Molecular Genetics and Mechanisms of Pigmentation and Melanoma

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2010

Acta Universitatis Agriculturae Sueciae
2010:76

Cover: *Dancing Melanocytes*. The beauty of nature in the shape of murine melanocytes in cell culture.
(Photo: Elisabeth Sundström)

ISSN 1652-6880
ISBN 978-91-576-7521-7

Print: SLU Service/Repro, Uppsala 2010

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Abstract

Ever since man domesticated the first wild animals several thousand years ago, many species have been living in close relationship with humans, being objects for our requests and wishes about any kind of phenotypic traits. As a result, we have by selective breeding created a fascinating spectrum of phenotypes within most species we have domesticated. The broad aim of this thesis has been to investigate the molecular mechanisms causing different colour and pattern phenotypes in domestic animals. In more detail, the mutation causing Greying with age and melanoma in horses and the mutations leading to the Sex-linked barring feather pattern in the domestic chicken have been studied.

Grey horses are born any colour, but gradually loose their hair pigmentation and usually become white by the age of 6-8 years. The pigmentation loss only affects the hair, and the skin stays dark throughout life. Melanomas and vitiligo-like depigmentation frequently occur among Grey horses, and it is claimed that 70-80% of Grey horses older than 15 years have melanocytic lesions. The Sex-linked barring pattern in chickens is characterized by black and white stripes on the feathers. Similar barring patterns are found among many bird species, such as the peregrine falcon and the zebra finch. The mutation causing Sex-linked barring has previously been mapped to the q arm of chromosome Z, which explains why the homogametic (ZZ) sex in chickens, males, develop a broader barring pattern.

In this thesis, the mutations causing both traits are identified, and their functional properties are investigated. The mutation causing Greying with age and melanoma in horses was identified as a 4.6 kb intronic duplication in the gene *STX17*, not previously linked either to pigmentation or melanoma. Moreover, it could be shown that the mutation in *STX17* has an elevated copy number in melanoma DNA, and that it contains a strong melanocyte specific activating regulatory element residing a MITF binding site. This finding sheds further light on the intriguing molecular processes of melanoma development. The mutations linked to Sex-linked barring could be mapped to the *CDKN2A/B* tumour suppressor locus, a genetic region commonly associated with familiar forms of melanoma and also implied in the molecular mechanisms of pigmentation regulation.

Keywords: pigmentation, melanoma, horse, hair greying, chicken, Sex-linked barring

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We inhabit ourselves without valuing ourselves, unable to see that here, now, this very moment is sacred; but once it's gone – its value is incontestable.

Joyce Carol Oates

Contents

List of Publications

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

I Rosengren Pielberg G, Golovko A*, **Sundström E***, Curik I, Lennartsson J, Seltenhammer MH, Druml T, Binns M, Fitzsimmons C, Lindgren G, Sandberg K, Baumung R, Vetterlein M, Strömberg S, Grabherr M, Wade C, Lindblad-Toh K, Pontén F, Heldin C-H, Sölkner J, Andersson L. (2008). A *cis*-acting regulatory mutation causes premature hair graying and susceptibility to melanoma in the horse. *Nature Genetics* 40, 1004-1009.
* These authors contributed equally to the work.

II Hellström AR, **Sundström E**, Gunnarsson U, Bed'hom B, Tixier-Boichard M, Honaker CF, Sahlqvist A-S, Jensen P, Kämpe O, Siegel PB, Kerje S, Andersson L. (2010). Sex-linked barring in chickens is controlled by the *CDKN2A/B* tumour suppressor locus. *Pigment Cell & Melanoma Research* 23, 521-530.

III **Sundström E**, Imsland F, Mikko S, Wade C, Sigurdsson S, Rosengren Pielberg G, Golovko A, Sölkner J, Lindblad-Toh K, Andersson L. Copy number expansion of the *STX17* duplication in melanoma tissue from Grey horses. (Manuscript)

IV **Sundström E**, Jiang L, Golovko A, Andersson L. Identification of a melanocyte-specific, MITF-dependent regulatory element in the intronic duplication causing hair greying and melanoma in horses. (Manuscript)

Related Publications by the Author

(Not included in the thesis)

Andersson, L, Andersson G, Hjälm G, Jiang L, Lindblad-Toh K, Lindroth AM, Markljung E, Nyström A-M, Rubin C-J, **Sundström E**. (2010). ZBED6 – the birth of a new transcription factor in the common ancestor of placental mammals. *Transcription* 1. (In press)

The contribution of Elisabeth Sundström to the papers (I – IV) included in this thesis is as follows:

I Responsible for genotyping the Lipizzaner population material, genotyped part of the markers in the Grey haplotype and analyzed allelic imbalance in melanoma tissue.

II The main person responsible for transcription factor binding site analysis, EMSA analysis, cell culture experiments and luciferase assays.

III Genotyped and further investigated the selected polymorphisms in the resequenced Grey haplotype, genotyped horses, designed and ran the copy number assay.

IV Responsible for the experimental design, performed luciferase reporter assays, bioinformatic analyses and site-directed mutagenesis.

Abbreviations

ACTH	Adrenocorticotropic Hormone
α-MSH	α-Melanocortin Stimulating Hormone
ARF	Alternative Reading Frame
ASIP	Agouti Signalling Protein
BRAF	v-raf Murine Sarcoma Viral Oncogene Homolog B1
cAMP	cyclic Adenosinemonophosphate
CDKN2A/B	Cyclin Dependent Kinase 2
cDNA	complementary Deoxyribonucleic Acid
CMV	Cytomegalovirus
DNA	Deoxyribonucleic Acid
E	Embryogenic Day
E2F1	E2F Transcription Factor 1
EDN3	Endothelin 3
EDNRB	Endothelin receptor B
EMSA	Electrophoretic Mobility Shift Assay
ERK	Extracellular-Signal-Regulated Kinase
ERP44	Endoplasmic Reticulum Protein 44
GFP	Green Fluorescent Protein
HSV-1 TK	Herpes Simplex Virus type 1 Thymidine Kinase
IBD	Identical By Descent
INK4A	Cyclin-Dependent Kinase 4 Inhibitor A
INVS	Inversin
kDa	kilo Dalton
kb	kilo bases
Mb	Mega bases
MC1R	Melanocortin 1 Receptor
MDM2	Mdm2 p53 Binding Protein Homolog (Mouse)
MITF	Microphthalmia-associated Transcription Factor

Nor-1	Neuron-derived Orphan Receptor 1 (protein from NR4A3)
NR4A3	Nuclear Receptor Subfamily 4, group A, member 3 (gene)
NRAS	Neuroblastoma RAS Viral (v-ras) Oncogene Homolog
p53	Tumour Protein 53
PAX3	Paired-box 3
POMC	Pro-Opiomelanocortin
RAS	Rat Sarcoma Viral Oncogene Homolog (gene family)
RB	Retinoblastoma Protein
ROS	Reactive Oxygen Species
SCP	Schwann Cell Pecursor
SNP	Single Nucleotide Polymorphism
SOX10	Sex-determining Region Y-box 10
STX17	Syntaxin 17
SV-40	Simian Virus-40
TXNDC4	Thioredoxin Domain–Containing-4 (endoplasmic reticulum) (currently known as Endoplasmic Reticulum Protein 44)
TYR	Tyrosinase
UTR	Untranslated Region
UV	Ultraviolet

Aims of this Thesis

The broad aim of this thesis has been to investigate the molecular mechanisms causing different colour and pattern phenotypes in domestic animals. The specific aims have been to:

I Characterize and functionally dissect the mutation causing Greying with age and melanoma in horses. (Papers I, III and IV)

II Elucidate the functional properties of the mutations in the *CDKN2A/B* locus causing the black and white Sex-linked barring feather pattern in the domestic chicken. (Paper II)

Introduction

Why study pigmentation in animals?

Since man domesticated the first wild animals several thousand years ago, predominantly in the fertile crescent of the Middle East, a continuous selection for many different traits has been performed. Through domestication, many animals have been living in close relationship with humans, becoming objects to mould phenotypically; from body size to coat colour or length of the nose or tail. By selective breeding, we have created a spectrum of phenotypes within most species we have domesticated. As an example, there are dogs varying in size from as tiny as a rat to as big as a small pony, and their fur colour and pattern can range between completely black, golden, white, spotted or patched. The fact that we see a large phenotypic variation indicates that the molecular pathways resulting in every trait must show biological differences from each other. By studying the molecular mechanisms behind phenotypic variants in animals, we can reach interesting conclusions about the basic molecular processes regulating size, colour and pattern. Additionally, mammals, including humans, have many genes in common, and consequently, many signalling pathways are conserved between different species and are operated in similar manners. By studying the phenotypic variation in animals, it is therefore possible to pinpoint molecular processes and key genes that play a similar role also in humans.

Melanocyte biology and the molecular basics of pigmentation

Pigment cell development

The cell type responsible for producing pigment, the melanocyte, is derived from the neural crest and migrates into various body tissues during embryonic development (Mayer, 1973). Recent findings also indicate that many skin melanocytes may derive from Schwann cell precursors (SCPs), which reside along nerve cells throughout the body (Adameyko et al., 2009). The neural crest origin is mirrored in the dendritic shape of the melanocyte, which highly resembles the stretched structure of a neuron.

During embryogenesis in mice, melanoblasts differentiate from pluripotent neural-crest cells at about embryogenic day (E) 8.5, migrating along the dorsolateral pathway and eventually proceeding ventrally through the dermis (Lin and Fisher, 2007). From the dermis, the melanocytes continue their migration to the epidermis at day E14.5, and at this point they also populate the developing hair follicles (Lin and Fisher, 2007).

Many different signaling pathways and transcription factors tightly regulate melanocyte development and migration. Some of the most crucial genes for melanocyte development include *PAX3* (paired-box 3), *SOX10* (sex-determining region Y (SRY)-box 10), *MITF* (Microphthalmia-associated transcription factor), *EDN3* (endothelin 3) and *EDNRB* (endothelin receptor B). Mutations in the key genes governing melanocyte development are manifested as hypopigmentation; a consequence of the lack of melanocytes, rather than the lack of pigment in viable melanocytes, which is the case in albinism (Lin and Fisher, 2007).

Inside the melanocyte, the pigment is produced in a specific organelle, the melanosome. There are two main types of melanin molecules produced in the mature melanocyte: the red/yellow pheomelanin and the brown/black eumelanin. The two different types of melanin differ in several other aspects in addition to their colour, ranging from shape and size to the packaging of their granules (Slominski et al., 2004).

Melanocytes in different tissues

We may only think of the melanocyte as a "one-purpose" cell type, being present in our skin and hair follicles where it gives the skin and hair colour. However, melanocytes and melanin can be found in many different parts of the body, where they pursue a wide variety of different functions (Figure 1). In addition to the skin and hair follicles, melanocytes reside in the heart,

where they play an anti-inflammatory role and protect the tissue against reactive oxygen species (ROS), in the inner ear and cochlea, where they affect balance and hearing, in the adipose tissue, where they promote cellular survival by reducing ROS and in the eye, where they protect against UV-radiation (Plonka et al., 2009). In the rare lung disease lymphangioleiomyomatosis, the hyperproliferating smooth muscle cells have been shown to contain premelanosomes, and to express the melanocyte-specific proteins PMEL17, TYRP1 and TYRP2 (Klarquist et al., 2009). One interpretaion of this phenomenon could be that these cells revert towards their developmental origins and express the melanocyte-specific markers (Klarquist et al., 2009, Plonka et al., 2009).

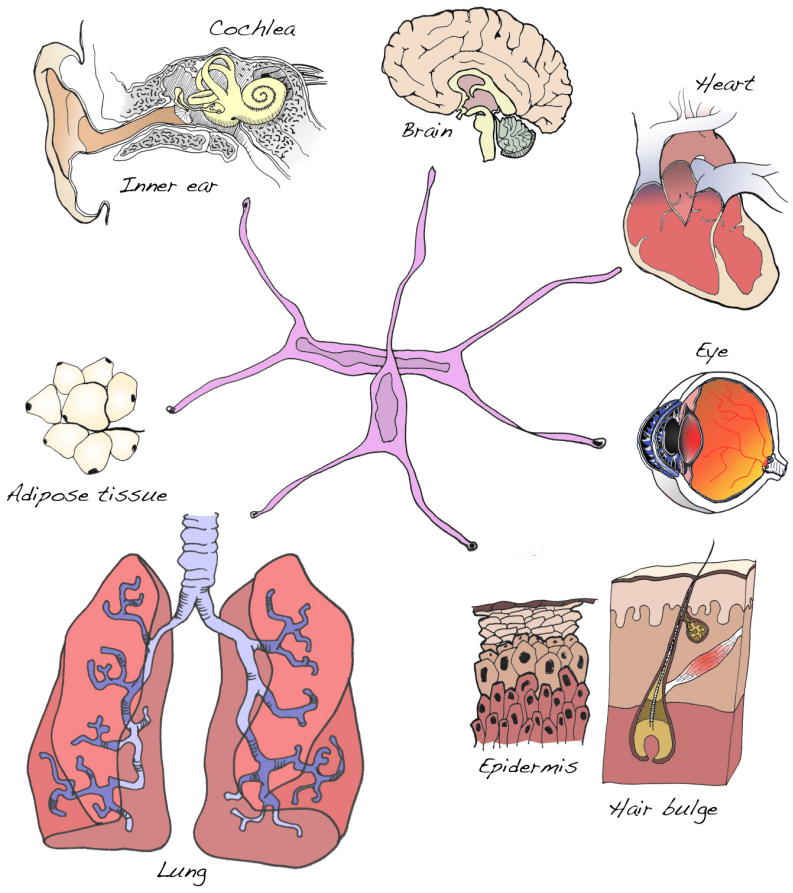

Figure 1. Different locations of melanocytes and/or melanin in the body. Illustration by Anders Sundström, modified from Plonka et al., 2009.

In the brain, melanocytes reside in the leptomeninges, a membrane sourrounding the brain and spinal cord, whilst a specific melanin molecule, called neuromelanin, exists in the dopaminergic neurons of the substantia nigra (Nordlund et al., 2006). Neuromelanin consists of a eumelanin covered pheomelanin core and is thought to be an auto-oxidative product of dopamine, generated by tyrosine hydroxylase synthezised in the neurons (Nordlund et al., 2006). Neuromelanin accumulates with age, and the function could be to remove ROS and metals that are highly toxic to neurons (Zecca et al., 2002). Neuromelanin has been detected in several mammals in addition to humans, namely gorillas, gibbons, baboons, horses and sheep (Marsden, 1961). In Parkinson's disease, the melanized neurons of the substantia nigra are more vulnerable to degeneration than the non-melanized ones, and the loss of dopaminergic neurons containing neuromelanin is highly associated with the disease (Zecca et al., 2002).

Regulation of melanogenesis

To date, more than 100 loci have been shown to be involved in mouse pigmentation (Bennett and Lamoreux, 2003). The seven-transmembrane G-protein-coupled melanocortin 1 receptor (MC1R) is one of the major determinants of pigmentary phenotypes. The extension locus (*Mc1r*$^{e/e}$), with mutants showing a light red coat colour and wild type individuals being black, was first identified in mice (Robbins et al., 1993). Since then, mutant alleles in homologs have been identified in many mammals, spanning from the extinct mammoths (Rompler et al., 2006) to extant living dogs (Everts et al., 2000, Newton et al., 2000), horses (Marklund et al., 1996) and humans (reviewed in Sturm, 2002).

The production of pigment starts with external signals acting on the melanocyte, and MC1R located in the membrane of the melanocytes serves as the main mediator of these signals (Figure 2). MC1R has two major agonists; the melanocortin peptide α-melanocortin stimulating hormone (α-MSH) that is cleaved from the pro-opiomelanocortin precursor (POMC) and the adrenocorticotropic hormone (ACTH), which originates from the same precursor. Agouti signalling protein (ASIP) functions as an antagonist to MC1R (Furumura et al., 1996).

When α-MSH or ACTH interacts with MC1R, the intracellular levels of cyclic AMP (cAMP) are elevated, and this results in an increase in tyrosine expression and eumelanin production (Barsh et al., 2000, D'Orazio

et al., 2006). Tyrosinase (TYR) is needed to catalyze the oxidation of tyrosine, producing dopaquinone, the precursor of both eumelanin and pheomelanin. High levels of cAMP also increases the expression of the tyrosinase-related enzymes TYRP1 and TYRP2, which catalyze steps in the oxidative pathway leading from the precursor dopaquinone to eumelanin. ASIP antagonizes the binding of α-MSH to MC1R, resulting in a shift from eumelanin to pheomelanin production, as the intracellular levels of cAMP are lowered (Barsh, 1996).

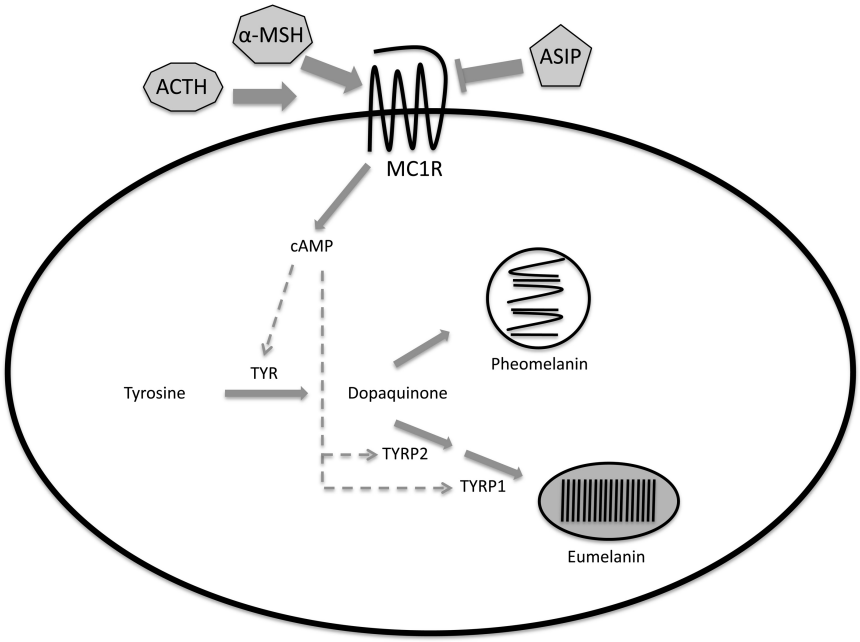

Figure 2. Regulation of melanogenesis in the melanocyte. Illustation by Elisabeth Sundström, modified from Barsh, 1996.

Melanogenesis in skin and hair

Both hair and skin contain melanocytes that provide the tissues with pigment. But the presence of one cell type in different tissues does not automatically suggest equal regulation of the cell populations, not even if they are as closely located as hair and skin.

Most of the melanocytes in the skin reside in the basal epidermis where the pigment is transferred to neighbouring keratinocytes by melanosomes. Melanocytes can also be present in the dermis, but to what extent varies

between mammalian species, between glabrous and non-glabrous skin and also between human ethnical groups (Nordlund et al., 2006). The melanocytes in the hair follicle are distinct from those in the skin, and are derived from a pool of pigment stem cells in the hair bulb (Figure 3).

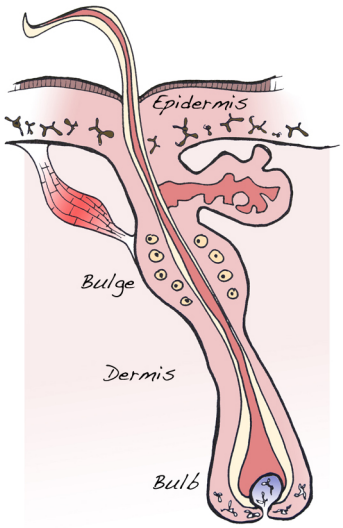

Figure 3. The epidermis, dermis and hair follicle. Mature melanocytes reside in the epidermis of the skin, occasionally also in the dermis, and in the hair bulb. Pigment stem cells mature from the bulge and migrate towards the bulb where they eventually enter the hair. Illustration by Anders Sundström, modified from Lin and Fisher, 2007.

Several studies have in the last decade indicated an independent regulation of skin and hair melanocytes (Fitch et al., 2003, Rosengren Pielberg et al., 2008, Van Raamsdonk et al., 2009). This was not entirely surprising, since the melanocyte populations are distinctly separated between hair and skin. Fitch and colleagues (2003) showed by the characterization of the ten mouse dark skin (Dsk) mutants that epidermal and dermal melanocyte populations could be regulated independently by a distinct set of genetic pathways (Fitch et al., 2003). This work was expanded by Van Raamsdonk and colleagues (2009), and the closer investigation showed that three specific mouse mutations in two different G-protein-coupled signalling pathways, the endothelin signalling pathway and the MC1R signalling pathway, affect hair and skin colour differently, and these mutations also have different outcomes in dermis versus epidermis. The two mutations affecting the endothelin signalling pathway are found in two closely related α-subunits of the

EDNRB receptor in melanocytes. These mutations darken hair and skin colour through different mechanisms: in the dermis, darkening is caused by an increased number of melanocytes, while in the hair, darkening is caused by an increase in pigment production, independent of the number of pigment cells. In turn, the *Mc1r*^{e} mutation does not couple the synthesis of eumelanin with pheomelanin in the epidermis as it does in hair follicles (i.e. when MC1R is blocked pheomelanin is produced, and when MC1R is active, eumelanin is produced), since eumelanin concentration is reduced in the tail epidermis, but pheomelanin is not increased (Van Raamsdonk et al., 2009). These findings would indicate that the production of pheomelanin in the epidermis is not regulated by MC1R or Agouti.

Melanoma

It may seem contradictory that the melanocytes, while playing an important role in protecting the skin from harmful UV-radiation, are also the precursors for one of the most common types of cancer today: melanoma. Out of 50000 diagnosed cancer cases in Sweden, 2000 concern malignant melanoma (Edqvist, 2008). The incidence of the disease is steadily rising in the western parts of the world, and during the last 20 years the number of cases has doubled (Gray-Schopfer et al., 2007). If diagnosed early, melanoma can be surgically removed, but in the later stages where metastases have occurred, the prognosis is very poor and the median survival time is estimated to only six months (Cummins et al., 2006). The fact that melanoma has a continuously increasing incidence, underlines the importance of investigating the basic molecular mechanisms regulating pigment production and pigment cell proliferation and differentiation.

Melanoma in animals

Melanoma is not a malignancy restricted only to humans, it occurs naturally in several other species like horses (Lerner and Cage, 1973), cows (Miller et al., 1995), sheep (Smith et al., 2002), goats (Parsons et al., 1990, Hamor et al., 1999), swine (Millikan et al., 1973, Oxenhandler et al., 1979), dogs (Cotchin, 1955, Conroy, 1967), cats (Luna et al., 2000), opossums (Robinson et al., 2000) and birds (Saunders and Saunders, 1991). Many domestic animals used predominantly for meat production, e.g. cows, sheep

and swine, are slaughtered at an early age before they develop melanocytic tumours, whilst horses, cats and dogs usually have longer life spans and live long enough to develop melanomas. Some animals are also used as laboratory models to study the molecular mechanisms of melanoma development, e.g. zebrafish (Ceol et al., 2008), rat and mouse, but in these species the melanoma is induced and not naturally occurring. While the predominating cause of melanoma in humans is mutations caused by UV-radiation, most melanomas in animals seem to have a strong genetic background. The alpaca goat and the opossum could be two exceptions where UV-induced melanoma has been found (Parsons et al., 1990, Robinson et al., 2000). The genetic predisposition to melanoma among certain animals makes them a valuable resource for identifying genes and mutations involved in the development and progression of melanoma.

Horse melanoma

Melanoma in horses commonly occurs among the so called Grey horses, which are born coloured, but become white by the age of 6-8 years. This process could be compared to the greying of human hair. The pigmentation loss only affects the hair, while the skin stays dark throughout life, indicating different cellular fates for the melanocytes in the hair follicle versus the skin. The Grey horse melanomas occur in many different breeds, usually in the later stages of life. It is generally claimed that 70-80% of Grey horses older than 15 years have melanomas (Sutton and Coleman, 1997, Fleury et al., 2000), and that the lesions start to develop early, by the age of 5-6 years (Lerner and Cage, 1973). The primary tumours arise in the dermis of the glabrous skin and are usually benign. Metastases, or lesions in different parts of the body like in skeletal muscle, heart, abdominal fat and lymph nodes, do occur (Figure 4), but as indicated by Sutton and Coleman (1997), the presence of tumours at these sites gives the impression of multicentric origins rather than metastases from a solitary primary tumour. The authors also noted that there is some correlation between the pattern of lesion distribution and the likely paths of neural crest cells as they migrate towards the skin during embryonic development.

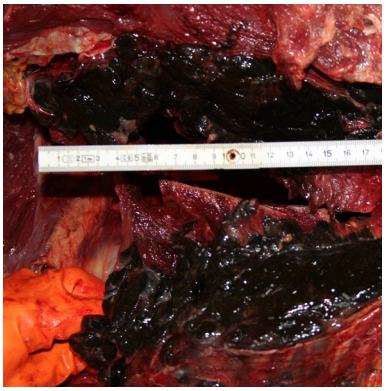

Figure 4. An internal tumour from a horse euthanized due to melanomas. The tumour is located in the shoulder cartilage and spans 18 cm. Photo by Monica Seltenhammer.

The tumours appear as black pigmented, hemispherical-shaped, well isolated nodules, and they are most frequently found in regions lacking hair, like underneath the tail, around the anus, perineum or genitalia, and occasionally also in the lips and the eyelids (Fleury et al., 2000). The tumours can grow big as an apple in size, and when cut into, the highly melanized content drips like black ink.

Grey horses with melanomas also develop vitiligo-like skin depigmentation, commonly seen on the muzzle and under the tail (Lerner and Cage, 1973). A possible cause for the depigmentation could be an auto-immune reaction against all melanocytes due to the tumour development. Melanocytic lesions also occur in non-Grey horses, but are very uncommon, and seldom show the same aggressiveness and growth ability as Grey horse melanomas. As shown by Rosengren Pielberg et al. (2008), the incidence of melanomas in Grey horses is significantly correlated to the presence of a 4.6 kb non-coding duplication in *STX17*. Melanomas can also occur in horses of the colour cremello, characterized by pink skin, blue eyes and creamed coloured or white hair. These horses have a very weak melanin production, but the ones with the duplication in *STX17* still get melanoma (Paper III). This fact supports the theory that the mutation is responsible for the development of tumours.

Genetics of melanoma

Both environmental and genetic factors contribute to the risk of developing melanoma. In humans, light skin colour, the inability to tan, blue eyes (Gilchrest et al., 1999) and the presence of many freckles and moles (Gandini et al., 2005) are correlated with an increased risk of melanoma

development. However, many individuals fulfilling the physical criteria never develop melanoma, while others not fulfilling them have a strong familial record of melanoma. This demonstrates that in certain types of melanoma, there is a genetic predisposition to the disease.

The *CDKN2A* locus has been denoted "the familial melanoma locus", due to its strong association to the familiar forms of the disease. Loss-of-function mutations in *CDKN2A* have been shown to be a major risk factor for familial forms of melanoma in humans (Randerson-Moor et al., 2001, Goldstein et al., 2008). *CDKN2A* encodes two different proteins, INK4A and ARF, through the usage of alternative promoters and first exons. Both proteins have crucial functions in cell cycle progression by indirectly regulating two tumour suppressors: the retinoblastoma protein (RB) and p53. INK4 inhibits cyclin-D-dependent kinases, causing cell cycle arrest in the G1 phase. The ARF-MDM2-p53 network both regulates the cell cycle and transition from G1 to S-phase and directs the cell to apoptosis (Quelle et al., 1995). When INK4A is lost, the cell cycle progression is consequently allowed to continue in an uncontrolled manner, and when ARF is lost, this results in the inactivation of the p53 pathway.

Two other genes of major importance in melanoma are *NRAS* and *BRAF*. *BRAF* has been shown to be mutated in 50-70% of human melanomas while *NRAS* is mutated in between 15-30% (Davies et al., 2002). The most common mutation in *BRAF* is the glutamic acid for valine substitution at position 600 (V600E), and in *NRAS* the replacement of leucine for glutamine at position 61 (Q61L) (Davies et al., 2002). Mutations in both *BRAF* and *NRAS* result in the activation of the ERK pathway, leading to increased cell proliferation and survival (Eskandarpour et al., 2005, Gray-Schopfer et al., 2005). *BRAF* is also mutated in up to 80% of benign naevi (Pollock et al., 2003), but the progression of naevi to melanoma is a rare event and mutated *BRAF* alone is not sufficient for initiating melanoma development (Gray-Schopfer et al., 2007).

Gene expression and regulation

Most cells within an organism have the same set of genes, yet the many cell types look very different. Melanocytes are small, brown, black, or yellow/red and have long, graceful arms, while erythrocytes, the red blood cells, are big, bright red and round and lack a nucleus. The gene setup is not the only factor determining the appearance of the cells, but what makes the

differences is which genes are expressed. Every cell type expresses a specific set of genes essential for its function and phenotype, and the expressed genes can also vary during different time points in the same cell type.

Non-coding DNA and regulatory elements

The regulation of gene expression is governed at many levels and by different means. Thirty years ago, the non-coding part of the genome was believed to be "junk DNA" with no dedicated function, a term introduced in 1972 by Susumu Ohno (Ohno, 1972). In 1980, Leslie Orgel and Francis Crick wrote in a review in Nature that junk DNA has "little specificity and conveys little or no selective advantage to the organism" (Orgel and Crick, 1980). Today, we know that much of the non-coding part of the genome is very important, since non-coding regulatory elements that regulate gene expression, both on the transcriptional and translational level, can be found there. Some non-coding regions could be of minor importance for the viability of organisms, as shown by Nobrega et al. (2004) through the deletion of megabases of non-coding DNA in mice, an experiment which did not rend any visible effects on the phenotype.

The non-coding part of the genome contains promoters, enhancers and repressors that regulate the expression of genes by binding specific transcription factors. The promoters are typically found directly upstream of a gene, while enhancers and repressor elements can be found both in the introns and in the near vicinity of a gene, but also megabases away. Transcription factors binding to regulatory DNA-elements and other factors interact with genes and the RNA polymerase machinery in order to regulate the transcription process. From this, it is determined how much gene product, protein, will be produced.

Luciferase assays

Why use glowing genes?

Reporter gene assays are widely used in cell biology to study gene expression or other cellular activities associated with gene expression. Two of the most commonly used reporter genes, Green Fluorescent Protein (GFP) (Shimomura et al., 1962, Chalfie et al., 1994, Heim et al., 1995) and firefly luciferase (de Wet et al., 1987), have been brought to laboratories

from nature, where in their native states these chemicals make jellyfish (*Aequorea victoria*) and fireflies (*Photinus pyralis*) glow. In the lab, the expression of reporter genes in eukaryotic cells is highly optimized, making it possible to fuse the DNA segment of interest with the reporter gene and to closely follow the effect on gene expression by measuring the fluorescence or luminescence, respectively.

The luciferase assay is a sensitive and easily measured method for exploring differences in gene expression. Two kinds of luciferases are used in a typical dual luciferase assay: the firefly luciferase and sea pansy (*Renilla reniformis*) luciferase. The substrate for *Renilla* luciferase is different from the substrate for firefly luciferase, and because of this, cross activation between the reactions catalyzed by the different luciferases cannot occur. The vector expressing the *Renilla* luciferase has consequently been established as a control vector cotransfected with the experimental firefly luciferase vector in order to normalize for differences in transfection efficiency, number of transfected cells or sample handling.

How to perform a luciferase assay

Both the firefly and the *Renilla* luciferase gene can be found in a variety of commercially available vectors, suitable for different aspects of gene expression studies, i.e. for investigating the effect of promoters, enhancers or other regulatory elements. The DNA fragment to be studied, often 100–2000 nucleotides long, is cloned in front of the luciferase gene into the vector, and if necessary combined with promoters or regulatory elements. When the vector is transfected into any kind of mammalian cells, a 61 kDa protein is translated from the luciferase gene, and the amount of this protein is regulated by the DNA fragment cloned in front of the gene. Between 24–48 hours after the transfection, the cell walls are destroyed so that the luciferase protein can be collected in a solution. Luciferase catalyses the bioluminescent reaction where beetle luciferin is converted to oxyluciferin and light. As such, beetle luciferin needs to be added to the solution in order for light to be produced. The luminescence is used as a measurement of the amount of luciferase produced from the vector with the cloned DNA fragment.

Performing the optimal luciferase experiment

Despite the relative simplicity of the assay, there are several points that need to be carefully considered and optimized when designing a luciferase experiment. Care has to be taken with respect to insert length and fragment composition. Too long inserts (over 2000 nucleotides) often give results that are hard to interpret, since many different regulatory regions in the insert could have synergistic effects on the expression of luciferase.

Another very important aspect in every experiment is to optimize the type of *Renilla* vector to be used. The *Renilla* luciferase gene can be found in combination with several different promoters, e.g. herpes simplex virus type 1 thymidine kinase (HSV-1 TK) promoter, simian virus-40 (SV-40) promoter and the cytomegalovirus (CMV) promoter, or without any promoter, but still with a low, basal expression from the gene. Different promoters have different activities in different cell types, and primarily a stable and sufficiently high expression from the *Renilla* gene must be ensured. A common problem with promoter-based *Renilla* vectors is the possibility of transactivation of the promoter, either from factors common in the cell type in use, or from your experimental vector or any factor cotransfected and expressed with your experimental vector. The transactivation results in a higher *Renilla* luciferase value than would normally have been observed, and often the transactivation, if occurring, could be different between different experimental vectors in the same assay. Some factors can also have a suppressing effect on the promoters in the *Renilla* vectors. Several studies have pointed out the effects of transactivation of promoter based *Renilla* control vectors, for example in the case of factors as Nor-1 (NR4A3) (Matuszyk et al., 2002) and Ras (Behre et al., 1999). Often this problem can be circumvented by using the promoterless *Renilla* vectors, provided that the luciferase gene is expressed to a satisfactory level in the cell type used.

Present Investigations

Identification and characterization of the mutation causing Greying with age and melanoma in horses (Paper I, III and IV)

Background

The Grey phenotype in horses has fascinated humans for several thousand years, judging from early historical records of the phenotype. Grey horses are mentioned in European, Asian and Native American mythology, and one of the oldest written records of the presence of white horses is by the Greek historian Herodotus, who describes the Persian emperor Xerxes (in reign from 485 to 465 BC) as keeping sacred white horses (Herodotus, 7.40) and another Persian emperor Cyrian being accompanied by white horses when he conquered Babylon (Herodotus, 1.189). Throughout history, the white horse has been commonly used to manifest purity, power and status for its owner or rider, and as a consequence of this, the phenotype has been favoured in breeding over hundreds of generations. Today, white horses are still very popular and exist in most breeds around the world, in particular within the Lipizzaner breed used in the Spanish riding school in Vienna, and also in Arabian horses.

Horses that become grey are born any colour, but they gradually loose the hair pigmentation and finally become white by the age of 6-8 years. The pigmentation loss only affects the hair, and the skin stays dark throughout life (Figure 5A). Melanomas are frequently occurring among Grey horses, usually in the later stages of life. It is generally claimed that 70-80% of Grey horses older than 15 years have melanomas (Sutton and Coleman, 1997, Fleury et al., 2000), and that the lesions start to develop early, by the age of 5-6 years (Lerner and Cage, 1973). Many Grey horses also develop vitiligo-

like skin depigmentation, commonly seen on the muzzle and under the tail (Lerner and Cage, 1973). A characteristic for many grey horses is spots of dark hair in the white fur. A horse with these spots is called "flea-bitten", due to the illusionary pattern of dark flies on the horse (Figure 5B). Some Grey horses develop large patches of red pigmentation in so called "blood marks" (Figure 5C).

Figure 5. Greying with age in horses. A. Partially paralyzed Grey horse diagnosed with multiple internal melanomas. The shaved areas show the dark skin pigmentation. B. Grey horse with characteristic speckling (small spots of pigmented hair, also called "flea-bitten" Grey). Photos by Monika H. Seltenhammer. C. Grey horse with red "blood marks". Photo by Emilie Kajle.

The Grey-causing mutation was assigned to horse chromosome 25 by three independent research groups in 2002 (Henner et al., 2002, Locke et al., 2002, Swinburne et al., 2002) and was later fine-mapped to a region corresponding to 6.9 Mb on human chromosome 9q (Pielberg et al., 2005). In paper I, we used more than 800 horses from 14 different breeds to narrow down the region with the mutation to 352 kb on horse chromosome 25, and subsequently also to identify the causative mutation to be a 4.6 kb intronic duplication in the *STX17* gene. This gene had not previously been linked to pigmentation. In paper III, we resequenced the entire 352 kb Grey haplotype, and excluded the occurrence of any additional mutations linked to the phenotype. In the same paper, we also showed a difference in copy number of the duplication between constitutional and tumour DNA, with the highest copy number in tumours classified as aggressive. The functional properties of the Grey duplication were investigated in paper IV, where with luciferase assays, we identified a strong melanocyte specific regulatory element in the duplication. This region contains a binding site for the transcription factor MITF, important for the development of melanocytes from stem cells. In summary, papers I, III and IV in this thesis describe the journey from fine-mapping and identification, to functional characterization of the mutation causing hair greying and melanoma in the horse.

Our initial hypothesis was that all Grey horses had inherited the mutated *G* (Grey) allele from a common ancestor, since the phenotype has been favoured in selective breeding for hundreds of generations. SNPs (single nucleotide polymorphisms) in the previously assigned 6.9 Mb region were then identified and screened on a panel of Grey and non-Grey horses. The SNPs in the crucial 350 kb interval showed complete linkage disequilibrium with the Grey phenotype across eight breeds, while SNPs flanking the interval did not, implying that historical recombination events had occurred and that regions outside these flanking markers could be excluded. The 350 kb Grey haplotype is surprisingly large, given that our material included populations as divergent as Icelandic and Arabian horses which have been separated for at least 1000 years. This implies a low rate of recombination in the region, which was also indicated in the previous linkage study (Pielberg et al., 2005). The conclusion from this was that the causal mutation was located in this interval, and that all 700 Grey horses, tested from eight different breeds, had inherited the mutation from a common ancestor. Interestingly, one non-Grey haplotype identical to the Grey haplotype for all tested SNPs, but not containing the duplication, was found in one Arabian horse. It is possible that this haplotype represents the "ancestral" haplotype for Grey, the haplotype present in the horse population before the occurrence of the mutation.

The 350 kb Grey haplotype contained four genes: *NR4A3* (nuclear receptor subfamily 4, group A, member 3), *STX17* (syntaxin 17), *ERP44* (endoplasmic reticulum protein 44, formerly known as *TXNDC4*: thioredoxin domain–containing-4′) and *INVS* (inversin). None of these genes had previously been indicated in any pigmentation disorder, making them interesting candidate genes for closer investigation. The four genes were first analyzed by Northern blot and quantitative PCR using liver and muscle tissue from one Grey and one non-Grey horse, and melanoma tissue from a heterozygous and a homozygous Grey horse. The results showed that all four genes were expressed in melanomas, and no variant transcripts unique to Grey horses were detected. However, the *NR4A3* expression in Grey melanoma tissue was strikingly high, compared to Grey and non-Grey muscle and liver, and compared to the other three genes. Southern blot analysis of genomic DNA, using full coding regions as probes, revealed no polymorphisms for *NR4A3*, *ERP44* or *INVS*, but a 4.6 kb insertion was present in *STX17* in Grey horse samples. Long-range PCR revealed that the insertion was a duplication located in intron 6 of *STX17*. By sequencing the intron, the exact position of the duplication could be determined, and a

diagnostic PCR-based test for the duplication was developed to screen more than 800 Grey and non-Grey horses from 14 different breeds. The duplication was detected in all Grey horses but in no non-Grey horses, and the statistical analysis of the genotype-phenotype correlation for close to 700 Lipizzaner horses showed that horses homozygous (*G/G*) for the mutation showed more rapid greying and were more homogenously white in the final stage of the process compared with Grey heterozygotes (*G/g*) (Figure 6). Homozygote Grey horses also had significantly higher incidence of melanoma and vitiligo and almost no speckling. We also tested if Grey horses carrying the loss-of-function mutation in *ASIP* were more prone to melanoma, and the statistical analysis of horses older than six years showed a highly significant association between *ASIP* genotype and the incidence of melanomas. This finding implies that increased MC1R signaling promotes melanoma development in Grey horses.

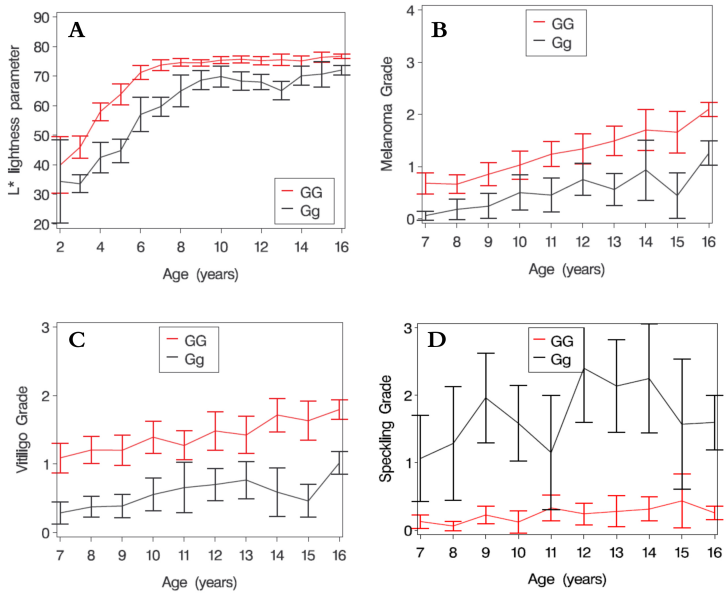

Figure 6. Analysis of phenotypic differences between heterozygous (*G/g*) (black lines) and homozygous (*G/G*) (red lines) Grey Lipizzaner horses. A. Degree of lightness, measured as light reflectance. B, C and D. Grade of melanoma, vitiligo and speckling.

Since the *STX17* duplication is located in intron 6, just upstream of the initiation of a short alternative transcript of the gene, we also aimed at studying the relative expression of the long and short transcripts of *STX17* in various tissues from Grey and non-Grey horses by quantitative PCR. The

long and the short form showed very similar expression patterns, and both showed high expression in Grey melanomas compared with liver and skin from both Grey and non-Grey horses. To study the differential expression more closely, we quantified the relative expression of alleles in melanomas from three *G/g* horses using SNPs located in the 5'UTR of the short transcript (this analysis was not possible for the long form due to the lack of suitable polymorphisms). cDNA sequences from all three melanoma samples revealed expression of only one allele, indicating differential expression of the short isoform. The same analysis was performed with *NR4A3* cDNA from two *G/g* horses, and this revealed *NR4A3* expression from only the Grey haplotype, indicating that a *cis*-acting regulatory mutation was underlying the upregulation of the expression. Genomic DNA from the same individuals was used as a control to ensure that the differential expression was not due to chromosome loss in the tumor.

In this study, we also showed that the 4.6 kb intronic duplication that causes greying with age in horses, only results in pigmentation loss in the hair, while the skin stays dark throughout life. Actually, the dermis of these horses, which go on to develop melanoma, instead accumulates melanocytes. Here, the exact mechanism by which the pigment loss and melanoma development occurs is not known, but the significant correlation of both phenotypes with the mutation shows that the hair and skin melanocytes in Grey horses are regulated independently.

Results and Discussion: Paper III

Here, we wanted to investigate whether the duplication in *STX17* showed copy number variation among Grey horses, since duplications are known to be genetically unstable, and we also wanted to exclude that other polymorphisms than the duplication were associated with *Grey* by using massively parallel resequencing of the Grey haplotype.

In order to investigate the possible copy number variation, we used a TaqMan Copy Number Assay approach where we designed test probes to quantify sequences inside and outside the duplicated region, and also over the 5'end of the duplicated sequence. First, 94 homo- and heterozygous Grey Lipizzaner horses were tested for the copy number of the duplicated sequence. Secondly, we analyzed DNA both from tumours and from several equine melanoma cell lines, When screening copy number expansion in constitutional DNA (Figure 7A), we found only one horse out of about 90 with an estimated copy number of 5 or higher, whereas the corresponding

figure for tumour DNA was 5 out of 8 (samples marked by asterisk in Figure 7B) ($P = 5x10^{-6}$; Fisher's exact test). In tumour DNA from one Grey horse and from two horse melanoma cell lines, the copy numbers were estimated to 3, as expected from their *Grey* genotypes. The elevated copy number in tumour DNA compared with constitutional DNA demonstrates the first set of proof for the duplication in *STX17* being a driving force in melanoma development in Grey horses.

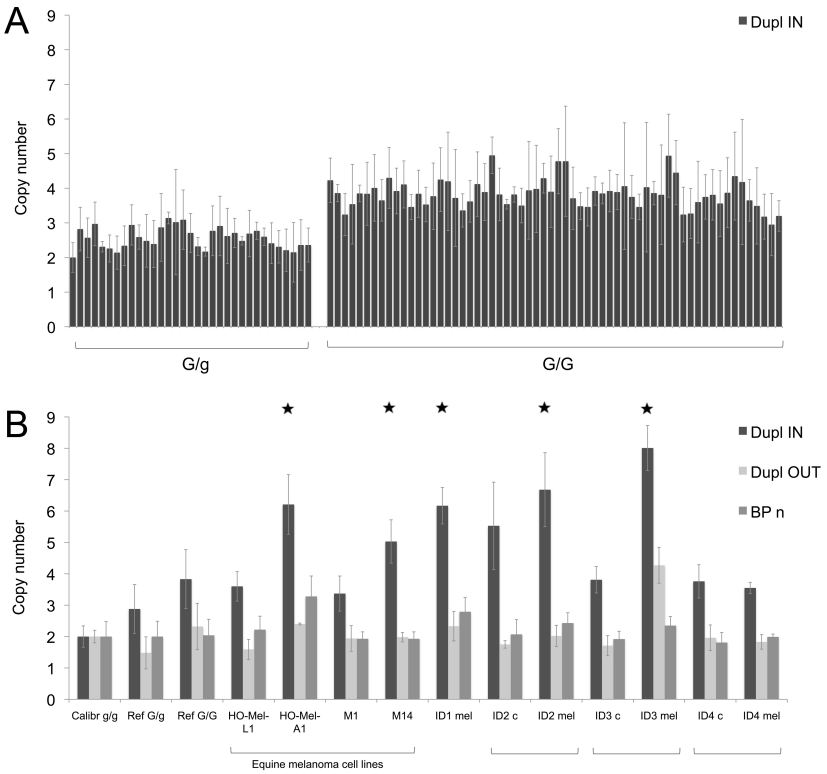

Figure 7. Copy number variation of the *STX17* duplication. (A) Copy number assay for the *STX17* duplication in constitutional DNA from 94 Grey Lipizzaner horses and one calibrator sample with known copy number of 2. (B) Copy number assay for the *STX17* duplication in constitutional DNA and/or melanoma DNA using three different probes. c = constitutional DNA, mel = melanoma DNA. The sample denoted 'Calibr' is a *g/g* individual with a known copy number of 2, used as a calibrator in the analysis. Constitutional DNA from one *G/g* and one *G/G* horse was tested in the assay and the results are shown as a reference for the copy number expected from each genotype. Brackets surround the equine melanoma cell lines or the paired constitutional and melanoma DNA samples. Error bars represent the copy number range from the CopyCaller™ Software analysis of quadruplicates in each assay.

The experimental design for the resequencing of the Grey haplotype was to use one horse homozygous for Grey, and the horse with the closely related haplotype identified in paper I, a haplotype identical to *Grey* for all tested polymorphisms except for the duplication. This experimental design was chosen to minimize the number of sequence polymorphisms uniquely associated with *Grey* in the sequence analysis. Five other non-Grey horses were also included in the experiment.

The analysis of the resequencing data identified 15 SNPs uniquely associated with the Grey homozygote, and these SNPs were further investigated by Sanger sequencing in three additional horses; Twilight, the heterozygous Grey horse used to generate the genome reference sequence, a second homozygous Grey Lipizzaner horse and the non-Grey horse carrying the closely related haplotype. This analysis excluded all but seven loci (SNP1-7), which were selected for further analysis by TaqMan SNP Genotyping Assays in 357 homozygous, heterozygous and non-Grey individuals, in order to investigate a possible association to the Grey phenotype. The results showed that none of the investigated polymorphisms showed a perfect concordance with the Grey phenotype, as the duplication does.

However, the analysis of the seven SNPs revealed other interesting results; the non-reference allele at the SNP1 and SNP3 loci was only found in Grey individuals, which means that these SNPs must have occurred subsequent to the *Grey* mutation. For SNP1, a fourth, distinct separate genotype cluster was detected in the TaqMan analysis (where ideally only three clusters, one for each genotype, should be observed). By sequencing the amplicon for the individuals in this cluster, a SNP in the TaqMan probe site was detected, resulting in a lower *G*-allele signal in the assay, thereby creating a fourth cluster. Since the *G* nucleotide is associated with *Grey*, this analysis revealed a third confirmed SNP that must have occurred on the *Grey* haplotype subsequent to the Grey mutation event.

SNP4 and SNP6 showed a nearly perfect association with the *Grey* genotype, but with the exception for two non-Grey and four heterozygous Grey individuals. These two SNP are located just outside the haplotype block showing complete LD with the *Grey* mutation. In conclusion, we failed to identify any other sequence polymorphism uniquely associated with *Grey*, where the genotype distribution perfectly matched the phenotype distribution at the Grey locus.

In this study, we aimed at functionally dissecting the duplicated region in Grey horses in order to reveal the mechanisms behind the *cis*-regulatory activity. We investigated potentially regulatory regions in the duplicated sequence by testing their regulatory activity in luciferase assays, and further characterized one of these regions.

We first identified the human region corresponding to the *STX17* 4.6 kb duplicated region by a BLAT search against the human genome sequence in the UCSC Genome Browser (http://genome.ucsc.edu/). From the track regPotential7X, only available in the Human Mar. 2006 assembly (NCBI36/hg18), seven well-defined potential regulatory regions were identified. All seven regions were found in the horse genome, and all were tested for their regulatory activity in both murine melanocytes and murine myoblasts. The results from the luciferase assay revealed that one of the elements, Reg3, had a strong melanocyte specific regulatory activity (Figure 8). When the element was duplicated in order to mimic the genetic landscape in Grey horse *STX17*, the activity increased by 2-fold.

Bioinformatic analyses of the equine sequence for Reg3 revealed one near-consensus NGFI-B-response element (NBRE), to which NR4A3 has been shown to bind (Paulsen et al., 1995), and two potential MITF binding sites separated by 83 bp, both containing the perfect core nucleotides CATGTG. When deleting the NBRE from the one-copy Reg3, the activity was dramatically elevated, from 8-fold to more than 20-fold. Deleting the MITF binding sites resulted in a reduced activation, for MITF site 1 from 8- to 2-fold, equal to the activity in the myoblasts, and for MITF site 2 the reduction in activity was from 8 to 5-fold. MITF has been shown to be crucial for melanocyte development and survival (Opdecamp et al., 1997), and is a very interesting candidate for the regulation of the Reg3 activity in melanocytes.

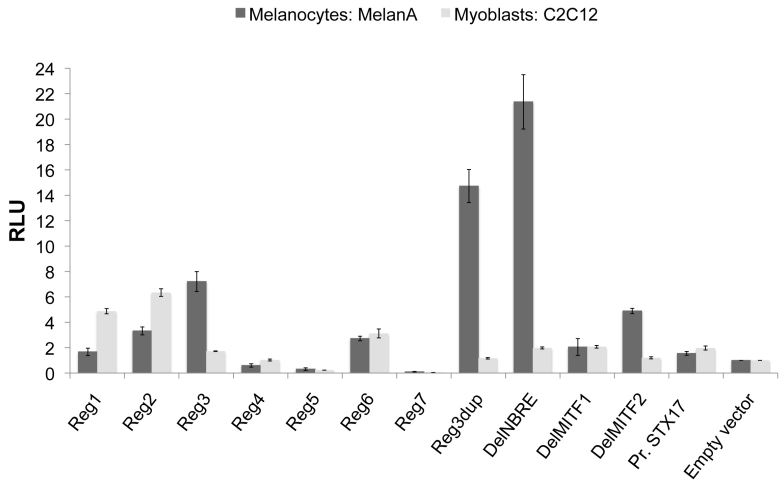

Figure 8. Luciferase activity of regulatory regions in the *STX17* duplication. Firefly luciferase reporter levels in relation to control *Renilla* luciferase levels. Error bars represent standard deviations of triplicates in one experiment. The results shown are representative for three repeated independent experiments. Results for the constructs containing regulatory regions 1-7 (Reg1-7), element 3 in two copies (Reg3dup) and regulatory element 3 with deleted NBRE, MITF1 or MITF2 binding sites (DelNBRE, DelMITF1, DelMITF2) are shown. In addition, the results from one construct containing the *STX17* promoter region (Pr. STX17) and the empty vector pGL3-Basic (Empty vector) are shown as references. RLU=Relative Luciferase Units.

Deletion of MITF site 1 showed a more dramatic effect on the reduction of the activation than deletion of MITF site 2. Both sites could be of importance for the melanocyte-specific activity, since the deletion of each of resulted in significantly lower activity.

Deletion of the near-consensus NBRE sequence in the melanocyte-specific regulatory element resulted in a melanocyte-specific increase of activation compared to empty vector activity; from 8-fold, when present, to more than 20-fold when deleted. This indicates that this sequence binds a transcriptional repressor present in melan-a cells, but it is unlikely that this repressor protein is NR4A3 since NR4A3 show only low expression in melan-a cells and NR4A3 has not previously been shown to act as a transcriptional repressor.

Future prospects

In paper I, III and IV of this thesis, the progress from mapping of the *Grey* mutation in horses, to the functional dissection of its mechanisms of action, is presented. Even if many of our initial questions have been answered, several question marks still remain. The mechanism by which the duplication in *STX17* causes hair greying and melanoma has not entirely been explained, but we have approached the goal from numerous angles.

To further elucidate the mechanism, and in order to see if other genes in addition to *STX17* and *NR4A3* have an altered expression, a transcriptome analysis between non-Grey and Grey melanocytes could be performed. Also, it would be very interesting to compare the gene expression profiles between hair follicles from the dark speckles and the white fur, and also the "blood marks" and the white fur in the same individuals.

The regulatory element 3 (Reg3) from paper IV, which shows a strong melanocyte specific activity, is currently being investigated in zebrafish enhancer assays in order to examine the extent to which the element shows expression in neural crest cells during development. A high expression in neural crest cells could indicate that this element affects the development and/or migration of melanocytes at an early stage. The effect of MITF binding to this element further supports a possible role during melanocyte development or migration. Also, it is possible that NR4A3 can promote transcriptional activation mediated by this regulatory region by interfering with the binding of a repressor protein to the NBRE site, but this needs to be investigated more in detail, for instance by overexpressing NR4A3 in melan-a cells.

The results from the research presented in paper I, III and IV could also eventually have practical repercussions for the diagnosis and treatment of Grey horse melanoma. Today it is possible to genotype horses for the *Grey* mutation, but the existing long-range PCR assay could be replaced by another genotyping method in order to perform less labour intensive routine genotyping. There is a high demand from breeders and owners for genotyping Grey horses in order to predict both the genotype and phenotype of the foal. According to the results from paper III, there is copy number variation of the duplication in melanoma tissue, and to some extent there appears to be higher copy numbers in tumours with a faster progression. Testing the copy number in tumours could be a valuable tool as an indication of the malignancy of the tumour, and the copy number of the duplication could also be used as a guide for surgical removal of putative malignant tumours.

Sex-linked barring in the domestic chicken (Paper II)

Background

The Sex-linked barring pattern in chickens is characterized by the pronounced black and white stripes on the feathers (Figure 9A and B). Similar barring patterns are found among many other bird species, like the peregrine falcon (Figure 9D) and zebra finch. The reason for the presence of these patterns in nature could be due to their good camouflaging properties. In chickens, a less pronounced barring pattern also exists, called autosomal barring. Sex-linked barring is characterized by bars with a complete absence of pigmentation, but in autosomal barring the bars vary between darker and lighter pigmentation, which results in a more irregular pattern.

Figure 9. A. An adult rooster (*B*B/B*B*) showing the Sex-linked barring feather pattern. B. A tail feather taken at 8 weeks of age from a *B*B* homozygous male (top) showing considerably wider white bands compared to a feather taken from a *B*B/W* hemizygous female (bottom). C. At hatch, Barred Plymouth Rock chicks display a white spot on their heads. The *B*B/W* female (left) has a smaller spot compared to the *B*B/B*B* male (right). D. Peregrine falcon exhibiting barred feathers. Photos A, B and C supplied by Christa F. Honaker and photo D by Jorma Luhta/naturepl.com.

The molecular mechanisms causing Sex-linked barring were first explored in the late 1920's and 1930's. The first theory was published in 1929 and relied on skin transplantation experiments. The authors performed skin transplants between one-day old barred and non-barred chickens, and saw that skin transplanted from one sex to the other grew feathers that in structure were like those of the host and, in colour and barring pattern, like those of the donor. From this they suggested that the Sex-linked barring pattern could be caused by a rhythmic variability in metabolism or hormonal secretions, and that a specific physiological "Sex-linked barring factor" must be present in the skin, since the skin was the determining factor for which pattern the feathers developed (Danforth, 1929). A few years later in 1934, Montalenti made the observation that two adjacent barring feathers did not necessarily develop the white and black bands simultaneously (Montalenti, 1934). Today, the conclusion that can be drawn from these two early examinations, is that a gene product causing barring must act in the feather follicle, since Danforth noticed that the barring pattern followed the transplanted skin (feather follicles), and Montalenti saw differences between different feathers in the same individual.

A decade later, in 1944, Nickerson was the pioneer of the theory claiming premature melanocyte death in the feather follicles of Sex-linked barred chickens, causing the white band development (Nickerson, 1944). This theory was confirmed 40 years later by Bowers and Asano (Bowers, 1984), and both Nickerson and Bowers suggested that the premature cell-death was the cause of the white band formation. Bowers continued his work on this theory, and published two sequential papers where he first proposed that the white bands formed due to an autophagic degeneration of the melanocytes at the edge of the black bands in the feather follicle (Bowers, 1988), and later his group suggested that the melanocytes in Sex-linked barred chickens would be more sensitive to oxidative stress than the melanocytes in the wild-type chickens. In the later work, the hypothesis was that the melanocytes would die due to high levels of reactive oxygen species (ROS) present during pigment production, and when the ROS levels would decrease, pigmentation could progress normally and produce the black bars until the next increase in ROS levels (Bowers et al., 1994).

The first mapping attempt of the Sex-linked barring locus was made by Bitgood in 1988, and placed the locus on the q arm of the male sex chromosome Z (Bitgood, 1988). In 2009, Dorshorst and Ashwell narrowed the locus to a 355 kb-region on the distal q arm of this chromosome (Dorshorst and Ashwell, 2009). In paper II, *Sex-linked barring* is mapped to the *CDKN2A/B* locus on chromosome Z, and the candidate mutations for

Sex-linked barring are presented and evaluated for their functional properties.

Results and Discussion

By using linkage analysis and a set of 675 F_2 chickens, we could assign the *Sex-linked barring* locus to a section on chromosome Z containing nine different genes. One of these genes stood out as an exceptional candidate; the *CDKN2A/B* locus. Loss-of-function mutations in this gene have previously been associated with familiar forms of melanomas in humans, indicating an important functional role in melanocyte biology (Hussussian et al., 1994, Dracopoli and Fountain, 1996, Monnerat et al., 2007). As a result of this, a new marker in *CDKN2A/B* was developed, and following screening in the F_2 individuals no recombination could be detected between this marker and *Sex-linked barring*. With the hypothesis that all individuals carrying *Sex-linked barring* would share the same mutation, a 12 kb identical by descent (IBD) region in the *CDKN2A/B* locus was defined following the sequencing of a number of individuals. As an IBD region, this is surprisingly small when compared for example to the 352 kb IBD region associated with *Greying with age* in horses (Rosengren Pielberg et al., 2008). This could be explained by the position of the region on the end of the Z chromosome q arm, a region that has been shown to have a high rate of recombination (Groenen et al., 2009).

Of the 320 SNPs found in the *Sex-linked barring* IBD region, two non-coding ones, one in the promoter region of the *CDKN2A/ARF* transcript (SNP1) and the next in the first intron of the same transcript (SNP2), showed nearly complete association with the phenotype. The mutations were genotyped in 285 individuals divided over 51 populations, and showed a very strong association across populations. In addition to the two non-coding SNPs, two missense mutations were identified at highly conserved sites in the ARF protein, V9D and R10C, and every bird tested with a confirmed Sex-linked barring phenotype carried one of these missense mutations. These mutations are found in a part of the ARF protein where mutations have been shown to be highly critical for the function of the protein (Kim et al., 2003, Moulin et al., 2008, di Tommaso et al., 2009).

Functional analysis did not conclude any proofs for the molecular mechanisms causing the barred feather pattern, but it did however clarify some important aspects of the two mutations, SNP1 and SNP2. Transcription factor binding site analysis showed that SNP1 was located in a

putative E2F1 binding site, with the B*B-allele matching slightly better than the B*N-allele. This finding was interesting because E2F1 is known to regulate *CDKN2A* expression in both mouse (Lomazzi et al., 2002) and chicken cell lines (Kim et al., 2006). SNP2 was found to be located in a NFκB binding site, with a better match for the B*N sequence than the B*B sequence. The putative binding sites were investigated for the binding of each transcription factor by Electrophoretic Mobility Shift Assays (EMSA). No DNA-protein complex was detected with the oligonucleotides corresponding to the two SNP1 alleles, but a specific complex was obtained when oligonucleotides for both alleles at the SNP2 site were used, however no obvious difference was seen between the alleles.

Luciferase reporter assays were also performed for SNP1 and SNP2 to test whether alleles at these loci influenced transcriptional activation, but there was no significant difference between alleles either for SNP1 or SNP2. The luciferase assay with cotransfection of the E2F1 transcription factor with the constructs harbouring the *CDKN2A* (*ARF*) promoter confirmed the results of Kim et al. (2006); that transcription of chicken *CDKN2A*, like mammalian *CDKN2A*, is activated by E2F1. However, we were unable to detect a significant difference in activity between the SNP1 alleles. When constructs containing all four combinations of SNP1 and SNP2 alleles were tested for possible interactions between SNPs, no significant differences in luciferase activity were detected.

Future prospects

The functional investigations of the candidate mutations turned out to be a difficult and not very straightforward task. The ARF proteins with the Sex-linked barring wild type allele and each of the two missense mutations have been studied in preliminary luciferase assays with the aim to investigate their possible interaction or absence of interaction with the p53 promoter. This was because the main function of ARF ($p14^{ARF}$ in humans, $p19^{ARF}$ in mice) is to activate p53 by inhibiting MDM2 (Figure 10), which results in induction of the expression of numerous apoptosis and cell cycle inhibitory genes (Lowe and Sherr, 2003). Preliminary results indicate a close to 2-fold lower activation of the p53 promoter in the two constructs expressing the proteins with V9D and R10C missense mutations, compared to the wild type protein, which showed the same fold activation of the p53 promoter as the $p14^{ARF}$ wild type form in humans (di Tommaso et al., 2009).

This is the first study showing that mutations in the *CDKN2A/B* locus can cause variation in coat or plumage colour. It would therefore be interesting to study the possible role for this locus in determining colour diversity both among birds and other species. Since *CDKN2A/B* is located on the Z chromosome in birds and does not show dosage compensation, this locus may contribute to sexual dimorphism in avian plumage colour. For instance, the zebra finch has zebra striped feathers, only present in males, and *CDKN2A/B* is an obvious candidate locus for this. According to the zebra finch genome assembly, *CDKN2A/B* encodes an ARF protein that just like the B2 allele differs from other vertebrate ARF proteins by not having an arginine residue at position 10. This raises many questions about the molecular processes behind fascinating coat and plumage colours found in nature, questions that urge to be further investigated.

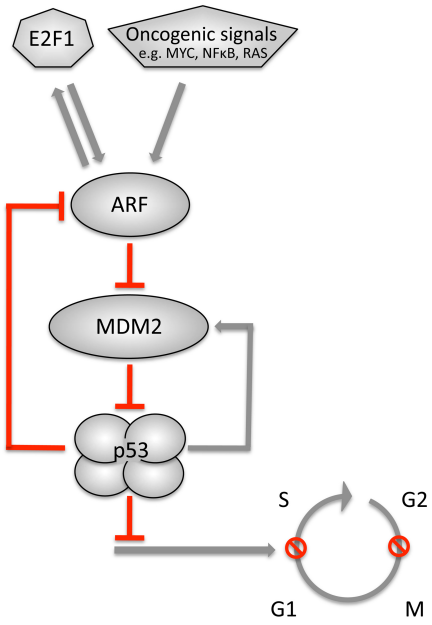

Figure 10. The ARF-MDM2-p53 network. Elevated expression of ARF, as a consequence of oncogenic signals or release of E2Fs, E2F1 in particular, results in the stabilization of p53 and upregulation of p53 target genes. ARF has been shown to regulate E2F1 expression (Parisi et al., 2002). The ARF protein binds to MDM2 and inhibit its ubiquitin ligase activity. MDM2 modulates p53 activity by blocking its transcriptional activation functions and by promoting its ubiquitilation and proteasome-mediated degradation. MDM2 is transcriptionally activated by p53, resulting in a feedback loop. p53 can cause transcriptional repression of ARF, creating a feedback loop which modulates p53 activity. ARF induction can induce apoptosis, but the usual outcome is cell-cycle arrest in the G1 and G2 phases. Illustration by Elisabeth Sundström, partly modified from Gil and Peters (2006).

Populärvetenskaplig sammanfattning

Genetiska studier av husdjur bidrar till förståelsen av pigmentering och melanom

Ända sedan människan tämjde sina första husdjur för tiotusentals år sedan, har vi genom avel skapat just de djur som vi har haft användning, nytta och nöje av. Detta har lett till en fantastisk mångfald av kroppsstorlekar, pälsfärger och kroppsformer bland våra husdjur, något vi idag med genetiska metoder kan studera på molekylnivå, och avgöra exakt vilka gener som ger upphov till vilka drag. I den här avhandlingen har vi genom att studera de genetiska egenheterna hos två framavlade utseenden i våra husdjur; avblekbar skimmel hos hästar och svart-vit randiga fjädrar hos höns, kunnat dra slutsatser om molekylära processer och signalvägar som leder till förändringar i pigmenteringen och uppkomst av pigmenttumörer, s.k. melanom. På denna mest grundläggande cellnivå är processerna ofta identiska mellan djur och människor, vilket gör att man genom den här forskningen också kan dra slutsatser om vad som händer i människor som har förändrad pigmentering eller får hudtumörer.

Avblekbara skimlar föds normalt färgade, till exempel bruna eller svarta. Med åldern försvinner pigmenteringen gradvis i håret, och vid 6-8 års ålder är de flesta hästar mer eller mindre vita. Förloppet kan liknas vid hur många av oss människor förr eller senare får grått hår, och precis som hos oss är det bara håret som förlorar sin pigmentering hos hästarna, hudens färg förblir oförändrad, vilket hos hästarna innebär svart. Till skillnad från gråhåriga människor, drabbas de avblekbara skimlarna i 8 fall av 10 av melanom, om de lever tillräckligt länge. Melanom är pigmentceller som förökar sig ohejdat, och dessa tumörer kan växa sig stora som en knuten hand hos skimlar. Oftast hittar man dem i hud som saknar behåring, t.ex. under svansen, vid munnen eller ögonen. I den här avhandlingen presenteras det arbete som startade med kartläggningen av den genetiska förändring som

orsakar förlusten av hårpigment och melanom, och som fortsatte med studier av hur förändringen påverkar pigmentcellerna. Vi kunde visa att alla vita hästar bär på samma förändring, och att den innehåller en region som har en starkt aktiverande effekt på andra gener, men bara i pigmentceller. Vi kunde också visa att förändringen finns i fler kopior i DNA från tumörvävnad jämfört med DNA från blod eller hår, och i flest kopior i tumörer som är aggressiva. Detta visar att den genetiska förändring vi hittade påverkar tumörutvecklingen.

Den svart-vit-randiga fjäderdräkten, som generellt kallas vattring hos fåglar, nedärvs hos höns på den könskromosom (Z) som finns i dubbel upplaga hos tuppar. Detta innebär att tuppar kan ha betydligt bredare vita ränder på fjädrarna än hönor. Vi ville veta var den eller de genetiska förändringar som orsakar mönstret fanns, för att kunna ta reda på närmare vad som händer i cellerna när fjädrarna utvecklar de svarta och ljusare banden. I den här avhandlingen presenteras det arbete som ledde fram till att vi exakt kunde peka ut var dessa genetiska förändringar fanns. De låg i en gen som tidigare har visat sig vara av stor betydelse för om människor utvecklar en ärftlig form av melanom. Genen har också visats vara viktig för hur pigmentering styrs.

Genom att i genetisk detalj studera två utseenden med annorlunda pigmenteringsprocesser hos djur, har vi genom vår forskning kunnat bidra med nya ledtrådar kring de mekanismer som styr pigmentering och uppkomsten av melanom, både hos djur och människor.

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Acknowledgements

LEIF, min huvudhandledare, tack för att du gav mig en plats i din forskargrupp och för att du från min första dag på labbet visade ett stort förtroende för mitt arbete. Genom att arbeta med dig har jag fått möjlighet att göra saker som jag inte skulle ha fått någon annanstans: bidra till en Nature Genetics-publikation, fått förtroendet att resa jorden runt för att presentera vår uppmärksammade forskning, och sist men verkligen inte minst har jag fått publicera en artikel i Forskning och Framsteg, något jag innan jag började i din grupp hade fått beskrivet för mig som ett ouppnåeligt mål. Men ingenting är omöjligt.

GERLI, min bihandledare, tack för att du i sann Baloo-anda har försökt att lära mig allt du kan! Att jag fick börja min tid som doktorand med att arbeta med dig var en starkt bidragande orsak till att jag valde att fortsätta.

I varje labb finns det också personer som mer eller mindre märkbart verkar som omistliga kugghjul i processen att hålla det dagliga arbetet rullande. Oändligt tack **GUDRUN** för att du inte bara med glans utförde dina kontraktsskrivna administrativa sysslor utan dessutom insisterade på att jag skulle begära traktamente vid varje utlandsvistelse. **ULLA**, labbets andra kugghjul, tack för att din hjälpande hand, ditt enorma laborativa kunnande och dina uppmuntrande ord alltid finns tillgängliga och har hjälpt mig ur många trånga återvändsgränder.

Ett stort tack vill jag också rikta till **GÖRAN ANDERSSON**, **JONAS ERIKSSON**, **ANDERS LINDROTH** och **ANNA-MAJA NYSTRÖM** för att ni ständigt delar med er av er tid och kompetens för att lösa både enkla och komplexa, laborativa liksom teoretiska problem.

Thanks to the members in the Grey horse project group; **LIN JIANG** and **ANJA GOLOVKO**, for fruitful monthly discussions about our work. Thanks LIN for teaching me the basics of chIP, and ANJA for contributing with Western Blots.

A profound thanks and the deepest bow to **JENNIFER MEADOWS** for spreading your native English language skills like little stars over this thesis! Thanks also for always having time for discussions about the jungle of copy number assays and anything else worth to chat about.

ÖVRIGA GAMLA OCH NYA KOLLEGOR I D11:3-KORRIDOREN, tack för hjälp i labbet och såklart ert trevliga sällskap!

ANDERS HELLSTRÖM, tack för att jag fick jobba tillsammans med dig i ditt randiga projekt. Efter och under alla våra timslånga spånanden kände jag mig verkligen som en självständig och ”riktig” forskare, och till slut resulterade det ju faktiskt i något publicerbart. Det har varit fantastiskt värdefullt att ha en pigmentkompis med sig på färden, såväl mot våra avhandlingar som kors och tvärs över världen.

VÄNNER, ni betyder alla mycket för mig, och i det här stycket vill jag i synnerhet tacka biotek-’99 gänget **GÖREL (OCH ANDERS)**, **KARIN**, **MARIA**, **STINA** och **VICTORIA (OCH FREDDIE)** för att er vänskap fick mig att fortsätta när grundkurserna kändes evighetslånga och för att ni i tiden efter vår examen alltid över en nationsburgare har velat dela både framgångar och motgångar.

MIN SLÄKT och FAMILJ, i norr och söder, Uppsala såväl som Öland, tack för att ni alltid finns där och är den lugnande oas dit vi alla då och då behöver dra oss tillbaka. **MAMMA** och **PAPPA**, att ni alltid har stått bakom mig och uppmuntrat mig att göra det jag vill har betytt mer för den här skriftens tillkomst än alla stipendier och dyra maskiners och reagensers närvaro tillsammans. Utan er hade jag inte varit där jag är idag, den här boken är lika mycket er som min. **ANDERS** och **DESPINA**, ibland är det skönt med någon som åtminstone förstår en bråkdel av vad man ägnar sina dagar åt! Tack bror för dina fantastiskt tjusiga illustrationer som min avhandling fick äran att prydas av.

JÖRGEN, tack för att du alltid tvivellöst tror på mig när jag själv inte gör det, och för att du orkade läsa igenom min avhandling och dessutom bidra med konstruktiva kommentarer. Jag är så glad och tacksam för varje dag som vi får gå tillsammans genom livet! Nu är vi ”**THE DOCTORS**” ☺