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Targeted re-sequencing of 29 candidate genes and mouse expression studies implicate *ZIC3* and *FOXF1* in human VATER/VACTERL association

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Abstract

The VATER/VACTERL association describes the combination of congenital anomalies including vertebral defects, anorectal malformations, cardiac defects, tracheoesophageal fistula with or without esophageal atresia, renal malformations, and limb defects. As mutations in ciliary genes were observed in diseases related to VATER/VACTERL, we performed targeted re-sequencing of 25 ciliary candidate genes as well as disease-associated genes (*FOXF1*, *HOXD13*, *PTEN*, *ZIC3*) in 123 patients with VATER/VACTERL or VATER/VACTERL-like phenotype. We detected no

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biallelic mutation in any of the 25 ciliary candidate genes however, identified an identical, probably disease-causing *ZIC3* missense mutation (p.Gly17Cys) in four patients and a *FOXF1 de novo* mutation (p.Gly220Cys) in a further patient. *In situ* hybridization analyses in mouse embryos between E9.5 and E14.5 revealed *Zic3* expression in limb and prevertebral structures, and *Foxf1* expression in esophageal, tracheal, vertebral, anal, and genital tubercle tissues, hence VATER/VACTERL organ systems. These data provide strong evidence that mutations in *ZIC3* or *FOXF1* contribute to VATER/VACTERL.

Keywords

VATER/VACTERL association; cilia; *ZIC3*; *FOXF1*

The acronym VATER/VACTERL association (MIM# 192350) refers to the rare, non-random co-occurrence of the following component features (CFs): vertebral defects (V), anorectal malformations (A), cardiac defects (C), tracheoesophageal fistula with or without esophageal atresia (TE), renal malformations (R), and limb defects (L) [Solomon et al., 2014]. Patients may present with additional congenital anomalies however, the clinical diagnosis requires the presence of at least three CFs [Solomon et al., 2014]. Patients presenting with two CFs have been termed VATER/VACTERL-like [Solomon et al., 2014]. The involvement of genetic factors in the development of this rare association is suggested by reports of familial occurrence, the increased prevalence of component features among first-degree relatives, high concordance rates among monozygotic twins, and chromosomal (micro-)aberrations or single gene mutations in affected individuals [Solomon et al. 2014].

Human ciliopathies such as McKusick-Kaufman syndrome (MIM# 236700), Meckel syndrome Type 1-11, and Kartagener syndrome (MIM# 244400) are clinically closely related to the VATER/VACTERL association as they may present with congenital heart defects (C), congenital anomalies of the kidney (R), and limb (L) defects. Additionally, a VATER/VACTERL-like phenotype was reported in the ciliary mutant mouse model of *Ift172* (*avc1*) [Bujakowska et al., 2015]. *Ift172* encodes a component of the intraflagellar transport (IFT) within the cilium.

Based on these observations, we hypothesized that ciliary candidate genes, notably members of the IFT complex, may also be defective in patients with a VATER/VACTERL phenotype. We therefore carried out targeted re-sequencing and mutation analysis of 25 ciliary candidate genes (Supp. Table S1) comprising two genes encoding proteins of intraflagellar transport subcomplex A (IFT-A) (*IFT139*/*TTC21B*, *IFT144*/*WDR19*), 14 genes encoding IFT proteins of the subcomplex B (IFT-B) (*IFT172*/*SLB*, *IFT81*/*CDV1*, *IFT57*/*HIPPI*, *IFT52*, *IFT54*/*TRAF3IP1*, *IFT46*, *IFT74*/*CCDC2*, *IFT22*/*RABL5*, *IFT20*, *IFT25*/*HSPB11*, *IFT27*, *TTC30B*/*IFT70*, *TTC30A*, *IFT88*/*TG737*), and one gene (*TTC26*) known for its association with IFT-B. Since the *avc1* mutant mouse, representing a hypomorphic *Ift172* mutation, mechanistically presents with perturbed sonic hedgehog (Shh) signalling [Bujakowska et al., 2015], we included eight ciliary candidate genes (*TULP3*, *SDCCAG8*, *GPR161*, *KIF3A*, *KIF3B*, *KIFAP3*, *WDR60*, and *KIF17*) for their association with the Shh-pathway. As human ciliopathies usually follow an autosomal-recessive mode of inheritance

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[Tobin and Beales, 2009], we assumed an underlying autosomal-recessive disease model for the selected ciliary candidate genes.

To investigate the contribution of previously disease-associated candidate genes, we included *HOXD13*, *PTEN*, *FOXF1*, and *ZIC3* in our analysis. Whereas *ZIC3* is X-linked, *HOXD13*, *PTEN*, and *FOXF1* have an underlying autosomal-dominant mode of inheritance. For both *PTEN* and *HOXD13*, a single point mutation was previously described in a VATER/VACTERL-like phenotype [Reardon et al., 2001; Garcia-Barceló et al., 2008]. While *HOXD13* mutation carriers usually exhibit limb defects (MIM# 186300) representing a VACTER/VACTERL CF, *PTEN* mutation carriers (MIM# 153480) usually do not exhibit any VATER/VACTERL CFs. Nevertheless, in order to re-evaluate these findings, we added both genes to our re-sequencing analysis. Four heterozygous point mutations were previously reported in *FOXF1* in four unrelated patients with alveolar capillary dysplasia and misalignment of the pulmonary veins (ACD/MPV) and additional malformations in VATER/VACTERL organ systems [Stankiewicz et al., 2009]. Several *ZIC3* mutations, including point mutations, deletions, and, in one instance, an intragenic polyalanine expansion, were reported for both VATER/VACTERL and VATER/VACTERL-like phenotypes with or without hydrocephalus [Wessels et al., 2010; Chung et al., 2011]. We did not include *FGF8* or *TRAP1* in our analysis, since both genes had been previously investigated by our group [Zeidler et al., 2014; Saisawat et al., 2014]. Furthermore, we did not include *LPP* in our analysis, since Hernandez-Garcia et al. [2011] when investigating 170 patients with isolated TE or TE as part of their VATER/VACTERL association by microarray, failed to detect any *LPP* deletions or disease-causing mutations in the Sanger sequencing analyses of 42 of these patients. Finally, we did not include disease-causing genes for Fanconi anemia, as VATER/VACTERL-like phenotypes have been described in less than 5% of patients with secured Fanconi anemia [Faivre et al., 2005].

Our study sample comprised 123 patients (78 males and 45 females). A VATER/VACTERL phenotype with at least three CFs was present in 80, a VATER/VACTERL-like phenotype with two CFs in 43 patients. Due to the recruitment procedure, all of our patients had anorectal malformations [Schramm et al., 2011]. Within our cohort was one affected sib pair, brother and sister, who were not previously reported. Here, only the sister was included in the initial study sample.

All 29 candidate genes were subjected to our re-sequencing approach using microfluidic Access-Array technology (Fluidigm). In two of these genes, namely *KIF17* and *SDCCAG8*, re-sequencing was not successful and no results could be obtained. In the other 27 candidate genes, PCR-based multiplex amplification of genomic target regions was successful and followed by molecular barcoding and next-generation re-sequencing on an Illumina HiSeq platform, as outlined in detail elsewhere [Halbritter et al., 2012]. In total, all 406 coding exons and splice sites were sequenced with this method (Supp. Table S1).

Read alignment and variant detection were carried out using CLC Genomics Workbench software (CLC-bio, Aarhus, Denmark) as described previously [Halbritter et al., 2012]. For variant calling, standard filtering criteria were used (Supp. Materials and Methods). The study was conducted in accordance with the Declaration of Helsinki, and ethical approval

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was obtained from the respective ethics committees at the recruitment centers. Written informed consent was obtained from all patients and parents prior to study entry. Mutations, unclassified variants, and phenotype data were submitted to the ClinVar, NCBI database (http://www.ncbi.nlm.nih.gov/clinvar/).

Mutation analysis of all 25 ciliary candidate genes did not identify homozygous or compound heterozygous disease causing variants in any of the investigated 123 patients. However, we identified 55 heterozygous variants of unknown significance in two ITF-A associated, nine IFT-B associated, and five Shh-pathway associated candidate genes (Supp. Table S1). Among these variants, 16 were found to be novel according to NCBI dbSNP (National Center for Biotechnology database for single nucleotide polymorphism) (Supp. Table S1). In nine patients, two mutations in different genes were identified, suggesting a possible digenic disease pathway. Nevertheless, since none of the combination of genes was found in more than one patient, these combinations remain of uncertain significance.

Mutation analysis of *FOXF1* (NM_001451.2), *HOXD13*, and *PTEN*, identified one *de novo* mutation in *FOXF1*, c.658G>T (p.Gly220Cys), in male patient no. 400. This patient presented with an anorectal malformation (A), left sided renal agenesis (R) and glandular hypospadias.

Mutation analysis of *ZIC3* (Ensembl ENST00000370606; NM_003413.3) identified a recurrent disease-causing mutation (c.49G>T, p.Gly17Cys) in three patients, including the sister of the above mentioned sib pair. Familial segregation analyses using Sanger sequencing revealed one further mutation in her affected brother. The male patient no. 347 (Supp. Table S2) presented with three CFs namely recto-vesical fistula (A), atrial septal defect (C), and right renal agenesis, grade IV-V vesicoureteral reflux (left) (R). Additional features were cryptorchidism and penoscrotal transposition. He inherited the *ZIC3* p.Gly17Cys mutation from his unaffected mother. In addition, this patient showed a heterozygous p.Asn411Ser variant in the *IFT57* gene, encoding the protein intraflagellar transport 57, *Clamydomonas* homolog. This variant was not detected in his mother and no paternal sample was available for testing.

In family no. 606, the p.Gly17Cys mutation in *ZIC3* was detected in the sister (no. 603b) and her brother (no. 603a; Supp. Table S2). The girl presented with vestibular fistula (A), higher grade vesicoureteral reflux (R) and 13 ribs on both sides. Her brother presented with recto prostatic fistula (A) and atrial septal defect (C). Because both siblings were affected to similar degree, we tested the hypothesis that skewed X chromosome inactivation in the sister led to expression of only the affected allele. Testing for the skewedness of X-chromosome inactivation in the family (Supp. Materials and Methods) showed indeed the allele transmitted from the mother to the daughter was the main active allele (only 9% inactivated) while the other allele was nearly 91% inactivated (Supp. Fig. S1). The same active allele in the sister was shared by her brother, which explains why both siblings were equally affected.

The male patient no. 633 (Supp. Table S2) presented with two CFs, namely recto-urethral fistula (A) and right sided ectopic kidney with grade I vesicoureteral reflux (R). In addition, he displayed penoscrotal transposition and glandular hypospadias. In this patient, aside from

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the *ZIC3* p.Gly17Cys mutation, an additional heterozygous variation p.Arg1311His in the *TTC21B* gene, encoding the protein tetratricopeptide repeat domain 21B, was found. *TTC21B* is a retrograde intraflagellar transport protein. Homozygous or heterozygous *TTC21B* mutations have been causally related to nephronophthisis-12 and/or the Joubert syndrome 11 (JBTS11; MIM# 613820). In our patient, both missense mutations were transmitted from the mother, affected with cleft lip and palate, an anomaly not associated with *ZIC3* or *TTC21B*.

To better describe the significance of the identified mutations and variants we used various online prediction tools (Mutation Taster (http://www.mutationtaster.org/), MutPred (http://mutpred.mutdb.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/) and PROVEAN (http://provean.jcvi.org/index.php). In patient no. 400, the p.Gly220Cys (c.658G>T) mutation in *FOXF1* substitutes a highly conserved small non-polar glycine with a polar cysteine. The introduction of a further cysteine residue may also allow formation of illegitimate disulfide bridges. This nucleotide transition is not listed in SNP database (build 141); however, only Mutation Taster classified it as disease-causing (Supp. Table S3). Previous *FOXF1* mutations have been described in patients with VATER/VACTERL-like phenotypes [Stankiewicz et al. 2009]. The authors suggested, that heterozygous invalidation of *FOXF1* could be considered in VATER/VACTERL cases for which there is a severe respiratory distress and/or neonatal lethality. Our patient with a probably disease-causing *de novo* mutation did not show any cardiac defects, MPV or any other large vessel anomaly, and his neonatal period was not complicated by respiratory distress. The Variant was submitted to ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/) (Supp. Table S3).

The *ZIC3* p.Gly17Cys mutation affects a highly conserved amino acid residue, substituting a small non-polar glycine with a polar cysteine. In accordance with most recent reports [D'Alessandro et al., 2013], four out of five publically available mutation prediction programs (Mutation Taster, MutPred, PolyPhen-2, SIFT and PROVEAN) predicted this mutation to be deleterious (Supp. Table S4). D'Alessandro et al. [2013] and De Luca et al. [2010] did not observe p.Gly17Cys in altogether 1129 controls, however, it is listed with database no. rs147232392 in the NCBI SNP database (Build 141), referring to data from the NHLBI ESP cohort. Here, a minor allele frequency (MAF) of 0.002 was observed in 2840 samples with no further data given. As outlined recently [D'Alessandro et al., 2013], p.Gly17Cys has also been deposited in the Exome Variant Server (EVS) [2012]; here, its presence was reported in 19 heterozygous females out of 4,053 individuals and 12 hemizygous males (total no. 2,414), resulting in a MAF of 0.0029. However, no phenotypic data were provided for these mutation carriers. Furthermore, the EVS data show that p.Gly17Cys is found slightly more often in European Americans (MAF 0.0039) than in African Americans (MAF 0.0012). This unequal distribution of the frequency might be due to a European founder mutation. The additional *IFT57* p.Asn411Ser (c.1232A>G) substitution observed in patient 347 represents a conservative exchange and affects a highly conserved carboxyterminal residue. There is no SNP database (Build 141) or Deep Catalog of Human Genetic Variation (www.1000genomes.org) entry and this variant is predicted to

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be possibly damaging by two (Mutation Taster, MutPred) out of five publically-available mutation prediction programs. The Variant was submitted to ClinVar (Supp. Table S3).

In patient no. 633, the additional p.Arg1311His (c.3932G>A) substitution also resides in a highly conserved carboxyterminal position in the TTC21B protein. A deleterious effect is predicted by four out of five publically available mutation prediction programs (Mutation Taster, Polyphen2, SIFT and PROVEAN). The variant has been deposited in the SNP database (Build 141) with ref. no. rs139327086 and was observed once in the NHLBI ESP cohort in 4542 samples (MAF: 0.001) with no further data available.

Based on their strict conservation and pathogenic prediction it could be that both additional variants: the *IFT57* p.Asn411Ser (c.1232A>G) substitution observed in patient no. 347, and the *TTC21B* p.Arg1311His (c.3932G>A) substitution in patient no. 633, might affect protein function. Although *TTC21B* has been implicated in nephronophthisis 12 (MIM# 613820) only in the mutated homozygous/compound heterozygous state, heterozygous *TTC21B* mutations have been observed in some patients with Joubert syndrome 11 [Davis et al., 2011], an allelic ciliopathy. Hence, as suggested by Davis et al. [2011], it could be that the additional variants in *TTC21B* and *IFT57* might have contributed to a mutational load in the described patients.

To attribute a functional effect to the p.Gly17Cys mutation, we performed a PROSITE motif search (www.Expasy.ch) which identified a potential N-myristoylation site in *ZIC3*, comprising amino acids 13-18 (GLgvGS). N-myristoylation refers to a co-translational modification involving the covalent attachment of the 14-carbon saturated fatty acid myristate to the N-terminal glycine of proteins [Farazi et al., 2001]. This irreversible acylation promotes protein-membrane and protein-protein interactions, thereby regulating protein targeting and function. Although other small uncharged residues, like Cys17, seem to be tolerated [Towler et al., 1988], a substitution of Gly17 might impair the covalent addition of myristate. In eukaryotic proteins, N-myristoylation has been shown to be essential for cell growth and survival [Farazi et al., 2001], and further studies should elucidate whether recruitment of this motif also governs ZIC3 function, thereby providing evidence for the functional consequences of this Glycine17 missense mutation, now observed in 11 (9 unrelated) patients (Supp. Table S2).

Overall the phenotypic spectrum of *ZIC3* mutation carriers is quite variable, with incomplete penetrance in males shown by D'Alessandro et al. [2013], who reported transmission of the mutation from unaffected males. Upregulation of *ZIC3* is detected in around 8% of female carriers [Chhin et al., 2007; de Luca et al., 2010]. This has, at least in one case, been attributed to a skewed (97:3) X-inactivation pattern [Chhin et al., 2007], a mechanism which also occurred in our female patient 606b. Penetrance variability has also been observed in the mouse, indicating that the penetrance of the *Zic3* null allele is sensitive to genetic background differences [Purandare et al., 2002]. To date, eight patients fulfilling the criteria of VATER/VACTERL association (at least three CFs being present) have been reported with *ZIC3* mutations (Supp. Table S4). None of these patients displayed hydrocephalus. *Situs* abnormalities were detected in four. Interestingly, aside from cardiac defects, ARM have been reported in at least 17 patients with *ZIC3* involvement [Purandare et al., 1999;

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Wessels et al., 2010; Chung et al., 2011]. Although knockout mice recapitulate the human heterotaxy and cardiac phenotype with occasional vertebral/rib anomalies, ARM or other VATER/VACTERL CFs were not reported in *Zic3* deficient mice [Purandare et al., 2002; Sutherland et al., 2013].

To assess the potential role of *ZIC3* and *FOXF1* for the VATER/VACTERL association, we investigated the expression of *Zic3* and *Foxf1* by *in situ* hybridization (ISH) on mouse embryos at embryonic days (E) 9.5 to 14.5, the equivalent of human gestational weeks 4-6 (postulated time of VATER/VACTERL organogenesis in humans) [Stevenson and Hunter, 2013]. *In situ* hybridization on mid-sagittal sections of E12.5 and E13.5 mouse embryos (corresponding to approximately week 5 of human gestation) was performed to examine *Foxf1* expression (Fig. 1). Strong expression of *Foxf1* could be detected in various VATER/VACTERL associated tissues, including trachea and esophagus, pre-vertebrae, and areas surrounding the urethra and rectum/anus within the genital tubercle. Staining was also apparent in the developing lungs, and in the tooth buds.

These ISH data for *Foxf1* in developing mouse embryos suggests a role for this gene in relevant VATER/VACTERL tissues, although whole-mount expression data from urogenital tissues from the GUDMAP database (http://www.gudmap.org) does not show significant staining in the kidneys. Nevertheless, *Foxf1* heterozygotes have been shown to display tracheo-esophageal atresia and fistulas (Mahlapuu et al., 2001), one hallmark of VATER/VACTERL association.

In situ hybridization for *Zic3* was performed on mouse embryos at gestational days (E) 9.5-14.5, with particular emphasis on the uro-rectal region (Fig. 2). Since *Zic3* is located on the X-chromosome, male and female embryos were examined separately to determine whether sex-specific differences in expression were present. No differences were noted. From E9.5 to E13.5 *Zic3* could be detected in discrete regions within the developing brain, eye, spinal column, and limbs. A stripe of *Zic3* expression was present at all stages within the anterior pre-somitic mesoderm (Fig. 2, top). Expression in the limbs was present in the autopod and apical ectodermal ridge at early stages, and then became confined to the presumptive wrist region and digital tips. A close-up examination of staining in the uro-rectal region of male and female embryos between E12.5 and E14.5 again revealed no sex-specific differences in expression. Staining was undetected in the cloaca/genital tubercle up to E12.5. From E13.5, there was weak expression in the lateral aspects of the genital tubercle, but *Zic3* could not be detected in the developing anal region (Fig. 2, bottom).

Taken together our WISH data do not suggest a role for *Zic3* in renal and urogenital development, since there was lack of expression in these mouse tissues at the relevant stages. The failure to detect *Zic3* expression in heart at the stages examined (E9.5-E13.5), is in agreement with the study from Sutherland et al. [2013], who found absent *Zic3* expression in mouse heart when using either WISH or a *Zic3-LacZ-BAC* reporter mouse during heart looping morphogenesis. These authors defined the temporal requirement of Zic3 for cardiac development between E7.0 and E8.0. However, *Zic3* expression could be detected in murine E10.5 heart tissue by RT-PCR [Bedard et al., 2011]. Nevertheless, we detected *Zic3* expression in the limbs and prevertebral structures, all tissues affected in the VATER/

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VACTERL association. Failure of staining renal and urogenital tissues might depend on low but relevant *Zic3* expression only detectable by RT-PCR.

In summary, we present novel families with VATER/VACTERL or VATER/VACTERL-like phenotypes with mutations in *ZIC3* and *FOXF1*, respectively. WISH/ISH expression studies in mouse embryos revealed *Zic3* and *Foxf1* expression in the presomitic mesoderm and limb, esophageal, tracheal, vertebral, anal and genital tubercle tissues, all belonging to VATER/VACTERL organ systems, providing independent corroboration for *ZIC3* and *FOXF1* as disease-causing genes for human VATER/VACTERL association.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Bedard JEJ, Haaning AM, Ware SM. Identification of a novel ZIC3 isoform and mutation screening in patients with heterotaxy and congenital heart disease. PLoS ONE. 2011; 6:e23755. [PubMed: 21858219]

Bujakowska KM, Zhang Q, Siemiatkowska AM, Liu Q, Place E, Falk MJ, Consugar M, Lancelot ME, Antonio A, Lonjou C, Carpentier W, Mohand-Saïd S, et al. Mutations in IFT172 cause isolated retinal degeneration and Bardet-Biedl syndrome. Hum Mol Genet. 2015; 24:230–242. [PubMed: 25168386]

Chhin B, Hatayama M, Bozon D, Ogawa M, Schön P, Tohmonda T, Sassolas F, Aruga J, Valard AG, Chen SC, Bouvagnet P. Elucidation of penetrance variability of a ZIC3 mutation in a family with complex heart defects and functional analysis of ZIC3 mutations in the first zinc finger domain. Hum Mutat. 2007; 28:563–570. [PubMed: 17295247]

Chung B, Shaffer LG, Keating S, Johnson J, Casey B, Chitayat D. From VACTERL-H to heterotaxy: variable expressivity of ZIC3-related disorders. Am J Med Genet A. 2011; 155A:1123–1128. [PubMed: 21465648]

D'Alessandro LCA, Latney BC, Paluru PC, Goldmuntz E. The phenotypic spectrum of ZIC3 mutations includes isolated d-transposition of the great arteries and double outlet right ventricle. Am J Med Genet A. 2013; 161A:792–802. [PubMed: 23427188]

Davis EE, Zhang Q, Liu Q, Diplas BH, Davey LM, Hartley J, Stoetzel C, Szymanska K, Ramaswami G, Logan CV, Muzny DM, Young AC, et al. TTC21B contributes both causal and modifying alleles across the ciliopathy spectrum. Nat Genet. 2011; 43:189–196. [PubMed: 21258341]

De Luca A, Sarkozy A, Consoli F, Ferese R, Guida V, Dentici ML, Mingarelli R, Bellacchio E, Tuo G, Limongelli G, Digilio MC, Marino B, Dallapiccola B. Familial transposition of the great arteries caused by multiple mutations in laterality genes. Heart. 2010; 96:673–677. [PubMed: 19933292]

Faivre L, Portnoï MF, Pals G, Stoppa-Lyonnet D, Le Merrer M, Thauvin-Robinet C, Huet F, Mathew CG, Joenje H, Verloes A, Baumann C. Should chromosome breakage studies be performed in

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patients with VACTERL association? Am J Med Genet A. 2005; 137A:55–58. [PubMed: 16015582]

Farazi TA, Waksman G, Gordon JI. The biology and enzymology of protein N-myristoylation. J Biol Chem. 2001; 276:39501–39504. [PubMed: 11527981]

Garcia-Barceló MM, Wong KK, Lui VC, Yuan ZW, So MT, Ngan ES, Miao XP, Chung PH, Khong PL, Tam PK. Identification of a HOXD13 mutation in a VACTERL patient. Am J Med Genet A. 2008; 146A:3181–3185. [PubMed: 19006232]

Halbritter J, Diaz K, Chaki M, Porath JD, Tarrier B, Fu C, Innis JL, Allen SJ, Lyons RH, Stefanidis CJ, Omran H, Soliman NA, Otto EA. High-throughput mutation analysis in patients with a nephronophthisis-associated ciliopathy applying multiplexed barcoded array-based PCR amplification and next-generation sequencing. J Med Genet. 2012; 49:756–767. [PubMed: 23188109]

Hernandez-Garcia A, Brosens E, Zaveri H, Yu Z, Fernandes C, Johnson A, Lalani S, Tibboel D, de Klein A, Scott DA. Mutation screening of the LPP gene in patients with esophageal atresia, tracheoesophageal fistula, and VACTERL association. Am J Hum Genet. 2011; 89(suppl) Abstract 986W.

Mahlapuu M, Enerbäck S, Carlsson P. Haploinsufficiency of the forkhead gene Foxf1, a target for sonic hedgehog signaling, causes lung and foregut malformations. Development. 2001; 128:2397–2406. [PubMed: 11493558]

Purandare SM, Ware SM, Kwan KM, Gebbia M, Bassi MT, Deng JM, Vogel H, Behringer RR, Belmont JW, Casey B. A complex syndrome of left-right axis, central nervous system and axial skeleton defects in Zic3 mutant mice. Development. 2002; 129:2293–2302. [PubMed: 11959836]

Reardon W, Zhou XP, Eng C. A novel germline mutation of the PTEN gene in a patient with macrocephaly, ventricular dilatation, and features of VATER association. J Med Genet. 2001; 38:820–823. [PubMed: 11748304]

Saisawat P, Kohl S, Hilger AC, Hwang DY, Gee HY, Dworschak GC, Tasic V, Pennimpede T, Natarajan S, Sperry E, Matassa DS, Stajić N, et al. Whole exome resequencing reveals recessive mutations in TRAP1 in individuals with CAKUT and VACTERL association. Kidney Int. 2014; 85:1310–1317. [PubMed: 24152966]

Schramm C, Draaken M, Bartels E, Boemers TM, Aretz S, Brockschmidt FF, Nöthen MM, Ludwig M, Reutter H. De novo microduplication at 22q11.21 in a patient with VACTERL association. Eur J Med Genet. 2011; 54:9–13. [PubMed: 20849991]

Solomon BD, Baker LA, Bear KA, Cunningham BK, Giampietro PF, Hadigan C, Hadley DW, Harrison S, Levitt MA, Niforatos N, Paul SM, Raggio C, Reutter H, Warren-Mora N. An approach to the identification of anomalies and etiologies in neonates with identified or suspected VACTERL (vertebral defects, anal atresia, tracheo-esophageal fistula with esophageal atresia, cardiac anomalies, renal anomalies, and limb anomalies) association. J Pediatr. 2014; 164:451–457. [PubMed: 24332453]

Stankiewicz P, Sen P, Bhatt SS, Storer M, Xia Z, Bejjani BA, Ou Z, Wiszniewska J, Driscoll DJ, Maisenbacher MK, Bolivar J, Bauer M, et al. Genomic and genic deletions of the FOX gene cluster on 16q24.1 and inactivating mutations of FOXF1 cause alveolar capillary dysplasia and other malformations. Am J Hum Genet. 2009; 84:780–791. [PubMed: 19500772]

Stevenson RE, Hunter AGW. Considering the embryopathogenesis of VACTERL association. Mol Syndromol. 2013; 4:7–15. [PubMed: 23653571]

Sutherland MJ, Wang S, Quinn ME, Haaning A, Ware SM. Zic3 is required in the migrating primitive streak for node morphogenesis and left-right patterning. Hum Mol Genet. 2013; 22:1913–1923. [PubMed: 23303524]

Tobin JL, Beales PL. The nonmotile cilipathies. Genet Med. 2009; 11:386–402. [PubMed: 19421068]

Towler DA, Gordon JL, Adams SP, Glaser L. The biology and enzymology of eukaryotic protein acylation. Annu Rev Biochem. 1988; 57:69–99. [PubMed: 3052287]

Wessels MW, Kuchinka B, Heydanus R, Smit BJ, Dooijes D, de Krijger RR, Lequin MH, de Jong EM, Husen M, Willems PJ, Casey B. Polyalanine expansion in the ZIC3 gene leading to X-linked heterotaxy with VACTERL association: a new polyalanine disorder? J Med Genet. 2010; 47:351–355. [PubMed: 20452998]

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Zeidler C, Woelfle J, Draaken M, Mughal SS, Große G, Hilger AC, Dworschak GC, Boemers TM, Jenetzky E, Zwink N, Lacher M, Schmidt D, et al. Heterozygous FGF8 mutations in patients presenting cryptorchidism and multiple VATER/VACTERL features without limb anomalies. Birth Defects Res A Clin Mol Teratol. 2014; 100:750–759. [PubMed: 25131394]

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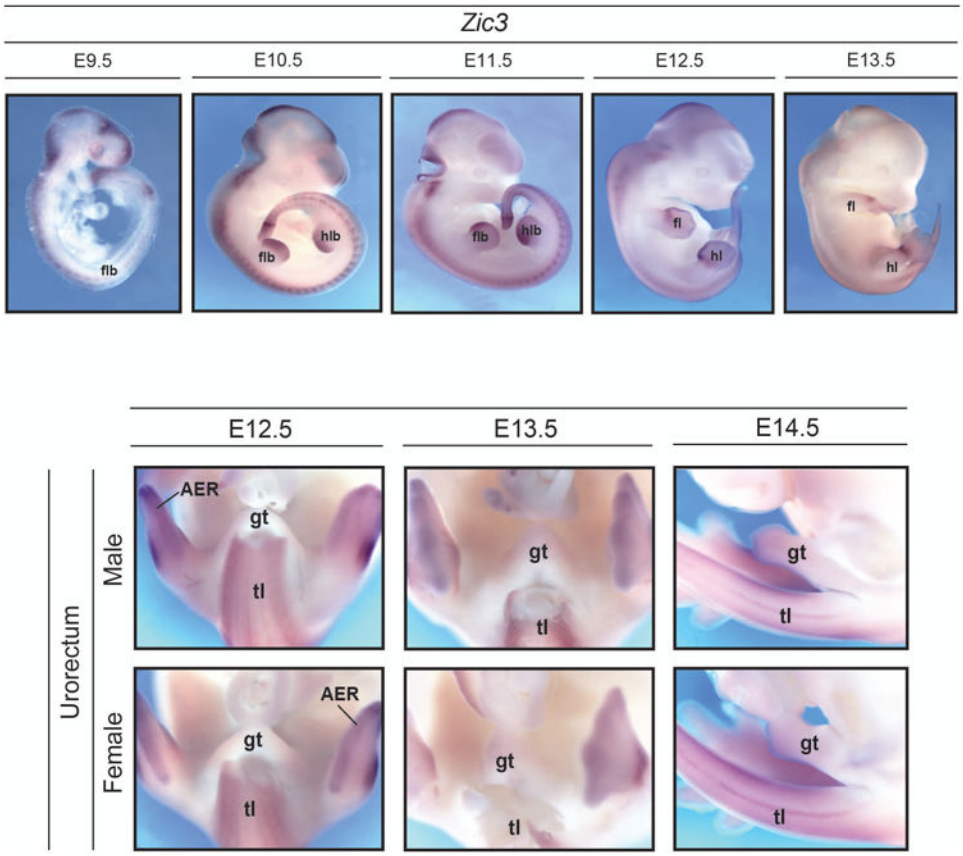

Figure 1.

In situ hybridization of mouse embryo sections for *Foxf1*. At E12.5 and E13.5 *Foxf1* could be detected in various tissues affected in VATER/VACTERL association, including trachea and esophagus, vertebrae, and urorectal structures. Staining was detected in mesenchyme surrounding the urethra and anus at both stages. *Foxf1* was also present in the lung and tooth buds, but was absent from the heart (a, anus; es, esophagus; gt, genital tubercle; ht, heart; lu, lungs; tr, trachea; ve, vertebra).

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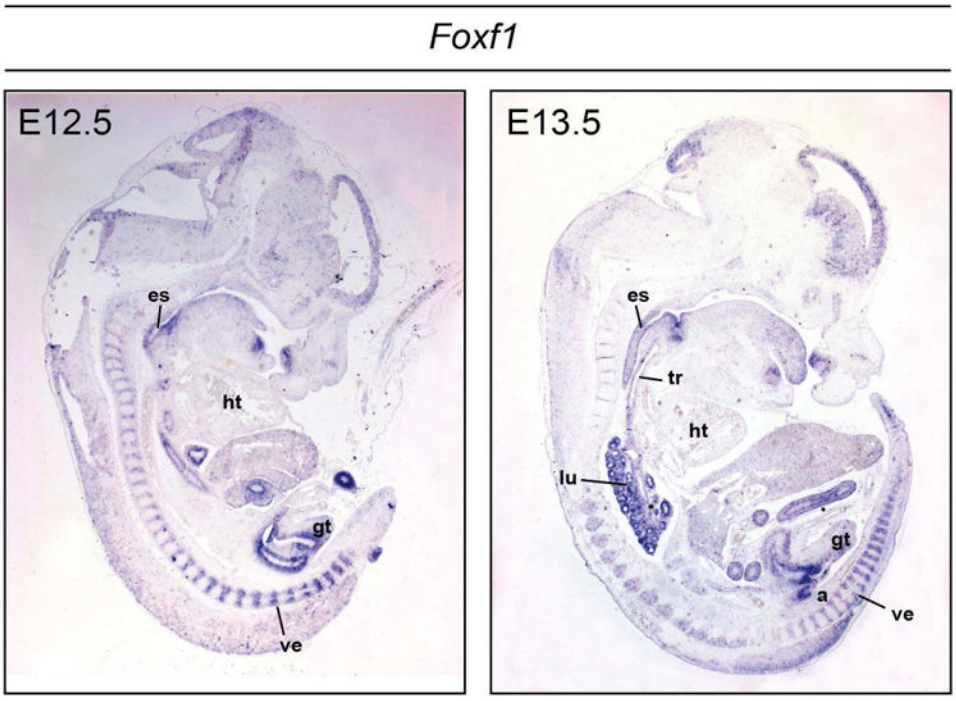

Figure 2.

Whole-mount *in situ* hybridization of mouse embryos for *Zic3*. (**top**) From E9.5 to E13.5 *Zic3* could be detected in discrete regions within the developing brain, eye, spinal column, and the developing limbs. A stripe of *Zic3* expression is present at all stages at the anterior pre-somitic mesoderm. No differences were noted in the expression pattern between males and females, and male embryos are shown. (**bottom**) Close-up examination of staining in the uro-rectal region of male and female embryos between E12.5 and E14.5 revealed weak expression in the lateral aspects of the genital tubercle, but *Zic3* was not detected in the developing anal region. Note that the tails have been removed at E12.5 and E13.5 to reveal the genital tubercle (flb, forelimb bud; hlb, hindlimb bud; fl, forelimb; hl, hindlimb; gt, genital tubercle; tl, tail).