The influence of the Y chromosome on autosomal DNA methylation

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Abstract

Sexual dimorphism between males and females of a given species ranges from gross phenotypes to gene expression and DNA methylation. Both gonadal sex and sex-chromosome complement contribute to sexual dimorphism in DNA methylation in somatic cells. However, the molecular mechanisms responsible for the establishment of sexually dimorphic DNA methylation levels in somatic cells are still poorly understood. Hence, this study focused on one of the factors that potentially contribute to sex bias in DNA methylation, the influence of the Y chromosome, using mice with different combinations of gonadal sex and sex-chromosome complement.

Whole genome bisulfite sequencing (WGBS) methylation data from livers and brains of adult mice with different combinations of gonadal sex and sex-chromosome complement were analyzed, and a list of 22 Y-chromosome dependent sex-associated differentially methylated regions (yDMRs) was generated. Approximately 90% of yDMRs overlapped with repetitive elements. Four yDMRs were validated using pyrosequencing methylation assays and used as reporter yDMRs for analyzing tissue-specificity of yDMRs and effects of genetic background on Y-dependent methylation.

We tested tissue-specificity of the reporter yDMRs by analyzing DNA methylation in spleen, heart, lung, brain, tail, and testes from the same mice and we demonstrated that yDMRs were present in all tested non-reproductive organs. This suggested that yDMRs either arise early in development or the Y-linked gene(s) responsible for influencing autosomal DNA methylation is expressed in all tested organs. Next, we examined the impact of genetic background including the subspecific origin of the Y chromosome by analyzing DNA methylation in mice from different laboratory strains carrying either the *Mus musculus musculus* or *Mus musculus domesticus* Y

chromosomes. We demonstrated that both genetic background and the origin of the Y chromosome had an effect on DNA methylation. Lastly, we hypothesized that Y-dependent DNA methylation was associated with a non-synonymous coding polymorphism in lysine demethylase 5D (*Kdm5d*) gene. To test this hypothesis, we analyzed methylation of reporter yDMRs in *Kdm5d* knock-out mice and found no effect. Therefore, we established that *Kdm5d* was unlikely to be affecting DNA methylation at the yDMRs.

Résumé

Le dimorphisme sexuel entre les mâles et les femelles d'une espèce donnée varie des phénotypes bruts à l'expression des gènes et à la méthylation de l'ADN. Le sexe gonadique et le complément du chromosome sexuel contribuent au dimorphisme sexuel à la méthylation de l'ADN dans les cellules somatiques. Cependant, les mécanismes moléculaires responsables de l'établissement de niveaux de méthylation de l'ADN sexuellement dimorphes dans les cellules somatiques sont encore mal compris. Par conséquent, cette étude s'est concentrée sur l'un des facteurs qui contribuent potentiellement au biais sexuel dans la méthylation de l'ADN, l'influence du chromosome Y, en utilisant des souris avec différentes combinaisons de sexe gonadique et de complément de chromosome sexuel.

Les données de méthylation du séquençage du bisulfite du génome entier provenant du foie et du cerveau de souris adultes avec différentes combinaisons de sexe gonadique et de complément de chromosome sexuel ont été analysées, et une liste de 22 régions méthylées différentiellement associées au sexe dépendantes du chromosome Y (yDMRs) a été générée. Environ 90 % des yDMRs se chevauchaient avec des éléments répétitifs. Quatre yDMRs ont été validés à l'aide d'essais de méthylation par pyroséquençage et utilisés comme rapporteurs yDMRs pour analyser la spécificité tissulaire des yDMRs et les effets du bagage génétique sur la méthylation dépendante de Y.

Nous avons testé la spécificité tissulaire des rapporteurs yDMRs en analysant la méthylation de l'ADN dans la rate, le cœur, les poumons, le cerveau, la queue et les testicules des mêmes souris et nous avons démontré que les yDMRs étaient présents dans tous les organes non reproducteurs testés. Cela suggère que les yDMRs apparaissent tôt dans le développement

embryonnaire ou que le(s) gène(s) présent(s) à chromosome Y responsables de l'influence de la méthylation autosomique de l'ADN sont exprimés dans tous les organes testés. Ensuite, nous avons examiné l'impact du bagage génétique, y compris l'origine sous-spécifique du chromosome Y, en analysant la méthylation de l'ADN chez des souris de différentes souches de laboratoire portant le chromosome Y *Mus musculus musculus* ou le chromosome Y *Mus musculus domesticus*. Nous avons démontré que le bagage génétique et l'origine du chromosome Y avaient un effet sur la méthylation de l'ADN. Enfin, nous avons émis l'hypothèse que la méthylation de l'ADN dépendante de chromosome Y était associée à un polymorphisme codant non-synonyme dans le gène lysine K déméthylase (*Kdm5d*). Pour tester cette hypothèse, nous avons analysé la méthylation des rapporteurs yDMRs chez des souris *Kdm5d* knock-out et n'avons trouvé aucun effet. Par conséquent, nous avons établi qu'il était peu probable que *Kdm5d* affecte la méthylation de l'ADN des yDMRs.

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Contribution of Authors

Organs from XX.FT, XY.MT, sex-reversed XY.FT, XX^{Paf}.F and XO.F mice were collected by Najla AlOgayil, a student in Dr. Anna Naumova's lab. Testes of XY.MT mice were collected by Dr. Naumova and Enkhjin Batdorj (the candidate). Najla AlOgayil also extracted genomic DNA from the livers for whole genome bisulfite sequencing methylation analysis (WGBS). WGBS analysis was performed by Qinwei Kim-Wee Zhuang, a student in Dr. Guillaume Bourque's lab (Zhuang et al, 2020).

Validated assays, which are *Caprin1*, *Chr2_CG1*, *Ch2qH1_2*, and *Ch6qA*, were designed by Najla AlOgayil. The *Chr9qA5* and *Chr15qE2* assays were designed by Enkhjin Batdorj. The *Chr16qB* assay was designed by Dr. Anna Naumova.

Fig S2 was made using Najla AlOgayil's data.

Klara Bauermeister, a student in Dr. Anna Naumova's lab, designed some of the primers used to genotype the mouse Y chromosome.

The abstract was translated into French with the help of Tserennadmid Bayasgalan and Chantal Cozian.

All other experiments described in the thesis were performed by the candidate.

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List of Abbreviations

5caC: 5-carboxylcytosine

5fC: 5-formylcytosine

5hmC: 5-hydroxymethylcytosine

5mC: 5-methylcytosine

ANOVA: analysis of variance

AR: androgen receptor

AREs: androgen response elements

B6.Y^{TIR}: Tirano mice

BRG1: brahma-releated gene-1 protein

Caprin1: cell cycle associated protein 1 gene

CG: CpG

Chr: chromosome

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

CXXC: Cysteine-X-X-Cysteine

DHT: dihydrotestosterone

DMR: differentially methylated region

DNA: deoxyribonucleic acid

DNMT: DNA methyltransferase

Dnmt1: DNA methyltransferase 1 gene

DNMT1: DNA methyltransferase 1 protein

Dnmt3a: DNA methyltransferase 3a gene

DNMT3A: DNA methyltransferase 3A protein

Dnmt3b: DNA methyltransferase 3b gene

DNMT3B: DNA methyltransferase 3B protein

Dnmt3c: DNA methyltransferase 3c gene

DNMT3C: DNA methyltransferase 3C protein

DNMT3L: DNA methyltransferase 3L protein

E: embryonic day

E_1: estrone

E_2: 17β-estradiol

E_3: estriol

ERs: estrogen receptors

EREs: estrogen response elements

EZH2: enhancer of zeste homolog 2 protein

FB: F_1 (C57BL/6J female x FVB/NJ male)

FGF9: fibroblast growth factor 9 protein

Fnbp1: forming binding protein 1 gene

H3K4: lysine 4 of histone H3

H3K4me2/3: di- and tri-methylated lysine 4 of histone H3

H3K27me3: tri-methylated lysine 27 of histone H3

HMG: high mobility group

ICR: Institure of Cancer Research mouse strain

IDAX: inhibition of the dvl and axin complex protein

JmjC: Jumonji C-terminal domain

JmjN: Jumonji N-terminal domain

KDM5C: lysine demethylase 5C protein

Kdm5d: lysine demethylase 5D gene

KDM5D: lysine demethylase 5D protein

KO: knock out

LINE: long interspersed element

LINE-1: long interspersed nuclear element-1

LTR: long terminal repeat

Mb: megabase

MSY: male-specific region of the Y chromosome

NS: nonsynonymous

P450arom: cytochrome P450 aromatase

Paf: patchy fur

PAR: pseudoautosomal region

PRC2: polycomb repressive complex 2

Rbmy: RNA-binding motif protein, Y chromosome

RFLP: restriction fragment length polymorphism

RNA-FISH: ribonucleic acid-fluorescence in situ hybridization

sDMR: sex-associated differentially methylated region

SINE: short interspersed element

SNP: single nucleotide polymorphism

SNV: single nucleotide variant

Sox9: sry-box transcription factor gene

SOX9: sry-box transcription factor protein

Sry: sex-determining region, Y gene

SRY: sex-determining region, Y protein

SETDB1: SET domain bifurcated histone lysine methyltransferase 1 protein

SWI/SNF: switch/sucrose nonfermenting

TE: transposable element

TET: ten-eleven translocation methylcytosine dioxygenase protein

TET1: ten-eleven translocation methylcytosine dioxygenase 1 protein

TET2: ten-eleven translocation methylcytosine dioxygenase 2 protein

TET3: ten-eleven translocation methylcytosine dioxygenase 3 protein

TESCO: testis-specific enhancer of *Sox9* core

Uba1y: ubiquitin activating enzyme E1, Y chromosome gene

UCSC: University of California, Santa Cruz

UHRF1: ubiquitin-like protein containing PHD and RING finger domains 1

Usp9y: ubiquitin specific peptidase 9, Y chromosome

Uty: ubiquitously transcribed tetratricopeptide repeat containing, Y-linked gene

WGBS: whole genome bisulfite sequencing

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

WT: wild type

XIC: X-inactivation centre

XistI: X-inactive specific transcript gene

XO.F: XO female mice from the cross between C57BL/6J female and $X^{Paf}Y$ male mice

XX.FT: XX female mice from the cross between C57BL/6J female and $B6.Y^{TIR}$ male mice

XX^{Paf}.F: XX^{Paf} female mice from the cross between C57BL/6J female and $X^{Paf}Y$ male mice

XY.FT: sex-reversed XY female mice from the cross between C57BL/6J female and B6.Y^{TIR} male mice

XY.MT: XY male mice from the cross between C57BL/6J female and B6.Y^{TIR} male mice

YDMR: Y-chromosome dependent sex-associated differentially methylated region

Yp: short arm of the Y chromosome

Yq: long arm of the Y chromosome

Y^{TIR}: Tirano Y chromosome

Zfy1: zinc finger protein 1, Y chromosome gene

Zfy2: zinc finger protein 2, Y chromosome gene

Chapter 1:

Introduction

1.1. Sexual dimorphism

Sexual dimorphism refers to the differences between males and females of a given species (reviewed in (Frayer & Wolpoff, 1985; Grath & Parsch, 2016; McCombe & Greer, 2014; Mesnick & Ralls, 2018)). Sexual dimorphism is observed as early as embryonic development and throughout mammalian lifetime, including humans and mice. During early embryonic development in mice, male embryos develop faster than female embryos (Tsunoda et al, 1985). At the time of birth in humans, male newborns weigh more than female newborns (Karn & Penrose, 1951). Males are more susceptible to certain infectious diseases, including bacterial, fungal, parasitic, and viral infections, compared to females (reviewed in (Klein, 2004; McClelland & Smith, 2011)). Males also have a higher risk of developing certain types of cancers that are not associated with reproductive function, such as liver, lung, colon, and skin cancers, and they are more likely to get diagnosed with invasive cancers compared to females (Siegel et al, 2015; 2022). However, females are more susceptible to autoimmune diseases, such as thyroiditis, systemic sclerosis, systemic lupus erythematosus, and Sjögren disease, compared to males (Pillemer et al, 2001; Steen et al, 1997; (reviewed in (Cooper & Stroehla, 2003; Kaminsky et al, 2006)). Additionally, older females have a higher chance of suffering from Alzheimer's disease compared to males of same age (reviewed in (Podcasy & Epperson, 2016; Viña & Lloret, 2010)). These phenotypic differences between mammalian males and females may arise due to their differences in sex-chromosome complement and gonadal sex, and hence differences in genetics and gonadal hormones, respectively. Males have XY chromosomes as sex-chromosome complement, testes as

gonads, and testosterone as the main gonadal hormone while females have XX chromosomes as sex-chromosome complement, ovaries as gonads, and estrogen as the main gonadal hormone.

1.2. Sex determination (the role of the Y chromosome) and gonadal hormones

1.2.1. Sex determination and the role of the Y chromosome

Sex determination in mice occurs during early embryonic development; around embryonic day 10.5 (E10.5), and if the fertilized egg contains XX or XY chromosomes, the embryo will develop as a female or a male, respectively (reviewed in (Eggers & Sinclair, 2012; Greenfield, 2015; Swain & Lovell-Badge, 1999)). However, when XX or XY gonads begin to form at E10.0, they are morphologically and functionally identical to each other and they have the potential to become either ovaries or testes, respectively, and hence they are termed bipotential gonads (reviewed in (Greenfield, 2015; Kashimada & Koopman, 2010; Mäkelä et al, 2018)). Sex determination in the bipotential gonads occurs when the Y-linked gene (*Sry*) is expressed and subsequently contributes to the initiation of testis determining pathway by upregulating factors required for testes to differentiate (Koopman et al, 1991; Welshons & Russell, 1959; (reviewed in (Arnold, 2017; Cameron & Sinclair, 1997; Capel, 2017; Greenfield, 2015; Lowell-Badge, 1993; Swain & Lovell-Badge, 1999)).

The testis-determining gene sex determining region, Y (*Sry*) resides on the short arm of the Y chromosome (Gubbay et al, 1990). *Sry* encodes a protein that contains a DNA-binding high mobility group (HMG) box domain and acts as a transcription factor (Harley et al, 1992; Sinclair et al, 1990; (reviewed in (Warr & Greenfield, 2012)). *Sry* is transiently expressed in the somatic cells, specifically pre-Sertoli cells, of XY bipotential gonad between E10.5 and E12.5 (reviewed in (Kashimada & Koopman, 2010; Warr & Greenfield, 2012)). SRY expression in the pre-Sertoli

cells leads to the activation of its immediate target termed sry-box transcription factor (*Sox9*) gene at E11.5 by binding to the testis-specific enhancer of *Sox9* core (TESCO) (Sekido & Lovell-Badge, 2008; reviewed in (Capel, 2017; Kashimada & Koopman, 2010; Mäkelä et al, 2018; Warr & Greenfield, 2012)). *Sox9* also contains a DNA-binding HMG box domain and is necessary for the differentiation of both Sertoli cells and testes (Barrionuevo et al, 2006; Chaboissier et al, 2004; Kent et al, 1996; Sekido et al, 2004; (reviewed in Barrionuevo et al, 2011)). Differentiation of testes requires high levels of continuous expression of *Sox9*, and hence SOX9 employs a positive feedback loop where it acts together with its target fibroblast growth factor 9 (FGF9) to maintain continuously its expression at high levels (reviewed in (Capel, 2017)). FGF9 functions to inhibit the expression of Wnt family member 4 (*Wnt4*) gene involved in the activation of genes important for the differentiation of ovaries, and hence female pathway (reviewed in (Capel, 2017; Kashimada & Koopman, 2010)).

1.2.2. Gonadal hormones

Testosterone is the main male sex hormone that is essential for the male reproductive system and male secondary sexual characteristics (reviewed in (Davey & Grossmann, 2016; Klein, 2004; Tyagi et al, 2017)). Testosterone, the main androgen, is synthesized from cholesterol in the mitochondria of the Leydig cells of the testes and then secreted from the testes (reviewed in (Luetjens & Weinbauer, 2012; Norman & Henry, 2015)). Testosterone is converted into its more potent form dihydrotestosterone (DHT) by 5α-reductase enzyme and DHT triggers the urogenital sinus and the genital tubercle to differentiate into the prostate and penis, respectively, which are the parts of the external male genitalia (reviewed in (Bagatell & Bremner, 2003; Jordan & Doncarlos, 2008; Marchetti & Barth, 2013; Norman & Henry, 2015; Roy et al, 1998)).

Testosterone can be further modified into E_2 or 17β-estradiol, a form of estrogen (reviewed in (Dong & Hardy, 2004)).

Testosterone is aromatized to E_2 in the brain by cytochrome P450 aromatase (P450arom) enzyme and both testosterone and E_2 are involved in the masculinization of the developing brain (reviewed in (Dong & Hardy, 2004; Lenz & McCarthy, 2010; McCarthy, 2009)). Estrogens including 17β-estradiol (E_2), estrone (E_1), and estriol (E_3) are also synthesized from cholesterol and are the primary female sex hormones important for the female reproductive system and female secondary sexual characteristics (reviewed in (Chen et al, 2019; Gruber et al, 2002; Leung et al, 2004)). E_2 is the most active form of estrogens in females and is produced and secreted by the granulosa cells of the ovaries (reviewed in (Chen et al, 2019; Cui et al, 2013; Gruber et al, 2002)). E_2 is also involved in the growth, differentiation, and function of nonreproductive tissues, such as skeletal, cardiovascular, and nervous systems both in males and females (reviewed in (Chen et al, 2019; Deroo & Korach, 2006)).

Androgens and estrogens mediate their effect on sexual differentiation by binding to their cognate receptors - androgen receptor (AR) and estrogen receptors (ERs), respectively - present on the surface of the target cells. Ligand bound AR and ligand bound ERs enter the nucleus of the target cells and regulate the expression of the target genes by binding to androgen response elements (AREs) and estrogen response elements (EREs), respectively, present within the promoter or enhancer regions of the target genes (reviewed in (Davey & Grossmann, 2016; Gruber et al, 2002; Hall et al, 2001; Kiyama & Wada-Kiyama, 2015; Wallach et al, 1996)).

1.3. The sex-chromosome complement

Sex-chromosome complement of mammalian males and females, including humans and

mice, are XY and XX, respectively. Sex-chromosome complement evolved from a pair of autosomes as a consequence of the emergence of the sex-determining gene *Sry* on one of the autosomes and that chromosome later became the Y chromosome (reviewed in (Arnold, 2019; Disteche et al, 2002; Ohno, 1966)). Regardless of X and Y chromosomes being homologs, they differ significantly in size. The evolving X chromosome preserved most of the ancestral genes while only 2% of the ancestral genes survived on the evolving Y chromosome (Soh et al, 2014). The X and Y chromosomes recombine only at the pseudoautosomal region (PAR) present at the end of their long arm during male meiosis (Soh et al, 2014; reviewed in (Arnold, 2019; Raudsepp & Chowdhary, 2015)). Testis-specific genes and genes important for fertility of males reside throughout the rest of the Y chromosome and this region is termed male-specific region of the Y chromosome (MSY) (Skaletsky et al, 2003; Soh et al, 2014).

1.3.1. X-inactivation

The human X chromosome contains approximately 800 and the mouse X chromosome contains approximately 850 protein-coding genes (Mueller et al, 2013). One of the two X chromosomes in XX females undergoes inactivation during early embryonic development (Lyon, 1961; (reviewed in (Balaton & Brown, 2016; Berletch et al, 2010; Posynick & Brown, 2019)). The purpose of X-inactivation is to balance X-linked gene dosage differences between males and females carrying one and two X chromosomes, respectively (reviewed in (Augui et al, 2011; Deng et al, 2014; Disteche & Berletch, 2015)). However, approximately 15% of X-linked genes in humans and 3-7% of X-linked genes in mice escape X-inactivation, and hence they are termed escape genes (Berletch et al, 2015; Carrel & Willard, 2005; (reviewed in (Balaton & Brown, 2016; Posynick & Brown, 2019)). Escape genes can reside within PAR or outside PAR (Lahn et al, 1997; (reviewed in (Disteche & Berletch, 2015)). Certain escape genes residing outside PAR have a Y-

linked homolog (Lahn et al, 1997; (reviewed in (Disteche & Berletch, 2015)). Escape genes residing outside PAR are expressed in higher dosage in individuals carrying two or more X chromosomes compared to those carrying one X chromosome, thus resulting in differences in gene expression and hence differences in phenotypes between males and females (reviewed in (Arnold, 2017; Berletch et al, 2011; Disteche, 2012; Fang et al, 2019; Posynick & Brown, 2019)). Differences in gene expression are also observed in mouse embryos carrying one X chromosome *versus* two X chromosomes (Schulz et al, 2014; Werner et al, 2017).

In mice, four days after fertilization (at two-cell stage), imprinted X-inactivation occurs on the paternally inherited X chromosome and this process is not random and does not occur in humans (Okamoto et al, 2005; (reviewed in (Augui et al, 2011; Deng et al, 2014)). The inactivated paternal X chromosome is then reactivated in the inner cell mass and then the random X-inactivation occurs at the blastocyst stage (Mak et al, 2014; (reviewed in (Avner & Heard, 2001; Disteche & Berletch, 2015)). X-inactive specific transcript (*Xist*) gene residing within a region termed X-inactivation centre (XIC) is an important regulator of X-inactivation because the X chromosome to be inactivated upregulates the expression of *Xist* (Kay et al, 1993; Penny et al, 1996; (reviewed in (Deng et al, 2014; Disteche & Berletch, 2015; Heard & Disteche, 2006)). *Xist* coats the inactive X chromosome in *cis* effect and one can observe this effect as a cloud within the nucleus of somatic cells using RNA-FISH (Clemson et al, 1996; (reviewed in (Disteche & Berletch, 2015; Heard & Disteche, 2006; Lu et al, 2017)). Next, *Xist* recruits repressive epigenetics marks, which deacetylate histone proteins, methylate lysine 27 of histone H3, and ubiquitinate lysine 119 of histone H3, to the promoter regions of the genes residing on the inactive X chromosome (reviewed in (Balaton & Brown, 2016; Disteche & Berletch, 2015; Galupa & Heard, 2018)). Further, DNA methylation plays a key role in stably maintaining the genes on the inactive

X chromosome in silent state and DNA methylation also recruits other repressive epigenetic marks (reviewed in (Deng et al, 2014; Disteche & Berletch, 2015; Heard & Disteche, 2006; Riggs, 1975)).

1.3.2. The Y chromosome

The mammalian Y chromosome, for a long time, was considered to be functionally inactive, gene poor, and highly heterochromatic due to the high contents of repetitive and non-coding regions residing within its MSY. However, it was demonstrated that the mouse MSY, particularly that of the C57BL/6J Y chromosome, contains approximately 700 protein-coding genes and is 99.9% euchromatic, of which 98% is ampliconic sequences different from repetitive sequences (Soh et al, 2014). It was also established that the C57BL/6J Y chromosome is 94.7 Mb long making it the largest mammalian Y chromosome compared to human, chimpanzee, and rhesus Y chromosomes (Bergstrom et al, 1998; Soh et al, 2014). Since only 2% of the ancestral genes survived on the evolving C57BL/6J Y chromosome, it is predictable that the mouse Y chromosome is composed of both ancestral and acquired genes. The former ones have housekeeping functions in transcription, translation, and protein degradation while the latter ones have testis-specific expression and hence suggesting their involvement in male gametogenesis (Bellott et al, 2014; Soh et al, 2014; (reviewed in (Disteche et al, 2002)). The long arm of the C57BL/6J Y chromosome (Yq) contains most of the acquired genes, except few of them dispersed between the ancestral genes residing on the short arm of the Y chromosome (Soh et al, 2014). The acquired genes are both non-coding and protein-coding genes and the protein-coding genes exist both in amplified copies and single copy (Soh et al, 2014). The ancestral genes, including *Sry*, are located within the short arm of the Y chromosome (Yp), and they all exist in single copy, except *Rbmy* that exists in 35 copies (Soh et al, 2014). Additionally, the ancestral genes are expressed either exclusively in the testis or ubiquitously throughout the body (Bellott et al, 2014; Soh et al, 2014).

Y-linked protein-coding genes that may either directly or indirectly influence DNA methylation are all located on Yp (**Fig 1.1**).

Zinc finger proteins 1 and 2, Y chromosome (*Zfy1* and *Zfy2*) genes have testis-specific expression (Bellott et al, 2014; Soh et al, 2014). *Zfy1* and *Zfy2* encode proteins of 782 and 783 amino acids, respectively, and both protein products contain 13 zinc-fingers, a characteristic of transcription factor and hence both genes have the ability to regulate gene expression by acting as transcriptional activators (Mardon & Page, 1989; Nagamine et al, 1990; Taylor-Harris et al, 1995).

Ubiquitin activating enzyme E1, Y chromosome (*Uba1y*) gene is expressed only in the testis (Bellott et al, 2014; Odorisio et al, 1996; Soh et al, 2014). This gene encodes a protein that activates ubiquitin and ubiquitination is one of the histone modifications, including phosphorylation, acetylation, sumolyation, and methylation, that are involved in gene regulation (reviewed in (Alaskhar Alhamwe et al, 2018; Bernstein et al, 2007; Bohnsack & Pandey, 2021; Gibney & Nolan, 2010; Weinhold, 2006)). Monoubiquitination and polyubiquitination of proteins have different effects on the target proteins in the sense that the former is involved in gene regulation while the latter is involved in protein degradation (reviewed in (Alaskhar Alhamwe et al, 2018)). It was demonstrated that monoubiquitination of histone H2A and histone H2B is normally associated with gene repression and gene activation, respectively (reviewed in (Alaskhar Alhamwe et al, 2018)).

Ubiquitin specific peptidase 9, Y chromosome (*Usp9y*) gene is expressed exclusively in the testis (Bellott et al, 2014; Brown et al, 1998; Soh et al, 2014). This gene encodes a protein that cleaves the ubiquitin moiety from ubiquitin-tagged precursors and/or ubiquitinylated proteins

(Bellott et al, 2014). It is, therefore, logical to assume that *Usp9y* is involved in gene regulation through the process of deubiquitination.

Lysine demethylase 5D (*Kdm5d*) gene is expressed ubiquitously in the adult mice (Agulnik et al, 1994; Bellott et al, 2014; Soh et al, 2014). *Kdm5d* encodes a protein that demethylates lysine 4 of histone H3 present on gene promoter regions and this histone modification is associated with gene repression (Barski et al, 2007; Bellott et al, 2014). KDM5D belongs to the Jumonji C-terminal domain (JmjC) family of histone demethylases that contain two domains, which are Jumonji N-terminal (JmjN) and JmjC domains (Kosugi et al, 2020; (reviewed in (Harmeyer et al, 2017; Hou & Yu, 2010)). The JmjN domain plays an important role in the activity and stability of the JmjC domain that carries out the catalytic activity of KDM5D (Kosugi et al, 2020; (reviewed in (Hou & Yu, 2010)). It was determined that *Kdm5d* was involved in the regulation of sex-biased genes in mice in that sex-biased gene expression was altered when *Kdm5d* was mutated (Mizukami et al, 2019). It was shown that mutations in the X-linked homolog of KDM5D, KDM5C was associated with loss of DNA methylation in humans and hence the mutations affected sex-biased DNA methylation (Grafodatskaya et al, 2013).

Ubiquitously transcribed tetratricopeptide repeat containing, Y-linked (*Uty*) gene is ubiquitously expressed throughout the body (Bellott et al, 2014; Greenfield et al, 1996; Soh et al, 2014). This gene is involved in gene regulation by associating with different complexes including H3K4 methyl transferase complex, the SWI/SNF chromatin complex, and heart transcription factor (Shpargel et al, 2012). For example, it was demonstrated that *Uty* was involved in the activation of *Fnbp1* by influencing the methylation level of lysine 4 of histone H3 (Shpargel et al, 2012).

Sry is present on the short arm of the Y chromosome and expressed in the embryonic gonad (Koopman et al, 1990; Soh et al, 2014). This gene encodes a protein containing a DNA-binding HMG box domain and acts as a transcription factor (Harley et al, 1992; Sinclair et al, 1990). Therefore, *Sry* can be considered as a potential regulator of gene expression.

1.3.2.1. Genetic variations in the Y chromosome

Genetic variations in DNA sequences often have a deleterious outcome and hence mutation is a way to introduce changes in DNA sequences (reviewed in (Alzu'bi et al, 2019; Choudhuri, 2014; Rohner, 2016)). There are different types of mutations, including a chromosomal mutation, a frameshift mutation, and a point mutation, that generate changes in DNA sequences (reviewed in (Cardoso et al, 2015; Choudhuri, 2014; Spencer et al, 2015)). A point mutation will generate a single nucleotide polymorphism (SNP) between individuals and a point mutation termed a non-synonymous SNP or a missense SNP changes the amino acid sequence of a protein, and hence results in a protein with different functions (reviewed in (Cartegni et al, 2002; Choudhuri, 2014; Spencer et al, 2015; Zheng et al, 2014)). A nonsense SNP, another type of point mutation, results in premature termination of translation or a shorter protein product (reviewed in (Cartegni et al, 2002; Choudhuri, 2014; Morais et al, 2020; Spencer et al, 2015)). Another way through which premature termination of translation can be generated is occurrence of mutations in splice site that change the joining of exons (reviewed in (Cartegni et al, 2002; Spencer et al, 2015)). However, a synonymous SNP can be not deleterious in the sense that the change in the amino acid sequence does not affect the protein function and hence results in same protein product (reviewed in (Bailey et al, 2021; Sauna & Kimchi-Sarfaty, 2011; Spencer et al, 2015)).

It was established for the first time that the mouse Y chromosome contains polymorphisms

and/or genetic variations between different mouse strains using restriction fragment length polymorphisms (RFLPs) (Bishop et al, 1985). It was demonstrated that differentially expressed genes between males and females of one mouse strain were not differentially expressed between males and females of another mouse strain (C57BL/6J *versus* CAST/EiJ strains) (Matthews et al, 2021). In addition to differences in genetic background, genetic variants in the Y chromosome could also be inducing differences in gene expression between the two mouse strains because it was established that *Mus musculus castaneus* (CAST/EiJ) Y chromosome diverged from *Mus musculus musculus* (C57BL/6J) Y chromosome 1 million years ago (Soh et al, 2014). Further, Morgan & Pardo-Manuel de Villena, (2017) identified thousands of single-nucleotide variants (SNVs) and hundreds of indels on the Y chromosome of different mouse strains (Morgan & Pardo-Manuel de Villena, 2017). It was also demonstrated that genetic variation in the Y chromosome affected the survival of male mice infected with influenza A virus and coxsackievirus B3 that leads to myocarditis (Case et al, 2012; Krementsov et al, 2017). Genetic variations in the Y chromosome also affected the susceptibility of male mice to autoimmune disease, multiple sclerosis (Case et al, 2013).

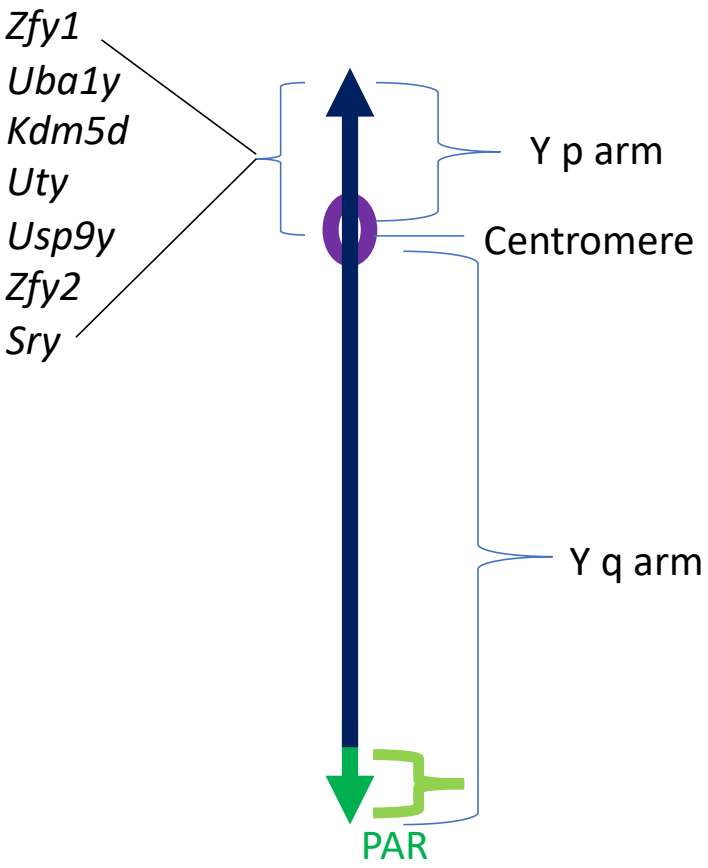

Fig 1.1. Map of the mouse Y chromosome with positions of Y-linked genes involved in gene regulation and/or histone modifications (not in scale). Yp arm and Yq arm represent the short arm and the long arm of the chromosome, respectively. The pseudoautosomal region (PAR) is the region of homology between the X and Y chromosomes where they recombine during meiosis.

1.4. DNA methylation

DNA methylation is a reversible but stable modification of DNA and involves the covalent addition of a methyl group to the fifth carbon of a cytosine base that is present next to a guanine base throughout the genome and they are termed CpG dinucleotides (reviewed in (Jaenisch & Bird, 2003; Kaminsky et al, 2006; Newell-Price et al, 2000; Suzuki & Bird, 2008; Zeng & Chen, 2019)). DNA methylation is a part of epigenetic modifications that regulate gene expression without causing alterations in the underlying DNA sequences (reviewed in (Esteller, 2008; Gibney & Nolan, 2010; Waterland, 2006; Weinhold, 2006)). DNA methylation is required for the normal development of an organism in that it is involved in cellular differentiation (reviewed in (Ambrosi et al, 2017; Huang & Fan, 2010; Miranda & Jones, 2007)). It can also arise randomly during lifetime or under the environmental influences in an adult organism and hence its involvement in many cancer types (reviewed in (Cedar & Bergman, 2009; Esteller, 2008; Jaenisch & Bird, 2003; Suzuki & Bird, 2008)). In brief, DNA methylation is involved in gene regulation, including X-inactivation (section 1.3.1) and genomic imprinting, as well as in the silencing of repetitive elements throughout the genome and hence DNA methylation plays an important role in maintaining genomic stability (reviewed in (Ambrosi et al, 2017; Greenberg & Bourc'his, 2019; Jansz, 2019; Smith & Meissner, 2013)).

1.4.1. Role of DNA methylation in transposable elements

DNA sequences that have the capacity to move within the genome is termed transposable elements (TEs) and TEs are major constituents of eukaryotic genomes, including humans and mice, and hence their significance in the mammalian genome diversity and expansion (Zhou et al, 2020; (reviewed in (Bourque et al, 2018; Jansz, 2019; Platt et al, 2018; Zamudio & Bourc'his,

2010)). There are two major types of TEs, which are DNA transposons and retrotransposons that use a DNA intermediate and an RNA intermediate, respectively, to change their positions within the genome (reviewed in (Bourque et al, 2018; Goerner-Potvin & Bourque, 2018; Jansz, 2019)). Retrotransposons are further classified into two different types, which are long terminal repeat (LTR) elements and non-LTR elements that include long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs), and DNA methylation mainly regulates the expression of retrotransposons compared to that of DNA transposons (Walsh et al, 1998; (reviewed in (Chuong et al, 2017; Greenberg & Bourc'his, 2019; Jansz, 2019; Platt et al, 2018)). Promoter regions of retrotransposons are CpG-rich and when they are methylated the expression of retrotransposons are repressed and hence their ability to move within the genome and cause genomic instability are inhibited (reviewed in (Greenberg & Bourc'his, 2019; Grundy et al, 2022; Smith & Meissner, 2013)). Hypomethylation of promoter regions of retrotransposons is associated with formation of many cancer types in the sense that when retrotransposons are expressed and inserted into different positions in the genome, they disturb genes such as tumor-suppressor genes and oncogenes (reviewed in (Bourque et al, 2018; Jansz, 2019; Yoder et al, 1997)). Additionally, hypomethylated promoter regions of retrotransposons is associated with DNA breaks and autoimmune diseases, and many more disorders (reviewed in (Bourque et al, 2018; Chénais, 2022; Grundy et al, 2022;)).

1.4.2. DNA methyltransferases

DNA methylation is catalyzed by DNA methyltransferases (DNMTs) that add a methyl group to the fifth carbon of a cytosine base (reviewed in (Gibney & Nolan, 2010; Greenberg & Bourc'his, 2019; Jansz, 2019; Miranda & Jones, 2007; Zeng & Chen, 2019)). There are four biologically active DNMTs, which are DNMT1, DNMT3A, DNMT3B, and DNMT3C, while

DNMT3L is the one that encodes biologically inactive protein (reviewed in (Ambrosie et al, 2017; Chédin, 2011; Greenberg & Bourc'his, 2019; Newell-Price et al, 2000; Zeng & Chen, 2019)). The catalytic methyltransferase domain of DNMTs is located in the C-terminal end while the N-terminal end is most variable between the different DNMTs (reviewed in (Ambrosie et al, 2017; Zeng & Chen, 2019)). DNMT1 is exclusively involved in the methylation of hemi-methylated DNA, which is generated after DNA replication, and hence it is termed maintenance enzyme (Lei et al, 1996; (reviewed in (Ambrosie et al, 2017; Gibney & Nolan, 2010; Miranda & Jones, 2007; Smith & Meissner, 2013)). DNMT3A, DNMT3B, DNMT3C, and DNMT3L are all involved in *de novo* DNA methylation, and hence they are termed *de novo* methyltransferases (Okano et al, 1998; (reviewed in (Ambrosie et al, 2017; Chédin, 2011; Greenberg & Bourc'his, 2019; Jansz, 2019; Lyko, 2018; Newell-Price et al, 2000; Zeng & Chen, 2019)). DNMT3L is an important cofactor for both DNMT3A/B and enhances their catalytic activities by directly interacting with them (Gowher et al, 2005; Suetake et al, 2004; (reviewed in (Ambrosie et al, 2017; Jansz, 2019; Zeng & Chen, 2019)). It was demonstrated that embryos lacking either *Dnmt1* or *Dnmt3b* were not viable while mice lacking *Dnmt3a* lived approximately 4 weeks postnatally (Okano et al, 1999; Ueda et al, 2006; (reviewed in (Ambrosie et al, 2017; Chédin, 2011; Zeng & Chen, 2019)). While *Dnmt3c*, once identified as a pseudogene, is a duplicated gene of *Dnmt3b*, with 70% of sequence identity, and specific to rodent genomes (Barau et al, 2016; Lees-Murdock et al, 2004; (reviewed in (Zeng & Chen, 2019)). DNMT3C methylates promoter regions of young retrotransposons in the male germ line and *Dnmt3c* mutant animals develop normally but only males are affected in the sense that they are sterile (Barau et al, 2016; (reviewed in (Jansz, 2019; Zeng & Chen, 2019)).

1.4.3. Interaction between DNA methylation and histone modifications

DNA methylation and chromatin modifications, such as histone methylation, interact with

each other to regulate gene expression (reviewed in (Cedar & Bergman, 2009; Jin et al, 2011; Li et al, 2021)). For example, DNMT1 is recruited to hemi-methylated DNA by UHRF1, E3 ubiquitin-protein ligase that preferentially recognizes and binds to hemi-methylated DNA (Liu et al, 2013; Sharif et al, 2007; (reviewed in (Moore et al, 2013)). During cellular differentiation, DNMT3A/B are recruited to the promoter regions of the target genes by the histone methyltransferases G9a that methylates lysine 9 of histone H3 to repress genes responsible for maintaining pluripotent state (Fuks et al, 2003; reviewed in (Casciello et al, 2015; Cedar & Bergman, 2009; Rose & Klose, 2014)). DNMTs interacts with another histone modification, lysine methyltransferase SETDB1 that methylates lysine 9 of histone H3 proteins, to silence repetitive elements in pre-implantation embryos (reviewed in (Ecco et al, 2017; Jansz, 2019)). DNA methylation and histone modifications also interact with each other to maintain genes on the inactive X chromosome stably in repressed state (section 1.3.1). Additionally, EZH2, a part of polycomb repressive complex 2 (PRC2) that methylates lysine 27 of histone H3, interacts with DNMTs to repress the expression of their target genes (Viré et al, 2006; (reviewed in (Jin et al, 2011)).

1.4.4. DNA demethylation

Methylated DNA can be demethylated or reversed and the process of DNA demethylation can occur in two ways; passive and active (reviewed in (Moore et al, 2013; Wu & Zhang, 2017)). Passive DNA demethylation is associated with loss and reduced activity of DNMT1 in the sense that methylation level of hemi-methylated DNA is reduced after each cell division and the methyl group is eventually removed (reviewed in (Ambrosi et al, 2017; Bayraktar & Kreutz, 2018; Wu & Zhang, 2010)). Active DNA demethylation involves ten-eleven translocation methylcytosine dioxygenases (TETs) that catalyze the conversion of 5-methylcytosine (5mC) to 5-

hydroxymethylcytosine (5hmC), which in turn is further modified into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (He et al, 2011; Ito et al, 2011; (reviewed in (Ambrosi et al, 2017; Bohnsack & Pandey, 2021; Kohli & Zhang, 2013)). There are three TET proteins, which are TET1, TET2, and TET3, and their catalytic domain is present within the C-terminus (reviewed in (Rasmussen & Helin, 2016)). The N-terminus of both TET1 and TET3 harbors a CXXC domain that binds to unmethylated CpGs while TET2 interacts with a protein containing CXXC domain, IDAX, to exert its effect on DNA demethylation (reviewed in (Ambrosi et al, 2017; Bohnsack & Pandey, 2021; Rasmussen & Helin, 2016)).

1.5. Sexual dimorphism in gene expression and DNA methylation

Sexual dimorphism in gene expression (also referred to as sex-biased gene expression) refers to the genes that are differentially expressed between males and females of a given species (reviewed in (Ellegren & Parsch, 2007; Grath & Parsch, 2016; Ingleby et al, 2014)). The genes that have higher expression in males compared to females are termed male-biased genes while the genes that have higher expression in females compared to males are termed female-biased genes (Ellegren & Parsch, 2007; Yang et al, 2006). All the analyzed non-reproductive tissues harbored differentially expressed genes between male and female mice but the differentially expressed genes were tissue-specific in that they had different functions depending on the tissues they were present (Yang et al, 2006). Sexual dimorphism in gene expression is mostly studied in the livers of different organisms, such as zebrafish, rats, mice, and humans, to better understand the underlying mechanisms responsible for the establishment of sex-biased gene expression between males and females (Kwekel et al, 2010; Yang et al, 2006; Zhang et al, 2011; Zheng et al, 2013). In addition, liver was determined to be a non-reproductive tissue that harbored the highest number of differentially expressed genes between male and female mice (Yang et al, 2006). It was

demonstrated that sex-chromosome complement, uncoupled from gonadal sex hormones, was enough to cause sexual dimorphism in gene expression between male and female mouse embryonic stem cells, and autosomes contained higher number of differentially expressed genes compared to sex chromosomes (Werner et al, 2017; (reviewed in (Engel, 2018)). Further, another study demonstrated that the number of X chromosomes, uncoupled from gonadal sex, had an impact on gene expression levels and hence sex-biased gene expression using a mouse model termed "Four Core Genotypes" (Wijchers et al, 2010). It was determined that gene expression was associated with hypomethylation (Anastasiadi et al, 2018; Zhuang et al, 2020). DNA methylation, therefore, could be one of the factors regulating and/or responsible for the establishment of sex-biased gene expression between males and females and hence sexual dimorphism in DNA methylation has been demonstrated in many organisms including insects, reptiles, chicken, zebrafish, mice, and humans (Grimm et al, 2019; Hu et al, 2019; Laing et al, 2018; Rancourt et al, 2018; Singmann et al, 2015; Xia et al, 2017). Both autosomes and sex chromosomes were shown to harbor sex-associated differentially methylated regions (sDMRs) between males and females, including humans and mice (García-Calzón et al, 2018; Ho et al, 2018; Zhuang et al, 2020). Same as in sex-biased gene expression, liver was determined to be a non-reproductive tissue that contained higher number of sDMRs in mice (McCormick et al, 2017). It was demonstrated in mouse liver that sDMRs were hypermethylated in females compared to males on autosomes (McCormick et al, 2017; Reizel et al, 2015; Zhuang et al, 2020). Same result was observed in humans in that sDMRs present on autosomes were hypermethylated in females compared to males when whole blood samples were analyzed (Grant et al, 2022). On the contrary, it was demonstrated that LINE-1 repetitive regions were hypomethylated in females compared to males when blood was analyzed (El-Maarri et al, 2011). Further, it was established that sDMRs were dependent on

sex phenotype, X-chromosome dosage, and the presence of the Y chromosome (also referred as Y-chromosome dependent sDMRs or yDMRs for short) in mouse livers (Zhuang et al, 2020). More recently, the role of gonadal sex in sex-biased DNA methylation has been studied (Alogayil et al, 2021) but the underlying mechanisms responsible for the establishment of sex-biased DNA methylation still remain poorly understood. Our study, therefore, wanted to explore the influence of sex-chromosome complement, especially that of the Y chromosome, on sex-biased DNA methylation using mouse as a model organism.

1.6. Mouse models that allow to separate the effects of the sex-chromosome complement and gonadal sex

There are mouse models that permit to distinguish the effects of the sex-chromosome complement from gonadal sex. Male mice harboring the patchy fur (*Paf*) mutation present close to the PAR of the X chromosome were used to study the impacts of X-chromosome dosage on sDMRs (Lane & Davisson, 1990). The *Paf* mutation interferes with the correct segregation of the X and Y chromosomes during male meiosis and XO female offspring carrying only the maternal X chromosome are generated (Lane & Davisson, 1990). B6.Y^{TIR} (Tirano) mice carrying a variant of *Mus musculus domesticus* Y chromosome on the C57BL/6J genetic background were used to generate mice with same sex-chromosome complement but different sex phenotypes (XY males vs. sex-reversed XY females) and mice with same sex phenotype but different sex-chromosome complement (XX females vs. sex-reversed XY females) (Nagamine et al, 1987). Therefore, through these mouse models, the effects of sex-chromosome complement and gonadal sex on sex-biased DNA methylation can be examined independently from each other (Zhuang et al, 2020).

1.7. Outstanding question to be solved, hypothesis, and objectives

The main scientific question that we are asking here is whether the presence of the Y chromosome influences autosomal DNA methylation.

In order to solve the main question of my project, we studied mice with different combinations of gonadal sex and sex-chromosome complement (XX females, XY males, sex-reversed XY females, and XO females). This allows to distinguish the effect of the Y chromosome from that of X-chromosome dosage and sex-phenotype on autosomal DNA methylation.

We hypothesized that the presence of the Y chromosome influenced DNA methylation due to the functions of one or several Y-linked genes that acted as epigenetic modifiers or transcription factors. To test the hypothesis, we set the following research objectives:

1. To identify yDMRs using WGBS data from mouse liver and brain, and design and validate pyrosequencing DNA methylation assays for 3-5 yDMRs;

2. To determine if the presence of yDMRs was limited to liver and brain by comparing organs derived from different germ layers;

3. To determine if genetic background and more specifically genetic variants of the Y chromosome influence yDMR methylation;

4. To test the impact of a specific Y-linked gene candidate, *Kdm5d* on autosomal DNA methylation

Chapter 2:

Methodology

2.1. Mouse strains and crosses

C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA).

B6.Y^{TIR} (Tirano) mice carry the Y chromosome from the wild derived mouse captured in Tirano (Italy) on the C57BL/6J genetic background (Nagamine et al, 1987). The Tirano Y chromosome carries a variant of the sex-determining region Y (*Sry*) gene that has delayed expression on the C57BL/6J genetic background (Coward et al, 1994; Park et al, 2011). SRY is essential for the regulation of the sry-box transcription factor (*Sox9*) gene, which in turn is necessary for testis differentiation (Chaboissier et al, 2004). The Tirano SRY is not effective on the C57BL/6J genetic background and hence delayed expression of *Sox9* (Park et al, 2011). As a consequence, testis development often fails in B6.Y^{TIR} mice and results in high rates of sex-reversed XY female offspring (Taketo-Hosotani et al, 1989). Therefore, we used B6.Y^{TIR} mice to generate XY males (XY.MT) as well as sex-reversed XY females (XY.FT) for our study (**Fig 2.1**). XX females (XX.FT) from the same cross were also included in the study. These mice were provided by Dr. Teruko Taketo (McGill University, QC, Canada).

B6.C3H/HeSn-*Paf* (referred to as *Paf* from here on) male mice carry the patchy fur (*Paf*) mutation located close to the pseudoautosomal region of the X chromosome. This mutation interferes with the correct segregation of the X and Y chromosomes during male meiosis and results in XO female offspring (Lane & Davisson, 1990). Approximately 20% of the female offspring from the crosses between C57BL/6J females and *Paf* (X^{Paf}Y) males are mice with monosomy X carrying only the maternal X chromosome (Burgoyne & Evans, 2000). The

B6.C3H/HeSn-*Paf* mice used in our study were the result of five to six generations of backcrossing of *Paf* males (purchased from the Jackson Laboratory) to C57BL/6J females and were used to generate XO (XO.F) and XX^{Paf} (XX^{Paf}.F) females (**Fig 2.1**). These mice were provided by Dr. Teruko Taketo (McGill University, QC, Canada).

FVB/NJ males (Jackson Laboratory, Bar Harbor, ME) were crossed with C57BL/6J females. The resulting F_1 (C57BL/6J female x FVB/NJ male) (FB) mice were analyzed in the study. The FVB/NJ males carry the *Mus musculus domesticus* Y chromosome (**Fig 2.1**) (Mardon et al, 1989). FVB/NJ male mice were a gift from Dr. Loydie Majewska (McGill University, QC, Canada).

Kdm5d knock-out mice. Exon 1 of *Kdm5d*, which encodes the Jumonji N-terminal (JmjN) domain, was targeted to knock out the gene and the mutation resulted in a deletion of two nucleotides, causing a frameshift mutation and premature termination of translation (Kosugi et al, 2020). DNA samples from livers and spleens of *Kdm5d* mutant mice and controls were provided by Dr. Tohru Kimura (Kitasato University, Minami-ku, Sagamihara, Kanagawa, Japan). The wild type (control) and *Kdm5d* mutant mice have the same genetic background (Kosugi et al, 2020).

Liver samples from other strains of mice (**C57BL/10J**, **C57BL/6N**, **A/J**, **C3H/HeN**, **DBA/1J**, and **MOLF/EiJ**) were a gift from Dr. Danielle Malo (McGill University, QC, Canada).

2.2. Organ collection

For the analysis of DNA methylation, liver, spleen, heart, lung, brain, and tails were collected from 8-week old XX.FT, XY.MT and sex-reversed XY.FT mice. Only livers were collected from 16-week old XX.FT, XY.MT and sex-reversed XY.FT mice. Testes were collected

from XY.MT.

For the analysis of DNA methylation, livers were collected from 8-week old XX^{Paf} female, XO female, C57BL/6J female, C57BL/6J male, C57BL/6N female, C57BL/6N male, FB female, and FB male mice.

2.3. DNA extraction, bisulfite conversion, and pyrosequencing analysis

Genomic DNA was extracted using the standard proteinase K/phenol-chloroform protocol. The concentration and integrity of the extracted genomic DNA were determined using nanodrop and 0.7% agarose gel electrophoresis, respectively.

Two hundred fifty to 1000 nanograms of DNA was converted using the EpiTect Bisulfite Kit (QIAGEN, NL), according to manufacturer's instruction. A modification was made to the incubation step, which was extended by five minutes at 95ºC and two hours at 60ºC.

Bisulfite-converted DNA was amplified using One*Taq* Hot Start DNA polymerase (New England BioLabs, Canada) in a PCR reaction of 30 µl. Pyrosequencing methylation assays were performed using the PyroMark Q24 Advanced platform and PyroMark Q24 Advanced CpG reagents (QIAGEN, NL), according to manufacturer's instructions. The analysis of the results was done using the Pyromark Q24 Advanced software (QIAGEN, NL).

2.4. Selection of target yDMRs and primer design for pyrosequencing methylation assays

The target yDMRs were selected using the list of autosomal sDMRs generated by whole genome bisulfite sequencing methylation analysis. We selected yDMRs that are not present within a region with high density of CGs and not overlapping with SNPs for pyrosequencing methylation

assays. Once the reporter yDMRs were selected, the corresponding DNA sequence was used for the primer design using PyroMark Assay Design 2.0 Software (QIAGEN, NL). The list of primers used for pyrosequencing methylation assays is provided in **Table 2.1**.

2.5. Statistical analysis

To test the significance of methylation differences between groups, we used ordinary one-way or two-way analysis of variance (ANOVA) followed by Tukey's or Šídák's multiple comparisons tests, respectively, on *GraphPad Prism 9* software.

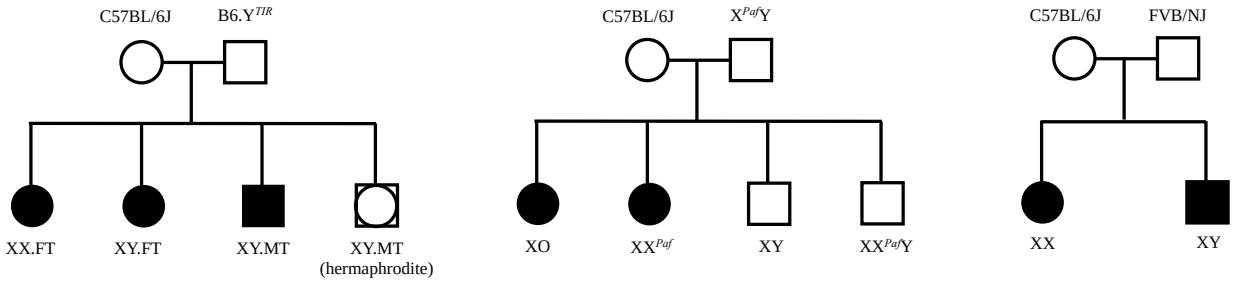

Fig 2.1. Crosses used in the study.

(From left to right) B6.Y^{TIR} male mice were backcrossed to C57BL/6J female mice to generate XX.FT, XY.MT, and sex-reversed XY.FT mice. X^{Paf}Y male mice were backcrossed to C57BL/6J female mice to obtain XX^{Paf}.F and XO.F mice. Lastly, FVB/NJ male mice were crossed with C57BL/6J female mice. Circles represent females and squares represent males. Filled shapes indicate the mice used in the study.

Table 2.1. List of yDMRs and their pyrosequencing methylation assay primers

Name	Position	Chr	Target CG	Forward primer	Reverse primer (Biotinylated)	Sequencing primer
Caprin1	Exon	2	103,766,583	TTAAGTAAGGGTGAGGAATTT	AAAAAAACTATTTTCATCCCATAAAAACAA	TGTATTGTGTGTAAGGTTT
Chr2_CG1	Intron	2	155,020,225	AGGTTGTAGAGAGGATTTTAGGTTATG	ACTTAAAACAATTTTTCTAAAAACCTACT	AATTGGTTTTTGAGAATGTTA
Ch2qH1_1	Intron	2	155,023,306	TGTGGTATAGTTGTTAAGTAGGAGAGGAT	TACTACTACCCAACAAACCCATAAT	GAGTGATGGAAAATTTAAGT
Ch6qA	Intergenic	6	13,714,975	GTTATGGAGGTTGGTAATAATGTT	ACCAATAAAAATCAAACAATTCACAAA	GGGGAATATATAGAAAGATATAAAA
Chr9qA5	Exon	9	42,505,444	GTAGAAGGGATTTTAGAAATAAAGATGGTAT	TCTACACCAAATCTTAATCCACTCCAA	GGATTTTAGAAATAAAGATGGT
Chr15qE2	Intergenic	15	84,155,936	AGTAGATTAGGTTTATATATGTGAGT	CCAACCAAAACTAAAAACCACTA	AGATTAGGTTTATATATGTGAG
Chr16qB	Intergenic	16	21,107,389	GTTTGTTAAGATTGATAATGGATTAGTTTA	CTCAACAATACCCTATCCTTAAAA	TTTAAAAATTTTAGTAGTTTTGT

Chapter 3:

Research findings

3.1. Selection of the target autosomal yDMRs

To study the influence of the Y chromosome on autosomal DNA methylation, we first had to isolate sDMRs that were dependent solely on the presence of the Y chromosome from sDMRs that were dependent on sex-phenotype and X-chromosome dosage. We, therefore, used four different comparison groups to identify autosomal yDMRs: 1) XX.FT versus XY.MT, 2) XY.FT versus XY.MT 3) XX.FT versus XY.FT, and 4) XX^{Paf}.F versus XO.F. Further, the sDMRs were called if they show methylation difference of at least 20% between the mice in each comparison group. We assumed that the overlapping sDMRs between XX.FT vs. XY.MT and XY.FT vs. XY.MT comparison groups represented the sex-phenotype dependent sDMRs because the mice in each comparison group have different sex phenotypes (female and male). The overlapping sDMRs between XX.FT vs. XY.MT, XX.FT vs. XY.FT, and XX^{Paf}.F vs. XO.F comparison groups represented the X-chromosome dosage dependent sDMRs because the mice in each comparison group have different number of X chromosomes. Lastly, the yDMRs were those that exclusively overlap between XX.FT vs. XY.MT and XX.FT vs. XY.FT comparison groups, but not the XX^{Paf}.F vs. XO.F comparison group (**Table 3.1**). As a result, we have identified a total of 46 autosomal yDMRs in mouse liver. The WGBS pipeline has limitations of yielding both false-negative and false-positive results. Therefore, to exclude false-positive yDMRs, we examined each one of the 46 yDMRs on UCSC genome browser (GRCm38/mm10). We excluded yDMRs containing differentially methylated CGs that had low quality sequence (or missing data) and inconsistent data from the list of yDMRs. Consequently, only 22 yDMRs were not excluded from the list of yDMRs (**Table S1**). Out of the 22 yDMRs, 13 were within long terminal repeats (LTRs),

five were in long interspersed elements (LINEs) and only four were in unique sequences (**Fig 3.1 a**).

Similar analysis of WGBS data from mouse brain led to the identification of 24 autosomal yDMRs, of which 14 yDMRs had high quality methylation data. Out of the 14 yDMRs, nine overlapped with LTRs, four overlapped with LINEs and only one had a unique sequence (**Fig 3.1 b**).

Table 3.1. The result of WGBS of DNA methylation in mouse liver

Type of sDMRs	**Number of autosomal sDMRs identified**
Sex-phenotype dependent	2,853
X-chromosome dosage dependent	803
Y-chromosome dependent	46

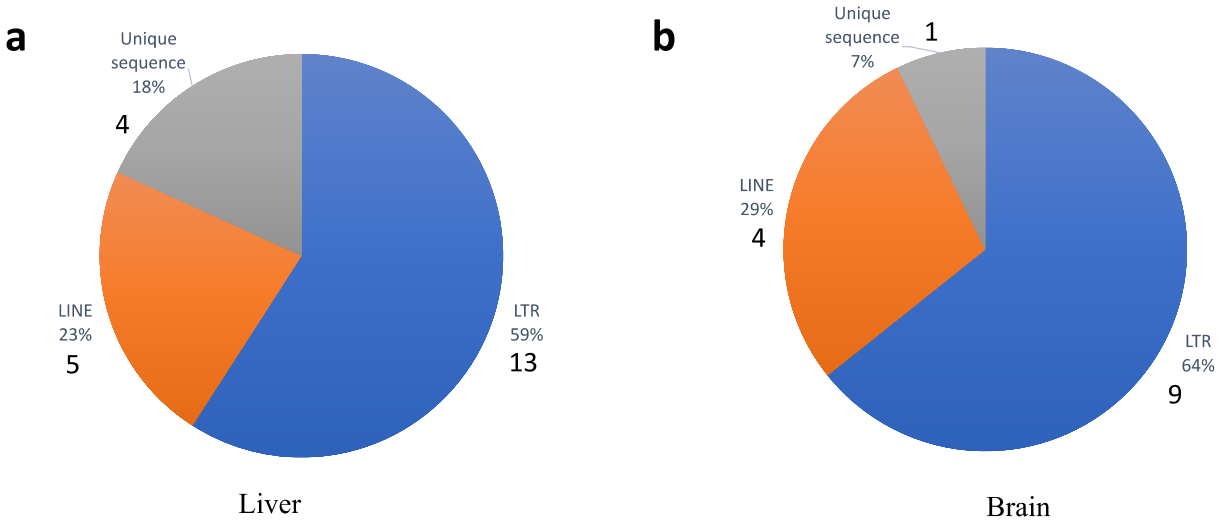

Fig 3.1. The distribution of yDMRs in mouse liver and brain.

Blue, orange, and gray colors represent the proportions of yDMRs that overlap with LTRs, LINEs, or unique sequences, respectively, in (**a**) liver and (**b**) brain.

3.2. Validation of yDMRs

To study how the presence of the Y chromosome influences autosomal DNA methylation, we selected seven yDMRs based on our selection criteria and designed pyrosequencing methylation assays (**Fig S1**). Next, we performed pyrosequencing DNA methylation assays using livers from 8-week old XX.FT, XY.MT, sex-reversed XY.FT, XX^{Paf}.F, and XO.F mice to validate that the reporter yDMRs were Y-chromosome dependent and not X-chromosome dosage dependent. Only four of the seven selected yDMRs had significantly different levels of DNA methylation in XX and XY mice (**Fig S1 a-d**). The remaining three yDMRs had statistically not significant DNA methylation in XX and XY mice, thus they were excluded from further studies (**Fig S1 e-g**).

We observed significantly lower levels of DNA methylation in XY mice compared to XX and XO mice at the *Caprin1*, *Ch6qA* and *Ch2qH1_2* yDMRs (**Fig 3.2 a-c**). For all three assays, XY.MT and sex-reversed XY.FT mice had similar levels of DNA methylation, thus indicating that these reporter yDMRs were not sex-phenotype dependent. Conversely, we observed significantly higher levels of DNA methylation in XY mice compared to XX and XO mice at the *Chr2_CG1* yDMR (**Fig 3.2 d**). We also found not significant DNA methylation difference between XY.MT and sex-reversed XY.FT mice. This shows that *Chr2_CG1* yDMR is not sex-phenotype dependent (**Fig 3.2 d**). Furthermore, we have determined that all the reporter yDMRs are not X-chromosome dosage dependent because XX^{Paf}.F and XO.F mice had similar levels of DNA methylation (**Fig 3.2 a-d**).

Our results suggest that our reporter yDMRs are indeed Y-chromosome dependent and not X-chromosome dosage dependent because XY mice had significantly different levels of DNA

methylation compared to XX and XO mice in liver.

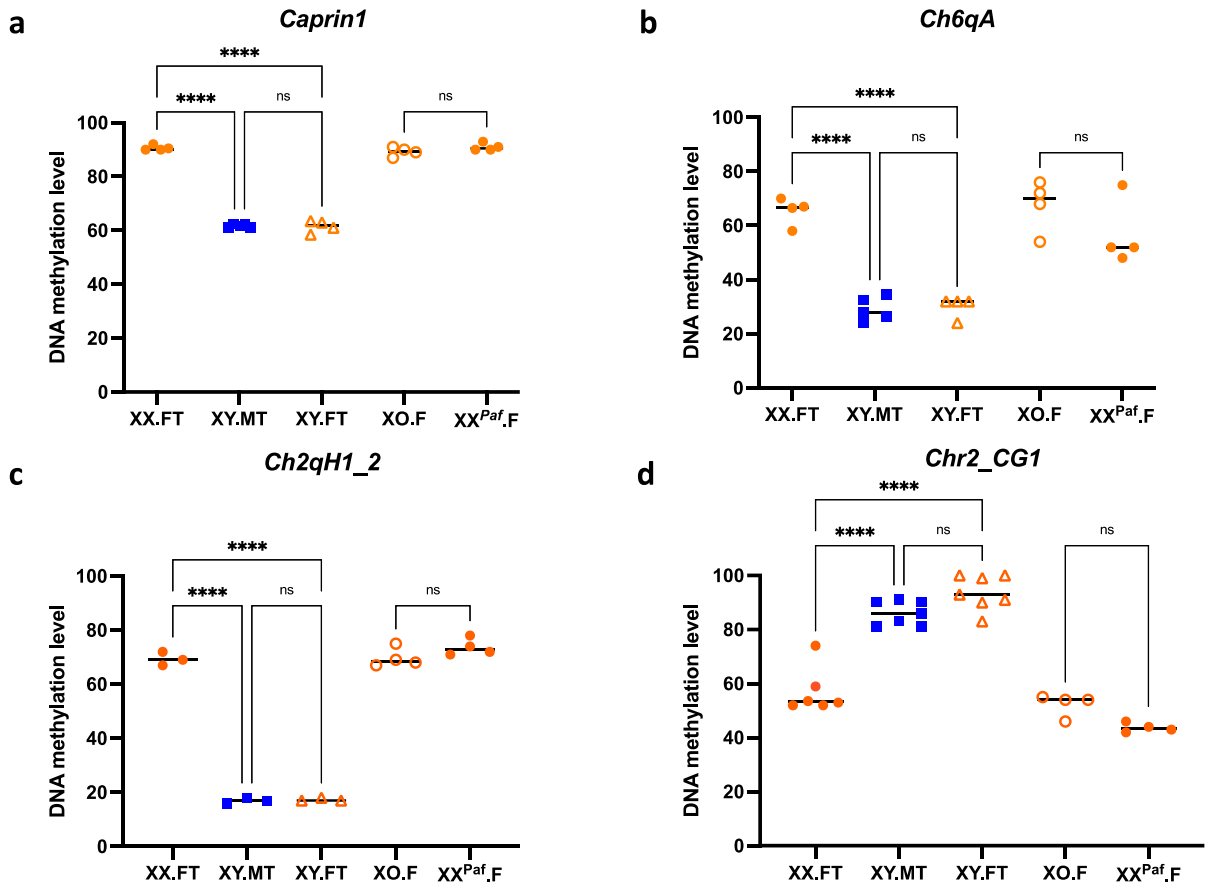

Fig 3.2. **Validation of yDMRs in mouse liver**.

(**a-d**). Methylation levels in mice carrying the Y chromosome. The Y-axis represents DNA methylation levels (in percentages) and the X-axis represents different sex/genotype groups: XX.FT (n=3-6), XY.MT (n=3-7), XY.FT (n=3-7), XX^{Paf}.F (n=4) and XO.F (n=4). Each point represents one DNA sample. Error bars represent standard deviation and asterisks represent statistically significant DNA methylation differences. (****), (**) and (*) indicate $p < 0.0001$, $p < 0.01$ and $p < 0.05$, respectively. ns – not significant. Significance was tested using ordinary one-way ANOVA followed by Tukey's multiple comparisons test.

3.3. Testing organ-specificity of yDMRs

Next, we asked whether the association between the presence of the Y chromosome and methylation levels was limited to liver and brain, or was common to multiple somatic tissues. We, therefore, analyzed spleen, heart, lung, brain, tail, and testis samples collected from the same mice. We chose these organs because they are derived from different germ layers and hence would show if methylation varies in different germ layers. Liver and lung are derived from the endoderm germ layer (Rosenquist, 1970 & 1971; Douarin, 1975). Spleen and heart are derived from the mesoderm germ layer (Thiel & Downey, 1921; Rawles, 1943). Testis consists of somatic cells derived from mesoderm germ layer and germ cells (Buehr et al, 1993; Merchant-Larios et al, 1993). Lastly, brain is derived from the ectoderm germ layer and tail consists of skin and bone that are derived from ectoderm germ layer and mesoderm germ layer, respectively (Waddington, 1932; Grüneberg, 1956). If the association is brain- and liver-specific, we will conclude that the Y-linked gene(s) responsible for influencing autosomal DNA methylation is expressed in brain and liver.

Caprin1, *Ch6qA* and *Ch2qH1_2* yDMRs had significantly lower levels of DNA methylation in XY mice compared to XX mice in all the tested organs (**Fig 3.3 a**-**c**). Conversely, *Chr2_CG1* yDMR had significantly higher levels of DNA methylation in heart, lung, liver, brain, and tail of XY mice (**Fig 3.3 d**). Interestingly, DNA methylation was high in both XX and XY mice in spleen (**Fig 3.3 d**).

At *Caprin1* and *Ch2qH1_2* yDMRs, testis had similar levels of DNA methylation as the other organs of XY.MT mice (**Fig 3.3 a** and **c**). However, at *Ch6qA* yDMR, testis had higher levels of DNA methylation compared to the other organs of XY.MT mice (**Fig 3.3 b**). At *Chr2_CG1*

yDMR, testis had lower levels of DNA methylation compared to the other organs of XY.MT mice (**Fig 3.3 d**).

Our results suggest that the Y-linked gene(s) responsible for influencing autosomal DNA methylation is expressed in all tested tissues or, alternatively, yDMRs arise early in development. However, DNA methylation difference observed between testis and other organs of XY.MT mice could be explained by methylation reprogramming that occurs in the germ line.

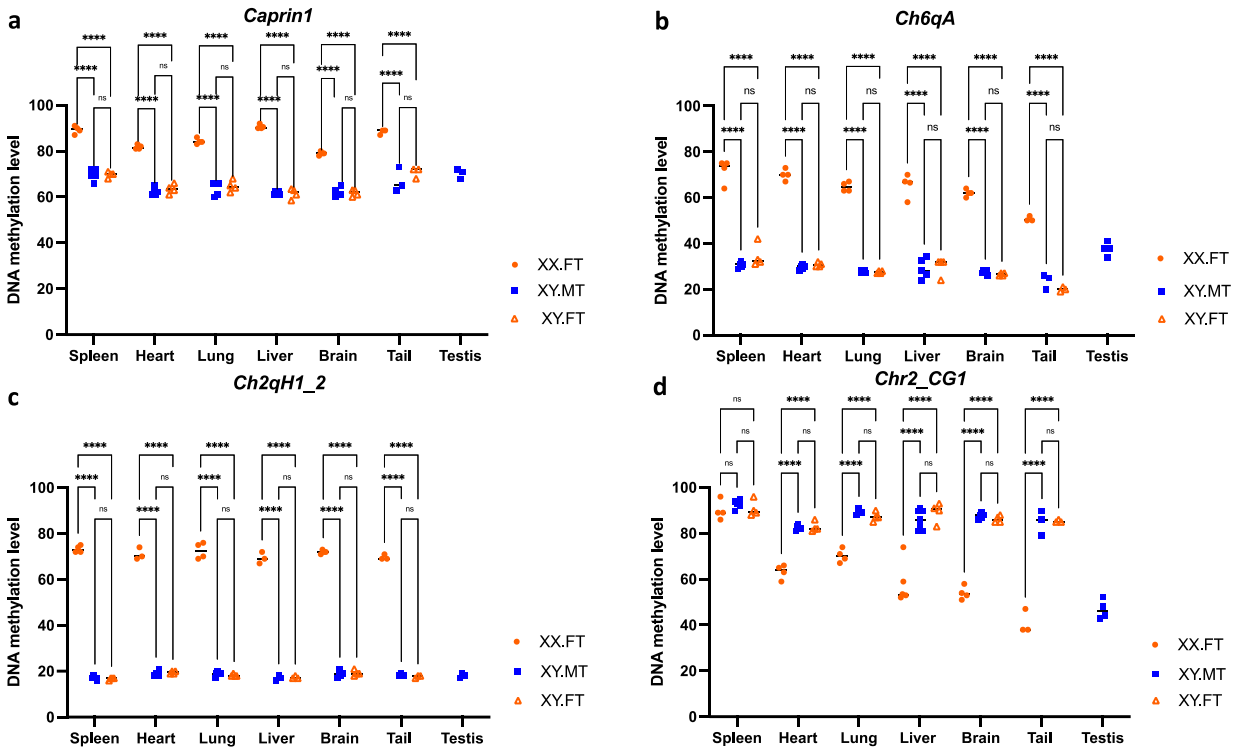

Fig 3.3. **yDMRs are present in most organs.**

(**a-d**). Methylation levels in different organs of XY mice. The Y-axis represents DNA methylation levels (in percentages) and the X-axis represents different organs. Each point represents one DNA sample: XX.FT (n=3-5), XY.MT (n=4-7), and XY.FT (n=3-7). Error bars represent standard deviation and asterisks represent statistically significant DNA methylation differences. (****) indicates p <0.0001. ns – not significant. Significance was determined using two-way ANOVA followed by Tukey's multiple comparisons test.

3.4. Analysis of genetic variants of the Y chromosome

Preliminary pyrosequencing methylation data for *Caprin1* and *Ch6qA* yDMRs generated by Najla AlOgayil, a student in Dr. Naumova's lab, showed that the effect of the Y chromosome on autosomal DNA methylation was present in B6.Y^{TIR} mice, which carry the Y chromosome of a *Mus musculus domesticus* origin, but not in C57BL/6J mice carrying the Y chromosome of a *Mus musculus musculus* origin (**Fig S2**) (Zhuang et al, 2020). Hence, a genetic variant(s) present on the Y^{TIR} chromosome causes lower methylation at certain autosomal regions. To determine if the effect was associated with the *domesticus* variant, we analyzed DNA methylation in livers from different inbred strains of mice carrying either the *Mus musculus domesticus* Y chromosome (FVB/NJ and MOLF/EiJ) or the *Mus musculus musculus* Y chromosome (C57BL/6J, C57BL/6N, C57BL/10J, A/J, C3H/HeN, DBA/1J) at the four previously validated yDMRs (Bishop et al, 1985; Nishioka et al, 1986; Nishioka et al, 1987). Additionally, we analyzed livers from F_1 (C57BL/6J female x FVB/NJ male) (FB) mice because the male mice carry the *Mus musculus domesticus* Y chromosome inherited from their father. Based on our preliminary data, we expected to observe different levels of DNA methylation between mice carrying the *Mus musculus musculus* Y chromosome and mice carrying the *Mus musculus domesticus* Y chromosome.

Caprin1 and *Ch2qH1_2* yDMRs had lower levels of DNA methylation in XY mice carrying the *Mus musculus domesticus* Y chromosome compared to XY mice carrying the *Mus musculus musculus* Y chromosome (**Fig 3.4 a and c)**. However, *Ch6qA* yDMR had lower levels of DNA methylation only in B6.Y^{TIR} mice compared to the other mouse strains (**Fig 3.4 b**). Lastly, *Chr2_CG1* yDMR had higher levels of DNA methylation in XY mice carrying the *Mus musculus domesticus* Y chromosome compared to XY mice carrying the *Mus musculus musculus* Y chromosome (**Fig 3.4 d**).

Our results demonstrate that genetic variation in the Y chromosome contributes to variation in DNA methylation, such that mice carrying the *Mus musculus domesticus* Y chromosome have different levels of DNA methylation compared to mice carrying the *Mus musculus musculus* Y chromosome at certain autosomal loci.

Next, to narrow the list of Y-linked gene candidates that could be influencing autosomal DNA methylation, we wanted to identify Y-linked protein-coding genes harboring genetic variation between the *Mus musculus domesticus* and *Mus musculus musculus* Y chromosomes.

We chose Y-linked protein-coding genes that have non-synonymous (NS) coding polymorphisms between strains of *domesticus* and *musculus* origins (**Table 3.2**). If a NS substitution occurs in a protein domain that carries out an important function for the protein product, then the protein will acquire an altered function. Next, we wanted to analyze DNA methylation in male mice carrying mutations in our Y-linked gene candidates.

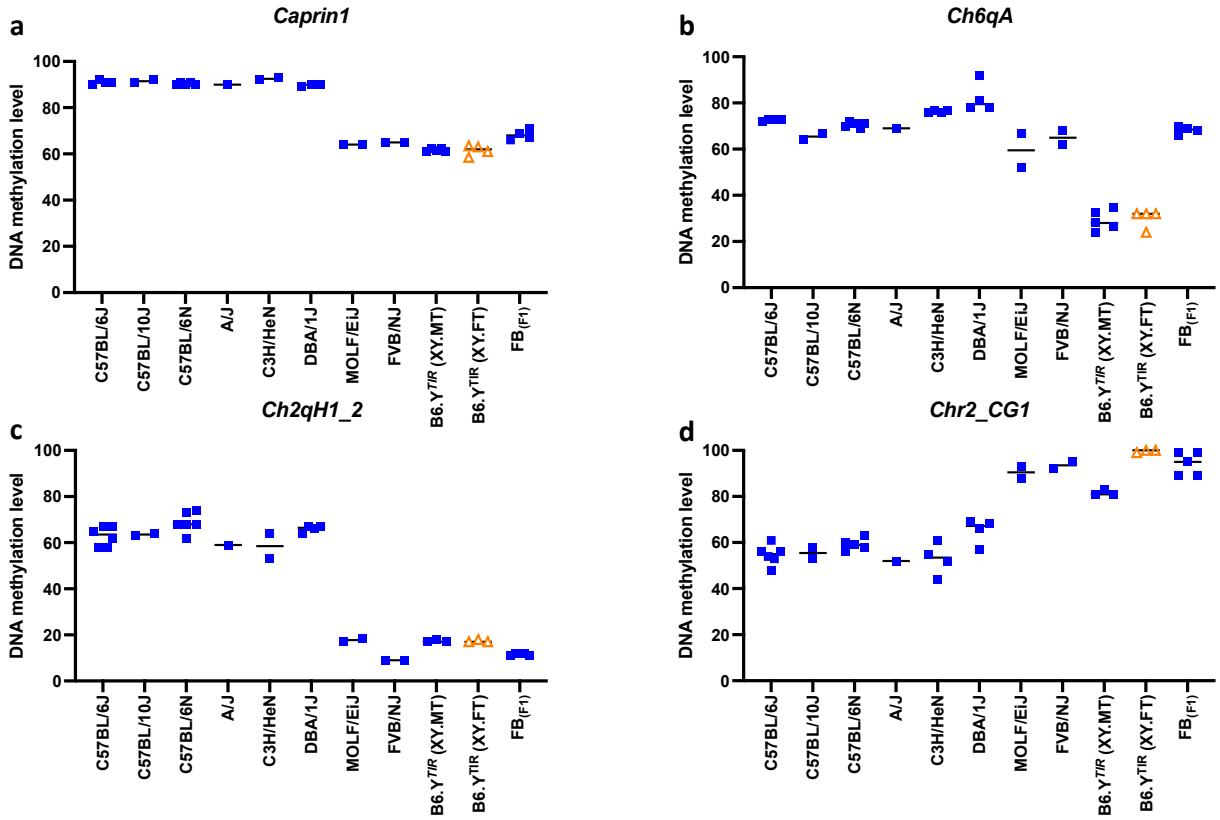

Fig 3.4. **Origin of the Y chromosome influences methylation of autosomal yDMRs.** (**a-d**). Methylation levels in livers of mice from different laboratory strains. The Y-axis represents DNA methylation levels (in percentages) and the X-axis shows the strains or crosses. Each point represents one DNA sample. Error bars show standard deviation. Blue rectangles represent XY male mice and empty orange triangles represent sex-reversed XY female mice.

Table 3.2. List of non-synonymous coding variants of Y-linked genes

Gene	Non-synonymous coding position	DNA Polymorphism*	Amino Acid position	Amino Acid Polymorphism*	Protein Domain (Source)	Function
Uba1y	ChrY:821197	T/C	60	V/A	Ubiquitin-activating enzyme E1 (https://www.ncbi.nlm.nih.gov/protein/NP_001343972.1)	Activates ubiquitin
Uba1y	ChrY:821486	G/T	127	A/S		
Uba1y	ChrY:825910	G/T	355	E/D		
Kdm5d	ChrY:900594	G/C	213	G/A	Disordered (https://beta.uniprot.org/uniprotkb/Q62240/entry)	Histone demethylase (H3K4me2/3)
Uty	ChrY:1158202	C/T	552	D/N	Disordered (https://beta.uniprot.org/uniprotkb/P79457/entry)	Histone demethylase (H3K27me3) independent gene regulation by associating with H3K4 methyl transferase complex, the SWI/SNF chromatin remodeler, BRG1, and heart transcription factors
Uty	ChrY:1170117	C/G	280	D/H	Tetratrico-peptide repeat 6	
Usp9y	ChrY:1303405	A/G	2503	Y/H	No domain is associated (https://beta.uniprot.org/uniprotkb/Q91XW2/entry)	Ubiquitin carboxyl-terminal hydrolase
Usp9y	ChrY:1324872	C/T	1728	G/S	Ubiquitin specific protease	
Usp9y	ChrY:1332445	T/G	1633	S/R		
Usp9y	ChrY:1340075	C/T	1435	V/I	No domain is associated	
Usp9y	ChrY:1391397	T/C	591	Q/R		
Usp9y	ChrY:1434349	C/T	371	G/E		
Usp9y	ChrY:1448948	A/C	49	Y/D	Disordered	
Zfy2	ChrY:2106304	C/G	777	A/P	No domain is associated (https://beta.uniprot.org/uniprotkb/P20662/entry)	Transcriptional activation
Zfy2	ChrY:2108497	G/T	388	Q/K	Disordered	

*The reference allele and/or residue is written first (the reference is the *Mus musculus musculus* Y chromosome).

DNA polymorphisms and related amino acid polymorphisms between the *Mus musculus musculus* and *Mus musculus domesticus* Y chromosomes were retrieved from UCSC genome browser (https://genome.ucsc.edu/) and MGI (http://www.informatics.jax.org/snp). Information on protein domain affected by NS coding polymorphism was retrieved from (https://beta.uniprot.org/).

3.5. *Kdm5d* knock-out does not influence yDMR methylation levels

Lysine-specific demethylase 5D (*Kdm5d*) gene demethylates di- and tri-methylated lysine 4 of histone H3 (H3K4me2/3) and is involved in gene regulation (Bellott et al, 2014; Barski et al, 2007; Heintzman et al, 2007). Therefore, *Kdm5d* is a good candidate that could be influencing DNA methylation. To test the impact of loss of function of *Kdm5d* on methylation, we analyzed methylation of reporter yDMRs in livers and spleens of *Kdm5d* mutant mice.

If *Kdm5d* was Y-linked gene responsible for Y-dependent methylation, we would observe significantly different levels of DNA methylation between *Kdm5d* knock-out (KO) and wild type (WT) male mice. However, at all four validated yDMRs, we observed similar levels of DNA methylation between WT and *Kdm5d* KO male mice both in liver and spleen (**Fig 3.5 a-b**). We also determined that the WT mice (ICR strain) have similar levels of DNA methylation as the mice carrying the *Mus musculus domesticus* Y chromosome at the four yDMRs, consistent with the result obtained by Aldinger and colleagues, who established that ICR mice carry the *Mus musculus domesticus* Y chromosome (**Fig S3**) (Aldinger et al, 2009).

Our results suggest that 1) *Kdm5d* is not inactive on the *Mus musculus musculus* Y chromosome and 2) *Kdm5d* is unlikely to be the Y-linked gene that influences autosomal DNA methylation.

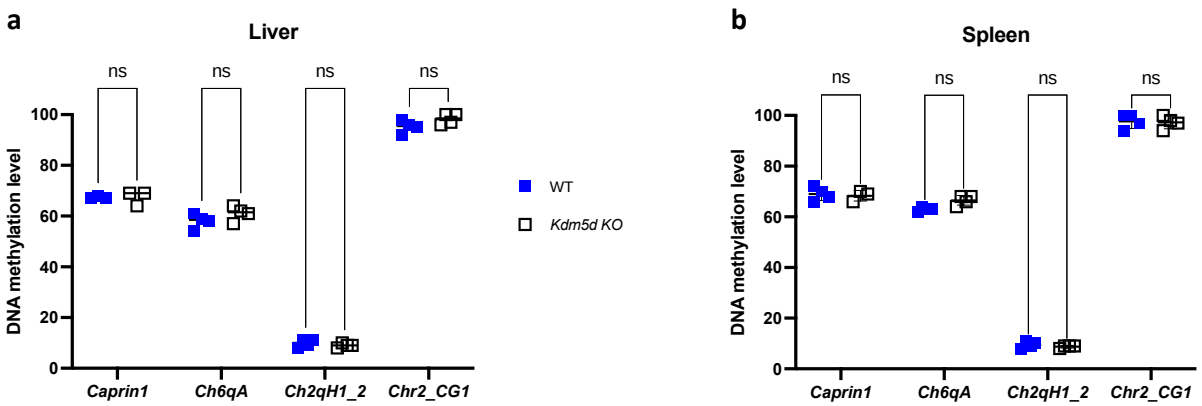

Fig 3.5. ***Kdm5d* does not influence yDMR methylation.**

(**a**-**b**). Methylation levels in livers and spleens of mice carrying a mutation in the *Kdm5d*. The Y-axis represents DNA methylation levels (in percentages) and the X-axis represents the four validated yDMRs. Each point represents one DNA sample (n=3-4). Error bars represent standard deviation. ns-not significant. Blue rectangles represent WT male mice and empty black rectangles represent *Kdm5d* KO male mice.

Chapter 4:

Discussion

In this study, we wanted to determine whether the presence of the Y chromosome influenced autosomal DNA methylation, and hence the role of the Y chromosome in sex-biased DNA methylation, by using mice with different combinations of gonadal sex and sex-chromosome complement (XX.FT, XY.MT, sex-reversed XY.FT, XX^{Paf}.F, and XO.F mice). Further, we wanted to find out whether yDMRs were tissue-specific by examining different mouse organs from the same mice. We also wanted to know whether different origins of the mouse Y chromosome had an effect on DNA methylation by studying different mouse strains.

We showed that most yDMRs were not tissue-specific in that they were present in all the tested organs in this study. We also established that *Mus musculus musculus* and *Mus musculus domesticus* Y chromosomes influenced DNA methylation differently, and *Kdm5d* was unlikely to be the Y-linked gene that influences autosomal DNA methylation.

4.1. The influence of the Y-chromosome on DNA methylation

Our lab, in collaboration with Dr. Taketo's and Dr. Bourque's labs (McGill University, QC, Canada), recently conducted whole genome bisulfite sequencing methylation analysis (WGBS) using livers and brains of adult mice with different combinations of gonadal sex and sex-chromosome complement (Zhuang et al, 2020). We determined that sex-phenotype, X-chromosome dosage, and the presence of the Y chromosome all resulted in differentially methylated regions (DMRs) (Zhuang et al, 2020). Through the analysis of Y-chromosome dependent sDMRs (yDMRs), our lab established that mice carrying the *Mus musculus domesticus* Y chromosome had lower levels of DNA methylation at certain autosomal regions compared to

mice carrying the *Mus musculus musculus* Y chromosome (Zhuang et al, 2020). In the present study, we determined that yDMRs can be both hyper- and hypo-methylated and most of the yDMRs were located on repeat elements both in mouse liver and brain (**Fig 3.1; Tables S1 and S2**). It suggested that the distribution of yDMRs in the mouse genome was not random. Furthermore, we established that mice carrying the Y chromosome, independent of gonadal sex, had significantly different yDMR methylation levels compared to XX and XO mice, indicating that the presence of the Y chromosome contributes to sex-biased DNA methylation (**Fig 3.2**). DNA methylation regulates both the expression and transposition of certain repetitive elements in the genome, thus contributing to genomic stability. Our results, therefore, suggest that the presence of the Y chromosome may play a role in the regulation of transposons throughout the genome and hence genomic stability. It was demonstrated that loss of the Y chromosome was associated with hepatocellular carcinoma in humans (Park et al, 2006).

4.2. yDMRs are not tissue-specific

Since the validation of our reporter yDMRs was done in liver of adult mice with different combinations of gonadal sex and sex-chromosome complement, we wanted to study whether yDMRs were tissue-specific by analyzing different organs, such as spleen, heart, lung, brain, and tails, collected from the same mice. In addition, testes were collected from XY.MT mice. We established that yDMRs were mostly not tissue-specific in the sense that they were present in all the tested organs (**Fig 3.3**). However, we observed similarly high DNA methylation at *Chr2_CG1* yDMR in the spleens of XX.FT, XY.MT, and sex-reversed XY.FT mice (**Fig 3.3 d**). Testes had different DNA methylation levels compared to the other organs of XY.MT mice at *Ch6qA* and *Chr2_CG1* yDMRs (**Fig 3.3 b** and **d**). This could be explained by methylation reprogramming that occurs in the germ line where somatic methylation patterns are erased so that sex-specific

methylation patterns can be established (reviewed in (Zeng & Chen, 2019; Seisenberger et al, 2013)). Overall, our results suggest that the Y-linked gene(s) responsible for influencing autosomal DNA methylation is expressed in all the tested organs and/or yDMRs arise early in development, i.e., before the separation of germ layers.

4.3. Different origins of the Y chromosome

Our lab previously determined that it was the male mice carrying a variant of *Mus musculus domesticus* Y chromosome on the C57BL/6J genetic background (B6.Y^{TIR}), not C57BL/6J male mice carrying the *Mus musculus musculus* Y chromosome, had lower levels of DNA methylation at certain sDMRs (Zhuang et al, 2020). We, therefore, analyzed yDMR methylation in different strains of mice carrying either the *Mus musculus musculus* Y chromosome or the *Mus musculus domesticus* Y chromosome. Our results showed that mice carrying the *Mus musculus domesticus* Y chromosome had different levels of DNA methylation compared to mice carrying the *Mus musculus musculus* Y chromosome at *Caprin1*, *Ch2qH1_2*, and *Chr2_CG1* yDMRs (**Fig 3.4 a** and **c-d**). However, *Ch6qA* yDMR was specific to B6.Y^{TIR} mice in that only B6.Y^{TIR} mice had lower levels of DNA methylation (**Fig 3.4 b**). In brief, our results suggest that genetic background and the origin of the Y chromosome influence DNA methylation and hence genetic variants in the Y chromosome must be involved.

4.4. Impact of Y-linked gene candidates on autosomal DNA methylation

Since our results suggested the possibility of yDMRs arising early in development or ubiquitous expression of the Y-linked genes responsible for influencing autosomal DNA methylation, we wanted to analyze Y-linked gene candidates that have the following characteristics – 1) expressed early in embryonic development or 2) expressed ubiquitously in

adults; and 3) contain non-synonymous coding polymorphism between *Mus musculus musculus* and *Mus musculus domesticus* Y chromosomes. Y-linked gene candidates of our study were mentioned in 1.3.2 section and **Table 3.2**. *Kdm5d* was one of the Y-linked gene candidates that fulfilled our criteria (Agulnik et al, 1994). We analyzed DNA methylation in livers and spleens of WT and *Kdm5d* KO mice at our reporter yDMRs and we expected to observe different levels of DNA methylation between the two groups of mice if *Kdm5d* had an influence on autosomal DNA methylation. However, we did not observe DNA methylation difference between WT and *Kdm5d* KO mice (**Fig 3.5**). Our results suggested that *Kdm5d* was unlikely to be having an impact on DNA methylation levels and hence this gene is unlikely to be one of the Y-linked genes responsible for influencing autosomal DNA methylation.

Chapter 5:

Final conclusions and future directions

Based on our observations and results, the conclusions of this study are as follows:

1. YDMRs are present mostly on transposable elements
2. YDMRs are not limited to the liver and observed in other organs. This suggests that they either arise early in development and/or the Y-linked gene(s) responsible for influencing autosomal DNA methylation is expressed ubiquitously throughout the body of the mouse
3. Genetic background and the origin of the Y chromosome influence DNA methylation of yDMRs
4. *Kdm5d* is unlikely to be the Y-linked gene responsible for yDMRs

In the future, to identify the Y-linked gene(s) responsible for sexual dimorphism in DNA methylation, genome-wide DNA methylation levels should be analyzed in mice with mutations in Y-linked gene candidates.

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Appendix: Supplementary materials

Table S1. Genomic locations of Y-chromosome dependent sDMRs in liver

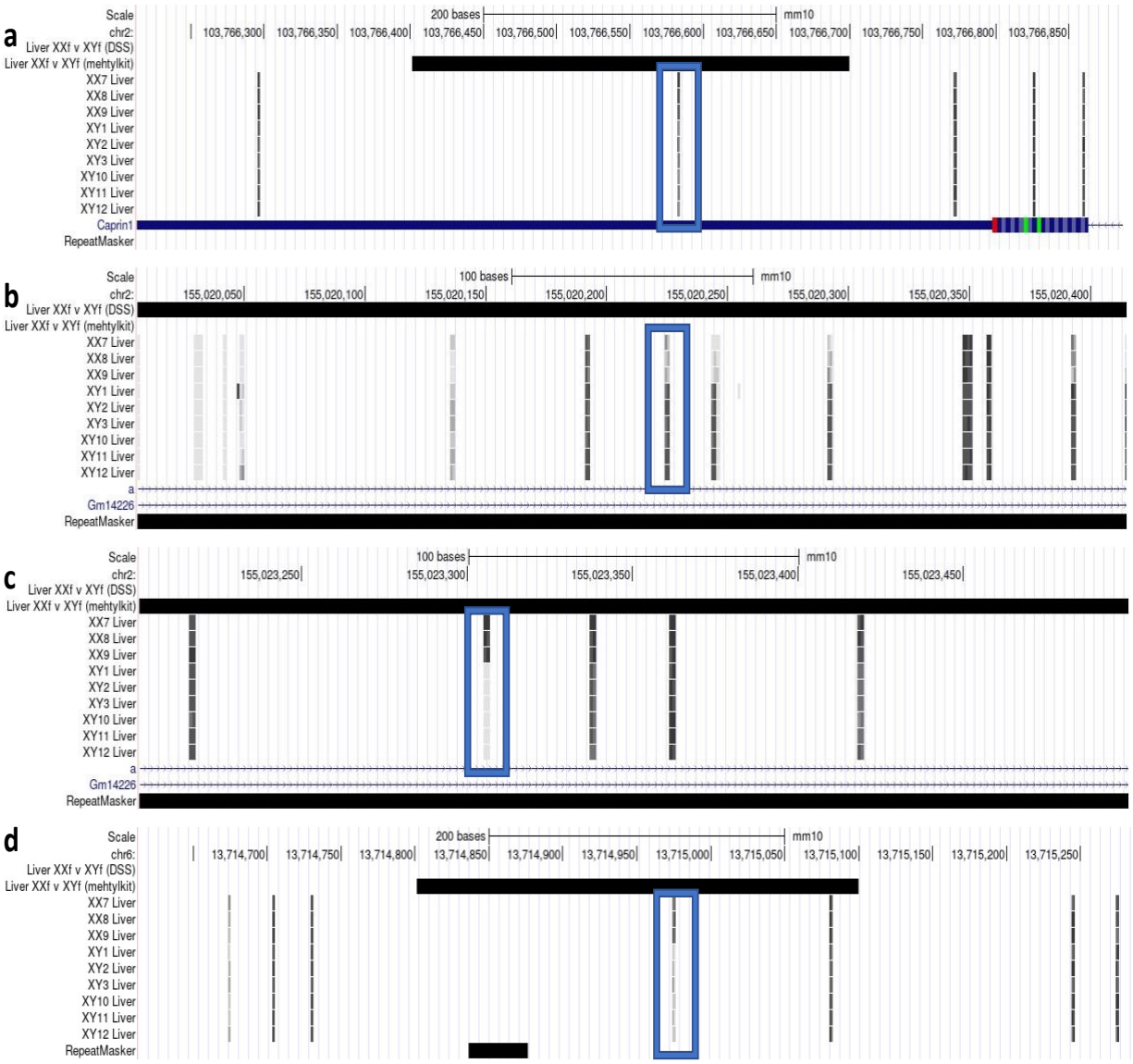
Chr	Start	End	Hyper- and hypo-methylation in XY mice	Repeat	yDMR (with complete data)	Assays
1	60424501	60424800	Hyper	LTR	Yes	
2	103766401	103766700	Hypo	no repeat	Yes	Assayed/validated (*Caprin1*) – a
2	155020006	155020415	Hyper	LTR	Yes	Assayed/validated (*Gm14226*) – c
2	155022601	155022900	Hyper	LTR	Yes	
2	155022901	155023200	Hypo	LTR	Yes	
2	155023201	155023500	Hypo	LTR	Yes	Assayed/validated (*Gm14226*) – d
2	155024101	155024400	Hypo	LTR	Yes	
2	155024401	155024700	Hypo	LTR	Yes	
2	155024701	155025000	Hypo	LTR	Yes	
3	129668867	129669272	Hypo	LTR	Yes	
3	129672722	129673189	Hypo	LTR	Yes	
5	9293101	9293400	Hypo	LINE	Yes	
6	13714801	13715100	Hypo	no repeat	Yes	Assayed/validated (*Ch6qA*) – b
8	89372101	89372400	Hyper	no repeat	Yes	
9	42505412	42505584	Hyper	no repeat	Yes	Assayed/not validated (showed no difference)
12	81394600	81394904	Hypo	LINE	Yes	
15	84,155,825-	84,156,079	Hyper	no repeat	Yes	Assayed/ not validated (showed no difference)
16	21107101	21107400	Hypo	LTR	Yes	Assayed/ not validated (showed no difference)
17	14014445	14014733	Hypo	LINE	Yes	
17	58762146	58762377	Hypo	LINE	Yes	
17	72438073	72438162	Hypo	LINE	Yes	
18	8141701	8142000	Hypo	LTR	Yes	
19	13140001	13140300	Hypo	LTR	Yes	

The gene name where the target yDMRs are present is written in parentheses.

Table S2. Genomic locations of Y-chromosome dependent sDMRs in brain

Chr	Start	End	Hyper- and hypo-methylation in XY mice	Repeat	yDMR (with complete data)	Assay progress
2	155020006	155020704	Hyper	LTR	Yes	Assayed/validated (*Gm14226*) – c
2	155024401	155024700	Hypo	LTR	Yes	
3	129668867	129669272	Hypo	LTR	Yes	
3	129672957	129673189	Hypo	LTR	Yes	
4	82014619	82014755	Hypo	LINE	Yes	
5	7429801	7430100	Hypo	LTR	Yes	
6	19585201	19585500	Hypo	no repeat	Yes	
10	24331208	24331405	Hypo	LTR	Yes	
12	81394600	81394904	Hypo	LINE	Yes	
13	100593828	100594433	Hyper	LTR	Yes	
14	52862581	52862746	Hyper	no repeat	Yes	
15	97458248	97459326	Hyper	LINE	Yes	
16	21107226	21107602	Hypo	LTR	Yes	
17	14014445	14014733	Hypo	LINE	Yes	

The gene name where the target yDMRs are present is written in parentheses.


a
Scale
chr2:
200 bases
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103,766,300
103,766,350
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103,766,550
103,766,600
103,766,650
103,766,700
103,766,750
103,766,800
103,766,850
Liver XXf v XYf (DSS)
Liver XXf v XYf (mehtylkit)
XX7 Liver
XX8 Liver
XX9 Liver
XY1 Liver
XY2 Liver
XY3 Liver
XY10 Liver
XY11 Liver
XY12 Liver
Caprin1
RepeatMasker
b
Scale
chr2:
100 bases
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155,020,050
155,020,100
155,020,150
155,020,200
155,020,250
155,020,300
155,020,350
155,020,400
Liver XXf v XYf (DSS)
Liver XXf v XYf (mehtylkit)
XX7 Liver
XX8 Liver
XX9 Liver
XY1 Liver
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Liver XXf v XYf (DSS)
Liver XXf v XYf (mehtylkit)
XX7 Liver
XX8 Liver
XX9 Liver
XY1 Liver
XY2 Liver
XY3 Liver
XY10 Liver
XY11 Liver
XY12 Liver
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Scale
chr6:
200 bases
mm10
13,714,700
13,714,750
13,714,800
13,714,850
13,714,900
13,714,950
13,715,000
13,715,050
13,715,100
13,715,150
13,715,200
13,715,250
Liver XXf v XYf (DSS)
Liver XXf v XYf (mehtylkit)
XX7 Liver
XX8 Liver
XX9 Liver
XY1 Liver
XY2 Liver
XY3 Liver
XY10 Liver
XY11 Liver
XY12 Liver
RepeatMasker

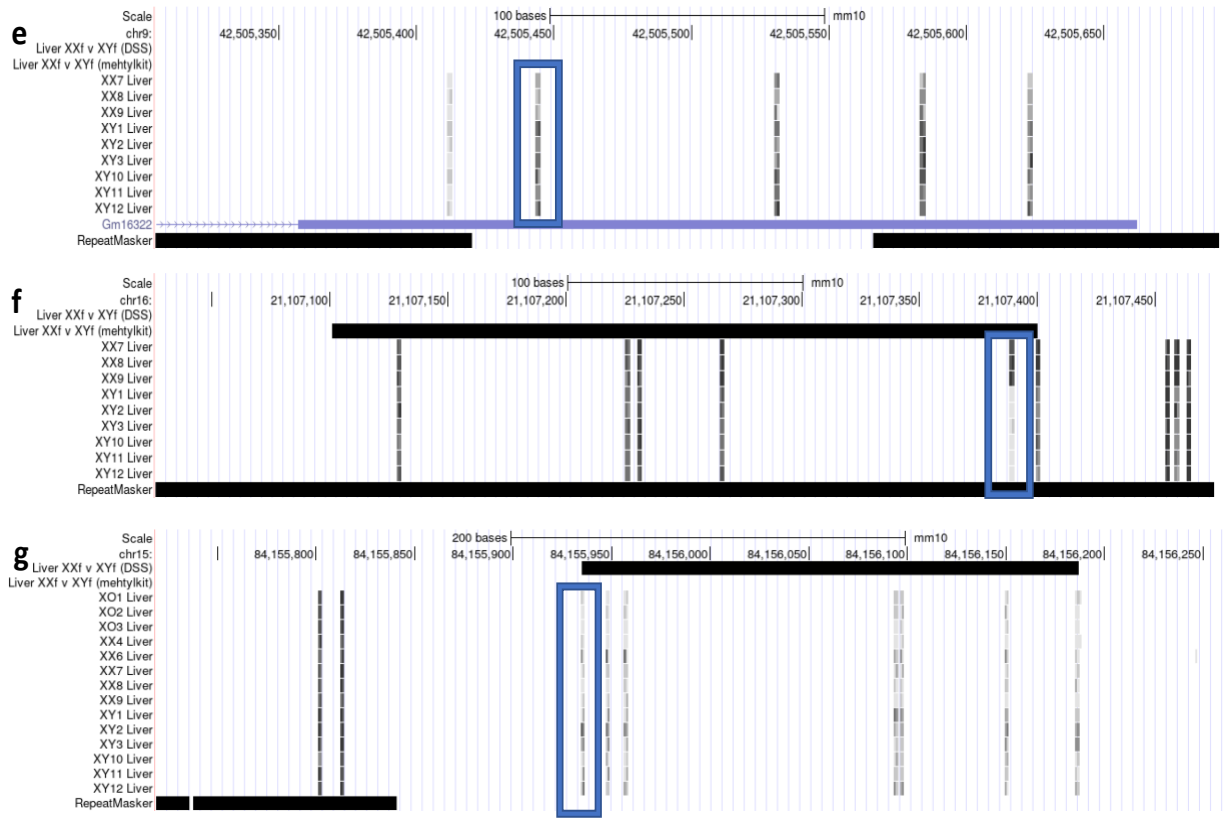

Fig S1. **Selected "reporter" Y-chromosome dependent sDMRs and their location in the genome**. (**a**) The yDMR resides in exon 9 of *Caprin1* on chromosome 2. (**b-c**) The yDMRs reside on repeat element (LTR) and in an intronic region of the agouti gene and on chromosome 2. (**d**) The yDMR resides in an intergenic region on chromosome 6. (**e-g**) The sDMRs were assayed but showed not significant methylation difference between XX and XY mice and were not analyzed in further studies. Blue rectangles indicate the selected "reporter" yDMRs.

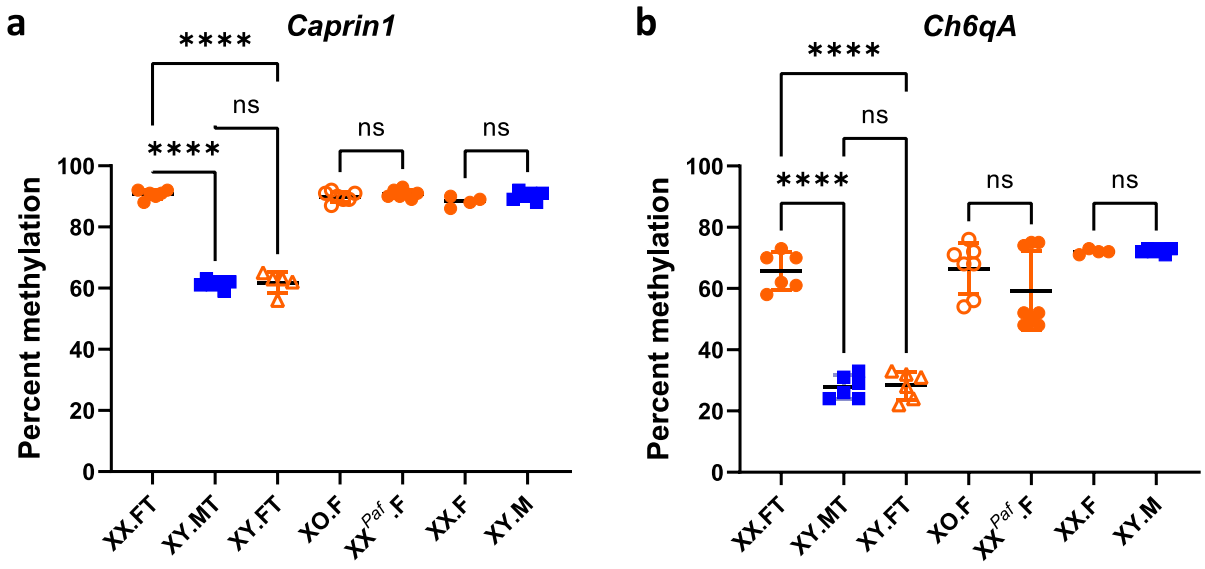

Fig S2. yDMRs are present in only B6.Y^{TIR} mice.

(**a-b**). Methylation levels in mice carrying the Y chromosome. The Y-axis represents DNA methylation levels (in percentages) and the X-axis represents different sex/genotype groups. Each point represents one DNA sample. Error bars represent standard deviation and asterisks represent statistically significant DNA methylation differences. (****) indicates $p < 0.0001$. ns – not significant. Significance was tested using ordinary one-way ANOVA followed by Tukey's multiple comparisons test.

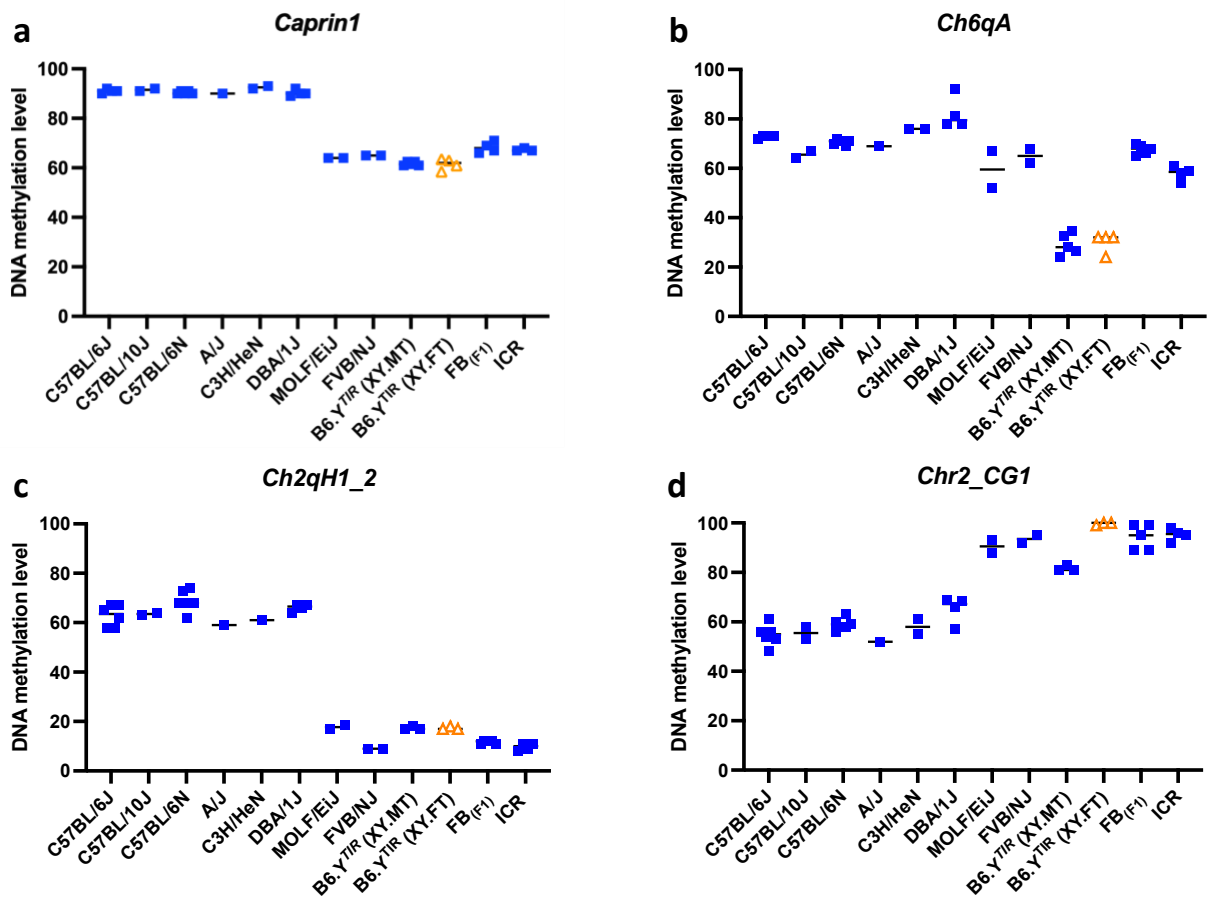

Fig S3. **Origin of the Y chromosome influences methylation of autosomal yDMRs.** (**a-d**). Methylation levels in livers of mice from different laboratory strains. The Y-axis represents DNA methylation levels (in percentages) and the X-axis shows the strains or crosses. Each point represents one DNA sample. Error bars show standard deviation. Blue rectangles represent XY male mice and empty orange triangles represent sex-reversed XY female mice.