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GENETIC CHARACTERIZATION OF CONGENITAL DEFECTS IN DOGS: CAUDAL DYSPLASIA, ECTODERMAL DYSPLASIA AND MUCOPOLYSACCHARIDOSIS VII

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ACADEMIC DISSERTATION

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Abstract

Since the sequencing of the *Canis lupus familiaris* genome the dog has become a powerful tool for scientists. Selective breeding has created more than 400 different breeds each representing genetic isolates with breed-specific morphological and behavioral characteristics. Unique population history, available genealogical records, veterinary diagnostics and novel genomic tools greatly facilitate gene mapping studies in dogs. Given that over 600 genetic disorders have been described in dogs and that most of them are similar to human conditions, dogs have emerged as a clinically relevant model for human inherited disorders.

This study explores the genetics of three different inherited developmental defects in dogs, caudal dysplasia, ectodermal dysplasia, and mucopolysaccharidosis VII, which all have counterparts in human. In this study, various clinical and pathological techniques have been used to characterize the phenotypes, and genetic methods such as genome-wide association studies and next-generation sequencing to resolve the genetics of the diseases. Moreover, functional studies in mice have been performed to explore the molecular role during embryonic development. The discoveries made here have established the affected breeds as models to further explore disease mechanisms and therapeutic methods, identified new disease pathways, and offered novel approaches for further developmental studies. Furthermore, this work has enabled the development of genetic tests for breeding purposes.

Three different phenotypes have been investigated in this study. First, we studied genetics of caudal dysplasia, which in its mildest form is presenting as short-tail phenotype in dogs. A mutation in *T* (brachyury homolog) was earlier identified to cause this phenotype in Pembroke Welsh Corgis. Homozygous mutations of *T* in mouse result in severe caudal dysplasia and embryonic lethality suggesting an essential role for the *T* gene during mammalian development. The presence of the documented *T* mutation, c.189C>G, was investigated in 23 other breeds demonstrating that short-tailed dogs from 17 breeds were heterozygous for the mutation that associated completely with the phenotype. The homozygous mutation was suggested to be lethal, as no dogs homozygous for the mutation were found and an approximately 30% decrease was seen in the size of Swedish Vallhund litters when both parents were short-tailed. However, short-tailed dogs were found from six breeds that did not carry the known substitution or any other mutations in the *T* coding regions and therefore other genetic factors are yet to be discovered that affect the development of the posterior mesodermal region. The short-tailed dogs which do not have *T* mutation will serve as models in future studies to identify possible novel genetic factors for caudal dysplasia and related medical conditions.

Second, a new gene was identified for a hairless phenotype and some of its upstream regulators were characterized. Hairless dog breeds show a breed characteristic which is in clinical terms an ectodermal dysplasia. In this study, the causative mutation for canine ectodermal dysplasia (CED) was sought and

subsequently the function of this novel gene in the ectodermal organ development was explored. Accordingly, a genome-wide association study was performed and CED was mapped to dog chromosome 17. Haplotype association testing revealed a 160-kb haplotype, which was fine-mapped using three different breeds. The causative genetic mutation for CED was identified as a 7-bp duplication producing a frameshift and premature stop codon in a previously uncharacterized canine gene forkhead box protein I3 (*FOXI3*). The study provided a novel gene focus to aid research into ectodermal development. Therefore, a detailed expression pattern of murine *Foxi3* during the development of the ectodermal organs was constructed and a series of tissue culture experiments and expression analyses with mouse embryos were performed to assess the function of Foxi3 in mammalian embryogenesis. The results suggest that Foxi3 regulates hair follicle and tooth formation as well as the development of mammary and salivary gland, nail, and eye. Ectodysplasin and activin A were identified as upstream regulators of *Foxi3*.

Third, Brazilian Terriers with severe skeletal defects at early puppyhood were identified through information provided by breeders. Subsequently, a major aim of this work was to describe the clinical and pathological features of the syndrome and to identify its genetic cause. Clinicopathological examinations and pedigree analysis demonstrated that the affected puppies had a recessive spondyloepihyseal dysplasia. The disease locus was mapped to chromosome 6 and a mutation leading to pathogenic p.P289L change in a conserved functional domain of β-glucuronidase (GUSB) was identified. Elevated glycosaminoglycans were detected in urine and only a residual β-glucuronidase activity was observed in the serum of the affected dogs, which confirmed the pathogenity of the mutation. GUSB defects result in mucopolysaccharidosis VII (MPS VII) in several species and thus the mutation defined the syndrome as MPS VII in Brazilian Terriers.

Overall, this study illustrates how unique morphological diversity and enriched genetic alterations in closed populations can be efficiently harnessed to gain new insights into developmental biology across species. For example, the identification of the CED mutation in *FOXI3* revealed a completely novel gene with a previously unknown essential function in ectodermal development. This work has established several novel large animal models to further explore disease mechanisms and to develop therapeutic methods. Moreover, several new DNA tests have been developed for different breeds of dogs to eradicate or, to control better, the conditions through improved breeding plans. This will improve the welfare of our beloved pets.

Contents

List of original publications

This thesis is based on the following publications:

I **Hytönen MK***, Grall A*, Hédan B, Dréano S, Seguin SJ, Delattre D, Thomas A, Galibert F, Paulin L, Lohi H, Sainio K and André C (2009). Ancestral T-box mutation is present in many, but not all, short-tailed dog breeds. Journal of Heredity 100(2):236-240.

II Drögemüller C, Karlsson EK, **Hytönen MK**, Perloski M, Dolf G, Sainio K, Lohi H, Lindblad-Toh K and Leeb T (2008). A mutation in hairless dogs implicates FOXI3 in ectodermal development. Science 12;321(5895):1462.

III Shirokova V*, Jussila M*, **Hytönen MK*,** Perälä N, Drögemüller C, Leeb T, Lohi H, Sainio K, Thesleff I and Mikkola ML (2013). Expression of *Foxi3* is regulated by ectodysplasin in skin appendage placodes. Developmental Dynamics 242(6):593-603.

IV **Hytönen MK**, Arumilli M, Lappalainen AK, Kallio H, Snellman M, Sainio K and Lohi H (2012). A novel *GUSB* mutation in Brazilian Terriers with severe skeletal abnormalities defines the disease as mucopolysaccharidosis VII. PLoS One 7(7):e40281.

*Equal contribution

The publications are referred to in the text by their roman numerals. Some unpublished data are also presented.

Abbreviations

BAC	bacterial artificial chromosome
CED	canine ectodermal dysplasia
CFA	*Canis lupus familiaris* (used as a prefix of canine chromosome)
CNV	copy number variant
CT	computed tomography
GAG	glycosaminoglycan
GUSB	β-glucuronidase
GWAS	genome-wide association study
E	embryonic day
EDA	ectodysplasin
EDAR	ectodysplasin A receptor
EDARADD	EDAR-associated death domain
ERT	enzyme replacement therapy
FOX	forkhead box
HED	hypohidrotic ectodermal dysplasia
HSCT	hematopoietic stem cell transplantation
HSPG	heaparan sulfate proteoglycan
ISH	*in situ* hybridization
LD	linkage disequilibrium
LOD	logarithm of odds
MPS	mucopolysaccharidosis
mtDNA	mitochondrial DNA
NGS	next generation sequencing
OMIA	Online Mendelian Inheritance in Animals
OMIM	Online Mendelian Inheritance in Man
PKP1	plakophilin-1
SNP	single nucleotide polymorphism
T	brachyury homolog (mouse)
UTR	untranslated region
VCP	variant calling pipeline
XHED	X-linked hypohidrotic ectodermal dysplasia

1 Review of the literature

Dog, *Canis lupus familiaris* is considered to be the first domesticated animal. It is commonly believed that domestication predates the beginning of agriculture, but the more accurate estimate of time and place remains controversial. Based on the fossil evidence and phylogenetic findings, canine domestication has been dated to at least 15,000 years ago, and was followed by the domestication of sheep, goat, cow, pig, and others thereafter. Since domestication, dogs have been selectively bred to form the numerous breeds existing today. Modern genetic technology has made it possible to explore the evolution and origin of the dog more accurately than using only archeological remains. Well supported evidence suggests that all dog breeds originate from the Eurasian gray wolf (*Canis lupus lupus*) and the latest studies indicate South-East Asia to be the initial domestication site, although the results have been somewhat controversial when considering the exact geographical location and the timing of the process. An initial study using canine mitochondrial DNA (mtDNA) that dated the domestication to more than 100,000 years ago [1] has been since challenged and more recent mtDNA analyses place the domestication process to approximately 16,000 years ago or less [2-4]. On the other hand, some recent studies based on archeological and some genetic evidence claim that dogs were present already more than 30,000 years ago [5-7]. It is obvious to hypothesize, regarding whether domestication occurred once or multiple times, that some crossbreeding might have happened during dog evolution. Indeed, this is true at least for some North Scandinavian/Finnish spitz breeds for which mtDNA studies indicate that a backcrossing of wolf to dog has happened a few hundreds or thousands years ago [8].

The domestic dog population is clearly ancient, although diverse breeds were established quite recently since the majority of the breeds are less than 200 years old. At the moment, over 400 breeds exist, each defined by specific physical and behavioral characteristics, which make the dog the most phenotypically diverse domesticated species [9]. Originally different types of dogs arose due to selection for behavioral traits for working purposes like hunting or herding and guarding the flock. However, the phenotypic variation seen in modern dogs today is mainly a result of recent strong artificial selection driven by dog breeders admiring distinctive features. The establishment of registering bodies (*e.g.* kennel clubs), together with breeding standards, has led to a controlled and restricted breeding. Many breeds have arisen from a limited number of founder animals and the use of popular sires has been common. As a consequence, each breed represents an isolated breeding population with high levels of phenotypic homogeneity and, importantly, reduced genetic diversity.

The breed creation process also has had unfavorable consequences producing high rates of specific diseases within breeds due to enrichment of the disease causing or predisposing alleles. This has happened, first of all, due to random fixation of risk alleles during the bottlenecks and use of popular sires harboring the disadvantageous

alleles. In addition, some destructive variants have been hitch-hiking along with the selected traits and some undesirable traits are even due to pleiotropic effects of selected variants, like the dominant mutation causing the dorsal hair ridge in Rhodesian and Thai Ridgeback dogs but also predisposing to dermoid sinus [10].

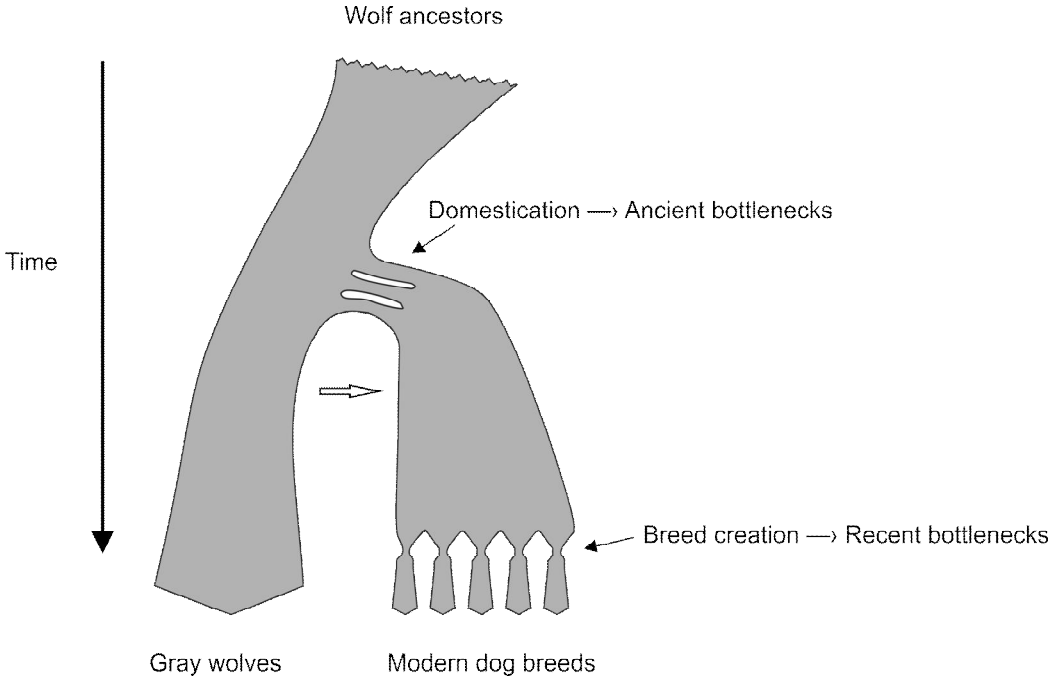


Figure 1. Creation of modern breeds through domestication (ancient bottlenecks) and breed creation (recent bottlenecks). Modified from [11].

1.1 Genetic discoveries in dogs benefit human medicine

Dogs have become an important genetic model organism for numerous heritable human diseases, as well as morphological and behavioral phenotypes. A large number of successful gene discoveries have confirmed that gene mapping can be powerfully performed in dogs. A definite advantage is gained from the canine population history which has resulted in the creation of hundreds of breeds, each representing an isolated population with breed-specific morphological and behavioral characteristics and limited locus and disease heterogeneity [11,12]. On the contrary, the genetic heterogeneity is high across different breeds. Dogs possess a unique genetic architecture, consisting of short-range linkage disequilibrium (LD) and short haplotype blocks resulting from ancient population bottleneck caused by the domestication process, in addition to long-range LD and long haplotype blocks arising from recent breed creation (Fig. 1) [11,13]. Moreover, artificial selection has outweighed the forces of natural selection and as a consequence some parts of the genome have undergone a relaxation of selective constraint [14]. Therefore, as dogs also have a remarkable phenotypic variation and excess of inherited diseases, they offer a unique opportunity for disease gene mapping. In addition, the dog genome is

less divergent from the human than the mouse genome and, therefore, more human genomic sequence can be syntenically aligned to the genome of dog than to mouse [11]. Dogs have approximately the same number of genes as humans, most of them being close orthologues.

But the canine genetic system offers other advantages as well. Given that most of the over 600 genetic disorders that have been described in dogs, have similarities to human disease, dogs have emerged as a clinically relevant model of human inherited disorders [15]. Dogs serve as a large animal model being both physiologically and clinically more similar to human than the mouse. Importantly, dogs used for genetic studies are pets that are living in similar environmental conditions along with humans and are thus affected not only by genetic traits but also "life style". This makes the dog far superior model for human disease than the laboratory mouse that lives mostly in pathogen-free and controlled conditions where the outcome or the severity of the genetic traits may be stabilized compared to corresponding dog or human conditions. A wealth of spontaneously occurring common diseases in dogs are analogous to human diseases such as diabetes, cancers, epilepsies, numerous eye diseases and autoimmune diseases not to mention high numbers of rare monogenic diseases. Heritable diseases, other single locus traits and identified mutations that have been demonstrated in dogs are recorded in a public database Online Mendelian Inheritance in Animals (OMIA) (http://omia.angis.org.au) [15], which is similar to human database Online Mendelian Inheritance in Man (OMIM) (http://www.omim.org) [16]. There are 240 mendelian traits or disorders recorded at OMIA database and for 165 of those traits the causative mutation has been identified. Numbers will be increasing as more and more novel traits and mutations are continuously being characterized. Mapping of these disease loci has proven that most of the genetic defects underlying the canine diseases are orthologues of the corresponding human conditions [17].

Moreover, there is well recorded genealogical data available. Purebred dogs have long been registered by kennel clubs or other equivalent organizations that record the pedigrees, and in some breeds the ancestors can be traced back more than a hundred years. The Finnish Kennel Club maintains an open access database of the pedigree dogs registrated in Finland. This Breeding Information Database (http://jalostus.kennelliitto.fi) contains useful information about each registered Finnish dog, *e.g.* pedigrees and results of health examinations. This can be taken advantage of when estimating the mode of inheritance of the phenotype or the relationships of the dogs studied. The dogs also have good-quality veterinary medical care available. Professional veterinary examinations are essential to achieve reliable and detailed diagnosis to help with proper phenotyping. In addition to the utility in genetics, the dogs may provide useful models for the development and validation of novel therapies for diseases. This has been verified in dogs with X-linked ectodermal dysplasia mutation of ectodysplasin (Eda); a single neonatal treatment with recombinant EDA prevents the respiratory disease in dogs [18,19]. At the moment, clinical trials with human patients having the similar X-linked mutation and ectodermal dysplasia are ongoing.

1.1.1 Genomic resources and methods for trait mapping in dogs

Interest in canine genetics has boomed after the sequencing and annotation of its genome. The first draft of dog genome was published in 2003 but containing only 1.5x whole-genome sequence of a Standard Poodle [20]. This was followed by a high quality version (7.5x) of a Boxer genome, CanFam1.0, and soon after an updated assembly CanFam2.0 [11]. These genomes revealed millions of single nucleotide polymorphisms (SNPs) that have been utilized in gene mapping studies thereafter. The newest genome build, CanFam3.1, was released in 2012. The dog genome, divided in 39 chromosomes, consists of approximately 2.4 Gb of nucleotide sequence and approximately 19,900 coding genes [11].

Traditional genome-wide association studies (GWAS), using unrelated cases and controls from the same population, have been extremely successful in identifying monogenic diseases in dogs. The first genome-wide SNP genotyping arrays from Affymetrix and Illumina contained less than 30,000 SNPs and were followed by the generation of higher density arrays, 50K Affymetrix array and the present high-density 172K Illumina array. From these, the 22K Illumina array and 50K Affymetrix array were used in this study.

Many of the phenotypic traits in dogs are fixed breed characteristics in particular breeds and, thus, classical genome-wide association analysis using cases and controls from the same breed cannot be applied for mapping these traits. An alternative approach is to perform GWAS by an across breed mapping, utilizing long-range LD blocks. The present 172K Illumina genotyping array has high SNP density, which enables a genomic survey with moderate resolution for copy number variants (CNVs) and for selective sweeps characterized by long regions of reduced heterozygosity [21].

Novel genetic methods utilizing next generation seguencing (NGS) technology such as targeted sequence capture, exome sequencing, transcriptome sequencing, mtDNA sequencing, and whole genome resequencing are becoming more and more routine and enable more efficient analyses with higher resolution data. Moreover, a recent release of the third draft of canine genome sequence CanFam 3.1 with improved sequence quality and annotation will likely facilitate the analysis of NGS data.

1.2 Developmental variation and defects in dogs

As a result of artificial selection, numerous morphological and behavioral characteristics have been enriched in breeds and even fixed as breed characteristics. This has led to a vast amount of morphological variation, which is greater than in any other species. Morphological features are formed during embryonic development and thus these characteristics offer a model for studying the genetic regulation of developmentally important molecular determinants. A growing number of genetic factors controlling the formation of canine morphological traits, such as body size, leg length and skull shape, have been characterized. Insulin-like growth

factor 1 (*IGF1*) and IGF1 receptor (*IGF1R*) have been indicated to be associated with canine body size [22,23]. These studies suggest that IGF1 pathway has major effect on controlling the size variation in dogs. Disproportionate dwarfism or chondrodysplasia is a distinguishable feature in numerous breeds such as Dachshund, Basset Hound, and Pembroke Welsh Corgi. Multibreed approach with GWAS across more than 70 breeds revealed a strong association on chromosome 18 (CFA18) and revealed an insertion of an extra functional copy of fibroblast growth factor 4 (*FGF4*) gene as the cause [24]. A mild recessive form of disproportionate dwarfism in Labrador Retrievers designated as skeletal dysplasia 2 was recently described and indicated to be caused by a mutation in collagen alpha-2(XI) chain (*COL11A2)* gene [25]. Abnormal growth of craniofacial bones leads to brachycephaly in Boxers, Bulldogs, and numerous other short muzzled breeds. This polygenic trait has been mapped to chromosome 1 but numerous other loci in several chromosomes have as well been described [17,26,27]. Fine-mapping of one of these loci locating in CFA32 revealed a mutation at highly conserved position of bone morphogenetic protein 3 (*BMP3*) [27]. Genes affecting the dog coat growth pattern, length, and curl were explored by GWAS using more than 1,000 dogs from 80 different breeds. As a result, mutations in three genes, R-spondin-2 (*RSPO2*), fibroblast growth factor 5 (*FGF5*) and keratin-71 (*KRT71*) were shown to explain more than 95% of the canine coat variation [28].

The following chapters will focus on the subjects of this thesis, including three inherited canine developmental defects: caudal dysplasia, ectodermal dysplasia, and mucopolysaccharidosis VII.

1.2.1 Caudal dysplasia

Caudal dysplasia, also referred to as caudal regression and sacral agenesis syndrome, is a rare (1-2.5/100,000 births) congenital malformation in human characterized by varying degrees of developmental failure of posterior body in early embryogenesis. It involves the lower extremities, the lumbar and coccygeal vertebrae, and corresponding segments of the spinal cord and sometimes major visceral anomalies. The etiology is unclear but maternal diabetes, genetic factors, and vascular hypoperfusion have been suggested to contribute. It has been hypothesized that T, brachyury homolog (mouse) (*T*) might be involved in sacral agenesis or congenital vertebral malformations in human patients but definitive evidence is lacking. A heterozygous c.1013C>T variant was found to be significantly associated in three unrelated patients with congenital vertebral malformations [29]. A clinically unaffected parent of each patient, however, also harboured the same variant. Earlier genetic studies have indicated that *T* gene is highly polymorphic. Several studies have identified non-synonymous variants in *T*, some implicating association with susceptibility to neural tube defects, whereas some excluding it. Another study also has excluded *T* locus as a major contributor for sacral agenesis [30-33].

1.2.1.1 Caudal dysplasia and tail length in dogs

Several dog breeds show very short tails (brachyury) or even complete absence of the tail vertebrae (anury). The dominant inherited trait is characterized by short tail, varying from a complete tailless to a half a tail with occasional kinks. A genetic cause of this dominant short-tail phenotype was originally identified in Pembroke Welsh Corgis [34]. The study demonstrated a c.189C>G mutation in exon 1 of the *T* gene that was shown to affect the DNA-binding property of the T protein and result in the bobtail phenotype in heterozygote animals. Embryonic lethality of the homozygous mutation was suggested as there were no dogs homozygous for the mutation found among the offspring of short-tailed parents. However, another study reported two malformed Welsh Corgi Pembroke puppies born to short-tailed parents and they were genotyped as homozygous for the *T* c.189C>G mutation [35]. One of the puppies was stillborn but the other stayed alive until put to death at one day old. The puppies had anorectal atresia and multiple serious spinal malformations. The study demonstrated that puppies homozygous for the *T* mutation can be born alive although it has been suggested that majority of the homozygote animals die early in fetal development. The phenotype of these homozygous dogs resembles human caudal dysplasia or caudal regression syndrome. Examination of 19 short-tailed Pembroke Welsh Corgi dogs showed no other spinal abnormalities than short tail [35] unlike heterozygous mice that have shown to manifest additional spinal defects [36,37].

There are several breeds with natural short-tailed dogs and also with variable phenotype and inheritance such as likely recessively inherited type of short tail in English and French Bulldogs, where all dogs in the breed have so called screw tail, short tail with multiple kinks. There are also occasionally short-tailed dogs born for long-tailed parents in some breeds, suggesting multiple patterns of inheritance or variations in penetrance. These dogs could serve as a model to explore the genetics of posterior vertebral development and caudal dysplasias in humans.

1.2.1.2 T gene

T encodes for a transcription factor containing the DNA-binding domain called T-box which is highly conserved among different metazoan species and defines the family of T-box genes. The members of T-box gene family have essential function in many developmental processes of both vertebrate and invertebrate embryos, like specification of the primary germ layers (ectoderm, mesoderm and endoderm) during gastrulation and assignment of cell identities in organogenesis. They have also been associated with several diseases in human: *e.g.* Holt-Oram syndrome, characterized by upper limb and cardiac malformations, is caused by mutations in T-box 5 (*TBX5*), while mutations in T-box 3 (*TBX3*) causes ulnar-mammary syndrome [38-40].

Mouse Brachyury (T) has an important function during early embryonic development being a key regulator of mesoderm formation [36,41-44]. T is needed for the correct specification of mesodermal identity in the epiblast during gastrulation and specification and cell survival in the notochord. In addition, it is

required for the development of the derivatives of posterior mesoderm including posterior somites and allantois. Mutations in *T* cause early embryonic lethality as homozygous. Defected T function results in abnormalities in the development of mesodermal tissues, including the tail and spine, thus suggesting an essential role for the T in mouse development [42]. In mice, *T* starts to be expressed at the onset of gastrulation in the nascent mesoderm at the primitive streak. As gastrulation proceeds, expression can be seen in the ectoderm next to the streak and in the newly formed mesoderm [41,45]. The expression is soonafter downregulated in the mesoderm when it separates into layers of paraxial and lateral plate mesoderm. Instead, *T* continues to be expressed in the mesoderm of the tailbud until embryonic day (E) 12.5-13.0. Tailbud is a structure which the posterior axis, namely lumbar, sacral and caudal vertebrae, is derived from [46]. In addition, the notochord precursor cells start to express *T* and the expression persists later in the notochord and its derivatives [41,45].

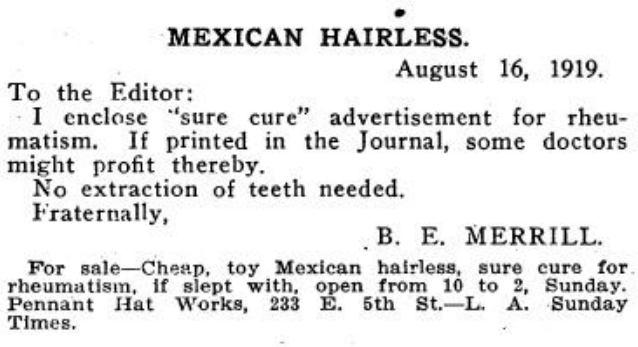
There are tens of *T* mutant-mouse lines; both spontaneous, chemically and radiation induced and genetically modified. The earliest phenotype caused by spontaneous mutation was already described in 1927 by Dobrovolskïa-Zavadskaïa [47], who reported the heterozygous mutant mice having a short and often kinked tail. Later it was shown that the homozygous animals had severe abnormalities in the development of posterior mesodermal organs leading to posterior truncation. The mice had thickened primitive streak, absent or abnormal somites posterior to somite 7, and absent notochord [36,48,49]. The embryos died at approximately 10.5 dpc (days post-coitum) due to the failure of the allantois to extend and connect with the placenta [50]. Recently, it has been demonstrated that inducible miRNA-based *in vivo* knockdown of *T* results in hypomorphic phenotype and causes axial skeletal defects and urorectal malformations resembling human caudal regression syndrome [51].

1.2.2 Ectodermal dysplasia

Human ectodermal dysplasias are a large group of congenital syndromes characterized by the abnormal development of two or more ectodermal appendages, teeth and hair follicles being the most commonly affected organs. The patients typically have sparse hair, absence of several deciduous and permanent teeth and diminished sweating. The remaining hair follicles are dysplastic, teeth are abnormal, and defects in various exocrine glands and nails are common. In addition, the typical features can occasionally be accompanied by various other dysmorphic features such as cleft lip or palate, limb dysplasia, or immunological aberrations and mental retardation. The most common form of ectodermal dysplasia in human is the X-linked hypohidrotic ectodermal dysplasia (HED) (OMIM #305100), which is caused by a mutation in ectodysplasin (*EDA*) gene. Autosomal dominant and recessive forms of HED are mostly results of mutations in other genes of the ectodysplasin signaling pathway, ectodysplasin A receptor (*EDAR*; OMIM #129490 and #224900), and intracellular adaptor protein EDAR-associated death domain (*EDARADD*; OMIM #614940 and #614941).

1.2.2.1 Canine ectodermal dysplasia

The three hairless breeds recognized by Fédération Cynologique Internationale (FCI) are Chinese Crested, Mexican Hairless and Peruvian Hairless dogs. The history of these hairless dogs dates back to more than 3,000 years ago, since statues looking identical to the Mexican Hairless dog ("Xoloitzcuintle") have been found in tombs of the Mayan, Colima and Aztec Indians. These dogs were raised by the native people and were considered sacred but are thought to have also been bred for their meat. Hairless dogs were described as *Canis aegyptius* by Linné [52] and also Darwin referred to the Turkish naked dog with defective teeth [53]. The Peruvian and Mexican Hairless dogs have also been used to relieve rheumatism throughout history (Fig. 2).



MEXICAN HAIRLESS.

August 16, 1919.

To the Editor:

I enclose "sure cure" advertisement for rheumatism. If printed in the Journal, some doctors might profit thereby.

No extraction of teeth needed.

Fraternally,

B. E. MERRILL.

For sale—Cheap, toy Mexican hairless, sure cure for rheumatism, if slept with, open from 10 to 2, Sunday. Pennant Hat Works, 233 E. 5th St.—L. A. Sunday Times.

Figure 2. California State Journal of Medicine advertised Mexican Hairless dog as "sure cure for rheumatism" in 1919 [54].

The hairless breeds are representatives of dogs with a phenotype considered as a breed characteristic that could be medically classified as a disease, ectodermal dysplasia. These dogs have missing or abnormally shaped teeth and absent or very sparse body hair with a variable amount and length of coat on top of the head, the toes, and the tip of the tail. Chinese Crested dogs, in particular, have a very characteristic appearance with long coat on these areas. Canine ectodermal dysplasia (CED) is inherited as a monogenic autosomal semidominant trait, since heterozygous dogs show the characteristic phenotype but homozygous mutants apparently die during embryogenesis [55].

Fukuta et al. [56] studied histologically skin and lymphoid organs of dogs derived from the Mexican Hairless breed. They demonstrated that the skin of newborn puppies comprised a thick epidermis with rudimental hair follicles but dogs older than 2 months of age had thin epidermis with only few epidermal ingrowths and no hair follicles or skin glands except in the hairy parts of the skin. The thymus of the newborn hairless puppies was normal but it had atrophied in older dogs, and lymphocyte accumulation was poor in the thymus as well as in the spleen and mesenteric lymph nodes [56].

A recent article reported a detailed histological analysis of the hair follicles and glandular organs of Chinese Crested dogs [57]. The dogs with forkhead box I3

(FOXI3) mutation had only simple primary hair follicles compared to the compound follicles of genotypically normal dogs. However, apocrine glands in the skin (sebaceous and sweat glands), respiratory mucous glands, nictitating membrane and the mammary gland were demonstrated to have no macroscopic or histopathological abnormalities [57].

Due to phenotypic similarity to HED, the possible involvement of candidate gene *EDAR* has been studied but excluded as causative gene [58]. CED was mapped by linkage analysis to canine chromosome 17 (CFA 17) in Chinese Crested Dogs [59].

1.2.2.2 Other canine ectodermal dysplasias

Canine X-linked HED has been clinically characterized in many breeds [60,61]. Similar to humans, a mutation in *EDA* was identified to cause the disease in a colony of dogs [62]. Canine X-linked HED is also clinically similar to the human disease. The clinical features include varying degrees of alopecia, oligodontia, abnormally shaped teeth and absence of certain exocrine glands, such as sweat and sebaceous glands. Affected dogs are also susceptible to pulmonary infectious diseases likely due to lack of tracheal and bronchial glands resulting in decreased mucociliary clearance.

Another ectodermal dysplasia type of disorder reported in dogs is an ectodermal dysplasia-skin fragility syndrome, which is a hereditary skin adhesion disorder belonging to the group of epidermolysis bullosa diseases. There is a clinically analogous disease in humans. In addition to the manifestations of the disease associated with the skin fragility arising from epidermal cell-cell separation (acantholysis), all human patients have been reported also to have hair abnormalities such as partial hypotrichosis to complete hairlessness, woolly hair, as well as nail dystrophies [63]. Recently, ectodermal dysplasia-skin fragility syndrome was demonstrated to also be genetically analogous to the human syndrome when plakophilin-1 (PKP1) deficiency was described in affected Chesapeake Bay Retriever dogs [64]. Candidate gene sequencing revealed a homozygous splice donor site mutation within the first intron of *PKP1* resulting in a premature stop codon as causative for this autosomal recessive disease.

1.2.2.3 Development of ectodermal organs

Skin appendages such as teeth, hairs, nails and many glands are all derivatives of the embryonic ectoderm. Although these mature fully-developed organs are highly divergent in shape and function, their early development is notably similar, both at the morphogenetic and molecular levels. The organogenesis of the ectodermal organs is regulated by interactions between ectodermal epithelium and mesenchyme that originates either from the mesoderm (*e.g.* in the case of body hairs and mammary gland) or from the neural crest (in the case of teeth and cranial hairs). The organ development can be divided into three stages: initiation, morphogenesis and cell differentiation. The initiation of appendage development is seen as a local

epithelial thickening, called the placode, which invaginates into the underlying condensating mesenchyme and forms an epithelial bud (Fig. 3). In mice, incisor and first molar placodes appear at embryonic day 12 (E12), the first set of hair placodes at E14 and mammary placodes at E11-E11.5 [65,66].

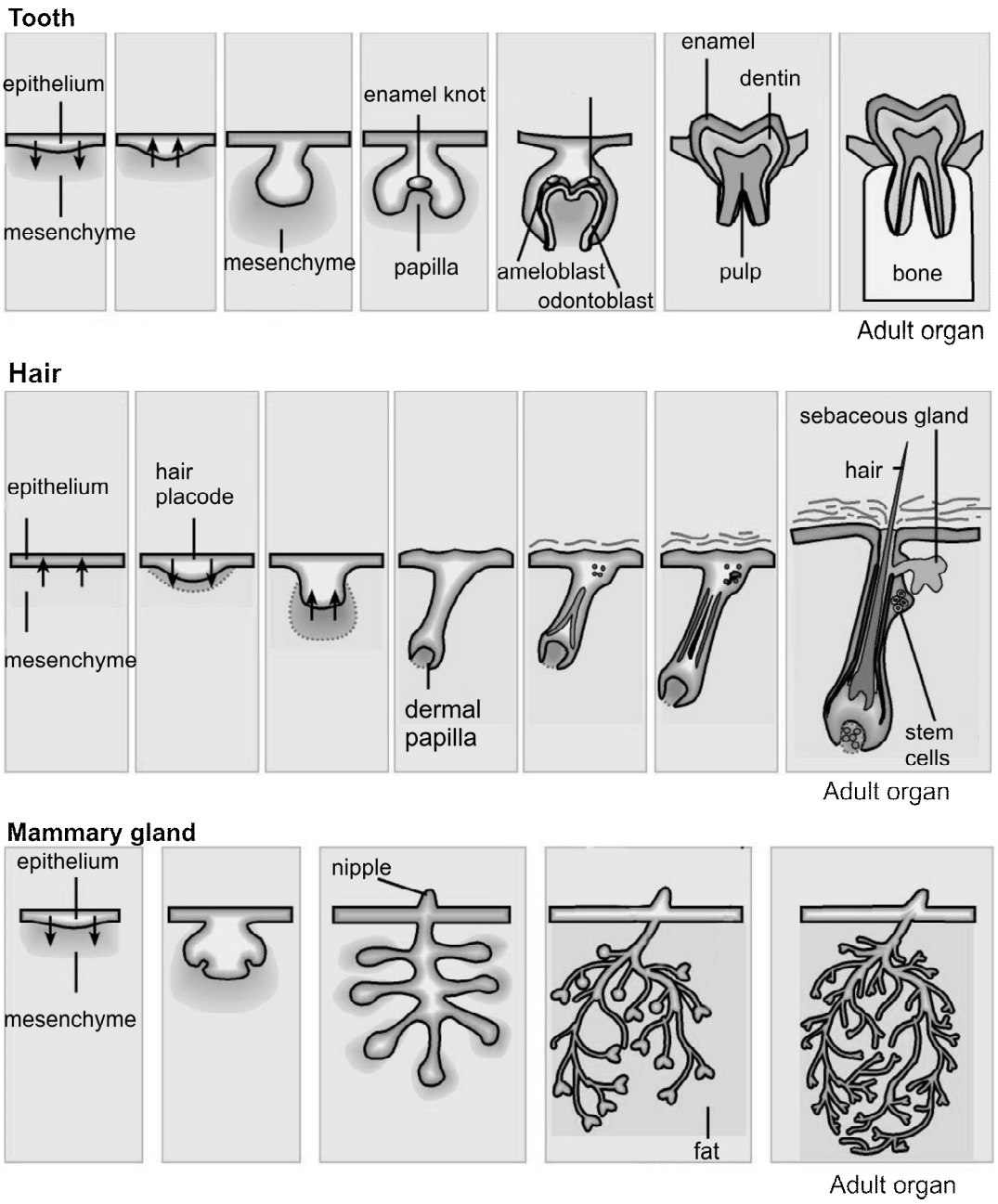


Figure 3. Development of ectodermal organs. The beginning of the development of different ectodermal organs is very similar. Epithelium thickens forming a placode and mesenchyme starts to condense around it. Subsequently, the placode grows forming a bud which invades into the underlying mesenchyme. Tightly controlled growth and branching of the epithelium and mesenchyme during morphogenesis determines the final shape of each specific organ. Modified from and used with permission [67].

In the following step, the epithelial bud grows and folds and as a consequence an organ specific shape is formed. Subsequently, the anatomical differences of the various ectodermal organs become more evident. The tooth epithelium grows extensively and undergoes complex folding morphogenesis through cap (at E14 in mouse) and bell (E16) stages. Consequently, the cusp morphology specific for each tooth type and later constituting the future tooth crown, is formed [68]. Distinct morphogenesis of the hair includes elongation of the hair germ and formation of a peg.

Tightly controlled reciprocal interactions between different tissue types regulate the formation of ectodermal appendage development. This crosstalk involve several families of signaling molecules the most essential being wingless-type MMTV integration site family (Wnt), transforming growth factor β (TGFb) (such as bone morphogenetic factors (BMPs) and activins), fibroblast growth factor (FGF), hedgehog (HH) and tumor necrosis factor (TNF) families [69,70]. Their functions are widely conserved between species and also between ectodermal appendages [66]. Consequently, mutations in several genes lead to defects in more than one ectodermal organ type. Signaling molecule Eda belongs to the family of TNF's and signals through its receptor Edar. Eda/Edar signaling pathway has a crucial role in the development of tooth and hair follicle [18,71]. Spontaneous Eda-deficient mice have missing and abnormally shaped teeth, and lack primary hair placodes [72], whereas overexpression of *Eda* in epithelium results in enlarged hair and/or ectopic hair, tooth and mammary placodes [73,74]. Eda is a key regulator of ectodermal development as the targets of Eda/Edar signaling pathway include several other important molecules such as BMP antagonists *Ccn2/ctgf* and follistatin, *Fgf20*, *Dkk4* and *Shh* [75]. BMPs, especially BMP4, have inhibitory function for placode formation and are expressed exquisitely in interfollicular region [76]. Several FGFs are expressed in epithelial compartments and signal to mesenchyme. For example *Fgf3, Fgf4, Fgf9, Fgf15* and *Fgf20* are expressed in tooth placode and/or enamel knot during tooth formation [77-80]. Activin A is a homodimer composed of two subunits encoded by inhibin βA *(Inhba)*. *Inhba* is expressed in the condensed mesenchyme in developing hair follicles and teeth, but epithelium is thought to be the target tissue [81,82]. Loss of *Inhba* leads to a developmental arrest of incisors and mandibular molars at the bud stage [81], and K14-Cre mediated conditional deletion of activin receptor 1b causes various degrees of hairlessness [83]. Wnt/ β-catenin signaling pathway is essential for the development of all skin appendages. Forced activation of this pathway in *β-cat*$^{\Delta ex3K14/+}$ embryos causes formation of supernumerary enamel knot signaling centers leading to continuous tooth generation, as well as precocious and ectopic hair follicle development [84,85]. Moreover, elevated Wnt/β-catenin signaling activity in *Sostdc1*-deficient mice result in extra incisors, premolar like teeth, enlarged hair placodes, and ectopic whisker buds [86-89].

1.2.2.4 Forkhead family of transcription factors

FOX proteins form a large family of fox transcriptional regulators characterized by an evolutionarily conserved DNA-binding domain (forkhead domain). FOX proteins have diverse functions ranging from embryonic development to regulation of metabolic processes and the immune system in the adult organism [90].

Ohyama and Groves [91] analyzed the expression of mouse *Foxi3* during early embryonic development from E6.5 to E10.5 and demonstrated that the expression covers the surface ectoderm adjacent to the neural plate, called panplacodal primordium, from late E6.5 to presomite stages. The panplacodal primordium is the origin for all cranial placodes giving rise to various sensory ganglia and contributing to the function of sensory organs and the pituitary gland [92]. Subsequently, during early somite stages, *Foxi3* expression is downregulated from the ectoderm and becomes restricted to branchial arches and later at E9.5 to E10.5 specifically to the region between maxilla and mandible and branchial pouches. *Foxi3* was shown to be expressed also in the dorsal part of the optic cup from E9.5 to E10.5. A very similar pattern of Foxi3 expression was recently demonstrated in chicken [93]. Based on the early embryonic expression pattern it has been suggested that Foxi3 is important in the establishment of the panplacodal domain and branchial pouch development [91].

1.2.3 Mucopolysaccharidoses

Deficiencies of lysosomal enzymes involved in the degradation of glycosaminoglycans (GAGs) cause a group of lysosomal storage diseases called mucopolysaccharidoses (MPSs). GAGs such as chondroitin, dermatan, heparan and keratin sulfates as well as hyalyronic acid, are long unbranched sulfated carbohydrates with repeating disaccharide units. While these heteropolysaccharides are synthetized they, with the exception of hyalyronic acid, are covalently attached to a specific protein core to produce molecules called proteoglycans (Fig. 4). Subsequently, proteoglycans are secreted forming a major component of the extracellular matrix. Proteoglycans are abundant in many tissues and have variety of functions being important for example in many developmental processes and tissue repair [94]. Many proteoglycans are structural, such as aggrecan that is a major proteoglycan in cartilage. In addition, they can control enzymatic activities and some even function as cell surface receptors. Heparan sulfate proteoglycans (HSPGs) have been extenxively studied in invertebrate and vertebrate development. A large number of growth factors, *e.g.* FGFs, Wnts and HHs, can bind HSPGs which serves as a way to concentrate ligands at the cell surface [95]. Heparan sulfates are essential in regulating the formation of morphogen gradients that are crucial in many differentiation events [96]. One family of HSPGs, namely syndecans, serves as transmembrane receptors which are able to transmit signals independently or together with other receptors [94].

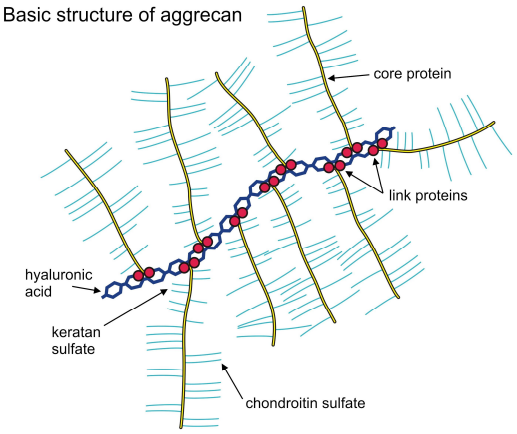


Figure 4. Structure of proteoglycans. The GAGs are covalently attached to core proteins at regular spaces and extend perpendicularly from the core forming a brush-like structure. Aggrecan, a major component of articular cartilage, is composed of large proteoglycan structures that are connected to a polysaccharide backbone, hyalyronic acid, via link proteins. The figure is used with permission [98].

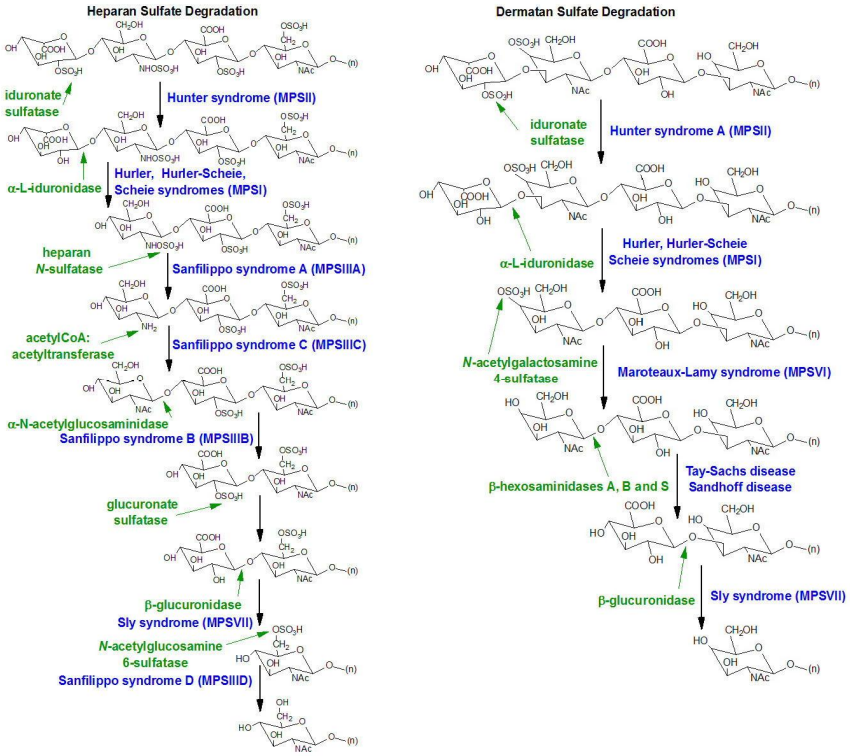


Figure 5. Degradation pathway of glycosaminoglycans. The stepwise degaradation of heparan and dermatan sulfate, and other GAGs, requires a cascade of enzymatic activity. Enzyme names are shown in green and the disorders caused by defective enzyme activity in blue. Modified and reproduced with permission of themedicalbiochemistrypage.org, LLC.

Depending on the GAG in question, one of the four different pathways is responsible for the degradation of these carbohydrate polymers: chondroitin sulfate, dermatan sulfate, heparan sulfate, or keratan sulfate degradation pathway. In each

pathway, several different lysosomal enzymes are required for the stepwise degradation of GAGs (Fig. 5). Deficiencies of these enzymes cause MPSs which have been categorized into eleven different forms in humans based on the underlying enzyme deficiency (Table 1) [97].

Table 1. *Summary table of different types of mucopolysaccharidoses*

Type	Alternative name	Deficient gene symbol	Deficient enzyme	OMIM	Dog model
MPS I	Hurler, Scheie	*IDUA*	α-L-iduronidase	607015	Plott Hound [99], Rottweiler, Boston Terrier [100]
MPS II	Hunter	*IDS*	Iduronate 2-sulfatase	309900	Labrador Retriever* [101]
MPS IIIA	Sanfilippo type A	*SGSH*	Heparan sulfate sulfamidase	252900	Dachshund [102], Huntaway Dog [103]
MPS IIIB	Sanfilippo type B	*NAGLU*	α-*N*-acetylglucosaminidase	252920	Schipperke [104]
MPS IIIC	Sanfilippo type C	*HGSNAT*	Acetyl-CoA transferase	252930	None
MPS IIID	Sanfilippo type D	*GNS*	*N*-acetylglucosamine 6-sulfatase	252940	None
MPS IVA	Morquio type A	*GALNS*	Galactose 6-sulfatase	253000	None
MPS IVB	Morquio type B	*GLB1*	β-Galactosidase	253010	None
MPS VI	Maroteaux-Lamy	*ARSB*	Arylsulfatase B (*N*-acetylgalactosamine 4-sulfatase)	253200	Miniature Pinscher [105], Miniature Poodle-type dog [106]
MPS VII	Sly	*GUSB*	β-glucuronidase	253220	mixed breed [107], German Shepherd [108]
MPS IX		*HYAL1*	Hyalyronidase	601492	None

*Not genetically identified but based on clinical diagnosis only

Due to deficient degradation of GAGs the characteristic feature of the MPSs is the accumulation of GAGs in lysosomes of various cell types such as fibroblasts, macrophages, leukocytes, chondrocytes and parenchymal cells of the liver. The

undegraded GAGs are also excessively excreted into urine. The excessive GAG accumulation leads to developmental disturbancies and dysfunction of many tissues and organs. Clinical characteristics of these progressive disorders are various and differ between MPS types but they also share features such as dwarfism, undeveloped epiphyseal centers, dysostosis multiplex, facial dysmorphia, corneal clouding and organomegaly [97]. Several MPS types have been identified clinically and genetically in dogs as well (Table 1). As in humans, all are inherited as autosomal recessive traits, except MPS II, which is X-linked.

1.2.3.1 Mucopolysaccharidosis VII

MPS VII (also referred to as Sly syndrome in human) is caused by deficient activity of β-glucuronidase (GUSB) enzyme leading to lysosomal accumulation of glucuronic acid containing GAGs (heparan, dermatan, chondroitin 4- and chondroitin 6-sulfates) [109]. GUSB mutations have been described in multiple species including human, mouse, cat, and dog.

Disease characteristics in human patients (OMIM #253220) include: mental retardation, skeletal deformities (dysostosis multiplex), corneal clouding, and hepatosplenomegaly. The clinical variability among human is extensive ranging from prenatal lethality to mild skeletal abnormalities with normal intelligence. The skeletal involvement is an early and prominent feature in almost all MPS disorders and leads to dysplastic skeletal features and short stature. The human *GUSB* gene contains 12 exons and encodes a 651-amino acid precursor. The precursor of GUSB undergoes cleavage and glycosylation and is transported into the lysosomes where its subunits form the mature enzyme [110].

Almost 50 unique mutations in *GUSB* have been described in human. The site of the mutation in the *GUSB* gene correlates with the residual enzymatic activity and related clinical severity [111]. It has been demonstrated that even a small percentage of normal GUSB activity (2-3%) can protect against a severe phenotype.

Seven spontaneous or induced murine MPS VII models are available [112-116]. All models present similar clinical, morphological, and histopathological characteristics but the severity of the deficits depends on the strain. The phenotypical features in mice are comparable with human MPS VII patients, including shortened life-span, dysmorphic facial features, skeletal dysplasia, and widespread lysosomal storage of GAGs in various tissues.

GUSB deficiency has been described in three separate cases of cats as well, but the causative mutation has been identified only in one of them [117-119]. All three feline models share typical clinical signs of MPS VII including a young age of onset, dwarfism, facial dysmorphism, walking difficulties, corneal clouding, epiphyseal dysplasia of the vertebrae and long bones, and vacuolization in several tissues. The causative mutation has been identified in one population of cats; p.E351K substitution affects a highly conserved residue of GUSB [119].

Canine MPS VII has been previously identified in two cases, in a mixed breed dog and a German Shepherd, having identical missense mutation, resulting in p.R166H substitution [107,108,120]. The first symptoms in these dogs appeared at 2

to 5 months of age and involved weakness of the hind legs followed by a progressive dysfunction of all limbs. The affected dogs also presented other typical MPS VII features including growth retardation, facial and skeletal dysmorphisms, and corneal clouding. Joints were extremely lax and easily subluxated, and radiographic examination showed severe epiphyseal dysplasia. Abnormalities in several other organs were also present, including hepatomegaly, tracheal dysplasia, and cardiac abnormalities.

2 Aims of the study

The overall objective of this study was to identify the genetic causes of three congenital developmental defects in dogs. Gene discoveries would then pave the way for further functional studies and pathway characterization and would help to develop new genetic tests for breeding purposes and aid in clarifying the etiology of corresponding conditions in humans. The specific aims of the study were the following:

1. To clarify the genetic cause of short tail in various short-tail breeds, starting from the known T gene mutation and extending the analyses to the whole coding region of the gene, if necessary (I).

2. To explore the eventual genetic heterogeneity behind the common short-tail phenotype to better understand development of caudal dysplasia (I).

3. To identify a causative mutation for canine ectodermal dysplasia in three hairless breeds (II)

4. To characterize the function of the novel ectodermal dysplasia gene in ectodermal organ development and identify its upstream regulators (III)

5. To describe the clinical and pathological features of the skeletal syndrome in Brazilian Terriers and to identify its genetic cause (IV)

3 Materials and methods

3.1 Study cohorts, pedigrees (I, II, IV)

Dr. Lohi's research group has established a large dog DNA bank in Finland currently including DNA samples of more than 40,000 dogs from 250 breeds. All samples used in these studies have been collected from privately owned pets. This significant resource has been used in all studies presented here. A large number of canine samples were also collected by the collaborators in Switzerland and France.

In Study I, the initial cohort consisted of 360 dogs from 23 breeds. Among these were 156 short-tailed and 204 long-tailed dogs. In addition, samples were collected from 80 dogs encompassing 9 breeds presenting only the long-tail phenotype. Pedigrees and tail phenotype information were collected from each sampled dogs. Tail phenotypes were recorded by the sample collector, the owner or from the public dog registry of the Finnish Kennel Club (http://jalostus.kennelliitto.fi).

In Study II, samples from altogether 195 partially related dogs were used (93 hairless and 49 coated Chinese Crested dogs, 39 hairless and 6 coated Peruvian Hairless dogs and 8 hairless Mexican Hairless dogs). From these, GWAS was performed using samples from 20 hairless and 19 coated Chinese Crested dogs, the rest were included in the fine-mapping.

In Study IV, samples were collected from a total of 202 Brazilian Terriers including 15 affected puppies from eight litters and 187 healthy controls. A large pedigree was established around the affected dogs using the GenoPro genealogy software (http://www.genopro.com/) and utilizing the public dog registry of the Finnish Kennel Club (http://jalostus.kennelliitto.fi) to evaluate the modes of inheritance.

Study I was conducted in collaboration with the research groups of Catherine André and Francis Galibert at the University of Rennes, France, the genetic testing laboratory Antagene, France and Lars Paulin at the Institute of Biotechnology, University of Helsinki. Study II was conducted in collaboration with the research groups of Tosso Leeb at the University of Bern, Switzerland and Kerstin Lindblad-Toh at the Broad Institute of Harvard and Massachusetts Institute of Technology, USA and at the Uppsala University, Sweden. Study III was conducted in collaboration with the research groups of Marja Mikkola and Irma Thesleff and at the Institute of Biotechnology, University of Helsinki and Tosso Leeb at the University of Bern, Switzerland. Study IV was conducted in collaboration with the veterinarians at the Animal Hospital of University of Helsinki (Anu Lappalainen, Heli Kallio and Marjatta Snellman).

3.2 Genomic DNA extraction (I, II, IV)

Samples were either ethylenediaminetetra-acetic acid (EDTA) -blood or buccal cell samples. Samples were collected either by a trained representative of the research laboratory or a licensed veterinarian. In Study I, samples were collected in Finland and by the collaborators in France. In Study II, samples were collected in Finland and by the collaborators in Switzerland. In Study IV, all samples were collected in Finland. Blood samples were stored at -20°C until genomic DNA was extracted. Genomic DNA was extracted from blood samples for Study I using Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN). Blood and buccal cells for Study I were extracted using either the NucleoSpin Kit (Macherey-Nagel, Hoerdt, France) or the BuccalAmp DNA Extraction Kit (Epicentre Biotechnologies, Madison, WI). Some samples with low DNA yields were amplified using the V2 Genomiphi Kit (GE Healthcare, Buckinghamshire, UK). DNA concentration was determined with the NanoDrop-1000 UV/Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Collection of blood samples was approved by the Animal Ethics Committee at the State Provincial Office of Southern Finland (ESLH-2009-07827/Ym-23). Owner consent was collected for each dog.

3.3 PCR and sequencing (I, II, IV)

PCR reactions were carried out in using a standard PCR protocol or in the case of GC-rich amplicons the Advantage GC Genomic LA Polymerase Mix (Clontech Laboratories, Inc., Mountain View, CA). The details of the primers used in studies I, II and IV have been described in the "Materials and Methods" section of each publication.

In Study I, the presence of the *T* mutation in the amplified PCR product was detected either by restriction enzyme assay with BstEII enzyme (New England Biolabs, Ipswich, MA) or sequencing.

PCR products were cleaned by ExoSAP-IT (GE Healthcare) and sequenced with either ABI PRISM 3130XL or 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA). The resulting sequencing data were analyzed using either Sequencher 4.6 (Gene Codes Corporation, Ann Arbor, MI), Variant Reporter v1.0 or DNA Sequencing Analysis v5.2 software (Applied Biosystems, Foster City, CA). Fragment size analyses were performed with the GeneMapper 4.0 software (Applied Biosystems, Foster City, CA).

3.4 Reference sequence and SNP databases (II and IV)

The dog genome build CanFam2.0 and human genome build 36 were used as references. The canine SNP databases are provided by the Broad Institute and are

available online: SNPs mapped on CanFam1.0 (http://www.broad.mit.edu/mammals/dog/snp) and CanFam2.0 (http://www.broad.mit.edu/mammals/dog/snp2).

3.5 Statistical analysis (I)

The possible effect of the homozygous *T* gene mutation for embryonic viability was estimated by following the litter sizes of short-tailed parents compared with long-tailed parents in Swedish Vallhund breed. Statistical significance of the variation between the study groups was measured by Student's t-test.

3.6 Genome wide association study (II and IV)

Study II: Samples (20 hairless and 19 coated Chinese Crested dogs) were genotyped using the 50K canine Affymetrix v2 SNP array (Affymetrix, Santa Clara, CA). Association analysis was performed by free, open-source analysis toolset PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) [121]. Only the 49,663 SNPs were included. SNPs with a genotyping rate less than 95% (9,730) were removed. Subsequently, the average genotyping rate per individual was 94.23%. Genotype data were further filtered with minor allele frequency (MAF) >20%, as the causative mutation must occur once in each affected dog. Based on this 34,175 SNPs were removed from the analysis. 12,355 SNPs remained for analyses after frequency and genotype pruning. Genome-wide significance was ascertained with permutation testing (n = 10,000). In order to analyze the haplotype association, the haplotype frequencies were determined for each set of 2-8 SNPs across the genome using PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) [121], and the logarithm of odds (LOD) score was calculated using the Haplotype Likelihood Ratio test [11].

In Study IV, the samples (seven cases and eleven controls) were genotyped using Illumina's CanineSNP20 BeadChip of 22,362 validated SNPs. Case-control association analysis was performed by PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) [121]. Genotype data were filtered with a SNP call rate of >95% and MAF of >5%. Based on these criteria 366 SNPs were removed for low genotyping efficiency and 5,647 SNPs for low MAF. No individual dogs were removed for low genotyping and no SNPs were removed because of significant deviations from the Hardy-Weinberg equilibrium ($p \leq 0.0001$). After frequency and genotype pruning, 16,595 SNPs remained for analyses. Genome-wide significance was ascertained with phenotype permutation testing (n = 10,000).

3.7 Fine-mapping (II)

A total of 111 SNPs located in the 1.7 Mb region between positions 40,197,592 – 41,904,861 on CFA 17 (CanFam2.0) were used for the fine-mapping. Of these, 48 SNPs were typed on a MassARRAY Analyzer (Sequenom, San Diego, CA) and 30 SNPs and one indel polymorphism were genotyped by re-sequencing of targeted PCR products using capillary sequencing technology. Finally, genotypes of 32 SNPs in this region were derived from the Affymetrix SNP microarray data explained in the previous chapter. Haplotypes were determined using linkage analysis package Merlin (http://www.sph.unich.edu/csg/abecasis/Merlin) [122].

3.8 Analysis of the canine *FOXI3* gene and mutation identification (II)

The bacterial artificial chromosome (BAC) clone S027P16A10 containing the canine *FOXI3* gene was isolated using PCR screening of hierarchical DNA pools of a canine BAC library (6). The *FOXI3* gene was subcloned in overlapping plasmids. A primer walking strategy with ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA) was performed to obtain the sequence of *FOXI3*. The sequence was submitted under accession AM998820 to the EMBL nucleotide database.

3.9 Mice (III)

Embryos aged from E9 to E19 from NMRI mice were collected to be used in the experiments where wild-type embryos were needed. The appearance of the vaginal plug was determined to be day 0.5 of embryogenesis (E0.5) and the age of the embryos was more precisely approximated by morphological criteria. The following transgenic and mutant mouse lines were used and genotyped as described in the referred articles: *Sostdc1* (ectodin) null [86], K14-*Eda* [73], and *Eda* null (Tabby) [72]. The β-cat$^{\Delta ex3K14/+}$ mice were generated by cross breeding K14-cre and β-catenin$^{lox(ex3)}$ mice [123,124]. Littermates were used as controls for all other transgenic mice except for *Eda* null which were maintained by breeding *Eda*$^{-/-}$ females with *Eda*$^{-/Y}$ males. Experiments on mice were approved by the Animal Ethics Committee at the State Provincial Office of Southern Finland (ESAVI/1181/04.10.03/201, KEK10-056 and VKL001-08).

3.10 *In situ* hybridization (II, III)

Non-radioactive *in situ* hybridization (ISH) on paraffin sections (5-7 µm) was performed with the Ventana Discovery ISH robot (Ventana Medical Systems, Oro Valley, AZ). Whole-mount ISH was carried out using the InsituProVS instrument

(Intavis Bioanalytical Instruments AG, Köln, Germany). Digoxigenin (DIG)-labeled cRNA probes were used for both non-radioactive applications and were following: *Foxi3* [91], *Dkk4*, *Fgf3*, *Sostdc1*, *Patched1*, and *Lef1* [82,125]. Sense probes were used as controls. The probes were detected with BM Purple AP Substrate Precipitating Solution (Roche Applied Science, Basel, Switzerland). The samples were visualized and photographed with Leica stereomicroscope equipped with a DC300F camera and IM1000 software (Leica Microsystems, Wetzlar, Germany). For vibratome sectioning, the samples were embedded into 5% low-melting agarose and cut to sections (20-25 μm) with Leica VT 1000S Vibratome (Leica Microsystems, Wetzlar, Germany). Radioactive ISH on paraffin sections was performed using a standard protocol with 35S-UTP labeled probes (PerkinElmer, Waltham, MA).

3.11 Tissue culture (III)

Wild-type NMRI mouse embryos were dissected in sterile Dulbecco's PBS pH 7.4 under a stereomicroscope. For bead experiments Affi-Gel agarose beads (BioRad, Hercules, CA) were incubated in one of the following protein solutions: activin A, BMP4 and Shh (100ng/ μl; R&D Systems, Minneapolis, MN). Heparin acrylic beads (Sigma, St Louis, MO) were used for the incubation of FGF4 (100ng/μl; R&D Systems, Minneapolis, MN). Control agarose and heparin beads were soaked in bovine serum albumin (BSA, 1μg/μl, Sigma, St Louis, MO). Beads were placed on top of the tissue explants using fine forceps. Tissue explants were cultured for 24 hours at 37˚C in a Trowell-type organ culture system on Nuclepore filters (0.1 μm) (Whatman, Kent, UK) in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum, glutamine and penicilline-streptomycin.

3.12 Hanging-drop experiments and quantitative RT-PCR (III)

Foxi3 mRNA levels in embryonic skin explants were analyzed substantially as described earlier [76]. In brief, the back skin of Eda-/- or wild type E14.5 mouse embryos was dissected and the explants were split along the midline: one half was used as a control while the other one was incubated with 0.25 μg/ml of recombinant Eda protein (Fc-Eda-A1; [126]) or 0.5 μg/ml of activin A [127] in a hanging drop of Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum, glutamine and penicillin–streptomycin. After 2 and 4 hours of culture, total RNA was isolated using RNeasy mini kit (Qiagen, Venlo, Netherlands), and reverse transcribed using 500 ng of random hexamers (Promega, Fitchburg, WI) and Superscript II (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. The hanging drop experiments were done twice each with minimum four replicates. Quantitative RT-PCR was performed using Lightcycler DNA Master

SYBR Green I with the Lightcycler 480 (Roche Applied Science, Penzberg, Germany). Gene expression was quantified by comparing the sample data against a dilution series of PCR products of RAN binding protein 1 (*Ranbp1*), follistatin, *Edar*, or *Foxi3*. Data were analyzed with the software provided by the manufacturer and normalized against *Ranbp1*. Statistical significance was tested by nonparametric Wilcoxon signed-rank test for paired samples, and p-value 0.05 was used as the significance threshold.

3.13 Clinical and histological examination of Brazilian Terriers with skeletal abnormalities (IV)

Seven affected Brazilian Terrier puppies, and a dam and three healthy littermates from five different litters were radiographically examined at the Animal Hospital of University of Helsinki to study the structural abnormalities. Laterolateral radiograph of the whole body and laterolateral as well as ventrodorsal radiographs of the skull were obtained. Affected and healthy puppies were photographed and video recorded. Ophthalmoscopic examination was performed for three affected puppies by a board-certified veterinary ophthalmologist. General post mortem examination was performed for three affected puppies from the same litter at the Animal Hospital of University of Helsinki. Autopsies were taken from different tissues including lung, kidney, spleen, heart, pituitary gland, brain, eye, long and short bones of the limbs and spine. In addition, histological analyses from tissue samples (limb bones, spine, skull and mandible with teeth) were later performed for four additional affected puppies. Histological samples from the Brazilian Terrier puppy that had died due to intestinal infection at 5 weeks of age and was without the skeletal disease was used as a control dog. Tissue samples were fixed in 10% formalin for 48 hours, bone samples were decalcified in Morse's solution or in 10% EDTA, dehydrated and embedded in paraffin. Paraffin blocks were cut into 5μm sections and stained with hematoxylin and eosin.

3.14 Biochemical studies of GAGs (IV)

Urinary samples were collected and GAG levels (expressed as GAG/creatinine ratios) were measured from three cases and controls using a protocol for colorimetric quantification of GAGs based on de Jong et al. [128]. Serum samples were collected from three cases and six controls (three heterozygous carriers and three healthy non-carriers). The samples were stored and shipped at -20°C to the laboratory of Oulu University Hospital for determination of the activity of β-glucuronidase and α-mannosidase according to routine protocols.

3.15 Prediction of the pathogeneity of a genetic variant (IV)

Web-based software PolyPhen-2 (Polymorphism Phenotyping v2) (genetics.bwh.harvard.edu/pph2) [129] was applied to evaluate the pathogenic effect of the mutation. The score range for PolyPhen-2 is from 0 to 1 with the threshold "probably damaging" at 0.85.

3.16 Target enrichment and next generation sequencing (IV)

A targeted sequence capture and next generation sequencing was performed to identify the disease-causing mutation. The NimbleGen's in-solution capture technology was used to enrich a 13.2-Mb target region (CFA6: 3,250,000-16,400,000) for sequencing using probes that were designed by Roche NibleGen (Roche NimbleGen, Madison, WI). The target enrichment and sequencing was performed for two Brazilian Terrier cases and controls with opposite haplotypes and, in addition, for 8 Border Terriers, 12 Nova Scotia Duck Tolling Retrievers, 8 Schipperkes, and 4 West Highland White Terriers, that were used as additional controls. Sample preparation, target enrichment experiments, sequencing and variant calling pipeline analysis [130] was performed by the core facility, Institute for Molecular Medicine Finland (FIMM, Technology Centre, University of Helsinki, Helsinki, Finland) and has been described in detail in Study IV. Further data analysis was performed using open source R environment (http://www.r-project.org) and ANNOVAR software [131]. Canine genome build CanFam2 was used as reference sequence.

4 Results and discussion

4.1 Genetic screening of *T* in multiple breeds with short-tail phenotype (I and unpublished data)

Natural short tail is a feature that exists in many breeds. Most of the breeds include both long and short-tailed dogs but there are also breeds in which the phenotype is fixed. Length of a short tail is variable (I/Fig. 1). The number of caudal vertebrae varies in short-tailed dogs and determines the length of the tail. A mutation causing dominantly inherited short tail in Pembroke Welsh Corgis and Corgi-Boxer crosses had been identified prior to this study to *T* gene belonging to a T-box transcription factor gene family [34]. The identified missense mutation (c.189C>G) (Fig. 6A,B) resulting in p.I63M substitution was suggested to be embryonic lethal in the homozygous condition. But as many different types of breeds include short-tailed dogs and some with apparently different mode of inheritance, we wanted to explore which breeds had the same mutation in the *T* gene and which breeds showed the short-tail phenotype without the known *T* mutation and could be thus used to identify novel mutations causing short tail, the mild form of caudal dysplasia.

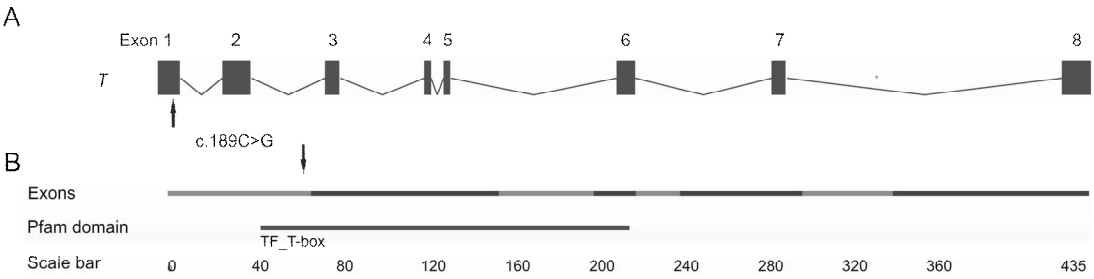


Figure 6. Heterozygosity for c.189C>G mutation in *T* gene causes short tail phenotype in dogs. The mutation is located in the end of exon 1 that codes for part of the DNA-binding domain of T protein (A and B).

4.1.1 Mutation in *T* is responsible for the short-tail phenotype in 17 breeds but excluded in 6 breeds

Short-tailed dogs were sought from breeds in which the short tail is a common and desired phenotype but also from breeds which had only occasional short-tailed dogs and the phenotype was not selected for. The *T* gene c.189C>G (p.I63M) mutation was genotyped from 360 dogs representing 23 breeds (I/Table 1). The study cohort included 156 short-tailed and 204 long-tailed dogs. The mutation was identified in short-tailed dogs within 17 breeds (I/Table 1). In the rest of the breeds (n=6) only the wild type allele was found (I/Table 1). In order to find out novel causative variations in *T* gene, all the coding exons, exon/intron boundaries, and untranslated regions (UTR) were sequenced but no variants associated with the phenotype were found. These results demonstrate that the *T* gene mutation is present in many short-tailed

dogs, but it does not explain all short tail phenotypes which indicates that other genetic alterations are likely involved in this phenotype in some breeds.

In this study, 17 breeds were identified, in addition to Pembroke Welsh Corgi, with the *T* c.189C>G mutation, suggesting an ancestral origin of this mutation. Indeed, these 17 breeds mainly belong to two groups: sheepdog and hunting breeds. Writings in the beginning of 19th century indicate that naturally short-tailed sheepdogs were common in Britain in those days. It is likely that the *T* mutation has its origin in these sheepdogs or their ancestors. Modern dog breeds have been created subsequently and therefore the mutation has presumably spread to several breeds originating from these sheepdogs. And indeed, these short-tailed sheepdogs have been mentioned in the history of several breeds. A more recent addition of the mutation into some breeds is likely as well since a short tail has been a desired characteristic in many breeds and as an autosomal dominant feature it is easily transmitted.

The studied short-tailed dogs with wild type *T* allele, c.189C, belong to six breeds, which are Boston Terrier, English Bulldog, King Charles Spaniel, Miniature Schnauzer, Parson Russell Terrier, and Rottweiler. The tail phenotype differs between the breeds suggesting heterogeneous genetic backgrounds. Boston Terriers and English Bulldogs all either lack or have very short and kinky tails, commonly referred as screw tail due to its characteristic appearance. In these breeds, the short tail is a breed characteristic and a fixed trait. The mode of inheritance has been suggested to be autosomal recessive [132]. Especially English Bulldogs could be studied further and compared genetically to other breeds of the same origin, namely the American and Continental Bulldogs (not recognised by FCI) that have normal long tails. On the other hand, short tail with multiple prominent kinks seems to be a very common, but not fixed, phenotype in King Charles Spaniel. Further analyses should be made to determine the mode of inheritance and the genetic alteration resulting in this short-tailed phenotype. Dogs with natural short tail were also found among Miniature Schnauzers, Parson Russell Terriers and Rottweilers. All the short-tailed dogs from these breeds in the study had normal long-tailed parents. This suggests that the traits are due to either a recessive single locus mutation, spontaneous developmental abnormality (congenital but not hereditary), or a sporadic *de novo* mutation. In mouse, mutations in several genes such as *Pax1*, *Wnt-3a*, *Dll3*, *Noto*, and *Ptf1a* have been associated with short and kinked tails and, thus, also remain as potential candidates in dogs [46].

4.1.2 Reduced litter size indicates lethality of homozygous embryos

Mice homozygous for the different T mutations die during embryogenesis and possess severe abnormalities in the posterior body [42,49]. Lethality of the homozygous mutation has been suggested in dogs as well, since no dogs homozygous for the *T* mutation were found within the study identifying the *T* c.189C>G mutation. Further evidence was gained when Pembroke Welsh Corgi puppies with severe anatomical defects were reported to be homozygous for the mutation [35]. Therefore, it has been suggested that the homozygous mutation results

in either embryonic or, in rare cases, early postnatal lethality due to serious developmental defects. Our results showed that all short-tailed dogs with the *T* c.189C>G allele were heterozygous for the mutation and none of the long-tailed dogs carried the mutation. This result is in concordance with autosomal dominant mode of inheritance with complete penetrance and supports the previous assumption of the lethality of the homozygous mutation. In order to provide further proof for the embryonic lethality of the homozygotes, we compared the average litter size of two breeding combinations (long tailed _ long tailed and short tailed _ short tailed) in Swedish Vallhunds. We calculated the litter sizes of 56 litters born between years 2000 and 2007 using the Breeding Database from the Finnish Kennel Club (2008) (jalostus.kennelliitto.fi). The average litter sizes were 5.5 puppies for long_long and 3.9 puppies for short_short breeding combinations (I/Fig. 2). Breeding short-tailed dogs together produced, on average, 29% (P = 0.0008) smaller litters compared to litters of long-tailed dogs of the same breed. This is close to the expected value (25%) when hypothesizing the *in utero* lethality and therefore, provides further support for the hypothesis that the *T* mutation is homozygous embryonic-lethal.

Although the short-tail phenotype as such has no counterpart in human, it is a posterior truncation and could be seen to represent a mild form of caudal dysplasia. The identification of the novel mutations might provide information regarding the genes that regulate the development of the caudal most structures and/or the vertebrae.

4.1.3 A puppy homozygous for *T* mutation with severe caudal dysplasia (unpublished data)

A malformed Swedish Vallhund puppy was delivered to the laboratory for examination. The puppy had been born alive but euthanized shortly after birth due to multiple severe malformations. Both sire and dam of the puppy were congenitally short-tailed dogs. The puppy was tailless and had hypoplastic lower limbs, abdominal hernia and anal atresia (absence of the anus). The radiography and computed tomography (CT) scan with volume rendering technique showed severe vertebral malformations including missing sacral and caudal vertebrae (Fig. 7). Genotyping revealed that the puppy was homozygous for the *T* c.189C>G mutation. The phenotype is comparable with the previously reported Welsh Corgi Pembroke puppies which also were homozygous for the *T* mutation [35]. This provides further support that the homozygotes might occassionally survive until birth. These homozygotes have features typical for caudal dysplasia or caudal regression syndrome, which is a congenital malformation in human characterized by varying degrees of developmental defects of posterior body in early embryogenesis. In addition, the canine phenotype is in concordance with the mouse hypomorphic phenotype caused by *in vivo* knockdown of *T* which was demonstrated to result in axial skeletal defects and urorectal malformations resembling human caudal regression syndrome [51]. Together these studies indicate that defected function of T can result in caudal dysplasia syndrome.

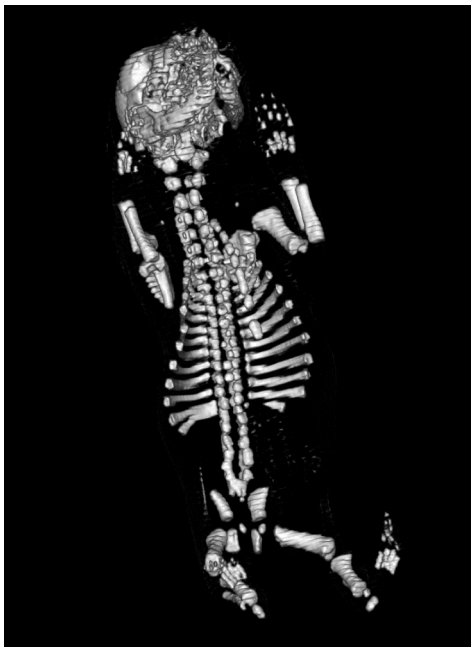


Figure 7. A whole body CT scan (ventral, slightly oblique view) of Swedish Vallhund puppy with severe spinal dysmorphism and caudal dysplasia.

4.1.4 T c.189C>G mutation is ancestral and causative for short tail phenotype in several, but not all, breeds

Short tail in dogs is a phenotype that has been favoured by man and therefore has been deliberately selected for. Short tail is a fixed breed characteristic in some breeds but in many breeds tail phenotype has remained variable. Screening of previously identified *T* c.189C>G mutation in 23 breeds revealed that the short tail is caused by the mutation in 17 different breeds. This demonstrates that the mutation is widely spread and suggests that the mutation occurred in a common ancestor quite long time ago. On the other hand causative variants in *T* were not found in short-tailed dogs belonging to six breeds. It will be interesting to see what the genes responsible for these traits are. Identification of genetic variant resulting in the short tail that is a fixed trait in several breeds could be successfully achieved using sweep mapping as these breeds likely share the same causative genetic variant. Alternatively, NGS approaches, namely exome or whole genome resequencing might be successful. Morover, it will be important to characterize the phenotypes of these short-tailed dogs thoroughly as the abnormalities in tail morphology may not be the only changes involved in the phenotype. Identification of the causative genes would then provide information about the function of these genes.

4.2 Identification of the *FOXI3* mutation as causative for the canine ectodermal dysplasia (II)

4.2.1 Mapping the CED to CFA17 in Chinese Crested dogs and fine-mapping with Mexican and Peruvian Hairless dogs

The characteristic hairless phenotype of three breeds, Chinese Crested, Mexican Hairless and Peruvian Hairless dogs, includes missing or abnormally shaped teeth and sparse or lacking hair (Fig. 8 and II/Fig. 1A,B). This autosomal dominant trait is classified as canine ectodermal dysplasia. CED was earlier mapped to large segment of CFA17 by linkage analysis using experimental matings [59]. With more recent techniques using substantially denser marker assays it was expected to be possible to map the disease more precisely in Chinese Crested dogs. We therefore decided to perfom GWAS and collected an initial cohort of 20 hairless and 19 coated Chinese Crested dogs. The samples were genotyped using Affymetrix 2.0 canine array with ~50k SNPs. A case-control association analysis detected strong association on CFA17 at position 41,039,521 ($p_{raw} = 3.5 \times 10^{-6}$, $p_{genome} = 0.044$) (II/Fig. S2A). No single SNP, however, segregated completely with the CED phenotype.

Combinations of genetic variants locating at adjacent regions on the same chromosome and inherited together are called haplotypes. Association methods based on haplotypes may sometimes provide additional power for gene mapping. Therefore, haplotype association approach was conducted. Haplotype frequencies were determined using sliding windows of two to eight SNPs across the genome and the LOD scores were calculated. Consequently, 8-SNP haplotype sized 160 kb was revealed on CFA17 at a position ranging from 41.04 Mb to 41.20 Mb and having perfect concordance (II/Fig. S2B).

As mentioned above, the CED phenotype segregates in three different breeds, which was taken advantage of in this study. We fine-mapped the locus in a cohort of 195 dogs (140 cases and 55 controls) including Chinese Crested dogs (93 cases and 49 controls), Mexcican Hairless dogs (8 cases) and Peruvian Hairless dogs (39 cases and 6 controls). The haplotypes were determined by exploiting the familial relationships of the dogs in the cohort. All hairless dogs contained identical marker alleles in 102-kb region (II/Fig. S2C). This 102-kb haplotype located between 41,045,331 and 41,147,100 bp on CFA17. Due to different recombination histories, the original extensive 1.7 Mb region of association, mapped in the Chinese Crested dog, was efficiently narrowed down with the other two breeds to only 102-kb region.

Figure 8. A hairless (CED affected) (left) and coated (right) Peruvian Hairless dog (A). A hairless Peruvian Hairless dog (B) and a head figure of another hairless dog demonstrating the sparse hair on top of the head (C). Malformed and missing incisors and canine teeth of a hairless dog (D). A normal dentition of a coated dog (E). All premolars are missing from the hairless dog (F). A normal dentition of a coated dog of same breed (G).

The region contained only two predicted genes LOC483074 and LOC483075. LOC483074 represents a processed pseudogene of the X-chromosomal NONO gene. LOC483075 showed homology to the forkhead box (FOX) transcription factor gene family. Several members of the FOX family of transcription factors have been shown to function in different developmental processes [90] and therefore, it was considered to be a good candidate gene.

There was one article published about mammalian Foxi3 reporting the expression of the mouse *Foxi3* during early murine embryogenesis [91]. The expression analysis included only the stages before the organogenesis of the ectodermal appendages and therefore, the expression in the developing murine tooth and hair follicles, which are the organs mainly defected in the dogs with CED, was assessed

here. Whole-mount ISH of the E13.5 and E14.5 mouse embryos revealed specific expression of *Foxi3* transcripts in the epithelium of the hair and whisker placodes and developing teeth (both molars and incisors) (II/Fig. S3). This suggested a functional role for Foxi3 in the development of these organs and triggered to the search for a mutation related to this gene.

The canine dog assembly CanFam2.0 did not cover the entire exon 1 of LOC483075 because of a sequence gap within that region. Therefore a BAC clone corresponding to this region was isolated and the complete genomic sequence of *FOXI3* and its upstream sequence (accession AM998820) were determined. This previously uncharacterized canine gene showed highest similarity to the mouse *Foxi3* gene. Like most other FOX genes, the coding sequence of the canine *FOXI3* gene is contained within two relatively large exons (II/Fig. S4). The first exon and the putative promoter region are GC-rich (>85%) (II/Fig. S4). Multiple alignments across mammalian FOXI3 protein sequences were performed (II/Fig. S5). The canine FOXI3 protein is predicted to contain 436 amino acids with 74% identity to the murine Foxi3 protein.

Sequence analysis of the *FOXI3* gene in hairless and coated Chinese Crested dogs revealed a 7-bp duplication (c.57_63dup7) within exon 1 leading to a frameshift and a premature stop codon (p.A23fsX219) (II/Fig. 1B and Fig. S4). In order to validate the mutation, an additional cohort of dogs was genotyped, including all three studied breeds (140 hairless and 55 coated dogs) and 19 other breeds (32 coated dogs). The duplication was found to segregate perfectly with the dominant CED phenotype. All the CED dogs were heterozygotes and no homozygotes for the mutation were found.

The identified duplication leads to a frameshift and a premature stop codon quite downstream. Consequently the encoded protein would be truncated and include 218 altered amino acids. More than 95% of the normal protein would be missing and therefore the protein would likely be disfunctional. Thus, the CED phenotype in hairless dogs is possibly caused by haploinsuffiency of FOXI3. The lack of dogs which are homozygotes for the mutation is consistent with the previous hypothesis of the lethality of the homozygous genotype and thus, the results also indicate that half the amount of the protein is not enough to fully accomplish the functions of FOXI3.

4.2.2 Identification of *FOXI3* mutation indicates an essential function for *FOXI3* in the development of ectodermal organs

Identification of the *FOXI3* mutation as a causative gene for the CED and its specific expression in the developing teeth and hair follicles suggested a novel and significant function for FOXI3 in the development of these ectodermal organs. It also provides a novel candidate gene for human ectodermal dysplasias.

FOXI3 mutation has been found only in three breeds of dogs with similar hair and tooth phenotype and other ectodermal anomalies that are not described by the breed standards. The three breeds also are presumed to be closely related, based on their history, and actually could be variations of the same common ancestor. This

further suggests that the mutation in *FOXI3* is again an ancestral mutation with a common origin.

4.3 Regulation of *Foxi3* expression during ectodermal development in mouse (III and unpublished data)

4.3.1 *Foxi3* expression in mouse

Prior to identification of the mutation in *FOXI3* as causative for CED, only one article was published about the mammalian *Foxi3* describing the expression during early mouse embryogenesis (E6.5 to E10.5) and suggesting a role for Foxi3 in pharyngeal organ development and specification of cranial placodes [91]. Therefore, the function of *Foxi3* was explored here, commencing with a detailed expression pattern of *Foxi3* during the developmental stages of tissues that are relevant for the development of the ectodermal organs focusing on developing teeth, hair follicles, and mammary glands.

4.3.1.1 Foxi3 expression during tooth morphogenesis

Mouse dentition differs from carnivores as it lacks the canines and premolars, and instead consists of one continuously erupting incisor and three molars separated by a toothless diastema region in each jaw quadrant. At E11.5, a thickening of the oral epithelium (dental lamina) marks the future site of the dental arch. ISH of E11.5 mouse embryos showed that *Foxi3* was strongly expressed throughout the dental lamina of the maxilla and mandible (III/Fig. 1A,B). At E12.5 *Foxi3* expression was downregulated in the toothless diastema region but was persistent in the emerging placodes of incisors and molars (III/Fig. 1C and Fig. 5E). As tooth formation progressed, *Foxi3* expression maintained in the entire dental epithelium until E13.5 and was not restricted to the enamel knot signaling center only. Subsequently at the cap stage of molar development, *Foxi3* expression became asymmetric, being more intensely expressed in the lingual side of the tooth bud (III/Fig. 1E). At later stages of molar morphogenesis (E17-E18), *Foxi3* was expressed at high levels in the inner enamel epithelium (III/Fig. 1F,G), which marks the precursors of enamel producing ameloblasts. In addition, a weaker expression was detected in the outer enamel epithelium and in stellate reticulum (III/Fig. 1F,G). By postnatal day 2, *Foxi3* expression was downregulated from the differentiated ameloblasts of the molars but remained in the stellate reticulum cells, in the stratum intermedium, and in the cervical loops (III/Fig. 1H). In conclusion, *Foxi3* expression was confined only to the epithelial compartments starting from the first developmental stages and continuing until the late stages of dental morphogenesis. The expression was downregulated, however, from the cells of the inner enamel epithelium when they differentiated into ameloblasts. This suggests that Foxi3 has an impact in the initiation and morphogenesis of teeth but not in enamel formation.

4.3.1.2 Foxi3 expression during hair follicle morphogenesis

In Study II, *Foxi3* was demonstrated to be expressed in the follicles of developing vibrissae and hair follicles by whole-mount ISH [133]. More thorough expression analysis with non-radioactive and radioactive ISH on paraffin sections showed that *Foxi3* expression was present in the hair placodes of all three hair follicle types (III/Fig. 2) which form in separate phases at E14.5, E16.5, and around birth [134]. In mouse, hair follicle development is initiated at E14-14.5 with the formation of the first, primary, hair placodes that give rise to guard hairs. At this stage, E14.5, *Foxi3* expression was clearly seen in the placodal epithelium of these primary hair follicles (III/Fig. 2A,B). At E16, the expression appeared to the secondary hair placodes that give rise to awl/auchene hairs, but also remained in the growing primary hair follicles (III/Fig. 2C). At E19, *Foxi3* expression was detected in all hair follicle types including further maturing primary and secondary, and nascent tertiary hair placodes that give rise to zig zag hairs (III/Fig. 2D). It is noteworthy that throughout the organogenesis of both teeth and hair follicles, the expression was confined only to the epithelial cells.

4.3.1.3 Foxi3 expression in other ectodermal organs

Foxi3 expression was also analyzed during other ectodermal organ development, namely during morphogenesis of mammary, salivary and sweat glands. In mice, mammary gland development is initiated at around E11 starting from the most posterior pair of glands. By E12.5 all five pairs of mammary buds have formed [135]. *Foxi3* expression was detected in the epithelium of all mammary buds by radioactive ISH at E12.5 (III/Fig. 3A and 4H). However, the expression was no longer detected at later stages when mammary gland morphogenesis had progressed (III/Fig. 3B and 4K). *Foxi3* expression was also detected in a subset of epithelial buds at E14.5 by radioactive ISH, apparently representing the sublingual gland, as well as in minor salivary gland buds (III/Fig. 3C,D). Low levels of *Foxi3* transcripts were detected in developing eccrine sweat glands at E18.5 similar to *Edar* expression (III/Fig. 3E,F).

Whole-mount ISH on whole mouse embryos and tissues revealed *Foxi3* expression in several other tissues as well. *Foxi3* expression had been previously demonstrated in the region between maxilla and mandible, in the branchial pouches and the dorsal part of the optic cup at E9.5 to E10.5 [91]. In addition to these sites, an intense expression around the anal opening was observed at E9 (III/Fig. 3G). Moreover, the expression in the optic cup was also maintained at later stages of eye development (until E16.5) (III/Fig. 3H-J). The expression was strongest in the dorsal and ventral regions of the optic cup at E11.5, but later became restricted to the most anterior parts. This spatiotemporal expression pattern of *Foxi3* in the eye suggests that it also has a function in the development of the neural retina. In addition, *Foxi3* was detected in the epithelium of the maxillary process and in the nasolacrimal groove at E11.5 (III/Fig. 3H,K) and at E14.5 at the sites of nail primordia and around

future tactile pads (III/Fig. 3L). The tactile pads begin to develop at this stage and will eventually give rise to footpads.

4.3.2 Foxi3 has a function in multiple organ systems (unpublished data)

In addition to teeth and hair follicles, these expression analyses indicate a possible function for Foxi3 in a few other ectodermal organs and together with the previously published expression analysis [91] in other organ systems as well. There is no scientific evidence that the canine *FOXI3* mutation would alter other ectodermal organs than tooth and hair follicle. A recent article described histological characteristics of hair follicles and glandular tissues of Chinese Crested dogs [57] demonstrating that the hairless dogs with *FOXI3* mutation had abnormal hair follicles whereas the apocrine glands in the skin and respiratory mucous glands were normal. However, the canine phenotype is caused by a heterozygous *FOXI3* mutant allele likely representing a haploinsufficiency and therefore it is expected that the canine phenotype does not correlate completely with the functions of FOXI3 in different organ systems. The homozygous *FOXI3* mutant dogs die during embryogenesis [136] and so do the *Foxi3*$^{-/-}$ transgenic mice (personal communication with Andrew K. Groves), which implies severe defects in vital organ systems due to complete loss of Foxi3 function. Interestingly, Letard [55] has reported that as a result of experimental breeding of two hairless dogs some non-viable puppies with severe orobuccal malformations were born. This raises a question as to whether these puppies were homozygous for *FOXI3* mutation or maybe just very severily affected heterozygotes. Most likely they, indeed, were homozygotes as the *Foxi3*$^{-/-}$ embryos have very severe inner, middle, and external ear as well as lower jaw phenotype (personal communication with Andrew K. Groves). Puppies with equally severe anomalies have not been reported by breeders of hairless dog breeds, altough mild malformations of the outer auditory canal (closed ear canal) and ear lobe are not rare findings (The Society of Finnish Chinese Crested Dog, http://www.kiinanharjakoirat.fi, and personal communication with breeders of Mexican and Peruvian Hairless Dogs). We have also performed a clinical examination, CT scan, and autopsy for one Peruvian Hairless dog that was euthanized at the age of 4 weeks due to auditory defects (unpublished data). The examinations showed that the the dog lacked an outer auditory ear canal and had small tympanic cavity on the right side but had normal hearing and ear anatomy on the left side (Fig. 9). This suggests that *FOXI3* might also have a role in the morphogenesis of external structures of ear. Interestingly, some dogs with missing anal opening also have been reported by the breeders in all three breeds. As mouse *Foxi3* was expressed around the anal opening as early as E9, it can be suggested that Foxi3 regulates the formation of this ectodermal structure as well.

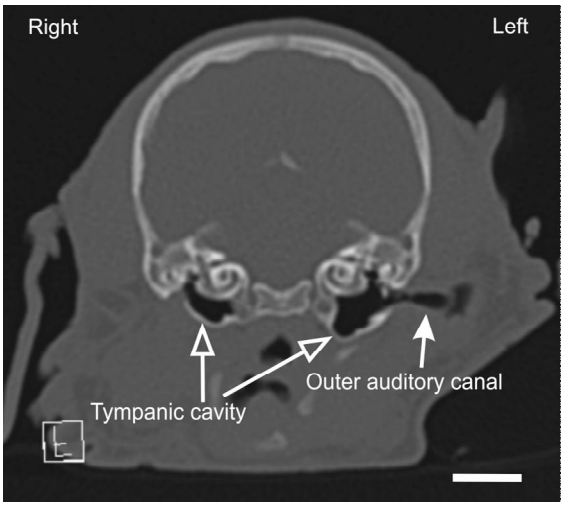


Figure 9. Auditory structures of a Peruvian Hairless puppy. CT scan demonstrates that the outer auditory canal is missing and tympanic cavity is smaller on the right side compared to the normal structures on the left side of the head. Scale bar is 1 cm.

4.3.3 *Foxi3* lies downstream of *Eda* in skin appendage placodes and buds

Eda/Edar signaling pathway has a significant role in the development of tooth and hair follicle and the mutations in these genes are the most common cause of human HED [18,71]. In dogs, the *FOXI3* mutation results in ED indicating Foxi3 to be essential in ectodermal organogenesis. Additionally, similar to *Foxi3*, expression of *Edar* and activity of transcription factor NF-κB, the essential downstream mediator of *Eda/Edar*, is high in all skin appendage placodes [71,137]. These similarities promoted the exploration of the interplay and hierarchy in the Foxi3 and Eda/Edar signaling pathway. *Eda*-deficient and overexpressing mouse lines were employed to explore this. *Eda*-deficient mice lack primary hair placodes and have hypodontia and abnormally shaped teeth [72], whereas *Eda*-overexpressing mice (K14-*Eda*) are characterized by enlarged hair and/or ectopic hair, tooth and mammary placodes [73,74]. The expression of *Foxi3* in tooth, mammary buds and hair follicles was assessed in *Eda* mutant embryos compared with wild type controls. *Foxi3* expression was reduced in the tooth buds of *Eda*-deficient and increased in K14-*Eda* embryos at E13.5 compared with control littermates (III/Fig. 4A–C). Similar correlation was seen in the mammary buds at E12.5 (III/Fig. 4G–I).At E13.5 when *Foxi3* expression was no longer detected in control and *Eda* null mammary buds, it was readily observed in the endogenous and ectopic mammary buds of K14-*Eda* mice (III/Fig. 4J–L).No *Foxi3* expression was detected in the dorsal skin of *Eda*-deficient embryos at E14.5 (III/Fig. 4D), which is not suprising as the primary hair placodes are missing in these mutants. Instead, the expression was slightly increased in the primary hair placodes of K14-*Eda* embryos compared with control littermates (III/Fig. 4E,F). These findings suggest that *Foxi3* lies downstream of *Eda* signaling but whether *Foxi3* is a direct target of Eda cannot be verified based on these experiments.

The ability of recombinant Eda protein to upregulate *Foxi3* expression in E14.5 *Eda*$^{-/-}$ skin explants was analyzed in order to determine whether *Foxi3* could be a direct target gene of *Eda* [76,138]. Skin explants dissected from E14.5 mouse embryos were treated with Eda protein added to the culture media and the *Foxi3* expression was measured by RT-PCR at two different time-points and compared to the *Foxi3* mRNA levels in untreated control explants. Only a 2h-exposure to Eda led to a 12-fold induction of *Foxi3* mRNA level and >20-fold induction after 4h-exposure (III/Fig. 4M). This identifies *Foxi3* as one of the most rapidly upregulated Eda-induced genes [76,125,138]. The swift and pronounced induction of *Foxi3* expression by Eda strongly suggests that *Foxi3* is a direct transcriptional target of the Eda pathway.

Possible NF-κB binding sites were sought from the *Foxi3* promoter region with the Consite program (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite/) in order to gain further evidence for the direct regulation of *Foxi3* by Eda signaling. Indeed, several putative NF-κB sites were found in the *Foxi3* promoter region in all mammalian species tested. One of these sites (GGTGCTTTCC in the mouse), located near the 5' end of the sole *Foxi3* intron, was conserved in multiple species (data not shown). However, all the found elements are solely putative binding sites and further studies will be required to determine their functional relevance.

4.3.4 *Foxi3* expression is unaffected in mice with increased Wnt signaling activity

Together with the Eda/Edar pathway, canonical Wnt signaling regulates hair follicle development and also has an essential function in tooth and mammary bud formation [139]. The expression pattern of *Foxi3* in the developing ectodermal appendages has been reported to overlap with the sites of high Wnt/β-catenin signaling activity *i.e.* hair, tooth and mammary placodes and buds [84,140,141]. Therefore, the possibility that Wnt signaling could regulate *Foxi3* was investigated by *Foxi3* expression analysis in the embryos of two transgenic mouse lines, *β-cat*$^{\Delta ex3K14/+}$ and *Sostdc1*$^{-/-}$. In *β-cat*$^{\Delta ex3K14/+}$ embryos forced stabilization of β-catenin in the embryonic ectoderm causes formation of supernumerary enamel knot signaling centers leading to continuous tooth generation, as well as precocious and ectopic hair follicle development [84,85]. *Foxi3* expression was observed both in the ectopic signaling centers in the oral epithelium and in the ectopic hair placodes at E14.5, but no differences were seen in the expression levels between the *β-cat*$^{\Delta ex3K14/+}$ and control embryos (III/Fig. 5A–D).

Sostdc1 (also known as ectodin and Wise) is an antagonist of both Bmp and Wnt signaling pathways [142,143]. *Sostdc1*-deficient mice feature extra incisors, premolar like teeth, enlarged hair placodes, and ectopic whisker buds that are thought to be caused by elevated Wnt/β-catenin signaling activity [86-89]. Whole mount ISH did not reveal changes in the expression level of *Foxi3* in the tooth and hair placodes of *Sostdc1*$^{-/-}$ embryos compared to the wild type embryos (III/Fig. 5E–H). These results together suggest that Foxi3 is not a target of the canonical Wnt pathway.

4.3.5 Activin A regulates *Foxi3* expression *in vitro*

Other molecular mechanisms that could regulate *Foxi3* expression, in addition to *Eda* signaling, were exlopred. The targets of interest were the signaling pathways with an important function in ectodermal organ development: activin, Bmp, Fgf and Shh signaling [69,70]. Protein-absorbing beads on wild-type mouse embryonic skin cultures were used and *Foxi3* expression was detected by whole mount ISH (III/Fig. 6A–E). The beads soaked in activin A, Bmp4, Fgf4, Fgf10, or Shh were placed on top of the skin explants separated from mouse embryos at E13.5, just prior to the induction of primary hair follicles, and the explants were cultured for 24 hours. The whole mount ISH of the cultured explants revealed a strong induction of *Foxi3* expression in the epithelium around the activin A -releasing bead (III/Fig. 6D). Fgf4, Fgf10, or Shh had no effect on *Foxi3* expression (III/Fig. 6A,B and data not shown). Bmp4-releasing beads have previously been shown to inhibit hair placode formation [76]. It has been, however, shown that Bmp4 is able to induce target gene expression in the tissue surrounding the bead [143]. As expected based on these previous results, there was no hair placode formation around Bmp4-soaked bead, but no ectopic induction of *Foxi3* expression in the tissue either (III/Fig. 6C).

In the bead experiments, the exposure time of the protein to the tissue is relatively long and the expression cannot be quantitatively measured by ISH. Thus, the observed results reveal a long-term overview of the effect of the tested protein. Therefore, a similar approach as with Eda was used to investigate whether the upregulation of *Foxi3* is due to a direct effect to activin A exposure. Skin explants from E14.5 mouse embryos were exposed to activin A and the *Foxi3* expression was measured by RT-PCR after two and four hours. The *Foxi3* mRNA levels were compared to corresponding levels of untreated skin explants. Follistatin was used as a positive control as it is a known transcriptional target of activin. The quantitative measurements were consistent with the bead expriments as the activin A exposure produced a statistically significant 1.6-fold and 2.2-fold increase in *Foxi3* mRNA levels after two and four hours, respectively (III/Fig. 6F). Follistatin showed an increase of 4.6-fold (III/Fig. 6F). Activin A has been proposed to regulate *Edar* expression [82,144], and thus the upregulation of *Foxi3* after activin A exposure might be caused as a secondary effect through Eda signaling. Therefore, *Edar* mRNA levels after activin A exposure were also assessed. Expression of *Edar* turned out to be unaffected (III/Fig. 6F). Subsequently, to further confirm that activin A can induce *Foxi3* expression independent of the Eda pathway, the activin A bead experiments were repeated using *Eda* null skin explants. As a result, activin A induced *Foxi3* expression in 5 out of 15 explants whereas BSA bead had no effect (0 out of 7 explants) (III/Fig. 6G,H).

The results from short- and long-term protein induction experiments indicate that activin A is a putative upstream regulator of *Foxi3*, which suggests a functional link between activin A and Foxi3 and imply that activin A can regulate the expression of *Foxi3* in hair placodes independent of Edar.

4.3.6 Future aspects of exploring the functions of Foxi3

The aim of this study was to explore the function of Foxi3 in mammalian embryogenesis. Therefore a detailed expression pattern of murine *Foxi3* during the development of the ectodermal organs was constructed as well as tissue culture experiments and expression analyses with mouse embryos. The results imply that Foxi3 functions in the development of several ectodermal appendages, namely hair follicle, tooth, mammary and salivary gland, nail, but also in some other tissue compartments such as eye. The findings of this study suggest that *Foxi3* lies downstream of *Eda* signaling and could even be a direct transcriptional target of the Eda pathway. In addition to Eda, activin A was indicated to be a putative upstream regulator of *Foxi3*.

The almost total lack of hair especially in Mexican and Peruvian Hairless dogs is more prominent than the phenotype of canine X-linked ectodermal dysplasia caused by *EDA* mutation [60]. This further suggests that FOXI3 is not solely a target of Eda/Edar signaling pathway but it is regulated by additional factors, such as Activin A, as shown in this study, but maybe other signaling pathways as well. Downstream targets of FOXI3 remain to be explored, but based on the CED phenotype caused by the haploinsuffiency of FOXI3, these targets should be essential for the ectodermal development, and based on the expression pattern in mouse, could regulate the development of the whole facial region as well.

In the future, the role and regulation of during the development of ectodermal organs will be more thoroughly explored. A transgenic mouse model, that has already been developed, will provide a valuble tool to unravel the function of Foxi3 and its regulators and targets. The knowledge about the function of Foxi3 in different organ systems using mouse model will be of great interest to developmental scientists. Moreover, a detailed characterization of dogs with CED could bring some new information. Great attention is paid to see whether any *FOXI3* mutations will be found in human ectodermal dyplasia or other disease patients.

4.4 Identification of a novel GUSB mutation defines the hereditary skeletal disease in Brazilian Terriers as mucopolysaccharidosis VII

A litter of seven Brazilian Terriers aged 4 weeks was examined in the Animal Hospital of the University of Helsinki because three of the puppies showed severe ambulatory defects. Later, several other related Brazilian Terrier litters with similar puppies were born and altogether 15 affected dogs from 8 different litters were confirmed to have similar clinical signs, although, the severity of the phenotype varied even among the affected littermates. The congenital and progressive features became evident within the first four weeks of life. The affected puppies showed normal behavioural activities and appetite but had severe ambulatory difficulties. All affected puppies presented similar phenotypic characteristics with brachycephaly, dwarfism, deformed legs with crooked radiocarpal joints and prominent joint

hyperlaxity of the limbs (IV/Fig. 1A,C). Due to poor prognosis the affected puppies were euthanized between 2 to 6 weeks of age except for two puppies that died spontaneously at the age of 1 and 3 weeks for unknown reasons. Both sexes were equally affected.

4.4.1 Clinical and histopathological examinations indicate spondyloepiphyseal dysplasia

Radiographs of seven affected dogs and three age- and sex-matched controls were compared to explore the cause for the structural abnormalities (IV/Fig. 2A-D). In addition, radiographs were taken from the dam of the first litter. Epiphyseal dysplasia of long bones and vertebral endplates with delayed ossification of the epiphyseal cartilages was evident in all affected dogs (IV/Fig. 2A,C). In addition, the ossification centers of the cuboid bones of the carpus and tarsus were reduced in size when compared to normal littermates (IV/Fig. 2A). Marked carpal and tarsal joint effusion was seen in all affected dogs. The craniofacial abnormalities included short maxilla (retrognathia superior) and widened calvaria (IV/Fig. 2E). Skeletal radiopacity was generally slightly decreased in the affected dogs, but the cortices of the long bones appeared normal. Ophthalmoscopic examination was performed for the three affected puppies from the first litter but revealed no pathological changes in the eyes. The affected puppies showed growth retardation weighing 35% less than their healthy littermates at the age of three weeks (IV/Fig. 1E).

Postmortem tissues were collected from eight euthanized puppies for histopathological analyses. Histological examination of the skeletal structures of the affected puppies showed that secondary ossification centers in the epiphyses were either small or completely lacking and occassionally containing regions of loose fibrous tissue (IV/Fig. 2G,I). Similarly, the ossification centers of the carpal and tarsal bones were either lacking or were small and included thin and irregular primary trabeculae. The vertebral bodies occasionally had irregular cartilage columns in the growth plate and inclusions of loose fibrous tissue (IV/Fig. 2J). No gross abnormalities in the other tissues were found.

4.4.2 GWAS maps the disease to CFA6

A large pedigree was established tracing all the affected dogs back to a common Brazilian ancestor (IV/Fig. 3). Both males (n=7) and females (n=5) were equally affected. The affected puppies were always born to healthy parents and many litters included several affected puppies. The proportion of the affected dogs in the litters was 31,6%, which is close to an average of 25% assumed for a recessive condition. Therefore, an autosomal recessive mode of inheritance was assumed to be the most feasible choice for the mode of inheritance.

We performed GWAS to map the disease locus and genotyped altogether 18 Brazilian Terriers including 7 cases and 11 controls using Illumina's 22K canine SNP array. A case-control association test revealed significant association on CFA6

with the best SNP (BICF2G630808208) at 7,265,846 bp (p_{raw} = 1.86 x 10^{-5}, p_{genome}= 0.03313) (IV/Fig. 4A). All affected dogs shared a long homozygous haplotype adjacent to the centromeric end of the chromosome (CFA6:3,341,099-16,324,133 bp) (IV/Fig. 4B). Moreover, the three obligate carriers (parents of the affected dogs) were heterozygous for the haplotype, as expected. The haplotype contained 137 SNPs of which none showed a complete segregation between the cases, obligate carriers and controls.

4.4.3 Next generation sequencing identifies a missense mutation in *GUSB*

The associated locus contains a large number of genes (>210) and many of them were considered as potential candidate genes (IV/Fig. 4B). Two of them, Procollagen C-endopeptidase enhancer (*PCOLCE*) and SMAD ubiquitin regulatory factor 1 (*Smurf1*) were selected for further investigation but sequencing did not reveal any segregating coding variants in either of the genes. Subsequently, a targeted sequence capture was performed, in addition to next generation sequencing for the whole 13-Mb associated region. Two cases and two controls with opposite haplotypes were selected for capturing (IV/Fig. 4B). After the variant calling pipeline (VCP) analysis of the sequencing data, altogether approximately 13,000-19,000 SNPs and approximately 5,000 indels were evident for each dog (IV/Table 1). In addition, sequence data for 32 healthy dogs from four different breeds were available to be used as controls. Comparison of the variants between cases and controls revealed two novel homozygous exonic SNPs, one deletion, and one insertion, which were all unique for cases. The 9-bp deletion was located in the monoacylglycerol O-acyltransferase 3 (*MOGAT3*) gene (at position BROADD2:6:11,681,043), which is an intestinal specific enzyme implicated in fat absorption [145]. The 3-bp insertion was located in the olfactory receptor, family 2, subfamily AE, member 1 (*OR2AE1*) gene (at position BROADD2:6:12,694,743). These as candidate genes were excluded due to their known specific function outside the skeletal system. One of the exonic SNPs was synonymous. The other was non-synonymous and located in the exon 5 (c.866C>T) of the β-glucuronidase (*GUSB*) gene; this missense mutation results in a proline to leucine substitution at amino acid position 289 (p.P289L) (Fig. 10A and IV/Fig. 4D,E).

The *GUSB* gene encodes a lysosomal β-glucuronidase, an enzyme that degrades several GAGs. In human, deficient β-glucuronidase activity causes a lysosomal storage disease, MPS VII. Mutations have, however, been described in many other species as well, including mouse, cat and dog [109,112,117,119,120]. In all of them, the enzyme deficiency leads to lysosomal storage of GAG's in several tissues and several common clinical signs such as skeletal deformities, corneal clouding and hepatosplenomegaly. Dwarfism, joint hyperlaxity and epiphyseal dysplasia have also been reported in the dogs with previously identified MPS VII and these features are consistent with the affected Brazilian Terriers. Therefore, the *GUSB* c.866C>T variant was a plausible mutation.

The GUSB enzyme is a lysosomal homotetramer [110]. Both human and canine monomers consist of 651 amino acids. The structure of human GUSB has been well characterized and shown to contain three functional domains, comprised of residues 22-223, 224-342 and 343-632 (Fig. 10B). The first domain of the GUSB monomer forms a distorted barrel-like structure with a jelly roll motif and two β-hairpin loops; the second domain is similar to the immunoglobulin constant domains, and the third forms the active enzyme site with α/β- or TIM-barrel motif [146]. The canine GUSB protein is 81% similar to human GUSB at the amino acid level [120] and we can assume that the domain structure is similar as well. The identified p.P289L substitution of GUSB in the affected Brazilian Terriers resides in the middle of the second domain (Fig. 10B,C). Multiple alignments performed across eukaryotic and prokaryotic species indicated that the substitution site and its surrounding residues are well conserved (IV/Fig. 5). The disruption of the conserved region by the substitution is likely to impair the GUSB structure and thus function. This was further supported by a bioinformatic analysis of the substitution with PolyPhen-2 software. The effect of the mutation was predicted to be "probably damaging" with a maximum score of 1.0.

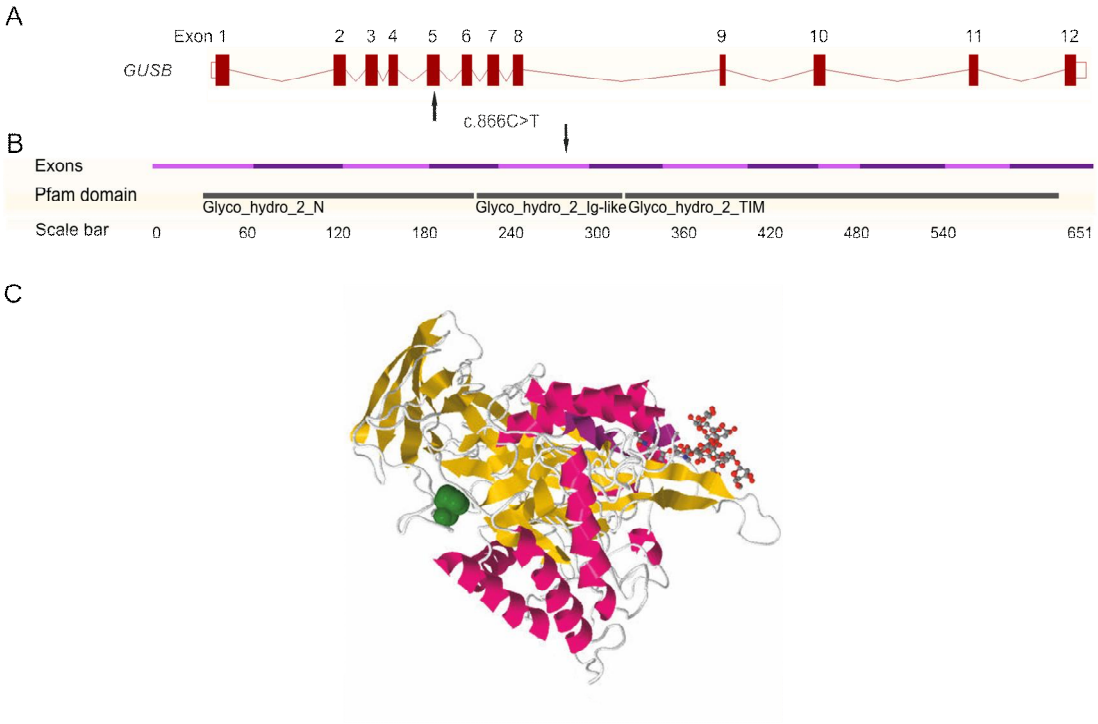


Figure 10. Canine *GUSB* has 12 exons. C.866C>T mutation (arrow) is located in the exon 5 (A) and forms a p.P289L substitution in the middle of the third domain of GUSB protein (B). The p.P289L substitution site is marked as green in a ribbon model of GUSB monomer (C).

A cohort of 202 Brazilian Terriers comprising of 15 cases and 187 controls was genotyped in order to confirm the association of the c.866C>T mutation with the disease (IV/Table S1). As expected, all affected dogs were homozygous and all obligatory carriers (n=12) were heterozygous for the variant. The carrier frequency

among the control population was 28.3%. The results confirmed a significant association of the c.866C>T variant with the disease ($p=7.71 \times 10^{-29}$). In addition, a set of 166 dogs from 36 different breeds was genotyped to further confirm the causality of the mutation (IV/ Table S1). None of these dogs carried the mutation. The complete segregation of the mutation according to the recessive mode of inheritance validates the disease locus identified through GWA.

4.4.4 Elevated urinary GAGs and substantially decreased β-glucuronidase activity in the the affected dogs confirm the pathogenity of the mutation

Deficiency of GAG degradating enzymes, including β-glucuronidase, leads to accumulation of GAGs and subsequently to elevated excretion of GAGs into urine. Therefore, the quantitative measurement of urinary GAGs is commonly used in screening patients for mucopolysaccharidosis. In order to confirm the detrimental effect of the mutation, urinary GAGs were measured from three affected puppies (~6 weeks of age) and three healthy age-and breed-matched wild-type controls (IV/Table S2). The affected dogs had a 2.7-fold increase in GAG levels. In addition, the activity of β-glucuronidase was determined from the serum of the three affected dogs and six control dogs (three carriers and three wild-types) (IV/Table 2). In the affected dogs, the enzyme activity was severely impaired with a mean residual activity of 0.2% compared with the wild-type dogs. Moreover, the carrier dogs also had a decreased level of enzyme activity (67%). These results confirmed the predicted pathogenic effect of the *GUSB* mutation and demonstrated that the disease in Brazilian Terriers is MPS VII.

4.4.5 Identification of the mutation led to the development of genetic test for dogs and provides a large animal model of MPSVII for human medicine

In conclusion, a canine disease causing prominent skeletal defects was described in this study and a mutation in the GUSB gene was identified to be causative. The evident features including multiple skeletal dysmorphisms, joint hyperlaxity and growth retardation in the affected Brazilian Terriers are consistent with the clinical signs in MPS VII affected dogs containing the p.R166H substitution in *GUSB*. Some typical signs of the disease such as corneal clouding, organomegaly and lysosomal vacuolization were not demonstrated in the MPS VII affected Brazilian Terriers in this study. This could be due to the early age of the puppies examined. The disease is progressive due to constantly increasing amount of undegraded GAGs in cells and tissues. Thus, systematic examination of the target organs and tissues of affected dogs at different ages might reveal additional features as well.

This study is an example of a gene mapping resulting in extensive association due to the use of closely related dogs of small inbred population. Number of affected dogs was limited and there was no possibility to take advantage of other breeds to be

used in fine-mapping. This challenged the mutation identification as the critical disease-associated region extended over 13 Mb with over 220 genes. However, using the latest gene technology to capture and resequence the entire associated region, the causative *GUSB* mutation was successfully identified. Consequently, the mutation defined the disease as mucopolysaccharidosis VII establishing a novel canine model for the disease. Enzyme replacement therapy (ERT) and hematopoietic stem cell transplantation (HSCT) have been used as a treatment for several MPSs in human patients [147]. For example, MPS I (Hurler disease) patients are successfully treated by both ERT and HSCT [148,149]. However, there is no efficient treatment available for MPS VII for the moment; there are very few data and no convincing results about the use of bone marrow transplantation for MPS VII [150], whereas ERT for MPS VII has been mentioned to be in development [147]. Interestingly, gene therapy has been quite successfully performed in dogs with MPS VII. It has been demonstrated that neonatal intravenous injection of gamma retroviral vector expressing *GUSB* in dogs significantly increases lifespan, reduces skeletal abnormalities, and improves the mobility throughout the lives of MPS VII dogs [151-154]. The dogs with the *GUSB* mutation identified in this study could as well be exploited for the development and validation of therapeutical methods. This could eventually be advantageous for both dogs and human patients.

The pedigree data indicated that all affected dogs had a common Brazilian ancestor, suggesting that the *GUSB* mutation has arrived to the Brazilian Terrier population in Finland along with the dogs imported from Brazil. Since the discovery of the mutation, affected puppies have also been reported in Brazil. The mutation has not been found in other breeds so far, suggesting that it has occurred inside the Brazilian Terrier population. The *GUSB* mutation is yet another example of an ancestral mutation that has spread throughout the whole population of the breed. Identification of the mutation enabled the development of a genetic test to eradicate the disease from the breed. The carrier frequency in the study cohort was high, nearly 30%. As a result of this study genetic testing of *GUSB* mutation in dogs is now possible. With the help of genetic testing it is possible to avoid the disease manifestations while retaining the carrier dogs in breeding and thus maintaining the genetic diversity.

5 Concluding remarks

This study is rich in methodology, covers different fields of science including genetics, molecular biology, developmental biology and veterinary medicine, and has several theoretical and practical implications. Unique canine genomic system has been efficiently utilized here, along with genomic resources, to identify new genes in developmental and disease traits. This study combines clinical and basic research to describe new disease phenotypes and to characterize their genetic causes. In addition to new genes, a new disease mechanism and biological pathway that regulate important developmental processes have been revealed. The clinical and genetic similarities of canine and human disease are demonstrated in this work, and as such, new large animal models have been established for possible therapeutic studies that may impact human medicine. Finally, this study has provided new tools for veterinary diagnostics and has changed breeding practices in the affected breeds for the improvement of their health.

In the beginning of this study, the high quality assembly of canine genome had recently been annotated, providing the necessary genomic resources for gene mapping studies, including the first low-density SNP arrays. Using less than 30K SNPs, many monogenic loci were identified in small sample cohorts but mapping of the susceptibility loci in polygenic traits remained more challenging [155]. Current high-density arrays provide even better resolution and may help to identify loci for complex traits in larger study chorts. Combination of unique canine population structure and fast developing genetic tools for canine research has turned out to be a powerful recipe for discovery of genes. The next generation sequencing approaches are becoming routine in many research laboratories with appropriate bioinformatics and computational resources and may further boost canine genetics by improving resolution in studies.

Many of the mutations causing breed characteristic phenotypes have arisen a long time ago and drifted to several different populations as these characteristics ended up being favoured by man in many breeds. Two examples of these ancestral mutations were included in this study; *T* mutation causing short tail in several breeds, and *FOXI3* mutation causing the hairless phenotype in three breeds. Both of these represent mutations, which result in the desired phenotype as heterozygous but lethality as homozygous. This study confirmed the lethality of these homozygous mutations and thus has had an impact on the breeding practices of the involved breeds. The breeders are now aware of the possible effects of these mutations and the inheritance. The breeding of two short-tailed dogs has been prohibited in the breeds with T mutation due to the possibility of producing severely defected puppies. What comes to the Mexican and Peruvian Hairless dogs, the coated representatives of the breeds have been accepted as part of the breeding program.

This study demonstrates the characteristics of the canine genomic system. The population bottlenecks created during domestication and creation of modern breeds established a specific population structure seen today in the canine genome. The

typical canine genome is featured by long LDs within the breed and short LDs across the breeds, which facilitates gene mapping if the studied phenotype exists in several breeds. This genomic feature could be exploited while searching the mutation for the CED in different hairless breeds. As expected the additional breeds improved the resolution in the associated region first mapped within a breed.

Inbred populations are powerful for gene mapping but may result in extensive association that challenges the mutation discovery. This was experienced in the small Brazilian Terrier breed, in which the critical disease-associated region extended over 13 Mb with over 220 genes. Without any possibility to use other breeds for fine mapping, it presented a remarkable challenge to identify the mutation in the gene rich region. However, using the latest gene technology to capture and resequence the entire associated region, the causative *GUSB* mutation was successfully identified.

This study has several practical implications. Two genetic tests have been developed to assist breeders to avoid the detrimental conditions in the breeds. Genetic testing for MPS VII enables the eradication of the lethal skeletal condition from Brazilian Terriers and represents a striking example of how a small breed with a limited genetic diversity can benefit from genetic research and genetic testing. The prevalence of the mutation carriers was very high in the breed (28%) and now affected dogs can be avoided. Moreover, the carrier dogs can be safely preserved in breeding programs to maintain the limited gene pool.

The genetic testing of *T* mutation has found its place in breeding plans, although the phenotype is a favored characteristic in many breeds. The reason for the use of the genetic testing is that tail docking is prohibited in several countries, including Finland. Therefore a dog that has a bobtail and belongs to one of the breeds that have the *T* mutation, has to have a certificate based on either a veterinary examination or now, a genetic test to prove its congenitaly. This study has endorsed changes in the breeding recommendations. Breeding of of two short-tailed dogs has been prohibited since 2009 by the Finnish Kennel Club, since it may result in the born of severely affected puppies. Genetic testing can now resolve uncertain cases, for instance if a short-tailed dog has been exported from a country where tail docking is allowed the genetic test is the only way to tell whether the dog is a natural bobtail. This study has also significantly extended the list of breeds that benefit from the genetic testing for short tail.

This study demonstrates the value of canine models to developmental biology. The extent of morphological variation in dogs is unique and different dog breeds carry numerous interesting phenotypes for developmental biology. Some of the traits are fixed in the breeds and can be efficiently mapped by across breed approaches such as sweep mapping. Some conditions are harmful or even lethal and often breed-specific as evidenced in this study. The causative mutation has been identified only for the fraction of the existing morphological traits and more traits are continuously being characterized. Dog breeds contain a wealth of valuable genetic information for developmental biologists and geneticists and offer possibilities to find new genes and pathways for comparative studies across species including human.

This study also has an impact on human medicine by providing new candidate genes for corresponding human conditions and by establishing a new large animal model for potential therapeutic purposes. The two genes studied here, *T* and *FOXI3*, do not yet have clear association to equivalent diseases in humans. However, both genes have been shown to play essential roles in several developmental processes. Furthermore, the detrimental effect of the mutations in these genes leading to embryonic lethality might even be the reason for the lack of identified human mutations. Instead, the canine MPS VII could be considered as a worthy model for human disease. Whereas some forms of mucopolysaccharidoses have been treated with enzyme replacement therapies, there is no efficient treatment for MPS VII. The affected Brazilian Terriers could potentially serve as models for the development of therapy for this type of MPS.

Future follow-up experiments are currently focused on the role and regulation of *Foxi3* in ectodermal processes. A transgenic mouse model has already been developed and several lines of functional analyses are ongoing. The knowledge about the role of Foxi3 in different organ systems will be of great interest to developmental scientists. Notably, histological examination of selected tissues of dogs with CED has also been performed very recently and more phenotyping studies could bring some new information of the correlations of the *Foxi3* genotype and phenotypes across species. It will also be interesting to see whether any *FOXI3* mutations will be found in human ectodermal dyplasia patients or perhaps in the cases of other diseases. Other future experiments may relate to the mapping the genes for the short-tail phenotype in the breeds not harbouring the *T* mutation. Identification of the causative genes, whether novel or not, could indicate their developmental roles not only in tail morphology but possibly behind other traits as well.

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