

# WNT Signaling Perturbations Underlie the Genetic Heterogeneity of Robinow Syndrome

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Locus heterogeneity characterizes a variety of skeletal dysplasias often due to interacting or overlapping signaling pathways. Robinow syndrome is a skeletal disorder historically refractory to molecular diagnosis, potentially stemming from substantial genetic heterogeneity. All current known pathogenic variants reside in genes within the noncanonical Wnt signaling pathway including *ROR2*, *WNT5A*, and more recently, *DVLI1* and *DVL3*. However, ~70% of autosomal-dominant Robinow syndrome cases remain molecularly unsolved. To investigate this missing heritability, we recruited 21 families with at least one family member clinically diagnosed with Robinow or Robinow-like phenotypes and performed genetic and genomic studies. In total, four families with variants in *FZD2* were identified as well as three individuals from two families with biallelic variants in *NXN* that co-segregate with the phenotype. Importantly, both *FZD2* and *NXN* are relevant protein partners in the *WNT5A* interactome, supporting their role in skeletal development. In addition to confirming that clustered -1 frameshifting variants in *DVLI1* and *DVL3* are the main contributors to dominant Robinow syndrome, we also found likely pathogenic variants in candidate genes *GPC4* and *RAC3*, both linked to the Wnt signaling pathway. These data support an initial hypothesis that Robinow syndrome results from perturbation of the Wnt/PCP pathway, suggest specific relevant domains of the proteins involved, and reveal key contributors in this signaling cascade during human embryonic development. Contrary to the view that non-allelic genetic heterogeneity hampers gene discovery, this study demonstrates the utility of rare disease genomic studies to parse gene function in human developmental pathways.

## Introduction

The study of rare disease informs human biology. Bridging the gap between disease and gene can often provide the initial insights into gene function and disease mechanism, drive experimental clinical or laboratory questions, and provide immediate practical application in clinical genomic diagnostics.<sup>1</sup> Robinow syndrome (RS) is a congenital skeletal dysplasia, with autosomal-dominant (DRS) and -recessive (RRS) inheritance and evidence for genetic

heterogeneity. Originally described by Meinhard Robinow in 1969, it is characterized by short stature, mesomelic limb shortening, broad thumbs and toes, genital hypoplasia, and distinctive and recognizable craniofacial features with frontal bossing, high, broad forehead, depressed nasal bridge, and prominent eyes; the tall forehead relative to the face leads to facial disproportion often termed the “fetal face.”<sup>2</sup> The clinically more severe recessive Robinow syndrome (RRS [MIM: 268310]) has been associated with biallelic variants in the tyrosine kinase-like orphan receptor,

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<https://doi.org/10.1016/j.ajhg.2017.10.002>

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ROR2 (MIM: 602337), in an estimated 80% of the case subjects described.<sup>3,4</sup> ROR2 is essential for noncanonical Wnt ( $\beta$ -catenin-independent) signaling that establishes cellular orientation via the Wnt/planar cell polarity pathway (PCP).<sup>5</sup> Ensuing discovery of heterozygous hypomorphic missense alleles in WNT5A (MIM: 164975), the gene that encodes the signal transducer and the putative ligand for ROR2, resulting in dominant Robinow syndrome (DRS1 [MIM: 180700]),<sup>6</sup> supports the hypothesis that a specific pathway could be involved in the disease etiology. More recently, *de novo* indels resulting in clustered protein-truncating variants affecting two out of the three human orthologs of the *Drosophila dishevelled* (*dsh*) gene, *DVL1* and *DVL3* (MIM: 601365, 601368), were shown to be another cause of dominant Robinow syndrome (DRS2 [MIM: 616331], DRS3 [MIM: 616894]).<sup>7–9</sup> This finding is consistent with the observation that human genes with two or more paralogs for the *Drosophila* ortholog are eight times more likely to be disease relevant.<sup>10</sup> DVL is a key downstream mediator of the Wnt pathway, including the Wnt/noncanonical PCP pathway via interaction with ROR2,<sup>11,12</sup> which further strengthens the link of the disease with a perturbed PCP signaling cascade during human development.

The mutational mechanism observed in *DVL1*- and *DVL3*-associated RS is exclusively clustered mutations in the penultimate or last exon resulting in  $-1$  frameshifting variants that result in a premature stop codon within the last exon. Those variants generate a stable mRNA that is predicted to translate into a mutant protein with a similarly truncated C terminus. This is remarkably specific and recurs independently in DRS-affected families.<sup>7–9</sup> Whether the pathogenicity of these truncated proteins results from a gain-of-function or dominant-negative effect is actively being investigated, but preliminary experimental data suggest that it is not driven by the loss of the C terminus alone.<sup>8</sup> Collectively, the pathogenesis of RS appears to be a result of perturbation of the WNT5A/ROR2 signaling cascade required for efficient PCP.

The Wnt signaling pathways control many critical early developmental and post-natal physiological processes. One fundamentally important function of noncanonical Wnt signaling is to provide directional information by regulating the evolutionarily conserved PCP pathway. The PCP pathway is essential for vertebrate embryogenesis, including convergent-extension movements and limb bud outgrowth and patterning.<sup>13</sup> The core system of Wnt/PCP signaling is composed of a set of highly conserved proteins that interact with each other to form opposing complexes at opposite sides of the cell, thus allowing cellular orientation.<sup>14</sup> Mutation in genes crucial for PCP have been linked to human diseases, including neural tube defects<sup>15,16</sup> and nearly identical mouse phenotypes,<sup>17,18</sup> most notably RS.<sup>3,6,7,9</sup> Moreover, despite discovery of some of the genes and variants underlying DRS, it is estimated that 70% to 80% of individuals with DRS have an unknown underlying molecular etiology.<sup>7,9</sup> Based on the fact that all the Robinow syndrome-associated proteins play a vital role

in noncanonical Wnt/PCP signaling, we hypothesized that studying subjects with Robinow syndrome would reveal other causative variants within genes important to the establishment of PCP and human development.

Herein we report the analyses of a Robinow syndrome-affected cohort where we applied a combination of targeted Sanger and whole-exome sequencing (WES) studies to 21 unrelated families diagnosed clinically with RS, some of whom have more than one individual affected. This study revealed *de novo* and/or private variants affecting four genes for which translated proteins directly interact with *DVL1* and *DVL3* or have a role in the Wnt/PCP pathway: *FZD2* (MIM: 600667), *NXN* (MIM: 612895), *RAC3* (MIM: 602050), and *GPC4* (MIM: 300168). Importantly, mutations of the Frizzled receptor, *FZD2*, alone accounts for 3/21 (14%) of the clinically ascertained RS-affected case subjects, two of which are due to substitution of an identical amino acid that maps adjacent to the domain that directly interacts with *DVL*. In addition, we report that *NXN*, which encodes a regulator of the Wnt pathway, is also associated with RRS.<sup>19,20</sup> In total, *WNT5A*, *DVL1*, *DVL3*, and *FZD2* explain 12/21 (57%) of our DRS-affected case subjects. In summary, these data strongly support the notion that RS results from perturbation of the Wnt/PCP pathway during human development and reveals specific gene paralogs, key proteins, and potential domains involved in human limb and craniofacial formation.

## Subjects and Methods

### Study Participants

We recruited 21 unrelated individuals with a clinical diagnosis of RS or presenting clinical features consistent with a Robinow-like syndrome. Of the 21 probands, 10 were ascertained during the 13th Biennial Robinow Syndrome Conference in Minneapolis, MN. All individuals were screened for pathogenic variants in *ROR2* before inclusion into this study, and therefore the cohort was biased toward individuals with the dominant form of RS. Inclusion was based on core clinical phenotypes including characteristic facial features, short stature, and limb abnormalities but not altogether meeting a strict criterion for DRS clinical diagnosis (see [Supplemental Note](#) for detailed clinical synopsis). RS was of sporadic occurrence, except for two families with an affected mother and child. DNA was obtained after all relevant family members provided written informed consent. Two additional families, which were not included in the original cohort, were subsequently ascertained via GeneMatcher. This study was approved by the institutional review board at Baylor College of Medicine (protocol no. H-29697).

### Sanger Sequencing

From our previous studies, we estimate that nearly 20% of DRS results from truncating alleles in either *DVL1* or *DVL3*.<sup>7,9</sup> Therefore, before performing WES, we first Sanger sequenced the penultimate and final exons of *DVL1* and *DVL3* from the 21 probands, since *DVL*-mediated RS is the unique result of clustered  $-1$  frameshifting indels in either *DVL1* or *DVL3*, the mRNAs being predicted to escape nonsense-mediated decay.<sup>7,9</sup> All PCR products containing candidate frameshift alleles detected by Sanger

sequencing were further confirmed using allele-specific sequencing via manually cloning both alleles into a standard TOPO TA cloning vector (Life Technologies) and then transforming the recombinant clones into chemically competent *Escherichia coli* to be grown overnight. Individual clonal colonies were then sequenced by a standard Sanger dideoxy capillary sequencing.

### Exome Sequencing

The DNA samples from “unsolved” individuals lacking pathogenic variants in either *DVL1* or *DVL3* were subjected to WES, through the Baylor-Hopkins Center for Mendelian Genomics initiative.<sup>21</sup> WES was performed at the Baylor College of Medicine-Human Genome Sequencing Center (BCM-HGSC), and pre-captured libraries were pooled and then hybridized in solution using the BCM-HGSC in-house VCRome 2.1 design<sup>22</sup> according to the manufacturer’s protocol *NimbleGen SeqCap EZ Exome Library SR User’s Guide* with minor revisions. All samples achieved 96% of the targeted exome bases covered to a depth of 20× or greater with an average depth of coverage of 95×. The choice of variant annotation software can influence the filtering and parsing of rare variants, thus affecting one’s ability to identify potential disease-relevant variants. Differing annotation software tools often provide distinct interpretations and varying levels of false-positive and false-negative findings; this appears particularly true for indels.<sup>23</sup> To maximize our variant discovery from the Illumina sequencing data, we used two variant discovery methods in parallel starting with the BCM-HGSC Mercury analysis pipeline,<sup>24</sup> which moves data from the initial sequence generation on the instrument to annotated variant call files (VCF) via various analysis tools. In addition, we used the Genome Analysis Toolkit (GATK) HaplotypeCaller to produce joint called files with indel realignment and base recalibration in all families that underwent WES. We identified *de novo* mutations *in silico* by using read-depth information extracted from the BAM files of either parent and proband using the in-house developed software DNM-Finder.<sup>25</sup> Candidate variants were filtered against exome data in publicly available databases, including the 1000 Genomes Project, the NHLBI Exome Sequencing Project (ESP) Exome Variant Server, the Atherosclerosis Risk in Communities Study (ARIC) database, and our internal Baylor-Hopkins Centers for Mendelian Genomics variant analyzer database of approximately 6,400 exomes. In addition, all affected individuals were screened for copy-number variants (CNV) with exome data, utilizing XHMM<sup>26</sup> and our in-house developed algorithm for detecting homozygous exonic deletions, HMZDelFinder.<sup>27</sup> We also used WES data to calculate a B-allele frequency and delineate genomic intervals for absence of heterozygosity consistent with identity by descent.<sup>27</sup> We verified and evaluated potential disease-associated variants identified via WES for co-segregation with the phenotype by using standard PCR amplification. PCR products were purified with ExoSAP-IT (Affymetrix) and sequenced with dideoxy nucleotide Sanger sequencing at the DNA Sequencing and Gene Vector Core at Baylor College of Medicine.

## Results

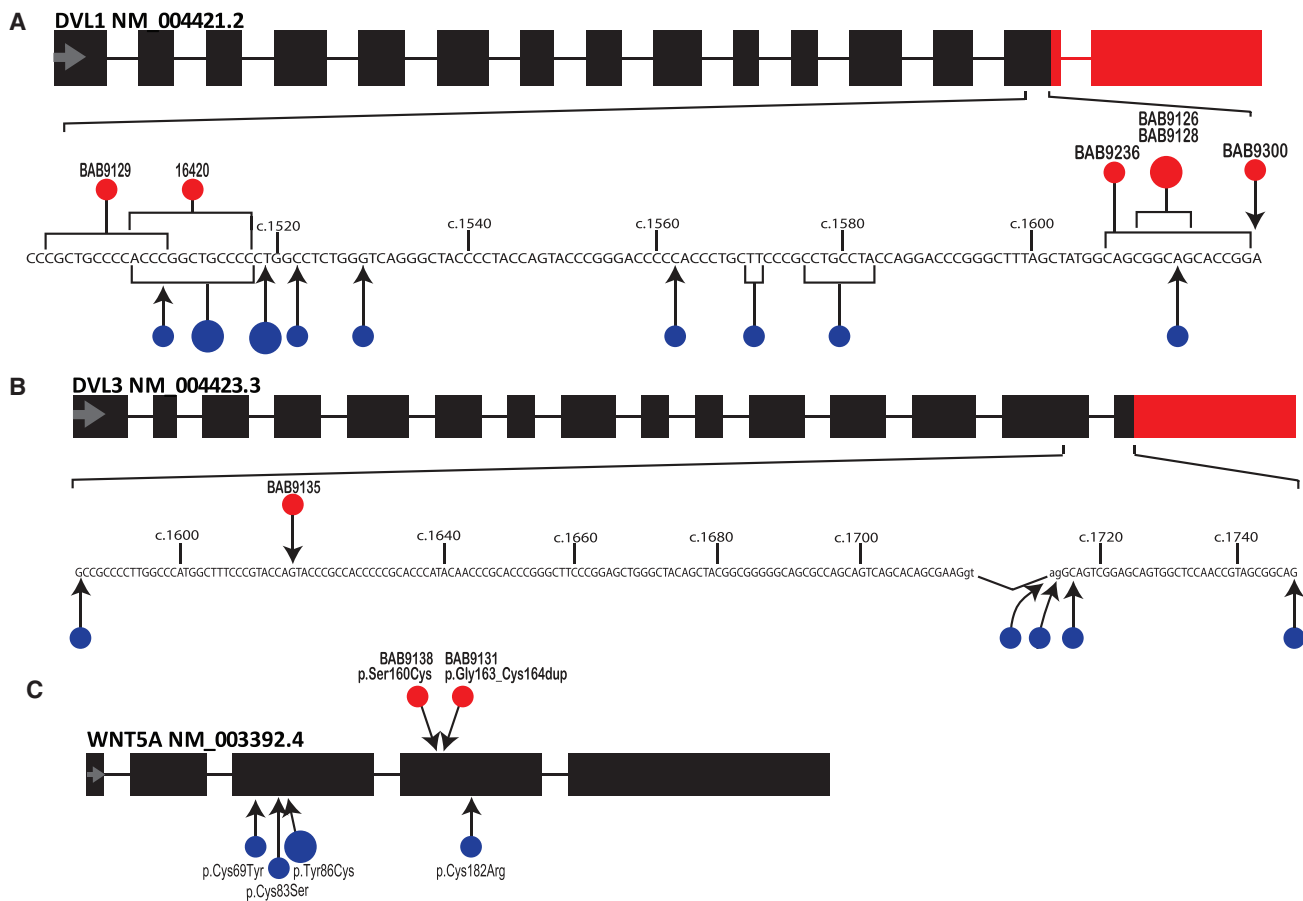
### Contribution of Genes Associated with Robinow Syndrome

We initially set out to confirm and assess the contribution of *DVL1* and *DVL3* to DRS by targeted Sanger sequencing

of the penultimate and final exons in *DVL1* and *DVL3* from our in-house database of clinically diagnosed RS-affected subjects (n = 21). Seven individuals (33%) presenting with DRS harbored likely pathogenic variants in either *DVL1* or *DVL3*, offering strong support to our assertion that at least 20% of DRS are caused by indels in *DVL1* or *DVL3*. The identified variants in *DVL1* and *DVL3* are shown in [Figures 1A, 1B, S1](#), and [Table S1](#). Five individuals had truncating alleles distributed within a highly GC-rich, 116-nucleotide stretch of the penultimate exon of *DVL1* according to RefSeq transcript GenBank: NM\_004421.2. Two unrelated individuals contained an identical 4-base-pair duplication (c.1612\_1616dup [p.Ser539Argfs\*112]), another individual harbored a single base-pair deletion (c.1623del [p.Ser542Valfs\*107]), and one individual had a 16-base-pair deletion (c.1608\_1623del [p.Ser537Valfs\*107]). Lastly, two unrelated individuals contained distinct 13-base-pair deletions (c.1496\_1508del [p.Pro499Argfs\*146], c.1505\_1517del [p.His502Profs\*143]) located in a region where half (9/17) of all reported variants in *DVL1* are tightly clustered, likely the result of sequence complexity of this region. The repeated sequence “GCTGCC” is potentially mediating the recurrently observed 13-bp deletion variants via secondary structure mutagenesis.<sup>28</sup> In total, the four newly identified pathogenic variants in *DVL1* are consistent with previous reports wherein variants are tightly clustered in the penultimate exon and result in a –1 frameshift.

In addition to the six subjects with variants in *DVL1*, one individual had a likely pathogenic variant in *DVL3*. According to the *DVL3* Refseq transcript GenBank: NM\_004423.3, this variant is located in the penultimate exon (c.1617del [p.Gln539Hisfs\*129]). Collectively, and in agreement with previous reports, the variants in *DVL1* and *DVL3* indicate that DVL-mediated RS is the unique result of clustered –1 frameshifting indels supporting the hypothesis that translation from this mutant reading frame is necessary for the pathogenic effect, due to either a gain-of-function or dominant-negative effect.<sup>7–9</sup>

The first gene to be associated with DRS was *WNT5A*, but hypomorphic missense alleles of *WNT5A* in DRS-affected individuals has only been reported in a small number of subjects with DRS (<10 individuals). All individuals without pathogenic variants in *DVL1* or *DVL3* were studied by WES and two likely pathogenic variants in *WNT5A* were found in two unrelated probands ([Figure 1C](#)). In addition to the annotated WES results, to ensure complete sequence read coverage, we manually visualized the coding regions of *WNT5A* in the Integrative Genomic Viewer (IGV) for all affected individuals studied. One individual, BAB9138, contained a private missense in *WNT5A* (GenBank: NM\_003392.4; c.479C>G [p.Ser160Cys]), but this individual was adopted and parental studies to segregate this allele are unavailable. Another individual, BAB9131, was found to carry a *de novo* non-frameshift 6-base-pair insertion affecting *WNT5A* (GenBank: NM\_003392.4; c.487\_492dup [p.Gly163\_Cys164dup]). The variant was



**Figure 1. Location of Identified Variants in *DVL1*, *DVL3*, and *WNT5A* Resulting in Dominant Robinow Syndrome**

The variants in *DVL1* and *DVL3* are mostly small insertions or deletions, except for two splicing variants in *DVL3*; all of them are predicted to lead to  $-1$  frameshifting. Black rectangles represent transcript segments identical to the reference *DVL1* (A), *DVL3* (B), or *WNT5A* (C) mRNAs. Red rectangles indicate the shared transcript regions affected by the  $-1$  frameshift in the predicted protein structure in all subjects. Part of exon 14 (*DVL1*) or exons 14 and 15 (*DVL3*) transcript sequence is shown in detail. Previously described variants are displayed by blue circles, whereas variants identified in this study are displayed by red circles. Larger circles represent identical variants in unrelated individuals. For complete description of all variants, see [Tables S1](#) and [S2](#).

observed in 16/70 (23%) mapped reads, which is consistent with a mosaic state of the mutant allele. The identification of only two individuals ( $\sim 9.5\%$ ) in our cohort of 21 RS-affected probands indicates that *WNT5A* is an infrequent cause for this disorder. All publicly available pathogenic *WNT5A* variants are demonstrated in [Figure 1C](#).<sup>6,29</sup>

The clinical phenotype of BAB9131 and BAB9138 were generally more severe than previous reports of individuals with *WNT5A*-mediated RS as both individuals presented with hemivertebrae and significant mesomelic limb shortening indicating that these variants, including one that appears to be mosaic in blood DNA, may result in a more severe RS phenotype (see [Supplemental Note](#) for the detailed clinical findings).

#### Differential Diagnosis of Robinow Syndrome

Approximately 57% of our cohort (12 out of 21) did not yield a pathogenic variant in *DVL1*, *DVL3*, or *WNT5A*. The remaining “unsolved” individuals were then studied by WES, thus allowing for gene discovery and a comprehensive and agnostic screening of all the genes within

the broader differential diagnosis of clinical observations consistent with RS.

Clinical findings in subjects diagnosed with RS can elicit a range of potential differential diagnoses, including other skeletal disorders, most notably Aarskog-Scott syndrome (MIM: 305400), Opitz G/BBB syndrome (MIM: 145410), and omodysplasia type 2 (MIM: 164745). In our cohort, four individuals were found to carry likely pathogenic single-nucleotide variants (SNVs) in genes associated with diseases considered within the context of the differential clinical diagnoses of DRS. In addition, one individual, BAB8836, has two large copy-number variants that were revealed by XHMM and/or HMZDelFinder and further confirmed by aCGH ([Figure S2](#)). The CNVs are constituted by an  $\sim 8.3$  Mb deletion of the short arm of chromosome X and a  $\sim 13$  Mb duplication spanning the long arm of chromosome 6. Array analysis in parental samples indicates a *de novo* event but chromosome analysis did not reveal any visible cytogenetic abnormalities in the proband, which supports the hypothesis that BAB8836



carries an unbalanced chromosomal translocation; karyotype 46,XY,der(X)t(X;6)(p22.31;q25.3).arr[GRCh37]6q25.3q27(157,870,814\_170,881,475)x3, Xp22.33p22.31(409,876\_8,199,541)x0. Parental samples were not available for further testing so we could not rule out the possibility that BAB8836 has inherited an unbalanced chromosome translocation that resulted from the transmission of a derivative chromosome involved in a balanced event in the mother. The Xpter deletion encompasses the pseudoautosomal gene *SHOX* (MIM: 312865), deletion of which causes Leri-Weill dyschondrosteosis (MIM: 127300). The partial duplication of the long arm of chromosome 6 and the hemizygous deletion of Xpter also encompasses several known contiguous gene deletion syndromes including chondrodysplasia punctata (MIM: 302950) and ichthyosis (MIM: 308100). Collectively, the clinical phenotype of BAB8836 includes short stature, developmental delay, distal phalangeal hypoplasia, dryness of the skin, scoliosis, hemivertebrae, and an increase of sclerosis in bones, representing a unique amalgamation resulting from these large copy-number variants (see [Supplemental Note](#)).

Two male subjects, BAB8747 and BAB8751, were found to have hemizygous frameshift mutations in *FGD1* (GenBank: NM\_004463.2; c.892dup [p.Cys298Leufs\*5] and c.527dup [Leu177Thrfs\*40]) ([Table S1](#)). Truncating alleles in *FGD1* (MIM: 300546) are an established cause of Aarskog-Scott syndrome, which has a wide phenotypic variability that largely overlaps with DRS. One individual, BAB8743, was found to have a previously reported pathogenic variant in *PTPN11* (MIM: 176876; GenBank: NM\_002834.4; c.836A>G [p.Tyr279Cys]).<sup>30</sup> The resulting disorder, Noonan syndrome (MIM: 163950), often includes facial features similar to those of DRS.

Lastly, in BAB8759, who was the offspring of consanguineous parents, homozygous pathogenic variants were identified in two genes mapping to distinct segments of absence of heterozygosity (AOH) on heterologous chromosomes. A homozygous frameshift deletion was detected in *SH3PXD2B* (MIM: 613293; GenBank: NM\_001017995.2; c.969del [p.Arg324Glyfs\*19]). Recessive loss-of-function mutations in *SH3PXD2B* cause Frank-Ter Haar syndrome (MIM: 249420)<sup>31</sup> and multiple skeletal anomalies and heart defects which likely explain these clinical observations in subject BAB8759. Additionally, the homozygous missense variant in *INPPL1* (MIM: 600829; GenBank: NM\_001567.3; c.1636G>A [p.Val546Ile]) likely contributes to the additional clinical features present in BAB8759, including nuchal edema and recurrent respiratory infections, which are observed in opsismodysplasia (MIM: 258480).<sup>32</sup> Therefore, the resulting clinical features of BAB8759 are likely a blended phenotype<sup>33</sup> resulting from a dual molecular diagnoses in which homozygous single gene defects in both *INPPL1* and *SH3PXD2B* contributed. From our cohort of 21 families, identification of one individual with a likely dual molecular diagnosis (4.8%) is consistent with previous estimates of the rate of dual

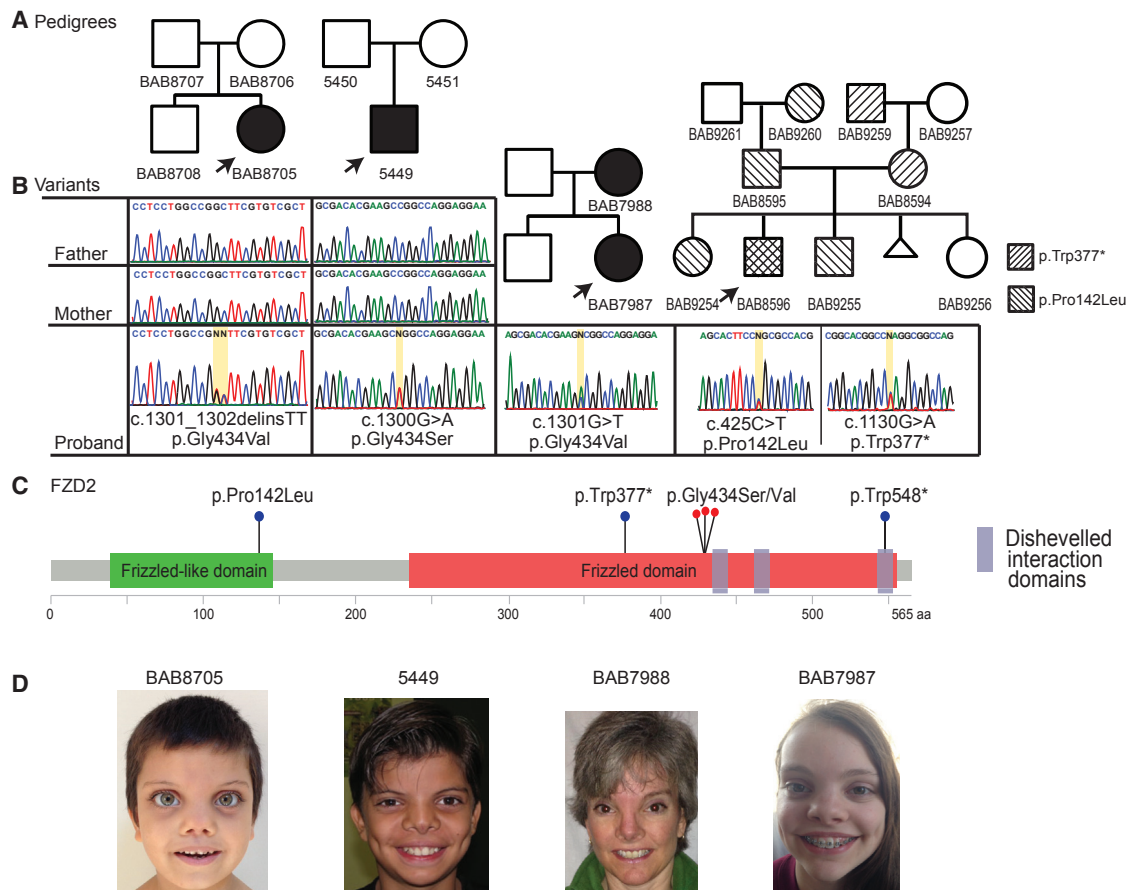
molecular diagnosis in clinical WES.<sup>33–35</sup> In total, five individuals from our cohort of 21 families who were ascertained due to a clinical impression of RS were found to contain likely pathogenic variants in other relevant genes that represent a broad range of overlapping features. This demonstrates the utility of exome sequencing and molecular diagnosis as an adjuvant approach to achieve characterization of affected individuals as well as detecting disease-gene associations in a complex clinical landscape.<sup>35,36</sup>

### Recurrent Missense and Truncating Variants in *FZD2* Cause Robinow Syndrome Features

Similar to the “solve-rate” for RS, after analyzing our cohort for known genes with a disease association, seven individuals from our cohort (7/21) remained without a molecular diagnosis. We then performed a personal genome analysis to identify rare and potentially damaging variants that might represent additional genes underlying DRS. Most notably, five affected individuals from three distinct families with DRS or Robinow-like traits had variants affecting *FZD2* either *de novo* or co-segregating with the disease. We then performed a GeneMatcher<sup>37</sup> search which led to the identification of a fourth family with a single simplex case of Robinow-like features and a *de novo* variant in *FZD2*. The four families, identified *FZD2* variants, and selected subjects are shown as [Figure 2](#) and described in [Table S1](#).

Of note, the same amino acid residue, glycine 434, is altered in three out of four families. One family with an affected mother and child co-segregate a missense variant (GenBank: NM\_001466.3; c.1301G>T [p.Gly434Val]) and one subject has a dinucleotide substitution (c.1301\_1302delinsTT [p.Gly434Val]), but the same modification at the protein level ([Figures 2A–2C](#)). Lastly, in one affected individual, a *de novo* variant (c.1300G>A [p.Gly434Ser]), which also changes the same codon, altering residue Gly434 was observed. Residue Gly434 is conserved throughout all vertebrates and is located at the edge of a transmembrane domain; the adjacent intracellular loop is one of the three motifs that were shown to be required for binding and stabilizing the interaction with Dvl1.<sup>38</sup> Therefore, alterations in Gly434 potentially interfere or enhance the affinity or stability of the *FZD2*-DVL interaction, which has been shown to occur via the C terminus of DVL1 including the DEP domain.<sup>39</sup>

In contrast to the above individuals with *FZD2* variants, one subject (BAB8596) carries a nonsense truncating allele (c.1130G>A [p.Trp377\*]) in addition to a variant of uncertain significance (VUS) (c.425C>T [p.Pro142Leu]) affecting *FZD2* *in trans* ([Figure 2](#)). The nonsense single-nucleotide variant is located upstream of the recurrent variant present in all other families and is predicted to result in truncation of nearly 200 aa at the C terminus. *FZD2* is a single-exon gene not subject to nonsense-mediated decay, thus indicating that this truncating allele may produce a stable mRNA product. BAB8596 inherited this allele from his



**Figure 2. Location of Identified Variants in *FZD2* Segregating with the Associated Phenotypic Features**

(A) Pedigrees of the four probands with Robinow syndrome features, carrying variants in *FZD2*.

(B) Sanger sequencing traces for index case subjects, demonstrating the detected variants at the nucleotide level. Family of BAB8596, far right, contains two variants in *FZD2*: p.Trp377\* and p.Pro142Leu. The stop gain was inherited from an affected mother and the missense represents a variant of uncertain significance inherited from father.

(C) Representation of the known functional domains of *FZD2* (green and red rectangles).<sup>86</sup> Location of protein-coding variants identified in our cohort: one stop gain and three recurrent variants all affecting glycine 434 located within the third intracellular loop (red dots). One additional variant (p.Trp548\*) from the literature, and reported in association with omodysplasia, is also included.<sup>46</sup>

(D) Photographs of consenting subjects with *FZD2* variants demonstrating shared facial characteristics consisting of a high, broad forehead, prominent eyes, broad and low nasal bridge, low-set ears, broad nasal tip, and anteverted nares.

affected mother, BAB8594, who has a milder form of DRS; her phenotype includes short stature, a high and broad forehead, and mesomelia. The proband presents with short stature, RS facial characteristics (more prominent in him than in his mother), and mesomelia early in life that improved with age as assessed both clinically and by radiographic measurement (Figure S3 and Supplemental Note). The VUS was inherited from his father, BAB8595, and is present in two siblings, BAB9254 and BAB9255, all of whom have short stature but no other features consistent with RS (Table 1). Therefore, we are unable to determine the potential genetic contribution of this VUS to the phenotypes observed in this family. An alternative interpretation of the *FZD2* variant data and segregation in this family is that the VUS may be benign and the short stature observed in this pedigree, a result of assortative mating, as both maternal and paternal lineages are significant for short stature with the proband's expected mid-parental height of 162 cm (<2<sup>nd</sup> centile).

Pertinent clinical information in all individuals with variants in *FZD2* is shown in Table 1 (see Supplemental Note for detailed clinical case reports). The clinical presentation of affected individuals could be considered a constellation of features representing a distinct disorder that includes short stature, limb defects, and shared facial characteristics that includes a high, broad forehead, flat face, low-set ears, broad nasal tip, and anteverted nares (Figure 2D). In addition, all affected individuals (6/6) have mesomelia or micromelia, 4/6 have brachydactyly, and 5/6 have significant short stature. Alternatively, as a result of the broad variable expressivity of associated RS features, individuals with *FZD2* variants could be considered as atypical RS case subjects. In fact, *FZD2* has been proposed in a single family as a candidate gene for omodysplasia<sup>40</sup> (MIM: 258315), a disorder that significantly overlaps with RS. Indeed, the clinical similarities between omodysplasia and DRS are striking. Given the observations that *FZD2*, *WNT5A*, and *DVL1* and *DVL3* interact molecularly and

**Table 1. Phenotypic Features of Individuals with FZD2 (GenBank: NM\_001466.3) Variants**

| Individual ID          | 5449                     | BAB8705             | BAB7987     | BAB7988                             | BAB8596                      | BAB8594                               | BAB9254*                     | BAB9255*                      |
|------------------------|--------------------------|---------------------|-------------|-------------------------------------|------------------------------|---------------------------------------|------------------------------|-------------------------------|
| Zygoty                 | het                      | het                 | het         | het                                 | comp het                     | het                                   | het                          | het                           |
| Variant                | c.1300G>A                | c.1301_1302delinsTT | c.1301G>T   | c.1301G>T                           | c.1130G>A, c.425C>T          | c.1130G>A                             | c.425C>T                     | c.425C>T                      |
| Effect                 | p.Gly434Ser              | p.Gly434Val         | p.Gly434Val | p.Gly434Val                         | p.Trp377*, p.Pro142Leu       | p.Trp377*                             | p.Pro142Leu                  | p.Pro142Leu                   |
| Age last examination   | 10 y 3 mo                | 5 y 8 mo            | 15 y        | 47 y                                | 6 y 7 mo                     | 30 y                                  | 8 y 4 mo                     | 4 y 3 mo                      |
| Inheritance            | <i>de novo</i>           | <i>de novo</i>      | inherited   | unknown; affected mother of BAB7987 | inherited                    | inherited; affected mother of BAB8596 | inherited; sister of BAB8596 | inherited; brother of BAB8596 |
| Gender                 | M                        | F                   | F           | F                                   | M                            | F                                     | F                            | M                             |
| <b>Growth</b>          |                          |                     |             |                                     |                              |                                       |                              |                               |
| Height SD              | 45 <sup>th</sup> centile | –2.9 SD             | –2.25 SD    | –1.7 SD                             | –3.5 SD                      | –2.1 SD                               | –4.5 SD                      | –2.6 SD                       |
| <b>Facial Features</b> |                          |                     |             |                                     |                              |                                       |                              |                               |
| Macrocephaly           | –                        | relative            | –           | +                                   | –                            | –                                     | –                            | –                             |
| Broad forehead         | +                        | +                   | +           | +                                   | +                            | +                                     | –                            | –                             |
| High forehead          | +                        | +                   | –           | –                                   | +                            | +                                     | –                            | –                             |
| Midface hypoplasia     | +                        | +                   | +           | +                                   | –                            | –                                     | –                            | –                             |
| Hypertelorism          | +                        | +                   | +           | +                                   | –                            | –                                     | –                            | –                             |
| Long eyelashes         | mild +                   | +                   | +           | +                                   | +                            | –                                     | –                            | –                             |
| Prominent eyes         | +                        | +                   | +           | +                                   | –                            | –                                     | –                            | –                             |
| Anteverted nares       | +                        | +                   | +           | +                                   | +                            | +                                     | –                            | –                             |
| Wide nasal bridge      | +                        | +                   | –           | ND                                  | –                            | –                                     | –                            | –                             |
| Thin vermilion border  | +                        | +                   | +           | +                                   | –                            | –                                     | –                            | –                             |
| Gingival hyperplasia   | +                        | +                   | +           | +                                   | ND                           | ND                                    | –                            | –                             |
| Bilobed tongue         | –                        | +                   | +           | +                                   | –                            | –                                     | –                            | –                             |
| Dental anomalies       | +                        | +                   | +           | +                                   | –                            | –                                     | –                            | –                             |
| Low set ears           | +                        | +                   | +           | +                                   | +                            | +                                     | –                            | –                             |
| <b>Skeletal</b>        |                          |                     |             |                                     |                              |                                       |                              |                               |
| Hand length            | 14.5 cm (–1 SD)          | ND                  | ND          | ND                                  | 11.5 cm                      | 15.5L 15.7R                           | 12 cm                        | 10 cm                         |
| Limbs                  | mesomelia                | micromelia          | mesomelia   | mesomelia                           | mesomelia, improved with age | mesomelia                             | micromelia                   | micromelia                    |

(Continued on next page)

| Table 1. Continued        |                            |               |         |         |         |         |          |          |  |
|---------------------------|----------------------------|---------------|---------|---------|---------|---------|----------|----------|--|
| Individual ID             | 5449                       | BAB8705       | BAB7987 | BAB7988 | BAB8596 | BAB8594 | BAB9254* | BAB9255* |  |
| Brachydactyly             | +                          | +             | +       | +       | -       | -       | -        | -        |  |
| Clinodactyly              | 5th fingers +              | 5th fingers + | +       | +       | -       | -       | -        | -        |  |
| Camptodactyly             | short low implanted thumbs | 4th fingers + | -       | -       | -       | -       | -        | -        |  |
| Broad thumb               | -                          | +             | -       | -       | -       | -       | -        | -        |  |
| Broad 1 <sup>st</sup> toe | -                          | +             | +       | +       | -       | -       | -        | -        |  |
| <b>Other Features</b>     |                            |               |         |         |         |         |          |          |  |
| Genital hypoplasia        | -                          | +             | +       | +       | -       | ND      | -        | -        |  |
| Renal anomalies           | -                          | -             | -       | +       | -       | -       | -        | -        |  |
| Cardiac anomalies         | -                          | -             | -       | -       | -       | -       | -        | -        |  |

Abbreviations: ND, no data; het, heterozygous. Asterisk (\*) indicates subjects not diagnosed with Robinow syndrome.

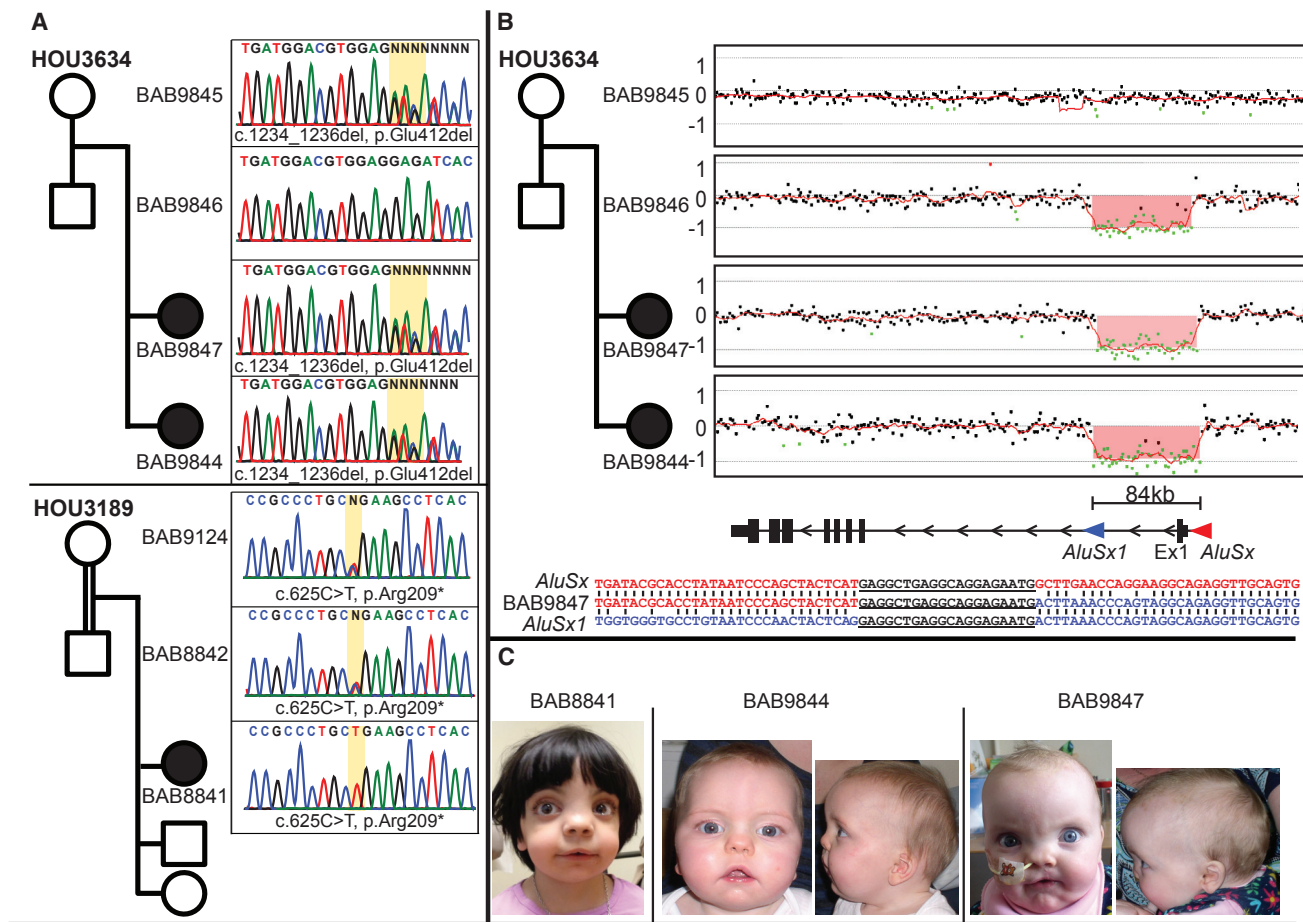
the shared phenotype between omodysplasia and RS, these data are consistent with the hypothesis that omodysplasia represents a subset of RS with predominant short humeri and radial head dislocation collectively representing a point on the spectrum of RS, rather than a distinct clinical syndrome. The characteristic “fetal face” is present in all affected individuals with additional skeletal and limb defects of varying severity but this would not meet a strict clinical diagnostic criterion for DRS, except for BAB7987, BAB7988, and BAB8596. Although there have been previous reports of RS-affected individuals without limb defects, it is unclear whether this cohort of affected individuals would constitute necessary phenotypic overlap to clinically describe this as RS or a distinct disorder of variable severity.<sup>41</sup>

#### Biallelic Variants in *NXN* Cause Recessive Robinow Syndrome

One individual clinically diagnosed with RS, BAB8841, carries a homozygous stopgain variant in *NXN* (GenBank: NM\_022463.4; c.625C>T [p.Arg209\*]) inherited from consanguineous parents (Figure 3A). GeneMatcher<sup>37</sup> identified a second family with two affected siblings (BAB9844, BAB9847) who shared compound heterozygous biallelic variants in *NXN*: a maternally inherited in-frame 3-bp deletion (GenBank: NM\_022463.4; c.1234\_1236del [p.Glu412del]) and a paternally inherited intragenic 84-kb deletion that encompasses the entire first exon: seq[GRCh37]del (17)(p13.3) chr17:g.805043::GAGG.....AATG::889090 (Figures 3A, 3B, and S4). Deletion CNVs can often account for disease-associated pathogenic alleles at both AD and AR disease trait loci.<sup>42–44</sup> This specific *NXN* exon 1 deletion CNV is mediated by two directly orientated *Alu* elements (*AluSx-AluSx1*) sharing 81% nucleotide identity (Figure 3B); such *Alu-Alu*-mediated exonic deletions often involve different *Alu* family members located within flanking introns. Remarkably, *NXN* maps to 17p13.3, which has a high *Alu* density and likely elevated rates of *Alu-Alu*-mediated rearrangements,<sup>45</sup> indicating that future diagnostic efforts should include copy-number analyses for this locus. The first and last exons of a multi-exon gene may be particularly prone to *Alu-Alu*-mediated deletion CNVs related to the abundance of *Alu* elements flanking the 5' and 3' ends of the gene which can be used as template switch substrates during microhomology-mediated break-induced replicative repair.<sup>46,47</sup>

*NXN* encodes an oxidative stress response protein, nucleoredoxin, which is highly expressed in the developing limb bud of mice.<sup>48</sup> Abnormal activation of the Wnt/ $\beta$ -catenin signaling in *Nxn*<sup>-/-</sup> knockout mice is hypothesized to lead to the observed craniofacial defects, a phenotype perhaps relevant to the subjects with RRS reported herein due to biallelic *NXN* variants that are likely loss-of-function alleles. Moreover, studies in *Xenopus laevis* have collectively shown that *Nxn* acts as





**Figure 3. Identified Biallelic Variants in *NXN***

(A) Pedigrees and Sanger sequencing traces for the two families with variants in *NXN*; a homozygous stop gain in BAB8841 (family HOU3189) and a heterozygous 3-bp in-frame deletion in BAB9847 and BAB9844 (family HOU3634).

(B) High-density arrays and breakpoint mapping showing the second mutant allele in BAB9847 and BAB9844. The deletion is mediated by two highly similar *Alu* elements in direct orientation that flank the first coding exon.

(C) Facial pictures demonstrating shared facial features including high forehead, prominent eyes, broad and low nasal bridge, broad nasal tip, anteverted nares, and micrognathia.

a negative regulator of the Wnt/PCP pathway potentially by blocking the ubiquitination and degradation of Dvl as well as inhibiting Dvl-induced *c-jun* phosphorylation via Rac, the crucial biochemical mechanism underlying the Wnt/PCP pathway.<sup>19,20</sup> Two independent *Nxn*<sup>-/-</sup> mouse models show abnormal craniofacial morphology with a shortened snout and cleft palate partially recapitulating the RS subjects' phenotype.<sup>49,50</sup> The individuals with biallelic variants in *NXN* present with a phenotype consistent with a diagnosis of RS, wherein all three identified individuals have typical facial characteristics, mesomelia, brachydactyly, and broad thumbs/toes (Figure 3C and Supplemental Note for detailed clinical case reports).<sup>51</sup>

#### Identification of Additional Robinow Syndrome Candidate Disease-Associated Genes

In two affected individuals we identified likely pathogenic rare variants in association with the rare RS phenotype in two distinct candidate genes whose function has been demonstrated to play an important role in Wnt/PCP

signaling: *RAC3* and *GPC4*. These include one sporadic Robinow-like individual (BAB8740) with a *de novo* missense variant in *RAC3* (GenBank: NM\_005052.2; c.176C>G [p.Ala59Gly]). Another individual (BAB8295) has a hemizygous nonsynonymous missense variant in *GPC4* (GenBank: NM\_001448.2; c.1235G>A [p.Arg412Lys]) inherited from a heterozygous unaffected mother, potentially responsible for an X-linked form of RS. We propose *RAC3* and *GPC4* to be candidate genes underlying Robinow-like features due to the shared interactome and signaling network of known (*WNT5A*, *ROR2*, *DVL1*, *DVL3*) and currently described (*FZD2*, *NXN*) RS disease-relevant genes. In addition, both affected subjects share the same rare phenotype and the exceedingly rare nature of the mutant alleles. *RAC3* is a member of the Rac subfamily of the Rho family of GTPases, which specifically interacts with DVL in response to Wnt ligand to activate downstream effects including JNK/*c-jun* phosphorylation essential for cytoskeletal reorganization.<sup>52</sup> *GPC4* is one of the six members of the heparin sulfate proteoglycans, which are key players in

endochondral ossification<sup>53</sup> with defects in *GPC6* (MIM: 604404) and *GPC3* (MIM: 300037) resulting in the skeletal disorders omodyspasia<sup>54</sup> and Simpson-Golabi-Behmel syndrome (MIM: 312870),<sup>55</sup> respectively. Specifically, *GPC4* has been shown to be a positive regulator of Wnt/PCP signaling by promoting the accumulation of dishevelled at the cell membrane<sup>56</sup> and can rescue *Drosophila* Dfz2 aberrant Wnt signaling phenotypes.<sup>57</sup> Indeed, duplications of *GPC4* have been associated with Simpson-Golabi-Behmel syndrome,<sup>58</sup> however, loss of *gpc4* in *Danio rerio* results in dwarfism phenotypes, which may represent an unspecific finding.<sup>59</sup> The clinical phenotype of individuals harboring candidate variants is consistent with earlier clinical descriptions of DRS with varying severity of associated traits;<sup>51</sup> shared phenotypes are shown in Table 2 (see Supplemental Note for detailed clinical descriptions).

## Discussion

The concept that mutation in distinct genes could lead to the same phenotype was first suggested almost a century ago by William Allan when he observed multiple modes of inheritance (AD, AR, XL) for Charcot-Marie-Tooth disease consistent with at least two genetic loci (autosomal and X-linked);<sup>60</sup> this was further demonstrated by N.E. Morton more than 60 years ago, wherein elliptocytosis was mathematically linked to two distinct human loci in different families.<sup>61</sup> Although genetic heterogeneity has been known for some time, only recently with comprehensive clinical phenotyping, syndrome characterization, and cataloguing of Mendelian disease traits through the efforts of OMIM can we begin to appreciate the frequency of genetic heterogeneity.<sup>62</sup> As of April 2017, an estimated 12.5% (395/4,931) of Mendelian phenotypes have had disease-causing mutations identified in two or more genes represented as a “phenotypic series.”<sup>63</sup> Genetic heterogeneity can be viewed as an obstacle to identifying disease-gene associations, although it is also recognized that overlapping phenotypes arising from distinct loci might reflect perturbed protein interactions or shared biological relationships.<sup>64</sup> Accordingly, genetically heterogeneous disorders provide an opportunity for insight into the pathways and biological underpinnings of disease, wherein a single syndrome provides evidence for the aberrant interactome, network, or biological signaling cascade.

Noncanonical Wnt signaling regulates, via the PCP pathway, convergent extension movements, an essential cell migration process during vertebrate gastrulation.<sup>65</sup> The aforementioned developmental processes in different organisms are largely controlled by the same set of core PCP proteins, which were originally identified in *Drosophila*. However, compared to *Drosophila*, the vertebrate PCP pathway has evolved and diversified by specification and emergence of multiple paralogs from a single fly gene, throughout nearly all of the core components of this pathway. Not surprisingly, recent studies

have demonstrated that genes that are essential in flies and have multiple human orthologs are more likely to be associated with human disease than are fly genes with a single human ortholog,<sup>10</sup> potentially indicating duplication and further paralog specialization during evolution from flies to human. Indeed, in vertebrates, Wnt/PCP signaling partially drives the patterning and formation of the limb-bud outgrowth and growth plate in skeletal formation.<sup>66,67</sup> Of particular interest is the organized cellular division and planar alignment of proliferating chondrocytes at the growth plate, as this process is dependent on Wnt/PCP activation and defects result in abnormal growth plate chondrocyte localization and morphology with skeletal defects analogous to RS.<sup>66,68,69</sup>

Genetic screening in *Drosophila* identified many of the components of the PCP pathway including both frizzled<sup>70</sup> and dishevelled.<sup>71</sup> We provide evidence that variants in multiple WNT/PCP signaling genes can be associated with RS including *FZD2* and *NXN* in autosomal-dominant and -recessive inheritance patterns, respectively. *FZD2* encodes the highly conserved seven-pass transmembrane protein of the Frizzled family of membrane receptors. Frizzled was originally identified by Vinson and Adler in *Drosophila melanogaster* with “clinical findings” of misoriented cuticle hairs, which they named *frizzled*.<sup>70</sup> Interestingly, similar to all other Robinow-associated proteins, *FZD2* is implicated in the Wnt/PCP pathway, in which studies have demonstrated Frizzled proteins functioning as Wnt receptors and co-receptors<sup>72,73</sup> and is required for the WNT5A-mediated recruitment of DVL2 (MIM: 602151) to the membrane.<sup>74</sup> WNT5A binding, in the presence of ROR1 (MIM: 602336), ROR2, and DVL2, induces clathrin-mediated internalization of *FZD2*, which is necessary for RAC1 activation in HEK293 cells.<sup>74</sup>

*NXN* demonstrates a specific redox-dependent interaction with dishevelled via the PDZ domain; in fact, pull-down assays of mouse fibroblasts indicate that *NXN* is a substantial interacting partner of DVL1, particularly under oxidative conditions,<sup>20</sup> often stimulated by growth factors.<sup>75</sup> *In vitro* association with DVL2 and DVL3 has also been demonstrated.<sup>76</sup> The interaction of *NXN* and DVL may be a key regulatory mechanism to maintain a spatial or temporal balance between canonical and noncanonical Wnt pathways during development. Evidence for this includes the fact that *NXN* specifically inhibits DVL function *in vitro* whereas it also blocks ubiquitination and degradation of DVL via KLHL12 in a tissue-specific manner; the latter helps to regulate transmission of Wnt/ $\beta$ -catenin signal upon Wnt stimulation.<sup>20,49</sup> In addition, tissue-specific dysregulation of Wnt/ $\beta$ -catenin signaling was experimentally demonstrated in *Nxn*<sup>-/-</sup> mice in which osteoblastic cells show excessive differentiation associated with strong Wnt/ $\beta$ -catenin signaling activation compared to osteoblasts of *Nxn*<sup>+/+</sup> mice whereas mouse embryonic fibroblast cells show impaired Wnt/ $\beta$ -catenin signaling.<sup>49</sup> Collectively, there is ample evidence in the literature that disruption of Frizzled and

**Table 2. Phenotypic Features of Individuals with Variants in RS Candidate Genes *NXN* (Recessive), *RAC3* (Dominant), and *GPC4* (X-Linked)**

| Individual ID          | BAB8841     | BAB9847  | BAB9844  | BAB8740        | BAB8295                 |
|------------------------|-------------|--|--|----------------|-------------------------|
| Gene                   | <i>NXN</i>  | <i>NXN</i>   | <i>NXN</i>   | <i>RAC3</i>    | <i>GPC4</i>             |
| GenBank                | NM_022463.4 | NM_022463.4  | NM_022463.4  | NM_005052.2    | NM_001448.2             |
| Zygoty                 | hom         | comp het   | comp het   | het            | hemizygous              |
| Variant(s)             | c.625C>T    | c.1234_1236del<br>chr17:g.805043::GAGG....AATG::889090 | c.1234_1236del<br>chr17:g.805043::GAGG....AATG::889090 | c.176C>G       | c.1235G>A               |
| Effect                 | p.Arg209*   | p.Glu412del, ?   | p.Glu412del, ?   | p.Ala59Gly     | p.Arg412Lys             |
| Consanguinity          | +           | -  | -  | -              | -                       |
| Age last examination   | 5 y         | 2 y 5 mo   | 4 wk   | 13 y 2 mo      | 8 y                     |
| Inheritance            | inherited   | inherited  | inherited  | <i>de novo</i> | inherited               |
| Gender                 | F           | F  | F  | F              | M                       |
| <b>Facial Features</b> |             |  |  |                |                         |
| Macrocephaly           | +           | relative   | relative   | -              | +                       |
| Frontal bossing        | -           | +  | +  | -              | +                       |
| High forehead          | +           | +  | +  | -              | +                       |
| Midface hypoplasia     | -           | +  | +  | +              | +                       |
| Hypertelorism          | +           | +  | +  | +              | +                       |
| Long eyelashes         | +           | -  | -  | +              | -                       |
| Prominent eyes         | +           | +  | +  | +              | -                       |
| Anteverted nares       | +           | +  | +  | +              | +                       |
| Wide nasal bridge      | -           | +  | +  | +              | +                       |
| Short nose             | -           | +  | +  | +              | -                       |
| Long philtrum          | +           | +  | +  | +              | -                       |
| Triangular mouth       | +           | +  | +  | -              | +                       |
| Gingival hyperplasia   | +           | +  | +  | -              | +                       |
| Absent uvula           | +           | -  | +  | -              | -                       |
| Cleft soft palate      | -           | -  | +  | -              | +                       |
| Dental anomalies       | +           | -  | ND   | +              | delayed dental eruption |
| Micrognathia           | +           | +  | +  | +              | -                       |

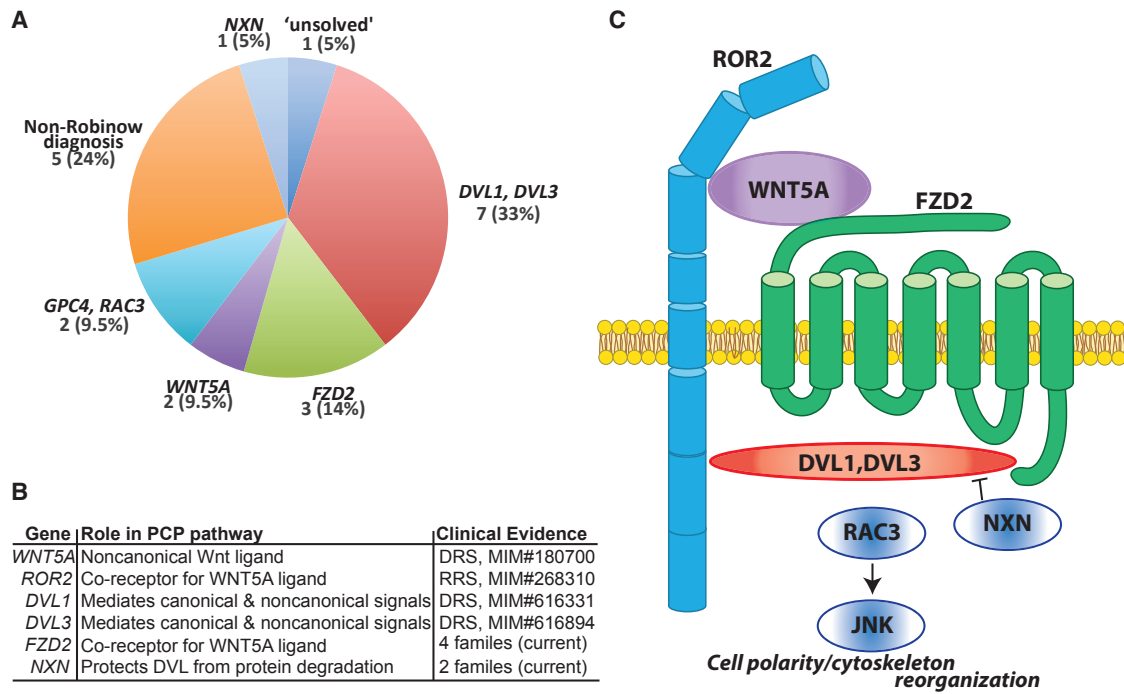
*(Continued on next page)*

| Individual ID         | BAB8841 | BAB9847             | BAB9844 | BAB8740 | BAB8295 |
|-----------------------|---------|---------------------|---------|---------|---------|
| <b>Skeletal</b>       |         |                     |         |         |         |
| Mesomelia             | +       | + improved with age | +       | -       | +       |
| Brachydactyly         | +       | +                   | +       | -       | +       |
| Clinodactyly          | +       | +                   | +       | +       | +       |
| Camptodactyly         | -       | +                   | +       | -       | +       |
| Broad thumb           | +       | +                   | +       | -       | +       |
| Fetal finger/toe pads | -       | +                   | +       | +       | -       |
| Broad 1st toe         | +       | +                   | +       | -       | +       |
| <b>Other Features</b> |         |                     |         |         |         |
| Genital hypoplasia    | ND      | -                   | -       | +       | -       |
| Renal anomalies       | +       | -                   | -       | -       | -       |
| Cardiac anomalies     | +       | -                   | -       | -       | PDA     |

Abbreviations: ND, no data; hom, homozygous; het, heterozygous; PDA, patent ductus arteriosus.

noncanonical Wnt signaling leads to delay or block in chondrocyte maturation and shortening of skeletal elements,<sup>67,76</sup> which could be the basis of short stature and mesomelia in individuals with RS. Pathogenic variants found in individuals with RS provide insights into the underlying biology of the disease, e.g., loss-of-function mutations affecting the WNT5A receptor ROR2 in ~80% of individuals with RRS<sup>3,4</sup> and hypomorphic mutations affecting WNT5A in ~9.5% of individuals with DRS<sup>6</sup> (Figure 4A), suggesting that the Wnt/PCP pathway is affected in those subjects. This hypothesis is strengthened with the discovery that recurrent truncating variants in both *DVL1* and *DVL3*, leading to C-terminal replacement of those proteins with a highly basic tail, is currently the major cause (33%) of DRS. Furthermore, it has been demonstrated that a subset of *DVL1*-mediated DRS-affected subjects present with osteosclerosis, a phenotype that is associated with perturbed Wnt signaling.<sup>8</sup> Such mutational specificity in both *DVL1* and *DVL3* is intriguing and suggest a specific role in the noncanonical pathway. Despite DVL being an important contributor in diverse signaling pathways,<sup>11</sup> its C terminus is characterized by (1) having a conserved DEP domain that works in the PCP signaling, (2) being required for membrane recruitment via Wnt-mediated signaling,<sup>77</sup> (3) carrying relevant phosphorylation sites that specify DVL functions and subcellular localization,<sup>78,79</sup> and (4) being necessary to establish and to stabilize protein-protein interactions with ROR2<sup>12</sup> as well as FZD2.<sup>38</sup> In fact, our current study indicates that variants affecting *FZD2* specifically lead to DRS or Robinow-like traits<sup>40</sup> when all the DVL interaction domains either are removed (p.Trp377\*) or are adjacent to them (p.Gly434Ser/Val) (Figure 2C). Moreover, *NXN*, a gene that encodes a redox-dependent regulator of DVL, was identified in 5% (one family) of this clinically ascertained RS cohort and appears to cause a new recessive form of RS. Compound heterozygous variants within this gene co-segregating with the disease were further observed in another family, confirming our initial observation. *NXN* is a potent regulator of DVL stability, particularly in conditions of high oxidative stress,<sup>49</sup> circumstances intriguingly similar to the high levels of reactive oxygen species during chondrocyte hypertrophy at the growth plate.<sup>80</sup> The relevance of *NXN* mutations for RRS requires further studies in other unsolved RS cases.

In aggregate, our data provide supportive experimental evidence that the clinical phenotype in RS results from subtle dysregulation of the Wnt/PCP pathway, contextually affecting the organized proliferation of chondrocytes at the growth plate. A delicate balance between canonical and PCP signaling requires precise regulation of DVL, so it appears that the pathogenesis of Robinow is not simply loss of Wnt/PCP signaling, but a context-specific perturbation in the Wnt signaling. These new findings also support the hypothesis that additional proteins underlying RS or overlapping genetic syndromes will also have a



**Figure 4. Robinow Syndrome-Associated Genes in the Wnt/PCP Pathway Identified in Human Subjects**

(A) Molecular diagnosis pie chart from the cohort of 21 Robinow syndrome-affected individuals who had a combination of direct Sanger screening and whole-exome sequencing to identify the molecular cause of their disorder highlighting the contribution of *DVL1*, *DVL3*, and *FZD2* variants to RS.

(B and C) The establishment of planar cell polarity is vital for vertebrate development; in humans, variants affecting genes in that pathway are linked to skeletal defects, including Robinow syndrome. *In vitro* studies from several model organisms have demonstrated that ROR2 binds to WNT5A and acts as a co-receptor with FZD2.<sup>5</sup> The downstream effect is routed by the dishevelled homologs, which are stabilized by NXN in a context-dependent manner.<sup>49</sup> The downstream readouts, which ultimately involves cytoskeletal reorganization, are a combination of small GTPases including RAC to activate JNK signaling.<sup>52</sup> Pathogenic variants in all of the aforementioned genes have been identified in individuals with Robinow syndrome, underscoring the notion that this disorder results from aberrant Wnt/PCP signaling, likely due to disturbance of the organized development of chondrocytes at the growth plate.

relevant role in this pathway (Figure 4B). Our data initially indicated that 3 of the 21 families from the RS-affected case subjects remained “unsolved” or without a plausible molecular diagnosis (Figure 4A). However, two of the three unsolved probands in our RS cohort have variants affecting genes biologically relevant in the context of Wnt signaling: a *de novo* mutation in an autosomal gene (*RAC3*) and an X-linked variant in a male from a heterozygous, unaffected mother (*GPC4*). Both genes *RAC3* and *GPC4* are established components of the Wnt/PCP pathway and therefore represent candidate genes that need to be confirmed by the ascertainment of additional affected individuals. In aggregate, personal genome analyses provide a plausible molecular diagnosis that parsimoniously explains the observed clinical phenotype in 95% of individuals clinically diagnosed with Robinow; i.e., 20/21 subjects investigated.

In summary, we demonstrate that the study of rare human syndromes and phenotypes can provide insight into human biology, a Garrodian principle stemming from the early pioneers of human genetics.<sup>1,81</sup> Figure 4C depicts a representative model for the physical interactions of all identified Robinow-associated proteins with a role in the Wnt/PCP pathway that have been identified in human mutational studies thus far. Of note, in this cohort all

variant types—single-nucleotide variants (SNVs), indels, and copy-number variants (CNVs)—were found to be pathogenic and a multitude of mutational processes, including *Alu-Alu*-mediated rearrangement resulting in exonic deletion<sup>82,83</sup> and secondary structure mutagenesis for recurrent indel formation,<sup>84</sup> were shown to be operative. In dissecting the etiology of non-allelic genetic heterogeneity in RS, we observe strong evidence that disturbed levels of a complex interplay of noncanonical and canonical Wnt signaling underlies the core phenotype exhibited by affected individuals. The relevance of this observation becomes clear when considering that canonical and noncanonical pathways use common intracellular components and that specificity of distinct Wnt signaling pathways is regulated by the combination of ligands and receptors in a particular cellular context. In this way, the same proteins in distinct cellular environments may inhibit or trigger a given signal branch. For example, FZD2 can either inhibit or trigger canonical and non-canonical  $\beta$ -catenin pathways depending on the presence of additional transmembrane receptors.<sup>74</sup> Ligation of WNT5A to FZD2 was shown to inhibit canonical  $\beta$ -catenin in addition to activate the noncanonical pathway in HEK293 cells, as measured by downstream RAC1 activation, the latter depending on the presence of ROR1, ROR2, and



DVL2. The discovery of the main gene contributors in RS will likely further clarify underlying molecular mechanism in other skeletal dysplasias with an overlapping phenotype, as for example, in omodysplasia.

As we progress in our molecular and genetic understanding of many Mendelian disease traits, it is important to understand and appreciate genetic heterogeneity, clinical variability, and overlap of clinical features (i.e., phenotypic signs/symptoms) with other disorders. Here we have approached gene discovery in a phenotype-driven manner, using an unbiased genomics rather than locus-specific approach, i.e., a Mendelian genomics versus Mendelian genetics single-gene/locus approach. As shown here, Mendelian genomics can readily investigate the underpinnings of genetic heterogeneity of disease traits as well as explore mutational burden and multi-locus variation models for disease.<sup>33,85</sup> This study has enabled the identification of pathway-specific candidate disease-associated genes in RS, the specific paralogs involved, assessed the contribution of known genes, and provided insights into underlying mechanisms of a disease marked by genetic heterogeneity, which ultimately revealed the contribution of key proteins to limb formation and human development.

#### Accession Numbers

The accession numbers for identified variants were deposited into ClinVar with the following identifiers: SCV000583563–SCV000583582.

#### Supplemental Data

Supplemental Data include four figures, two tables, and Supplemental Notes of case reports and can be found with this article online at <https://doi.org/10.1016/j.ajhg.2017.10.002>.

#### Conflicts of Interest

J.R.L. has stock ownership in 23andMe, is a paid consultant for Regeneron Pharmaceuticals, has stock options in Lasergen, Inc., and is a co-inventor on multiple United States and European patents related to molecular diagnostics for inherited neuropathies, eye diseases, and bacterial genomic fingerprinting. The Department of Molecular and Human Genetics at Baylor College of Medicine derives revenue from molecular genetic testing offered in the Baylor-Genetics Laboratories.

#### Acknowledgments

The authors would like to thank the individuals and their families who contributed to this study and the Robinow Syndrome Foundation for facilitating collaboration. Supported by the US National Human Genome Research Institute/National Heart Blood Lung Institute jointly funded Baylor Hopkins Center for Mendelian Genomics (UM1 HG006542). J.J.W. was funded in part by the Smith-Magenis Syndrome Research Foundation (SMSRF). A.M.V.-M. is funded by FAPESP (CEPID 2013/08028-1). The

content is solely the responsibility of the authors and does not necessarily represent the official views of the NHGRI/NHBLI, NIH.

Received: May 26, 2017

Accepted: October 6, 2017

Published: December 21, 2017

#### Web Resources

1000 Genomes, <http://www.internationalgenome.org/>  
Atherosclerosis Risk in Communities Study (ARIC) Database, <http://www2.csc.ccc.unc.edu/aric/>  
Baylor Genetics Laboratory, <http://bmgf.com/>  
BCM HGSC Mercury, <https://www.hgsc.bcm.edu/software/mercury>  
ClinVar, <https://www.ncbi.nlm.nih.gov/clinvar/>  
dbGaP, <http://www.ncbi.nlm.nih.gov/gap>  
ExAC Browser, <http://exac.broadinstitute.org/>  
GenBank, <https://www.ncbi.nlm.nih.gov/genbank/>  
OMIM, <http://www.omim.org/>  
RefSeq, <http://www.ncbi.nlm.nih.gov/RefSeq>  
Robinow Syndrome Foundation, <http://www.Robinow.org>

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