Roles of sex phenotype and sex chromosome dosage in sex-biased DNA methylation

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Abstract

Females and males have different sets of sex chromosomes and produce different levels of gonadal sex hormones. They also differ in many aspects of their anatomy and physiology, susceptibility to disease, drug metabolism, gene expression levels, and DNA methylation patterns. The liver is a sexually dimorphic organ showing major sex differences in steroid and drug metabolism profiles, as well as sex differences in gene expression and DNA methylation.

We have recently generated a catalogue of sex-biased differentially methylated regions (sDMRs) for mouse liver and demonstrated that sex-chromosome complement and the sex phenotype influence autosomal DNA methylation. The mechanisms by which the Y chromosome and the sex phenotype influenced DNA methylation remained unclear. In this project, we had two aims: first, to understand how the Y chromosome influences DNA methylation, and second, how the sex phenotypes influence DNA methylation.

For our first aim, we hypothesized that Y-chromosome dependent sDMRs were specific for the B6.TIR strain, and genetic variation in the Y chromosome between B6.TIR and C57BL/6J (B6) influenced DNA methylation. To test this hypothesis, we compared the methylation levels of two Y-chromosome dependent sDMRs in B6 and B6.TIR animals. We also compared the expression of Y-linked genes that are expressed in the liver between the two strains.

For our second aim, we hypothesized that androgen (testosterone) and estrogen (estradiol) signaling influenced DNA methylation through their receptors (androgen and estrogen receptors, respectively). To test this hypothesis, we examined the age dynamics of sex-biased methylation in B6 male and female mice, true hermaphrodites, and sex-reversed XY females. We also tested sex-biased methylation in the liver of androgen receptor knockout (ARKO) and estrogen receptor 1 knockout (ESR1KO) mice.

Liver DNA samples were extracted from (1) B6 female and male mice at three different ages: embryonic day 14.5 (E14.5), 4 weeks, and 8 weeks after birth; (2) B6.TIR mice: XY males, hermaphrodites, and sex-reversed females, as well as XX females at 8 and 16 weeks of age; (3) XY ARKO and wild type littermates at 10-11 weeks of age; (4) homozygous and heterozygous ESR1KO mice and wild type littermates at 8 weeks of age. We examined DNA methylation levels using targeted pyrosequencing methylation assays at six sex-phenotype dependent sDMRs that were selected for their association with sex-biased expression: three with higher expression and lower methylation in male livers (male-biased sDMRs) and three

with higher expression and lower methylation in female livers (female-biased sDMRs). To test if the timing of sex-biased methylation coincides with sex-biased gene expression, we performed RT-qPCR for two male-biased and two female-biased genes.

We show that Y-chromosome dependent sDMRs are specific to the B6.TIR strain and Y-linked genes may contribute to the TIR-specific sex-biased methylation. We show that sex bias in DNA methylation and gene expression varies with age. At eight weeks, hermaphrodites showed either intermediate or female-like methylation levels, and they established a male-like methylation level at 16 weeks. Our results suggest that the presence of an ovary and a testis in the same mouse affects DNA methylation in an age-dependent manner. AR loss in male mice leads to the feminization of DNA methylation at sDMRs. Loss of ESR1 changes methylation levels of both females and males at male-biased sDMRs, and only females at female-biased sDMRs.

In summary, we demonstrate that signaling of both estrogen and testosterone through their receptors contributes to sex-biased methylation in the mouse liver.

Résumé

Les mâles et les femelles ont des chromosomes sexuels différents et produisent des niveaux différents d'hormones sexuelles. Ils diffèrent aussi au niveau de l'anatomie, la physiologie, le métabolisme des médicaments, l'expression des gènes et aussi les patrons de méthylation d'ADN.

Nous avons récemment généré un catalogue de régions du génome dont les patrons de méthylation diffèrent entre mâles et femelles (sDMRs) chez le foie de souris et nous avons démontré que les chromosomes sexuels ainsi que le phénotype sexuel influencent la méthylation de l'ADN autosomique. Le mécanisme par lequel le chromosome Y et le phénotype sexuel impactent la méthylation de l'ADN demeure peu connu. Ce projet comportait deux objectifs : le premier, comprendre comment la méthylation de l'ADN est influencée par le chromosome Y et le deuxième, comment elle est affectée par le phénotype sexuel.

Pour le premier objectif, nous avons posé l'hypothèse que les sDMRs dépendants du chromosome Y étaient spécifiques à la souche de souris B6.TIR et que la variation génétique du chromosome Y entre les souches B6.TIR and C57BL/6J (B6) influençait la méthylation de l'ADN. Pour tester cette hypothèse, nous avons comparé les niveaux de méthylation de deux sDMRs dépendants du chromosome Y chez les souches B6 et B6.TIR. Nous avons aussi comparé l'expression de deux gènes liés au chromosome Y dans le foie des deux souches de souris.

Pour le deuxième objectif, nous avons posé l'hypothèse que les voies de signalisation des androgènes (testostérone) et des œstrogènes (estradiol) influencent la méthylation de l'ADN via les récepteurs des androgènes et des œstrogènes, respectivement. Cette hypothèse fut testée en examinant la dynamique de la méthylation spécifique au sexe en fonction de l'âge des souris de souche B6 males, femelles, hermaphrodites et avec réversion du sexe XY. Nous avons aussi étudié la méthylation spécifique au sexe chez le foie de la souris knock-out pour le récepteur des androgènes (ARKO) et pour le récepteur des œstrogènes (ESR1KO).

L'ADN fut extrait du foie des souches de souris suivantes: (1) B6 mâle et femelle au jour embryonnaire 14.5, à 4 semaines et à 8 semaines; (2) B6.TIR males, femelles, hermaphrodites, avec réversion du sexe XY, âgées de 8 et 16 semaines; (3) knockout ARKO XY et les types sauvages de la même portée, âgées de 10-11 semaines et (4) ESR1KO homozygotes, hétérozygotes et les types sauvages de la même portée, âgées de 8 semaines. Nous avons utilisé le pyroséquencage ciblé pour examiner les niveaux de méthylation d'ADN

de six sDMRs sélectionnés : trois faiblement méthylés et fortement exprimés dans le foie du mâle (spécifiques au male) et trois faiblement méthylés and fortement exprimés dans le foie de la femelle (spécifiques à la femelle). Nous avons ensuite vérifié si les patrons de méthylation coïncidaient avec ceux de l'expression pour deux gènes spécifiques à la femelle et deux spécifiques au mâle, par PCR quantitatif en temps réel (RT-PCR).

Nous avons démontré que les sDMRs dépendants du chromosome Y sont spécifiques à la souche B6.TIR et que les gènes lies au chromosome Y peuvent contribuer à cette spécificité. Nous avons montré que les patrons de méthylation et d'expression des gènes étudiés varient en fonction de l'âge des souris. A huit semaines, les hermaphrodites ont un niveau de méthylation intermédiaire, similaire aux femelles, qui devient ensuite semblable au niveau des mâles, à 16 semaines. Ces résultats suggèrent que la présence d'un ovaire et d'un testicule chez la même souris affecte la méthylation de l'ADN différemment en fonction de l'âge, la perte du récepteur AR chez le mâle entraînant la féminisation du patron de méthylation aux sDMRs. La perte du récepteur ESR1 semble aussi modifier les niveaux de méthylation aux sDMRs spécifiques aux mâles chez les deux sexes, alors que les sDMRs spécifiques aux femelles sont affectés seulement chez les femelles.

En résumé, nous avons démontré que les voies de signalisation des androgènes et des œstrogènes, de par leur récepteur respectif, contribuent aux patrons de méthylation spécifiques à chaque sexe.

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List of Abbreviations

5caC: 5-carboxycytosine

5fC: 5-formylcytosine

5hmC: 5-hydroxymethylcytosine

5mC: 5-methylcytosine

A4: androstenedione

ACTH: adrenocorticotrophic hormone

AIS: androgen insensitivity syndrome

Aldh3b3: aldehyde dehydrogenase 3 family, member B3

ANOVA: analysis of variance

AR/*Ar*: androgen receptor gene

AR: androgen receptor protein

ARE: androgen receptor response elements

ARKO: androgen receptor knockout

B6: C57BL/6J

Bcl6: B cell leukemia/lymphoma 6

bp: base pair

Caprin1: cell cycle associated protein 1

cDNA: complementary DNA

CGIs: CpG islands

COMT/*Comt*: catechol O-methyl transferase

CpG: 5' – cytosine – phosphate – guanine – 3'

CTD: carboxyl-terminal domain

Cux2: cut like homeobox 2 gene

CUX2: cut like homeobox 2 protein

CYP: cytochromes P450

Cyp2b9: cytochrome P450, family 2, subfamily b, polypeptide 9

Cyp7b1: cytochrome P450, family 7, subfamily b, polypeptide 1

DBD: DNA-binding domain

Ddx3y: DEAD box helicase 3, Y-linked

DHEA: dehydroepiandrosterone

DHEA-S: dehydroepiandrosterone sulfate

DHT: dihydrotestosterone

DME: drug metabolizing enzymes

DMR: differentially methylated region

DNA: deoxyribonucleic acid

DNMT1: DNA methyltransferase 1

Dnmt1: DNA methyltransferase 1 gene

DNMT3A: DNA methyltransferase 3A

Dnmt3a: DNA methyltransferase 3A gene

DNMT3B: DNA methyltransferase 3B

Dnmt3b: DNA methyltransferase 3B gene

DNMT3L: DNA methyltransferase 3L

DNMTs: DNA methyltransferase enzymes

E: embryonic day

E_1: estrone

E_2: 17β-estradiol

E_3: estriol

E_4: estetrol

ERE: estrogen receptor response elements

Ergic1: endoplasmic reticulum-Golgi intermediate compartment

ESC: embryonic stem cell

ESR: estrogen receptor proteins

ESR1/*Esr1*: estrogen receptor 1 (alpha) gene

ESR1: estrogen receptor 1 (alpha) protein

ESR1KO: estrogen receptor knockout

ESR2/*Esr2*: estrogen receptor 2 (beta)

Fez2: fasciculation and elongation protein zeta 2

FMO: flavin-containing monooxygenases

Foxa1: forkhead box A1 gene

FOXA1: forkhead box A1 protein

Foxl2: forkhead box L2

FSH: follicle-stimulating hormone

GH: growth hormone

GnRH: gonadotropin-releasing hormone

GPR30: membrane-bound receptor G protein-coupled receptor 30

GST: glutathione S-transferases

Gstp1: glutathione S-transferase, pi 1

H: hinge region

H3K3me3: trimethylation of histone H3 at lysine 4

HPA: hypothalamic-pituitary-gonadal

Hsd3b5: hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 5

Kdm5d: lysine (K)-specific demethylase 5D

KF: klinefelter syndrome

LBD: ligand-binding domain

LH: luteinizing hormone

MBP: Methyl-CpG-binding proteins

mRNA: messenger RNA

NAFLD: non-alcoholic fatty liver disease

NR: nuclear receptor

NTD: amino-terminal domain

P: postnatal day

Paf: patchy fur

PCR: polymerase chain reaction

PGC: primordial germ cell

Pgk1: phosphoglycerate kinase 1

PRL: prolactin

RNA: ribonucleic acid

RNA-seq: RNA sequencing

Rpl19: ribosomal protein L19

Rspo1: R-spondin 1 gene

RSPO1: R-spondin 1protein

RT-qPCR: quantitative reverse transcription PCR

SAM: s-adenosyl methionine

sDEG: sex-biased differentially expressed gene

sDMR: sex-biased differentially methylated region

Shbg: sex-hormone binding globulin gene

SHBG: sex-hormone binding globulin protein

Snrpn: small nuclear ribonucleoprotein N

Sox9: Sry-box transcription factor 9

Sry: sex determining region of Chr Y

SULT: sulfotransferases

Tet: ten-eleven translocation

TET1: tet methylcytosine dioxygenase 1

TET2: tet methylcytosine dioxygenase 2

TET3: tet methylcytosine dioxygenase 3

TF: transcription factor

TIR: Tirano

TS: Turner syndrome

TSH: thyroid-stimulating hormone

TSS: transcription start site

UGT: uridine diphosphate glucuronosyltransferases

Uty: ubiquitously transcribed tetratricopeptide repeat containing, Y-linked

WGBS: whole-genome bisulfite sequencing

Wnt4: wingless-type MMTV integration site family, member 4 gene

WNT4: wingless-type MMTV integration site family, member 4 protein

XCI: X-chromosome inactivation

Xist: inactive X specific transcripts

XO.F: female with monosomy X

XX.FT: XX female from the Tirano cross

XX^{Paf}F: XX^{Paf} female

XY.FT: XY^{TIR} sex-reversed female

XY.HT OV+T: XY^{TIR} hermaphrodite with one testis and one ovary

XY.HT S+T: XY^{TIR} hermaphrodite with one testis and a streak gonad

XY.HT: true XY^{TIR} hermaphrodite

XY.MT: XY^{TIR} male

Zfy1: zinc finger protein 1, Y-linked

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Format of the thesis

This is a traditional format thesis that follows the traditional format thesis guidelines of the Department of Human Genetics at McGill University. This thesis is composed of six chapters. **Chapter I** is an introduction of the relative background for the thesis and contains the hypotheses and objectives of my project. **Chapter II** contains the materials and methods used in the project. **Chapter III** describes the results. **Chapter IV** provides a discussion of the main findings presented. **Chapter V** contains the conclusions and discusses future directions. **Chapter VI** has a list of references in the entire thesis.

Contribution of Authors

All the experiments presented in this thesis were performed and written by the candidate, except for the following contributions:

The abstract for this thesis was translated into French by Josée Martel.

The B6.TIR and *Paf* mice were provided by Dr. Teruko Taketo (Department of Surgery, McGill University, Canada).

The liver DNA samples of androgen receptor knockout mice and wild type littermates were provided by Dr. Rachel Davey (Department of Medicine, University of Melbourne, Australia).

RNA samples from the liver of estrogen receptor knockout mice and wild type littermates were extracted by another member of the Naumova lab, Klara Bauermeister. RT-qPCR for *Elovl3* in the liver of B6.TIR mice in **Figure 3.6A** was performed by Klara Bauermeister as well.

Chapter I: Introduction

1.1. Sexual dimorphism

Sexual dimorphism historically was understood as the morphological differences between females and males of the same species. Currently, the term sexual dimorphism refers to all sex differences on both molecular and phenotypic levels. Sex is the biological characteristics of females and males. The concept of 3G sex consists of genetic, gonadal, and genital sex. In normal development, mammalian females have XX as their genetic sex, ovaries as their gonadal sex, and female external genitalia. Mammalian males have XY genetic sex, testes as their gonadal sex, and male external genitalia.

1.1.1. Sex determination and gonadal formation

The mammalian sex determination and development start at fertilization, where the genetic sex of the zygote is established as XX or XY (Ford et al., 1959). This genetic sex leads to the differentiation of primitive gonads (bipotential) into ovaries or testes. The commitment of XY gonads toward the male pathway depends on activation of the testis pathway and repression of the ovary pathway, while in the XX gonads, the ovarian fate is mostly driven by activation of the ovary pathway and female-promoting genes (Munger et al., 2013). Finally, the sex phenotype of the fetus develops mainly due to gonadal hormones (Gardiner & Swain, 2015).

The sex-determining region of chromosome Y (*Sry*) gene residing on the Y chromosome is responsible for the initiation of testes determining mechanisms in mammals (Koopman et al., 1991; Pannetier et al., 2006; Sinclair et al., 1990). SRY is a transcription factor that binds to downstream target regions and genes, thus regulating sex determination (Goodfellow & Lovell-Badge, 1993; Harley et al., 1992). In mice, *Sry* is expressed in the XY gonads around embryonic day (E) 11, peaks at E12, and starts declining at E13 (Gubbay et al., 1990; Hacker et al., 1995; Jeske et al., 1995; Koopman et al., 1990; Stévant, Neirijnck, et al., 2018). This is in line with the testicular growth rate, as it increases around E12 (Palmer & Burgoyne, 1991a). SRY activates the expression of Sry-related HMG box-containing gene 9 (*Sox9*) that leads to the differentiation of Sertoli cells (Sekido et al., 2004; Sekido & Lovell-Badge, 2008). Activation of *Sox9* is sufficient for testicular development in the absence of *Sry* (Qin & Bishop, 2005; Vidal et al., 2001). Sertoli cells then regulate the differentiation of other male-specific cell lineages in the gonad (Palmer & Burgoyne, 1991b; Swain & Lovell-Badge, 1999).

The fetal testis produces anti-Müllerian hormone that prevents the formation of female genitalia, and insulin-like factor 3 and testosterone that induce the differentiation of the male phenotype (external and internal gonads) (Giuili et al., 1997; Mahendroo et al., 2001; Zimmermann et al., 1999).

Female sex determination is less well understood. There are few identified genes required for the initiation of ovary development, such as wingless-type MMTV integration site family, member 4 (*Wnt4*), R-spondin 1 (*Rspo1*), and forkhead box L2 (*Foxl2*) (Loffler et al., 2003; Smith et al., 2008). WNT4 and RSPO1 are signaling molecules involved in the canonical β-catenin signaling pathway that is required for ovarian development (Chassot et al., 2008; Liu et al., 2009; Maatouk et al., 2008; Smith et al., 2008). *Wnt4* is expressed in the bipotential gonad before it becomes female-specific around E12.5 (Vainio et al., 1999). Disruption of *Wnt4* expression in the XX gonad partially initiates testis development and does not completely block ovarian development (Jeays-Ward et al., 2003; Kim et al., 2006; Yao et al., 2004). A similar mouse phenotype is seen when *Rspo1* expression is disrupted (Chassot et al., 2008; Tomizuka et al., 2008). Female-specific genes are upregulated in pregranulosa cells between E12.5 and E16.5 (Stévant, Kühne, et al., 2018). The differentiation of granulosa cells of the ovary is then completed postnatally (Pepling et al., 2010). The fetal ovary does not produce estrogen in culture (Weniger, 1993). However, recent observations show possible estrogen synthesis in the fetal ovaries and the possibility that both maternal and fetal estrogen regulate ovary development (Dutta et al., 2014).

1.1.2. Sex-related hormone signaling

1.1.2.1. Androgens

Androgens are lipophilic steroid hormones synthesized from cholesterol, mainly in the gonads (testes and ovaries) and adrenal gland (Henley et al., 2005). The main androgens are testosterone, dihydrotestosterone (DHT), and androstenedione (Hall, 2010). Testosterone is synthesized in the Leydig cells of the testes (Lipsett et al., 1966). Testosterone is converted to the more potent androgen, dihydrotestosterone, by the enzyme 5α-reductase (Andersson et al., 1989). This conversion occurs in the prostate, liver, skin, and brain. During embryogenesis, both testosterone and DHT stimulate the formation of internal and external male genitalia (Jansson et al., 1985). Testosterone directs the differentiation of Wolffian ducts into the epididymis, seminal vesicles, and vas deferens (Morohashi et al., 2013). DHT is involved in urogenital sinus and urogenital tubercle differentiation into the penis and prostate (Morohashi

et al., 2013). In addition, testosterone can be aromatized to estrogen and act through the estrogen receptor (Bulun et al., 2004). The aromatase enzyme is active in the testes, ovaries, brain, adipose tissue, and bone in humans and is restricted to the brain and gonads in rodents (Rommerts et al., 1982; Simpson, 2004; Stocco, 2008). In humans, almost 98% of testosterone found in plasma is bound to albumin or sex-hormone binding globulin (SHBG) that is mainly secreted from the liver (Baker, 2002; Laurent et al., 2016; Rosner et al., 1991; Södergard et al., 1982). In rodents, *Shbg* is expressed in the testes, but hepatic *Shbg* expression and SHGB secretion are lacking (Gunsalus et al., 1978; Sullivan et al., 1991). This is thought to be the reason for low and fluctuating plasma testosterone in rodents (Jänne et al., 1998; Laurent et al., 2016).

In humans, during the first trimester of gestation, androgens are important for the formation of fetal male genitalia (Kuiri-Hänninen et al., 2014). The placenta secretes chorionic gonadotropin hormone that stimulates testosterone production by the fetal testes (Abdallah et al., 2004). The fetal and neonatal exposure to testosterone is essential for male brain masculinization (Breedlove et al., 1982; Döhler et al., 1984; Simerly et al., 1985). After birth, testosterone level remains high in the infant plasma for a few weeks before it decreases and remains low until puberty (Andersson et al., 1998; Forest et al., 1974, 1976). During puberty, the pituitary gland secrets gonadotropic hormone that stimulates tissue development and maturation of testes to produce androgens and sperm (for more details, please refer to section 1.1.2.6).

In mice, the Leydig cells secret testosterone shortly after their differentiation around E13. The plasma testosterone surges to higher levels at three time points (Clarkson & Herbison, 2016; Pointis et al., 1979, 1980) (**Figure 1.1**). The first testosterone surge occurs around E16-17 and gradually decreases until birth (**Figure 1.1**). The second testosterone surge occurs soon after birth, and as mentioned before, it's important for brain masculinization (Corbier et al., 1992). Finally, the largest surge is at puberty, and it continues through adulthood (Pointis et al., 1979, 1980) (**Figure 1.1**).

The above data show that androgens have roles in males' sexual differentiation and are important throughout their development. Androgen has two modes of action to exert its phenotypic effect, genomic and non-genomic. In the non-genomic action, androgens bind to androgen receptor protein and initiate cellular events (e.g., protein phosphorylation) by rapid induction of 2nd messenger pathways without binding to DNA (Foradori et al., 2008; Gill et al., 2004). The genomic action of androgen occurs by binding to androgen receptor protein that

binds to DNA and modulates gene transcription (Beato, 1989; Brinkmann, 2011; De Vos et al., 1991; Rennie et al., 1993; Roy et al., 1998).

1.1.2.2. Androgen receptor (AR)

The androgen receptor (*AR*) gene is located on the X chromosome (in humans in chromosomal region Xq11-12), and it is highly conserved across mammals (Spencer et al., 1991). *AR* is widely expressed in a variety of organs, both reproductive and non-reproductive (Fagerberg et al., 2014; Yue et al., 2014).

The AR shares the common structure of steroid nuclear receptors (NR) that consist of five major domains: a variable amino-terminal domain (NTD), a highly conserved DNA-binding domain (DBD) that contains two zinc fingers, a less conserved ligand-binding domain (LBD), a hinge region (H) that connects the DBD to the LBD, and a carboxyl-terminal domain (CTD) that varies in length between different members of the nuclear receptors (Kumar & Thompson, 1999) (**Figure 1.2**). The two zinc fingers recognize specific DNA sequences, termed androgen response elements (ARE) (5′-AGAACAnnnTGTTCT-3′), and facilitate the binding of AR to its target genes (Nelson et al., 2002). Cytoplasmic AR binds to testosterone or DHT, and a conformational change occurs, allowing the receptor to translocate from the cytoplasm to the nucleus, where it binds to AREs and regulates transcription (MacLean et al., 1997; Shang et al., 2002; van Royen et al., 2012; Wang et al., 2005). DHT has a higher activation potential of AR than testosterone (Grino et al., 1990).

Mutations in *AR* cause a wide range of sexual differentiation impairments in genetic males, clinically known as androgen insensitivity syndrome (AIS) (Brinkmann, 2001; Lobaccaro et al., 1995; McPhaul et al., 1991, 1993). Individuals with complete androgen insensitivity syndrome have XY genotype and develop external female genitalia (Brinkmann, 2001).

1.1.2.2.1. Androgen receptor knockout mouse model

To better understand AIS and the roles of AR, global and tissue- or time- specific androgen receptor knockout (ARKO) mouse models have been developed. These mice have a female phenotype (external genitalia), and they don’t develop male organs (vas deferens, epididymis, seminal vesicle, and prostate gland) (Yeh et al., 2002). In these mice, the testis develops, but it is hypoplastic.

Models with frameshift mutations produced AR-null mice that do not express AR (De Gendt et al., 2004; Sato et al., 2004; Yeh et al., 2002). In our study described in this thesis, we focus on a mouse model that carries an in-frame deletion of exon 3 of the *Ar* that encodes the second zinc finger of the DNA binding domain, resulting in loss of DNA binding activity of the AR. The model was generated on a C57BL/6J (B6) background, and the ARKO mice develop a complete androgen insensitivity phenotype (female external genitalia, intra-abdominal small testes, and lower testosterone levels) (Notini et al., 2005).

1.1.2.3. Estrogen

There are four main estrogens: estrone (E_1), 17β-estradiol (E_2), estriol (E_3), and estetrol (E_4). E_2 is the most biologically active estrogen, and it is produced by the granulosa cells of the ovary.

Estrogens are the predominant sex steroid hormones in females. Although they are considered female sex hormones due to their role in developing the female reproductive system and establishing female secondary sexual characteristics, they have other physiological functions in both females and males. Estrogens have a role in regulating lipid homeostasis and carbohydrate, bone mineralization, and cardiovascular functions in both sexes (Gennari et al., 2004; Heine et al., 2000; Jones et al., 2000; Öz et al., 2000; Smith et al., 1994). In males, estrogens are also involved in the reproduction system, specifically germ cell development and maintenance, sexual behavior, and brain masculinization (Pentikäinen et al., 2000; Robertson et al., 1999; Sharpe, 1998).

In humans, ovaries are the main source of estrogen production in premenopausal women. In men, testes synthesize estrogens (Hess et al., 1997). Estrogen is also produced by extragonadal tissues in both sexes, such as adipose tissue, adrenal gland, smooth muscle cells, bone, vascular endothelium, and different parts of the brain (Bruch et al., 1992; Naftolin et al., 1975; Sasano et al., 1999). Like testosterone, most of the plasma estrogen in humans is bound to SHBG or serum albumin (Knochenhauer et al., 1998).

Estrogen levels vary during development. In female mice, plasma estrogen concentration increases around E17.5, declines after birth, and remains low until postnatal day (P) 26 to 30 (Ahima et al., 1997; Bell, 2018; Dutta et al., 2014). After puberty, the level of plasma estrogen fluctuates with the phases of the estrous cycle (Nilsson et al., 2015) (**Figure 1.1**).

1.1.2.4. Estrogen receptor

Most estrogens' actions are mediated through their binding to estrogen receptors (Beato, 1993). There are three estrogen receptors (ESRs): the estrogen receptor alpha 'ERα' (ESR1), estrogen receptor beta 'ERβ' (ESR2), and the membrane-bound receptor G protein-coupled receptor 30 'GPER' (GPR30) (Koike et al., 1987; Kuiper et al., 1996). The ESR1 and ESR2 are encoded by two different genes located on the 6th and 14th chromosomes in humans, respectively (the 10th and 12th chromosomes in mice) (Enmark et al., 1997; Menasce et al., 1993). ESR1 and ESR2 have the five main NR domains (Kumar & Thompson, 1999) (**Figure 1.2)**. Estrogen binds to ESR1 and ESR2, and they form homo- or heterodimers and bind to estrogen receptor response elements (EREs) (the consensus: 5′-GGTCAnnnTGACCT-3′). This is known as the genomic signaling of ESRs (Klein-Hitpaß et al., 1986; Klinge, 2000). ESRs also have non-genomic signaling 'tethering' where ESRs bind to other transcription factors and stabilize their DNA binding. In this mechanism, ESRs do not bind to DNA (Paech et al., 1997; Scholz et al., 1998; Webb et al., 1995). GPER is a seven-transmembrane domain G protein-coupled receptor and is structurally unrelated to ESR1 and ESR2. GPER is located at the cell membrane and endoplasmic reticulum membrane (Zimmerman et al., 2016).

The distribution of ESR1 and ESR2 is different in different tissues and cells. ESR1 is the predominant ESR in the pituitary gland, skeletal muscles, adipose tissue, liver, mammary gland, and uterus (Hamilton et al., 2014; Hewitt et al., 2016; Yue et al., 2014), while ESR2 is the predominant ESR in the ovary, prostate, lung, and cardiovascular system (Harris, 2007; Yue et al., 2014).

1.1.2.4.1. Estrogen receptor 1 knockout mouse model

Mouse models with global deletion of ESR1 were developed in order to understand the physiological and biological influence of ESR1 (Dupont et al., 2000; Hewitt et al., 2010; Lubahn et al., 1993). A mouse model with a deletion in exon 3 of *Esr1* generated *Esr1* knockout (ESR1KO) mice with complete depletion of ESR1 (Hewitt et al., 2010). Female homozygous ESR1KO mice have abnormal ovarian stimulation due to the dysregulation of the hypothalamic-pituitary-ovarian axis, and the homozygous mutant female and male mice are infertile (Couse et al., 2003; Hewitt et al., 2010). They have higher levels of plasma estrogen and testosterone than wild-type females (Hewitt et al., 2010).

1.1.2.5. Peripheral androgen and estrogen synthesis

The adrenal gland in humans secretes inactive C_{19} steroids; dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEA-S), and androstenedione (A4) that are converted to potent estrogen or androgen in peripheral target tissues (Labrie et al., 1997). DHEA and DHEA-S secretion increase between the age of 6 and 8 years in humans, and their elevated circulating levels are maintained through adulthood (Kushnir et al., 2010; Remer et al., 2005). The estrogen and androgen levels in each peripheral tissue depend on the expression levels of the enzymes needed to convert the precursor steroids to estrogen or androgen (e.g., aromatase and hydroxysteroid dehydrogenase, respectively). Rodents' adrenal gland lacks the enzymes necessary to synthesize C_{19} steroids. Thus the main source of sex hormones in rodents is their gonads (van Weerden et al., 1992).

1.1.2.6. Hypothalamic-pituitary-gonadal axis

Development and maintenance of sexual characteristics and the secretion of sex hormones from gonads are regulated by the hypothalamic-pituitary-gonadal (HPA) axis (**Figure 1.3**). In humans, the HPA axis is activated in three periods of life: the midgestational fetus, newborn, and at puberty (Lanciotti et al., 2018).

The hypothalamus produces growth hormone-releasing and growth hormone inhibitory hormones that regulate the release of growth hormones from the pituitary gland (Hall, 2010). It also produces gonadotropin-releasing hormone (GnRH) that regulates luteinizing hormone and follicle-stimulating hormone secretion from the pituitary (Amar & Weiss, 2003).

The pituitary gland secretes peptide hormones, namely, thyroid-stimulating hormone (TSH), corticotropin or adrenocorticotrophic hormone (ACTH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), growth hormone (GH), and prolactin (PRL) (Amar & Weiss, 2003). PRL acts on the breast tissue to stimulates milk production (Hall, 2010). All the other five hormones act on other glands and regulate their secretions (Hall, 2010). The release of GH stimulates protein synthesis, and it’s responsible for the growth of cells and tissues. FSH stimulates granulosa cells in ovaries to produce estrogen, and promotes sperm maturation and the proliferation of the Sertoli cells of testes (Abel et al., 2008; Goodman, 2009; O’Shaughnessy et al., 2012; Oduwole et al., 2018). In females, LH stimulates theca cells to produce A4 and progesterone in ovaries and ovulation (Goodman, 2009). In males, LH stimulates testosterone release by the Leydig cells (Ramaswamy & Weinbauer, 2014).

The secretion of gonadal hormones is regulated by a negative feedback mechanism where the hormone itself prevents its over-secretion (Couse et al., 2003; McCormick et al., 2002) (**Figure 1.3**).

1.1.3. Sexual dimorphism in disease

Females and males show differences in the prevalence, severity, and pathophysiology of many diseases. Men are at higher risk of infectious diseases and arteriosclerosis, while women have higher risk of autoimmune disorders, such as multiple sclerosis and rheumatoid arthritis (Beeson, 1994; Kalin & Zumoff, 1990; Klein, 2000; Klein & Flanagan, 2016; McBride et al., 2005; Muenchhoff & Goulder, 2014; Whitacre et al., 1999). Many neuropsychiatric disorders, such as anxiety and depression, are influenced by sex and gonadal hormones. Women show higher incidence of stress-related physiological complaints like chronic pain and fibromyalgia (Verhaak et al., 1998; Wolfe et al., 1995). Women are more responsive than men to acute stressors, such as CO2 exposure or construction noise, and have higher cortisol levels after exposure (Paris et al., 2010). Changes in estrogen levels during the estrous cycle in rodents and menstrual cycle in humans alter stress response, as higher estrogen levels associate with increased stress response (Carey et al., 1995; McCormick & Teillon, 2001; Shors et al., 1998). On the other hand, the risk of autoimmune diseases such as lupus erythematosus is influenced by X chromosome dosage (Chitnis et al., 2000). Men with Klinefelter syndrome (XXY) have high risk similar to that seen in women (Scofield et al., 2008; Souyris et al., 2018). Sex-reversed female mice (XY) have less severe lupus compared to XX females (Sasidhar et al., 2012).

The prevalence of non-alcoholic fatty liver disease (NAFLD) is greater in white men compared to white women (42% and 24%, respectively) (Browning et al., 2004). It's worth noting that NAFLD is twice as common in postmenopausal women as in premenopausal women, suggesting that sex hormones, most likely estrogen, have a protective role (Lazo & Clark, 2008; Polyzos et al., 2013). This estrogen protective effect is further supported by NAFLD development in women treated with tamoxifen, and the better liver enzyme profiles in postmenopausal women taking estrogen replacement treatment (Bruno et al., 2005; Florentino et al., 2013).

Females and males also show differences in their responses to pharmacological treatment (Alomar, 2014; de Vries et al., 2019; Ekhart et al., 2018; Fattinger et al., 2000; Yu et al., 2016; Zopf et al., 2008). They show differences in both drug pharmacokinetics and pharmacodynamics (Anderson, 2005, 2008). Pharmacokinetics is the absorption, distribution,

metabolism, and excretion of drugs. The sex differences in the drug pharmacokinetics include differences in renal and gastrointestinal blood flow, gastric acid secretion, and percentage of body fat and muscles (Chen et al., 2000; Wahl et al., 2003).

Pharmacodynamics refers to the effects and side effects of drugs. Female sex is considered a risk factor for adverse drug reactions and side effects (Fattinger et al., 2000; Nakagawa & Kajiwara, 2015). Endogenous and exogenous sex hormones can affect pharmacodynamics and pharmacokinetics directly and indirectly, and some drugs modify hormonal signaling pathways (Franconi et al., 2007; Spoletini et al., 2012).

The sex differences in drug metabolism are the primary reason behind sexual dimorphism in pharmacokinetics and pharmacodynamics of the drugs.

1.1.4. Sexual dimorphism in liver metabolism

The liver has many essential functions, including metabolism and breakdown of endogenous substances and xenobiotics and synthesis of blood clotting factors and lipids (cholesterol and triglyceride). The liver is involved in maintaining lipid, glucose, and protein homeostasis (Cherrington, 1999; Ekberg et al., 1999; Klover & Mooney, 2004; Zammit, 2002). The term xenobiotics refers to all extrinsic chemicals found in the organism that are not naturally produced or present in that organism, such as drugs, plant-derived secondary metabolites, pesticides, and environmental pollutants (Juchau & Chen, 1998; Testa & Krämer, 2006).

Drug metabolism is catalyzed by different reactions classified into two phases (Nelson & Gordon, 1983). Phase I includes oxidation, reduction, and hydrolysis, and phase II includes conjugation reactions such as acetylation, sulfation, glucuronidation, methylation, and glutathione conjugation (Brunton et al., 2008). Phase I and II do not necessarily occur in this order. The common families of phase I hepatic enzymes are the cytochromes P450 (CYPs) and flavin-containing monooxygenases (FMOs), whereas N-acetyltransferases (NATs), sulfotransferases (SULTs), uridine diphosphate glucuronosyltransferases (UGTs), catechol O-methyl transferase (COMT), and glutathione S-transferases (GSTs) are phase II enzymes (Brunton et al., 2008; Jancova et al., 2010). Hepatic drug-metabolizing enzymes (DMEs) have a critical role in drug bioavailability and elimination, and the metabolism of endogenous compounds. In humans, sex, age, hormones, genetic polymorphisms, and epigenetic variation, as well as environmental factors, play important roles in shaping interindividual differences in

DME expression and activity (Cotreau et al., 2005; Court et al., 2002, 2004; Daly et al., 1991; Kennedy, 2008; Moon et al., 2006; Murray, 2007; Szyf, 2007).

CYPs are heme proteins that oxidase their substrates and include three major families CYP1, CYP2, and CYP3. The CYPs are involved in metabolism of pharmaceuticals and exogenous chemicals, endogenous bile acid and cholesterol biosynthesis, and steroids and vitamin D3 synthesis and catabolism (Nebert & Russell, 2002). In mice, the hepatic expression of *CYP* family members differs between females and males, partly reflecting sexual dimorphism in metabolism (Waxman, 1988; Waxman & O'Connor, 2006). There are well-characterized examples of male and female predominant liver CYPs, such as the female-biased *Cyp2b9*, and the male-biased *Cyp7b1* (Jarukamjorn et al., 2002). In humans, sex differences in CYPs are documented but are less pronounced than in rodents (Scandlyn et al., 2008; Yang & Li, 2012). Here, I'll give a couple of examples of documented sex differences in CYP enzyme activity that causes a sex difference in therapeutic effects. CYP3A4 is a predominant enzyme for phase I metabolism (Soldin et al., 2011; Waxman & Holloway, 2009; Zanger & Schwab, 2013). Women have higher level of hepatic CYP3A4 protein activity compared to men (Wolbold et al., 2003). The clearance of CYP3A4 substrates (e.g., cortisol) is 20-30% greater in women compared to men (Greenblatt & Von Moltke, 2008; Wolbold et al., 2003). CYP1A2 is the primary enzyme that metabolizes antipsychotic drugs (Pirmohamed et al., 1995). CYP1A2 shows higher activity in men, and they have higher clearance of antipsychotic drugs (Bigos et al., 2008).

The contribution of glucuronidation to drug metabolism is the highest among other phase II reactions (Brunton et al., 2008). There are 22 different UGT enzymes in humans with two main families UGT1A and UGT2B (Mackenzie et al., 2005; Miners et al., 2004). Men have higher rates of glucuronidation compared to women (Court et al., 2004). They also have a higher clearance rate of a widely used analgesic 'acetaminophen' compared to women, which is mainly metabolized by glucuronidation (Cummings et al., 1967; Lowenthal et al., 1976; Miners et al., 1983). The activity of UGT2B15 is 67% higher in the male liver, and the clearance of its substrate oxazepam is 40% higher in males than females (Court, 2010; Court et al., 2002, 2004; Greenblatt et al., 1980; Walkenstein et al., 1964).

COMT catalyzes the transfer of methyl group from S-adenosylmethionine to catecholamines, including the neurotransmitters norepinephrine, epinephrine, and dopamine, and catechol drugs such as Levodopa in treating Parkinson's disease (Guldberg & Marsden,

1975). The hepatic activity of COMT is 30% higher in men compared to women (Boudíková et al., 1990). A polymorphism in *COMT* that results in an amino acid change (valine to methionine) is linked to a decrease in COMT activity by around four fold (Boudíková et al., 1990; Dawling et al., 2001). One major pathway of estrogen metabolism is through the hydroxylation of estrogen to catecholestrogens, which are inactivated by COMT. COMT genotype is significantly correlated with the serum level of estrogen in postmenopausal women receiving hormonal replacement therapy (Worda et al., 2003).

In conclusion, sex differences in the expression and/or the activity of metabolizing enzymes in the liver could lead to the sex difference in metabolic phenotype.

1.1.5. Sexual dimorphism in gene expression

Somatic non-gonadal tissues including peripheral blood, heart, liver, brain, kidney, pancreas, and muscle show sex bias in gene expression (Davegårdh et al., 2019; Hall et al., 2014; Jansen et al., 2014; Kang et al., 2011; Mayne et al., 2016; Melé et al., 2015; Naqvi et al., 2019; Trabzuni et al., 2013; Tullis et al., 2003; Vawter et al., 2004; Waxman & Celenza, 2003; Weickert et al., 2009; Zhang et al., 2011).

The sex-biased gene expression is tissue specific, and the number of genes that are differentially expressed between the sexes differs from one tissue to the other (Yang et al., 2006). Seventy two percent of liver genes and around 14% of brain genes show sex bias in expression (Yang et al., 2006). Genes with sex-biased expression are involved in important biological pathways specific for the organ expressing those genes, such as lipid metabolism in the liver and ATPase activity in the brain (Yang et al., 2006). The sex phenotype and testosterone influence sex-biased gene expression in adult mouse liver (Delić et al., 2010; Gatti et al., 2010; Reizel et al., 2015; Van Nas et al., 2009). On the other hand, the differential gene expression in adult thymus and heart depends on the sex-chromosome complement (Deegan et al., 2019; Wijchers et al., 2010). Sex hormones regulate gene expression in the mesenteric arteries, suggesting that the cardiovascular system is influenced by both sex genotype and sex hormones (Deegan et al., 2019; Eyster et al., 2007). Autosome and sex-chromosome linked genes are differentially expressed between sexes in early development before the influence of hormones based on data from mouse embryonic stem cells and early embryo (8-cell stage) (Lowe et al., 2015; Werner et al., 2017). The effect of sex-chromosome complement on blood gene transcription is seen in humans. Individuals with Turner syndrome (TS) (45,X) and Klinefelter syndrome (KF) (47,XXY) have abnormal sex chromosome numbers. The

transcriptional profile of blood from those individuals is different from karyotypically normal females and males (46, XX, and 46, XY, respectively) (Huang et al., 2015; Manotas et al., 2020; Skakkebæk et al., 2018; Trolle et al., 2016; Zitzmann et al., 2015). The majority of differentially expressed genes between TS vs. females, and KF vs. males are autosomal (Zhang et al., 2020).

DNA methylation and histone modification are important in the regulation of gene transcription (Berger et al., 2009). Therefore, sex bias in gene expression may reflect sex bias in DNA methylation and/or histone modifications. In this work, we focus on the sex bias in DNA methylation and factors that may modify it.

1.2. DNA methylation

The hypothesis that DNA methylation plays a role in the regulation of gene expression was first proposed by (Holliday & Pugh, 1975; Riggs, 1975). They suggested that DNA methylation influenced gene expression by altering the binding of transcription factors to DNA. It is now well established that DNA methylation, along with other epigenetic factors, has a major role in gene regulation. Thus, DNA methylation serves as a non-genetic chemical modification regulating transcription and gene activity (Holliday & Pugh, 1975; Jones, 2012). Since 1975, the study of DNA methylation changed as more technologies evolved to investigate DNA methylation across the genome and its relation to gene transcription.

1.2.1. CpG methylation

DNA methylation is a mitotically heritable epigenetic modification that consists of a covalent addition of a methyl group to the carbon at position five of the cytosine ring of DNA (Hotchkiss, 1948; Wigler et al., 1981). In most mammalian cells, methylation takes place at a cytosine followed by guanine (CpG dinucleotide) (Doskočil & Šorm, 1962; Lister et al., 2009; Zemach et al., 2010). The mammalian genome has less CpG dinucleotides than expected, and they occur at only 25% of the expected frequency in DNA (Josse et al., 1961; Smallwood et al., 2011). The CpG dinucleotides are not evenly distributed throughout the genome. CpG rich genomic regions are termed CpG islands (CGIs), the regions adjacent to CGIs are termed CGI shores (0 to 2 kb up/downstream of CGIs), whereas regions 2 to 4 kb up/downstream of CGIs are termed CGI shelves (Gardiner-Garden & Frommer, 1987). In the mammalian genome, about half of CGIs are not associated with gene promoters and found in intergenic or intragenic regions, and they are termed orphan CGIs (Illingworth et al., 2010). Transposon sequences

(SINE, LINE, and LTR) are abundant in the mammalian genome, and they are densely methylated (Edwards et al., 2017; Lister et al., 2009).

In vertebrates, almost 75% of the annotated gene promoters contain CGIs that are generally low in methylation in somatic cells (Antequera & Bird, 1993; Bird et al., 1985; Deaton & Bird, 2011; Edwards et al., 2010; Lister et al., 2009; Saxonov et al., 2006). The majority of housekeeping genes have CGIs at their promoter regions, as well as a proportion of developmental regulator genes and tissue-specific genes (Cooper & Gerber-Huber, 1985; Larsen et al., 1992; McKeon et al., 1982; Stein et al., 1983; Zhu et al., 2008). Transcription start sites are flanked by nucleosomes marked with trimethylation of histone H3 at lysine 4 (H3K4me3), a histone mark, and the histone variant antagonistic to methyltransferases (H2A.Z). The unmethylated CGIs are substrates for activating transcriptional factors, and they are associated with transcriptional activity (Deaton & Bird, 2011; Weber et al., 2007; Weinmann et al., 2002). On the other hand, methylated CGIs usually associate with long-term stable repression of gene expression, such as in X-chromosome inactivation, cell-specific gene expression, and genomic imprinting (Larsen et al., 1992).

DNA methylation levels in CGI shores and shelves are dynamic (Irizarry et al., 2009; Wang et al., 2013). CGI shores act as enhancers, and they are involved in tissue-specific methylation associated with gene expression (Ehrlich et al., 1982; Li & Zhang, 2014).

Recent evidence suggests that DNA methylation in gene bodies is involved in transcriptional elongation and can impact splicing (reviewed in (Jones, 2012)). Methylation in gene bodies positively correlates with gene expression, as shown by studying genes on the active X-chromosome (Hellman & Chess, 2007).

1.2.2. CpG methylation outside CGIs; in enhancers

DNA methylation can alter the activity of regulatory elements such as enhancers and insulators. The methylation patterns at enhancers vary depending on cell type and the developmental time. Several observations suggest the association of DNA methylation and enhancer activity (Saluz et al., 1986; Wiench et al., 2011). Worth noting, DNA methylation of enhancer regions shows a stronger association with gene regulation than DNA methylation of promoters in cancer cells (Aran et al., 2013). It has been shown that regions with low methylation (30%) in mouse embryonic stem cells (ESCs) are usually distal regulatory regions (Stadler et al., 2011). Those regions undergo demethylation by binding to transcription factors (TFs) (Stadler et al., 2011). However, the relationship between methylation and transcription

factor binding is complicated. DNA methylation inhibits gene expression by preventing activator TFs from binding to their target sequences and/or recruiting repressor proteins, such as Methyl-CpG-binding proteins (MBPs), leading to gene-silencing (Campanero et al., 2000; Iguchi-Ariga & Schaffner, 1989; Watt & Molloy, 1988).

1.2.3. Non-CpG methylation

In mammals, non-CpG (CpH; H is A, C, or T) methylation is found in adult glia and neurons, ESCs, pluripotent stem cells generated by induced pluripotent stem cells (iPSC) and somatic cell nuclear transfer stem cells, and oocytes (Guo et al., 2014; Lister et al., 2009, 2011, 2013; Ma et al., 2014; Tachibana et al., 2013; Tomizawa et al., 2011). CpH methylation is found to a lesser extent in adipose tissue, skeletal muscle tissue, adrenal gland, gastric system, and the heart (Barres et al., 2013; Barrès et al., 2009; Schultz et al., 2015; Varley et al., 2013). Upon differentiation of ESCs, methylation at CpH decreases dramatically, and it can be restored by stem cell reprogramming (Ziller et al., 2011). In neurons, CpH methylation accumulates with age as synaptogenesis progresses (Lister et al., 2013). Thus, CpH methylation has a cell-specific regulation that does not depend on CpG methylation.

Because the project studies DNA methylation in the liver where CpH methylation is uncommon, the following sections focus on CpG methylation.

1.2.4. DNA methyltransferases

DNA methylation is established and maintained by DNA methyltransferases (DNMTs). There are five DNMTs in humans: DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L. DNMT1, DNMT3A, and DNMT3B are canonical methyltransferases that catalyze DNA methylation by transferring methyl groups from S-adenosyl methionine (SAM) to the fifth carbon of the cytosine ring (Fatemi et al., 2002; Wu & Santi, 1985, 1987). DNMT2 and DNMT3L are non-canonical methyltransferases, and they do not possess catalytic DNMT activity.

DNMT2 has a weak DNA methyltransferase activity in vitro (Hermann et al., 2003). However, deletion of *DNMT2* in ESC did not affect global DNA methylation, suggesting that DNMT2 has little involvement in establishing DNA methylation patterns (Masaki Okano et al., 1998). DNMT2 has a methyltransferase activity toward cytosine of RNA (Goll et al., 2006; Schaefer et al., 2010).

DNMT3A and DNMT3B function in *de novo* DNA methylation (*de novo* methyltransferases) (Okano et al., 1998). *Dnmt3a* and *Dnmt3b* are expressed at high levels during embryonic development and in mouse embryonic stem cells (Chen et al., 2003; Masaki Okano et al., 1999). *Dnmt3a* knockout mice appear normal at birth but die at four weeks (Masaki Okano et al., 1999). In contrast, there are no viable *Dnmt3b* knockout mice at birth (Masaki Okano et al., 1999). The double knockout of *Dnmt3a/Dnmt3b* in ESCs completely lacked *de novo* methylation, and the embryos died before E11.5 (Masaki Okano et al., 1999).

DNMT1 acts as a maintenance methyltransferase for hemi-methylated DNA (Bestor, 1992) and also has *de novo* methyltransferase activity (Gowher et al., 2005; Liang et al., 2002). *Dnmt1* expression is activated by cell-cycle dependent regulators and is present at high levels in proliferating cells (Kishikawa et al., 2003). DNMT1 is found in the developing embryo cells and the nuclei of the postimplantation embryo's cells (Trasler et al., 1996). The homozygous mutation of *Dnmt1* causes a reduction in DNA methylation in embryonic stem cells and embryo lethality before midgestation (Li et al., 1992). *Dnmt1* null embryos show reduced global DNA methylation, loss of imprinting, defects in X inactivation, and activation of retrotransposons (Beard et al., 1995; Dahlet et al., 2020; Min et al., 2020; Panning & Jaenisch, 1996; Sado et al., 2000; Walsh et al., 1998). DNMT1O is an oocyte-specific DNMT1 isoform that lacks 118 amino acids of the N-terminal domain of somatic DNMT1 and is found in the cytoplasm of mature oocytes and preimplantation embryos, and the nuclei of 8-cell embryos (Carlson et al., 1992; Mertineit et al., 1998; Ratnam et al., 2002). DNMT1O acts to maintain CpG methylation of imprinted genes in the early embryo (8-cell stage) (Cirio et al., 2008).

DNMT3L is physically associated with DNMT3A and DNMT3B and guides their *de novo* DNA methylation activity (Suetake et al., 2004). *Dnmt3l* is expressed in high levels in developing germ cells, early embryo, and embryonic stem cells (Hata et al., 2002). DNMT3L regulates the establishment of maternal imprinting during gametogenesis (Bourc'his et al., 2001). DNMT3L recognizes unmethylated histone H3 at lysine 4 and recruits DNMT3A2, which will catalyze DNA methylation (Ooi et al., 2007). Female mice deficient in DNMT3L produce non-viable embryos due to aberrant acquisition of DNA methylation during oogenesis, while males are infertile (Bourc'his et al., 2001).

1.2.5. DNA demethylation

The ten-eleven translocation proteins (TET) catalyze the oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), and oxidize 5hmC to the unstable 5-

formylcytosine (5fC) and 5-carboxycytosine (5caC) (He et al., 2011; Tahiliani et al., 2009). The oxidized form of cytosine can be further removed passively through DNA replication or by DNA repair mechanisms. TET proteins in mammalian cells include TET1, TET2, and TET3. The three TET enzymes share a similar catalytic function, but they differ in their expression and activity in different tissues (Zhu et al., 2020). The expression of *Tet1* and *Tet2* is shown during pre-implantation development (blastocyst) and in ESC, whereas *Tet3* is expressed in the oocyte and zygote, but decreases in the blastocyst (Gu et al., 2011; Iqbal et al., 2011; Wossidlo et al., 2011). *Tet2* and *Tet3* expression increases during differentiation of the germ layer (Rasmussen & Helin, 2016). *Tet2* and *Tet3* are expressed in a wide range of adult organs, including the liver and the central nervous system (Dawlaty et al., 2013; Yue et al., 2014).

TET proteins have diverse roles in biological processes such as embryonic development by regulating DNA methylation reprogramming (discussed in section 1.2.6.).

1.2.6. Roles of DNA methylation during development

DNA methylation has a key role in different processes, including X-chromosome inactivation, genomic imprinting, and transcriptional repression of retrotransposons (Hackett et al., 2012; Reik, 2007).

X-chromosome inactivation (XCI) in mammals is a process where one X chromosome is silenced in female somatic cells during early embryonic development (Lyon, 1961). XCI balances X-linked gene dosage between female (two X-chromosomes) and male (one X-chromosome) cells. In humans, 15-25% of X-linked genes escape XCI, while in mice, only 3-7% escape XCI (Balaton et al., 2015; Berletch et al., 2010, 2015; Peeters et al., 2018).

In both mice and humans, the primary regulator of XCI is the long non-coding RNA X-inactive specific transcript (*Xist/XIST*) expressed by the inactive X chromosome (Brockdorff et al., 1991; Brown et al., 1991; Penny et al., 1996). XCI is associated with specific DNA methylation patterns (Mohandas et al., 1981). Chromatin modifications and DNA methylation contribute to the maintenance of XCI and repression (Blewitt et al., 2008; Csankovszki et al., 2001; Hernández-Muñoz et al., 2005).

Genomic imprinting is a phenomenon where imprinted genes are expressed from only one parental allele, either the maternal or paternal (Morison et al., 2005). DNA methylation is associated with the establishment and maintenance of genomic imprinting (Bourc'his et al.,

2001; Caspary et al., 1998; Hata et al., 2002; Hirasawa et al., 2008; Kaneda et al., 2004). Loss of DNMT1 leads to silencing or biallelic expression of imprinted genes (Li et al., 1993).

The epigenome undergoes reprogramming in primordial germ cells (PGCs) and early embryos (Smith et al., 2012). This includes erasure of DNA methylation and reprogramming of histone modifications (Guibert et al., 2012; Hajkova et al., 2002, 2008; Monk et al., 1987; Rougier et al., 1998; Seki et al., 2005; Sekl et al., 2007).

PGCs undergo genome-wide DNA demethylation from E8 to E13.5 (Guibert et al., 2012; Seisenberger et al., 2012). After PGCs reprogramming, the establishment of DNA methylation in germ cells is sex-specific. The process of remethylation of imprinted regions in female oocytes occurs during the oocyte growth phase after birth (Gahurova et al., 2017; Hiura et al., 2006; Kono et al., 1996; Lucifero et al., 2002, 2004; Seisenberger et al., 2012; Walsh et al., 1998). In contrast, in male germ cells, methylation of imprinted regions is initiated in prospermatogonia (E15 to E16) and maintained through many mitotic division cycles (Coffigny et al., 1999; Davis et al., 2000; Kafri et al., 1992; Lees-Murdock et al., 2003; Seisenberger et al., 2012; Ueda et al., 2000).

The second wave of DNA methylation reprogramming occurs during early embryogenesis after zygote formation (Santos et al., 2002). The somatic cells of embryos undergo demethylation that is completed by the blastocyst stage (Howlett & Reik, 1991; Santos et al., 2002; Smith et al., 2012). Demethylation of the paternal genome occurs a few hours after fertilization via an active and rapid process, whereas the maternal genome undergoes passive demethylation through a replication-dependent dilution process (Mayer et al., 2000; Rougier et al., 1998). Unlike demethylation in PGCs, somatic cell demethylation does not affect the DNA methylation of imprinted genes. TET3 mediated oxidation is involved in the demethylation of the paternal genome and, to a lesser degree, the maternal genome (Iqbal et al., 2011; Peat et al., 2014; Tsukada et al., 2015).

1.2.7. Sex bias in DNA methylation

Since DNA methylation affects gene expression that regulates cellular phenotype, sex differences in DNA methylation may result in sexually dimorphic phenotypes. The contribution of DNA methylation and other epigenetic marks to sex differences in gene regulation is relatively undiscovered. Sex difference in DNA methylation is found on both X chromosome and autosomes in different tissues (García-Calzón et al., 2018; Singmann et al., 2015).

Data from human blood show sex differences in DNA methylation (El-Maarri et al., 2007, 2011; Ho et al., 2018; Shah et al., 2014; Singmann et al., 2015). Females have lower DNA methylation levels at repetitive elements compared to males (El-Maarri et al., 2007), and the sex-biased methylation is independent of menstrual cycle or age (El-Maarri et al., 2011). Data from human saliva show that females have higher methylation at X-linked and autosomal CpGs (Liu et al., 2010). In human pancreatic islets, the global autosomal methylation shows no sex difference, however, 470 autosomal CpGs display sex-biased DNA methylation. Out of the 470 CpGs, 18 are associated with sex-biased differentially expressed genes (Hall et al., 2014). Data from human livers show sex-biased DNA methylation at both X chromosome and autosomes, with men having higher methylation at autosomal regions and women having higher methylation at X-chromosome regions (García-Calzón et al., 2018).

In principle, the sex-biased DNA methylation may be influenced by the sex phenotype (sex hormones) and/or sex-chromosome complement. The role of the sex-chromosome complement has been established in DNA methylation studies of the blood methylomes of individuals with TS or KF. Data from individuals with TS and KF show global changes in DNA methylation in the blood and leukocytes of these individuals in comparison to individuals with normal karyotypes (Sharma et al., 2015; Skakkebæk et al., 2018; Trolle et al., 2016; Wan et al., 2015; Zhang et al., 2020). Analyzing DNA methylation using the 450K -Illumina Infinium assay in leukocytes of individuals with TS show lower global methylation than karyotypically normal females and males (Trolle et al., 2016). This hypomethylation is seen in different genomic regions, including repetitive elements (Trolle et al., 2016). Global DNA methylation (X chromosome and autosomes) is lower in females with TS compared to females, but higher than in males (Sharma et al., 2015; Zhang et al., 2020). The X chromosome's methylation level in KF is higher than males, while DMRs on autosomes have a similar proportion of lower and higher methylation (Zhang et al., 2020).

1.2.7.1. Sex-biased DNA methylation in mouse liver

Sex-specific DNA methylation differences have been extensively studied in the mouse liver. In livers, male mice have lower DNA methylation compared to females (McCormick et al., 2017; Reizel et al., 2015; Takasugi et al., 2013; Zhuang et al., 2020). Furthermore, data from castrated mice show that male-specific demethylation in the liver is influenced by postnatal testosterone exposure, which suggests an interplay between DNA methylation and testosterone (Reizel et al., 2015). Re-administration of testosterone restored the original pattern

of lower methylation in males (Reizel et al., 2015). Although sex-biased differentially methylated regions (sDMRs) detected between female and male adult livers were absent after male castration, a new set of sDMRs emerged independently of testosterone (McCormick et al., 2017). Comparing sDMRs from different tissues showed a minimal number of overlapping regions, which indicates that sex-biased DNA methylation is tissue specific (McCormick et al., 2017).

To further delineate the distinct roles of the sex-chromosome complement and sex phenotype in DNA methylation of the mouse liver, Dr. Naumova's lab, in collaboration with Dr. Taketo and Dr. Bourque's lab, conducted whole-genome bisulfite sequencing (WGBS) and RNA-seq experiments on liver samples from mice with different combinations of sex-chromosome complement and sex phenotype to distinguish the effect of gonadal hormones and sex chromosomes on DNA methylation and gene expression (Zhuang et al., 2020).

To generate mice with monosomy X, the patchy fur (*Paf*) mutation carriers were used. The *Paf* mutation causes abnormal segregation of X and Y chromosomes during meiosis I in hemizygous males (Lane & Davisson, 1990). Due to the XY nondisjunction, *Paf* males produce XO females when mated to B6 females (Lane & Davisson, 1990). To generate mice with the same sex-chromosome complement but different sex phenotype, B6.TIR mice that have the Y chromosome of *Mus musculus domesticus* were used (Nagamine et al., 1987). These mice carry an *Sry* gene variant that causes a high rate of sex reversal in XY animals when placed on a B6 genetic background (Coward et al., 1994). This is due to SRY protein inefficiency in upregulating its target *Sox9* gene, which plays an essential role in testicular differentiation (Park et al., 2011).

Liver samples were collected from XO (XO.F) and XX females ($XX^{Paf}F$) from the *Paf* cross and XX females (XX.FT), XY males (XY.MT), and sex-reversed females (XY.FT) from the B6.TIR cross. Four pairwise comparisons were used to identify and generate lists of sDMRs and sex-biased differentially expressed genes (sDEGs) that depended on different factors (Zhuang et al., 2020). SDMRs that depended on sex-chromosome complements (X dosage and Y-chromosome presence) and sDMRs that depended on the sex phenotype were identified **(Figure 1.4**). Almost 80% of sex-phenotype dependent sDMRs had lower methylation in males compared to females (Zhuang et al., 2020). The majority (96%) of sDMRs identified when comparing groups with the same sex-chromosome complement, but different sex phenotypes (XY.FT vs. XY.MT) were autosomal. However, even when comparing groups with different

sex-chromosome complements, many sDMRs resided on autosomes, whether the mice had the same or different sex phenotype (XX.FT vs. XY.MT, XX.FT vs. XY.FT and XX^{Paf}F vs. XO.F) (53%, 34%, and 31%, respectively). Similar to sDMRs, our results showed that more sDEGs were found when comparing groups with different sex phenotype than groups with the same sex phenotype, but different sex-chromosome complements. We found that around 24% of autosomal sex-phenotype dependent sDEGs overlapped with genes containing sDMRs. As expected, most of these genes showed an opposite direction to DNA methylation in the sDMRs.

In summary, sex phenotype, X-chromosome dosage, and Y-chromosome presence shape autosomal sex-biased DNA methylation in the mouse liver (Zhuang et al., 2020) (**Figure 1.4)**. The sex phenotype is the major player in autosomal sDMRs, and it is associated with sex-biased gene expression. I used the WGBS and RNA-seq data from our published work as a starting point for my study.

Hypotheses and Objectives

Our WGBS shows that sex difference in DNA methylation in mouse liver depends on the sex-chromosome complement and sex phenotype. However, the mechanisms by which the sex-chromosome complement or the sex phenotype influenced DNA methylation remained unclear. In this project, we attempted to answer the following scientific questions: (i) how does the Y chromosome influence DNA methylation? and (ii) how does the sex phenotype influence sex-biased DNA methylation?

To address the first question, we tested the **hypothesis** that Y-chromosome dependent sDMRs were specific for the B6.TIR strain. Our **objective** was to determine if genetic variation in the Y chromosome (TIR vs. B6) influenced DNA methylation and attempt to identify the Y-linked gene(s) responsible for the Y-effect on methylation.

To address the second question, we tested the **hypothesis** that sex hormone signaling through their receptors influenced DNA methylation at autosomal regions in the mouse liver. To test our hypothesis, we set two **objectives**: (a) to establish the developmental timing of sex bias in DNA methylation, and (b) to investigate the roles of AR and ESR1 in establishing sex-biased DNA methylation using knockout mouse models.

Figures:

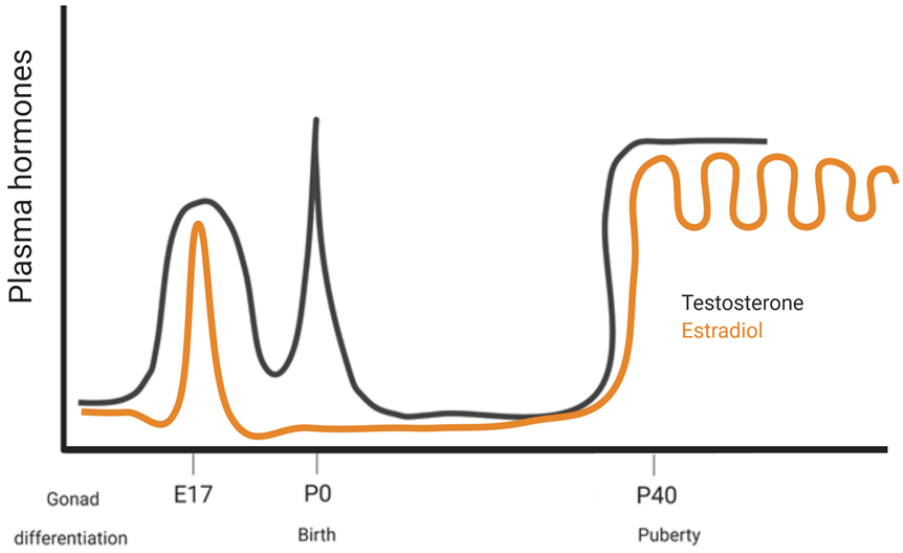

Figure 1.1. Plasma hormones level during mouse development.

In black, plasma testosterone levels during male mouse development (adapted from (Clarkson & Herbison, 2016)). In orange, estradiol levels during female mouse development. E17: embryonic day 17, P0: day of birth, and P40: postnatal day 40. Created with Biorender. This figure illustrates the pattern of plasma hormones and does not reflect the accurate levels.

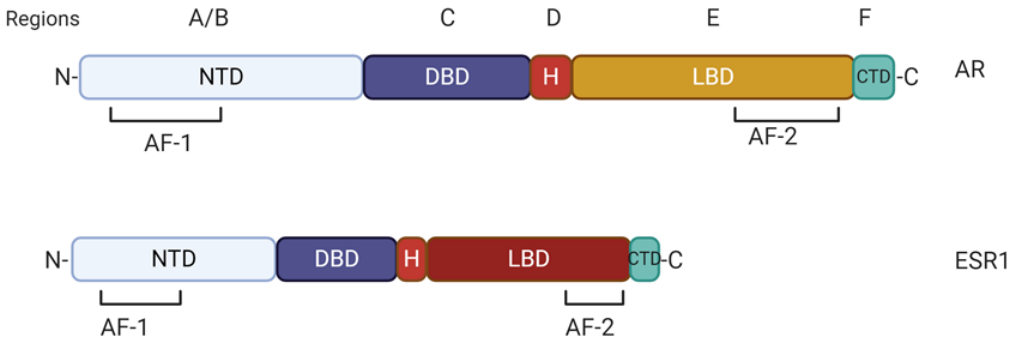

Figure 1.2. Structure of androgen and estrogen receptors.

The receptors are divided into six regions (A-F) and correspond to five structural domains. The A and B regions correspond to the N-terminal domain (NTD) that contains activation function 1 (AF-F). The C region corresponds to the DNA-binding domain (DBD). The D region corresponds to the hinge region (H). The E region corresponds to the ligand-binding domain (LBD) that contains activation function 2 (AF-2). The F region corresponds to the C-terminal domain (CTD). Created with Biorender.

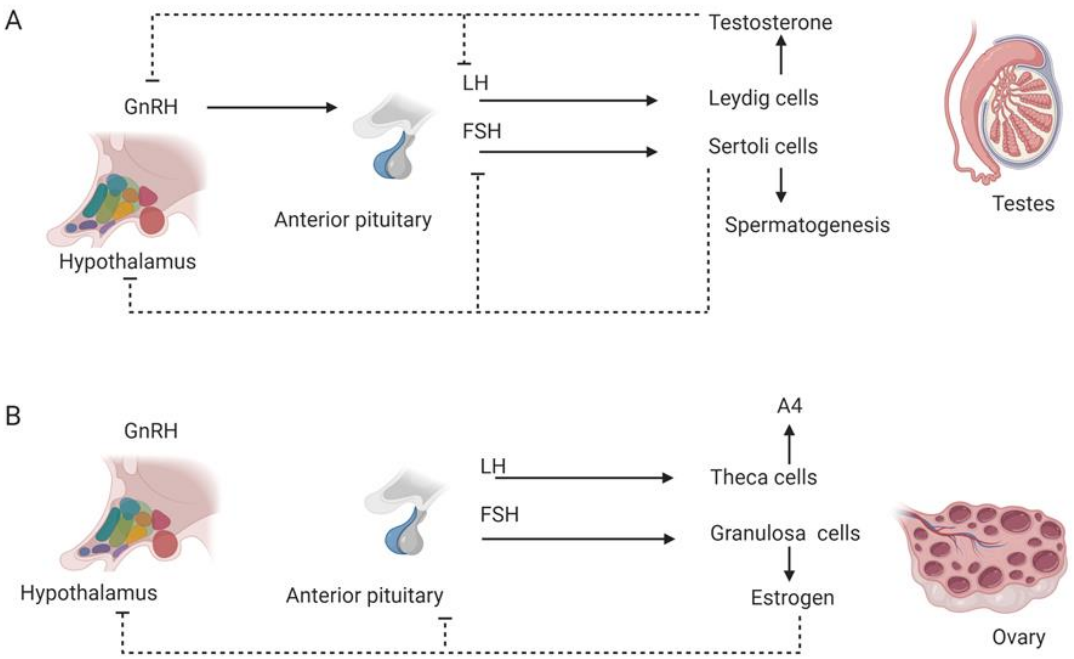

Figure 1.3. Hypothalamic-pituitary-gonadal axis.

A. Hypothalamic-pituitary-testicular axis. **B**. Hypothalamic-pituitary-ovarian axis. Dotted arrows represent negative feedback. GnRH: gonadotropin-releasing hormone, FSH: follicle-stimulating hormone, LH: luteinizing hormone, A4: androstenedione. Created with Biorender.

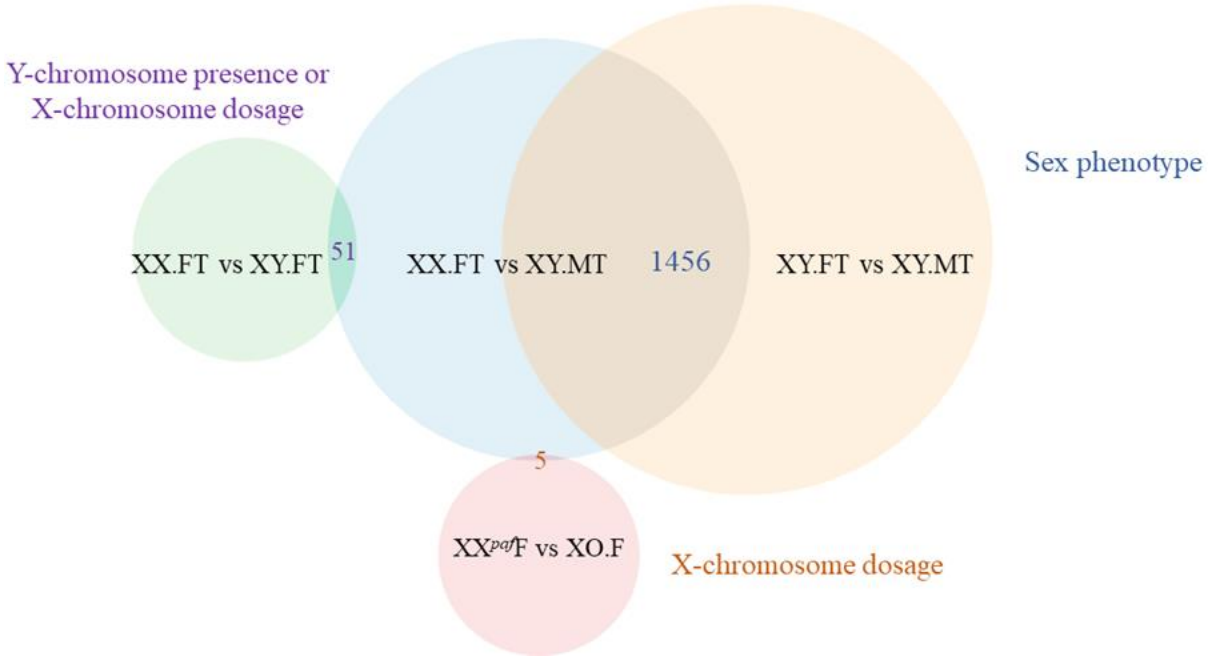

Figure 1.4. Autosomal sDMRs in each comparison and their overlap with XX.FT vs. XY.MT from WGBS.

The numbers are sDMRs overlapped with XX.FT vs. XY.MT and represents sDMRs influenced by sex phenotype (XY.FT vs. XYT.M), X-chromosome dosage (XX^{Paf}F vs. XO.F), and Y-chromosome presence or X-chromosome dosage (XX.FT vs. XY.FT).

Chapter II: Materials and Methods

Ethics statement

All animal work was conducted in accordance with the guidelines set by the Canadian Council on Animal Care (Ottawa, ON, CA) and an approved protocol by the Animal Care Committee of the McGill University Health Center (Montreal, QC, CA).

2.1. Mouse strains and crosses

B6 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA).

B6.C3H/HeSn-*Paf* male carriers of *Paf* mutation (referred to as *Paf* from this point on) were backcrossed to B6 mice for several generations to place the mutation into the B6 genetic background and generate females with monosomy X (XO females) (**Figure 2.1A**). Liver samples from N6 to N8 XO females and their XX^{Paf} female littermates were collected at eight weeks of age for methylation and expression analyses. Female offspring were genotyped using RT-qPCR of *Xist*, which is expressed in XX females (Alton et al., 2008; Kay et al., 1993). The list of genotyping primers is provided in **Table 2.1.**

The B6.TIR mouse was established by placing the Y chromosome from *Mus musculus domesticus* onto B6 genetic background (Eicher et al., 1982). Mating XY^{TIR} male to a B6 female generates XY.FT, XY.MT, XX.FT, and true hermaphrodites (XY.HT) (Taketo-Hosotani et al., 1989) (**Figure 2.1B**). Liver samples from XY.FT, XY.MT, XX.FT, and XY.HT were collected at 8 and 16 weeks of age for methylation and expression analyses. Female offspring were genotyped using PCR amplification of the zinc finger protein 1, Y-linked (*Zfy1)* sequence from ear punches (as described in (Amleh et al., 2000). The list of genotyping primers is provided in **Table 2.1.** Mice from the *Paf* and B6.TIR crosses were provided by Dr. Teruko Taketo (McGill University, QC, Canada).

ARKO mice, these mice carry a global deletion of exon 3 of the androgen receptor gene on a B6 genetic background. Heterozygous ARKO female mice were generated by mating CMV-Cre mice to mice carrying the floxed AR allele (Notini et al., 2005). Heterozygous ARKO female mice were mated to B6 males to produce hemizygous XY ARKO (Notini et al., 2005) (**Figure 2.1C)**. DNA from livers of 10-11 week old XY ARKO mice and the wild type (WT) littermates was provided by Dr. Rachel Davey (University of Melbourne, Victoria, Australia).

B6N(Cg)-*Esr1*$^{tm4.2Ksk}$/J mice (stock# 026176) (referred to as ESR1KO from this point on) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). These mice carry

a deletion of exon 3 of the estrogen receptor 1 gene on a C57BL/6N genetic background (Hewitt et al., 2010). Heterozygous ESR1KO male and female mice were mated to produce homozygous and heterozygous ESR1KO, as well as WT mice (**Figure 2.1D**). Genotype was determined by standard PCR (**Table 2.1).** Livers were collected from homozygous and heterozygous ESR1KO mice and their WT littermates at eight weeks of age for methylation and expression analyses.

For the study of the developmental timing of sex-biased methylation and expression, B6 mice were mated, and livers were collected from female and male offspring at E14.5, four weeks, and eight weeks of age.

To generate E14.5 embryos, mice were mated, and the morning of detection of the vaginal plug was considered E0.5. The pregnant females were sacrificed at E14.5, and embryos were collected. Livers were isolated from embryos using a stereomicroscope and genotyped using PCR of *Sry* to determine the genetic sex. The list of genotyping primers is provided in **Table 2.1.**

2.2. DNA extraction

DNA from mouse livers was extracted using a standard proteinase K phenol/chloroform procedure or the QIAamp Fast DNA Tissue Kit (Qiagen, NL). Crude lysates of ear punches using sodium hydroxide lysis were used for genotyping. For more details regarding genotyping, refer to section **2.1**.

2.3. Sodium Bisulfite Treatment and Pyrosequencing

One µg of DNA per sample was treated with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen, NL) according to the manufacturer's protocol with modification to the incubation step extending it by a five minutes cycle at 95°C and two hours at 60°C.

PCR was done in 30 µl with 50 ng of bisulfite converted DNA using one standard primer and one HPLC-purified 5' biotinylated primer (IDT). Pyrosequencing was then carried out using the PyroMark Q24 Advanced platform and PyroMark Q24 Advanced CpG Reagents (Qiagen, NL). Results were analyzed by the PyroMark Q24 Advanced software (Qiagen, NL).

2.3.1. Assay and primer design for pyrosequencing methylation analysis

Pyrosequencing assays for sDMRs (50-70 bp) were designed using the PyroMark Assay Design 2.0 Software (Qiagen, NL). Specific criteria were maintained when selecting sDMRs for pyrosequencing methylation assays design: (1) avoiding genomic regions with high density of CpG as they can form stable secondary structures; (2) avoiding sDMRs located within repetitive elements to prevent amplification of non-specific regions; (3) no CpGs sites or SNPs within primers to stabilize their binding and avoid allele bias; (4) pyrosequencing can read around 50-75 bp after the sequencing primer, so the target sequence should not exceed 70 bp. The list of pyrosequencing methylation assay primers is provided in **Table 2.2**.

2.4. Expression analysis by RT-qPCR

Total RNA was isolated using TRIzol Reagent (Thermo Fisher Scientific) and followed by purification using the RNeasy® MinElute® Cleanup Kit (Qiagen, NL). CDNA was synthesized using one µg of RNA, Oligo dT 12-18 primers, and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MuLV RT) (Thermo Fisher Scientific, MA, US). Quantitative reverse transcription PCR (RT-qPCR) was performed using Power SYBR Green PCR master mix (Thermo Fisher Scientific, MA, US) and Eco^{TM} Real-Time PCR System (Illumina, CA, US). Each cDNA sample was assayed in replicate, and the average Cq value was used to calculate the relative RNA (expression) levels using the delta Cq method ($2^{-\Delta Cq}$). At least three biological replicates were used in each group (genotype/sex/age). Gene expression levels were normalized to the housekeeping gene ribosomal protein L19 (*Rpl19*).

2.4.1. Expression primer design

Primers for expression analysis were designed using Primer3 software (version 4.1.0) (http://primer3.ut.ee/) and checked with Bisearch, UCSC genome browser, and IDT OligoAnalyzer (Arányi & Tusnády, 2007; Remm et al., 2012) (https://www.idtdna.com/pages/tools/oligoanalyzer). In order to detect DNA contamination, all primer sets were designed into two adjacent exons flanking an intron. The Oligo dT primer used for cDNA synthesis binds to the poly-A tail (3' end) of the messenger RNA (mRNA). Therefore, the RT-qPCR primers were designed closer to the 3' end and covered as many isoforms as possible. The primers should only amplify the targeted genomic region. The annealing temperatures for the primers were optimized using standard PCR. The list of expression primers is provided in **Table 2.3**.

2.5. Statistical analyses

Statistical significance was calculated using a Student's t-test, one-way ANOVA with multiple correction testing (Tukey's multiple correction test), or two-way ANOVA with multiple correction testing (Sidak's multiple correction test) using GraphPad Prism 8. Data are shown as mean ± standard deviation.

Tables:

Table 2.1. List of genotyping primers.

Gene	Mouse cross	Technique	Forward primer	Reverse primer
Esr1	ESRKO	Standard PCR	ATCCCATGTGCTTGAGTGGT	CCACTTCTCCTGGGAGTCTG
Sry	B6	Standard PCR	GCAGGCTGTAAAATGCCACT	ATGCAGGTGGAAAAGCCTTA
Xist	*Paf*	RT-PCR	ACTGCCAGCAGCCTATACAG	GTTGATCCTCGGGTCATTTA
Zfy	B6.TIR	Standard PCR	AAGATAAGCTTACATAATCACATGGA	CCTATGAAATCCTTTGCTGCACATGT

Table 2.2. List of sDMRs and their pyrosequencing methylation assay primers.

Gene symbol	Functional annotation	Chr.	Position of the assayed CG	Primer 1 (5'-3')	Primer 2 (5'-3') Biotinylated	Sequencing primer (5'-3')
Aldh3b3	3kb upstream of TSS	19	3,955,095	TTTTGGTGGTTGTAGATAGTGGT	TAATTACCCCCCCCCCTACAAT	GTAGATAGTGGTTGGTA
Bcl6	Intergenic	16	24,010,090	AGTAAGTATTTGAAGGTTTGTTAGAGT	ATTCAAAATTAAACCACTCATAAACATAC	GGTTTGTTAGAGTTTTAGTATATT
Caprin1	Exon	2	103,766,583	TTAAGTAAGGGTGAGGAATTT	AAAAAAACTATTTTCATCCCATAAAAACAA	TGTATTGTGTGTAAGGTTT
Ch6qA1	Intergenic	6	13,714,975	GTTATGGAGGTTGGTAATAATGTT	ACCAATAAAAATCAAACAATTCACAAA	GGGGAATATATAGAAAGATATAAAA
Comt	Exon, enhancer	16	18,407,984	AAGGGGAAGGTGTTTTTAGTTGATAATG	TTCTCCAAACCCTCCACCACTTTCATA	GGTGTTTTTAGTTGATAATGT
Cux2	Intron	5	121,999,270	TAGGTATGGAATAGGATTTTATGTGTT	ATAATAAATAACTCTCACCACCTTTACT	ATGTGTTTTTAAAAGGTTAAAGAT
Cyp2b9	Exon	7	26,210,279	GGTAAGTTTTGTTGTTTTAAAGGATATTGA	ATAACACCTAACTCCCTCAC	AAATATTTTTAGTATATTAGATT
Cyp7b1	Intron	3	18,239,446	GGTTATAAGGTTTGTGATATGTTGTTA	ATTCTTAACCAACTCTCTAAATATACAAT	GGTTTGTGATATGTTGTTATAG
Ergic1	Intron, enhancer	17	26,634,198	AAAGAAGTATTAGGGATAATTTAGGGTAAG	TAACTCAAAATACACCCCTCACC	GGGATAATTTAGGGTAAGAG
Esr1	Intron	10	4,729,743	TTGGGGTTAATTATTTATTTGTGAGT	TCCCAAAAACACATTCCAAAAC	TGAGTTATTGGGTTGG

Fez2	Intron, enhancer	17	78,407,054	AGGGGAGGTTTATTTGGAAAA	CTTCCTAAACCCCTTTCATTCACTAACTT	ATGGGATATATTTAAGTTGTAGA
Fmo3	Intron	1	162,982,506	GATAAAGGTATATTTGTTTATGGATATGT	AATTACTCTCTAACCAAACAATTAAAC	ATTTTATTTTGTGAGGTTGAA
Gstp1	500bp upstream gstp1, enhancer	19	4,034,872	GTTTTGGTTGTTTTGGAATTTATTATGT	AAATTTCTCTCCTTAACCTCAATATTCT	ATTTATTATGTAAATTAGGTTGG
Hsd3b5	Intron	3	98,626,053	TTGTAGATATTGAATAGATATTAGGGAATT	CTTCCCCAACTTACTTCTTAATCATA	ATTGAATAGATATTAGGGAATTTT
Pgk1	Intron, promoter	X	106,187,981	TTTAGTAATTTTTTTAGGTAAGGGAGAAGT	TTAAACTCCTAACCCTAAAATTTACATCA	GTGAGGTTATAGGTTTTTAAT
*Snrpn**	Exon, promoter	7	60,005,146	TTGGTAGTTGTTTTTTGGTAGGAT	TCCACAAACCCAACTAACCTTC	GTGTAGTTATTGTTTGGGA
Xist	Exon, promoter	X	103,481,082	GTAATAGTTATGGGGTAGATTTTGGA	CTTAACCTCTAATTTAACCAACACTAA	ATTTAGTAGGTTTAGAGAAT

CG position:

UCSC genome browser - mouse (GRCm38/mm10) assembly

Functional annotation:

Based on genomic region and histone marks enrichment (H3K4me1, H3k27ac for enhancers, and H3k4me3 for promoters).

Information obtained from UCSC genome browser (Bing Ren's laboratory - LIRC histone track).

*Primers from (Whidden et al., 2016).

Table 2.3. List of gene expression primers.

Gene	Forward primer	Reverse primer
Ar	AGAATCCCACATCCTGCTCA	AAGTCCACGCTCACCATATG
Cyp2b9	TGAGCACTTTCTAGATGCCAAT	GGCAATGCTTTCACCAAGAC
Cyp7b1	GCCCTCTTTCCTCCACTCAT	CCTCCTTTGAAAAACGTGCT
Cux2	CCCACTCGGGTCAAAGTC	GCTGCTCTCCTTCCAACTCA
Ddx3y	AGCAGCCGAAGTAGTGGTAG	ATTCAATTGCCCCACCAGTC
Elovl3	TTCTCTTTCTTCTCAGCAAGGT	GTGGTACCAGTGGACAAAGA
Esr1	CAGACACTTTGATCCACCTGA	CGTTCTTGCATTTCATGTTGTAG
Fmo3	TGATGAGAAAATGGGGGAAA	GCTTTGCACCAATGAAGGAG
Foxa1	ACTGTGAAGATGGAAGGGCA	CCGGAGTTCATGTTGCTGAC
Kdm5d	AGAATCCCAATCTAGAGCGCA	CAGAACCACCTTTTGCCTCC
Rpl19	GATCATCCGCAAGCCTGTGA	GCATCCGAGCATTGGCAGTA
Uty	TGTCAGAACTGTGCACGAAA	TGCAGAAGATAACGAAGGAGC

Figures:

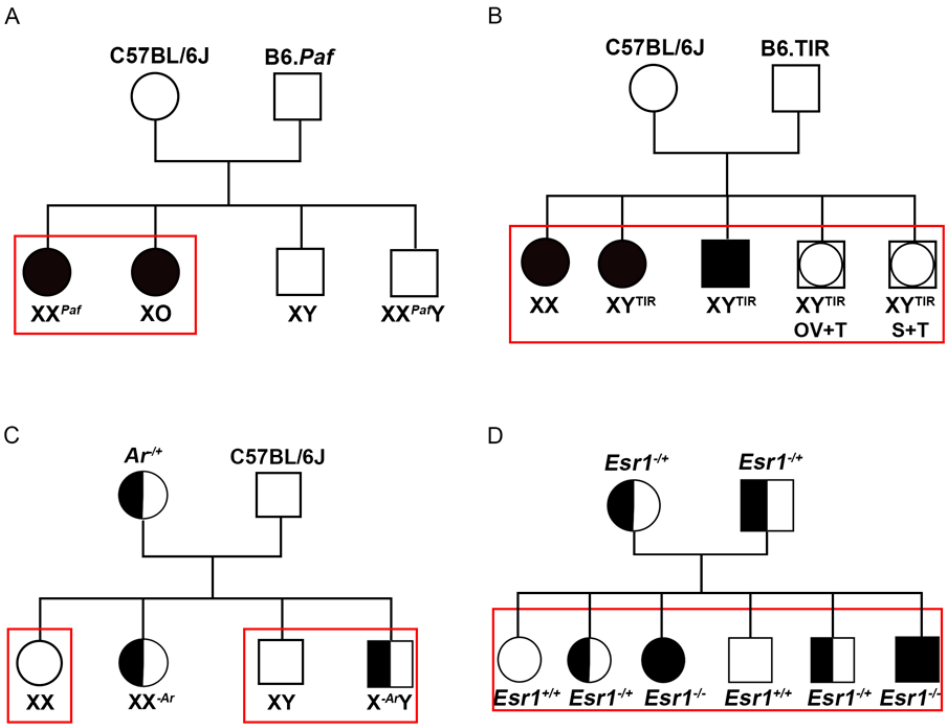

Figure 2.1. Mouse crosses

A. The *Paf* cross. **B**. The B6.TIR cross. **C**. ARKO cross. **D**. ESR1KO cross. Circles represent females, squares represent males, and combined circle and square represents hermaphrodites. **A-B.** Filled shapes represent groups used in whole-genome analyses. **C-D**. Filled shapes represent mutant, half-filled represent heterozygotes/hemizygotes, empty shapes represent WT. Red rectangles represent groups used for further analyses (pyrosequencing and RT-qPCR). OV = ovary, T = testis, and S = streak gonad.

Chapter III: Results

3.1. SDMR validation and selection of reporter sDMRs for further studies

To better understand the mechanisms responsible for sex-biased DNA methylation levels, we needed to select reporter sDMRs that could be used in future low-throughput studies and further investigations of sex-biased methylation mechanisms. For this purpose, autosomal sDMRs with sex-phenotype dependent methylation located near sDEGs were selected (Zhuang et al., 2020). Eight with lower methylation in males; three associated with sDEGs (*Cyp7b1*, *Gstp1*, and *Hsd3b5*), and five with no sDEGs (*Bcl6*, *Comt*, *Ergic1*, *Esr*, and *Fez2*) (**Figure 3.1A, Figure S1**). Two with lower methylation and higher expression in females (*Aldh3b3* and *Cyp2b9*) (**Figure 3.1B, Figure S1**). Two autosomal sDMRs with Y-chromosome dependent methylation (*Caprin1* and Ch6qA1) were also selected (**Figure 3.2A**). We also tested sDMRs on the X chromosome with methylation levels that depended on X-chromosome dosage (promoters of X-linked genes *Pgk1* and *Xist*) (**Figure 3.1C**) and a DMR located in the promoter of imprinted gene small nuclear ribonucleoprotein N (*Snrpn*) (**Figure 3.1D**).

We performed pyrosequencing methylation analyses using additional liver DNA samples from *Paf* and B6.TIR mice (n= 4-8 samples per each sex/genotype group). We validated the sex-biased methylation at the ten sex-phenotype dependent sDMRs and two Y-chromosome dependent sDMRs ($P<0.0001$, one-way ANOVA) (**Figure 3.1 and 3.2**). As expected, mice with two X chromosomes had higher and lower methylation levels compared to groups with one X chromosome at *Pgk1* and *Xist* sDMRs, respectively ($P<0.0001$, one-way ANOVA) (**Figure 3.1C**). All five groups had similar methylation levels at *Snrpn* DMR ($P=0.07$, one-way ANOVA) (**Figure 3.1D**).

3.2. SDMRs that depend on the presence of the Y-chromosome are specific to the B6.TIR cross

Our WGBS data analyses showed that a subset of 50 sDMRs were influenced by the presence of the Y chromosome rather than sex phenotype or X-chromosome dosage (Zhuang et al., 2020). Most of these sDMRs were located within repetitive elements, and therefore, it was possible to design pyrosequencing assays for only 4 regions. We validated two Y-chromosome dependent autosomal sDMRs, one located in the last exon of *Caprin1* and the other in an intergenic region of chromosome 6qA1 (Ch6qA1) using pyrosequencing assays. We asked whether the Y-chromosome dependent sDMRs were specific for the B6.TIR strain or were present in the WT B6 mice as well. Methylation of the two sDMRs was tested in B6.TIR mice: XX.FT, XY.FT, and XY.MT, *Paf* mice: XX^{Paf}F and XO.F, and B6 mice: WT.F

and WT.M. At both sDMRs, XY.FT and XY.MT had lower methylation than XX.FT, XX^{Paf}F, XO.F, and WT.F. In contrast, WT.M showed methylation levels similar to females at both sDMRs (P<0.0001, one-way ANOVA) (**Figure 3.2A**). At Ch6qA1, there was high inter-individual variance in XX^{Paf}F and XO.F methylation levels, which could be due to the genetic variation in these mice. The *Paf* cross may have portions of the C3H genome in a largely B6 background that led to increase in inter-individual variation in methylation.

The B6.TIR and B6 mice differ in their Y chromosome, the B6.TIR mice have the Y chromosome of *Mus musculus domesticus*, while the B6 mice have the Y chromosome of *Mus musculus musculus* (Tucker et al., 1992). We hypothesized that methylation levels at these two sDMRs are influenced by Y-linked genes expressed in the liver. Since we had the liver RNA-seq data for B6.TIR mice, we looked at the expression levels of Y-linked genes and identified genes expressed in the liver of XY.FT and XY.MT (Zhuang et al., 2020). There were four genes expressed in the liver of XY.MT and XY.FT: *Eif2s3y*, involved in the early steps of protein synthesis; *Ddx3y*, an ATP-dependent RNA helicase; *Uty*, catalyzes the demethylation of trimethylated lysine 27 of histone 3; and *Kdm5d*, demethylates lysine 4 of histone 3. Interestingly, *Uty* and *Kdm5d* have epigenetic regulatory functions as histone demethylases, and *Ddx3y* has a role in cellular processes, including RNA degradation (Bellott et al., 2014; Shpargel et al., 2012). Therefore, we hypothesized that the difference we see between the two strains was due to differences of those Y-linked genes that may influence DNA methylation, i.e., *Uty*, *Kdm5d*, or *Ddx3y*. In principle, genetic differences in the Y-linked genes in the two strains could lead to differences in their regulation or protein function, which in turn may affect DNA methylation of autosomal regions in *trans*. To test if there was a difference in these genes' expression levels, we performed RT-qPCR for *Ddx3y*, *Uty*, and *Kdm5d* in livers of WT.M, XY.MT, and XY.FT. WT.M had a higher relative RNA level of *Ddx3y* and a lower RNA level of *Uty* compared to XY.MT and XY.FT (P<0.001 and P<0.05, respectively, one-way ANOVA). The relative expression of *Kdm5d* was not significantly different between the three groups (P=0.55, one-way ANOVA) (**Figure 3.2B**).

Taken together, sDMRs that depend on the Y-chromosome presence are specific to the B6.TIR cross. Two Y-linked genes, *Ddx3y* and *Uty*, may contribute to this TIR-specific sex-biased methylation.

3.3. Sex bias in DNA methylation and gene expression varies with age

The production of testosterone starts at mid-gestation in the testes of male mice, and they have three plasma surges in their lifetime. The first peak occurs around E16-17, the second peak is a few hours after birth, and the largest surge is at puberty and continues through adulthood (Pointis et al., 1979, 1980). To investigate how sex bias in DNA methylation arises and elucidate the role of testosterone exposure on DNA methylation, we compared methylation levels of validated sex-phenotype dependent sDMRs in B6 female and male livers at three different developmental stages: fetal at E14.5, prepubescent at four weeks of age, and adult at eight weeks of age, using pyrosequencing methylation assays. If testosterone exposure is sufficient to cause sex bias in DNA methylation, we would expect that sex-biased methylation would be present at all three ages.

DNA methylation at *Cyp7b1*, *Ergic1*, *Fez2*, *Gstp1*, and *Hsd3b5* sDMRs showed no sex bias at both E14.5 and 4 weeks, but showed lower levels of methylation in 8-week old males compared to females (*Cyp7b1* and *Ergic1* $P<0.001$, *Fez2*, *Gstp1* and *Hsd3b5* $P<0.0001$, for interaction between sex and age, two-way ANOVA) (**Figure 3.3A**). *Comt* and *Esr1* sDMRs showed significant sex differences at 4 and 8 weeks. They had significantly lower methylation in 4-week and 8-week old males compared to females ($P<0.0001$, for interaction between sex and age, two-way ANOVA) (**Figure 3.3A**).

At *Fmo3* sDMR, DNA methylation showed no sex bias at both E14.5 and 4 weeks, and showed lower methylation levels in 8-week old females compared to males ($P<0.0001$, for interaction between sex and age, two-way ANOVA) (**Figure 3.3B**). *Cux2* and *Cyp2b9* sDMRs showed significant sex differences at both 4 and 8 weeks. They had lower methylation levels in 4- and 8-week old females compared to males ($P<0.0001$, for interaction between sex and age, two-way ANOVA) (**Figure 3.3B**). The *Aldh3b3* sDMR showed significant sex differences at every age we tested (**Figure 3.3B**). We detected a small (3%) but significant difference in E14.5 livers. At 4 and 8 weeks, sex differences in methylation had an opposite direction to E14.5, as females showed significantly lower methylation levels than males ($P<0.0001$, for interaction between sex and age, two-way ANOVA) (**Figure 3.3B**).

At the *Xist* sDMR, females had lower methylation levels compared to males of all three ages ($P<0.0001$, for interaction between sex and age, two-way ANOVA) (**Figure 3.3C**). Both females and males had lower methylation at E14.5 compared to 4 and 8 weeks, which was not expected. However, the expected sex difference is still present at E14.5 (**Figure 3.3C**). Finally,

no sex differences in methylation levels were detected at *Snrpn* DMR (P=0.71, for interaction between sex and age, two-way ANOVA) (**Figure 3.3D**).

Next, we wanted to see if the timing of sex-biased expression of genes containing the sDMRs coincides with sex-biased methylation. We performed RT-qPCR for two genes with lower methylation and higher expression in males (male-biased genes, *Cyp7b1* and *Elovl3*), and two genes with lower methylation and had higher expression in females (female-biased genes, *Cyp2b9* and *Fmo3*). We determined their expression levels during development in B6 female and male livers at E14.5, four weeks, and eight weeks of age. *Cyp7b1* showed higher expression in 8-, but not 4-week old males (P<0.001, for interaction between sex and age, two-way ANOVA) (**Figure 3.4A**). For *Elovl3*, *Cyp2b9,* and *Fmo3* significant sex bias in expression was found at 4- and 8- weeks of age (*Elovl3* P<0.01, *Cyp2b9* and *Fmo3* P<0.0001, for interaction between sex and age, two-way ANOVA) (**Figure 3.4**). Interestingly, *Cyp7b1* and *Cyp2b9* showed significant sex differences in expression at the same time as sex-biased DNA methylation (**Figure 3.3 and 3.4**).

Our data show that both sex-biased methylation and expression show different onset timing at different regions, suggesting the involvement of multiple factors in establishing sex bias in DNA methylation and gene expression. In conclusion, at the majority of tested sex-phenotype dependent sDMRs, we did not detect sex differences in DNA methylation levels in fetal and prepubescent mouse livers. Our results show that testosterone production by fetal and prepubescent testes is not sufficient to cause sex bias in methylation at certain sDMRs, and other factors must be involved.

3.4. Presence of ovaries and testes in hermaphrodites affects DNA methylation of sDMRs in an age-dependent manner

Our developmental timing data for sex-phenotype dependent sDMRs suggest that testosterone alone is not sufficient to cause sex-biased DNA methylation. We hypothesized that testosterone influenced sex-biased DNA methylation in a dose-dependent manner, or prolonged exposure to testosterone was needed to establish sex-biased methylation.

In our B6.TIR cross, 36% of XY mice developed as females, 46% as males, and 18% as hermaphrodites with one testis and either an ovary or a streak gonad (**Table 3.1**). Hermaphrodites have about 10-20% of males' testosterone levels (Houle & Taketo, 1992).

The hermaphrodites had intersex external genitalia, shorter anogenital distance compared to males, and often showed signs of mammary gland development (**Figure 3.5A**). Hermaphrodites had smaller testes compared to males. Moreover, males had a high variation in testis weight (**Figure 3.5B and C**).

Therefore, to test our hypothesis, we decided to compare methylation levels in B6.TIR mice with the three different sex phenotypes using a panel of six sDMRs, three sDMRs with lower methylation in males *Cyp7b1, Gstp1,* and *Hsd3b5* (male-biased sDMRs) and three with lower methylation in females *Aldh3b3, Cyp2b9,* and *Fmo3* (female-biased sDMRs).

We investigated methylation levels in the livers of 8- and 16-week old XX.FT, XY.FT, XY.MT, and XY.HT. At eight weeks, hermaphrodites with an ovary and a testis (XY.HT OV+T) had female-like or intermediate methylation levels (**Figure 3.5D and F**). Hermaphrodites with a streak gonad and a testis (XY.HT S+T) show methylation levels similar to males, however, we could not perform statistical tests due to the low sample number (n=2) and the absence of this group in 16 weeks animals (**Figure 3.5D and F**). So, from this point onward, we report the methylation levels in XY.HT OV+T.

At 8 weeks, XY.HT showed significantly higher methylation levels compared to XY.MT and lower methylation levels compared to XY.FT at the *Hsd3b5* sDMR ($P<0.0001$, one-way ANOVA) (**Figure 3.5D**). At *Cyp7b1* and *Gstp1* sDMRs, XY.HT mice showed significantly higher methylation levels than XY.MT ($P<0.0001$, one-way ANOVA) (**Figure 3.5D**). At female-biased sDMRs, XY.HT showed significantly higher methylation than XY.FT and lower methylation than XY.MT ($P<0.0001$, one-way ANOVA) (**Figure 3.5F**).

Interestingly, at 16 weeks, XY.HT had the same methylation levels as XY.MT at all sex-phenotype dependent sDMRs. At the three male-biased sDMRs, XY.HT showed significantly lower methylation compared to XY.FT ($P<0.0001$, one-way ANOVA) (**Figure 3.5E**). At the three female-biased sDMRs, XY.HT showed significantly higher methylation compared to XY.FT ($P<0.0001$, one-way ANOVA) (**Figure 3.5G**).

Next, we performed RT-qPCR to test *Cyp7b1, Elovl3, Cyp2b9,* and *Fmo3* expression in 8-week old XX.FT, XY.MT, XY.FT and XY.HT. The relative RNA levels of *Cyp7b1* were significantly higher in XY.MT and XY.HT compared to XY.FT, and higher in XY.MT compared to XY.HT ($P<0.0001$, one-way ANOVA) (**Figure 3.6A**). *Elovl3* expression was higher in XY.MT and XY.HT compared to XY.FT, and there was no significant difference between XY.MT and XY.HT ($P<0.0001$, one-way ANOVA) (**Figure 3.6A**). *Cyp2b9* and *Fmo3*

had the opposite direction, where XY.FT showed higher relative RNA level compared to XY.MT and XY.HT (P<0.0001, one-way ANOVA) **(Figure 3.6B**).

In conclusion, hermaphrodites showed methylation levels different from both males and sex-reversed females at 8-weeks and similar methylation levels to males at 16-weeks. However, hermaphrodites had either male-like or intermediate expression levels of sex-biased differentially expressed genes at 8-weeks.

3.5. Dynamic expression of TFs during development in the mouse liver

Testosterone exerts its effects by binding to a ligand-dependent TF, the androgen receptor. This binding mediates testosterone function on its target regions and activates downstream genes (Davey & Grossmann, 2016). Estrogen regulates gene expression by binding to estrogen receptor (Yaşar et al., 2017). Thus, the effect of sex hormones on sex-biased methylation is mediated by their receptors. Our RNA-seq analysis showed that of the estrogen receptor subtypes, only *Esr1* is expressed in the liver (Zhuang et al., 2020). Moreover, motif enrichment analysis on autosomal sDMRs show enrichment of FOXA1 and CUX2 TFs (Grimm et al., 2019; Zhuang et al., 2020). These TFs are known for their regulation of gene expression in the liver (Conforto et al., 2012; Reizel et al., 2020).

To better understand the developmental profiles of TFs expression in the mouse liver, we performed RT-qPCR for *Ar*, *Esr1*, *Cux2*, and *Foxa1* in WT mice at three different ages. *Ar* expression was low at E14.5 and 4 weeks and increased by 5-fold between 4- and 8- weeks mice (P<0.05 for interaction between sex and age, two-way ANOVA) (**Figure 3.7A**). *Esr1* expression was low at E14.5 in both females and males. In females, *Esr1* expression increased 150-fold between E14.5 and 4 weeks and 2-fold between 4- and 8- weeks. In males, *Esr1* expression increased 200-fold between E14.5 and 4 weeks and did not change between 4- and 8- weeks (P<0.01 for interaction between sex and age, two-way ANOVA) (**Figure 3.7A**). *Cux2* expression was low at all three ages in males. *Cux2* expression increased 24-fold between E14.5 and 4 weeks and 2.5-fold between 4- and 8- weeks in females (P<0.0001 for interaction between sex and age, two-way ANOVA). In contrast, *Foxa1* expression increased with age in both males and females. Males had a bigger increase from E14.5 to 4 weeks than females resulting in a significant sex difference at 4 weeks (almost 2-fold difference) (**Figure 3.7A**). This sex difference was lost at 8 weeks (P=0.07 for interaction between sex and age, two-way ANOVA) (**Figure 3.7A**). Sex difference in *Ar* and *Esr1* expression was observed at 8 weeks, with females showing higher expression levels (2-, and 2.5- fold difference), while sex

difference in *Cux2* expression was observed at 4- and 8- weeks, with higher expression in females (17-, and 141- fold, respectively).

Next, we wanted to test the expression of the 4 TFs in hermaphrodites in comparison to females and males. We performed RT-qPCR for the 4 genes in 8 weeks B6.TIR mice. *Ar*, *Esr1*, and *Cux2* had higher relative RNA levels in XY.FT compared to XY.MT and XY.HT, and no difference was observed between XY.MT and XY.HT (*Ar* and *Cux2* $P<0.0001$, *Esr1* $P<0.01$, one-way ANOVA) (**Figure 3.7B**). As expected, there was no significant difference between the groups in *Foxa1* expression ($P=0.12$, one-way ANOVA) (**Figure 3.7B**).

Our data show dynamic expression of TFs at different ages in the liver, with increased expression at 4 or 8 weeks in one or both sexes.

3.6. Loss of DNA binding of androgen receptor results in feminization of DNA methylation at sDMRs in XY animals

To better understand the role of AR in sex-biased methylation, we tested DNA methylation levels of sDMRs in ARKO mice. XY ARKO mice develop androgen insensitivity, and they have female external genitalia and small intraabdominal testes (Notini et al., 2005).

We analyzed DNA methylation levels in ARKO and WT mice in the same panel of six sDMRs (**Figure 3.8**). At both male-biased and female-biased sDMRs, ARKO mice had female-like methylation levels (**Figure 3.8**). At the male-biased sDMRs, ARKO mice showed significantly higher methylation compared to WT males ($P<0.0001$, one-way ANOVA) (**Figure 3.8A**). At female-biased sDMRs, ARKO mice showed significantly lower methylation compared to WT males (*Aldh3b3* $P<0.001$, *Cyp2b9* and *Fmo3* $P<0.0001$, one-way ANOVA) (**Figure 3.8B**).

3.7. ESR1 depletion changes methylation levels of both females and males at male-biased sDMRs, and only females at female-biased sDMRs

To better understand the role of estrogen receptor in sex-biased DNA methylation, we used ESR1KO mice. We confirmed the reduction of *Esr1* expression in ESR1KO female and male livers by RT-qPCR (**Figure 3.9B**). The expression of *Esr1* is 8% and 28% in adult ESR1KO females and males, respectively, compared to their WT littermates ($P<0.01$ for interaction between sex and genotype, two-way ANOVA) (**Figure 3.9B**).

We analyzed DNA methylation levels in ESR1KO mice and their WT littermates using our panel of six sDMRs (**Figure 3.10**). Sex-biased methylation was lost or reduced in homozygous ESR1KO mice at all six sDMRs. At male-biased sDMRs, both females and males were affected by ESR1 loss (**Figure 3.10A**). At *Gstp1* and *Hsd3b5* sDMRs, ESR1KO females gained methylation while ESR1KO males lost methylation compared to their WT littermates ($P<0.0001$ for interaction between sex and genotype, two-way ANOVA) (**Figure 3.10A**). At *Cyp7b1*, ESR1KO males show higher methylation compared to WT males ($P<0.0001$ for interaction between sex and genotype, two-way ANOVA) (**Figure 3.10A**). At female-biased sDMRs, ESR1KO females showed higher methylation levels than WT females and similar to males ($P<0.0001$ for interaction between sex and genotype, two-way ANOVA) (**Figure 3.10B).** Heterozygous ESR1KO mice show methylation levels similar to the WT mice (**Figure 3.10**).

To test if ESR1 loss influenced sex-biased gene expression, we checked the expression of two sex-biased differentially expressed genes (*Cyp7b1* and *Cyp2b9*) and three TFs (*Ar*, *Cux2*, and *Foxa1*) in homozygous ESR1KO and WT mice using RT-qPCR. The sex-biased expression of *Cyp7b1* and *Cyp2b9* was lost in ESR1KO mice ($P<0.0001$ for interaction between sex and genotype, two-way ANOVA) (**Figure 3.9A**). Also, the sex-biased expression of TFs *Ar* and *Cux2* was lost in ESR1KO mice ($P<0.05$, and $P<0.01$, respectively for interaction between sex and genotype, two-way ANOVA) (**Figure 3.9B**). The expression of *Foxa1*, which did not show sex bias at 8 weeks, was not affected by ESR1KO (**Figure 3.9B**).

These data suggest that estrogen signaling through ESR1 contributes to establishing or maintaining sex-biased methylation and expression levels in mouse liver. This could be mediated by interacting and influencing other TFs such as *Ar* and *Cux2*.

Tables:

Table 3.1. Distribution of genotypes and sex phenotypes among the offspring of XY^{Tir} males.

	Total	XX.FT	XY.FT	XY.MT	XY.HT	XY.HT OV+T	XY.HT S+T
Number	105	49	20	26	10	8	2
Percent of total		47%	19%	25%	9%	n/a	n/a
Percent of XY	n/a	n/a	36%	46%	18%		

OV = ovary, T = testis, and S = streak gonad**.**

Figures:

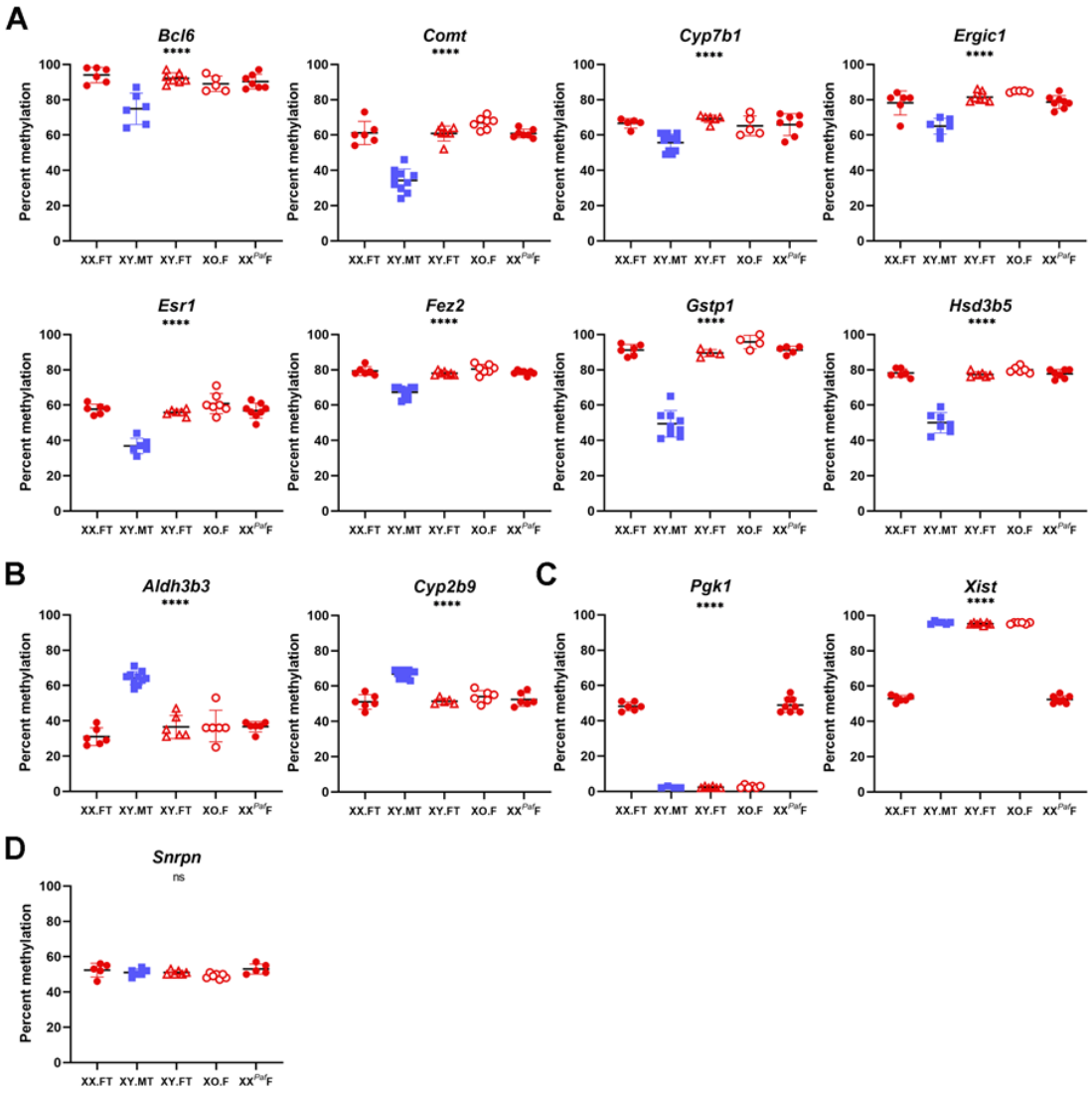

Figure 3.1. Validation of sDMRs using pyrosequencing methylation assays.

A-B. Autosomal sex-phenotype dependent sDMRs with lower methylation in males (*Bcl6*, *Comt*, *Cyp7b1*, *Ergic1*, *Esr1*, *Fez2*, *Gstp1*, and *Hsd3b5*) (**A**); lower methylation in females (*Aldh3d3* and *Cyp2b9*) (**B**). **C.** X-linked sDMRs (*Pgk1 and Xist*) where methylation levels depend on X-chromosome dosage. **D**. Methylation levels at the imprinted *Snrpn* DMR are similar across all groups. Each point corresponds to one DNA sample. Error bars show standard deviation. Statistically significant results of one-way ANOVA represented by asterisks ****p<0.0001, ns: non-significant.

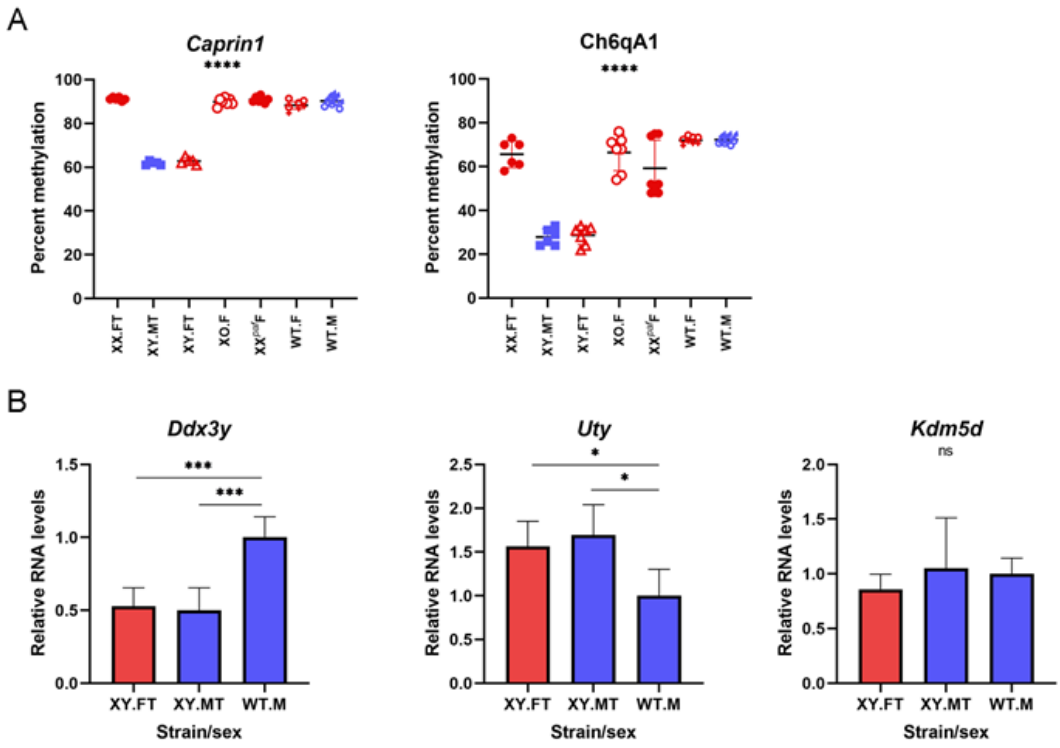

Figure 3.2. Characterization of sDMRs associated with the presence of the Y chromosome.

A. Methylation levels of Y-chromosome dependent sDMRs in adult mice from the B6.TIR, *Paf*, and B6 crosses. **B**. Relative RNA levels of Y-linked genes (*Ddx3y*, *Uty*, and *Kdm5d*) in livers of XY^{TIR} females and males, and B6 males. Error bars show standard deviation. Statistically significant results of one-way ANOVA results (**A**); or one-way ANOVA followed by multiple testing with Tukey's correction (**B**) represented by asterisks * P<0.05, *** P<0.001, **** P<0.0001, ns: non-significant.

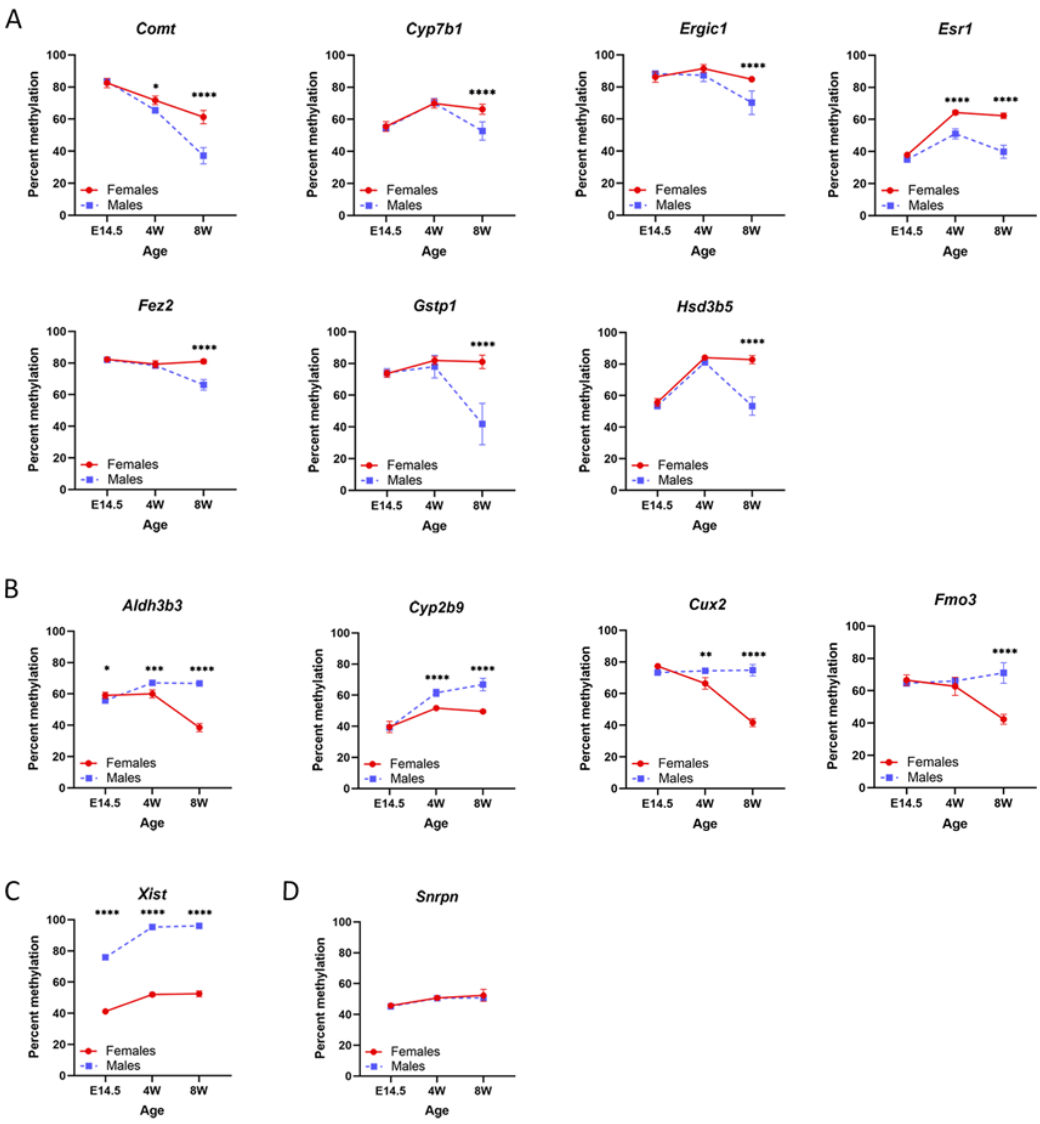

Figure 3.3. Developmental profile of sDMRs in mouse liver.

A-D. Methylation levels in the liver of female and male B6 mice at three developmental stages by pyrosequencing (n= 4-7 per sex and stage). **A**. SDMRs with lower methylation in males (*Comt*, *Cyp7b1*, *Ergic1*, *Esr1*, *Fez2*, *Gstp1*, and *Hsd3b5).* **B**. SDMRs with lower methylation in females (*Aldh3b3*, *Cyp2b9*, *Cux2*, and *Fmo3*). **C**. Methylation levels depend on X-chromosome dosage at *Xist* sDMR. **D**. Methylation levels at the imprinted *Snrpn* DMR. Error bars show standard deviation. Statistically significant results of two-way ANOVA followed by multiple comparisons and Sidak's correction for multiple testing represented by asterisks * P<0.05, *** P<0.001, **** P<0.0001.

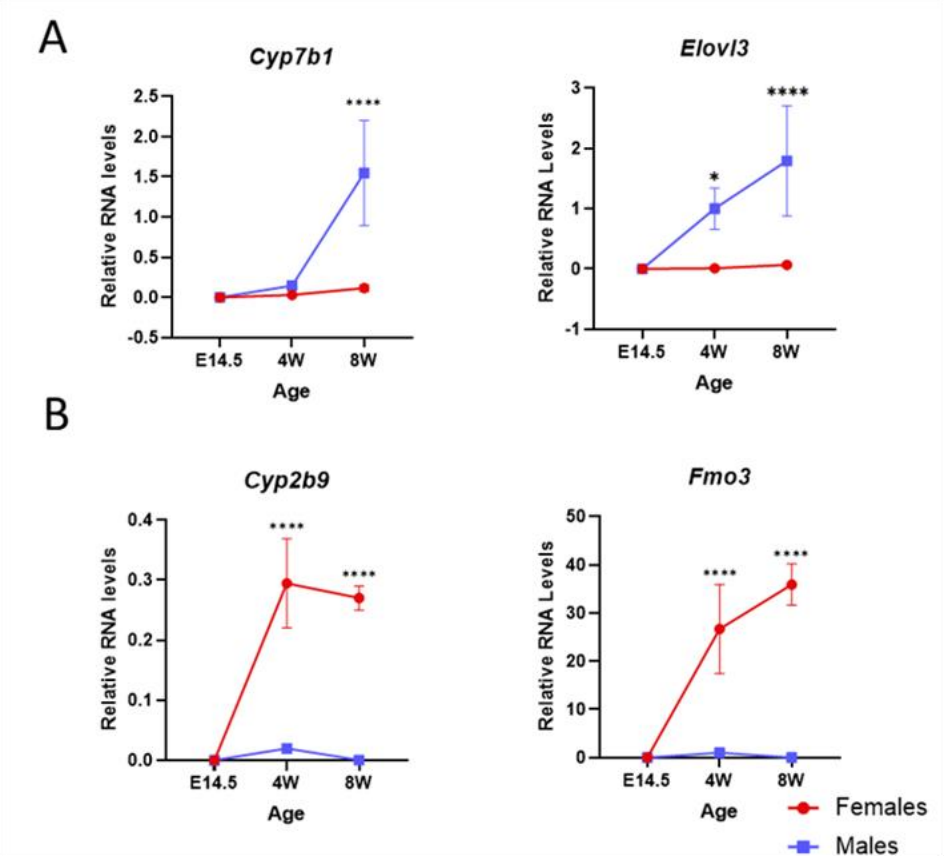

Figure 3.4. Developmental profile of sex-biased differentially expressed genes in mouse liver.

A-B. Relative RNA levels were normalized by *Rpl19* in the liver of B6 female and male mice at three developmental stages, by RT-qPCR (n= 4-7 per sex and stage). **A**. Genes with higher RNA levels in males (*Cyp7b1* and *Elovl3*). **B**. Genes with higher RNA levels in females (*Cyp2b9* and *Fmo3*). Error bars show standard deviation. Statistically significant results of two-way ANOVA followed by multiple comparisons and Sidak's correction for multiple testing represented by asterisks * P<0.5, **** P<0.0001.

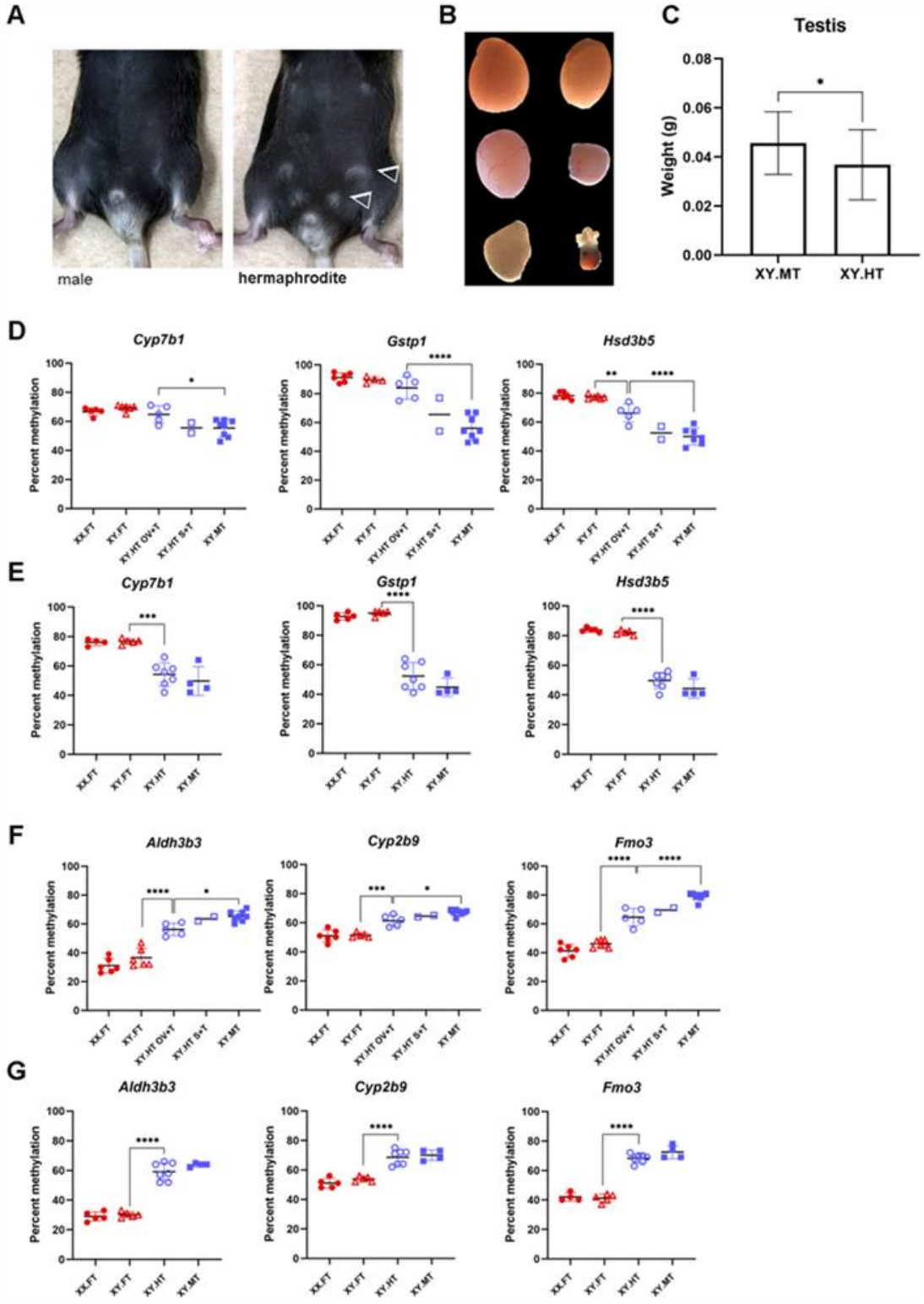

Figure 3.5. Characterizing the methylation of selected sDMRs in hermaphrodites.

A-C. Male (XY.MT) and hermaphrodite (XY.HT) phenotypes. (**A)** Different anogenital distance and mammary gland development in males and hermaphrodites (arrowheads point to mammary glands); (**B)** Males show variable testes weight with either two testes of similar

weight (top row) or one testis larger than the other (middle row), hermaphrodites have unilateral small testis and contralateral ovary (XY.HT OV+T) (bottom row) or a streak gonad (XY.HT S+T) (not shown); (**C)** Testes weight in males and hermaphrodites. **D**-**E**. DNA methylation levels at autosomal sDMRs with lower methylation in males. Each point corresponds to one DNA sample. **(D)** Livers of 8-week old; **(E)** 16-week old XY^{TIR} animals and XX females. **F**-**G**. DNA methylation levels at autosomal sDMRs with lower methylation in females. **(F)** Livers of 8-week old; **(G)** 16-week old XY^{TIR} animals and XX females. **C**-**G**. Error bars show standard deviation. Statistically significant results of one-way ANOVA followed by multiple testing with Tukey's correction represented by asterisks * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. Comparisons to XY.HT are shown.

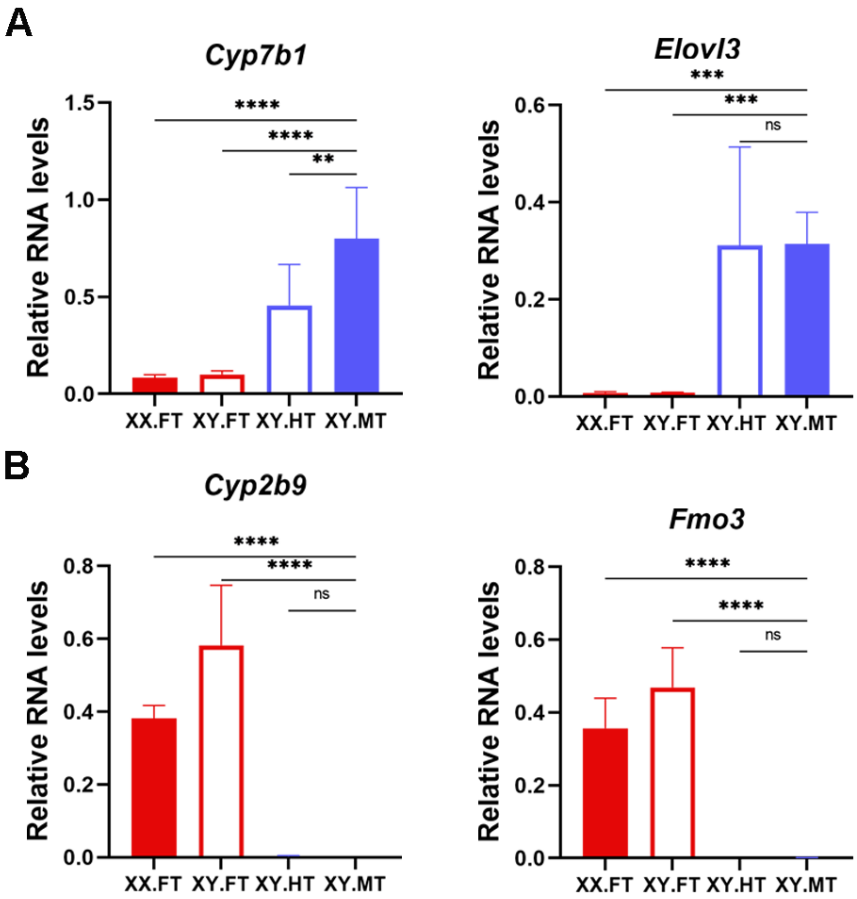

Figure 3.6. Expression levels of sDEGs in the liver of 8-week old B6.TIR mice

A-B. Relative RNA levels were normalized by *Rpl19* in the livers of XY^{TIR} animals and XX females (n= 4-8 per group) **A.** *Cyp7b1* and *Elovl3*. **B.** *Cyp2b9* and *Fmo3*. Error bars show standard deviation. Statistically significant results of one-way ANOVA followed by multiple testing with Tukey's correction represented by asterisks * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Comparisons to XY.MT are shown.

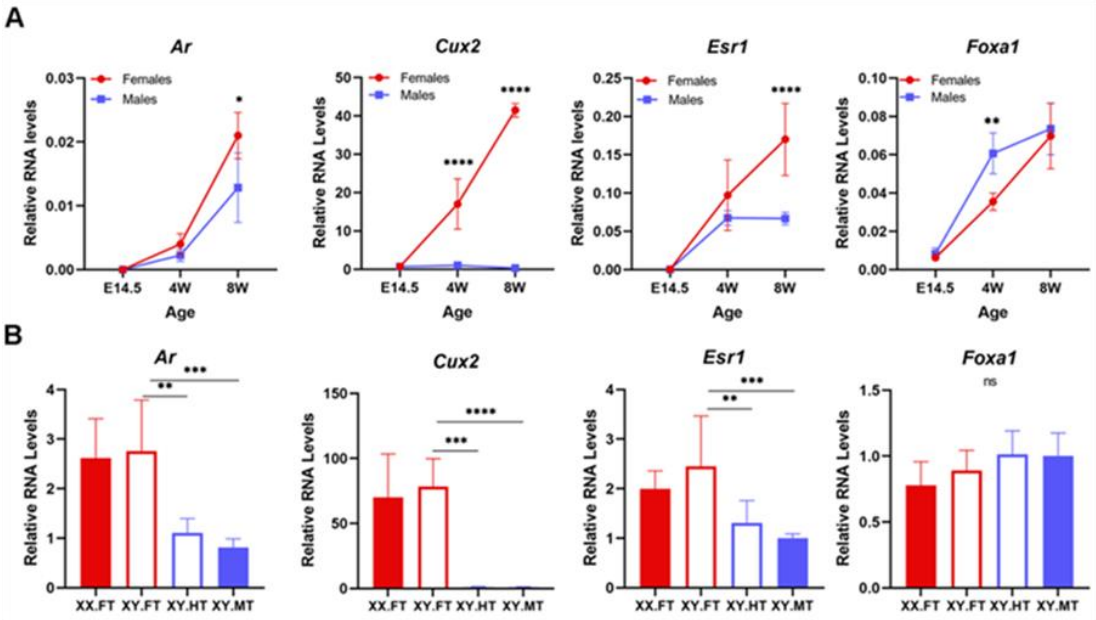

Figure 3.7. **Expression profiles of TFs in the liver of B6 and B6.TIR mice**

A-B. Relative RNA levels were normalized by *Rpl19* in the liver of; **A**. B6 mice at three developmental stages (n= 4-7 per sex and stage). Error bars show standard deviation. Statistically significant two-way ANOVA followed by multiple comparisons and Sidak's correction for multiple testing represented by asterisks * P<0.05, ** P<0.01, **** P<0.0001. **B**. 8-week old XY^{TIR} animals and XX females (n= 4-8 per group). Error bars show standard deviation. Statistically significant one-way ANOVA followed by multiple testing with Tukey's correction represented by asterisks ** P<0.01, *** P<0.001, **** P<0.0001, ns: non-significant.

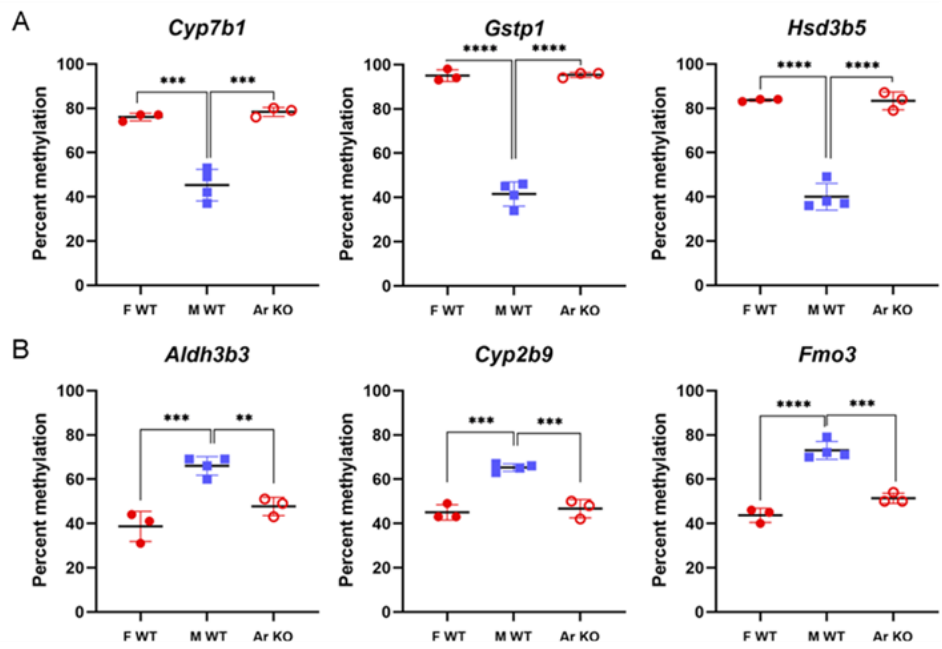

Figure 3.8. Methylation levels in livers of ARKO and WT littermates at six sDMRs.

A. Male-biased sDMRs. **B**. Female-biased sDMRs. Error bars show standard deviation. Each point corresponds to one DNA sample. Statistically significant results of one-way ANOVA followed by multiple testing with Tukey's correction represented by asterisks ** P<0.01, *** P<0.001, **** P<0.0001.

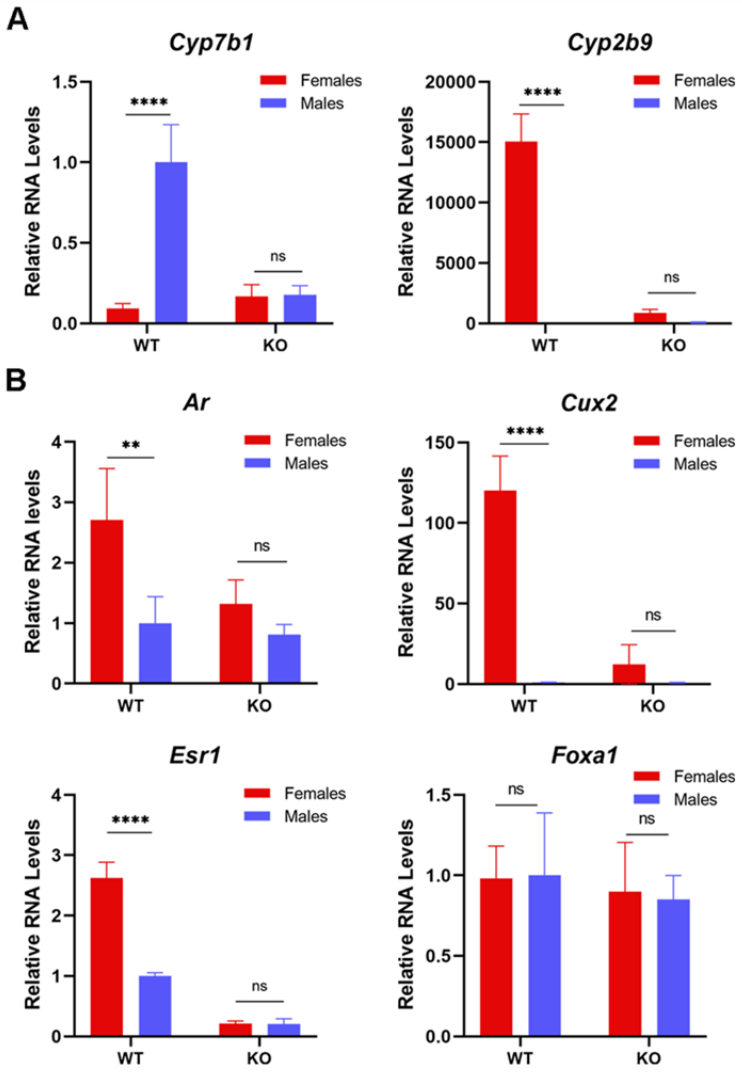

Figure 3.9. Expression levels of sDEGs and TFs in the liver of ESR1KO and WT littermates

A-B. Relative RNA levels were normalized to *Rpl19* (n= 3-4 per sex and genotype)**. A.** *Cyp7b1* and *Cyp2b9*. **B.** *Ar*, *Cux2*, *Esr1*, and *Foxa1*. Error bars show standard deviation. Statistically significant results of two-way ANOVA followed by multiple comparisons and Sidak's correction for multiple testing represented by asterisks ** P<0.01, **** P<0.0001, ns: non-significant.

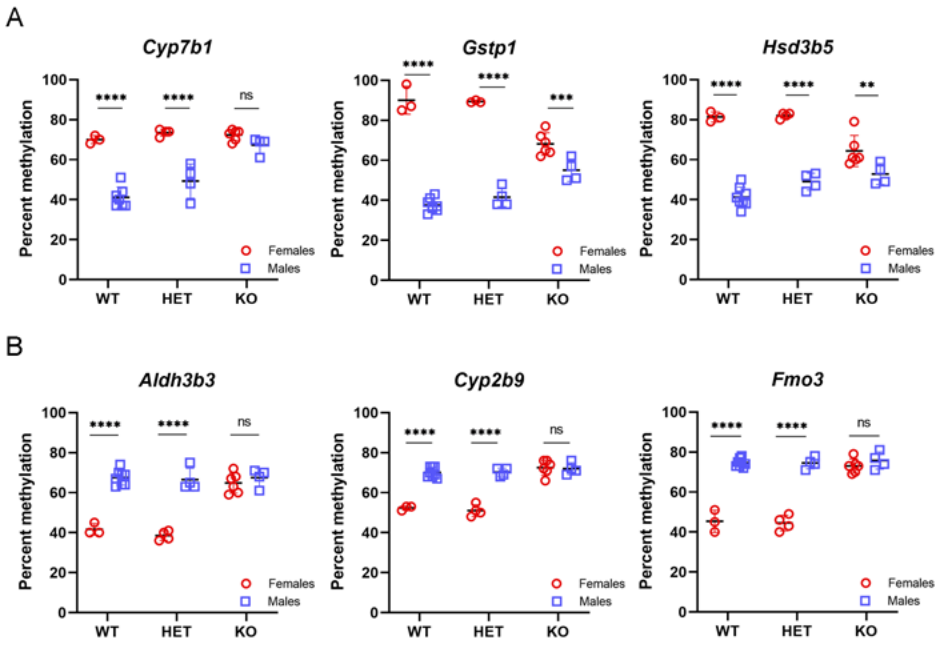

Figure 3.10. Methylation levels in livers of ESR1KO mice and WT littermates at six sDMRs.

A. Male-biased sDMRs. **B**. Female-biased sDMRs. Error bars show standard deviation. Each point corresponds to one DNA sample. Statistically significant results of two-way ANOVA followed by multiple comparisons and Sidak's correction for multiple testing represented by asterisks ** P<0.01, *** P<0.001, **** P<0.0001, ns: non-significant.

Chapter IV: Discussion

Discussion

In this study, we tested the hypothesis that the influence of sex phenotype on sex-biased DNA methylation is mediated by the signaling of sex hormones through their receptors. We established the developmental timing of sex-biased DNA methylation and gene expression at autosomal regions. We also tested the hypothesis that Y-chromosome dependent sDMRs are specific to the B6.TIR strain.

We show that Y-chromosome dependent sDMRs are specific to the B6.TIR cross and Y-linked genes may contribute to this TIR-specific sex-biased methylation. We show that both testosterone and estrogen signaling through their receptors influence methylation at sex-phenotype dependent sDMRs.

4.1. Role of the Y chromosome in sex-biased DNA methylation

We show that the Y chromosome from *Mus musculus domesticus* (B6.TIR) and not from B6 causes lower DNA methylation at certain autosomal regions in mouse liver in XY mice independent of their sex phenotype or X-chromosome dosage (**Figure 3.2A**). This indicates that Y chromosome genes influence DNA methylation at certain autosomal loci. There are two potential mechanisms, genetic differences in the Y-linked genes between B6.TIR and B6 mice could lead to differences in their regulation or protein function.

We tested the possibility that differences in methylation resulted from differences in Y-linked gene expression levels and selected Y-linked genes expressed in the liver based on our previously published RNA-seq data (Zhuang et al., 2020). Four genes were expressed in the liver of XY^{TIR} females and males: *Eif2s3y*, *Ddx3y*, *Uty*, and *Kdm5d*. Of those genes, *Ddx3y*, *Uty*, and *Kdm5d* were of particular interest for their involvement in histone modification and cellular processes. The expression levels of both *Ddx3y* and *Uty* are different in B6 males compared to XY.MT and XY.FT (**Figure 3.2B**). There was no difference in the expression of *Kdm5d* between the two strains, however, we cannot exclude the possibility of differences in protein function. Histone modification and DNA methylation are interdependent and can influence each other (Jia et al., 2007; Ooi et al., 2007). Our results suggest that two Y-linked genes, *Ddx3y* and *Uty*, maybe the contributing factors to B6.TIR-specific methylation levels at autosomes.

Although B6.TIR and B6 autosomal and X-linked genes are from the same genetic background (B6), their interaction with Y-genes, hence their expression, may vary depending

on the Y chromosome's genetic background. So, sex-biased DNA methylation at TIR-specific sDMRs could be an indirect effect of autosomal or X-linked downstream targets of Y-linked genes.

4.2. The developmental timing of sex-biased DNA methylation

At most sDMRs, the sex bias in DNA methylation was associated with loss of methylation in one of the sexes at 8 weeks of age (**Figure 3.3**). These results are consistent with the observation from another group where liver methylation was compared using RRBS in mouse livers and concluded that loss of methylation in males occurs around puberty as a result of testosterone surge (Reizel et al., 2015). However, in our targeted approach, we find that in a couple of sDMRs (*Cyp2b9*, and *Esr1*) one sex had less gain of methylation before puberty at 4 weeks, which may be associated with sex-biased expression of TFs FOXA1 or CUX2, for example (**Figure 3.7A**). Hence, our data show variation in the age of onset of sex-biased DNA methylation and different profiles in establishing sex difference in methylation, suggesting the involvement of several factors.

We observed changes in DNA methylation with age included but not limited to in sDMRs in gene bodies, which made it difficult to predict its association with gene transcription. To test if the timing of sex-biased expression of genes containing sDMRs coincides with sex-biased methylation, we performed gene expression analysis of four genes that harbor sDMRs. We observed some overlap between the timing of sex bias in DNA methylation and gene expression (**Figure 3.3 and 3.4**). The role of DNA methylation in intergenic regions on transcription is complex making the nature of their association hard to predict. Many of our male-biased sDMRs reside in enhancer regions (**Table 2.2**). Interestingly, in the mouse liver, lower methylation at enhancer elements occurs after birth and is required to establish postnatal gene expression patterns (Reizel et al., 2018). A survey of DNA methylation and expression in human tissues reveals a negative correlation between methylation and gene expression (Schultz et al., 2015). The correlation becomes stronger closer to the transcription starting site (TSS) but reaches the strongest correlation downstream TSS (Schultz et al., 2015).

4.3. Age-dependent change in DNA methylation in hermaphrodites

XY hermaphrodites showed female-like or intermediate methylation levels at 8 weeks (**Figure 3.5**). This could be due to the lower testosterone levels in hermaphrodites compared to males or the presence of ovaries (Houle & Taketo, 1992). As to my knowledge, the possible effect of ovaries on DNA methylation seen in our study has not been reported before. Hermaphrodites establish male-like methylation levels at 16 weeks (**Figure 3.5**). This could be an effect of prolonged testosterone exposure or the deterioration in ovarian function. These findings provide evidence that both testosterone and estrogen signaling contribute to sex-biased DNA methylation. The expression of three out of four sex-biased genes in hermaphrodites was not significantly different from males at 8 weeks (**Figure 3.6**).

4.4. The roles of sex hormone receptors in sex-biased DNA methylation

The effects of sex hormones on sex-biased DNA methylation may depend on the availability of their receptors, androgen and estrogen receptors (Zheng et al., 2018). We demonstrate that sex-phenotype dependent DNA methylation levels are influenced by testosterone signaling through AR and estrogen signaling through ESR1. We also find an interaction between those TFs, as the DNA methylation at the 6 sDMRs is affected by either AR or ESR1 loss (**Figure 3.8 and 3.10**). We also show that depletion of ESR1 decreases the expression of *Ar* in the female liver (**Figure 3.9**).

The effect of AR and ESR1 could be by direct binding to their target region and/or by more complex mechanisms. In the liver, only 26% and 15% of sex-biased differentially expressed genes have ESR1 and AR binding sites, respectively (Zheng et al., 2018). It is possible that AR and ESR1 action outside of the liver is contributing to sex-biased DNA methylation. In our mouse models, the ARKO mice have AR protein that is unable to bind to DNA (Notini et al., 2005). This suggests that the feminization of methylation patterns in the XY ARKO mice is due to the genomic activity of AR. However, the ESR1KO mice have a complete global depletion of ESR1, so the effect of ESR1 on methylation is caused by either the genomic or nongenomic ESR1 activity in both sexes (Hewitt et al., 2010). ESR1 regulates the secretion of gonadal hormones by a negative feedback mechanism on the pituitary and hypothalamus (Messinisi, 2006). Thus, it is possible that ESR1 action on the hypothalamic-pituitary-gonadal axis plays a role in establishing or maintaining sex bias in DNA methylation. It is also possible that AR and ESR1 influence other TFs that regulate DNA methylation.

Our recently published motif analysis shows enrichment of CUX2 and FOXA1 at autosomal sex-phenotype dependent sDMRs (Zhuang et al., 2020). CUX2 is a female-specific repressor of male-biased genes and activator of female-biased genes (Conforto et al., 2012; Sugathan & Waxman, 2013). ESR1 binds to multiple regions at the *Cux2* gene (Zheng et al., 2018). We show that *Cux2* expression is higher in females compared to males at 4 and 8 weeks (**Figure 3.7A**). Moreover, ESR1 depletion reduces the expression of *Cux2* in the adult female liver to a similar level to the male (**Figure 3.9**). Thus, the influence of ESR1 on DNA methylation at sDMRs could be mediated by CUX2. This interaction between ESR1 and CUX2 could explain the loss of both higher methylation at male-biased sDMRs and lower methylation at female-biased sDMRs in ESR1KO female liver.

FOXA1 is a pioneer TF with a pivotal role in liver development that is recruited to enhancer regions and associates with lower methylation (Reizel et al., 2020; Sahu et al., 2011). FOXA1 depletion reverses the lower methylation of its target sites, indicating a role of FOXA1 in changing DNA methylation levels (Zhang et al., 2016). This observation was confirmed by FOXA1 overexpression (Zhang et al., 2016). We show that the expression of *Foxa1* increases with age in both females and males, and sex-biased expression is seen at 4 weeks, but not in the adult liver (**Figure 3.7A**). Our data show that TFs involved in the regulation of sex differences in the liver have dynamic expression profiles, suggesting that sex-biased expression of TFs may be associated with sex-biased DNA methylation seen at different ages.

In this thesis, we selected a panel of 11 sDMRs for the study of developmental profiles, and 6 of these sDMRs for the study of sex hormones receptors, which may or may not capture the genomic profile of sex-biased DNA methylation. However, we selected sDMRs at different genomic regions to try to reflect the wider picture. We selected these sDMRs for their association with sex-biased expression of their nearby genes. Unfortunately, due to the high conservation between different genes from the same family, we validated the results of RNA-seq for three sDMR-proximal genes only. We tested the expression of one additional male-biased gene *Elovl3*, that also harbors an sDMR, but the methylation profile was not tested.

Chapter V: Conclusions and Future Directions

Conclusions

Based on our observations described in this thesis, we came to the following conclusions:

1- SDMRs that depend on the Y-chromosome presence are specific to the B6.TIR strain of mice.

2- Sex-biased DNA methylation and gene expression in mouse liver vary with age.

3- Hermaphrodites have female-like or intermediate methylation levels at sDMRs at 8 weeks, and they develop male-like methylation by 16 weeks. Hermaphrodites have male-like or intermediate expression levels of sex-biased differentially expressed genes at 8 weeks.

4- Signaling of gonadal sex hormones through their receptors contributes to sex-biased methylation in mouse liver. The genomic activity of AR in males contributes to both lower methylation and higher methylation at male-biased and female-biased sDMRs, respectively. ESR1 contributes to sex-biased methylation and expression. Loss of ESR1 induces loss/reduction of sex differences in DNA methylation at male-biased sDMRs by increasing methylation levels in males and decreasing methylation levels in females. At female-biased sDMRs, loss of ESR1 induces loss of sex bias in DNA methylation by increasing methylation in females.

Future Directions

We demonstrate that the signaling of sex hormones through their receptors influences sex-biased DNA methylation in the mouse liver. We used global androgen and estrogen receptor knockout mouse models, thus the effect of these receptors on DNA methylation might be outside the liver. It would be interesting to compare the results from the global knockout models to liver-specific knockouts.

In our study, we show that ESR1 influences DNA methylation in both female and male livers. For the AR, we had only the XY KO. Therefore, in future studies, we can examine the effect of AR on sex-biased methylation in the liver of females.

Finally, we have shown that ESR1 loss influences both sex-bias in DNA methylation and gene expression. However, we did not test the expression of sex-biased genes in ARKO. Future work could examine the effect of AR loss on sex-biased gene expression in the mouse liver.

Chapter VI: References

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Appendix

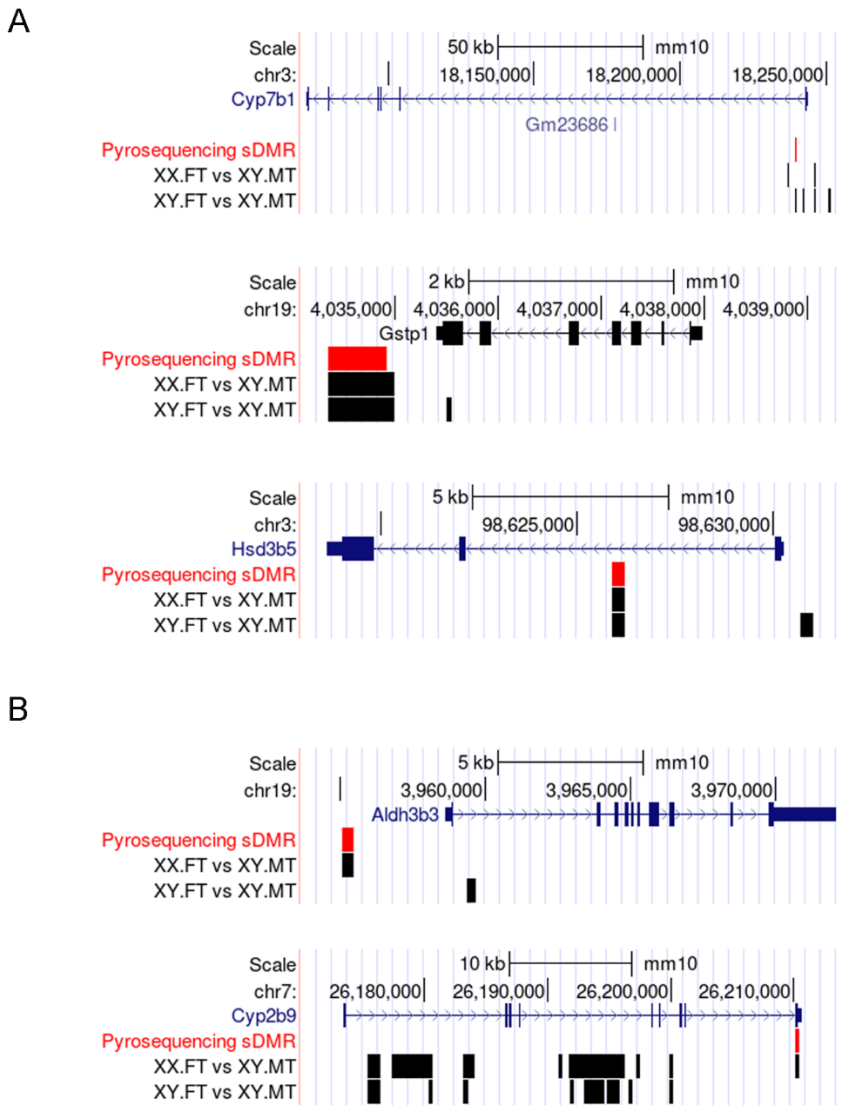

Figure S1. SDMRs associated with sDEGs.

All tracks are shown in the context of the UCSC genome browser (mm10). The 'Pyrosequencing sDMR' track shows the sDMR used for pyrosequencing methylation analysis. The 'XX.F vs XY.M' and 'XY.F vs XY.M' tracks show the locations of sDMRs based on WGBS data from (Zhuang 2020). **A.** Male-biased sDMRs. **B.** Female-biased sDMRs.

McGill

November 19, 2020

Animal Certificate

This is to certify that **Dr. Anna K. Naoumova, Department of Obstetrics and Gynecology, (RI MUHC) Glen site,** currently holds an approved **Animal Use Protocol # 2001-4037** with McGill University and its Affiliated Hospital's Research Institutes for the following project:

Animal Use Protocol Title: Sexual dimorphism in the mammalian epigenome

Start date: November 1, 2020

Expiration date: October 31, 2021

McGill University and Affiliated Hospitals Research Institutes recognize the importance of animal research in our efforts to further our knowledge of natural processes, diseases and conservation. Research, educational and testing projects are conducted with full commitment to the wellbeing of the animal subjects. In order to limit animal use to meritorious research or educational projects, the institution relies on stringent peer review processes, along with assessment of ethical issues by the Animal Care Committee. McGill University recognizes that the use of animals in research, teaching and testing carries significant responsibilities. The institution will continue to develop and maintain guidelines and regulations, following the high standards established by the Canadian Council on Animal Care. It is committed to conducting the highest-quality research and to providing animals with the best care.

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THIS IS TO CERTIFY THAT

Najla Alogayil

Department of Human Genetics

SUCCESSFULLY COMPLETED CORE TRAINING IN

Introduction to Biosafety

ON 07-Mar-19

Joseph Vincelli
EHS Operations Manager

Wayne Wood
Director, EHS

Valid Until Sunday, March 06, 2022

Centre universitaire de santé McGill
Institut de recherche

McGill University Health Centre
Research Institute

This is to certify

Najla Alogayil

Has completed the following workshop courses at the Research Institute of the McGill University Health Center (RI-MUHC):

Mouse Module 1
On
August 13, 2019

Techniques successfully completed:
Handling/Restraint
Isoflurane/CO2 euthanasia
Cardiac Puncture (blood collection) under anesthesia
Cervical Dislocation under anesthesia
Decapitation under anesthesia
Pneumothorax
Mouse Neonate Decapitation without Anesthesia

Melanie Houston
RI-MUHC Training Coordinator