WNT3 involvement in human bladder exstrophy and cloaca development in zebrafish

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Abstract

Bladder exstrophy, a severe congenital urological malformation when a child is born with an open urinary bladder, is the most common form of bladder exstrophy-epispadias complex (BEEC) with an incidence of 1:30,000 children of Caucasian descent. Recent studies suggest that WNT genes may contribute to the etiology of bladder exstrophy. Here, we evaluated WNT-pathway genes in 20 bladder exstrophy patients using massively parallel sequencing. In total 13 variants were identified in WNT3, WNT6, WNT7A, WNT8B, WNT10A, WNT11, WNT16, FZD5, LRP1 and LRP10 genes and predicted as potentially disease causing, of which seven variants were novel. One variant, identified in a patient with a de novo nonsynonymous substitution in WNT3 (p.Cys91Arg), was further evaluated in zebrafish. Knock down of wnt3 in zebrafish showed cloaca malformations, including disorganization of the cloaca epithelium and expansion of the cloaca lumen. Our study suggests that the function of the WNT3 p.Cys91Arg variant was altered, since RNA overexpression of mutant Wnt3 RNA does not result in embryonic lethality as seen with wild-type WNT3 mRNA. Finally, we also mutation screened the WNT3 gene further in 410 DNA samples from BEEC cases and identified one additional mutation c.638G>A (p.Gly213Asp), which was paternally inherited. In aggregate our data support the involvement of WNT-pathway genes in BEEC and suggest that WNT3 in itself is a rare cause of BEEC.

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Introduction

The bladder extrophy-epispadias complex (BEEC) is a severe congenital urological malformation where a child is born with an open urinary bladder. The phenotype differs in severity from an open urethra only (epispadias), classic bladder extrophy (CBE) and cloaca extrophy, the most severe form with involvement of the intestines (1). Bladder extrophy is a rare malformation with a birth incidence of 1:30,000 in children of European descent (2). The patients are usually operated neonatally, and on several occasions thereafter, and they generally need a life-long medical follow-up.

Most BEEC cases are sporadic and non-familial. Recent studies suggest that periconceptive folic acid supplementation may have a protective effect, whereas maternal smoking and medical radiation during the first trimester may be risk factors for bladder extrophy (3). The risk for siblings to be born with BEEC has been estimated to be 1:70 and the risk for offspring is 1:300, which is much higher than in the general population (4). Moreover, twin studies have shown a higher concordance rate in monozygotic twins compared with dizygotic twins, 62 and 11% respectively, which suggests an underlying genetic effect (2). BEEC is thus considered to be a multifactorial disorder, caused by both environmental and genetic factors.

The underlying predisposing genetic factors for BEEC are still largely unknown although chromosomal aberrations have been associated with BEEC (5). We have previously shown that the recurrent ~3 Mb 22q11.2 duplication is associated with BEEC and hearing loss (6-8). The 22q11 duplication syndrome shows highly variable both inter- and intra-family expressivity between carriers; some individuals are asymptomatic/subclinical while others present with a combination of cardiovascular anomalies, velopharyngeal insufficiency with or without a cleft palate, hearing loss, global developmental delay and abnormal facial features (9).

Several lines of evidence suggest that WNT gene family members may be involved in the etiology of BEEC. First, in an attempt to identify new bladder extrophy genes, a Genome-Wide Expression Profiling study was performed using normal and extrophian human bladder tissue, as well as human and mouse embryonic bladder tissues. In total 162 differentially expressed genes were identified, including genes from the WNT-pathway, p63, PERP and SYNPO2 (10). Second, recent genome-wide association studies have identified an association between a highly conserved 32 kb regulatory region (top SNP: rs98990413) located between WNT3 and WNT9b genes on chromosome 17q21.32 (11) and an association with a region of approximately 220 kb (top SNP: rs9291768) on chromosome 5q11.1 harboring the Wnt/β-catenin signaling regulated ISL1 (ISL LIM homeobox 1) gene (12). Finally, Wnt3 and Wnt9b were shown to be expressed in genital tubercle in mouse embryos, E9.5-E11.5 and E9.5-E13.5 respectively, which correspond perfectly to the period of bladder development in humans (11).

The WNT gene family encodes 19 highly conserved secreted glycoproteins that activate cell surface receptors (13,14). The different proteins are involved in different WNT-signaling pathways with different ligands, receptors and intracellular mediators. In this way the WNT proteins are involved in multiple intracellular functions that are critical for many developmental processes, among others organogenesis and organization of the body plan during embryonic development, as demonstrated by animal models (13–16). For example, Wnt3 mutant (MT) mice present defects in axis formation and gastrulation.

Zebrafish have been previously used with success to model defects of the cloaca (proctodeum) (17–19), making it a suitable model organism for studying developmental mechanisms underlying BEEC. The mechanism underlying BEECs are unknown, but likely arise from abnormal morphogenesis of the cloaca. Zebrafish do not have a urinary bladder, but the kidney (proephros) is thought to be homologous to the mammalian nephric duct, which inserts into the bladder (20). Cells contributing to both the proephros and the cloaca arise from the ventral mesoderm with specification of cloaca domain requiring BMP signaling (18). At 24 h post-fertilization (hpf) the ectodermal cells of the cloaca are clearly marked by the expression of transcription factors such as pdml1 and eve1. The tubular cells of the proephros actively migrate towards these cells in a process requiring the cilia gene nephrocystin-4 (19). Around 24–30 hpf the proephros has reached its ventral target, and the cloaca channel opens to its environment (17). At this stage the hindgut is a mass of endodermal cells (17), however 48 hpf the hindgut too has reached the cloaca and starts to fuse with it. At 96 hpf it has reached the ventral limit of the embryo, but does not open to the environment until around 120 hpf (17). Cloacal malformations as seen in sonic hedgehog pathway MTs can interfere with this developmental process, leading to anorectal abnormalities in both fish and mouse (17,21,22).

The aim of this study was to explore the mutation spectrum of the WNT gene family network in an extensive Swedish BEEC patient material using next generation sequencing technology and to evaluate the identified candidate genes in the zebrafish model.

Results

Massive parallel sequencing identifies coding changes in WNT genes

We screened the WNT family network genes (Supplementary Material, Table S1) for mutations in a massive parallel sequencing experiment. Five of the genes (WNT7A, WNT11, DKK1, BMP1 and DVL1) showing differential expression in the study by Qi et al. 2011 were also screened for variants in the current study. We analyzed 20 CBE patients without the 22q11 duplication, which has previously been associated with CBE (6). The analysis identified a total of nine coding changes in seven different WNT genes in eight CBE cases, one coding change in Fzd5 in a CBE patient and three variants in two different LRP genes in four CBE cases (Table 1). Two different in silico prediction softwares, MutationTaster (23) and Polymorphism Phenotyping v2 (PolyPhen-2) (24), were used to assess pathogenicity and eight variants were predicted to be pathogenic by both programs, whereas remaining variants were recognized as pathogenic by one of the programs. PolyPhen-2 only provides an estimate for missense- and nonsense variants but not for silent substitutions or frameshift mutations. All variants were confirmed by Sanger sequencing (Table 1). Family segregation showed that two variants were de novo in two patients with classical bladder extrophy: one heterozygous missense mutation in exon 2 of WNT3 (c.271T>C, p.Cys91Arg) and one silent base pair substitution affecting exon 3 of WNT6 (c.570G>A). Remaining variants were maternally or paternally inherited. In total seven variants were novel. Neither the c.271T>C (success rate > 91%) nor the c.570G>A (success rate > 99%) variants were found in 376 placenta samples used as controls. WNT3 was chosen for further studies since it was the only gene with a de novo variant predicted to be pathogenic.

WNT3 mutations are uncommon in BEEC patients

To further explore the frequency of WNT3 mutations in BEEC, we sequenced the promoter region and protein coding exons of
Table 1. Genetic variants in WNT family genes evaluated in 20 CBE patients

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Gene</th>
<th>Reference transcript</th>
<th>Chromosome</th>
<th>cDNA change</th>
<th>Protein change</th>
<th>SNV type</th>
<th>dbSNP</th>
<th>Mutation taster</th>
<th>PolyPhen</th>
<th>Confirmation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEEC11</td>
<td>WNT3</td>
<td>NM_030753</td>
<td>17q21.31</td>
<td>c.271T&gt;C</td>
<td>p.Cys91Arg</td>
<td>Nonsynonymous SNV</td>
<td>n.a.</td>
<td>+</td>
<td>+</td>
<td>de novo</td>
</tr>
<tr>
<td>BEEC13</td>
<td>WNT16</td>
<td>NM_016097</td>
<td>7q31.31</td>
<td>c.1_2insCCCA</td>
<td>p.Metfs</td>
<td>Frameshift insertion</td>
<td>rs75556099</td>
<td>+</td>
<td>−</td>
<td>Paternally inherited</td>
</tr>
<tr>
<td>BEEC18</td>
<td>WNT16</td>
<td>NM_016097</td>
<td>7q31.31</td>
<td>c.1_2insCCCA</td>
<td>p.Metfs</td>
<td>Frameshift insertion</td>
<td>rs75556099</td>
<td>+</td>
<td>−</td>
<td>Paternally inherited</td>
</tr>
<tr>
<td>BEEC5</td>
<td>WNT16</td>
<td>NM_057168</td>
<td>7q31.31</td>
<td>c.66G&gt;C</td>
<td>p.Leu22=</td>
<td>Synonymous SNV</td>
<td>rs35391640</td>
<td>+</td>
<td>−</td>
<td>Paternally inherited</td>
</tr>
<tr>
<td>BEEC9</td>
<td>WNT16</td>
<td>NM_016097</td>
<td>7q31.31</td>
<td>c.1_2insCCCA</td>
<td>p.Metfs</td>
<td>Frameshift insertion</td>
<td>rs75556099</td>
<td>+</td>
<td>−</td>
<td>Paternally inherited</td>
</tr>
<tr>
<td>BEEC3</td>
<td>LRP1</td>
<td>NM_002332</td>
<td>12q13.3</td>
<td>C.9259C&gt;T</td>
<td>p.His3087Tyr</td>
<td>Nonsynonymous SNV</td>
<td>rs2229279</td>
<td>+</td>
<td>−</td>
<td>Paternally inherited</td>
</tr>
<tr>
<td>BEEC9</td>
<td>LRP1O</td>
<td>NM_014045</td>
<td>14q11.2</td>
<td>c.1685G&gt;A</td>
<td>p.Arg562His</td>
<td>Nonsynonymous SNV</td>
<td>rs142131185</td>
<td>+</td>
<td>−</td>
<td>Paternally inherited</td>
</tr>
<tr>
<td>BEEC8</td>
<td>FZD5</td>
<td>NM_003468</td>
<td>2q33.3</td>
<td>c.267G&gt;A</td>
<td>p.Met89Ile</td>
<td>Nonsynonymous SNV</td>
<td>n.a.</td>
<td>+</td>
<td>+</td>
<td>Maternally inherited</td>
</tr>
</tbody>
</table>

All patients present with classical bladder exstrophy, all variants are exonic. Disease causing +; Benign −.

Loss of predicted disulfide bond in p.Cys91Arg variant of WNT3 does neither alter the protein resultant surface, nor the intracellular distribution, concentration and cytoplasmic mobility in NIH 3T3 cells

Structural modeling of the p.Cys91Arg variant in WNT3 showed a loss of a predicted disulfide bond compared with wild-type (WT) protein, although, there was no major steric change in the overall model. Modeling of the proteins resultant surface showed that there does not seem to be an interference with the substitution and the structure.

Studies in NIH 3T3 cells in culture showed that there are no significant differences between the WT and MT protein variants, neither with respect to their intracellular localization (Supplementary Material, Fig. S1A and B), nor with respect to their concentration and cytoplasmic mobility (Supplementary Material, Fig. S1C and D). FCS analysis revealed that protein concentration was similar in all tested cells (n = 12 WT and 16 MT), ranging from 20 to 100 nM, as evident from the amplitudes of autocorrelation curves recorded in different cells (Supplementary Material, Fig. S1C). Fitting analysis yielded two characteristic decay times, $\tau_1 = (0.7 \pm 0.1) \mu s$ and $\tau_2 = (750 \pm 70) \mu s$, with relative contributions of the components $x_1 = (0.7 \pm 0.1)$ and $x_2 = (0.3 \pm 0.1)$, suggesting that about 70% of molecules is freely diffusing in the cytoplasm, whereas about 30% is transiently binding to intracellular targets and thus pausing. The autocorrelation curves normalized to the same amplitude (1 at 10 µs; Supplementary Material, Fig. 1D), showed that there are no significant differences in the cellular dynamic of the MT and the WT protein, as evident from the overlap of these curves.

Knockdown of Wnt3 results in abnormal formation of the cloaca in zebrafish

To test the involvement of the WNT3 in cloacal morphogenesis underlying BEECs in vivo we turned to the zebrafish model. Reciprocal blast identified one Wnt3 zebrafish orthologue with 90% protein identity to human WNT3.

In order to knockdown wnt3, we used a previously published morpholino oligonucleotide (MO) (26). Pod-cad embryos (cadherin17:mCherry) were injected with 3 ng of Wnt3 MO, stained with the nuclear stain DAPI and FITC-phalloidin to visualize filamentous actin (F-actin). Confocal microscopy was used to analyze cloaca morphology between 24 and 96 hpf (Fig. 2). At 48 hpf the distal pronephros and the cloaca epithelium appeared disorganized (Fig. 2A and D) with the lumen of the cloaca slightly expanded, despite normal specification of the cloaca tissue as indicated by expression of the protodeum marker prdm1 (Fig. 2I and J). Similarly, tbx2b staining at 48 hpf was evident in the WNT3 gene in 410 BEEC patients without the 22q11 microduplication. One additional mutation, c.638G>A p.Gly213Asp, was identified in a male patient, that was paternally inherited. Since our pilot group of 20 patients initially showed a higher allele frequency compared with the 1000 Genomes project (25), we genotyped rs142131185, a single nucleotide normal variant (SNV) located upstream of the WNT3 gene in 95 BEEC patients (MAF T = 0.032) and 362 controls (MAF T = 0.0138). However, no statistically significant difference was detected between patients and controls (Chisquare p-value 0.091, permuted p-value (10,000) = 0.1547). Finally, we also analyzed rs9890413, located between WNT3 and WNT9b, in both BEEC patients (MAF G = 0.321) and controls (MAF G = 0.296), but no statistically significant association was detected (Chisquare p-value 0.407, p-value 0.5233, permuted p-value (10,000) = 0.7847).
distal pronephros but appeared disorganized and some ectopic expression was seen in the surrounding tissue (70%, $n = 14/20$) compared with WT controls (11%, $n = 2/16$). By 72 hpf a clear expansion of the cloaca lumen (Fig. 2B, E and Unfilled arrows B′, E′) or expansion of the cloaca and distal pronephros was observed in a significant number of injected embryos (19%, $n = 6/31$ and 36%, $n = 11/31$ Table 2). Thus, the combined number of embryos that showed an expansion of the cloaca lumen at 72 hpf was $n = 17/31$ (55%). By 96 hpf the cloaca is widened (doubled headed arrow, Fig. 2C and F) and a severe expansion of the hindgut lumen was observed (asterisk, Fig. 2C′ and F′). The normally highly polarized cells of the cloaca appear dissociated (arrowheads, Fig. 2C′ and F′). Taken together these results show that loss of Wnt3 during development affects cloaca development.

Wnt3 MT RNA shows reduced activity compared with WT RNA

Next we assayed the effect of WT Wnt3 and MT wnt3p.Cys91Arg mRNA during zebrafish development. Injection of 5–100 pg WT
wnt3 mRNA results in a large proportion of embryos dying by 24 hpf (Table 3). In contrast, injection of even higher doses of wnt3p.Cys91Arg mRNA does not result in cell death (Table 3).

Embryos injected with 5–100 pg of MT RNA survive up to 2 days post-fertilization (dpf), but lack forebrain structures and the eye (data not shown) similar to overexpression of Wnt8b (27) and show malformations of the cloaca in a significant percent of embryos as assessed by bright-field microscopy on anesthetized embryos (Table 3, Fig. 3C and D) with an absence or severe reduction of tbx2b (Fig. 3E and F) and an expansion of prdm1 (Fig. 3G–J). Due to the high level of dead embryos following WT Wnt3 mRNA injection we could not confirm whether overexpression of WT RNA had a significant effect on cloaca development. Titration of RNA dose to low levels showed that both overexpression of WT or MT Wnt3p.Cys91Arg RNA results in a small percent of embryos with cloaca malformation and reduced forebrain structures (Table 3), however this was not significant compared with WT controls.

Discussion

Previous work has suggested that WNT3 may be involved in isolated CBE. Here we use massive parallel sequencing of the WNT-pathway genes and zebrafish knockdown to further support a link between WNT3 and BEEC. We identified a patient with a de novo amino acid substitution in WNT3 (p.Cys91Arg) predicted to be pathogenic, and showed that knockdown of Wnt3 in zebrafish induces clear cloaca abnormalities. An expansion of the cloaca lumen is observed at 72 hpf and 4 dpf, we also see an expansion of the hindgut (asterisk in C′ and F′) and a widening of the cloaca (double headed arrow C and F). Epithelial cells of the normally polarized structure of the cloaca (filled arrowheads C) appear dissociated (filled arrowheads F). Abbreviations: cloaca (cl), hindgut (hg), pronephric duct (pd).

Table 2. Penetrance of knockdown phenotypes at 72 hpf

<table>
<thead>
<tr>
<th>Tissue severely distorted</th>
<th>Distal pronephros, shortended lumen</th>
<th>Cloaca lumen expanded</th>
<th>Normal cloaca, normal pronephros</th>
<th>Distal pronephros shortended, lumen expanded/cloaca lumen expanded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>22</td>
<td>9% (2)</td>
<td>91% (17)</td>
<td>19% (6)*</td>
</tr>
<tr>
<td>5 ng std-cont MO</td>
<td>0</td>
<td>0</td>
<td>91% (17)</td>
<td>0</td>
</tr>
<tr>
<td>3 ng Wnt3 MO</td>
<td>3% (1)</td>
<td>36% (11)**</td>
<td>29% (9)</td>
<td>13% (4)</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with UI controls, **P < 0.001 compared with std-control MO-injected embryos.
show that the function of the identified WNT3 variant was lost, reduced or altered as RNA overexpression of MT Wnt3 RNA does not result in embryo death as seen with WT Wnt3 mRNA.

Interestingly, we identified six CBE individuals with multiple WNT-pathway variants. Patient BEEC11 with a de novo mutation in WNT3 (p.Cys91Arg) also inherited three polymorphisms in WNT7A (p.Ala163Thr), WNT8B (p.Ala141 =) and WNT11 (p.Arg316His) genes. Moreover, WNT11 showed differential expression in the study by Qi et al. 2011, which is the only gene that appears in both studies. Patient BEEC13 with a de novo synonymous change in WNT6 (p.Arg190Arg) also inherited one polymorphism in WNT10A (p.Phe228Ile). Similarly, four other CBE patients (BEEC3, BEEC9, BEEC10 and BEEC18) are carriers of a combination of several variants in WNT-pathway genes (WNT10A, WNT16, LRP10 and LRP1). Further studies in both cases and controls will be necessary to fully determine whether specific combinations of multiple WNT-pathway variants contribute to BEEC.

The importance of WNT3 in bladder exstrophy has been indicated by expression studies performed in mice and humans (10,11) as well as by the association of a highly conserved 32 kb regulatory region in the vicinity of the WNT3 gene (11). Moreover, a homozygous nonsense mutation, c.366C>T (p.Gln83*), in WNT3 most likely resulting in a truncated protein has been described in four affected fetuses of a consanguineous family with autosomal recessive tetra-amelia (28). The authors concluded, based on the phenotypic findings in the fetuses that WNT3 is crucial in early limb formation as well as in urogenital and craniofacial development. Interestingly the Wnt antagonist Dkk, expressed in the procloacal mesenchyme, has recently been linked to genitourinary and anorectal defects in mouse (29). These defects are thought to arise from elevation of Wnt/b-catenin activity, supporting the cloaca malformations seen in our overexpression studies.

In zebrafish both Wnt3 and Wnt3a are expressed at the 18 somite stage in the tailbud, a region caudal to the developing cloaca (30). Unfortunately, we were unable to ascertain the level of Wnt3 expression at later stages of cloaca development. Expression analysis of predicted Wnt receptors fzd1, fzd5, fzd7a, fzd7b and fzd8a revealed that the transmembrane receptor fzd7a, previously shown to mediate Wnt3 signaling (31), was expressed in the mesenchyme adjacent and rostral to the cloaca (Supplementary Material, Fig. S2).

Due to the high level of embryo death following overexpression of WT RNA, it was difficult to assay loss or gain of function of the MT directly in relation to cloaca development. However, we were able to assay a change in activity between MT RNA and WT RNA due to the reduction in embryo death. Ubiquitous overexpression of Wnt3 has previously been shown to induce the dorsalizing factor chordin throughout the early gastrula (30).

Table 3. Penetration of overexpression phenotypes at 48 hpf

<table>
<thead>
<tr>
<th></th>
<th>Grossly malformed/severely dorsIALIZED</th>
<th>Posterized head/cloaca abnormal</th>
<th>Posterized head/normal cloaca</th>
<th>Normal</th>
<th>Dead</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>UI</td>
<td>0</td>
<td>0</td>
<td>88% (66)</td>
<td>12% (9)</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Wnt3 WT 100 pg</td>
<td>0</td>
<td>0</td>
<td>3% (2)</td>
<td>18% (13)</td>
<td>7% (5)</td>
<td>74</td>
</tr>
<tr>
<td>Wnt3 MT 100 pg</td>
<td>27% (12)</td>
<td>36% (54)**</td>
<td>0</td>
<td>52% (42)</td>
<td>12% (10)*</td>
<td>81</td>
</tr>
<tr>
<td>Wnt3 WT 50 pg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>73% (32)</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Wnt3 MT 50 pg</td>
<td>0</td>
<td>36% (29)**</td>
<td>0</td>
<td>52% (42)</td>
<td>12% (10)*</td>
<td>81</td>
</tr>
<tr>
<td>Wnt3 WT 5 pg</td>
<td>39% (30)</td>
<td>7% (5)</td>
<td>14% (11)</td>
<td>3% (2)</td>
<td>37% (28)</td>
<td>76</td>
</tr>
<tr>
<td>Wnt3 MT 5 pg</td>
<td>0</td>
<td>13% (9)*</td>
<td>1% (1)</td>
<td>79% (56)</td>
<td>7% (5)*</td>
<td>71</td>
</tr>
</tbody>
</table>

*P < 0.01 compared with UI controls, **P < 0.001 compared with UI controls, 'P ≤ 0.01 compared with WT injected embryos.
We predict that this results in perturbation of the dorsal-ventral patterning and hence the embryos do not survive gastrulation following overexpression of WT RNA. The reduction in embryo death following injection of MT RNA suggests that the function of Wnt3 in early development is lost, reduced or altered due to the arginine to cysteine substitution. The mutation in Wnt3p.Cys91Arg is predicted to cause disruption of the disulfide bond between residue C91 and C131 (32), which may potentially affect protein folding and may subtly alter the kinetics of Wnt3p.Cys91Arg interactions with other targets, causing changes in lateral diffusion that are too small to be identified by FCS. Still, such minute changes could cause the observed effects during early gastrulation.

We show for the first time the importance of Wnt3 signaling in cloaca development in vivo, and confirm a change of Wnt3 activity due to a cysteine to arginine mutation identified in a BEEC patient, supporting the pathogenicity of this mutation. Thus, a WNT-signaling pathway including WNT3 may be associated with the etiology of BEEC; however, mutations in WNT3 do not seem to be a common cause in BEEC cases presented in the current study. Further studies in mammals are now needed to explain how WNT3 dysregulation lead to bladder extrophy.

Materials and Methods

The local Ethics Committees approved this study.

Patients

Ninety-five sporadic BEEC patients were recruited from the Pediatric Surgery Departments in Stockholm, Gothenburg, Uppsala and Lund, Sweden. DNA samples from 376 placenta, acquired after normal delivery of healthy newborns in 2006 at the Karolinska University Hospital, where used as controls. All parents were unaffected. For WNT3 sequence analysis we investigated an additional 315 duplication 22q11.21 negative BEEC patients. Written informed consent was obtained from all these patients or their parents prior to study entry, and the study was approved by the Ethics Committee of the Medical Faculty of the University of Bonn.

Massive parallel sequence analysis

Massive parallel sequencing was performed on a pilot sample set that consisted of 20 CBE cases that were negative for the 22q11 microduplication and with available parental samples for further verification. They were chosen from a total of 60 BEEC samples which were available at the time the experiment was performed. Libraries for sequencing on Illumina HiSeq2000 (Illumina) were prepared from DNA samples and exome sequences were enriched with Agilent SureSelect Human All Exon 50 M (Agilent), according to manufacturer’s instructions. Post-capture libraries were sequenced as 2 × 100 bp paired end reads on the Illumina sequencer. Reads were base called using Illumina OLB (v 1.9, Illumina). Sample library preparation, sequencing, and initial bioinformatics up to base calling and demultiplexing were performed at the Science for Life Laboratory, Stockholm.

An in-house pipeline was used to process reads, call variants and annotate them. It is freely available under a GPL license (http://github.com/dnil/etiologica). Briefly, reads were mapped to the human reference genome (hg19) using Mosaik (v0.1.18) (33), quality filtered (Q ≥ 20) and further annotated using ANNOVAR (version 2012 May 25) (34) to estimate allele frequencies in large databases, predict, pathogenicity, evolutionary conservation and segmental duplication status (Supplementary Material, Table S2).

Sanger sequencing and genotyping

Sanger sequencing was performed according to standard procedure using the Big Dye® terminator v3.1 cycle sequencing kit (Applied Biosystems, CA, USA) and ABI 3730 or 3130XL DNA Analyzer (Applied Biosystems) to sequence the protein coding region including promoter of WNT3 gene (five exons and 1580 bp upstream of exon 1) in a total of 410 BEEC patients (primers available upon request). The same method was used to sequence candidate mutations in 376 placenta controls. Sequences were analyzed using CodonCode Aligner software package (CodonCode Corporation, MA, USA).

TaqMan® SNP genotyping assays were ordered for rs9890413 and rs142121185 (Life Technologies Europe BV, Netherlands). Rs9890413 was analyzed in 95 BEEC patients and 285 placenta controls; rs142121185 was analyzed in 94 BEEC patients and 362 placenta controls according to the manufacturer’s recommendation, using the ABI 7900HT instrument and the SDS software (v2.4, Applied Biosystems).

Association analyses, case/control, were performed using the Haplovie software 4.2. Permuted P-value was obtained by performing 10 000 iterations.

Reporter constructs, cell culturing and transfection

WNT3 (NM_030753) human cDNA ORF construct tagged with C-terminal TurboGFP in pCMV6-AC-GFP vector was purchased (RG211115, Origene, MD, USA). Plasmid was cloned, transformed and grown according to manufacturer’s instructions (One Shot® MAX Efficiency® DH10B-T1® Competent Cells, Invitrogen, Carlsbad, CA, USA). Site-directed mutagenesis (GENEART Site-Directed Mutagenesis System, Invitrogen) of the WT construct was performed to generate the MT WNT3c.271T>C, followed by sequencing of the entire insert. Plasmids were purified with either Plasmid Mini Kit or Endo-Free Maxi prep (Qiagen, Hilden, Germany). Sequences were analyzed using CodonCode Aligner software package (CodonCode Corporation, MA, USA).

Mouse adherent fibroblasts, NIH 3T3 (ATCC® CRL-1658™) were purchased and grown according to manufacturers recommendation in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% bovine calf serum and 0.5% Penicillin-Streptomycin (ATCC, Wesel, Germany). Cells were tested negative for mycoplasma (Venor GeM Mycoplasma Detection Kit, Minerva Biolabs, Berlin, Germany).

Transient transfection was performed at approximately 60–70% confluence on 8-well Nunc™ Lab-Tek™ Chambered Coverglass (Thermo Scientific, MA, USA) in triplicates. Transfections were performed using Xfect™ Transfection Reagent according to manufactures recommendations (Clontech, CA, USA).

Protein in silico modeling

Three-dimensional protein structure and surface modeling for WT and MT WNT3 was obtained as a homology (comparative) protein structure with (PS)2-v.2 based on Protein Data Bank entry c40AB (crystal structure of Xenopus Wnt8 in complex with the cysteine-rich domain of frizzled 8) as template (35). Structures were
processed with JSmol with 83% of residues (amino acids 60–354) modeled with a reliability >90% for WT and MT.

Confocal laser scanning microscopy (CLSM) and fluorescence correlation spectroscopy (FCS)

CLSM imaging and FCS measurements were performed using the LSM510 ConfoCor3 instrument (Carl Zeiss, Jena, Germany) individually modified to enable fluorescence imaging with silicone avalanche photodiodes (SPCM-AQR-1X; PerkinElmer, USA) (36). These detectors are characterized by higher sensitivity and lower noise levels, which makes it possible to visualize fluorescent molecules at very low concentrations and using low excitation intensities, which is favorable for live cells imaging. The C-Apochromat 40×, NA = 1.2, water immersion UV-VIS-IR objective was used throughout. The Green Fluorescent Protein (TurboGFP) was excited using the 488 nm line of the Ar/ArKr laser. Emitted light was separated from the incident light using the main dichroic beam splitter HFT KP 700/488 and split using the secondary dichroic beam splitter NFT 545. Further spectral separation of the emitted light splitter was achieved using specialized filters in front of the detectors. For TurboGFP imaging, emitted light was collected using the band pass filter BP 505–530 (pseudo-colored green). Autofluorescence was visualized using the long pass filter LP 580 (pseudo-colored red). Images were acquired without averaging, using a scanning speed of 51.2 µs/pixel and a 512 × 512 or 1024 × 1024 pixels frame. Pinhole of 1 Airy (70 µm) was used.

FCS is a quantitative method with single molecule-sensitivity that uses statistical approaches in the form of temporal autocorrelation and/or photon counting histogram (PCH) analysis of fluorescence intensity fluctuations recorded over time to obtain information about molecular numbers and their transporting properties (temporal autocorrelation) or molecular brightness distribution (PCH) (37,38). In this study, fluorescence intensity fluctuations recorded over time were analyzed using temporal autocorrelation in order to extract information about the average number of molecules in the observation volume element, i.e. the concentration, and the diffusion times. For this purpose, the normalized autocorrelation function $G(t)$ was first derived:

$$G(t) = 1 + \frac{\langle I(t) I(t + \tau) \rangle}{\langle I(t) \rangle^2}. \quad (1)$$

$G(t)$ gives the correlation between the deviation of fluorescence intensity measured at a certain time point $t$, $\delta I(t) = I(t) - \langle I(t) \rangle$, which is given as the difference in fluorescence intensity $I(t)$ and the mean fluorescence intensity over the recorded time-series $\langle I(t) \rangle$, and its intensity measured at a later time, $\delta I(t + \tau) = I(t + \tau) - \langle I(t) \rangle$. $G(t)$ is then plotted as a function of different lag times $\tau$, yielding the experimental autocorrelation curve.

To extract the molecular numbers ($N$) and diffusion times ($\tau_d$), the experimental autocorrelation curves were fitted using a model with the lowest number of components, in this case a model for free 3D diffusion of two components (37):

$$G(t) = 1 + \frac{1}{N} \left( \sum_{i=1}^{N} \frac{y_i}{(1 + (t/\tau_{d,i}))} \right) + \left( \frac{1 + (w_{d,y}/w_{d,x})}{(1 + (t/\tau_{d,x}))} \right). \quad (2)$$

In Eq. (2), $i$ is the number of components ($i = 2$), $\tau_{d,i}$ is the diffusion time of the $i$-th component and $y_i$ is its relative amplitude ($y_i = 1$). $w_{d,y}$ and $w_{d,x}$ are the distances from the center of the laser beam focus in the radial and axial directions, respectively, at which the collected fluorescence intensity has dropped by a factor of $e^2$ compared with its peak value for the Gaussian beam profile.

The optical setting for FCS was identical as for CLSM, with the main dichroic beam splitter HFT KP 700/488 and the band pass filter BP 505–530 for TurboGFP, except that the light was not split in order to collect autofluorescence. Instrument calibration was performed using aqueous solution of rhodamine 6G (Rh6G), yielding a diffusion time $\tau_d = (27 ± 2)$ µs. Fluorescence intensity fluctuations were recorded in arrays of 10 consecutive measurements, each measurement lasting 10 s. Autocorrelation curves recorded on the same cell were averaged and analyzed using the dedicated ConfoCor3 Zeiss LSM software (Carl Zeiss, Jena, Germany).

Morpholino and RNA injections

A previously published MO shown to specifically knockdown zebrafish Wnt3 (Wnt3MO; GAT CTC TTA CCA TTC GTC CTG C) (26) along with a control MO (Std-cont MO; CCT TCT ACC TCA GTT ACA ATT TAT A) were purchased from Genetools (Philomath, OR, USA). For RNA injection experiments human WT WNT3 mRNA and MT WNT3p.Cys91Arg mRNA were cloned into the pc52+ vector by Genescript (Piscataway, NJ, USA). The plasmids were linearized at the 3‘ end via enzymatic digestion with NotI and used as templates for the generation of Capped poly-adenylated mRNA using the Sp6 mMessage Machine kit (Ambion, TX, USA). MOs and RNA were diluted to the desired concentration and injected at the one-cell stage into newly fertilized TL WT embryos or transgenic fish line (Pod-cad) expressing m-cherry under the cadherin 17 promoter in cells of the pronephros. All fish were maintained on a 12-h day/night cycle at the Karolinska institute zebrafish core facility situated in the faculty of comparative medicine. Embryos were produced via light-induced spawning, and raised at 28°C to 2 dpf.

F-actin staining and confocal analysis

Injected Pod-cad transgenic embryos were fixed in 4% PFA for 2 h at room temperature (RT) or overnight at 4°C. Embryos were then washed 3 × 5 min in PBST and 1 × 5 min in PBS-Tx (0.2% Triton X) before being incubated for 2 h in PBS-Tx to allow permeabilization. Embryos were subsequently stained in 0.25 µM FITC-phalloidin (Sigma) overnight at 4°C. The staining solution was briefly washed off in PBST before being mounted in a solution of gelvatol containing DAPI as a nuclear stain. A total of 0.5 µm thick optical sections were collected through the entire thickness of the specimen on an Olympus FX1000 CLSM or Zeiss LSM 700 CLSM. FITC, DAPI and mcherry were excited using the 488 nm, 405 nm and 555 nm wavelengths of a Diodolaser Images and were then analyzed in Image J and compared with control embryos. Significance in difference of phenotypic penetrance was analyzed using the Fisher exact test both in the separate experimental groups and in the pooled samples. All experiments were repeated twice.

In situ hybridizations

For in situ hybridizations, plasmids containing previously described probe sequences for prdm1a and tbx2b (19) were linearized and used in a Dioxigenin labeling reaction using a commercially available RNA labeling kit (Hoffman-La Roche Ltd, Basel, Switzerland). In situ hybridizations were carried out as described previously (39). Gene expression patterns were visualized using
Nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl phosphate (Hoffman-La Roche Ltd).

Supplementary Material
Supplementary Material is available at HMG online.

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