Chapter 5

Summary and General discussion

The rough coat mutation spontaneously developed in the C57BL/6J mouse strain and was identified in 1966 (Dickie, 1966) and mapped in 1977 at the 9th chromosome close to the *Mpi-1* gene (Eicher and Reynolds, 1977). The next few decades the rough coat strain was not studied but in 2004 the laboratory of Dr. Csiszar focused on identifying the causative gene of the rough coat phenotype, as it was hypothesized that it might be allelic with Lysyl oxidase like gene (*loxl*), a matrix component. It turned out that rough coat is not allelic with *loxl* (Hayashi et al., 2004) but the interest in the mutation remained. The main aim of my study described in this thesis was to answer two questions about the rough coat mutation: where is the exact location of the mutation in the mouse genome, and what kinds of mechanisms underlie the complex rough coat phenotype? To answer these questions we used forward and reverse genetic approaches, and investigated the mutation both by *in vivo* and *in silico* approaches.

For identification of the mutation we first investigated in detail the phenotype of the rough coat mice and determined the phenotypic characteristics for the positional cloning analyses in Chapter 2. The mutated mice developed cyclic and progressive hair loss by the weaning age and ulcers appeared on the ventral skin of the neck (63%) over the first year. Histological examination of the skin showed enlargement of the glands that secrete a waxy substance called sebum that functions to lubricate the skin and hair, and also showed excessive proliferation of the cells (sebocytes) involved in sebum production. Histological dissection of the ulcers revealed typical signs of chronic wounds. Previously the linkage of the rough coat locus (*rc*) with two microsatellite markers *D9Mit162* at 49.954 Mb and *D9Mit104* at 65.953 Mb was described (Hayashi et al., 2004). Using two different mice strains and published as well as novel polymorphic microsatellites we identified the mapping interval of the *rc* locus and by sequence analyses we identified a point mutation in the open reading frame within a novel gene (ENSMUSG00000070305) located at 44.989~45.009 Mb. The mutation is a G→A transition in exon 3 of this gene, resulting in an Arg100→Gln (R100Q) substitution. EST analyses revealed that at least two different transcripts exist in mice. One longer transcript consists of 6 exons and encodes a 237 amino acid polypeptide and the shorter one consists of 2 exons and encodes a 96 amino acid polypeptide. The protein was predicted as a cell adhesion molecule with the highest homology to Myelin Protein Zero (MPZ), Myelin Protein Zero-like 1 (MPZL1), and Myelin Protein Zero-like 2 (MPZL2, also called Epithelial V-like Antigen, EVA1). We therefore named this gene *Mpzl3* (Myelin Protein Zero-like 3). All three proteins belong to a conserved protein family called Myelin Protein Zero. The major characteristic of this family is that all the members have immunoglobulin V type and myelin Po domains. We analyzed the expression pattern of *Mpzl3* both at the transcript level using Reverse transcriptase-PCR and at the protein level using Western blot analysis. We found that *Mpzl3* was expressed in all the investigated organs including brain, esophagus, heart, intestine, kidney, liver, lung, muscle, skin and spleen. Closer examination of the skin by immunofluorescence staining of skin sections using a

polyclonal antibody specific for extracellular domain of MPZL3 revealed that MPZL3 is expressed in the keratinocytes in the epidermis, hair follicles, and sebocytes in the sebaceous glands. By examining staining at high magnifications, it was clear that the staining was strong around the plasma membrane, consistent with the prediction of a transmembrane protein involved in cell adhesion. We also detected staining in the cytoplasm, but not in the nuclei.

To extend our knowledge of MPZL3, in Chapter 3 we addressed the question whether this protein exists only in mammals or is an older evolutionary conserved protein. We were also interested in its domain structure and its possible role in human. Therefore, we analyzed the MPZL3 orthologue proteins in the available public databases and we investigated the sequence and domain structure with web based bioinformatics tools. We identified orthologues of MPZL3 in other mammals with 79–99% identity at the protein level, and also found the protein to be conserved in other vertebrates, including chicken and zebrafish, with 30-55% amino acid identity. All the putative orthologue proteins possess the myelin Po protein signature and immunoglobulin V-Type domain. Based on EST counts in the UniGene database of NCBI, MPZL3 is expressed in wide variety of organs in human. Similar to our expression data in mouse, *MPZL3* expression was detected in human brain, esophagus, heart, kidney, liver, lung, muscle, spleen, and in addition in blood, colon, eye, lymph node, mammary gland, mouth, ovary, parathyroid, pharynx, pituitary gland, prostate, stomach, testis, uterus and vascular tissues. Furthermore, EMBL-EBI Array Express microarray database analysis showed *MPZL3* expression in dendritic cells, and in CD4 and CD8 central memory and effector T cells. The R100Q mutation we identified in rc mice is in the Ig-domain recognition loop that has known functions in T-cell receptors and cell adhesion. The homologous MPZ and MPZL2/EVA1 also play roles in cell adhesion and in the immune response. NCBI Entrez database analysis revealed multiple SNPs and mutations within the MPZL3 gene suggesting that humans with homozygous or compound heterozygous mutations may develop symptoms similar to the anomalies observed in rc mice. To investigate whether MPZL3 has a similar expression pattern in the skin as we observed in mice we performed Western blot analysis and detected a 54 kDa and a fainter 56 kDa band in cultured primary human dermal fibroblasts, which may result from dimerization and/or from posttranslational modifications of MPZL3 (predicted MW 25.98 kDa). Using indirect immunofluorescence we localized MPZL3 in similar regions of the human skin as in the mouse skin (Cao et al., 2007). It can be hypothesized that, based on our in silico data, MPZL3 might be involved in immune-mediated hereditary disorders presenting with hair loss, like alopecia area or alopecia universalis.

As the rough coat point mutation in *Mpzl3* already has strong effects, it is possible that the complete lack of *Mpzl3* function may lead to more drastic changes in the affected organ systems and result in a lethal phenotype in mice. Considering the time investment and the possible outcome of the mouse knock-out experiment, in Chapter 4 we decided first to investigate the *mpzl3* gene function in the zebrafish

embryo model, in which rapid knock-down studies are possible. First we investigated the zebrafish *mpzl3* sequence in the public databases, and analyzed the domain structure of the predicted protein with EBI-InterProScan web based software. We identified its evolutionary related counterparts by phylogenetic analysis using the neighbor joining method and found that closely related genes in zebrafish included *mpz*, *mpzl1*, *eva1*, and *scn4b*. By using quantitative reverse transcriptase PCR (qRT-PCR) we observed high *mpzl3* expression in the embryos within the first 5 hours post fertilization, likely derived from maternal RNA, but at later time points *mpzl3* expression became gradually reduced. We analyzed the localization of *mpzl3* expression by whole mount *in situ* hybridization and recognized specific expression in the head and in neuromasts of the lateral line of larvae at 5 days post fertilization. To investigate the role of *mpzl3* in zebrafish we synthesized *mpzl3* mRNA and injected this into embryos at the 1-2 cell stage. We observed a general retardation of development, bent and shortened tails or edema of the heart cavity in some embryos, but we also observed consistent anophthalmia. Some embryos showed aberrant development of both eyes and others showed asymmetric development of the two eyes, with one developing normally and the other being highly abnormal or completely undeveloped. Based on this unexpected phenotype, we decided to investigate the phenotype of the eye in rough coat mouse and we observed clear eye abnormalities in the mutant too (Figure1). Homozygous rough coat mice have been noted to be prone to develop an eye phenotype that could described in lay terms as "cloudy", or as a whitish haze. It may affect one eye, sometimes both eyes. It has not been investigated whether these mice are able to see with those affected eyes. The onset has not been seen before four months.

To analyze the effect of *mplz3* knock-down on development of zebrafish we used translation blocking morpholinos. The morphant fish obtained with two different morpholinos showed lower survival rates, immobility in response to touch stimuli, and cardiovascular system defects. They also showed a deficiency in the expression of recombination activating gene 1 (*rag1*) in the thymus, a gene essential for the development of the adaptive immune system. The morphant phenotypes could be rescued by co-injection of *mpzl3* mRNA. To identify the molecular pathways underlying the *mpzl3* morphant phenotype we performed a preliminary microarray analysis to analyze the gene expression pattern changes at 8 hours post fertilization, prior to the appearance of the phenotypic abnormalities. Based on gene ontology term analysis of the overlapping gene set showing altered expression with both morpholinos we identified several genes that play a role in developmental processes, including components of the Wnt and Notch signaling pathways. These microarray data provide useful leads for further investigation of *mpzl3* function in zebrafish and mouse models.

In conclusion, in this study the gene responsible for the rough coat phenotype in mouse was identified and shown to encode a member of the Myelin Protein Zero family that we named *Mpzl3*. The hypomorphic mutation in the rough coat mice was

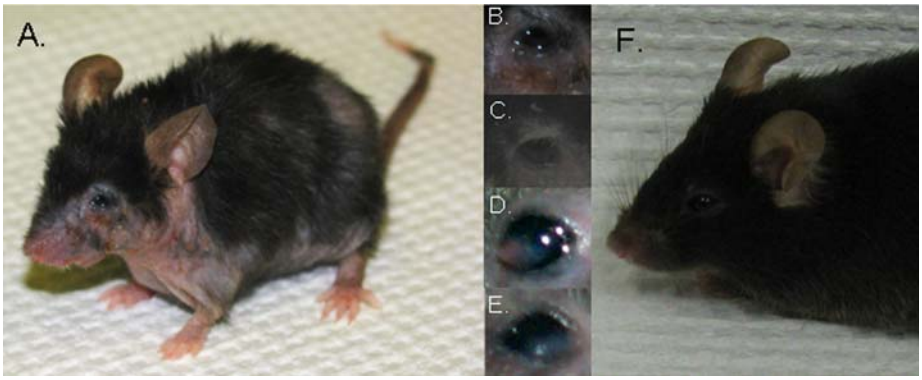

Figure 1. Eye phenotypes of rough coat mice. Figure **(A)** shows a 6 months old rc/rc male mouse with affected eyes. In Figure **(B)** and **(C)** it can be observed that both eyes are affected; however, the appearance of the abnormalities is different. Figure **(D)** and **(E)** shows the cloudiness of the eyes. Figure **(F)** shows a 6 months old rc/+ control male littermate.

shown to be due to an amino acid substitution in the conserved immunoglobulin domain of *Mpzl3* and resulted in severe skin abnormalities. We argue that mutations in the human orthologue of *Mpzl3* may be involved in immune-mediated heriditary hair loss diseases. Furthermore, we identified the *mpzl3* gene in zebrafish and we present here the first functional study of this gene. Our overexpression and loss-of-function experiments indicated a role for *mpzl3* in developmental processes during zebrafish embryogenesis, which based on microarray analysis might involve Wnt and Notch signaling. In future work, it will be of great interest to study the connection of *mpzl3* with these signaling pathways and the possible role of the gene in skin development, myelination and immune functions using zebrafish and mouse models