# An epigenetic basis for essential hypertension

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# An epigenetic basis for essential hypertension

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Faculty of Medicine

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Thesis/Dissertation Sheet

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**Abstract 350 words maximum: (PLEASE TYPE)**

Essential hypertension – high blood pressure – is responsible for more disease and deaths worldwide than any other single health risk factor. Despite its thoroughly researched and confirmed heritability, essential hypertension lacks a genetic explanation. An underexplored cause for essential hypertension could be related to *epi*genetics. Epigenetic states dictate gene expression independent of variation in the underlying DNA sequence, and as such an aberrant epigenetic state would be invisible to conventional genetic association studies.

In this work I proposed to investigate the hypothesis that epigenetic changes could contribute to the development of hypertension. In order to investigate this, I utilised the canonical model for human hypertension, the spontaneously hypertensive rat (SHR). Comparison of cytosine methylation patterns of SHR to the related normotensive Wistar Kyoto rats (WKY) identified thousands of methylation differences between the kidneys of the two strains. Further exploration of the brain and liver of the same animals showed that some of these methylation differences were represented in all three germ layers, and thus hold the potential to be both inborn and transmissible events. Even though the vast majority of differences were located in yet to be annotated regions of the rat genome and could not be explored at this point, at least two candidates for so called "germline epimutations" were identified in *Arhgap11a* and *Tomm20*. Neither of the two had previously been linked to hypertension but are involved in biological pathways that can be associated with blood pressure regulation. In a complementary experiment I confirmed that transient exposure to Captopril caused reversion to normotension in the SHR. In contrast to previous reports, lowering of blood pressure was not heritable. However methylation

differences induced by transient Captopril treatment were subtle and less numerous than those seen in the comparison of WKY and SHR. Nevertheless, hundreds of methylation differences were found and 41 were the same as seen in all three tissues between SHR and WKY. The results of this thesis are consistent with some epigenetic involvement in blood pressure regulation, and provide a platform for future studies into the investigation of the role of epigenetics in human hypertension.

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## Abstract

Essential hypertension – high blood pressure – is responsible for more disease and deaths worldwide than any other single health risk factor. Despite its thoroughly researched and confirmed heritability, essential hypertension lacks a genetic explanation. An underexplored cause for essential hypertension could be related to *epi*genetics. Epigenetic states dictate gene expression independent of variation in the underlying DNA sequence, and as such an aberrant epigenetic state would be invisible to conventional genetic association studies.

In this work I proposed to investigate the hypothesis that epigenetic changes could contribute to the development of hypertension. In order to investigate this, I utilised the canonical model for human hypertension, the spontaneously hypertensive rat (SHR). Comparison of cytosine methylation patterns of SHR to the related normotensive Wistar Kyoto rats (WKY) identified thousands of methylation differences between the kidneys of the two strains. Further exploration of the brain and liver of the same animals showed that some of these methylation differences were represented in all three germ layers, and thus hold the potential to be both inborn and transmissible events. Even though the vast majority of differences were located in yet to be annotated regions of the rat genome and could not be explored at this point, at least two candidates for so called "germline epimutations" were identified in *Arhgap11a* and *Tomm20*. Neither of the two had previously been linked to hypertension but are involved in biological pathways that can be associated with blood pressure regulation. In a complementary experiment I confirmed that transient exposure to Captopril caused reversion to normotension in the SHR. In contrast to previous reports, lowering of blood pressure was not heritable. However, methylation differences induced by transient Captopril treatment were subtle and less numerous than those seen in the comparison of WKY and SHR. Nevertheless, hundreds of methylation differences were found and 41 were the same as seen in all three tissues between SHR and WKY. The results of this thesis are consistent with some epigenetic involvement in blood pressure regulation, and provide a platform for future studies into the investigation of the role of epigenetics in human hypertension.

## List of publications, presentations and awards

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Poster presentation: “An epigenetic basis for essential hypertension” EMBL Australian PhD Course, Canberra, Australia. 2014.

Oral presentation: “The role of epigenetics in high blood pressure”, Paul Korner Lecture Series, Victor Chang Cardiac Research Institute, Sydney, Australia. 2014.

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Oral presentation: “An epigenetic basis for essential hypertension”, Paul Korner Lecture Series, Victor Chang Cardiac Research Institute, Sydney, Australia. 2015.

Poster presentation: “The role of epigenetics in high blood pressure – rats under pressure”, EMBL Student Symposium, Sydney, Australia. 2015.

Poster presentation: “An epigenetic basis for essential hypertension”, Epigenetics 2015, Hobart, Australia. 2015.

Oral presentation: “An epigenetic basis for essential hypertension – rats under pressure”, Paul Korner Lecture Series, Victor Chang Cardiac Research Institute, Sydney, Australia. 2016.

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*"I am just a child who has never grown up. I still keep asking these 'how' and 'why' questions. Occasionally, I find an answer." – Stephen Hawking*

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## Abbreviations and symbols

| | |
|---|---|
| bp | base pairs |
| kb | kilobase pairs |
| nt | nucleotides |
| nm | nanometres |
| mm | millimetres |
| µM | micromolar |
| mM | millimolar |
| M | molar |
| µl | microlitres |
| ml | millilitres |
| ng | nanograms |
| µg | micrograms |
| mg | milligrams |
| g | grams |
| d | days |
| h | hours |
| min | minutes |
| s | seconds |
| × *g* | times gravity |
| V | volts |
| °C | degree Celsius |

| | |
|---|---|
| 5’ | 5’ end of nucleic acid |
| 3’ | 3’ end of nucleic acid |
| A | adenine |
| T | thymine |
| C | cytosine |
| G | guanine |
| U | uracil |
| Chr | Chromosome |
| CpG | CG dinucleotide |
| CGI | CpG island |
| CGI shore | CpG island shore |
| RNAi | RNA interference |
| dsRNA | double-stranded RNA |
| ssRNA | single-stranded RNA |
| PCR | polymerase chain reaction |
| RT-PCR | reverse transcription PCR |
| q-RT-PCR | quantitative RT-PCR |
| 5mC | 5-methylcytosine |
| DNA | deoxyribonucleic acid |
| RNA | ribonucleic acid |
| mRNA | messenger RNA |
| DNMT | DNA methyltransferases |
| ncRNA | non-coding RNA |
| RNase | ribonuclease |
| DNase | deoxyribonuclease |
| UV | ultraviolet |
| GSC | germline stem cell |
| PGC | primordial germ cell |
| EtOH | ethanol |
| EtBr | ethidium bromide |
| RT | room temperature |
| DMEM | Dulbecco’s modified Eagle’s medium |
| DMC | Differentially methylated cytosine |
| DMR | Differentially methylated region |
| RRBS | Reduced representation bisulfite sequencing |
| SHR | Spontaneously hypertensive rat (descendant from Wistar rats) |
| WKY | Wistar Kyoto rat (descendant from Wistar rats) |
| ACE | Angiotensin-converting-enzyme |
| CAP | Captopril |
| SHR-CAP | SHR animals exposed to Captopril |
| CAP-F2 | Offspring of SHR animals exposed to Captopril |
| UNSW | University of New South Wales |

## Contribution

Throughout this work bioinformatics analysis and file conversion as well as sequencing data processing and application of annotation scripts was performed with the help of Paul Young. He further conducted file conversion and applied annotation scripts.

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# Chapter 1. Introduction

## 1.1 Hypertension

Hypertension, commonly known as high blood pressure, is a chronic medical condition in which the blood pressure exceeds 140/90 mmHg (systolic/diastolic). Hypertension can be classified as either of two different types: primary/essential hypertension (from here on referred to as essential hypertension) or secondary hypertension.

Secondary hypertension is observed in about 5-10% [1] of all patients who present to their doctor with a sudden onset of high blood pressure [2, 3]. It is frequently referred to as treatable or curable hypertension [4] since it is defined as being caused by another medical condition. These conditions are many, including renal malfunctions, endocrine disease, and pregnancy, and some are attributable to a Mendelian genetic lesion [5]

Essential hypertension, however, lacks any apparent associated underlying condition and as such has been the focus of scientific investigation for decades. The term, first coined in 1911 by Eberhard Frank (cited in [6]), describes a form of hypertension that is often familial despite no confirmed genetic cause being identified. It furthermore presents sexual dimorphism, with men displaying higher blood pressure than women throughout most of their lives, whilst women present with more rapidly developing hypertension later in life [7, 8]. These idiosyncrasies of essential hypertension can be observed all over the world, despite varying ethnicities and cultural backgrounds [9].

The danger of essential hypertension lies in the long-term damage that can be caused if high blood pressure is either not discovered or cannot be controlled through medication. Essential hypertension is the main contributor for cardiovascular disease (CVD) such as damage to the vasculature of the eye, atherosclerosis, renal failure, stroke, heart failure and heart attacks [10].

### 1.1.1 Occurrence and global impact

Essential hypertension is a global health burden and from 2010 became the number one health risk factor, as quantified by Disability-adjusted life years (DALYs) [11]. DALYs are a global measure of the overall disease burden (years affected or lost) due to a medical condition.

When the World Health Organization published 'A global brief on hypertension' in April 2013 [12] they reported that worldwide, in 2008, approximately 40% of all adults aged 25 and above had been diagnosed with essential hypertension. They also estimated that essential hypertension accounts for over 9 million deaths worldwide every year. The number of people globally affected by essential hypertension has been dramatically increasing over time from 600 million people in 1980, to 972 million in 2000, to over 1 billion in 2008. It is estimated to rise even further to 1.5 billion by 2025 [12].

The prevalence of essential hypertension is highest in developing countries, with 639 million people affected compared to 333 million in developed countries in 2000 [12, 13]. This observation is particularly concerning given that only few cases of hypertension were recorded in developing countries across the world in the past century [14, 15], by 2025 approximately 75% of the world's hypertensive population are estimated to be living in these regions [12].

Interestingly, studies have found the prevalence of hypertension to also be greater in urban areas compared to rural areas within the same country [13, 16]. This, much like the increase in essential hypertension in developing countries, may be due to Western lifestyle-related factors such as obesity, diabetes, stress etc. that are quickly becoming commonplace in urbanized and more developed areas. It should be mentioned, however, that in both developed countries as well as urban areas, medical intervention of essential hypertension is more easily accessible which may explain the different distribution of essential hypertension in areas lacking these benefits.

When investigating the overall prevalence of hypertension it is important to note that, especially in developing countries, the 'rule of halves' is applicable, due to the fact that hypertension is

asymptomatic for most of the affected individual's life. Coined in the 1970s the 'rule of halves' states that: 'half the people with hypertension are not known, half of those known are not under treatment, and half of those treated do not have their blood pressure under control despite the medication' [17]. This rule has held true over time and in all countries tested [18-20], particularly in developing countries where improvements are needed in accessibility and advances in medical treatment and care [21, 22].

Apart from the personal cost, hypertension also carries a huge global financial burden. For example, in 2013 the annual average economic burden caused by hypertension on the United States was estimated to be at $51.2 billion. Projections for 2030 suggest for the U.S. alone the total direct costs could increase to an estimated $200 billion [23].

### 1.1.2 Environmental factors and heritability

Right from the initial investigations into blood pressure it became apparent that the environment and lifestyle are key factors affecting essential hypertension (reviewed in [24]). The environmental factors found to negatively impact blood pressure regulation the most are chronic stress [25], salt intake [26, 27], excessive alcohol consumption [28-30], obesity [31-33] and physical inactivity [34, 35].

Interestingly environmental effects on essential hypertension are most clearly shown between populations rather than between individuals within a population. For example, the absence of high blood pressure in some societies may reflect different geography, diet, and lifestyle [36]. Nevertheless there are also obvious influencing factors within populations. Copious research over decades has elucidated a strong heritability component to the essential hypertension. Numerous investigations of nuclear families were carried out to reveal the degree of heritability. However, given that both parent-offspring and sibling pairs share on average only 50% of their genetic material, it was difficult to distinguish genetic from shared environmental influences. One approach used to overcome this was to compare these results to adoption studies [37]. The more elegant and common study designs, however, include twin studies examining

monozygotic (MZ) and dizygotic (DZ) twin pairs. The following tables adapted from a review by Wang and Snieder [38] show that in pediatric twin studies the estimated heritability ($h^2$) for systolic blood pressure (SBP) ranges widely from 11 to 78%, with the average closer to 50%, throughout both genders and different ethnicities (Table 1-1).

**Table 1-1. Pediatric twin studies estimating heritability ($h^2$) in systolic (SBP) and diastolic blood pressure (DBP)**

| Study | Pairs of twins | Age | | Race | Sex | $h^2$ | |
|---|---|---|---|---|---|---|---|
| | | Mean (SD) | Range | | | SBP | DBP |
| Yu *et al.* **[39]** | 274 MZ, 65 DZ | ? (?) | 0.0 – 1.0 | Chinese | Male & Female | 0.29 – 0.55 | 0.27 – 0.45 |
| Levine *et al.* **[40]** (supported by Kramer **[41]**) | 67 MZ, 99 DZ | ? (?) | 0.5 – 1.0 | Black & White | Male & Female | 0.66 | 0.48 |
| Havlik *et al.* **[42]** | 72 MZ, 40 DZ | | | Black | Male & Female | 0.46 | 0.51 |
| | 43 MZ, 42 DZ | | | White | Male & Female | 0.11 | 0.71 |
| | 115 MZ, 82 DZ | 7.0 (?) | ? | All | Male & Female | 0.23 | 0.53 |
| Wang *et al.* **[43]** | 75 MZ, 35 DZ | ? (?) | 7.0 – 12.0 | Chinese | Male & Female | 0.32 | 0.46 |
| Schieken *et al.* **[44]** | 71 MZM, 74 MZF | 11.1 (0.25) | ? | White | Male | 0.66 | 0.64 |
| | 23 DZM, 31 DZF, 52 DOS | | | | Female | 0.66 | 0.51 |
| McIlhany *et al.* **[45]** | 40 MZM, 47 MZF | 14.0 (6.5) | 5.0 – 50.0 | Black & White | Male | 0.41 | 0.56 |
| | 32 DZM, 36 DZF, 45 DOS | | | | Female | 0.78 | 0.61 |
| Snieder *et al.* **[46]** | 75 MZM, 91 MZF | 14.9 (3.0) | 10.0 – 26.0 | White | Male | 0.57 | 0.45 |
| | 33 DZM, 31 DZF, 78 DOS | | | | Female | 0.57 | 0.45 |
| | 52 MZM, 58 MZF | 14.6 (3.2) | 10.0 – 26.0 | Black | Male | 0.57 | 0.58 |
| | 24 DZM, 39 DZF, 50 DOS | | | | Female | 0.57 | 0.58 |
| Snieder *et al.* | 35 MZM, | 16.8 (2.0) | 13.0 – 22.0 | White | Male | 0.49 | 0.69 |

| | | | | |
|---|---|---|---|---|
| **[47]** | 33 MZF | | | |
| | 31 DZM, 29 DZF, 28 DOS | Female | 0.66 | 0.50 |

MZF monozygotic females, MZM monozygotic males, DZF dizygotic females, DZM dizygotic males, DOS dizygotic opposite sex, '?' unknown/undisclosed.

The same degree of heritability was observed in adult twin studies where $h^2$ was found to range from 17 – 74% (Table 1-2).

**Table 1-2. Adult twin studies estimating heritability ($h^2$) in systolic (SBP) and diastolic blood pressure (DBP).**

| Study | Pairs of twins | Age | | Race | Sex | $h^2$ | |
|---|---|---|---|---|---|---|---|
| | | Mean (SD) | Range | | | SBP | DBP |
| Sims *et al.* **[48]** | 40 MZM, 45 DZM | 19.4 (3.0) | ? | White | Male | 0.68 | 0.76 |
| Ditto **[49]** | 20 MZM, 20 MZF | 20.0 (5.0) | 12.0 – 44.0 | White | Male | 0.63 | 0.58 |
| | 20DZm, 20 DZF, 20 DOS | | | | Female | 0.63 | 0.58 |
| McCaffery *et al.* **[50]** | 129 MZ, 66DZ | 21.3 (2.8) | 18.0 – 30.0 | 94% White | Male & Female | 0.48 | 0.51 |
| Bielen *et al.* **[51]** | 32 MZM | 21.7 (3.7) | 18.0 – 31.0 | White | Male | 0.69 | 0.32 |
| | 21 DZM | 23.8 (3.9) | | | | | |
| Fagard *et al.* **[52]** | 26 MZM | 23.8 (4.2) | 18.0 – 38.0 | White | Male | 0.64 | 0.73 |
| | 27 DZM | 24.7 (4.8) | | | | | |
| Busjahn *et al.* **[53]** | 100 MZ, 66DZ | 29.8 (12.0) | ? | White | Male & Female | 0.74 | 0.72 |
| Slattery *et al.* **[54]** | 77 MZM, 88 DZM | ? (?) | 22.0 – 66.0 | White | Male | 0.60 | 0.66 |
| Vinck *et al.* **[55]** | 150 MZ, 122 DZ | 34.9 (?) | 18.0 – 76.0 | White | Male & Female | 0.62 | 0.57 |
| Jedrusik *et al.* **[56]** | 39 MZ, 37 DZ | 35.0 (8.0) | 18.0 – 45.0 | White | Male & Female | 0.53 | 0.62 |
| Williams *et al.* **[57]** | 14 MZM, 44 MZF | 36.4 (?) | 17.0 – 65.0 | White | Male | 0.60 | 0.52 |
| | 9 DZM, 31 DZF, 11 DOS | | | | Female | 0.60 | 0.43 |
| Austin *et al.* **[58]** | 233 MZF, 170 DZF | 41.0 (?) | ? | 90% White | Female | 0.35 | 0.26 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Baird *et al.* **[59]** | 30 MZM, 28 MZF | 43.7 (1.4) | 40.5 – 46.5 | White | Male | 0.48 | 0.30 |
| | 35 DZM, 45 DZF, 60 DOS | | | | Female | 0.48 | 0.76 |
| Snieder *et al.* **[47]** | 43 MZM, 47 MZF | 44.4 (6.7) | 34.0 – 63.0 | White | Male | 0.40 | 0.42 |
| | 32 DZM, 39 DZF, 39 DOS | | | | Female | 0.63 | 0.61 |
| Snieder *et al.* **[60]** | 213 MZF, 556 DZF | 45.4 (12.4) | 18.0 – 73.0 | White | Female | 0.17 | 0.22 |
| Feinleib *et al.* **[61]** | 250 MZM, 264 DZM | ? (?) | 42.0 – 56.0 | White | Male | 0.60 | 0.61 |
| Hong *et al.* **[62]** | 41 MZM, 66 MZF | 63.0 (8.0) | > 50.0 | White | Male | 0.56 | 0.32 |
| | | | | | Female | 0.56 | 0.32 |
| Wu *et al.* **[63]** | 332 MZM, 111 DZM, 288 MZF, 103 DZF, 200 DOS | 37.8 (9.8) | 19.1 – 81.4 | Chinese | Male & Female | 0.46 | 0.30 |

MZF monozygotic females, MZM monozygotic males, DZF dizygotic females, DZM dizygotic males, DOS dizygotic opposite sex, '?' unknown/undisclosed.

On average the heritability in the adults was therefore estimated to be over 55%, independent of gender. The quoted studies did not reflect much ethnic diversity, as they were mainly performed with Caucasian twins. Nevertheless, this meta-analysis reveals some clear heritability of essential hypertension which leads to the next question as to the origins of this heritability.

### 1.1.3 Investigation of potential genetic origins of essential hypertension

To determine the origins of the observed heritability of hypertension researchers initially turned to candidate gene approach studies, and family linkage studies. The candidate gene approach focused on genes already identified in several major pathways involved in blood pressure regulation, and attempted to identify single-nucleotide polymorphisms (SNPs) between healthy and hypertensive individuals. The results of most of these investigations ([64-66]) revealed only a small number of SNPs with a very a limited contribution to the phenotype in only a small number of individuals. The major limitation of this approach is that it relies on existing

knowledge about blood pressure homeostasis [67]. Therefore, candidate gene approach studies cannot identify novel genes or chromosomal regions associated with essential hypertension. Furthermore the reproducibility of these studies have proven to be either challenging [66] or inconsistent [64, 65].

The field subsequently moved on to family linkage studies which, while overcoming the above obstacles, presented with shortcomings of their own. Linkage studies identify candidate genes by linking inherited genomic regions to similarities in individual phenotypes within a family. Many of these studies have identified regions of interest but reproducibility outside of the original family unit is most often lacking [68]. Additionally, the small sample size restricts the statistical power of these investigations: only candidate genes with a large contribution to the phenotype can be uncovered.

With the advent of genome-wide association studies (GWAS) an improved platform became available to investigate the underlying genetics of essential hypertension. GWAS are able to utilize upwards of a few hundred thousand SNPs, which are then interrogated in order to determine any association between these genetic markers and a given complex trait, such as essential hypertension. The advantage here is that family cohorts are unnecessary and that the unifying element is the disease or the lack of it. A significance threshold of $p \leq 5 \times 10^{-8}$ [69-71] has been commonly used based on the estimation that approximately 1 million independent SNPs are present in a population of European decent [72].

When the first two essential hypertension GWAS were performed in 2007 they failed to reach this level of significance for any locus. The Wellcome Trust Case Control consortium investigated approximately 500,000 SNPs for seven common diseases (this was approximately 2,000 individuals in essential hypertension alone) and only essential hypertension failed to reach the significance threshold [73]. The other GWAS, conducted by the Framingham Heart Study, specifically focused on quantitative BP phenotypes, SBP and DBP, and analysed approximately 71,000 SNPs genome-wide in just under 1,400 individuals. This study also failed to find

significant results [74]. The lack of tangible findings from these two comprehensive studies suggested that the underlying mechanisms for BP regulation must be far more complex than anticipated and future GWAS were performed with far greater samples sizes. The following Table 1-3 gives an excerpt of the extensive research that has been undertaken in essential hypertension GWAS and the number of individuals that have been screened.

**Table 1-3. Excerpt of genome-wide association studies (GWAS) performed since 2007 to the present.**

| Study | Year | SNPs investigated | No of individuals | | Ethnicity | Loci identified | |
|---|---|---|---|---|---|---|---|
| | | | discovery | replication | | total | new |
| Wellcome Trust Case Control **[73]** | 2007 | 500,568 | ~2,000 | | European | no significance[a] | |
| Levy *et al.***[74]** | 2007 | 70,987 | 1,260 | | European | no significance[a] | |
| Saxena *et al.***[75]** | 2007 | 386,731 | 2,931 | | European | no significance[a] | |
| Adeyemo *et al.***[76]** | 2009 | ~800,000 | 1,017 | | African American | no significance[a] | |
| Levy *et al.***[77]** | 2009 | ~2.5 million | 29,136 | 34,433 | European | 11 | |
| Newton-Cheh *et al.***[78]** | 2009 | ~2.5 million | 34,433 | 71,225 | European | 8 | 3 |
| Wang *et al.***[79]** | 2009 | 79,447 | 7,125 | | European ancestry (Amish) | 1 | |
| Kang *et al.***[80]** | 2010 | 1,253 | 756 | | African ancestry | no significance[a] | |
| Padmanabhan *et al.***[81]** | 2010 | 551,629 | 3,320 | 36,386 | European | 1 | |
| Ehret *et al.***[82]** | 2011 | ~2.5 million | 69,395 | 233,662 | European | 16 | 10 |
| Fox *et al.***[83]** | 2011 | ~2.5 million | 8,591 | 81,781 | African American | 3 | |
| Ho *et al.***[84]** | 2011 | > 360,000 | 28,345 | | European | 1 | |
| Kato *et al.* **[85]** | 2011 | | 30,126 | | Asian | 11 | 4 |
| Wain *et al.***[86]** | 2011 | | 74,064 | 48,607 | European | 7 | |
| Kidambi *et al.***[87]** | 2012 | | 2,474 | | African American | no significance[a] | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Franceschini *et al.* **[88]** | 2013 | ~2.42 million | 29,378 | 99,383 | African American, European, east Asian | 5 | 3 |
| Ganesh *et al.***[89]** | 2014 | ~2.5 million | 46,629 | 39,489 | European | 19 | 4 |
| Simino *et al.***[90]** | 2014 | ~2.5 million | 99,241 | 8,682 | European and Asian | 20 | |
| Tragante *et al.***[91]** | 2014 | ~50,000 | 87,736 | 68,368 | European | 11 | 27 |
| Lu *et al.***[92]** | 2015 | 2,485,448 | 11,816 | 69,146 | Chinese | 4 | |
| Ehret *et al.***[93]** | 2016 | 128,272 | 201,529 | 140,886 | European | 66 | 17 |
| Liang *et al.***[94]** | 2017 | | 31,968 | 54,395 | African ancestry and multi-ethnic | 12 | |
| Park *et al.***[95]** | 2017 | 500,568 | 8,839 | | Korean | 2 | |
| Salvi *et al.***[96]** | 2017 | | 1,739 | | European ancestry | no significance[a] | |
| Sofer *et al.***[97]** | 2017 | | 12,278 | | Latino/ Hispanic | no significance[a] | |
| Wain *et al.* **[98]** | 2017 | ? | 150,134 | 228,245 | European | 8 | 6 |
| Warren *et al.* **[99]** | 2017 | ~9.8 million | 152,249 | 330,965 | European | 107 | 11 |

[a] no loci reached $p \leq 5 \times 10^{-8}$.

This list makes no claim for completeness.

The GWAS listed here alone, interrogated over 2.5 million individuals and only identified a few hundred loci that held any potential to regulate essential hypertension, SBP or DBP. However, all these studies are in agreeance that the associated genetic variation identified thus far explains only up to 4% variability in blood pressure [82, 99, 100]. In regards to the observed heritability of around 50%, stated above, this is of great surprise.

To put this into some perspective: height, another classical complex trait similar to BP, has an estimated heritability of ~80% [101]. The international Genetic Investigation of Anthropometric Traits (GIANT) Consortium has been able to identify 697 genome-wide significant variants, identified in 253,288 individuals, to explain 20% of the variance in height [102].

One proposed explanation as to why essential hypertension GWAS have not been as successful as other GWAS is that essential hypertension develops slowly over time and develops in response to the environment. As such an individual with a normal blood pressure measurement may carry the essential hypertension causal loci but may not have yet developed the disease due to lack of exposure to external factors for essential hypertension such as age, persisting stress, excessive salt intake, inactivity and obesity.

While shortcomings of the essential hypertension GWAS, such as potentially incorrectly categorizing individuals in their cohorts persist, there could also be other explanations for the accumulation of essential hypertension in families, independent of a genetic cause. Given that both lifestyle choices and external influencers can trigger and worsen essential hypertension [24, 103, 104], more and more research is emerging, pointing towards epigenetics to have an impact on essential hypertension and calling for investigations into that direction.

## 1.2 Epigenetics

The term ‘epigenetics’ refers to a system of changes in gene expression that are independent of alterations to the underlying DNA sequence [105]. Epigenetic states are heritable through cell division, maintaining epigenetic patterns faithfully throughout the soma during the lifetime of an organism, and can sometimes even be passed on through the germline from one generation to the next. Epigenetic gene regulation is essential for eukaryotic life and epigenetic states are typically represented in a binary form of genetic transcription: ‘on’ or active versus ‘off’ and silent.

### 1.2.1 Epigenetic gene regulation

As mentioned, epigenetic gene regulation is fundamental to eukaryotes. In metazoans in particular, it plays a crucial role in cell differentiation and organ formation [72, 106], parental imprinting and fetal development [107, 108], silencing of repetitive elements (which has a positive effects on the genome stability) as well as maintenance of X inactivation [109].

The basic concept is that epigenetics enables the utilization of a single underlying genome to create multiple "epigenomes" in order to give rise to the wide range of cell diversity that can be found in complex organisms. Epigenetic gene regulation works through the principle that transcription is either enabled or inhibited. This inaccessibility of the genetic information can manifest in numerous ways: from the way DNA is packaged as either heterochromatin – tight and inaccessible – or euchromatin – open and often under active transcription, to direct molecular alterations to the DNA facilitating gene expression, as well as more recently discovered forms of gene regulation that involve RNA pre- and post-transcription. These mechanisms will be described in more detail below. However, given the complexity of epigenetic regulation, it is not surprising to find that sometimes mistakes occur in this system (effectively analogous to mutations, but without changing the DNA sequence). Such errors, called epimutations, incur phenotypes that are impossible to be explained by traditional Mendelian rules of heredity.

### 1.2.2 Epigenetic mechanisms

As previously mentioned there are three major epigenetic mechanisms that influence gene regulation by either permitting or obstructing transcription. Often two or even all three of these mechanisms can work synergistically.

Histone modification

The first of these is histone modification. DNA, when packaged within the nucleus, is wrapped around numerous histone octamers forming so called nucleosomes. These nucleosomes make up the fundamental subunits of the chromatin. The N-terminal 'tail' of the histone proteins can be equipped with different covalent modifications (including methylation, acetylation, phosphorylation, ubiquitination and others), either facilitating or repressing DNA repair, replication or even transcription by altering the DNA accessibility to transcription enzymes [110]. Histone modifications can impact all loci and are conserved throughout the domain of eukaryotes.

Non-Coding RNAs

More recently, non-coding RNAs (ncRNAs) have been found to be a key epigenetic mechanism for regulating gene expression by directing the initiation of the silent, heterochromatin state. After being transcribed from DNA these RNA molecules are not then translated into protein; rather they regulate gene expression both at the transcriptional and post-transcriptional level. They play roles in gene silencing, DNA methylation targeting, heterochromatin formation, as well as histone modification [111]. To date the best understood epigenetic functions of ncRNAs are via mRNA interference and facilitating its cleavage as well as through recruitment of protein complexes that initiate the heterochromatin state of the DNA itself. No doubt there are further epigenetic actions of ncRNA that remain to be discovered.

DNA methylation

The best studied epigenetic mechanism, however, is DNA methylation. Since being described as early as 1948 by Hotchkiss [112] in mammalian DNA - interestingly calling it ‘epicytosine’ - research on methylation has progressed considerably (Figure 1).

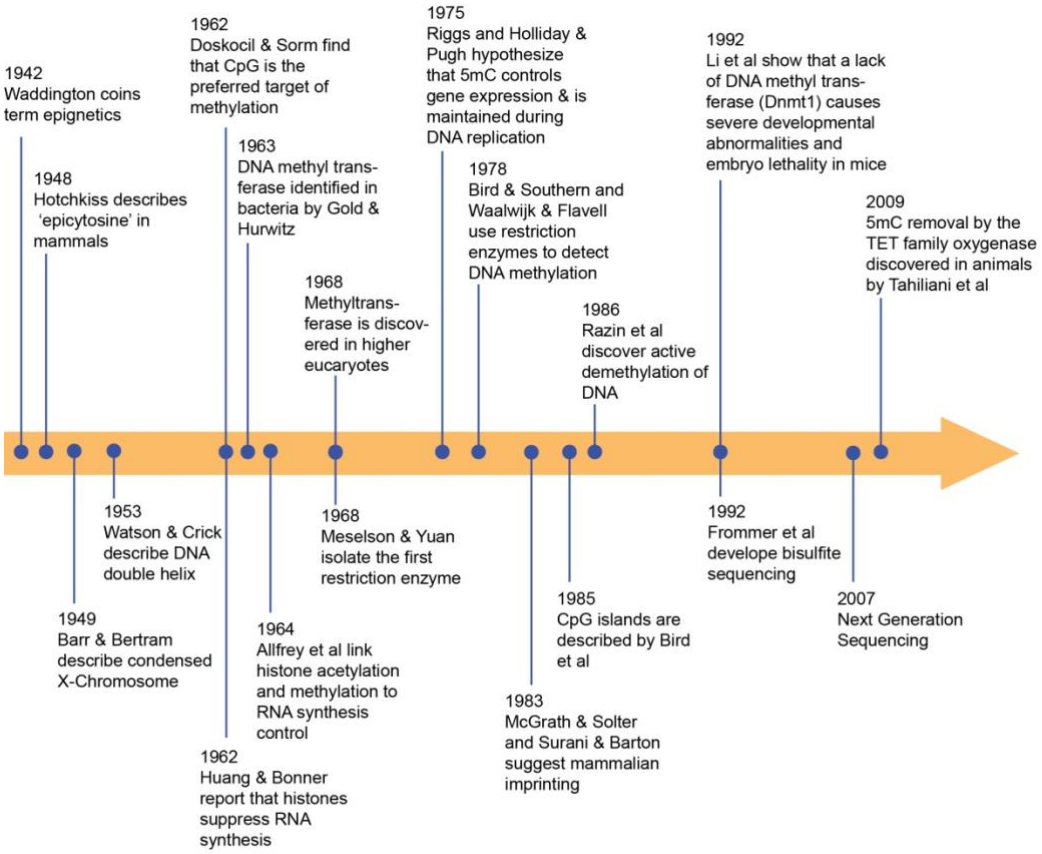



**Figure 1. Milestones in cytosine methylation research** [107, 112-129]

Typically, this covalent modification of the DNA is broadly distributed in nature. When present in metazoans, as Hotchkiss proposed, the pyrimidine derivative cytosine is usually the one targeted to be unmethylated or methylated. In the latter, DNA methyltransferases (DNMTs) transfer a methyl group to the C-5 position of the cytosine ring (Figure 2).

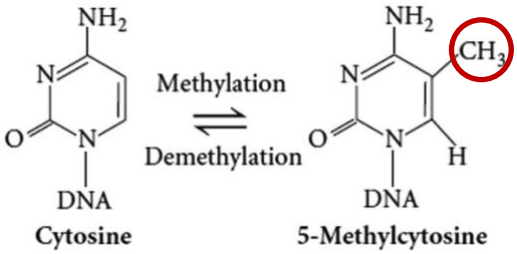



**Figure 2. Cytosine methylation**

In mammals, greater than 98% of cytosine methylation occurs in the context of an immediately following 3'guanine nucleotide [130]. These CpG sequences are, however, not randomly

distributed in the mammalian genome but are mostly clustered in regions of CG enrichment, so called CpG islands [125]; such islands often contain the promoter of the adjacent gene. Due to so far unknown mechanisms CpG islands generally remain unmethylated. If methylation occurs however, as suggested by both Riggs [123] and Holliday & Pugh [131] as early as 1975, gene expression is inhibited and the gene is effectively silenced. In other words, by just a covalent molecular attachment to the DNA sequence and without any mutation, genes can be equally disrupted in their expression as an actual, detectable lesion to the underlying DNA strand in form of mutation, deletions, insertions, SNPs etc.

### 1.2.3 Methylation maintenance and epigenetic reprogramming

Cytosine methylation in mammals is maintained via three DNA methyltransferases (DNMTs): DNMT1, DNMT3a and DNMT3b [132].

As a general rule DNMT1 is responsible for maintenance of methylation while DNMT3a and 3b are *de novo* DNMTs [133, 134]. DNMT1 furthermore is expressed the highest of the three and mutations in DMNT1 will cause the most severe phenotype. DNMT1 functions by binding to hemi-methylated DNA, usually the parental strand after mitosis [135] or post DNA repair [134], and then establishes methylation on the cytosine of the counter strand [136]. Experiments in mice have thus far demonstrated that loss of DNMT1 leads to genome-wide hypomethylation, loss of monoallelic expression of imprinted genes and embryonic lethality [127, 137]. DNMT3a and b are essential for early development and particularly the establishment of *de novo* methylation following embryo implantation as well as for parental imprinting (explained below) [138, 139]. A loss of these enzymes has also been found to be lethal [140].

When discussing the importance of methylation and its maintenance, the process of epigenetic reprogramming also needs to be examined (Figure 3 [141]).

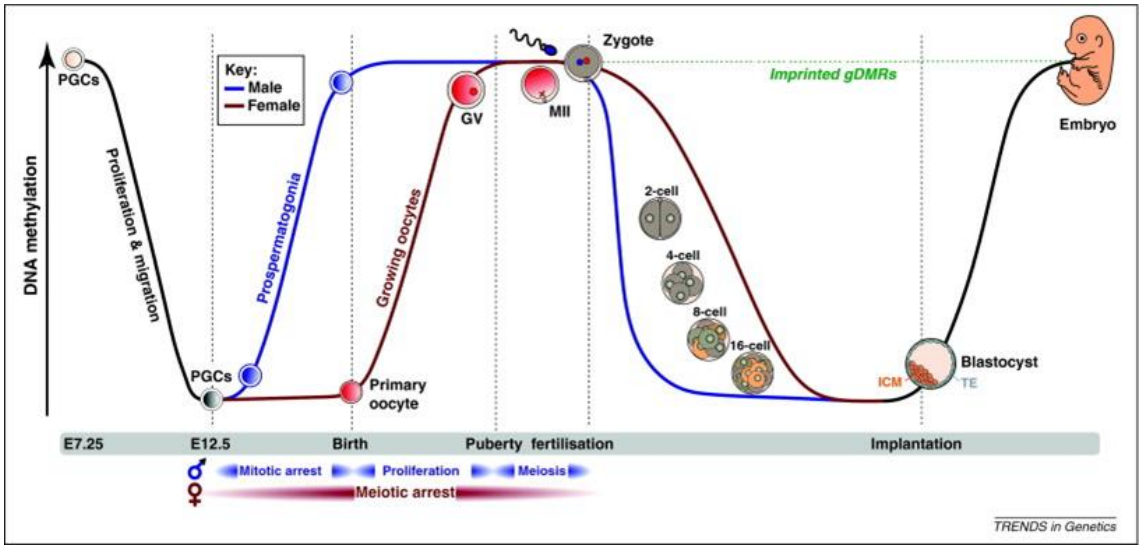



**Figure 3. DNA methylation changes during developmental epigenetic reprogramming.**

In mice, primordial germ cells (PGCs) emerge in embryos at E7.5 and DNA methylation is globally erased (black line). Following sex-determination, new DNA-methylation landscapes are established in germ-cell precursors in an asymmetrical fashion in male and female embryos. In the male embryo (blue line), *de novo* methylation takes place before meiosis in mitotically arrested cells (G1-phase; pro-spermatogonia) and is completed before birth. In the female embryo (red line), primary oocytes enter meiosis and arrest in prophase-I (diplotene stage); DNA methylation is established after birth during the follicular/oocyte growth phase. At puberty, under specific endocrine triggers, fully-grown germinal vesicle (GV) oocytes resume the first meiotic division. After extrusion of the first polar body, oocytes arrest in metaphase of the second meiotic division (MII oocytes) and meiosis is completed only upon fertilisation. Following fertilisation, a new wave of DNA demethylation takes place that is distinct on the parental genomes. In the zygote, DNA methylation of the paternal genome is rapidly erased by an active mechanism (blue line). Demethylation of the maternal genome is slower (red line) and is dependent on DNA replication (passive demethylation). These post-fertilisation demethylation events do not include imprinted gDMRs (green dotted line), resulting in parental-allele-specific methylation of these elements in early embryos and consequent parental-allele-specific expression of associated imprinted genes. Concomitant with blastocyst implantation and cell-lineage determination, new methylation landscapes become established, associated with cellular differentiation [141].

Essentially two events of active erasure of methylation as well as *de novo* establishment can be found in mammalian development. One takes place prior to implantation in the zygote and the

other in the germ line of the developing embryo. Generally speaking both restore a level of totipotency, establish the necessary parental imprinting and are assumed to facilitate the erasure of potential epimutations [142].

Interestingly some areas of the chromosomes remain methylated and silenced even during the two events of methylation remodeling most likely due to their 'nature' that they would otherwise disrupt the necessary events in the DNA and the cells as a whole. These areas escaping epigenetic reprogramming include centromeres, repetitive elements [143] and transposons [144, 145]. There are clearly mechanisms that help maintain silence of these regions throughout the life cycle, raising the possibly that other regions may be captured by this system.

### 1.2.4 Scientific investigation of cytosine methylation

As a result of the papers by Riggs [123] and Holliday and Pugh [131], identifying that cytosine methylation is in fact capable of influencing gene expression, methods were developed over time to measure and investigate these epigenetic alterations to the DNA.

Early quantitative approaches to measure methylation were restricted to high-performance liquid chromatography [146] or radioactive labeling of DNA samples [147]. Broadly speaking these early studies were limited to global methylation comparisons and did not reveal much about the epigenetic state of single genes.

With the publication of the first genomes, research became more locus specific, eventually down to single nucleotide resolution. Once aware of the underlying sequence, researchers were able to focus on specific areas in the genome by methods that identify methylation and demethylation through site-specific cleavage of DNA by restriction enzymes which lost their specificity in the presence or absence of methylation [148]. Analysis of methylation in these instances was restricted to the relatively few restriction enzymes. New approaches were required to broaden the analysis of methylation across a given locus. A development to achieve

this was made when Frommer *et al.* [128] described the method of bisulfite conversion of the DNA.

Under optimal conditions sodium bisulfite deaminates unmethylated cytosines into uracil, while methylated cytosines remain unchanged. Amplification of DNA by PCR after treatment in this way, leads to the unmethylated cytosines, now present as uracil, to be replaced with a thymine. In comparison, the methylated cytosines are not converted and remain the same after PCR. Once sequenced and compared to the original sequences the ratios of cytosines and thymidines represents unmethylated and methylated cytosines respectively. In other words the epigenetic methyl-mark is converted into an actual change in the DNA sequence that can be read out after sequencing.

This pre-treatment to the DNA allowed new PCR approaches with primers specific to the presence or absence of cytosines due to their methylation status prior to bisulfite conversion [149]. It also opened up the utilisation of additional restriction enzymes with the specificity of their cutting sites dependent on the presence of either a thymidine or a cytosine after bisulfite PCR [150]. In other words, in methods utilising these enzymes the cut of the DNA was either enabled or hindered due to potential changes in the sequence.

However, in disease approaches, such as proposed in this thesis, where it remains to be uncovered if cytosine methylation holds the potential to be the basis for a disease, two specific factors are needed: the genome as a whole needs to be assessed while still giving single base resolution. Today this can be done through either microarrays or next generation sequencing combined with a treatment of the genomic DNA in one of following three ways.

The first method utilises immunoprecipitation with antibodies specific for methyl cytosine or methyl binding proteins, with enriched samples compared to input [151]. The second method, exploits restriction enzymes which are either dependent or blocked by methylation thereby creating DNA fragments with ends reflective of the methylation state [152]. The third is

bisulfite conversion, which remains the gold standard in the determination of methylation at high resolution.

The two most commonly used methods to assess cytosine methylation genome-wide while ensuring single base resolution, are reduced representation bisulfite sequencing (RRBS; [153]) as well as whole genome bisulfite sequencing (WGBS) [154]. As their name suggests, both methods take advantage of bisulfite conversion as explained above.

The method of RRBS, however, couples a restriction enzyme digest, with CpG specific enzymes such as *Msp1*, to the bisulfite treatment before sequencing. The *Msp1* digest, recognizing the palindromic sequence of CCGG (with an asymmetrical cut behind the first cytosine), occurs often in CpG rich areas of the genome, the previously described CpG islands. This approach enriches for these areas and so limits the cost of sequencing per sample while focusing on the elements of the genome of the greatest interest. However other regions of interest potentially remain underexplored. Furthermore a certain shotgun-effect can be seen, where representation of all the areas of the genome can be quite variable.

Both these disadvantages are overcome by WGBS where the entire DNA methylome is assessed at a single nucleotide resolution post bisulfite treatment. However, the nature of bisulfite treated DNA to be less diverse makes the sequencing, particularly in repeats, as well as the necessary bioinformatics steps that need to follow, rather challenging. Together with the cost of whole genome sequencing, this approach is not yet economically feasible for mammalian genomes or larger numbers of samples [155, 156]. In the absence of array based platforms for non-human species, RRBS remains the method of choice for epigenomic studies of large genomes, such as the rat as studied here.

### 1.2.5 Epigenetics in human disease

With the advances in investigation of the epigenetic mechanism of methylation, as well as the other two, histone modifications and ncRNA, the involvement of epigenetics in human disease has become clearer.

One mentioned previously is genomic imprinting, representing an epigenetic event in which genes are expressed in a parent-of-origin-specific manner. It has been shown to involve histone modifications as well as cytosine methylation and the additional involvement of ncRNA is more than likely. However, when the imprinting is atypical in form of an epimutation, severe disease phenotypes manifest. The first to ever be characterized were Prader-Willi and Angelman syndrome. Strikingly both the paternal and the maternal genetic information of the chromosome 15q11-q13 region are needed to have the correct imprinting to prevent each of these syndromes. Prader-Willi syndrome, associates with hypotonia, obesity and hypogonadism amongst other characteristics, occurs when the paternal imprinting is disrupted. This can either be due to the loss of the entire region on the paternal chromosome or in about 25% of the cases [157] due to a lack of the appropriate, parental specific epigenetic marks being in place.

Interestingly the opposite occurs in Angelman syndrome in regards to the necessity of the maternal imprinting for the same chromosome 15q11-q13 region. Specifically, patients presenting with this disorder show intellectual and developmental impairments. This can again either be caused by a lack of the maternal chromosomal region altogether, or incorrect silencing of that region through defective epigenetic mechanisms [158]. Other similar diseases that can either be caused by a genetic mutation or a lesion in the imprinting pattern are Beckwith-Wiedemann syndrome [159], Silver-Russel syndrome [160] and pseudohypoparathryoidism [161].

Apart from imprinting abnormalities that present with these kinds of severe phenotypes, methylation has been shown to have negative impacts in cancer. A notable example is that of *MLH1*, a mismatch repair gene associated with hereditary nonpolyposis colorectal cancer (HNPCC). While a germline mutation in this gene often causes this syndrome, it has been shown multiple times [162-168] that germline *epimutation* of this gene can produce a phenocopy of the disease, in the absence of any underlying mutation. An epimutation is defined as aberrant silencing of a gene that should normally be active (or less commonly, abnormal activation of a silent gene), in the absence of any underlying genetic change. In the case of

*MLH1*, the epimutation silences this gene (which should be pan-somatically active), thus mimicking the effects of an inactivating genetic mutation. Epimutations are common features of tumours, however, if an epimutation is present in all somatic tissues it suggests that it occurred either very early in development or was inherited via the gametes. This is then called a "constitutional" or "germline" epimutation and can thus be present in somatic tissues derived from all three germ layers of the affected individual. Interestingly, like germline epimutations seen in plants, the *MLH1* germline epimutation has been shown in some cases to be inherited to a subsequent generation. This implies that the epimutation can evade the two waves of epigenetic reprogramming in mammals [164].

The case of *MLH1* demonstrates that a germline epimutation can give rise to a pattern of disease that mimics a genetic syndrome, although because of epigenetic reprogramming, the patterns of inheritance are non-Mendelian. There is no reason to suppose that *MLH1* is the only gene susceptible to germline epimutation, or that cancer is the only complex disease with an epigenetic basis. This raises the important question of whether essential hypertension might similarly be due to defects in epigenetic marks.

Essential hypertension lacks a genetic explanation at this point; despite decades of genetic research only up to 4% variability in blood pressure can be explained by genetic variants. Therefore, it is a clear candidate disease to investigate an epigenetic contribution. In humans this would be challenging, primarily due to the late development of the phenotype as well as the overwhelming genetic and environmental diversity in human populations, which can confound epigenetic studies. This is why model organisms under a controlled environment as well as a uniform genetic background are the best substitute to investigate potential epigenetic lesions.

## 1.3 The spontaneously hypertensive rat – a canonical model for hypertension

In order to investigate if there might be an epigenetic basis for essential hypertension, in particular in the form of distinctly different methylation patterns between individuals with

essential hypertension and those normotensive, I looked into the traditional model organisms for essential hypertension.

Commonly rats and mice are chosen as model organisms for human hypertension, given the fact that rats especially show a similar range in SBP as humans do with a healthy 120 mmHg. One of the longest standing rat models of hypertension stood out as the best candidate for assessing the epigenetic contribution to essential hypertension: the spontaneously hypertensive rat (SHR). The SHR is the only strain of rats that naturally develops hypertension without any causal induction (e.g. surgery, drug exposure, dietary changes, stress). All other rat models of hypertension only develop the phenotype after such influences and could therefore be considered secondary hypertensive. The history of how this strain was derived, as well as some key publications of experiments performed with these animals, made the SHR the obvious choice for this thesis.

### 1.3.1 Breeding and disease development in the SHR

The SHR, one of the most used model species for essential hypertension, was first described by the founders of the strain, Okamoto and Aoki in 1963 [169]. The strain was founded on a single male from the Wistar strain that had presented with spontaneous hypertension. Breeding this male to a female with slightly elevated blood pressure and successive brother-sister-mating, resulted in 100% of the animals presenting with essential hypertension as early as the third generation (see Fig 5). From the start the researchers observed that SHRs are pre-hypertensive at a young age but start to develop essential hypertension from 10 to 15 weeks on and eventually exceed a systolic blood pressure (SBP) of over 200 mmHg later in life. Like in humans, animals present with a range of end organ problems later in life, ranging from damage to the vascular system of the eyes to kidney problems, strokes, and heart attacks.

Ever since the establishment of the strain, the SHR has been used as a canonical model of essential hypertension, given that their essential hypertension occurs spontaneously and does not need to be induced [169]. This observation, together with the development of the essential

hypertension over time [169, 170] as well as the range in end organ complications [171], most closely mimic the human disease phenotype of essential hypertension and sets the strain apart from other rat models of essential hypertension. Also, just as in humans, genomic analyses have failed to uncover a genetic basis: sequencing of the SHR has not been successful to find a genetic cause for the hypertension phenotype, neither when compared to the genome of the Brown Norway Rat [172] nor when compared to more than two dozen strains including the traditionally used control strain to the SHR, the Wistar Kyoto rat (WKY) [173].

The inbred WKY strain had been derived from the same ancestral Wistar rat colony as the SHR. WKYs, however, remain normotensive through their lifetime and have thus been used as the closest genetic control for the SHR. The way the WKY strain was developed, has caused some problems however. The breeding stock was distributed to different research facilities prior to being fully inbred [174-177] and almost 10 years after the breeding of the SHR had been initiated (Figure 4; [176]). This causes the WKY and SHR to be more genetically heterogeneous than initially anticipated [178].

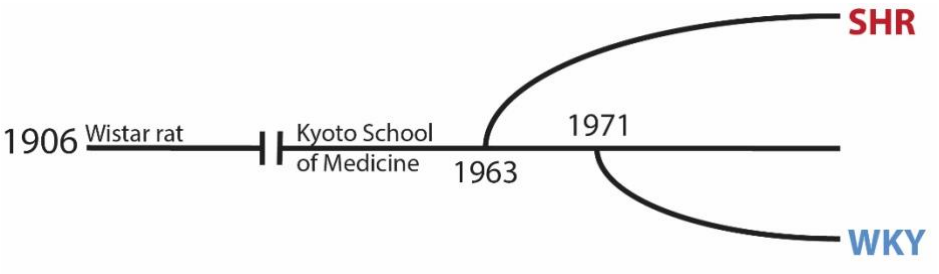



**Figure 4. Timeline of creation for the SHR and WKY Strain.**

### 1.3.2 Epigenetic clues in the SHR

Even though it has come into question if SHR and WKY are a good model organism and control pairing for each other, since the establishment of the two strains diverse research has been performed to explore the essential hypertension phenotype. The most remarkable findings will

be summarised here, particularly with regards to the proposed work.

The SHR strain in particular provides a range of findings that make an epigenetic cause plausible. One was the rapid propagation throughout the SHR strain when it was first created (Figure 5, [169]).

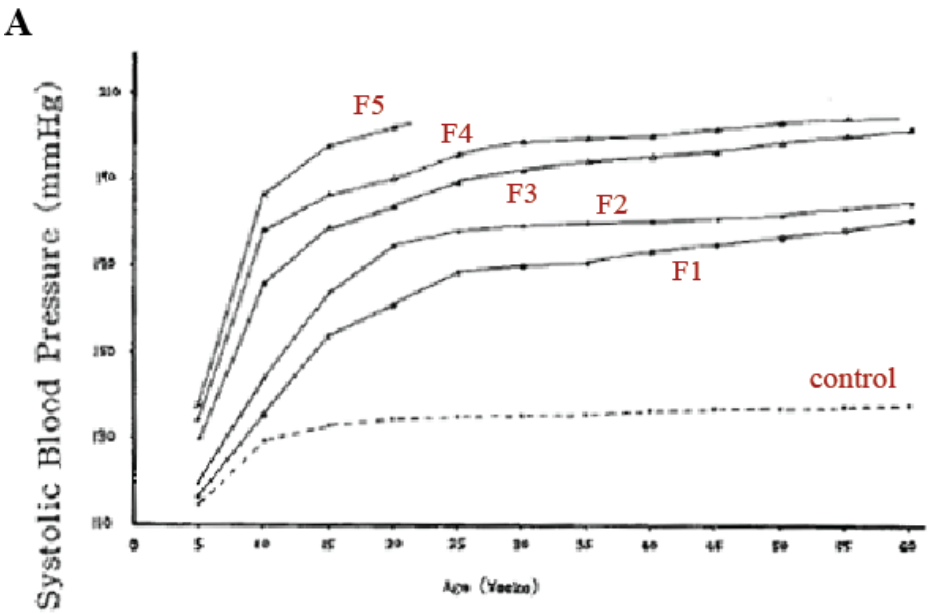



**Figure 5. Original figure of accumulation SBP in males of subsequent generations in the process of the creation of the SHR strain [169].**

The fact that there was an observed accumulation of the severity of essential hypertension within the first five generation makes it likely that one or only a few genes at most are involved in causing such a severe phenotype [179, 180]. However, combined with a lack of an observed genetic lesion, even though the SHR has now been sequenced twice [172, 173], these genes would have to be affected in a different way than a traditionally genetic one, pointing towards an epimutation as a possible explanation.

Other experiments have shown that, just as in humans, environmental factors can impact the hypertension of SHR e.g. diet [181, 182] or stress [183], or even maternal condition. For example, cross-suckling of pups born of SHR to normotensive rats delays hypertension in the

offspring. [184-186]. Di Nicolantonio *et al.* [187] went even further by performing embryo transfer as well as cross-suckling. They described an additive effect of *in utero* and postnatal maternal BP had the strongest effect in causing full blown hypertension later in the life of SHR offspring. However, if both the *in utero* and postnatal factors were missing, as in SHR cross-gestated and cross-suckled on normotensive WKY, these offspring still developed hypertension but more slowly and less pronounced. This "modifiable" nature of essential hypertension in the SHR is something that cannot be explained via genetic lesions like mutations, as the genome remained unchanged.

A final area of research performed with the help of the SHR strain and that needs to be mentioned here, particularly for the Chapter 5, involves the effects of transient medical treatment of the hypertension phenotype.

A number of studies have found that exposure to angiotensin-converting-enzyme inhibitors (ACE inhibitor), unlike other hypertension drugs like β-blockers, alternative vasodilators or calcium antagonists [188-192] can have a long-lasting effect on SHR, diminishing their hypertension. This seems to be especially the case when animals were exposed in a crucial time window during their development, leaving them with long-lasting protection from the fully hypertensive phenotype into adulthood [193-196].

The most compelling study, taking advantage of this phenomenon, was performed by Wu and Berecek [197], using the first ever discovered ACE inhibitor Captopril [198] and exposing pregnant females to its effects. The offspring were further kept on the medication till eight weeks of age and then Captopril exposure was ceased. Wu and Berecek observed that animals, exposed to Captopril during gestation and until eight weeks of age, showed significantly lower mean arterial blood pressure (MAP) even 27 weeks after the medication treatment had been stopped. They further reported that offspring of the Captopril exposed animals, even though never been exposed to the medication themselves, also had a significantly lower MAP compared to untreated, age matched SHR.

Despite multiple other studies reporting similar results after oral administration of ACE inhibitors, the actual causes for these effects remain unclear to this day.

Given all these discoveries that the hypertension phenotype in the SHR is highly modifiable through environmental factors – stress, diet, *in utero* and postnatal factors as well as long lasting through medication – alongside the fact that the two previous whole genome sequencing attempts were not fruitful, make the SHR even more interesting for the proposed research.

## 1.4 Hypothesis and aims

The central hypothesis to be tested in this thesis is that essential hypertension has an epigenetic, as opposed to genetic, basis. In other words, that the underlying defect predisposing to hypertension relates to an aberration in epigenetic state at one or more genes, rather than a change in the DNA sequence. This hypothesis can be tested by comparing epigenetic profiles of hypertensive and normotensive individuals and identifying epigenetic differences between the two.

Epigenetic states are determined by a complex interplay of chemical modifications to the DNA itself, and proteins that associate with DNA. One of the best characterised epigenetic modifications is cytosine (DNA) methylation. Cytosine methylation is stable, and can be assessed by a variety of methods at a genome scale.

In this thesis I have examined cytosine methylation patterns in a long-standing model of human hypertension, the spontaneously hypertensive rat (SHR). The SHR has a disease course that mimics the human condition, and despite decades of research, a genetic defect underpinning their phenotype has remained elusive. My hypothesis predicts that the SHR carries an epigenetic lesion, or lesions, that promote the development of hypertension. The SHR have a highly penetrant hypertension, suggestive of a germline defect. Interestingly, their hypertension can be modified by early and transient pharmacologic intervention, consistent with an underlying

epigenetic basis. This thesis will attempt to identify potential germline epimutations in the SHR that may underlie their hypertension.

In Chapter 3 I determine the nature and extent of cytosine methylation differences in the kidney of SHR relative to a related normotensive strain, WKY. The kidney is closely associated with blood pressure regulation and epigenetic lesions in this organ may have direct relevance to hypertension. I studied the kidney of pre-hypertensive animals to avoid any confounding influence of hypertension itself.

In Chapter 4 I extend my examination of cytosine methylation patterns in SHR and WKY to tissues derived from different embryonic germ layers in an attempt to identify epigenetic variants that may represent a germline epimutation. Methylation patterns in the liver (endoderm) and brain (ectoderm) are analysed along with the data from the kidney (mesoderm) to identify germline epigenetic variants associated with hypertension.

In Chapter 5 I exploit the modifiable nature of hypertension in the SHR to perform epigenetic profiling in SHR who do not develop hypertension. SHR treated with the ACE inhibitor Captopril *in utero* and for the first eight weeks of life never go on to develop hypertension. I profiled cytosine methylation in the kidney of these animals (and their progeny) and untreated animals. This strategy allows identification of pure epigenetic variants associated with hypertension in the absence of any genetic variation.

# Chapter 2. Material and methods

## 2.1 Animal husbandry

All animals for this work were handled in accordance with the guidelines of the 'Animal Research Act 1985', the '2010 Animal Research Legislation' and the '8th Edition of the Australian code of practice for the care and use of animals for scientific purposes from 2013' (National Health and Medical Research Council, Australian Government). All animal work was approved by the Garvan /St Vincent's Animal Ethics Committee under Animal Research Authority numbers 14_09 and 14_37.

### 2.1.1 Strains and purchase

Rats were initially purchased from the Animal Research Centre (ARC) (Canning Vale, Western Australia), and were descended from animals originally sourced from the Charles River Institute (Massachusetts, United States). The rats were from the substrains SHR/NCrlArc with the MHC haplotype $RT1^k$ (SHR), and WKY/NCrlArc with the MHC haplotype $RT1^l$ (WKY).

### 2.1.2 Housing and weighing

Rats were housed at an ambient temperature of 19-23°C and humidity between 40-60%. Lighting intensity was kept under 350 lux and a light/dark cycle of 12 hrs/12 hrs was maintained. Rats had *ad libitum* access to 'Gordons rat and mouse breeder diet premium' (23% protein) which had been sterilised by irradiation, as well as fresh tap water, filtered to 0.2 microns and acidified to pH 2.5-3.0 with sulphuric acid.

Cages had a floor area of 1500 $cm^2$ and an interior height of 235 mm. A maximum of four rats per cage were housed together during the process of the experiments.

Rats' weights were monitored weekly using a Mettler Toledo PL1501-S (Port Melbourne, Victoria, Australia).

### 2.1.3 Breeding

Breeding pairs were set up with a male to female ratio of 1:1. Rats were a minimum of eight weeks old when paired, and chosen from different litters to eliminate potential epigenetic bias from either of the parents. At weaning two males from each litter were randomly assigned to either be sacrificed for the reduced representation bisulfite sequencing (RRBS; see 1.3) assessment at four weeks, or for long-term blood pressure monitoring and sample collection.

### 2.1.4 Blood pressure measurement

Blood pressure of all experimental animals was measured using the non-invasive tail-cuff method with a BP-2000 from Visitech Systems, Inc. (Apex, North Carolina, USA). The setup consisted of a Control Unit (Model BP-2000-CU), a rat platform with four channels (Model BP-2000-RP4), to measure four rats simultaneously, and a computer with the most current version of the BP-2000 Analysis Software (starting with Version 19/2014).

Measurements were performed as follows: rats were restrained in a rat holder (sizes BP-RH1 -3 with rat holder baseplate RHB-Small) and placed onto the rat platform. The platform had been preheated to 37°C to ensure that the rats were brought to an appropriate temperature, in order to dilate tail blood vessels and facilitate accurate assessment of blood pressure and pulse rate. Once an even curve for the pulse was visually observed in all rats by the user, 20 consecutive measurements were performed.

In order to ensure the most accurate assessment, blood pressure was measured after several practice measurements in order to accustom the rats to the restraint, noises of the machinery and physical pressure on the tail by the cuff. A five day regime was established, with three days of practice measurements followed by two days of recorded measurements.

On the fourth and fifth consecutive day the experimental blood pressure measurements were performed, with one measurement run in the morning and one in the afternoon of each day. An average of these four measurements was calculated for both the systolic blood pressure as well as the pulse. Rats were systematically rotated through all channels, being measured at a different

position on the platform each session, in the process of the four measurements to even out potential variations in the sensors that measured the blood pressure.

### 2.1.5 Captopril treatment

For this experiment eight week old male and female rats were paired as described above and exposed to 1.84 mM Captopril (Sigma-Aldrich Chemie Gmbh, Munich, Germany) dissolved in their drinking water. Captopril solution was refreshed every 2-3 days. Pups born to these breeding pairs were weaned at four weeks of age but maintained on Captopril drinking water until they were eight weeks old, after which Captopril water was replaced with standard acidified water.

Rats for the CAP-F2 generation were generated from breeding pairs of animals that had been exposed to Captopril till eight weeks of age. Breeding pairs were then matched at 10 weeks.

CAP-F2 animals were generated to assess their blood pressure. The hypothesis based on previous research was that Captopril had not only altered the F1's disease phenotype but potentially also given rise to factors inheritable to their offspring lowering their blood pressure.

### 2.1.6 Culling and tissue collection

All rats were sacrificed by $CO_2$ asphyxiation. Tissues were harvested and individually placed into labelled 7x5 cm zip lock bags. All bags were snap-frozen in liquid nitrogen and, placed into -70°C for long term storage. For the 35-week animals, the left kidney was placed in 4% paraformaldehyde (PFA; Redox, Minto, New South Wales, Australia) and stored separately at 4°C.

## 2.2 Molecular biology: general

### 2.2.1 Tissue preparation for DNA and RNA extraction

Due to the large size of rat organs tissue preparations were performed with the Cellcrusher$^{Tm}$ (Cork, Ireland). Snap frozen tissues were pulverized in the Cellcrusher$^{Tm}$ chamber, pre-chilled in

liquid nitrogen. The pulverized sample was removed using a chilled recovery spoon and distributed into tubes that were kept frozen at -70°C until nucleic acid extraction.

### 2.2.2 DNA extraction from tissue

Approximately 100 mg of pulverized tissue was lysed with 500 µl of Heavy TESS buffer (50 mM Tris-HCl pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS). 20 µl of Proteinase K (20 mg/ml) was added and the sample incubated overnight at 55°C with gentle agitation. After incubation the lysate was diluted 1:2 with water to compensate for the amount of salt present in the heavy TESS and then DNA extraction performed as described for blood in 1.2.1.

### 2.2.3 RNA extraction from tissue

Approximately 100 mg of pulverized tissue was lysed with 1 ml of TRIzol (Thermo Fisher, North Ryde, New South Wales, Australia) and left to incubate for 5 minutes. After incubation, 200µl of chloroform was added to the lysate and left to incubate for 3 minutes. The sample was then centrifuged at 12,000 *g* for 15 minutes at 4°C and the aqueous phase was then transferred into a new tube. 500 µl of isopropanol was added and the lysate incubated for 10 minutes at room temperature. The lysate was then centrifuged at 12,000 *g* for 10 minutes at 4°C. The supernatant was discarded and the RNA pellet resuspended in 1 ml of 75% ethanol. After briefly vortexing the sample, it was once again centrifuged at 7,500 *g* for 5 minutes at 4°C. The RNA pellet was air dried and resuspended in 50 µl of RNAse-free water.

### 2.2.4 Determination of nucleic acid concentration

DNA and RNA concentration and purity were assessed using a UV spectrophotometer (Nanodrop 2000; Thermo Fisher, North Ryde, New South Wales, Australia). DNA samples with an $A_{260/280}$ of ≥1.8, and RNA samples with an $A_{260/280}$ of ≥2.0 were deemed suitable for further analysis.

### 2.2.5 Agarose gel electrophoresis and nucleic acid visualisation

PCR products were visualized on a horizontal 1.5% agarose gel containing 0.5 µg/ml Ethidium Bromide. Samples were loaded with an appropriate amount of 6x Blue/orange loading dye

(Promega, Alexandria, New South Wales, Australia). Gels were run at 100 V until DNA bands were sufficiently distinct. As a reference, 100 bp DNA ladder and/or the 1 kb DNA ladder from Promega (Alexandria, New South Wales, Australia) were used.

DNA bands were visualized using long wavelength UV (302nm) on a FLA-5100 (Fujifilm, Brookvale, New South Wales, Australia) and processed using Image Gauge software version 4.0 (Fujifilm, Brookvale, New South Wales, Australia).

### 2.2.6 Nucleic acid gel extraction and purification

DNA fragments were extracted from the agarose gel using the MinElute Gel Extraction Kit (Qiagen, Chadstone Centre, Victoria, Australia).

The desired DNA fragment was cut out of the gel with a scalpel and weighed. Three volumes of the Buffer QG were added and the sample incubated at 50°C until the gel slices had completely dissolved. The sample was thoroughly vortexed and 10 µl of 3 M sodium acetate pH 5.0 was added when necessary. One gel volume of isopropanol was added and the sample then transferred into a MinElute spin column to be centrifuged at maximum speed for 1 minute. The flow-through was discarded and 500 µl of Buffer QG added to the column. After this washing step, 750 µl of Buffer PE was added to the column. Following this second wash with PE, the sample was centrifuged once more for 1 minute to remove all residual of Buffer PE. To finally elute the DNA, 10 µl of Buffer EB or water were added directly to the membrane of the MinElute column and left to incubate for 1 minute before centrifuging for a final minute.

## 2.3 Reduced Representation Bisulphite Sequencing

Reduced representation bisulphite sequencing (RRBS) is a cost-efficient technique to examine methylation patterns at single nucleotide resolution. The methods relies on restriction digest to enrich for CpG rich regulatory regions of the genome, resulting in sequencing libraries enriched for likely areas of interest. These libraries can then be sequenced at reasonable depth at a fraction of the cost of performing whole genome bisulphite sequencing. Meissner *et al*, who

published this method in 2005, suggested that sequencing approximately 1% of the genome by RRBS is sufficient to robustly represent the epigenetic methylation patterns of an individual with an adequate depth of coverage [153].

In this thesis, RRBS was carried out with two different protocols: either an in house method, or a kit-based method that became available during my candidature. Samples analysed in Chapter 3 were prepared using the in-house protocol; samples in Chapters 4 and 5 were prepared with the Ovation RRBS Methyl-System from NuGEN (Leek, Netherlands).

### 2.3.1 In-house RRBS protocol

Samples that were prepared with the in-house method were handled largely in accordance with the method of Meissner [153]. Specifically, genomic DNA (1 µg) was digested for 16 hours with $60x10^3$ U of the restriction enzyme *Msp*I (NEB, Arundel, Queensland, Australia) in a volume of 30 µl, according to the manufacturer's instructions. End repair was then performed by addition of 5 U of Klenow (NEB, Arundel, Queensland, Australia) and 1 µl dNTP mix (10 mM dATP, 1 mM dGTP, 1 mM dCTP; Sigma-Aldrich, Castle Hill, New South Wales, Australia) for 20 minutes at 30°C followed by 20 minutes at 37°C. Unincorporated dNTPs were then removed with the Qiagen nucleotide removal kit (Qiagen, Chadstone Centre, Victoria, Australia) as per manufacturer's protocol and the resulting sample ligated with a unique adaptor by adding 2000 U T4 DNA ligase, 4.5 µl 10x ligase buffer, 6 µl of a 1:10 diluted index adapter from the Truseq kit and 3.5 µl of nuclease free water. This solution was incubated at 16°C overnight. The next day the Qiagen GeneReads kit (Qiagen, Chadstone Centre, Victoria, Australia) was used as per manual by Qiagen to remove adapter dimers.

Once this clean-up had been performed the bisulfide conversion was prepared as advised by the Qiagen EpiTect kit (Qiagen, Chadstone Centre, Victoria, Australia). 35 µl of DNA protection buffer was added to the cleaned and indexed sample as well as 85 µl of Bisulfide mix. Incubation, and the following clean up, was performed as per protocol and the sample eluted in 20 µl of the provided elution buffer. Bisulfide conversion was once again repeated in the same

manner to increase the conversion rate. Once the second bisulfide clean-up had been performed, the sample was amplified via PCR.

| | |
|---|---|
| 10x PFU Buffer | 10 µl |
| dNTP | 0.2 mM |
| Forward Primer | 200 nM |
| Reverse Primer | 200 nM |
| PFU Turbo | 5 U |
| Prepared sample | 10 µl |
| $H_20$ | to 100 µl |

The thermo cycler settings were as followed:

| | | |
|---|---|---|
| 94 °C | 2 minutes | 1 cycle |
| 94 °C<br>60 °C<br>74 °C | 10 seconds<br>10 seconds<br>20 seconds | 16 -18 cycles |
| 74 °C | 4 minutes | 1 cycle |

An 8% Page gel 1x TBE was prepared as described in 1.3.2 and the PCR product then loaded onto the gel with a filament pipette tip alongside the 1kb DNA ladder (Promega, Alexandria, New South Wales, Australia) was used to identify sample pieces of 150-500 bp size post run and 1x SYBR gold staining (Sigma-Aldrich, Castle Hill, New South Wales, Australia). The gel part representing those 150-500 bp fragments was cut out of the gel and the library sample extracted as described previously.

### 2.3.2 PAGE gel electrophoresis and nucleic acid visualisation

For RRBS library preparation, samples were run on a vertical 8% Polyacrylamide (PAGE) gel. The gel chamber used for this was able to contain 35 ml of PAGE gel composed of the following chemicals:

| PAGE gel | |
|---|---|
| 30% Polyacrylamide | 9 ml |
| 1X TBE | 26 ml |
| 10% APS | 260 µl |
| TEMED | 26 µl |

Once the gel had set, samples were loaded, containing the required volume of 6X Blue/orange loading dye, with filament pipette tips. Gels were run on 150 V for approximately 90 minutes. Gels were subsequently stained with 1X SYBR gold in TBE for approximately 10 minutes. After being washed in 1X TBE the visualization was performed on the FLA-5100 and processed using Image Gauge software version 4.0.

### 2.3.3 PAGE gel extraction and purification

DNA fragments of desired size were cut out of a PAGE gel with a scalpel. The gel was then minced and incubated overnight in PAGE Elution buffer.

| PAGE gel elution buffer | |
|---|---|
| 1X TBE | 0.5X |
| Ammonium Acetate | 2.5 M |

Following incubation, the gel debris was removed by centrifugation in a spinX column (Sigma-Aldrich, Castle Hill, New South Wales, Australia). Subsequently, 2 volumes of 100% ethanol as well as 1 µl of glycogen were added, and the sample incubated at -70°C for an hour. Following incubation, the sample was centrifuged and the pellet washed with 70% ethanol, with a final

centrifugation step prior to air dry. Finally DNA was resuspended in 20 µl water and quantified using the Agilent DNA 1000 chip (Agilent, Mulgrave, Victoria, Australia).

### 2.3.4 Ovation RRBS Protocol

After trialing several commercial RRBS library preparation kits over the course of this work, the Ovation RRBS kit (NUGEN, Leek, Netherlands) was found to be superior to our in-house method in terms of speed and simplicity, and was thus adopted. Samples were digested with MspI, specific adapters ligated onto the sample and a final end repair performed with no clean up steps in between necessary. Bisulfide conversion was performed in the same way as the in house method with the use of the Qiagen EpiTect kit. After the final amplification PCR a single bead clean-up was performed and the library sample checked on the Agilent chip (Agilent, Mulgrave, Victoria, Australia) for quality.

### 2.3.5 Sequencing

RRBS libraries were sent to the Ramaciotti Centre for Gene Function Analysis (University of New South Wales, Sydney, NSW, Australia). Sequencing was performed in 50pb single end reads for indexed libraries. Due to the loss of diversity within the DNA sequence after bisulfite conversion, libraries needed to be multiplexed with samples of other, non-bisulfite treated libraries. The platform used for sequencing was the Illumina HiSeq.

## 2.4 Bioinformatics analysis

### 2.4.1 Genomic alignment and methylation calling

Bisulfite sequencing reads received from Ramaciotti were preprocessed prior to genomic alignment. The Ovation diversity trim python script was used to remove the 5’ diversity bases left after library preparation (trimRRBSdiversityAdaptCustomers.py). Conversion efficiency was assessed by the percentage of non-bisulfite converted cytosines at the C position filled-in during end repair after MspI digestion (see above); libraries were only considered in further analyses if conversion rates were ≥98%. Then, cutadapt version 1.8.3 was used to remove the adapter sequence AGATCGGAAGAGC. The resulting trimmed fastq files were then passed to

bismark version 0.14.5 for alignment using bowtie version 1.1.2. with the parameters of 1 mismatch in a 28 base seed. The resulting SAM file was processed with Bismark methylation extractor to extract the methylation call for each cytosine in the context of CpG. This file was conflated using a custom perl script for preparation of the methylkit input file.

### 2.4.2 Analysis for differentially methylated cytosines

Cytosines were included for statistical analysis if they were present in at least 3 samples per treatment group with a minimum coverage of 20X. Statistical analysis (logistic regression) was performed using The R Bioconductor package methylkit. Differentially methylated cytosines were defined when the methylation difference between treatment groups was at least 20% and the adjusted p-value (adjusted with the SLIM method) was <= 0.01.

## 2.5 Gene array

### 2.5.1 Gene array preparation

RNA samples from the kidneys of 12 week old rats were submitted to Ramaciotti Centre for Gene Function Analysis (University of New South Wales, Sydney, NSW, Australia) and microarray analysis was performed there using the Affymetrix Clariom S array Rat. The Clariom S for the rat covers >22,900 genes and >129,800 transcripts with >231,800 probes. Transcriptome Analysis Console (TAC) Version 4.0.0.25 was used for further Data analysis.

# Chapter 3. Epigenetic differences in the four week old kidney of SHR and WKY

## 3.1 Introduction

This chapter details the first steps in investigating the potential involvement of epigenetic mechanisms in essential hypertension. Here I describe the analysis of the patterns of cytosine methylation in kidney tissue from the spontaneously hypertensive rat (SHR), and the closely related Wistar Kyoto (WKY) strain.

The SHR rat has been the canonical animal model for essential hypertension since the strain was established in the 1960s [169]. This is likely because their hypertension does not demand an experimental induction, and their disease progression closely resembles that of essential hypertension in humans. Both the SHR and the most commonly used normotensive control strain to the SHR, the WKY, were originally derived from the Wistar strain.

Rats and humans surprisingly have the same threshold of healthy blood pressure, which is 140 over 90 mmHg for systolic and diastolic blood pressure respectively. The SHR strain, just as many humans, however, develops hypertension, with males exhibiting a slightly earlier onset of the phenotype than females [199-206]. SHR are pre-hypertensive at four weeks of age, with hypertension starting from around six weeks and eventually plateauing at a systolic blood pressure of ~200 mmHg in adulthood. Due to this exceedingly high blood pressure, animals show a range of end organ damage later in life, from kidney failure to stroke and cardiac hypertrophy [171]. In this way, the SHR very much mimic the progression and complications of human essential hypertension. Also, just like in humans, the underlying cause remains unknown.

Genetic investigations by whole genome sequencing have so far failed to identify any genetic variant that explains the hypertensive phenotype in the inbred SHR [172, 173]. This is particularly surprising, given that the rapid propagation of the hypertensive phenotype in the

development of the strain [169] suggested only a few loci (at most) are involved. As described in detail in Chapter 1, this lack of a genetic explanation for their highly penetrant phenotype raises the possibility that hypertension in this canonical model may be epigenetic in nature. An epigenetic basis for hypertension is a possibility that has so far not been explored in humans, or any model system.

Epigenetic marks vary among cell types as cell differentiation is an epigenetic process. I chose to begin my investigation of epigenetic changes in the SHR by examining DNA from whole kidney, a tissue with a key role in arterial blood pressure regulation [207]. The kidney is directly responsible for sodium homeostasis through water excretion and retention [208, 209], and can impact the vascular tone throughout the body via sympathetic signals to the central nervous system [210-212]. Furthermore, clinical research in the 1970s and '80s showed in multiple cases that "….*hypertension moves with the kidney*" [213]. Not only did previously normotensive patients who received kidneys from hypertensive donors develop hypertension [214-218], the opposite has also been reported, whereby hypertensive recipients transplanted with a normotensive donor's kidney experienced remission of their hypertension [213].

In the SHR specifically, similar allograft transplantation experiments demonstrated that the kidney plays a causal role in the hypertension phenotype and therefore is a pivotal target for investigation. As early as 1974, Bianchi *et al.*[219] showed that kidney transplantations from hypertensive SHR of 3-4 months of age into normotensive WKY led to hypertension in the WKY. Conversely, normotensive WKY kidneys transplanted into SHR reduced the SHR blood pressure. A follow up experiment illustrated that young WKY, receiving kidneys from pre-hypertensive SHR, still developed significantly higher blood pressure than animals that had received kidneys from normotensive donors, demonstrating that even pre-hypertensive kidneys were able to initiate hypertension. Other studies utilizing F1 hybrids of SHR and WKY [220, 221] or other normotensive strains [222] showed similar findings. In 2004 Smallegange et al. [223] described, that once transplanted, the kidneys of 15 week old SHR that had previously been under two week exposure of the angiotensin-enzyme inhibitor drug Enalapril as well as a

low-salt diet, caused a decrease in blood pressure in the untreated recipient SHR. The opposite was true, once the kidneys of untreated SHR were transplanted into previously treated SHR; these animals displayed a rise in blood pressure. Taken together, there is strong evidence linking the kidney to the etiology of hypertension, and thus it was chosen for my initial analysis of methylation patterns in the SHR and WKY.

Cytosine methylation patterns can be assessed by a variety of methods, as discussed in Chapter 1. Here I have chosen to use reduced representation bisulfite sequencing (RRBS) for its ability to produce snapshots of genomic methylation patterns with single-base resolution at reasonable sequencing cost. I aimed specifically to:

1. Produce RRBS maps of cytosine methylation from the kidneys of four week old pre-hypertensive SHR males and age-matched WKY males.
2. Compare methylation patterns between the two strains at the level of individual CpG sites, as well as genomic regions.
3. Correlate any observed differences in methylation between the strains with differences in gene expression in the kidney.

## 3.2 Results

### 3.2.1 Confirmation of strain phenotypes

I measured the blood pressure of SHR and WKY rats of my cohort to confirm the phenotype of both strains in the colonies maintained at the VCCRI. This is particularly important since there have been a small number of reports that some SHR substrains develop hypertension before they are four weeks old [224-226]. In regards to the WKY, which were also derived from the same strain of Wistar rats, it needed to be confirmed that none of the animals in my colony spontaneously showed unusually high blood pressure, confounding the findings.

Blood pressure of male SHR and WKY over their life course is shown in Figure 6A. At four weeks of age, during the pre-hypertensive stage, SHR showed an average blood pressure of 135.3 ± 1.8 mmHg (mean ± SEM) and the WKY an average of 141.3 ± 2.2 mmHg. At 10 weeks the SHR had developed significantly higher blood pressure compared to the WKY ($p \leq 0.0001$), with means of 193.9 ± 2.6 mmHg and 149.1 ± 4.6 mmHg respectively. This significantly higher blood pressure plateaued in the SHR at 197.1 ± 3.4 mmHg [WKY 148.9 ± 3.1 mmHg] at the 16 week blood pressure assessment ($p \leq 0.0001$), and remained significantly higher at 35 weeks with 198.3 ± 2.3 mmHg for the SHR compared to 154.1 ± 2.3 mmHg for the WKY ($p \leq 0.0001$).

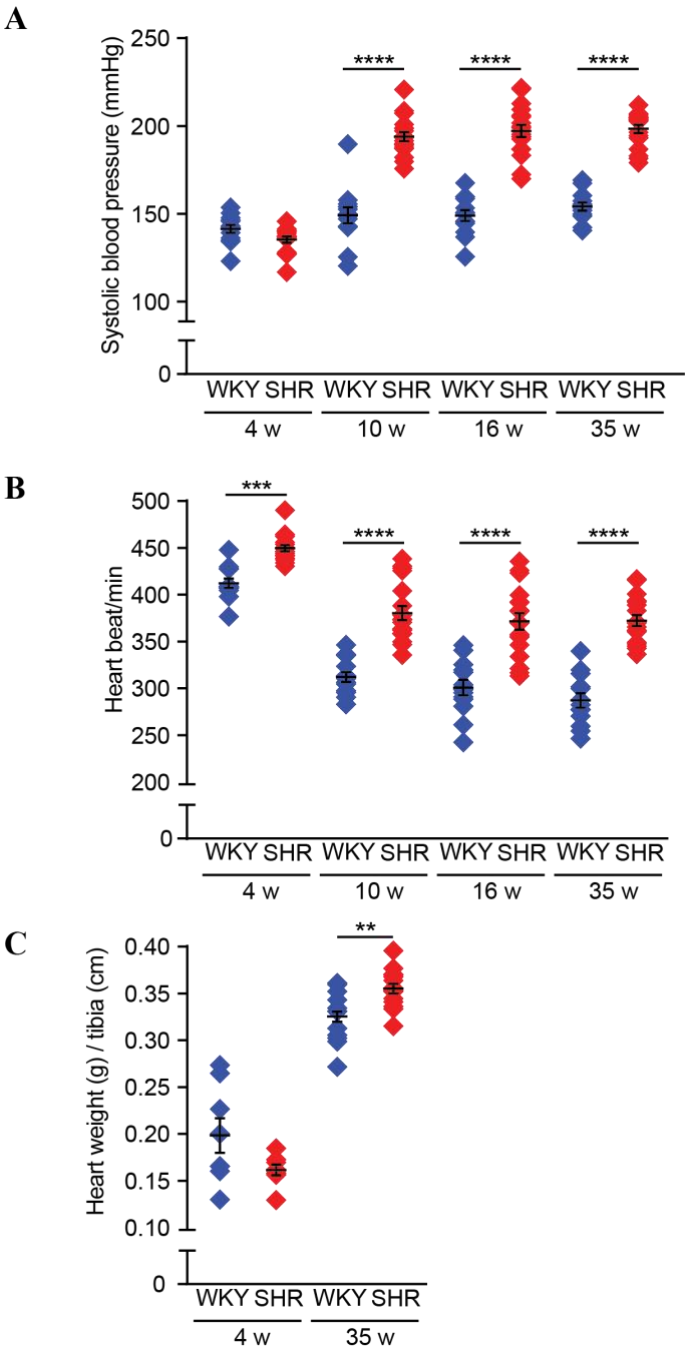



**Figure 6. Blood pressure, heart rate and heart weight over the life course of male SHR and WKY.** **A** Systolic blood pressure of WKY (blue) and SHR (red) at pre-hypertensive (4 weeks), developing (10 weeks), plateau (16 weeks) and end-stage hypertension (35 weeks) (SHR $n$ = 18, WKY $n$ = 13). **B** Heart rate of WKY (blue) and SHR (red) at the same time points (SHR $n$ = 18, WKY $n$ = 13)**. C** Heart weight to tibia length ratio for WKY (blue) and SHR (red) at pre-hypertensive (4 weeks; SHR and WKY $n$ = 8) and end-stage (35 weeks; SHR $n$ = 15; WKY $n$ = 12) (Error bars indicate mean ± SEM; two-tailed Student's unpaired t-test ** $p \leq 0.01$, **** $p \leq 0.0001$).

I also measured the heart rate of WKY and SHR (Figure 6B). Consistent with reports in the literature [6, 227], SHR showed a significantly higher heart rate across the life course than the WKY, with an average of up to 100 beats per minute more (increase of 130%) in SHR compared to the WKY at week 35 ($p \leq 0.0001$).

Long term hypertension often leads to cardiac hypertrophy, a thickening of the heart muscle which amongst other adverse effects, decreases the volume of blood that can be circulated through the body as well as increasing the risk of heart failure [228, 229]. To assess the presence of cardiac hypertrophy in my cohort of animals, I assessed the heart weight to tibia ratio in four week old and 35 week old rats, as shown in Figure 6C. I found that while there was no difference between four week old pre-hypertensive SHR and WKY, 35 week old SHR had a significantly higher heart weight to tibia length ratio ($p \leq 0.01$; SHR $n$= 15, WKY $n$ = 12). These findings are consistent with the published findings that SHR develop cardiac hypertrophy as a consequence of sustained hypertension [230-232].

### 3.2.2 Analysis of cytosine methylation in WKY and SHR kidney

#### SHR can be distinguished from WKY by kidney DNA methylation patterns

Once the essential hypertension phenotypes of the respective strains were confirmed within my colonies, I sacrificed seven males from separate litters per strain for cytosine methylation analysis (Table 3-1). Due to financial constraints and to avoid sex-related differences in DNA methylation I decided to only analyze males over females, as the males develop hypertension earlier in life, and also develop more severe disease. Animals were chosen from separate litters to exclude any potential confounding influence from a particular parent, or induced by a particular intrauterine environment. Four week old kidneys were collected and DNA extracted from one whole organ, ensuring that information on cytosine methylation was not biased by variations in cellular composition.

I assessed genome-wide methylation patterns between SHR and WKY kidney DNA using an in-house RRBS protocol. RRBS libraries were sequenced on the NextSeq500 Illumina platform (75 bp; single-end read) at the Ramaciotti Centre for Genomics (Sydney) and mapped to the rat genome assembly rn5 publicly available through UCSC Genome Browser. Table 3-1, below, presents sequencing summary statistics.

**Table 3-1. RRBS sequencing summary statistics of the kidney for pre-hypertensive SHR and WKY at four weeks of age.**

| Animal ID | Strain | Number of unmapped reads | Number of mapped reads | Mapping efficiency (%) | Number of CpGs captured | Number of CpGs at 20x coverage |
|---|---|---|---|---|---|---|
| **1000558** | **WKY** | 56,944,130 | 35,237,127 | 61.9 | 2,290,713 | 1,493,451 |
| **1000566** | **WKY** | 49,556,434 | 31,370,539 | 63.3 | 2,398,255 | 1,481,762 |
| **1000630** | **WKY** | 69,750,615 | 44,020,128 | 63.1 | 2,276,812 | 1,555,628 |
| **1000638** | **WKY** | 44,521,361 | 27,223,545 | 61.1 | 2,105,532 | 1,335,598 |
| **1000650** | **WKY** | 26,263,935 | 16,701,668 | 63.6 | 2,261,925 | 935,451 |
| **1000691** | **WKY** | 30,913,734 | 18,533,625 | 60.0 | 2,151,513 | 1,043,556 |
| **1000699** | **WKY** | 33,980,230 | 21,553,427 | 63.4 | 2,086,584 | 1,198,250 |
| **1000583** | **SHR** | 57,678,877 | 35,666,907 | 61.8 | 2,892,796 | 1,514,422 |
| **1000604** | **SHR** | 61,828,348 | 39,748,739 | 64.3 | 2,244,414 | 1,537,145 |
| **1000615** | **SHR** | 55,913,132 | 35,689,188 | 63.8 | 2,163,950 | 1,517,485 |
| **1000620** | **SHR** | 82,403,179 | 52,236,103 | 63.4 | 2,412,835 | 1,583,454 |
| **1000624** | **SHR** | 60,284,692 | 38,788,487 | 64.3 | 2,341,008 | 1,531,259 |
| **1000674** | **SHR** | 46,744,198 | 29,564,775 | 63.2 | 2,162,739 | 1,338,952 |
| **1000710** | **SHR** | 51,470,372 | 32,623,154 | 63.4 | 2,039,578 | 1,338,881 |

On average, RRBS captured 2,273,475 CpGs, which is consistent with RRBS, where only about 1% of the genome is sequenced. However, this 1% is enriched for CpG sites such that ~80-90% of all CpG sites and more than half the CpG islands and promoter regions are represented [233]. The rat genome contains about 17,000 CpG [234] islands, in this dataset I found 12,007 CpG islands with at least one cytosine covered at 20X coverage.

Based on previous RRBS studies and sequencing analysis performed by my laboratory I set the parameters to be used in this analysis, that CpGs had to have at least 20X coverage and be represented in this depth by at least three of the seven animals. This gave 1,458,086 kidney CpGs (~ 64% of all the CpGs captured by RRBS) which were considered in further analyses.

The mapping efficiency against the reference rat assembly rn5 was 62.9% on average. Given that rn5 is based on neither the SHR or WKY genome but rather the Brown Norway rat strain, I attempted to increase overall mappability by identifying potential strain specific CpGs. With the assistance of Paul Young I approached this by mapping RRBS reads against an enhanced SHR-SNP_rn5 (an updated version of the rn5 informed by SNPs identified in the sequencing advances of Atanur *et al.* [172, 173]). However, this did not yield significantly greater mappability, with an increase of only 0.4% of reads. Further analysis of cytosine methylation was thus carried out on the alignments to the original rn5 reference.

Unsupervised hierarchical clustering of the RRBS data at 20X coverage neatly separated the WKY and SHR into their strains, as shown in the dendrogram in Figure 7A. Principal components analysis (PCA; Figure 7B) also separated SHR and WKY, with the first principal component contributing to ~18% of all methylation variance.

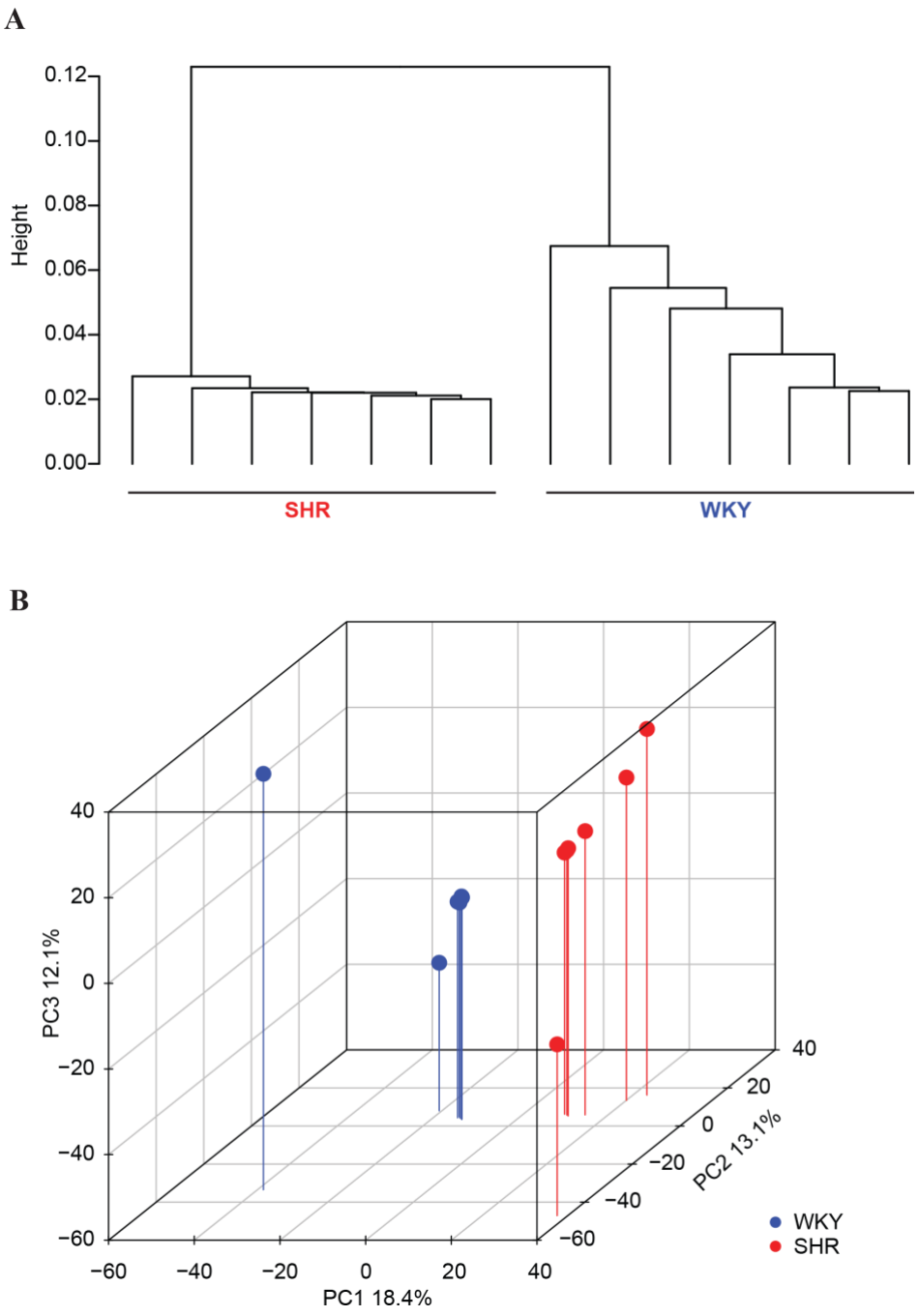



**Figure 7. Unsupervised hierarchical clustering and PCA of RRBS data of the kidney of pre-hypertensive SHR and WKY at four weeks of age.**

**A** Dendrogram showing unsupervised hierarchical clustering of RRBS data of the kidney of pre-hypertensive SHR and WKY at four weeks of age (Ward's Method). **B** Pseudo-3D principal components analysis (PCA) plots of the RRBS data of the kidney of pre-hypertensive SHR (red) and WKY (blue) at four weeks of age.

## methylKit identifies thousands of differently methylated cytosines between the two strains

Since hierarchical clustering indicated that SHR and WKY can be distinguished based on patterns of cytosine methylation in the kidney, I next sought to determine which CpG sites differed in their degree of methylation between the strains. To do this, I was assisted by Paul Young using the methylKit package in R [235].

For statistical identification of differentially methylated CpGs (DMCs) we chose the following stringent parameters that are in consensus with other current publications [233, 236]: coverage of 20X or greater, a significance threshold of $q < 0.01$, and $\geq 20\%$ average methylation difference between SHR and WKY. This analysis defined 8011 DMCs between SHR and WKY (Figure 8). It is interesting to note that 4775 of the 8011 (59.6%) DMCs are hypomethylated in the SHR. This shift towards one of the two methylated states is unlikely to be seen by chance with a $p$-value < 0.0001 (Chi-square test).

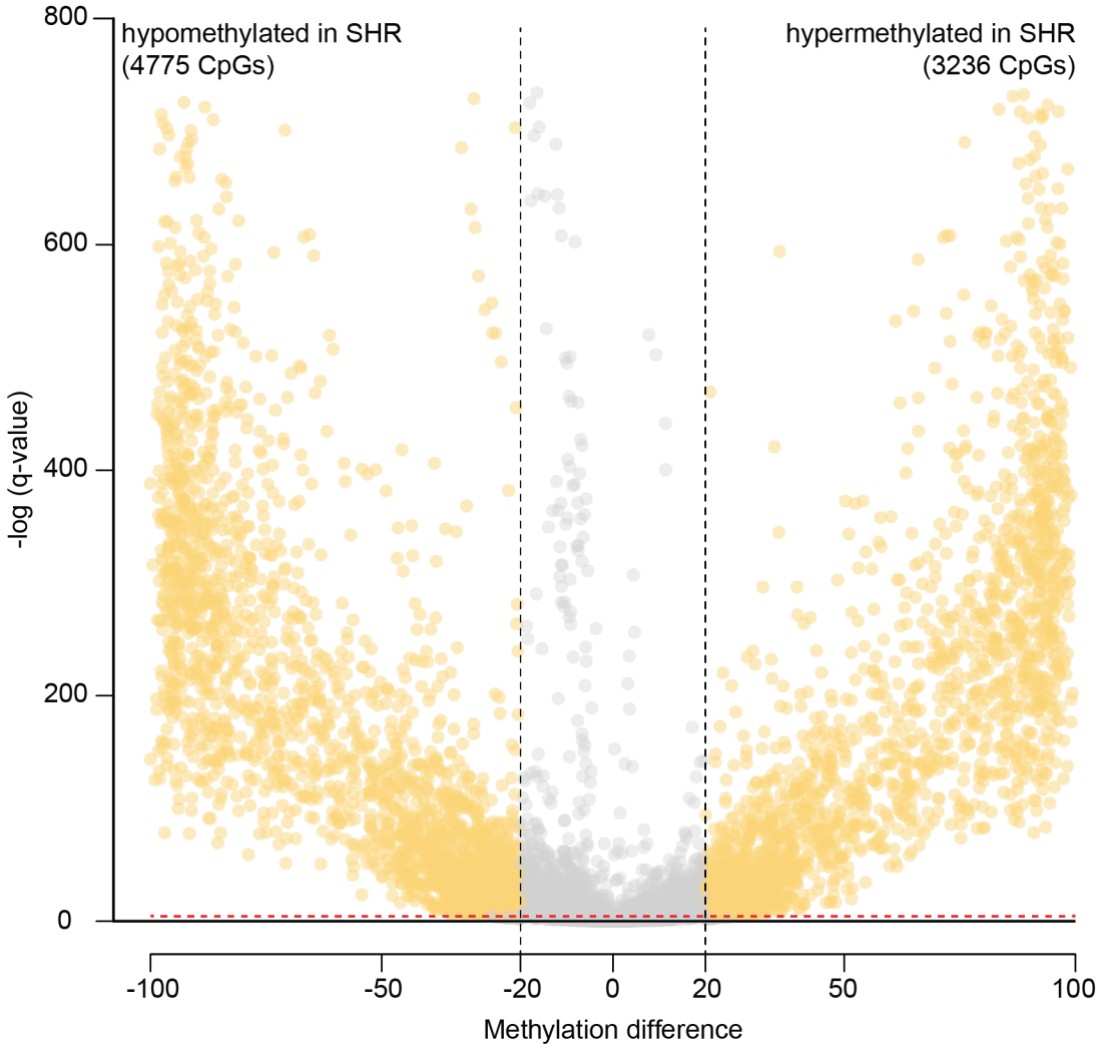



**Figure 8. Thousands of differentially methylated cytosines (DMCs) identified in the kidney between pre-hypertensive SHR and WKY at four weeks of age.**

Volcano plot depicting DMCs. DMCs, meeting the parameters of significance threshold of $q \leq 0.01$, and $\geq$ 20% average methylation difference between SHR and WKY, are shown in yellow (red dotted line represents $q = 0.01$).

In order to determine whether certain genomic regions were enriched in the DMCs, I examined the genomic distribution of the DMCs, and compared this with the genomic distribution of all CpGs with 20X coverage (Figure 9A and B). In the main dataset of all analyzed CpGs, more than half of CpGs resided in CpG islands or their shores (2000 bp from CpG islands), but for

DMCs only ~15% were in CpG islands or shores (Figure 9A). It therefore appears that the DMCs are depleted in CpG islands and their shores.

Regarding the DMCs proximity to genes, I observed an overall decrease in the proportion of DMCs residing within genic regions, relative to all CpGs (Figure 9B). Within genic regions, there were also specific decreases in the proportions mapping to 5' untranslated regions (5'UTR) and around (<1 kb upstream) transcription start sites (TSS; Figure 9B) when compared to all CpGs. Both 5'UTR and TSS regions are linked to gene promoter function and, given the well-established gene silencing effect of cytosine methylation in promoters, they are of particular interest. While 18% of all CpGs were in 5'UTR and TSS, only 2% of DMCs fell into these subgroups.

At present it is difficult to interpret the functional significance of differential methylation outside of gene regions, but those within genes or in close proximity can be analyzed further. The DMCs found in gene annotated regions (Figure 9C; n=3250) could be assigned to 1552 different genes (Figure 9D). The majority of these genes carried DMCs in intergenic and intronic regions, while only 3.7% showed methylation in 5'UTR and TSS.

In order to determine if any of the genes that harbored a DMC fell into biological pathways related to blood pressure regulation, I performed gene ontology with g:Profiler and DAVID, two commonly used annotation platforms. The gene ontology on all 1552 genes revealed enrichment for 46 biological pathways with B-H $p$-value $\leq 0.05$. The top ten identified in g:Profiler are shown in Figure 9E with the three most significant ones representing single-organism cellular processes, synapse pathways and ion binding. None of the ten identified pathways, containing the identified genes, were related to blood pressure regulation but interestingly a few seem to be involved in the brain and its functions.

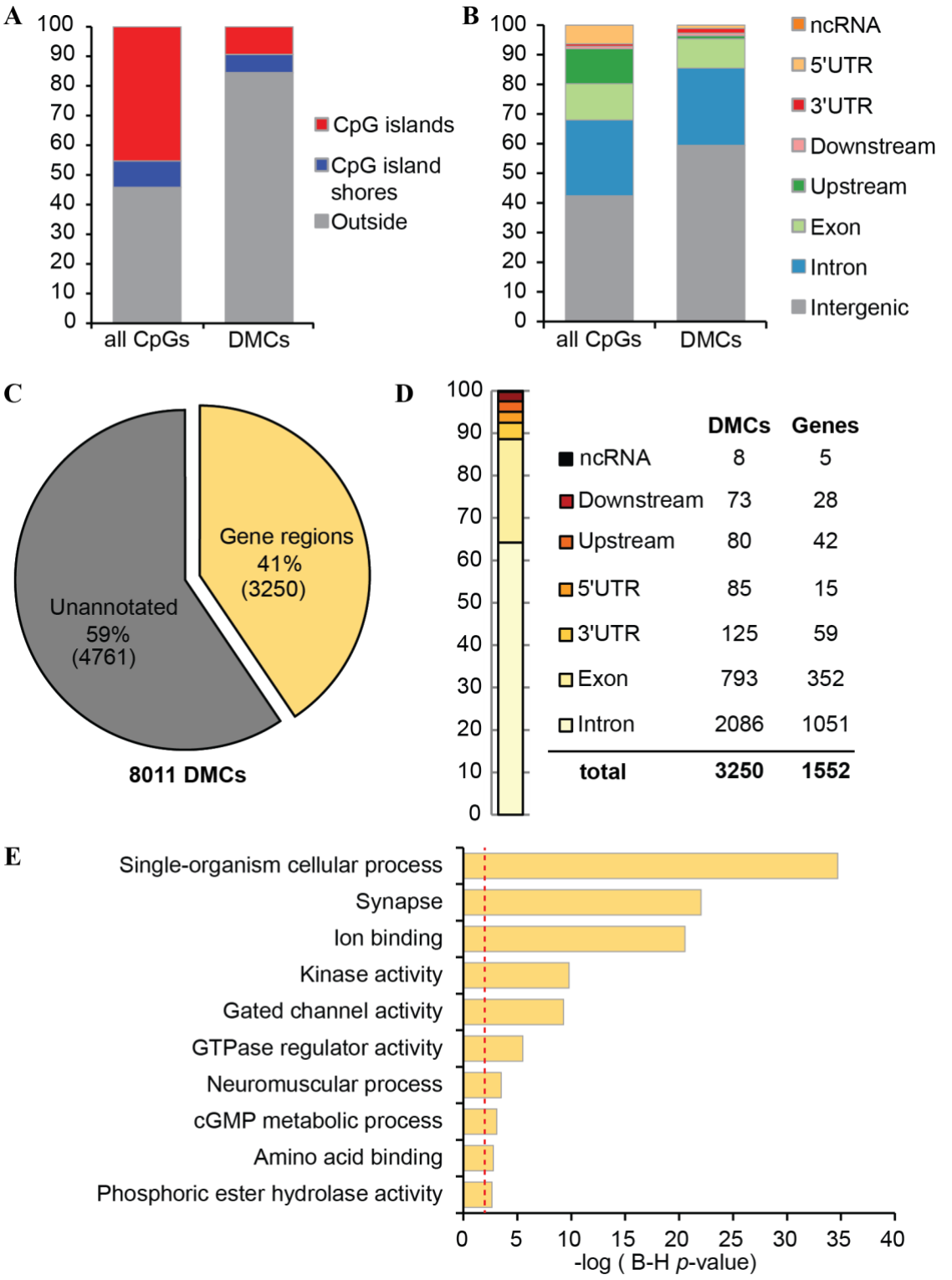



**Figure 9. Differently methylated cytosines (DMCs) between pre-hypertensive SHR and WKY in the kidney at four weeks of age.**

**A, B** Annotation of all analyzed CpGs and DMCs to CpG islands **(A)** and genic location **(B). C** Annotation ratio of DMCs. **D** Identified DMCs that can be linked to genes. **E** Gene ontology of all genes identified to contain the DMCs (red dotted line represents B-H $p$ = 0.01; g:Profiler).

## DMCs can be conflated into differentially methylated regions

Methylation of contiguous CpGs is known to be associated robustly with epigenetic silencing. Regions of differential methylation were defined using methylKit's subprogram eDMR. The parameters for eDMR identification were that DMCs were allowed to have a maximum distance to the next CpG of up to 100bp. Furthermore, there needed to be a minimum number of three CpGs, two of which were required to be differentially methylated ≥ 20% between the SHR and WKY with a corrected significance value of $q \leq 0.01$. Using these parameters 462 regions were identified. Out of the 462 eDMRs, 63% were in intergenic regions, remote from any annotated gene, making further analysis impossible. The remaining 37% (Figure 10A) were mainly assigned to introns and exons, and less so to 3'UTR, TSS and 5'UTR (Figure 10B). Interestingly a search within the database of the most recent hypertension GWAS study [99] for all of the 164 genes containing eDMRs revealed that eight of these genes (*Prdm11*, *Sdccag8*, *Wnt3a*, *Fbn2*, *Cdh13*, *Dpep1*, *Mapk4* and *Arghap24*) were identified in this GWAS, and had also been previously linked to hypertension. None of these eDMRs were in promoter regions; all eight resided in introns or exons of their respective genes.

However another five of my gene containing eDMRs also identified in the GWAS were assigned to the promoter region of the genes: *Arghap11a*, *Armc9*, *Larp1b*, *Nek2* and *Zdhhc7* which is where the majority of known DMRs that regulate gene expression are located. These genes could be potential candidates involved in the hypertension phenotype.

Gene ontology analysis on all 164 genes, identified in the 173 eDMRs that fell within genes, was performed using g:Profiler (Figure 10C). The three most significant pathways enriched were for single-multicellular organism processes, membrane regions and single-organism cellular processes. Notably the fifth identified pathway inferred involvement in blood pressure regulation, specifically involving the genes: *ABAT*, *EMP2*, *TAC4*, *TAC1*, *SLC4A5*, *BBS4* and *DRD3*. *EMP2* and *SLC4A5* are genes responsible for membrane composition, while *BBS4* is known to be involved in both renal malformation as well as mental retardation. Most interestingly however, the other four genes (*ABAT*, *TAC4*, *TAC1*, and *DRD3*) are expressed in

the brain and involved in GABA catabolism and Dopamine receptor formation. Given that the brain is another key organ for blood pressure regulation, particularly through the hypothalamus, this suggests that the brain is an organ that requires further investigation.

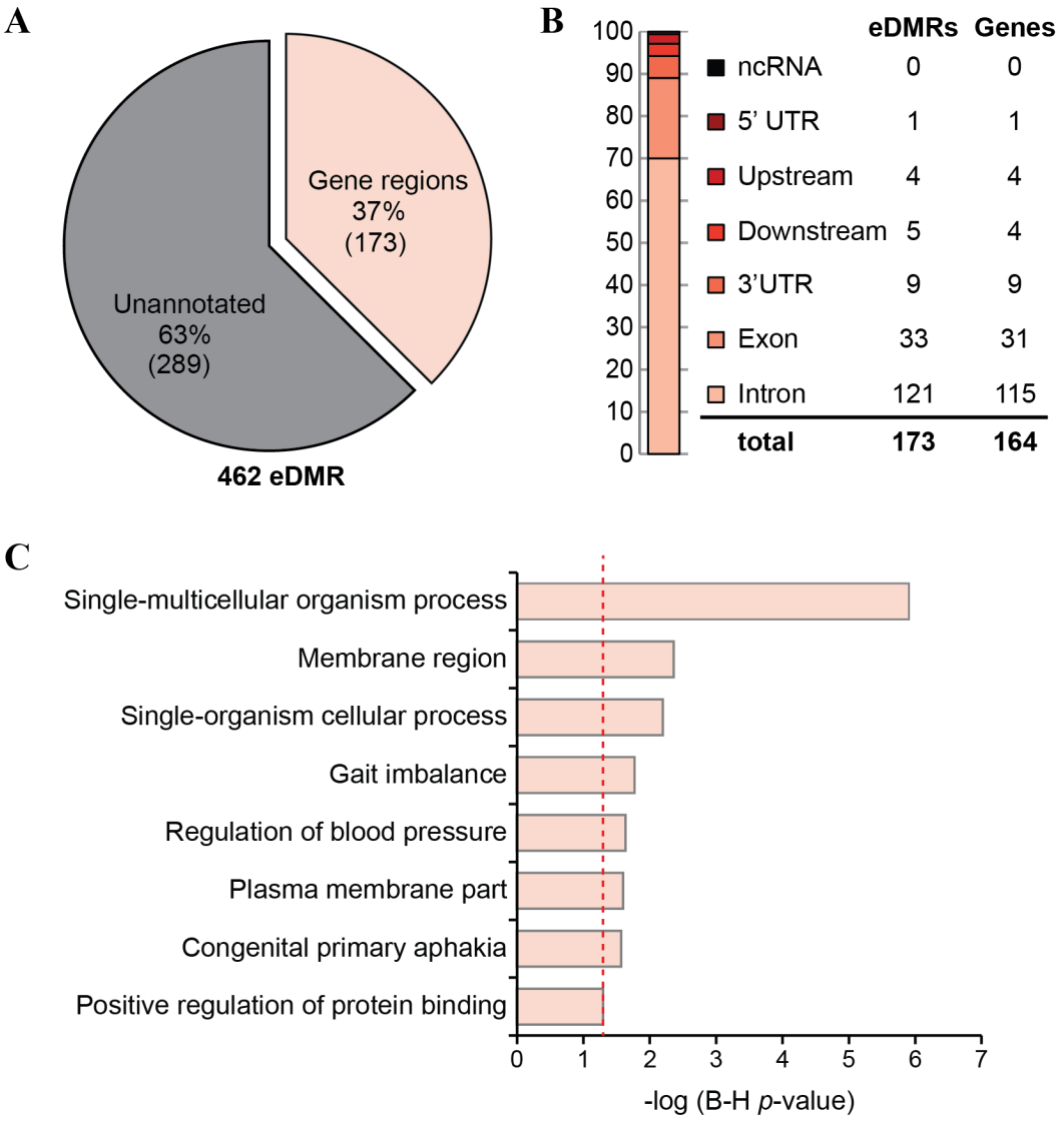



**Figure 10. Differently methylated regions (eDMR) defined by methylKit between pre-hypertensive SHR and WKY in the kidney at four weeks of age.**

**A** Annotation rate of eDMR data. **B** eDMRs that can be linked to genes. **C** Gene ontology for genes identified via eDMR (red dotted line represents B-H $p = 0.05$; g:Profiler).

## Empirical determination of DMRs

Given that the eDMR program identified only five promoter-associated DMRs, I employed a bespoke method to empirically identify additional DMRs associated with known genes. I collated all DMCs that were found within the 5'UTR and TSS and noted all genes that had at least two DMCs present. I identified 19 genes with this approach that showed differential methylation in the promoter region between the two strains (Table 3-2). The five genes called by eDMR were also included in this list.

Gene ontology analyses on these 19 genes was not possible due the limited number of genes (as the set was too small). Nevertheless, these DMRs are potential candidates that may be involved in regulation of gene expression which leads to the hypertensive phenotype observed in the SHR compared to the WKY later in life.

**Table 3-2.** Differentially methylated promoter regions between the pre-hypertensive SHR and WKY in the four week old kidney.

| | Gene | DMCs | Methylation difference SHR vs WKY (%)[a] |
|---|---|---|---|
| Both eDMR and empirically identified DMRs | *Arhgap11a* | 11 | 35.4 |
| | *Armc9* | 2 | -33.4 |
| | *Larp1b* | 10 | 35.9 |
| | *Nek2* | 3 | 46.1 |
| | *Zdhhc7* | 6 | -31.5 |
| Empirically identified DMRs only | *Avpi1* | 5 | 25.3 |
| | *C1qtnf3* | 2 | -21.7 |
| | *Cmss1* | 3 | 34.9 |
| | *Ddx3y* | 19 | -26.2 |
| | *Dnajc11* | 3 | -26.3 |
| | *Golt1b* | 2 | 43.3 |

| | | | |
|---|---|---|---|
| | *Kbtbd8* | 2 | -22.2 |
| | *Rnf2* | 15 | -37.8 |
| | *Sipa1l2* | 2 | -22.2 |
| | *Stk3* | 12 | 32.9 |
| | *Tbc1d5* | 2 | -42.9 |
| | *Tomm20* | 16 | -29.9 |
| | *Tymp* | 3 | -22.3 |
| | *Znrf4* | 2 | -22.1 |

[a] positive numbers represent hypermethylation in SHR vs WKY, negative numbers represent hypomethylation in SHR vs WKY

### 3.2.3 Correlation of differential methylation with gene expression in the kidney

#### Differences in gene expression in the kidney between SHR and WKY

To determine if the cytosine methylation differences within genes were associated with a change in expression of these genes, I examined gene expression in the kidney of SHR and WKY using microarray. Total RNA was extracted from the whole kidney from three 12 week old males of each strain, and used in an Affymetrix Clariom S™ rat microarray at the Ramaciotti Centre at UNSW. Principal components analysis of the resulting data with Transcriptome Analysis Console software showed SHR and WKY clearly separate by gene expression alone, with the first principal component accounting for more than 92% of all variance in gene expression (Figure 11).

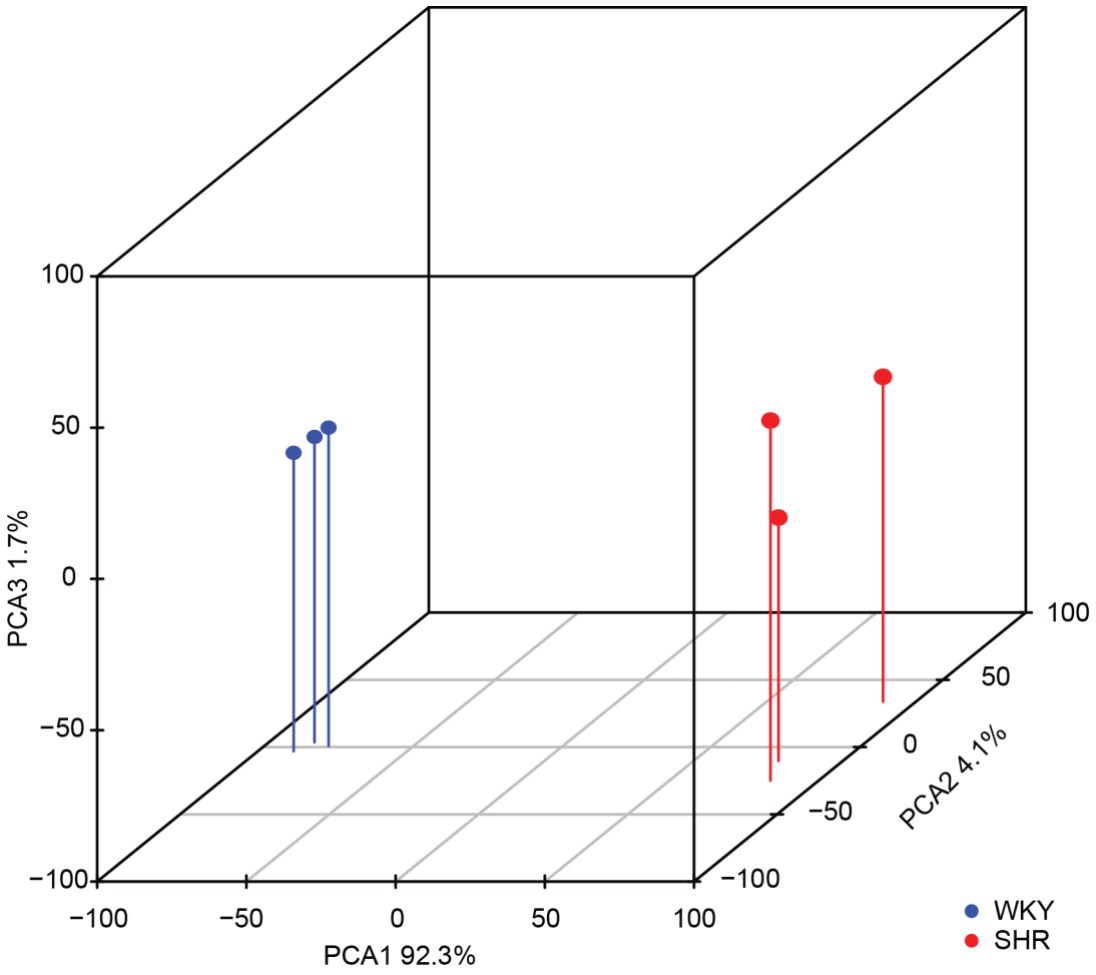



**Figure 11. Transcriptome Analysis Console (TAC) PCA output of expression array data of SHR (red) and WKY (blue) in the 12 week old kidney.**

Pseudo-3D Principal components analysis (PCA) plots of the expression array data from SHR and WKY.

Analysis of the differential expression between the strains revealed 223 genes with a greater than two-fold expression change between the strains at a corrected significance level of $q < 0.01$. Of these, expression in 126 genes was higher in the SHR while the expression in the remaining 97 genes was higher in the WKY (Figure 12A).

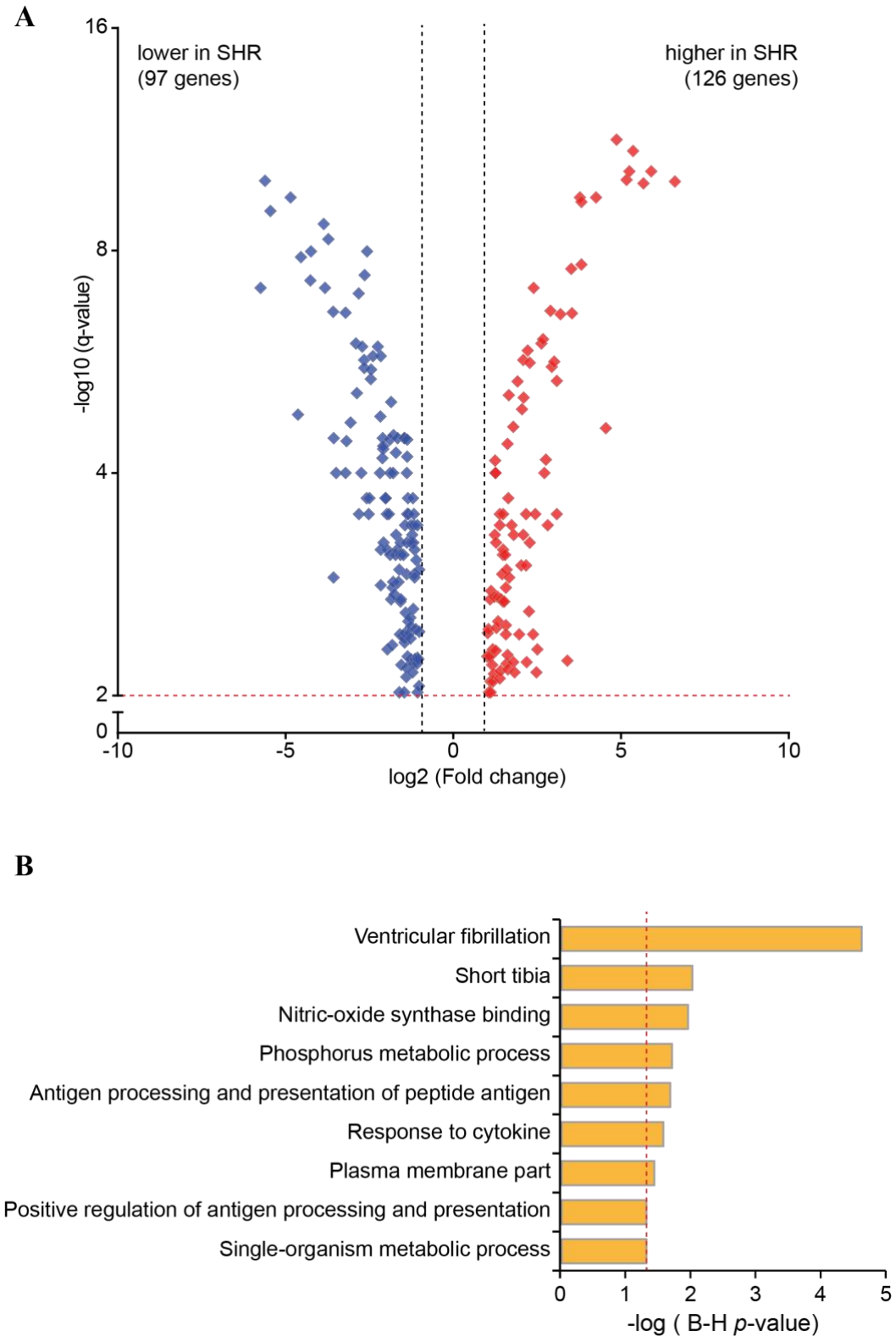



**Figure 12. Differently expressed genes in the kidney of SHR and WKY at 12 weeks.**

**A** Volcano plot depicting 223 genes differently expressed between SHR and WKY in the kidney at 12 weeks of age (all dots represent ≥ 2 fold change; red dotted line represents $q = 0.01$). **B** Gene ontology for genes with differential expression (red dotted line represents B-H $p = 0.05$; g:Profiler).

Gene ontology analysis of the 223 genes displaying differential expression using g:Profiler found nine significant pathways with $p \leq 0.05$ (Figure 12B). Surprisingly none of them inferred direct involvement in blood pressure regulation, but ventricular fibrillation and the known effect of Nitric-oxide on vascular tone could indirectly influence the blood pressure. It is interesting that no direct involvement of genes was identifiable despite the fact that SHR at 12 weeks already showed significantly higher blood pressure than WKY. The full list of genes is shown in Table in the Appendix.

## Concordance of findings in the kidney

I next examined the union between genes harboring DMCs and those genes which differed in expression between WKY and SHR. In doing so I found 36 genes containing DMCs which also exhibit changes in gene expression; the DMCs in five of these 36 genes were called as DMRs. (Figure 13A).

Upon investigation of those DMCs and DMRs located only in promoter associated regions, I found two genes exhibiting both methylation and gene expression changes: *Spock2* and *Cntnap4* (Figure 13B). *Spock2* showed 2.18 fold higher expression levels in SHR while *Cntnap4* expression in SHR was 5.19 fold times higher compared to WKY. Both genes were hypomethylated less than 1000 bp from TSS in the SHR but only contained a single DMC, which are uncommonly associated with gene expression changes.

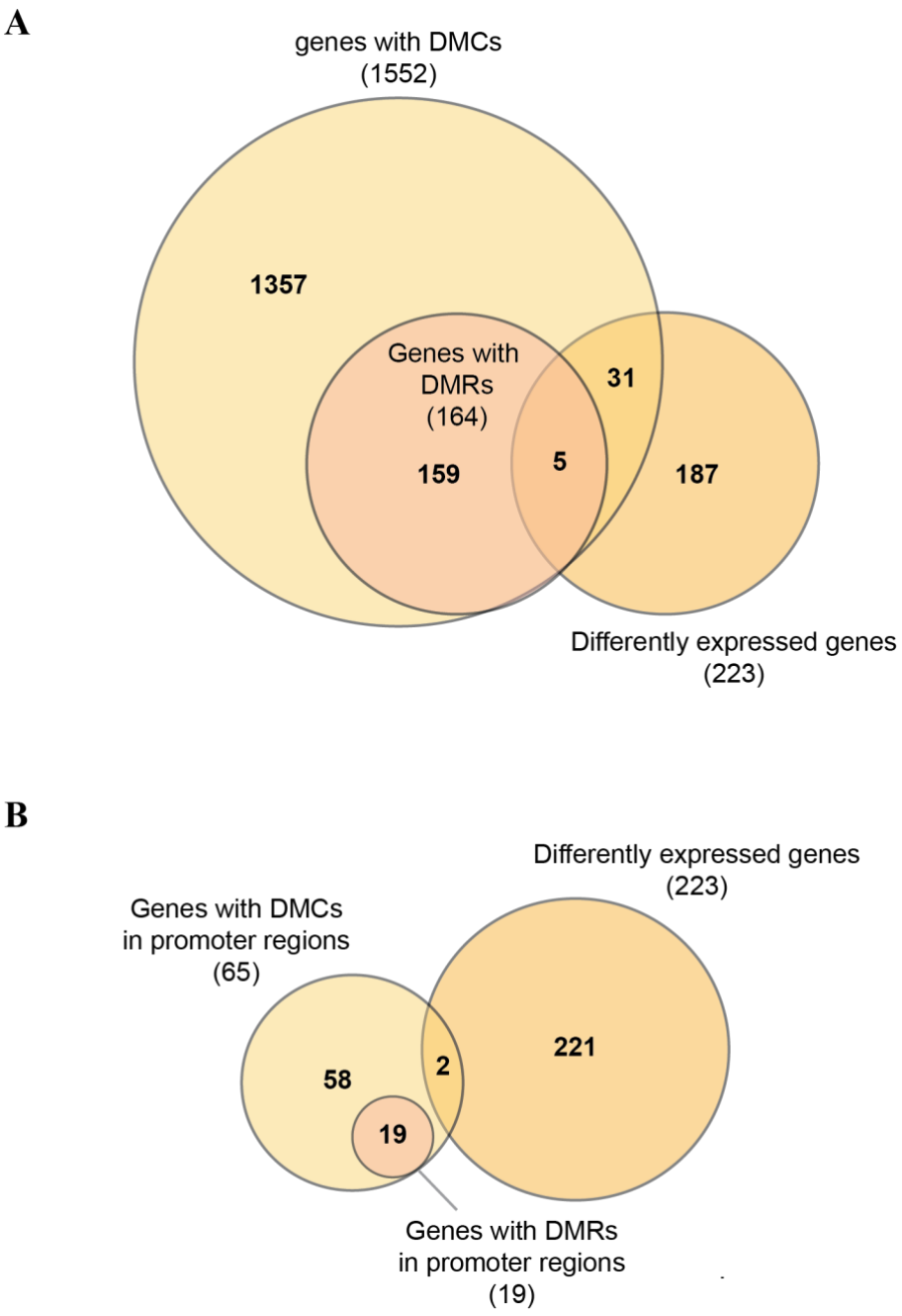



**Figure 13. Union between genes found in the RRBS DMCs and DMRs of four week old pre-hypertensive SHR and gene expression analysis of 12 week old hypertensive SHR and normotensive WKY.**

**A** Overlay of filtered DMCs (1152) and eDMR (164) with the expression array data (223). **B** Overlay of specific DMCs (65) and eDMR (5) in 5'UTR and upstream of coding regions with the expression array data (223).

The modest overlap between differential methylation and differential expression was surprising, particularly in the cases where methylation changes occurred at the promoter. It is possible however, that this subset of genes is not expressed (or very lowly expressed) in the kidney. If any of the changes in promoter methylation are indicative of a germ line event (addressed in the next chapter), it is possible that expression changes will only manifest in tissues in which those genes are expressed. This prompted me to examine published gene expression data for the rat at the 'Expression Atlas – EMBL-EBI' (Table 3-3). From this I was able to determine that two of the genes harboring DMRs in the promoter, are in fact not expressed in the kidney at all, and with the exception of *TOMM20* and *C1qtnf3*, most others are expressed at low to modest levels.

**Table 3-3. Published gene expression of DMRs that were in potential promoter regions.**

Transcripts per kilobase million (TPM) reported in at least four replicates and with a default minimum expression value of 0.5 TPM (shades of blue represent the fold changes of each gene between the different tissues).

| Gene | | kidney | brain | liver | heart | lung | spleen | thymus | adrenal | muscle | testis |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Armc9* | hypomethylated in SHR | 11 | 22 | 4 | 3 | 16 | 9 | 14 | 9 | 3 | 96 |
| *C1qtnf3* | | 52 | 3 | | | 3 | | | 1 | 6 | 2 |
| *Ddx3y* | | | | | | | | | | 1 | 143 |
| *Dnajc11* | | 28 | 36 | 25 | 16 | 33 | 29 | 49 | 48 | 18 | 42 |
| *Kbtbd8* | | 3 | 8 | 3 | 1 | 4 | 9 | 11 | 3 | 2 | 35 |
| *Rnf2* | | 16 | 23 | 15 | 12 | 33 | 32 | 89 | 15 | 21 | 64 |
| *Sipa1l2* | | 7 | 16 | 1 | 12 | 39 | 8 | 3 | 14 | 4 | 8 |
| *Tbc1d5* | | 16 | 24 | 10 | 8 | 31 | 41 | 26 | 26 | 8 | 55 |
| *Tomm20* | | 171 | 199 | 64 | 74 | 145 | 117 | 210 | 102 | 102 | 84 |
| *Tymp* | | 12 | 16 | 156 | 6 | 25 | 16 | 32 | 16 | 7 | 18 |
| *Zdhhc7* | | 16 | 12 | 7 | 12 | 27 | 24 | 22 | 16 | 11 | 18 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Znrf4* | | | 1 | | | 1 | 1 | | 2 | | 31 |
| *Arhgap11a* | hypermethylated in SHR | 3 | 2 | 3 | 2 | 8 | 20 | 41 | 4 | 2 | 19 |
| *Avpi1* | | 19 | 20 | 14 | 15 | 100 | 13 | 14 | 25 | 6 | 10 |
| *Cmss1* | | 5 | 13 | 5 | 1 | 8 | 9 | 19 | 8 | 5 | 61 |
| *Golt1b* | | 14 | 32 | 5 | 10 | 39 | 30 | 35 | 15 | 13 | 24 |
| *Larp1b* | | 29 | 14 | 38 | 20 | 25 | 17 | 16 | 18 | 46 | 210 |
| *Nek2* | | 3 | | 1 | | 2 | 8 | 13 | 1 | 1 | 152 |
| *Stk3* | | 21 | 16 | 10 | 9 | 41 | 19 | 20 | 24 | 8 | 13 |

Given this discovery I then reasoned it would be interesting to see if the methylation changes identified in the kidney, and particularly the genes that showed methylation differences in the promoter regions between SHR and WKY, could be found in other tissues as well. If this was indeed the case then the gene expression changes driving the SHR hypertensive phenotype could be originating from these other tissues rather than our initial candidate, the kidney. (This would open up the possibility that gene expression could be affected in those tissues and not be observed in the kidney, despite its strong impact on blood pressure regulation.)

The way I approached this in the following chapter was to assess the methylation patterns in the brain and liver of the same animals analyzed here and to take each of those tissues as a representative of the three germ layers: kidney – mesoderm, brain – ectoderm, liver – endoderm.

## 3.3 Discussion

Although essential hypertension is heritable with a strong familial penetrance, and a genetic determinant of the disease has been under investigation for decades, a cause for the disease remains unknown. One potential explanation for this is that hypertension has an underlying *epigenetic* (as opposed to genetic) basis. I chose to explore this hypothesis in a rodent model system employing the well-characterized spontaneously hypertensive rat (SHR), and the closely related but normotensive Wistar Kyoto rat (WKY). In this chapter I began my investigations by examining a stable and heritable epigenetic modification, cytosine methylation, in a hypertension-relevant organ: the kidney.

The animals in my study displayed the expected progressive hypertensive and heart rate phenotypes, as well as the previously described effects of these two measures on cardiac hypertrophy. Most importantly, no SHR animals were hypertensive at the time point chosen for study (four weeks), and no animals in the WKY colony exhibited higher than normal BP throughout their development. However, it needs to be noted that WKY animals in my experiments showed a higher systolic blood pressure than the expected ~120 mmHg. This is most likely due to the method of measurement. While not invasive, and certainly the method of choice for epigenetic studies, the tailcuff method requires restraint which can induce stress that impacts BP. However, because all animals (SHR and WKY) were trained and measured in the same way, the impact of this potential confounder is minimised.

This initial phenotyping was a fundamental starting point for the investigation of epigenetic causality in hypertension, as many epigenetic changes may result from hypertension itself, confounding interpretation. Perhaps surprisingly, cytosine methylation patterns at this pre-hypertensive stage could easily distinguish the two strains. This segregation was due to large differences in the degree of methylation at thousands of CpG sites (DMCs), many of which formed larger differentially methylated regions (DMRs).

Of the ~8,000 DMCs identified in the kidney, 60% were located in intergenic regions and were not able to be ascribed to genes for further investigation. These intergenic DMCs (which encompass 462 DMRs) may be functionally relevant however: they could be located in yet to be annotated genes or, in regulatory regions that hold the potential to affect gene expression. Identification of such loci will only be possible with better annotation of the rat genome. Unlike for mice and humans there is as yet no rat database for histone modifications or chromatin accessibility. These databases provide tools which would provide insight into whether the DMCs and DMRs in regions outside of genes might have enhancer or silencer functions. In the following chapter, I will investigate if intergenic DMCs and/or DMRs are present in multiple tissues. This would give these regions more gravitas to be worthy of further analysis regarding their possible function in the hypertensive phenotype.

The remaining 40% of DMCs however, were associated with genes; although only about 4% of DMCs were found within gene promoters, where methylation is known to have a direct impact on transcription.

The genes harboring DMCs showed no enrichment in ontologies related to hypertension. But this does not necessarily mean that any of the methylation changes are not relevant to hypertension. It may be that only a few – even just one – of these genes could be responsible for the predisposition for hypertension in the SHR. Narrowing this list of candidates therefore becomes the focus of the remainder of this thesis.

While there are reports of methylation at single CpG sites regulating gene expression, such as those that prevent the binding of a specific transcription factor [237], it is more likely that any gene expression change will be due to methylation of multiple CpGs across a large region [238, 239]. For this reason I set out to identify DMRs. As with individual DMCs, the majority of DMRs lie in unannotated or intergenic regions, which posed the same impediment to further investigation as I had for DMCs. Interestingly gene ontology for all genes with DMRs revealed a potential involvement in blood pressure regulation (Figure 10C). Furthermore, a search of the

most recent GWAS data [99], revealed that eight of the 164 genes had been linked to hypertension before. This is noteworthy given that the animals are not hypertensive at four weeks of age, and suggests that one or more of these genes may be involved in the SHR predisposition to hypertension.

What proportion of the methylation differences might be linked to genetic variation is currently unknown. It could be argued that many, even the majority, of methylation differences between the two strains could be due to genetic variation: Johnson *et al.* have suggested that WKY and SHR may be as genetically distinct as two unrelated humans [178, 240]. The use of WKY as an appropriate control for SHR experiments has also been brought into question [174]. This is because WKY as a strain was not fully inbred prior to distribution, and shows high variability in phenotype when purchased from different distributors [241]. Subsequent genetic analysis has confirmed that this phenotypic diversity does have a genetic basis [174, 242, 243]. To attempt to compensate for this I incorporated known SHR SNPs into my mapping strategy but this had little, if any, effect on the overall mappability. This could mean that the majority of epigenetic differences between the strains are not genetically determined. Ideally it would be preferable to have a more genetically similar normotensive control strain, but such a strain does not currently exist. This is a potential confounder that I attempt to address in Chapter 5. It would also help to have a more representative reference genome to map reads to: the rn5 rat reference genome is based on the Brown Norway rat. The most recent release in August 2017, 'Rnor_6.0', could provide better mappability as it includes for the first time a mix of the whole genome of SHR male rats with the genome of female Brown Norway rats.

As I observed for cytosine methylation, gene expression patterns in the kidney clearly separated the two strains by Principle Components Analysis (Figure 11). Gene expression microarrays were performed on 12 week old animals – by this age the SHR are already hypertensive. Therefore the distinct transcriptomes may in large part be due to the effects of hypertension itself on the SHR kidney. This is a confounder that could be addressed in future studies, but for

the purposes here, any changes due to hypertension itself would more likely result in false positives, rather than false negatives.

Interestingly, despite a strong hypertensive phenotype at 12 weeks of age, gene ontology analysis of differentially expressed genes between SHR and WKY, did not reveal any significant pathways related to blood pressure regulation. This might be due to the fact that in the early stages of hypertension development the kidney is not yet severely impacted or causal for the phenotype.

Other studies which have analyzed high throughput differential gene expression via microarray analysis between SHR and WKY both in pre-hypertensive or hypertensive animals have been equally surprising. Seubert *et al* [241, 244] observed no significant differences in the majority of the genes thought to be involved in blood pressure regulation when between SHR and WKY at three and nine weeks, while Koo *et al* [245] only observed pathways such as insulin resistance, signal transduction and lipid and glucose metabolism but not blood pressure regulation were altered when comparing hypertensive SHR rats to WKY.

It is therefore unsurprising that I was only able to identify a very modest overlap between differential methylation and expression, and most notably, that there was no overlap at all between differentially expressed genes and those with a DMC or DMRs in their promoter region.

Given that gene expression analysis in the kidney was not very informative, one logical avenue to explore would be to assess genes that display methylation differences but physiologically have little to no expression in the kidney. If a similar methylation pattern is present in other tissues where the pertinent gene is expressed, then alterations in gene expression could affect those organs resulting in lesions that lead to the hypertensive phenotype. In the following chapter I therefore assessed the brain and liver to determine changes in CpG methylation between SHR and WKY. Concurrently, given that these organs represent the ectoderm (brain) and the endoderm (liver), whilst the kidney is a tissue derived from the mesoderm, I could asses

if DMCs and DMRs in common between all three tissues are potential germ line epimutations that could be heritable.

In summary, this chapter identified many thousands of epigenetic differences between the kidney of normotensive WKY and the pre-hypertensive SHR. The majority of these were found in either unannotated or intergenic regions and one can only speculate whether or not they might contain inhibiting or enhancing effects on gene expression which can only be revealed upon further interrogation of the rat genome in the future. Those few differentially methylated CpGs that were found in genes and particularly promoter regions represent a set of candidate loci that may contribute or be causal of hypertension in the SHR and demand further investigation. If these epigenetic variants are also present in other tissues they could be representative of a germline epigenetic change perpetrating gene expression changes in any tissue or time point throughout the SHR and causal for their hypertensive phenotype. In order to assess this in the following chapter I will investigate two more tissues, the brain and the liver, and thereby also two additional germ layers. Together with the data from the kidney, representing the mesoderm, those methylation changes in common between the three tissues could be potential germ line epimutations.

# Chapter 4. Identification of potential germline epimutations in the SHR when compared to the WKY

## 4.1 Introduction

Chapter 3 revealed thousands of methylation differences between the pre-hypertensive kidney of SHR and the normotensive WKY, but which of these differences might be specifically associated with hypertension was not yet possible to determine. Given that the penetrance of hypertension in the SHR is 100%, it is most likely that if hypertension is due to an epigenetic variant, then this variant must have arisen in the germline, become stable and able to be faithfully passed from one generation to the next. In other words, the epigenetic lesion would meet the defining characteristics of a stable 'germline epimutation' [246, 247].

Germline epimutations manifest in form of aberrant epigenetic silencing of a gene that should normally be active (or vice versa) [248, 249]. In the case of deviant silencing, a germline epimutation can mimic an inactivating genetic mutation. There are multiple examples of such epimutations in plant biology as detailed in Chapter 1. In terms of mammalian systems there are far fewer clear examples than in plants, perhaps because plants do not segregate their germline in the same way animals do. Nevertheless, there is precedent in humans for germline epimutation to be a basis for complex disease. This is the case of the *MLH1* germline epimutation.

*MLH1* is a well-characterised tumour suppressor gene that when mutated predisposes an affected individual to hereditary nonpolyposis colorectal cancer (HNPCC), a very common inherited cancer syndrome associated with mismatch repair deficiency [250]. In 2004, Suter *et al*. [162] identified two individuals with apparent HNPCC that did not carry a germline mutation in *MLH1*, or any other mismatch repair gene, but rather had a germline *epi*mutation of *MLH1*. The epimutation was characterised by dense soma-wide hypermethylation of the promoter of the gene, leading to loss of *MLH1* expression. Since 2004 there have been further

cases of *MLH1* germline epimutation described, and in each case the epimutation leads to a syndrome that is a complete 'phenocopy' of the genetic mutation – in the absence of a mutation.

In the majority of cases of *MLH1* germline "pure" epimutation, the associated phenotype appears in a single isolated generation [164, 251], but there have been several cases in which the epimutation has been transmitted from parent to offspring, along with the disease risk it imparts [164, 252, 253]. This nonmendelian pattern of inheritance is typical of epigenetic phenomena [254]; why this is so often the case is unknown, but in mammals it is probably related to the epigenetic resetting events in germ cells and embryos (as discussed in Chapter 1 [141]). But the *MLH1* epimutation is only one example of germline epimutation. While, the germline stability of this particular epimutation seems weak, in other systems the heritability of germline epigenetic silencing can be very stable, and in some cases (such as the b1 locus in maize) completely irreversible [255]. It is worth noting that some sequences in the mammalian germline, such as centromeres [256-258], and some repetitive elements [259, 260], are stably silenced throughout the mammalian life cycle. This implies that mechanisms that elicit stable and permanent silencing are active throughout the life cycle. There is thus good reason to suppose that it is possible for any gene to be affected by germline epimutation, and that such a change might be stably heritable in mammals.

Germline mutations are usually sought in potentially affected individuals by assessing DNA from a somatic tissue – blood is commonly used. However, identification of a germline epimutation is more complex as each tissue has its own epigenome. This necessitates the study of multiple tissues, ideally tissues from distinct germ layer origins.

In order to ask whether any of the methylation differences identified in Chapter 3 might have arisen in the germline (and thus potentially represent a germline epimutation), in this chapter I sought epigenetic variants in the SHR that were common to tissues derived from all three distinct germ layers. I again employ reduced representation bisulfite sequencing (RRBS) to

generate snapshots of genomic methylation patterns in relevant tissues from SHR and WKY. I aimed specifically to:

4. Produce RRBS maps of cytosine methylation from the liver (derived from endoderm) and brain (derived from ectoderm) of the same four week old pre-hypertensive SHR males and age-matched WKY males as used in Chapter 3.
5. Compare methylation patterns between the two strains at the level of individual CpG sites, as well as regions, in both tissues.
6. Determine which methylation variants are present in all three germ layers and thus may represent a germline epimutation.

## 4.2 Results

### 4.2.1 Analysis of cytosine methylation in WKY and SHR liver

**SHR can be distinguished from WKY by liver DNA methylation patterns**

Subsequent to the kidney analysis, I discovered and implemented a more efficient and cost effective protocol for preparation of RRBS libraries using the Nugen Ovation RRBS kit (NuGen; Chatswood, Australia). I randomly selected five of the seven animals that had their kidneys assessed in the previous chapter and extracted DNA from whole livers to prepare RRBS libraries. As with the kidney in the previous chapter the whole liver was analyzed to prevent bias in the libraries due to differences in cellular composition. Sequencing and mapping were performed as for the kidney. A summary of the sequencing statistics for the liver are presented below (Table 4-1).

**Table 4-1.** Summary of the RRBS sequencing statistics of the liver of pre-hypertensive SHR and WKY at four weeks of age.

| Animal ID | strain | Number of unmapped reads | Number of mapped reads | Mapping efficiency (%) | Number of CpGs captured | Number of CpGs at 20x coverage |
|---|---|---|---|---|---|---|
| **1000566** | **WKY** | 13,015,002 | 8,936,382 | 68.7 | 2,842,020 | 140,265 |
| **1000630** | **WKY** | 40,438,301 | 28,230,640 | 69.8 | 3,258,667 | 1,780,601 |
| **1000638** | **WKY** | 19,553,715 | 13,519,407 | 69.1 | 3,052,504 | 635,029 |
| **1000650** | **WKY** | 22,294,362 | 15,376,224 | 69.0 | 2,989,393 | 489,712 |
| **1000691** | **WKY** | 45,576,170 | 31,082,865 | 68.2 | 3,051,836 | 1,391,019 |
| **1000583** | **SHR** | 10,219,890 | 7,143,847 | 69.9 | 2,743,300 | 57,381 |
| **1000604** | **SHR** | 22,066,829 | 15,608,036 | 70.7 | 3,095,034 | 838,367 |
| **1000615** | **SHR** | 25,261,782 | 17,906,567 | 70.9 | 3,122,556 | 1,141,323 |
| **1000620** | **SHR** | 3,752,658 | 2,622,117 | 69.9 | 2,185,913 | 7,384 |
| **1000674** | **SHR** | 30,388,472 | 21,451,652 | 70.6 | 3,090,551 | 1,455,175 |

There was high variability in the RRBS data between animals, ranging from a WKY rat with 1,780,601 CpGs to a SHR rat with 7,384 CpGs sequenced to a 20X coverage depth. However, one of the parameters we established for analysis of differential methylation was that CpGs with at least 20X coverage were required to be represented in only a minimum of three animals per strain. For the liver that left 344,983 CpGs for further downstream assessment. This was only

about 24% of the number of CpGs that had been analyzed in the kidney which should be considered in interpretation of the results.

As was observed with the kidney RRBS data, unsupervised hierarchical clustering of the RRBS liver data at 20X coverage neatly separated the WKY and SHR into their strains, as shown in the dendrogram in Figure 14A. Principal components analysis (PCA; Figure 14B) also separated SHR and WKY, with the first principal component contributing to 54.6% of all methylation variance; almost three times higher than that observed in the kidney at 18.4%. This is remarkable, given that the liver has not previously been implicated in the SHR phenotype of hypertension as a disease, nor would be expected to be different between the SHR and WKY. Furthermore, it is noteworthy that this is quite surprising for two strains that have only been separated from their common parent strain for 60 years [241], and while the SHR are hypertensive later in life, animals at this age displayed no hypertensive phenotype.

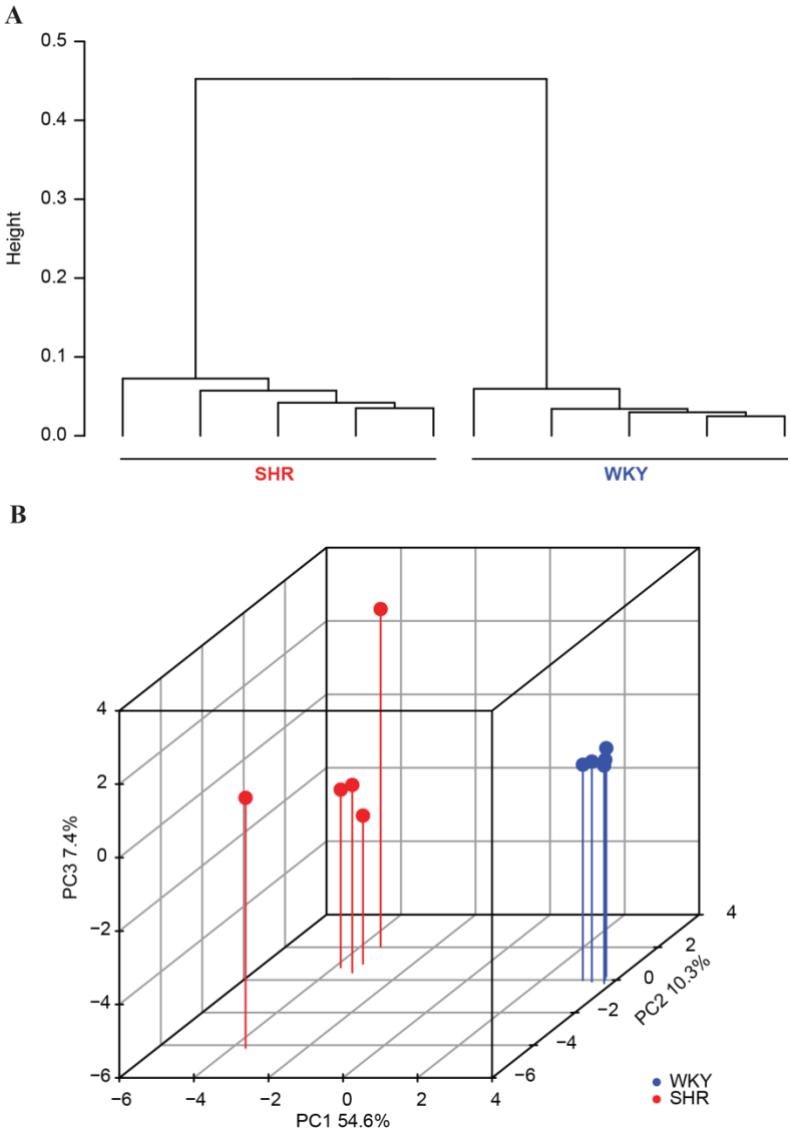



**Figure 14. Unsupervised hierarchical clustering and PCA of RRBS data of the liver of pre-hypertensive SHR and WKY at four weeks of age.**

**A** Dendrogram depiction of unsupervised hierarchical clustering of RRBS data of the liver of pre-hypertensive SHR and WKY at four weeks of age (Ward's Method). **B** Pseudo-3D principal components analysis (PCA) plots of the RRBS data of the liver of pre-hypertensive SHR (red) and WKY (blue) at four weeks of age.

## methylKit identifies thousands of differently methylated cytosines (DMCs) between pre-hypertensive SHR and WKY in the liver

As in the previous chapter, the R package methylKit was used to identify differentially methylated CpGs between four week old livers of pre-hypertensive SHR and WKY rats.

In order to statistically identify differentially methylated CpGs (DMCs) the same stringent parameters as for the kidney analysis were applied: coverage of 20X or greater, a significance threshold of $q < 0.01$, and $\geq 20\%$ average methylation difference between SHR and WKY. This analysis defined 2340 DMCs between SHR and WKY (Figure 15). As I previously observed in the kidney, the majority of DMCs, (1493 of the 2340; 63.8%) were found to be hypomethylated in the SHR relative to WKY with a $p$-value $< 0.00001$ (Chi-Square test).

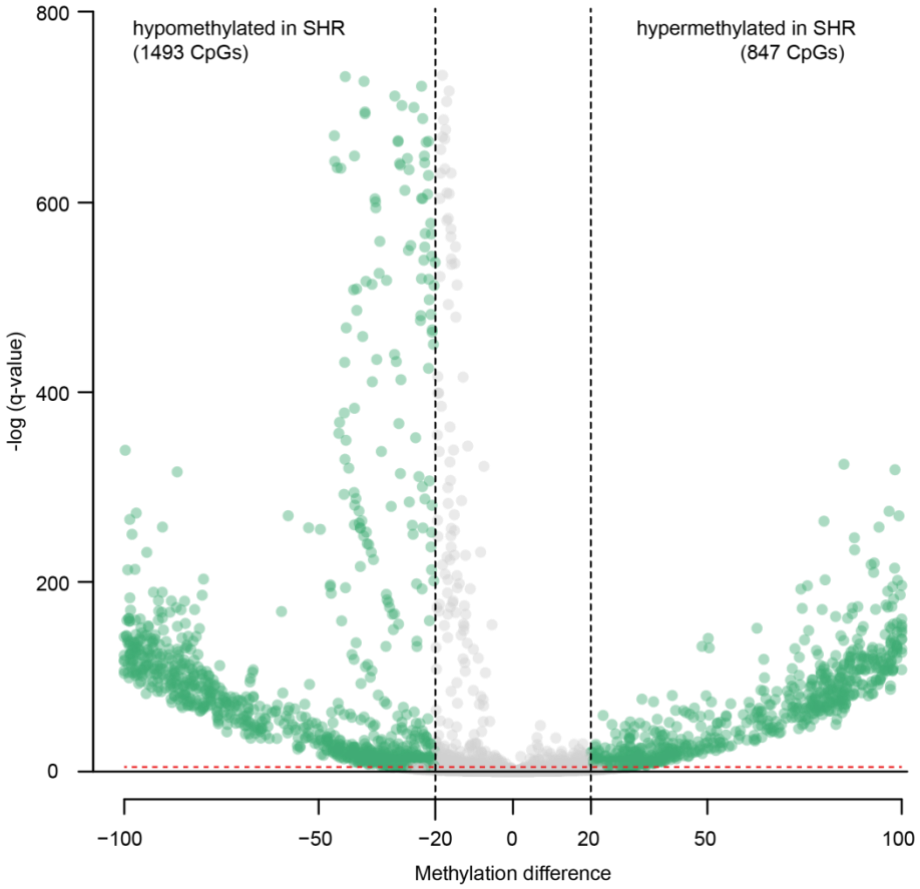



**Figure 15. Thousands of differentially methylated cytosines (DMCs) identified in the liver between pre-hypertensive SHR and WKY at four weeks of age.**

Volcano plot depicting DMCs. DMCs that passed a significance threshold of $q \leq 0.01$, and $\geq$ 20% average methylation difference between SHR and WKY, are shown in green (red dotted line represents $q = 0.01$).

Upon evaluation of the genomic location of the methylated cytosines, I discovered that the majority of all analyzed CpGs and DMC were found to be located outside of defined CpG islands. Whilst about 30% of all CpGs in the analysis were found in CpG islands, only about 10% of the DMCs were located here. The percentage of DMCs found in CpG island shores remained approximately the same to the distribution of all CpGs, with just under 10% of all DMC or CpGs located there (Figure 16A). These were similar percentages to what I had observed in the kidney, suggesting that DMCs were equally depleted in CpG islands and shores in the liver as in the kidney.

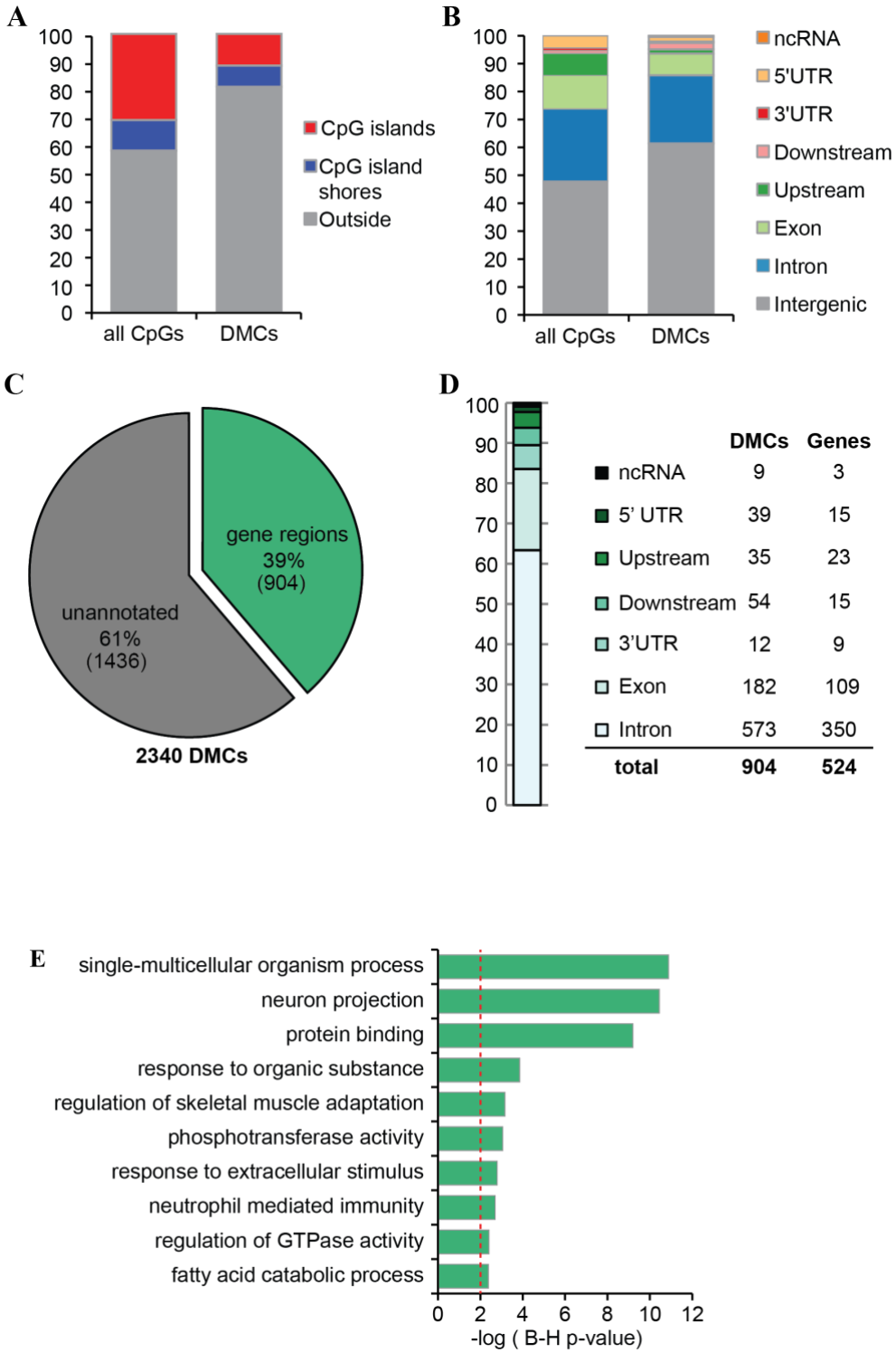



**Figure 16. Differently methylated cytosines (DMCs) between pre-hypertensive SHR and WKY in the kidney at four weeks of age.**

**A, B** Annotation of all analyzed liver CpGs and DMCs to CpG islands **(A)** and genic location **(B). C** Annotation ratio of DMCs. **D** Identified DMCs that can be linked to genes. **E** Gene ontology of all genes identified to contain the DMCs (red dotted line represents B-H $p = 0.01$; g:Profiler).

As observed in the kidney, the proportion of liver DMCs within genic regions was smaller compared to all CpGs in the analysis (Figure 16B), this was mainly due to depletion in both 5'UTR and regions ≤ 1000 bp upstream of protein coding regions. These two genic regions are termed here as transcription start sites (TSS) and are usually associated with promoter regions. Thus, the majority of DMCs in the liver were not found in promoter regions.

The remaining DMCs that were located in gene annotated regions (Figure 16C; n=904) were able to be assigned to 524 genes (Figure 16D), with the majority located in introns and exons. Just 7.3% of the annotated DMCs were found in 5'UTR and TSS, which was about half that found in these regions in the kidney.

I next investigated whether any of these 524 genes which contained DMCs fell into biological pathways that potentially could be linked to blood pressure regulation, by performing gene ontology analysis via g:Profiler. From these genes, 57 biological pathways with B-H *p*-value ≤ 0.05 were identified. Figure 16E identifies the top ten pathways; the top three consisted of single-multicellular organism processes (also seen in the kidney), neuron projection and protein binding. As I had previously observed in the kidney none of the pathways identified suggested an involvement in blood pressure regulation.

## Differentially methylated regions (DMRs) identified in the pre-hypertensive liver

In order to identify potential regions of contiguous CpGs with differential methylation between the strains, hereafter named eDMRs, we next applied methylKit's subprogram eDMR as previously described in Chapter 3 for the kidney. The eDMR parameters were defined as follows: at least three CpGs to be present within 1000 bp section and at least two of them defined as DMCs; this analysis identified 143 eDMRs. Of these 143 eDMRs 87 (61%) were found in intergenic regions and as such further interpretation of their causal effect could not be made at this point. The remaining 56 (39%) eDMRs that were harbored by gene annotated regions (Figure 17A) were, like the DMCs, mainly assigned to introns and exons (Figure 17B).

These 56 eDMRs were found to be located within 51 genes, with three within promoter regions, either in the 5'UTR or ≤ 1000 bp upstream from a transcription start site (Table 4-2).

Gene ontology on either the 51 genes containing DMRs or the three genes harboring DMRs in promoter regions was unable to be performed due to the limited number of genes.

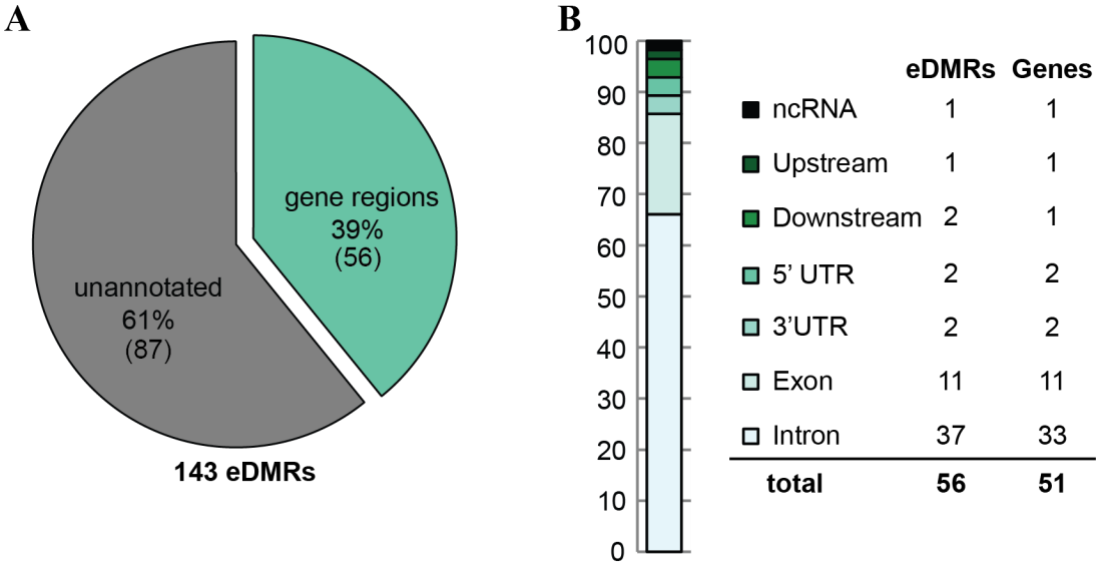



**Figure 17. Differently methylated regions (eDMR) defined by methylKit between pre-hypertensive SHR and WKY in the liver at four weeks of age.**

**A Broad** annotation of eDMR data. **B** Annotation of eDMRs that can be linked to genes.

As for the kidney analysis in Chapter 3, I again performed an empirical determination of additional DMRs to capture any DMRs not identified by eDMR/methylKit. I identified an additional six genes with DMRs in their promoter regions which merited further investigation (Table 4-2).

**Table 4-2.** Differentially methylated promoter regions between the pre-hypertensive SHR and WKY in the four week old liver.

| | Gene | DMCs | Methylation difference SHR vs WKY (%) [a] |
|---|---|---|---|
| Both eDMR and empirically identified DMRs | *Eif1a* | 4 | 37.5 |
| | *RGD1562114* | 6 | -30.9 |
| | *Stk3* | 4 | 32.7 |
| Empirically identified DMRs only | *Arhgap11a* | 6 | 40.1 |
| | *Dnajc11* | 2 | -29.6 |
| | *Insl6* | 2 | -21.5 |
| | *Ddx3y* | 7 | -28.6 |
| | *Tomm20* | 9 | -43.1 |
| | *Trim52* | 2 | 53.0 |

[a] positive numbers represent hypermethylation in SHR vs WKY
negative numbers represent hypomethylation in SHR vs WKY

Next I move on to RRBS analysis of the brain, representing the last of the three germ layers, the ectoderm. I will then merge the liver data with that from the kidney and brain and perform further analysis to find common methylation differences in all three germ layers.

### 4.2.2 Analysis of cytosine methylation in WKY and SHR brain

#### SHR can be distinguished from WKY by brain DNA methylation patterns

DNA from the whole brain of the same five animals chosen for the liver and kidney analyses was used to prepare RRBS libraries that were sequenced on the Illumina platform at the Ramaciotti Centre for Genomics (Sydney) and mapped to rn5. A summary of the sequencing statistics is shown in Table 4-3.

**Table 4-3. A summary of RRBS sequencing statistics in the brain of pre-hypertensive SHR and WKY at four weeks of age.**

| Animal ID | Strain | Number of unmapped reads | Number of mapped reads | Mapping efficiency (%) | Number of CpGs captured | Number of CpGs at 20x coverage |
|---|---|---|---|---|---|---|
| **1000566** | **WKY** | 12,204,145 | 8,308,364 | 68.1 | 2,857,878 | 113,039 |
| **1000630** | **WKY** | 10,169,772 | 6,894,001 | 67.8 | 2,802,421 | 62,925 |
| **1000638** | **WKY** | 34,342,578 | 24,030,956 | 70.0 | 3,001,058 | 509,507 |
| **1000650** | **WKY** | 18,825,803 | 13,108,979 | 69.6 | 3,162,750 | 1,617,265 |
| **1000691** | **WKY** | 14,445,774 | 9,829,947 | 68.0 | 2,857,601 | 196,319 |
| **1000583** | **SHR** | 13,625,510 | 9,368,897 | 68.8 | 2,841,399 | 377,849 |
| **1000604** | **SHR** | 7,975,318 | 5,474,374 | 68.6 | 2,699,758 | 50,539 |
| **1000615** | **SHR** | 13,725,515 | 9,706,561 | 70.7 | 2,911,796 | 185,273 |
| **1000620** | **SHR** | 33,758,639 | 23,685,415 | 70.2 | 3,113,424 | 1,592,453 |
| **1000674** | **SHR** | 29,860,580 | 21,325,456 | 71.4 | 3,166,165 | 1,431,047 |

Overall less CpGs were captured by RRBS of the brain compared to liver and kidney. Sequencing analysis from the brain revealed a range from 1,617,265 CpGs reaching a depth of 20X coverage in a WKY animal to only 50,539 CpGs in a SHR. This left only 97,066 CpGs that were able to be analyzed, after taking into consideration that at least three of the five animals per strain had to contain the same CpG with 20X coverage in order to be represented in the analysis. This is less than a third of the number of CpGs with 20X coverage analyzed in the liver and less than 7% of the number of CpGs with 20X coverage seen in the kidney in the previous chapter.

Unsupervised hierarchical clustering of the RRBS brain data at 20X coverage nevertheless neatly separated the WKY and SHR into their strains, as shown in the dendrogram in Figure 18A. Principal components analysis (Figure 18B) also separated SHR and WKY, with the first principal component contributing to ~33% of all methylation variance. As a comparison the kidney separated with the first principal component contributing ~18%, while it was ~55% in the liver.

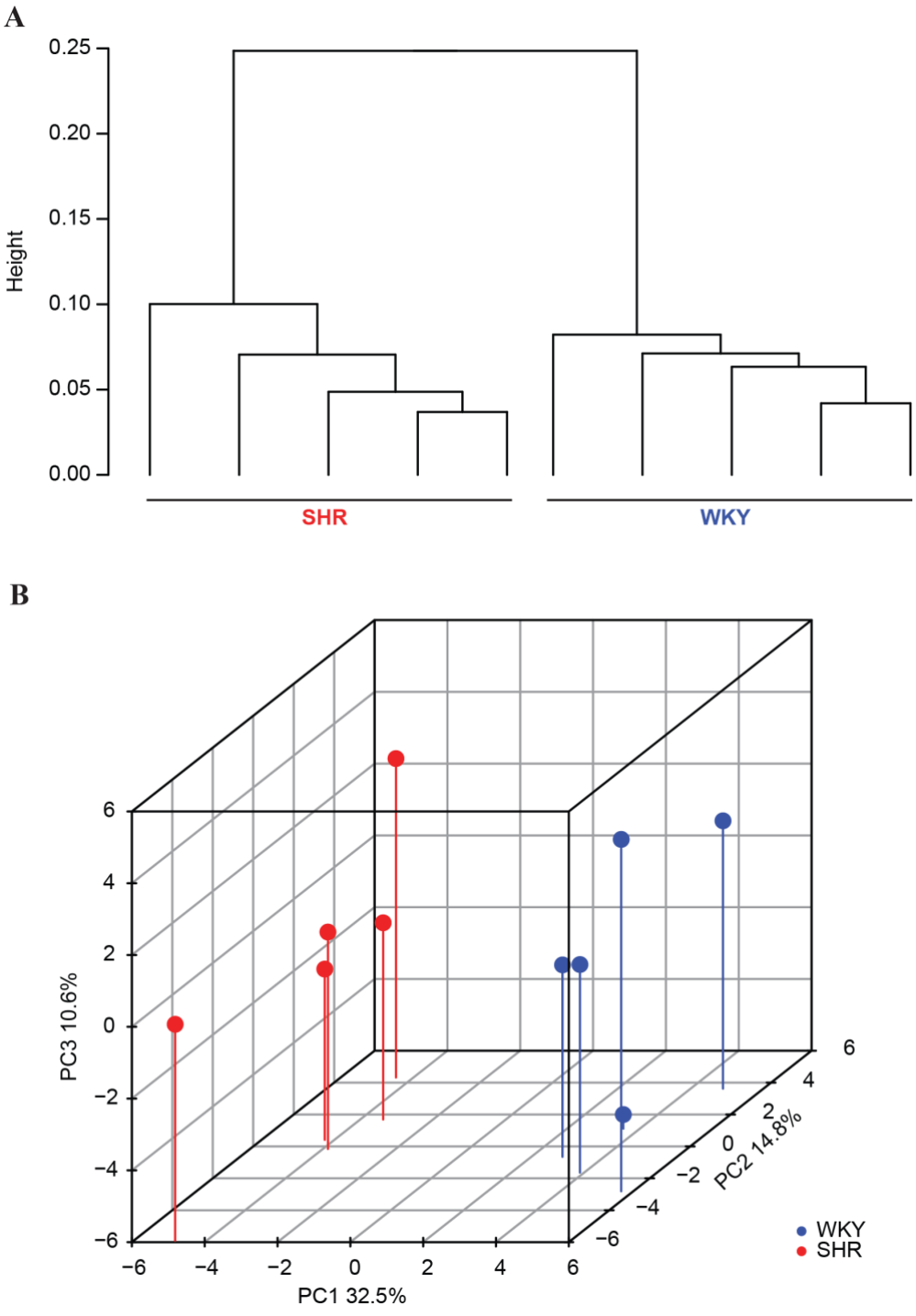



**Figure 18. Unsupervised hierarchical clustering and PCA of RRBS data of the brain of pre-hypertensive SHR and WKY at four weeks of age.**

**A** Dendrogram depicting unsupervised hierarchical clustering of RRBS data of the brain of pre-hypertensive SHR and WKY at four weeks of age (Ward's Method). **B** Pseudo-3D principal components analysis (PCA) plots of the RRBS data of the brain of pre-hypertensive SHR (red) and WKY (blue) at four weeks of age.

## methylKit identifies hundreds of differently methylated cytosines (DMCs) between pre-hypertensive SHR and WKY in the brain

In the brain methylKit analysis defined 878 DMCs between SHR and WKY (Figure 19). As observed in the kidney and the liver before, the majority of DMCs, 647 of the 878 (73.7%), were found to be hypomethylated in the SHR with a *p*-value < 0.00001 (Chi-Square test).

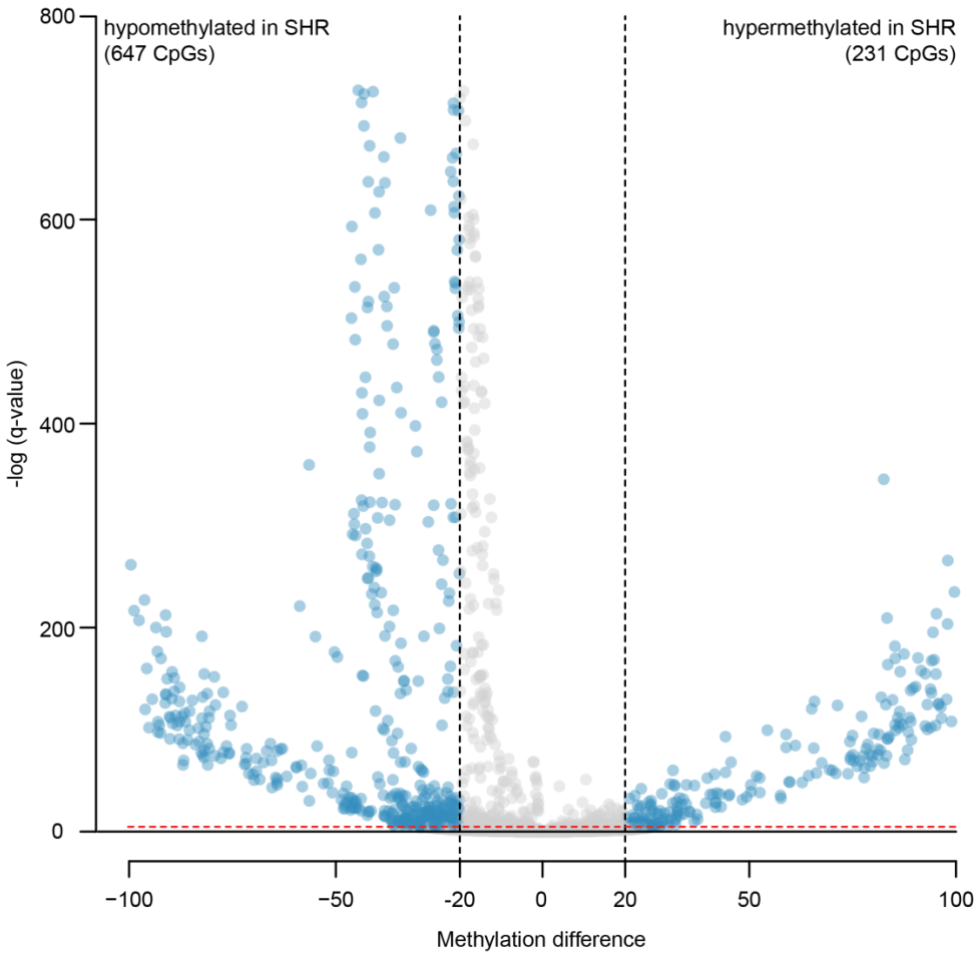



**Figure 19. Hundreds of differentially methylated cytosines (DMCs) identified in the brain between pre-hypertensive SHR and WKY at four weeks of age.**

Volcano plot depicting DMCs. DMCs, meeting the parameters of significance threshold of $q \leq 0.01$, and $\geq$ 20% average methylation difference between SHR and WKY, are shown in blue (red dotted line represents $q = 0.01$).

The majority of all CpGs covered at 20X, as well as the DMCs, were located outside of CpG islands. Whilst about 25% of all CpGs were in CpG islands, only ~15% of the DMCs were

identified here. The percentage of those found in CpG island shores dropped from about 15% in all CpGs to about 10% in the DMCs (Figure 20A). These results were similar to those observed in the kidney and liver suggesting that DMCs were equally depleted in CpG islands and shores, as witnessed in the other two germ layers.

In addition, as observed in the other two tissues, the proportion of DMCs within genic regions was smaller relative to all CpGs (Figure 20B) and further depleted in both 5'UTR and TSS.

The DMCs that were found in gene annotated regions (Figure 20C; n = 317), could be assigned to 165 genes (Figure 20D), with the majority located in introns and exons. Of these genes 14 (8.5%) contained DMCs in the 5'UTR or TSS.

I next interrogated which biological pathways were associated with these genes containing DMCs by performing gene ontology analysis via g:Profiler. From these 165 genes seven biological pathways with B-H $p$-value $\leq 0.05$ were identified (Figure 20E), however, as observed in the kidney and liver, none of these pathways, with the exception of episodic sleep apnea, were found to be involved in blood pressure regulation.

Taken together, these data indicates that while hundreds of cytosines are differentially methylated between pre-hypertensive SHR and WKY in the brain, most DMCs are located outside of regions where methylation is obviously associated with regulation of gene expression such as 5'UTR and upstream of the TSS.

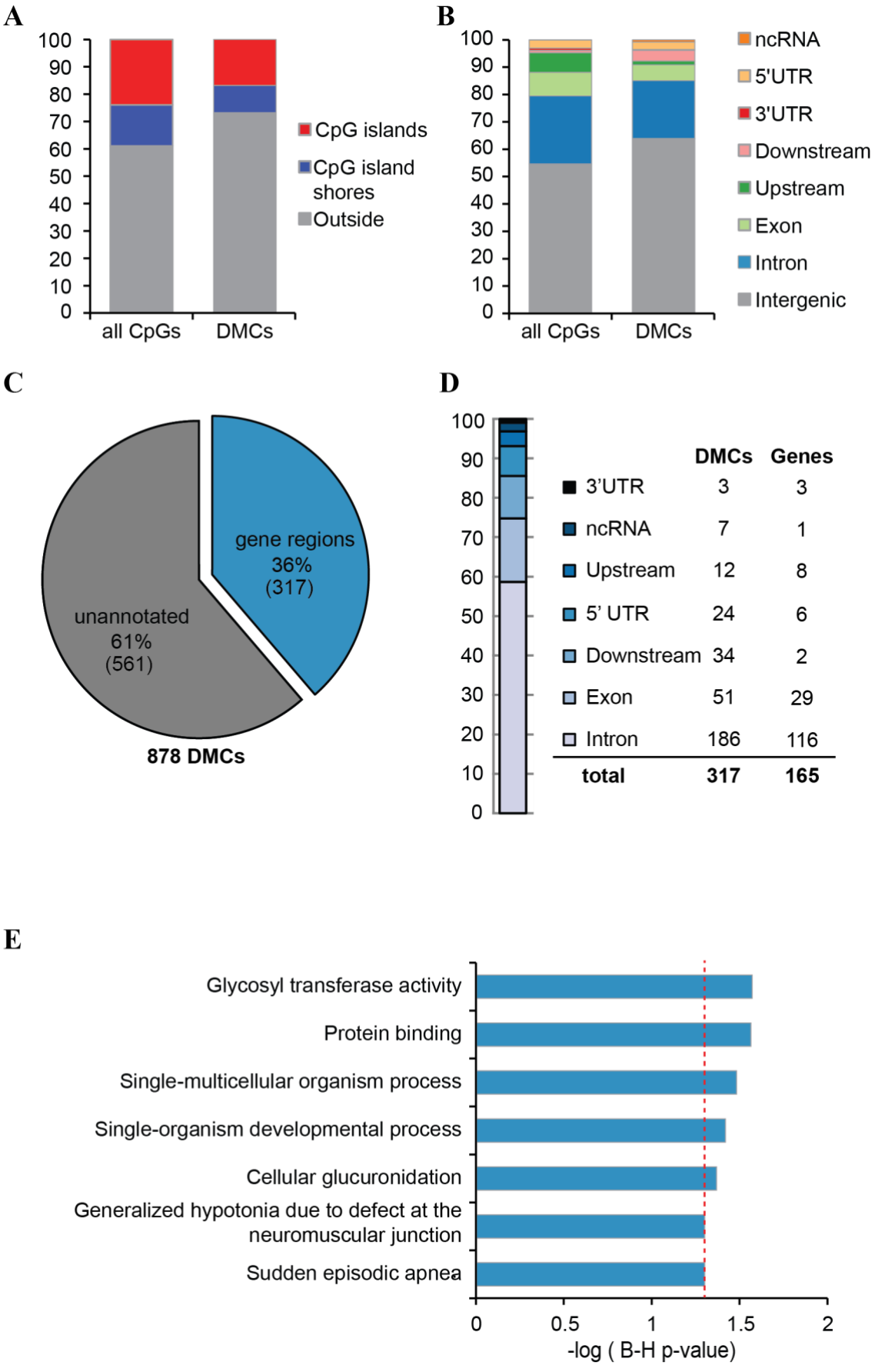

A
100
90
80
70
60
50
40
30
20
10
0
all CpGs
DMCs
CpG islands
CpG island shores
Outside
B
ncRNA
5'UTR
3'UTR
Downstream
Upstream
Exon
Intron
Intergenic
C
gene regions 36% (317)
unannotated 61% (561)
878 DMCs
D
DMCs
Genes
3'UTR 3 3
ncRNA 7 1
Upstream 12 8
5' UTR 24 6
Downstream 34 2
Exon 51 29
Intron 186 116
total 317 165
E
Glycosyl transferase activity
Protein binding
Single-multicellular organism process
Single-organism developmental process
Cellular glucuronidation
Generalized hypotonia due to defect at the neuromuscular junction
Sudden episodic apnea
0
0.5
1
1.5
2
-log ( B-H p-value)

**Figure 20. Differently methylated cytosines (DMCs) between pre-hypertensive SHR and WKY in the brain at four weeks of age.**

**A, B** Annotation of all analyzed CpGs and DMCs to CpG islands **(A)** and genic location **(B). C** Annotation ratio of DMCs. **D** Identified DMCs that can be linked to genes. **E** Gene ontology of all genes identified to contain the DMCs (red dotted line represents B-H $p = 0.05$; g:Profiler).

## Differentially methylated regions (DMRs) identified in the pre-hypertensive brain

I next utilized methylKit's subprogram eDMR in order to identify DMRs for the brain, as I had for the previous two tissues. The program identified 29 eDMRs in total, of which 13 (45%) were in intergenic regions. The remaining 16 eDMRs (55%) within gene annotated regions (Figure 21A) were, as the DMCs before, mainly assigned to introns and exons (Figure 21B). These genic eDMRs were located within 14 genes, three of which contained a DMR in the promoter region (Table 4-4).

Again, due to the small number of genes, it was not possible to perform gene ontology.

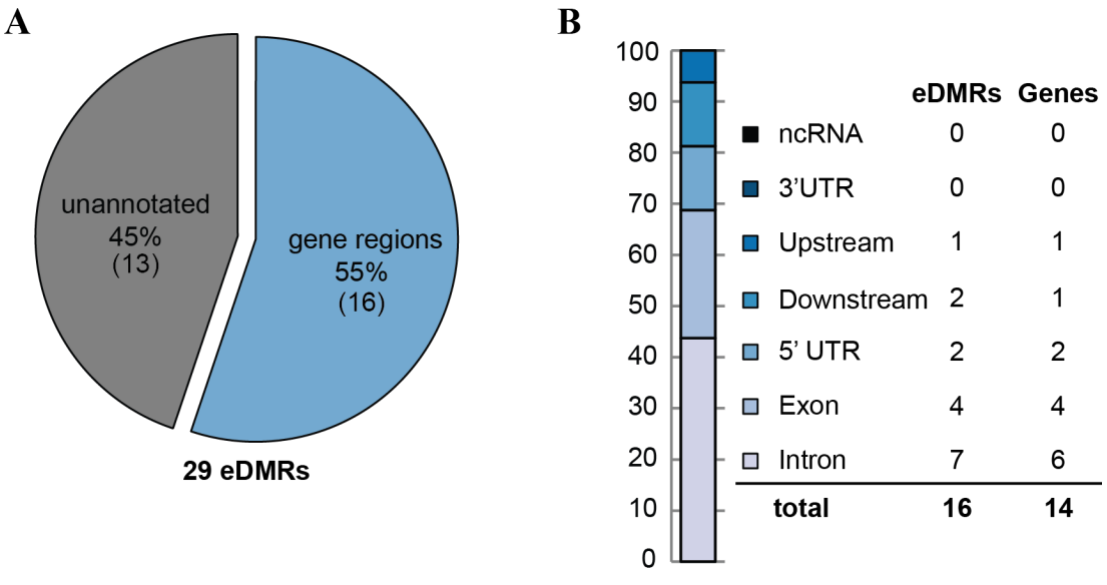



**Figure 21. Differently methylated regions (eDMR) defined by methylKit between pre-hypertensive SHR and WKY in the brain at four weeks of age.**

**A** Broad annotation of eDMR data. **B** Annotation of eDMRs that can be linked to genes.

I next manually determined empirical DMRs as described before with the other two tissues in order to overcome the short comings of the eDMR subprogram. Using previously described parameters I was able to identify an additional two genes with DMRs in their promoter regions (Table 4-4).

**Table 4-4. Differentially methylated promoter regions between SHR and WKY at a pre-hypertensive four weeks of age in the brain.**

| | Gene | DMCs | Methylation difference (SHR vs WKY) [a] |
|---|---|---|---|
| Both eDMR and empirically identified DMRs | *Rnf2* | 8 | -41.1 |
| | *Tomm20* | 11 | -42.0 |
| | *Zdhhc7* | 3 | -31.5 |
| Empirically identified DMRs only | *Arhgap11a* | 2 | 38.3 |
| | *Insl6* | 2 | -30.1 |

[a] positive numbers represent hypermethylation in SHR vs WKY
negative numbers represent hypomethylation in SHR vs WKY

### 4.2.3 Identification of overlaps between the tissues of all three germ layers

#### DMCs in common between all three germ layers

In order to determine potential germline epimutations I next investigated the union of all three tissues representing the three germ layers. Any methylation patterns in common between all three germ layers and different between the two strains could represent germline epimutations, and thus hold the potential to be causally linked to the hypertensive phenotype in the SHR.

I investigated DMCs in common between the three tissues, and the results are presented in Figure 22.

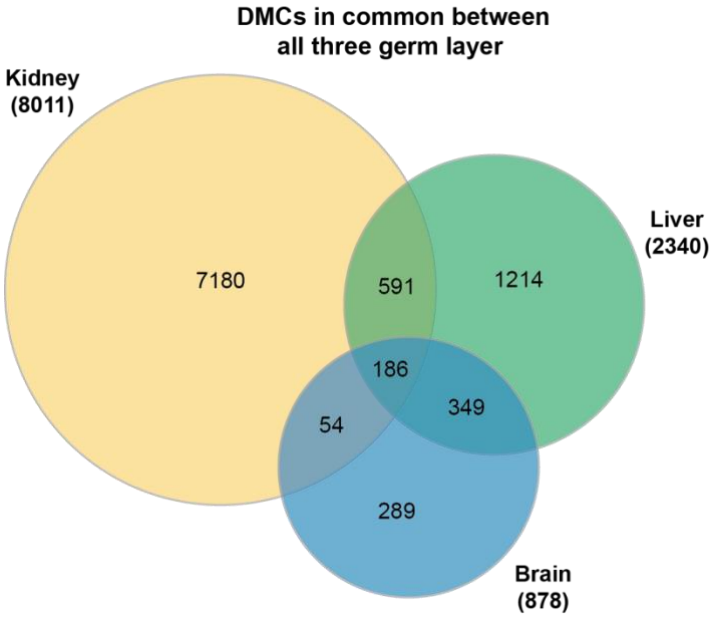



**Figure 22. Venn diagram representing the union of DMCs in common between all three germ layers at pre-hypertensive four weeks of age.**

A subset of 186 DMCs were found to be in common between all three tissues derived from distinct germ layers and these DMCs were able to be assigned to 36 genes. Of these 36 genes some contained just a single DMC, present in each tissue and so were excluded from further analysis as they were unlikely to regulate gene expression, particularly those which were also located outside of promoter regions. Other genes which did harbor more than one DMC but showed inconsistent methylation between the DMCs with both hyper- and hypomethylation identified were also excluded from further analysis. This left 18 genes which contained multiple DMCs as well as a consistent hyper- or hypomethylation between all neighboring DMCs (Table 4-5).

**Table 4-5. Genes harboring DMCs identified to be in common between all three germ layers at pre-hypertensive four weeks of age.**

| Gene | Genic region | Kidney | | Liver | | Brain | |
|---|---|---|---|---|---|---|---|
| | | DMCs | Methylation difference SHR vs WKY[a] (%) | DMCs | Methylation difference SHR vs WKY[a] (%) | DMCs | Methylation difference SHR vs WKY[a] (%) |
| ***Arhgap11a*** | 5'UTR | 11 | 35.43 | 6 | 40.07 | 2 | 38.3 |
| ***Bmp7*** | Intron | 2 | -46.48 | 2 | -70.63 | 1 | -64.16 |
| ***Epb41l4a*** | Intron | 3 | -84.98 | 1 | -40.9 | 1 | -71.72 |
| ***Kcnk2*** | Intron | 2 | 61.49 | 1 | 100 | 1 | 98.9 |
| ***Krt80*** | Exon | 7 | -38.81 | 5 | -31.38 | 4 | -36.78 |
| ***LOC310926*** | downstream protein coding/ intron | 33 | -35.85 | 118 | -30 | 84 | -31.59 |
| ***Maml3*** | Intron | 5 | 68.57 | 1 | 86.61 | 1 | 79.72 |
| ***Npr2*** | Intron | 2 | 59.26 | 1 | 83.04 | 1 | 91.56 |
| ***Parp14*** | Exon | 2 | 58.13 | 1 | 98.18 | 1 | 94.44 |
| ***Pcdhga10*** | Exon | 2 | -44.58 | 1 | -48.69 | 1 | -75.76 |
| ***Ppp1r14b*** | Exon | 5 | -22.03 | 4 | -27.68 | 7 | -24.56 |
| ***Sept11*** | Intron | 3 | 28.83 | 1 | 79.52 | 1 | 25.69 |
| ***Sgk2*** | Intron | 3 | 69.18 | 1 | 78.06 | 1 | 74.35 |
| ***Slco3a1*** | Intron | 2 | 87.81 | 1 | 73.59 | 1 | 67.54 |
| ***Snx24*** | Intron | 2 | 54.05 | 2 | 71.65 | 2 | 67.42 |
| ***Tomm20*** | 5'UTR | 17 | -29.81 | 10 | -42.63 | 11 | -42.03 |
| ***Ubb*** | Exon | 4 | 28.92 | 6 | 28.41 | 4 | 29.46 |
| ***Unc5cl*** | Intron | 2 | 71.23 | 1 | 74.06 | 1 | 54.38 |

[a] positive numbers represent hypermethylation in SHR vs WKY

negative numbers represent hypomethylation in SHR vs WKY

Both *Arhgap11a* and *Tomm20* are of particularly interest as the DMCs from both these genes were located in the 5'UTR. Since DMRs that regulate gene expression are most likely to be

found in the 5'UTR the identified DMCs associated with these two genes are good candidates for also regulating their associated gene's expression. A third gene of interest is *LOC310926*. Despite the DMCs being located downstream of the protein coding region, the number of DMCs found in each of the tissues is noteworthy. Given that *LOC310926* is at present only a hypothetical protein coding region, future investigations of this region will further illuminate the potential of this differentially methylated region found between SHR and WKY.

As previously mentioned DMCs located in intergenic regions cannot currently be further interrogated. Nevertheless, these DMCs may still affect gene regulation if for example they were located in enhancers, suppressor regions or in regions for unannotated long noncoding RNAs. The current state of the rat genome's annotation prevents further analysis at this time. However, if methylation differences are present in the tissues derived from all three germ layers, this could indicate that the DMC/DMR affects a functional genetic element. In order to take all DMCs into consideration and the lack of annotation, we decided to generate differentially methylated regions informed by our data sets of the three tissues. This was achieved by identifying differently methylated regions where the distance between two consecutive DMCs was constrained to 100bp or less. These regions were then conflated if they overlapped. Furthermore, DMCs had to be consistent within their methylation state. A change from hypermethylated to hypomethylated (and vice versa) terminated the defined region.

By this method 1,620 regions of differential methylation were identified between all three tissues datasets. I subsequently analyzed the union between all three germ layer and found 52 of those regions were in common between all three germ layers (Figure 23).

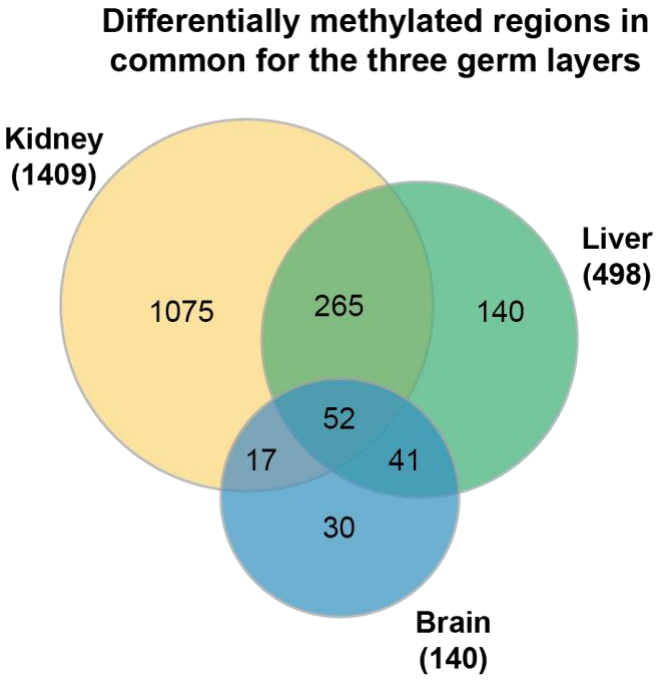



**Figure 23. Venn diagram depicting the union of differentially methylated regions in common between all three germ layers at pre-hypertensive four weeks of age.**

I once again decided to exclude DMRs that harbored a single DMC. This resulted in 39 regions which are represented in Table 4-6.

**Table 4-6.** Regions containing DMRs in common between all three germ layers at pre-hypertensive four weeks of age.

| Chromosomal Region | Gene | Genic region | Kidney | | Liver | | Brain | |
|---|---|---|---|---|---|---|---|---|
| | | | DMCs | Methylation difference SHR vs WKY[a] (%) | DMCs | Methylation difference SHR vs WKY[a] (%) | DMCs | Methylation difference SHR vs WKY[a] (%) |
| Chr1: 13499473 - 13499553 | | | 6 | -34.7 | 15 | -32.5 | 17 | -30.8 |
| Chr1: 13499858 - 13499956 | | | 18 | -34.0 | 39 | -39.1 | 39 | -40.1 |
| Chr1: 13500338 - 13500366 | | | 3 | -24.8 | 5 | -30.4 | 5 | -29.0 |
| Chr1: 13501085 - 13501181 | ***LOC310926*** | downstream protein coding | 16 | -47.2 | 23 | -36.7 | 16 | -42.5 |
| Chr1: 13502915 - 13502955 | ***LOC310926*** | intron | 3 | -42.6 | 3 | -42.6 | 3 | -42.3 |
| Chr1: 13503550 - 13503786 | ***LOC310926*** | intron | 7 | -21.3 | 28 | -24.0 | 21 | -21.4 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Chr1: 13504610 - 13504671 | ***LOC310926*** | intron | 2 | -23.1 | 6 | -24.3 | 1 | -27.6 |
| Chr1: 13509618 - 13509683 | | | 1 | -24.0 | 5 | -24.9 | 3 | -23.2 |
| Chr1: 13560973 - 13561004 | | | 1 | -22.0 | 2 | -25.4 | 1 | -22.1 |
| Chr1: 229220716 - 229220875 | ***Ppp1r14b*** | exon | 4 | -22.4 | 4 | -27.7 | 6 | -25.2 |
| Chr1: 285845445 - 285845446 | | | 2 | 77.0 | 1 | 71.7 | 1 | 22.0 |
| Chr2: 118405 - 118431 | | | 3 | -24.8 | 3 | -21.9 | 3 | -25.5 |
| Chr2: 209185569 - 209185601 | | | 2 | -31.2 | 1 | -28.7 | 2 | -24.8 |
| Chr2 - 226680926 - 226680927 | | | 1 | 56.0 | 2 | 62.0 | 1 | 83.0 |
| Chr2: 228608567 - 228608579 | | | 2 | -34.9 | 2 | -37.1 | 2 | -36.2 |
| Chr3: 111874779 - 111874821 | ***Arhgap11a*** | 5'UTR | 2 | 22.1 | 2 | 37.5 | 2 | 38.3 |
| Chr3: 174376308 - 174376536 | | | 10 | -54.6 | 6 | -36.1 | 2 | -39.0 |
| Chr4: 208475541 - 208475568 | ***Tac1*** | intron | 13 | -32.6 | 1 | -22.3 | 2 | -28.9 |
| Chr5: 168565261 - 168565308 | | | 3 | -33.4 | 3 | -31.3 | 1 | -56.4 |
| Chr6: 40316536 - 40316537 | | | 2 | -79.2 | 1 | -66.8 | 2 | -54.6 |
| Chr6: 138093579 - 138093653 | | | 6 | -37.4 | 19 | -31.8 | 20 | -29.8 |
| Chr7: 140797816 - 140797875 | ***Krt80*** | exon | 3 | -52.4 | 4 | -33.4 | 4 | -36.8 |
| Chr8: 80039509 - 80039543 | | | 2 | 23.6 | 1 | 46.5 | 1 | 32.7 |
| Chr9: 59789232 - 59789233 | | | 1 | -65.1 | 2 | -70.9 | 1 | -82.5 |
| Chr10: 1036724 - 1036725 | | | 1 | 84.9 | 1 | 83.4 | 2 | 90.5 |
| Chr10: 48350436 - 48350531 | | | 3 | 29.2 | 1 | 24.7 | 1 | 22.1 |
| Chr10: 48664598 - 48664716 | ***Ubb*** | exon | 4 | 28.9 | 6 | 28.4 | 4 | 29.5 |
| Chr12: 328465 - 328513 | | | 2 | -20.8 | 4 | -20.9 | 2 | -20.9 |
| Chr12: 345905 - 346084 | | | 6 | -27.4 | 5 | -25.1 | 2 | -27.3 |
| Chr14: 46811246 - 46811312 | | | 2 | 56.1 | 2 | 46.2 | 2 | 50.1 |
| Chr14: 46811440 - 46811503 | | | 1 | -21.7 | 4 | -26.4 | 3 | -23.6 |
| Chr14: 107175874 - 107175908 | | | 5 | -47.7 | 6 | -43.2 | 6 | -39.6 |
| Chr15: 107519073 - 107519182 | ***Cldn10*** | intron | 1 | 24.9 | 3 | 30.6 | 2 | 29.3 |
| Chr19: 63498814 - | | | 4 | -32.5 | 1 | -37.4 | 1 | -43.9 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 63498913 | | | | | | | | |
| Chr19: 70540282 - 70540370 | ***Tomm20*** | 5'UTR | 17 | -29.8 | 10 | -42.6 | 11 | -42.0 |
| Chr20: 8747187 - 8747188 | | | 1 | -24.2 | 2 | -26.8 | 2 | -26.0 |
| Chr20: 42611991 - 42612010 | | | 1 | -89.1 | 2 | -89.6 | 1 | -93.0 |
| ChrX: 114905293 - 114905340 | ***Ccdc65*** | exon | 1 | -27.1 | 3 | -21.5 | 1 | -25.2 |
| ChrUn_JH620584: 12655 - 12669 | | | 1 | -27.1 | 1 | -21.3 | 3 | -23.9 |

[a] positive numbers represent hypermethylation values in SHR
negative numbers represent hypomethylation values in SHR

These regions may contain novel areas of differential gene regulation which could be revealed in the future as the rat genome's annotation advances. At the same time, unsurprisingly but nevertheless noteworthy, I found *Arhgap11a*, *Tomm20*, and *LOC310926* in the regions as three of only nine annotated genes.

## DMRs in common between all three germ layers

Finally, I investigated the union of the DMRs identified by methylKit, which revealed four genes in common between all three germ layers: *Krt80*, *LOC310926*, *Tomm20* and *Ubb* (Figure 24A). Of these four only *Tomm20* harbored the DMR in the promoter region.

Further investigation of DMRs that I had empirically defined and limited to the promoter regions (Figure 24B), once again identified both *Arhgap11a* and *Tomm20*.

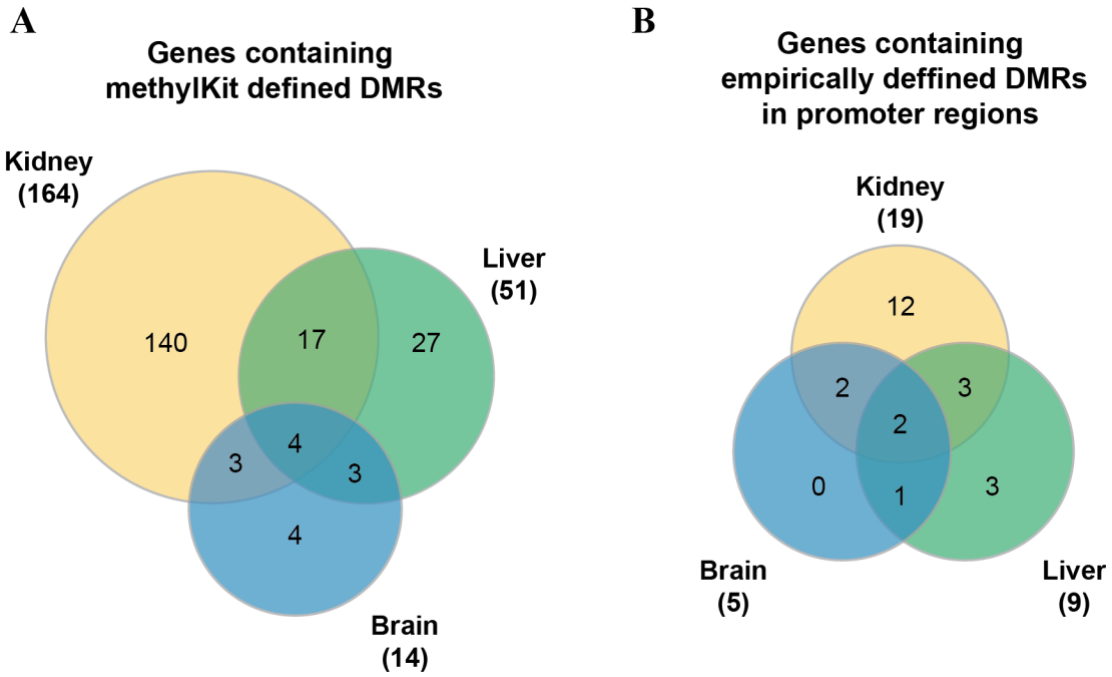
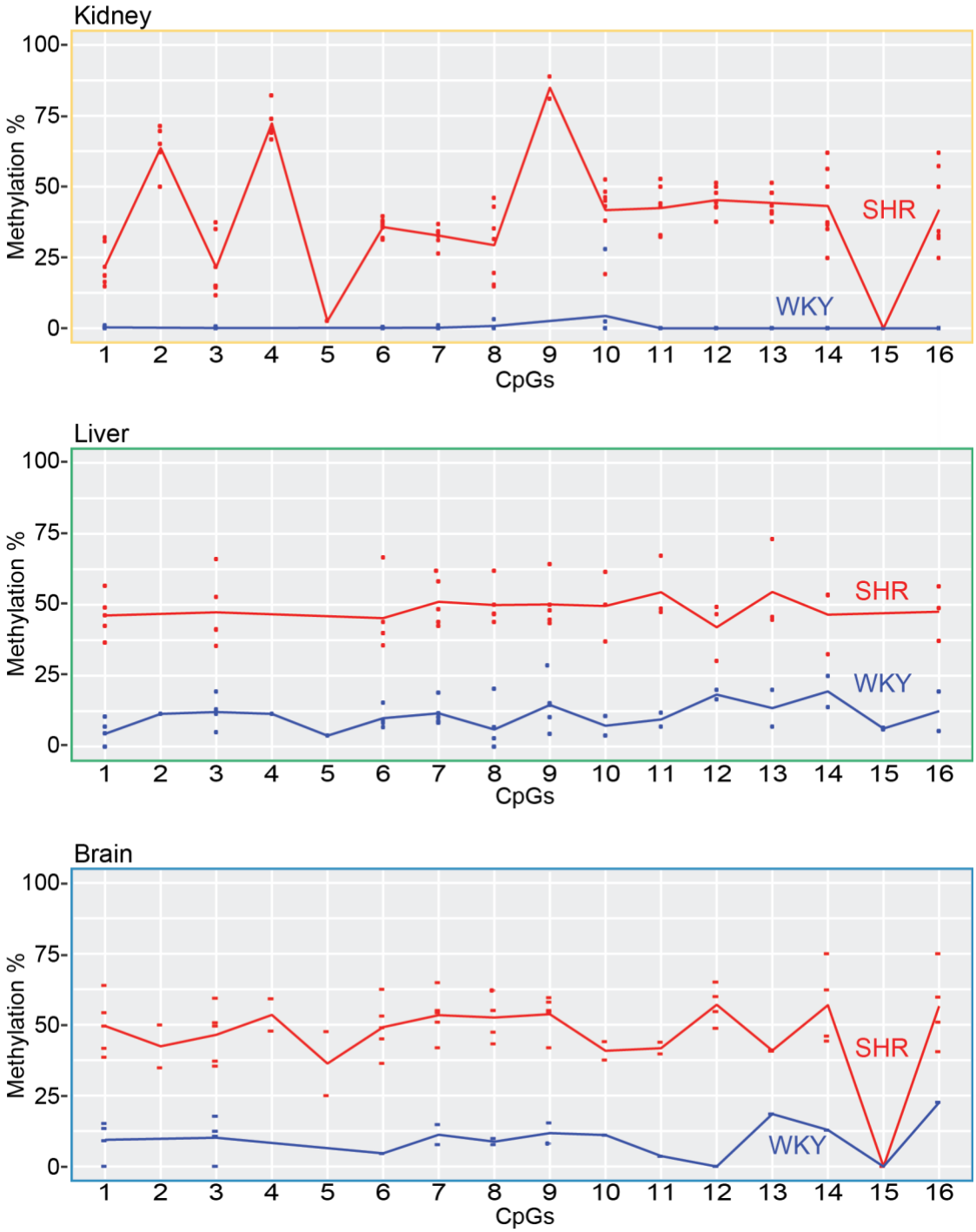



**Figure 24. Overlay of genes containing DMRs (A) and empirically identified genes containing DMRs in the promoter region (B) for all three germ layers at pre-hypertensive four weeks.**

Both *Arhgap11a* and *Tomm20* emerged as strong candidates from my analysis and as such I then investigated the actual methylation patterns for all three germ layers (Figure 25). The gene *Arhgap11a* was found to be hypermethylated in the SHR whilst *Tomm20* was hypomethylated in the SHR in comparison to the WKY.

**A**

***Arhgap11a*** (Chr.3: 111,874,779 - 111,875,130)



**B**

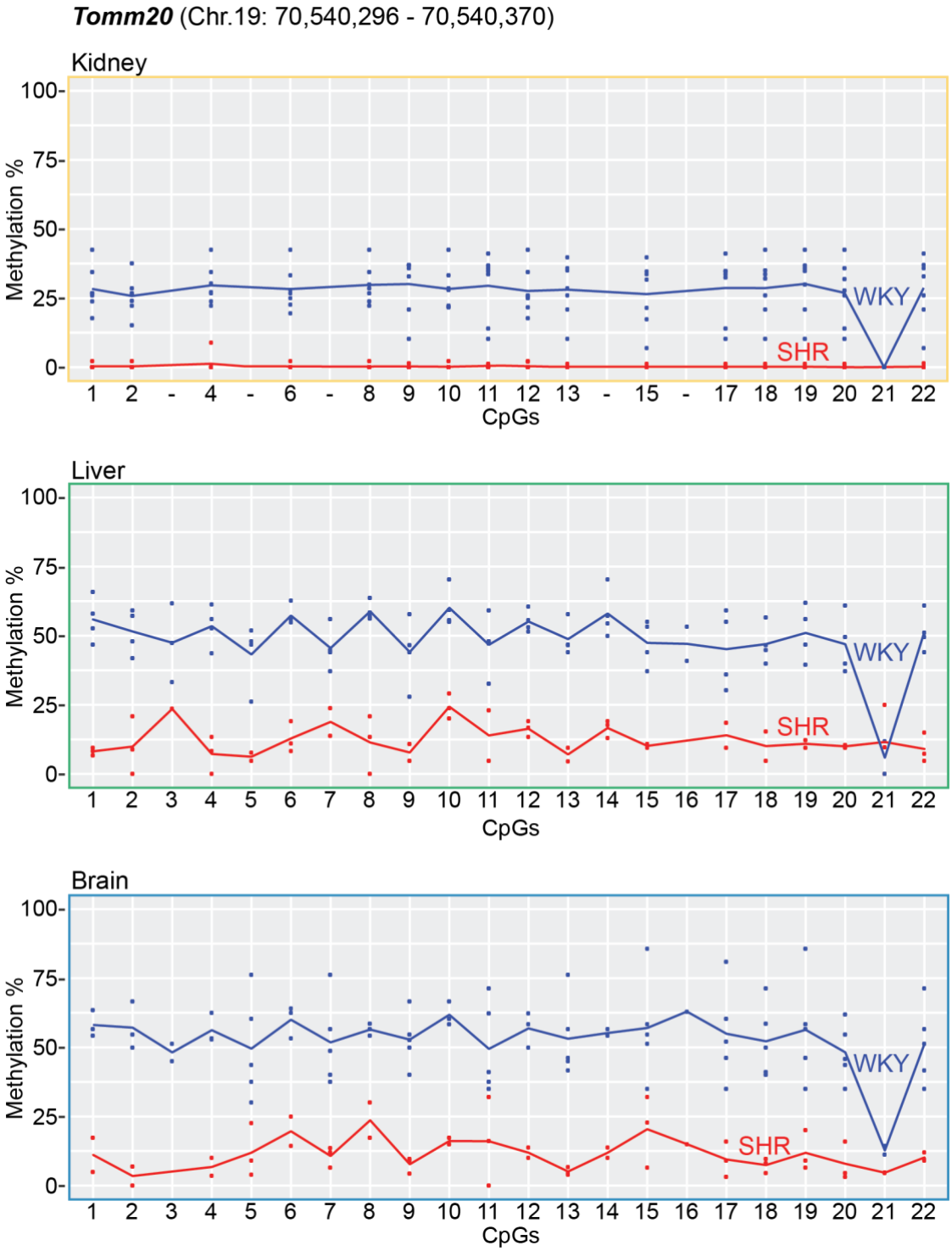



**Figure 25. RRBS 'Methylation fingerprint' for the genes Arhgap11a (A) and Tomm20 (B).**

Lines represent the average methylation of the CpGs in the region, while dots show the values for individual animals of the SHR (red) and WKY (blue) analysed by RRBS.

## 4.3 Discussion

In this chapter I investigated methylation patterns between SHR and WKY in the liver and brain of the same animals whose kidney had been assessed prior, in order to explore whether any differences in cytosine methylation observed in Chapter 3 could potentially be germline epimutations. Germline epimutations are epigenetic lesions that occur very early in the development of an organism: they may be inherited via the gametes or occur stochastically in the very early embryo. Given their early developmental origins, germline epimutations are persistent throughout all three germ layers. Differences in methylation between SHR and WKY kidney, representing the mesoderm, were considered alongside those of the liver and brain, representing the endo- and ectoderm respectively. Any commonalities in methylation difference between SHR and WKY in these tissues representing all three germ layers suggest that these differences may be representative of a germline epigenetic change that could be involved in blood pressure homeostasis.

The approach of performing RRBS to analyse methylation differences on a genome scale is not without limitations (as discussed later) however, the differences that were in common between all three tissues were noteworthy. Of particular interest are those differences across regions (DMRs) of potential regulatory function. Many such DMRs occurred in intergenic regions and as such were difficult to interpret given the current state of annotation for the rat genome. The lack of knowledge regarding potential sites of histone modification, as well as enhancers, silencers or transcription sites of non-coding RNA, made it particularly challenging to draw conclusions for many of the intergenic areas that showed a distinctive difference in methylation between SHR and WKY. Such regions will be of interest in the future when the annotation of the rat genome has progressed further, but nevertheless, some of the regions that I was able to identify were associated with annotated genes and thus hold the potential to regulate expression of those genes. The strongest candidates for germline epimutation resulting from the analyses performed in this chapter were: *LOC310926*, *Arhgap11a*, and *Tomm20*.

Little is known about *LOC310926*. In the latest rat genome build it is annotated as a

hypothetical protein coding gene, with no known or putative conserved domains. While the differences in methylation at *LOC310926* between SHR and WKY are compelling, this candidate germline epimutation is not a top candidate for follow up studies in hypertension. This is because in addition to there being very little known about *LOC310926*, it seems to be poorly conserved with no obvious orthologue in humans, or mice.

*Arhgap11a* is a member of the Rho GTPase activating protein family that has previously been implicated as an oncogene [261, 262]. Whilst not much is known about Arghap11a function specifically, it is known that Rho GTPase activating proteins are one of the major regulators of Rho GTPases, and crucial in cell cytoskeletal organization, growth, differentiation, neuronal development and synaptic functions [263]. This wide range of biological pathways involving Rho GTPase activating proteins makes it possible that a change in *Arhgap11a* gene expression could be influential for one or maybe even both phenotypes associated with the SHR strain: hypertension and ADHD-like behaviour.

The hypermethylation of *Arhgap11a* may be particularly relevant for hypertension: Rho proteins influence on contractibility of vascular smooth muscle which has long been suggested to play a key role in hypertension [264]. That the *Arhgap11a* promoter carried on average ~50% methylation, while the WKY had little to none, suggests that only one allele is affected. In terms of expression this would cause haploinsufficiency for Arhgap11a protein in tissues in which it is expressed. In this regard it is interesting to note that in humans a chromosomal deletion or hypermethylation of the paternal allele of chromosome 15, including *Arhgap11a*, underlies some cases of Prader-Willi syndrome. There are no specific gene knockout models for *Arhgap11a* and it remains to be investigated what the loss of *Arhgap11a* would mean for an individual.

Marginally more is known about *Tomm20*. It is often referred to as the central component of the receptor complex responsible for the recognition and translocation of pre-proteins synthesized in the cytosol into mitochondria [265]. While the composition of the protein import complex of mitochondria is quite well known and highly conserved from fungi and yeast up to mammals

[266], functionality of the machinery in mammalian mitochondria remains less well understood [266, 267]. *Tomm20* knock-down as well as overexpression are thought to alter the morphology of mitochondria [267] . This may have an adverse effect on the well described crucial role of mitochondria in the synthesis of ATP, the energy currency of the body.

Research has shown that mitochondrial dysfunction contributes to the organ damage caused by hypertension [268, 269]. Oxidative stress within mitochondria can also be linked directly to hypertension itself [270, 271]. These findings raise the possibility that altered expression of *Tomm20* may not only influence the end-organ effects of hypertension, but also be one of its causes.

If the cytosine methylation differences that were identified between the SHR and WKY in this work, give rise to an epimutation of *Tomm20*, which potentially changes the expression levels of the gene, then these changes could affect any tissue and subsequently influence blood pressure regulation. An example for the different methylation of *Tomm20* to have a direct effect on health was performed in the work by Toro-Martin *et al.*[272]. In this study, severely obese subjects presenting with a certain methylation quantitative trait loci of *Tomm20*, also showed an associated elevation in metabolic syndrome-related complications. Since hypertension can be highly impacted by weight and metabolism, this could be an indirect impactor on blood pressure homeostasis. At this point, however, the direct impact of *Tomm20* on hypertension is only speculative.

RRBS has proven to be an effective technique for studying cytosine methylation patterns across many different cell types and species [153], but it does have limitations that are relevant to this study. RRBS captures only a fraction of the genome – on average 1% - albeit enriched for regions of interest such as CpG islands. But a major disadvantage is that there is some inconsistency between what CpG rich regions of the genome are captured at the recommended sequencing coverage. This variability is most likely due to technical variation in the library

preparation process, in particular the efficiency of enzyme digestion and the size-selection process.

This disadvantage of RRBS is very relevant to the results of this chapter. While the kidney data had acceptable coverage of the genome, the liver and brain RRBS data were less extensive and so did not capture every region that was captured in the kidney analysis. The regions covered in only one or two tissues (and thus non-informative for germline epimutation candidacy where information from all three tissues is required) means that there may be candidate germline epimutations that were missed by my approach. For example, only 52 loci overlapped in all three tissues but many (323 loci) had overlap in two of the three tissues. While this risk of false negative results will underestimate the potential occurrence of germline epimutations in the SHR, it also drastically reduces the risk of false positive findings. In other words, the findings of methylation differences meeting the threshold of >20% within at least three animals/group at a coverage of >20X is highly unlikely to occur in all three tissues by chance.

So notwithstanding the limitations of RRBS, the studies performed here suggest some of the first targets for future research in genes that might cause the hypertension phenotype. Epigenome-wide studies (EWAS) of any complex human disease are in their infancy, and at the time of writing this thesis the first EWAS of hypertension in humans was published [273]. This study identified a modest number of genes ($n$=6) with correlations between methylation, gene expression and blood pressure. While *LOC310926*, *Arhgap11a*, and *Tomm20* were not listed among these genes, it is worth noting that the approach used by the study was array based, and only captured single CpG sites in blood. More comprehensive profiling of cytosine methylation in humans (such as with RRBS or WGBS) will be required to understand the full extent of association of methylation variants with hypertension.

A final factor that needs to be addressed regarding the findings of this Chapter is the impact that genetic differences may have on methylation. Despite the fact that the WKY is the most commonly used control for the SHR when it comes to hypertension, it is undeniable that past

research has shown that there are significant genetic differences between the two strains. It is therefore possible that when utilizing these two rat strains for this research, said genetic differences of the strains could have been one of the reasons for the methylation differences found in both this and the prior Chapter.

In order to eliminate the confounding effects of potential genetic differences from the analysis of methylation, in the next Chapter I exploited the SHR response to early and transient exposure to Captopril. In this experiment all animals studied were offspring of the same SHR colony and thus with identical genetic background, largely eliminating potential genetic effects.

# Chapter 5. Epigenetic differences in the four week old kidney of SHR transiently exposed to the medication Captopril and unexposed SHR

## 5.1 Introduction

In the previous two chapters I identified many differences in methylation patterns between the SHR and WKY; these included differences across multiple tissues which made them candidate germline epimutations. However, given that SHR and WKY appear to have also a large number of genetic differences [178] it is difficult to assign hypertensive causality to any of these epigenetic lesions. In order to illuminate whether any of the differences in phenotype can be identified despite these genetic differences, I next took advantage of some rather intriguing studies of the SHR, described below, which made it possible to eliminate any genetic contribution to the epigenetic changes.

Several studies using the SHR have found that transient exposure to the angiotensin-converting enzyme (ACE) inhibitor drug group can have a long-lasting effect, diminishing the hypertensive phenotype. This is particularly pronounced when the animals are exposed during a crucial time window during their pre-hypertensive phase (before 10 weeks of age), bestowing long-lasting protection from the full hypertensive phenotype well into adulthood [193-196].

Of particular relevance to my investigations was the work performed by Wu and Berecek in 1993 [197], using the first ever developed ACE inhibitor, Captopril [198]. In this study pregnant females were treated with the drug from time of mating, throughout pregnancy and lactation, and their offspring were kept on the medication until eight weeks of age when Captopril exposure was ceased. Wu and Berecek observed that F1 animals exposed to Captopril from gestation until eight weeks of age presented with significantly lower mean arterial blood pressure (MAP), even up to 27 weeks after the medication treatment had been withdrawn. More surprisingly, they further demonstrated that ‘unexposed’ CAP-F2 offspring of the Captopril

exposed animals, also had significantly lower MAP compared to untreated, age matched SHR. In addition, Captopril exposed SHR and their offspring both exhibited lower levels of plasma angiotensin II (ANGII) and less signs of hypertrophy [197].

This heritable response to Captopril from one generation to the next, entirely changing a phenotype, is very unlikely to be due to a genetic change. It however aligns with the findings of environmental stimuli altering epigenetic markers and thereby gene expression. In other words, if hypertension had a purely genetic basis then one would assume that BP would elevate as dictated by the aberrant genes after the ACE inhibitor gets withdrawn. However, Captopril appears to induce changes in either the fetal and or early stages of development that gene expression is altered into a persisted state of hypertension relief.
If it were possible to identify changes in cytosine methylation in a RRBS analysis of Captopril treated SHR kidneys compared to untreated SHR, it would strongly suggest an epigenetic component of hypertension.

In this chapter of my thesis I recapitulated the experiment of Wu and Berecek in order to explore if methylation changes have been induced through the transient exposure to the ACE inhibitor Captopril that then lead to the rescue of the exposed SHR animals from their hypertensive fate. I analyzed cytosine methylation patterns in untreated SHR, SHR animals exposed to Captopril (SHR-CAP) and their offspring (CAP-F2) that had never been directly exposed to the drug. Again, I used RRBS to interrogate the kidney in four week old animals (i.e. at the usually pre-hypertensive stage), due to the key role accredited to the kidney when it comes to controlling blood pressure.

As such my specific aims for this chapter were to:

1. Generate SHR-CAP animals that had been exposed to Captopril from conception to 8 weeks of age
2. Generate a CAP-F2 generation of animals from SHR-CAP animals (after medication was ceased in SHR-CAP).

3. Produce RRBS maps of cytosine methylation from the kidneys of four week old pre-hypertensive male SHR-CAP as well as age-matched unmedicated CAP-F2 and SHR animals.
4. Comparison between methylation patterns of the three different groups at the level of individual CpG sites, as well as regions.
5. Comparison of the DMCs from the comparison of SHR and WKY with DMCs from the SHR and the SHR-CAP.
6. Correlate any observed differences in methylation between the SHR and the SHR-CAP with disparity in gene expression.

## 5.2 Results

### 5.2.1 Experimental design

To generate SHR-CAP animals for this arm of the research, male and female SHR from different litters were paired for mating at eight weeks of age (Figure 26). By selection of the parents from different litters I ensured that no crossing was duplicated to exclude potential accumulation of epigenetic bias. Breeding pairs were exposed to 1.84mmol/l Captopril dissolved in their drinking water. The Captopril solution was made fresh and replenished every 2-3 days. Mating pairs were allowed to breed until 30 weeks of age or until three litters had been produced. Dams were maintained on Captopril through lactation, and SHR-CAP pups weaned at four weeks of age and maintained Captopril until eight weeks of age.

Captopril has been reported to have a teratogen effect in other species [274, 275], although not confirmed in the rat, as such the number of SHR-CAP litters was kept at a minimum and two males per litter were chosen for blood pressure measurements at four weeks and then immediately sacrificed for tissue collection. Another two males from the litter were kept on the Captopril regime for long term blood pressure assessment: They were then withdrawn from the medication at eight weeks with their blood pressure was measured and blood was drawn from their tail veins at 10, 16 and 35 weeks of age. At the final time point of 35 week they were sacrificed and their tissues collected.

CAP-F2 were generated from SHR-CAP males and females from separate litters; mating commenced from 10 weeks of age (i.e. two weeks after withdrawal from Captopril). One male per litter was used for the four week blood pressure measurement and subsequent sacrifice for tissue collection. One additional male per litter followed the above long term assessments at 10, 16 and 35 weeks before being sacrificed for tissue collection as well. Animals were weighed weekly for the duration of the experiment.

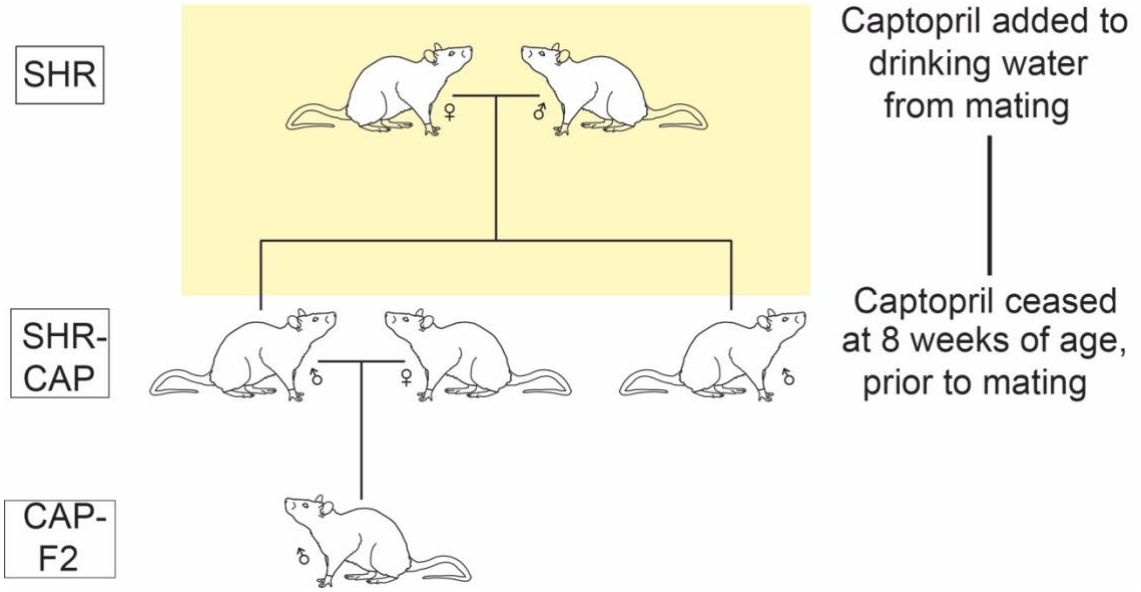



**Figure 26. Experimental schematic for breeding of SHR-CAP and CAP-F2.**

SHRs from separate, but related, pedigrees were mated and exposed to Captopril in their drinking water from the day of pairing. Their offspring formed the SHR-CAP and were exposed to the medication up to eight weeks of age. SHR-CAP were then mated 10 weeks post cessation of medication to generate the CAP-F2. Four males from each litter of SHR-CAP and two of CAP-F2 were selected for a four week blood pressure measurement. Two SHR-CAP and one CAP-F2 animals were subsequently culled for tissue collection and the remaining animals subjected to a blood pressure and blood draw time course at 10, 16 and 35 weeks. At 35 weeks tissues of the second male were also collected.

### 5.2.2 Physiological results in the SHR-CAP

#### Blood pressure and heart weight over the life course of SHR-CAP

In order to address the effects of early life exposure to Captopril in the SHR, blood pressure and signs of hypertrophy were assessed. SHR-CAP animals displayed significantly lower blood pressure throughout their lives compared to untreated SHR (Figure 27A). It is clear upon observation of Figure 27A that SHR-CAP animals have significantly lower blood pressure than untreated SHR, however they are still not normotensive like the WKY at all stages of their lives measured. The WKY data here was from Chapter 3 and only shown as a reference to what is considered normotensive.

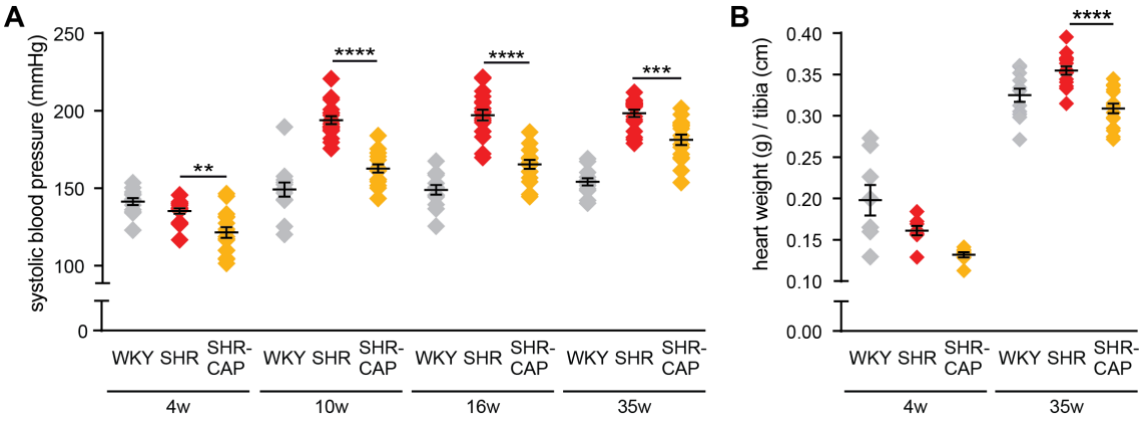



**Figure 27. Animals that were exposed to Captopril early in life display lower systolic blood pressure, even after cessation from the drug, as well as reduced signs of cardiac hypertrophy later in life.**

SHR-CAP were exposed to Captopril from gestation up to 8 weeks of age. **A** Systolic blood pressure in mmHg of WKY (grey), untreated SHR (red) and SHR-CAP (yellow) across the life course (WKY *n* = 13, SHR *n* = 18, SHR-CAP *n* = 16). **B** Heart weight to tibia length ratio for WKY (grey), SHR (red) and SHR-CAP (yellow) at pre-hypertensive (4 weeks; WKY, SHR and SHR-CAP *n* = 8) and hypertensive state (35 weeks; WKY *n* = 12, SHR *n* = 15, SHR-CAP *n* = 16) in life (Error bars indicate mean ± SEM; one-way ANOVA between SHR and SHR-CAP $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p < 0.0001$(****); WKY only serves as reference & therefore statistics are not shown).

At four weeks of age, when SHR are pre-hypertensive, the untreated animals showed a systolic blood pressure of 135.3 ± 1.76 mmHg (mean ± SEM) whereas SHR-CAP displayed significantly lower blood pressure of 121.5 ± 3.45 mmHg (SHR *n* = 18; SHR-CAP *n* = 16; $p \leq 0.01$). This effect is likely due to the continued presence of Captopril in the drinking water at this time point.

At 10 weeks, when SHR are developing hypertension, blood pressure was 193.9 ± 2.58 mmHg for the untreated group; SHR-CAP had significantly lower blood pressure of 162.7 ± 2.69 mmHg ($p \leq 0.0001$). It should be noted that at this time point animals had ceased medication for two weeks.

At 16 weeks of age SHR displayed a blood pressure of 197.1 ± 3.41 mmHg while SHR-CAP maintained a significantly lower blood pressure of 165.3 ± 2.82 mmHg ($p \leq 0.0001$).

At the final blood pressure measurement, at 35 weeks of age, SHR measured a systolic blood pressure of 198.3 ± 2.33 mmHg while the SHR-CAP had risen to 181.1 ± 3.35 mmHg but was still significantly lower than the blood pressure of the untreated SHR ($p \leq 0.0001$).

Given that the transiently treated SHR-CAP consistently displayed lower blood pressure than the untreated SHR throughout their life I also compared the risk of hypertrophy between the two groups. For this measurement both heart weight and tibia length were assessed for animals sacrificed at either four or 35 weeks for tissue collection (Figure 27B). At four weeks of age there was no significant difference in the heart weight to tibia length ratio between SHR and SHR-CAP; however at 35 weeks SHR displayed a significantly higher ratio than the SHR-CAP (0.3549 ± 0.0051 vs 0.309 ± 0.0058 (mean ± SEM; one-way ANOVA $p \leq 0.001$); 4 weeks: SHR and SHR-CAP $n = 8$; 35 weeks: SHR $n = 15$, SHR-CAP $n = 16$).

Taken together these results indicate that early and transient exposure to Captopril results in sustained lowering of blood pressure in the SHR, along with a concomitant reduction in hypertension-related cardiac hypertrophy. This effect cannot be attributed to any genetic difference between the groups as the animals are genetically homogenous.

### Heart rate over the life course of SHR-CAP

It is possible that the effect of transient Captopril may be related to indirect effects on heart rate. I thus also assed heart rate throughout the lives of the SHR-CAP and compared them to the unmedicated SHR (Figure 28). SHR-CAP showed no significant difference in their heart rate over the life span compared to the untreated SHR. Clinically, Captopril has not been found to decrease the heart rate [276]; these reports in conjunction with my findings suggest that the observed change in blood pressure in SHR-CAP must be via means other than diminished heart rate.

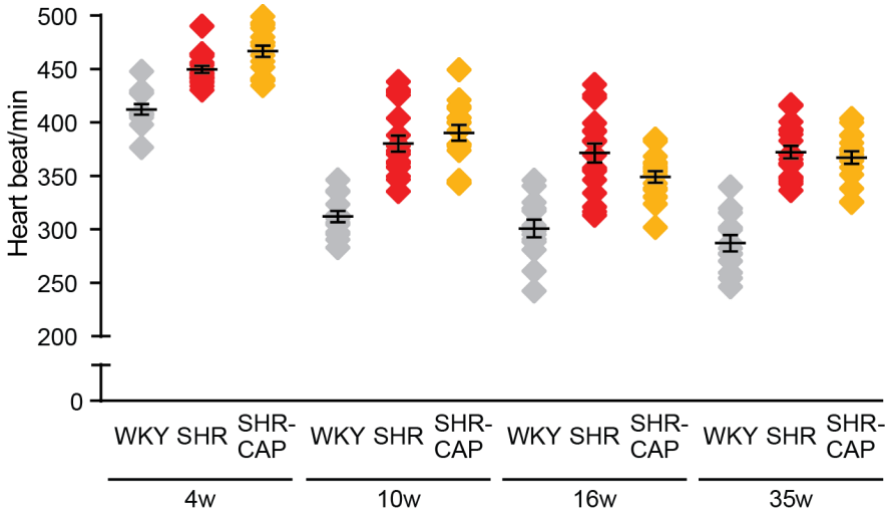



**Figure 28. Animals exposed to Captopril in early life display no change in heart rate compared to unexposed SHR controls.**

SHR-CAP were exposed to Captopril from gestation up to 8 weeks of age. The graph presents heart rates of WKY (grey), untreated SHR (red) and SHR-CAP (yellow) over the course of their life. (Error bars indicating SEM; one-way ANOVA showed no significance between SHR and SHR-CAP; WKY only serves as reference and therefore statistics are not shown; WKY $n$ = 13, SHR $n$ = 18, SHR-CAP $n$ = 16).

## Body weight over the life course of SHR-CAP

Animals were weighed on a weekly basis over their life course as a matter of course. Surprisingly, compared to the untreated SHR, SHR-CAP displayed a significantly lower mean body weight from eight weeks of life onward (Figure 29). On average SHR-CAP were ± 20 g lighter than SHR ($p \leq 0.01$). These findings were of particular interest in light of investigation into the CAP-F2 generation below.

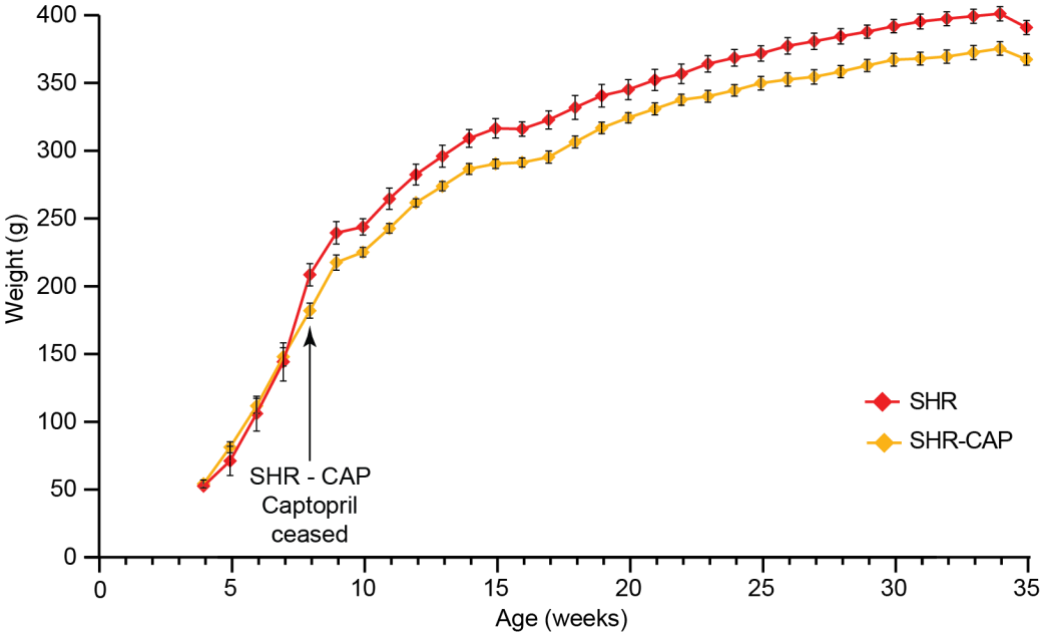



**Figure 29. Effect of Captopril exposure on bodyweight.**

Shown is a graph of body weight (y-axis) over time (x-axis) (Error bars indicate mean ± SEM; one-way repeated measure ANOVA $p \leq 0.01$;SHR $n = 13$, SHR-CAP $n = 16$).

### 5.2.3 Physiological results in CAP-F2

Wu and Berecek reported that the transient exposure to Captopril and the beneficial effects of blood pressure reduction were passed on to the following unexposed generation [197]. I set out to confirm this in order to see if this protection against the hypertension phenotype can be linked to methylation patterns in the four week old kidney.

#### Blood pressure and cardiac hypertrophy in CAP-F2

CAP-F2, conceived after SHR-CAP had been withdrawn from medication, generally displayed similar blood pressure to untreated SHR over their life course, although their development to full blown hypertension was clearly time delayed (Figure 30A). This finding was somewhat unexpected and contradicted the previous report of Wu and Berecek [197].

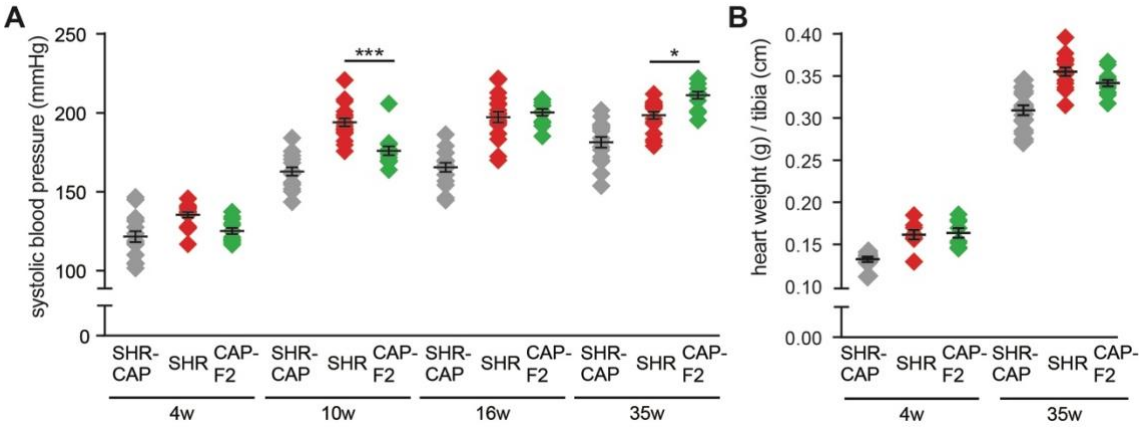



**Figure 30. Offspring of animals that had been exposed to Captopril early in life show reversion to the high blood pressure phenotype, as well as signs of cardiac hypertrophy later in life.**

**A** Systolic blood pressure in mmHg of SHR-CAP (grey), untreated SHR (red) and CAP-F2 (green) across the life course (SHR-CAP *n* = 16, SHR *n* = 18, CAP-F2 *n* = 12). **B** Heart weight to tibia length ratio for SHR-CAP (grey), untreated SHR (red) and CAP-F2 (green) at pre-hypertensive (4 weeks; SHR-CAP, SHR and CAP-F2 *n* = 8) and hypertensive state (35 weeks; SHR-CAP *n* = 16, SHR *n* = 15, CAP-F2 *n* = 12) in life (Error bars indicate mean ± SEM; one-way ANOVA between SHR and SHR-CAP $p \leq 0.05$ (*), $p \leq 0.001$ (***), $p<0.0001$(****); SHR-CAP only serve as reference and statistics therefore not shown).

At four weeks there was no significant difference between SHR and CAP-F2 with a mean systolic blood pressure of 135.3 ± 1.76 mmHg and 125 ± 1.92 mmHg respectively. At 10 weeks SHR presented with a mean blood pressure of 193.9 ± 2.58 mmHg while CAP-F2 animals still had a significantly lower mean blood pressure of 175.8 ± 2.81 mmHg. By 16 weeks untreated SHR were measured with a blood pressure mean of 197.1 ± 3.41 mmHg, matched by the blood pressure of CAP-F2, whose mean had raised to 200.1 ± 2.15 mmHg. At the final time point of measurement of 35 weeks SHR blood pressure mean of 198.3 ± 2.33 had significantly been exceeded by the mean blood pressure of CAP-F2 measuring 211 ± 2.26 mmHg.

As in the previous section, signs of cardiac hypertrophy were assessed in form of the ratio of heart weight (g) to tibia length (cm) and are depicted in Figure 30B. CAP-F2 and SHR displayed no statistically significant differences in this ratio, both at four weeks and later in life

at 35 weeks. This is consistent with the reversion of CAP-F2 to the hypertension phenotype typical of SHR.

## Heart rate over the life course of CAP-F2

CAP-F2 displayed the same mean heart rate as SHR during their life (Figure 31). This is noteworthy given that SHR-CAP had the same heart rate despite a very different hypertension phenotype.

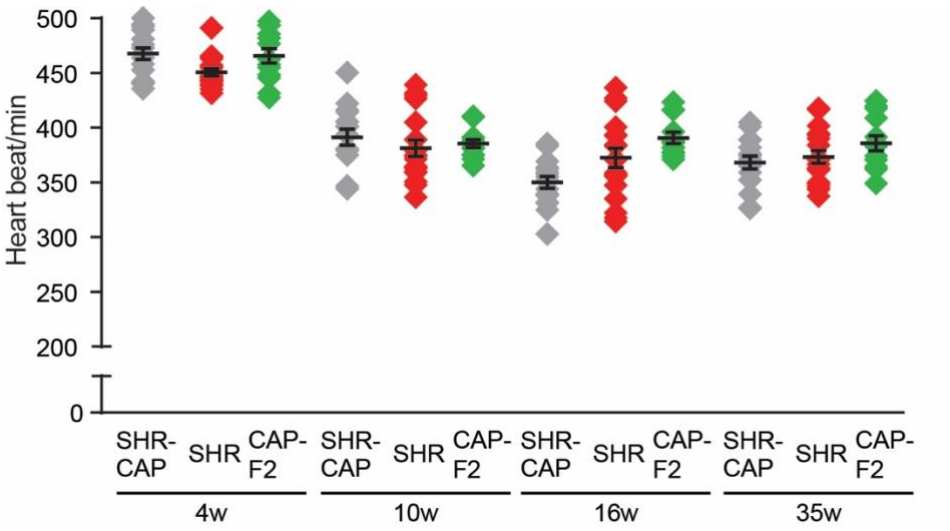



**Figure 31. Offspring of animals exposed to Captopril in early life display no change in heart rate compared to control.**

Graph showing heart rate of SHR-CAP (grey), untreated SHR (red) and SHR-CAP offspring CAP-F2 (green) (Error bars indicating SEM; one-way ANOVA showed no significance between the three groups; SHR-CAP $n$ = 16, SHR $n$ = 18, CAP-F2 $n$ = 12).

## Body weight over the life course of CAP-F2

Even though the reduced blood pressure in SHR-CAP returned to normal SHR levels in CAP-F2, surprisingly, CAP-F2, just like CAP-SHR exhibited significantly lower body weights compared to the untreated SHR (Figure 32). This implies that while the effects of transient Captopril on blood pressure are not heritable, other changes leading to a reduction in weight are able to passed on in the absence of Captopril.

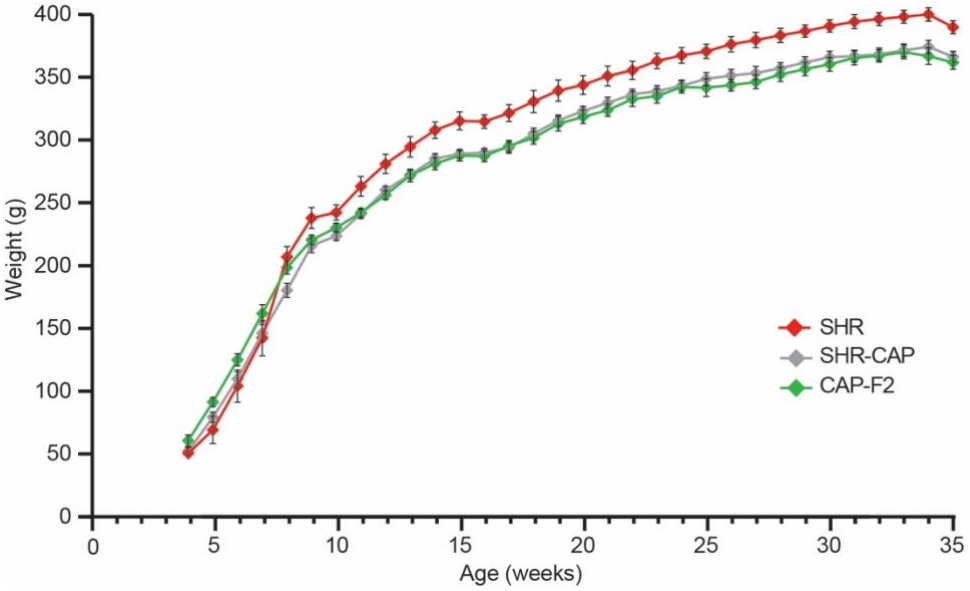



**Figure 32. Adverse effect of Captopril exposure on bodyweight is observed over two generations.** Shown is a graph of body weight (y-axis) over time (x-axis) (Error bars indicate mean ± SEM; one-way repeated measure ANOVA $p \leq 0.01$; SHR $n = 13$, CAP-F2 $n = 13$)

### 5.2.4 Cytosine methylation analyses of the SHR, SHR-CAP and CAP-F2 kidney

After the phenotypic evaluation between the different SHR cohorts had been completed, I then proceeded to analyze tissues from seven SHR-CAP as well as four CAP-F2. All these animals were four weeks old as in the previous investigations. In case of the SHR-CAP this time point meant that at time of tissue collection animals were still under the direct influence of Captopril.

As with the analysis in the other chapters only males were selected due to financial constraints and to avoid the observed sexual dimorphism in hypertension. Whole kidneys of the animals were collected and DNA extracted. Since the libraries were prepared with the Nugen Ovation RRBS kit (NuGen; Chatswood, Australia), I decided to also prepare new libraries of the SHR kidneys from Chapter 3 with this kit to eliminate any batch effects that would be otherwise expected between the in-house and commercial RRBS library preparation methods.

Once again RRBS libraries were sequenced on the NextSeq500 Illumina platform (75 bp; single-end read) at the Ramaciotti Centre for Genomics (Sydney) and mapped to the rat genome

assembly rn5. Table 5-1, below, presents sequencing summary statistics for each of the samples. Mapping efficiency was 68.9% on average, slightly better than in the analysis of the previous chapters. On average, RRBS captured 3,079,521 CpGs, which was 1.4-fold more than the successful RRBS analysis performed in Chapter 3.

**Table 5-1. RRBS sequencing summary statistics of the kidney at 4 weeks.**

| Animal ID | strain | Number of unmapped reads | Number of mapped reads | Mapping efficiency (%) | Number of CpGs unfiltered | Number of CpGs at 20x coverage |
|---|---|---|---|---|---|---|
| **1000583** | **SHR** | 31,706,783 | 21,871,897 | 69.0% | 3,104,875 | 1,385,994 |
| **1000604** | **SHR** | 31,892,479 | 22,249,551 | 69.8% | 3,030,922 | 1,347,108 |
| **1000615** | **SHR** | 32,239,415 | 20,922,093 | 64.9% | 2,925,324 | 1,394,599 |
| **1000620** | **SHR** | 31,505,219 | 21,440,045 | 68.1% | 2,991,809 | 1,375,598 |
| **1000624** | **SHR** | 31,688,103 | 20,842,740 | 65.8% | 2,846,649 | 1,174,089 |
| **1000674** | **SHR** | 29,651,527 | 19,831,168 | 66.9% | 2,921,274 | 1,415,767 |
| **1000710** | **SHR** | 29,269,019 | 20,392,590 | 69.7% | 3,016,072 | 1,251,690 |
| **1000901** | **SHR-CAP** | 30,305,170 | 20,855,756 | 68.8% | 2,992,431 | 1,360,861 |
| **1000952** | **SHR-CAP** | 27,758,505 | 18,972,259 | 68.3% | 3,025,059 | 1,312,351 |
| **1000968** | **SHR-CAP** | 34,695,807 | 24,186,414 | 69.7% | 3,206,355 | 1,538,707 |
| **1000995** | **SHR-CAP** | 42,383,318 | 29,730,262 | 70.1% | 3,145,897 | 1,864,487 |
| **1001006** | **SHR-CAP** | 42,951,899 | 29,898,452 | 69.6% | 3,262,773 | 1,733,503 |
| **1001031** | **SHR-CAP** | 27,649,488 | 19,500,352 | 70.5% | 3,160,189 | 1,119,720 |
| **1001065** | **SHR-CAP** | 36,263,946 | 24,722,558 | 68.2% | 3,114,824 | 1,608,446 |
| **1001320** | **CAP-F2** | 29,558,513 | 20,589,497 | 69.70% | 3,070,720 | 1,169,564 |
| **1001324** | **CAP-F2** | 35,412,530 | 24,723,896 | 69.80% | 3,096,781 | 1,430,323 |
| **1001331** | **CAP-F2** | 30,600,595 | 21,465,761 | 70.10% | 3,150,812 | 1,213,513 |
| **1001338** | **CAP-F2** | 46,954,870 | 33,200,129 | 70.70% | 3,368,620 | 1,817,393 |

For differential methylation analysis, once again CpGs were required to have at least 20X coverage and be represented at this depth by at least three animals. This resulted in 1,222,210 CpGs (~ 40% of the average) being interrogated between SHR and SHR-CAP, 1,494,557 CpGs (~ 49% of the average) between SHR-CAP and their CAP-F2, and 1,130,112 CpGs (~ 37% of

the average) between SHR and CAP-F2 animals. The numbers of CpGs analyzed are similar to those of Chapter 3, and greater than those in Chapter 4.

Unsupervised hierarchical clustering of RRBS data at 20X was unable to clearly separate the SHR and SHR-CAP from each other (Figure33) and was not even attempted for the comparison of SHR and CAP-F2. The finding that SHR and SHR-CAP could not be separated in the clustering is perhaps not surprising as the animals in this experiment have the same genetic background. At the same time this implies that any methylation differences between groups are likely to be subtle.

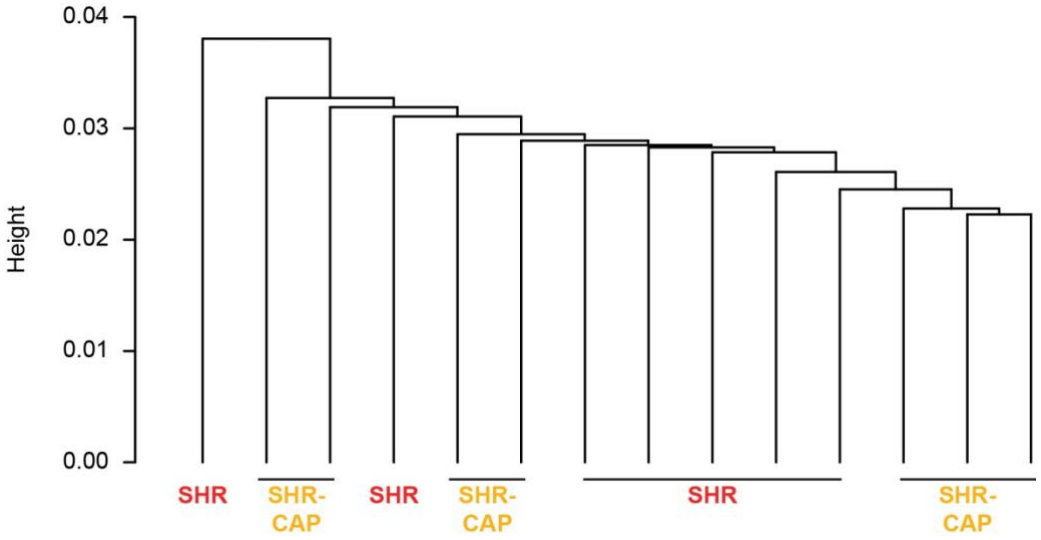



**Figure 33. Unsupervised hierarchical clustering of RRBS data of the kidney of pre-hypertensive SHR and SHR-CAP at four weeks of age.**

Dendrogram showing unsupervised hierarchical clustering of RRBS data of the kidney of pre-hypertensive SHR and SHR-CAP at four weeks of age (Ward's Method).

## methylKit identifies a small number of differently methylated cytosines between the three SHR groups

I next sought to determine whether there were any differentially methylated CpGs (DMCs) between the groups. Because any such changes are likely to be subtler than those observed in previous chapters (based on unsupervised clustering and PCA) I altered the stringency of my

analysis parameters accordingly. For this analysis CpGs still needed to be present in at least three animals with a coverage of 20X or greater, with a significance threshold of $q < 0.01$, but the threshold of difference was lowered to ≥ 5% average methylation difference.

This analysis defined 192 DMCs between SHR and SHR-CAP (Figure 34) and 164 DMCs between SHR and CAP-F2 (Figure 35). It is interesting to note that SHR-CAP were found overall to be more hypomethylated than SHR, while their offspring the CAP-F2 became hypermethylated in comparison to both SHR-CAP and even the SHR. Furthermore the DMCs found between SHR and SHR-CAP showed a lower value of significance.

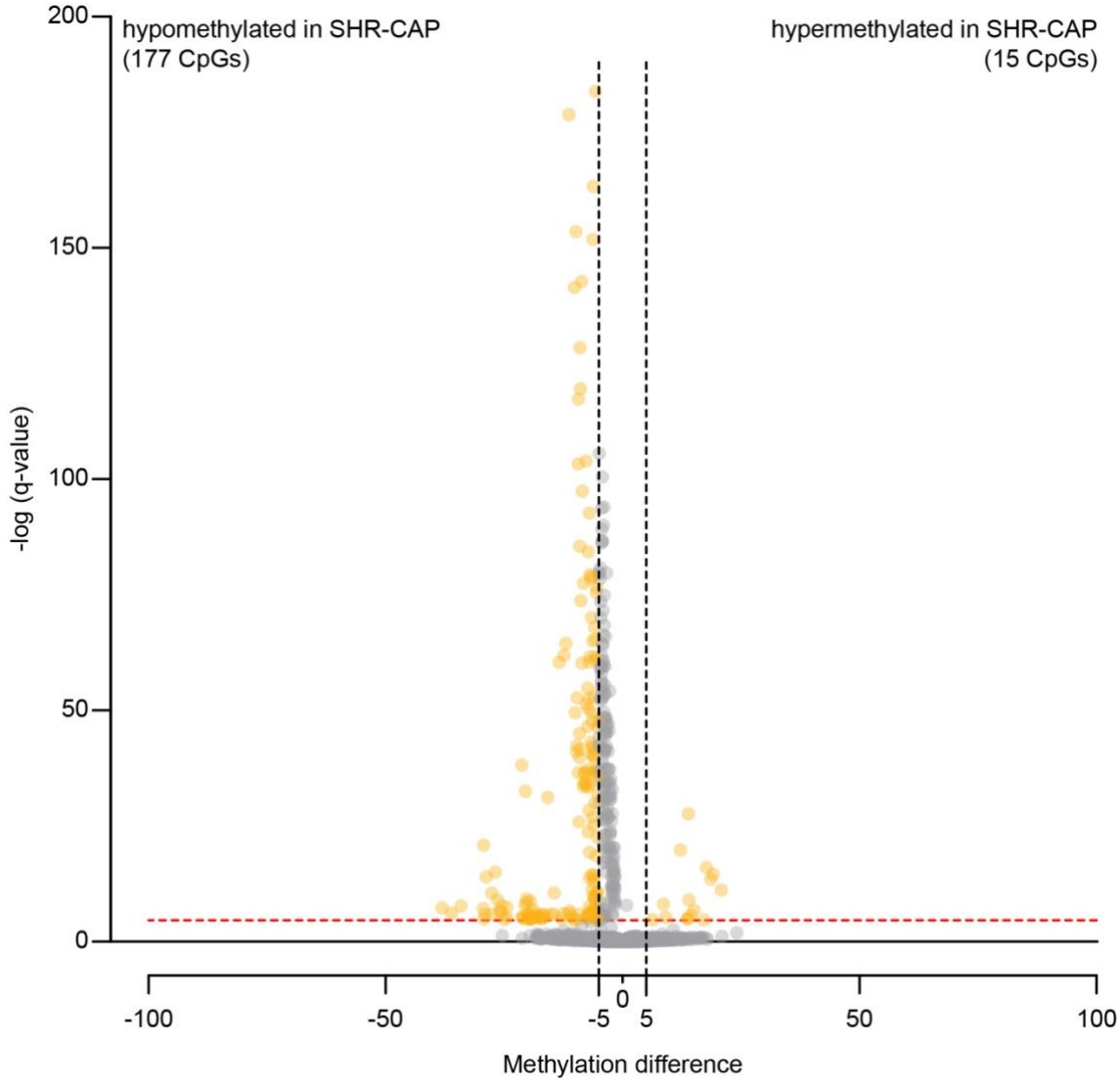



**Figure 34. Differentially methylated cytosines (DMCs) identified in the kidney between pre-hypertensive SHR and ACE inhibitor exposed SHR-CAP at four weeks of age.**

Volcano plot depicting DMCs. DMCs, meeting the parameters of significance threshold of $q \leq 0.01$, and $\geq$ 5% average methylation difference between SHR and SHR-CAP, are shown in yellow (red dotted line represents $q = 0.01$).

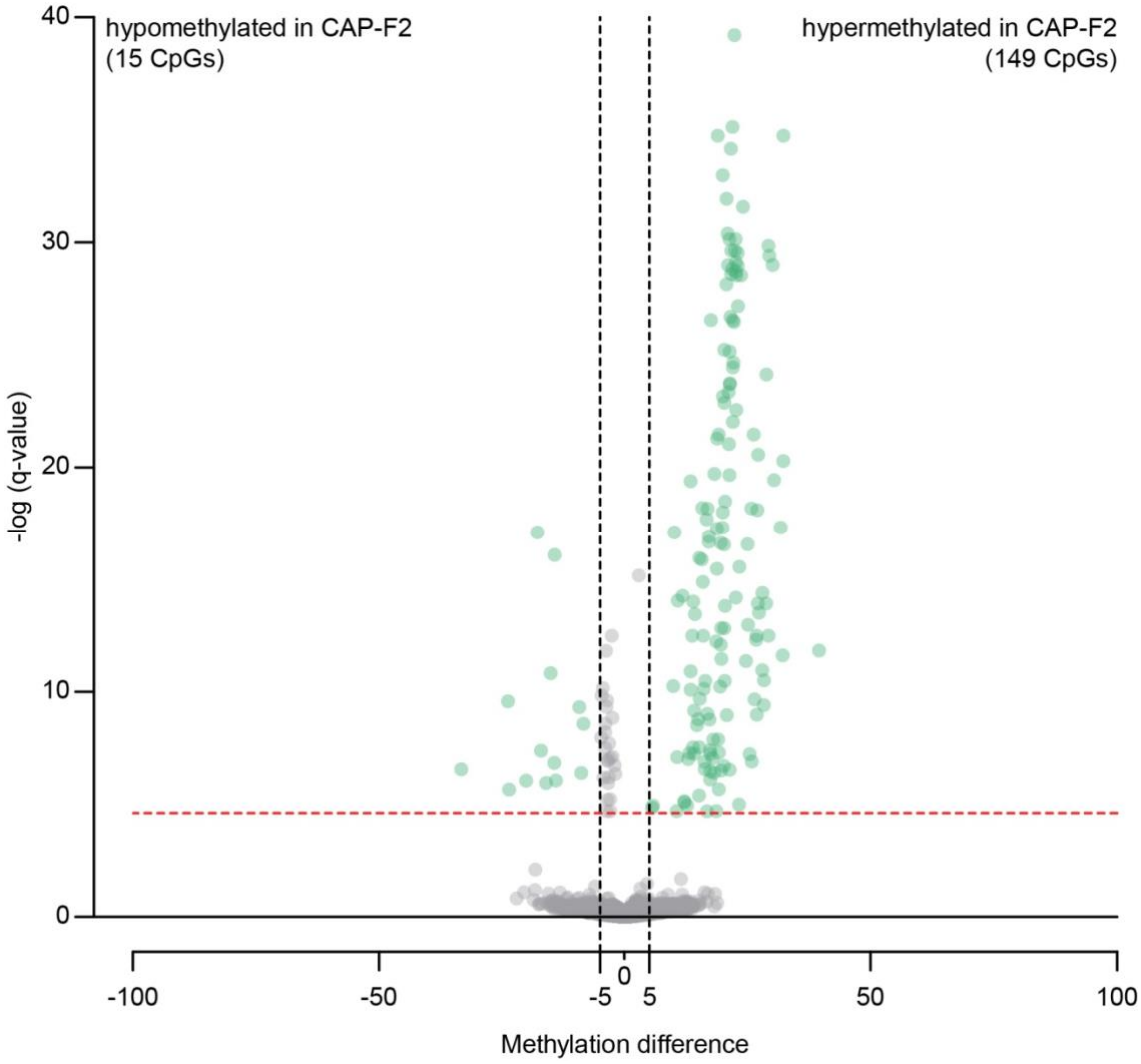



**Figure 35. Differentially methylated cytosines (DMCs) identified in the kidney between pre-hypertensive SHR and CAP-F2 at four weeks of age.**

Volcano plot depicting DMCs. DMCs, meeting the parameters of significance threshold of $q \leq 0.01$, and $\geq$ 5% average methylation difference between SHR and CAP-F2, are shown in green (red dotted line represents $q = 0.01$).

In order to determine whether certain genomic regions were enriched in the DMCs, I examined the genomic distribution of the DMCs, and compared this with the genomic distribution of all CpGs with 20X coverage for each of the three comparisons.

For the comparison of SHR and SHR-CAP (Figure 37) about half of all analyzed CpGs were found in CpG islands or their shores. However, for the DMCs only ~15% were in CpG islands

or shores (Figure 37A). As I had observed in the previous data DMCs appeared to be depleted in these areas of interest.

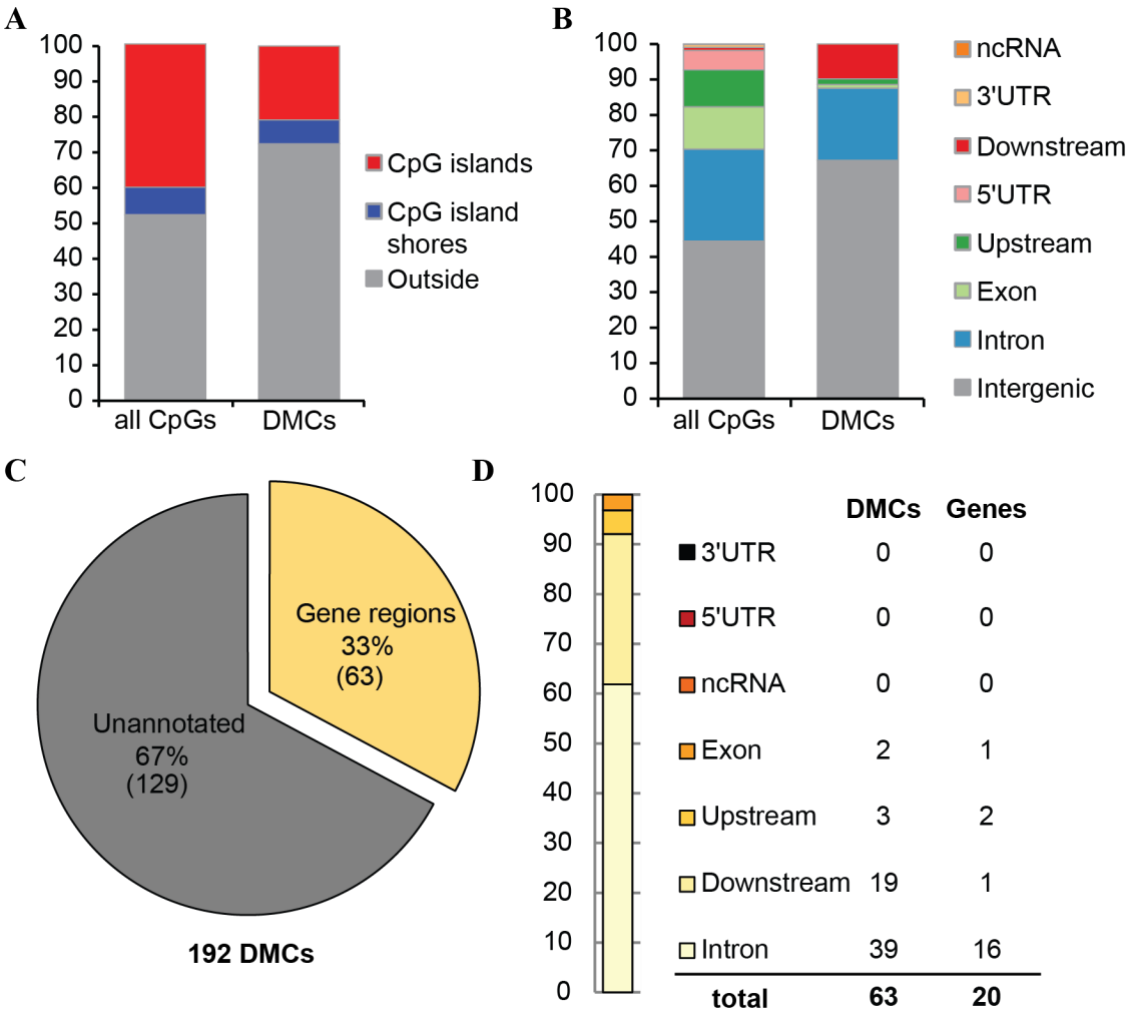



**Figure 36. Differently methylated cytosines (DMCs) between pre-hypertensive SHR and ACE inhibitor treated SHR-CAP in the kidney at four weeks of age.**

**A, B** Annotation of all analyzed CpGs and DMCs to CpG islands **(A)** and genic location **(B). C** Annotation ratio of DMCs. **D** Identified DMCs that can be linked to genes.

In respect to the DMCs proximity to genes, I observed an overall decrease in the proportion of DMCs residing within genic regions, relative to all CpGs (Figure 37B). In particular those regions of interest in regards to transcription, 5'UTR and upstream of protein coding regions, were depleted in the DMCs compared to all CpGs.

The 63 DMCs identified in gene annotated regions (Figure 37C) were able to be assigned to 20 distinct genes (Figure 37D). However, only two genes – *Apob* and *Gstm4* - harbored DMCs upstream of their protein coding region while the rest of the genes were found to have their DMCs in intergenic and intronic regions. Given that *Apob* was associated with only two DMCs and *Gstm4* only one, and there were no other DMCs in proximity, it is questionable if these would have an effect on gene expression. Gene ontology analyses were not possible with such a limited number of affected genes.

In the comparison between SHR and CAP-F2 (Figure 38) about 40% of all analyzed CpGs were found in CpG islands or their shores. However, of the DMCs ~90% were in CpG islands or shores (Figure 38A). This is remarkable and had not been observed in any other data comparisons in this work. In this instance DMCs appeared to be enriched in the areas of CpG islands and shores.

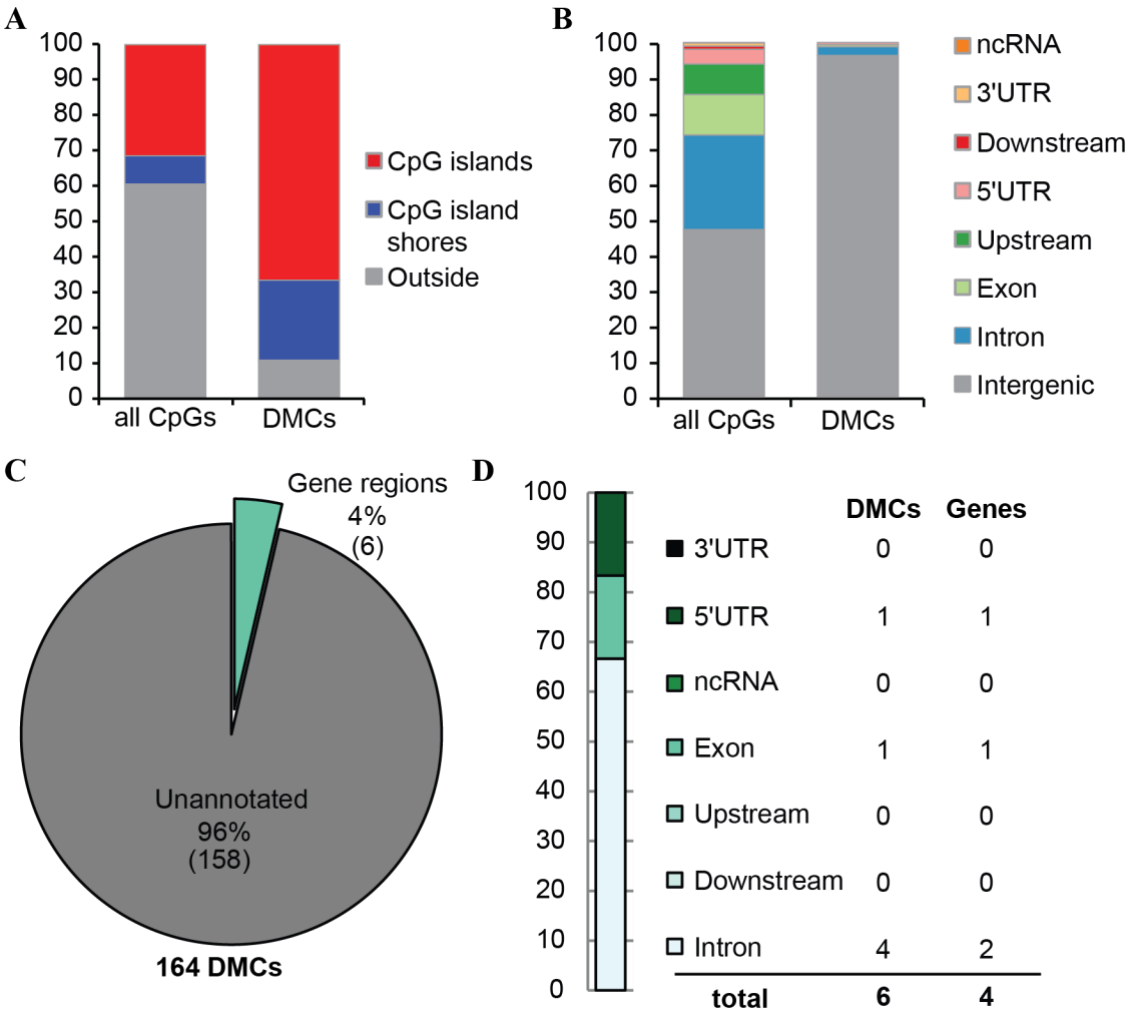



**Figure 37. Differently methylated cytosines (DMCs) between pre-hypertensive SHR and CAP-F2 in the kidney at four weeks of age.**

**A, B** Annotation of all analyzed CpGs and DMCs to CpG islands **(A)** and genic location **(B). C** Annotation ratio of DMCs. **D** Identified DMCs that can be linked to genes.

In regards to the DMCs between SHR and CAP-F2 in proximity to genes I observed a drastic decrease in the proportion of DMCs residing within genic regions, relative to all CpGs (Figure 38B). Almost all DMCs were in intergenic regions.

Only six DMCs were found in gene annotated regions (Figure 38C) and these were able to be assigned to four different genes (Figure 38D). Of these four genes harboring DMCs only one – *Tspan12* – harbored the DMC in a 5'UTR. However, *Tspan12* only contained a single DMC and

therefore was not further investigated. Gene ontology was not attempted as only four genes were identified.

It became apparent that the data did not reveal many genes with changes in cytosine methylation. However, I was interested to explore if *any* of the DMCs that were found in the previous chapters were represented in the comparison of the SHR to the hypertension rescued SHR-CAP. What I found was that 41 DMCs of the 192 DMCs identified in the comparison of SHR to SHR-CAP, were also found in the overlay of the DMCs of all three tissues explored in Chapter 4. This is a surprisingly high 21% of all DMCs found for the SHR to SHR-CAP comparison. Even more interestingly these 41 DMCs were confined into three areas, two in intergenic regions and one annotated to a gene. The two intergenic regions were located one on Chromosome 1: 13,499,544 – 13,499,907, containing 14 of the DMCs and the other on Chromosome 14: 46,811,246 – 46,811,503 with six DMCs. The genic region, containing 19 of the DMCs, was linked to the gene *LOC310926.* Since we had already established in Chapter 4 that not much can be said about this gene, I was nevertheless curious to then manually explore the data for the other two genes that had been previously identified as candidate germline epimutations: *Arhgap11a* and *Tomm20*.

### *Arghap11a* and *Tomm20* in the three SHR cohorts

As I had previously identified both *Arhgap11a* and *Tomm20* as candidate germline epimutations related to blood pressure regulation, I was curious to see whether the methylation patterns of these two genes were altered in the SHR-CAP and CAP-F2, particularly since the SHR-CAP maintained a significantly lower blood pressure throughout their life.

I discovered that both genes had enough animals with coverage of 20X or greater but patterns of methylation across the region of interest were almost identical among groups (Figure 40).

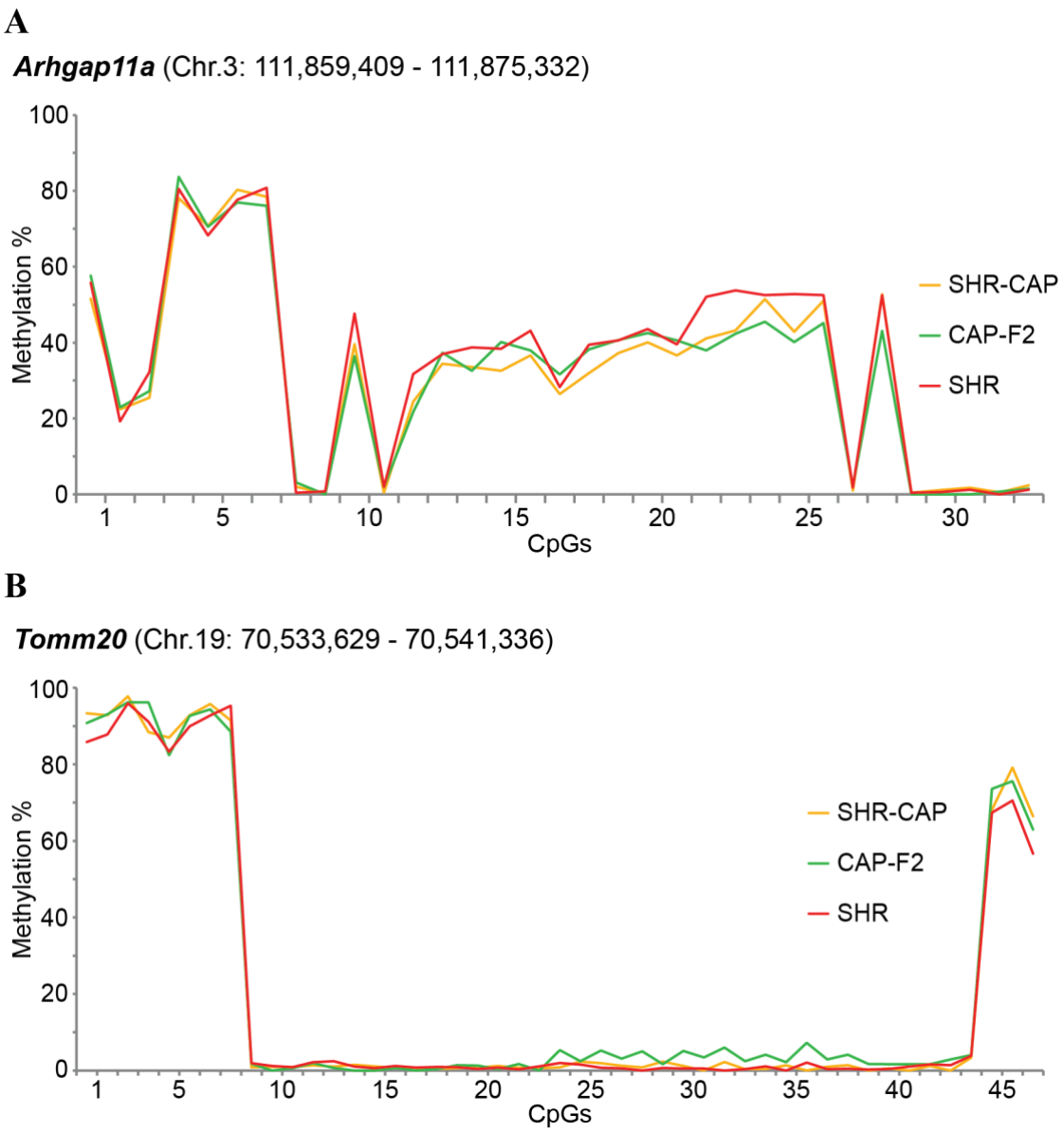



**Figure 38. Methylation percentages over multiple CpGs for the genes *Arhgap11a* (A) and *Tomm20* (B) in the four week kidney of pre-hypertensive SHR, ACE inhibitor treated SHR-CAP and medically unexposed CAP-F2.**

## Dynamics of Captopril methylation patterns across generations

Even though there were relatively few Captopril-induced DMCs, I was interested to determine whether the changes induced in SHR-CAP were maintained in CAP-F2, or alternatively had reverted to the naïve SHR levels (as did the hypertensive phenotype). In this analysis all DMCs

were included, even the ones outside of gene annotated regions. The parameters for the DMCs remained the same. The results are shown in Figure 39.

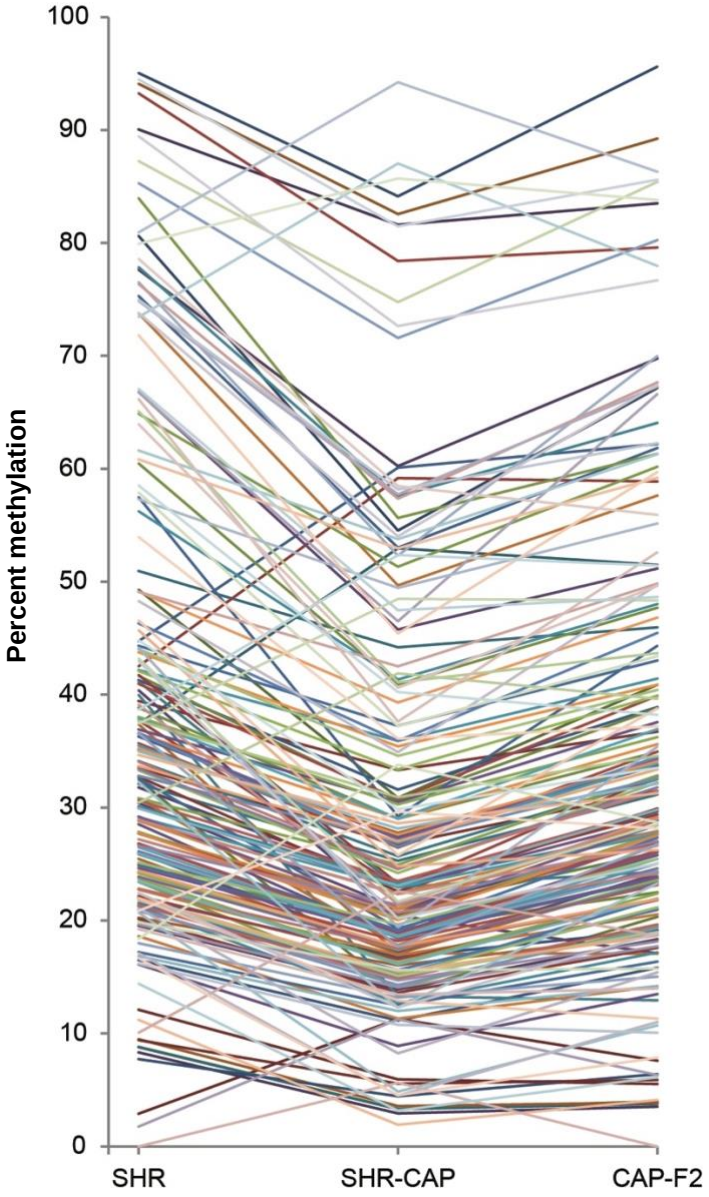



**Figure 39. Changes in methylation in the four week old kidney of SHR, SHR-CAP and CAP-F2.** Each line represents the methylation trajectory of a single CpG site that was identified as a DMC induced by Captopril in SHR-CAP. The majority of DMCs appear to be hypomethylated in response to Captopril but reverted to the SHR methylation percentage in the unexposed CAP-F2.

What becomes very apparent is that in the majority of methylation differences between the three groups, there appears to be a change of methylation, either to a more hyper- or more often hypo-

methylated state, from the SHR to the SHR-CAP that is reversed in the CAP-F2. Given that the CAP-F2 revert back in their blood pressure phenotype and ultimately even surpass that of the hypertensive SHR at 35 weeks this is unlikely to be entirely coincidental.

However, given that the majority of these sites were outside of regions that are associated with gene annotation, an in-depth analysis of these sites will only be possible in the future with advances in annotation of the rat genome.

### 5.2.5 Differences in kidney gene expression between SHR and SHR-CAP

The SHR-CAP animals presented with a significantly lower level of blood pressure throughout their lives and as such, despite the lack of clear candidate genes that display differences in methylation between them and untreated SHR, I performed gene expression analysis of the kidney of each three SHR males and three SHR-CAP males. It should be noted that animals were 12 weeks at the time of tissue collection. Therefore, SHR-CAP had ceased Captopril four weeks prior. Total RNA was extracted from the whole kidney from three males of each cohort and used in an Affymetrix Clariom S$^{TM}$ rat microarray at the Ramaciotti Centre at UNSW.

Principal components analysis of the resulting data with Transcriptome Analysis Console software showed SHR and SHR-CAP barely separate by gene expression alone, with the first principal component accounting for just ~27% of all variance in gene expression (Figure 40).

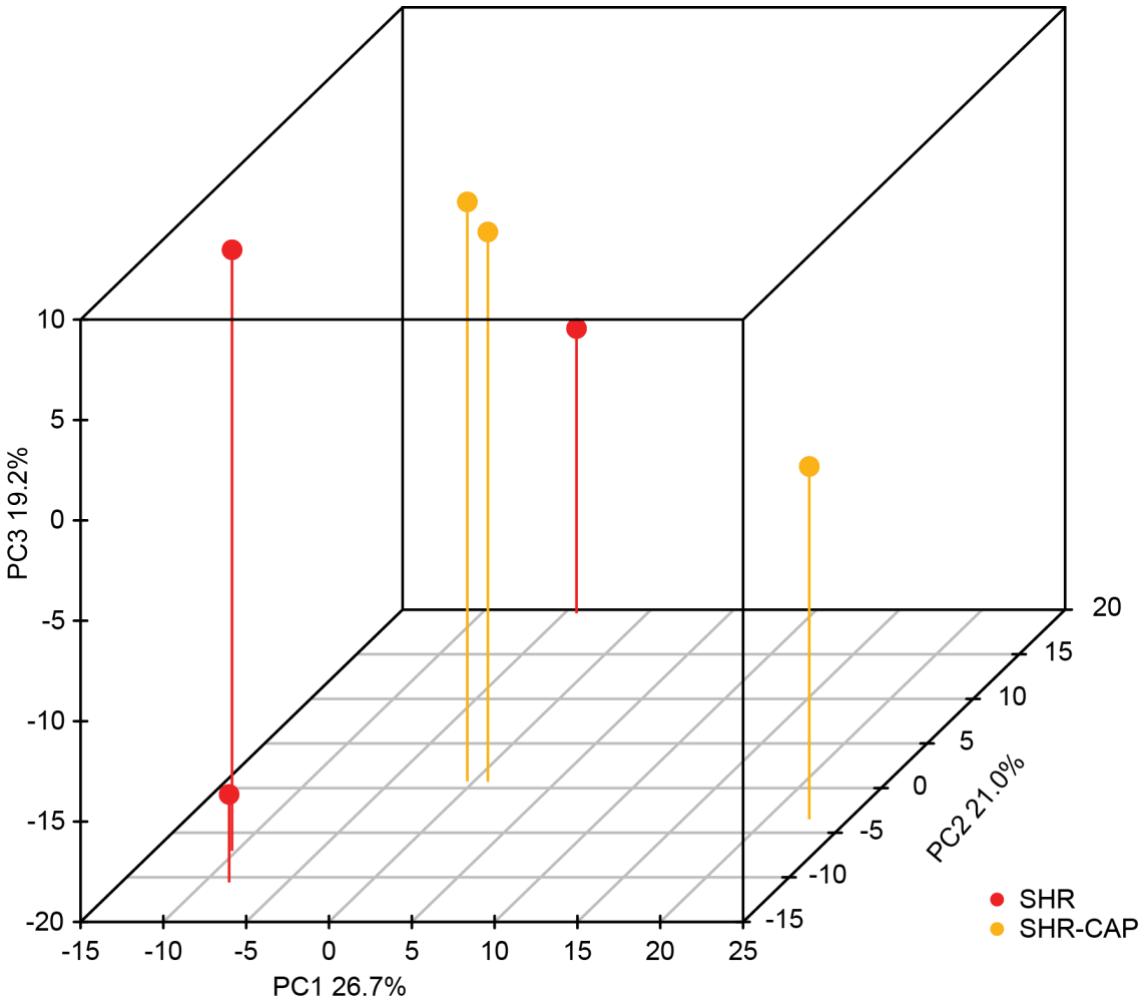



**Figure 40. Transcriptome Analysis Console (TAC) PCA output of expression array data of SHR and SHR-CAP in the 12 week old kidney.**

Pseudo-3D Principal components analysis (PCA) plots of the expression array data from SHR (red) and SHR-CAP (yellow).

Analysis of differential expression between the strains revealed not a single gene with a greater than two-fold expression change between the groups (at a corrected significance level of $q < 0.01$). No differences were detected even by lowering the fold-change to 1.5. This is somewhat surprising given the fact that the SHR and SHR-CAP present with very different blood pressure levels at this time point. At the same time it puts the methylation analysis of the four week old kidney, usually seen as the target organ for hypertension, performed in this chapter into

question. It would be of interest to follow up this expression analysis in other tissues, like the brain [277], that also have a large capacity in influencing blood pressure homeostasis.

## 5.3 Discussion

Wu and Berecek, along with others, have shown that the SHR can have their innate fate of pronounced hypertension altered through short term exposure to an ACE inhibitor, such as Captopril [193-196, 278]. The reason for this phenomenon is to date a mystery, and so in this chapter I explored whether DNA methylation might be involved. The previous observation of the apparent heritability of the effects of Captopril [197] led me to ask whether the inherited modification of disease risk might be related to germline changes in methylation patterns. I once again selected the kidney for this analysis, principally due to its suspected key role in maintaining blood pressure homeostasis, but also the large number of methylation differences identified between the kidney of SHR and WKY in Chapter 3. It would have been helpful here in this chapter to have studied multiple tissues (for the same reason that multiple tissues were studied in Chapter 4), but budgetary constraints did not permit this.

From a purely phenotypic perspective, the results of this chapter confirmed that transient early exposure to Captopril results in SHR maintaining a significantly lower blood pressure than untreated SHR. In addition, SHR-CAP animals also had a reduction in body weight (across the adult life course), a finding that has not previously been reported. However, the offspring of SHR-CAP animals that had ceased Captopril two weeks prior to mating (CAP-F2), reverted back to a hypertension phenotype; this result stands in contrast to that of the Wu and Berecek. It is difficult to reconcile this difference, but it may be related to a number of factors, including the way that BP was measured (Wu and Berecek used telemetry) and the breeding strategy employed. Given the number of CAP-F2 rats ($n$ = 12) in my experiment I believe my findings to be robust, and unlikely the result of statistical chance.

However, unlike the reversion of the blood pressure phenotype, the weight phenotype (reduction in total body weight in SHR-CAP) persisted into the unexposed CAP-F2. This could

mean that at least in regard to the weight, Captopril could possibly have given rise to the inheritance of another environmentally-induced trait. This is a phenomenon that has been seen multiple times and is assumed to have an epigenetic basis. Particularly maternal effects, like nutrition, access to water etc., have been reported to cause epigenetic inheritance of certain phenotypes [279, 280]. The fact that these findings can last to offspring in the F2 generation can on one hand be due to a true epigenetically inherited alteration or, one the other hand, due to the phenomenon that the germ cells in developing F1 individuals are also being exposed to the effector. In this second scenario, not only the F1 generation is being altered by the maternal effect but also the germ cells that will give rise to the following generation F2. Only if a change in phenotype persists into F3 and beyond, it can be assumed to be an event of epigenetic inheritance [281]. In regard to paternal effects, already the F2 generation would be an indicator for this to have occurred. This is due to the fact that sperm cells and their epigenetic make-up are being established later in life [282].

Returning to the actual work of this thesis, cytosine methylation in the kidney of all three groups (SHR, SHR-CAP, CAP-F2) was assessed at four weeks of age; this age was chosen because it precedes hypertension in SHR, but a potential confounder is that the SHR-CAP animals are still exposed to Captopril, and so methylation differences may be a result of direct exposure to the drug. That being said, the CAP-F2 were not under direct influence of the drug at the time of measurement and still showed a lower blood pressure till at 10 weeks of age.

However, despite their different fate in hypertension development and even the fact that SHR-CAP were still under medical exposure at time of analyses, the degree of methylation difference at any of the DMCs in SHR-CAP was small. Out of the several hundred differences in methylation sites found between the SHR and SHR-CAP the majority were less than a 10% difference, and were also found in unannotated intergenic regions. Gene expression analysis was similarly unenlightening, despite being performed on 12 week old animals. At this age the SHR display significant signs of hypertension development (when compared to the normotensive WKY) while the SHR-CAP display significantly lower blood pressure than the unmedicated

SHR. Nevertheless, no significant gene expression differences were identified in the kidney. This was somewhat surprising and puzzling, but perhaps suggests that the kidney is not the organ of origin of hypertension. It would have been very interesting to study the brain in this regard, given that more and more research emerges that suggests that there is a correlation between the nervous system and essential hypertension [277, 283, 284].

The goal of this experiment was to identify epigenetic lesions related to blood pressure without the confounding variable of genetic variance between the normo- and hypertensive animals analyzed. As such the data produced complement the previous analyses comparing WKY and SHR (Chapter 3 and 4), but also stands on its own as a means of assessing the epigenetic contribution to hypertension. While germline epimutation candidates Arghap11a and Tomm20 did not appear affected by Captopril, this does not preclude them being bona-fide candidates for blood pressure regulation. It does appear, however, that the kidney was not the ideal tissue to do this experiment. Despite finding methylation differences in the kidney, they were few and subtle. Nevertheless, this approach exploiting the long-term response to short transient Captopril exposure, holds potential for future investigations targeted to other tissues. At the very least it demonstrates that early environmental signals can not only worsen hypertension (e.g. stress, diet etc.) but are also potentially positive environmental effectors.

# Chapter 6. Conclusions and outlook

While there are a number of well-established risk factors, essential hypertension lacks a causal explanation. This is particularly surprising given that a strong heritability has been proven, millions of people suffer from the disease worldwide, and decades of research have been performed. However, the possibility of an 'epigenetic' cause has only been raised relatively recently [285-287] and needs to be explored. This work was a first attempt of doing so, by utilising the spontaneously hypertensive rat (SHR) as a model animal. Unlike other rat strains that develop hypertension, SHR do so entirely without experimental induction and the penetrance throughout the strain is at 100%. Furthermore, these animals show a development of the hypertension just like humans do, by being pre-hypertensive at a young age, developing hypertension over time and eventually plateauing at a high blood pressure. The diversity in end organ failure after long-term elevated blood pressure, which has been described for the SHR, also represents the human condition quite closely. Finally, the fact that the disease occurred spontaneously and propagated rapidly to create this strain, and that two attempts of whole genome sequencing have not been successful [169, 170] in identifying a genetic cause, made the SHR even more attractive for the proposed analysis.

I focused on methylation as the epigenetic mechanism to be explored as a potential cause for the hypertension. Methylation, particularly in the promoter region of genes, has been associated with gene silencing [105]. I therefore compared genome wide snapshots of SHR methylation pattern to those of their cousin strain and commonly used control, the normotensive Wistar Kyoto rat (WKY). My goal was to potentially identify candidate genes that may be silenced or have altered expression due to differences in methylation between the two strains. The method chosen to explore methylation was RRBS, which comes with the advantage of a producing a genome scale picture of methylation at single nucleotide resolution, in a cost-efficient manner.

I focused Chapter 3 of this work on RRBS analysis of the kidney. The kidney has been implicated as one of the key regulators of blood pressure homeostasis. In the SHR in particular, transplantation experiments have shown that kidneys of hypertensive SHR can cause

hypertension when transplanted into normotensive recipients, and vice versa [219]. Thousands of differences in methylation were identified in the kidney, but the majority were located in unannotated regions. The number of potential candidate genes was further depleted when taking into consideration that methylation differences of single nucleotide are rather unlikely to have a large impact on expression. Nevertheless, several genes were identified with differences in promoter methylation between the two strains.

In order to narrow down potential germline epimutations from these candidates identified in the kidney, I performed RRBS on the liver and brain of the same animals in Chapter 4 of this work. The idea was to represent an organ that originated from each one of the three germ layers with those changes found in all three deemed potential germline changes. Germline epimutations are epigenetic changes that occur so early in an individual's development, that they become established throughout every germ layer and thus tissue of the individual and are accompanied by resulting gene expression changes.

The commonalities between all three tissues were modest, even at the level of individual CpGs, but three candidate genes nevertheless were consistent throughout the analysis: *Arhgap11a*, *Tomm20* and *LOC310926*. In future work it would be interesting to observe the methylation patterns of these loci in sperm or eggs; if they are also aberrantly methylated here an obvious subsequent experiment would be an investigation as to how these methylation patterns are able to evade the epigenetic resetting of both germ cells and the early embryo. While none of these three genes have previously been referred to in the context of hypertension, there are plausible links to blood pressure regulation (at least for *Arhgap11a* and *Tomm20*) and as such they would be prime candidates for future investigations into the epigenetic contribution to hypertension.

One factor that confounds the identification of 'pure' epimutations between SHR and WKY is that there are significant underlying genetic sequence differences between the two. It may be that the epimutations described in this thesis are due to the influence of a genetic change, although no changes to the local sequence were apparent in this work.

In Chapter 5 of this thesis, I attempted to circumvent the effects of genetic sequence variations by exploiting the reports that transient Captopril treatment early in life can have long-lasting preventative effects towards hypertension in the SHR. One report even suggested that *in utero* exposure would facilitate an inheritable effect of hypertension protection in the SHR. This would not be explicable from a genetic standpoint, given that the exposure to medication would not be expected to genetically alter all the offspring in the same way. But epigenetic mechanisms have been proven to be easily modifiable during critical time points in development, and therefore this experiment provided a unique opportunity to assess epigenetic changes induced by Captopril that result in sustained reduction of blood pressure. In my experiment, control SHR animals developed hypertension as expected, while animals exposed *in utero* and up to eight weeks of life (SHR-CAP) showed a lifelong protection from the disease. Unlike in one previous report [197], however, the second generation animals (CAP-F2), did not remain on a lower blood pressure long term.

Despite the dramatic change in phenotype, I observed few methylation differences between SHR and SHR-CAP or SHR and the CAP-F2 throughout and even less so in gene annotated regions. Interestingly some of the methylation differences between the SHR and SHR-CAP were in sites, that had been found to be in common with methylation differences between all three tissues in the Chapter 4 which suggests that may be related to blood pressure regulation.

## Limitations of this work

All the approaches in this work were carefully considered and based on the current knowledge about the SHR, hypertension, and epigenetics. However, experiments could have been performed differently in hindsight or without financial limitations.

Since there had not really been any epigenetic research regarding hypertension to this date, it would have been beneficial to assess methylation changes in the development of hypertension in the SHR over time. This would have made it easier to uncover true inborn methylation differences, i.e. those that are simply a reflection of hypertension within the SHR animals.

Ideally this would also involve gene expression analyses at different time points in the animals' lives. Furthermore, organs other than the kidney should have been examined more thoroughly. The brain with its influence on hormonal regulation and even critical areas within the brain (such as the medulla oblongata with its impact on the autonomous nervous system and thereby blood pressure homeostasis [288, 289]) could have undergone further investigation, particularly since SHR are also a known model for Attention Deficit Hyperactivity Disorder (ADHD) [290]. As a side note it should be mentioned that just like the hypertensive phenotype, the ADHD phenotype in the SHR lacks a causal explanation. Therefore, any of the findings in this work should also be considered as a potential epigenetic elicitor on the animals' ADHD.

Another improvement relates to the molecular methodology. This thesis has found that RRBS is quite inconsistent in which parts of the genome are covered, raising the risk of failing to reach sufficient coverage for areas of interest. Furthermore, while RRBS is very cost effective, whole genome bisulfite sequencing (WGBS) would have allowed for a more complete picture of methylation differences, especially between SHR and SHR-CAP. As WGBS will become less costly in the future, it will become a viable contender. Both techniques, RRBS and WGBS, are superior for methylation analysis when compared to antibody-based approaches or those utilizing restriction enzyme specificity because bisulfite conversion allows for single CpG resolution. WGBS allows one to analyse cytosine methylation globally but repetitive regions in the genome make both sequencing and mapping extremely challenging. This also leads to a need for greater average coverage compared to RRBS (ENCODE standards require at least 10X average coverage for RRBS and 30X average coverage for WGBS), which in turn necessitates a higher cost for large whole genomes such as that of the rat assessed in this thesis.

In summary, this thesis revealed that there are extensive epigenetic differences between the SHR and the related WKY rats supporting the idea that epigenetics may have a role to play in essential hypertension. Furthermore, the temporal medical exposure to Captopril and its resulting effects on hypertension in the SHR have so far been underutilised and hold potential for future studies on hypertension. Overall the results of this thesis will be published to guide

further research into the role of epigenetics in hypertension, in particular in concert with human cohort studies.

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## Appendix

**Appendix Table 3-1** Genes with differential expression between SHR and WKY in the whole kidney at 10 weeks of age

| Gene | Fold Change | SHR | WKY | FDR *p*-value |
|---|---|---|---|---|
| ***2510049J12Rik; LOC100361147*** | -2.39 | 78.25 | 568.10 | 0.0058 |
| ***Abca16*** | 5.45 | 16.56 | 12.64 | 0.0003 |
| ***Abcb10*** | -3.66 | 116.97 | 526.39 | 0.0001 |
| ***Acot12*** | 4.15 | 17.88 | 250.73 | 0.0000131 |
| ***Acsm3*** | 29.12 | 45.57 | 13969.57 | 5.03E-12 |
| ***Acss1*** | -6.53 | 1398.83 | 20452.65 | 0.00000117 |
| ***Ak2*** | 2.75 | 268.73 | 2646.74 | 0.0012 |
| ***Aldh3a2*** | 3.07 | 675.59 | 1024.00 | 0.0000419 |
| ***Amigo2*** | -3.11 | 10155.69 | 209.38 | 0.0014 |
| ***Ankfn1*** | 4.5 | 154.34 | 367.09 | 0.001 |
| ***Anxa10*** | -2.96 | 18.90 | 15.35 | 0.0008 |
| ***Apoc2*** | 3.49 | 20.97 | 18.25 | 0.0005 |
| ***Apoh*** | 7.05 | 48.84 | 10.27 | 0.0004 |
| ***Aqp3*** | 2 | 8.17 | 1016.93 | 0.0055 |
| ***Asb9*** | -2.73 | 240.52 | 330.84 | 0.0044 |
| ***Azgp1*** | -2.86 | 270.60 | 304.44 | 0.0039 |
| ***Bdh1*** | -2.26 | 18.25 | 1287.18 | 0.0004 |
| ***Bin2*** | -3.47 | 30.48 | 54.19 | 0.0014 |
| ***Bmpr1a*** | -2.12 | 9.45 | 2076.59 | 0.0061 |

| | | | | |
|---|---|---|---|---|
| ***Cbr1*** | 6.21 | 31.12 | 5.58 | 0.00000102 |
| ***Ccdc88a*** | -4.26 | 83.29 | 4420.52 | 0.0000487 |
| ***Cckar*** | -5.48 | 9.65 | 125.37 | 0.00000301 |
| ***Cd244*** | -3.36 | 3125.78 | 166.57 | 0.0018 |
| ***Cd36*** | -3.51 | 66.26 | 867.07 | 0.0016 |
| ***Cd74*** | 2.38 | 699.41 | 18053.61 | 0.0000691 |
| ***Cdk14*** | -2.17 | 200.85 | 2091.03 | 0.0035 |
| ***Cdk18*** | 2.39 | 471.14 | 1541.37 | 0.0001 |
| ***Cdkn1a*** | 3.18 | 699.41 | 71.51 | 0.0013 |
| ***Cep295*** | 4.27 | 2957.17 | 41.36 | 0.00000863 |
| ***Ces2*** | 2.86 | 93.70 | 26.91 | 0.0021 |
| ***Ces2g*** | -5.75 | 15.67 | 16961.78 | 0.0003 |
| ***Cfh*** | 2.44 | 22.94 | 1652.00 | 0.0006 |
| ***Ch25h*** | 2.44 | 20.68 | 151.17 | 0.0034 |
| ***Chchd5*** | -2.96 | 128.00 | 259.57 | 0.0006 |
| ***Cldn10*** | -2.14 | 28.25 | 49667.00 | 0.0009 |
| ***Cldn3*** | -5.98 | 33.59 | 2817.11 | 0.0002 |
| ***Cldn6*** | 7.45 | 108.38 | 95.01 | 0.000000234 |
| ***Clec7a*** | -2.72 | 8.06 | 372.22 | 0.0041 |
| ***Cmc1*** | -3.04 | 64.89 | 512.00 | 0.0096 |
| ***Cml5*** | 2.23 | 885.29 | 321.80 | 0.0063 |
| ***Cntfr*** | 2.8 | 724.08 | 60.13 | 0.0007 |
| ***Cntnap4*** | 5.19 | 1038.29 | 17.27 | 0.0038 |
| ***Cpxm2*** | -2.62 | 261.38 | 249.00 | 0.0012 |
| ***Crot*** | -2.09 | 163.14 | 1082.39 | 0.0096 |

| | | | | |
|---|---|---|---|---|
| ***Csad*** | -2.01 | 26.35 | 7696.57 | 0.0011 |
| ***Ctnna3*** | -2.6 | 837.53 | 694.58 | 0.0006 |
| ***Cxcl13*** | -4.76 | 191.34 | 608.87 | 0.00000118 |
| ***Cyp2c24*** | -19.22 | 10.78 | 5148.73 | 5.15E-08 |
| ***Cyp8b1*** | 59.52 | 337.79 | 13.27 | 5.81E-11 |
| ***Dapk2*** | -4.53 | 10.78 | 128.00 | 0.0001 |
| ***Decr2*** | 2.15 | 9.92 | 5220.60 | 0.0081 |
| ***Dnm1*** | -12.03 | 88.03 | 250.73 | 0.000000248 |
| ***Dnm3*** | 6.61 | 143.01 | 51.63 | 0.0001 |
| ***Dtna*** | -2.29 | 93.70 | 843.36 | 0.0002 |
| ***Dusp15*** | -48.92 | 776.05 | 809.00 | 1.14E-10 |
| ***Efr3b*** | -2.19 | 9.78 | 274.37 | 0.0057 |
| ***Endog*** | -5.24 | 247.28 | 108.38 | 0.00000173 |
| ***Entpd2*** | -2.32 | 15.67 | 3795.30 | 0.007 |
| ***Ephx2*** | 14.08 | 30.91 | 85.04 | 0.000000022 |
| ***Errfi1*** | 5.58 | 8079.22 | 3019.30 | 0.007 |
| ***Etnk2*** | -2.28 | 49.52 | 59.71 | 0.0006 |
| ***Exnef*** | 38.05 | 222.86 | 13.64 | 5.81E-11 |
| ***F13b*** | 8.49 | 20.39 | 317.37 | 0.0003 |
| ***F2*** | -11.81 | 374.81 | 96.34 | 0.0000348 |
| ***Fam189a2*** | -3.7 | 451.94 | 36.76 | 0.0000365 |
| ***Farp1*** | 2.07 | 100.43 | 215.27 | 0.0055 |
| ***Fbxo22*** | -2.09 | 67.65 | 1820.35 | 0.0056 |
| ***Fitm1*** | 2.81 | 1144.10 | 12.82 | 0.0003 |
| ***Fmo2*** | 11.58 | 167.73 | 3236.01 | 0.000000265 |

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|---|---|---|---|---|
| ***Gabrp*** | -3.96 | 186.11 | 754.83 | 0.0003 |
| ***Galnt3*** | -6.3 | 5.17 | 4389.98 | 0.00000283 |
| ***Gapdh-ps1*** | 36.21 | 87.43 | 32.22 | 1.07E-10 |
| ***Gem*** | 3.07 | 25.81 | 50.56 | 0.0054 |
| ***Glp1r*** | -4.48 | 13.18 | 36.00 | 0.0007 |
| ***Gng13*** | -4.49 | 22.16 | 484.38 | 0.00000173 |
| ***Gpr110*** | -6.99 | 106.89 | 584.07 | 0.0003 |
| ***Grik2*** | -7.08 | 21321.18 | 160.90 | 0.0000001 |
| ***Grpr*** | -2.95 | 64.89 | 38.85 | 0.002 |
| ***Gstm1*** | -18.97 | 377.41 | 12765.83 | 1.05E-08 |
| ***Gtf2h3*** | -2.32 | 1746.20 | 139.10 | 0.0005 |
| ***Gtpbp4*** | 41.13 | 121.10 | 1260.69 | 1.22E-11 |
| ***Gucy1b3*** | -2.28 | 136.24 | 372.22 | 0.0024 |
| ***Gulp1*** | -2.58 | 59.30 | 786.88 | 0.0003 |
| ***Hao1*** | -3.78 | 25.11 | 40.79 | 0.0003 |
| ***Hba-a1*** | -24.8 | 1234.75 | 1128.35 | 0.000016 |
| ***Hist1h4b*** | 2.07 | 9.32 | 132.51 | 0.0035 |
| ***Hmgcs2*** | 3.56 | 18432.96 | 424.61 | 0.007 |
| ***Hmgn3*** | -2.26 | 95.01 | 1217.75 | 0.0003 |
| ***Hpgd*** | -4.04 | 1770.57 | 3373.43 | 0.0002 |
| ***Hpgds*** | 2.04 | 266.87 | 29.24 | 0.0037 |
| ***Hpse2*** | -2.64 | 1509.65 | 24.59 | 0.0076 |
| ***Icoslg*** | -3.6 | 304.44 | 2797.65 | 0.0000102 |
| ***Idi2*** | 2.94 | 3040.30 | 26.54 | 0.0008 |
| ***Ifi44l*** | -9.03 | 14066.74 | 276.28 | 0.0000382 |

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|---|---|---|---|---|
| ***Ifit1*** | 23.42 | 1782.89 | 7.31 | 0.0000252 |
| ***Ighg; Igh-6*** | -3.9 | 15608.02 | 1314.23 | 0.0049 |
| ***Igkc*** | -5.65 | 71.51 | 530.06 | 0.0002 |
| ***Igsf5*** | 3.16 | 17.51 | 2005.85 | 0.00000796 |
| ***Il20ra*** | -2.45 | 131.60 | 43.11 | 0.0037 |
| ***Inmt*** | 9.19 | 5.06 | 198.09 | 0.000000278 |
| ***Kcne1*** | -2.2 | 421.68 | 91.14 | 0.0057 |
| ***Kcnk5*** | 3 | 263.20 | 362.04 | 0.0011 |
| ***Klhl29*** | 2.51 | 237.21 | 23.10 | 0.0019 |
| ***Klrk1*** | -4.22 | 183.55 | 689.78 | 0.0006 |
| ***Krt19*** | 2.97 | 59.71 | 306.55 | 0.0032 |
| ***Lgals1*** | -3.06 | 1640.59 | 3492.39 | 0.0011 |
| ***Lifr*** | -2.62 | 367.09 | 48308.85 | 0.0066 |
| ***Lmbr1*** | 3 | 347.29 | 596.34 | 0.0061 |
| ***LOC100362814*** | -6.66 | 26.17 | 64.45 | 0.0001 |
| ***LOC100365047*** | 3.47 | 163.14 | 22.94 | 0.006 |
| ***LOC100910134*** | -3.53 | 572.05 | 34.54 | 0.0046 |
| ***LOC100910566*** | -23.21 | 541.19 | 32541.65 | 1.45E-08 |
| ***LOC100911315*** | 8.47 | 63.12 | 20.39 | 0.00000467 |
| ***LOC100912165*** | 2.18 | 1144.10 | 404.50 | 0.0017 |
| ***LOC100912416*** | -2.93 | 31.78 | 64.89 | 0.0063 |
| ***LOC102548978*** | 2.15 | 41.36 | 20.82 | 0.002 |
| ***LOC102551064*** | -3.92 | 124.50 | 42.22 | 0.0007 |
| ***LOC102551184*** | -2.74 | 962.07 | 4803.93 | 0.0000348 |
| ***LOC102553917*** | 3.1 | 23170.48 | 171.25 | 0.0067 |

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|---|---|---|---|---|
| ***LOC361914*** | -14.54 | 982.29 | 147463.67 | 2.02E-09 |
| ***LOC499469*** | 2.08 | 95.01 | 21.26 | 0.0096 |
| ***LOC679605*** | 4.55 | 867.07 | 10.13 | 0.006 |
| ***LOC681366*** | 2.97 | 519.15 | 56.49 | 0.0016 |
| ***LOC683573*** | 3.92 | 16.34 | 306.55 | 0.0038 |
| ***LOC685203; LOC102556334*** | -3.02 | 1807.78 | 560.28 | 0.0038 |
| ***LOC690955*** | 2.65 | 3821.70 | 16.00 | 0.0069 |
| ***Lss*** | -2.7 | 19619.49 | 68.12 | 0.0004 |
| ***Map3k5*** | 3.78 | 2048.00 | 194.01 | 0.00000479 |
| ***Mdc1*** | -2.74 | 59.71 | 177.29 | 0.0000348 |
| ***Mdfi*** | 2.34 | 445.72 | 40.22 | 0.0019 |
| ***Mep1a*** | -2.01 | 274.37 | 39511.91 | 0.0036 |
| ***MGC105567*** | -8.3 | 44.32 | 78.79 | 0.000021 |
| ***MGC108823*** | -3.18 | 374.81 | 1438.15 | 0.0007 |
| ***Mocs1*** | 2.29 | 44.63 | 714.11 | 0.0082 |
| ***Ms4a6b*** | -3.44 | 11268.44 | 106.89 | 0.0001 |
| ***Mthfr*** | -3.26 | 16.22 | 1217.75 | 0.0005 |
| ***Nat8*** | 2.23 | 885.29 | 321.80 | 0.0063 |
| ***Ncbp2*** | -53.46 | 719.08 | 4182.07 | 7.67E-08 |
| ***Ndufb8*** | -2.03 | 1.00 | 3666.02 | 0.0087 |
| ***Nebl*** | -4.06 | 729.11 | 106.89 | 0.0002 |
| ***Nostrin*** | 3.13 | 1629.26 | 224.41 | 0.0002 |
| ***Npas2*** | -11.88 | 2856.44 | 580.04 | 0.0013 |
| ***Nt5c3a*** | -5.93 | 93.70 | 4153.18 | 1.05E-08 |
| ***Olr1668*** | -4.53 | 487.75 | 151.17 | 0.0000169 |

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|---|---|---|---|---|
| ***Olr321*** | -2.22 | 42938.97 | 70.52 | 0.0013 |
| ***Olr322*** | -2.96 | 2998.45 | 76.64 | 0.0021 |
| ***Orai2*** | -2.43 | 3691.52 | 319.57 | 0.0028 |
| ***Oscp1*** | -2.24 | 1323.37 | 141.04 | 0.0012 |
| ***Pcdh9*** | -28.8 | 4011.71 | 515.56 | 3.66E-10 |
| ***Pck1*** | 2.98 | 369.65 | 87076.75 | 0.0038 |
| ***Pecr*** | 4.25 | 58.08 | 23821.89 | 0.00000206 |
| ***Per1*** | 5.71 | 955.43 | 634.73 | 0.0049 |
| ***Phldb2*** | -2.22 | 280.14 | 2538.92 | 0.0007 |
| ***Pigz*** | -43.67 | 166.57 | 5113.16 | 8.98E-10 |
| ***Pitpnm2*** | 2.63 | 93326.55 | 106.89 | 0.0077 |
| ***Plod2*** | -2.29 | 42.52 | 792.35 | 0.0061 |
| ***Ppic*** | 2.64 | 157.59 | 35610.13 | 0.0004 |
| ***Ppp1r16b*** | 4.07 | 7281.40 | 213.78 | 0.001 |
| ***Ppp2r4*** | 5.27 | 167.73 | 219.79 | 7.67E-08 |
| ***Pqlc1*** | -3.28 | 75.06 | 734.19 | 0.0008 |
| ***Prima1*** | -11.26 | 168.90 | 2702.35 | 0.0001 |
| ***Prnp*** | 2.78 | 36.00 | 60.13 | 0.0021 |
| ***Ptprn*** | 19.19 | 77.17 | 60.13 | 3.66E-10 |
| ***Pus7l*** | -5.5 | 77.71 | 86.22 | 0.00000437 |
| ***Qrich2*** | -2.42 | 910.17 | 12.21 | 0.0041 |
| ***Rad51d*** | -2.4 | 167.73 | 630.35 | 0.0006 |
| ***Rdh16*** | 2.27 | 258534.99 | 321.80 | 0.0049 |
| ***Reep6*** | -2.57 | 1089.92 | 36107.23 | 0.0055 |
| ***Ren*** | -2.71 | 3147.52 | 159.79 | 0.0037 |

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|---|---|---|---|---|
| ***Retsat*** | -3.43 | 155.42 | 27746.15 | 0.0000316 |
| ***RGD1309540; LOC102546523; LOC102555364*** | -2.1 | 530.06 | 199.47 | 0.0004 |
| ***RGD1564347*** | 97.73 | 699.41 | 17.51 | 1.2E-10 |
| ***RGD1564999*** | 51.16 | 6338.83 | 10.93 | 1.39E-10 |
| ***RGD1564999; LOC102550988*** | 8.06 | 227.54 | 22.63 | 0.00000217 |
| ***RGD1565117*** | 4.81 | 625.99 | 45.89 | 0.0025 |
| ***Rimbp2*** | 2.31 | 1652.00 | 1234.75 | 0.0072 |
| ***Ros1*** | -6.32 | 79.34 | 418.77 | 0.00000204 |
| ***RT1-A1*** | 14.12 | 63.56 | 137.19 | 4.87E-10 |
| ***RT1-Bb*** | -7.29 | 1509.65 | 78.25 | 0.00000749 |
| ***RT1-EC2*** | 7.67 | 734.19 | 40.50 | 0.00000269 |
| ***RT1-N2*** | -3.28 | 1200.98 | 66.72 | 0.0000546 |
| ***Rtel1*** | 4.65 | 873.10 | 179.77 | 0.00000139 |
| ***Rxfp2*** | 2.63 | 1038.29 | 63.56 | 0.0003 |
| ***Scn1b*** | 2.39 | 101421.22 | 1251.98 | 0.0001 |
| ***Sdr9c7*** | 4.27 | 247.28 | 58.08 | 0.0005 |
| ***Sema4g*** | -2.53 | 177.29 | 181.02 | 0.003 |
| ***Sema6a*** | -2.58 | 4329.55 | 7858.29 | 0.0000622 |
| ***Shmt1*** | -2.55 | 1652.00 | 4544.80 | 0.0002 |
| ***Slc11a1*** | 4.86 | 46.21 | 70.52 | 0.00000229 |
| ***Slc17a3*** | -4.31 | 837.53 | 33456.53 | 0.000065 |
| ***Slc22a24*** | -2.67 | 219.79 | 3304.00 | 0.0026 |
| ***Slc23a1*** | -2.53 | 77.17 | 39511.91 | 0.0003 |
| ***Slc34a2*** | -2.74 | 342.51 | 1038.29 | 0.0095 |

| | | | | |
|---|---|---|---|---|
| ***Slc43a3*** | 3.46 | 89.88 | 477.71 | 0.0000241 |
| ***Slc7a12*** | -2.78 | 1160.07 | 59475.18 | 0.0008 |
| ***Slc9a9*** | 3.34 | 69.07 | 187.40 | 0.0004 |
| ***Slco3a1*** | 2.41 | 16844.62 | 548.75 | 0.005 |
| ***Snip1*** | -3.69 | 3615.55 | 324.03 | 0.0008 |
| ***Sorcs1*** | -4.47 | 25531.66 | 290.02 | 0.0015 |
| ***Spock2*** | 2.18 | 340.14 | 7.46 | 0.0096 |
| ***Srek1ip1*** | -2.41 | 1795.29 | 1009.90 | 0.0033 |
| ***Sult1a1*** | 2.54 | 72.50 | 377.41 | 0.003 |
| ***Sv2a*** | 2.8 | 709.18 | 26.91 | 0.0008 |
| ***Tas1r1*** | -14.08 | 310.83 | 2179.83 | 7.67E-08 |
| ***Tcaim*** | -2.58 | 182.28 | 3902.01 | 0.0000365 |
| ***Ten1*** | -6.26 | 173.65 | 1251.98 | 3.87E-08 |
| ***Thumpd1*** | 4.48 | 2702.35 | 962.07 | 0.0003 |
| ***Tmed6*** | -13.17 | 1820.35 | 249.00 | 5.16E-09 |
| ***Tmem169*** | 4.85 | 3125.78 | 15.89 | 0.0006 |
| ***Tmem207*** | -9.23 | 89.26 | 2503.97 | 0.000000258 |
| ***Tmem252*** | 6.76 | 37640.55 | 265.03 | 0.0000674 |
| ***Tmem53*** | -2.39 | 89.88 | 439.59 | 0.0004 |
| ***Topaz1*** | -7.49 | 1200.98 | 232.32 | 0.00000102 |
| ***Tspan4*** | 2.14 | 1937.53 | 174.85 | 0.0055 |
| ***Tspyl4*** | -3.17 | 1152.06 | 317.37 | 0.0000348 |
| ***Ttc21b*** | -2.61 | 171.25 | 4608.24 | 0.0001 |
| ***Ttyh2*** | -4.25 | 408506.64 | 1112.82 | 0.000045 |
| ***Ugt2b7*** | 2.69 | 1160.07 | 58.49 | 0.002 |

| | | | | |
|---|---|---|---|---|
| ***Usp2*** | 10.61 | 519.15 | 296.11 | 0.0059 |
| ***Vrk2*** | -2.06 | 51776.15 | 33.59 | 0.0058 |
| ***Wdr46*** | -4.28 | 556.41 | 3104.19 | 0.0000348 |
| ***Wdr86*** | -9.21 | 792.35 | 168.90 | 0.0001 |
| ***Zfp819*** | 2.37 | 1710.26 | 206.50 | 0.0005 |