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A massive alteration of gene expression in undescended testicles of dogs and the association of *KAT6A* variants with cryptorchidism

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Cryptorchidism is the most common form of disorder of sex development in male dogs, but its hereditary predisposition is poorly elucidated. The gonadal transcriptome of nine unilaterally cryptorchid dogs and seven control dogs was analyzed using RNA-seq. Comparison between the scrotal and inguinal gonads of unilateral cryptorchid dogs revealed 8,028 differentially expressed genes (DEGs) (3,377 up-regulated and 4,651 down-regulated). A similar number of DEGs (7,619) was found by comparing the undescended testicles with the descended testicles of the control dogs. The methylation status of the selected DEGs was also analyzed, with three out of nine studied DEGs showing altered patterns. Bioinformatic analysis of the cDNA sequences revealed 20,366 SNP variants, six of which showed significant differences in allelic counts between cryptorchid and control dogs. Validation studies in larger cohorts of cryptorchid (n = 122) and control (n = 173) dogs showed that the TT genotype (rs850666472, p.Ala1230Val) and the AA genotype in 3′UTR (16:23716202G>A) in *KAT6A*, responsible for acetylation of lysine 9 in histone H3, are associated with cryptorchidism (P = 0.0383). Both the transcript level of *KAT6A* and H3K9 acetylation were lower in undescended testes, and additionally, the acetylation depended on the genotypes in exon 17 and the 3′UTR. Our study showed that the massive alteration of the transcriptome in undescended testicles is not caused by germinal DNA variants in DEG regulatory sequences but is partly associated with an aberrant DNA methylation and H3K9 acetylation patterns. Moreover, variants of *KAT6A* can be considered markers associated with the risk of this disorder.

cryptorchidism | DNA methylation | histone H3 acetylation | RNA-seq | transcriptome

Significance

Cryptorchidism, which has a polygenic nature, is frequently diagnosed in mammals, including humans and dogs. The dog is a valuable large animal model in studies of human genetic disorders, so elucidation of the molecular mechanism responsible for canine cryptorchidism has a broader significance. It is known that thousands of genes are expressed in mammalian testes, and we show that gene expression in the inguinal testicles of unilateral cryptorchid dogs is massively altered (approximately 8,000 genes), which can be partly explained by aberrant DNA methylation and histone H3 acetylation patterns. Moreover, two cosegregating DNA variants of the *KAT6A* gene, encoding the enzyme responsible for lysine 9 acetylation of histone H3, were associated with cryptorchidism and decreased levels of histone acetylation.

The descent of testicles proceeds in two gonad migration phases, transabdominal and inguinoscrotal. These two phases are mainly controlled by the INSL3 hormone and its receptor RXFP2 (in the first phase) as well as by testosterone and its receptor AR (in the second phase). The failure of testicular descent (cryptorchidism) is generally considered nonsyndromic (an isolated condition), but there are also cases with comorbid disorders, such as hypospadias (1).

Cryptorchidism is a complex disorder of sex development (DSD) caused by an unknown number of gene variants and nongenetic factors altering testicular descent to the scrotum (2). Its prevalence in dogs varies from 1 to 11% (3, 4), and it is most common in small breeds (5, 6). Cryptorchidism is also a major form of DSD in humans, with an incidence in newborn boys that varies between 1.6% and 9% (7). Since the majority of canine genetic diseases and disorders have human counterparts, the dog can serve as a valuable large animal model in biomedical studies (8).

There has to date been only a single report on the heritability (h^2) of canine cryptorchidism, which showed a rather low value of 0.23 for this coefficient (9). Recently, a large-scale genome-wide association study (GWAS) revealed the presence of an SNP in the region of *HMGA2* associated with cryptorchidism (10). Another risk variant has been identified in the promoter of *RXFP2* (11).

Retained testicles face an unusual environment (e.g., elevated temperature) that does not affect scrotal gonads. This may alter gene expression, as has been revealed by RNA-seq analysis of gonads collected from dogs with experimentally induced cryptorchidism (12). There are also reports showing the altered expression of *INSL3, RXFP2*, and *CYP17A1* in the undescended testes of cryptorchid dogs (11, 12).

We hypothesized that DNA variants and aberrant DNA methylation in regulatory sequences of genes involved in testicular descent are responsible for their altered expression and an increased risk of cryptorchidism. The aim of our study was to perform a comparative analysis of the transcriptome in the descended and undescended testicles of dogs with congenital nonsyndromic unilateral cryptorchidism and in the scrotal gonads of

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control males. We additionally sought for the associated DNA variants in the transcripts isolated from the gonads of the affected dogs.

Results

Global Transcript Level Analysis. We studied the transcriptome from three groups: undescended (UD) and descended (D) testes from postpubertal dogs diagnosed with nonsyndromic unilateral inguinal cryptorchidism, and the right scrotal testes (C) from healthy control males. The experimental design was verified using principal component analysis (PCA). The samples were clearly grouped into two clusters based on the location of testes: scrotal (D and C samples) and inguinal (UD), while the breed had no effect on the grouping of the samples (Fig. 1).

Altogether, the expression of 16,676 genes was analyzed. Differentially expressed genes (DEGs) must concurrently fulfill two criteria: FDR < 0.05 and |log2FC| > 1.5. The analysis of DEGs indicated a massively altered transcription profile in the UD testes (Dataset S1). In the UD vs. D comparison, a total of 8,028 genes showed a disturbed transcript level (with 3,377 up-regulated and 4,651 down-regulated in the UD gonads). At the same time, in the UD vs. C comparison the number of DEGs was 7,619 (with 3,099 up-regulated and 4,520 down-regulated in UD gonads). Of these, 7,395 DEGs were common in both comparisons (4,410 down-regulated and 2,985 up-regulated; *SI Appendix*, Fig. S1). The ten DEGs, common in both comparisons (UD vs. D and UD vs. C), with the most significant FDR between-group values, are shown in Fig. 2; all 10 were down-regulated in the undescended gonads. There were no transcripts designated as DEGs when the scrotal gonads of the cryptorchid group (D) and testes from the control dogs (C) were compared (Dataset S1). The number of genes whose expression did not differ significantly (FDR > 0.05 or |log FC| < 1.5) was 8,731 for UD vs. C and 8,369 for UD vs. D comparisons (*SI Appendix*, Table S1). The DEGs also included known candidate genes for human or canine cryptorchidism (*INSL3, RXFP2,* and *AR*), as well as *KAT6A*, which is a candidate described in this study (*SI Appendix*, Table S2).

Functional Enrichment Pathway Analysis. We tested the enrichment of GO terms with DEGs fulfilling the conditions: FDR < 0.05 and |log2FC| > 1.5. An enrichment test based on the hypergeometric distribution was applied. Only those processes with at least 10 genes involved in the process were considered. The significance threshold of each GO, based on the returned corresponding Bonferroni-corrected *P*-values, was set at the 0.01 level. In the UD vs. D comparison, 148 significant processes were identified in down-regulated DEGs, whereas 392 GO terms were found when the analysis was restricted to up-regulated DEGs only (Dataset S2). In the UD vs. C comparison, there were 352 significant GO terms (Dataset S2) triggered by up-regulated DEGs only and 142 significant GO terms were found for down-regulated genes. The most significant GO terms for each comparison (UD vs. D and UD vs. C) are shown in *SI Appendix*, Figs. S2 and S3.

Validation of RNA-seq Data. Based on RNA-seq data analysis, we selected eleven genes classified into three categories for validation in larger samples: 1) out of the 30 genes with the most significant DEGs of known function in the regulation of spermatogenesis and reproduction, which were also common for UD vs. D and UD vs. C comparisons (*SPIRE2*, *TSSK4*, *FAM221B,* and *PIH1D2*); 2) genes involved in development of seminoma (*TP53*, *KLF4,* and *KITLG);* and 3) candidate genes for cryptorchidism based on studies in humans and other mammals (*SERPINH1, ACTN3, AMH,* and *COL2A1*).

For all the studied genes, we used real-time PCR to confirm the changes in mRNA levels between UD and scrotal testes (D and C), with no significant differences in the comparison of D vs. C. The relative mRNA level of all the validated genes from the list of the 30 top DEGs was strongly decreased in UD testes. The *TP53* and *KITLG* genes were up-regulated in contrast to *KLF4*, which was down-regulated in the retained gonads. Among the candidate genes for canine cryptorchidism, the relative transcript levels of *SERPINH1* and *AMH* were higher in retained gonads, but *ACTN3* and *COL2A1* had lower expressions in the undescended testes (Fig. 3 and *SI Appendix*, Table S3).

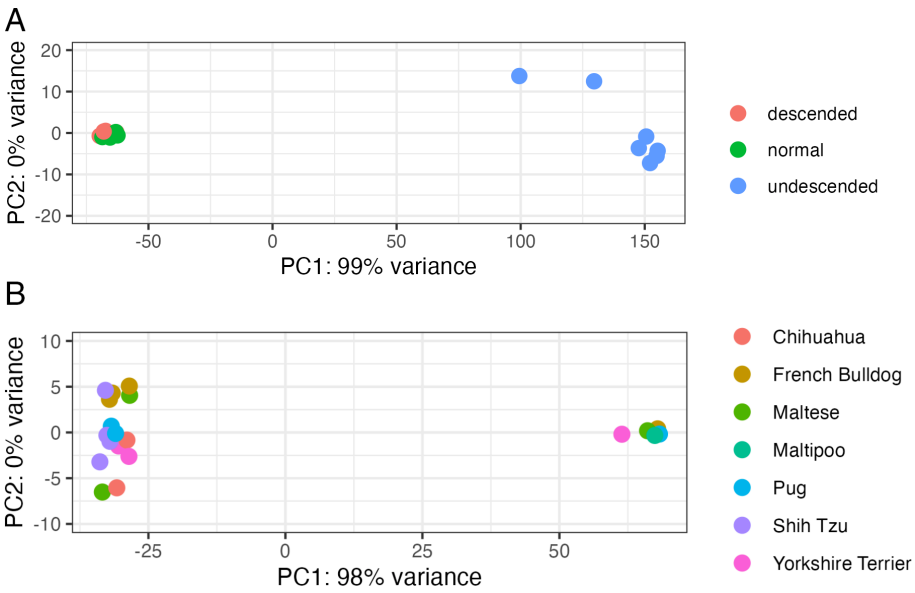

Fig. 1. PCA plots: (*A*) different colors represent samples belonging to the following groups: D testes (red), contralateral UD testes of cryptorchids (blue), and normal testes (C) of control dogs (green); (*B*) different colors represent samples belonging to the following breeds: French Bulldog, Chihuahua, Maltese, Maltipoo, Pug, Shih Tzu, and Yorkshire Terrier.

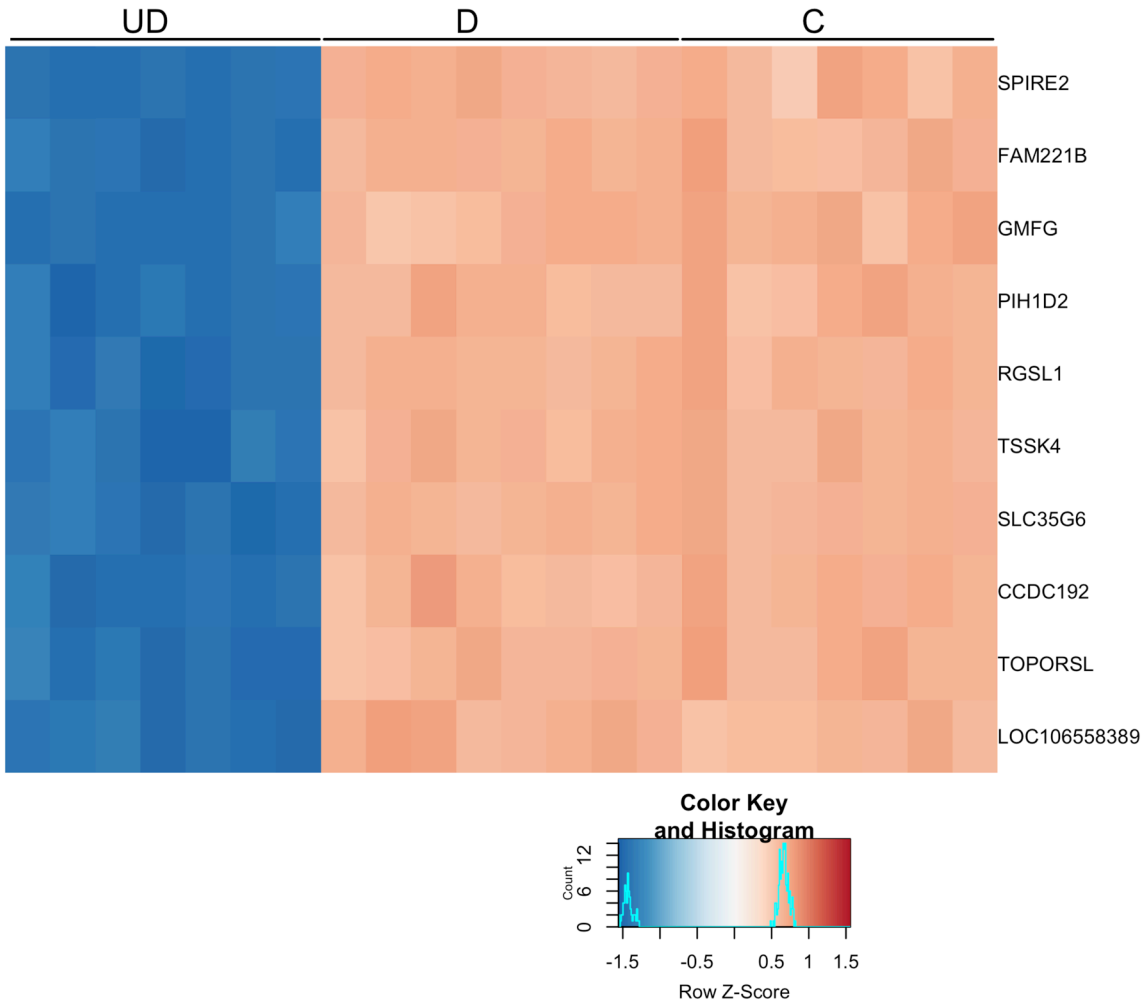

Fig. 2. Top 10 differentially expressed genes commonly identified by comparing: undescended (UD) vs. descended (D) testes of cryptorchids and undescended testes of cryptorchids (UD) vs. scrotal testes of control dogs (C).

We also investigated whether the *KAT6A* gene, which showed significant differences in RNA-seq data (*SI Appendix*, Table S2), is differently expressed depending on the genotypes in exon 17 (rs850666472) and the 3′UTR (16:23716202G>A). To this end, we selected animals with opposite homozygote genotypes (CC/GG vs. TT/AA in exon 17/3′UTR, respectively) and verified the transcript level by real-time PCR. We found that the transcript level was decreased in the undescended testes (regardless of genotypes), whereas there were no differences between undescended testes in animals with CC/GG and TT/AA genotypes, indicating that the genotypes had no effects on the transcript level of the *KAT6A* gene (Fig. 4*A* and *SI Appendix*, Table S3).

DNA Methylation. Out of 11 DEGs validated by real-time PCR, the CpG methylation level was determined for nine genes (*SPIRE2, FAM221B*, *PIH1D2*, *TP53*, *KLF4*, *KITLG*, *SERPINH1*, *ACTN3*, and *AMH*) that possessed CpG islands. Two genes could not be studied due to the lack of CpG (*TSKK4*) or because of unsuccessful amplification (*COL2A1*). The number of cytosines analyzed in the CpG context varied from two (*KITLG*) to nine (*ACTN3*), and the tested CpGs were located in the 5′-flanking or 5′-untranslated regions (5′UTR) with the exception of *AMH*, in which the CpGs were located in the coding sequence in exon 3 (*SI Appendix*, Table S4). Significant differences were found for the three studied genes in UD vs. D and UD vs. C comparisons.

The most interesting results were obtained for the *KLF4* gene, in which all eight analyzed cytosines showed significantly increased methylation levels in UD testes (Fig. 5). In silico analysis of the 5′-flanking region of this gene revealed that two cytosines (CpG7 and CpG8) were located within the consensus sequence for E2F-1 transcriptional factor (*SI Appendix*, Fig. S4), which acts as a transcriptional regulator of the *KLF4* gene. The hypermethylation of the 5′-flanking region was associated with a decreased transcript level of *KLF4* (Fig. 3).

In *KITLG*, another gene involved in carcinogenesis, two cytosines located in the 5'-flanking region had abnormal methylation levels in the retained gonads (*SI Appendix*, Fig. S5), though this effect was uncommon. Hypermethylation in the promoter was associated with elevated *KITLG* mRNA levels (Fig. 3).

CpG methylation analysis of the *AMH* gene included eight cytosines located in the coding sequence. The elevated methylation level of all the tested cytosines in the UD testes (*SI Appendix*, Fig. S5) was found to be associated with increased transcript levels of this gene (Fig. 3).

The methylation levels of CpGs located in the 5′-regulatory regions of *SPIRE2*, *FAM221B*, *PIH1D2*, *TP53*, *SERPINH1*, and *ACTN3* were low (median value < 15%) and there were no significant changes depending on the location of gonads (*SI Appendix*, Fig. S6).

There was no difference in CpG methylation levels between the descended testes of cryptorchid dogs and the control testes in our

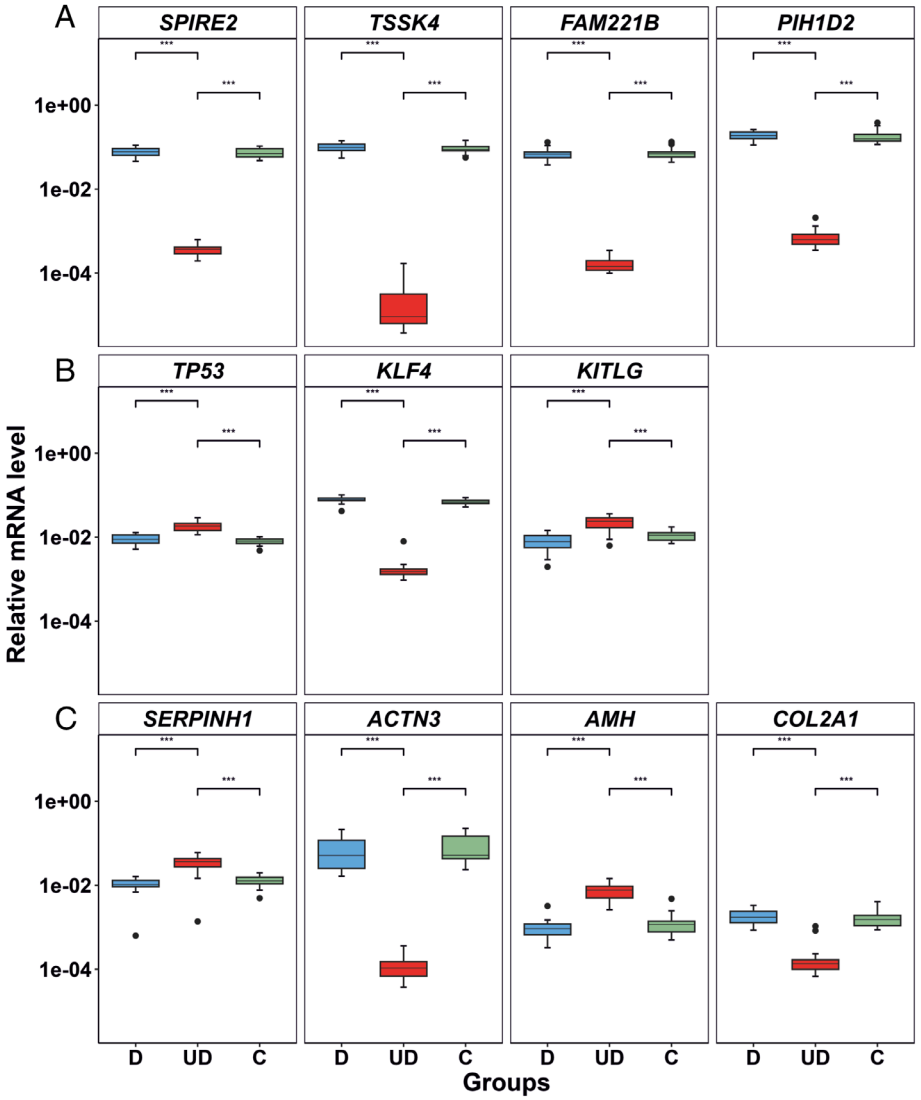

Fig. 3. Relative mRNA expression in UD, D, and C testes for 11 genes: (*A*) selected from the list of the 30 top DEGs; (*B*) involved in seminoma development; and (*C*) known candidate genes for cryptorchidism. ***P_{adj} < 0.001.

study. We were thus unable to indicate any epigenetic marker for canine cryptorchidism in terms of DNA methylation.

We previously found significant hypomethylation of a single CpG site in *INSL3* (15 bp upstream of the translation start codon) in both the UD and D gonads of unilateral cryptorchids, as compared to the gonads of normal dogs (11). In the present study, we tested whether this hypomethylation is also present in easily accessible tissue (blood cells) collected from the cryptorchid and control dogs. It was found that methylation at this CpG site was similar in both cohorts (23.4% and 23.7%, respectively). This analysis shows that the studied CpG in the *INSL3* gene is not a universal epimarker detectable in blood cells.

Protein Level. Western blot analysis in gonadal tissue was performed for four proteins (AMH; SCF, encoded by *KITLG*; TP53; and KLF4). The encoded protein level was not detectable in the case of the other genes studied by real-time PCR due to the low specificity of the antibodies to canine proteins. Two isoforms were detected for AMH and SCF (*KITLG*); the larger AMH isoform (70 kDa) showed decreased levels in the UD testes as compared to the D and C gonads ($P < 0.01$). The shorter AMH isoform (59 kDa) did not display any significant difference between the study groups, but when results of both isoforms were analyzed together the reduction in the UD group was also significant ($P < 0.05$) (*SI Appendix*, Table S6). Similar results were obtained for SCF: the shorter isoform (35 kDa) and joint analysis of both isoforms revealed significantly lower levels in UD gonads (*SI Appendix*, Table S6). The TP53 protein level was significantly increased in the UD testes (*SI Appendix*, Fig. S7 and Table S6), while the KLF4 protein level was not altered.

Global Acetylation of Lysine 9 in Histone 3 (H3K9ac). Since the *KAT6A* gene encodes a lysine acetyltransferase 6A which is part of the histone acetyltransferases protein complex, we determined whether the animals (unilateral cryptorchids vs. controls) with different genotypes within exon 17 and 3′UTR of the *KAT6A* gene (CC/GG and TT/AA in exon 17/3′UTR, respectively) differed in terms of global H3K9ac. We found a reduction in global H3K9ac in undescended testes as compared with descended and control testes, but this effect was more significant in cryptorchid dogs (77.5%) with TT/AA genotypes than in control animals with CC/GG genotypes (100%) (Fig. 4*B*).

Screening for Variants Associated with Cryptorchidism. Analysis of RNA-seq data facilitated identification of 20,366 SNPs annotated in the transcribed regions of the dog genome.

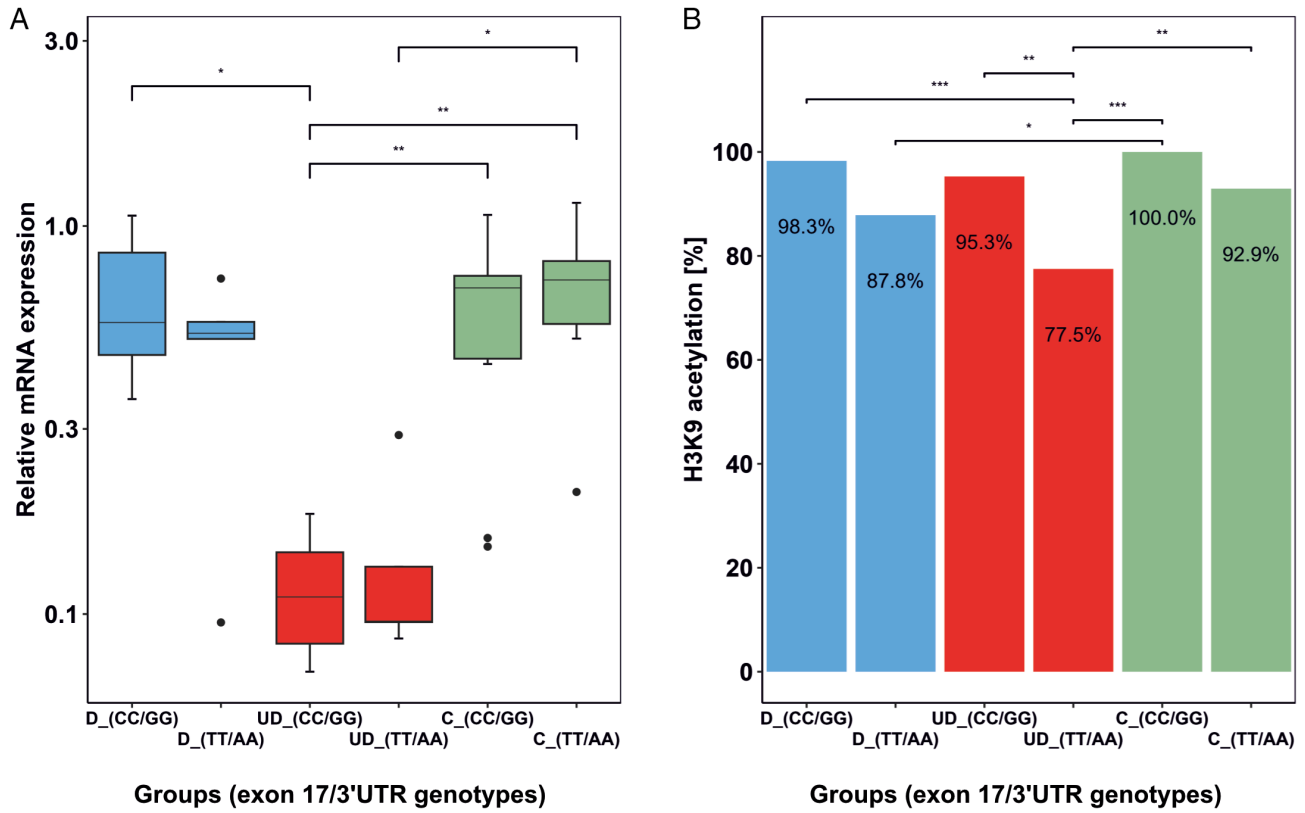

Fig. 4. Relative mRNA expression for *KAT6A* gene (*A*); and global H3K9ac in UD, D, and C testes (*B*)—depending on genotypes: CC/GG and TT/AA in exon 17 and the 3′UTR of *KAT6A*, respectively. *P_{adj} < 0.05; **P_{adj} < 0.01; ***P_{adj} < 0.001.

These included a large number (13,187) of synonymous and 364 nonsynonymous variants, as well as 805 and 6,010 nucleotide substitutions located respectively in the 5′- and 3′-untranslated regulatory regions (Dataset S3).

Comparison of the allele counts between the small groups of cryptorchid and control dogs used for the RNA-seq study revealed six missense variants with significant differences (P < 0.05) in their frequencies (Dataset S3). Of these, three SNPs (rs22661209, rs851133825, and rs851361715) were annotated in transcripts that are not available in the current gene set (ENSCAFT00000046132, ENSCAFT00000046132, and ENSCAFT00000048896, respectively). Further analysis was therefore performed only for three significant SNPs: rs23171989 (c.112G>C, p.Ala38Pro) in *TP53RK* (P < 0.0238), rs850666472 (c.3689C>T, p.Ala1230Val) in *KAT6A* (P < 0.0266), rs23375230 (c.5635G>A, p.Val1879Met) in *WNK1* (P < 0.0279). An additional variant located in the 3′ untranslated region (3′UTR) of the *KAT6A* gene (16:23716202G>A) was also studied. Of these, *TP53RK* and *KAT6A* were designated as DEGs and both were down-regulated in the undescended gonads (Dataset S1 and *SI Appendix*, Table S2). The distribution of these four variants was studied in larger multibreed cohorts of 122 cryptorchids and 173 control dogs. Variants of two SNPs in exon 17 (C > T) and in the 3′UTR (G > A) in *KAT6A* co-segregated perfectly (C-G and T-A). When allele counts were compared between these cohorts, none of the genes showed significant results, but the distribution of variants in *KATA6* was close to significance (*SI Appendix*, Table S7). A recessive model of inheritance was tested for these variants. The odds-ratio test for cryptorchids vs. controls was performed by comparing a combined cohort of wild-type homozygotes and heterozygotes (CC + CT for exon 17 and GG + GA for 3′UTR) vs. alternative homozygotes (TT for exon 17 and AA for 3′UTR). The analysis showed an association of cryptorchidism (P = 0.0383) with TT and AA genotypes in exon 17 and the 3′UTR, respectively. A similar analysis for the genotypes in the *WNK1* and *TP53RK* genes did not confirm any association (Table 1).

The *KAT6A* variants were evaluated in silico. Using the SNAP2 tool, we found that substitution (A > V) is not considered a deleterious mutation; this substitution involves nonpolar amino acids, the only difference being an additional methyl group (CH_3) in valine. Next, protein structure prediction using the PredictProtein tool revealed slight differences in the composition of secondary structures as a result of this variation. The variation occurs in a region of the protein that is natively unstructured, indicating flexibility and an absence of stable structures. The structural modeling conducted with the Swiss-Model tool confirmed that the canine KAT6A protein is an intrinsically disordered protein and that A > V substitution is localized within this flexible region. Moreover, since the second SNP was identified in the 3′UTR of the *KAT6A* gene (16:23716202G>A), we found that the position of this SNP could potentially serve as a target for microRNAs, e.g., cfa-miR-135a-3p and cfa-miR-486; however, the G > A variant was located outside the seed sequences for both microRNAs. To verify the stability of the microRNA's binding to the 3′UTR of the *KAT6A* gene, we calculated the minimal free energy (MFE), finding that the binding of cfa-miR-135a with this fragment of the 3'UTR is more stable for the G allele (wild type), with an MFE of −24.4 kcal/mol, than for the A allele (alternative variant), with −19.0 kcal/mol. No differences for MFE were observed in the case of cfa-miR-486.

In addition, based on the GWAS results reported by Blades et al. (10), we considered *HMGA2* a candidate gene for canine cryptorchidism. Our analysis revealed that this gene was not differentially expressed in the UD and D or C gonads (Dataset S1).

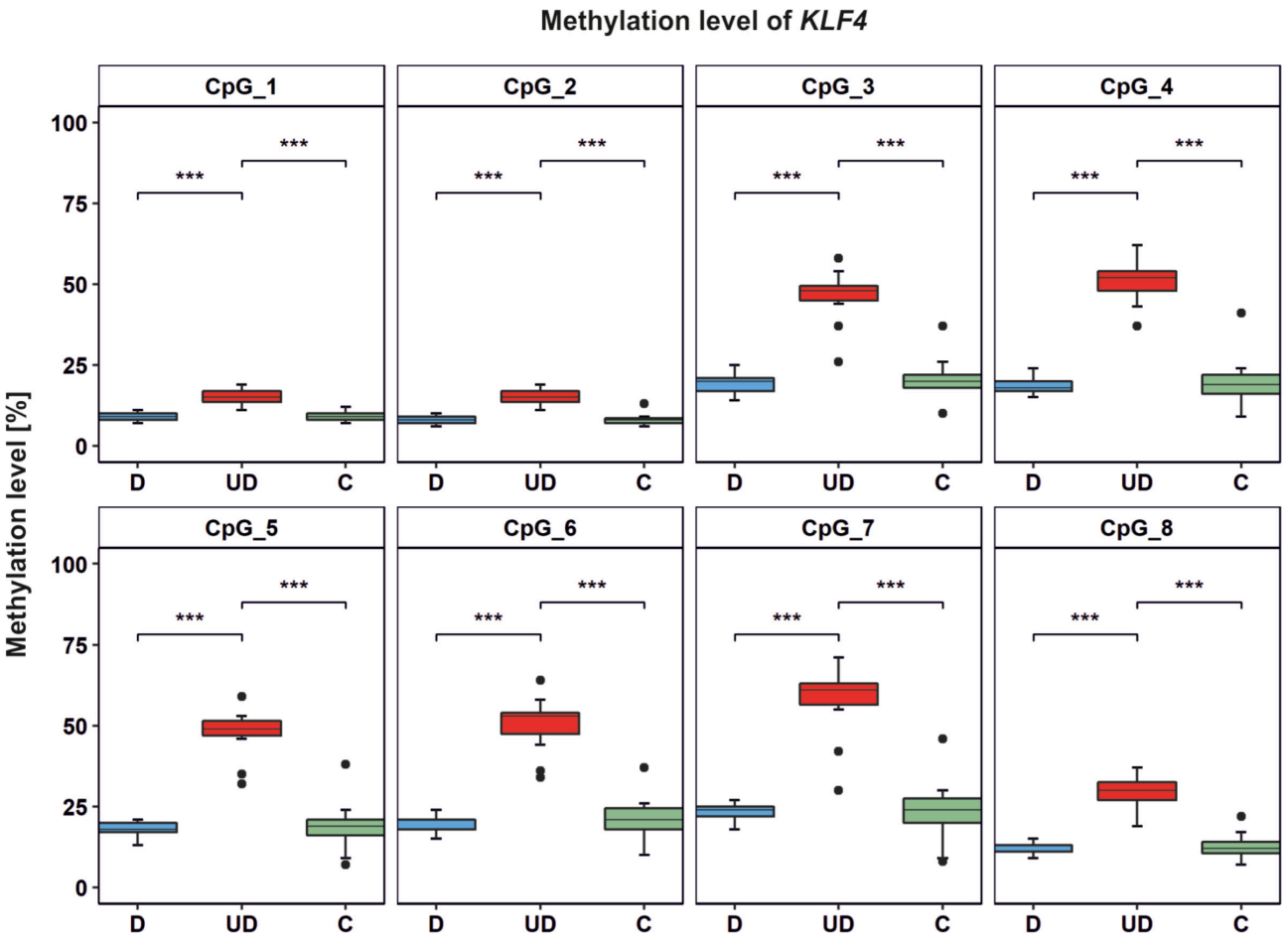

Fig. 5. Methylation level of eight CpG sites in the 5′-flanking region of *KLF4*. ***$P_{adj} < 0.001$.

The sequencing of the coding sequence and exon/intron junctions revealed only eight intronic variants, but none of these were associated with cryptorchidism (*SI Appendix*, Table S8).

Discussion

Cryptorchidism is responsible for sterility in the case of bilateral cryptorchidism or decreased fertility in unilateral cryptorchidism, as well as an increased risk of gonadal cancers. Thus, the molecular elucidation of hereditary predispositions to this disorder is an important task for human and veterinary medicine. It is assumed that human cryptorchidism is moderately controlled by genes (13), and a slightly lower estimate was reported for dogs (9). The environment during fetal development is thus considered a crucial risk factor.

We showed that the transcriptome of inguinal testicles was massively altered in comparison to contralateral descended gonads. However, the transcriptome of the descended testicles of the unilateral cryptorchid dogs and of the scrotal testicles of the control dogs did not differ. This observation strongly indicated that altered gene expression in undescended gonads is not governed by germinal DNA variants in the regulatory sequences. This is in agreement with the extensively affected transcriptome profile in testicles, which were experimentally relocalized to the abdomen cavity for a short time (30 d). Comparative analysis of the RNA-seq, performed for the relocalized and scrotal testicles, revealed 4,448 DEGs, with 2,102 up-regulated and 2,346 down-regulated (12). In our study, which was performed on the gonads of dogs with congenital cryptorchidism, the number of the DEGs was much higher (8,028 DEGs) and included genes showing associations with human cryptorchidism or carcinogenesis, as well as genes not yet associated with this disorder. Among the DEGs, there were also genes whose altered expression has been reported in undescended testes—namely, *INSL3*, *RXFP2*, *CYP17A1*, and *CYP19* (11, 14). On the other hand, no change was seen in the expression of *HMGA2,* which is located near an SNP previously associated with cryptorchidism (10).

The identified DEGs enriched the GO terms; the 10 top terms in both comparisons are shown in *SI Appendix*, Figs. S2 and S3. When the GO terms common to both comparisons (UD vs. D and UD vs. C) were examined, we found that 331 were up-regulated while 135 were down-regulated. The up-regulated GO terms included two involved in hormone metabolic processes (58 DEGs) and in response to hormones (140 DEGs). Of the genes involved in these terms, some were responsible for testicular steroidogenesis (e.g., *CYP17A1*, *HSD17B1*, *CYP11A1*, and *HSD3B2*). Moreover, the *NR5A1* encoding SF-1 transcription factor, which plays a crucial role in the sex determination process and in the activation of numerous genes of the cytochrome P450 family was also found in these GO terms. The proteins in P450 family are involved in the synthesis of cholesterol and steroids. Our previous studies have shown that the altered expression of such genes is associated with canine cryptorchidism (14). Our present study confirms that aberrant expression of genes involved in hormonal balance may play a role in the pathogenesis of cryptorchidism. Moreover, the *AR* gene—which encodes the androgen receptor responsible for the transduction of the dihydrotestosterone signal to its target cells—was also involved in the GO term for hormonal processes. The up-regulated GO terms also

Table 1. Association analysis between genotypes for SNP variants in *KAT6A*, *WNK1*, and *TP53RK* and cryptorchidism

Gene	Number of genotypes		OR, *P*-value and 95% CI
KAT6A (C > T, rs850666472), p.Ala1230Val	CC + CT	TT	OR = 1.80
Cryptorchids (*n* = 122)	43 + 43	36	*P* = 0.0383
Controls (*n* = 173)	70 + 70	33	95% CI: 1.03 to 3.06
KAT6A (16:23716202G>A), 3′UTR	GG + GA	AA	OR = 1.78
Cryptorchids (*n* = 122)	43 + 43	36	*P* = 0.0383
Controls (*n* = 173)	70 + 70	33	95% CI: 1.03 to 3.06
WNK1 (C > T, rs23375230), p.Val1879Met	CC + CT	TT	OR = 1.66
Cryptorchids (*n* = 122)	99 + 15	8	*P* = 0.3381
Controls (*n* = 173)	140 + 26	7	95% CI: 0.59 to 4.72
TP53RK (G > C, rs23171989), p.Ala38Pro	GG + CG	CC	OR = 1.42
Cryptorchids (*n* = 122)	16 + 26	80	*P* = 0.1489
Controls (*n* = 173)	28 + 46	99	95% CI: 0.88 to 2.30

included processes referred to as reproductive structure or system development with 95 DEGs, including *NR5A1*, *MAMLD1*, *NR0B1*, *WT1,* or *GATA4*, whose mutations cause disorders of sex development (15). Moreover, the down-regulated GO terms also included general reproduction pathways (>300 DEGs), as well as gamete generation (184 DEGs), spermatogenesis (151 DEGs), and spermatid differentiation and development (76 DEGs). The DEGs associated with sexual reproduction pathways included the *RXFP2* gene, which together with *INSL3* plays a crucial role in the first stage of testicular descent (11), and *SRD5A*, which catalyzes the conversion of testosterone to dihydrotestosterone. There were also GO terms related to meiotic cleavage as well as sperm structure and motility.

Western blot analysis could be performed for four proteins only, since the availability of canine-specific antibodies is limited. When compared with the transcript levels the results of this analysis were ambiguous. The elevated protein level of TP53 reflected its higher transcript level in undescended testes. For AMH and KITLG, which possess two isoforms, the opposite result was observed—transcript level was elevated and total protein level was decreased in the undescended testes—while the level of the KLF4 protein did not differ between samples. Elevated serum levels of the AMH protein have been reported in dogs diagnosed with bilateral abdominal cryptorchidism compared to normal dogs (16). Our study did not confirm this finding in testicular tissue. It should be mentioned that the serum level of AMH is decreased (17, 18) or unchanged (19) in cryptorchid boys compared with a control cohort. This may suggest that other regulatory mechanisms (e.g., RNA interference) are involved in posttranscriptional expression control of the *AMH*, *KITLG*, and *KLF4* genes.

Since the transcriptome of the scrotal testes of the unilateral cryptorchid dogs did not differ from that of the scrotal gonads of the control dogs, we analyzed the methylation pattern of selected DEGs. It is known that elevated methylation of CpG islands near the transcription start site (TSS) is usually associated with decreased transcript level, while methylation in coding sequences can be associated with elevated gene expression (20). Of the nine analyzed DEGs, altered methylation was observed in three (*AMH, KLF4,* and *KITLG*), confirming that inguinal localization of the gonads affects methylation of CpG sites of some genes, as has already been shown for *INSL3*, *RXFP2, CYP17A1*, and *CYP19A1* in dogs with unilateral cryptorchidism (11, 14).

Cryptorchidism is a significant risk factor for gonadal carcinogenesis in humans (21) and dogs (22). Our study has found that, in undescended gonads, some oncogenes and suppressor genes (*KLF4* and *KITLG*) that were recognized as DEGs had unusual methylation patterns in the inguinal gonads. This suggests a possible role of this mechanism in gonadal carcinogenesis.

Seeking DNA variants associated with cryptorchidism in the gonadal transcriptomes of the studied dogs revealed an association of a missense SNP (rs850666472) and an SNP located in the 3′UTR of the *KAT6A* gene encoding the acidic domain of lysine acetyltransferase 6A protein. This gene belongs to the family of histone acetyltransferases. The encoded protein plays a role in epigenetic control of chromatin organization and in the expression of genes involved in various developmental processes. It catalyzes acetylation of lysine 9 of histone H3, as well as of non-histone proteins involved in other processes, including cellular proliferation, signal transduction, protein folding, and autophagy. There are over eighty known cases of *KAT6A* syndrome in humans, caused by pathogenic mutations of this gene (23–25). This syndrome, presently referred to as Arboleda-Tham syndrome (ARTHS; OMIM, 616268), is manifested by intellectual disability, speech delay, microcephaly, neonatal hypotonia, etc. There have also been reports of five patients with cryptorchidism as a co-existing disorder. It is known that *KATA6A* controls expression of some homeotic genes (*HOX*) (26) and that knock-out of two of them (*HOXA10* and *HOXA11*) causes cryptorchidism in mice (27). The above suggests that *KAT6A* variants can contribute to altered descent of testes.

We also found that, besides the association of *KAT6A* variants with the risk of cryptorchidism, an association with global levels of lysine 9 acetylation in histone H3 was observed. The reduced acetylation in the gonads of the TT/AA dogs may be a result of a reduced level of the *KAT6A* transcript, due to the genotype at the SNP in the 3′UTR or due to reduced functionality of the encoded enzyme as a result of a missense SNP in exon 17 of this gene. However, the transcript level was reduced independently from the genotypes in exon 17 and 3′UTR and the in silico analysis did not indicate deleterious effects of these variants in terms of amino acid properties change or microRNA binding ability. On the other hand, it is worth to notice that KAT6A protein functions as a histone acetyltransferase (HAT), forming a larger complex with BRPF1/2/3, ING5, and EAF6 proteins (28). It is documented that KAT6A syndrome is frequently triggered by nonsense mutations affecting the C-terminal unstructured region. Importantly, these interactions may play a significant role in modulating KAT6A enzymatic activity (29) and regulation of recruitment of KAT6A to the target genes (28). In our study, we identified a substitution in the canine *KAT6A* gene (p.A1230V) within this unstructured region; thus, we assume that this alteration may influence the ability

of KAT6A interaction with other HAT proteins and in consequence reduce global acetylation levels in animals with TT/AA genotypes. Further investigations are warranted to elucidate the precise mechanisms underlying this relationship.

There are several candidate genes, including *INSL3, RXFP2,* and *AR*, associated with human cryptorchidism (7, 30). Studies of this type are less advanced in dogs, though recent reports showed an association of SNPs located near the *RXFP2* (11) and *HMGA2* (10) genes. In our study, we observed altered expression of *RXFP2*, but not of *HMGA2.* To reveal the possible function of *HMGA2,* we sequenced its exons, but did not find missense variants. We thus suggest that the potential functional DNA variant responsible for the increased risk of cryptorchidism is not located in the coding sequence of the *HMGA2* gene. On the other hand, variants of genes encoding proteins that control testicular descent (*INSL3* and *AR*) or enzymes involved in steroid hormones' synthesis (*CYP17A1* and *CYP19*) did not show this association (14, 31).

In conclusion, we demonstrated a large number of DEGs in the undescended testicles of unilateral cryptorchid dogs, while such differences were not observed when the transcriptomes of the scrotal testes of the unilateral cryptorchid and control dogs were compared. This allows us to suggest that the altered transcript levels in inguinal gonads are due to their abnormal localization, associated with exposure to elevated temperature, but not due to germinal DNA variants in regulatory sequences of DEGs affecting their transcript level. On the other hand, analysis of the testicular transcript sequences revealed an association of SNP variants in *KAT6A* with decreased acetylation level of lysine 9 in histone H3 and cryptorchidism. Summarizing, our study shows that an epigenetic mechanism, namely the reduced global level of H3K9ac and the altered DNA methylation of the studied genes, plays a significant role in the pathogenesis of cryptorchidism.

Materials and Methods

Animals. UD and D testes from postpubertal dogs diagnosed with nonsyndromic (lacking other health issues) unilateral inguinal cryptorchidism, and right scrotal testes (C) from healthy control males, were collected during routine castration, immediately frozen in liquid nitrogen, and stored at −80 °C until further analysis.

Peripheral blood samples from cryptorchid and control dogs were collected during a standard clinical examination. All animals included in the study were tested for the presence of the chromosome Y-linked *SRY* gene (32). Altogether nine panels of dogs (Dataset S4 and *SI Appendix*, Table S4) were used.

Blood and tissue samples were collected at veterinary clinics located in the west of Poland, in line with standard veterinary protocols and with the owners' consent, after approval of the local Bioethical Commission for Animal Care and Use in Poznan, Poland (Approval No. 1/2019).

Methods.

RNA isolation and RNA sequencing. Total RNA from testicular tissue was isolated using an RNeasy Mini Kit (Qiagen). The concentration of RNA was measured with the Qubit RNA BR Assay (Invitrogen) on a Qubit 2.0 fluorometer (Invitrogen), and its purity was analyzed using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The RNA integrity number (RIN) values were checked on a 2100 Bioanalyzer instrument (Agilent) using the RNA 6000 Nano kit (Agilent). For each sample, 100 ng of total RNA was used for library preparation with the TruSeq Stranded mRNA kit (Illumina). RNA sequencing (RNA-seq) was performed on a NovaSeq 6000 (Illumina) platform using 2 × 100 bp read length by CeGaT GmbH. High-throughput sequencing data were deposited in the NCBI Sequence Read Archive (SRA) database, accession number PRJNA901164.

Bioinformatic analysis. The quality of the sequencing data was first checked with FastQC (33). Adapters were trimmed using a custom program prepared with Awk software (34). Reads were mapped using STAR (35) to reference dog genome (CanFam3.1, Ensembl database) (36) and counted using the Rsubread (37) package. Low-expression genes were discarded, retaining those with CPM > 0.5 in more than two samples. TMM normalization and differential expression analysis used edgeR package (38) with adjusted *P*-value (FDR) < 0.05 and $|\log_2 FC| > 1.5$ criteria. The functional enrichment analysis employed the systemPipeR (39) and clusterProfiler (40) packages.

To perform PCA, the count data were transformed to a log2 scale to minimize differences between samples for rows with small counts and to normalize for the library size. This transformation was useful in checking for outliers and for input to machine learning techniques such as clustering or linear discriminant analysis. PCA was used to visualize the similarity between samples based on gene expression, as it can help to identify patterns and clusters in the data.

The variant analysis focused on SNPs. NGS reads were aligned using BWA (41), and GATK (42) performed the variant calling. Samtools software (43) was used to mark duplicates. Variant reports and annotations utilized VariantAnnotation (44) and snpStats (45) packages. All SNPs with missing data, low quality, or low frequency were discarded from further analysis. The ensembl variant effect predictor (VEP) software (46) provided SNP function information. Fisher's exact test assessed differences between SNP groups with a significance level of 0.05 for the FDR-adjusted *P*-value.

All visualizations were prepared using the ggplot2 package (47). All data preprocessing was performed with the tidyverse ecosystem of R packages (48), and the statistical analysis was carried out using R software (49).

In silico analysis included 1) predicting the functional effects of the identified amino acid substitution using the SNAP2 tool (https://bio.tools/snap2); 2) predicting the structure of the KAT6A protein for the wild type amino acid sequence and the p.A1230V variant, using the Swiss-Model (https://swissmodel.expasy.org/) and PredictProtein (https://predictprotein.org/) tools; 3) predicting microRNA binding sites within the 3'UTR of the *KAT6A* sequence overlapping the G>A variant (16:23716202G>A) using miRDB https://mirdb.org/custom.html and miRBase (https://mirbase.org/); 4) estimating the stability of microRNA binding using the RNAHybrid tool (https://bio.tools/rnahybrid); and 5) searching for the transcription factor binding sites in the DNA sequence that were predicted with the PROMO tool (https://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3).

cDNA synthesis and real-time PCR. Real-time PCR was used to validate the RNA-seq results for twelve genes. First, 1,000 ng of RNA was reversely transcribed (Transcriptor First Strand cDNA Synthesis Kit, Roche). Real-time PCR primers (*SI Appendix*, Table S6) were designed (Primer3 tool; https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) using the CanFam3.1 dog reference sequence. The PCRs were performed in triplicate on a Light Cycler 480 II (Roche) system with the use of the Light Cycler 480 SYBR Green I Master Kit (Roche). The relative mRNA levels of the tested genes were quantified using the second derivative maximum method (Roche), and the results were normalized to the geometric mean of the expression of *ACTB* and *GAPDH* reference genes. The transcript levels were then compared between the tested groups using a nonparametric Kruskal–Wallis and a post-hoc Dunn's test with Bonferroni correction in R software 4.2.1 (Package stats and FSA, respectively).

Analysis of DNA methylation by pyrosequencing. Genomic DNA was isolated from the testes using a Genomic Mini Kit (A&A Biotechnology). Then 500 ng of DNA was bisulfite-converted using an EZ DNA Methylation Gold Kit (Zymo Research). The PyroMark Assay Design 2.0 software (Qiagen) was used for assay design (*SI Appendix*, Table S2) and the PCRs were performed using the PyroMark PCR Kit (Qiagen). The PyroMark CpG Kit (Qiagen) was used for the pyrosequencing reactions conducted on PyroMark Q48 system (Qiagen). The CpG methylation level (%) was compared between studied groups using a nonparametric Kruskal–Wallis test and a post-hoc Dunn's test with the Bonferroni correction, using R software 4.2.1 (Package stats and FSA, respectively).

Western blot. Total proteins were isolated from testicular tissue using RIPA lysis buffer (Sigma-Aldrich) and quantified with a Protein Assay Kit (Thermo Fischer Scientific) on a Qubit fluorometer (Invitrogen). The analysis was performed for four selected proteins, AMH (Abcam; rabbit antibody ab229212, dilution 1:1,000), SCF/KITLG (Abcam; rabbit antibody ab64677, dilution 1:1,000), TP53 (Abcam; mouse antibody ab26, dilution 1:1,000), and KLF4 (GeneTex; rabbit antibody GTX101508, dilution 1:500). A reference protein, GAPDH (Abcam; mouse antibody ab8245, dilution 1:5,000), and HRP-conjugated secondary antibodies (Abcam, anti-rabbit ab97051 and anti-mouse ab6728, dilution 1:5,000) were also included. The protein samples (25 μg for AMH, SCF/KITLG, and TP53 and 75 μg for KLF4) were separated on Bolt 12% Bis-Tris Plus gel by electrophoresis

(120 V, 180 min.) and subsequently transferred to a nitrocellulose membrane (Life Technologies). The membranes were then blocked at room temperature for 60 min using 5% nonfat dried milk in TBST solution. Immunodetection was performed as previously described (14), with the exception that Restore Western Blot Stripping Buffer (Life Technologies) and an additional membrane-blocking step were used prior to incubation with the anti-GAPDH antibody.

Global acetylation of lysine 9 in histone H3 (H3K9ac) by colorimetric assay. The histone proteins were isolated using the Histone Extraction Kit (Abcam) from frozen testes that came from unilateral cryptorchids and control animals (30 samples in total; 5 samples per group). The procedure was performed following the manufacturer's instructions and with quantification using a Qubit fluorometer with the Qubit Protein Assay Kit (Life Technologies). The total histone H3 lysine 9 acetylation level was measured by colorimetric reaction using the Histone H3 (acetyl K9) Quantification Kit (Abcam). Briefly, 150 ng of histone proteins (each sample in duplicate) was applied to eight-well strips coated with a specific anti-acetyl H3K9 antibody. The standard curve was also prepared using a 1:1 dilution series (from 100 ng to 1.562 ng and a negative control). The samples were incubated in wells with antibody buffer for 1.5 h at room temperature. Next, the wells were washed three times with 1× wash buffer. Afterward, the wells were incubated with detection antibody in dilution 1:1,000 for 1 h at room temperature, following six washes with 1× wash buffer. The color development reagent was then added for 5 min following the stop buffer addition. Within the next 5 min, the intensity of absorbance was read at a wavelength of 450 nm on a microplate reader (Tecan). This test was performed for animals differing in terms of their genotypes in exon 17 and 3′UTR of the *KAT6A* gene: for the CC/GG genotypes: control testes (C, $n = 5$), for the descended (D, $n = 5$) and undescended (UD, $n = 5$) samples from cryptorchid dogs, as well as from animals with TT/AA genotypes: control testes (C, $n = 5$), descended (D, $n = 5$), and undescended (UD, $n = 5$) from cryptorchid dogs. The results are shown as percentage of acetylation in the study groups in relation to the control animals with CC/GG genotypes (as a 100%). Parametric ANOVA was performed to determine whether there are any differences between the groups in terms of average acetylation levels. Assumptions as to the normality and homogeneity of the data were checked using the Shapiro–Wilk test and Bartlett's test, respectively. The ANOVA gave significant differences between groups (P value < 0.05). The post-hoc Tukey test was used to gain more insight into comparisons between specific groups. The corrected p-values for Tukey's test after adjustment for multiple comparisons are shown.

Variant detection by Sanger sequencing. Three candidate missense SNPs, rs850666472 in *KAT6A*, rs23171989 in *TP53RK*, and rs23375230 in *WNK1*, selected after variant calling analysis of the RNA-seq data, were genotyped by Sanger sequencing in larger cohorts of cryptorchid and control dogs (Dataset S4). We additionally screened for potentially functional DNA variants in the *HMGA2* gene, recently reported as associated with the risk of cryptorchidism in dogs (10). Moreover, we also determined whether there is any other SNP variant outside the coding sequence; we found that a variant G > A in the 3′UTR region is present in the cohort. Genomic DNA was isolated from peripheral blood using the MasterPure DNA Purification Kit for Blood (Epicentre). The PCR primers were designed to cover all tested SNPs in *KAT6A*, *TP53RK*, and *WNK1* and to flank all coding exons of *HMGA2* (*SI Appendix*, Table S6). Prior to cycle sequencing, the amplicons were purified with alkaline phosphatase (Thermo Fisher Scientific) and exonuclease I (Thermo Fisher Scientific). The sequencing reactions were performed using a BigDye Terminator v3.1 kit (Thermo Fisher Scientific). After filtration on a Sephadex g50 (Sigma Aldrich), the DNA fragments were separated and detected using a 3500 Genetic Analyzer (Applied Biosystems).

Allele and genotype counts were compared between cohorts using the odds-ratio test. Two inheritance models were tested for the cosegregation of SNP genotypes (missense SNP and SNP in the 3′UTR), following the approach of the study on a candidate variant for canine cryptorchidism in the *HMGA2* gene (10). The first approach assumed the dominance of the polymorphic variants (TT/AA) over the wild variants (CC/GG), while the second model assumed that the polymorphic variants (TT/AA) are recessive.

Data, Materials, and Software Availability. The RNA-sequencing data were deposited in the NCBI SRA database, accession number PRJNA901164 (50). The identified SNP variant within 3′UTR (16:23716202G>A) is deposited in European Nucleotide Archive Database, accession number PRJEB70515 (51).

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