DNA methylations: A comparison of four genes between Red Junglefowl and White Leghorn

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Domestication of animals has given rise to a great phenotypic divergence in selected animals and has rapidly generated species of animals more accustomed to human contact and social interactions within the species. Previous studies in chickens (*Gallus gallus*) have managed to find behavioral and adaptive differences between Red Junglefowl (RJF) and White Leghorn (WL), differences inherent to the domestication process. These phenotypic changes could spawn from a variety of different genomic factors, including an epigenetic gene expression regulatory mechanism known as CpG methylation, a DNA modification of CpG dinucleotides that in turn affect nucleosome formation. In this study we investigated the methylation differences between RJF and WL. This was done by selecting genes that has previously been shown to be both differentially expressed (DE) and differentially methylated (DM) between RJF and WL, and had shown the same kind of differences in both parental animals and their offspring. By using methylation-sensitive high-resolution melting (MSHRM) we tried to confirm previous DM result, and four genes; *FUCA1, RUFY3, PCDHAC1* and *TXNDC16* were tested and verified to be DM between RJF and WL.
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1 Abstract
Domestication of animals has given rise to a great phenotypic divergence in selected animals and has rapidly generated species of animals more accustomed to human contact and social interactions within the species. Previous studies in chickens (Gallus gallus) have managed to find behavioral and adaptive differences between Red Junglefowl (RJF) and White Leghorn (WL), differences inherent to the domestication process. These phenotypic changes could spawn from a variety of different genomic factors, including an epigenetic gene expression regulatory mechanism known as CpG methylation, a DNA modification of CpG dinucleotides that in turn affect nucleosome formation. In this study we investigated the methylation differences between RJF and WL. This was done by selecting genes that has previously been shown to be both differentially expressed (DE) and differentially methylated (DM) between RJF and WL, and had shown the same kind of differences in both parental animals and their offspring. By using methylation-sensitive high-resolution melting (MSHRM) we tried to confirm previous DM result, and four genes: FUCA1, RUFY3, PCDHAC1 and TXNDC16 were tested and verified to be DM between RJF and WL.

Keywords: DNA methylation, domestication, High-resolution melting, White Leghorn, Red Junglefowl

2 List of abbreviations
CGI – CpG Island
DE – Differentially expressed
MSHRM – Methylation-sensitive high-resolution melting
WL – White Leghorn

CpG – Cytosine-phosphate-Guanine
DM – Differentially methylated
RJF – Red Junglefowl
SNP – single nucleotide polymorphisms

3 Introduction
Domestication of animals and artificial selection of their phenotypes, along with adaptation of the animals towards their captive environment have rapidly driven the evolution of the selected animals. Price and King (1968, cited by Price, 1999) defined domestication as “that process by which a population of animals becomes adapted to man and to the captive environment by genetic changes occurring over generations and environmentally induced developmental events reoccurring during each generation”. A well studied example is the domestication of the silver fox (Trut et al, 2009), and the behavioral, morphological and physiological changes that these animals show after less than half a century of captive breeding. At a molecular level, Trut suggests that genes associated with tame behavior and hormonal regulation can become fixated within 10 generations, and further destabilize other genes in their pathways which could alter the expression profiles in unpredictable ways, leading to new phenotypes. This tremendously rapid domestication over the span of just a few generations and the genetic changes causing gene expression differences can most likely not be explained solely by single random mutations in all affected phenotypes.

DNA methylation is a common genomic modification among eukaryotes, and in vertebrates and plants with large genomes the phenomenon of global methylation of the genome is widespread. DNA methylation means that the genomic residue cytosine is modified to contain an extra methyl group, a trend independent of eukaryote organism studied (Suzuki and Bird, 2008). Methylation generally occur when a cytosine residue is followed by a guanine, creating pairs known as CpG dinucleotides. CpG is an abbreviation for cytosine-phosphate-genome.
guanine, as contrast to C/G base pairing, in order to emphasize that they are on the same DNA strand. Single methylated dinucleotides dispersed through the genome are likely to be removed through spontaneous deamination (Millar et al, 2002), with the exception for those in formation of CpG islands (CGI), which are small areas in the genome with high CpG density (Illingworth and Bird, 2009). These CGIs vary in the degree of methylation, and studies on a large number of loci in human chromosomes found approximately 40 % of all loci being hypermethylated (>80 % methylation) and 30 % hypomethylated (<20 % methylation) (Eckhardt et al, 2006).

Another discovery is that CGIs are distributed in a non-random manner, and that they frequently span the promoter region and the first exon of protein coding genes. This way cells have the possibility to regulate expression by regulating the methylation of these sequences. Silencing of transcription can be achieved by methylating individual promoter region CGIs which in turn induce formation of inactive chromatin by the recruitment of histone deacetylases (Wojdacz and Dobrovic, 2007). Genome-wide studies of methylation patterns in eukaryotes reveal a trend towards hypermethylation in gene bodies and hypomethylation in active promoters, and this trend has been shown to be inversely linked to deposition of core histone protein H2A2 (Zemach et al, 2010). Chromatin structural changes can be the response to even very subtle variations in surroundings and a process mediating the effects of that variation, as suggested by a study on the effects of maternal behavior in rats (Weaver et al, 2004).

Low CpG content in promoters and enhancers are often connected with methylation differences in different tissues, and studies indicate that there are dynamic changes of methylation patterns during cell differentiation at the CGIs in promoters of lineage-specific and pluripotency genes (Rottach et al, 2009). The mechanisms behind the inheritance of epigenome factors such as methylation are poorly understood, but fall into the category of epigenetics, a form of soft inheritance (Richards, 2006), as they would carry on and signal changes in expression by modifying the structure of chromosomal regions (Bird, 2007). Any environmental changes will require the genome to carefully orchestrate its transcriptional output in order for the organism to be able to adapt (Bonasio et al, 2010), and unlike the rigid DNA sequence, the genome's epigenetic status can change much more dynamically (Jaenisch and Bird, 2003).

Since the definition of epigenetic inheritance varies between authors a distinction has to be made between somatic cells which only divide by mitosis, and germ cells which can divide by both mitosis and meiosis, the latter thereby being able to carry on their genetic code to the offspring of the organism. Epigenetics however, as defined by Bird (2007), include both types of cells. Crews (2011) states that the only real transgenerational epigenetic modifications are germ-line-dependent. This in contrast with context-dependent epigenetic modifications, which are affecting somatic cells through continuous stimulations over generations, which makes it impossible to determine any true inheritance.

Waddington (1961, cited by Pigliucci and Murren, 2003) proposed that acquired characters, or phenotypically plastic traits as we know them today, may become converted into inherited characters if selection acts on them for many generations (Pigliucci and Murren, 2003). Several transgenerational epigenetic inheritance studies have been made, and the most well known are on the Agouti mice, which is an example of a study on genetically identical mouse which produce offspring with variable coat colors depending on the variation in the epigenome (Morgan et al, 1999). Trut et al (2009) discovered silencing of alleles in their silver foxes, and this heritable gene activation and reactivation seems to work in a manner similar to Axin-fused genes in mice, implicated to be regulated by DNA methylations (Rakyan et al, 2003). A study on humans served as a demonstration of transgenerational
responses in a population of people in northern Sweden, indicating environmental factors such as food supply might have an effect on grandchildren and that these effects may be mediated through the sex chromosomes (Pembrey et al, 2006). Diet can affect the offspring and its expression patterns in mice, and is an example of how the heritable genome is reprogrammed by environment. Even though no major DNA methylation change was discovered between dietary groups in mice, some specific changes in lipid class genes and their pathways were indicated (Carone et al, 2010).

In light of these findings, investigating the possibility of DNA methylation as an epigenetic marker involved in the gene expression difference and inheritance of behavioral changes seems reasonable. Lindqvist et al. (2007) studied the transmission of a stressful environment from the parental generation to its offspring and found that White Leghorns (WL) transmit both an altered gene expression profile as well as altered behavior (decreased spatial learning ability) as a response to unpredictable light-dark rhythm to their offspring while Red Junglefowl (RJF) offspring remained unaffected. Despite showing stress-affected gene expression patterns being inherited across generations, the genetic mechanisms behind it are still not yet clear. Further, albumen corticosterone levels were measured in eggs as an indicator of parental hormonal levels during 10-12 hours before the laying of eggs, and these tests showed no alteration of baseline secretion, indicating that this specific hormone did not contribute to possible context-dependent alterations in a major fashion at that stage of development (Lindqvist et al, 2007). Nätt et al (2009) performed a similar study with the same form of induced stress, looking at feeding behavior of parents and their offspring. This stress-related behavior also showed transmission across generations. Both studies eliminated animal traditions, suggesting that transgenerational epigenetic inheritance are responsible for the expression differences. The most likely candidates are context-dependent effects (hormonal influences) or germ-line dependent effects, such as aforementioned methylations. Previous genetic studies on chickens have shown tissue-specific methylation and significant differences in the degree of CCGG-site methylations across tissues (Xu et al, 2007), and distinctly different CpG methylation profiles in genes with high and low expression levels due to dietary variations (Xing et al, 2009).

In order to detect methylations, the most common practice is to use sodium bisulphite. The basic principle behind this method is to use sodium bisulphite in order to selectively convert only unmethylated cytosine to uracil whilst methylated cytosines remain unchanged (Clark et al, 1994). The chemical process uses sulphonation/desulphonation to enable the deamination of cytosine to uracil in single-stranded DNA. These subtle sequence differences can be detected utilizing a methylation-sensitive high resolution melting (MSHRM) to denature double-stranded DNA and comparing melting curves (Wojdacz 2007 and 2008). By amplifying the strands of both artificially unmethylated and fully methylated DNA using PCR and MSHRM, and primers that do not discriminate between the two types of DNA, it is possible to analyze the degree of methylation in small samples of DNA.

Another method for detecting gene-specific methylations is methylation-sensitive restriction enzyme mapping, which is based on size fractionation of DNA that has been fragmented using methylation-sensitive restriction enzymes, revealing differently sized fragments detectable using a Southern hybridization assay (Selker et al, 2003; Ashikawa, 2001). Other methods include sensitive melting analysis after real-time methylation-specific PCR (SMART-MSP) and methylation-specific fluorescent amplicons generation (MS-FLAG) (Kristensen et al, 2008; Bonanno et al, 2007). SMART-MSP is a complementary method to the MSHRM, more accurate and sensitive but might still need the HRM step to control amplification. The purpose of this method is to combine a quantitative PCR method with a
HRM, in addition to the stand-alone MSHRM. The MS-FLAG method uses a restriction endonuclease recognition sequence added to a methylation-sensitive primers 5’-end, and as more recognition sequences are generated, the stronger the fluorescence signal gets. If the primer target sequence is unmethylated the primer will not be able to bind, resulting in the absence of a detectable signal.

A third alternative would be using a standard Sanger based sequencing with a bisulphite pretreatment; these methods however are generally too extensive for the purpose of this study (Rabinowicz et al, 1999; Cokus et al, 2008).

In this study a previously tested MSHRM protocol was applied in the investigation of methylation differences between White Leghorn and Red Junglefowl. This is part of a larger study on expression and methylation differences between WL and RJF, and the inheritance of such differences within each race. The setup was based on data from an oligonucleotide expression array (Affymetrix) of hypothalamus-thalamus tissue from both WL and RJF, and a subsequent methylation tiling array (Nimblegen). This data provided a number of both differentially expressed (DE) and methylated (DM) promoter regions overlapping in two generations of animals. For this study, a new set of offspring WL and RJF was provided from the same parental group as previously tested. The animals were sacrificed and hypothalamus-thalamic (diencephalic) as well as cerebellum samples were taken in order to test the same tissue type as the arrays were based on and also to examine different parts of the brain. A set of 23 primer pairs for a total of 13 genes were screened and from these, four primer pairs were chosen for further MSHRM tests of the genes **FUCA1**, **RUFY3**, **PCDHAC1** and **TXNDC16**.

The hypothesis behind this study was that the methylation differences observed between WL and RJF on the methylation array would be reproducible using MSHRM. This would work as a proof of principle, confirming the array results and strengthen them, and also provide a tool for small scale testing of a large group of test animals and tissues.

4 Materials and methods

4.1 Animal handling and DNA preparations

Parental generations (P0) of WL and RJF from the Wood-Gush facility outside Linköping were bred within each group to obtain a second generation (F1) WL and RJF chicks. These (eight WL and eight RJF, four of each sex within the subgroups) were hatched in incubators at the Krujit hatchery in Linköping University and allowed to grow five weeks old before they were sacrificed through decapitation and their brains subsequently dissected. A seven-part dissection of the brain was performed, dissecting it into telencephalon, optic tectum, cerebellum, diencephalon (hypothalamus–thalamus), mesencephalon, pons, and medulla. Parts from diencephalon weighed approximately 150-180mg and parts from cerebellum 150-200mg. Samples were frozen in liquid nitrogen and stored at -80 °C until used for tissue homogenization and DNA isolation. Homogenization of samples was achieved in a FastPrep instrument (MP Biomedicals) with Fast Prep tubes containing ceramic beads (Lysing Matrix D, MP Biomedicals). Entire tissue samples were mixed with 500µl ATL buffer (DNeasy Blood & Tissue Kit, Qiagen) and homogenized in the FastPrep machine at the setting 6 m s⁻¹ for 40s.

4.2 DNA isolation from homogenized tissue

The DNA isolation was performed using the DNeasy Blood & Tissue Kit (Qiagen), following the manufacturers Animal Tissues Spin-Column Protocol. From each FastPrep tube, samples containing 25mg tissue in 180µl ATL were prepared and to these was added 20µl (0.9 U µl⁻¹)
Proteinase K, followed by 3h incubation at 56 °C. After incubation 20µl RNase were added to each sample and they were allowed to incubate at room temperature for 2min. Afterwards, 200µl Buffer AL and 200µl ethanol (99 %) was added to each sample, followed by vortexing for 15s. The complete mixture was then transferred into DNeasy Mini spin columns and centrifuged at 8000rpm for 1min. The flow-through was discarded and 500µl Buffer AW1 added to the column, followed by centrifugation at 8000rpm for 1min. The flow-through was discarded and 500µl Buffer AW2 was added to each spin column, followed by centrifugation at 13300rpm for 3min. The flow-through was discarded and the spin columns placed in 1.5ml microcentrifuge tubes. Three subsequent elutions were performed by pipetting 50µl, 150µl and 100µl Buffer AE directly onto the column membrane. Samples in all three steps were first let to incubate for 1min at room temperature followed by centrifugation at 8000rpm for 1min. New microcentrifuge tubes were used for each elution. A Nanodrop ND1000 (Thermo Scientific) spectrophotometer was used to determine DNA concentrations in samples, and sample purity was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies).

4.3 Replication of positive and negative controls
Two controls, negative and positive, were made from DNA of all animals pooled together. The controls are the two extremes from which a calibration curve was made in order to estimate the degree of methylation of samples and to aid in the determining the optimal annealing temperature for primers. The positive control was methylated with M. SsSI CpG Methyltransferase (New England Biolabs). The protocol provided with the kit was modified for 1µg DNA, and mixtures were prepared containing; 2µl 10X NEBuffer 2, 2µl SAM (1.6 mM), 1µl SssI methylase (4 U µl⁻¹), 1µg pooled DNA and nuclease-free water to a total volume of 16.5µl. Samples were incubated at 37 °C for 2h. 2.5µl more SAM was added to each sample and they were allowed to incubate for another 2h. The reaction was stopped by heating samples to 65 °C for 20min.

For the negative control a REPLI-g Mini Kit (Qiagen) was used, following the manufacturers protocol. DNA from the pool was diluted to 10-20ng µl⁻¹ and 2.5µl diluted DNA was mixed with 2.5µl Buffer D1 and incubated for 3min at room temperature. Afterwards the samples were mixed with 5ml Buffer N1. A 40µl mastermix containing 10µl nuclease-free water, 29µl Reaction buffer and 1µl DNA polymerase was added to each of the negative control samples. The samples were incubated at 30 °C for 16h, and the reaction and polymerase was then inactivated by heating the samples to 65 °C for 3min. A cleanup protocol was developed using DNEasy Blood & Tissue Kit (Qiagen) to purify the amplified negative control. The whole 50µl negative control mixtures was pipetted to 1.5ml microcentrifuge tubes, and to each tube 200µl Buffer AL and 200µl ethanol (99 %) was added to. The samples were vortexed and transferred to DNeasy Mini spin columns and centrifuged at 8000rpm for 1min. The flow-through was discarded and 500µl Buffer AW1 added to the column, followed by centrifugation at 8000rpm for 1min. The flow-through was discarded and 500µl Buffer AW2 was added to each spin column, followed by centrifugation at 13300rpm for 3min. Samples were eluted with 20µl Buffer AE, centrifuging at 8000rpm for 1min.

4.4 Bisulfite treatment of DNA
Samples and controls were bisulfite treated using EpiTect Bisulfite Kit (Qiagen) following the manufacturers protocol. A mastermix containing 1µg DNA (max 20µl), 85µl Bisulfite mix, 35µl DNA protection buffer and RNase-free water to a total volume of 140µl were prepared in PCR-tubes for each sample and control. The bisulfite conversion was performed using a thermal cycler on a 5h long program with the following steps; Denaturation 5min at 95 °C, Incubation 25min at 60 °C, Denaturation 5min at 95 °C, Incubation 85min at 60 °C, Denaturation 5min at 95 °C and a final Incubation for 175min at 60 °C. After the cycle
completed the samples were moved to 1.5ml microcentrifuge tubes and mixed with 560 Buffer BL (containing 10µg ml⁻¹ carrier RNA) and were vortexed. Samples were then transferred to spin columns and placed in 2ml collection tubes. The columns were centrifuged at 13300rpm for 1min and the flow-through was discarded. 500µl Buffer BW was added to the spin columns and they were centrifuged at 13300rpm for 1min. The flow through was discarded and 500µl Buffer BD was added to the spin columns, and the columns were let to incubate at room temperature for 15min before they were centrifuged at 13300rpm for 1min. The flow-through was discarded and 500µl Buffer BW was added and the tubes centrifuged at 13300rpm for 1min. This last step was repeated once more and the flow-through and collection tubes were discarded. The spin columns were placed in new collection tubes and centrifuged at 13300rpm and the collection tubes were discarded. The columns were placed with open lids in 1.5ml microcentrifuge tubes and put in a heating block set at 56 °C. The samples were let to incubate for 5min and the microcentrifuge tubes were then discarded. The spin columns were placed in new 1.5ml microcentrifuge tubes and 20µl Buffer EB was placed on the column membrane. The tubes were centrifuged at 12000rpm for 1min and the columns were discarded. The samples were then diluted by adding 19µl Buffer EB to the microcentrifuge tubes.

4.5 HRM run on DNA
The real time PCR and MSHRM was performed using a Rotor-Gene 6000 thermocycler (Corbett Research), combined with an EpiTect HRM PCR Kit (Qiagen) containing HotStarTaq Plus polymerase and EvaGreen fluorescent dye. The manufacturer’s protocol was followed for 10µl reaction mixes containing 5µl 2X HRM PCR master mix, 0.75µl of each primer (10µM), 2.5µl RNase-free water and 1µl DNA sample. A mixture of positive and negative control was used to make a calibration series consisting of 100 %, 75 %, 50 %, 25 %, 2.5 % and 0 % of methylated DNA.

The following program was run in the thermocycler: Activation 5min at 95°C, 45 cycles of: Denaturation 10s at 95 °C, Annealing at 30s 53-57 °C (primer dependent) and Extension 10s at 72 °C. Fluorescent measurement of amplification was made through the Green channel available in the Rotor-gene. HRM was run between the interval of 70 °C to 90 °C, with a 0.05 °C increase for each step, and each step held for 2s. Fluorescence data was acquired on the HRM channel.

4.6 PCR screening of primers
Screening of primer pairs was performed using a HotStarTaq Plus DNA Polymerase (Qiagen). Following the manufacturers protocol, and using the 10x CoralLoad PCR Buffer, each sample contained the following reagents: 1µl 10x CoralLoad PCR Buffer, 0.8mM dNTP (0.2mM of each in final concentration), 0.75µl of each primer (10µM), 5.65µl RNase-free Water, 1µl DNA sample (negative or positive control samples) and 0.05µl HotStarTaq polymerase for a total sample volume of 10µl.

The following program was run in the PCR : Activation 5min at 95 °C, 45 cycles of; Denaturation 10s at 95 °C, Annealing at 30s 55.5 °C and Extension 10s at 72 °C. PCR products where examined via gel electrophoresis using 2 % gels: 200ml TBE 0.5x, 4g Agarose and 5µl SYBRSAFE. The gels where studied with UV-light and a Benchtop UV Transilluminator (UVP).

4.7 Data analysis
The Rotor-Gene 6000 thermocycler (Corbett Research) comes with a software (Rotor-Gene Series Software Version 1.7) that allows data analysis of the melting curves, and normalized
MSHRM melting can be calculated. Graphical and raw data can also be exported to be analyzed in other programs for statistical tests. The calibration series was used to calculate methylation fraction in the samples, and the methylation rate were helpful when trying to increase resolution for melting curves. Standard deviation and the standard error of the mean were calculated in order to determine the sample variations. The Kolmogorov-Smirnov test was used to test the normal distribution of the sums of differences. For normally distributed samples the Student's t-test was used to compare mean values of RJF and WL, and the Mann–Whitney test was applied when the assumption of normality could be rejected. The two-tailed significance level was $p = 0.05$. Statistical tests were performed in Minitab.

### 4.8 Bioinformatics, gene selection and primers design

Candidate genes for the study were selected by comparing data from an expression array and a methylation array (manuscript, Daniel Nätt, IFM, Linköping University). Genes that differed in expression and could be linked to differentiation in methylation between races were selected as candidate genes for primer design. Primer design was performed by analyzing predicted promoters and CpG-content in annotated genes using slightly modified settings published by Wojdacz et al (2008), creating CpG-containing primers. Regions from parents and offspring that both showed significantly differentially expressed spots on an expression array were selected for a methylation array. In the methylation array, 145 regions showed differentially methylated spots overlapping between offspring and parentals, and candidate genes where chosen from these. Candidate genes where annotated using the WashU 2.1 release (May 2006) of the chicken genome through the Ensemble gene browser. Primers were designed to capture spots in the 70-200bp amplicons, or to be in close proximity to the spots to analyze CpGs in the same region. Most spots were located to the upstream region off annotated genes (5'-end), correlating with promoter regions and in some cases CpG islands. Thirteen candidate genes were selected from the list of most significantly differentially methylated genes. In total, 23 primer pairs were designed for these 13 genes (Table 1), with the aim to have at least two primers for each candidate gene. Primers were designed using the web-based Primer3, and were allowed to contain CpGs as long as there was no 3' CpG dinucleotides or if number of CpGs per primer did not exceed three. All primers have a melting temperature ($T_m$) of approximately 59-60 °C. A Python program script was used in order to easily simulate bisulfite sequence conversions of target DNA sequences. All primers were designed to the bisulfite converted DNA regions, where the only allowed cytosines were present in CpG dinucleotides. The AutoDimer software was used to evaluate primer dimer formation properties.
Table 1. Primer pair sequence and properties data for all designed primers. Tm and CG % data from primer design program Primer3. CpGs indicate product CpG count for each primer pair. CpG dinucleotides underlined in primers where those were included.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Tm (°C)</th>
<th>CG (%)</th>
<th>Primer length</th>
<th>Product length</th>
<th>CpGs</th>
</tr>
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<tbody>
<tr>
<td>ABHD7 Forward-1</td>
<td>5'-TTTGTTCATTATGGGATCATCGTCCGA-3'</td>
<td>58.4</td>
<td>30.4</td>
<td>23</td>
<td>123</td>
<td>1</td>
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<td>ABHD7 Reverse-1</td>
<td>5'-TCGAATCCATACCTTCCCTCCTCA-3'</td>
<td>59.8</td>
<td>36.3</td>
<td>22</td>
<td>82</td>
<td>1</td>
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<tr>
<td>ABHD7 Forward-2</td>
<td>5'-TTAGTTATGAAGGCTTATATGGG-3'</td>
<td>58.3</td>
<td>30.4</td>
<td>23</td>
<td>82</td>
<td>1</td>
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<tr>
<td>ABHD7 Reverse-2</td>
<td>5'-CCCTCCTATACCAACACAAATC-3'</td>
<td>58.4</td>
<td>45.2</td>
<td>22</td>
<td>82</td>
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<tr>
<td>FUCA1 Forward-1</td>
<td>5'-TGCTTTGTTAGGTTAGGTGGTAC-3'</td>
<td>59.3</td>
<td>50.0</td>
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<td>70</td>
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<td>FUCA1 Reverse-1</td>
<td>5'-ACCACATTCCTCCCTAATAC-3'</td>
<td>59.8</td>
<td>50.0</td>
<td>20</td>
<td>70</td>
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<td>5'-GGCGGGTGTGGTTTTGTTG-3'</td>
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<td>64.7</td>
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<td>FUCA1 Reverse-2</td>
<td>5'-ACAGCCCAATACACAGA-3'</td>
<td>59.3</td>
<td>55.0</td>
<td>20</td>
<td>145</td>
<td>15</td>
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<td>OF1 Forward</td>
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<td>47.6</td>
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<td>17</td>
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<td>OF1 Reverse</td>
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<td>59.7</td>
<td>50.0</td>
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<td>5'-CTCAACCAAGCAGAATCTCC-3'</td>
<td>59.3</td>
<td>57.1</td>
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<td>LRRC28 Forward-1</td>
<td>5'-GTTTTTGTGCTCTGCGCCTG-3'</td>
<td>59.0</td>
<td>45.0</td>
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<td>LRRC28 Reverse-1</td>
<td>5'-ACTACCTCCCTCCCTCACC-3'</td>
<td>58.6</td>
<td>57.8</td>
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<td>5'-TTTGTAAAAGTGGGCTAAAG-3'</td>
<td>58.4</td>
<td>42.8</td>
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<td>LRRC28 Reverse-2</td>
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<td>59.1</td>
<td>52.6</td>
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<td>5'-GTTACACGAGGGAGTACCC-3'</td>
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<td>57.8</td>
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</tr>
<tr>
<td>RLA1 Reverse-1</td>
<td>5'-CAACCCAGACAAACCCTAAA-3'</td>
<td>59.4</td>
<td>47.6</td>
<td>21</td>
<td>181</td>
<td>22</td>
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<tr>
<td>RLA1 Forward-2</td>
<td>5'-GGCGAGTGGGCTGCTTT-3'</td>
<td>59.3</td>
<td>64.7</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RLA1 Reverse-2</td>
<td>5'-CTCTCTCCCTTCGTCTCAC-3'</td>
<td>60.0</td>
<td>65.0</td>
<td>20</td>
<td>139</td>
<td>9</td>
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<tr>
<td>RLA1 Forward-3</td>
<td>5'-AGACGAGGATATGGGTGGG-3'</td>
<td>59.3</td>
<td>55.0</td>
<td>20</td>
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<td></td>
</tr>
<tr>
<td>RLA1 Reverse-3</td>
<td>5'-GGAACAGAAAAGAGAGCACC-3'</td>
<td>59.5</td>
<td>40.9</td>
<td>22</td>
<td>141</td>
<td>12</td>
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<tr>
<td>CDHR3 Forward</td>
<td>5'-TTTTAGGAGGTGGTCTAGAG-3'</td>
<td>58.9</td>
<td>45.8</td>
<td>24</td>
<td></td>
<td></td>
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<tr>
<td>CDHR3 Reverse</td>
<td>5'-TCACACAGATCTTCTTTCTCCAAA-3'</td>
<td>58.3</td>
<td>36.0</td>
<td>25</td>
<td>172</td>
<td>3</td>
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<tr>
<td>RUFY3 Forward-1</td>
<td>5'-TGAAAGGGATATGATGCTTGGTA-3'</td>
<td>59.9</td>
<td>37.5</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RUFY3 Reverse-1</td>
<td>5'-CTCAACATCTCTTTATAACCAACAA-3'</td>
<td>59.2</td>
<td>33.3</td>
<td>24</td>
<td>143</td>
<td>3</td>
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<tr>
<td>RUFY3 Forward-2</td>
<td>5'-GGGATTCTGGATGTTATTCGGAGG-3'</td>
<td>59.0</td>
<td>48.0</td>
<td>25</td>
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<tr>
<td>RUFY3 Reverse-2</td>
<td>5'-GAGACGCTCTATTCCTCCAAAC-3'</td>
<td>59.0</td>
<td>55.0</td>
<td>20</td>
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<tr>
<td>PCDHAC1 Forward-1</td>
<td>5'-GGTATCGAGGCTGGTATGTTG-3'</td>
<td>59.0</td>
<td>57.1</td>
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<tr>
<td>PCDHAC1 Reverse-1</td>
<td>5'-CAACCTCATAACTTCTCATCCAC-3'</td>
<td>59.2</td>
<td>50.0</td>
<td>22</td>
<td>95</td>
<td>9</td>
</tr>
<tr>
<td>PCDHAC1 Forward-2</td>
<td>5'-GTGATGCTTGTTGGTTCTT-3'</td>
<td>59.1</td>
<td>55.0</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSMB Forward</td>
<td>5'-GTTAGGGTTGTTGTTT-3'</td>
<td>59.3</td>
<td>65.0</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSMB Reverse</td>
<td>5'-CCAGAAAACAGACACTAACAAC-3'</td>
<td>59.5</td>
<td>47.6</td>
<td>21</td>
<td>150</td>
<td>16</td>
</tr>
<tr>
<td>TXNDC16 Forward-1</td>
<td>5'-TATCTCTCTCCTCCTATACGTC-3'</td>
<td>59.6</td>
<td>45.8</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TXNDC16 Reverse-1</td>
<td>5'-CCCTACACATGACACAACT-3'</td>
<td>59.3</td>
<td>45.4</td>
<td>23</td>
<td>123</td>
<td>4</td>
</tr>
<tr>
<td>TXNDC16 Forward-2</td>
<td>5'-GGGTTATGTTGTGTGTGTGTC-3'</td>
<td>58.0</td>
<td>47.8</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TXNDC16 Reverse-2</td>
<td>5'-TTTCTCCCAATTTCCATCTTCCA-3'</td>
<td>58.5</td>
<td>42.8</td>
<td>21</td>
<td>139</td>
<td>3</td>
</tr>
<tr>
<td>GAS6 Forward</td>
<td>5'-GAGAGTCTGTTATGTTATGTTCT-3'</td>
<td>58.6</td>
<td>40.0</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAS6 Reverse</td>
<td>5'-ATATCTCCTCAACCCACCTTCTTA-3'</td>
<td>59.2</td>
<td>41.6</td>
<td>24</td>
<td>120</td>
<td>2</td>
</tr>
<tr>
<td>TESC Forward-1</td>
<td>5'-TAAAGGGTGGTGGAGGAGGGA-3'</td>
<td>59.7</td>
<td>55.5</td>
<td>18</td>
<td>80</td>
<td>6</td>
</tr>
<tr>
<td>TESC Reverse-1</td>
<td>5'-GGGCGTTTCATCGATGTGTTT-3'</td>
<td>58.4</td>
<td>45.0</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TESC Forward-2</td>
<td>5'-CGACCACCCCGACACAC-3'</td>
<td>58.4</td>
<td>70.5</td>
<td>15</td>
<td>110</td>
<td></td>
</tr>
</tbody>
</table>

5 Results
In total, 23 primer pairs were designed, covering 13 genes. One primer pair each for four genes, two primer pairs each for eight genes and three primer pairs for one gene. This distribution was an effect of the size of gene region and not of the importance of the individual genes. All primer pairs were screened using regular PCR with two DNA samples per primer pair, one fully methylated and the other demethylated, in order to screen for amplification efficiency of both strands. Of all primers pairs, eleven managed to display good enough amplification of both DNA samples to go on to further trials with qPCR and MSHRM.
Only four of the primer pairs selected for qPCR showed the ability to differentiate amplification at varying temperatures, also known as temperature dependent differences in amplification. Primers containing CpGs have experimentally been shown to change binding affinity with temperature (Wojdacz et al, 2009) which aid in finding the equilibrium between amplification of both the methylated and unmethylated strands, and in turn aid in increasing resolution during HRM.

Table 2. Primer pair sequence and properties data. Tm and CG % data from primer design program Primer3. CpG dinucleotides underlined in primers where those were included.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Tm(° C)</th>
<th>CG%</th>
<th>CpGs</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUCA1 Forward-1</td>
<td>5'-TCGGTGTTAGGTTAGTGCCTAG-3’</td>
<td>59.3</td>
<td>50.0</td>
<td>2</td>
</tr>
<tr>
<td>FUCA1 Reverse-1</td>
<td>5'-AACCCCATCCCCCTCAAAATAC-3’</td>
<td>59.8</td>
<td>50.0</td>
<td>0</td>
</tr>
<tr>
<td>RUFY3 Forward-1</td>
<td>5'-TGAAAAAGGTAGTGCCTATTGA-3’</td>
<td>59.9</td>
<td>37.5</td>
<td>1</td>
</tr>
<tr>
<td>RUFY3 Reverse-1</td>
<td>5'-TCTCAAACTCTTTTATCAAAACCA-3’</td>
<td>59.2</td>
<td>33.3</td>
<td>0</td>
</tr>
<tr>
<td>PCDHAC1 Forward-1</td>
<td>5'-GGCTAAGGGTGGTATT-3’</td>
<td>59.0</td>
<td>57.1</td>
<td>2</td>
</tr>
<tr>
<td>PCDHAC1 Reverse-1</td>
<td>5'-CACCATCAAATCGCTTTACC-3’</td>
<td>59.2</td>
<td>50.0</td>
<td>1</td>
</tr>
<tr>
<td>TXNDC16 Forward-1</td>
<td>5'-TTGTTAAAGTGAAGGACGGCTTG-3’</td>
<td>59.6</td>
<td>45.8</td>
<td>1</td>
</tr>
<tr>
<td>TXNDC16 Reverse-1</td>
<td>5'-CCCCAATCTACAACACACACCA-3’</td>
<td>59.3</td>
<td>43.4</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3. Primer amplicons information, and annealing temperatures used to collect data.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product length (bp)</th>
<th>Product CpGs</th>
<th>Temperature (° C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUCA1</td>
<td>70</td>
<td>3</td>
<td>55.0</td>
</tr>
<tr>
<td>RUFY3</td>
<td>143</td>
<td>3</td>
<td>54.0</td>
</tr>
<tr>
<td>PCDHAC1</td>
<td>95</td>
<td>9</td>
<td>55.0</td>
</tr>
<tr>
<td>TXNDC16</td>
<td>123</td>
<td>4</td>
<td>54.0</td>
</tr>
</tbody>
</table>

The four working primer pairs were designed against the genes FUCA1, RUFY3, PCDHAC1 and TXNDC16 (table 2 and 3). Values for statistical tests were taken at the melting temperature where the positive and negative controls differed the most from each other, which was individual for each gene. A graphical illustration of the basis of data collection in FUCA1 can be seen in figure 1, where the thick black line represent the temperature chosen and the fluorescence values are collected where the lines cross the line at the x-axis. Genes were tested in both diencephalon and cerebellum, and the melting point for data selection was almost the same in both tissues.

Methylation differences between RJF and WL were identical in both tissues, and sample animals that deviated from the racial group in diencephalon showed the same methylation degree and group divergence in cerebellum. The animal test group consisted of a total of 16 animals. From these, 16 (8 WL and 8 RJF) diencephalon and 14 (7 WL and 7 RJF) cerebellum samples were retrieved, meaning the cerebellum tests were performed on slightly smaller groups.

RUFY3, PCDHAC1 and TXNDC16 were all significantly differentially methylated between WL and RJF in both tissues, whilst FUCA1 only showed trends indicating DM in both diencephalon (p = 0.08) and cerebellum (p = 0.20) (figure 2 and 3).

The methylation distribution for FUCA1 was the following: All RJF samples displayed 0 % to 2.5 % methylation, and six of the eight WL samples had 50 % methylation at the gene. The remaining two WL samples had 0 % methylation in both diencephalon and cerebellum, meaning these two individuals had the same methylation pattern as RJF. The result of this was a large internal variation in the WL sample groups (figure 1).
In *RUFY3*, RJF samples all displayed 100 % methylation whilst WL samples all showed 2.5 % to 25 % methylation except for one sample showing a methylation level of 25 % to 50 %. The calibration curve for *PCDHAC1* (data not presented) showed a clean separation, and RJF samples displayed methylation at 0 % and slightly above, up to below 2.5 %. For WL samples, the methylation level was between 2.5 % to 25 %, and easily distinguishable from RJF.

Both RJF and WL samples in *TXNDC16* showed 50 % methylation, barely distinguishable from each other when examining the raw data visually.

![Melting curves for primer pair FUCA1 in cerebellum. Graphs show positive (100 %) and negative (0 %) controls, and Red Junglefowl (RJF) and White Leghorn (WL) samples.](image)

*Figure 1. Melting curves for primer pair FUCA1 in cerebellum. Graphs show positive (100 %) and negative (0 %) controls, and Red Junglefowl (RJF) and White Leghorn (WL) samples. Standard error of means are included for RJF and WL samples. The error bars show the big sample variation in WL, compared to the very small variation in RJF. The black line cutting the graphs vertically at approximately 77.4 °C marks the temperature from which fluorescence data were selected for this specific gene and tissue.*
Figure 2. Mean value fluorescence data from diencephalon for Red Junglefowl (RJF) and White Leghorn (WL), with standard error calculations represented as error bars. Three genes show significantly differential methylation. As seen, both FUCA1 and RUFY3 show a larger sample variation in WL than in RJF. The normalized fluorescence is specific within each tested gene, so methylation degrees are not comparable between genes in this figure. (* = p<0.05; ** = p<0.01; *** = p<0.001).

Figure 3. Mean value fluorescence data from cerebellum for Red Junglefowl (RJF) and White Leghorn (WL), with standard error calculations represented as error bars. RUFY3, PCDHAC1 and TXNDC16 show significant differential methylation. Normalized fluorescence is specific within each tested gene, so methylation degrees are not comparable between genes. (** = p<0.01).
Test of the normal distribution of the sums of differences indicated no normal distribution for samples for genes \textit{FUCA1}, \textit{RUFY3} and \textit{PCDHAC1}. The assumption of normal distribution was not rejected for \textit{TXNDC16}, meaning Student's t-test was applied when calculating differences between RJF and WL in \textit{TXNDC16} compared to the Mann–Whitney test used for the other three genes.

\textbf{6 Discussion}

In this study, an already tested MSHRM method was used in the verification of differential DNA methylation of promoter regions in cerebellum and diencephalon from chickens. Promoter regions in four genes were analyzed in both diencephalon and cerebellum in a comparative study between WL and RJF, based on previous expression microarray and methylation tiling array data. Two of the genes, \textit{FUCA1} and \textit{PCDHAC1}, showed high methylation in WL compared to RJF, corresponding to downregulation of gene expression in the WL animals. The two other genes, \textit{RUFY3} and \textit{TXNDC16}, displayed low methylation in WL which indicates an upregulation of gene expression. Three genes showed significantly DM patterns between RJF and WL, and the fourth gene showed a clear trend towards methylation differences between RJF and WL.

In a previous study by Nätt et al (unpublished data), 145 genes were found to be DM between RJF and WL, and these methylation differences showed an overlap between offspring and parental animals. We sought to examine 13 genes, roughly 9 \% of all DM genes. Out of these only four primer pairs worked, i.e. four genes could be examined. This indicate a drawback of the used method that has to be improved, perhaps by finding out why a majority of primer pairs did not work as intended and thereby trying to increase robustness of designed primers. In regard of tissue specificity, both diencephalon and cerebellum tissue were tested, and all genes except \textit{FUCA1} were significantly DM in both tissues. \textit{FUCA1} showed a trend towards DM; however the large variation in WL made it unable to statistically establish significance between RJF and WL in either of the tissues. The diencephalic p-value was lower than in cerebellum, and this is most likely due to the sample size variation. Two animals deviated from the rest of the group, and the methylation pattern causing this was present in all tissues taken from these two individuals. Had the test group been slightly larger, containing one or two more animals with the same methylation degree as the rest of the WL animals, statistical significance might have been achieved when looking at racial differences.

The genes tested in this study were compared with resequenced data (Rubin et al, 2010) in order to check for sequence differences such as single nucleotide polymorphisms (SNPs), since methylations are sequence dependent and in need of CpGs. The \textit{RUFY3} promoter region contained three SNPs, whilst the three other genes regions contained no SNPs.

As for transmission of epigenetic signals, Bonasio (2010) states three independent criteria in order to classify a mechanism as truly epigenetic. Briefly, these are “a mechanism for propagation”, ”evidence of transmission” and ”effect on gene expression”. DNA methylation fulfills these: after cell division hemimethylated DNA is converted into fully methylated DNA with the help of methyltransferase; the cells keep methylated DNA methylated even after several cell divisions, and; transcription has been shown to be affected by methylation (Chen et al, 2007; Takebayashi et al, 2007; Wigler et al, 1981; Kass et al, 1997). DNA methylation would be a cis-epigenetic signal, a feature that might have arisen with increased complexity and as an alternative to trans-epigenetic signals and all complex pathways used in order to orchestrate them (Bonasio et al, 2010).

The four working genes showed the same methylation distribution as on the tiling array, as indicated by the fold change values in table 4, an indicative of the results being reproducible.
DE and DM do correlate for the four genes studied here, and in the pool of 145 genes from which they were selected. Still, this correlation does not have to imply causality, as methylations are not the only epigenetic factor. The epigenetic status of methylations is becoming unquestionable, but some studies question the role DNA methylation plays in the regulation of upstream CGI promoters in autosomal genes, and speculate that histones and their posttranslation modifications might have an equally relevant role (Maunakea et al, 2010).

Table 4. Data from the methylation tiling array from which the four genes in this study were selected. Fold change and correlating significance correspond to values for the offspring. Positive fold change indicates hypermethylation in White Leghorn, and negative values hypermethylation in Red Junglefowl.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUCA1</td>
<td>1.82</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RUFY3</td>
<td>-0.86</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>PCDHAC1</td>
<td>1.25</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TXNDC16</td>
<td>-1.36</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Those primers that did not generate results were discarded for various technical reasons, such as non-specific amplification, primer dimer formation or because they could not compensate for PCR bias, and therefore favored amplification of either the methylated or unmethylated DNA strands. This was tried to be avoided using Wojdacz’s (2008) primer design with CpGs. As seen, the gene methylations do not always show absolute or lack of any methylation, but end up on partial methylations in relation to the calibration series. This could occur if the dissection does not generate a homogenous cell tissue. Previous studies have found both expression and methylation differences across different brain tissues in humans, and heterogeneous tissues present an obstacle when trying to correlate expression rates to CpG methylation (Lister et al, 2009; Gibbs et al, 2010). However, since two separate parts of the brain were examined and the results acquired indicated no tissue differences, heterogeneous tissue is not a likely explanation. This could indicate that the four studied genes in this study have the same function in different part of the brain.

**FUCA1** encodes a lysosomal hydrolase, alpha-L-fucosidase, which is an enzyme that is involved in the degrading of fucose-containing glycoproteins (Occiodoro et al, 1996). RJF showed low methylation whilst a majority of the WL samples showed 50 % methylation. A small samples group as the one tested in this study makes it hard to determine how widespread this difference is among WLs. A possible explanation for the variation might be that epialleles have not yet been fixed in the population, or may never be if they are naturally occurring variations that are not selected upon. Methylations play a role in regulation of monoallelic gene expression in mammals and are a heritable factor connected to genomic imprinting (Keverne, 2009), and different methylation status on alleles could also explain why WL seems to have 50 % methylation. The variation in methylation could also be caused by trans-regulatory mechanism, and not at all be the effect of an epiallelic variation.

**RUFY3**, also known as a Singar (Single-axon-related), is a gene that as the name indicates suppresses formation of additional axons and thereby secures the robustness of neuronal polarity (Mori et al, 2007). A significant difference was found between RJF with 100% methylation and WL with 2.5 % to 25 % methylation. One WL sample deviated from the others, with a melting temperature corresponding to the 25 %-50 % methylation interval in the melting curves, and the explanation for this could as mentioned before be the lack of allelic fixation.
The genomic structure of PCDHAC1 (protocadherin alpha-C1) is that of clustered protocadherins (Morishita et al, 2007). Cadherins play a role in calcium-dependent selective cell-cell interactions, and it has been indicated to do so during development and in tissue morphogenesis, and also in the adult brain (Wu et al, 1999; Gumbiner 1996; Takeichi, 1991). There is a large molecular diversity in this gene family in the human population, and PCDH genes are suggested as candidate genes for determining functional characteristics in the brain (Yagi, 2008). PCDHAC1 had the best separation of melting curves and therefore the highest resolution. This could be because it had the most number of CpGs in the product which would give the biggest sequence difference between methylated and unmethylated strands after conversion. RJF samples were basically unmethylated at 0 % to 2.5 %, compared to WL samples with 2.5 % to 25 % methylation.

TXNDC16 encodes for a thioredoxin domain-containing protein which has been implied to affect DNA binding by redox regulation of transcription factors NF-κB and AP1 (Schenk et al, 1994; Matthews et al, 1992). NP-κB in turn has been shown to play a role in memory formation and learning capability (Meffert et al, 2003). Both RJF and WL groups showed 50 % methylation, but are significantly different from each other, probably because the groups do not overlap and the small internal variations. This does however exemplify how statistics don’t account for biological relevance, as this small difference for TXNDC16 probably is nowhere near as important as the large variation we have in FUCA1, a gene promoter which still show no significant methylation difference.

**Conclusion**

DNA methylations provide the genetic machinery with additional ways to regulate gene expression, and possibly new factors to select on during domestication. Here we show that MSHRM is a valuable tool for methylation detection and quantification in a smaller sample of animals. Three genes; RUFY3, PCDHAC1 and TXNDC16 were found to be significantly differently methylated between WL and RJF, correlating with differential expression. The fourth gene, FUCA1, showed a clear trend towards differential methylation between the races and with a larger sample group those differences could be enhanced. Even though no differences were found between cerebellum and diencephalon, primers designed for this experiment provide a ready to use tool for further testing in other tissues or generations in chicken.

**7 Acknowledgements**

I would like to thank Professor Per Jensen and Daniel Nätt for supervising me and allowing me to be a part of your project. I would also like to thank Martin Johnsson for all your technical support and guidance in the laboratory.

**8 References**


