12-2005

Cloning and Characterization of Three Alleles of the Mouse Furloss (Frl) Gene

Heather Marie Dech
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Heather Marie Dech entitled "Cloning and Characterization of Three Alleles of the Mouse Furloss (Frl) Gene." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

Edward J. Michaud, Major Professor

We have read this thesis and recommend its acceptance:

Dabney Johnson, Bruce D. McKee, Brynn Voy, Carla Sommardahl

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Vice Chancellor and Dean of Graduate Studies

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Cloning and Characterization of Three Alleles of the Mouse *Furloss* (*Frl*) Gene

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Heather Marie Dech
December 2005

Dedication

This thesis is dedicated to my wonderful family. Thank you, John, for always supporting me in all that I do, for being there the many times that I thought I couldn't make it, and most of all, for loving me. To my precious children, Sean, James and Matthew, I love you more than anything in this world, and everything that I have accomplished has been done with the hope that I can make the world a better place for you to live in. Never let life interfere with reaching for your dreams.

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Lastly, I would like to thank my family and friends whose suggestions and encouragement made this work possible.

Abstract

Single-gene mutations in the mouse serve as useful models for understanding the pathophysiology and molecular mechanisms of human diseases. Three dominant autosomal mutations affecting skin, hair and eye development in the mouse were recovered in mutagenesis experiments at the Oak Ridge National Laboratory. Previous work demonstrated that these mutations constitute an allelic series at the mouse *Furloss* (*Frl*) locus, and they were given the symbols Frl^a, Frl^b and Frl^c. The heterozygous phenotypes of all three mutants are similar; the mice appear normal at birth but gradually lose their hair until the hair follicles and sebaceous glands are completely absent. Mice also develop corneal opacities at about six months of age. Homozygous mice exhibit the same skin, hair and eye abnormalities, but Frl^a and Frl^b homozygotes have reduced viability. The phenotype of *Frl* mice closely resembles seven other dominant mouse mutations (*Bareskin*, *Bsk*; *Rex denuded*, Re^{den}; *Recombination-induced mutation 3*, *Rim3*; *Defolliculated, Dfl*; *Reduced Coat 2, Rco2*; *Finnegan, Fgn*; and *Hairloss*), which all map to the distal portion of chromosome 11. Genetic linkage analysis with *Frl* and *Rex* (*Re*) demonstrated that *Frl* also maps to this same region. Recent work by others demonstrated that *Rim3* mice have a mutation in the *Gasdermin 3* (*Gsdm3*) gene. Due to the striking similarity of phenotypes between *Rim3* mice and *Frl* mice, as well as the *Frl* mapping data, the *Gsdm3* gene was sequenced in the *Frl* mice, and through collaboration, in *Bsk* mice. The mutations for all three *Frl* alleles were identified in exon 12, and the mutation for *Bsk* was identified in exon 10, of *Gsdm3*. In addition to *Rim3*, Frl^a, Frl^b, Frl^c and *Bsk*, other groups have recently determined that *Bsk*, Re^{den}, *Rco2*, *Dfl*, *Fgn* and *Hairloss* (unpublished) are all the result of mutations in *Gdm3*, bringing the allelic series of unique mouse mutations in the *Gasdermin 3* gene to nine. Histological analysis revealed acanthosis, hyperkeratosis and hypergranulosis in the epidermis of the skin and the cornea of the eye. In addition, it also showed a prolonged catagen stage, which resulted in abnormally long hair follicles during the next anagen stage, and the eventual complete destruction of hair follicles. *Gsdm3* does not have an ortholog in humans or rats, and is believed to be the result of a unique duplication event of *Gasdermin1* in the genus *Mus*. The *Furloss* mice will serve as useful models for studying related sebaceous gland defects and scarring alopecia in humans. The allelic series of nine mutations in *Gsdm3* will be valuable for studying the structure and function of the *Gsdm3* protein.

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List of Abbreviations

Arg	Arginine
Asp	Aspartic acid
bp	Base pair
BrdU	Bromodeoxyuridine
Bsk	*Bareskin*
^{o}C	Degrees Celsius
cDNA	Complimentary DNA
Chr	Chromosome
cm	Centimeter
cM	Centimorgans
Den	*Denuded*
df	Degrees of freedom
Dfl	*Defolliculated*
DHGP	German Human Genome Project
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
ENU	*N*-ethyl-*N*-nitrosourea
EtOH	Ethanol
Fgf5	*Fibroblast growth factor 5*
Fgn	*Finnegan*
Frl	*Furloss*
Glu	Glutamic acid
Grb7	*Growth factor receptor-bound protein 7*
Grn	*Granulin*
Gsdm	*Gasdermin*
GSDML	*Gasdermin like*
Gsdml1	*Gasdermin-like 1*
Gsdml2	*Gasdermin-like 2*
H&E	Hematoxylin and eosin
Ile	Isoleucine
IP	Intraperitoneally
Jup	*Junctional plakoglobin*
kb	Kilobases
kg	Kilogram
Leu	Leucine
Lys	Lysine
Mbp	Mega basepairs
mg	Milligram
MHC	Major histocompatability complex
ml	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid

NEB	New England Biolabs
ORNL	Oak Ridge National Laboratory
P	Probability
P	Post partum
PCR	Polymerase chain reaction
pH	Potential of hydrogen
Pro	Proline
Psmd3	*Proteasome 26S non-ATP-ase subunit 3*
Rarα	*Retinioc acid receptor alpha*
Rco2	*Reduced Coat 2*
Re	*Rex*
Re^{den}	*Rex denuded*
Re^{wc}	*Rex wavy coat*
Rim3	*Recombination induced mutation 3*
rpm	Revolutions per minute
Scd1	*Stearoyl-CoA desaturase*
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SINE	Short interspersed nuclear elements
Trp	Tryptophan
UCSC	University of California Santa Cruz
µg	Micrograms
µl	Microliters
µm	Micrometers
V	Volts
Znfn1a3	*Zinc finger protein Aiolos*

Chapter 1

Introduction and history of several mouse models that exhibit fur loss

Introduction

The mouse is an ideal organism for the study of human diseases not only because it is physiologically very similar to humans, but also because of the completion of the sequencing of the mouse genome and the relative ease by which its genome is manipulated to create potential disease models. These mouse models provide us with a better understanding of the human diseases, which can then be used to develop strategies for prevention and treatment. One field where mouse models have proved very useful is in the analysis of skin and hair development.

During the last decade, substantial progress has been made in our understanding of the development and homeostasis of the mammalian epidermis and hair follicle. Key to this understanding has been the molecular characterization of mouse mutants with defects in the epidermis and hair follicle (Nakamura et al., 2001; O'Shaughnessy and Christiano, 2004; Porter, 2003; Randall et al., 2003; Tong and Coulombe, 2003). A search of the Mouse Genome Informatics web site (http://www.jax.org) for mutant phenotypes that contain the terms 'skin OR coat' resulted in the identification of 1138 matching alleles in 800 genes/markers. In addition to the numerous engineered mouse models on this list, there are a significantly larger number of mutant mice with skin and hair phenotypes that arose spontaneously, or as a result of random mutagenesis experiments (e.g., radiation- or chemical-based mutagenesis screens). The reason for these large numbers of skin and hair mutant mice is that these phenotypes are generally very easily recognized by investigators and animal caretakers. With the completion of the mouse genome sequence, the pace at which many of these mutations are being mapped, cloned and characterized is increasing dramatically, as might be expected. In this thesis I will present the mapping, cloning and characterization of three alleles at the mouse *Furloss* locus that arose spontaneously or in a radiation-based mutagenesis screen at the Oak Ridge National Laboratory (ORNL).

Composition of the mouse hair follicle and stages of the hair-growth cycle

The mouse hair follicle is a complex cylindrical structure made up of several distinct layers. The outermost layer is the outer root sheath. The next layer is the companion layer. Following that is the inner root sheath, which is made up of Henley's layer, Huxley's layer and the inner root sheath cuticle. Innermost in the hair follicle is the hair shaft which is made up of the hair shaft cuticle (serves to protect the cortex), the hair shaft cortex (provides strength and both color and texture to the hair) and the hair shaft medulla (usually only located in large thick hairs). Each of these layers is a distinct lineage of epithelial cells. An additional group of epithelial cells forms the sebaceous

gland, which occurs above the bulge region near the apex of the hair follicle. The function of the sebaceous gland is to secrete a lipid mixture called sebum, which acts to lubricate the infundibulum (the upper portion of the hair follicle, beginning above the sebaceous gland) as well as the emerging shaft of hair. The bulge contains stem cells that differentiate to form the hair follicle and the epidermis (Fig. 1).

In the mouse, hair growth occurs in a distinctive cycle which consists of three stages: anagen, catagen and telogen (Figs. 2 and 3). Anagen is the stage of the hair-growth cycle when hair is actively growing. In most hair follicle types, the lower part of the hair follicle extends downward, and epithelial cells begin to differentiate to form the inner root sheath and the hair shaft. The length of this phase determines the length of the hair. There are six sub stages of anagen: anagen I – anagen VI, with anagen III being divided into anagen IIIa – IIIc. Catagen is the regression phase of the hair-growth cycle. During this stage of hair growth, the proximal end of the hair shaft keratinizes and a club-shaped structure is formed. This is followed by cellular apoptosis, with the base of the follicle and dermal papilla moving upward. There are eight sub stages of catagen: catagen I – catagen VIII. Telogen is the resting phase of the hair-growth cycle. It is during this stage that the club hair is usually shed. Each of these stages and phases contain distinctive features that can be determined histologically (Müller-Röver et al., 2001), allowing a researcher to exactly pinpoint when alterations in hair growth occur, and helping him determine what may be causing the changes.

The complete hair-growth cycle takes approximately four weeks in newborn mice, or in mice where the hairs have been plucked, however, the cycle is longer in adult mice due to longer stages of anagen and telogen (Chase et al., 1951; Müller-Röver et al., 2001). Many factors affect the hair-growth cycle, such as numerous growth factors, cytokines, hormones, neuropeptides and pharmaceutical products (Paus and Cotsarelis, 1999; Stenn and Paus, 1999; Stenn and Paus, 2001; Stenn et al., 1996), and there are many well studied mouse models that show how alterations in these factors affect the coat of the mouse. One example, a mouse mutation called *Angora*, is an autosomal recessive mutation caused by a deletion of approximately 2 kb in the *fibroblast growth factor 5* (*Fgf5*) gene. This mutation results in abnormally long hair due to a prolongation of the anagen phase of the hair cycle of approximately 3 days (Sundberg et al., 1997). Another example, a mouse mutation called *Asebia*, is the result of a mutation in the *stearoyl-CoA desaturase* (*Scd1*) gene. The mutation of this very important sebaceous gland gene results in perifollicular inflammation, sebaceous gland destruction, hair shaft granuloma and cicatricial follicle drop-out (Stenn, 2001). The last example I will give, a group of mouse mutations called *Hairless*, are the result of mutations in the *hairless* (*Hr*) gene. These mutations result in progressive irreversible hair loss soon after birth, wrinkled skin and often a shorter life span (Cochon-Gonzalez et al., 1994).

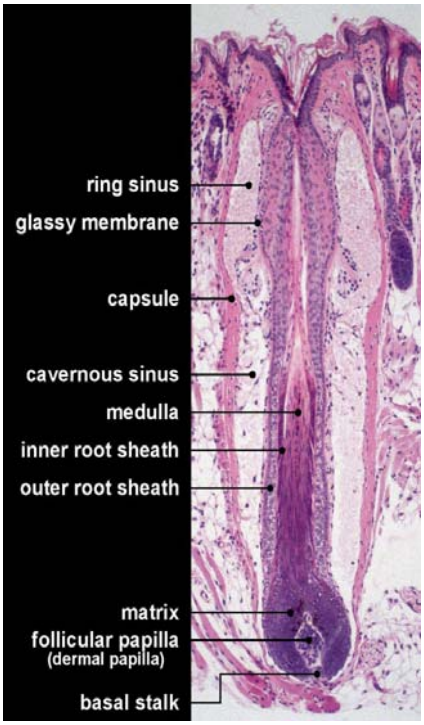

Figure 1: The mouse hair follicle. Shown are the structures of the mature hair follicle. [Used with permission from John Sundberg and the Iowa State University Press (Sundberg and King, 2000).]

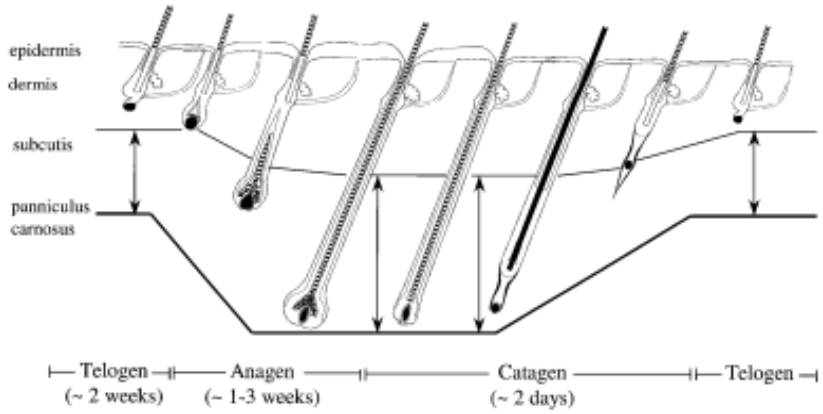

Figure 2: Schematic representation of the increasing and decreasing length of the hair follicle during the hair-growth cycle. Arrows between panniculus carnosus and the border dermis/subcutis indicate the hair cycle-associated changes in the thickness of the subcutis. The approximate duration of each phase is indicated in parentheses. Note changes of the dermal papilla shape and size throughout the cycle as well as the increasing size of the sebaceous gland during anagen IV – VI. [Used with permission from Ralf Paus, Department of Dermatology, Universitätsklinikum Hamburg-Eppendorf, Martinsried, Germany (Müller-Röver et al., 2001).]

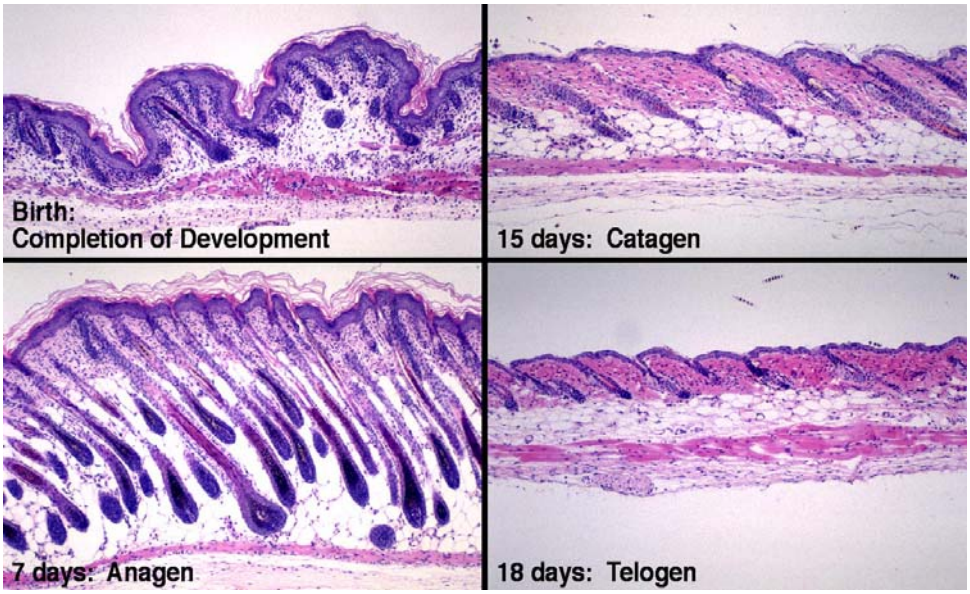

Figure 3: The mouse hair cycle. At birth, the hair follicles are completing their development. By 7 days of age the hair follicles are in the middle of anagen. By 15 days of age the hair follicles are undergoing catagen. By 18 days of age the hair follicles are undergoing telogen. Hair follicles proceed through the stages of anagen (growth), catagen (regression), and telogen (resting) throughout life. [Used with permission from John Sundberg and the Iowa State University Press (Sundberg and King, 2000).]

Background

Furloss

Furloss (*Frl*) is a mouse mutation in which the mutant mice develop a full coat of fur, but lose their hair irreversibly following three to five hair-growth cycles. Mice also develop corneal opacities at approximately six months of age (Fig. 4). The first mutation arose during a specific locus test (Russell, 1951) in the DFiOD stock on March 20, 1963 in the fourth litter of an X-irradiated male at ORNL. The mutation was originally given the name of "*4DFiOD*," and is most likely radiation induced. The second mutation originated spontaneously in the 19DFiOD stock on February 28, 1965 at ORNL and was originally given the name of "*Poor Fur*." The third mutation arose spontaneously in the 17ZB stock and was born on February 25, 1967 at ORNL. In 1971 it was separated from the 17ZB stock and officially given the name "*Furloss*" (Raymer, 1971). All three of the mutations are dominant and autosomal, and are currently congenic on the BALB/cRl background (BALB/cRl is an Oak Ridge subline of the standard inbred strain BALB/c).

In the early 1980's the *Furloss*, *4DFiOD* and *Poor Fur* mutations were tested for allelism by Kathren Stelzner (Stelzner, 1983). She performed allelism testing by producing animals doubly heterozygous for two of the three mutations, backcrossing

Figure 4: Photo of an adult heterozygous *Frl*^{c} mouse exhibiting hairloss, excessive wrinkling and thickening of the skin, and corneal opacity of the eye.

them to wild-type mice and scoring the offspring for loss of fur. If the mutations are allelic (or very tightly linked), no wild-type animals (crossovers) are expected in the backcross. If the mutations are unlinked, approximately three quarters of the offspring should have a furloss phenotype and one quarter of the offspring should have a wild-type phenotype. If the mutations are linked (close on the same chromosome, but not the same gene), there should be a slightly to significantly higher percentage of furloss animals as compared to the wild type (Stelzner, 1983).

In the test cross of *Furloss* and *4DFiOD*, 416 offspring were produced, all of them with furloss (no crossover occurred), indicating that the two mutations were very closely linked or allelic. In the test between *Furloss* and *Poor Fur*, 139 offspring were produced, all of them with furloss, indicating that the two mutations were very closely linked or allelic. In the test between *4DFiOD* and *Poor Fur*, 391 offspring were produced, all of them with furloss, indicating that the two mutations were very closely linked or allelic. With the confirmation that the three mutations were likely allelic, Stelzner renamed the mutations: *Frl*^{a} (*Furloss*), *Frl*^{b} (*4DFiOD*) and *Frl*^{c} (*Poor Fur*) (Stelzner, 1983).

The heterozygous phenotype of all three *Furloss* alleles is nearly identical. Mutant mice appear normal at birth. At eight days of age, there is a distinct difference

between mutant mice and their wild-type littermates due to the sparse appearance of the mutant fur and slightly wrinkled skin. The mice develop a full coat of fur by day 15, which is slightly thinner than that of a wild-type mouse. By day 20 the mice begin to lose their fur in patches, first on the forehead, followed by the back and the abdomen. Regrowth of the fur occurs by day 25 and is followed by a second shedding cycle at approximately 35 days of age. Some regrowth of fur occurs, resulting in a sparse coat by 8 weeks. The final shedding cycle begins a few days later, with a slowly increasing loss of fur until the mouse is completely hairless by 12 to 14 weeks of age, with the exception of the muzzle. The vibrissae are unaffected by this process (Raymer, 1971; Stelzner, 1983). In addition to the loss of fur, mutant mice exhibit thickened, scaly, slightly wrinkled skin, and most mice develop corneal opacities by six months of age.

The loss of fur correlates with the mouse hair cycle. At 15 days of age the hair follicles are undergoing their first catagen, which would result in the slightly thinner coat that is seen in mutant mice. By day 20, the first telogen has commenced, resulting in the random loss of fur. Around day 28, anagen begins correlating with the regrowth of fur, and catagen occurs by day 42, close to the time that the second shedding occurs. Following this time period, the hair cycle is no longer synchronized, and it becomes difficult to correlate hair loss with a specific stage (Müller-Röver et al., 2001).

Heterozygous *Furloss* mice appear to be slightly smaller than their wild-type litter mates from about 16 days of age. At weaning they were found to weigh an average of five percent less than normal litter mates, and as adults they weigh an average of ten percent less than controls (Stelzner, 1983). Mature heterozygotes are vigorous and fertile.

Homozygous animals are difficult to produce, and for Frl^a and Frl^b there is considerable lethality prior to the time at which animals can be test mated; lethality often occurs before birth. Homozygotes have a phenotype identical to that of the heterozygotes and are fertile, but their viability is poor to fair (Stelzner, 1983).

Other similar mutations

Literature research prior to the molecular characterization of these *Frl* mutations revealed two other classical mouse mutations that are phenotypically very similar to *Frl*: *Bareskin* (*Bsk*) and *Rex denuded* (Re^{den}).

Bareskin is an *N*-ethyl-*N*-nitrosourea (ENU)-induced mutation first published in 1984 (Lyon and Glenister, 1984). The mutation is inherited dominantly and is autosomal, and it is maintained at The Jackson Laboratory on the C57BL/6J background. It has heterozygous and homozygous phenotypes identical to the description of *Frl*, but unlike *Frl*, *Bsk* homozygotes are described as being vigorous and fertile (Fig. 5).

Rex denuded is a spontaneous mutation first published as *Denuded* (*Den*) in 1968 (Snell and Bunker, 1968). It also has a dominant mode of inheritance and is autosomal, and is maintained at The Jackson Laboratory on the C57BL/6By background. In 1986, results were published for a backcross between *Den* and a wavy-hair mutation called *Rex* (*Re*), which is located on mouse chromosome 11. The authors stated that out of 504 offspring produced from the backcross, no crossover occurred between the two

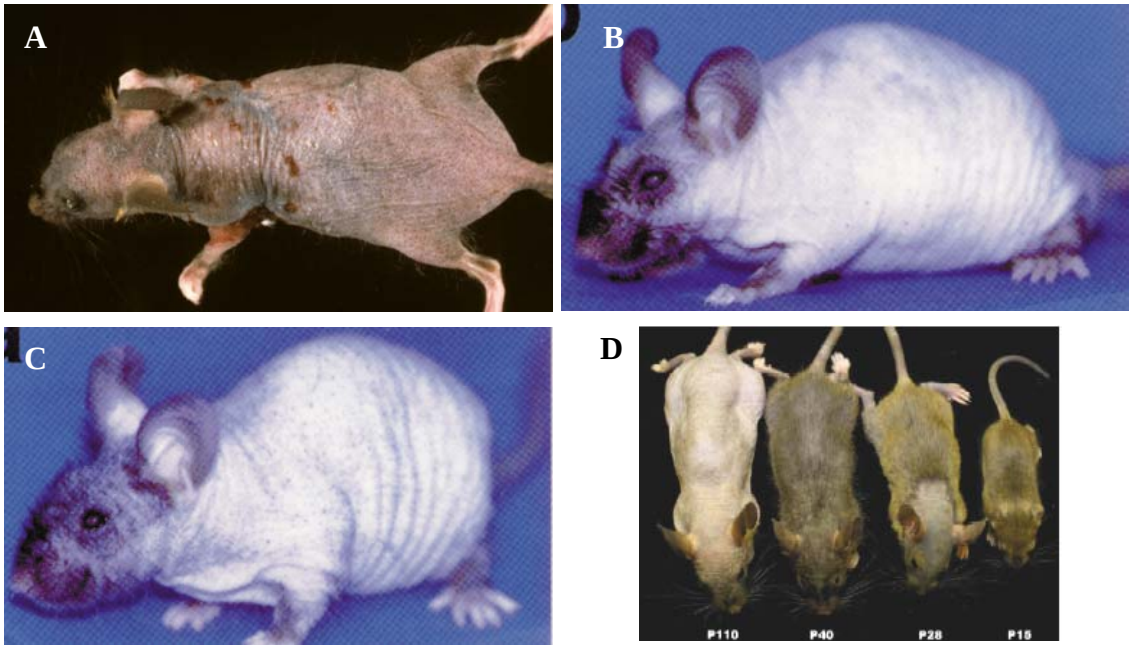

Figure 5: Four lines of mutant mice with modes of inheritance and phenotypes similar to *Frl*. (A) *Bsk*, (generously supplied by John Sundberg, The Jackson Laboratory, Bar Harbor, ME); (B) Re^{den} [used with permission from Toshihiko Shiroishi, National Institute of Genetics, Shizuoka-ken, Japan (Sato et al., 1998)]; (C) *Rim3* [used with permission from Toshihiko Shiroishi, National Institute of Genetics, Shizuoka-ken, Japan (Sato et al., 1998)]; (D) *Rco2* at different age intervals [used with permission from M. Augustin, Ingenium Pharmaceuticals AG, Martinsried, Germany (Porter et al., 2002)]. (Not pictured: *Dfl* and *Fgn*.)

mutations, and they renamed *Den* as an allele of *Re* (Eicher and Varnum, 1986). Like *Bsk*, Re^{den} has heterozygous and homozygous phenotypes identical to *Frl*, with homozygotes described as being "healthy and virile" (Snell and Bunker, 1968) (Fig. 5). In 1986 and 1987, linkage testing results were published for *Bsk* and *Re* (Lyon and Zenthon, 1986; Lyon and Zenthon, 1987). The findings showed that while *Bsk* and *Re* were very tightly linked, they were not mutations in the same gene (one crossover animal that was phenotypically wild type was produced in a backcross of 811 animals). With this in mind, the authors stated that there was a very good possibility that *Den* also recombines with *Re* at a very low frequency, and that *Den* is as likely to be an allele of *Bsk* as of *Re*, especially considering the closer similarity of the mutant phenotypes.

Prior to and during the course of the present investigation of the three *Frl* alleles, several more highly similar mutations arose: *Recombination induced mutation 3* (*Rim3*), *Defolliculated* (*Dfl*), *Finnegan* (*Fgn*) and *Reduced Coat 2* (*Rco2*).

Recombination induced mutation 3 occurred spontaneously in an intra-MHC (major histocompatability complex) recombinant strain in 1994 and was first published in 1998 (Shiroishi et al., 1991; Sato et al., 1998). It is a dominant autosomal mutation with

heterozygous and homozygous phenotypes being identical to that of *Frl*, and is currently maintained at the National Institute of Genetics on the C57BL/10 background (Fig. 5).

Defolliculated is a spontaneous mutation that occurred in a BALB/c colony and was first published in 2002 (Porter et al., 2002). It is a dominant autosomal mutation that is phenotypically identical to *Frl*. On the BALB/c background *Dfl* animals reproduced very poorly and homozygous animals were difficult to produce, so the strain was made congenic on C57BL/6. On the new background, homozygous animals are said to be viable (Porter et al., 2002). This information leads us to believe that the reason there is so much difficulty producing homozygous animals in the *Frl* stocks is due to their BALB/c genetic background, and not, as Stelzner suggested (1983), due to preterm homozygous lethality.

Reduced Coat 2 is an ENU-induced mutation that occurred in the course of the ENU-Mouse Mutagenesis Screen Project within the German Human Genome Project (DHGP), and is on a C3HeB/FeJ background (Runkel et al., 2004). It is an autosomal dominant mutation with heterozygous and homozygous phenotypes identical to *Frl* (Fig. 5).

Finnegan is also an ENU-induced mutation that arose at Harwell and was published in 2005 (Lunny et al., 2005). It is on a BALB/cOlaHsd X C3HeH background and is a dominant autosomal mutation with a heterozygous phenotype identical to that of *Frl*.

Phenotyping and molecular characterization of Bareskin, Rex denuded, Recombination induced mutation 3, Reduced coat 2, Defolliculated and Finnegan

Extensive studies have been performed by others on all of the mutations that are phenotypically similar to *Frl*. In 1994, John Sundberg published the histological phenotype of *Bsk* skin in his book *Handbook of Mouse Mutations with Skin and Hair Abnormalities: Animal Models and Biomedical Tools* (Sundberg, 1994). In it he showed that the cycle of the *Bsk* loss of fur was identical to that of *Frl*. He found that microscopically in 10-month-old male mice the epidermis was fairly acanthotic, with hair follicles essentially being absent. He saw linear tracks of melanocytes angled toward the epidermis near arrector pili muscles, which indicated remnants of hair follicles. He also found that in the areas where hair remained (the muzzle, eyelids and distal limbs), the infundibulum above the level of the sebaceous gland was filled with cornified debris, and sebaceous glands consisted of only rudimentary buds. Cells in the sebaceous glands were undergoing abnormal cornification rather than sebaceous differentiation.

When *Rim3* was introduced in 1998, the authors published histological analysis of the skin and eye phenotypes of not only *Rim3*, but also of Re^{den}. The skin phenotypes of these two mutants mirror that of *Bsk*. The authors found that the eye "exhibited hyperkeratosis, acanthosis, hypergranulosis in the epithelial layer, contraction of collagen fibers, infiltration of inflammatory cells in the stromal layer and severe granulation at the subepithelial portion," with homozygous animals showing signs of corneal lesions earlier than heterozygous ones (Sato et al., 1998). These two mutants were found to be indistinguishable. It was determined that the mutant phenotypes become histologically

evident in the skin at one week of age due to slight epithelial thickening, and in the eye at three months of age due to the flattening of epithelial cells in the center of the cornea.

In addition to the histological data, fine mapping of *Rim3* was also presented (Sato et al., 1998). Two large-scale intersubspecific backcrosses were performed in which 2212 progeny were obtained. Haplotype analysis of 15 genes and microsatellite markers was performed on these offspring, and genetic distances between the genes and the marker loci were calculated. The results of this mapping placed *Rim3* on mouse Chromosome (Chr) 11 in a region syntenic with human chromosome 17q12, centromeric to genes such as *Retinioc acid receptor alpha* (*Rarα*), the Keratin type I complex, *Granulin* (*Grn*), and *Junctional plakoglobin* (*Jup*), all of which regulate the growth and differentiation of epithelial cells (Fig. 6). While they did not map Re^{den}, the authors proposed that *Rim3* and Re^{den} are allelic. Based on these mapping data, and on my observation that the phenotype of *Frl* mice appears to be identical to that of *Rim3* and Re^{den}, I performed linkage analysis with *Frl* and *Re*, which placed *Frl* in this same region of mouse Chr 11 (Chapter 2). These data suggested that *Frl*, *Rim3* and Re^{den} may all be allelic.

The critical interval for the location of the *Rim3* mutation was eventually narrowed down (by Sato and colleagues in the laboratory of T. Shiroishi at the National Institute of Genetics in Japan, presented in a poster at the 16th International Mouse Genome Conference) to a 200 kb region between microsatellite marker *D11Mit14* and *Growth factor receptor-bound protein 7* (*Grb7*), which contained, at that time, approximately seven known genes (unpublished data, Tanaka, 2002). Following the initial mapping of *Rim3* on mouse Chr 11 by Sato et al. (1998), Saeki et al. (2000) cloned several new genes in this region in the search for the *Rim3* candidate, including a gene they named *Gasdermin* (*Gsdm*). The gene was named *Gasdermin* because of the highly restricted expression to both the upper gastrointestinal tract (specifically the esophagus and stomach) and the skin. This gene covers an area of approximately 10 kb in genomic DNA, which spans 12 exons with the nucleotide sequence of the cDNA being 2730 basepairs (bp). The gene encodes a protein consisting of 446 amino acids with a leucine zipper motif near the center (leucine zippers are often implicated in DNA binding) (Fig.

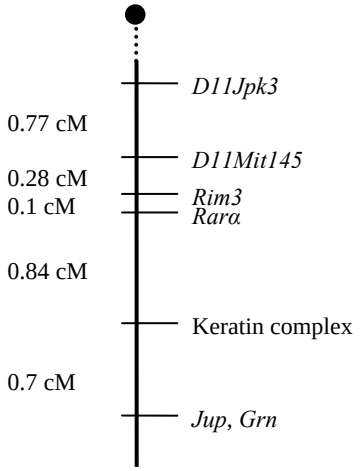

Figure 6: Map position of *Rim3* on mouse Chromosome 11. This map of Chr 11 is a representation of the haplotype analysis of *Rim3* by Sato et al. (1998). One cM is defined as the distance between two loci that recombine with a frequency of 1% and is approximately 2000 kb (Silver, 1995). The mutations *Bsk* and Re^{den} were previously mapped to this same region.

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GAGTCATTCCCTCACCCTATAGACAGAACGAGCTCTGGTTCCTCACCACACACCAGCTGCCAGACAGGATCC     72
AGCATCACCCTTCCCTGAGACAATGACTATGTTTGAGAATGTCACCCGGGCCCTGGCTAGACAGCTGAACCC    144
                        M  T  M  F  E  N  V  T  R  A  L  A  R  Q  L  N  P     17

TCGAGGGGATCTGACACCCCTAGACAGCCTCATCGACTTCAAACGCTTCCATCCCTTCTGCCTGGTGCTGAG    216
  R  G  D  L  T  P  L  D  S  L  I  D  F  K  R  F  H  P  F  C  L  V  L  R     41

GAAGAGGAAGAGCACACTGTTCTGGGGAGCCCGCTATGTGCACACCGACTACACTCTCCTGGATGTGCTGGA    288
  K  R  K  S  T  L  F  W  G  A  R  Y  V  H  T  D  Y  T  L  L  D  V  L  E     65

GCCGGGCAGCTCCCCCTCAGATCCGACAGACAGTGGCAACTTTAGCTTTAAGAATATGCTGGATGCTCGAGT    360
  P  G  S  S  P  S  D  P  T  D  S  G  N  F  S  F  K  N  M  L  D  A  R  V     89

AGAGGGAGATGTGGATGTGCCAAAGACAGTGAAGGTAAAGGGGACTGCGGGTCTGTCACGGAGCAGCACACT    432
  E  G  D  V  D  V  P  K  T  V  K  V  K  G  T  A  G  L  S  R  S  S  T  L    113

GGAGGTGCAGACGCTCAGCGTGGCTCCCACGGCTCTGGAGAACTTGCACAAGGAGAGGAAACTGTCAGCAGA    504
  E  V  Q  T  L  S  V  A  P  T  A  L  E  N  L  H  K  E  R  K  L  S  A  D    137

CCACCCATTCCTGAAGGAGATGCGGGAACGCGGGGAGAACCTCTATGTGGTGATGGAGGTGGTGGAAACCCT    576
  H  P  F  L  K  E  M  R  E  R  G  E  N  L  Y  V  V  M  E  V  V  E  T  L    161

ACAGGAAGTCACTCTGGAGCGAGCCGGCAAGGCAGAGGGCTGCTTCTCTCTCCCCTTCTTTGCCCCACTGGG    648
  Q  E  V  T  L  E  R  A  G  K  A  E  G  C  F  S  L  P  F  F  A  P  L  G    185

ACTACAGGGATCCGTGAACCACAAGGAGGCTGTAACCATCCCCAAGGGCTGTGTTCTGGCCTATCGAGTGAG    720
  L  Q  G  S  V  N  H  K  E  A  V  T  I  P  K  G  C  V  L  A  Y  R  V  R    209

ACAACTGATGGTCAACGGCAAAGATGAGTGGGGCATTCCACACATTTGCAATGACAGCATGCAAACCTTCCC    792
  Q  L  M  V  N  G  K  D  E  W  G  I  P  H  I  C  N  D  S  M  Q  T  F  P    233

TCCTGGAGAAAAGCCAGGAGAAGGGAAGTTCATATTGATCCAGGCATCTGATGTTGGGGAGATGCACGAAGA    864
  P  G  E  K  P  G  E  G  K  F  I  L  I  Q  A  S  D  V  G  E  M  H  E  D    257

CTTCAAGACATTAAAGGAAGAGGTTCAGCGAGAGACTCAGGAAGTGGAGAAGTTAAGTCCAGTGGGGCGAAG    936
  F  K  T  L  K  E  E  V  Q  R  E  T  Q  E  V  E  K  L  S  P  V  G  R  S    281

CTCACTACTCACTTCCCTCAGCCATCTCCTAGGAAAGAAGAAAGAGCTCCAGGACCTTGAGCAGACGCTTGA   1008
  S  L  L  T  S  L  S  H  L  L  G  K  K  K  E  L  Q  D  L  E  Q  T  L  E    305

AGGGGCTCTAGACAAGGGACACGAAGTGACCCTGGAAGCACTCCCCAAAGATGTCCTGCTGTCAAAGGACGC   1080
  G  A  L  D  K  G  H  E  V  T  L  E  A  L  P  K  D  V  L  L  S  K  D  A    329

TATGGACGCCATCCTTTACTTCCTCGGGGCTCTGACAGTGCTAAGTGAAGCCCAACAGAAGCTTCTAGTAAA   1152
  M  D  A  I  L  Y  F  L  G  A  L  T  V  L  S  E  A  Q  Q  K  L  L  V  K    353

ATCCTTGGAGAAAAAGATCCTACCGGTGCAACTGAAGCTGGTTGAAAGCACCATGGAGAAGAACTTCCTGCA   1224
  S  L  E  K  K  I  L  P  V  Q  L  K  L  V  E  S  T  M  E  K  N  F  L  Q    377

AGATAAAGAGGGTGTTTTCCCCCTGCAACCTGATCTGCTCTCCTCCCTCGGGGAGGAGGAACTGATCCTAAC   1296
  D  K  E  G  V  F  P  L  Q  P  D  L  L  S  S  L  G  E  E  E  L  I  L  T    401

AGAAGCACTGGTGGGACTAAGCGGCCTGGAAGTCCAGAGATCAGGCCCCCAGTACACGTGGGATCCGGACAC   1368
  E  A  L  V  G  L  S  G  L  E  V  Q  R  S  G  P  Q  Y  T  W  D  P  D  T    425

GCTCCCCCACCTTTGTGCCCTCTATGCTGGCCTCTCCCTCCTTCAACTGCTAAGCAAGAATTCCTAATGCAC   1440
  L  P  H  L  C  A  L  Y  A  G  L  S  L  L  Q  L  L  S  K  N  S  *         446

CTTCTTTGCCTGCTGCCCTAAAGCCTTCCCAGCCTTACTGTGCTCCATCTGTAACACTGCAAGACACTACAG   1512
AGCCTCCAGGCTGAGGACAACTGAAATGCCAGCTCAAAAATCAGTCTCCAAGTTCCTTCTGCCTCACCATCT   1584
ATCTCTCTCCTTCTGTCCTCCAGCTCAGCACCCACTAAGCCCATCTCCTGATGCTTAAATCCTGAAGATACA   1656
GAATCATTCAAACCCTTACTACTTTGAGTCACTTCATTAAGAGGGGTTGGGGGGAGGCTTGGGGTGGTGATG   1728
GTGGTGGTTATGGTGTGAGGCAGAGGCAGGTGAATCTCTTAAGTTCGAGGCCAGCCTGGTCTGCAGAGTGAA   1800
CTCCAGGACAGCCAGGGCAACACAGAGAAACCTTGTCTTGAAAACAACAAAACCACATTTATTTCCTAGTCT   1872
GATCCTTCCCATGTGTGCCTCAAAAAAAAAAAAAAAAAAAAAAAAAAAACTGGTCTTACAAGAATGAATGGAG   1944
AATACTATGTCAATCCTAGAAGAAGCTGTCTTCTGGTTTTTCTGTTTAAGGGAATTGTCCCAGGTAAAGAAA   2016
CAGCCATGATCCATACATACACGTTACTGCCCACACTGTTCCTCAGGCCTGCTGGAAAATGCTCCCAGTGCT   2088
CAGAGGGTTAGAATCCAGAACATCTGCTGTACACTGGAAAGATCATCCAAGCTGCCTGACCTCACCAAAGCG   2160
CCCATGTTCTCCAGCCCTGGCTGAGGGCTTGGGAGAGGAAAGGAAGGTCTAGGGGACTAAGATACAAGCAGA   2232
AAACAAGACAATAGATTTCATGTTGATTAAGTAGAAAATGAAGGTGAGGTTCTTTCTCAAAATAAATAAATA   2304
AATAAATAAATAAATTAGAGGAAGAGGGAGGGGGCACCCAAGAATAAGACCTAAACCTTTAAATCAGTGGTT   2376
TGACCTGTGAGTCGCGACCCCGGTGGGAGGTCAGATGACTCACAGGAGTTGCATATCAGATATCCTTCATAT   2448
CAAATATTTACATTATGACTTCTAACAGGAGCAAAATTGGAGCTATGAATTTACAATGAAAATAATTCTATG   2520
GTTGGGGGTCACCACAGCATGAGGAACTGGATCAAAGGGTCGCAGTGTTAGGAAGGCTGAGAGCCGCTGCTT   2592
CAGTTTTGTTTTCAACCCTCAACTCAGGATGAGTGTGGATGAGCTTGGCTTGTATATTATAATCTCATTAAA   2664
CATCTGTATATTATAATCTCATTAAACGTCTGGGATCTCATCACCCTCTGCCCAACGCTGGCTTTG         2730
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Figure 7: mRNA and protein sequence of mouse *Gsdm* (NM_021347). The *Gsdm* nucleotide sequence is 2730 bp in length. There are 446 amino acids contained in the protein. The leucine zipper motif [L(6)L(6)L(6)L] is shaded gray.

7). In addition, the human homolog, *GSDM*, shares a high sequence similarity with that of the mouse.

Only one *Gsdm* gene was published or mentioned by Sato et al. (1998). However, during my investigation of the *Rim3* critical interval for candidates for the *Frl* gene, I discovered that *Gsdm* is one of three closely related genes that occur in a tandem gene cluster on Chr 11. I performed a BLAT search of the *Gsdm* published sequence against the mouse genome in the UCSC (University of California Santa Cruz) Genome Browser (http://genome.ucsc.edu) and discovered that not only did it identify *Gsdm*, but that there appeared to be two more genes with approximately 98% identity that are in a cluster and centromeric to *Gsdm*. At the time, these other genes were completely unknown (Fig. 8). This *Gsdm*-gene cluster spans approximately one-quarter of the *Rim3* critical interval, making these genes excellent candidates for *Frl*. To rule out the possibility that this cluster was an artifact generated in the construction of this region of the genomic sequence, I generated several genomic DNA probes and performed Southern blot analysis of this region. I determined that there are, in fact, three *Gsdm* genes in this region, although there were no alterations in the genomic structure of the *Frl* mutants at the level of Southern blot analysis (Chapter 2). Based on the published sequence of the original *Gsdm* gene and on the highly conserved portions of the other two genes identified by the BLAT search, I began sequencing these genes in *Frl* mutant mice and wild-type controls (Chapter 2).

The publication of *Dfl* included in-depth diagnostic histopathology of the mutant phenotype and the mutation was mapped by means of haplotype analysis to the same region of Chr 11 as *Bsk*, *Re*^{den} and *Rim3* (Porter et al., 2002). The authors found "abnormal differentiation of sebaceous glands with little or no sebum" produced, along with some cornification. They also found decreased lipid production in the preputial gland and Meibomian gland of the inner eyelid. Because the function of the Meibomian

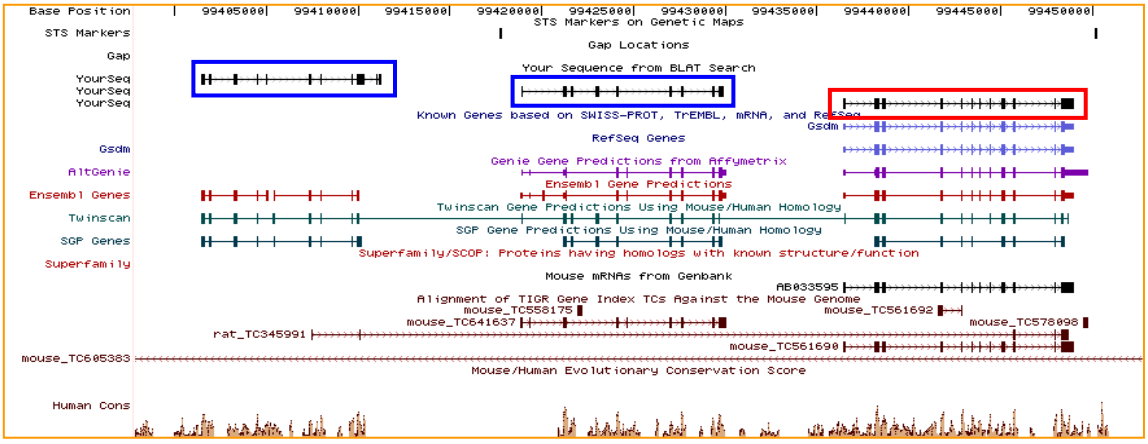

Figure 8: *Gsdm* triplet on mouse Chromosome 11. Boxed in red is the original *Gsdm1* gene. Centromeric to *Gsdm1* are two unknown genes with 98% identity (boxed in blue). (Taken from Mouse February 2002 Assembly of the UCSC database.)

gland is to prevent evaporation of tears that cover the surface of the eye, they suggested that "the reduced lipid production in the cells of this gland is the likely cause of the corneal opacities in the older mice". The acanthotic and hyperkeratotic epidermis that was previously described by Sundberg (1994) for *Bsk* and by Sato et al. (1998) for Re^{den} and *Rim3* was attributed to increased proliferation of the skin, as evidenced by increased staining in the skin of the proliferation marker, Ki67. Additionally, the *Dfl* mutants were found to have a defect in catagen, in which their hair follicles fail to regress, resulting in abnormally long follicles in the second hair cycle. The phenotypic characterization of *Bsk*, Re^{den} *Rim3* and *Dfl* aided in my own histological analysis of the *Frl* phenotype, which appears to be nearly identical to these other mutants (Chapter 2). Together, these observations reinforced the possibility that all of these mutations may be allelic.

In May of 2004, Katoh and Katoh published a paper that gave credence to the tandem cluster of three related *Gsdm* genes. Based solely on a bioinformatics approach, they submitted a proposed sequence for both of the related *Gsdm* genes and called them *Gsdm-like 1* (*Gsdml1*) and *Gsdm-like 2* (*Gsdml2*). The order of the genes in the cluster from centromere to telomere is *Gsdml1-Gsdml2-Gsdm*. The current nomenclature for these three genes is *Gsdm3 – Gsdm2 – Gsdm1* (see below). The *Gsdm3* (*Gsdml1*) gene consists of 12 exons spanning approximately 10 kb. The cDNA is 1523 bp and encodes a protein with 456 amino acids (Fig. 9). The *Gsdm2* (*Gsdml2*) gene consists of 10 exons spanning approximately 10 kb. The cDNA is 1494 bp and encodes a protein with 443 amino acids (Fig. 10). Both genes also contain the leucine zipper motif observed in *Gsdm1*.

I performed a BLAT search with the cDNA sequences of *Gsdml1* and *Gsdml2* in order to define their exon-intron boundaries. I used these data to design PCR primers in genomic DNA that would uniquely amplify all the exons in these two genes. I then continued my ongoing DNA sequence analysis of both genes in all three *Frl* alleles and determined that all three alleles are point mutations in the *Gsdml1* gene (Chapter 2). I also obtained genomic DNA of *Bsk* mutant mice from Dr. John Sundberg at The Jackson Laboratory and determined that, like the three *Frl* alleles, *Bsk* is also the result of a point mutation in the *Gsdml1* gene (now called the *Gsdm3* gene, see below) (Chapter 2).

In November of the same year (2004), one month after I determined that *Frl* and *Bsk* are both mutations in the *Gsdm3* (*Gsdml1*) gene, the *Rco2* phenotype, as well as the *Rco2*, *Bsk* and Re^{den} mutations were published by Runkel et al. (2004). *Rco2* was finely mapped to the same area of Chr 11 as all of the previously mentioned mutations by means of positional cloning. The authors sequenced their entire critical interval for the location of the mutation. They found the *Rco2* mutation in a previously unknown gene and named it *Gsdm3* (=*Gsdml1*). They also identified the other related *Gsdm* gene in the cluster and named it *Gsdm2* (=*Gsdml2*). The *Gsdm3* mutation in *Rco2* is a result of nucleotide position 1110 being changed from T to C, causing a codon change from CTC to CCC in exon 10, which results in changing leucine 343 to proline. In addition to sequencing the mutation for *Rco2*, the authors sequenced the *Gsdm3* gene in *Bsk*, Re^{den} and Re^{wc} (*Rex wavy coat*, another allele of *Re* with a wavy-hair phenotype more similar to

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CAACCTATAGACCGAAAGAGCTCTGATTTCTCACCAAACACCTGCTGCTGAACAGGACCTAGCATCACCCTT     72
CCCTGAGACAATGCCTGTGTTTGAGGATGTCACCCGGGCCCTGGTTAGAGAGCTGAACCCTCGAGGGGATCT    144
          M  P  V  F  E  D  V  T  R  A  L  V  R  E  L  N  P  R  G  D  L     21

GACACCCCTAGACAGCCTCATCGACTTCAAACACTTTCGTCCCTTCTGCCTGGTGCTGAGGAAGAGGAAGAG    216
  T  P  L  D  S  L  I  D  F  K  H  F  R  P  F  C  L  V  L  R  K  R  K  S     45

CACATTGTTCTGGGGAGCCCGCTATGTGCGCACCGACTACACTCTCCTGGATTTGCTGGAGCCGGGCAGCTC    288
  T  L  F  W  G  A  R  Y  V  R  T  D  Y  T  L  L  D  L  L  E  P  G  S  S     69

CCCCTCAGATCTGACAGACAGTGGCAACTTTAGCTTTAAGAATATGCTGGATGTCCAAGTACAGGGACTTGT    360
  P  S  D  L  T  D  S  G  N  F  S  F  K  N  M  L  D  V  Q  V  Q  G  L  V     93

GGAAGTGCCAAAGACAGTGAAGGTAAAGGGGACTGCGGGTCTGTCACAAAGCAGCACACTGGAGGTGCAGAC    432
  E  V  P  K  T  V  K  V  K  G  T  A  G  L  S  Q  S  S  T  L  E  V  Q  T    117

ACTCAGCGTGGCTCCCTCGGCTCTGGAGAACTTGAAGAAGGAGAGGAAACTGTCAGCAGACCACTCGTTCCT    504
  L  S  V  A  P  S  A  L  E  N  L  K  K  E  R  K  L  S  A  D  H  S  F  L    141

GAACGAGATGAGGTATCATGAGAAGAACCTGTATGTGGTGATGGAGGCAGTAGAAGCCAAGCAGGAAGTTAC    576
  N  E  M  R  Y  H  E  K  N  L  Y  V  V  M  E  A  V  E  A  K  Q  E  V  T    165

TGTGGAGCAAACTGGCAACGCAAATGCCATCTTCTCTCTCCCCAGCTTGGCTCTACTGGGACTACAGGGATC    648
  V  E  Q  T  G  N  A  N  A  I  F  S  L  P  S  L  A  L  L  G  L  Q  G  S    189

CTTGAACAACAACAAGGCTGTAACCATCCCCAAGGGCTGTGTCCTGGCCTATCGAGTGAGACTACTGAGAGT    720
  L  N  N  N  K  A  V  T  I  P  K  G  C  V  L  A  Y  R  V  R  L  L  R  V    213

CTTTTTGTTCAATCTTTGGGATATTCCGTACATTTGCAATGACAGCATGCAAACCTTCCCTAAGATCAGGCG    792
  F  L  F  N  L  W  D  I  P  Y  I  C  N  D  S  M  Q  T  F  P  K  I  R  R    237

TGTACCTTGCAGTGCCTTCATATCTCCTACCCAGATGATATCTGAAGAGCCAGAAGAAGAGAAGCTCATTGG    864
  V  P  C  S  A  F  I  S  P  T  Q  M  I  S  E  E  P  E  E  E  K  L  I  G    261

GGAGATGCACGAAGACTTCAAGACATTAAAGGAAGAGGTTCAGCGAGAGACTCAAGAAGTGGAGAAGTTGAG    936
  E  M  H  E  D  F  K  T  L  K  E  E  V  Q  R  E  T  Q  E  V  E  K  L  S    285

TCCAGTGGGTCGAAGCTCCCTACTCACTTCCCTCAGCCATCTCCTAGGAAAGAAGAAAGAGCTCCAGGACCT   1008
  P  V  G  R  S  S  L  L  T  S  L  S  H  L  L  G  K  K  K  E  L  Q  D  L    309

TGAGCAGAAGCTTGAAGGGGCTTTAGACAAGGGTCAGAAAGTGACCCTGGAAGCACTCCCCAAAGATGTCCT   1080
  E  Q  K  L  E  G  A  L  D  K  G  Q  K  V  T  L  E  A  L  P  K  D  V  L    333

GCTGTCAAAGGACGCTATGGATGCCATCCTCTACTTCCTCGGGGCTCTGACAGAGCTAACTGAAGAACAACT   1152
  L  S  K  D  A  M  D  A  I  L  Y  F  L  G  A  L  T  E  L  T  E  E  Q  L    357

GAAGATTCTAGTAAAATCCTTGGAGAAAAAGATCTTACCAGTGCAACTGAAGCTGGTTGAAAGCACCTTGGA   1224
  K  I  L  V  K  S  L  E  K  K  I  L  P  V  Q  L  K  L  V  E  S  T  L  E    381

GCAGAACTTCCTGCAAGATAAAGAGGGTGTTTTCCCCCTGCAACCTGATCTGCTCTCCTCCCTCGGGGAGGA   1296
  Q  N  F  L  Q  D  K  E  G  V  F  P  L  Q  P  D  L  L  S  S  L  G  E  E    405

GGAACTGACCCTAACGGAAGCACTGGTGGGACTAAGCGGCCTGGAAGTCCAGAGATCAGGCCCCCAGTACGC   1368
  E  L  T  L  T  E  A  L  V  G  L  S  G  L  E  V  Q  R  S  G  P  Q  Y  A    429

GTGGGATCCAGACACTCGCCACAACCTTTGTGCCCTCTATGCTGGCCTCTCCCTCCTTCACCTGCTAAGCAG   1440
  W  D  P  D  T  R  H  N  L  C  A  L  Y  A  G  L  S  L  L  H  L  L  S  R    453

GAAATCTAATGCACTTACTTATTGTGCTCTATCTTAACACTCCAAGACACTACAGAGCCTCCAGTCTGAGGA   1512
  K  S  N  A  L  T  Y  C  A  L  S  *                                        464

TAACTGAAATGCCTGCTCATCTTTG                                                   1537
```

Figure 9: mRNA and protein sequence of mouse *Gsdm3* (NM_001007461) (*Gsdml1*). The *Gsdm3* nucleotide sequence is 1537 bp in length. There are 464 amino acids contained in the protein. The leucine zipper motif [L(6)L(6)L(6)L] is shaded gray.

```
AGTCATTCTCTCACCCTATAGACAGAACGAGCTCTGGTTCCTTACCACACATCTGCTGCCAGACAGGACCCA     72
GCATCACCCTTCCCTGAGACAATGTCTATGTTTGAGGATGTCACCCGGGCCCTGGCTAGACAGCTGAACCCT    144
                     M  S  M  F  E  D  V  T  R  A  L  A  R  Q  L  N  P      17

CGAGGGGATCTGACACCCCTAGACAGCCTCATCGACTTCAAACGCTTCCATCCCTTCTGCCTGGTGCTGAGG    216
 R  G  D  L  T  P  L  D  S  L  I  D  F  K  R  F  H  P  F  C  L  V  L  R      41

AAGAGGAAGAGCACACTGTTCTGGGGAGCCCGCTATGTGCGCACCGACTACACTCTCCTGGATGTGCTGGAG    288
 K  R  K  S  T  L  F  W  G  A  R  Y  V  R  T  D  Y  T  L  L  D  V  L  E      65

CCGGGCAGCTCCCCCTCAGATCCAACACTCCTTGGCAATTTTAGCTTTAAGAATATGCTGGATGTCAGAGTA    360
 P  G  S  S  P  S  D  P  T  L  L  G  N  F  S  F  K  N  M  L  D  V  R  V      89

GAGGGAGATGTGGAGGTGCCAACAATGATGAAGGTAAAGGGGACTGTGGGCCTGTCACAAAGCAGCACGCTG    432
 E  G  D  V  E  V  P  T  M  M  K  V  K  G  T  V  G  L  S  Q  S  S  T  L     113

GAGGTACAGATGCTCAGTGTGGCTCCCACGGCTCTGGAGAACTTGCACATGGAGAGGAAACTGTCAGCAGAC    504
 E  V  Q  M  L  S  V  A  P  T  A  L  E  N  L  H  M  E  R  K  L  S  A  D     137

CACCCGTTCCTGAAGGAGATGCGGGAATACAAGCAAAACCTGTATGTGGTGATGGAGGTGGTGAAAGCCAAG    576
 H  P  F  L  K  E  M  R  E  Y  K  Q  N  L  Y  V  V  M  E  V  V  K  A  K     161

CAGGAAGTCACTCTGAAGCGAGCTAGCAACGCAATTTCCAAATTCTCTCTCAACCTTCCCTCACTGGGACTA    648
 Q  E  V  T  L  K  R  A  S  N  A  I  S  K  F  S  L  N  L  P  S  L  G  L     185

CAGGGATCCGTGAACCACAAGGAGGCTGTAACCATGCCCAAGGGCTGTGTTCTGGCCTATCGAGTGAGACAA    720
 Q  G  S  V  N  H  K  E  A  V  T  I  P  K  G  C  V  L  A  Y  R  V  R  Q     209

CTGATCATCTATGGGAAAGATGAGTGGGGCATTCCATACATTTGTACTGACAACATGCCAACCTTTAACCCC    792
 L  I  I  Y  G  K  D  E  W  G  I  P  Y  I  C  T  D  N  M  P  T  F  N  P     233

CTGTGTGTGCTTCAGAGACAAGGCAGTACTGTCCAGATGATATCTGGGGAGATGCACGAAGACTTCAAGACA    864
 L  C  V  L  Q  R  Q  G  S  T  V  Q  M  I  S  G  E  M  H  E  D  F  K  T     257

TTAAAGAAAGAGGTTCAGCAAGAGACTCAAGAAGTGGAGAAGTTGAGTCCAGTGGGGCGAAGCTCCCTACTC    936
 L  K  K  E  V  Q  Q  E  T  Q  E  V  E  K  L  S  P  V  G  R  S  S  L  L     281

ACTTCCCTCAGCCATCTCCTAGGAAAGAAGAAAGAGCTCCAGGACCTTGAGCAGATGCTTGAAGGGGCTCTA   1008
 T  S  L  S  H  L  L  G  K  K  K  E  L  Q  D  L  E  Q  M  L  E  G  A  L     305

GACAAGGGACACGAAGTGACCCTTGAAGCACTCCCCAAAGATGTCCTGCTGTTAAAGGACGCTATGGACGCC   1080
 D  K  G  H  E  V  T  L  E  A  L  P  K  D  V  L  L  L  K  D  A  M  D  A     329

ATCCTCTACTTCCTCGGGGCTCTGACAGAGCTAAGTGAAGAACAACTGAAGATTCTAGTAAAATCCTTGGAG   1152
 I  L  Y  F  L  G  A  L  T  E  L  S  E  E  Q  L  K  I  L  V  K  S  L  E     353

AACAAGGTCCTACCGGTGCAACTGAAGCTGGTTGAGAGCATGTTGGAACAGAACTTCCTGCAAGATAAAGAG   1224
 N  K  V  L  P  V  Q  L  K  L  V  E  S  I  L  E  Q  N  F  L  Q  D  K  E     377

GATGTTTTCCCCCTGCGACCTGATCTGCTCTCCTCCCTCGGGGAGGAGGACCAGATTCTAACAGAAGCACTG   1296
 D  V  F  P  L  R  P  D  L  L  S  S  L  G  E  E  D  Q  I  L  T  E  A  L     401

GTGGGACTAAGCGGCCTGGAAGTCCAGAGATCAGGGCCCCAGTACACGTGGAATCCAGACACTTGTACAAAC   1368
 V  G  L  S  G  L  E  V  Q  R  S  G  P  Q  Y  T  W  N  P  D  T  C  H  N     425

CTCTGTGCCCTCTATGCTGGCCTCTCCCTCCTTCACCTGCTAAGCAGGGATTCCTAATGCAACTTCTCTGCC   1440
 L  C  A  L  Y  A  G  L  S  L  L  H  L  L  S  R  D  S  *                    456

TGCTGCCCTAAAGCCTTCCCGGCCTTACTGTGCTCTGTCTGAAACACTCCAAGA                      1494
```

Figure 10: mRNA and protein sequence of mouse *Gsdm2* (Katoh and Katoh, 2004, *Gsdml2*). The *Gsdm2* nucleotide sequence is predicted to be 1494 bp in length. There are 456 amino acids predicted in the protein. The leucine zipper motif [L(6)L(6)L(6)L] is shaded gray.

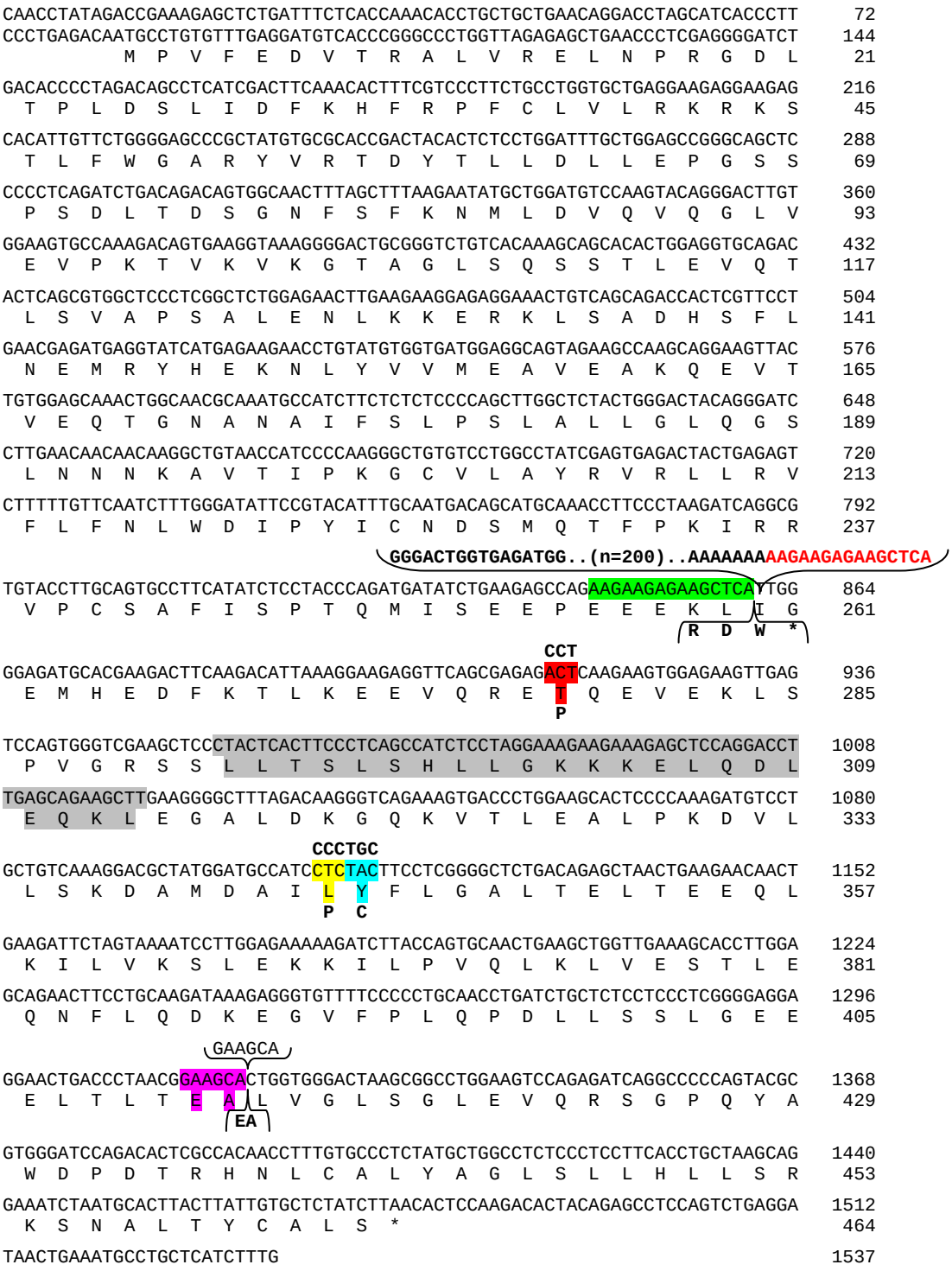
the original allele of *Re*). They found the *Bsk* mutation to be a substitution of nucleotide 1113 (A to G), which changed codon TAC to TGC and tyrosine 344 to cysteine in exon 10. This finding is in agreement with my own sequence analysis of *Bsk* (Chapter 2). The Re^{den} mutation was determined to be a 6 bp insertion (AAGCGG) in exon 12 beginning at nucleotide 1314, which results in a duplication of codons 411 (GAA – glutamic acid) and 412 (GCG – alanine) to produce Glu Ala Glu Ala (EAEA) 411-414 (Fig. 11). However, no *Gsdm3* mutation was found in Re^{wc} mice. These data demonstrate that *Rco2*, *Bsk* and Re^{den} are alleles of *Gsdm3* and suggest that *Re* and Re^{wc} are allelic, but a separate gene that is tightly linked to *Gsdm3*.

The *Rco2* phenotype differs from all the other similar mutations in that their hair loss does not progress in a patchy fashion, but rather, it progresses cranially to caudally (Runkel et al., 2004). Runkel et al. (2004) provide a very detailed description of *Gsdm3* expression, though no difference was found in the expression of *Gsdm3* between mutant and wild-type littermates in mice of various ages. In spite of this fact, there are very distinct differences between the *Rco2* and wild-type hair follicles as there were in the other mutant mice. The authors found that "the main defect in hair follicle morphogenesis caused by the *Rco2* mutation in the *Gsdm3* gene is an abnormal and protracted catagen" (Runkel et al., 2004) as was the case in *Dfl* mice (Porter et al., 2002). Additionally, they saw complete loss of the sebaceous glands by day P24, which may indicate that the main effect of the *Gsdm3* mutation in *Rco2* mice is to abolish the normal development of the sebaceous glands in the skin, "with the phenotypic effects on the hair follicle and epidermis being secondary to the absence of sebaceous glands" (Runkel et al., 2004). Investigation of the Meibomian gland at two months, however, showed normal holocrine secretion, making it unlikely that *Gsdm3* has a direct effect on holocrine gland cells at large, or on sebum production (Runkel et al., 2004).

Although mice have three *Gsdm* genes, rats have only a single *Gsdm* gene. Humans also have one *GSDM* gene that is highly conserved with the orthologous *Gsdm* gene in rats and *Gsdm1* gene in mice. Additionally, humans have an added gasdermin-like gene, identified as *GSDML*, which is only 27% similar at the amino acid level to human *GSDM*. There is no ortholog for human *GSDML* in the rat or mouse. Thus, *Gsdm2* and *Gsdm3* in the mouse most likely arose from duplications of *Gsdm1*, and are restricted to the genus *Mus* (Runkel et al., 2004).

The *Dfl* and *Fgn* mutations were published in 2005 (Lunny et al., 2005). The *Dfl* mutation was also located in the *Gsdm3* gene. It consists of an "insertion of a *Mus musculus* B2 element near the 3' splice site of exon 7" (Lunny et al., 2005). B2 elements are one of the main families of short interspersed nuclear elements (SINE). A SINE is approximately 190-bp long, and is repeated several thousand times throughout the mouse genome (Hasties, 1989). In *Dfl*, the B2 insertion "generates an in-frame stop codon changing the wild-type amino acid sequence encoded by exon 7 from 253-GluProGluGluGluLysLeu**Ile**-260 to 253-GluProGluGluGluLysLeu**ArgAspTrp***-262. Additionally, the insertion results in a 15-bp duplication of nucleotides 846-860 (AAGAAGAGAAGCTCA)" (Lunny et al., 2005). The premature truncation of the protein occurs prior to the location of the leucine zipper motif (Fig. 11). The authors state that based on their research and on the mode of inheritance of the mutation, the phenotype is not likely due to haploinsufficiency, but rather to a dominant-negative effect

Figure 11: mRNA and protein sequence of mouse *Gsdm3* (NM_001007461) and the mutations in the *Rco2*, *Bsk*, Re^{den}, *Dfl* and *Fgn* alleles. The mutations are highlighted, with the codon changes indicated above the nucleotide sequence and amino acid changes indicated below the protein sequence. The *Rco2* mutation (nucleotide T1110C, amino acid L343P) is highlighted in yellow. The *Bsk* mutation (nucleotide A1113G, amino acid Y344C) is highlighted in blue. The Re^{den} mutation (duplication of nucleotide sequence 1314-1319 GAAGCA and amino acids 411-412 EA) is highlighted in purple. The *Dfl* mutation (insertion of a B2 element that causes a premature stop codon and duplicates nucleotides 846-860) is highlighted in green. The *Fgn* mutation (nucleotide A914C, amino acid T278P) is highlighted in red.



of the mutated protein.

The *Fgn* mutation was found to be caused by a point mutation (A914C) in exon 8 of *Gsdm3* which changes threonine 278 to proline (Lunny et al., 2005) (Fig. 11). This particular mutation lies in the region of the protein that the authors contend contains a coiled coil domain, and is expected to create a major change in the protein's final structure.

In addition to finding the *Dfl* and *Fgn* mutations, Lunny et al. (2005) found that *Gsdm* proteins normally reside in the cytoplasm of a cell, but a small amount also co-localizes with the nucleus of cells located in the granular layer of the epidermis. This information supports the idea that the leucine zipper motif contained in the *Gsdm3* gene may indeed be associated with DNA binding. They also state from their observations that *Gsdm* seems to be primarily involved with cells that are about to undergo programmed cell death, and is likely a late differentiation marker.

This brings the total number of published *Gsdm3* mutations to five (*Rco2*, *Bsk*, Re^{den}, *Dfl* and *Fgn*). My impending publication of the two *Frl* alleles (Chapter 2) will make seven. An additional allele of *Gsdm3*, *hairloss*, was presented at the 18th International Mouse Genome Conference (unpublished data, Weber et al., 2004), which makes eight, and it is likely that a forthcoming publication of *Rim3* will make nine. The *Rim3* mutation was identified in the *Gsdm3* gene and presented at the 17th International Mouse Genome Conference, but it has not yet been published (unpublished data, Tanaka, 2003). The reason for the identification of this large allelic series of *Gsdm3* mutations is likely due to the strikingly obvious phenotype and the dominant mode of inheritance.

Chapter 2

Cloning and phenotypic characterization of the mouse *Furloss* locus

Introduction

Based on the similar phenotypes and modes of inheritance of mice carrying the *Bsk*, *Re*^{den}, *Rim3* and *Frl* mutations, I hypothesized that all of these mutations may be allelic. This hypothesis was supported by genetic mapping experiments presented here, which demonstrate that *Frl* maps to the same interval of distal mouse Chr 11 as *Bsk*, *Re*^{den} and *Rim3*. Therefore, based on the nearly identical phenotypes of *Frl* and *Rim3*, and their similar map positions, I initiated a candidate gene search in the 0.38 cM interval of Chr 11 containing *Rim3* (Sato et al., 1998), which is flanked by the marker *D11Mit145* and the gene *Rarα* (Fig. 6). The February 2002 freeze of the mouse genome sequence on the UCSC Genome Browser indicated that these markers flanked 1.26 Mbp of genomic DNA and contained approximately 73 genes. A number of these genes have known roles in the growth and differentiation of epithelial cells, such as *Rarα*, numerous keratin genes, *Grn* and *Jup*, making them excellent candidates for *Frl*. Many additional genes with unknown functions are expressed in the skin, providing additional candidates for *Frl*. As I was considering long-range physical mapping of this region in *Frl* mice, and sequencing candidate genes, investigators in the laboratory of Dr. T. Shiroishi at the National Institute of Genetics in Japan, who published the *Rim3* genetic map, presented a poster at the 16th International Mouse Genome Conference that narrowed the *Rim3* critical interval to a 200-kb region between microsatellite marker *D11Mit14* and *Growth factor receptor-bound protein 7* (*Grb7*). These data allowed me to quickly narrow my candidate gene search to this 200-kb interval that contained eight known and putative genes: *Growth factor receptor-bound Protein 7* (*Grb7*), *Zinc finger protein Aiolos* (*Znfn1a3*), Ensmus 17339.1 (similar to human *zona pellucida binding protein*), *Ormdl-3*, Ensmusest 18305.1 (putative nuclear protein), Ensmusest 19133.1 (similar to human *splicing factor, arginine/serine rich 3*), *Gasdermin* (*Gsdm*) and *Proteasome 26S non-ATP-ase Subunit 3* (*Psmd3*).

During the course of considering each of these eight genes as candidates for *Frl*, I discovered that the *Gsdm* gene may actually be one of a tandem cluster of three nearly identical genes (Fig. 8). The fact that *Gsdm* is expressed in the upper gastrointestinal tract and skin, and the cluster covers one-quarter of the *Rim3* critical interval, makes these genes excellent candidates for *Frl*. Since the three *Gsdm* genes are nearly identical, even in their non-coding regions, I considered the possibility that the *Frl* mutations could have arisen by illegitimate recombination between these genes. To test this possibility, and to verify that there actually are three *Gsdm* genes, Southern blot analysis of this region was performed in *Frl* mice.

Southern blot experiments demonstrated that there are three closely related *Gsdm* genes, but that there are no gross alterations in the *Gsdm* triplet in *Frl* mice. However, these data did not rule out the *Gsdm* genes as candidates for the *Frl* mutations.

Therefore, PCR primers were designed that would amplify every exon in the three *Gsdm* genes. At the time this experiment was initiated, only one *Gsdm* gene was known (*Gsdm*, now *Gsdm1*) and none of the *Gsdm3* mutations had been identified and published. I relied on the DNA sequence of *Gsdm1* in the UCSC Genome Browser to identify the exon-intron boundaries and to design PCR primers for this gene. For the other two genes (I will refer to them by their current names, *Gsdm2* and *Gsdm3*), I relied on the sequence similarity from the BLAT analysis I performed with the *Gsdm1* cDNA sequence (Fig. 7) to design primers to amplify the hypothetical exons for DNA sequence analysis.

As DNA sequence analysis of the three *Gsdm* genes was under way, S. Tanaka and colleagues in the laboratory of T. Shiroishi presented a poster at the 17th International Mouse Genome Conference (Tanaka, 2003) stating that they had identified a tandem cluster of three *Gsdm* genes and that the *Rim3* mutation was in one of the genes of the *Gsdm* triplet, but that it was not in the original *Gsdm1* gene. Shortly after this time, Katoh and Katoh (2004) published the predicted cDNA sequence of the other two genes in the *Gsdm* triplet, now known as *Gsdm2* and *Gsdm3*, which aided me in designing PCR primers for exon sequences that I had not already designed based on the BLAT analysis. Based on the discovery of the *Rim3* mutation by Tanaka et al. (2003) and my hypothesis that the *Rim3* and *Frl* mutations were allelic, the complete *Gsdm2* and *Gsdm3* genes were sequenced in all three alleles of *Frl*. In addition, *Bsk* DNA was obtained through collaboration with Dr. John Sundberg at The Jackson Laboratory and was sequenced as well. DNA sequence data presented here demonstrate that all three alleles of *Frl*, and *Bsk*, each contain single basepair substitutions in the *Gsdm3* gene.

Diagnostic histopathology and immunocytochemical detection of bromodeoxyuridine (BrdU) incorporated into DNA demonstrate that the phenotype of *Frl* mice closely resembles the phenotypes exhibited by other *Gsdm3* mutant mice (*Rim3*, *Bsk*, Re^{den}, *Dfl*, *Rco2* and *Fgn*). This allelic series of mutations should prove useful in future studies for discovering the mechanism of action of *Gsdm3* in hair follicle morphogenesis and sebaceous gland development.

Materials and methods

Mice and DNA samples

Frl^a, Frl^b and Frl^c are all dominant mouse mutations that originated at ORNL. Frl^a and Frl^c are spontaneous mutations, and Frl^b originated in the offspring of an X-irradiated male. These mice were maintained as heterozygotes at ORNL by outbreeding, and then were made congenic on BALB/cRl (mice are currently preserved as sperm and ovary pieces). Although these mice are congenic on BALB/cRl, the mutations arose in offspring from the mating of C3H and 101 mice. C3H, 101 and C57BL/6J stocks are all maintained as inbred lines at ORNL. FVB/N mice are maintained at ORNL as a small, random-bred, closed colony stock. Homozygous *Re* mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were maintained on an Rsv/Le background. DNA samples of *Bsk* were a gift from Dr. John Sundberg at The Jackson Laboratory

(C57BL/6J-Bsk 30492, C57BL/6J-Bsk 30493, B6.Cg-Bsk/+ 34517, B6.Cg-Bsk/+ 34518).

Linkage analysis of Furloss

Heterozygous *Frl*^{a} mice were mated to homozygous *Re* mice. Compound mutant offspring, characterized by wavy fur that was subsequently lost, were backcrossed to wild-type FVB/N. Offspring from these matings were individually classified as having one of four possible phenotypes: wavy hair with furloss, wavy hair with no furloss, straight hair with furloss and straight hair with no furloss. A χ^2 test was performed to ascertain the significance of the results.

Preparation of genomic DNA

Genomic DNA was prepared from the livers of mice carrying each of the three *Frl* alleles, and from BALB/cRl, C3H, 101 and C57BL/6J control mice. The tissues (approximately 1 cm^3 each) were ground by mortar and pestle in liquid nitrogen. The tissue powder was added to 20 ml of tissue lysis buffer (10 mM Tris-HCl, pH 7.6; 10 mM EDTA; 100 mM NaCl; 0.5% SDS; 0.1 mg/ml Proteinase K) and incubated at 65^{o}C for three hours. Following incubation, 20 ml of phenol was added, gently mixed, and the mixture was centrifuged using a Beckman TJ-6 centrifuge with a TH-4 rotor at 3000 rpm for 10 minutes. The upper aqueous phase was added to 20 ml of chloroform, gently mixed, and the mixture was centrifuged using a Beckman TJ-6 centrifuge with a TH-4 rotor at 3000 rpm for 10 minutes. The upper aqueous phase was added to 2 volumes (30 ml) of absolute EtOH. The precipitated DNA was transferred to a fresh tube, any residual EtOH was removed, and the DNA was redissolved in 1.5 ml of TE buffer and stored at 4^{o}C.

Preparation of probes for Southern blot analysis

Probes for Southern blot analyses were isolated from genomic DNA of a wild-type BALB/cRl mouse by PCR using the TaKaRa kit (TaKaRa Bio Inc.) following the manufacturer's specifications. Primer set Probe1 (5'-CTGGTCTTACAAGAATGAATGG-3' and 5'-CCTCACCTTCATTTTCTAC-3') amplifies a 311 bp product that hybridizes to the 3' UTR of the *Gsdm1* gene and to the 3' end of *Gsdm3*. Primer set Probe2 (5'-GCTTTGAGAAACTGACCGCTGTATC-3' and 5'-CCTACTGACCTCCACAAGGGGG-3') amplifies a 505 bp product that hybridizes approximately 3 kb from the 3' end of the *Gsdm1* gene. Primer set Probe3 (5'-GTTAGGCAGTGTGTGGATGATGC-3' and 5'-CCTCCTACACCTCAAGGTTCTTGG-3') amplifies a 557 bp product that hybridizes to the 5' end of the *Gsdm1* gene. Primer set Probe4 (5'-AAAGGAAGAGGTTCAGCGAGAGAC-3' and 5'-

AAACTGGGTGACTAATACTTGCGG-3') amplifies a 582 bp product that hybridizes between the middle and 3' end of each of the *Gsdm* genes. The PCR products were run on a 1.5% agarose gel and purified using the QIAquick Gel Extraction Protocol from the Qiagen PCR Purification Kit (Qiagen Inc.) following the manufacturer's instructions. The amplified fragments were labeled with α-^{32}P dCTP utilizing the Stratagene Prime-It II kit (Stratagene) following the manufacturer's instructions.

Southern blot analysis of Furloss

DNA (10 μg) from each of the three *Frl* alleles, as well as the BALB/cRl, C3H, 101 and C57BL/6J controls, were digested with the following enzymes obtained from NEB following the manufacturer's instructions: Southern 1 (Probe1) – BssSI; Southern 2 (Probe2) – XhoI; Southern 3 (Probe3) – EcoRV and HindIII; and Southern 4 (Probe4) – SspI. Following digestion, the DNA was loaded into a 0.7% agarose gel and electrophoresed at 25 V overnight. The gel was then denatured and neutralized, and the DNA was transferred to a nylon membrane. The *Gsdm* probes were hybridized with the appropriate membrane. The final wash conditions were 68^{o}C for 30 minutes in 0.2x SSC, 0.1% SDS.

PCR and sequencing of genomic DNA

In order to analyze the *Gsdm2* and *Gsdm3* genes for mutations, all of the exons and the adjacent intronic splice junctions were amplified from three separate animals for each *Frl* allele, as well as from BALB/cRl, 101 and C3H mice. PCR was performed using the TaKaRa kit (TaKaRa Bio Inc.) following the manufacturer's recommendations. The primers that were used, the exons that they amplified and the amplicon size are listed in Tables 1 and 2. The annealing temperatures for each primer set are as follows: Gsdm3-1, 67.2^{o}C; Gsdm3-2, 71.0^{o}C; Gsdm3-3, 57.1^{o}C; Gsdm3-4, 53.8^{o}C; Gsdm3-5, 55.5^{o}C; Gsdm3-6, 55.5^{o}C; Gsdm3-7, 63.9^{o}C; Gsdm3-8, 63.9^{o}C; Gsdm3-9, 60.9^{o}C; Gsdm3-10, 63.9^{o}C; Gsdm3-11, 63.9^{o}C; Gsdm3-12, 63.9^{o}C; Gsdm2-1, 59.9^{o}C; Gsdm2-2, 60.9^{o}C; Gsdm2-3, 59.2^{o}C; Gsdm2-4, 63.9^{o}C; Gsdm2-5, 59.2^{o}C; Gsdm2-6, 70.0^{o}C; Gsdm2-7, 59.9^{o}C; Gsdm2-8, 59.2^{o}C; Gsdm2-9, 67.2^{o}C; Gsdm2-10, 71.5^{o}C; Gsdm2-11, 65.6^{o}C. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen Inc.) and DNA was eluted from the columns with 30 μl of elution buffer. The purified DNA and primers were submitted to the DNA sequencing core at the University of Tennessee, Knoxville, where DNA was labeled and fluorescent automated DNA sequencing was performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) according to standard protocols.

Table 1: *Gsdm3* primer sets. Listed are the primers used to amplify each exon of *Gsdm3* and the amplicon size of the expected products.

Primer Name	Forward	Reverse	Exon	Amplicon Size
Gsdm3-1	5'-CCAGTCCTGGTAAGGGACAG-3'	5'-ACGGATTGAGGGGGACTGAC-3'	1	353 bp
Gsdm3-2	5'-TCTAGAGCTCGGGATTGTCC-3'	5'-GTACCCCCACTCATCAGCAC-3'	2	562 bp
Gsdm3-3	5'-TCTCAAGCTCTCCCCACTG-3'	5'-GATTCCCTTGCTGAGCATTC-3'	3	568 bp
Gsdm3-4	5'-GTGGCCTTGGTGAAGAAATG-3'	5'-TTTCCACTGGGCTTTAACTG-3'	4	563 bp
Gsdm3-5	5'-CCAGCCGTGTACTTGAATCC-3'	5'-TCCTTCTTCAGCCTCAAACC-3'	5	570 bp
Gsdm3-6	5'-GGTTGCTCTCGATGAAGGAC-3'	5'-TTGGAACACATCCCATCTCC-3'	6	597 bp
Gsdm3-7	5'-ACAAAGAGACAGGGCCAAAG-3'	5'-TGATGACAGCAAAGGTCTGG-3,	7	380 bp
Gsdm3-8	5'-AGCCCCCGTATTAGTTCTGC-3'	5'-GCTTGTGCAGCAAAGTCTTG-3'	8	455 bp
Gsdm3-9	5'-GTCCACCTCTCTGCTCCATC-3'	5'-GTGCACTTGGGAAGGACATC-3'	9	495 bp
Gsdm3-10	5'-CTTGGCTGCTGGACTACCAC-3'	5'-AAGCCATCTGGAATCCACTC-3'	10	538 bp
Gsdm3-11	5'-TTCCCTCTGTACCAGCAACAC-3'	5'-GCAGGAAGTTCTGCTCCAAG-3'	11	413 bp
Gsdm3-12	5'-TCACAGGACCCAGCCTTATC-3'	5'-TCCTCAGACTGGAGGCTCTG-3'	12	435 bp

Table 2: *Gsdm2* primer sets. Listed are the primers used to amplify each exon of *Gsdm2* and the amplicon size of the expected products.

Primer Name	Forward	Reverse	Exon	Amplicon Size
Gsdm2-1	5'-GTGGGGCACTTCAAACAGAC-3'	5'-AGGGCTGCCTCTTTTCTCTC-3'	1	357 bp
Gsdm2-2	5'-GCCACTCTGGTGAAGGGTAG-3'	5'-AGCTTGGTCTTGGGAAACAC-3'	2	370 bp
Gsdm2-3	5'-TGGGATTGTCCATCACAAAAG-3'	5'-AAATTGCCAAGGAGTGTTGG-3'	3	623 bp
Gsdm2-4	5'-AGTGCTGTGCTGATGAGTCG-3'	5'-CTCTGTGCATTAGCCCTCTG-3'	4	423 bp
Gsdm2-5	5'-GGGAGGTAGTTGGCATCATC-3'	5'-GTCTGTGGCCTATGGAGACC-3'	5	652 bp
Gsdm2-6	5'-CCTGTACCTGCATCCTGCTC-3'	5'-AATCACCAATCCAGGTCTGC-3'	6	466 bp
Gsdm2-7	5'-TGTGGGGAGTGACCCTAAAG-3'	5'-GACCCAGGAAACAAGAGCAG-3'	7	438 bp
Gsdm2-8	5'-CACACCCCTTGGTAAAGAG-3'	5'-GTAGCCTAGCAGCCAAGGTG-3'	8	471 bp
Gsdm2-9	5'-AAGTGGTGTGTGCCCTCTG-3'	5'-CTATAAGGCACCTGGCATCC-3'	9	469 bp
Gsdm2-10	5'-GAGCAGGTGTTCATCTGTGG-3'	5'-AGCAGAGCAGGCTGAGTACC-3'	10	478 bp
Gsdm2-11	5'-ACTCAGCCTGCTCTGCTAGG-3'	5'-CATGCGACCTGTGTCCTG-3'	11	608 bp

Histology

The right eye and sections of dorsal skin were taken from sex matched and age matched littermates from *Frl*^{a} mutant and BALB/cRl control mice. Tissues were taken at the following time points: day 8, day 14, day 21, day 28, day 35, 8 months, 11 months and one year. At least two mutant mice and one control mouse were examined for each time point with the exception of the ones occurring at eight months, eleven months and one year. Day 8 genotypes were verified by PCR and DNA sequence analysis. The sections were preserved in acid alcohol formalin, with one change in 70% EtOH. The skin and eye samples were processed for embedding in paraffin. Section thickness was 4 μm. The slides were stained with hematoxylin and eosin (H&E) according to standard protocols. (Section processing and staining performed by Jim Wesley at Ridge Microtome, Oak Ridge, TN, USA.)

Immunohistochemical detection of BrdU

BrdU analysis was performed on sex matched and age matched littermates of *Frl*^{a} and *Frl*^{c} mutant and BALB/cRl control mice in order to detect the proliferation rate of epithelial cells in the skin and eye of mutant animals. BrdU was injected IP into five-month-old mice at a concentration of 100 mg/kg. Mice were sacrificed at 2 hours or 30 hours following injection, and the dorsal skin and right eye were harvested. Tissues were preserved in Davidson's solution. The dorsal tissues were processed for paraffin embedding. Section thickness was 4 μm. The sections were stained with anti-BrdU utilizing the Anti-BrdU Kit I from Zymed Laboratories (San Francisco, CA). The staining was performed according to the manufacturer's recommendations with the exception of digesting tissues for 6 to 8 minutes with proteinase K rather than with trypsin. In addition, the tissues were stained with H&E according to standard protocols. (Section processing and H&E staining performed by Jim Wesley at Ridge Microtome, Oak Ridge, TN, USA.)

Cell counting

Anti-BrdU stained slides were viewed using a 40X objective. A total of 1250 cells, both stained and unstained, were counted per slide and three slides were examined per BrdU incubation sample set. The sample sets were as follows: two hour control, two hour mutant, 30 hour control and 30 hour mutant. Because the mutant mice lack hair follicles, only the cells in the interfollicular regions of the control mouse epidermis were counted. Individual slide data for each of the sample sets were compared between the mutants and their respective controls, and Student's t-tests were calculated in order to determine whether or not the differences were statistically significant.

Results and discussion

Linkage analysis of Furloss

A linkage test was performed between *Frl* and *Re* to determine if *Frl* maps to the distal end of mouse Chr 11 in the same region as *Bsk*, Re^{den} and *Rim3*. The data available at the time of this experiment (Lyon and Zenthon, 1986; Lyon and Zenthon, 1987; Eicher and Varnum, 1986; Sato et al., 1998) suggested that *Bsk*, Re^{den} and *Rim3* were all alleles of a single gene, and that *Re* was a separate gene, but very tightly linked to *Bsk*, Re^{den} and *Rim3*. However, since *Bsk* and Re^{den} mice were frozen at The Jackson Laboratory, and *Rim3* was in Japan and not readily available, we purchased live *Re* mice from The Jackson Laboratory to perform the linkage analysis with *Frl* (Fig. 12).

Five hundred seventy-eight mice were produced from the backcross between the Frl^{a}/+;*Re*/+ compound mutant mice and wild-type mice. Of these offspring, 302 had a phenotype of wavy hair without furloss, 274 had a phenotype of straight hair with furloss and 2 had a phenotype of straight hair without furloss (wild-type crossovers). These data are significantly different (see below) from what one would expect if *Frl* and *Re* were not linked, and support the hypothesis that *Frl* is tightly linked to *Re* (not allelic) and may be allelic with *Bsk*, Re^{den} and *Rim3*. This finding permitted me to initiate a candidate gene search for *Frl*, utilizing the *Rim3* mapping data presented by Sato et al. (1998).

$$\chi^2 = \frac{(576-2)^2}{578}$$

$$\chi^2 = 570.03$$

$$P << 0.0001 \quad (\mathrm{df} = 1)$$

Southern analysis of Furloss

Figure 13 shows the predicted hybridization sites and fragment sizes for four probes and five restriction enzymes based on the genomic DNA sequence of the C57BL/6J mouse. These combinations of probes and enzymes were selected to verify that there are three *Gsdm* genes in this region of Chr 11, and to determine if there are any deletions or other alterations in this genomic region in *Frl* mice. The results confirm that there are three nearly identical *Gsdm* genes in this region of Chr 11 (Fig. 14). They further show that there are no chromosomal alterations in *Frl* mice, indicating that the *Frl* alleles did not arise from recombination between any of the three *Gsdm* genes. Based on these results, PCR primers were designed in order to sequence these *Gsdm* genes in mice carrying the three *Frl* alleles to look for more subtle mutations.

Cloning of Furloss *and* Bareskin

The *Gsdm2* and *Gsdm3* genes were sequenced in their entirety in Frl^{a}, Frl^{b} and Frl^{c} mice. The mutations in all three *Frl* alleles were identified in exon 12 of *Gsdm3*.

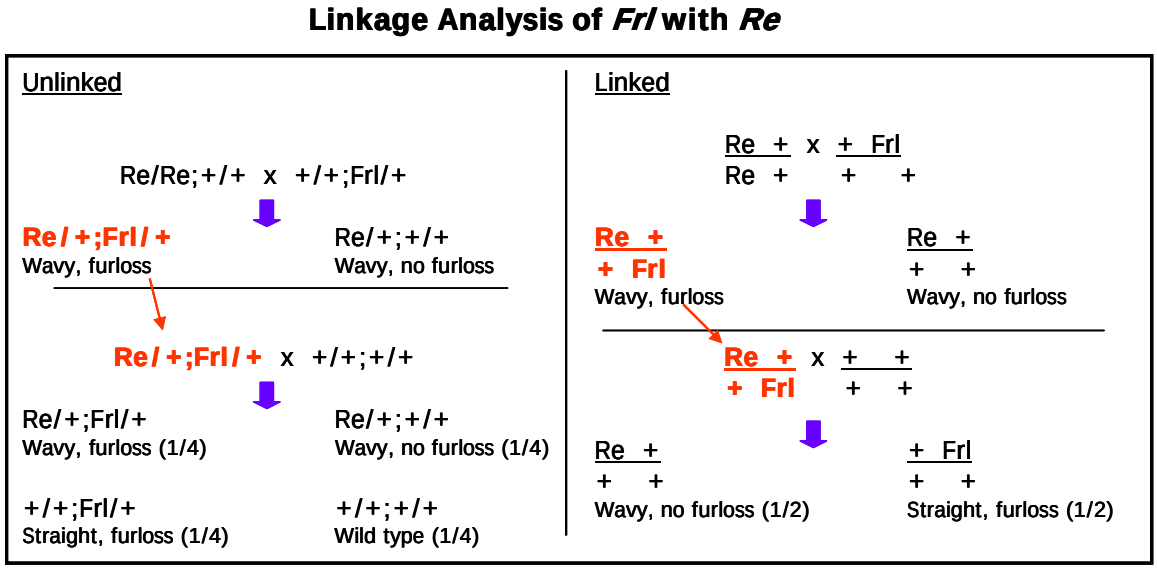
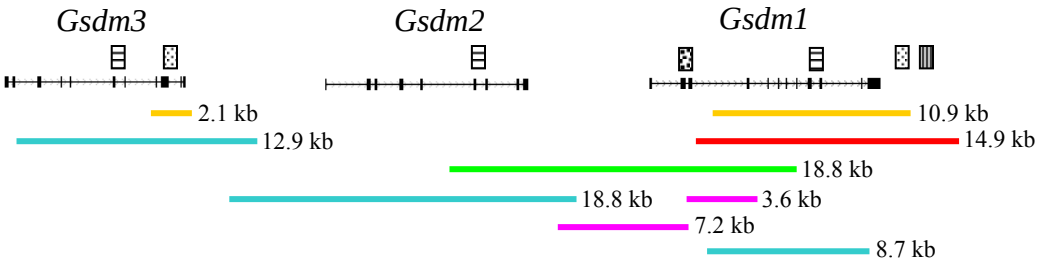


Figure 12: Linkage analysis of *Frl* and *Re*. Shown is a diagram of the matings that were performed and the ratios of expected phenotypes/genotypes of the offspring if *Frl* and *Re* are unlinked (left panel) or linked (right panel). Homozygous *Re* mice were mated to heterozygous *Frl*^{a} mice to generate compound heterozygous mice (highlighted in red). The compound heterozygous mice were mated to wild-type mice and the offspring were visually scored for presence of wavy (*Re*/+) or straight (+/+) hair at one week old, and furloss (*Frl*/+) or no furloss (+/+) at three weeks old. If *Frl* and *Re* are unlinked and assort independently, each of the four possible indicated phenotypes will be represented by one quarter of the offspring. If the mutations are allelic or very tightly linked, only the original parental phenotypes will be represented by one half of the offspring each. If the mutations are very tightly linked and a sufficient number of offspring are produced, it is expected that the vast majority of mice will exhibit wavy hair/no furloss or straight hair/furloss phenotypes in equal proportions, with only a few mice exhibiting wild-type or compound mutant phenotypes, which would result from rare crossovers between the tightly linked mutations. The strength of the linkage will be shown by the proportion of the crossover mice produced to the mice exhibiting the original parental phenotypes.



Probe Number	Enzyme(s) Used for Digestion
Probe 1	BssSI
Probe 2	XhoI
Probe 3	EcoRV
Probe 4	SspI
	HindIII

Figure 13: Diagram of the tandem cluster of three *Gsdm* genes. Shown are four probes, their sites of hybridization, the restriction enzymes used, and the expected restriction fragment sizes based on the sequenced C57BL/6J mouse genome.

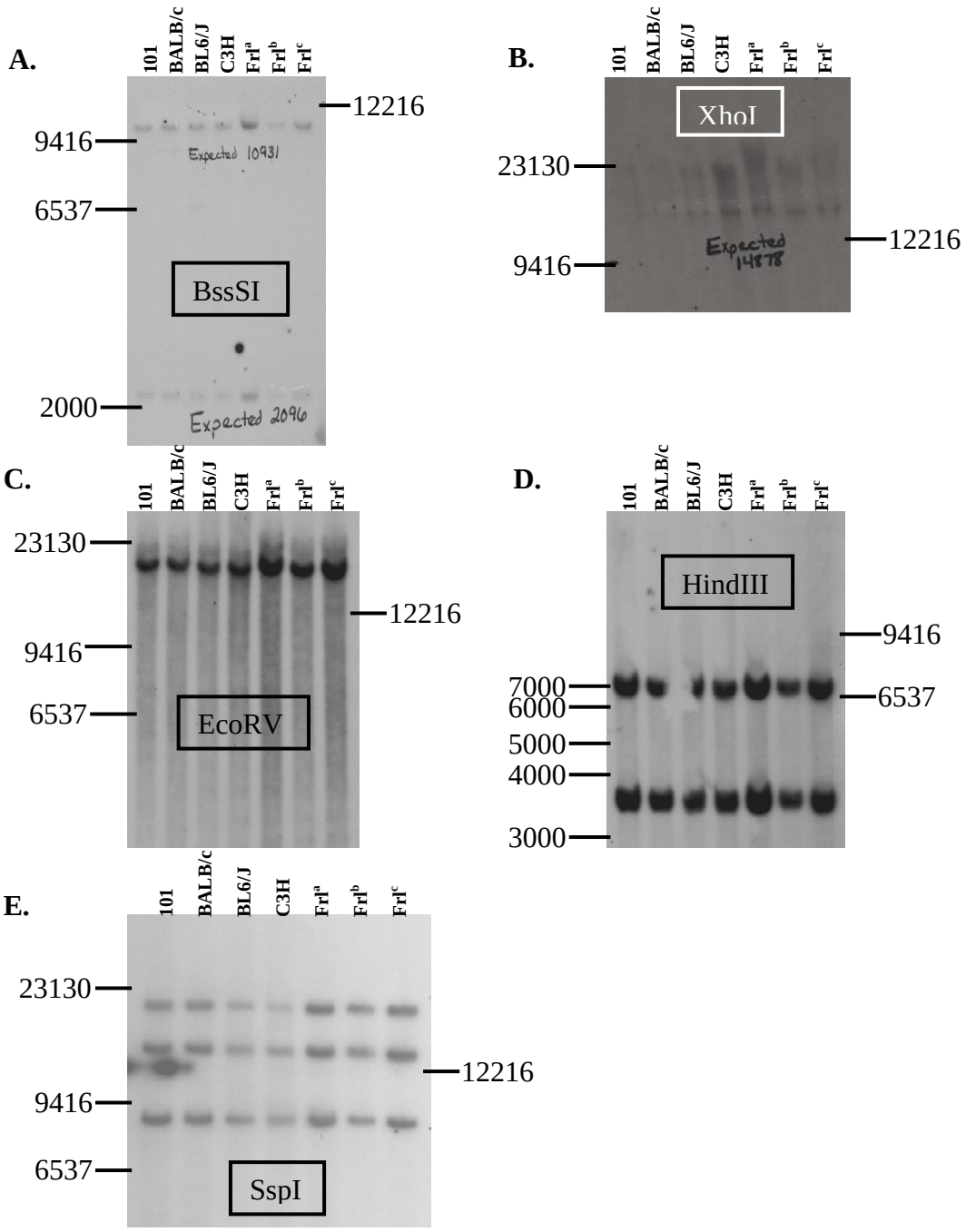


Figure 14: Southern blot analysis of the *Gsdm* locus in *Frl* and control mice. (A) Probe 1 hybridized with 10.9 kb and 2.1 kb fragments in DNA digested with BssSI. (B) Probe 2 hybridized with a 14.8 kb fragment in DNA digested with XhoI. (C) Probe 3 hybridized with an 18.8 kb fragment in DNA digested with EcoRV. (D) Probe 3 hybridized with 7.2 kb and 3.6 kb fragments in DNA digested with HindIII. (E) Probe 4 hybridized with 18.8 kb, 12.9 kb and 8.7 kb fragments in DNA digested with SspI. All fragments are of the expected sizes (see Fig. 13).

The *Frl*^{a} mutation is a single base pair change (C1366A) that causes tyrosine 428 to change into a premature stop codon, resulting in a truncated protein (Fig. 15). Sequence analysis revealed that *Frl*^{b} and *Frl*^{c} have identical mutations, which is a single base pair substitution (G1398T) that changes cysteine 439 into a phenylalanine (Fig. 15). The mutation in *Bsk* was also identified in the *Gsdm3* gene in exon 10 and is a single base pair change (A1113G), which results in a Y344C amino acid substitution, in agreement with the finding of Runkel et al. (2004) (Fig. 15).

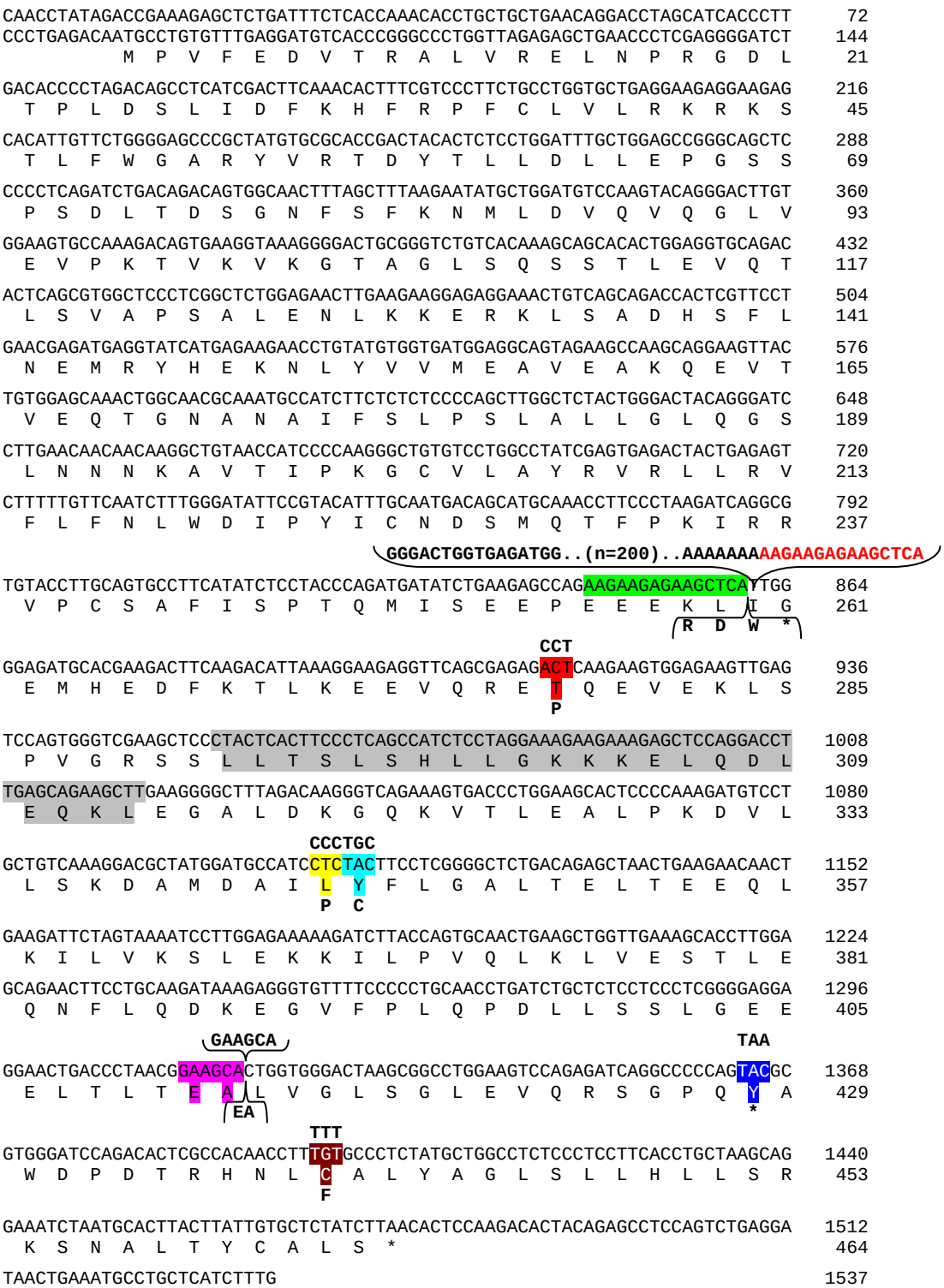
The *Frl*^{b} mutation was originally thought to have been induced by X-irradiation. However, given that mutations induced by X-irradiation are usually large chromosomal alterations, such as deletions, inversions or translocations (Russell, 1951; Russell, 1989; Russell and Russell, 1992), it is probable that the point mutation in *Frl*^{b} may have arisen spontaneously, even though the original mutant mouse was the offspring of an X-irradiated male.

It was determined that the mutations in *Frl*^{b} and *Frl*^{c} are identical. Although it is possible that these two identical mutations arose independently, it is more likely that these mice represent a single mutation. Discussions with technical staff members who know the history of these stocks indicated that *Frl*^{c} arose two years after *Frl*^{b}, in the same mouse room and in close proximity to *Frl*^{b}. It is possible that a mouse from the *Frl*^{b} colony was accidentally placed in a cage with mice from the 19DFiOD colony, and when fur loss was later observed in this animal, it was assumed that this was a new spontaneous mutation. Although this possibility was entertained at the time, the mutations were considered to have arisen independently because of the different degrees of severity of the mutant phenotypes in homozygous individuals. However, this could also be explained by the different genetic backgrounds of the two colonies. Another possibility is that there really were independent *Frl*^{b} and *Frl*^{c} mutations, but that an error in stock maintenance occurred during the allelism testing of these two stocks, and two independent lines of the same mutation were subsequently maintained. Therefore, while it is possible that *Frl*^{b} and *Frl*^{c} are independent occurrences of the same mutation, it is more likely that they are independent stocks of the same original mutation.

Histological analysis of the Furloss *alleles*

Basic histology of the *Frl* animals showed very drastic differences between mutant and wild type phenotypes, as was seen with all of the other *Gsdm3* mutants. In the skin, thickening of the epidermis was visible by 8 days of age (Fig. 16). At all time points, the mutant phenotype was characterized by a mildly thickened epidermis (acanthosis) with a thickened stratum corneum (orthokeratotic hyperkeratosis) and a thickened dermis. The infundibulum of the hair follicle was often dilated and plugged with cornified material (Fig. 17). *Frl* undergoes a prolonged catagen stage, resulting in abnormally long hair follicles during the next anagen. This is very easily seen at the three, four and five week time points (Figs. 18, 19 and 20). In addition to this, destruction of the hair follicles begins at about nine weeks, with the hair follicles being completely absent by four months (Figs. 21, 22 and 23). The eye has many of the same characteristics that are associated with the skin, such as thickening of the corneal

Figure 15: mRNA and protein sequence of mouse *Gsdm3* (NM_001007461) and the mutations identified in Frl^{a}, Frl^{b} and Frl^{c}. Shown in addition to the five published *Gsdm3* mutations in *Dfl* (B2 insertion, premature STOP), *Fgn* (T278P), *Rco2* (L343P), *Bsk* (Y344C) and Re^{den} (EA411-412 duplication), are the Frl^{a} (Y428STOP) (highlighted in dark blue) and the Frl^{b} and Frl^{c} (C439F) (highlighted in brown) mutations.



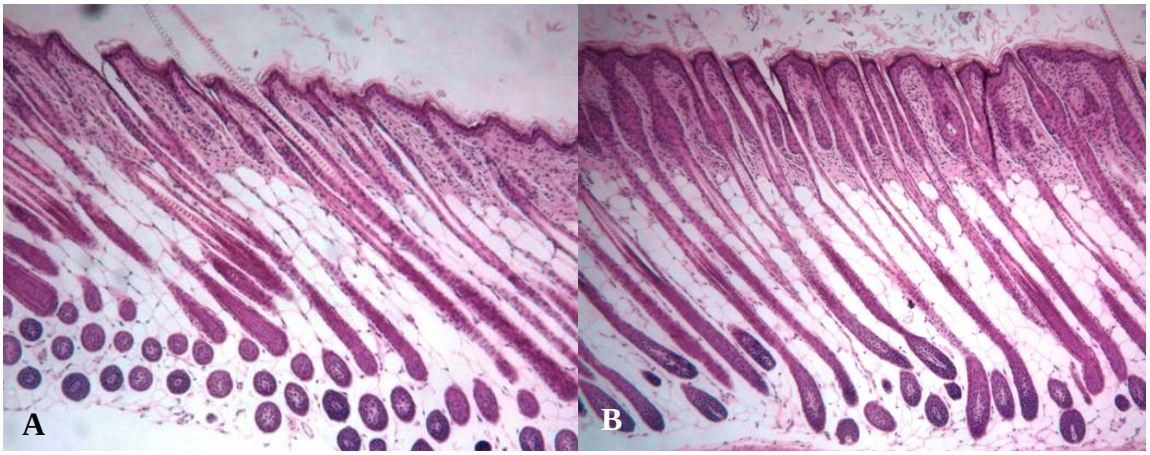


Figure 16: Dorsal skin of eight day old control and *Frl*^{a} mutant mice. The hair follicles of both the control mouse (A) and the mutant littermate (B) are in the middle stage of anagen. The mutant mouse has a slightly thickened epidermis. (Pictures taken at 10x magnification.)

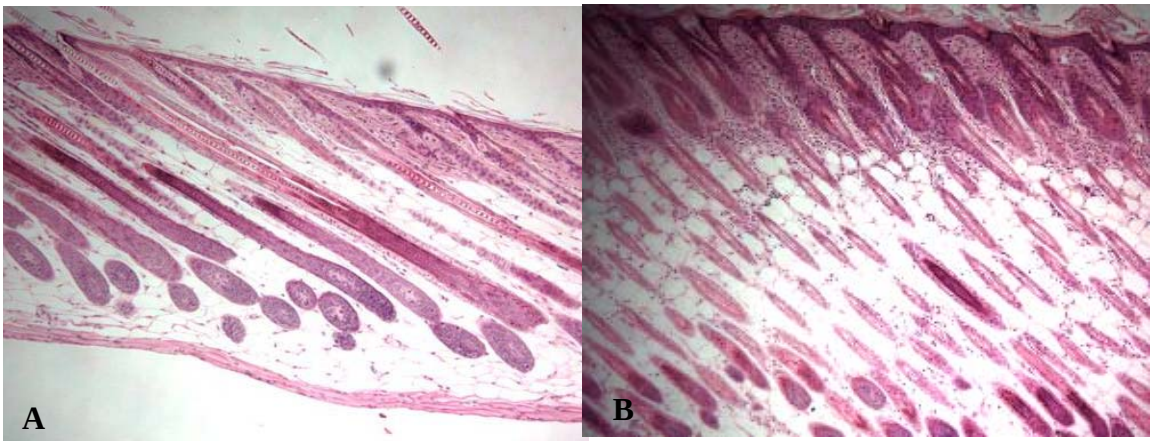


Figure 17: Dorsal skin of two week old control and *Frl*^{a} mutant mice. The hair follicles of both the control mouse (A) and the mutant littermate (B) are in the final stage of anagen. The mutant mouse has a thickened epidermis and cornified material building up in the hair follicle where the hair emerges from the shaft. (Pictures taken at 10x magnification.)

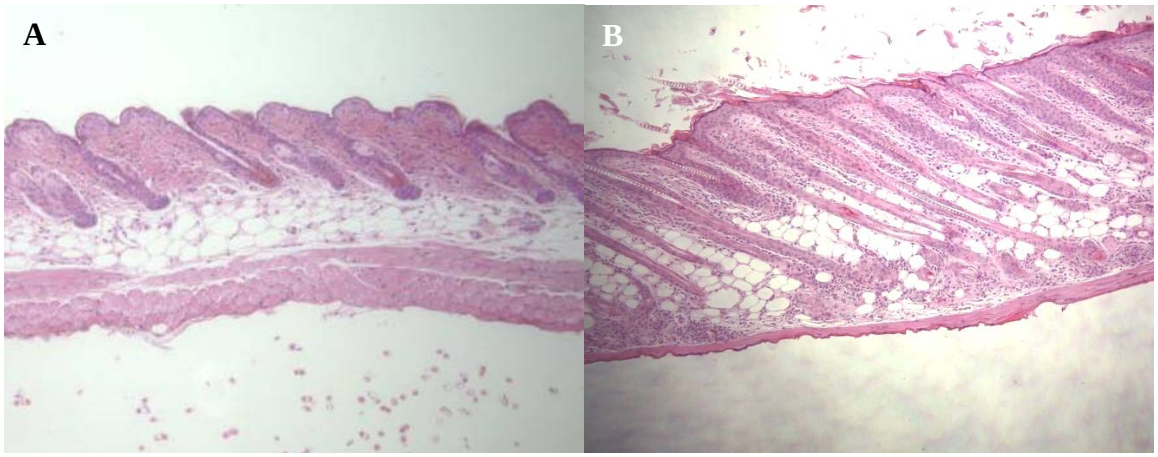


Figure 18: Dorsal skin of three week old control and *Frl*^{a} mutant mice. The hair follicles of the wild-type control mouse (A) are in early anagen, while the hair follicles of the mutant littermate (B) are in the middle stage of catagen. In addition, the mutant mouse has a thickened epidermis and hyperkeratinization of the skin compared to the control. (Pictures taken at 10x magnification.)

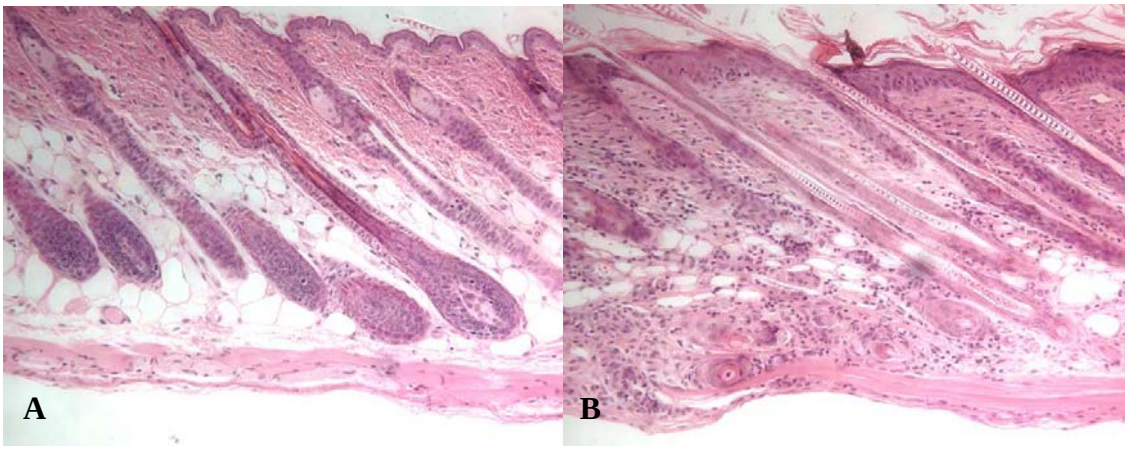


Figure 19: Dorsal skin of four week old control and *Frl*^{a} mutant mice. The wild-type hair follicles (A) are in the middle stage of anagen, while the mutant hair follicles (B) are in the beginning of anagen. In addition, the mutant mouse has a thickened epidermis and hyperkeratinization of the skin compared to the wild type littermate. (Pictures taken at 10x magnification.)

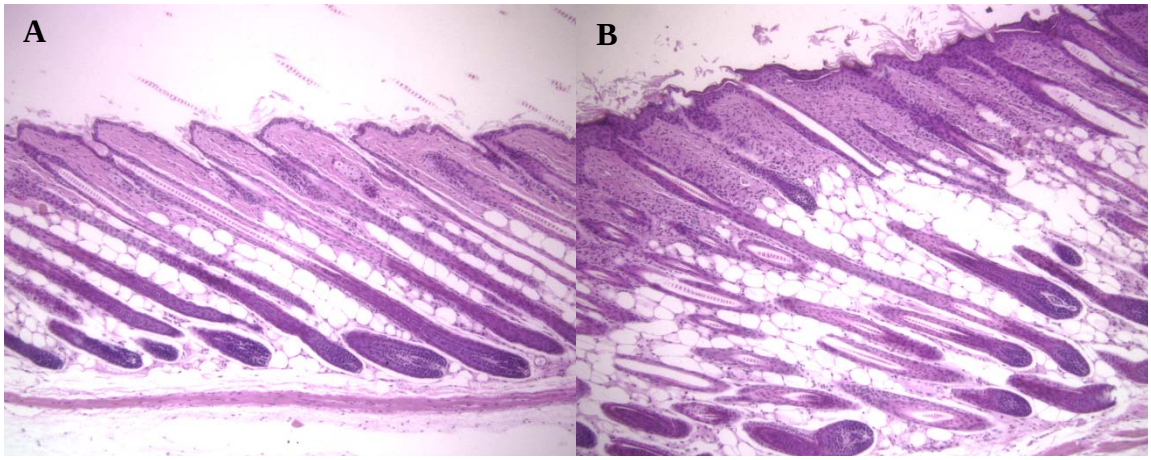


Figure 20: Dorsal skin of five week old control and *Frl*^{a} mutant mice. The wild-type hair follicles (A) are in early catagen, while the mutant hair follicles (B) are in the middle stage of anagen. The mutant hair follicles are much longer than they were in the first anagen cycle as a result of the prolonged catagen stage. In addition, the mutant mouse has a thickened epidermis and hyperkeratinization of the skin compared to the wild type littermate. (Pictures taken at 10x magnification.)

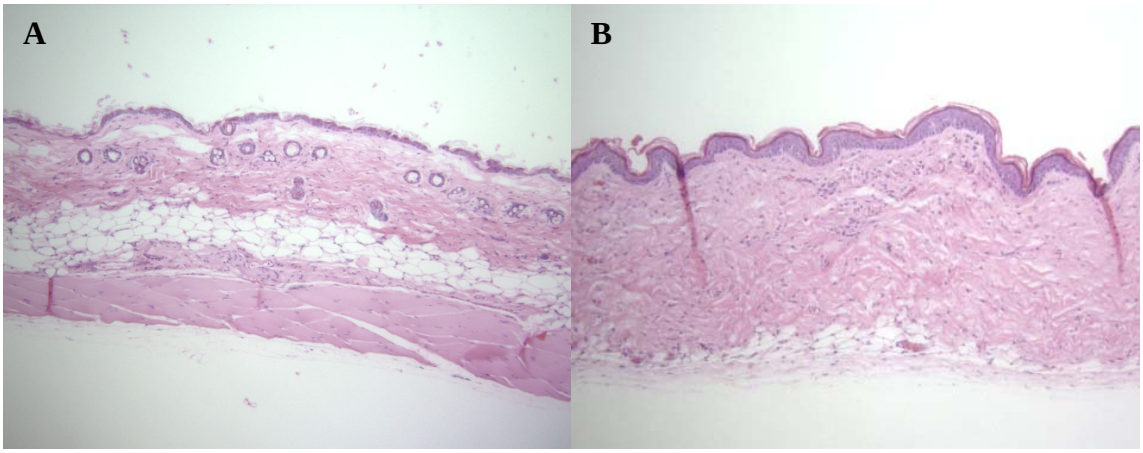


Figure 21: Dorsal skin of eight month old control and *Frl*^{a} mutant mice. The sections are cut perpendicular to the hair follicles resulting in clusters of hair follicle circles in the epidermis of the control mouse (A). There is a complete lack of hair follicles in the skin of the mutant mouse (B), as well as thickening in the epidermis, stratum corneum and dermis, and hyperkeratinization. (Pictures taken at 10x magnification.)

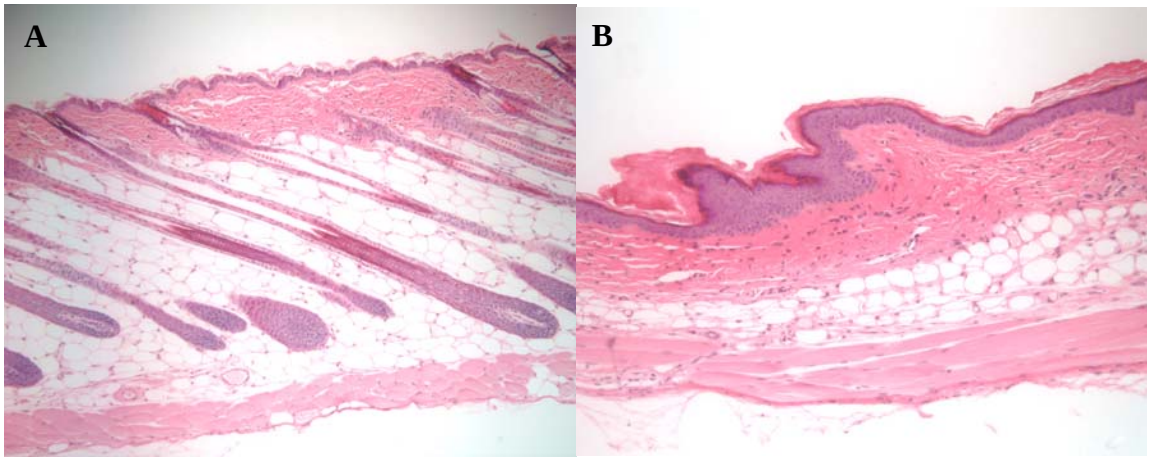


Figure 22: Dorsal skin of eleven month old control (A) and *Frl*^{a} mutant (B) mice. There is a complete lack of hair follicles in the skin of the mutant mouse, as well as thickening in the epidermis, stratum corneum and dermis, and hyperkeratinization. (Pictures taken at 10x magnification.)

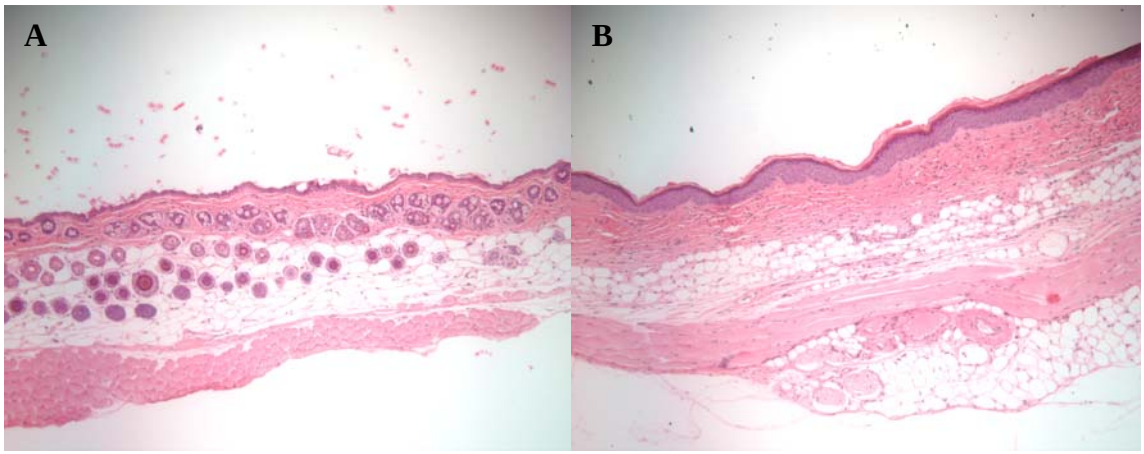


Figure 23: Dorsal skin of one year old control and *Frl*^{a} mutant mice. The sections are cut perpendicular to the hair follicles resulting in clusters of hair follicle circles in the epidermis of the control mouse (A). There is a complete lack of hair follicles in the skin of the mutant mouse (B), as well as thickening in the epidermis, stratum corneum and dermis, and hyperkeratinization. (Pictures taken at 10x magnification.)

epithelium and hyperkeratinization, but it also exhibits inflammation and neovascularization (Fig. 24).

BrdU analysis of Furloss

In addition to determining the histological phenotype of *Frl*, I wanted to determine if the thickened epithelial tissues of the skin and eye are caused by rapid division of the cells, or by failure of the cells to die. In order to ascertain if the thickening is due to rapid division of the cells, histological analysis utilizing anti-BrdU staining was performed. BrdU (bromodeoxyuridine) is a chemical that, when injected into an animal, incorporates into replicating DNA in the place of thymidine, one of the four types of nucleotides that make up DNA. This chemical can then be detected using a biotinylated monoclonal antibody specific to the BrdU (called anti-BrdU), containing Streptavidinperoxidase with DAB (3,3-diaminobenzidine), which darkly stains cells undergoing replication, allowing an investigator to make an accurate count of proliferating cells and compare differences between animals.

Anti-BrdU staining revealed that there was indeed a difference in the proliferation rate between mutant and control mice for both tested BrdU incubation periods (Fig. 25). Student's *t*-tests show that the different proliferation rates are statistically significant, which supports the hypothesis that the thickening of the skin in the mutant mouse is due to rapid division of epithelial cells rather than failure of the cells to die (Table 3).

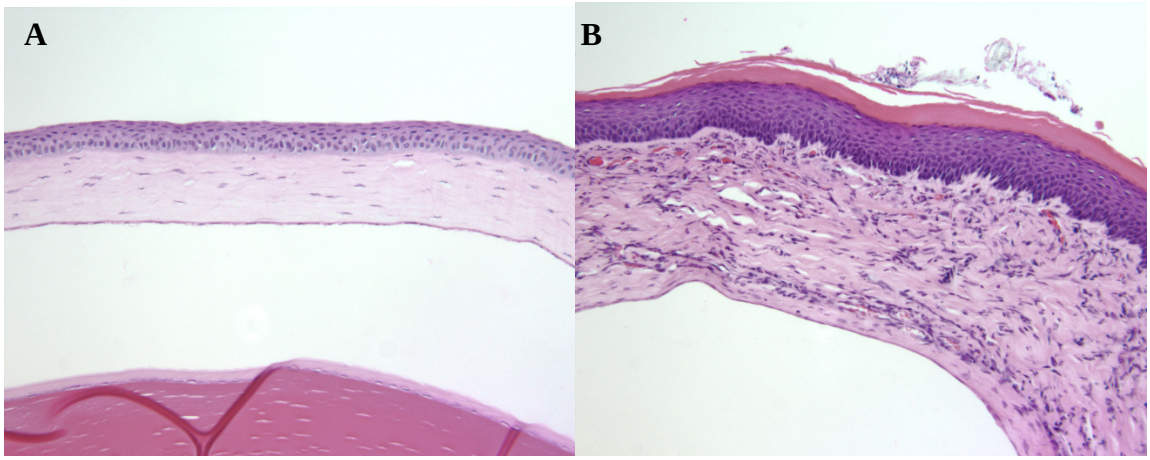


Figure 24: Eye of eight month old control (A) and Frl^{a} mutant (B) mice. The mutant eye has a central thickening and hypercellularity of the corneal stroma, with marked hyperplasia, keratinization and neovascularization. There is also cellular hypertrophy in the corneal epithelium, as well as inflammation throughout the eye. (Pictures taken at 20x magnification.)

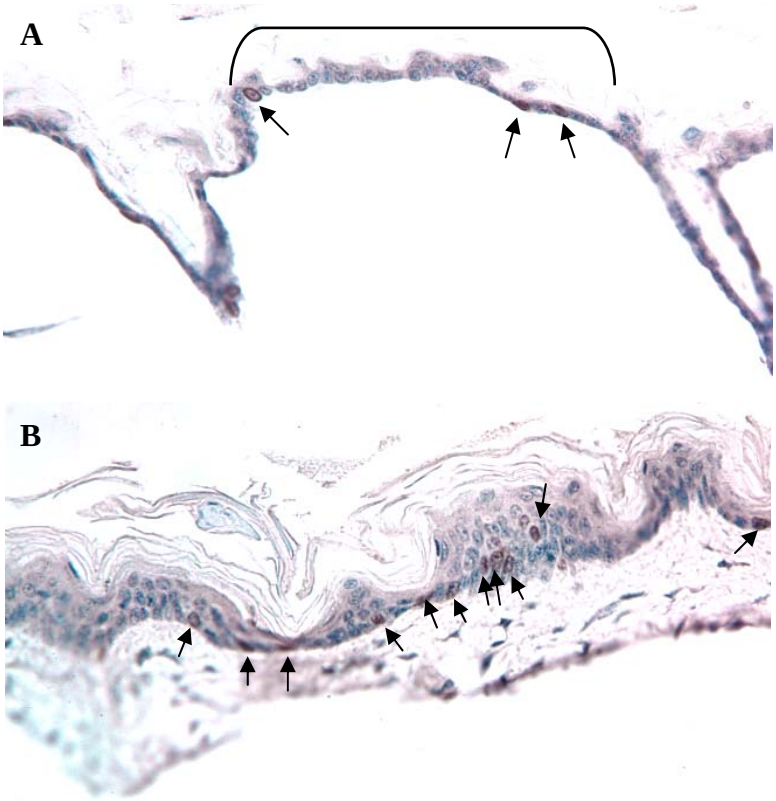


Figure 25: Dorsal skin of six month old control (A) and *Frl*^{a} mutant (B) mice stained with anti-BrdU following two hour BrdU incubation. The BrdU cells are stained dark brown. The interfollicular region in the control mouse (the area where the cells were counted) has been marked. Black arrows point to some of the anti-BrdU stained cells. There is increased staining in the mutant epithelium as compared to the control. There is also a complete lack of hair follicles in the skin of the mutant mouse, as well as thickening in the epidermis. (Photos taken at 40x magnification.)

Table 3: Measure of relative cellular proliferation in the dorsal skin of five month old control and *Frl*^{a} mutant mice as determined by counting the numbers of cells stained with anti-BrdU at 2 hour and 30 hour post-BrdU injection. The number of cells stained with anti-BrdU was significantly greater in *Frl*^{a} mice than in the controls at both time points.

	2 Hour		30 Hour	
	Control	**Mutant**	**Control**	**Mutant**
Slide 1	62	123	103	268
Slide 2	67	131	108	287
Slide 3	74	142	114	309
Mean	67.7	132.0	108.3	288.0
SD	6.03	9.54	5.51	20.5
P	0.001		<0.001	

Conclusions

To date, there are nine known (but only five published) mutations in the *Gsdm3* gene. These mutations range from simple point mutations resulting in single amino acid substitutions, to a very large insertion that disrupts the reading frame of the gene resulting in a prematurely truncated protein. However, there is relatively little difference in the morphological and histological phenotypes of the different mutant mice. It seems that even the simplest alteration disrupts the normal function of the *Gsdm3* gene. The protein sequence of the *Gsdm* genes contains a leucine zipper motif. This type of motif is often implicated in DNA binding following dimerization of another molecule containing a leucine zipper. Most of the alterations in the mutant sequences produce significant amino acid changes that would disrupt the tertiary folding of the protein. Perhaps, if the gasdermins are DNA binding proteins, the mutations in *Gsdm3* are preventing the protein from interacting with other leucine zippers or from binding the DNA appropriately. With this in mind, we hypothesize that the *Gsdm3* mutations may be acting as dominant negative mutations, whose gene products adversely affect the normal, wild-type gene products within the same cell.

So far there have been no reported mutations in either of the other two *Gsdm* genes. The fact that *Gsdm3* expression is restricted to the skin while the other two gasdermins are expressed in the gut as well may have something to do with the lack of *Gsdm1* and *Gsdm2* mutants (Saeki et al., 2000; Runkel et al., 2004). Perhaps these genes provide such a vital role in the upper gastrointestinal tract that mutations result in embryonic or early postnatal lethality.

The histology of the *Frl* mutants as well as of mice carrying the other *Gsdm3* alleles also provides us with an interesting puzzle. *Gsdm3* specifically localizes to cells in the companion layer, the inner root sheath, the hair shaft of the hair follicle and to cells in the sebaceous gland that are closest to the sebaceous gland duct (Lunny et al., 2005). Perhaps the expression of the altered protein in these areas changes how the cells differentiate, which in turn alters the makeup of the hair follicle itself.

Chapter 3

Future work to be performed and usefulness of the *Gsdm3* mutations as mouse models of human hairloss disease

Future work

Many questions have been answered about the *Furloss* mutations during the course of this investigation, but those answers have led to even more questions, and there is much more work to be done.

Many different events are occurring as shown by the *Furloss* histological phenotype, and we have only just begun to see what is taking place with the few major time points that have been classified. Several time points in between those that have already been done are needed in order to determine exactly what changes are taking place in the hair follicle during the first two hair cycles. In addition, more information is needed for adult and geriatric mice. The studies need to be aimed at finding out why the catagen stage of the hair cycle is lengthened and what types of cells are involved, and determining what causes the sebaceous gland to disappear. Immunohistochemical analysis of mutant and control skin with markers that identify specific cell layers of the skin and hair will aid in this investigation. Lipid staining should also be performed in order to assess the internal health of the hair follicle.

Because there is a very good possibility that the *Gsdm* mutations act as leucine zipper molecules and may be involved in protein dimerization, it is very important that protein interaction studies be done. Yeast two hybrid screening utilizing an epidermal specific library would be a good choice for this type of study. Even if it is found that the leucine zipper is not utilized, it will be important to find out what other proteins *Gsdm3* may form a complex with, and what effect the mutations have on the complex formation.

While the gasdermins do contain a leucine zipper motif, it is also purported that there is a coiled coil region contained in *Gsdm3* (Lunny et al. 2005). An amino acid threading assay should be carried out in order to get a prediction on the way the protein is folded into its tertiary structure, and see what impact the different mutations have on the final product.

I think that the *Furloss* mutations should be made congenic on a different background. I believe that on an altered background, vigorous and fertile homozygous animals will be able to be produced and compared in depth with the heterozygotes, as was the case with the *Dfl* mice (Porter et al., 2002). In addition, placing *Frl* on different mouse backgrounds could allow the investigator to map modifiers of the *Frl* mutant phenotype.

Finally, mutations and conditional knockouts should be made in the other two remaining *Gsdm* genes. In that way one would be able to determine if the reason no mutations exist in those genes is due to prenatal death, or perhaps mutations in those genes do not result in an observable phenotype or are recessive in inheritance.

Gsdm3 as a mouse model

There exists a human hair loss disease called primary cicatricial (scarring) alopecia. This is a group of hair disorders that are linked by the potential permanent loss of scalp hair follicles in affected areas. In the early stages of this disorder, there is an inflammatory cell infiltrate associated with the mid follicular region and sebaceous gland destruction. In the late stages, the follicular structures are replaced by fibrous tissues (Stenn 2001). Currently, researchers are using a mouse model called *Asebia* to study this disorder. *Asebia* is a recessive mouse mutation that has a one-gene sebaceous gland defect that leads to sebaceous gland hypoplasia and as a result, cicatricial alopecia. The study of this mouse has shown that in the absence of normal sebaceous gland functions, the sheath adheres to the shaft, prevents shaft exit and leads to follicular destruction. These secondary responses observed in *Asebia* are identical to what we see in the *Frl* mice, and we believe that the *Gsdm* mutations would make another good mouse model for this disease.

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Vita

Heather Marie Pratto-Dech was born in Southfield, MI on May 10, 1977. She moved to Tennessee at the age of 13 with her family. She graduated from Clinton High School in 1995 in the top twelve in her class of 252. From there, she attended the University of Tennessee, Knoxville, where she participated in the Threshold Honors Program and received a B.S. degree in Ecology and Evolutionary Biology in 2001. She also attended the University of Tennessee, Knoxville and participated in the Genome Science and Technology graduate program, and received a M.S. degree in Life Sciences, with a concentration in Genome Science and Technology, in 2005.

Heather is currently working towards obtaining a teaching certificate from the University of Tennessee, Knoxville, in order to pursue a career of teaching biology.