The origin of the Ridge and Associated Anomalies in Rhodesian Ridgebacks

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Abstract


The thesis presents studies on the inheritance of the dorsal hair ridge and Dermoid Sinus (DS) in Rhodesian Ridgeback dogs. DS is classified as a neural tube defect in humans. Thus, the dog is proven to be an excellent comparative model regarding neural tube defects. It was shown that the hair ridge is caused by an autosomal dominant mutation that predisposes for DS. Collection of material from DS-affected Rhodesian Ridgeback puppies, their parental animals and littermates was performed. Evaluation by histopathology to confirm the presence of DS was conducted. Results revealed that DS (in dogs) were located in the cervical region and that a novel skin lesion (previously referred to as DS), denoted Lipoma of the terminal filum (with skin-dimple and extra-spinal connection) (LTF) was located in the sacral region. A common genetic origin between DS and LTF was suggested. It was proposed that different types of DS and LTF may be caused by differences in FGF levels in combination with different genetic backgrounds and environmental interactions. Samples from eleven DS-affected Rhodesian Ridgebacks and nine ridgeless Rhodesian Ridgebacks were genotyped by a dog-specific genome-wide association analysis utilizing an array of 26,500 SNPs. Association between a 750 kb region and the ridge phenotype was identified. The region contained five genes \( FGF3, FGF4, FGF19, ORAOV1 \) and the 3'-end of \( CCND1 \). Further fine-mapping of the identified region, utilizing the recently developed multiple ligation-dependent genome amplification (MLGA) technique, enabled identification of the mutation causing the ridge. It was shown that the dorsal hair ridge in ridgeback dogs is caused by a 133 kb duplication of three fibroblast growth factor genes \( FGF3, FGF4 \) and \( FGF19 \) and the \( ORAOV1 \) gene. Dogs homozygous for this copy number variation mutation have an increased risk of developing DS, a neural tube-like defect. The hair ridge and development of DS is most likely caused by a gene dosage effect of increased FGF expression during a critical phase of dermal development. Nucleotide sequence analysis of the internal breakpoint of the duplication further showed that the ridge mutation was identical in Rhodesian- and Thai Ridgebacks, revealing a common origin of the mutation in the two breeds. Ridgebacks homozygote for the ridge mutation, have an increased susceptibility to develop DS and/or LTF. Further studies regarding the genetic complexity of DS and LTF will shed light on the biological complexity of these dermal lesions.

Keywords: dermoid sinus, neural tube defect, lipoma of the terminal filum with skin-dimple and extra-spinal connection, genome-wide association analysis, duplication, MLGA.

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To my husband Anders and
the Rhodesian Ridgeback puppies that were autopsied.
Appendix

This thesis is based upon the following papers, which are referred to in the text by their Roman numerals:


Abbreviations

- **bp**: Base pairs
- **CCND1**: Cyclin D1
- **cDNA**: Complementary DNA
- **Ct**: Cycle threshold
- **DNA**: Deoxyribonucleic acid
- **DS**: Dermoid sinus
- **FGF3**: Fibroblast growth factor 3
- **FGF4**: Fibroblast growth factor 4
- **FGF19**: Fibroblast growth factor 19
- **GWAA**: Genome-wide association analysis
- **LEF**: Lymphoid enhancer factor
- **LTF**: Lipoma of the terminal filum with skin-dimple and extra-spinal connection
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<th>Term</th>
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<td>mtDNA</td>
<td>Mitochondrial DNA</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>NTD</td>
<td>Neural tube defect</td>
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<tr>
<td>ORAOV1</td>
<td>Oral Cancer Over-expressed Gene 1</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>RTQ-PCR</td>
<td>Real-time quantitative PCR</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>Homozygote ridgeless</td>
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<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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Introduction

The ridge phenotype and breed history of the Rhodesian Ridgeback dog

The dorsal hair ridge is found in two dog breeds registered by the The Fédération Cynologique Internationale – The Rhodesian Ridgeback and the Thai Ridgeback. The ridge, in the Rhodesian Ridgeback constitutes of three components; two symmetrical crowns, a box and a tail. The hair of the tail grows in the opposite direction of the normal coat, the left crown rotates counter-clockwise and the right crown rotates clockwise (Fig. 1).

The ridge phenotype is congenital, i.e. no changes regarding ridge phenotype occur in the growing dog, and should be dorsally located between withers and the hips according to breed standard for Rhodesian Ridgeback dogs. A breed standard is often a very detailed description set to ensure that dogs are bred according to the specifics of the breed. Individuals deviating from the breed standard are classified as faulty, which in many instances results in exclusion from breeding. Thus, the most common faulty ridges found in Rhodesian Ridgebacks are the ones displaying three crowns (Salmon Hillbertz, data not shown). Ridgeless individuals are produced, thus less common are the ridges in which four or more crowns are present (Fig. 2). The ridge, according to the 1926 breed standard, was a fiddle ridge (3-4 crowns). Further, it was stated that dogs; “without a clearly defined ridge” were not recognized. During 1948, the breed standard was rephrased in some aspects, whereas one was the first mentioning of two symmetrical crowns; “should contain two identical crowns opposite each other” (Murray, 1989).

A dorsal ridge may infrequently be found in other breeds, such as the Boxer and the Bavarian Mountain scent hound, thus the ridges are most commonly located in the cranial/cervical region (Fig. 3). The historical origin of the ridge has been largely debated. Gwatkin (1934) hypothesized that the ridge originated from Asia, i.e. the extinct Phu-Quoc dog. Hubbard (1948) hypothesized that the ridge was of African origin, i.e. the extinct Hottentot Khoi dog, which was involved in the creation of the modern Rhodesian Ridgeback. Epstein (1937) discussed the possibility of parallel mutations which could have occurred in both Asia and Africa. Available English literature regarding the origin of the Thai Ridgeback is extremely scarce. Around year 1660, “powerful dogs” were imported to South Africa by the Europeans from Java to protect the fenced cattle (Theal, 1900). In the early 1860, hound dogs (Bloodhounds such as Cuban Bloodhound, and Schweishunden such as the Bavarian and Hanoverian types, Staghound, Greyhound, Deerhound and Foxhound), Great Dane, Bulldogs, Terriers, Mastiffs, Labradors and Pointers were found at European military posts in South Africa (Hubbard, 1948; Hawley, 1984; Helgesen, 1991). The typical Boer farm dogs (some carried the ridge, others were ridgeless) were steekbaard- (stick beard) and vuilbaardhonde (dirty beard) (Hawley, 1984) which originated from the Boerhond. It is most likely that several of the European dog breeds were bred to the indigenous Hottentot Khoi dog, as many European dogs were not as adaptable to the African environment as the indigenous dogs (Helgesen, 1991).
During the 1930ies, two reports emerged from England and South Africa, which described a congenital skin-condition identified in Rhodesian Ridgeback families. The condition was described as tubular growths with openings on the skin surface (Hare, 1932; Steyn et al., 1939). The congenital nature and its clinical appearance suggested a developmental origin of the skin lesion.

Extensive registries of the Swedish population of Rhodesian Ridgeback dogs

An extremely successful long-term collaboration between the Swedish Kennel Club and dog breeders/owners has resulted in an impressive register containing extensive pedigrees, veterinary information (such as Hip- and elbow status, dog show results, field trial results and behavioural test results). All information is available to the public. Until 1989, neutering/spaying of dogs was exclusively allowed due to specific medical conditions according to Swedish law, which has resulted in a Swedish dog population where intact dogs are more common than sterilized dogs. The Swedish Rhodesian Ridgeback Club keeps a complementary and more comprehensive register (1964 to current date) as the Swedish Kennel Club register, thus it is far more informative in aspects such as number of stillbirths, number diseased and euthanized puppies, phenotypic deviation, gender distribution in born litters and tooth status of parental animals.
Figure 1a. The dorsal ridge of a Rhodesian Ridgeback. The ridge constitutes of three parts, 1) the box, 2) the two crowns of which one rotates counter-clockwise (left) and the other rotates clockwise (right) and 3) the tail. Image by Salmon Hillertz.
Figure 1b. Dorsal ridge in a male (left) and female (right) Thai Ridgeback. Variation in ridge phenotype is acceptable according to Thai Ridgeback breed standard. Images by Salmon Hillbertz (left) and Selin M (right).
Figure 2. According to the Rhodesian Ridgeback breed standard, more than two crowns are classified as a faulty ridge. In the left image, a female Rhodesian Ridgeback displays six crowns. Ridgeless Rhodesian Ridgebacks are also produced (right image). Images by Salmon Hillbertz.
Canine development

The nature of the ridge phenotype implies a mutation that influence the embryonic development of the skin and hair follicles, both which originate from the neural tube of the ectoderm. Here I will briefly discuss the gestation and embryonic development in dogs.

Normal embryogenesis and neurulation

The gestation period of dogs is normally 63 days, but with variation (Willis, 1989). Four days after mating, the fertilized egg has divided into two cells and by the 8-9th day the morula (early embryo) is transported, via the uterine tube, to the uterus (Fig. 4). On the 20-21st day, the embryo is implanted to the epithelium of the uterine horns (Evans & White, 1997; Concannon, 2000).

Neurulation is the process in which the neural plate develops and forms the neural tube. The formation of the neural tube is initiated as the surrounding cells of the neural plate signal proliferation, invagination, fusion and detaching leading to the formation of a cylinder. Between days 21 and 28, the formations of the brain, spinal cord and skin occur (ectoderm) in dogs (Evans & White, 1997). The mesoderm (which contributes to the formation of connective tissue, bone, muscle) and the endoderm (giving rise to the epithelium lining of the digestive, urinary- and respiratory systems as well as the liver, pancreas and thyroid glands) are
developed (Sjaastad et al., 2004; Wolpert et al., 2004). The three germ layers give rise to all the additional tissues/organisms besides those stated above. Van der Put et al. (2001) described multiple neural tube closure sites in man. As similar closing sites have been suggested to occur in other vertebrates (Golden & Chernoff, 1993; Peeters et al., 1998), the sites may be applicable to dogs. Primary closure of the neural tube has been suggested to occur initially in the high cervical region in a caudal and cranial direction. A secondary closure point occurs bi-directionally in the fore- and midbrain region, followed by the third closure site initiated at the primitive mouth with a caudal direction to convene to the second closure site. The fourth closure site (cranial direction) occurs between the first and second closure site (Fig. 5). Van der Put et al. (2001) indicate a fifth closure site, occurring in the sacral region (secondary neurulation). In dogs, the spinal cord ends at lumbar disc 6/7. Below the cauda equine (sacral and caudal region) the remaining spinal nerve roots are collected (Kainer & McCracken, 2003).

Figure 4. The development of a fertilized egg of a dog. Fifteen days after fertilization, the blastocyst is free-floating in the uterus and by the twentieth to twenty-first day the egg is implanted to the wall of the uterus horn. Image by Salmon Hillbertz.
Neural tube defects

Neural tube defects (NTD) are a wide range of congenital malformations which occur due to incomplete closure of the neural tube during early embryogenesis. NTD’s are suggested to occur between the third and fourth week after conception in human (Martinez-Lage et al., 1995) which correlates to week three during dog embryonic development according to Evans & White (1997). NTD’s are classified as i) open (spina bifida aperta (Fig. 6) and spina bifida cystica) - and ii) closed defects (spina bifida occulta, a vertebrae bone fusion) (Chesney, 1973; Clayton, 1983; van den Broek et al., 1991). Another anomaly classified as a NTD is Dermoid sinus (DS). DS occurs due to incomplete separation between de cutaneous ectoderm and the neuroectoderm, resulting in isolation of cutaneous elements (Booth, 1998). DSs may be associated with spina bifida occulta or exist as sole entities (Hofmeyer, 1963; Chesney, 1973; Fatone et al., 1995).
Figure 6. A stillborn German shepherd puppy affected by an open neural tube defect. Note the “ridge” associated with the congenital defect. Image from Clayton (1983) with permission from Veterinary Records.

Dermoid sinus

Dermoid sinus (DS) is as discussed above a disease affecting both the Rhodesian and Thai Ridgeback dogs. DS frequencies in breeds other than Ridgeback dogs are currently unknown, as many breeders/veterinarians only associate DS occurrence with Ridgeback dogs. Therefore, the number of euthanized dogs (across breeds) due to incorrect/failed diagnosis is currently unidentified.

The term dermoid sinus, also known as dermal sinus/sacral dimple/dermoid cyst, in Rhodesian Ridgebacks was originally used by Steyn et al. (1939), followed by Hofmeyer (1963) regarding Rhodesian Ridgeback crosses. Both authors described the identical skin disorder as originally described by Hare (1932). Five types of DS have been described (Lord et al., 1957; Mann & Stratton, 1966; Booth, 1998; Tshamala & Moens, 2000; Pratt et al., 2000), classified according to their association to the nuchal/supraspinous ligament or dura mater. The nuchal ligament overlays the cervical vertebrae continuing over the thoracic and sacral vertebrae as the supraspinous ligament (Goody, 2002). Three layers of protective membranes which cover the spinal cord and brain compose the meninges; i) the outer and most fibrous membrane, dura mater ii) the arachnoid mater and iii) the vascular pia mater (inner membrane) located closest to the neuroaxis (Kainer & McCracken, 2003). DSs occur along the extremes of the midline axis (dorsal). The anatomical locations correspond to the regions of late neural tube closure (Fig. 7). Skin palpation is the most widely used diagnosis method to detect DS in young
puppies. The procedure is mainly performed by dog breeders. The most common clinical diagnosis is one or multiple skin-openings. Skin-dimples (lacking a skin-opening) located by the root of the tail have also been classified as DSs. Currently, conclusive clinical diagnosis of DS requires histopathological examination (Lord et al., 1957; Antin, 1970; Gammie, 1986; Marks et al., 1993; Penrith, 1994; Cornegliani et al., 2001). The inheritance pattern has been described as either autosomal recessive, dominant or complex (Mann & Stratton, 1966; Gammie, 1986; Angarano & Swain, 1993; Scott et al., 1995, Lord et al., 1957). The high incidence of DS in Rhodesian Ridgebacks leaves no doubt as to its heritable nature, but there is still some uncertainty concerning the mode of inheritance as well as the underlying genetics (Hathcock et al., 1979; Kása et al., 1992). The uncertainty of reaching a conclusive definition of the mode of inheritance is influenced by the difficulty to stringently phenotype DS. Finally, genetic interactions between the major disease causing mutation and the genetic background of multiple genes with minor effects are likely to influence the DS phenotype.
Figure 7. Five different types of Dermoid sinus have been reported. The types are classified according to vertebrae association and extension, anatomical location (dorsal) and if a skin-opening is present or not. Image by Gessbo, with permission.

Features of canine genomics and its role in comparative studies

The domestic dog (Canis familiaris) originated from wolves 10,000-15,000 years ago (Vilà et al., 1997; Savolainen et al., 2002) and the long history of human/dog companionship has resulted in various modern dog breeds, which display vast phenotypic diversity (compare the Pug and the Great Dane for example). The breeding of pure-bred dogs for specific traits has resulted in highly inbred dog breeds with high frequency of genetic disease. In fact, high frequency of particular diseases that are specific for some or a few breeds is very common in the dog.
Therefore, the dog is an excellent model species for identifying genes underlying genetic disease (see below).

In an effort to breed healthier dogs and to use the dog as a model for genetic disease in human, the structure and organization of the dog genome has been of interest since the 1990ies resulting in an international collaboration denoted the DogMap (Binns et al., 1998; Breen et al., 1999; Breen et al., 2001). The DogMap research teams established the platform for future canine research. The National Human Genome Research Institute decided in 2002 to sequence the complete dog genome. The main reason for the choice of sequencing the dog, prior to commercially important domestic species such as cattle (*Bos taurus*), was the realisation of the dog as an excellent model for genetic diseases, which also affect humans.

In 2003, Kirkness et al. presented 1.5-fold coverage of the poodle genome sequence. Parker et al. (2004) identified sequence variation in 120 dogs (60 breeds) via a ~2 kb non-contiguous genome sequence analysis; they found 14 breed-specific Single Nucleotide Polymorphisms (SNPs). To further investigate genetic variation within and between breeds, a microsatellite approach was conducted utilizing 414 dogs representing 85 breeds. Each breed was represented by four to five individuals that were unrelated (3rd generation). The study presented two distinctive clusters of dog groups consisting of i) ancient breeds (including the grey wolf, *Canis lupus*) and ii) more recently founded breeds (such as herding and hunting breeds). Two months after the Parker et. al., publication (July 2004), a ~7.5-fold coverage of the dog (*CanFam1.0*) genome became public to the scientific community. The choice of breed was Boxer and the draft was assembled by a group, headed by Kerstin Lindblad-Toh, at the Broad Institute of MIT and Harvard (Cambridge, MA, USA) and Agencourt Bioscience Corp (USA), enabling a variety of comparative studies between dog/human and thus initiating the major transformation regarding dog research project planning, technology development and methodological choices currently available and applied. By 2005, an improved assembly (*CanFam2.0*) was launched covering 99% of the dog genome (Lindblad-Toh et al., 2005). The International HapMap (human) Consortium (2005) provided ample evidence of efficacy regarding a recently developed high-throughput genomic method; a SNP-based genome-wide association array. During this time-frame, Lindblad-Toh et al. (2005) presented a SNP map (http://www.broad.mit.edu/mammals/dog.snp/), which contained more than 2.5 million unique SNPs, representing coverage of ~1 SNP/kb. With such a dense SNP coverage available, in combination with extensive linkage disequilibrium found within dog breeds, the development of a dog-specific SNP array became possible.

Many dog breeds are predisposed for specific diseases, *i.e.* one breed may be severely affected by a specific disease whilst another breed may not. Not only DS but also several other diseases seen in dogs resemble those in man *i.e.* Atopic dermatitis, heart diseases such as dilated cardiomyopathies, autoimmune diseases such as lymphocytic thyroiditis and several forms of cancers. Purebred dogs have been subjected to several bottlenecks such as small founder populations, high inbreeding for specific phenotypic traits and also the first and second world wars.
Diminishing access to breeding-stocks and/or the quest of attaining representative breeding animals would most likely have influenced distribution of disease-causing mutations due to indirect selection, i.e. breeding animals displaying desired phenotypes may have carried disease-causing mutations. Purebred dogs have for many generations also been subjected to different levels of inbreeding, resulting in relatively low genetic variation within breed (SNP rate $\sim 1/1600$ base pairs (bp)) and higher genetic variation (SNP rate $\sim 1/900$ bp) between breeds (Lindblad-Toh et al., 2005). Another explanation of breed-specific disease segregation is that only 10-20% of a dog population is used for breeding purposes of which the majority of the breeding animals are selected due to successful showing results. Historic breeding strategies have resulted in long haplotypes (0.5-1Mb) as well as high degree of linkage disequilibrium within dog breeds, far more extensive than reported in humans (Sutter et al., 2004; Lindblad-Toh et al., 2005). Note that LD-pattern between breeds resembles that of human populations (Lindblad-Toh et al., 2005). Mutations being identical by descent (disease genes originates from a common ancestor) therefore enable scientists to trace mutation-segregation throughout several generations. Relatively short generation intervals, fairly large litters (breed-specific), shared environments with humans, access to extended pedigrees and phenotypic data are aspects of consideration regarding genetic research. Following, a study in which the Rhodesian Ridgeback was utilized, is presented. The study provides ample evidence of the grandness of using the dog regarding disease-genetic studies.
Aims of the thesis

- to indicate the mode of inheritance for DS (paper I)

- to establish the inheritance for the Ridge and indicate its relation to DS (paper II)

- to improve the diagnostic classification of Dermoid sinus (paper III)

- to reveal mutation(s) underlying the Ridge and DS phenotypes (paper IV & V)

Summary of investigations

The summary of investigations provides a broad overview, discussion and comments of the work included in this thesis. The coat-colour study presented in paper VI will not be discussed herein. Detailed information may be found in each of the separate papers included in this thesis.

Materials & Main methods

Dogs and collection of material

The collection procedures of Rhodesian Ridgeback material were:
- collection of whole blood and tissue from DS-affected (identified by breeders) puppies aged 1-56 days as identified by breeders.
- whole-blood from their parental animals and littermates.
- collection of DS-tissue and whole-blood from individuals where the DSs were surgically removed.
- collection of material from ridgeless dogs, their parental animals and littermates.
- collection of whole-blood or cheek-swabs from Norwegian-, Danish-, German-, English-, South African-, North American- and Brazilian Rhodesian Ridgeback families in which DS-affected puppies were identified as well as random collection of healthy individuals.
- collection of whole-blood or cheek-swabs of DS-affected or healthy Thai Ridgeback individuals from Europe and Thailand.
- collection of a family of cervical ridged Bavarian Mountain scent hound
- collection of ~20 Boxers with crowns located at the scapula.
All collections of dog material were conducted in accordance with the animal ethics regulations of the Swedish National Board for Laboratory Animals and the Swedish Board of Agriculture. The DS-affected puppies were brought to the Swedish University of Agricultural Sciences (SLU), where they were subjected to euthanazation followed by diagnostic classifications, whole-blood extraction and blunt autopsy. For the majority of the Ridgeback samples, pedigrees are available as well as information regarding the ridge phenotypes.

Statistical analysis of inheritance

To identify if DS and the ridge followed Mendelian inheritance patterns, a Chi-Square test was employed. DS was tested to evaluate an autosomal recessive mode of inheritance where the expected ratios would be 1:2:1 (paper I). The dorsal ridge was tested for an autosomal dominant inheritance pattern where the ratios 3:1 would be expected (paper II). For paper I, data from a total of 372 litters was selected from the Swedish Rhodesian Ridgeback Club register, of which 243 litters were excluded to avoid bias (such as breeders not palpating all stillborn/destroyed puppies for DS, lack of information). The degree of freedom was one, as two classes were of interest; DS-affected vs. healthy individuals. In paper II, data from a total of 508 litters was selected for the segregation analyses. Lack of information from 106 of the selected litters, resulted in exclusion from the statistical test. Two classes were to be evaluated; ridged vs. ridgeless individuals. To avoid bias in segregation ratios, correction of estimated data was employed. Bias may occur as observed ratios not always correlate with expected ratios. Further, in paper II a possible association between DS and the ridge was evaluated.

Histopathology

Extracted tissues (blunt dissection) were fixated in 4% buffered formalin and paraffin-embedded according to standard procedures. Cut sections were stained with hematoxylin and eosin. An E1000 microscope (Nikon Eclipse) was used for digital light micrograph images.

Genome-wide association analysis

A SNP array is a chip with a large number of minute wells (features) attached to a coated quartz surface and is used to simultaneously analyze different genotypes in a single individual. Each feature contains covalently immobilized SNP-specific oligonucleotides (probes). PCR amplifications of genomic DNA with fluorescent marked primers are performed. The PCR amplicons are digested and denatured (fragmentation step to allow increased hybridization efficacy), followed by hybridization of the fluorescent-marked strands to the probes located in features on the array. The hybridization signal intensities are detected and recorded, resulting in a vast number of base calls for further computational analysis. Approximately 26,500 informative SNPs distributed across the chromosomes were selected for the genome-wide association study with the aim of proving the
principle that only relatively small amounts of genetic markers as well as cases and controls are necessary to obtain significant results.

**Pyrosequencing**

Pyrosequencing is a cost-efficient sequencing method based on synthesis of a complementary strand to a single stranded DNA template by the addition of nucleotides (real-time pyrophosphate detection). As the complementary synthesis elongates, chemical enzymatic reactions occur resulting in light signals which are detected and analyzed. Signal intensity reveals the number of added nucleotides (Nordfors et al., 2002). The high-throughput method is often used for SNP genotyping. The SNP of interest was SNP_51,399,353 utilizing primer-pairs (of which one primer was biotinylated) in the near vicinity of the SNP.

**Multiple ligation-dependent genome amplification**

The recently developed multiple ligation-dependent genome amplification (MLGA) technique uses the spelling of the base pairs to determine if portions of chromosomes or genes are changed or mutated, by amplifying gDNA instead of probe molecules (Isaksson et al., 2007). A set of restriction fragments are generated by using a restriction enzyme. Single fragments for each target sequence are then chosen in such a way that fragments in each pool are between 100 & 400 nucleotides in length (each fragment with different lengths) with a minimum size difference of six nucleotides (Fig 8, Step 1). The selector technique is employed regarding probe and vector designs (Fig 8, Step 2). Each selector consists of two synthetic oligonucleotides (selector constructs); a target-specific selector probe and a vector oligonucleotide. The vector is designed with target-specific ends flanking the motif of interest. The central part of each selector probe is complementary to the vector oligonucleotide so that hybridization between the two generates the recognition sequence for the HindIII restriction enzyme and a universal primer pair site for parallel PCR amplification. The ends of the selector probes each have sequences complementary to the ends of the restriction fragments targeted for selection.
Step 1.

Step 2. Selector Technique

UCSC.edu database
Dog sequence Chr18

Restr. Fragment Sequence cut
100-400nt

TGAGTGGGTCGACCCCCTGGGGCATACGATAACGGTAGAAAGCTTTGCTAACGGTCGAGGTACACCCAAGCTGGGGG

Figure 8. Step 1; Principle of MLGA methodology (image by Isaksson et al., 2007, with permission from the authors). Step 2; The selector technique is applied regarding the selector constructs (image by Salmon Hillbertz).
Real-Time Quantitative PCR

To evaluate gene expression, the real-time quantitative PCR (RTQ-PCR) technique is applied. RTQ-PCR is a sensitive technique used for identification of gene expression and copy number variation for which a reporter (attached the 5'-end) and quencher (attached to the 3'-end) dyed probe is used. The probe is designed to be complementary to the target sequence and is flanked by forward and reverse primers. Upon hybridization, the probe and complementary DNA template bind. When polymerization reaches the site of probe, the polymerase degrades the probe resulting in the release of the reporter dye, followed by single nucleotide releases and the release of the quencher dye. Due to the release of the reporter- and quencher dye detectable light signals are generated. If no signals are detected, the gene is not expressed in the tissue type from which mRNA was extracted and cDNA synthesized. Detected light signals generated during the exponential phase of the run are plotted against cycle numbers on a logarithmic scale and a detection threshold is determined. During a cycle when the light signal from an experimental sample crosses the threshold (cycle threshold, C_T) the gene of interest is expressed. Each experimental sample (specific genotype or tissue type) per gene is run in duplicate or triplicate to obtain a mean C_T value per experimental sample. For quantification purposes, the mean C_T value of each experimental sample/gene is divided by the mean C_T value of a control gene (often a house-keeping gene).

Results and discussion

Mode of inheritance

Segregation analysis did not support a simple autosomal recessive inheritance pattern regarding Dermoid sinus (DS), thus suggesting a more complex mode of inheritance (paper I). However, statistical support was provided regarding an autosomal dominant inheritance of the dorsal ridge as well as a significant association between ridge and DS (paper II). Frequency estimations regarding DS- and ridgeless prevalence in produced litters (Swedish Rhodesian Ridgeback population) were 8-10% and 5.6%, respectively.

Segregation analyses regarding DS, the ridge and ridge/DS association were only possible due to the impressive register kept by Swedish Rhodesian Ridgeback club register. Thus, even though the register contains information regarding produced litters from 1964 to the current date, full breeder participation did not occur until the beginning of 1981. However, a drawback regarding information from the 1980ies was the lack of reports of DS-affected puppies. Non-reporting of DS-affected individuals occasionally still takes place. Further, anatomical locations of DS (cervical vs. caudal locations) and phenotypic recordings (skin-opening/skin-dimple) were most often not available. Information regarding caudally located DSs was available from merely fourteen litters (between 1964 and 2006 a total of seven hundred sixty-seven litters were reported).
The majority of DS-affected offspring are euthanized, according to breed-club recommendations and no autopsies are performed. DS examinations of stillborn and ridgeless Rhodesian Ridgebacks which are euthanized are normally not conducted. The frequency of DS type V within the Swedish Rhodesian Ridgeback population is currently not identified and only two scientific case-reports describing DS type V are available (Booth, 1998; Tshamala & Moens, 2000). Even though no reports are available regarding ridgeless Rhodesian Ridgebacks affected by DS, it may theoretically occur as a stochastic event. The Thai Ridgebacks share not only the ridge phenotype but also an increased frequency of DS occurrence with the Rhodesian Ridgeback. Strangely, DS occurrence in the Thai Ridgeback has previously not been scientifically reported. DS frequency in the Thai Ridgeback is yet unknown. The evidence of clear association between the ridge and DS proved to be a novel finding.

**Histopathological evaluation**

During autopsies morphological differences were observed between DS type I-IV and DS type VI, which were histopathologically confirmed. Thus, similarities between partially pigmented DS with skin-openings and DS type VI were also observed. Based on histopathology, a new definition of DS type VI was suggested: Lipoma of the terminal filum with skin-dimple and extra-spinal connection (LTF). In figure 9, a DS-type II is shown and in Figure 10, a LTF. Criteria of LTFs were the presence of skin-dimple and mesoderm tissue (adipose-, connective and muscle tissue).
Figure 9. a) Dermoid sinus (DS) in a three weeks old Rhodesian Ridgeback male, where a skin-opening is marked (circle). Inserted is the DS morphology in subcutis. b) The extracted DS in full length. The DS was classified as a type II. c) A cross-section of the DS. The lumen is lined by stratified squamous keratinized epithelium and contains keratin and hair debris. Hematoxylin and eosin stain, objective lens x 20. Image 10a-b by Salmon Hillbertz. 10c by Hellmén E.
Figure 10. a) Skin-dimples at the root of the tail in a two weeks old Rhodesian Ridgeback male. Upon *In toto* removal morphological appearance displayed a non-pigmented funnel-like structure. b & c) Histopathological results revealed presence of muscle-, connective- and adipose tissues. Histopathology and the anatomical location of the condition in Rhodesian Ridgebacks resulted in a new definition; Lipoma of the terminal filum with skin-dimple and extraspinal connection (LTF). In figure b, objective lens x 4 was used and in c) objective lens x 10 was used. Image a by Salmon Hillbertz and images b and c by Hellmén E, objective lens x 4.
Extraction of DS/LTF tissue was not successful in four cases thus anatomical locations, phenotypic criteria and autopsy observations supported DS/LTF appearance. Major emphasis was made on quick and efficient extraction followed by instant tissue freezing in liquid nitrogen in an attempt to limit tissue degradation, as the samples were prepared for gene expression studies. Unfortunately, Computer tomography and Magnetic resonance imaging technologies were not available. Therefore, identification of possible Spina Bifida Occulta in the euthanized puppies was not achievable. According to the dog owners, abnormal movement patterns were not observed. An interesting observation during autopsy was that none of the observed DSs were identical in regards of growth direction, DS extension vs. age of the puppy and morphology. In contrast, the majority of the observed LTFs showed very low divergence in morphology and length. Autopsy observations in combination with morphological differences and histopathological analysis of extracted tissues, led to the conclusion that DSs and LTFs ought to be distinguished, hence the suggested descriptive denotation “Lipoma of Termile Filum with skin-dimple and extra-spinal connection”. Anatomical locations of DS/LTF in Rhodesian Ridgebacks seem to correlate with primary neurulation respectively secondary neurulation development described in human studies. Further, classification, anatomical locations, skin markings and histopathological results regarding DS and LTF identified in dogs seem to be in concordance with findings presented in human literature. Finn & Walker (2007) mentioned a possible genetic factor regarding LTFs. Even though the Swedish Rhodesian Ridgeback club-register cannot provide the quantity of data required for statistical analysis, a heritable nature of LTF is generally accepted (hence the lay definition “tail-DS”) by breeders. A three generation pedigree including six of fourteen litters, produced over a twelve year period, highlights litters in which both ridgeless and DSs with cervical/caudal locations were produced (Fig. 11). The high resemblance of skin lesion presentations between two species (dog/human) presents additional motivation regarding selection of utilizing the dog as model organism in human disease research.

The findings presented in paper III, led to the conclusions that histopathology must be employed to confirm bona fide DS prior incorporation of samples to molecular genetic approaches. Further, the combination of DS anatomical locations, extensions, morphology and histopathological information will most likely play an important role regarding the enablement of genetically distinguishing between different DS types. Association between DS and LTF has previously been discussed in human literature as both lesions originate during neurulation. Thus, the suggestion provided herein, a shared genetic origin between DS and LTF is most likely original and is supported by the three-generation pedigree provided herein.
Figure 11. Pedigree of three generations of Rhodesian Ridgebacks displaying litters in which ridgeless and Dermoid sinus affected puppies were produced. Red crosses (+) are cervical DS, blue crosses (♦) are caudal DS, triangles (▲) are ridgeless puppies (gender unknown) and squares (Square) indicate euthanized puppies (reason and gender unknown). All information regarding litter outcome were reported by breeders to the Swedish Rhodesian Ridgeback Club. Image by Salmon Hillertz.

Genome-wide association analysis

The ridgeless-locus was mapped to a 750-kb region followed by identification of a haplotype defined by three SNPs in which ten (n=11) DS-affected Rhodesian Ridgebacks were homozygous. The haploblock was not present in ridgeless Rhodesian Ridgebacks. Within the identified haploblock, five genes were located; FGF3 (fibroblast growth factor 3), FGF4 (fibroblast growth factor 4), FGF19 (fibroblast growth factor 19), ORAOV1 (oral cancer over-expressed gene1) and CCND1 (cyclin D1).

The GWAA approach proved to extremely successful, utilizing only 11 DS-affected and nine ridgeless Rhodesian Ridgebacks. The mapped locus on chromosome 18 left no doubt regarding the presence of a region associated with the ridge phenotype. The region contained five genes FGF3, FGF4, FGF19, ORAOV1 and CCND1 of which all are involved during early development (Table 1).

The power of the GWAA approach will from now on have a large impact regarding future molecular genetic studies. The time consuming and costly days of sample collections of entire families will most likely be replaced by the GWAA approach as only cases and controls are necessary for association analysis. The low number of animals necessary to obtain reliable results is remarkable; monogenic traits, ten cases and ten controls and polygenic traits, predicted 100 cases and 100 controls. This, in combination with the genetic makeup of purebred dogs (low genetic diversity within breed, high genetic diversity between breeds) will lead to identification of loci, associated with phenotypes of interest, in a more rapid mode than anyone could have expected just a few years ago.
Table 1. Description of the role of FGF3, FGF4, FGF19, ORAOV1 and CCND1. The genes were included in the loci which was associated with the dorsal hair ridge in Ridgeback dogs by Genome wide association analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Role</th>
<th>Reference</th>
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<tbody>
<tr>
<td>FGF3</td>
<td>Cerebellum, hindbrain and forebrain, tail retina/tooth and inner ear development, Extra-embryonic endoderm,</td>
<td>Wilkinson et al. (1988)/(1989); Murakami et al., (2004); Galdemard et al., (2000); Pasqualetti et al., (2001); Tekin et al. (2007)</td>
</tr>
<tr>
<td>FGF4</td>
<td>Limb development, inner cell mass proliferation, promote neural stem cell proliferation and neuronal differentiation in the postnatal brain</td>
<td>Zúñiga et al. (1999); Feldman et al., (1995); Kosaka et al., (2006); Zhong et al., (2006)</td>
</tr>
<tr>
<td>FGF19</td>
<td>Fetal brain development, fetal cartilage, skin, and retina bile acid synthesis and gallbladder filling, mesoderm expression during neural groove closure, involvement in steroid hormone activity</td>
<td>Nishimura et al., (1999); Xie et al., (1999); Inagaki et al., (2005); Choi et al. (2006); Ladher et al. (2000); Fu et al. (2004).</td>
</tr>
<tr>
<td>ORAOV1</td>
<td>Unknown</td>
<td>Huang et al., (2002)</td>
</tr>
<tr>
<td>CCND1</td>
<td>Regulates G1 (gap) phase of cell division cycle, developing brain, involved in mitochondrial function and nuclear DNA synthesis</td>
<td>Sherr &amp; Roberts, (1999); Ishibashi &amp; McMahon, (2002); Wang et al., (2006)</td>
</tr>
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Fine-mapping

One SNP (SNP_51,399,353) located within the identified haploblock, revealed significant deviations (p<0.001) from Hardy-Weinberg expectations as additional ridged individuals were genotyped. Via MLGA, ~87% of the DS-affected ridged Ridgebacks (n=15) were scored as homozygote, ~66% of the healthy ridged individuals (n=29) were scored as heterozygote and all ridgeless individuals homozygote wild-type.

Genotyping, utilizing Pyrosequencing (SNP_51,399,353), results confirmed the presence of a duplication (Table 2). Via MLGA, a ~133 kb region was identified as the duplication breakpoints (Fig. 11). CCND1 was initially (via GWAA) a candidate gene regarding the ridge phenotype. Further fine-mapping analysis (MLGA) revealed the only the 3’-end of CCND1 was included in the duplication, suggesting that CCND1 most likely does not contribute to the ridge phenotype. Utilization of additional probes located in the near vicinity of the internal breakpoint revealed the internal breakpoint. Sequencing results of 700 bp up-respectively downstream of the internal breakpoint showed complete identity between the two Ridgeback breeds compared to the available Boxer sequence. Several purebred dogs were sequenced, from which results confirmed the duplication as Ridgeback-specific. Gene expression (RTQ-PCR) was not detectable in post-natal tissue regarding the three FGF-genes of interest. However,
two-fold and one-and-a-half fold \textit{ORAOV1} mRNA expression was identified in homozygote and heterozygote ridged individuals compared to ridgeless dogs. The control gene ($\beta$-actin) was expressed in all analysed tissue-types. Histopathological analysis of dorsal skin derived from ridged and ridgeless Rhodesian Ridgebacks, confirmed phenotypic differences regarding hair bulb orientation. Further, it was identified that the hair bulbs in ridged Rhodesian Ridgebacks were located in a more distal mode (deeper into dermis) than those of a ridgeless Rhodesian Ridgeback. mtDNA analysis did not reveal a close association between Rhodesian and Thai Ridgebacks, \textit{i.e.} the divergence between the breeds indicated an ancient history of the ridge mutation.

\textbf{Table 2}. SNP genotyping by pyrosequencing. The SNP of interest (SNP 51,399,353) was found to be heterozygote in DS-affected Rhodesian Ridgeback and homozygote in Thai Ridgebacks.

<table>
<thead>
<tr>
<th>No.</th>
<th>Individuals</th>
<th>Genotype</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS+/RR Rhodesian Ridgeback</td>
<td>22</td>
<td>A C</td>
<td>1 1</td>
</tr>
<tr>
<td>DS+/RR Rhodesian Ridgeback</td>
<td>5</td>
<td>A C</td>
<td>1 2</td>
</tr>
<tr>
<td>DS+/RR Rhodesian Ridgeback</td>
<td>1</td>
<td>A C</td>
<td>1 3</td>
</tr>
<tr>
<td>DS+/RR Thai Ridgeback</td>
<td>3</td>
<td>C</td>
<td>0 1</td>
</tr>
<tr>
<td>DS+/rr Bloodhound</td>
<td>1</td>
<td>C</td>
<td>0 1</td>
</tr>
<tr>
<td>DS+/RR Cambodian Local</td>
<td>1</td>
<td>C</td>
<td>0 1</td>
</tr>
<tr>
<td>Ridgeless (rr) Rhodesian Ridgeback</td>
<td>19</td>
<td>C</td>
<td>0 1</td>
</tr>
<tr>
<td>Ridgeless (rr) Rhodesian Ridgeback</td>
<td>4</td>
<td>A</td>
<td>1 0</td>
</tr>
<tr>
<td>Ridgeless (rr) Rhodesian Ridgeback*</td>
<td>1</td>
<td>A C</td>
<td>1 1</td>
</tr>
<tr>
<td>Ridgeless (rr) Rhodesian Ridgeback*</td>
<td>1</td>
<td>A C</td>
<td>1 2</td>
</tr>
<tr>
<td>Ridged Rhodesian Ridgeback</td>
<td>6</td>
<td>A C</td>
<td>1 1</td>
</tr>
<tr>
<td>Ridged Rhodesian Ridgeback</td>
<td>9</td>
<td>A C</td>
<td>1 2</td>
</tr>
<tr>
<td>Ridged Rhodesian Ridgeback</td>
<td>1</td>
<td>A C</td>
<td>1 3</td>
</tr>
<tr>
<td>Ridged Thai Ridgeback</td>
<td>11</td>
<td>C</td>
<td>0 1</td>
</tr>
</tbody>
</table>

* Phenotyped by breeder
The molecular mechanism of FGF3 is yet to be further investigated, thus Miyoshi et al. (2002) implicated involvement with several signalling pathways, including the WNT signalling pathway. Transcription of the FGF4 gene is activated by a β-catenin/TCF/LEF complex, which forms in the presence of a WNT signal (Ishibashi & McMahon, 2002; Kratochwil et al., 2002; Miyoshi et al., 2002). Four FGF receptor (FGFR1-4) genes have been identified, thus alternative splicing of FGFR1-3 mRNAs generate additional receptor isoforms (Murakami et al., 1999; Zhang et al., 2006). The FGF3 ligand binds to FGFR1b and FGFR2b (Ornitz et al., 1996). Both “b” splice forms are involved in epithelial lineages (Zhang et al., 2006). Kawano et al. (2005) mention FGFR2b as keratinocyte-specific. The FGF4 ligand binds to FGFR1c, FGFR2c and FGFR3c (Ornitz et al., 1996). The “c” receptor splice forms are involved in mesenchymal lineages (Zhang et al., 2006). FGF19 has been suggested to be a component in the WNT pathway (Ohyama et al., 2006) and binds to the FGFR4 receptor (Harmer et al., 2004; Tamimi et al., 2006; Abraira et al., 2007), which has been associated with endoderm tissue development (Jung et al., 1999).

The WNT- and FGF-signalling pathways have been suggested to play crucial roles during different stages of development (Böttcher & Niehrs, 2005; Katoh & Katoh, 2006). Merrill et al. (2001) discussed TCF (T-cell factor), LEF (lymphoid enhancer factor) and β-catenin involvement regarding epidermis, sebaceous gland and hair follicle development. LEF/TCFs are transcription factors which form complexes to activate target genes (Jamora et al., 2003; de Lau & Clevers, 2001). β-catenin is involved in intercellular junction formation and regulates transcription (such as by binding to LEF/TCFs) in the WNT-signalling pathway (Merrill et al., 2001).

Figure 11. Results from MLGA technique.
ORAOV1 has been associated with oral cancer and Huang et al. (2002) reported ORAOV1 expression in several types of tissue. The expression levels correlated to CNV. Herein, ORAOV1 mRNA expression was identified in adult skin derived from Rhodesian Ridgeback dogs, which has previously not been reported.

Over-expression of many FGF genes has been associated with different cancer forms (Tai et al., 2005; Xie et al., 1999; Nishimura et al. 1999; Sherr & Roberts 1999). Tekin et al. (2007) reported hereditary deafness in humans associated with mutations in FGF3. The FGF3-FGF4-FGF19-ORAOV1-CCND1 locus identified in dogs corroborate with findings reported across species. Katoh & Katoh (2003) reported that mouse FGF15 was the ortholog of human FGF19 and that the FGF4-FGF19-ORAOV1-CCND1 locus is evolutionary conserved between human, zebra fish and rodents. Additional comparative studies showed that FGF3 was positioned upstream of FGF4 (Katoh & Katoh, 2005). The FGF3 enhancer (located upstream of FGF3) was not included in the duplicated region reported herein.

The proteins encoded by the Fz (Frizzled) genes function as WNT receptors. Guo et al. (2004) presented results in which Fz6-/- mice displayed alterations in hair patterning, such as additional hair whorls. The patterns identified in the Fz6 knockout mice, highly resemble the ridge phenotype identified in dogs. Knockout of Fz3, a gene involved in the developing CNS and neural tube closure, and Fz6 displayed failure in neural tube closure and abnormal tail development (Wang et al., 2006).

Twenty two FGF genes have previously been described in humans. FGF3, FGF4 and FGF19 are only expressed during embryological development (Ornitz & Itoh, 2001), which confirms the absence of identifiable gene expression in postnatal tissue described herein. Hypothetically, there might be a common factor between the FGF- and WNT-signalling cascades downstream of β-catenin which very well may explain the phenotypic deviations in hair patterning and deficiency in neural tube closure reported by Guo et al. (2004) and Wang et al. (2006) as well as the hair ridge and DS in Ridgeback dogs. To investigate the hypothesis, knock-out mice models could be utilized in an effort to identify the common gene/genes involved in the development of NTDs. Furthermore, gene-environment interactions may also play a role in NTD development, as discussed in human literature (Johnston, 2008) as well as interactions between several loci.

Based on the findings herein, there is little doubt that homozygote ridged Ridgebacks are subjected to a highly increased risk of developing DS/LTF. The situation becomes complicated for breeders, as the ridge is the trademark of the breed. When the genetic complexity of DS/LTF has been identified, a DNA-test could be developed. Such a test would aid breeders as parental animals that carry the DS/LTF-causing mutation could be identified. To avoid decrease in genetic variation (i.e. decreasing the number of breeding animals), a long-term breeding program could be established with the aim of diluting the DS/LTF-causing mutation over several generations. This approach is only applicable if there is an open communication between breeders on a global aspect. Another approach would be the introduction of ridgeless individuals as breeding animals during
some generations (with careful monitoring of the produced offspring). Thus, if breeders wish to maintain the ridge in the Ridgebacks, this approach also requires open communication between breeders. In both scenarios, all results from DNA-testing (future DS/LTF specific DNA-test or ridge-specific DNA-test) of individuals must be available to the public.

Certain important aspects ought to be considered such as selection of cases/controls within breed and selection within geographically distinct populations. For fine-mapping approaches, the availability of other breeds, which do and do not display similar phenotypes, is necessary upon identifying ancestral haplotype blocks associated with phenotypes of interest. Therefore, an important issue to address is the awareness of the origin of breeds. Many breeds are not more than 200-150 years old and during breed creations, multiple breeds (whereas some are now extinct) were commonly used. This may most likely have an important impact for dog research from two aspects; 1) selection of control breeds, i.e. breeds that do not display phenotypes of interest and are genotypically as diverse as possible and 2) selection of control breeds in which mutations of interest would be expected to be present, i.e. lower genotypic diversity between breeds.

Herein, only Rhodesian Ridgebacks derived from the Swedish population were utilized for the GWAA approach to avoid population stratification. All cases (homozygote ridged and DS-affected) were histopathologically confirmed to avoid bias and all controls were defined as ridgeless. Initially, exclusively Swedish Rhodesian Ridgebacks were used for identification of the 133 kb duplicated region (MLGA). Subsequently, additional Rhodesian Ridgeback cases/controls and their parental animals were used as well as ridged Thai Ridgebacks. Screening of homozygote ridged (DS-affected) Ridgebacks was performed regarding internal breakpoint identification, which enabled detection of the ancient ridge-causing mutation present in both Rhodesian- and Thai Ridgebacks, as both breeds showed identical nucleotide sequences in the near vicinity of the internal duplication breakpoint and nucleotide sequence diversity 5’ and 3’ of the 133 kb duplicated region. Multiple non-ridged breeds were utilized to precisely define/confirm the ancient haplotype block associated with the ridge phenotype. Rhodesian Ridgeback family material (parental animals and littermates) was used for segregation analyses to confirm obtained results regarding the ridge-causing mutation.

The geographical origin of the ridge present in Ridgeback dogs is still a mystery, thus solid proof has now been provided that the ridge mutation in Ridgeback dogs is identical by descent and the likelihood of parallel mutations occurring in Asia and Africa (Epstein, 1937) can therefore be rejected. Unravelling the mystery of the ridge’s geographic origin, may shed new light on the pathway of dog domestication and distribution. Further, identification of the DS and LTF complexity may also reveal a previously unknown ancient background.

As both DS and LTF-affected Ridgeback puppies may be present within a produced litter, several possible explanations are plausible regarding the genetic complexity of DS/LTF origin. One explanation could be CNV where the number of duplicated copies could correlate to different types of DS or LTF. Another explanation could be allelic variants within the duplication (genetic heterogeneity).
which was elegantly shown by Pielberg et al. (2002). Within the near future, the enigma of the occurrence of DS versus LTF will be unravelled and a new era of knowledge regarding genes involved during development will be initiated.

**Conclusions**

- The mode of inheritance for DS was evaluated (paper I).

- Inheritance of the Ridge was defined. Further, it was shown that the Ridge predisposes to DS (Paper II).

- The diagnostic classification was shown to be improved by histopathology and a new terminology “Lipoma of the terminal filum with skin-dimple and extra-spinal connection was suggested (paper III).

- The mutation underlying the Ridge phenotype was identified (paper IV & V).

The knowledge gained from histopathological analysis of extracted tissues via blunt dissection revealed the necessity of correct disease diagnosis which became evident as results from the GWAA were obtained and analyzed.

The proof-of-principle study was proven to be extremely cost- and time-efficient. The combinations of i) high linkage disequilibrium and long haplotypes, *i.e.* high degree of inbreeding within breeds (low genetic diversity), ii) high genetic diversity between breeds (up to three-fold higher than described in human populations according to main reference IV), iii) access to genealogical material, iv) shared environmental factors and v) similar disease morphology and diagnosis clearly provide support for the notion of the supremacy of dogs as model organism for human oriented research fields. The overall impact of the proof-of-principle approach described herein, will most likely have a major influence regarding future project designs for genome-wide studies, *i.e.* previously family-based approaches will most likely be substituted by case/control approaches.
Future prospects

- Further elaboration on the genetic complexity of DS and LTF origin in Ridgeback dogs.

- Screen other breeds which display cervical ridges, such as Boxers and Bavarian Mountain scent hounds, to identify if the ridge mutation is identical or variable to that found in the Ridgebacks.

- Studies on transcription regulatory pathways associated with the phenotypes identified in $Fz6^{-/-}/Fz3^{-/-}$ mice (Guo et al., 2004; Wang et al., 2006) and Ridgeback dogs reported herein, utilizing knock-out mice.
Populärvetenskaplig sammanfattning

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