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Identification of Novel Mutations and Molecular Pathways for Canine Neurodegeneration and Chondrodysplasia

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Abstract

Inherited diseases occur across different species. This thesis work has provided insights into the molecular genetic background of three autosomal recessive diseases that affect specific dog breeds. The studied phenotypes comprised two neurodegenerative diseases and a type of skeletal dysplasia. Genome-wide methods, such as SNP chip genotyping, were used to identify disease-associated gene variants. In all three disease phenotypes, the likely causative variant was found in a gene that had not been previously associated with a monogenic disorder.

The Norwegian Elkhound and Karelian Bear Dog breeds are affected with inherited chondrodysplasia that causes short-stature dwarfism of varying severity. A genome-wide association study in Norwegian Elkhounds revealed a disease-associated locus on canine chromosome 17 and a nonsense mutation in the *ITGA10* gene. The identified mutation was homozygous in all affected dogs from both breeds, and may have been introduced to Karelian Bear Dogs from Norwegian Elkhounds. The *ITGA10* gene encodes an α10-integrin protein that assembles into a collagen-binding α10β1 integrin. The α10β1 integrin is a cell surface receptor, found in growth plate chondrocytes, where it mediates the cell's attachments with the surrounding matrix. Due to the mutation, a full-length α10-protein is not produced, disturbing the growth of long bones.

Early-onset cerebellar degeneration occurs in the Finnish Hound dog breed. Neurological examination of affected dogs revealed quickly progressing cerebellar ataxia and failure to thrive. Genome-wide association analyses mapped the disease to a 1.5-Mb locus on canine chromosome 8. Sequencing of the *SEL1L* gene from the locus identified a homozygous missense mutation in all affected dogs. The mutation causes a serine to proline amino acid change within a highly conserved functional domain of the encoded SEL1L protein. The SEL1L protein is found in the endoplasmic reticulum, where it functions within a protein quality control and degradation pathway. Cerebellar tissue samples from affected dogs showed signs of endoplasmic reticulum stress, which may be the cause of premature cell death.

A novel neurological disease, characterized by juvenile to adult onset cerebellar ataxia, was recognized in the Lagotto Romagnolo breed. Through linkage analysis, homozygosity mapping and whole-genome sequencing, the disease was associated with a homozygous missense change in the autophagy-related *ATG4D* gene. Pathological examination of affected dogs revealed progressive cerebellar degeneration, and intracellular vacuolar changes both in neuronal and extraneuronal tissues. The *ATG4D* gene encodes a cysteine protease, which is thought to function in the macroautophagy pathway. The autophagy process degrades and recycles damaged or obsolete cellular materials via membrane-enclosed autophagosomes. In line with this, the neuronal tissues of affected dogs showed signs of altered autophagic flow.

Overall, this study has revealed three new disease-linked genes in dogs, which may be associated to similar disorders in other species. On the basis of the results, DNA-tests have been developed for veterinary diagnostic and breeding purposes. Importantly, by shedding light into disease-causing pathways, the results of this study could prove beneficial not just for canine health but for human medicine as well.

Tiivistelmä

Perinnöllisiä sairauksia tavataan ihmisten lisäksi myös muissa lajeissa, kuten kotieläimissä. Tässä väitöskirjatyössä tutkittiin kolmea koirissa esiintyvää peittyvästi periytyvää sairautta. Tutkimuksen kohteena oli lyhytkasvuisuutta aiheuttava luustosairaus sekä kaksi erityyppistä hermostorappeumasairautta. Tutkimuksessa käytettiin hyväksi koko perimän laajuisia tutkimusmenetelmiä, joiden avulla paikannettiin sairauksien taustalla olevat geenivirheet.

Harmaissa norjanhirvikoirissa sekä karjalankarhukoirissa esiintyy kondrodysplasiaa, eli rustonsisäisen luutumisen häiriötä, joka ilmenee lyhytkasvuisuutena. Sairaiden koirien raajat ovat huomattavan lyhyet ja näillä esiintyy myös muita vakavuudeltaan vaihtelevia luustomuutoksia. Kummankin rodun lyhytkasvuisilta koirilta löytyi homotsygoottinen geenivirhe *ITGA10*-geenistä. Tutkimustulosten perusteella geenivirhe on saattanut siirtyä karjalankarhukoiriin harmaista norjanhirvikoirista. *ITGA10*-geeni koodaa α10-intergriini proteiinia, joka on osa kasvulevyjen rustosoluissa toimivaa, kollageeneja sitovaa α10β1-integriini reseptoria. Geenivirhe estää α10-proteiinin normaalin tuoton, minkä seurauksena kasvulevyn toiminta sekä luuston kasvu häiriintyy.

Suomenajokoirilla tavataan pentuiän pikkuaivorappeumaa, jota aiheuttava geenivirhe löytyi *SEL1L*-geenistä. Sairaat ajokoiranpennut kärsivät nopeasti etenevästä pikkuaivoataksiasta, eli liikkeiden säätelyn vaikeudesta. Pennuilta tunnistettiin *SEL1L*-geenistä homotsygoottinen yhden emäksen muutos, joka johtaa yhden aminohapon vaihtumiseen toiseksi SEL1L-proteiinin toiminnallisesti tärkeässä rakenteessa. SEL1L-proteiini toimii solulimakalvostossa proteiinien laaduntarkkailussa. Tunnistetun geenivirheen oletetaan aiheuttavan häiriöitä solulimakalvossa, mikä puolestaan vaikuttaa pikkuaivojen hermosolujen solunsisäinen tasapainoon ja johtaa ennenaikaiseen solukatoon.

Lagotto romagnolo -rodusta tunnistettiin uuden tyypin neurologinen sairaus, jonka pääasiallinen oire on etenevä, nuoruus- tai aikuisiällä alkava pikkuaivoataksia. Patologiset tutkimukset paljastivat solutason muutoksia paitsi keskushermostosta, mutta myös muualta elimistöstä. Pikkuaivoissa nähtiin etenevää hermosolukatoa. Sairauteen liitettiin homotsygoottinen yhden emäksen muutos *ATG4D*-geenissä. *ATG4D*-geenin toiminta liittyy autofagosytoosiin, joka on solunsisäinen puhdistus- ja ravinteiden kierrätys mekanismi. Sairaiden koirien keskushermostosta löytyikin viitteitä autofagosytoosin häiriintymisestä.

Kyseisiä geenejä ei ole aiemmin liitetty vastaaviin sairauksiin muissa lajeissa, mikä on antanut uutta tietoa sairauksiin liittyvistä molekyylireiteistä. Tästä tiedosta saattaa tulevaisuudessa olla hyötyä myös ihmislääketieteessä. Tutkimustulokset ovat lisäksi mahdollistaneet geenivirheitä kantavien koirien tunnistamisen DNA-testeillä. Testauksen avulla voidaan estää sairaiden koirien syntyminen sekä saada tukea eläinlääketieteelliseen diagnostiikkaan.

Table of contents

List of original publications

This thesis is based on the following publications:

I **Kyöstilä K**, Lappalainen AK and Lohi H. Canine chondrodysplasia caused by a truncating mutation in collagen-binding integrin alpha subunit 10. *PLoS One*. 2013 Sep;8(9):e75621.

II **Kyöstilä K**, Cizinauskas S, Seppälä EH, Suhonen E, Jeserevics J, Sukura A, Syrjä P, Lohi H. A *SEL1L* mutation links a canine progressive early-onset cerebellar ataxia to the endoplasmic reticulum-associated protein degradation (ERAD) machinery. *PLoS Genet*. 2012 Jun; 8(6):e1002759.

III **Kyöstilä K**, Syrjä P[*], Jagannathan V[*], Chandrasekar G, Jokinen TS, Seppälä EH, Becker D, Drögemüller M, Dietschi E, Drögemüller C, Lang J, Steffen F, Rohdin C, Jäderlund KH, Lappalainen AK, Hahn K, Wohlsein P, Baumgärtner W, Henke D, Oevermann A, Kere J, Lohi H[†], Leeb T[†]. A Missense Change in the *ATG4D* Gene Links Aberrant Autophagy to a Neurodegenerative Vacuolar Storage Disease. *PLoS Genet*. 2015 Apr 15;11(4):e1005169.

[*]Equal contribution

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The publications are referred to in the text by their roman numerals.

Author's contribution

Author's contribution to each publication:

I **Canine chondrodysplasia caused by a truncating mutation in collagen-binding integrin alpha subunit.**

Apart from the radiographic examinations, the author planned and performed the experiments, analyzed the data and wrote the paper under the supervision of Professor H. Lohi.

II **A SEL1L mutation links a canine progressive early-onset cerebellar ataxia to the endoplasmic reticulum-associated protein degradation (ERAD) machinery.**

The author designed and performed the genetic experiments and analyzed the data under the supervision of Professor H. Lohi. The author also wrote the paper, aside from with the clinical and pathological characterization.

III **A Missense Change in the ATG4D Gene Links Aberrant Autophagy to a Neurodegenerative Vacuolar Storage Disease.**

The author was involved in organizing and performing the genotyping of Finnish sample cohorts under the supervision of Professors H. Lohi and T. Leeb. The author performed the RNA-level experiments and participated in the design of the immunohistochemical and zebrafish experiments. The author had the main responsibility in combining the results and writing the publication.

Abbreviations

AD	Alzheimer disease
ALS	Amyotrophic lateral sclerosis
ATG	Autophagy-related
ATG4D	Autophagy-related 4D, cysteine peptidase
CFA	Canine chromosome
CNS	Central nervous system
Dpf	Day(s) post-fertilization
EA	Episodic ataxia
ECM	Extracellular matrix
EM	Electron microscopy
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated protein degradation
FFPE	Formalin-fixed, paraffin-embedded
FH	Finnish Hound
FRDA	Friedreich ataxia
HD	Huntington disease
HE	Haematoxylin-eosin
IHC	Immunohistochemistry
ITGA10	Integrin, alpha 10
KBD	Karelian Bear Dog
LD	Linkage disequilibrium
LR	Lagotto Romagnolo
LSD	Lysosomal storage disorder
MRI	Magnetic resonance imaging
NCL	Neuronal ceroid lipofuscinose
NE	Norwegian Elkhound
NMD	Nonsense-mediated mRNA decay
OMIM	Online Mendelian Inheritance in Man
PAS	Periodic-acid-Schiff´s
PC	Purkinje cell
PD	Parkinson disease
PolyQ	Polyglutamine
qPCR	Real-time quantitative PCR
RT-PCR	Reverse transcriptase PCR
SCA	Spinocerebellar ataxia
SCAR	Spinocerebellar ataxia, autosomal recessive
SEL1L	Sel-1 suppressor of lin-12-like (C. elegans)
SMO	Splice-morpholino
SNP	Single nucleotide polymorphism
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
Ya	Years ago

1 Introduction

The genome sequence of the domestic dog (*Canis lupus familiaris*) was published in 2005 (Lindblad-Toh *et al.* 2005), only a few years after the completion of the initial sequence draft of the human genome in 2001 (Lander *et al.* 2001, Venter *et al.* 2001). At present, the number of sequenced genomes is expanding at a very rapid rate, owing to advancements in the next generation high-throughput sequencing technologies. This progress has provided researchers valuable tools to conduct genetic studies in various different species and model organisms.

The domestic dog has proved to be a very useful model to delineate the molecular genetic background of different diseases and traits. Due to decades of artificial selection and inbreeding, inherited diseases have become common in the modern dog breeds. Recent discoveries have shed light into the genetic basis of several canine inherited diseases, but many remain to be studied further, and there likely are many more that have not yet been recognized. Apart from being beneficial to canine health, genetic studies in dogs can have broader significance, for instance by providing basic understanding of the pathways and mechanisms involved in disease pathogenesis. Furthermore, given that many canine disorders correspond with human conditions, both in regard to the clinical phenotype and the underlying molecular defect, the dog is a highly relevant model for human diseases.

There are several practical advantages in studying inherited diseases in purebred dogs. These include the availability of samples from several generations, as well as the availability of extensive pedigree records that can be utilized in determining inheritance patterns. Moreover, the population history of modern breeds had an impact on the dog genome, namely in the form of extensive linkage disequilibrium (LD) within breeds, which enables genome-wide studies to be performed using low sample and marker numbers (Karlsson *et al.* 2007).

During the course of this thesis work, breed clubs and dog owners have participated in the canine genetic research in large numbers. Starting from 2006, DNA-samples from over 50,000 pet dogs have been donated to the dog DNA bank maintained at the University of Helsinki. Alongside the physical DNA repository, a digital database holds comprehensive health information from the sampled animals. By utilizing these resources, this study has provided insights into the genetics of three canine recessive disorders, a short-stature dwarfism, and two types of progressive neurodegenerative disease.

2 Review of the literature

2.1 The domestic dog in genetic research

2.1.1 Dog domestication and the origin of breed structure

The dog is believed to have been domesticated from the gray wolf (*Canis lupus*) before the rise of agriculture (Vila *et al.* 1997, Lindblad-Toh *et al.* 2005). Recent genetic studies estimate that the domestication event took place somewhere between 11,000 to 30,000 years ago (ya) (Pang *et al.* 2009, Thalmann *et al.* 2013, Freedman *et al.* 2014). However, the question of exactly when, where and how has remained controversial (Savolainen *et al.* 2002, Pang *et al.* 2009, Boyko *et al.* 2009, Vonholdt *et al.* 2010, Ding *et al.* 2012, Thalmann *et al.* 2013, Freedman *et al.* 2014). For instance, mitochondrial and Y-chromosomal data from modern dogs support a single origin in Southeast Asia less than 16,300 ya (Pang *et al.* 2009, Ding *et al.* 2012), whereas mitochondrial genomes of ancient canids suggest an older origin in Europe between 18,800 to 32,100 ya (Thalmann *et al.* 2013). Likewise, analysis of whole genome sequence data from present-day dogs and wolves implicates a time of divergence between 11,000 and 16,000 ya (Freedman *et al.* 2014), whereas the genome sequence of an ancient wolf suggests that the ancestors of modern dogs and wolves diverged at least 27,000 ya (Skoglund *et al.* 2015). Some of the earliest dog-like fossils, dating more than 30,000 years, have been found in Siberia (Ovodov *et al.* 2011, Druzhkova *et al.* 2013) and Belgium (Germonpré *et al.* 2009, Thalmann *et al.* 2013), but whether these remains belong to early domesticated dogs or other canids is unsure (Ovodov *et al.* 2011, Drake *et al.* 2015). It is also possible that several domestication attempts may have taken place at different locations in different times and some of the resulting lineages have gone extinct (Thalmann *et al.* 2013). In addition, there is evidence of later admixture with wolf populations, which might also complicate the scenario (Vonholdt *et al.* 2010, Skoglund *et al.* 2015). However, regardless of the exact time and location of domestication, the dog has since spread to all corners of the world, serving various roles among human populations.

The current global dog population consists not only of purebred dogs, but also of different kinds of local dog types, mixed-breed dogs and free-ranging dogs that live in rural and urban environments. Semi-feral indigenous “village dogs” are for instance found in different parts of Africa and Asia (Boyko *et al.* 2009). At the moment, over 400 dog breeds are recognized worldwide, most of which are of European origin and have been created during the last few hundreds of years (Parker *et al.* 2004, Larson *et al.* 2012). After domestication, human selection is thought to have acted largely on behavior, such as herding, hunting or guarding, whereas more recently, the emphasis has shifted to appearance. The current breeds are the result of intense artificial selection, which has acted on different behavioral and morphological characteristics and resulted

in a tremendous amount of phenotypic variation within a single species (Galibert & André 2008). However, the phenotype of animals within each breed is typically very homogeneous. Detailed breed standards have been employed to define the desired behavior and appearance, which has led to the fixation of these traits within breeds. The "breed barrier" rule states that for a dog to be registered to a particular breed, both of its parents need to be registered to the same breed (Schoenebeck & Ostrander 2014). In practice, this has made each breed a genetic isolate, preventing gene flow across breed borders. Furthermore, the mating of close relatives with each other and the use of "popular sires" with hundreds of offspring has been common practice in the recent past (Calboli *et al.* 2008).

Present-day breeds have been formed through several bottlenecks; the major ones being the domestication and breed formation processes (**Fig. 1A**) (Lindblad-Toh *et al.* 2005, Freedman *et al.* 2014). Catastrophic events, such as the World Wars, have also had a large impact on the population sizes of several breeds (Galibert & André 2008, Larson *et al.* 2012). As a consequence of the bottlenecks and strict breeding practices, the phenotypic variation and genetic diversity is higher across breeds than within breeds. Dogs from a given breed are genetically more similar to each other than to dogs from other breeds, and breeds that are morphologically similar or originate from the same geographical region tend to cluster together on the basis of genetic data (Parker *et al.* 2004, Vonholdt *et al.* 2010). The population history of breeds has also given rise to a particular genomic structure, which is characterized by long-range LD within breeds and short-range LD across breeds, the latter of which reflects the genomic structure and short haplotype blocks of the pre-breed dog populations (**Fig. 1B**) (Lindblad-Toh *et al.* 2005). Within the breeds, LD can extend to megabases, when the size of the short ancestral haplotype blocks is only 5-15 kb (Sutter *et al.* 2004, Lindblad-Toh *et al.* 2005). In comparison, the LD of human populations is typically measured in a few tens of kilobases (Reich *et al.* 2001, Wall & Pritchard 2003).

2.1.2 Dogs can be used to understand inherited diseases

Dogs are reported to suffer from the second largest number of naturally occurring inherited diseases after humans (Patterson 2000, Sargan 2004). Mutations that cause monogenic diseases, and risk alleles that predispose to complex disorders, have been enriched within breeds as a consequence of the population bottlenecks and breeding practices. Diseases are often breed-specific or found only in a few related breeds. Autosomal recessive disorders are particularly frequent, as it has been easier for breeders to eradicate dominant than recessive conditions. Furthermore, the use of popular sires has very effectively spread recessive disease alleles in many breeds, a problem that has become apparent after a few generations (Patterson 2000, Ostrander & Kruglyak 2000). In some cases, artificial selection has favored monogenic phenotypes, which in dogs are referred to as traits, but in humans are considered pathological conditions, such as chondrodysplasia (causing short-stature) (Parker *et al.* 2009) or ectodermal dysplasia (causing hairlessness) (Drogemuller *et al.* 2008). The OMIA

database (Online Mendelian Inheritance in Animals) currently lists ~650 diseases and traits in dogs, over 260 of which are simple Mendelian phenotypes. Furthermore, nearly 200 of the phenotypes have been molecularly delineated, including traits such as coat color and type, and diseases such as developmental disorders, eye diseases, skeletal disorders, neurodegenerative phenotypes and metabolic diseases (http://omia.angis.org.au/).

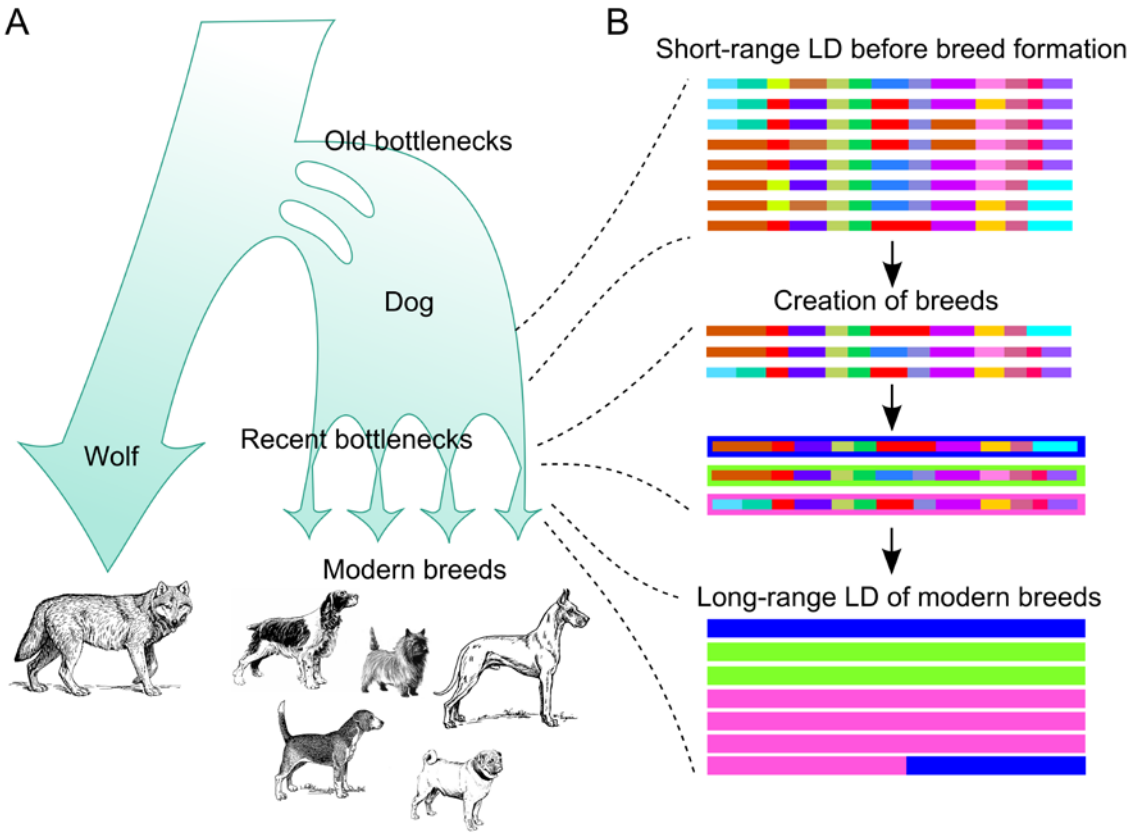

Figure 1. ***Domestication and haplotype structure of modern dog breeds.*** *Both old and recent bottlenecks have shaped the haplotype structure and LD in current dog breeds.* ***A)*** *Bottlenecks have been introduced by ancient domestication events, as well as the creation of modern breeds in the recent past.* ***B)*** *Before modern breeds were created, the dog population had a short-range LD structure. When subsets of dogs from this population were used to form the current breeds, the short ancestral haplotypes, which happened to be carried out on to the new breeds, formed the long-range LD pattern and haplotypes seen today. Because breed creation is a recent event, recombination has not yet had a large impact on the haplotype structure. The figure is adapted from Lindblad-Toh et al. 2005 and Karlsson & Lindblad-Toh 2008. Image sources: Wolf and dog drawings (Wikimedia Commons, Archives of Pearson Scott Foresman).*

The dog is not a replacement for traditional model organisms, such as the mouse, but it can be used as a platform to understand the molecular basis of hereditary diseases in humans and other species. Dogs are close to humans in size and physiology and they also share our environment, and in these respects, can be considered a good model for human diseases (Ostrander & Kruglyak 2000). In many cases, inherited diseases in dogs have proved to be caused by mutations in genes that have already been linked to similar disease phenotypes in humans (Katz *et al.* 2005, Goldstein *et al.* 2010, Ahonen *et al.* 2013). However, new disease-linked genes and pathways have also emerged (Patterson *et al.* 2008, Seppälä *et al.* 2011, Olsson *et al.* 2011, Grall *et al.* 2012), and some of these novel findings have already helped human medicine. For instance, the discovery of a hypocretin receptor 2 (*HCRTR2)* mutation in an autosomal recessive canine narcolepsy (Lin *et al.* 1999) has provided insights into the mechanism involved in human narcolepsies (Nishino *et al.* 2000), and contributed to the development of narcolepsy therapies (Hoyer & Jacobson 2013). Furthermore, dogs may be used to test gene therapy options, as has already been done with inherited eye diseases (Acland *et al.* 2001, Komaromy *et al.* 2010).

The canine genome is organized into 38 pairs of autosomes and one pair of sex chromosomes (Switonski *et al.* 1996). The initial draft of the canine genome sequence was published in 2003 (Kirkness *et al.* 2003), which was followed by a high-quality genome assembly in 2005 (CanFam1.0 and CanFam2.0) (Lindblad-Toh *et al.* 2005). The current CanFam3.1 assembly was made available in 2011. Alongside the high-quality genome sequence, a dense map of over 2.5 million single nucleotide polymorphisms (SNPs) was catalogued (Lindblad-Toh *et al.* 2005). This enabled the development of SNP arrays that can be utilized in genome-wide study approaches, such as linkage and association analyses. Importantly, the particular genomic architecture of dog breeds can be of use in genome-wide studies. The long-range LD within breeds makes it possible to map disease loci in genome-wide studies with only a limited number of samples and markers (Karlsson *et al.* 2007). However, on a downside, pinpointing the actual causative variant from the mapped locus may sometimes prove challenging because of the long haplotype blocks. Finally, recent advances in next-generation sequencing technologies and the subsequent drop in their cost, has made it possible to use these techniques more routinely also in canine genetics. Exome sequencing and whole-genome sequencing can be used either independently or in parallel with association and linkage studies to identify disease-causing gene variants.

2.2 Inherited skeletal disorders affect bone and cartilage tissues

Genetic skeletal disorders are diseases of the bone and cartilage tissues, arising from mutation in genes that govern skeletal patterning, development, growth and homeostasis (Warman *et al.* 2011). In humans, these disorders are individually rare but their collective birth prevalence is estimated to be around 1/5,000 (Orioli *et al.* 1986, Rasmussen *et al.* 1996). The most recent classification effort concerning human genetic skeletal diseases recognized altogether 456 distinct disease phenotypes that were placed into 40 different subcategories, depending on radiographic, biochemical and molecular features. For 316 of these disease entities, the molecular basis had been identified in altogether 226 different genes (Warman *et al.* 2011). Collectively, skeletal disorders are characterized by abnormalities in the shape, size and composition of the skeletal system. These changes can be either generalized or focal, and can vary greatly in severity (Mortier 2001, Superti-Furga *et al.* 2001). Disorders affecting individual bones, or a group of bones, have been called dysostoses, whereas generalized disorders of cartilage and bone have been referred to chondrodysplasias and osteodysplasias, respectively, or more generally as osteochondrodysplasias (Krakow & Rimoin 2010). Osteodysplasia is associated with reduced bone mineral density or excess bone deposition, and chondrodysplasia with cartilage defects, such as disturbed endochondral ossification (Newman & Wallis 2003). In addition to bone and cartilage defects, skeletal dysplasias can be accompanied by a variety of other clinical manifestations, such as auditory, neurologic, cardiac and ocular dysfunction (Krakow & Rimoin 2010). Although molecular information has now been incorporated into the categorization of human skeletal dysplasias, the naming of phenotypes has relied largely on different types of clinical and radiographic aspects. For instance, the terms rhizomelic, mesomelic and acromelic are used to refer to the involvement of the proximal, middle and distal limb segments, respectively, whereas ephiphyseal, metaphyseal, diaphyseal and spondylo (vertebral) determine the affected bone regions (Krakow & Rimoin 2010, Alanay & Lachman 2011). Another approach has been to use Greek terminology to define diseases such as thanatophoric ("death bringing"), campomelic ("bowing" of legs) and diastrophic ("twisting" of bones) dysplasia (Mortier 2001). **Table 1** lists osteochondrodysplasia subclasses in humans and gives examples of disease phenotypes and disease-linked genes.

Table 1. ***Human osteochondrodysplasia subcategories and disease examples.***

Disease Group / Disease phenotype (OMIM#)	Gene (Protein)
• **FGFR3 chondrodysplasias** Achondroplasia, AD (#100800)	*FGFR3* (Fibroblast growth factor receptor 3)
• **Type 2 collagen disorders** Spondyloepiphyseal dysplasia congenita, AD (#183900)	*COL2A1* (Type II collagen)
• **Type 11 collagen disorders** Stickler syndrome type 2, AD (#604841)	*COL11A1* (Type XI collagen)
• **Sulfation disorders** Diastrophic dysplasia, AR (#222600)	*SLC26A2* (Sulfate transporter)
• **Perlecan group** Dyssegmental dysplasia, AR (#224410)	*HSPG2* (Perlecan)
• **Aggrecan group** Spondyloepiphyseal dysplasia, AD (#612813)	*ACAN* (Aggrecan)
• **Filamin- and related disorders** Frontometaphyseal dysplasia, XLD (#305602)	*FLNA* (Filamin A)
• **TRPV4 group** Metatropic dysplasia, AD (#156530)	*TRPV4* (Cation channel)
• **Short-ribs dysplasias group** Chondroectodermal dysplasia, AR (#225500)	*EVC1* (Ellis van Creveld protein)
• **Multiple epiphyseal dysplasias** Multiple epiphyseal dysplasia 1, AD (#132400)	*COMP* (Cartilage oligomeric matrix protein)
• **Metaphyseal dysplasias** Cartilage-hair hypoplasia, AR (#250250)	*RMRP* (RNA component of RNAse MRP)
• **Spondylometaphyseal dysplasias** Spondyloenchondrodysplasia, AR (#607944)	*ACP5* (Tartrate-resistant acid phosphatase)
• **Spondylo-epi-(meta)-physeal dysplasias (SE(M)D)** SEMD, short limb-abnormal calcification, AR (#271665)	*DDR2* (Discoidin domain receptor 2)
• **Severe spondylodysplastic dysplasias** Achondrogenesis type 1A, AR (#200600)	*TRIP11* (Golgi-microtubule-associated protein)
• **Acromelic dysplasias** Acrocapitofemoral dysplasia, AR (#607778)	*IHH* (Indian hedgehog)
• **Acromesomelic dysplasias** Chondrodysplasia, Grebe type, AR (#200700)	*GDF5* (Growth and differentiation factor 5)
• **Mesomelic and rhizo-mesomelic dysplasias** Dyschondrosteosis, pseudo-AD (#127300)	*SHOX* (Short stature homeobox)
• **Bent bones dysplasias** Campomelic dysplasia, AD (#114290)	*SOX9* (SRY-box 9)
• **Slender bone dysplasias** 3-M syndrome, AR (#273750)	*CUL7* (Cullin 7)
• **Dysplasias with multiple joint dislocations** Desbuquois dysplasia, AR (#251450)	*CANT1* (Calcium-activated nucleotidase 1)
• **Chondrodysplasia punctata (CDP)** Rhizomelic CDP type 1, AR (#215100)	*PEX7* (Peroxisomal biogenesis factor 7)
• **Neonatal osteosclerotic dysplasias** Chondrodysplasia, Blomstrand type, AR (#215045)	*PTHR1* (PTH/PTHrP receptor 1)

The table is modified from Warman et al. 2011. Abbreviations: AD = autosomal dominant; AR = autosomal recessive; XLD = X-linked dominant; XLR = X-linked recessive

2.2.1 Chondrodysplasias are caused by defects in endochondral ossification

Intramembranous and endochondral ossification are the two principal mechanisms of bone formation. Intramembranous ossification transforms mesenchymal tissue directly into bone, whereas endochondral ossification occurs via a cartilaginous template (Newman & Wallis 2003, Krakow & Rimoin 2010). Endochondral ossification is the major mechanism responsible for longitudinal bone growth and its dysfunction typically results in disproportionate short stature (Newman & Wallis 2003). Endochondral ossification takes place at the cartilaginous growth plates (also known as physes and ephiphyseal plates), which are located at the ends of the growing long bones, between the epiphysis and metaphysis (**Fig. 2A**). The growth plates are composed of extracellular matrix (ECM) and longitudinally stacked chondrocyte columns, which can be divided into morphologically distinct chondrocyte zones based on the functional states of the cells (**Fig. 2B**) (Mackie *et al.* 2008). Within the growth plate, the chondrocytes first proliferate and then differentiate into large, non-dividing, hypertrophic cells that eventually die and become replaced by bone tissue (**Fig. 2B**) (Yeung Tsang *et al.* 2014). The chondrocytes also deposit the surrounding ECM, which is composed of collagens, proteoglygans and other structural proteins (Myllyharju 2014). However, the components of the growth plate ECM have not only structural importance, but can also mediate important functional signals via cell surface receptors (Mackie *et al.* 2008). Overall, the increase in bone length is thought to be the combined result of chondrocyte division, hypertrophy and matrix synthesis (Wilsman *et al.* 1996). The conversion of cartilage to bone is governed by several different transcription factors, growth factors and cell surface receptors that mediated signaling cascades related to growth plate physiology (Myllyharju 2014). Not surprisingly, many chondrodysplasias are caused by defects either in ECM components or in proteins that govern chondrocytic differentiation and proliferation (**Table 1**).

SOX9 transcription factor, Indian hedgehog (IHH), parathyroid hormone-related protein (PTHrP) and fibroblast growth factor receptor 3 (FGFR3) are examples of proteins that control the chondrocyte journey within the growth plate. SOX9 is needed for the differentiation of mesenchymal cells into chondrocytes, and in the zone of chondrocyte proliferation, it regulates the expression of several genes important for growth plate function, such as type II collagen (Bell *et al.* 1997, Bi *et al.* 1999). Heterozygous *SOX9* mutations cause campomelic dysplasia, which is a severe and often lethal disorder, characterized by congenitally bowed, thick long bones and XY sex reversal (Foster *et al.* 1994, Wagner *et al.* 1994). Once the chondrocytes mature and enter the prehypertrophic stage IHH and PTHrP form a negative feedback loop that tightly regulates chondrocyte differentiation and prevents premature hypertrophy (Vortkamp *et al.* 1996). *IHH* mutations cause autosomal recessive short-stature dysplasia known as acrocapitofemoral dysplasia (Hellemans *et al.* 2003) and dominantly inherited type A1 brachydactyly, characterized by short or absent middle phalanges (Kirkpatrick *et al.* 2003). The parathyroid hormone receptor 1 (PTH1R) serves as a receptor for both parathyroid hormone and PTHrP. Heterozygous *PTH1R*

mutations cause Jansen metaphyseal chondrodysplasia (Schipani *et al.* 1995), whereas homozygous *PTH1R* mutations result in two other types of skeletal dysplasia, Blomstrand chondrodysplasia (Jobert *et al.* 1998) and Eiken syndrome (Duchatelet *et al.* 2005).

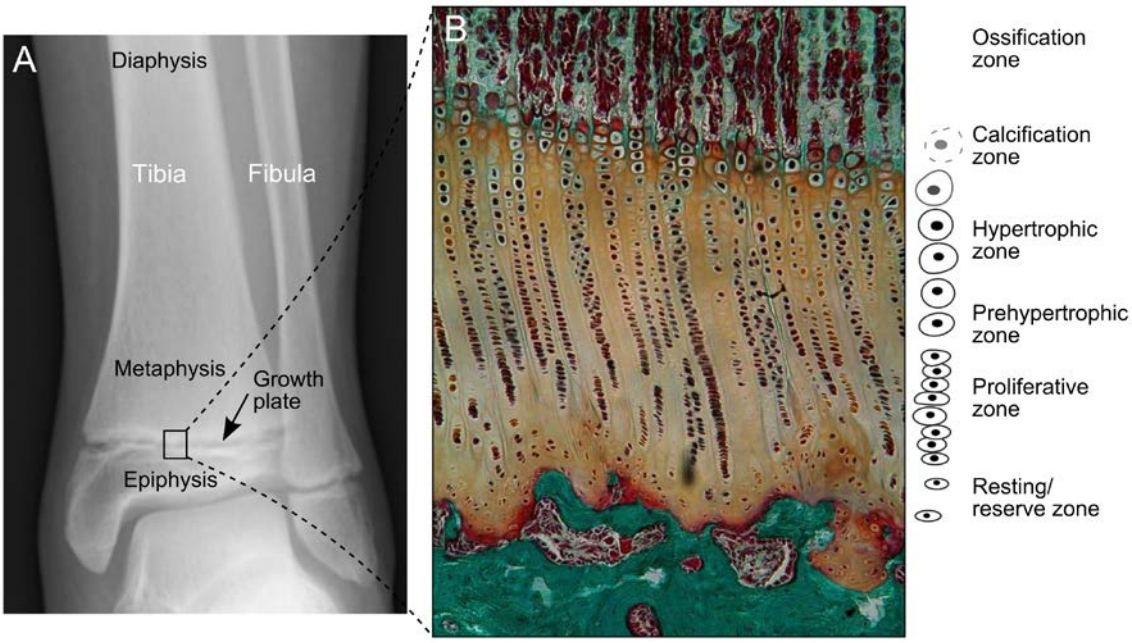

Figure 2. ***The growth plate. A)*** *The epiphyseal growth plates are found in the ends of long bones and are responsible for bone growth.* ***B)*** *Within the growth plate, the chondrocytes are organized in longitudinal columns, and can be separated into different horizontal zones that reflect the cellular states. The reserve zone contains resting chondrocytes. In the zone of proliferation, the chondrocytes form distinctive stacks of rapidly-dividing flattened cells. Once the chondrocytes enter the prehypertrophic zone, they exit the cell cycle and initiate differentiation. In the hypertrophic zone, the chondrocytes become massively enlarged and eventually die. Finally, the growth plate cartilage is calcified and replaced by bone tissue, through the invasion of blood vessels, osteoclasts and osteoblasts. The figure is adapted from Mackie et al. 2008 and Yeung Tsang et al. 2014. Image sources: Radiograph, the lower leg of a 12-year-old child (Wikimedia commons, Gilo1969); Growth plate histology, rabbit, Masson-Goldner trichrome stain (Wikimedia commons, Jeppe Achton Nielsen).*

FGFR3 is a negative regulator of growth plate chondrocyte proliferation and differentiation (Deng *et al.* 1996). The most frequent and well-known form of short-limbed dwarfism, achondroplasia, is caused by heterozygous *FGFR3* mutations (Shiang *et al.* 1994, Rousseau *et al.* 1994). The condition is characterized by rhizomelic shortening of limbs and particular facial features, including prominent forehead and mid-face hypoplasia (Horton *et al.* 2007). The large majority of achondroplasia cases result from de novo mutations, which typically are of paternal origin and associated with advanced paternal age (Wilkin *et al.* 1998). Achondroplasia mutations are thought

to cause abnormal FGFR3 activation, which results in exaggerated inhibition of bone growth (Horton *et al.* 2007). In addition to achondroplasia, *FGFR3* mutations cause a spectrum of other skeletal disorders, such as thanatophoric dysplasia, which is a severe short-limbed dysplasia that typically leads to death at the perinatal period (Tavormina *et al.* 1995).

The integrity of the growth plate matrix can be compromised through mutations in ECM proteins, but also via other pathogenic mechanisms, such as disturbed ECM remodeling. Collagens constitute a key component of cartilage ECM. The major collagen in growth plate cartilage is type II collagen but collagens IX, X and XI are also present (Mendler *et al.* 1989, Myllyharju 2014). Type II collagen is encoded by the *COL2A1* gene, which is defective in a range of autosomal dominant skeletal disorders (Kannu *et al.* 2012) that vary from mild disorders of the adulthood, such as premature osteoarthritis (Ala-Kokko *et al.* 1990), to more severe short-stature dysplasias and perinatally lethal syndromes, such as Strudwick type spondyloepimetaphyseal dysplasia (Tiller *et al.* 1995) and achondrogenesis type II (Vissing *et al.* 1989), respectively. In addition to collagen type II disorders, various skeletal dysplasia are caused by mutations in the type IX, X and XI collagen genes, and also by mutations in other ECM proteins, such as cartilage oligomeric matrix protein (COMP) and proteoglycans aggrecan and perlecan (Warman *et al.* 2011). The matrix metalloproteinases MMP9 and MMP13 work together in degrading type II collagen and aggregan within growth plate ECM, allowing subsequent vascular invasion and ossification (Stickens *et al.* 2004). *MMP9* and *MMP13* mutations cause metaphyseal anadysplasia, which is a type of chondrodysplasia characterized by short-stature and severe metaphyseal bone irregularities that typically resolve in early childhood (Lausch *et al.* 2009, Li *et al.* 2015). A subgroup of skeletal dysplasias result from impaired sulfation of ECM proteoglycans. The *SLC26A2* gene encodes a sulfate transporter that maintains adequate chondrocyte sulfate levels, which are required for proteoglycan sulfation (Park *et al.* 2014). Similar to many other skeletal dysplasia genes, mutations in *SLC26A1* cause a spectrum of phenotypes (Hästbacka *et al.* 1994, Superti-Furga *et al.* 1996, Superti-Furga *et al.* 1999), ranging from lethal achondrogenesis (Superti-Furga *et al.* 1996) to a mild form of epiphyseal dysplasia (Superti-Furga *et al.* 1999).

2.2.2 Inherited chondrodysplasia in dogs

Chondrodysplastic phenotype can occur in dogs either as a desired, breed-defining characteristic (Martinez *et al.* 2007, Parker *et al.* 2009), or as an unwanted condition in otherwise non-chondrodysplastic breeds (Carrig *et al.* 1977, Bingel & Sande 1982, Sande *et al.* 1982, Breur *et al.* 1989). Similar to humans, canine chondrodysplasias are characterized by disproportionate short-limbed dwarfism. In many cases, asynchronous growth of the radius and ulna results in curved front limbs and carpal valgus deformity (bending and outward rotation of the distal limbs). Other clinical features may include bone malformations, vertebrate abnormalities, joint defects and secondary osteoarthritis (Sande & Bingel 1983). Overall, the clinical phenotype can vary from a mild shortening

of long bones (Frischknecht *et al.* 2013) to a more severe skeletal deformity and lameness (Riser *et al.* 1980, Neff *et al.* 2012). Flaring, widening or otherwise irregular appearance of epiphyses, growth plates and metaphyses are often seen in radiographic examination (Sande *et al.* 1982, Bingel & Sande 1982, Breur *et al.* 1989, Bingel & Sande 1994, Martinez *et al.* 2007). Histological characteristics vary depending on the disorder, but can include abnormal structure of forming metaphyseal bone and different types of growth plate disturbances, such as disorganized chondrocyte columns, abnormal chondrocyte appearance, reduced chondrocyte numbers and abnormal wide bars of ECM (Sande *et al.* 1982, Bingel & Sande 1982, Breur *et al.* 1989, Bingel & Sande 1994, Martinez *et al.* 2007).

The molecular genetic background of five canine chondrodysplasias has been delineated thus far (**Table 2**). The first genetic discovery shed light into an autosomal dominant breed-defining chondrodysplasia, fixed into breeds such as the Dachshund, Basset Hound, Pekingese and Corgi (Martinez *et al.* 2007, Parker *et al.* 2009). By using a multi-breed genome-wide association approach, the phenotype was associated with an expressed extra copy of fibroblast growth factor 4 (*FGF4*) gene that was found to be inserted within a long interspersed element (LINE) (Parker *et al.* 2009). The expression of the *FGF4* retrogene was suggested to cause an abnormal activation of fibroblast growth factor receptors, such as FGFR3. In all affected breeds, a common haplotype surrounded the retrogene, indicating that the retrotransposition event had occurred only once before the modern breeds were created (Parker *et al.* 2009).

The four other genetically characterized canine chondrodysplasias are autosomal recessive conditions. The Samoyed and Labrador Retriever breeds are both affected with a very similar type of oculo-skeletal dysplasia, characterized by short-limbed dwarfism and severe ocular defects, such as cataracts and retinal detachment (Carrig *et al.* 1977, Meyers *et al.* 1983, Aroch *et al.* 1996). These disorders were found to be caused by homozygous defects in the type IX collagen genes, *COL9A2* and *COL9A3* (Goldstein *et al.* 2010). Homozygous mutations in the human *COL9A2* gene result in Stickler syndrome type V, which resembles the canine phenotype, as it is also characterized by ocular abnormalities and short stature (Baker *et al.* 2011). Another type of chondrodysplasia that affects the Labrador Retriever breed causes only a mild shortening of long bones (Frischknecht *et al.* 2013). The phenotype is associated with a homozygous missense mutation in the type XI collagen gene, *COL11A2* (Frischknecht *et al.* 2013), which is also linked with several human skeletal phenotypes (**Table 2**) (Vikkula *et al.* 1995). Finally, a severe form of osteochondrodysplasia in the Miniature Poodle breed is caused by a 130-kb homozygous deletion in the *SLC13A1* gene, encoding a sodium/sulfate symporter that regulates serum sulfate levels (Neff *et al.* 2012). The affected dogs present with several skeletal defects, such as short and curved long bones, malformed femoral heads and necks, misshapen paws and a flattened rib cage (Riser *et al.* 1980). Similar to the human *SLC26A2*-linked dysplasias, the canine phenotype was suggested to result from disturbed sulfation of ECM proteoglycans (Neff *et al.* 2012). Besides these genetically characterized conditions, several other canine osteochondrodysplasias with an unknown genetic cause have been described (**Table 3**).

Table 2. ***Canine chondrodysplasias with an identified genetic basis.***

Affected Breed	Clinical features	Radiographic/ histologic features	Gene Protein	Human skeletal disease (OMIM#)
Labrador Retriever	Short-limbed dwarfism, bowing of front limbs, carpal valgus, ocular defects	Curved radii, ununited anconeal and coronoid processes, hip dysplasia, delayed development of epiphyses (Carrig *et al.* 1977)	*COL9A3* Type IX collagen	•Multiple epiphyseal dysplasia 3 (#600969)
Labrador Retriever	Mild short-limbed dwarfism	Slightly shortened long bones, widened epiphyses (Frischknecht *et al.* 2013)	*COL11A2* Type XI collagen	•Fibrochondrogenesis 2 (#614524) •Otospondylomega-epiphyseal dysplasia (#215150) •Stickler syndrome III (#184840) •Weissenbacher-Zweymuller syndrome (#277610)
Miniature Poodle	Short-limbed dwarfism, bowed limbs, enlarged joints, flattened rib cage, lameness, secondary arthritis	Short vertebral bodies, maformed ribs, stippled and poorly outlined epiphyses, abnormal hyaline cartilage, delayed ossification (Riser *et al.* 1980)	*SLC13A1* Sodium/ sulfate symporter	*Not reported*
Samoyed	Short-limbed dwarfism, carpal valgus, ocular defects	Metaphyseal flaring; curved radii; abnormal femoral heads (Meyers *et al.* 1983, Aroch *et al.* 1996)	*COL9A2* Type IX collagen	•Multiple epiphyseal dysplasia 2 (#600204) •Stickler Syndrome V (#614284)
Several short-stature breeds (Dachshund, Gorgi, Basset Hound etc.)	Breed defining short-stature dwarfism, bowed limbs	Wide metaphyses, irregular epiphyses, disorganized chondrocyte columns, premature ossification (Martinez *et al.* 2007)	*FGF4* Fibroblast growth factor 4	*Not reported*

Table 3. *Canine chondrodysplasias with an unknown genetic etiology.*

Affected Breed	Clinical features	Radiographic/ histologic features	Similar human disease
Alaskan Malamute	Short-limbed dwarfism, bowing of limbs, carpal valgus, anemia	Flared, wide and irregular growth plates, excess growth plate matrix, metaphyseal microfractures (Smart & Fletch 1971, Sande *et al.* 1982)	•Metaphyseal dysplasia
Danish-Swedish Farm Hound	Short-limbed dwarfism, abnormally short limbs and body, lameness	Short vertebral bodies, abnormal vertebral epiphyses, narrow articular spaces, spondylosis, osteoarthritis of joints (Arnbjerg *et al.* 2007)	•X-linked spondylo-epiphyseal dysplasia tarda
English Pointer	Short-limbed dwarfism, bowed limbs, flattened sternum, enlarged growth plates, lameness	Flared and wide growth plates, widened zone of chondrocyte hypertrophy, degeneration of articular cartilage (Whitbread *et al.* 1983)	*Not available*
Great Pyrenees	Short-limbed dwarfism, deafness	Short ribs, abnormal vertebrae, wide metaphyses, dizorganization of chondrocyte columns and wide areas of matrix, thick and irregular trabeculae in spongiosa (Bingel & Sande 1994)	•Spondylometa-physeal dysplasia
Irish Setter	Short-limbed dwarfism, bowing of front limbs	Shortened vertebral bodies, subtle histological abnormalities in epiphyses, growth plates and spongiosa (Hanssen *et al.* 1998)	•Hypochondroplasia
Labrador Retriever	Short-limbed dwarfism, bowed front limbs	Absence of the anconeal process, wide metaphyses, disorganized growth plate chondrocyte columns (Smit *et al.* 2011)	•Multiple epiphyseal dysplasia •Spondylo-epiphyseal dysplasia congenital •Stickler syndrome
Norwegian Elkhound	Short-limbed disproportionate dwarfism, bowing of limbs, carpal valgus	Flared and wide growth plates, delayed ossification of carpal cuboid bones, short vertebral bodies, abnormal chondrocyte morphology, disorganization of chondrocyte columns (Bingel & Sande 1982)	•Spondylometa-physeal dysplasia
Scottish Deerhound	Short-limbed disproportionate dwarfism, bowing of limbs, joint laxity, exercise intolerance	Short vertebrae, abnormal carpal and tarsal bones, malformed hip and elbow joints, histological irregularities in epiphyses, growth plates and metaphyses (Breur *et al.* 1989)	•Spondyloepimeta-physeal dysplasia

2.3 Inherited neurodegenerative diseases

Neurodegenerative disorders encompass a variety of conditions, in which the primary characteristic is progressive neuronal degeneration that has irreversible and debilitating effects on motor and cognitive functions (Przedborski *et al.* 2003). Certain neurodegenerative diseases can occur without a clear family history, such as sporadic forms of Alzheimer disease (AD), Parkinson disease (PD) and amyotrophic lateral sclerosis (ALS) (Mitsui & Tsuji 2014), whereas others are inherited in a monogenic fashion, including the cerebellar ataxias (Mancuso *et al.* 2014), Huntington disease (HD) (The Huntington's Disease Collaborative Research Group 1993) and familial ALS (Rosen 1993), AD (Goate *et al.* 1991) and PD (Polymeropoulos *et al.* 1997). Depending on the disorder, the principal neurodegenerative lesions are characteristically found in selected neuronal subpopulations within specific anatomical regions of the nervous system, giving rise to a particular set of neurological symptoms (Hardy & Gwinn-Hardy 1998, Przedborski *et al.* 2003). For instance, the inherited ataxias are characterized by cerebellar degeneration (Manto & Marmolino 2009), ALS by lower and upper motor neuron loss (Foerster *et al.* 2013) and PD by atrophy of the substantia nigra (Dickson *et al.* 2009). However, despite these distinctions, there can be significant overlap between the clinical signs and the affected nervous system regions, which can make diagnosis challenging. When the disease manifests clinically, marked pathological lesions are usually already present (Hardy & Gwinn-Hardy 1998, Przedborski *et al.* 2003).

2.3.1 Protein homeostasis and degradative pathways in neurodegeneration

Regardless of the underlying genetic defects or risk factors, common molecular cascades have emerged both in familial and complex neurodegenerative disorders (Jellinger 2010). These include disturbances in protein homeostasis (proteostasis) (Lim & Yue 2015), mitochondrial dysfunction and oxidative stress (Federico *et al.* 2012, Dasuri *et al.* 2013), altered calcium signaling (Marambaud *et al.* 2009) and endoplasmic reticulum stress (Hetz & Mollereau 2014). A recurrent finding in many neurodegenerative diseases is the accumulation of proteinaceous aggregates within the nervous system. For example, PD is characterized by α-synuclein aggregation (Lewy bodies), AD by deposition of β-amyloid (senile plaques) and tau (neurofibrillary tangles), and HD by aggregation of the mutated huntingtin protein (Forman *et al.* 2004, Lim & Yue 2015). Certain causative mutations, such as coding polyglutamine (polyQ) repeat expansions, directly result in aberrant and aggregation-prone protein structures (Weber *et al.* 2014). However, neuronal proteostasis may also be disturbed through dysfunction of the pathways that are involved in protein trafficking, degradation and quality control.

The autophagy-lysosome route and the ubiquitin-proteasome system (UPS) are the two major intracellular proteolytic mechanisms that are involved in maintaining cellular homeostasis under basal and stressful conditions (**Fig. 3**) (Nedelsky *et al.* 2008). The

UPS has been implicated to selectively target short-lived and misfolded proteins, which are first polyubiquitinated by E3 ubiquitin ligases, and then degraded via the proteasome complex (Korolchuk *et al.* 2010). The UPS pathway is closely linked with the molecular chaperones and the endoplasmic reticulum (ER) -associated protein degradation (ERAD) machinery. The chaperone proteins function in ensuring correct protein folding and recognizing misfolded protein states (Smith *et al.* 2015), while the ERAD machinery delivers aberrantly folded proteins from the ER to the cytosolic proteasome machinery (Smith *et al.* 2011). In comparison to the UPS, the autophagy pathway is thought to constitute more of a bulk degradation and recycling system, targeting organelles, as well as long-lived and aggregated proteins (Korolchuk *et al.* 2010). Macroautophagy, which is the major autophagy pathway and usually referred to simply as autophagy, engulfs its targets inside the double-membraned autophagic vesicles, autophagosomes (**Fig. 3**). The autophagosomes then fuse with the lysosomes, enabling the degradation of the captured materials via lysosomal hydrolases (Hyttinen *et al.* 2013). The endocytic trafficking pathways also deliver material for lysosomal degradation. Late endosomes that carry degradation-bound materials fuse with lysosomes, either directly or through intermediate vesicular structures, amphisomes, which are the fusion products of endosomes and autophagosomes (**Fig. 3**) (Berg *et al.* 1998).

As a whole, neurons appear to be very sensitive to perpetuations in catabolic pathways and increasing evidence suggests that these intertwined degradative routes are either directly disturbed or otherwise affected in a range of neurodegenerative diseases (Lehman 2009, Nixon 2013, Dantuma & Bott 2014, Wong & Holzbaur 2015). For instance, several forms of familial PD result from mutations that disrupt protein trafficking and degradation through the endosomal pathway (Perrett *et al.* 2015). Dysfunction of molecular chaperones causes inherited cerebellar ataxia (Engert *et al.* 2000, Anttonen *et al.* 2005, Senderek *et al.* 2005, Shi *et al.* 2013), and defects in autophagy-linked receptor proteins have been implicated in familial ALS (Majcher *et al.* 2015).

The lysosome is the end-point of several degradation routes, it is not therefore surprising that primary lysosomal defects also cause neurodegeneration. Lysosomal dysfunction gives rise to a specific disease entity known as lysosomal storage disorders (LSDs). LSDs are typically caused by loss-of-function mutations in lysosomal enzymes, leading to an excessive accumulation of their unprocessed substrates within storage vacuoles (Boustany 2013). The majority of LSDs involve the nervous system (Boland & Platt 2015), in particular the neuronal ceroid lipofuscinoses (NCLs), which are characterized by neurodegeneration (Bennett & Rakheja 2013). Interestingly, most NCLs do not result from defects in lysosomal enzymes, but are linked to genes that function within vesicular trafficking pathways, related to the ER, Golgi, endosome and autophagy-lysosome axis (Bennett & Rakheja 2013, Kollmann *et al.* 2013).

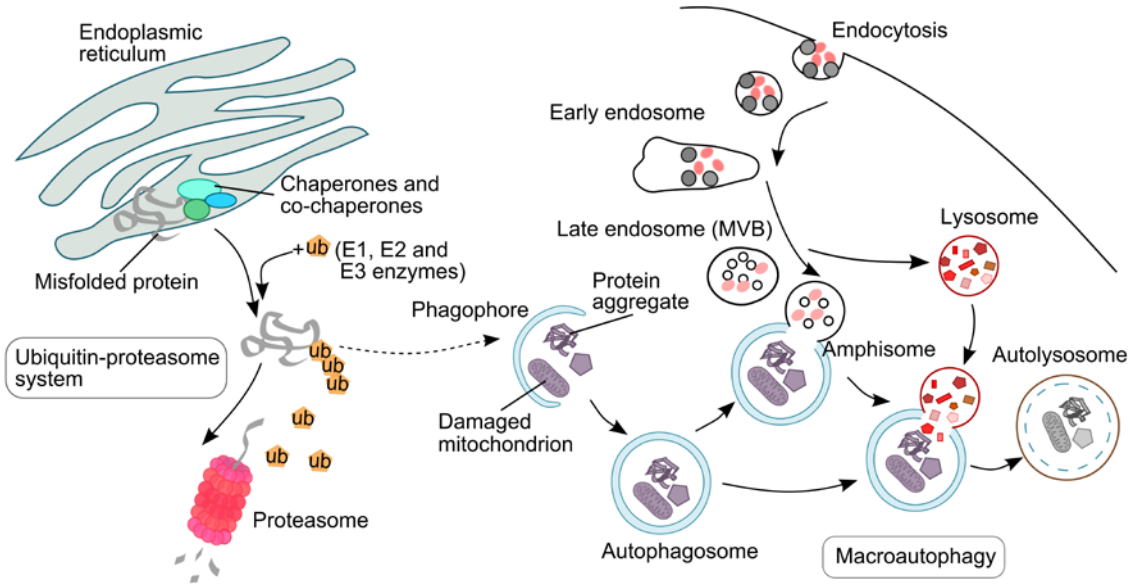

Figure 3. ***Cellular degradative pathways.*** *The two principal degradative pathways are the ubiquitin-proteasome system (UPS) and the autophagy-lysosome route. The UPS can receive target proteins from the endoplasmic reticulum, where molecular chaperones recognize misfolded proteins. The misfolded or unfolded proteins are then ubiquitinated through the actions of the E1, E2 and E3 enzymes, which function canonically in ubiquitin activation, conjugation and ligation, respectively. Polyubiquitination targets proteins for the proteasome, but may also serve as an autophagosomal signal. The macroautophagy pathway captures degradation-bound materials within autophagosomal vesicles that eventually fuse with the lysosomes, either directly, or alternatively via formation of amphisomes that are fusion products of late endosomes (also known as multivesicular bodies, MVBs). The figure is adapted from Nedelsky et al. 2008 and Lim & Yue 2015.*

2.3.1 Inherited ataxias are caused by cerebellar dysfunction

Inherited cerebellar ataxias are rare neurological diseases that result from degeneration and dysfunction of the cerebellum and its connections. The predominant clinical sign is progressive ataxia, but other neuronal and extraneuronal tissues may also show various degrees of involvement (Manto & Marmolino 2009, Klockgether & Paulson 2011, Mancuso *et al.* 2014). Ataxia itself is a neurological symptom of motor incoordination, which typically manifests as impaired gait, caused by difficulty in coordinating limb movements. In addition to gait difficulties, cerebellar dysfunction typically causes tremors, balance problems, poor posture, abnormal eye movements and speech difficulties. Patients that suffer from cerebellar dysfunction may also suffer from mild cognitive deficits (Schmahmann 2004, Grimaldi & Manto 2012).

The cerebellum is an anatomically distinct region at the back of the brain (**Fig. 4A-B**). Besides its well-established role in motor coordination, increasing evidence suggests involvement in cognitive functions, such as those relating to working memory and language (Ramnani 2006, Gordon 2007, Stoodley 2012). The gross cerebellar

anatomy can vary considerably between different animal species but the cellular architecture and circuitry are largely conserved in evolution (Voogd & Glickstein 1998). The tightly-folded cerebellar cortex has a uniform composition of three different cell layers: the molecular layer, the Purkinje cell (PC) layer and the granular cell layer (**Fig. 4C**). The PCs are considered the most important information-processing units within the cerebellar cortex. They provide the sole output of the cortex by synapsing with the deep cerebellar nuclei and certain brain stem nuclei (Voogd & Glickstein 1998, Ramnani 2006). Inherited cerebellar ataxias typically result from PC loss. As the main functional units of the cerebellar cortex, the PCs are very sensitive to different types of disturbances affecting the cerebellum (Grüsser-Cornehls & Bäurle 2001). At the same time, PC loss and dysfunction can cause secondary degeneration of other cerebellar neurons, such as the granule cells and the deep cerebellar nuclei (Sarna & Hawkes 2003).

The human inherited ataxias have a highly heterogeneous disease etiology and clinico-pathological characteristics (Pandolfo & Manto 2013). Most hereditary ataxias in humans are autosomal dominant or recessive diseases, while a few are inherited in an X-linked manner. In general, the dominant ataxias tend to be restricted to the central nervous system (CNS) and have an onset in adulthood, whereas the recessive ataxias can involve multiple organs and typically have an earlier onset (Taroni & DiDonato 2004, Wolf & Koenig 2013). The autosomal dominant ataxias have been systematically designated as spinocerebellar ataxias (SCAs), and currently include at least 35 different disease phenotypes, with a molecular genetic cause identified for the large majority (Kim & Cho 2015) (http://neuromuscular.wustl.edu/ataxia/domatax.html). In comparison to the dominant ataxias, the autosomal recessive ataxias represent a less well-defined disease entity that can include anywhere from 20 to well over 50 disorders, depending on the classification criteria (Mancuso *et al.* 2014, Kim & Cho 2015) (http://neuromuscular. wustl.edu/ataxia/recatax.html).

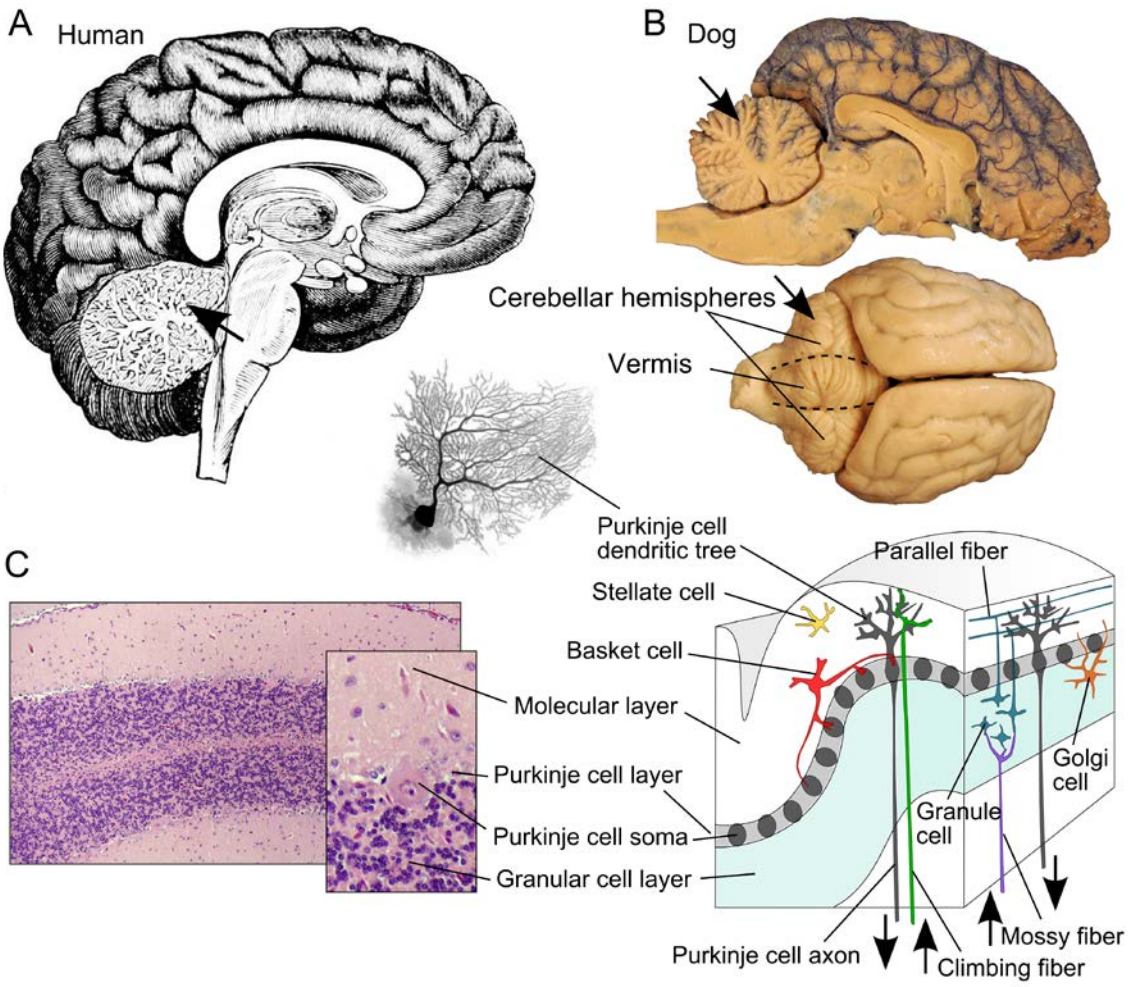

Figure 4. ***The cerebellum and its cellular composition. A)*** *The human and* ***B)*** *canine brain, with arrows denoting the cerebellum. The major anatomical subdivisions within the cerebellum include the vermis and the cerebellar hemispheres.* ***C)*** *The cerebellar cortex is composed of three distinct layers: the molecular layer, Purkinje cell (PC) layer and granular cell layer. The molecular layer is relatively cell poor, whereas the small granule cells within granule cell layer are the most numerous neuron type within the whole brain. The somas of the large PCs form a monolayer between the molecular and granular cell layers and their intricate dendritic trees extend to the molecular layer. The PC dendrites synapse with two major afferents, the climbing fibers, which originate from the inferior olive, and mossy fibers, which originate from the peripheral nervous system, spinal cord and brainstem. The climbing fibers synapse directly with the PC dendrites, whereas the mossy fibers pass information through the excitatory granule cells, whose axons constitute the parallel fibers (Ito 2006). Other cell types within the cortex include the Golgi-, stellate- and basket cells, which are inhibitory interneurons (Voogd & Glickstein 1998). The schematic structure of the cerebellar cortex is adapted from Ramnani 2006. Image sources: Human brain (Wikimedia Commons, Popular Science Monthly Volume 1); Canine brain (Pixabay, Katja); PC image (Wikimedia Commons, http://ccdb.ucsd.edu); Cerebellar cortex histology, dog, HE (Pernilla Syrjä).*

The genetic background of the dominantly inherited SCAs includes expansion of both coding and non-coding microsatellites, as well as more conventional mutations (Hekman & Gomez 2015). Several SCAs are polyQ diseases, caused by expanded glutamine-codon (CAG) repeats within protein coding regions of the disease-linked genes (Fan *et al.* 2014). Translation of the polyQ tracts is thought to disrupt protein folding, which eventually results in accumulation and aggregation of the aberrant polypeptides. It is currently not clear whether the large end-point aggregates are the primary cause of neuronal death, or if their formation is a protective mechanisms to help cells cope with smaller soluble oligomers that may be toxic for the cell (Takahashi *et al.* 2010, Todd & Lim 2013). The expanded CAG-repeats are unstable and tend to expand further, resulting in anticipation, which refers to a more severe disease with an earlier onset in successive generations (Fan *et al.* 2014). At the moment, coding CAG-repeat expansions have been reported in six SCAs (SCAs 1, 2, 3, 6, 7 and 17) (Orr *et al.* 1993, Kawaguchi *et al.* 1994, Pulst *et al.* 1996, Lindblad *et al.* 1996, Zhuchenko *et al.* 1997, Koide *et al.* 1999), whereas untranslated repeat expansions are found in five diseases (SCAs 8, 10, 12, 31 and 36) (Koob *et al.* 1999, Holmes *et al.* 1999, Matsuura *et al.* 2000, Sato *et al.* 2009, Kobayashi *et al.* 2011). The pathogenic effects of the non-coding repeat expansions have been implicated to include both RNA and protein level toxicity (Moseley *et al.* 2006, Daughters *et al.* 2009, Zu *et al.* 2011, White *et al.* 2012, Niimi *et al.* 2013). The causative mutation is reported for at least 16 SCAs that do not result from repeat expansions (http://neuromuscular.wustl.edu/ataxia/domatax.html). The pathogenic mechanisms in these diseases include mitochondrial dysfunction (SCA28) (Di Bella *et al.* 2010) and ion channel disturbances (SCAs 13, 19/22, 27, 41) (van Swieten *et al.* 2003, Waters *et al.* 2006, Duarri *et al.* 2012, Lee *et al.* 2012, Tempia *et al.* 2015, Fogel *et al.* 2015).

The recessive ataxias include diseases with various etiologies, such as mitochondrial dysfunction, impaired DNA repair, metabolic disease and molecular chaperone dysfunction (**Table 4**) (De Michele *et al.* 2004, Vermeer *et al.* 2011). The autosomal recessive Friedreich ataxia (FRDA) is estimated to be the most common form of inherited ataxia in Caucasian populations (Cossee *et al.* 1997, Schulz *et al.* 2009). The large majority of FRDA cases are caused by a homozygous GAA-triplet expansion in the first intron of the frataxin (*FXN)* gene, resulting in reduced RNA and protein levels (Campuzano *et al.* 1996, Campuzano *et al.* 1997). The frataxin protein localizes to the mitochondria (Campuzano *et al.* 1997), and may have roles in iron homeostasis, respiratory control and oxidative stress resistance (Wong *et al.* 1999, Delatycki *et al.* 1999, Lodi *et al.* 1999). In addition to early-onset ataxia, FRDA patients typically suffer from cardiomyopathy, diabetes and scoliosis (Parkinson *et al.* 2013). Ataxia-telangiectasia and ataxia-telangiectasia-like disorder 1 represent recessive ataxias resulting from defective DNA repair. The diseases are caused by mutations in the double-strand break repair genes *ATM* (Savitsky *et al.* 1995) and *MRE11* (Stewart *et al.* 1999), respectively. In both conditions, cerebellar ataxia is accompanied by chromosomal instability and immune deficiency, while ataxia-telangiectasia patients also present with cancer susceptibility and small dilated blood vessels (telangiectases) (Taylor *et al.* 2004). Molecular chaperone dysfunction has been implicated for instance

Table 4. ***Autosomal recessive human ataxias and disease genes[1].***

Disease (OMIM#)	Gene	Protein
• DNA repair defects		
Ataxia-telangiectasia (#208900)	*ATM*	Serine/threonine kinase
Ataxia-telangiectasia-like disorder 1 (#604391)	*MRE11A*	Double strand break repair nuclease
Ataxia-telangiectasia-like disorder 2 (#615919)	*PCNA*	Proliferating cell nuclear antigen
Ataxia-oculomotor apraxia 1 (#208920)	*APTX*	Aprataxin
Ataxia-oculomotor apraxia 2 (#606002)	*SETX*	Senataxin
Ataxia-oculomotor apraxia 4 (#616267)	*PNKP*	Polynucleotide kinase 3'-phosphatase
Spinocerebellar ataxia with axonal neuropathy 1 (#607250)	*TDP1*	Tyrosyl-DNA phosphodiesterase 1
• Mitochondrial dysfunction		
Friedrich ataxia (#229300)	*FXN*	Frataxin
Infantile-onset spinocerebellar ataxia (#271245)	*C10orf2*	DNA helicase (Twinkle)
Mitochondrial recessive ataxia syndrome (#607459)	*POLG*	Polymerase gamma
Primary coenzyme Q10 deficiency 4 (#612016)	*ADCK3*	AarF domain containing kinase
• Metabolic ataxias		
Abetalipoproteinaemia (#200100)	*MTP*	Microsomal triacylglycerol transfer protein
Ataxia with isolated vitamin E deficiency (#277460)	*TTPA*	Alpha-tocopherol transfer protein
• Chaperone defects		
Spastic ataxia of Charlevoix- Saguenay (#270550)	*SACS*	Sacsin molecular chaperone
Marinesco-Sjögren syndrome (#248800)	*SIL1*	Nucleotide exchange factor
SCAR16 (#615768)	*STUB1*	Ubiquitin ligase/co-chaperone
• Congenital ataxias		
Cayman ataxia (#601238)	*ATCAY*	Caytaxin
SCAR5 (#606937)	*ZNF592*	Zinc finger protein 592
SCAR13 (#614831)	*GRM1*	Metabotropic glutamate receptor 1
SCAR17 (#616127)	*CWF19L1*	CWF19-like 1, cell cycle control
• Endosomal-lysosomal dysfunction		
SCAR7 (#609270)	*TPP1*	Tripeptidyl peptidase I
SCAR15 (#615705)	*KIAA0226*	Rubicon
SCAR20 (#616354)	*SNX14*	Sorting nexin 14
• Other recessive ataxias		
Ataxia-oculomotor apraxia 3 (#615217)	*PIK3R5*	Phosphoinositide-3-kinase, subunit 5
SCAR8 (#610743)	*SYNE1*	Synaptic nuclear envelope protein 1
SCAR10 (#613728)	*ANO10*	Anoctamin 10
SCAR11 (#614229)	*SYT14*	Synaptotagmin XIV
SCAR14 (#615386)	*SPTBN2*	β-III spectrin
SCAR18 (#616204)	*GRID2*	Ionotropic glutamate receptor
Lichtenstein-Knorr syndrome (#616291)	*SLC9A1*	Na+/H+ antiporter

[1]The list does not include all known recessive ataxia syndromes.
Abbreviations: SCAR = spinocerebellar ataxia, autosomal recessive

in Marinesco-Sjögren syndrome, which is an ataxia syndrome characterized by cerebellar atrophy, cataracts and myopathy. The disease is caused by mutations in the co-chaperone gene *SIL1* (Anttonen *et al.* 2005, Senderek *et al.* 2005). SIL1 is a nucleotide exchange factor that functions as a co-chaperone for BiP (HSP70) (Chung *et al.* 2002), which in turn promotes protein folding and assembly within the ER, and targets misfolded proteins for proteasomal degradation (Otero *et al.* 2010).

In recent years, several novel recessive ataxia genes have been identified, largely by utilizing next-generation sequencing methods. The resulting phenotypes are referred to as SCARs (spinocerebellar ataxia, autosomal recessive) (**Table 4**). Although the exact pathological mechanisms remain to be delineated for many of the conditions, some interesting links to the degradative pathways have emerged. The new disease-associated genes include the co-chaperone/E3 ligase *STUB1* (Shi *et al.* 2013), as well as the sortin nexin gene *SNX14* (Akizu *et al.* 2015) and *KIAA0226*, which encodes the rubicon protein (Assoum *et al.* 2010, Assoum *et al.* 2013). Both sortin nexin-14 and rubicon localize to endosomes and lysosomes and have been indicated to function in autophagy regulation (Matsunaga *et al.* 2009, Zhong *et al.* 2009, Akizu *et al.* 2015). Ataxia-causing mutations were also found in the lysosomal enzyme gene tripeptidyl peptidase 1 (*TPP1*) (Sun *et al.* 2013). *TPP1* mutations have been previously reported in late-infantile NCL (Sleat *et al.* 1997), and the new link to cerebellar ataxia implicates shared genetic etiology in these different types of neurodegenerative diseases.

2.3.2. Inherited cerebellar ataxia in dogs

Inherited cerebellar ataxia occurs in several dog breeds. Both spinocerebellar (Higgins *et al.* 1998, Gilliam *et al.* 2014) and pure cerebellar disorders (Steinberg *et al.* 1981, Yasuba *et al.* 1988, Gandini *et al.* 2005) have been described, along with some more systemic diseases (deLahunta & Averill 1976, Chieffo *et al.* 1994, Carmichael *et al.* 1996). In veterinary medicine, degeneration of the cerebellar cortex has traditionally been referred to as cerebellar cortical abiotrophy (de Lahunta 1990), which has been further classified according to the primarily affected cell type, either the PCs or granule cells (granuloprival degeneration) (Urkasemsin & Olby 2014). However, loss of granule cells, with relative sparing of PCs, is encountered more rarely (Sisó *et al.* 2006). In many breeds, autosomal recessive mode of inheritance has been either suspected (Carmichael *et al.* 1996, van Tongern *et al.* 2000, Urkasemsin *et al.* 2010) or confirmed (Zeng *et al.* 2011, Forman *et al.* 2012, Forman *et al.* 2013, Agler *et al.* 2014, Gilliam *et al.* 2014, Forman *et al.* 2015), while possible dominant conditions have not yet emerged. Similar to humans, dogs affected with progressive cerebellar disorders develop significant difficulties in controlling their movements. The clinical signs in affected dogs include gait impairment, truncal swaying, broad-based stance, intention tremors and abnormal eye movements (nystagmus) (Summers *et al.* 1995). The onset of clinical signs can vary from a few weeks (Yasuba *et al.* 1988, Coates *et al.* 1996, Coates *et al.* 2002), to months (deLahunta & Averill 1976, Urkasemsin *et al.* 2010, Forman *et al.* 2015), or several years (Steinberg *et al.* 1981, Higgins *et al.* 1998, Steinberg *et al.*

2000). A large majority of the described conditions have an early onset and a fairly quick disease progression (Sisó *et al.* 2006). In recent years, significant progress has been made in identifying disease-associated gene variants (**Table 5**). However, there are still many conditions in which the genetic cause remains to be uncovered (**Table 6**).

Currently, the causative or associated gene variant has been reported in six autosomal recessive canine ataxias, four of which are linked to genes that have been implicated in similar human phenotypes (**Table 5**). In Coton de Tulear dogs, a retrotransposon insertion disrupts the metabotropic glutamate receptor 1 (*GRM1*) gene, causing neonatal cerebellar ataxia with synaptic abnormalities, but no apparent degenerative changes (Zeng *et al.* 2011). In humans, *GRM1* mutations result in infantile-onset autosomal recessive spinocerebellar ataxia (Guergueltcheva *et al.* 2012). Another type of neonatal ataxia, affecting the Beagle breed, is caused by an 8-bp exonic deletion in the *SPTBN2* gene (Forman *et al.* 2012). The encoded β-III spectrin protein has been reported to function in Golgi-related protein transport (Salcedo-Sicilia *et al.* 2013) and glutamatergic neurotransmission (Jackson *et al.* 2001). Its defects have been linked with both autosomal dominant and recessive ataxia in humans (Ikeda *et al.* 2006, Lise *et al.* 2012). Early-onset spinocerebellar ataxia within the Italian Spinone breed is associated with an intronic GAA-repeat expansion in the inositol 1,4,5-trisphosphate receptor 1 (*ITPR1*) gene (Forman *et al.* 2015). The ITPR1 receptor has been implicated to function in autophagy regulation (Criollo *et al.* 2007, Vicencio *et al.* 2009) and calcium signaling in the ER (Mikoshiba 2007). In humans, *ITPR1* mutations cause dominant spinocerebellar ataxia (van de Leemput *et al.* 2007, Huang *et al.* 2012). The Parson Russel Terrier breed is affected with at least two different forms of spinocerebellar ataxia, associated with mutations in the *KCNJ10* (Gilliam *et al.* 2014) and *CAPN1* (Forman *et al.* 2013) genes, encoding a potassium channel and a calcium dependent cysteine protease, respectively. The *KCNJ10* mutation is also found in affected dogs from other related terrier breeds (Gilliam *et al.* 2014, Rohdin *et al.* 2015). In humans, defects in the *KCNJ10* gene are linked to an autosomal recessive neurological syndrome, characterized by ataxia, seizures and hearing loss (Scholl *et al.* 2009). Finally, a late-onset cerebellar ataxia in Old English Sheepdogs and Gordon Setters was recently associated with a missense change in the autophagy-linked GTPase gene, *RAB24* (Agler *et al.* 2014). The role of RAB24 in autophagy is unclear, but it has been shown to localize to autophagosomal membranes (Munafo & Colombo 2002).

Table 5. ***Canine inherited ataxias with an identified genetic cause.***

Affected Breed	Clinical features (age of onset)	Pathological features	Gene Protein	Human disease (OMIM#)
Beagle	Neonatal cerebellar ataxia (3 weeks)	PC loss, secondary lesions in granular cell layer and molecular layer, swollen dendritic processes (Yasuba *et al.* 1988, Forman *et al.* 2012)	*SPTBN2* β-III spectrin	•SCA5 (#600224) •Early-onset SCAR14 (#615386)
Coton de Tulear	Neonatal cerebellar ataxia (2 weeks)	Synaptic abnormalities in the cerebellar cortex (Coates *et al.* 2002)	*GRM1* Glutamate receptor	•Early-onset SCAR13 (#614831)
Italian Spinone	Spinocerebellar ataxia (4 months)	No clear degenerative changes, dendritic abnormalities in PCs (Forman *et al.* 2015)	*ITPR1* Inositol trisphosphate receptor	•SCA15 (#606658) •SCA29 (#117360)
Old English Sheepdog, Gordon Setter	Cerebellar ataxia (6 months - 4 years)	Neuron loss within the PC layer, granular cell layer and molecular layer (Steinberg *et al.* 1981, Steinberg *et al.* 2000)	*RAB24* GTPase	*Not reported*
Parson Russell Terrier, Jack Russell Terrier, Russell Terrier, Smooth-Haired Fox Terrier, Toy Fox Terrier	Spinocerebellar ataxia, seizures, myokymia (2-12 months)	Generalized axonopathy, axonal swelling and myelin loss in spinocerebellar tracts (Wessmann *et al.* 2004, Bhatti *et al.* 2011, Gilliam *et al.* 2014)	*KCNJ10* Potassium channel	•SeSAME and EAST syndromes (#612780) (ataxia, seizures, sensorineural deafness)
Parson Russell Terrier	Spinocerebellar ataxia (6-12 months)	Presently unclear[1]	*CAPN1* Calcium dependent cysteine protease	*Not reported*

[1]*The histopathological changes are unclear, specifically in comparison to KCNJ10-linked ataxia in the same breed (Gilliam et al. 2014). Abbreviations: PC = Purkinje cell; SCA = Spinocerebellar ataxia, dominant; SCAR = Spinocerebellar ataxia, autosomal recessive*

Table 6. ***Canine cerebellar ataxias with an unknown molecular basis.***

Affected Breed	Clinical features	Age of onset (progression)	Pathological features
Australian Kelpie	Early-onset cerebellar ataxia	5-12 weeks (quick to moderate)	PC and granule cell loss, degeneration of the cerebellar white matter (Thomas & Robertson 1989)
Bavarian Mountain Dog	Early-onset cerebellar ataxia	Around 3 months (slow)	Granule cell loss (Flegel *et al.* 2007)
Belgian Shepherd Malinois	Early-onset cerebellar ataxia	4-7 weeks (quick)	Degeneration of the cerebellar nuclei, vacuolization of granular cell layer and white matter, vacuoles in medulla, pons and midbrain (Kleiter *et al.* 2011)
Bernese Mountain Dog	Early-onset cerebellar ataxia	4-6 weeks (quick)	PC and granule cell loss, thinning of molecular layer, liver degeneration and fibrosis (Carmichael *et al.* 1996)
Border Collie	Early-onset cerebellar ataxia	6-8 weeks (quick)	PC and granule cell loss (Gill & Hewland 1980)
Border Collie	Early-onset cerebellar ataxia	4 months (quick to moderate)	Granule cell loss, thinning of molecular layer (Sandy *et al.* 2002)
Brittany Spaniel	Late-onset spinocerebellar degeneration	5-10 years (slow)	PC loss, axonal degeneration within the spinal cord (Higgins *et al.* 1998)
Coton de Tulear	Early-onset cerebellar ataxia	7-8 weeks (quick)	Granule cell loss, thinning of molecular layer (Tipold *et al.* 2000)
English Bulldog	Early-onset cerebellar ataxia	10-12 weeks (slow)	PC and granule cell loss (Gandini *et al.* 2005)
Finnish Hound	Early-onset cerebellar ataxia	4-12 weeks (quick)	PC and granule cell loss, thinning of the molecular layer (Tonttila & Lindberg 1971)
Irish Setter	Neonatal quadriplegia and amblyopia (ataxia, tremors, nystagmus)	Present from birth (quick)	PC and granule cell loss (Palmer *et al.* 1973, Sakai *et al.* 1994)
Jack Russel Terrier	Neonatal spinocerebellar ataxia	2 weeks (quick)	Granule cell loss, PC vacuolization, vacuolar degeneration of the cerebellar white matter and dorsolateral tracts of the spinal cord (Coates *et al.* 1996)
Kerry Blue Terrier	Early-onset cerebellar ataxia	9-16 weeks (quick)	PC and granule cell loss, degeneration of the olivary nucleus, subtantia nigra and caudate nucleus (deLahunta & Averill 1976)
Labrador Retriever	Early-onset cerebellar ataxia	9-17 weeks (quick)	PC and granule cell loss (Perille *et al.* 1991)
Portuguese Podengo	Neonatal cerebellar ataxia	2-3 weeks (quick)	PC loss, thinning of granular cell and molecular layers, cerebral white matter loss and hydrocephalus (van Tongern *et al.* 2000)
Rhodesian Ridgeback	Neonatal cerebellar ataxia, growth retardation and coat color dilution	2 weeks (quick)	PC and granule cell loss, uneven distribution of macromelanosomes within hair safts (Chieffo *et al.* 1994)
Scottish Terrier	Juvenile to adult onset cerebellar ataxia	2 months - 6 years, median 7 months (slow)	PC loss; degeneration of the granular and molecular layers (Urkasemsin *et al.* 2010)

Abbreviations: PC = Purkinje cell

3 Aims of the study

The principal objective of this PhD study was to identify causative mutations in canine inherited diseases, focusing on neurodegenerative and skeletal disorders. The studied disease phenotypes were present within particular breeds and many similarly affected dogs were reported. This was suggestive of underlying genetic causes, and prompted us to pursue genetic examinations. As well as identifying causative gene variants, the aim was to recognize disease-linked molecular pathways and gain understanding of the biology of the studied diseases.

The specific aims were:

i. To identify the genetic cause of dwarfism in the Norwegian Elkhound and Karelian Bear Dog breeds (**Study I**).

ii. To reveal the causative mutation behind cerebellar ataxia in the Finnish Hound breed (**Study II**).

iii. To study the genetic background of a neurodegenerative vacuolar storage disease in the Lagotto Romagnolo breed (**Study III**).

4 Materials and methods

4.1 Ethics statement

All dogs that were used in the clinical, pathological and genetic experiments were privately owned pet dogs, enrolled in the studies with their owners' consent (**Study I-III**). The experiments were carried out with authorization from the "Animal Ethics Committee at the State Provincial Office of Southern Finland" (**Study I-III**: permits ESLH-2006-08207/Ym-23, ESHL-2009-07827 and ESAVI/6054/04.10.03/2012) and with permission from the "Cantonal Committee For Animal Experiments" (**Study III**: permit 23/10, Canton of Bern). Zebrafish experiments were approved by "Stockholm North Experimental Animal committee" (Dnr N29-12).

4.2 Study cohorts

Blood samples, buccal swabs and tissue samples were collected from canine subjects in order to isolate genomic DNA (**Study I-III**). Sample collection was focused on affected dogs, their parents, unaffected siblings, and other close relatives. Samples were stored in the canine DNA bank maintained by the research group of Dr. Lohi at the University of Helsinki. The DNA repository currently holds over 50,000 samples from more than 300 different dog breeds, representing a highly valuable resource for genetic research in dogs. In addition to the samples obtained from affected dogs and their healthy relatives, the DNA bank was utilized to retrieve larger sample cohorts from unaffected dogs that had been collected for other ongoing research projects. These cohorts were used to corroborate initial findings and to estimate mutation carrier frequencies within the examined breeds, and to screen the disease-associated variants in other breeds. The full sample cohorts that were used in **Studies I-III** are listed in **Table 1**. More detailed information about the cohorts can be found in each individual study.

A large majority of the DNA samples were acquired as ethylenediaminetetraacetic acid (EDTA)-blood, a small proportion as buccal swabs, and a few as tissue samples. Blood samples were collected at veterinary clinics or by trained research personnel. Buccal swabs could be collected at home by dog owners. Tissue samples were harvested during post-mortem examinations.

In **Study III**, blood samples were received from a variety of different countries, whereas samples in **Studies I** and **II** were predominantly from Finland. **Study III** was performed in collaboration with the research group of Dr. Tosso Leeb at the University of Bern, Switzerland, and some of the samples used in this study were collected in the group's DNA repository at the University of Bern.

Table 1. *Study cohorts.*

Study #	Affected breed	Studied Phenotype	Affected dogs	Control dogs	Controls from other breeds	Total dogs
I	Norwegian Elkhound	Chondrodysplasia	17	197	192	761
	Karelian Bear Dog	Chondrodysplasia	4	351		
II	Finnish Hound	Cerebellar ataxia	14	274	349	637
III	Lagotto Romagnolo	Neurodegenerative storage disease	22	2,330	642	2,994

4.3 Pedigrees

Pedigrees were established around the affected dogs by using the GenoPro genealogy software (http://www.genopro.com/) (**Study I-III**). The pedigrees of Finnish dogs were obtained from the Finnish Kennel Club's pedigree database KoiraNet (http://jalostus.kennelliitto.fi/). Other utilized pedigree databases included the Swedish Kennel Club's Hunddata (https://hundar.skk.se/hunddata/) and the Lagotto Pedigree database (http://lagotto.hu/database.htm). In addition, pedigree information was received from individual dog owners and breeders.

4.4 Clinical and pathological examinations

Clinical examinations were performed on affected dogs in **Studies I-III** and pathological examinations in **Studies II** and **III**. All pathologically examined dogs were euthanized on their owners' request.

In **Study I**, radiographs were taken from altogether three dogs, including two dogs affected with chondrodysplasia: a 3-year-old Norwegian Elkhound (NE) and 5-month-old Karelian Bear Dog (KBD). The third examined dog was an unaffected littermate of the 5-month-old chondrodysplastic KBD.

In **Study II**, detailed neurological examinations, along with general clinical and orthopedical examinations, were performed on ten ataxic Finnish Hound (FH) puppies and on one healthy littermate. Brain magnetic resonance imaging (MRI) scans and cerebrospinal fluid samples were acquired from all examined dogs. The affected puppies were euthanized because of progressive clinical signs and underwent a full post-mortem examination. The brain of one additional affected dog was also received for pathological studies. Formalin-fixed, paraffin-embedded (FFPE) tissue samples from the CNS, liver, lungs, spleen, kidney and heart were stained with haematoxylin-eosin (HE) and examined under a light microscope. CNS sections were stained with

luxol fast blue-cresyl echt violet (LFB-CEV) to examine chromatolysis and myelin loss. Immunohistochemistry (IHC) was utilized to detect possible infections by using antibodies raised against the canine distemper virus (MCA1893, Serotec; Bio-Rad Laboratories Inc., Hercules, CA, USA) and canine parvovirus (MCA2064, Serotec; Bio-Rad Laboratories Inc., Hercules, CA, USA). Astrogliosis was assessed via a glial fibrillary acidic protein (GFAP) antibody (MCA1909, Serotec; Bio-Rad Laboratories Inc., Hercules, CA, USA).

In **Study III**, general clinical and neurological examinations were performed on 16 Lagotto Romagnolo (LR) dogs affected with a neurodegenerative disease. Brain MRI scans were obtained from 11 affected LRs. A pathological examination was performed on seven affected LRs that were euthanized due to worsening clinical signs. FFPE tissue samples, collected from internal organs, skin, and the central and peripheral nervous system, were stained with HE and periodic-acid-Schiff´s (PAS) stains. Cerebellar cortical tissue samples from one affected LR were processed for electron microscopy (EM). The EM samples were fixed in 2.5% glutaraldehyde, embedded in epoxy resin and stained with the Reynolds lead citrate stain. IHC was used to get insights into the disease-causing pathological mechanisms. For this purpose, FFPE tissue samples from affected and control dogs were stained using primary antibodies against ubiquitin (ab7780, Abcam, Cambridge, UK), the astrogliosis marker GFAP (MCA1909, Serotec, Bio-Rad Laboratories Inc., Hercules, CA, USA), the authophagy markers LC3B (ab48394, Abcam, Cambridge, UK) and p62/SQSTM1 (P0067, Sigma-Aldrich, St. Louis, MO, USA), the lysosome membrane protein LAMP2 (LS-B3144, Lifespan Biosciences Inc., Seattle, WA, USA) and the disease-associated ATG4D protein (SAB1301447, Sigma-Aldrich, St. Louis, MO, USA).

4.5 Tissue samples

Whenever possible, fresh tissue samples were harvested from euthanized animals during pathological examination to be utilized in possible functional experiments. The tissue samples were stabilized in RNAlater reagent (Ambion, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions and stored in -80 °C until further use.

In **Study I**, a 3-year-old chondrodysplastic NE was euthanized at its owner's request, due to aggressive behavior towards people and other dogs. A panel of tissue samples was collected from this dog after euthanasia. Control tissue samples were obtained from a 2-year-old Australian Kelpie that was euthanized due to epileptic seizures.

In **Study II**, tissue samples were collected from five affected FH puppies' cerebellar cortex. The puppies were euthanized at around 3 months of age. Control samples were obtained from an 11-day-old Saluki puppy that had suffered from a peritoneo-pericardial hernia.

In **Study III**, tissues were collected from two 2-year-old affected LRs, and from four control LRs. The control dogs comprised an 11-month-old dog that suffered from

seizures, a 10-year-old dog with progressive cognitive decline, and a 9-year-old and a 10-year-old LR that were both euthanized due to severe hip dysplasia.

4.6 Reference sequences and databases

The CanFam2.0 dog genome build was used as the reference genome in **Studies I** and **II**, whereas the CanFam3.1 assembly was used in **Study III**. The NCBI GenBank database (http://www.ncbi.nlm.nih.gov/) was used to retrieve mRNA and protein sequences for the studied genes. The Ensembl database was used in **Study III** to obtain zebrafish reference sequences (http://www.ensembl.org/index.html). In **Study II**, the UCSC Genome Browser (http://genome-euro.ucsc.edu) was utilized to identify microsatellite markers near ataxia candidate genes.

4.7 Molecular biology methods

Several different molecular biology methods were employed. SNP arrays (**Study I-III**) and whole-genome sequencing (**Study III**) were used to obtain genome-wide genotype data. Sanger sequencing was used to screen for possible causative mutations in candidate genes (**Study I, II**), to genotype candidate variants in bigger sample numbers (**Study I-III**), and to verify mRNA sequences (**Study I-III**). In **Study II**, fragment analysis was utilized to determine allele sizes for microsatellite markers adjacent to known human and murine ataxia genes. Taqman genotyping chemistry was used to screen the disease-associated variants in large sample cohorts (**Study I-III**). Gene expression differences between affected and healthy dogs were examined by semi-quantitative PCR and/or real-time quantitative PCR (qPCR) (**Study I-III**). In **Study I**, immunoblotting was utilized to examine the protein level effects of the identified gene mutation.

4.7.1 DNA, RNA and protein extraction

Genomic DNA was isolated from most blood samples by using a semi-automated Chemagen extraction robot (PerkinElmer Chemagen Technologie GmbH, Baesweiler, Germany), which was also used to extract DNA from tissue samples. In **Study II**, the Puregene DNA Purification Kit (Gentra, Qiagen, Hilden, Germany) was used for a subset of samples, and in **Study III**, a part of the sample cohort was extracted by using the Nucleon Bacc2 kit (GE Healthcare, Little Chalfont, UK). DNA was extracted from buccal swabs by using the QiaAmp DNA Mini Kit (Qiagen, Hilden, Germany).

Total RNA was extracted from RNAlater preserved tissue samples by using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The samples were homogenized with a TissueRuptor rotor-stator (Qiagen, Hilden, Germany), and a DNase I digestion step was

included in the protocol (RNase-Free DNase Set; Qiagen, Hilden, Germany). In **Study I**, RNA was extracted from tracheal and bronchial tissue samples of one affected and one control dog. In **Studies II** and **III**, RNA was isolated from cerebellar cortical samples of five affected and one unaffected dog, and from two affected and four unaffected dogs, respectively.

The concentration of both DNA and RNA samples was determined by using a NanoDrop-1000 UV/Vis Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The DNA samples were stored at -20 °C and the RNA samples at -80 °C until further use.

In **Study I**, total protein lysates were extracted from RNAlater preserved tracheal tissue samples of one affected and one control dog. The samples were homogenized in Pierce T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) by using a TissueRuptor rotor-stator (Qiagen, Hilden, Germany). Pierce Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific Inc., Waltham, MA, USA) was added to the extraction buffer. Protein concentrations were measured by using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the lysates were stored at -80 °C.

4.7.2 Primer design

The Primer3 program (http://frodo.wi.mit.edu/primer3/) was used to design primers for fragment analysis, Sanger sequencing and quantitative PCR (**Study I-III**). Primers used in mRNA experiments were designed to span multiple exons in order to control for possible contamination with genomic DNA. The used primer sequences are listed in the supplementary materials of each individual study.

4.7.3 PCR, Sanger sequencing and fragment analysis

Most PCR reactions were performed using Biotools' DNA Polymerase (Biotools B&M Labs S.A., Madrid, Spain) under standard thermocycling conditions. In **Study III**, the AmpliTaq Gold 360 Mastermix (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and the Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Fisher Scientific Inc., Waltham, MA, USA) were used in a subset of PCR reactions. A PTC-225 Peltier Thermal Tetrad Cycler or a T100 Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used to carry out the reactions. PCR products were run on a 1% agarose gel stained with either 0.5 μg/ml ethidium bromide (Amresco Inc., Solon, OH, USA) or GelRed (Biotium, Inc., Hayward, CA, USA).

PCR products were purified for Sanger sequencing by using the ExoSAP-IT enzyme mix (GE Healthcare, Little Chalfont, UK) or a combination of Exonuclease I and FastAP Thermosensitive alkaline phosphatase (Thermo Fisher Scientific Inc., Waltham, MA, USA). Sequencing reactions were carried out on a 3730xl DNA Analyzer (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), and the obtained sequence data

was analyzed using either Variant Reporter v1.0 (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) or Sequencher 5.1 (GeneCodes Corp., Ann Arbor, MI, USA).

In **Study II**, AmpliTaq Gold Polymerase (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) was used to amplify the microsatellite markers for fragment analysis. The PCR reactions were carried out either with fluorescence-labeled forward primers (HEX or FAM label) or with a FAM-labeled third primer. The intensity of PCR products was evaluated from agarose gel, and the products were diluted for the fragment analysis runs, which were performed on a 3730xl DNA Analyzer (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). Applied Biosystems' GeneScan-500 LIZ was used as a size standard, and the fragment sizes were determined using the Peak Scanner software (Applied Biosystems, Life Technologies, Carlsbad, CA, USA).

4.7.4 Taqman genotyping

In **Studies I-III**, custom Taqman SNP Genotyping Assays were used to discriminate between different alleles via fluorescent-labeled probes (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The primer and probe sequences were custom-designed for each genotyping assay, and the sequences can be found in the individual studies. The genotyping reactions were run in 96-well format, using Taqman Genotyping Master Mix and 7500 Fast Real-Time PCR instrumentation (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). Control samples with known genotypes were included in each run to facilitate genotype calling.

4.7.5 Semi-quantitative and real-time quantitative PCR

In **Studies I-III**, total RNA isolated from tissue samples was reverse-transcribed to cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). In each experiment, equal amounts of RNA template were added to the reverse transcription reactions. The reverse transcriptase PCRs (RT-PCR) were performed on cDNA samples using three different cycle numbers (27, 32 and 37), to ensure the detection of logarithmic amplification phases. The RT-PCR products were run on 1.5% agarose gel to evaluate possible expression differences. The qPCR reactions in **Studies I** and **II** were run on a 7500 Fast Real-Time PCR instrument (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The reactions were carried out in triplicates, using FastStart Universal SYBR Green Master (Hoffmann La Roche, Basel, Switzerland). The obtained expression data was normalized against two housekeeping controls, *GAPDH* and *YWHAZ*. Reaction efficiencies were calculated from multi-point dilution series and the comparative ΔΔCt-method was used to determine relative expression levels (Livak & Schmittgen 2001).

4.7.6 Immunoblotting

In **Study I**, total protein lysates from tracheal tissue samples of an affected and healthy dog were immunoblotted against a polyclonal anti-ITGA10 antibody (SAB1411763, Sigma-Aldrich, St. Louis, MO, USA). An anti-GAPDH antibody (39-8600, Invitrogen, Life Technologies, Carlsbad, CA, USA) was used as a loading control. Equal amounts of protein lysates were run on a 9% SDS-PAGE gel and the separated proteins were blotted on a nitrocellulose membrane (Bio-Rad Laboratories Inc., Hercules, CA, USA), using a standard western blotting protocol. A horseradish peroxidase-conjugated anti-mouse IgG antibody (NA931, GE Healthcare, Little Chalfont, UK) and Pierce SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc., Waltham, MA, USA) were used to detect the antibody signals.

4.8 SNP arrays and genotyping cohorts

SNP chip genotyping arrays were utilized to identify disease associated chromosomal loci. CanineSNP20 BeadChip of 22,362 validated SNPs (Illumina, San Diego, CA, USA) was used as the genotyping platform in **Studies I** and **II**, whereas in **Study III**, the genotyping was carried out on the newer CanineHD BeadChip of 173,662 SNPs (Illumina, San Diego, CA, USA). In **Studies I** and **II**, genotyping was performed at the Institute for Molecular Medicine Finland (FIMM) Technology Center (Helsinki, Finland), and in **Study III**, at the GeneSeek laboratory (Neogen Corporation, Lincoln, NE, USA).

In **Study I**, nine chondrodysplastic and nine normal-height NEs were genotypes with the 22K SNP chips. The affected dogs were from different litters and the control dogs were case-matched relatives, either second-degree or more distant.

In **Study II**, altogether 13 affected FHs and 18 control FHs were genotyped for genome-wide analyses using the 22K arrays. The affected dogs came from seven different litters. The control samples comprised 11 parents of affected dogs and seven healthy littermates.

In **Study III**, three affected and four healthy LRs were genotyped with the 173K SNP arrays. The genotyping cohort included one separate affected dog from Switzerland and 6 dogs from a Finnish nuclear family, two affected and two healthy offspring and both parents.

4.9 Analysis of SNP chip data

4.9.1 Quality control

The quality of the SNP array data was assessed using the PLINK software (**Study I-III**) (Purcell *et al.* 2007). As a basic quality control measure, the sample gender was evaluated from X-chromosomal markers. In family data, the inheritance pattern of marker alleles was checked and SNPs with Mendel errors were removed. To remove SNPs that showed significant deviation from the Hardy-Weinberg equilibrium, a threshold of $p \leq 0.0001$ was applied. A > 95% call rate was utilized for both SNPs and samples to exclude markers and individuals with low genotyping efficiency. Finally, markers with a < 5% minor allele frequency were removed from analyses.

4.9.2 Association analysis and homozygosity mapping

In **Studies I** and **III**, the PLINK software package (Purcell *et al.* 2007) was used to perform allelic association tests. In **Study I**, SNP genotypes from nine affected and nine healthy NEs were used to perform the Fisher's exact test, and the genome-wide significance of the results was ascertained through phenotype permutation testing (n = 50,000). In **Study II**, a basic case-control association test was performed using 13 affected FHs and seven unaffected siblings. Genome-wide significance was ascertained through 100,000 phenotype permutations. In **Study III**, the PLINK software (Purcell *et al.* 2007) was used to carry out homozygosity mapping in three affected LRs. This approach was employed to identify regions of extended homozygosity that were shared by the affected dogs.

4.9.3 Family-based analyses

In **Study II**, family-based tests were performed on 13 affected and 18 unaffected FHs. The analyses were performed only on canine chromosome 8 (CFA8), which had shown a significant association signal. The family-based testing, which included parametric single-point linkage, association (LD|Linkage) and a joint test of association and linkage (LD + Linkage), was carried out under a recessive inheritance model using the Pseudomarker program (Hiekkalinna *et al.* 2011). In **Study III**, the Merlin software (Abecasis *et al.* 2002) was used to perform parametric linkage analysis on one nuclear LR family comprising two parent dogs, two affected and two healthy siblings. The linkage analysis was performed under a recessive inheritance model.

4.10 Next-generation sequencing

In **Study III**, the whole genome of one affected LR was sequenced using next-generation high-throughput sequencing technology. The sequencing was performed on Illumina's HiSeq2500 instrumentation, which yielded roughly 15.5x coverage of the genome. The obtained paired-end reads were mapped to the CanFam3.1 reference genome using Burrows-Wheeler Aligner (BWA) (Li & Durbin 2009). The mapped reads were sorted and the PCR duplicates labeled using the Picard tools (http://sourceforge.net/projects/picard/). The Genome Analysis Tool Kit (GATK) (McKenna *et al.* 2010) was used to call sequence variants. The protein level effects of the variants were annotated using the snpEFF software (Cingolani *et al.* 2012). Genomes of 118 dogs from various different breeds sequenced for other projects were used as controls to identify homozygous variants unique to the affected LR.

4.11 Statistical tests

Statistical tests were performed using the PASW Statistics 18 software (IBM, IBM, Armonk, NY, USA). In **Study I**, several body-length measurements were acquired from nine chondrodysplastic and 25 unaffected NEs. Statistical testing was used to determine whether the measurements were significantly different between the affected and unaffected animals. The Levene's test was used to evaluate the equality of variances ($p > 0.05$), and if fulfilled, the Student's t-test was used, otherwise the Mann-Whitney U-test was applied. In **Study II**, the statistical significance of qPCR results determined using the Student's t-test on normalized mean cycle threshold (Ct) -values.

4.12 Bioinformatic protein analyses

In **Study II**, the Pfam database (http://pfam.sanger.ac.uk/) (Finn *et al.* 2010) and the SMART tool (http://smart.embl-heidelberg.de/) (Schultz *et al.* 1998, Letunic *et al.* 2009) were used to examine protein domain structure. In **Studies II-III**, multiple-species protein alignments were constructed to evaluate the evolutionary conservation of the disease-linked amino acid positions. The ClustalW2 (**Study II**) (http://www.ebi.ac.uk/Tools/clustalw2/) and the Clustal Omega (**Study III**) (http://www.ebi.ac.uk/Tools/msa/clustalo/) sequence alignment algorithms were used for this purpose. Pathogenicity prediction tools were used to evaluate the impact of the identified missense variants. In **Study II**, three different programs were used: PANTHER (http://www.pantherdb.org/tools/) (Thomas *et al.* 2003), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) (Adzhubei *et al.* 2010) and SIFT (http://sift.jcvi.org/) (Ng & Henikoff 2001, Kumar *et al.* 2009). In **Study III**, the PredictSNP 1.0 program was utilized to obtain a consensus pathogenicity estimate from several prediction programs (http://loschmidt.chemi.muni.cz/predictsnp/) (Bendl *et al.* 2014).

4.13 Zebrafish experiments

In **Study III**, a zebrafish (*Danio rerio*) knock-down model was utilized to study the developmental role of the disease-associated *ATG4D* gene. The knock-down of the zebrafish orthologue *atg4da* (ENSDART00000152289) was carried out via an *atg4da* splice-morpholino (*atg4da*SMO) (Gene Tools, LLC, Philomath, OR, USA). The splice-morpholino oligo was designed to bind to intron 1 and exon 2 of the atg4da pre-mRNA, thus disturbing normal splicing. The used morpholino sequences are listed in **S7 Table** of **Study III**. To obtain morphant zebrafish, 1-cell stage embryos from a wild-type AB strain were injected with ~2-3 ng of the *atg4da*SMO or a control morpholino. The splicing effect and specificity of the *atg4da*SMO was evaluated by RT-PCR. The RT-PCR products were run on a 3% agarose gel and extracted from the gel for Sanger sequencing by using the GenElute Gel Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA). The phenotypes of the morphant and control embryos were examined by performing whole-mount immunostaining as described previously (Bae *et al.* 2009). The integrity of the developing cerebellum was assessed by using antibodies raised against the PC marker proteins Pvalb7 (1:1000; mouse ascites) and zebrin II (1:200; hybridoma supernatant), and the granule cell marker Vglut1 (1:1000; purified antibody). Immunostaining signals were detected via an anti-mouse Alexa 488 secondary antibody (Molecular Probes, Life Technologies, Carlsbad, CA, USA) and a spinning disk confocal microscope (Andor Technology Ltd., Belfast, UK).

5 Results and discussion

5.1 A nonsense mutation in the integrin gene *ITGA10* causes canine chondrodysplasia (Study I)

5.1.1 Disproportionate dwarfism of varying severity in two dog breeds

In 2009, we set out to identify the genetic cause of disproportionate short-stature dwarfism that was affecting the Finnish NE population. A corresponding condition had been described in a litter of inbred NEs from the United States already in the 1980s (Bingel & Sande 1982). Similar to the present-day dwarf dogs, the previously examined affected animals had been short-stature dwarfs, with disproportionately short and curved limbs. Pathological examinations had revealed several growth plates abnormalities, such as disorganized chondrocyte columns and unusual chondrocyte morphology (Bingel & Sande 1982).

During the course of our genetic study, a similar type of chondrodysplasia was recognized in the KBD breed, which is another Nordic hunting breed. Both the NE and KBD represent "primitive-type" dog breeds with long limbs, the affected dogs having considerably shorter limbs in comparison (**Fig. 1**). We obtained three different body length measurements from nine adult chondrodysplastic NEs ($n_{males} = 6$; $n_{females} = 3$) and from 25 unaffected adult NEs ($n_{males} = 14$; $n_{females} = 11$). Statistical analyses revealed significant differences in forearm length and in height at withers between cases and controls in both sexes (**I/Fig. 2, Table 1**).

Radiographic examination of a 3-year-old dwarf NE and a 5-month-old dwarf KBD revealed skeletal changes that varied in severity. The radiographs of the adult NE showed only relatively mild abnormalities such as slightly bowed radii, whereas more distinct changes were present in the KBD (**I/Fig. 4**). When compared to its unaffected sibling, the 5-month-old KBD had significantly shorter and curved radii and ulnae, malformed femoral heads, subluxated hip joints, irregular length of carpal and phalangeal bones, irregular and flared growth plates and widened vertebral epiphyses. The radiographic findings were in line with the general clinical status of the dogs. The adult NE did not suffer from noticeable movement difficulties, unlike the 5-month-old KBD, which presented with outward pending wrists (carpal valgus), knock knees (genu valgus) and preferred not to stand up because of the hip dysplasia (**I/Fig. 1**). However, the majority of dwarf dogs in our study were not as severely affected. Overall, the radiographic findings in our study were in accordance with the initial disease characterization from 1982 (Bingel & Sande 1982).

Figure 1. ***Affected and healthy dogs. A)*** *An unaffected adult Norwegian Elkhound (NE) (Photograph by Arto Rantanen).* ***B)*** *A chondrodysplastic adult NE (Photograph by Saija Nieminen)* ***C)*** *A normal-height 5-month-old male Karelian Bear Dog and* ***D)*** *its affected male sibling.*

5.1.2 Chondrodysplasia locus maps to canine chromosome 17

A pedigree that was established around the affected Finnish NEs was consistent with an autosomal recessive mode of inheritance (**I/Fig. 3A**). To identify the genetic cause of the condition, nine affected and nine healthy NEs were selected for a genome-wide association study. The 18 samples were genotyped using Illumina's canine 22K SNP arrays, resulting in 14,626 informative SNPs. The Fisher's exact test revealed a genome-wide significant association on CFA17 ($p_{raw} = 7.42x10^{-6}$; $p_{genome\text{-}wide} = 0.013$). Within the associated region, all nine cases shared a 2-Mb homozygous haplotype block that spanned from 60 to 62 Mb (CanFam2.0 assembly) and contained altogether 33 genes (**I/Fig. 5**). A careful inspection of the 33 genes uncovered a highly promising

candidate gene, the integrin alpha 10 (*ITGA10*). The *ITGA10* gene was found to encode the α10-integrin protein, which is a subunit of the heterodimeric α10β1 integrin receptor that binds collagens and is expressed in growth plate chondrocytes (Camper *et al.* 1998, Tulla *et al.* 2001, Camper *et al.* 2001). Moreover, *Itga10* knockout mice had been reported to present with a mild chondrodysplastic phenotype (Bengtsson *et al.* 2005).

5.1.3 A nonsense mutation in *ITGA10* causes loss of α10 integrin

The protein coding regions and exon-intron junctions of the *ITGA10* gene were sequenced in two affected and two healthy NEs, revealing altogether four intronic and five exonic variants (**I/Table 2**). However, only one of the variants segregated with the phenotype, when the other eight were homozygous in all four dogs. This variant was a single nucleotide change c.2083C>T in exon 16, predicted to introduce a premature stop codon p.Arg695* to the encoded protein. The c.2083C>T change was genotyped in larger sample cohorts, including the KBDs and a NE family cohort from the United States. The genotyping revealed a complete segregation of the nonsense variant with the chondrodysplasia phenotype (**Table 2**). Samples were obtained from altogether 21 affected dogs, all of which were homozygous for the c.2083C>T change. As further proof of pathogenicity, the variant was absent in 192 dogs from 12 other breeds (**I/Table S1**). Mutation carrier frequencies were calculated from separate population cohorts, indicating a 24% and an 8% frequency within the NE and KBD breeds, respectively (**Table 2**).

Table 2. ***ITGA10 c.2083C>T genotypes in the studied sample cohorts.***

Breed, country	Cohort/ status	Number of dogs	Wild-type (C/C)	Heterozygous (C/T)	Homozygous (T/T)
Norwegian Elkhounds, Finland	Unaffected relatives	32	15 (47%)	17 (53%)	-
	Affected dogs	13	-	-	13 (100%)
	Population cohort	156	119 (76%)	37 (24%)	-
Norwegian Elkhounds, USA	Unaffected relatives	9	2 (22%)	7 (78%)	-
	Affected dogs	4	-	-	4 (100%)
Karelian Bear Dogs, Finland	Unaffected relatives	64	44 (69%)	20 (31%)	-
	Affected dogs	4	-	-	4 (100%)
	Population cohort	287	263 (92%)	24 (8%)	-

The *ITGA10* gene is composed of altogether 30 exons. The p.Arg695* nonsense mutation in exon 16 introduces a premature stop-codon that eliminates nearly half of the protein body (**I/Fig. 6**). As a consequence of the premature stop-codon, the mutated ITGA10 transcript was expected to undergo nonsense-mediated mRNA decay (NMD) (Nagy & Maquat 1998). However, our RT-PCR and qPCR analyses, carried out with tissue samples from one affected and one control dog, did not provide evidence of NMD (**I/Fig. 7A, Fig. S1**). To examine this further, we evaluated the α10-protein levels in cartilaginous tracheal tissue lysates using a polyclonal ITGA10 antibody. The immunoblotting experiments revealed a strong band of the expected ~150 kDa size in the control lysate, whereas the sample from an affected dog showed no signal (**I/Fig. 7B**), indicating that either the entire α10-subunit is lost due to the mutation, or the used antibody does not recognize a possible truncated polypeptide. Either way, the identified nonsense mutation most likely results in loss of protein function.

5.1.4 Collagen-binding α10β1-integrin is needed for bone growth

In summary, this study identified a nonsense mutation in the *ITGA10* gene as the cause of chondrodysplasia in the NE and KBD breeds. The affected dogs suffer from short-stature dwarfism that varies in severity. Our immunoblotting results indicate that the canine phenotype is caused by loss of protein function, as the full-length α10-protein was not detected in the affected tissue.

The integrin α10-subunit, encoded by the *ITGA10* gene, assembles into the α10β1 receptor, a collagen-binding integrin predominantly found in cartilage chondrocytes (Camper *et al.* 2001). Altogether four collagen-binding integrins, α1β1, α2β1, α10β1 and α11β1 have been recognized in vertebrates (**Fig. 2A**) (Leitinger 2011, Heino 2014). Neither the *ITGA10* gene nor the other collagen-binding integrins have been previously implicated in monogenic diseases. Collectively, the integrin family of transmembrane cell surface receptors constitutes 24 distinct αβ-heterodimers that mediate ECM and cell-cell interactions in a variety of different tissues and pathways (**Fig. 2B**) (Barczyk *et al.* 2010, De Franceschi *et al.* 2015). The α10β1 integrin has been indicated to influence chondrocyte proliferation and the assembly of collagen fibrils in the surrounding ECM (Bengtsson *et al.* 2005). The nonsense mutation in affected dogs is likely to severely disrupt these functions. The α10β1 integrin is only the second collagen receptor that has been linked with inherited chondrodysplasia. Mutations in the discoidin domain receptor 2 (*DDR2*) have been reported to cause short-limbed dwarfism both in mice (Labrador *et al.* 2001, Kano *et al.* 2008) and in humans (Bargal *et al.* 2009). Similar to α10β1, the DDR2 receptor serves as a collagen-binding cell surface receptor, which is thought to be involved in regulating chondrocyte proliferation (Labrador *et al.* 2001, Kawai *et al.* 2012).

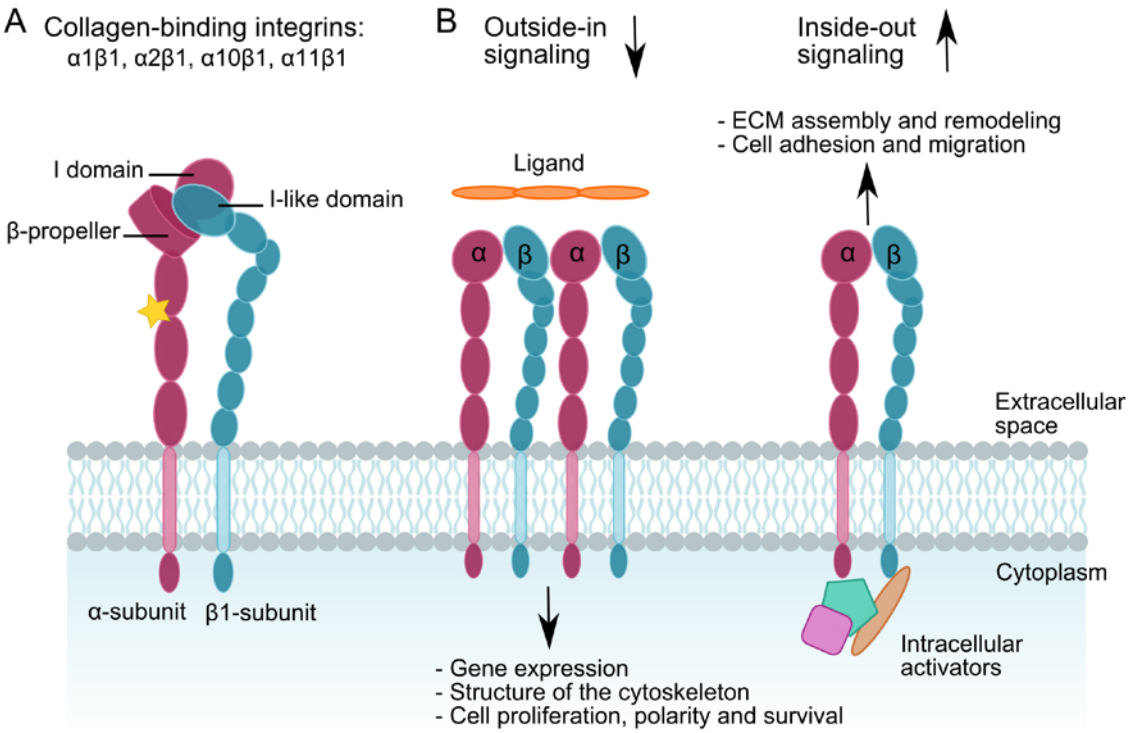

Figure 2. ***Integrin structure and signaling. A)*** *A schematic presentation of the structure of the four collagen-binding integrins, α1β1, α2β1, α10β1 and α11β1. The collagen-binding integrins constitute a subgroup of integrin receptors that share the β1-subunit and possess an I-domain within the α-subunit. The yellow star denotes the approximate position of the identified nonsense change in the canine α10-subunit.* ***B)*** *The basic modes of integrin signaling. Binding of extracellular ligands gives rise to conformational changes, and may result in clustering of integrin heterodimers. Ligand binding can have an effect on various intracellular processes, such as cell proliferation and gene expression. The integrin receptors can also be activated through intracellular proteins, such as Talin-1, which increase affinity for extracellular ligands. However, although conceptually separate, the two signaling modes are dynamic and interlinked. The figure is adapted from Askari et al. 2009, Shattil et al. 2010, and Leitinger 2011.*

The canine chondrodysplasia phenotype suggests a critical role for the α10β1 receptor in endochondral ossification. The biological importance of the four collagen-binding integrins has been explored in mouse-models. Inactivation of all β1 integrins in murine chondrocytes causes perinatal lethality and severe chondrodysplasia (Aszodi *et al.* 2003), but only a relatively mild phenotypes result from the knockdown of the individual α10-subunits of the collagen-binding integrins (Gardner *et al.* 1996, Chen *et al.* 2002, Holtkotter *et al.* 2002, Zemmyo *et al.* 2003, Bengtsson *et al.* 2005, Popova *et al.* 2007). Deficiency of the murine α10 causes a subtle reduction in the length of long bones (Bengtsson *et al.* 2005), whereas the affected dogs present with marked skeletal changes. It is plausible that the loss of α10 function in humans would resemble the canine phenotype, rather than the murine one. Then again, other types of mutations in the *ITGA10* gene could result in milder or otherwise different skeletal phenotypes in humans, as well as in dogs. In addition to the α10β1 receptor, cartilage chondrocytes also express other integrins (Salter *et al.* 1995), and although these have not been associated with inherited chondrodysplasias, they may be involved in the pathogenesis of other cartilage-related diseases, such as osteoarthritis (Zemmyo *et al.* 2003, Loeser 2014, Tian *et al.* 2015).

The full segregation of the *ITGA10* mutation with the chondrodysplasia phenotype in two different dog breeds is a strong indicator of its causative nature. It is likely that the mutation is identical by descent in the two breeds. The chondrodysplasia phenotype was described already a few decades ago in the American NE population (Bingel & Sande 1982), and our results confirm the presence of the mutation in a present-day family cohort from the United States. However, we did not have the samples to estimate the overall carrier frequency in the American NE population. The current high carrier frequency of the mutation in the Finnish NEs is probably due to chance and breeding practices. The mutation was less frequent in the Finnish KBD population, and may have been introduced to KBDs through crossbreeding with NEs in the recent past. Pedigree analysis in the KBDs suggested that a single popular sire from the 1980's highly increased the frequency of the mutated allele in KBDs (**I/Fig. 3C**). In our KBD sample cohort, all except one heterozygous animal were descendants of this particular popular sire, which was suspected of having been part NE.

As a result of this study, a gene test is now available to help recognize mutation carriers, which is particularly important for the NE breed, in light of the high carrier frequency within the breed. As a whole, this study shows that larger-sized animals, such as the dog, may be more suitable to model human skeletal disorders than small mammals. Our findings establish the *ITGA10* gene as a highly plausible candidate gene in human chondrodysplasias.

5.2 A missense change in the *SEL1L* gene implicates defective protein degradation in canine cerebellar ataxia (Study II)

5.2.1 Early-onset cerebellar degeneration in Finnish Hound dogs

In 2006, our research group initiated a genetic study to identify the molecular genetic cause of cerebellar ataxia in FH dogs. In 1971, a case study in the Finnish Veterinary Journal had described a FH puppy affected with cerebellar ataxia (Tonttila & Lindberg 1971). The puppy had presented with progressive ataxia, starting around the age of three months. The condition progressed quickly to a point at which eating became increasingly difficult due to uncontrolled head movements. The affected dog was euthanized at the age of 5 months, and post-mortem examination revealed marked cerebellar cortical atrophy. The authors proposed an underlying genetic cause, as another puppy from the same litter had presented with corresponding neurological problems. Furthermore, subsequent inquiries revealed that puppies with similar clinical signs had been previously encountered in at least eight different FH litters (Tonttila & Lindberg 1971).

During 2006-2008, detailed clinical examinations were performed on ten ataxic FH puppies (**Fig. 3**) and on one unaffected littermate. The examinations took place when the puppies were from 3 to 4 months old. The owners of the affected dogs had noticed the first clinical signs at the mean age of 9 weeks, ranging from 4 to 12 weeks **(II/Table 1)**. General clinical and orthopedical examination did not uncover abnormalities. Serum biochemistry profiles, complete blood cell count and cerebrospinal fluid cell count were within normal limits. However, neurological examination revealed generalized cerebellar ataxia, intention tremors and deficits in postural reactions in all examined affected dogs (**II/ Videos S1-S3**). MRI of the brain indicated reduced size of the cerebellum in nine out of ten affected dogs (**II/Fig. 1**) Due to rapid worsening of the clinical signs, all affected puppies were euthanized upon the owners' agreement and underwent post-mortem examination.

Pathological examinations did not reveal significant changes outside of the nervous system. Within the CNS, histological changes were confined to the cerebellum, and characterized by marked PC degeneration and subsequent loss of granule cells (**II/Fig. 2**). The most profoundly affected cerebellar regions were the vermis and the paramedian lobules, whereas some areas, such as the paraflocculi and flocculus, were mostly unaffected. As a whole, the findings reported in the 1970's case study were consistent with the clinicopathological findings in our study, implicating that the disease phenotype has been present in the breed for several decades.

Figure 3. ***Affected Finnish Hound dogs. A)*** *A 3-month affected male puppy during the testing of postural reactions.* ***B)*** *Another 3-month-old affected male puppy. Note the abnormal posture in both dogs.*

5.2.2 Ataxia locus maps to canine chromosome 8

Analysis of FH pedigree data strongly supported our hypothesis of an autosomal recessive inheritance mode. All parents of affected dogs were healthy, both sexes were equally affected and the proportion of affected dogs within the disease pedigree was close to 25% (**II/Fig. 3**).

The genetic examinations in FH ataxia were initiated with a candidate gene approach. For this purpose, we selected 24 genes that were known to cause ataxia either in humans or in mice (**II/Table S1**). The possible involvement of these genes in FH ataxia was studied through segregation of microsatellite markers in three nuclear families. However, none of the markers co-segregated with the phenotype in the examined families.

As the next step, we employed the genome-wide approach to shed light onto the underlying genetic cause. Illumina's 22K canine SNP chips were used as a genotyping platform for a cohort of 31 dogs, comprising 13 affected dogs, seven healthy siblings and 11 obligate carrier parents. A basic case-control test was carried out using the unaffected siblings as controls. This revealed a genome-wide significant association on CFA8 ($p_{raw} = 1.1x10^{-7}$ and $p_{genome} = 7.5x10^{-4}$), which was confirmed by family-based analyses in the full cohort (LOD score = 3.3, association $p = 2.2x10^{-7}$ and joint analysis $p = 4.0x10^{-10}$) (**II/ Fig. 4A, B**). The CFA8 locus contained a 1.5-Mb homozygous haplotype that was shared by all affected dogs, spanning from 56.0 to 57.5 Mb (CanFam2.0 assembly) (**II/Fig. 4C**). Within this haplotype, a single SNP (BICF2P948919) co-segregated fully with the phenotype. This SNP was intronic to the sel-1 suppressor of lin-12-like (*SEL1L*) (**II/Fig. 4D**), which was considered the strongest candidate gene within the associated locus due to its function in a protein quality control and degradation pathway (Lilley & Ploegh 2005, Mueller *et al.* 2006, Mueller *et al.* 2008). This was thought promising, as problems in protein degradation are found in many neurodegenerative diseases. However, six other genes were also present in the

haplotype (**Table 3**). According to the CanFam2.0 genome assembly, two of the genes were probable pseudogenes, and these were therefore excluded from detailed analysis. The remaining five genes were screened for possible causative mutations by sequencing their exons and exon-intron boundaries in two affected dogs and in two obligate disease carriers. Out of the five studied genes, only *SEL1L* contained variants that were within the protein coding regions, and only one of these variants was a non-synonymous change with a protein level effect (**II/Table S2**).

Table 3. ***Genes within the ataxia locus on canine chromosome 8.***

Gene symbol	Gene name	Chr8 position (Mb)[1]	Gene function	Screened	Reference (mRNA)
CEP128	Centrosomal protein 128kDa	55.954-56.346	Unknown	Yes	XM_547936.3
TSHR	Thyroid stimulating hormone receptor	56.364-56.520	Controls thyroid cell metabolism	Yes	NM_001003285.1
GTF2A1	General transcription factor IIA	56.562-56.607	Transcription initiation	Yes	XM_849814.1
STON2	Stonin 2	56.635-56.739	Regulation of endocytic complexes	Yes	XM_547937.3
SEL1L	Sel-1 suppressor of lin-12-like	56.808-56.861	ER-associated protein degradation	Yes	XM_537530.2
LOC612107	Uncharacterized hypothetical gene	57.034-57.111	Possible pseudogene[2]	No	XM_863603.3
LOC100687147	NIMA-related kinase 6 pseudogene	57.396-57.397	Pseudogene	No	XM_846279.1

[1]*The positions refer to the CanFam2.0 genome assembly.* [2]*The gene was later annotated as a non-coding RNA gene in the CanFam3.1 assembly.*

5.2.3 Affected dogs have a missense mutation in the *SEL1L* gene

Mutation screening of the candidate genes revealed a homozygous c.1972T>C single nucleotide alteration in exon 19 of the *SEL1L* gene, predicted to cause a p.Ser658Pro amino acid change in the encoded protein (**II/Fig. 4E, 5**). In our entire FH family cohort, the c.1972T>C change showed full segregation with the ataxia phenotype. All 13 affected dogs were homozygous for the variant and all 13 obligate carrier parents were heterozygous. Out of 20 unaffected siblings, 12 were heterozygous for the variant and the remaining eight were homozygous for the reference allele. A 10% mutation carrier frequency was indicated in a population cohort of 241 unaffected FHs, whereas in 349 dogs from 51 other breeds, the variant was completely absent.

The SEL1L protein localizes to the ER, where it attaches to the ER membranes by its carboxy-terminal transmembrane domain. The amino-terminal protein body lies in

the ER lumen and is composed of a fibronectin type II domain and three clusters of α/α-helical Sel1-like repeat motifs, which are a type of tetratricopeptide repeats (Biunno *et al.* 2006) that have been suggested to mediate protein-protein interactions (Blatch & Lassle 1999, Mittl & Schneider-Brachert 2007). The SEL1L p.Ser658Pro change was positioned on one of the evolutionary conserved Sel1-repeats within the carboxy-terminus of the protein (**II/Fig. 5**). The pathogenicity of the amino acid change was assessed trough the bioinformatics prediction tools PANTHER, PolyPhen and SIFT, all of which estimated it to have a damaging effect. Collectively, the complete segregation of the *SEL1L* c.1972T>C variant with the phenotype, the position of the amino acid change in a conserved protein domain, and the pathogenicity prediction results offer strong support for causality.

Since the pathological changes were restricted to the cerebellum, we used RT-PCR to confirm that the *SEL1L* gene is expressed in cerebellar tissue. The entire *SEL1L* transcript was successfully amplified and sequenced from cerebellar cortical tissue samples of affected and healthy dogs, verifying the cerebellar gene expression. Apart from the c.1972T>C variant, the obtained sequence data did not deviate from the reference sequence and there was no indication of differential mRNA splicing. As a next step, we wanted to examine whether there are signs of increased ER stress in the affected dogs' cerebellar cortex. The SEL1L protein functions in an ERAD pathway as a part of a protein quality control and degradation machinery (Mueller *et al.* 2008, Iida *et al.* 2011). Its loss has been reported to cause ER stress in murine models (Francisco *et al.* 2010, Sun *et al.* 2014). We therefore studied the relative expression levels of several genes that are a part of the unfolded protein response (UPR), a mechanism that promotes cell survival during ER stress by upregulating several ERAD-related genes (Hetz 2012). Our qPCR results revealed increased expression levels for the studied UPR genes in affected dogs (**II/Fig. 6B)**. An especially high 14-fold increase was found in the expression of a proapoptotic transcription factor CHOP, known to be highly upregulated during ER stress (Ron & Habener 1992, Okada *et al.* 2002, Nishitoh 2012). Prolonged UPR activity has been linked to cell death in neurodegenerative diseases and in other pathological states (Lindholm *et al.* 2006, Sano & Reed 2013). Our findings suggest that the *SEL1L* mutation in affected dogs causes ER stress and activates the UPR pathway.

5.2.4 *SEL1L* mutation links an impaired ERAD pathway to cerebellar degeneration

In summary, this study revealed a homozygous missense mutation within a conserved functional domain of the *SEL1L* gene in FH dogs with cerebellar ataxia. The affected dogs presented with early-onset, quickly-progressing movement incoordination. The cerebellum of affected dogs was atrophied and showed marked PC loss.

The role of *SEL1L* in protein quality control within the ER makes it a highly plausible candidate gene in a neurodegenerative phenotype. The ER is a cellular organelle made up of a network of membranes that serve as a major site of protein

synthesis, folding, post-translational modification and transport (Ellgaard & Helenius 2003). ER stress, UPR activation and accumulation of aberrant proteins have been reported in several neurodegenerative phenotypes (Hetz & Mollereau 2014). The ER-membrane-bound SEL1L has been suggested to function as an adaptor protein within a multiprotein ERAD complex that includes the E3 ubiquitin ligase HRD1 **(Fig. 4)** (Mueller *et al.* 2006, Christianson *et al.* 2008, Hosokawa *et al.* 2008, Cormier *et al.* 2009, Iida *et al.* 2011, Sun *et al.* 2014). Mouse models and cell line experiments have implicated a critical role for the SEL1L protein in cellular homeostasis. Its loss results in altered ER morphology, ER stress, protein degradation defects, reduced translation efficiency, impaired protein secretion and premature cell death (Francisco *et al.* 2010, Sun *et al.* 2014). Accordingly, our qPCR results were indicative of ER stress within in the cerebellar cortex of affected dogs. Prolonged ER-stress and alterations in ER homeostasis may therefore be the cause of premature neuron loss in FH dogs.

Bioinformatic prediction tools estimated that the *SEL1L* missense change in affected dogs is deleterious. However, since *Sel1l*-deficiency causes embryonic lethality in mice (Francisco *et al.* 2010), it is possible that the canine mutation does not completely disrupt protein function. Furthermore, SEL1L isoforms that lack some of the carboxy-terminal protein regions have been indicated to function in parallel or complementary to the full-length protein (Harada *et al.* 1999, Biunno *et al.* 2006, Cattaneo *et al.* 2009, Cattaneo *et al.* 2011). Overall, the partial function of the mutated protein and/or the existence of isoforms that do not contain the mutation position could help explain the cerebellum- restricted pathology in affected dogs. Moreover, since the cerebellar PCs have been suggested to be especially sensitive for disruptions in proteostasis (Hekman & Gomez 2015), they may also be more vulnerable to defects in the HRD1-SEL1L pathway than other cell types. *SEL1L* gene expression has been reported in several different fetal and adult tissues, with a particularly strong expression levels in the pancreas (Biunno *et al.* 1997, Donoviel *et al.* 1998, Biunno *et al.* 2006). Accordingly, previous studies have implicated a role for SEL1L in pancreatic function (Li *et al.* 2010, Sun *et al.* 2014). Unfortunately, the pancreas was not subject to histological examination in affected FHs. However, neither did the clinical picture, nor the gross pathological examination suggest pancreatic malfunction in affected dogs. In support of the neuronal phenotype in FHs, our RNA-level experiments indicate that *SEL1L* is expressed in the cerebellar cortex of affected and healthy animals. This is in accordance with publicly available protein expression data obtained from the adult human cerebellum, which shows SEL1L signals in the PCs, as well as in the cells of the molecular layer (http://www.proteinatlas.org).

As a whole, the results of this study are in line with previous knowledge on the importance of protein quality control and degradation to neuronal survival. However, our findings directly link the HRDI-SEL1L complex to inherited cerebellar degeneration, which suggests that the pathway is critical for post-natal PC survival and cerebellar function. Furthermore, our results implicate *SEL1L* as a promising new candidate gene for human early-onset ataxias. In addition, the findings of this study have already helped prevent the birth of affected dogs through genetic testing.

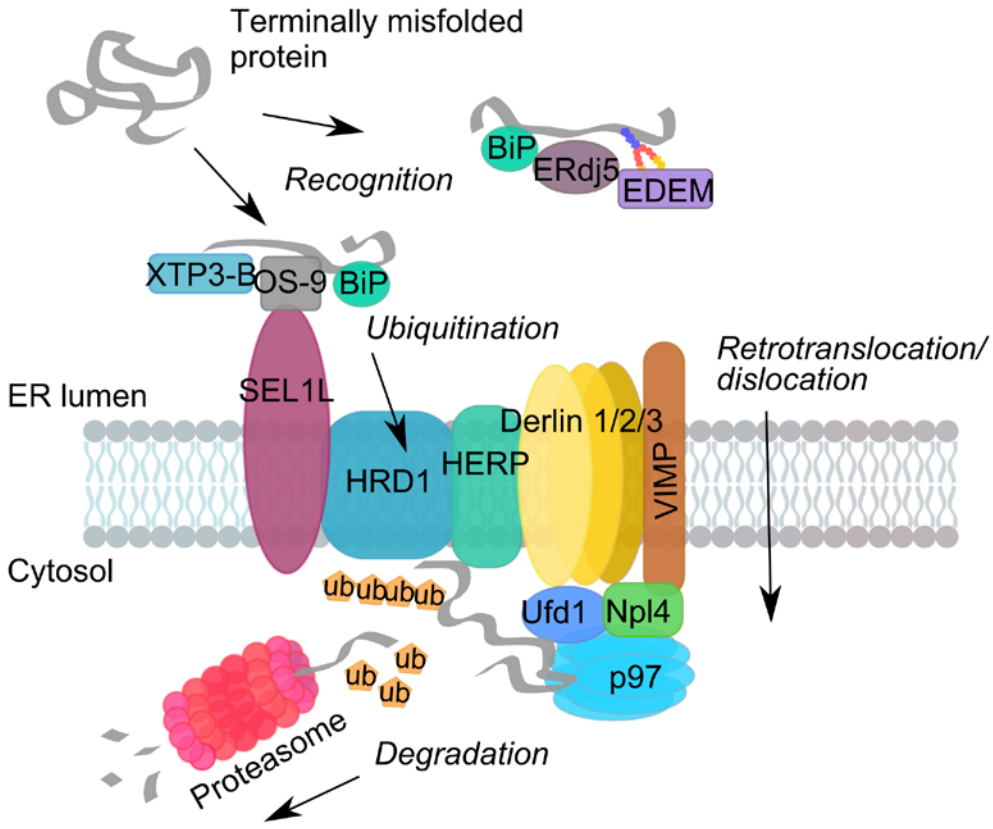

Figure 4. ***A schematic presentation of the mammalian HRD1-SEL1L complex****. The HRD1-SEL1L complex serves as a scaffold for different components of the mammalian ER-associated degradation (ERAD) pathway. The initial recognition of terminally misfolded proteins is carried out by several ERAD components, such as the chaperone proteins BiP and ERdj5, the EDEM family of lectins, and the XTP3-B and OS-9 proteins. SEL1L is thought to function as an adaptor protein. It may facilitate the delivery of ERAD substrates to the HRD1-ligase complex by binding to recognized target proteins. After being recognized and delivered to the ER-membrane, the misfolded proteins are ubiquitinated by the E3 ligase HRDI, translocated to the cytosol, and finally, degraded by the proteasome. The transportation of client proteins through the membrane is powered by the VIMP-anchored ATPase p97, which forms a complex with the Ufd1 and Npl4 proteins. The identity of the actual retrotranslocon channel is not currently clear but the process may involve the Derlin proteins, HERP and HRD1. The figure is adapted from Hoseki* et al. *2010, Kadowaki & Nishitoh 2013 and Christianson & Ye 2014.*

5.3 The autophagy-related *ATG4D* gene is associated with a canine neurodegenerative disease (Study III)

5.3.1 Cerebellar ataxia and cellular vacuolization in a novel canine disease

The LR is a dog breed that originates from Italy, where it was initially utilized as a water retriever (**Fig. 5**). A previous clinical study described two isolated LR cases that presented with a very early-onset cerebellar degeneration (Jokinen *et al.* 2007). In this study, we recognized a novel neurodegenerative disease with distinct pathological changes that affects the breed. The presently studied disorder was first encountered in 2013 when three adult LRs were euthanized due to progressive ataxia.

During the genetic study, altogether 22 affected LRs were identified. The predominant clinical sign in the affected dogs was progressive cerebellar ataxia (**III/S1 Video**). A subset of dogs presented with episodes of abnormal eye movements (nystagmus), and as the disease progressed, some developed behavioral changes. The first clinical signs were noticed in the affected dogs at the mean age of two years, ranging from 4 months to 4 years. The rate of disease progression varied from months to several years. Detailed neurological examination was performed on 16 affected animals. The examined dogs presented with cerebellar ataxia of varying severity, and showed abnormal responses when tested for hopping reactions, patellar reflexes and menace reaction. Brain MRI was performed on eleven affected dogs, revealing signs of cerebellar atrophy in nine examined dogs.

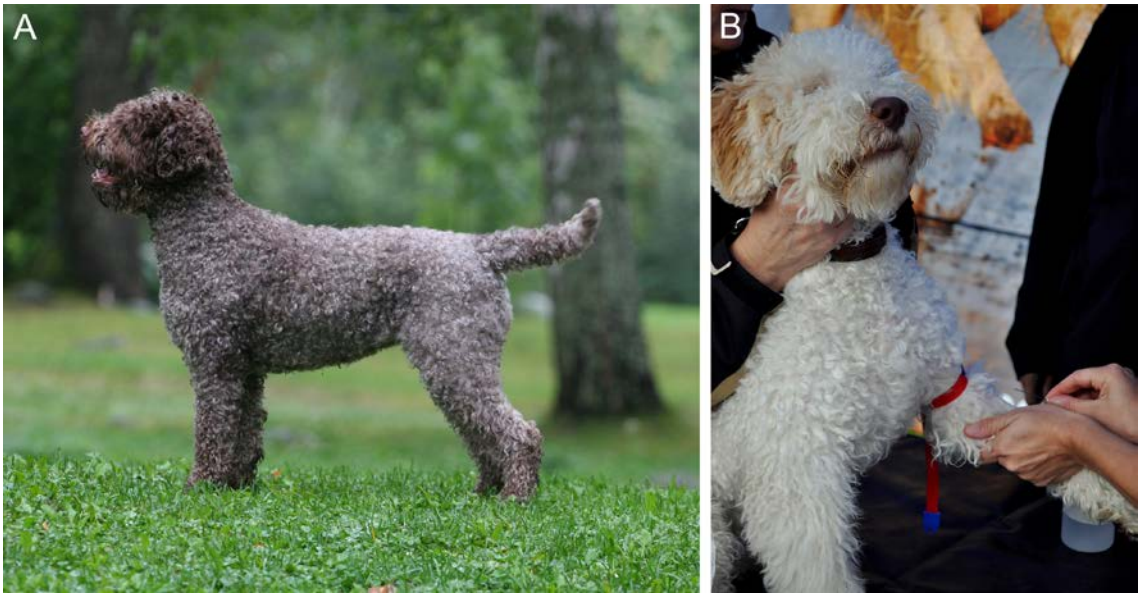

Figure 5. ***Lagotto Romagnolo dogs. A)*** *An unaffected Lagotto Romagnolo (LR) (Photograph by Marko Saren).* ***B)*** *A blood sample is drawn from another unaffected LR dog for the genetic study.*

Pathological examination was performed on seven affected LRs, all euthanized due to worsening clinical signs. The most notable histological finding in the affected dogs was clear cytoplasmic vacuolization that ranged from fine vesiculation to large vacuoles (**III/Fig. 1A**). The vacuolar change was present in the nervous system, but also in other tissues. Within the CNS, the cerebellar cortex was one of the most consistently and severely affected tissues (**III/Fig. 1B**), along with several specific nuclei, such as the deep cerebellar nuclei and nucleus vestibularis. Milder changes were seen in other regions, including the cerebral cortex. Axonal spheroids were indicative of disturbed axonal transport within the cerebellum and brainstem (**III/Fig. 1E**). Marked PC and granule cell loss was seen in the cerebellar cortex (**III/Fig. 1C, D**), which was likely the major cause of clinical signs in affected dogs. In extraneuronal tissues, vacuolization was present in tissues of mesodermal origin, such as the smooth muscle cells, and in several different secretory epithelial tissues, including the salivary glands, pancreatic acinar cells and the parathyroid gland (**III/Fig. 1F**). The vacuolar change was also found within apocrine sweat glands in skin biopsies of three symptomatic dogs. The possible content of the vacuoles could not be determined in HE or PAS stainings. EM sections of cerebellar PCs revealed single-membrane-bound vacuoles, which appeared either empty or contained small amounts of membranous or floccular material (**III/Fig. 1G**). Overall, the pathological changes in affected LRs did not directly correspond to previously characterized disorders. The vacuolization was indicative of a LSD, but the lack of specific, identifiable storage materials, such as lipofuscin or glycogen, did not rule out alternate disease etiologies.

5.3.2 A missense change in the autophagy-related *ATG4D* gene

A genome-wide approach was utilized to get insights into the genetics of the disorder. The genetic study was initiated when DNA-samples had been obtained from three pathologically confirmed affected dogs, comprising two LR siblings from Finland and one distantly related LR from Switzerland. The three affected dogs were genotyped using Illumina's 170K canine SNP chips, along with the unaffected parents and two healthy siblings of the Finnish cases (**III/ Fig. 2A**). In addition, we performed whole-genome sequencing on the affected LR from Switzerland.

The genetic data was analyzed under the assumption of a shared founder mutation and an autosomal recessive mode of inheritance (**III/Fig. 2B**). Parametric linkage analysis within the Finnish LR family revealed positive LOD scores for 25 genomic loci, comprising altogether 276 Mb of sequence (**III/S1 Table**). Homozygosity mapping in the three affected dogs identified simultaneous allele sharing across 38 Mb of sequence, within 11 genome regions (**III/S2 Table**). Three chromosomal segments on canine chromosomes 11, 13 and 20 overlapped in these two analyses, and had a combined size of 19 Mb (**III/S3 Table**). The whole genome sequence of the affected LR contained ~2.9 million homozygous variants, altogether 31,016 of which were found within the three critical loci on chromosomes 11, 13 and 20 (**III/Table 1**). However, when the variants present on the three critical regions were filtered against variants that

were present in the genomes 118 dogs from various different breeds, only five LR specific variants remained, one of which was predicted to have a protein-level effect (**III/Table 2**). This particular variant was a missense change, c.1288G>A; p.Ala430Thr, in the autophagy-related 4D cysteine peptidase (*ATG4D*). The other four variants comprised three intronic single nucleotide changes and one intergenic small deletion (**III/Table 2**).

The *ATG4D* c.1288G>A variant was genotyped in our entire LR sample cohort of 2,352 dogs. Within this cohort, 2,061 (88%) dogs had a wild-type genotype, 266 (11%) dogs were heterozygous, and 25 (1%) dogs were homozygous for the variant (**III/S5 Table**). The overall frequency of the variant allele was 7%. Compatible neurological signs were present in 22 out of the 25 homozygous animals, seven of which were confirmed as affected through post-mortem examination and three through a skin biopsy (**III/ S6 Table**). Clinical signs could not be ascertained in three adult dogs that were homozygous for the missense change. Two of these, a 4-year-old and a 7-year-old dog, had possible mild neurological signs, but one 12-year-old animal appeared completely unaffected when reviewed through video material. The variant was not present in 642 dogs from 40 other breeds (**III/S5 Table**). In the LR cohort, the statistical association between the *ATG4D* variant and disease was highly significant ($p = 3.8 \times 10^{-136}$).

The mammalian ATG4D protein is one of four ATG4 family members (ATG4A-D) that function as cysteine proteinases in the macroautophagy pathway (Marino *et al.* 2003). The ATG4 proteins are thought to play a role in the biogenesis of autophagosomes (Kaufmann *et al.* 2014). The identified c.1288G>A variant was located in the last exon of the *ATG4D* gene, and the resulting p.Ala430Thr amino acid change was positioned in the carboxy-terminus of the protein, immediately downstream of the principal functional C54 peptidase domain (**III/Fig. 3A, B, C**). The 430-alanine position was found to be moderately conserved in evolution, which may indicate functional significance (**III/Fig. 3D, E**). However, the p.Ala430Thr change in affected dogs was not estimated to have a damaging effect on protein function by bioinformatics tools. Analysis of the *ATG4D* transcript in cerebellar tissue through RT-PCR and sequencing did not reveal differences between affected and healthy dogs, either in mRNA splicing or in expression levels (**III/S2 Fig.**).

5.3.3 Affected tissues show signs of altered autophagic flow

The function of the *ATG4D* gene in macroautophagy prompted us to examine whether the autophagy-lysosome pathway is disturbed in the affected nervous tissue. For this purpose, we utilized immunohistochemistry and several different autophagy and lysosomal markers. The autophagosome membrane marker LC3B was detected in several PCs within the affected cerebellar cortex (**III/Fig. 4E**) and in the axonal spheroids (**III/Fig. 4A**), suggesting either induction of autophagy or blockage in the autophagy pathway. The granular core of the axonal spheroids showed positivity for ubiquitin and p62 (**III/Fig. 4B, C**). Given that ubiquitin marks degradation-bound cellular material and p62 in turn binds ubiquitinated materials destined for autophagy,

these findings may also indicate disturbed autophagy. Some neuronal vacuoles were positive for the lysosome marker LAMP2 (**III/Fig. 4F**), suggesting these could be secondary lysosomes or autolysosomes. However, some of the vacuoles did not stain with any of the used antibodies, leaving their characterization open. Overall, these results suggested alterations in the autophagic flux within affected neurons, but further examinations are required to obtain a more detailed picture concerning the nature of the pathological changes.

5.4.4 Zebrafish knockdown of *atg4da* reveals CNS abnormalities

Given that the functional importance of *ATG4D* is not well established, we utilized the zebrafish model to explore its role during development. For this purpose, we used the splice morpholino technique to knockdown the zebrafish homologue *atg4da*. The efficiency and transcript level consequence of the *atg4da* splice morpholino (*atg4da*SMO) was evaluated by RT-PCR, revealing skipping of exon three in the morphant embryos (**III/Fig. 5G**). Inspection of the morphant phenotype at 1 day post-fertilization (dpf) indicated severe CNS malformations and widespread neurodegeneration in comparison to the control embryos (**III/Fig. 5A-D**). The affected CNS regions corresponded to the cerebellum, hindbrain and midbrain-hindbrain boundary. At 2 dpf, the morphant embryos exhibited small-sized brain and eye, mild hydrocephalus and occasional pericardial edema (**III/Fig. 5E and F**). Within the cerebellar cortex, immunostaining with PC and granule cell markers revealed marked loss of both cell types at 4.5 dpf (**III/Fig. 6**). Overall, the severe phenotype caused by the knockdown of the zebrafish *atg4da* gene implicates an important role for the gene during CNS development.

5.4.5 *ATG4D* mutation strengthens the link between autophagy and neurodegeneration

The results of this study suggest that a previously unknown neurodegenerative disease in the LR breed is caused by a missense change in the autophagy-related *ATG4D* gene. The disorder is characterized clinically by cerebellar ataxia with a juvenile or adult onset, and pathologically by cytoplasmic cellular vacuolization. In support of the genetic association, immunohistochemical findings in affected canine brain tissues suggested altered autophagic flux, and a knockdown of the zebrafish homologue resulted in marked neurodevelopmental alterations and neurodegeneration.

Our genetic data revealed a highly significant association between the canine disease and the *ATG4D* c.1288G>A variant, representing the first link between the *ATG4D* gene and an inherited disease. Sequencing of the *ATG4D* transcript did not indicate differences in transcript splicing or expression levels between affected and healthy dogs, suggesting a possible protein-level functional change. However, on the basis of the pathogenicity prediction results and the severe phenotype of the morphant zebrafish,

it is probable that the resulting p.Ala430Thr amino acid change does not cause a complete loss of ATG4D function. Furthermore, clinical information obtained from LRs homozygous for the *ATG4D* variant indicates variable expressivity and possible incomplete penetrance. The age of onset and rate of disease progression varied considerably in the affected dogs, and clinical signs could not be confirmed in three homozygous animals. It is therefore conceivable that other genetic factors and/or environmental modifiers influence the phenotype. However, since direct proof of pathogenicity is still lacking, it remains a possibility that the actual causative variant is in LD with the presently identified *ATG4D* change. As this thesis was being written, two new affected LRs homozygous for the *ATG4D* variant were identified. Both dogs presented with progressive cerebellar signs, and the other underwent post-mortem examination, revealing similar vacuolization that was present in other pathologically examined dogs.

The evolutionary conserved macroautophagy pathway maintains cellular homeostasis both under normal and stressful conditions by recycling nutrients and disposing damaged cellular materials via the double-layered autophagosomes (Boya *et al.* 2013). The pathway is orchestrated by an array of autophagy-related (ATG) proteins (Kaufmann *et al.* 2014), whose roles have been delineated in yeast studies. The yeast possesses only a single Atg4 protein, which functions as a processor of the autophagosome membrane protein Atg8 (**Fig. 6**) (Lang *et al.* 1998, Kirisako *et al.* 2000, Ichimura *et al.* 2000). Unlike yeast, higher organisms possess several homologs of the yeast Atg4 and Atg8 proteins (**Fig. 6**) (Xin *et al.* 2001, Marino *et al.* 2003, He *et al.* 2003, Shpilka *et al.* 2011). Their particular roles and degree of redundancy are not yet well-established, although there are indications of differential functions (Xin *et al.* 2001, Weidberg *et al.* 2010, Li *et al.* 2011).

Dysfunction of the autophagy pathway has been associated with several neurodegenerative diseases, but reports of disease-causing mutation in the core autophagy components are rare. A recent study links an ATG protein with a neurodegenerative disorder in humans. Disease-causing mutations were identified in the human *WDR45* gene, which is a mammalian homolog of the yeast *Atg18* gene (Saitsu *et al.* 2013). The resulting disease phenotype is a severe autosomal recessive disorder, known as static encephalopathy of childhood with neurodegeneration in adulthood (Saitsu *et al.* 2013). In mice, neuronal deficiency of the autophagy proteins Atg5 and Atg7 causes neurodegeneration (Hara *et al.* 2006, Komatsu *et al.* 2006), and the loss of Atg5 results specifically in PC degeneration (Hara *et al.* 2006).

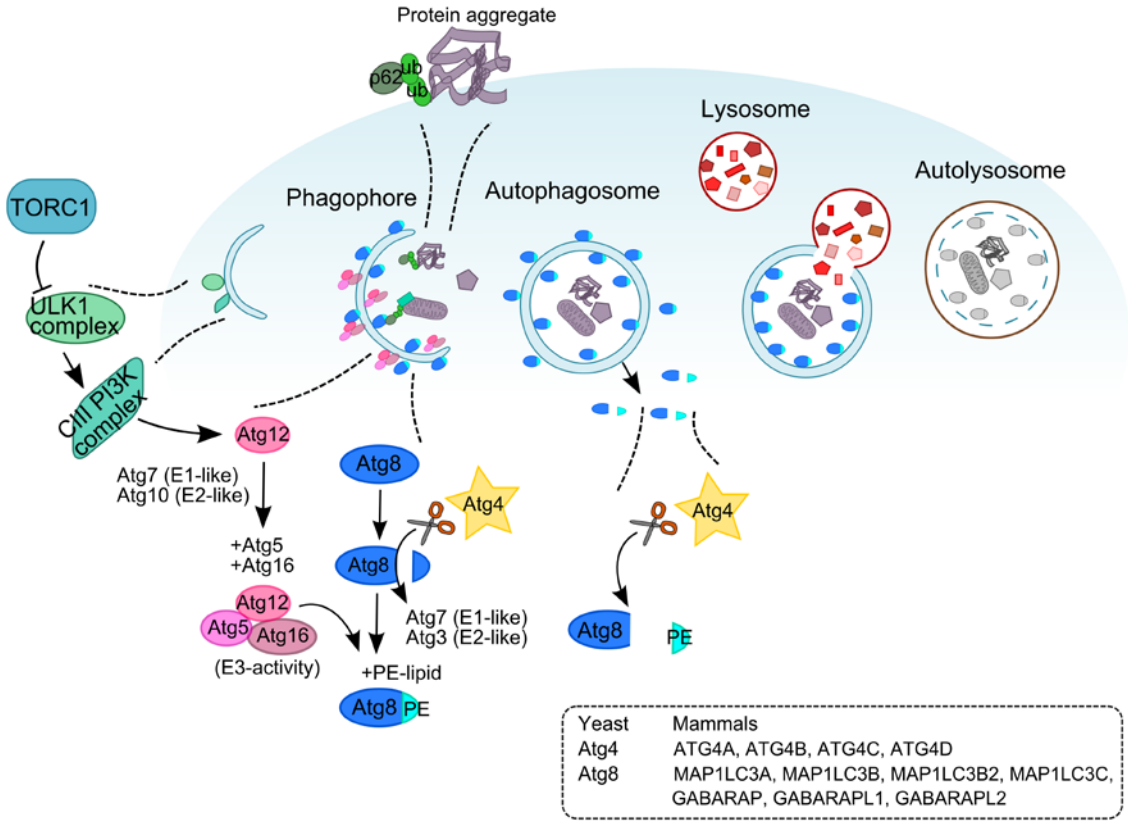

Figure 6. ***The ATG4 proteins function in the macroautophagy pathway.*** *Mammals possess four homologs of yeast Atg4 and at least seven homologs of the yeast Atg8. Atg8 and its mammalian homologs are needed for autophagosome formation and are also thought to facilitate cargo recognition through interaction with autophagic adaptors, such as the p62, which binds ubiquitinated targets. The macroautophagy pathway is negatively regulated by mTOR (via the TORC1 complex), whose suppression activates downstream regulators, such as the ULK1 and PI3 kinase complexes. Two ubiquitin-like conjugation systems are needed for the elongation of the forming autophagosomal membranes: the Atg12-Atg5 system and the Atg8 system. The Atg12-Atg5-Atg16 complex forms via the E1-like and E2-like activities of the Atg7 and Atg10 proteins, respectively. The Atg4 protease functions in the Atg8 conjugation system. Atg8 is cleaved by Atg4, enabling a covalent attachment of the lipid phosphatidylethanolamine (PE) to Atg8. This process is carried out by the enzymatic activities of Atg7 (E1-like), Atg3 (E2-like) and the Atg12-Atg5-Atg16 complex (E3-like). As the autophagosome fuses with the lysosome to form the autolysosome, the Atg8 proteins at the inner membrane are degraded, whereas those at the outer membrane can be recycled, via delipidation of Atg8-PE by Atg4. Figure is adapted from Weidberg* et al. *2011, Fleming* et al. *2011 and Nixon 2013.*

The most striking histological change in affected dogs was cytoplasmic vacuolization, present in both neuronal and extraneuronal tissues. Within the CNS, the cerebellum was one of the most severely affected organs and showed degenerative changes. In line with this, publicly available *ATG4D* expression data from mice indicates especially high expression levels in the adult cerebellum in comparison to other brain regions (Magdaleno *et al.* 2006) (http://www.informatics.jax.org/assay/MGI:4945783). The intracellular vacuolization in affected dogs was reminiscent of LSDs. However, we were unable to recognize any specific material within the intracellular vacuoles, which for the most part, appeared empty. Some of the neuronal vacuoles in affected dogs stained positive for the lysosomal marker LAMP2, suggesting an alteration in lysosomal homeostasis, which is not surprising as the lysosomal and autophagosomal systems are closely connected (Korolchuk *et al.* 2010). Many of the extraneuronal findings in affected LRs were found within secretory epithelial tissues, although they did not appear to result in specific clinical signs. It remains to be uncovered, whether the affected neurons and other affected tissues simply have a high basal demand for autophagy or whether ATG4D plays some other roles in these tissues, for instance, in secretion-related vesicular trafficking. Interestingly, recent studies have implicated the autophagy proteins to function in different secretion pathways (DeSelm *et al.* 2011, Torisu *et al.* 2013, Ohman *et al.* 2014, Ponpuak *et al.* 2015). Overall, the neurodegenerative disease in LRs does not seem to fall into the category of classical LSDs, but could rather be placed among a broader spectrum of neurodegenerative diseases that are characterized by defective clearance of cellular cargo through the network of secretory, endosomal, autophagic, lysosomal and exocytic pathways (Boland & Platt 2015).

As a whole, our findings suggest that the autophagy-related *ATG4D* gene is important for neuronal homeostasis and survival. The results of this study strengthen the link between the autophagy-lysosome pathway and neurodegeneration. It is plausible that similar phenotypes in other species may also result from *ATG4D* mutations. The canine phenotype serves as a platform to study the physiological role of the ATG4D in more detail, and to delineate the pathological mechanisms that lead to the observed vacuolar changes and neuronal degeneration. Finally, our results have provided a genetic test that can be used for both veterinary diagnostic and breeding purposes.

6 Concluding remarks

By utilizing the canine model and its unique genetic features, this thesis has provided insights into the molecular genetics of three inherited diseases in dogs. The results of this study have had both basic biological and practical significance by revealing new disease-associated genes and pathways, by implicating three novel candidate genes for corresponding human diseases, and by providing diagnostic help and breeding tools for veterinarians and dog owners.

Dwarf dogs from two different breeds were found to have a nonsense mutation in the *ITGA10* gene. The mutation is likely to result in a complete loss-of-function of the heterodimeric collagen-binding α10β1 integrin. The loss of the α10-integrin subunit was previously reported to cause mild chondrodysplasia in mice, whereas the affected dogs presented with more pronounced skeletal changes. Our results suggest that the α10β1 integrin has a critical role in skeletal development especially in larger-sized mammals.

The *SEL1L* gene was implicated in an early-onset ataxia and the *ATG4D* gene in a later onset neurodegenerative disease. Interestingly, both genes function in degradative pathways, *SEL1L* in ER-associated protein degradation, and *ATG4D* in the autophagy pathway. The involvement of degradative pathways in neurodegeneration has emerged as a common theme, and our findings may help understand their role in disease pathogenesis. Further studies are necessary to delineate exact the mechanisms through which the disease-associated *SEL1L* and *ATG4D* variants cause neuronal degeneration. More detailed characterization of the pathological mechanisms could, for instance, have implications for the development of therapy options. In addition, the canine models may prove helpful in studying the functional importance of the defective genes outside of the nervous system. Collaboration with researchers from the Columbia University has already explored the role of *SEL1L* in liver function in affected FH dogs (Wu *et al.* submitted).

The genetic studies in this thesis were performed in close collaboration with veterinary clinicians and pathologists. Especially in the neurodegenerative diseases, neurological and pathological examinations were necessary to ascertain the affection status of the studied animals. Overall, this collaboration between clinicians and basic researchers was instrumental in the successful identification of the disease-associated genetic variants.

This thesis showcases the benefits of the dog in identifying disease-associated loci and gene variants by using genome-wide approaches and small sample numbers. The genome sequence of the domestic dog was released shortly before this thesis work was initiated, and during the study, the available genomic tools became more sophisticated. The genetic examinations in this thesis started with a candidate gene approach, and from there, the utilized methods evolved from 22K canine SNP chips to 170K SNP arrays, and finally, to whole-genome sequencing. In studies I and II, genome-wide significant disease loci were first mapped by using SNP arrays, and the causative variants were then identified through Sanger sequencing. In study III, several suggestive disease loci were identified by performing linkage analysis and homozygosity mapping in a limited number of samples. Combining these results with genome-wide sequence data from one

affected dog led to the identification of the disease-associated variant. At the moment, next-generation sequencing technologies are rapidly becoming a predominant method in identifying causative variants in dogs, as well as in other species. However, association and linkage information can still significantly aid in identifying causative mutations, particularly in dogs, which currently lack the comprehensive variation databases that are available for human genetic studies.

A direct practical application of this thesis has been the development of genetic tests for the affected breeds. The tests can be of use in veterinary diagnostics, as well as in breeding programs. A large number of dogs were already tested as a part of this study. The owners of over 4,300 dogs received a test result based on the finding of this thesis work. The current commercial tests have been well accepted by breed clubs, and are used as part of the breeding programs. As a general guideline, breeders have been instructed not to discard all disease carriers from the breeding population. For instance, our results indicate that over 20% of Norwegian Elkhounds carry the *ITGA10* mutation, and exclusion of all carriers from the breeding population could therefore impact the breed's genetic diversity. However, with the help of the genetic testing, the disease-causing alleles are likely to eventually disappear from the breeds. In the meantime, the birth of affected puppies can be prevented by only mating carrier dogs with non-carriers.

This thesis work was critically dependent on the willingness of the dog owners to participate in the research, and to provide blood samples and health information from their pet dogs. Blood samples were received from various different countries, from as far away as New Zealand, which is an indication of the importance the canine genetic research holds for individual owners and breeders. During the course of this thesis work, the number of samples within the research group's DNA bank increased by tens of thousands. Many of the samples used in this work were initially collected for other projects, and samples obtained for this study will hopefully be of help in other research projects in the future.

To conclude, the genetic research performed on dogs has great potential to uncover the genetic basis of various different disease phenotypes, which can be of use to canine health, human health and basic research alike. This is achievable through the collaboration and shared efforts of dog owners, breed clubs, veterinary specialists and researchers.

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Helsinki, October 2015

Kaisa

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