Increased gene copy number of VAMP7 disrupts human male urogenital development through altered estrogen action

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Despite the fact that genitourinary defects are among the most common birth defects in newborns, little is known about their etiology. Here we analyzed children born with congenital genitourinary tract masculinization disorders by array-comparative genomic hybridization, which revealed in 1.35% of cases the presence of de novo copy number gains at Xq28 encompassing the VAMP7 gene, which encodes a vesicle-traffic protein that is part of the SNARE complex. Transgenic mice carrying a bacterial artificial chromosome encoding human VAMP7 mimicked the defective urogenital traits observed in boys with masculinization disorders such as cryptorchidism, urethral defects and hypospadias. Transgenic mice also exhibited reduced penile length, focal spermatogenic anomalies, diminished sperm motility and subfertility. VAMP7 colocalized with estrogen receptor α (ESR1) in the presence of its cognate ligand, 17β-estradiol. Elevated levels of VAMP7 markedly intensified ESR1-potentiated transcriptional activity by increasing ESR1 protein cellular content upon ligand stimulation and upregulated the expression of estrogen-responsive genes including ATF3, CYR61 and CTGF, all of which have been implicated in human hypospadias. Hence, increased gene dosage of VAMP7, and thus higher expression levels of its protein product, enhances estrogen receptor action in male genitourinary tissues, affects the virilization of the reproductive tract and results in genital abnormalities in humans.

Estrogens are generally perceived to be inhibitory in male development or at least to antagonize androgen action1, which is essential for the acquisition of a normal male phenotype2. Following the early steps of bipotential gonadal formation and testis determination, androgens are required for the stabilization of the Wolffian duct system and its differentiation into the epididymes, vasa deferentia and seminal vesicles. Their action is also critical for proper virilization of the external genitalia and migration of the testes through the inguinal canal into the scrotum2. The dependence of the masculinization of the reproductive system on androgens, however, renders this process inherently susceptible to destabilization by factors interfering with hormone synthesis, metabolism or action. Such disturbances represent major etiological determinants that potentially contribute to the high prevalence of human masculinization disorders, such as hypospadias (male urethral dysmorphogenesis) and cryptorchidism (failure of testicular descent).

Epidemiological studies have provided links between inappropriate estrogen exposure and increased incidence of reproductive abnormalities in men (see refs. 3, 4 for reviews on this topic). Similarly, reproductive tract lesions, such as cryptorchidism and penile defects, have been observed in male mice after prenatal and neonatal exposure to the potent nonsteroidal synthetic estrogen diethylstilbestrol5–8. Furthermore, transgenic male mice overexpressing the enzyme aromatase, necessary for the conversion of androgens to estrogens, display several genital abnormalities, including undescended testes, subtle effects on penile development and spermatogenic arrest9.

The inter-relationship between the androgens and estrogens working to regulate the differentiation of the male reproductive system relies on the balance between androgen and estrogen action rather than on the absolute circulating concentrations of these hormones. Any change in this homeostasis may disturb the internal milieu required for normal development and function of the male genital tract and may translate into a wide range of genital abnormalities that can subsequently affect normal fertility. To delineate disease-causing factors that may perturb proper hormone signaling during human urogenital development, we analyzed children born with masculinization disorders using a forward-genetics approach and identified in a subset of cases a structural genomic variant affecting the gene dosage of VAMP7. VAMP7 is a highly conserved gene10 that encodes a transmembrane protein belonging to the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) family that localizes in late endosomes and lysosomes. VAMP7 is required for heterotypic fusion of late endosomes with lysosomes and homotypic lysosomal fusion11–13, calcium-regulated lysosomal exocytosis14 and focal exocytosis of late endocytic vesicles during phagosome formation15,16. We generated humanized bacterial artificial chromosome (BAC) transgenic mice

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to infer biologically meaningful relationships between VAMP7 copy number variants and human genitourinary birth defects.

RESULTS

VAMP7 copy number gain in a subset of undermasculinized boys

We used a clinically validated comparative genomic hybridization microarray platform to analyze DNA from 116 children presenting with idiopathic cases of 46,XY disorders of sexual development (DSD), including anomalies of testicular descent and defects of penile morphogenesis. We hypothesized previously that structural DNA variation is an underlying etiology for human disorders of sexual development and showed that frequent submicroscopic gains and losses of DNA segments are strongly associated with defective urogenital traits. Among the clinically significant imbalances identified, we observed an identical copy number gain at the distal tip of the long arm of Xq28 in two unrelated subjects (Fig. 1a,b and Supplementary Fig. 1a,b). One child had bilateral cryptorchidism with a right inguinal testis and a left intra-abdominal gonad. The second child presented with midscrotal hypospadias with chordee and penoscrotal transposition. Comparative genomic hybridization (CGH) array analysis of DNA from unaffected parents revealed the de novo occurrence of the terminal Xq28 gain (Supplementary Fig. 1c,d). This structural variant was secondarily confirmed by fluorescence in situ hybridization (FISH) analysis (Fig. 1c). When we applied a two-tailed Fisher’s exact test, this copy number change was more frequent in subjects presenting with congenital genitourinary defects (2 out of 116) than in control individuals without urogenital abnormalities (n = 8,951) run on the same array CGH platform (P = 2.2 × 10−3). Whereas the Xq28 region had contiguous coverage on the microarray, the distal bacterial artificial chromosome (BAC) clone RP11-479B17 was the only segment showing a copy number gain (Supplementary Fig. 1a,b). The maximum size of the defect exclusively included the pseudoautosomal region 2 (PAR2), whereas the minimal critical interval (RP11-479B17) solely encompassed VAMP7 (Supplementary Fig. 1e). An analysis of published literature revealed that there are additional subjects who presented with syndromic cryptorchidism and/or micropenis among their clinical features together with larger Xq28 terminal duplications that also encompassed VAMP7 (Fig. 1d and Supplementary Table 1).

On the basis of these observations, we hypothesized that duplication of the Xq28 region and, more specifically, of VAMP7 plays a role in human disorders of masculinization of the urogenital tract. Quantitative PCR analysis of VAMP7 copy number in the genome of a distinct cohort of 180 individuals presenting with isolated hypospadias (n = 83), cryptorchidism (n = 79) or both urogenital conditions (n = 18) identified two additional subjects bearing VAMP7 copy number gain (one with isolated inguinal cryptorchidism and one with glandular hypospadias and chordee) (Supplementary Fig. 2a). A second independent replication study of primary cultures of human genital skin fibroblasts from 28 subjects with congenital genitourinary defects revealed 1 instance of mid-shaft hypospadias with a copy number gain of VAMP7 (3.6%) (Supplementary Fig. 2b), convincingly supporting the association of VAMP7 genomic gains with developmental disorders of the human genital tract.

VAMP7 is expressed in the human and mouse genital tract

We detected VAMP7 protein in cytoplasmic lysates from human fetal testes and ovaries (Fig. 2a). In adults, we detected VAMP7 mRNA throughout the human male genital tract, including the testes, epididymides, seminal vesicles and prostatic and penile tissues (Fig. 2b). A punctuate pattern of VAMP7 staining was present in the Sertoli cells, as well as in the germ cells of the seminiferous tubules of human adult testes with histologically normal spermatogenesis (Fig. 2c). The cytoplasm of Leydig cells also showed VAMP7-positive immunoreactive staining (Fig. 2c). In the adult human external genitalia, VAMP7 was...
strongly expressed in the urethral epithelium (Fig. 2d). Moreover, the genital mesenchyme, including corpus cavernosa and the androgen-dependent penile spines of the preputial epithelium, showed high levels of VAMP7 protein (Fig. 2e). Similarly, fetal testes and developing genital tubercles in mouse embryos at gestational day 16.5 were positively stained for VAMP7 (Fig. 2f,g). Thus, VAMP7 mRNA and protein are present in the human and mouse genitourinary tract during development and at maturity.

Increased VAMP7 gene dosage leads to genitourinary defects

We sought to investigate in vivo the impact of genetically elevated VAMP7 levels on genitourinary development and reproductive physiology. We produced a mouse model that expresses human VAMP7 under its endogenous regulatory sequences to recapitulate the genetic gain observed in patients. We generated mutant mice through microinjection of a 159-kilobase linearized BAC (clone RP11-479B17) including the human VAMP7 gene into fertilized oocytes of FVB mice and obtained two independent VAMP7 founder lines, 7 and 21. VAMP7–BAC transgenic mice were viable and had normal growth and lifespan. Western blot analysis using an antibody that specifically recognizes the human VAMP7 protein confirmed the expression of the transgene in the testes of mutant mice (Supplementary Fig. 3a). Using primers that recognize both human and mouse transcripts, we quantified mRNA levels of VAMP7 by quantitative RT-PCR in the two lines (21 and 7) and found them to be only two to three times higher than the messenger levels of the endogenous VAMP7 in wild-type (WT) mice (Fig. 3a). Throughout the adult and fetal testes and external genitalia, the spatial expression pattern of the transgene mirrored that of the endogenous product in the WT animals and localized to cytoplasmic compartments as expected (Fig. 3b). For the rest of the study, the two transgenic lines 7 and 21 were evaluated in parallel to control for potential insertion effects. Data from strain 7 are presented out of the scrotal sac, inguinal when superior to the scrotum but inferior to the bladder at the inguinal ring, and low abdominal or high abdominal, respectively, when they were at or superior to the level of the bladder. The testicular location in uni- or bilaterally cryptorchid VAMP7–BAC transgenic mice was variable, predominantly inguinal in both strains (strain 7: 7.1% scrotal, 64.3% inguinal and 28.6% low abdominal; strain 21: 21.4% scrotal, 42.9% inguinal and 35.7% low abdominal) (Fig. 3d). None of the cryptorchid gonads were located in a high intra-abdominal position (Fig. 3d,e), implying that the androgen-dependent inguinoscrotal phase of testicular descent was defective in VAMP7–BAC transgenic mice. Examination of the gubernaculum, a mesenchymal tissue connecting the fetal testis to the developing scrotum, revealed a slightly feminized appearance with a thinner and elongated gubernacular cord connected to the lower cauda epididymis, as compared to the rudimentary gonadal ligament in WT male siblings (Fig. 3f). Notably, thin and elongated gubernaculum cords have previously been seen in estrogen-treated mouse embryos18–21.

Histological analysis of genital tubercles from VAMP7–BAC transgenic mice revealed variable penile defects (Fig. 3g). Half of the analyzed male transgenic embryos had normally developed genital tubercles with the ventral epithelial surfaces forming a closed urethral groove where epithelial surfaces fused to create the urethral seam and resulted in a true urethra. However, 16.7% (one out of six) exhibited hypospadias, an anomaly of the ventral urethral tube closure (Fig. 3g). Underdeveloped baculum, abnormalities of the epithelial-lined prepuce housing the penis and reduced thickness of the tunica albuginea capsule surrounding the corpora cavernosa were observed in 33.3% (two out of six) of VAMP7–BAC transgenic male embryos (Fig. 3g). Many of the penile defects in VAMP7–BAC transgenic animals resembled those observed following the prenatal or neonatal administration of estrogen compounds in male mice6–8. Furthermore, anogenital distance tended to decrease in mutant VAMP7–BAC transgenic male mice (Fig. 3h), but, more strikingly, penile length was significantly shorter in all adult transgenic animals than in their WT littermates (Fig. 3i).

Thus, higher levels of VAMP7 expression in the mouse male genitourinary tract mimicked the urogenital phenotypic traits observed in patients with VAMP7 copy number gain, i.e., cryptorchidism, hypospadias and micropenis. The VAMP7–BAC transgenic mouse models suggest a causative link between VAMP7 duplication and congenital genitourinary defects in humans.

Modest impact of VAMP7 on androgen receptor action

As a VAMP7 genomic defect was present in male subjects presenting with cryptorchidism, hypospadias or micropenis, and because...
normal androgen action is crucial for penile development and testicular descent, we asked whether elevated levels of VAMP7 interfere with androgen action. To assess the impact of VAMP7 on androgen receptor (AR) transcriptional activity, we used a cell culture–based assay in HeLa cells using a firefly luciferase reporter gene driven by an androgen-responsive element (ARE) in the promoter of the kallikrein-related peptidase 3 (KLK3 gene, which encodes a protein more commonly known in clinical diagnostics as prostate-specific antigen). Ligand-dependent AR transcriptional activity was suppressed by 60% with VAMP7 overexpression after transient transfection with a VAMP7 expression vector (Fig. 4a). This observed inhibition was not a result of a decrease of AR protein levels (Supplementary Fig. 3b). We observed the physical presence of VAMP7 and AR in the same protein complexes by coimmunoprecipitation assays (Fig. 4b), suggesting possible functional interactions between these two proteins. As VAMP7 is involved in cellular sorting, we questioned whether VAMP7 overexpression alters the subcellular localization of AR at the time of ligand-dependent stimulation. Immunofluorescent staining after transient transfection of AR and/or VAMP7 in the presence or absence of dihydrotestosterone (DHT) revealed colocalization of VAMP7 and AR in the cytoplasm upon DHT administration (Fig. 4c), implying a failure of efficient translocation of AR to the nucleus despite the presence of DHT. As VAMP7 localizes in the endosomes, we used RAB5A, a marker of early endosomes, to demonstrate a colocalization of AR and RAB5A in the presence of DHT (Fig. 4c).

To determine whether AR may be mislocalized in vivo, we then analyzed...
the cellular distribution of AR in the testes of VAMP7-BAC transgenic mice, which express the transgene in ranges far below the high doses of our transfection conditions but similar to those in the human genomic gains. In vivo, we observed AR in the cytoplasm of few, but not all, cells of testes that stained positively for AR from VAMP7-BAC transgenic mice (Fig. 4d), contrasting with its exclusive nuclear localization in the male gonads of WT littermates (Fig. 4d). We concluded that the observed mosaic pattern of AR subcellular distribution in the testes of VAMP7-BAC transgenic mice reflects a partial interference of ligand-dependent shuttling of AR in vivo, probably the result of VAMP7 overexpression.

Under these conditions of VAMP7 overexpression in the VAMP7-BAC transgenic mice, we examined AR recruitment to its genomic occupancy sites in the presence of increased VAMP7 gene dosage. In vivo chromatin immunoprecipitation in testicular tissues of VAMP7-BAC transgenic mice was performed using the ARE of several genes involved in AR signaling, including Fkbp4, Mafb and Fkbp5 (refs. 23–25). Although we observed a clear in vivo binding of AR in WT testis, binding was significantly reduced in the gonads of male VAMP7-BAC transgenic mice (Fig. 4e). Consistent with the observed AR occupancy, the immunoreactivity of the androgen-responsive genes Fkbp5 and Mafb was lower in the fetal and adult genital tracts of VAMP7-BAC transgenic male mice than in those of WT littermates (Fig. 4f,g). The expression of other testicular androgen target genes such as Rhox5, Drd4, Eppin, Tubb3 and Cldn11 was examined. With the exception of a significant decrease in Cldn11 (encoding a tight junction protein at the blood-testis barrier), we did not observe any dramatic changes in the expression of these genes in the testes of VAMP7-BAC transgenic mice compared to those in WT animals (Supplementary Fig. 3c).

Thus, the impact of VAMP7 on AR transcriptional activity, although...
Elevated VAMP7 dosage potentiates estrogen receptor action

In vivo, the balance between androgen and estrogen receptor action, as opposed to androgen action alone, is an important factor for male reproductive tract development. Consequently, we focused our further investigations on the impact of VAMP7 overexpression on estrogen signaling. With a classic estrogen-responsive element driving ESR1 target genes

Figure 5 VAMP7 enhances estrogen receptor transcriptional activity. (a) Luciferase assays following transfection with a reporter construct containing estrogen receptor–responsive element and with VAMP7, ESR1 or VAMP7 plus ESR1 in HeLa cells incubated in absence (vehicle) or presence of 17b-estradiol (1 × 10−8 M) for 24 h. n = 3 independent experiments for each condition. RLU, relative light units. Data are presented as means ± s.e.m. One-way ANOVA with post hoc Bonferroni test was used for statistical analyses. ***P < 0.001. (b) Western blot analysis of ESR1 and lamin A/C (LMNA) in nuclear protein extracts of HeLa cells cotransfected with ESR1 or ESR1 plus VAMP7 (V7) in the absence (EtOH) or presence of 17b-estradiol (1 × 10−8 M) for 24 h. (c) Reciprocal coimmunoprecipitation of ESR1 and VAMP7 following their cotransfection in HeLa cells. IP, immunoprecipitation. (d) qRT-PCR analysis of key genes of ESR1 signaling in testes from WT (n = 3) and V7BAC mice (line 7; n = 3), v1 and v2, transcript variant 1 (NM_207707.1) and transcript variant 2 (NM_010157.3) of Esr2, respectively. Data are expressed as mean ± s.e.m. Mean differences between WT and V7BAC mice were determined by unpaired, two-tailed Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.001. (e) ATF3 immunostaining of testis and external genitalia at fetal and adult stages of WT and V7BAC mice. For fetal tissues: scale bars, 250 μm. For adult urethra and testis: scale bars, 100 μm. Data are representative of three independent experiments. (f) qRT-PCR of VAMP7 and ATF3 gene expression after incubation with nontargeting (scramble) or VAMP7 siRNA in NT2/D1 cells. n = 3 independent experiments for each condition. Mean differences were determined by unpaired, two-tailed Student’s t-test. *P < 0.05; **P < 0.01. (g) Left, heatmap of testis gene expression profiles in NT2/D1 cells transiently transfected with nontargeting (scramble) or VAMP7-specific siRNA. Yellow and blue colors indicate increased and decreased expression, respectively, relative to scramble. Right, Ingenuity Pathway Analysis (IPA 8.5) of top canonical pathways significantly altered (20% false discovery rate) after VAMP7 knockdown in NT2/D1 cells. Data are representative of three independent experiments. (h) Pictures of inguinal hernia in V7BAC males and H&E staining of extensive granulomatous inflammatory response of entrapped tissues. Scale bar, 100 μm. Data are representative of four independent experiments. (i) Schematic representation of VAMP7 impact on estrogen receptor signaling and male phenotypic development.
post-translational mechanisms, as endogenous ESR1 mRNA levels remained unaffected after a knockdown of VAMP7 in the human testicular embryonic carcinoma cell line NT2/D1 (Supplementary Fig. 3d) or after VAMP7 overexpression in the testes of VAMP7-BAC transgenic mice (Fig. 5). The potential for a functional interaction between VAMP7 and ESR1 was further substantiated by the fact that these two proteins were physically found in the same protein complexes (Fig. 5c) and colocalized in identical subcellular compartments (Supplementary Fig. 3e).

To examine the in vivo functional interaction between VAMP7 and ESR1, we assessed the gene expression of known players involved in the estradiol-mediated action in VAMP7-BAC transgenic mice. Whereas expression levels were unchanged for the estrogen-dependent co-regulators, including p160 family (Ncoa1, Ncoa2, Ncoa3), Crebbp, Kat2b, Med1, Ncor2, Carm1 and the nuclear receptors Ar and Esr1 (Fig. 5d and Supplementary Fig. 4a), we observed a significant upregulation of mRNA levels of three estrogen-responsive genes, including activating transcription factor 3 (Aft3), connective tissue growth factor (Ctgf) and cysteine-rich angiogenic inducer 61 (Cyr61), in the testes of the VAMP7-BAC transgenic mice (Fig. 5d and Supplementary Fig. 4a). These observations are of key importance, as Aft3, Ctgf and Cyr61 are estrogen-responsive genes that are strongly upregulated in patients with hypospadias29–33. Moreover, variants of Aft3 are associated with hypospadias in humans34,35. Consistent with this, Aft3 protein levels were higher in fetal and adult testicular and penile tissues of VAMP7-BAC transgenic animals than in the tissues of WT littermates (Fig. 5e). Conversely, selective knockdown of VAMP7 in the human testicular embryonal carcinoma NT2/D1 cell line led to a significant decrease of Aft3 gene expression (Fig. 5f), confirming that VAMP7 is a positive regulator of Aft3 gene expression. Concomitantly, the expression levels of additional estrogen-responsive genes were altered in the testes of VAMP7-BAC transgenic mice compared to WT gonads (Fig. 5d). Indeed, Nrip1 mRNA levels, directly induced by estrogen receptor action36, were higher in VAMP7-BAC transgenic mice than in WT littermates (Fig. 5d). The expression of Cyp19a1, which is downregulated by estradiol in the testis37, was lower in the testes of VAMP7-BAC transgenic mice than in WT gonads (Fig. 5d and Supplementary Fig. 4a). Additionally, we observed higher mRNA levels of estrogen sulfotransferase (Sult1e1), an enzyme involved in the maintenance of the functional integrity of male-specific tissues against local estrogen activity38, in the testes of VAMP7-BAC transgenic mice than in WT animals (Fig. 5d and Supplementary Fig. 4a). In line with these in vivo data, estrogen receptor signaling was among the top pathways affected following acute VAMP7 knockdown in NT2/D1 cells, as revealed by a functional analysis of gene expression microarrays data by Ingenuity Pathway Analysis (Fig. 5g).

Finally, the occurrence of inguinal hernias in some VAMP7-BAC transgenic mice further supports our conclusions (Fig. 5h), as this phenotypic trait is observed with estrogen overstimulation in animal models, i.e., after administration of estrogens in male rodents39,40 and in aromatase-overexpressing male mice.41

Collectively, increased gene dosage of VAMP7, a SNARE-encoding gene, has a positive impact on ESR1-mediated transcriptional activity (Fig. 5i) and exerts a partial and inhibitory effect on AR action, ATF3, although positively induced by ESR1 action, acts as an AR co-repressor by preventing its binding to target genes and inhibiting transcriptional activation.42 This may explain the effects of VAMP7 overexpression on the altered expression of several androgen-responsive genes, including Cldn11, FkbP5 and MafB. The dual differential action of VAMP7 may partially ‘estrogenize’ males and subtly reduce AR action, leading to slightly feminized male reproductive development in VAMP7-BAC transgenic mice. This observation provides a molecular explanation for the clinical picture phenotype found in our cohort of human subjects with genitourinary anomalies.

Mice with elevated levels of VAMP7 are subfertile
As androgen and estrogen action are also important for spermatogenesis, we examined 6-month-old male VAMP7-BAC transgenic mice. Transgenic animals had small testes and focal spermatogenic deficiency...
resulting in subfertility (Fig. 6 and Supplementary Fig. 4b–h). Testis weight was reduced to about 50% compared to that of WT scrotal testes (Fig. 6a). Epididymal and seminal vesicle weights were similar to those of WT controls (Fig. 6b,c and Supplementary Fig. 4c,d). The histological pattern of the adult mouse cryptorchid testes was less uniform, showing discrete foci of disrupted spermatogenesis (Fig. 6d and Supplementary Fig. 4e). Arrest of spermatogenesis, sloughing of germ cells at various stages of maturation and vacuolization of Sertoli cells were evident within the affected seminiferous tubules (Fig. 6d and Supplementary Fig. 4e). Large multinucleated round cells were often present in the lumen of the tubules (Fig. 6d and Supplementary Fig. 4e). Serum testosterone, 17β-estradiol, luteinizing hormone (Lh) and follicle-stimulating hormone (Fsh) levels were not significantly different from those in WT males (Fig. 6e–h and Supplementary Fig. 4f). To quantify the functional consequences of the damage to the seminiferous epithelium, we measured sperm count and motility (Fig. 6i and Supplementary Fig. 4g). Although the number of caudal sperm was not significantly decreased, the total motility of sperm isolated from caudal epididymes of 6-month-old VAMP7-BAC transgenic mice was reduced by 75% for strain 7 and 70% for strain 21, suggesting deficient epididymal function, perhaps owing to altered hormone action (Fig. 6i and Supplementary Fig. 4g). Histological examination of the reproductive tissue of male VAMP7-BAC transgenic mice revealed morphological alterations of the epididymis in the mice, including vacuolization of the epididymal epithelium as well as the presence of numerous round cells in the lumen of caput and cauda epididymides (Fig. 6d and Supplementary Fig. 4e).

To assess whether the reduced motility of caudal sperm from the VAMP7-BAC transgenic males was associated with reduced fertility, we conducted a controlled mating experiment in which we mated 6-week-old WT or transgenic males with 8-week-old WT females. During a 4-month period, VAMP7-BAC transgenic males sired a similar number of litters to the WT mice. However, the litter size in the transgenic group was significantly smaller (Fig. 6j and Supplementary Fig. 4h).

**DISCUSSION**

Copy-number variants account for considerable human phenotypic variation and disease risk. They exert their influence by modifying the expression of genes mapping within or close to the rearranged regions. A de novo copy number variant in 46,XY individuals with mild masculinization defects suggested a causal link of VAMP7 gene duplication to the congenital genitourinary defects. We did not find any copy number gain of VAMP7 in 8,951 control individuals, highlighting the high degree of significance for the VAMP7 copy number duplications and the strong association between VAMP7 gene dosage changes and human cryptorchidism, hypospadias and micropenis ($P = 2.2 \times 10^{-3}$, Fisher's exact test). We initially detected a gain of VAMP7 in 4 cases out of a total of 296 patients (1.35%); then, in a second replication study, we found 1 case in 28 distinct primary cultures of genital skin fibroblasts (3.6%). The incidence of VAMP7 gains represents the highest frequency reported to date, to our knowledge, for a genetic or genomic cause of congenital genitourinary defects. Even previously identified pathogenetic mutations in genes such as INSL3, RXFP2 and AR exhibited lower frequencies ranging from 0.3% to 0.8% in individuals with disorders of sexual differentiation. Additionally, the Gene Expression Omnibus (GEO) database repository of high-throughput gene expression data shows that VAMP7 mRNA levels were significantly upregulated in human cryptorchid testes in two independent studies (GSE16191 and GSE25518). These data substantiate our findings and support the fact that VAMP7 copy number change is a clinically significant factor in the etiology of human congenital genitourinary defects.

Although our data support a genomic basis for genitourinary birth defects, they also shed light on an unexpected molecular determinant affecting male phenotypic development. Under normal conditions, VAMP7 ensures proper membrane fusion and transport between various organelles. This study shows that VAMP7 is a dosage-sensitive gene, as we detected slight differences in the magnitude of the phenotypic defects between the two VAMP7-BAC transgenic lines. Indeed, line 7 mice, which had a higher level of VAMP7 expression than line 21 mice, exhibited a higher incidence of bilateral cryptorchidism, as well as a slightly more marked decrease of testis weight and litter size. Similarly, line 7 mice displayed a significant upregulation of select genes, with Atf3, Sult1e1 and Cyr61 showing the biggest differences, and Ctgf and Nrip1 revealing modest impairment. Mice from strain 21 also displayed a significant upregulation of Atf3 and Sult1e1 and a trend of increase of Cyr61 ($P = 0.09$), whereas expression of Ctgf and Nrip1 genes was unchanged. Our observations provide evidence that higher protein levels of VAMP7 correlate with a more severe phenotype in mice.

Elevated levels of VAMP7 enhanced estrogen receptor–driven transcription of genes including Atf3, Ctgf and Cyr61, all of which are upregulated in individuals with hypospadias. The dual function of VAMP7 in membrane sorting and transcriptional action was previously reported for a subset of factors involved in endocytosis and endosomal trafficking such as huntingtin-interacting protein 1, auxilin-2, clathrin heavy chain, β-arrestin-1 and β-arrestin-2, APPL1 and APPL2 (adaptor protein containing PH domain, PTB domain and leucine zipper motif 1 and 2) and CALM (clathrin assembly protein lymphoid myeloid). Several of these endocytic proteins exert AR co-regulatory characteristics, mainly through association with transcription factors and/or facilitation of chromatin modifications. Our study reinforces the concept that vesicular transport and endosomal factors are able to perturb key nuclear functions and transcriptional regulation.

We have demonstrated that elevated levels of VAMP7 can lead to an increase in ligand-dependent ESR1 protein content without affecting its mRNA levels and can result in a subsequent stimulation of transcriptional activity similar to the action of other previously reported proteins. ESR1 stability and protein turnover are finely controlled by ubiquitination and proteosomal degradation to ensure efficient transcriptional activity of the nuclear receptor. Recent evidence highlights the existence of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems. Perturbations in the flux through either pathway could affect the activity of the other system. Given that VAMP7 is a regulator of autophagosome formation, one of the potential effects of increased VAMP7 might be the perturbation of the ubiquitin-proteasome pathway leading to abnormal processing of select proteins, including ESR1. Although beyond the scope of this present study, the exact underlying molecular mechanisms merit further clarification.

In conclusion, genomic defects encompassing the gene encoding VAMP7 are present in a subset of humans with congenital genitourinary disorders. Alterations of the gene dosage of this vesicular trafficking factor are responsible for abnormal human and mouse male genitourinary tract development, mainly due to a marked enhancement of estrogen receptor transcriptional action. VAMP7 overexpression underlies the etiology of a subset of common disorders of male sexual differentiation.
METHODS

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AUTHOR CONTRIBUTIONS

Both senior authors, M.T.-L. and D.J.L., conceived and supervised the study, conducted experiments, analyzed data and wrote and revised the manuscript. S.H. and J.-F. L. performed experiments and analyzed data. B.Z., K.R., J.A. and A.S. performed experiments. S.W.C. conducted the human CGH array studies.

COMPETING FINANCIAL INTERESTS

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1. Sharpe, R.M. & Skakkebaek, N.E. Are oestrogens involved in failing sperm counts and disorders of the male reproductive tract? Lancet 341, 1392–1395 (1993).
2. Wilhelm, D. & Koopman, P. The makings of maleness: towards an integrated view of male developmental disorders. Nature 426, 15–16 (2003).
3. Vidaeff, A.C. & Sever, L.E. Male reproductive disorders in humans and developmental disorders of the rat penis involve both estrogen receptor (ESR)- and androgen receptor (AR)-mediated pathways. Biol. Reprod. 81, 507–516 (2009).
4. Simon, L. et al. Exposure of neonatal rats to anti-androgens induces penile maldevelopments and infertility comparable to those induced by oestrogens. Int. J. Androl. 35, 364–376 (2012).
5. Li, X. et al. Altered structure and function of reproductive organs in transgenic male mice overexpressing human aromatase. Endocrinology 142, 2435–2442 (2001).
6. Kloepper, T.H., Kienle, C.N. & Fasshauer, D. An elaborate classification of SNAREs involved in synaptotagmin VII–regulated lysosomal exocytosis. J. Biol. Chem. 279, 20471–20479 (2004).
7. Fader, C.M., Sanchez, D.G., Mestre, M.B. & Colombo, M.I. Ti-VAMP/VAMP3 cellubrevin: two v-SNARE proteins involved in specific steps of the autophagy/multivesicular body pathways. Biochim. Biophys. Acta 1793, 1901–1916 (2009).
8. Moorea, K., Ravikumar, B., Renna, M., Puris, C. & Rubinstein, D.C. Autophagosome precursor maturation requires homotypic fusion. Cell 146, 303–317 (2011).
9. Tannour-Louet, M. et al. Identification of de novo copy number variants associated with human disorders of sexual development. PLoS ONE 5, e15392 (2010).
10. Kloepper, T.H., Kienle, C.N. & Fasshauer, D. An elaborate classification of SNAREs involved in synaptotagmin VII–regulated lysosomal exocytosis. J. Biol. Chem. 279, 20471–20479 (2004).
11. Chen, H. et al. Estrogen receptor α is a major contributor to estrogen-mediated fetal testis dysgenesis and cryptorchidism. Endocrinology 148, 5507–5519 (2007).
12. Kent, H.M. et al. Structural basis of the intracellular sorting of the SNARE VAMP7 by the AP3 adaptor complex. Dev. Cell 22, 979–988 (2012).
13. Magee, J.A., Chang, L.W., Stormo, G.D. & Milbrandt, J. Direct, androgen receptor–mediated regulation of the FKBP5 gene via a distal enhancer element. Endocrinology 147, 590–598 (2006).
14. Makkonen, H., Kaulanen, M., Paakinaho, V., Jaakkelainen, T. & Palvimo, J.J. In utero exposure of neonatal rats to anti-androgens induces penile maldevelopment and cryptorchidism. J. Steroid Biochem. Mol. Biol. 95, 3190–3202 (2005).
15. Thenot, S., Charpin, M., Bonnet, S. & Cavailles, V. Estrogen receptor cofactors in the etiology of hypospadias. Pediatr. Res. 58, 1280–1283 (2005).
16. Liu, B. et al. Estradiol upregulates activating transcription factor 3, a candidate gene in the etiology of hypospadias. Pediatr. Dev. Pathol. 10, 446–454 (2007).
17. Liu, B. et al. Activating transcription factor 3 is up-regulated in patients with hypospadias. Pediatr. Res. 58, 1280–1283 (2005).
18. Liu, B., Agras, K., Willingham, E., Vilela, M.L. & Baskin, L.S. Activating transcription factor 3 is estrogen-responsive in utero and upregulated during sexual differentiation. Horm. Res. 65, 217–222 (2006).
19. Gurbuz, C. et al. Is activating transcription factor 3 up-regulated in patients with hypospadias? Korean J. Urol. 51, 561–564 (2010).
20. Kafka, N. et al. Genomic variants of ATF3 in patients with hypospadias. J. Urol. 180, 2183–2188, discussion 2188 (2008).
21. Belenky-Meireles, A. et al. (1996). Activating transcription factor 3: a hormone responsive gene in the etiology of hypospadias. Eur. J. Endocrinol. 158, 729–739 (2008).
22. Thonot, S., Charpin, M., Bonnet, S. & Cavailles, V. Estrogen receptor cofactors expression in breast and endometrial human cancer cells. Mol. Cell. Endocrinol. 156, 85–93 (1999).
23. Lambard, S. et al. Aromatase in testis: expression and role in male reproduction. J. Steroid Biochem. Mol. Biol. 95, 63–69 (2005).
24. Song, W.C., Qian, Y., Sun, X. & Negishi, M. Cellular localization and regulation of expression of testicular estrogen sulfotransferase. Endocrinology 138, 5006–5012 (1997).
25. Hazay, S. & Gardner, W.U. Influence of sex hormones on abdominal musculature and the formation of inguinal and scrotal hernias in mice. Anat. Rec. 136, 437–443 (1960).
26. Reindert, R.B. et al. Tamoxifen-induced Cre-loxP recombination is prolonged in pancreatic islets of adult mice. PLoS ONE 7, e33529 (2012).
27. Li, X. & Rahman, N. Impact of androgen/estrogen ratio: lessons learned from the aromatase over-expression mice. Gen. Comp. Endocrinol. 159, 1–9 (2008).
28. Wang, H. et al. The stress response mediator ATF3 represses androgen signaling by binding the androgen receptor. Mol. Cell. Biol. 32, 3190–3202 (2012).
29. Girirajan, S., Campbell, C.D. & Eichler, E.E. Human copy number variation and complex genetic disease. Annu. Rev. Genet. 45, 203–226 (2011).
30. Reinert, A. et al. Genetic alterations associated with cryptorchidism. J. Am. Med. Assoc. 300, 2271–2276 (2008).
31. Hadziselimovic, F. et al. EGR4 is a master gene responsible for fertility in cryptorchidism. Sex Dev. 3, 253–263 (2009).
32. Hadziselimovic, F., Hadziselimovic, N.D., Denoguine, P. & Oakley, E.J. Testicular expression in cryptorchid boys at risk of azospermia. Sex Dev. 5, 49–59 (2011).
33. Mills, I.G. et al. Huntingtin interacting protein 1 modulates the transcriptional activity of nuclear hormone receptors. J. Cell Biol. 170, 191–200 (2005).
48. Ray, M.R. et al. Cyclin G–associated kinase: a novel androgen receptor–interacting transcriptional coactivator that is overexpressed in hormone refractory prostate cancer. Int. J. Cancer 118, 1108–1119 (2006).
49. Jasavala, R. et al. Identification of putative androgen receptor interaction protein modules: cytoskeleton and endosomes modulate androgen receptor signaling in prostate cancer cells. Mol. Cell. Proteomics 6, 252–271 (2007).
50. Kang, J. et al. A nuclear function of β-arrestin1 in GPCR signaling; regulation of histone acetylation and gene transcription. Cell 123, 833–847 (2005).
51. Yang, L. et al. APPL suppresses androgen receptor transactivation via potentiating Akt activity. J. Biol. Chem. 278, 16820–16827 (2003).
52. Enari, M., Ohmori, K., Kitabayashi, I. & Taya, Y. Requirement of clathrin heavy chain for p53-mediated transcription. Genes Dev. 20, 1087–1099 (2006).
53. Wei, X., Xu, H. & Kufe, D. MUC1 oncoprotein stabilizes and activates estrogen receptor α. Mol. Cell 21, 295–305 (2006).
54. Reid, G. et al. Cyclic, proteasome-mediated turnover of unliganded and liganded ERα on responsive promoters is an integral feature of estrogen signaling. Mol. Cell 11, 695–707 (2003).
55. Lonard, D.M., Nawaz, Z., Smith, C.L. & O’Malley, B.W. The 26S proteasome is required for estrogen receptor-α and coactivator turnover and for efficient estrogen receptor-α transactivation. Mol. Cell 5, 939–948 (2000).
56. Nawaz, Z., Lonard, D.M., Dennis, A.P., Smith, C.L. & O’Malley, B.W. Proteasome-dependent degradation of the human estrogen receptor. Proc. Natl. Acad. Sci. USA 96, 1858–1862 (1999).
57. Korolchuk, V.I., Menzies, F.M. & Rubinsztein, D.C. Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems. FEBS Lett. 584, 1393–1398 (2010).
58. Korolchuk, V.I., Mansilla, A., Menzies, F.M. & Rubinsztein, D.C. Autophagy inhibition compromises degradation of ubiquitin-proteasome pathway substrates. Mol. Cell 33, 517–527 (2009).
ONLINE METHODS

Ethics statement, human subjects and sample collection. This study was approved by the Institutional Review Board Committee at the Baylor College of Medicine (Houston, TX, USA). Probands affected with idiopathic cryptorchidism and/or hypospadias were enrolled through Texas Children’s Hospital and Ben Taub General Hospital, (Houston, TX, USA). Known causes of these birth defects, such as anomalies in the synthesis of testosterone or adrenal steroid hormones or exogenous modifiers, or karyotypic abnormalities, were excluded etiologies after examination by pediatric urologists and/or neonatologists. Written informed consent was obtained from the parents of our subjects. Blood was collected from the children during surgery for correction of cryptorchidism or hypospadias. Parents provided saliva specimens for DNA isolation. Primary cultures of genital skin fibroblasts (GSF) isolated from the foreskin of male neonates at the time of circumcision or surgical correction were obtained from patients with hypospadias and/or cryptorchidism after approval from the Baylor College of Medicine Institutional Review Board (Houston, TX, USA). GSF cell lines were maintained in MEM with 10% FBS and 1% penicillin and streptomycin.

CGH-based microarray analysis (CMA). High molecular weight genomic DNA isolated from peripheral blood or saliva was submitted for chromosomal microarray analysis (CMA) to the Clinical Cytogenetics Laboratory at Baylor College of Medicine. CMA is a clinically validated targeted CGH array that covers over 150 distinct human clinically relevant chromosomal loci (http://www.bcm.edu/geneticlabs/?pmid=162070). CMA V6 OLIGO microarray, a custom-targeted array manufactured by Agilent Technologies (Santa Clara, CA) using approximately 44,000 oligonucleotides to emulate the CMA V6 BAC arrays49, was used. CMA procedures and data analyses using an in-house analysis package for copy number analysis were used as described previously60. One unique DNA reference served as a control for CMA analysis and was from a pregnancy-proven fertile gender-matched individual without any familial history of congenital genitourinary defects. The presence of imbalances was quantified by calculating the ratio of signal intensities of test DNA from patients compared with reference DNA extracted from a pregnancy-proven fertile gender-matched individual with no familial history of congenital genitourinary defects. The log2 (Cy5/Cy3) scaling has the consequence of centering the ratios at approximately 0, making DNA copy number gains appear positive (positive shift of the probes signal).

FISH analysis. The BAC clone of interest (RP11-479817B) and a control probe RP11-815E21 (at Xq22.3) were grown in TB medium with 20 mg ml-1 chloramphenicol. DNA was extracted from the BAC clones (Eppendorf Plasmid Mini Prep kit, Hamburg, Germany), directly labeled with fluorochrome-conjugated dUTP by nick translation (Vysis, Downer Grove, IL) according to the manufacturer’s instructions and hybridized to PHA-stimulated patient lymphocyte cultures. Digital FISH images were captured using MacProbe version 4.4 software (Applied Imaging, San Jose, CA).

CNV Taqman assays. Copy Number Variant TaqMan assays (Applied Biosystems) were performed according to the manufacturer’s protocol using One Step Plus Real-Time PCR. TaqMan Copy Number Reference Assay RNase P was used as the standard reference assay for copy number analysis. Relative quantitation analysis was done to estimate copy numbers for each sample by using the Copy-Caller-Software, v.1.0 (Applied Biosystems, Grand Island, NY, USA).

Cell culture and transfections. For transfection experiments, HeLa cells were grown in DME without phenol red in the presence of 10% stripped FCS. Cells were plated at 70% confluence in six-well plates for luciferase assays or 50% for immunofluorescence. Cells were transfected with several reporter-gene plasmids containing either the androgen-responsive element of PSA promoter or a multimerized ERE sequence. These constructs were cotransfected as indicated in each experiment with different expression vectors encoding VAMP7 in pCMV6-XL5 vector (OriGene, Rockville, MD) as well as PCMV-AR and PCR3.1-ESR1. Treatment with 1×10-8 M DHT or 17β-estradiol was done 24 h after transfection, and reporter gene levels were determined 24 h after hormonal induction using Promega reagents. For knockdown experiments, NT2/D1 (ATCC CRL-7973) cells were grown in a modified Dulbecco’s medium supplemented with 10% FBS and l-glutamine in an atmosphere of 5% CO2. Cells were plated in six-well plates (1×105 per well) for RNA extraction. The next day, cells were transfected for 72 h with nonspecific (scramble) siRNA or siRNA for human VAMP7 (Dharmacon, Lafayette, CO, USA) according to the manufacturer’s procedure.

Immunofluorescence. Cells were rinsed twice in ice-cold phosphate-buffered saline (PBS) and fixed with a freshly prepared solution of 4% paraformaldehyde in PBS for 20 min and permeabilized for 10 min with 0.1% digitonin in PBS. After several rinses in ice-cold PBS, cells were incubated in 3% normal goat serum in PBS (blocking solution) for 30 min at room temperature, followed by an overnight incubation at 4°C with primary antibodies. Cells were extensively washed with PBS containing 0.1% digitonin and then incubated with Alexa-conjugated secondary antibodies (Alexa Fluor 488 goat anti-rabbit, A-11008, Molecular Probes; Alexa Fluor 594 goat anti-rabbit, A-11012, Molecular Probes; Alexa Fluor 594 goat anti-mouse, A-11005, Molecular Probes; dilutions 1:600) for 60 min at room temperature. Coverslips were mounted in a mounting medium. Images were captured with an API Delta Vision deconvolution microscope using SoftWorx software (Applied Precision, Issaquah, USA). Primary antibodies used were anti-AR (Sc-816, 1:200, Santa Cruz Biotechnology Inc.), anti-VAMP7 (NB100-91356, 1:1,000, Novus Biologicals), ESR1 (MA1-310, 1:200, Thermo-Fisher) and anti-RABS (R2413, 1:100, Cell Signaling).

Quantitative RT-PCR analysis. Total RNA was purified using the RNeasy Kit including an optional DNase I treatment according to the manufacturer’s instructions (Qiagen). cDNA was prepared from 500 ng of total RNA by reverse transcription. Real-time PCR was performed with TaqMan PCR Master Mix on an ABI StepOnePlus Realtime PCR System (Applied Biosystems). PCR conditions were 50°C for 2 min and 94°C for 2 min, followed by 40 cycles of 94°C for 15 s and 60°C for 30 s. For each experimental sample, the relative abundance value was normalized to the value derived from the endogenous control (GAPDH) of the same sample. Relative mRNA levels were quantified by using the comparative 2-ΔΔCT method.

Generation of VAMP7-BAC transgenic mice. All described procedures were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine (Houston, TX, USA) and conducted in compliance with the Guide for the Care and Use of Laboratory Animals in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). BAC (RP11-479817B) containing human VAMP7 gene was purified using the NucleoBond BAC 100 Kit (Clontech). The purified transgene DNA was introduced into the pronuclei of fertilized oocytes from FVB/N mice through the service of Genetically Engineered Mouse Core Facility at the Baylor College of Medicine (https://www.bcm.edu/research/advanced-technology-core-labs/lablisting/genetically-engineered-mouse/home.htm).

Fertility studies and semen evaluation. The fertility and fecundity of 6-week-old FVB male VAMP7 and their WT littermates were determined in a continuous mating study in which each male was mated with two FVB WT females (6 weeks of age) for 4 months. The females were monitored for pregnancy during and after the mating periods, and the number of litters and offspring was recorded. Penile length was measured after exposing the penis up to the ischial arch, and its stretched length measured from the tip of the glans penis to the midpoint of the ischial arch. The cauda epididymides of 3- to 8-month-old VAMP7-BAC transgenic and WT mice were collected in M2 medium. Cuts were made and sperm were allowed to swim out into the medium for 15 min at 37°C temperature. Motility was manually assessed using a hemocytometer.

Hormone assays. Mice under anesthesia were exsanguinated by closed cardiac puncture. Serum samples were collected and stored frozen at −20°C until further use. RAs for LH and FSH were performed using US National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) hormone assay kits according to the standard protocols of the Ligand Assay and Analysis Core Laboratory (University of Virginia, Charlottesville, VA, USA).
Histological analysis. Bouin’s fixed, paraffin-embedded testes were cut in cross-sections and longitudinal sections and stained with hematoxylin and eosin. For immunohistochemistry analysis, slides of paraffin-embedded samples were deparaffinized and dehydrated. Antigen retrieval was performed by heat inactivation in 0.1 M sodium citrate for 30 min. Primary antibodies specific to VAMP7 (HPA036733, 1:25, Sigma Prestige or NB100-91356, 1:200, Novus Biologicals), AR (sc-816, 1:200, Santa Cruz), ATF3 (sc-188, 1:400, Santa Cruz), FKBP5 (AF4094, 1:200, R&D Systems), MAFB (ABE55, 1:200, Millipore) and desmin (NB110-56931, 1:500, Novus Biologicals) were used for immunodetection. The staining was performed using the avidin-biotin peroxidase system (ABC-peroxidase), and positive signals were visualized as brown precipitates using 3,3′-diaminobenzidine tetrahydrochloride. Control staining was conducted by omission of the primary antibody. Hematoxylin was used for counterstaining.

Western blot and coimmunoprecipitation. 20 µg of protein aliquots from cell lysates of testes from VAMP7-BAC transgenic (V7BAC) mice were separated by 15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with primary antibodies to VAMP7 (sc-166394, 1:100, Santa-Cruz or sc-67060, 1:50, Santa-Cruz), AR (sc-816, 1:1,000, Santa-Cruz), ESR1 (MA1-310, 1:200, Thermo-Fisher), LMNA (sc-20681, 1:200, Santa-Cruz), GAPDH (sc-32233, 1:200, Santa-Cruz) and ACTB (AC-40, 1:5,000, Sigma). HeLa cells were cotransfected with AR or ESR1 or VAMP7 or in combination. After 24 h of transfection, cell lysates were prepared, and immunoprecipitation was carried out using antibodies raised against AR (sc-816, Santa-Cruz), ESR1 (MA5-13065, Thermo-Fisher) or VAMP7 (sc-67060, Santa-Cruz) or control IgG antibody. The samples were later subjected to western blotting with the indicated antibodies. Input was 5% of the lysates.

Chromatin immunoprecipitation. In vivo ChIP was performed on single-cell collagenase-treated testis from VAMP7-mutant mice. Formaldehyde (1%) was added to produce cross-linking during 10 min at room temperature. The rest of the ChIP procedure was performed using the EZ ChIP kit (Millipore) following the manufacturer’s protocol. AR antibody was from Santa-Cruz (sc-816, Santa-Cruz). qPCR for ChIP was performed using the SYBR-Green technology (Applied Biosystems). Results were normalized to input in each case. Primer sequences are available upon request.

Gene expression microarray hybridization and analysis. NT2/D1 (ATCC CRL-1973) cells were grown in a modified Dulbecco’s medium supplemented with 10% FBS and L-glutamine in an atmosphere of 5% CO2. Cells were plated in 6-well plates (1 x 10⁶/well) for RNA extraction. The next day, cells were transfected for 72 h with nonspecific (scramble) siRNA or siRNA for human VAMP7 (Dharmacon, Lafayette, CO, USA) according to the manufacturer’s procedure. Three samples treated with siRNA targeting VAMP7 in NT2 cells were compared, with three samples of cells treated with scrambled siRNA using Affymetrix Human U133 2.0 Plus Array, which contains 1,300,000 unique oligonucleotides, 47,000 transcripts and variants that represent about 39,000 of the best-characterized human genes. The probe set design was based on databases: GenBank, dbEST and RefSeq. Microarray results were normalized using Robust Multichip Average (RMA). Statistical analysis approach of SAM (Significance Analysis of Microarray) was applied using Multiple Experiment Viewer (MeV). Data have been deposited in the GEO database under the accession number GSE56102. We identified a list of about 600 genes that are significantly altered at a 20% False Discovery Rate (FDR) when VAMP7 was knocked down in NT2/D1 cells. This list was then analyzed using Ingenuity Pathway Analysis (IPA) to identify pathways and biological functions involved.

Statistical analyses. Samples and animals were evaluated in a randomized manner by six investigators (M.T.-L., S.H., J.-F.L., B.Z., K.R. and J.A.) who were blinded to the treatment conditions. No statistical method was used to predetermine sample size for animal or cell studies. No inclusion or exclusion criteria were defined for samples and animals. At least 3 independent experiments were performed for mouse tissues and cell lines and included in the statistical analyses, as reported in all figure legends. Each replicate represents an independent experiment. Data are presented as means ± s.e.m. One- or two-way ANOVA with post hoc Bonferroni’s test was run for all statistical analyses except where a Student’s t-test was used. Significance threshold was set at P = 5.0 x 10⁻². To analyze the frequency of de novo Xq28 copy number change in affected genitourinary patients compared to unaffected genitourinary individuals (nongenitourinary controls), two-tailed Fisher’s exact test was performed and statistical significance determined using SPSS software.

59. Ou, Z. et al. Branchiootorenal syndrome and oculoauriculovertebral spectrum features associated with duplication of SIX1, SIX6, and OTX2 resulting from a complex chromosomal rearrangement. Am. J. Med. Genet. A 146A, 2480-2489 (2008).
60. Ou, Z. et al. Bacterial artificial chromosome-emulsion oligonucleotide arrays for targeted clinical array-comparative genomic hybridization analyses. Genet. Med. 10, 278-289 (2008).