

Genome-wide association study and mouse expression data identify a highly conserved 32 kb intergenic region between *WNT3* and *WNT9b* as possible susceptibility locus for isolated classic exstrophy of the bladder

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Bladder exstrophy-epispadias complex (BEEC), the severe end of the urorectal malformation spectrum, has a profound impact on continence as well as sexual and renal functions. It is widely accepted that for the majority of cases the genetic basis appears to be multifactorial. Here, we report the first study which utilizes genome-wide association methods to analyze a cohort comprising patients presenting the most common BEEC form, classic bladder exstrophy (CBE), to identify common variation associated with risk for isolated CBE. We employed discovery and follow-up samples comprising 218 cases/865 controls and 78 trios in total, all of European descent. Our discovery sample identified a marker near *SALL1*, showing genome-wide significant association with CBE. However, analyses performed on follow-up samples did not add further support to these findings. We were also able to identify an association with CBE across our study samples (discovery: $P = 8.88 \times 10^{-5}$; follow-up: $P = 0.0025$; combined: 1.09×10^{-6}) in a highly conserved 32 kb intergenic region containing regulatory elements between *WNT3* and *WNT9B*. Subsequent analyses in mice revealed expression for both genes in the genital region during stages relevant to the development of CBE in humans. Unfortunately, we were not able to replicate the suggestive signal for *WNT3* and *WNT9B* in a sample that was enriched for non-CBE BEEC cases ($P = 0.51$). Our suggestive findings support the hypothesis that larger samples are warranted to identify association of common variation with CBE.

INTRODUCTION

The Bladder exstrophy-epispadias complex (BEEC; OMIM %600057) represents the severe end of the urorectal malformation spectrum and has a profound impact on continence as well as sexual and renal functions. The BEEC comprises three levels of severity ranging from epispadias (E) (Supplementary Material, Fig. S1A and B) and classic bladder exstrophy (CBE) (Supplementary Material, Fig. S1C and D), to the most severe form, cloacal exstrophy (CE), often referred to within the OEIS complex (omphalocele, exstrophy, imperforate anus and spinal defects; Supplementary Material, Fig. S1E) (1,2). In approximately, one-third of all BEEC patients, there are also associated urological malformations (e.g. ectopic kidney, renal agenesis and hydronephrosis). Management of the BEEC is primarily surgical, and the main aims are the achievement of secure abdominal wall closure, urinary continence with preservation of renal function and adequate cosmetic and functional genital reconstruction (3). Following reconstructive surgery of the bladder during the neonatal period, continence rates of around 80% are expected during childhood. Additional surgery might be needed to optimize bladder storage and emptying function. Psychosocial and psychosexual outcome and adequate health-related quality of life depend on long-term multidisciplinary care (4–7). Among children of European descent, the overall birth prevalence for the entire spectrum has been estimated to be 1 in 10 000 (2). The birth prevalence for the specific subtypes, including terminated pregnancies, has also been estimated and established values for E of 1 in 117 000 for males and 1 in 484 000 for females (2), along with 1 in 37 000 for CBE (8) and 1 in 200 000 to 1 in 400 000 for CE (9).

Although the BEEC can occur as part of a complex malformation syndrome, the majority of cases (~98.5%) are classified as isolated (10–12). Formal genetic studies have suggested the involvement of genetic factors in the etiology of BEEC. For CBE, the recurrence risk among siblings in families with non-consanguineous and non-affected parents ranges between 0.3

and 2.3%, and the recurrence risk for the offspring of affected patients was reported to be 1.4% (13,14). Hence, the recurrence risk for the offspring of affected CBE patients and the risk of having a second affected child for parents who are non-consanguineous and non-affected exhibits an ~400-fold increase as compared with the general population. Further evidence for genetic factors underlying the BEEC comes from a classic twin study which clearly showed higher concordance rates among monozygotic (62%) as compared with dizygotic (11%) twin pairs (15).

Despite the overall rare occurrence of these congenital malformations, a total of 30 families with multiple affected individuals have been reported. In some of these, the pattern of BEEC incidences within single families resembles a Mendelian mode of inheritance (15–18); however, the general consensus in the field is that, in the majority of patients, the genetic basis appears to be multifactorial (11). To further support formal evidence for a genetic contribution to BEEC, recent studies have identified various molecular genetic factors including chromosomal aberrations such as trisomy 21 and micro-duplications on chromosome 22q11.21, as well as the association of polymorphisms within the $\Delta Np63$ promoter with an increased incidence of BEEC phenotypes (19–22). However, these associated chromosomal risk factors account for only a small portion of the BEEC, whereas most patients represent idiopathic cases.

To the best of our knowledge, we report here the first study using genome-wide association methods to analyze a BEEC cohort sample, comprising of patients presenting the most common form, CBE. Our aim was to identify genetic susceptibility loci for isolated CBE presentation. We therefore conducted a genome-wide association study (GWAS), and subsequently followed up the most promising genomic regions in independent replication samples, with the goal of identifying common single-nucleotide polymorphisms (SNPs) in the genome that are associated with risk for isolated CBE in patients of European descent.

RESULTS

For both GWASs, we followed the same stringent quality control (QC) protocol that has been successfully applied to other datasets (23,24). Details on the analytical pipeline can be found in Supplementary Material, Figure S2. The post-QC datasets (comprising 43 cases and 259 controls for GWAS1 and 55 cases and 267 controls for GWAS2, respectively) were subjected to imputation based on 1000 Genomes Project and HapMap 3 reference panels (25,26). After applying postimputation QC, single marker analyses were conducted for 4 510 680 markers each using a logistic regression (additive model) as implemented into SNPTEST (27). The subsequent meta-analysis of both datasets was performed using a fixed-effects model as implemented into YAMAS (28). The most significant results were obtained for two markers in (i) an intergenic region flanked by *CYLD* and *SALL1* for rs4785484 on chromosome 16q12.1, $P = 4.55 \times 10^{-8}$ and (ii) in an intergenic region flanked by *EEF1E1* and *SLC35B3* for rs73374907 on chromosome 6p24.3, $P = 6.13 \times 10^{-8}$ (more information in the Supplementary Material, Table S1; Figs 1 and 2). Follow-up on the most promising genomic regions based on the results of the meta-analysis was performed for a total of 75 SNPs that were successfully genotyped and (meta) analyzed after pruning for linkage disequilibrium in the initially obtained SNP list. The post-QC dataset for our first follow-up sample comprised 27 index cases with their parents and 120 cases/339 controls (Supplementary Material, Table S1 and Fig. S2; for more details on the follow-up samples, see the Materials and Methods). Based on the results from our first follow-up sample, a second independent sample (Follow-up 2, 51 index cases and their parents; Supplementary Material, Table S1 and Fig. S2) was analyzed for four markers that showed evidence of association. Evidence for replication (nominal significant association with same directionality based on effect allele across GWAS, Follow-up 1 and Follow-up 2) was found for two SNPs (Table 1) located in an intergenic region on chromosome 17 flanked by *WNT3* and *WNT9B* (rs9890413, $P_{USA} = 0.0095$, $P_{EU+USA} = 0.0025$) and intronically to *MECOM* (rs56273700, $P_{USA} = 0.0423$, $P_{EU+USA} = 0.0045$). The result for rs9890413 remained significant after correcting for the number of tests in the second follow-up sample. Subsequent meta-analysis of all samples tested in the discovery and follow-up steps showed P -values of 1.09×10^{-6} (rs9890413) and 1.24×10^{-6} (rs56273700), respectively. It is of note that for rs9890413 all samples (i.e. both GWAS

samples and all four follow-up samples) shared the effect allele and the direction of the effect. Unfortunately, we were not able to replicate our findings for the *WNT3-WNT9B* locus in a final sample of 78 cases and 336 controls that was enriched for non-CBE BEEC cases ($P = 0.51$; more information in the Supplementary Material). However, it is of note that the MAF in the control cohort was higher than expected from observations in the GWAS samples (0.36 versus 0.29 and 0.30, respectively), and that a test for deviation from Hardy–Weinberg equilibrium (HWE) in controls did show a trend towards significance ($P = 0.067$). Expression of *Wnt3* and *Wnt9b* was detected in the genital region of mouse embryos at all stages examined (Fig. 3). While *Wnt3* was found to be widely expressed within the epithelia, including that of the cloacal membrane and genital tubercle, *Wnt9b* was found to be strongly expressed in the cloacal membrane between E9.5 and E10.5, and the epithelia of the genital tubercle at later stages.

DISCUSSION

Presumably our constrained sample size did not allow us to find association of genetic variation with CBE (or BEEC) beyond reasonable doubt. Our results are therefore mainly to be regarded as suggestive evidence for association and larger studies are needed to confirm our findings. In the following, we would like to discuss these findings in light of the statistical power for GWAS of rare disorders such as BEEC (birth prevalence is 0.0001).

As outlined above, we found suggestive evidence for association with CBE across our study samples (rs9890413; discovery: $P = 8.88 \times 10^{-5}$; follow-up: $P = 0.0025$) in a highly conserved 32 kb intergenic region containing regulatory elements between *WNT3* and *WNT9B*. However, we were not able to replicate this finding in an independent sample that was enriched for non-CBE BEEC cases. It is reasonable to assume that (at least in part) the inability to find stronger evidence for association in the intergenic region between *WNT3* and *WNT9b* is based on a lack in statistical power: the risk allele frequency in our discovery samples was $\sim 30\%$ in controls and the effect size was estimated with an OR of ~ 2 . Under the assumption of an additive model and genome-wide significance (5×10^{-8}) power of 80% to see association at this significance level would require a sample of 357 cases (and equal number of controls). While our combined study samples (comprising 218

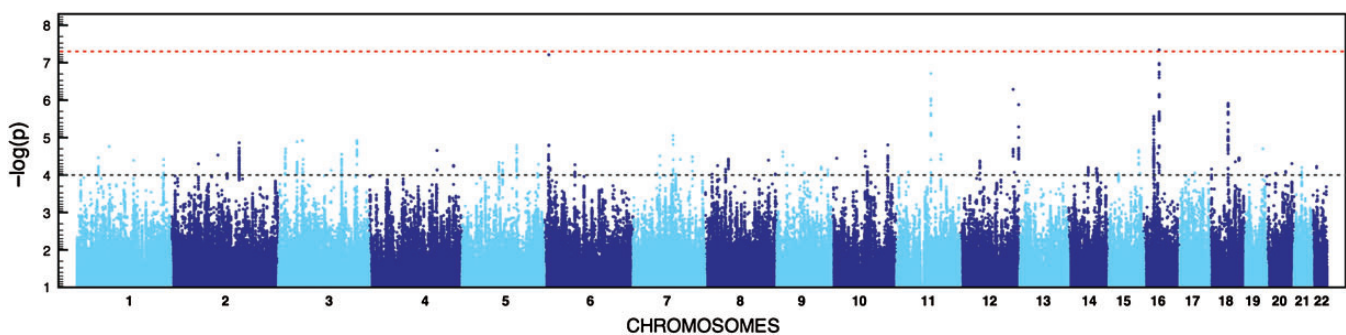


Figure 1. Manhattan plot for meta-analysis. Results based on fixed effect meta-analysis of GWAS1 and GWAS2 signals. Dashed red line shows threshold for genome-wide significance ($P < 5 \times 10^{-8}$) and dashed gray line shows cut off for the selection of SNPs for follow-up genotyping ($P < 1 \times 10^{-4}$).

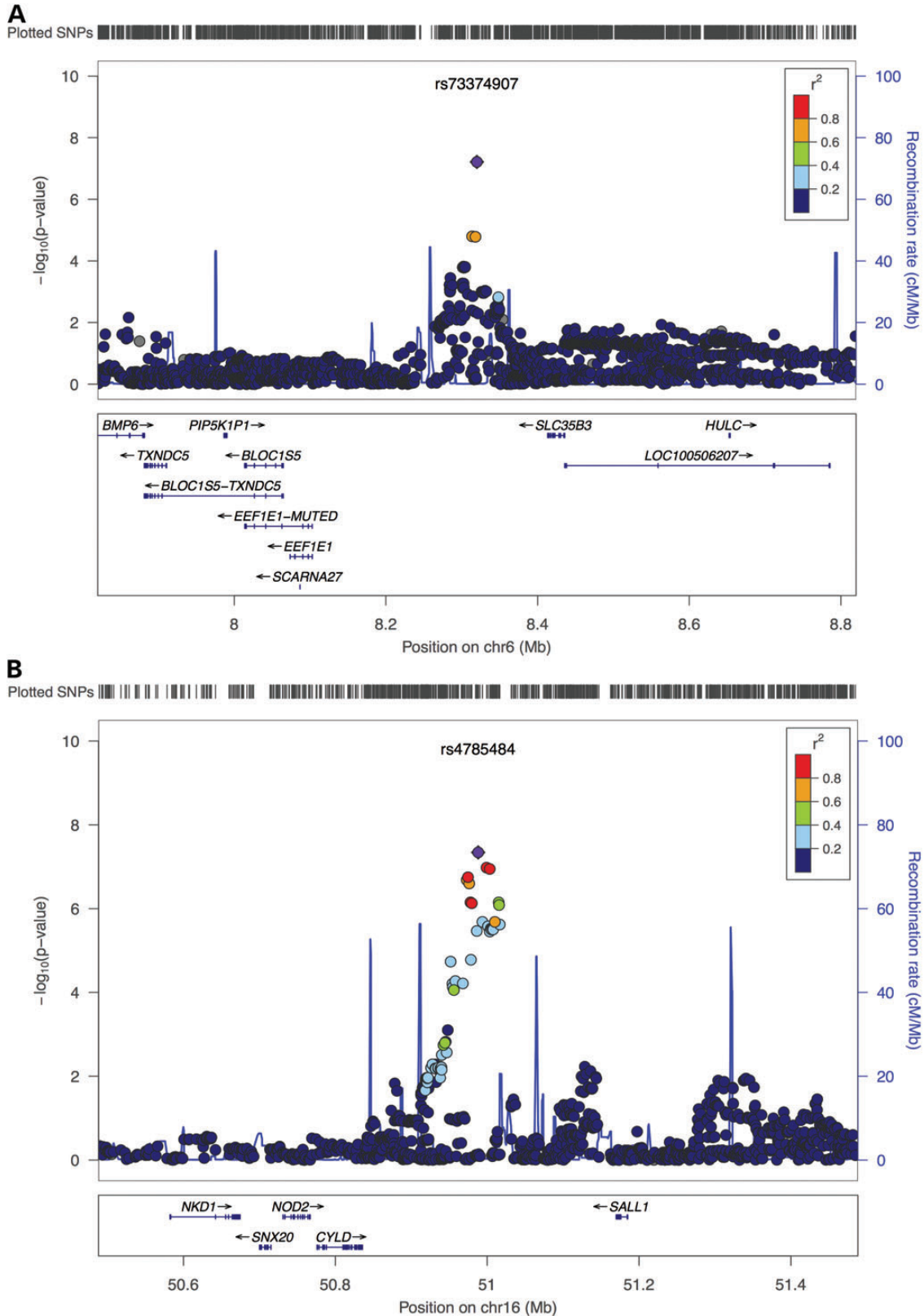


Figure 2. Regional association plots for regions on chromosome 6 (EEF1E1/SLC35B3) and chromosome 16 (CYLD/SALL1). The P -values from all imputed SNPs in the regions that passed post-imputation QC procedures are plotted against positions from the February 2009 human reference sequence, annotated by Ref Seq genes. The most associated marker (chromosome 6: rs73374907, $P_{\text{meta}} = 6.13 \times 10^{-8}$; chromosome 16: rs4785484, $P_{\text{meta}} = 2.65 \times 10^{-8}$) from the meta-analysis is indicated by a purple dot which is centered in a genomic window of ~ 1 Mb. The strength of LD (in r^2) between the top SNP and its adjacent markers is demonstrated by the red (high) to dark blue (low) color bar (top right corner). The recombination rate (second y-axis) is plotted in light blue, according to 1000 genomes project data. Plots were generated using Locuszoom (<http://csg.sph.umich.edu/locuszoom/>) (53).

Table 1. Association results for rs9890413 (WNT3/WNT9b) and rs56273700 (MECOM) at all stages of the analysis: discovery, Follow-up 1 and Follow-up 2

SNP	Sample	N		Test, <i>P</i>	OR	EA	MAF	
		Cases	Controls				Cases	Controls
rs9890413	Meta GWAS	98	526	Z-score, 8.88×10^{-5}				
	GWAS1	43	259	LOGISTIC, 0.0239	1.86	G	0.40	0.29
	GWAS2	55	267	LOGISTIC, 0.0013	2.11	G	0.45	0.30
	Follow-up 1	147	339	Z-score, 0.0390				
	Germany	94	278	LOGISTIC, 0.0995	1.32	G	0.39	0.32
	Sweden	26	61	LOGISTIC, 0.4915	1.24	G	0.40	0.34
	Spain–Italy	27	N/A	TDT, 0.2752	1.63	G	N/A	N/A
	Follow-up 2	51	N/A	TDT, 0.0095	2.31	G	N/A	N/A
rs56273700	Meta GWAS	98	526	Z-score, 5.15×10^{-5}				
	GWAS1	43	259	LOGISTIC, 0.0074	0.48	G	0.15	0.28
	GWAS2	55	267	LOGISTIC, 0.0013	0.49	G	0.15	0.29
	Follow-up 1	147	339	Z-score, 0.0317				
	Germany	94	278	LOGISTIC, 0.0171	0.62	G	0.22	0.31
	Sweden	26	61	LOGISTIC, 0.2897	0.66	G	0.23	0.31
	Spain–Italy	27	N/A	TDT, 0.4913	1.38	G	N/A	N/A
	Follow-up 2	51	N/A	TDT, 0.0423	0.52	G	N/A	N/A

N, number of cases and controls in study sample (for trio samples, the number of index patients is given in the case column); test, *P*, test statistic (LOGISTIC, logistic regression; TDT, transmission disequilibrium test) used and *P*-value for association; EA, effect allele; OR, odds ratio (based on effect allele); MAF, minor allele frequency (same as effect allele for both SNPs, no MAF given for trio samples).

cases/865 controls and 78 trios in total) comes reasonably close to this number it has to be taken into consideration that the estimated effect size is presumably inaccurate and the true effect size is likely to be smaller ('winner's curse'). The lower boundary of the 95% confidence interval (CI) for rs9890413 is 1.57 (which is still higher than the OR of the European replication sample) and results in a requirement of 866 cases and an equal number of controls to obtain genome-wide significance. Considering a recessive mode of inheritance even further increases sample size requirements (1702 cases). On top of the above outlined limitations our final replication sample, comprising a patient sample with a broader defined BEEC phenotype (including patients with CE and E), did show evidence for deviation from the expected allele frequencies in the controls (36 versus 31% in the independent German controls from discovery and replication; test for deviation from HWE: $P = 0.067$).

Similar observations with regards to the power considerations can be made for our initial genome-wide significant hit at the *SALL1* locus. Under the assumption of valid effect size estimation (OR = 2.89), a power of 80% for observing this locus with genome-wide significance (additive model, minor allele frequency 20%) is reached with ~100 cases (which is the size of our discovery sample). However, at the lower boundary of the 95% CI (OR = 2.20), an estimate that is still likely to be higher than the true effect size, a sample size of 200 cases (additive model) and 410 cases (recessive model) are required. For our replication study, we attempted replication for 75 SNPs, i.e. a $P < 6.7 \times 10^{-4}$ was required for experiment-wide significance. Hence, a sample of 150 cases (equal number of controls) would have been sufficient under the assumption of an additive model and requiring 80% power to see this experiment-wide significant at *SALL1* (369 cases for a recessive model). In sum, our datasets could have provided enough power to detect association of *SALL1* with CBE at the level of genome- or experiment-wide significant. However, it remains to be seen whether overestimation of the effect size did hinder a reasonable assessment of power and therefore could have hindered a valid interpretation of the results.

The effect size for common variation associated with CBE/BEEC seems to be not higher than those observed with other (more common) congenital disorders in human (e.g. orofacial clefts). Association of markers at 8q24.21 with non-syndromic cleft lip with or without cleft palate was found with an effect size similar to the one observed with our *SALL1* association (OR = 2.57, frequency of effect allele 20% in controls, 462 cases and 954 controls (29)). Accounting for winners curse (and taking into account effect size estimates of replication studies for 8q24.21), it is therefore reasonable to assume that future successful GWAS in CBE/BEEC will (at least) require a (combined) sample size of ~400 cases and equal number of controls (additive model, OR = 2, risk allele frequency 20%, power = 80%). Including more controls (e.g. three times the number of cases) potentially reduces the required number of cases (~250).

Although we consider our findings as suggestive evidence for association, we would like to briefly put them into context with earlier studies. We hope that insights from this discussion might help to guide future studies. A homozygous nonsense mutation in *WNT3* has been associated with tetra-amelia and urorectal malformations, which include persistent cloaca (30), and loss of *Wnt9b* causes urogenital defects (31). Our most associated SNP in this region (rs9890413) resides ~4 kb next to the *WNT3* promoter (<http://promoter.cdb.riken.jp/>), a region highly conserved among amniotes. Moreover, this transcriptional regulatory region is CpG enriched (ObsCpG/ExpCpG:0.718) and likely to be regulated by methylation status. Nakamura *et al.* identified several potential transcription factor-binding motifs to exist within the *WNT3* promoter region (32), several of which were previously found to be differentially expressed in human newborn bladder exstrophy tissue and known to be important for promotion of the embryonic urorectal septation process (33,34). Furthermore, this region has been shown to contain regulatory elements which regulate Wnt signaling via p63 (35). Thus, it is tempting to speculate that there are also regulatory domains within this intergenic region able to modulate Wnt

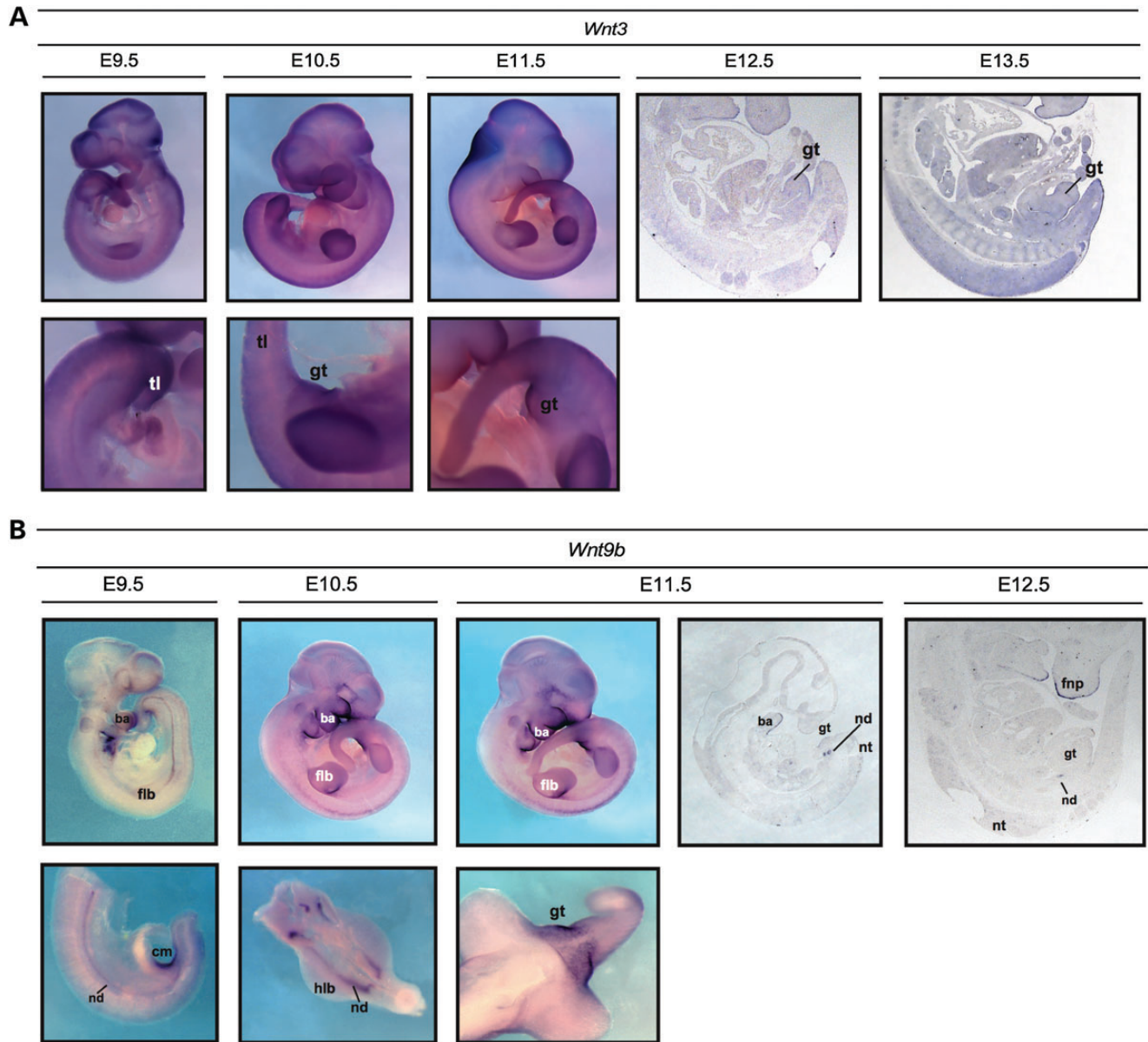


Figure 3. Analysis of *Wnt3* and *Wnt9b* expression in mid-gestational mouse embryos by *in situ* hybridization. **(A)** Expression of *Wnt3* was widely detected from E9.5 to E15.5, with stronger expression observed in the skin epithelia, limb buds and neural tissues. *Wnt3* is also present in the genital tubercle at these stages. Expression at E14.5 and E15.5 is not shown. gt, genital tubercle; tl, tail. **(B)** Expression was detectable between E9.5 and E13.5 in the epithelia of the branchial arches, frontonasal process, limb buds and genital tubercle (including the cloacal membrane between E9.5 and E10.5). Strong expression was also found in the nephric ducts. The expression pattern of E13.5 (not shown) was identical to that at E12.5. ba, branchial arch; cm, cloacal membrane; flb, forelimb bud; fnp, frontonasal process; gt, genital tubercle; hlib, hindlimb bud; nd, nephric duct; nt, neural tube; tl, tail.

signaling via a conserved WNT3-WNT9B-p63 regulatory module in the context of urorectal and urogenital development. Indeed, expression profiling of human urinary bladder exstrophy tissue showed dysregulation of both the *WNT* and *p63* pathways (34,36,37). Furthermore, the only known BEEC-associated animal knockout model to date is the $\Delta Np63^{-/-}$ mouse described by Cheng *et al.* (38), who were recently able to show association of a 12 bp deletion within the *TP63* promoter in BEEC patients of Canadian, U.S. American, Spanish origins and a 4 bp insertion in BEEC patients of Indian, Bangladeshi and Chinese origins (19).

We also found suggestive evidence for association of CBE with genetic variation at the *MECOM* gene locus. Data from the GenitoUrinary Development Molecular Anatomy Project (<http://www.gudmap.org>) (39,40) and the GenePaint.org project (41) suggest that *Mecom* is strongly expressed at E14.5 in the metanephros, the ureter, the urinary bladder and the urethra (39,40), and at E15.5 in the female reproductive system, the renal interstitium, the ureteric trunk and female urethra (41). Although E14.5–15.5 represents a developmental timeframe later than the critical period for the urorectal septation process, a modifier role for *Mecom* in the manifestation of the BEEC

cannot be excluded, as a recent study revealed zebrafish *mecom* expression in the cloacal chamber, a space proximal to the urogenital pore where the pronephric duct, intestine and the oviduct or sperm duct empties (42).

The initial genome-wide significant marker rs4785484 ($P = 4.55 \times 10^{-8}$) resides close to *SALL1*. Patients with *SALL1* mutations (Townes–Brocks syndrome, OMIM #107480) frequently present with urogenital anomalies (43) and *Sall1* mutant mice exhibit neural tube defects, which can be affiliated with the OEIS complex (44). A recent study proposed heterochromatin localization of *SALL1* as a new mechanism for the activation of Wnt signaling (45) which was also shown to be affected by *SALL1*-dependent signals in the context of ureter tip fate to initiate kidney development (46). Future studies are warranted to shed light on the potential interplay of *SALL1* and the conserved WNT3-WNT9B-p63 regulatory module in the etiology of BEEC.

MATERIALS AND METHODS

Ethics statement and subjects

Written informed consent was obtained from all subjects or their proxies, in case of minors. Demographic information was collected from both patients and controls through a structured questionnaire. This study was approved by each participating center's Institutional Ethics Committee and was conducted according to Declaration of Helsinki principles. Experienced physicians trained in the diagnosis of the BEEC personally recruited all BEEC patients included in this study. More details about the recruitment process (for discovery and follow-up samples) can be found elsewhere (20,47) and in the Supplementary Materials, Methods.

Genetic analyses (GWAS and follow-up)

For the purposes of phenotypic homogeneity, the GWAS sample consisted only of isolated, non-syndromic CBE, the most common form based on the definition described in detail elsewhere (2). Patients with additional malformations or congenital anomalies, not associated with the CBE or the BEEC, respectively, were excluded from the analysis. DNA was extracted from blood or saliva samples and genotyping of 107 isolated CBE patients of Central European ancestry was performed in two batches (51 and 56, respectively). Due to discontinuation of the genotyping array utilized for the first batch, different arrays were used for Batches 1 and 2. For case–control comparison, we also obtained genotypes of 538 (270 and 268, respectively) ethnically matched population-based controls that have been described elsewhere (48). All QC and subsequent procedures were applied to both batches separately (due to insufficient overlap in the SNP content of the utilized genotyping arrays). For both GWASs, we followed the same stringent QC protocol that has been successfully applied to other datasets (23,24). A detailed description of the protocol can be found in the Supplementary Materials, Methods. The post-QC datasets (comprising 43 cases and 259 controls for GWAS1 and 55 cases and 267 controls for GWAS2, respectively) were subjected to imputation based on 1000 Genomes Project and HapMap 3 reference panels (25,26). After applying postimputation QC, single

marker analyses were conducted for 4 510 680 markers each using a logistic regression (additive model) as implemented into SNPTEST (27). The subsequent meta-analysis of both datasets was performed using a fixed-effects model as implemented into YAMAS (28). A detailed overview on the analytical pipeline is provided in Supplementary Material, Figure S2. Results from our meta-analysis (for all 4 510 680 markers) are publicly available and can be downloaded from <http://www.sharing.biostats.info> after registration with the website. Follow-up on the most promising genomic regions was performed in a first independent sample (Follow-up 1) comprising three samples of European ancestry from Germany (94 cases and 278 controls post-QC), Sweden (26 cases and 61 controls post-QC), Spain and Italy (a total of 27 trios post-QC). All analyses for the downstream steps were performed using PLINK (49). In order to be selected for downstream analyses, SNPs were required to show $P < 0.0001$ in the meta-analysis of the two GWAS samples and $P < 0.05$ in the individual GWASs (with same effect allele and direction of effect; see Supplementary Material, Table S1). A total of 75 SNPs were successfully genotyped and (meta) analyzed after pruning for linkage disequilibrium in the initially obtained SNP list. Based on the results from our first follow-up sample, a second independent sample (Follow-up 2) was analyzed for four markers that showed evidence for association (see Supplementary Material, Table S2 for results of all 75 markers). The second follow-up sample comprised of 49 trios of European ancestry from North America with an isolated non-syndromic CBE patient as index (post-QC number). Finally, we attempted to replicate the most associated marker in the discovery and follow-up samples in a final sample of European ancestry. Due to a limited number of samples available for genetic studies on CBE, and in order to study the potential impact of rs9890413 on a broader defined phenotype of BEEC (including also patients with CE and E), we decided to study a sample comprising 78 samples (32 with CBE, 19 with CE and 27 with E; along with 336 controls from a cohort of ethnically matched blood donors).

Mouse expression data

Mouse embryos were fixed overnight in 4% paraformaldehyde (PFA)/PBS and processed for *in situ* hybridization as described elsewhere (50). For hybridization on sections (E12.5–E15.5), embryos were processed into paraffin wax and sections (5 μ m) were made using a microtome. Antisense RNA probes were transcribed from PCR products generated either from our in-house MAMEP collection (*Wnt9b*) (51) or as previously described for *Wnt3* (52), which was kindly made available by Andy McMahon. Riboprobes were synthesized using the appropriate RNA polymerases and a nucleotide mix containing digoxigenin-11-UTP (Roche Diagnostics, Mannheim, Germany) and were purified using G-50 sephadex columns (GE-Healthcare, Solingen, Germany). Following probe hybridization and washes, an anti-DIG antibody conjugated to alkaline phosphatase (AP) (Roche Diagnostics, Mannheim, Germany) was incubated with embryos overnight at 4°C, and detection of AP activity was carried out using BM Purple (Roche Diagnostics, Mannheim, Germany). For each probe, embryos were processed concurrently and staining reaction times were maintained between embryos in order to limit variations in signal intensity. For whole mounts, at

least three embryos were examined for each gene and stage. For slides, three sections from at least two different embryos were analyzed for each stage shown and figures depict representative staining. Images were captured using AxioVision software (Zeiss, Jena, Germany) with a Zeiss AxioCam and SteREO Discovery.V12 microscope.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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