corpus callosum, Chiari I malformation, tethered spinal cord, and a low vertebral deformity (Figure 1A, 1B, and 1K). She was also found to have congenital bilateral dysplastic kidneys, and subsequently developed bilateral VUR, pylonephritis, a ureterovesical junction diverticulum, and hydronephrosis, and required ureteral reimplantation surgery at age 2 y (Figure 1L; Tables 1 and S1). Chromosome analysis in the neonatal period revealed an apparent de novo balanced chromosome translocation between 1p31 and 20q13.

A second individual, DGAP089, is an 8-y-old male whose clinical profile was described previously (Table 1, S1) [22]. He has both an interstitial deletion on 2q and a balanced translocation involving 1p and 2q [22]. As an infant, he had poor fetal movement, and a CT scan revealed a vertical orientation of the ventricles consistent with a primary defect of the corpus callosum. At age 2 y, a CT scan revealed ventriculomegaly, and at age 6.5 y a brain MRI showed a hypoplastic corpus callosum, nonprogressive ventriculomegaly, and a gray matter heterotopia (Figure 1C and 1D; Tables 1 and S1). Renal ultrasound revealed no major abnormalities.
but a definitive evaluation for urinary reflux (i.e., a voiding cystourethrogram, or VCUG) was not performed.

A third individual, DGAP174, who exhibited complete ACC and enlarged ventricles by second trimester ultrasound, had both a de novo translocation of 1p31.1 and 3q25.1 and an interstitial deletion of 1p31. A postnatal brain CT scan confirmed these findings and also revealed ventriculomegaly and a tethered spinal cord. A brain MRI at age 3 y revealed a Chiari type I malformation, a downward displacement of the tip of the cerebellar tonsils below the foramen magnum, was found in DGAP104 (arrowhead in A), DGAP174 (arrowhead in E), and DGAP205-1s (arrowhead in I). Congenital ventriculomegaly is present in DGAP089 (D), DGAP174 (F), and DGAP205-1s (J), and hydrocephalus was found in DGAP104 (B) and DGAP205–1 (H). An occipital shunt (arrow in B) was placed in DGAP104 to relieve severe hydrocephalus.

(K) DGAP104 MRI shows a tethered spinal cord, with the extremity of the conus medullaris (arrow) at the level of the L4 vertebral body. Arrowhead shows a fishhook deformity of the lower sacral and coccygeal vertebrae.

(1) VCUG of DGAP104 depicts left vesicoureteral reflux with retrograde tracking of dye through the ureter into the renal pelvis (arrow) and a right diverticulum at the ureterovesical junction (arrowhead).

(M) Spine MRI of DGAP205–1 shows a tethered spinal cord with conus lying at the L3/L4 level (arrow).

(N) VCUG of DGAP205–1 shows left vesicoureteral reflux (arrow).

(O) Spine MRI of DGAP205–1s depicts a tethered spinal cord with conus lying at L5 (arrow).

doi:10.1371/journal.pgen.0030080.g001

Cytogenetic and Genetic Analyses of the Chromosomal Rearrangements

To determine whether a common genetic defect underlay the phenotypes of these individuals, we analyzed the 1p31 region in all five patients and found that the NFI gene was either disrupted or deleted in each case. DGAP104 has a de novo balanced translocation, 46,XX,t(1;20)(p31.3;q13.31)dn (Figure 2A, 2B). By metaphase fluorescence in situ hybridization (FISH), we identified a bacterial artificial chromosome (BAC) clone (RP4-802A10) that mapped to 1p31.3 and hybridized to the breakpoints of the der(1) and der(20) chromosomes (Figure 2C). The translocation breakpoint at 1p31.3 disrupts intron 2 of NFI, which is composed of 11...
phenotype deletion interval may thus also contribute to the DGAP089 breakpoint, for which the karyotype is 46,XY,t(1;2) interstitial deletion in 2q proximal to the 2q translocation. However, by RT-PCR, these intervals is interstitial deletion, del(1)(p31.3p32.1)dn. The 1p31.1 and DGAP174, who has both a t(1;3)(p31.1;q25.1)dn and an observed in DGAP104 and DGAP089, we next investigated primarily associated with the congenital CNS anomalies possible. On the other hand, the chromosome deletion in hence, a contribution to the DGAP174 CNS phenotype is hence localizing the breakpoint to 1p31.3 breakpoint-spanning BAC, RP5-902P15, which hybridized to Chromosome 1 and to both der(1) and der(2) chromosomes (Figure 2D). Subsequent FISH and Southern blot analyses (Figure S3) refined the breakpoint to ~9.9 kb between exons 7 and 8 of NFIA (Figure 2I). Similar analyses of the 2q breakpoint revealed a split signal for BAC RP11-745P9, thus localizing the breakpoint to ~138 kb in 2q22.1, which contains no annotated genes. However, metaphase FISH followed by aCGH at 1 Mb resolution revealed a ~12-Mb interstitial deletion in 2q proximal to the 2q translocation breakpoint, for which the karyotype is 46,XY,t(1;2)(p31.3q22.1),del(2)(q14.3q21)dn. The 39 genes within this deletion interval may thus also contribute to the DGAP089 phenotype [22].

To establish further whether disruption of NFIA is primarily associated with the congenital CNS anomalies observed in DGAP104 and DGAP089, we next investigated DGAP174, who has both a t(1;3)(p31.1q25.1)dn and an interstitial deletion, del(1)(p31.3p32.1)dn. The 1p31.1 and 3q25.1 translocation breakpoints were refined to 150 and 180 kb, respectively. The other potentially relevant gene in these intervals is NEGR1, which is disrupted by the 1p31.1 breakpoint. In rat, NEGR1 protein is expressed only after E16, with peak expression occurring postnatally, after corpus callosum formation [24]. However, by RT-PCR, NEGR1 transcript is expressed in human cerebral cortex, hippocampus, corpus callosum, and cerebellum (unpublished data); hence, a contribution to the DGAP174 CNS phenotype is possible. On the other hand, the chromosome deletion in DGAP174, which was delimited by FISH and aCGH to 2.2 Mb at 1p31.3–1p32.1 (Figures 2E and S4), also results in the complete deletion of NFIA and of eight additional genes (Figure 2I).

Lastly, we performed metaphase FISH analyses on chromosomes isolated from the two half-siblings DGAP205–1 and DGAP205–1s and their mother DGAP205–2 (Figure 2F–I). The 1p31.3–1p32.3 region, encompassing ~12 Mb and containing the entire NFIA gene and ~47 additional genes, is deleted in both DGAP205–1 and DGAP205–2 (Figure 2G–2I). The mother, DGAP205–2, is phenotypically normal but has an apparent balanced rearrangement in which 1p31.3p32.3 is inserted into Chromosome 4 with no loss of genetic material, and her karyotype is therefore designated 46,XX,ins(4;1)(q35;p31.3p32.3) (Figure 2H) [23].

NFIA Haploinsufficiency Is Common to All Five Cases
All five individuals share strikingly similar CNS phenotypes, including abnormalities of the corpus callosum, hydrocephalus, and ventriculomegaly. All also share disruption or deletion of NFIA, and in each case, the nonrearranged or nondeleted NFIA allele was subjected to DNA sequencing and no mutations were identified (unpublished data). Therefore, all five cases have NFIA haploinsufficiency in common. NFIA is highly expressed in multiple regions of the human brain, including the embryonic and adult corpus callosum (Figure S5) [25], and ACC and hydrocephalus were observed in Nfia−/− mutant mice [18].

In each DGAP case, one or more additional genes were also directly affected as a consequence of either the translocation or deletion. These additional genes may contribute to or modify the nature of the phenotype attributable to NFIA disruption or deletion. However, the identities of these genes differ among the five DGAP cases, except for 205–1 and 205–1s, who share the same 12-Mb deletion; and 205–1, 205–1s, and 174, who share a common 2.2-Mb deletion region (Figure 2F). C20orf32 is disrupted in addition to NFIA in DGAP104, whereas in DGAP089 disruption of NEGR1 and deletion of 39 genes in del(2)(q14.3q21) occurred. Besides the disruption or deletion of NFIA, none of the other of these genetic aberrations is shared by more than three cases. Therefore,

Table 1. Common Clinical Features in Five Individuals with NFIA Haploinsufficiency

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>DGAP089</th>
<th>DGAP104</th>
<th>DGAP174</th>
<th>DGAP205–1</th>
<th>DGAP205–1s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Karyotype</td>
<td>46,XY,t(1;2)(p31.3q22.1),del(2)(q14.3q21)dn</td>
<td>46,XX,t(1;20)(p31.3q13.31)dn</td>
<td>46,XY,t(1;3)(p31.1q25.1),del(1)(p31.3p32.1)dn</td>
<td>46,XY,del(1)(p31.2p32.3)mat</td>
<td>46,XX,del(1)(p31.2p32.3)mat</td>
</tr>
<tr>
<td>Phenotype</td>
<td>Abnormal corpus callosum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Ventriculomegaly or hydrocephalus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Developmental delay</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Tethered spinal cord</td>
<td>nd</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Chiari I malformation</td>
<td>nd</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Seizures</td>
<td>+</td>
<td>+</td>
<td>nd</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Urinary tract defects</td>
<td>nd</td>
<td>+</td>
<td>nd</td>
<td>+</td>
</tr>
</tbody>
</table>

d, not determined
doi:10.1371/journal.pgen.0030080.t001
Figure 2. *NFIA* is Deleted or Disrupted in All Five Individuals with 1p31.3 Rearrangements

(A and B) Partial karyogram (A) and ideogram (B) of DGAP104 show the chromosome translocation t(1;20)(p31.3;q13.31)dn. (C) FISH analysis of DGAP104; BAC RP4-802A10 (red signals) hybridizes to the normal Chromosome 1 and der(1) and der(20) chromosomes, thus spanning the 1p31.3 breakpoint. (D) FISH analysis of DGAP089 depicts BAC RP5-902P15 (red signals) hybridization to the normal Chromosome 1 and der(1) and der(2) chromosomes, thus spanning the 1p31.3 breakpoint. (E) FISH analysis reveals that BAC RP5-902P15 (overlapping with *NFIA*, orange color) is deleted from the der(1) in DGAP174. The nondeleted BAC RP11-134C1 (green) is present on both the normal and derivative Chromosome 1. (F) DGAP104 pedigree with *NFIA* deletion (arrow indicates the DGAP205–1 proband). DGAP205–1 and half-sister DGAP205–1s have an unbalanced interstitial microdeletion, del(1)(p31.3p32.3), while their phenotypically normal mother DGAP205–2 has a balanced chromosome rearrangement due to an insertion of 1p31.3-p32.3 into Chromosome 4, ins(4;1)(q35;p31.3p32.3). del-*NFIA*, deletion of *NFIA*; ins-*NFIA*, insertion of *NFIA*. (G and H) FISH analyses show that BAC RP5-902P15 (overlapping with *NFIA*, orange color) is absent from the der(1) in DGAP205–1 (G) and der(1) in the mother DGAP205–2 (H), but present in the der(4) in the latter (H). The nondeleted BAC RP4-654H19 (green) is present on both the normal and derivative Chromosome 1. (I) *NFIA* exon–intron structure is shown in the upper part with select exons numbered, and the relevant BAC contig below. Locations of the 1p31.3 translocation breakpoints in DGAP104 and DGAP089 are indicated by red dotted vertical lines. The lower part of (I) depicts 1p31.3–1p32.3 genomic regions with the cytogenetic bands on the short arm of Chromosome 1. TEL represents telomeric orientation, and CEN represents centromeric orientation. Known genes in this region are represented by dots and gene names. FISH-verified BAC clones are represented by horizontal bars. The full names of the BAC clones are listed in Materials and Methods. A 2.2-Mb genomic region deleted in DGAP174 and a ~12-Mb genomic region deleted in DGAP205–1 and 205–1s are shown. Deleted BAC clones tested by FISH are designated in red and nondeleted clones in blue. BAC 802A10 overlaps with *NFIA* and is deleted.

doi:10.1371/journal.pgen.0030080.g002
NFIA Haploinsufficiency in Human and Mouse

To gain further evidence for the assignment of NFIA as the gene responsible for the CNS and urinary tract defects observed in the five individuals, we re-investigated the previously described Nfia−/− knockout mouse [18]. Because syringomyelia, a persistent patency of the central canal within the spinal cord, is often associated with Chiari type I malformation [26] and was observed in DGAP205-1s but not previously noted in Nfia−/− mice [18], we first examined Nfia expression in the developing spinal cord (Figure 3A–3D). From E11.5–13.5, the expression of Nfia in the spinal cord moves dynamically from rostral to caudal as development proceeds, coincident with the timing of central canal closure (Figure 3A, 3B). Moreover, Nfia expression resides in the basal ventricular zone surrounding the central canal (Figure 3C, 3D). Four of six (66%) Nfia−/− newborns exhibited syringomyelia that was manifest as an enlarged central canal and principally confined to the lumbar region (Figure 3E, 3F). Syringomyelia has been proposed to result from progressive, mechanical overload and dissection of the rostral spinal cord by elevated cerebral spinal fluid hydrostatic pressure such as that associated with hydrocephalus [27]. Indeed, all five individuals with NFIA haploinsufficiency exhibited hydrocephalus or ventriculomegaly. However, mouse Nfia expression in the spinal cord surrounds the central canal and defective canal closure in Nfia−/− mutants precedes the development of hydrocephalus. This suggests that syringomyelia may result from a developmental defect in the dynamic process of canal closure.

NFIA Expression and Abnormal Kidney Development in Nfia Mutants

DGAP104, 205–1 and 205–1s share similar urinary tract phenotypes. Although the CNS defects in all five individuals are consistent with findings in mouse Nfia mutants [18], it remained possible that the urinary tract defects might result from the genetic lesion at 20q13.31 (in DGAP104), from haploinsufficiency for other genes in the 1p31.3-p32.3 deletion interval (in DGAP205–1 and DGAP205-1s), or from incidental mutations in other loci. Previously, it was noted that Nfia homozygotes died shortly after birth [18]. Based upon prior experience with mouse mutants with renal agenesis and dysplasia [28–32], we hypothesized that some part of this perinatal lethality might be explained by defects in renal development.

To determine whether the urinary tract phenotypes in DGAP104, 205–1, and 205–1s were linked to NFIA disruption, we investigated Nfia expression in the developing murine kidney and renal morphology in Nfia−/− mice. By in situ hybridization, Nfia was abundantly expressed in the developing nephric duct and metanephros from E9.5 to E16.5, correlating with the stages of ureteric bud outgrowth, hydrocephalus, and urinary tract defects (Table S2). Sequence analysis was also performed on a group of 96 individuals that included both syndromic and nonsyndromic ACC, and on another group of 39 individuals with nonsyndromic tethered cord syndrome. Although several known SNPs were detected, no intragenic mutations were identified. Thus, intragenic mutation in NFIA is not a frequent cause of the CNS defects described here.

Further Analysis of Mouse Nfia Mutants Reveals Additional Phenotypes

The most parsimonious explanation for the observed CNS phenotypes is NFIA haploinsufficiency, which is the only common genetic defect shared by all five individuals.

To test whether intragenic mutations in NFIA are associated with abnormal callosal development and other CNS phenotypes, we sequenced the 11 exons and intron-exon boundaries of NFIA in 84 patients with various combinations of syndromic CNS phenotypes, including abnormal corpus callosum, tethered spinal cord, Chiari I malformation,
metanephric induction, and rapid nephron morphogenesis; expression began to be downregulated at E17.5 (Figure 4A–4I). At E9.5, Nfia expression appeared in the nephric duct and persisted at E10.5 and 11.5 in the developing ureter, becoming more restricted to the distal ureter by E12.5 (Figure 4A and 4B). Nfia was expressed in both ureteric bud epithelium and the surrounding mesenchyme at these time points (Figure 4E and 4F). By E16.5, Nfia expression became restricted to stromal mesenchyme, and was downregulated a day later (Figure 4C, 4D, 4G, and 4H).

We next examined the kidney morphology of Nfia\textsuperscript{+/−}\textsuperscript{−}/C0 newborn mice. Remarkably, 13 of 19 (68\%) Nfia\textsuperscript{+/−}\textsuperscript{−}/C0 newborns displayed agenesis, dysplastic, cystic, or duplex kidneys (Figure 5A–5K). A small number of Nfia\textsuperscript{+/−}\textsuperscript{−} mutants exhibited a partial duplex kidney phenotype. This was apparent from both the elongated kidney morphology with a central constriction, and from histological evidence of an ectopic nephrogenic zone that demarcated discrete rostral and caudal nephric poles (Figure 5D, 5H, and 5I).

The presence of kidney defects was not confined to Nfia\textsuperscript{+/−}\textsuperscript{−} mutants. Four of 18 (22\%) of Nfia\textsuperscript{+/−}\textsuperscript{−} newborn mice also expressed hydronephrosis (Figure 5B). The possibility that these Nfia\textsuperscript{+/−}\textsuperscript{−} newborns might be Nfia\textsuperscript{−/−} mice that were misgenotyped was excluded because the Nfia\textsuperscript{+/−}\textsuperscript{−} affecteds were offspring of an Nfia\textsuperscript{+/−}\textsuperscript{−} x Nfia\textsuperscript{−/−} cross. The sensitivity of mouse kidney development to Nfia gene dosage supports the conclusion that kidney defects can occur in humans carrying disruption or loss of a single NFIA allele. Therefore, disruption or deletion of NFIA in DGAP104, 205–1, and 205–1s likely explains the kidney phenotypes observed in these individuals.

We next sought to characterize further the renal defects in Nfia\textsuperscript{−/−} embryos by marker experiments. Markers analyzed included Wt1 (glomerular podocytes), DBA (distal tubule and collecting duct), LTL (proximal tubule), and E-cadherin (cell adhesion and aggregation). No consistent changes from wild type that would reflect a deficiency of a particular cell type were observed (unpublished data). These results suggest that the renal defects in Nfia mutants are not lineage- or segment-specific.

NFIA Haploinsufficiency in Human and Mouse

NFIA Kidney Defects Involve Defective Ureteral Development

The relative preservation of tubular and glomerular markers in Nfia mutant kidneys, the diverse spectrum of renal abnormalities, and the presence of hydronephrosis suggested that the renal parenchymal changes might be secondary to ureteral reflux or obstruction. To test this hypothesis, we crossed a Hoxb7 promoter-directed GFP reporter allele into the Nfia\textsuperscript{+/−}\textsuperscript{−} and Nfia\textsuperscript{−/−} backgrounds. The Hoxb7-GFP transgene is specifically expressed in ureteral epithelium as early as E10.5, and thereafter in the ureteric

Figure 4. Nfia Expression in the Developing Mouse Urinary Tract

(A–D) Whole mount in situ hybridization shows Nfia expression in the developing nephric duct (arrow in A), ureter (arrow in B), and metanephros between E9.5 (not shown) and E16.5, becoming downregulated at E17.5. Reticular pattern of Nfia expression is in the kidney from E14.5–16.5 (C). (E–H) Section in situ hybridizations shows Nfia expression in the ureteral epithelium (arrow in F) and surrounding mesenchyme (arrowhead in F) at E11.5 (F is the enlarged view of boxed region in E), and in stromal mesenchyme (arrows in G and H) at E16.5–17.5. (I) RT-PCR of the developing mouse kidney showing that Nfia is abundantly expressed up to E16.5 and begins to be downregulated at E17.5. \(\beta\)-actin is used as RNA loading control.

NB, newborn.
doi:10.1371/journal.pgen.0030080.g004
bud and its derivatives, and eventually in renal tubular epithelium [33].

Remarkably, when assayed by GFP expression, four of 20 (20%) Nfia\(^{+/+}\) and two of six (33%) Nfia\(^{+/−}\) newborns exhibited clear abnormalities of ureteral development (Figure 6A–6F). These abnormalities fell into three classes. First, we observed a partial duplication of the ureter (Figure 6C), which correlates with the duplex kidney phenotype. A second class
consisted of mutant ureters that were dilated, either as a consequence of obstruction or from reflux (Figure 6C and 6D). In the third class, we observed abnormal flexure of the rostral ureter, with the site of flexion near the renal pelvis (Figure 6E), which could lead to obstruction. To determine whether prolonged reflux and obstruction in Nfia−/− mutants affected postnatal kidney development, we analyzed two rare Nfia−/− postnatal survivors. Both P16 Nfia−/− developed severe hydronephrosis, whereas kidneys in their wild-type littermates were normal (Figure S6).

Lastly, we analyzed the histology of two key ureteral structures, the ureteropelvic junction (UPJ), which connects the ureter to the kidney, and the ureterovesical junction (UVJ), which connects the ureter to the bladder. Both UPJ and UVJ histological defects were noted in Nfia−/− newborns, while only UPJ defects were identified in Nfia−/− newborns (Figure 7A–7F). The presence of UPJ and UVJ dilation in Nfia mutant mice (Figure 7B, 7C, and 7E) is consistent with the observations of VUR and hydronephrosis in DGAP104, 205–1, and 205–1s. Thus, abnormalities in ureteral development comprise a significant part of the Nfia mutant phenotype. Moreover, because proper formation of the vertebrate kidney depends upon induction by the ureteric bud, ureteral abnormalities could account for aspects of the kidney defects in Nfia haploinsufficient patients and mutant mice.

Discussion

Nfia Haploinsufficiency as a Pathogenetic Mechanism

The five individuals studied here share Nfia haploinsufficiency caused by translocation (DGAP089 and 104) or deletion (DGAP174, 205–1, and 205–1s). All five also share abnormalities of the corpus callosum, and partly share other CNS phenotypes, including ventriculomegaly, congenital hydrocephalus, Chiari type I malformation, and tethered spinal cord. All five individuals also have developmental delay and three exhibited seizure disorders. Three of the five also have urinary tract defects, including VUR. Prior work established that Nfia loss of function in the mouse results in ACC and abnormal development of the hippocampal commissure, two major axonal tracts that connect the cerebral hemispheres, and an associated hydrocephalus that develops in rare postnatal survivors [18,34]. We also found that the mouse Nfia mutant recapitulates the VUR phenotype in these humans. Therefore, although other affected genes may contribute to the overall phenotype, these cases suggest that Nfia haploinsufficiency can account for the observed CNS and renal defects.

It is important to acknowledge that a contribution to the developmental phenotypes identified here from additional genes that reside within various deletion intervals, or that also suffer disruption by breakpoints, is not excluded. In all five cases, additional genes besides Nfia are also disrupted or deleted, so that in no single case is a defect in Nfia the only genetic abnormality. While DGAP104 only inactivates Nfia and C20orf32 and the latter is an unlikely contributory factor, the most extreme cases are DGAP089 and the DGAP205 half-siblings, which contain deletions that involve 59 and 47 genes, respectively. Because these two deletions involve different chromosomes, none of the deleted genes are shared. However, in both cases, many more genes are affected than just Nfia, and some may participate in the observed phenotypes. For example, two cases described in the literature report 2q deletions that overlap with the del(2)(q14.3q21) in DGAP089, and these also involve ACC [35,36]. Therefore, in the absence of intragenic mutations in Nfia, the definitive argument that Nfia is the gene responsible for the CNS and renal phenotypes in these five patients cannot be made.

The inability to identify intragenic mutations in Nfia in cases involving ACC, hydrocephalus, tethered cord syndrome, and urinary tract defects could suggest that the phenotype of heterozygous intragenic loss-of-function Nfia mutations might differ from that described here. Indeed, as noted, it is quite plausible that in any individual DGAP case, the

![Figure 7. Ureter Defects at UPJ and UVJ in Nfia Mutant Mice](Image)
observed phenotype represents the additive effect of NFIA haploinsufficiency plus other loci that are deleted or disrupted. On the other hand, at least 20 discrete loci have been implicated in ACC alone, so that the failure to detect intragenic mutations in NFIA is not surprising. Ultimately, formal definition of the NFIA hemizygous loss-of-function phenotype would be strengthened by identification of intragenic loss-of-function NFIA mutations.

Lastly, it is well recognized that both chromosomal translocations and deletions may engender position effects that alter gene expression at considerable distances from the site of a chromosomal aberration ( reviewed in [1,2] ). For example, Shh expression in the limb bud mesenchyme is controlled by a regulatory region located ~1 Mb upstream within the unrelated Lmbr1 gene [37]. Position effects on neighboring genes for mouse knockouts have been described [38]. The mouse Nfia mutant results from a small exon 2 deletion, yet still accurately recapitulates many features of the human phenotype. Therefore, one would have to posit the existence of a conserved regulatory element within the exon 2 deletion region that would act on genes 3' to Nfia, which exhibit conservation of synteny between mouse and human. However, the genes immediately neighboring NFIA are not known to play a role in CNS or kidney development. These include C1orf87 ( GeneID 127795 ) and TM2D1 (beta amyloid binding protein, GeneID 83941), which reside approximately 1 Mb 5' and 200 kb 3' of NFIA, respectively. Additional genes that reside at larger distances from NFIA might be affected by a position effect, but none are obvious candidates. Taking these factors into consideration, we conclude that a true position effect is unlikely to explain the observed phenotypes.

Nature of the CNS Phenotype

Formation of the corpus callosum causes inversion of the cingulate gyri, which gives the medial surface of the brain its characteristic pattern. In ACC, the cingulum remains reverted at sites of agenesis, and the sulci of the medial brain extend into the third ventricle. The findings in DGAP174 of an reverted cingulate gyrus and longitudinal bundles of Probst are consistent with primary dysgenesis of the corpus callosum. Three midline populations contribute to formation of the corpus callosum: the glial sling, the glial wedge, and glia within the indusium griseum and its precallosoal extension, the hippocampal continuation [39–41]. NFIA protein is expressed in all three midline populations, which fail to develop properly in Nfia−/− mice [34]. These populations normally form the cortico septal boundary that prevents callosal axons from entering the septum.

The function of NFIA in formation of these neuronal populations places it within the class of genes that regulate axonal midline crossing. The prototypical regulatory gene in this class is roundabout or robo, which was originally identified in Drosophila. Roundabout encodes a transmembrane receptor expressed by migratory axons after they cross the CNS midline. Robo binds the extracellular ligand, Slit, which is expressed by midline glia and functions as a chemorepulsive cue that prevents axons from midline recrossing. This function extends to mammals, as mice lacking Robo1 or Robo2 exhibit CNS phenotypes that include abnormal midline commissural axonal guidance, and Robo1 mutants in particular exhibit callosal dysgenesis [42,43]. Similarly, Slit ligands also play a role in callosal development. Slit2 mutants display a small corpus callosum with a reduced number of traversing axons [44–46], while Slit2 glial expression during callosal development in Nfia mutants is reduced [34].

Interestingly, Nfia, Robo2, and Slit2 mouse mutants share not only axonal midline crossing defects, but also phenotypically related renal and ureteral defects. Mice deficient for Robo2 or Slit2 exhibit duplex kidney and megaureter phenotypes [32,47] that in some ways resemble those in Nfia mutants. Our recent study also implicated ROBO2 signaling in the pathogenesis of a subset of human VUR [32]. These related phenotypes raise the possibility that Nfia and SLIT–ROBO signaling are functionally linked in both CNS and ureteral development.

Relationship between Kidney and Ureter Phenotypes

Based on Nfia expression in the developing kidney and the presence of kidney hypoplasia in DGAP104, we identified several distinct kidney phenotypes, including renal dysplasia and hydronephrosis in Nfia mutant mice. Hydronephrosis usually results from an obstruction in the flow of urine at the level of the UPJ or UVJ. This results in an obstructive uropathy in which back pressure from the accumulation of urine in the ureter and renal pelvis results in destruction and distortion of the renal parenchyma. The presence of hydronephrosis in Nfia−/− and Nfia+−/− mutants therefore suggests that the observed renal defects reflect a primary disturbance in ureteral development.

A striking finding in Nfia mutant embryos and newborns is the presence of clear ureteral abnormalities: megaueter, abnormal ureteral folding, abnormalities at the UVJ and UPJ, and in a small number of cases, partial duplication of the ureter. These findings are consistent with the strong expression of Nfia in the developing nephric duct and ureter at E9.5–13.5. The ureteral duplication phenotype, distinct from normal patterns of ureteric bud branching [48], very likely explains the finding of a partial duplex kidney. Because ureteric bud contact with uninduced metanephric mesenchyme triggers the inductive cascade [49], contact by two separate ureteral branches should produce a partial duplex kidney. In addition to abnormal ureteral development, the VUR in DGAP104 and in the DGAP205 half-siblings may also develop as a consequence of the tethered spinal cord defect [50,51].

In DGAP089 and 174, renal ultrasound revealed no major abnormalities and urinary reflux was not observed. However, subtle anatomic defects in the ureter or kidney are often subclinical, and may exist below the limit of detection. In addition, these results are consistent with those in Nfia−/− and Nfia+/− mutants, where the penetrance of overt kidney or ureteral defects was only 22% and 68%, respectively. One explanation for the incomplete penetrance of ureteral or kidney defects in Nfia mutants could be functional redundancy with Nfib. In mice, Nfib is strongly expressed in the developing nephric duct, kidney, and ureter at E10.5–11.5, where its expression overlaps with that of Nfia (unpublished data). The other two Nuclear Factor I family members, Nfic and Nfia, are expressed only at lower levels in the developing urerter and kidney. In addition, Nfib−/− mice exhibit callosal agenesis and forebrain defects similar to those seen in Nfia−/− mice [21]. Thus, partial redundancy may exist between Nfia and Nfib in both CNS and urinary tract development. Genetic combinations of mutant alleles for Nfia and Nfib will be
required to address this question definitively, and to further disclose the roles of Nfia factors in development.

In sum, our results suggest that Nfia haploinsufficiency in humans results in a thin, hypoplastic or absent corpus callosum, and define the spectrum of defects attributable to Nfia loss of function to include additional CNS and urinary tract defects that were not previously apparent in the Nfia mouse mutant. These results illustrate the powerful synergy that occurs when corresponding human and mouse disorders are investigated in parallel.

Materials and Methods

DGAP individuals studied. DGAP104. DGAP104 is the product of in vitro fertilization via intracytoplasmic sperm injection, whose parents of European descent were unrelated with no reported medical problems. Amniocentesis demonstrated a 46,XX,t(1;20) (p32.3q13.3)1, but ultrasound revealed no organ malformations at 20 wk of pregnancy. Because of placenta previa and persistent vaginal bleeding, DGAP104 was delivered at 31 wk via elective cesarean section. She weighed 1,180 g with Apgar scores of 5/10. She was diagnosed with prematurity, hydropsphrosis, Chiarri I malformation, tethered spinal cord, congenital hydrencephalus, left hypoplastic kidney, bilateral inguinal hernia, hyaline membrane disease grade 2, and gastroesophageal reflux. Imaging studies indicated a thin posterior corpus callosum and an open aqueduct with progressive ventricular enlargement. She was delayed in reaching developmental milestones; at 2 y, she exhibited major motor delay with inhibited movement and was wheelchair bound. Speech was also delayed, and limited to a few words. At 6 y and 7 mo of age, DGAP104 received a performance IQ score of 42, verbal IQ score of 68, and global IQ score of 52 on the Weschler Preschool and Primary Scale of Intelligence-Revised test (WPPSI-R).

To relieve hydrocephalus, which caused progressive macrocephaly, seven neurosurgical operations were performed over 3 y, including a flow-regulated valve, and several revisions because of hyperdrainage and blockage of the shunt. At 7 d of age, abdominal ultrasonography revealed bilateral hypoplastic kidneys and bilateral dilatation of the renal pelvis. At 1 y, ultrasound showed left and right kidney lengths of 45 mm and 51 mm, respectively (mean length for age, 52 mm), and both kidneys lacked corticomedullary differentiation. At 2 y, the left kidney length was 49 mm and the right 58 mm (mean length for age, 55 mm), but both lacked discernable corticomedullary differentiation. At 3 y, the left kidney was 53 mm and the right 61 mm (mean length for age, 66 mm). DGAP104 first exhibited left grade II VUR by VCUG. VUR increased to grade III with a right vesioureteral junction diverticulum, and pylonephritis developed that required ureteral reimplantation surgery (Cohen operation). Urea and creatinine levels were normal at 5 y of age. DGAP104 was no longer wearing leg braces, was able to walk at 20 mo for cognition, and 17 mo for fine motor. At age 5 y, DGAP104 was functioning at an age equivalent of 2.5 y. At age 10 y he was functioning at an age equivalent of ~7 y, and was 4 ft tall and weighed ~65 lb. DGAP104–1s has limited verbal skills and uses a combination of words and signs for communication. He has not had any formal testing at 10 y of age, but can only read and spell three-letter words. DGAP104–1 was functioning at an age equivalent of 2.5 y. At age 10 y he was functioning at an age equivalent of ~7 y, and was 4 ft tall and weighed ~65 lb. DGAP104–1s has limited verbal skills and uses a combination of words and signs for communication. He has not had any formal testing at 10 y of age, but can only read and spell three-letter words. DGAP104–1s received the Bayley Scales of Infant Development test (BSID) at 2 y of age, and the Stanford-Binet test at 9 y. Both tests demonstrated borderline global cognitive impairment (scores not available). At 9 y of age he was functioning at a kindergarten level. Other clinical data for the affected sibs are summarized in Table S1, and described in more detail elsewhere [23].

DGAP174. DGAP174 was born to a 20-y-old mother at 37 wk by cesarean section and weighed 2,770 g (95th percentile) with length 49.5 cm (25th percentile) and occipitofrontal circumference 37.5 cm (75th percentile). Pregnancy was uncomplicated by teratogenic exposures or maternal illness. A second trimester prenatal ultrasound showed agenesis of the corpus callosum and enlarged ventricles. A CT scan of the brain in the immediate postnatal period confirmed the prenatal findings, and revealed ventriculomegaly with parallel lateral ventricles representing longitudinal bundles of Probst, and a tethered spinal cord. A small ventricular septal defect was also noted at birth. Chromosome analysis of peripheral blood lymphocytes revealed 46,XY,t(13q;22q)21,dn. The neonate was discharged and seen again at 13 d of age in the genetics clinic. He was found to have metopic stenosis and bitemporal narrowing that was surgically corrected at 5 mo of age. Neurological exam revealed normal tone and reflexes. At 8 mo of age, he was noted to be gaining weight rapidly, unrelated to any changes in feeding pattern. At 12 mo of age, he was noted to have significant motor development, macrocephaly without hydrocephalus, and height, weight, and length above the 95th percentile for his age. Additionally, a dimple on the posterior aspect of the right cheek, creases behind each earlobe, and esotropia secondary to telecanthus and epicanthal folds were noted. A left inguinal hernia was detected at 24 mo. At 24 mo, glasses were prescribed to correct hyperopia and strabismus, and all milestones were on track except for speech. At 36 mo, expressive language was still delayed. Due to concerns about the patient’s development, he was referred at 24 mo of age appropriate. A brain MRI at 36 mo of age revealed Chiarri I malformation and dysplasia of the anterior aspect of the left temporal fossa in addition to complete agenesis of the corpus callosum. At 57 mo of age, DGAP174 underwent several developmental tests. His IQ score was 68 on a Leiter International Performance Scale (LIPS) and his Performance IQ was 57 at 36 mo of age. He received a standard score of 60 in the Peabody Picture Vocabulary Test (Revised, Form M, PPVT-R) corresponding to an age equivalent of 35 mo. His standard score for the Developmental Test of Visual-Motor Integration (VMI) was 67, which also corresponded to an age equivalent of 35 mo. At 6 y of age, he underwent a successful Chiarri decompression and repair of the tethered spinal cord. At that time, he was diagnosed with attention deficit and hyperactivity disorder and is currently on Ritalin. He was noted to have right hemihypertrophy and scoliosis, for which he was referred to an orthopedics clinic. At age 8 y and 4 mo (January 2007), DGAP174 was functioning at the kindergarten level, and received occupational and physical therapy for speech.

DGAP205–1, 205–2, 205–3, and 205–4. DGAP205–1 and DGAP205–1s are two half-siblings with an interstitial microdeletion, del(1p)31.3p32.3; that was inherited as an unbalanced segregant resulting from a balanced rearrangement in their mother, DGAP205–2. Both half-siblings had congenital CNS and urinary tract defects while their mother was phenotypically normal. DGAP205–1 had the Bayley Scales of Infant Development test (BSID) at 4.5 y of age and received scores corresponding to an age equivalent of 18 mo of development, 20 mo for cognition, and 17 mo for fine motor. At age 5 y, DGAP205–1 was functioning at an age equivalent of ~2.5 y. At age 10 y he was functioning at an age equivalent of ~7 y, and was 4 ft tall and weighed ~65 lb. DGAP205–1s has limited verbal skills and uses a combination of words and signs for communication. He has not had any formal testing at 10 y of age, but can only read and spell three-letter words. DGAP205–2s received the Bayley Scales of Infant Development test (BSID) at 2 y of age, and the Stanford-Binet test at 9 y. Both tests demonstrated borderline global cognitive impairment (scores not available). At 9 y of age he was functioning at a kindergarten level. Other clinical data for the affected sibs are summarized in Table S1, and described in more detail elsewhere [23].

FISH, aCGH, and mutation screening. Metaphase FISH was performed according to standard methods. BAC clones were obtained from BACPAC Resources (http://bacpac.chori.org), labeled as FISH probes, and hybridized to metaphase chromosomes prepared from lymphoblastoid cell lines established from all five individuals. The full BAC names provided in Figure 2I are: RP4-654H19, RP11-707S7, RP5-1078M7, RP5-1084R6, RP11-89K19, RP11-131O15, and RP11-89K2. aCGH experiments were performed with the Spectral Genomics 2600 BAC array by the CytoGenetics Core Facility of the Dana-Farber/ Harvard Cancer Center for DGAP089 and DGAP174, and by Spectral Genomics for DGAP104. NFIA mutation screening employed PCR amplification of the exons and flanking boundaries, followed by purification and bidirectional DNA sequencing. NFIA cDNA sequence AB037860 (http://www.ncbi.nlm.nih.gov/ entrez/viewer.fcgi?db=nucleotide&val=7245273) was used to calculate
nucleotide positions. MLPA analysis of the NFIA coding region was performed in a subset of syndromic and nonsyndromic ACC samples, and no copy number changes were identified.

**RT-PCR analysis.** RT-PCR analyses were performed by routine protocols. RT-PCR primers used to amplify the Nfia 334 bp cDNA were forward primer: 5'-AGGCCACCTACGACAACTG-3' and mNfia-rR (5'-CCTGGTTGACGATGTTTTCCTG-3'). RT-PCR primers used to amplify the C20orf32 436 bp cDNA were mC20orf32-F (5'-GGGCCAACCTACGACAACTG-3') and mC20orf32-R (5'-TCTGGGAAGACACAGAGG-3').

**Southern and blot analysis.** Southern blotting was performed by standard methods. Probes were labeled using the MegaPrime labeling kit (Amersham/GE Healthcare, http://www.amershamhealth-us.com). Genomic DNA from the DGAP089 cell line and from a karyotypically normal control were digested with DraI, PstI, and SspI and hybridized overnight with a 700-bp probe amplified from RPS-902P15. This probe (AL096888, 66436–67143) was amplified by the following primers: forward primer: 5'-CAGCGCTTCTCCTCAAGAAG3' and reverse primer: 5'-GGTCCTTTCACGTGCATCTT-3'. Southern blots of Bmpl, BspHI, DraI, EcoRI, HindIII, PvuII, and Xhol-digested DNA from DGAP104 and genomic DNA from a karyotypically normal male control were hybridized with a 623-bp probe that was amplified from RP4-802A10 (AC096947.2; 50191–50687). This probe was amplified by the following primers: forward primer: 5'-AGGCCACCTACGACAACTG-3' and reverse primer: 5'-AGGCCACCTACGACAACTG-3'. A northern blot containing poly A+ RNA from multiple regions of human brain (Human Brain V blot, Clontech, http://www.clontech.com) was probed with a probe corresponding to exons 2–6 of NFIA following a standard protocol.

**Analysis of Nfia mutant mice.** The generation and analysis of brain defects in Nfia knockout mice in a C57BL/6 background has been previously described [18]. Nfia−/− mice analyzed in Figure S6 were C57Bl/6X129S6 F1 hybrids that have longer postnatal survival than C57BL/6 inbred mice. The Nfia−/− allele was genotyped by PCR amplification using the mutant allele specific forward primer Nfia-in1-F2 (5'-CGTGAATGCTGATGTCGAGCA-3') in the intron 7 region. Histological analyses were performed on mouse specimens fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. To examine ureter and kidney defects, Hoechst-GFP transgenic mice (gift from Dr. Frank Costantini, Columbia University) were bred with Nfia knockout mutants. GFP fluorescence illumination of the mouse urinary tract was evaluated using a Nikon SMZ-1500 epi-fluorescence stereomicroscope (http://www.nikon.com.ua).

**In situ hybridization and immunohistochemistry.** Tissue in situ hybridization of whole mount and cryosections was performed according to standard protocols using cRNA probes complementary to the 3'-UTRs of Nfia and C20orf32. For WT1 immunostaining, kidney sections were fixed in 4% paraformaldehyde, embedded in paraffin, and stained with anti-WT1 antibody (Santa Cruz Biotechnology, http://www.scbt.com). Dolichos Biflorus Agglutinin (DBA; Vector Labs, http://www.vectorlabs.com) and Lotus Tetragonolobus Lectin (LTL; Vector Labs) stainings were performed on paraffin-embedded kidney sections.

**Ethics.** All human studies were performed under informed consent protocols approved by the Human Research Committee of Partners HealthCare System, Boston. Mouse protocols were approved by the Institutional Animal Care and Use Committee at Harvard Medical School or at Boston University Medical Center.

**Supporting Information**

**Figure S1.** Southern Blot Analysis of 1p31.3 Breakpoint in DGAP089 NFIA is disrupted in DGAP089 and the breakpoint lies within intron 2. (A) Southern blot analysis of DGAP104 (P) and normal control (C) genomic DNA using the designated restriction enzymes and the probe A4 shown in panel B. Aberrant bands (arrows in A) are present only in DGAP104 DNA digested with Bmpl, BspHI, DraI, EcoRI, and Xhol. (B) Restriction map surrounding the NFIA intron 2 region. The base-pair position of BAC RP4-802A10 (AC096947, within intron 2 of NFIA, see BAC contig in Fig. 2I) was used to calculate the distance between restriction enzyme sites. BAC RP4-802A10 was used in FISH and contains the breakpoint, which is between boxed Bpml and Bpml sites based on the aberrant bands detected by Southern blot analysis.

**Figure S2.** In Situ Hybridization of Mouse C20orf32 C20orf32 is not expressed in the mouse embryonic spinal cord and kidney at E10.5 and E11.5.

**Figure S3.** Southern Blot Analysis of 1p31.3 Breakpoint in DGAP089 NFIA is disrupted in DGAP089 and the breakpoint lies within intron 7. (A) Southern blot analysis of DGAP089 (P) and normal control (C) genomic DNA using the designated restriction enzymes and the probe A1 shown in panel B. Aberrant bands (arrows in A) are present in DGAP089 DNA digested with DraI, PstI and SspI. (B) Restriction map surrounding the NFIA intron 7 region. The base-pair position of BAC RP5-902P15 (AL096888, within intron 7 of NFIA, see BAC contig in Fig. 2I) was used to calculate the distance between restriction enzyme sites. BAC RP5-902P15 was used in FISH and contains the breakpoint, which is between boxed Bpfl and Drall sites and based on the aberrant bands detected by Southern blot analysis.

**Figure S4.** Array Comparative Genomic Hybridization (aCGH) for DGAP174 Array CGH at a 1-Mb resolution defines the deletion interval in DGAP174. Spectral Genomics profile indicates the deletion interval (arrow) on 1p from p13.1 to p13.3.

**Figure S5.** Northern Blot Analysis of NFIA in Different Regions of the Human Brain Northern blot of multiple human brain tissues was hybridized with a probe containing NFIA exons 2–6.

**Figure S6.** Hydronephrosis in P16 Nfia−/− Mutant Severe hydronephrosis (* in B) is shown in a P16 Nfia−/− mutant kidney, whereas the kidney in its wild-type littermate (A) is normal.

**Table S1.** Clinical Findings in Five Individuals with Chromosome Abnormalities Involving 1p31

**Table S2.** Phenotypes of 84 Patients with Callosal and other CNS Malformations and Urinary Tract Defects Subjected to NFIA Intragenic Mutation Screening

**Accession Numbers** The GeneID numbers for the Entrez Genes (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene) discussed in this paper are AK3L2 (387851), ALG6 (29929), ANGPTL3 (27329), ANKR3D (163782), ATGHC (84938), BSN (7809), Clorf87 (127759), Clorf141 (400757), Clorf168 (169920), CCA (731), C8B (732), C20orf32 (57091), C20orf32 (329064), CAGHDI (57685), CYP2J2 (1573), DABI (1600), DNAJC6 (16092), DOCK (85440), FIV19867 (55277), FLJ14357 (400754), FOXD3 (27092), HOOK1 (51361), IL23R (149233), INADL (10970), INSIL5 (10022), ITGB3BP (23421), JAK1 (3716), JUN (3725), KIAA1799 (84455), LITD1 (54596), LEPR (3953), LEPROT (4741), MIER (5708), NEGR1 (257194), NFIA (4774), NFIB (18027), OMA1 (115209), PCSK9 (255738), PEDE4B (5142), PCMI (5226), PIPAP2 (8613), PRIKAA2 (5563), RAVER2 (59225), ROR1 (4191), SGIP1 (84251), SLCD35 (23169), TACSTD2 (4070), TCEX1DI (200132), TM2DI (89341), UBE2U (148581), USP1 (7398), and WDR78 (79819).

The disease identifiers for the OMIM (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM) genetic disorders discussed in this paper are an identifier of the corpus callosum (OMIM 217799), hydrocephalus and ventriculomegaly (OMIM 236600), Chiari malformation type I (OMIM 118420), and vesicoureteral reflux (OMIM 193000 and 610878).
Acknowledgments

We are indebted to the patients and families for participating in this study. We thank Robert Eisenman, Juan Liu, Mary Anne Anderson, Patricia Crawford, Francesca Puglisi, and the MGH Genomics Core Facility for technical support; Steven Moore for initial FISH experiments; Frank Costantini for providing Jnk-7-GFP transgenic mice; Wellington Cardoso and Jingi Lu for help with fluorescence stereomicroscopy; Caroline Robson for assistance in evaluating brain imaging; Kira Ape for help in collecting nonsyndromic ACC samples; Len A. Penacchio for sequencing analysis; and Natalia Leach, Irfan Saadi, Gail Bruns, Helmut Renkke, William Dobyns and David Salant for helpful suggestions.

Author contributions. WL, FQR, YF, CCM, JFG and RLM conceived and designed the experiments. WL, FQR, YF, SFA, DJD, DJH, HF, CK, AH, AHL, BJQ, CCM, JFG and RLM analyzed the data. WL, CGC, ALS, EHS, AA, BR, PP, AGB, CAM, RW, KG, KD, CCM, and RLM contributed reagents/materials/analysis tools. WL, FQR, JFG, and RLM wrote the paper.

Funding. This work was supported by NIH grants P01GM061354 (CCM) and R01DK068316 (RLM), and by the Cytogenetics Core Facility of the Dana-Farber/Harvard Cancer Center (P30CA06516). WL is supported by a National Kidney Foundation Young Investigator Grant, a BUMC DOM Pilot Project Grant and the Evans Medical Foundation. YF is supported in part by a fellowship from the Canadian Institute of Health Research.

Competing interests. The authors have declared that no competing interests exist.

References


