Assessing the Impact of the X-Chromosome and Sex Phenotype on Sex-Biased Gene Expression in the Mouse Brain and Liver

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Abstract

Understanding how sex differences in gene expression arise and how regulators of gene expression, such as DNA methylation, contribute to these differences is an important step to furthering comprehension of sexual dimorphism. Three groups of factors, X-linked genes, Y-linked genes, and gonadal sex hormones are the major contributors to sexual dimorphism in gene regulation. However, the extent of their contribution to sex bias in gene regulation in different tissues and the underlying mechanisms are still unclear. Recently, our lab used mice with different combinations of sex-phenotype and sex-chromosome complement to catalog sex-biased differentially expressed genes (sDEGs) and sex-biased differentially methylated regions (sDMRs) in the mouse liver and identify those dependent on sex-phenotype and those dependent on sex-chromosome complement. The goals of the follow-up study presented here were a) to test the contribution of estradiol signaling through estrogen receptor alpha (ESR1) to sex bias in gene expression in the mouse liver; b) to determine if the effects of sex-hormone signaling pathways on liver methylation could be explained by direct binding of transcription factors to sDMRs, and c) characterize sex-biased expression in whole brain and compare it to liver.

We performed RNA-seq on the mouse livers from global ESR1 knock-out mice and their wild type littermates. Subsequent analysis of the transcriptomes showed that loss of ESR1 reduced sex-biased gene expression in both male and females. This suggests that estradiol action, through ESR1, affected both male-and female-biased DEGs, either through direct or indirect mechanisms.

Next, by intersecting whole genome bisulfite sequencing (WGBS) data from our previous study with publicly available ChIP-seq data, we demonstrate that ESR1, B-cell leukemia/lymphoma 6 (BCL6), and androgen receptor (AR) bind DNA in close proximity to

sDMRs. This suggests that direct binding of TFs proximal to sDMRs is a potential mechanism through which these transcription factors regulate sex-biased DNA methylation or gene expression in mouse liver.

Finally, we conducted RNA-seq and analysis of the brain transcriptomes of the mice with different combinations of sex-phenotype and sex-chromosome complement to identify sDEGs in the mouse brain and compare that with previous liver data. We show that in contrast to liver, which shows a major effect of the sex phenotype on multiple DEGs, there were very few DEGs found in the mouse brain, and those that were detected were mainly located on the Y-chromosome.

Résumé

Une étape importante de l'amélioration de notre compréhension du dimorphisme sexuel est de comprendre comment surviennent les différences sexuelles dans l'expression des gènes ainsi que comment les régulateurs de l'expression génique, tel que la méthylation de l'ADN, contribuent à ces différences. Les contributeurs majeurs au dimorphisme sexuel dans l'expression génique sont les stéroïdes sexuels et les gènes liés au chromosomes X et Y. Cependant, le degré de leur contribution ainsi que les mécanismes par lesquels ils contribuent aux différences sexuelles dans l'expression génique dans les différents tissus sont encore nébuleux. Récemment, nous avons utilisé des souris avec de différentes combinaisons de phénotypes sexuels et de compléments de chromosomes sexuels afin de cataloguer les gènes différentiellement exprimés en fonction du sexe (DEG) et les régions différentiellement méthylées en fonction du sexe (sDMRs) dans le foie de souris. Nous avons aussi identifié lesquels dépandaient du phénotype sexuel et lesquels dépendaient du complément de chromosome sexuel. Les objectifs de cette étude de suivi étaient de : 1) tester la contribution de la signalisation de l'estradiol par le récepteur d'estrogène alpha (ESR1) aux différences sexuelles dans l'expression génique dans le foie de souris; 2) déterminer si les effets des voies de signalisation des hormones sexuelles sur la méthylation dans le foie pourraient être expliqués par la liaison directe des facteurs de transcription aux sDMRs; et 3) caractériser les différences sexuelles dans l'expression génique dans le cerveau entier et le comparer au foie.

Nous avons complété l'ARN-seq et l'analyse des transcriptômes de souris knock-out ESR1 global et de souris de type sauvage. Par ce, nous avons démontré que la perte de l'ESR1 réduit les différences sexuelles dans l'expression des gènes dans les DEG biasés vers les mâles et

les femelles. Ceci suggère que l'action de l'estradiol par l'ESR1 à affecté les DEG biasés vers les mâles et les DEG biasés vers les femelles, soit par des mécanismes directes ou indirectes. Ensuite, par l'intersection de données de séquençage de génome bisulfité (WGBS) de notre étude précédente avec des données ChIP-seq accessibles au public, nous avons démontré que ESR1, BCL6 et AR se lient à l'ADN à proximité de sDMRs. Ceci suggère que la liaison directe de facteurs de transcription à proximité de sDMRs est un mécanisme potentiel par lequel ces facteurs de transcription régulent la méthylation de l'ADN différentielle liée au sexe et l'expression des gènes dans le foie de souris.

Finalement, nous avons complété l'ARN-seq et l'analyse des transcriptômes de cerveau de souris avec de différentes combinaisons de phénotypes sexuels et de compléments de chromosomes sexuels pour identifier des DEGs dans le cerveau de souris et comparer ces données aux données de foie de souris. Nous avons démontré qu'il y a très peu de DEGs retrouvés dans le cerveau de souris comparé au foie de souris, où le phénotype sexuel a un effet majeur sur plusieurs DEGs. De plus, les DEGs retrouvés dans le cerveau de souris étaient principalement situés sur le chromosome Y.

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List of Abbreviations

1700030C10Rik: RIKEN cDNA 1700030C10 gene

17-OH-P4: 17-hydroxy-progesterone

17-OHP5: 17-hydroxy-pregnenolone

Adgrg2: adhesion G protein-coupled receptor G2

AF: activation function domains

AR: androgen receptor

ARE: androgen response element

Arhgap6: Rho GTPase activating protein 6

Barhl1: BarH-like Homeobox 1

BCL6 - B cell leukemia/lymphoma 6

Cfp - complement factor properdin

Chic1 - cysteine-rich hydrophobic domain 1

Cnpy1 - Canopy FGF signaling regulator 1

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

Cyp450s- cytochrome p450 enzymes

CYPs - cytochrome P450

DBD - DNA binding domain

DEGs - differentially expressed genes

DHEA - dehydroepiandrosterone

DNMTs - DNA methyl transferases

E1 - estrone

E2 - estradiol

E3 - estriol

E4 - estretrol

ERE - estrogen response element

Erα - estrogen receptor alpha

Erβ - estrogen receptor beta

Esr1 - gene coding for ESR1

ESR1 - estrogen receptor alpha

ESR2 - estrogen receptor beta

ESR1KO - B6N(Cg)-Esr1$^{tm4.2Ksk}$/J mice

FDEGs - female biased differentially expressed genes

FKO - female ESR1KO mice

FSH - follicle-stimulating hormone

FWT - female ESR1KO wildtype littermates

GH - growth hormone

Gm21064 - predicted gene 21064

Gm26652 - predicted gene 26652

Gm28587 - predicted gene 28587

GnRH - gonadotrophin releasing hormone

GPER - G protein-coupled estrogen receptor

Hccs - holocytochrome C synthase

HPG axis - hypothalamic-pituitary-gonadal axis

Ikbkg - inhibitor of nuclear factor kappa B kinase subunit gamma

lncRNA - long non-coding RNA

Klhl13 - kelch like family member 13

LBD - lipid binding domain

LH - gonadotropins luteinizing hormone

log2FC - log2 fold change

MDEG - male biased differentially expressed gene

MKO - male ESR1KO mouse

MWT - male ESR1KO wildtype littermate

NAFLD - non-alcoholic fatty liver disease

NTD - amino terminal domain

padj - adjusted p-value

Paf - C3H/HeSn-Paf/J mice

PAR - pseudo-autosomal region

PC - principal component

PCA - principal component analysis

REAC - reactome database

RT-PCR - reverse transcription PCR

SAM - s-adenyl methionine

sDEGs - sex-biased differentially expressed genes

sDMRs - sex-biased differentially methylated regions

Serpina1e - serine (or cysteine) peptidase inhibitor, clade E, member 1

Slc6a8 - solute carrier family 6 member 8

Sox9 - SRY-box transcription factor 9

Sry - sex-determining region Y

STAT5 - Signal transducer and activator of transcription 5 signaling

TF - transcription factor

Uba1y - ubiquitin-like modifier-activating enzyme 1 Y

Vsig8 - V-set and immunoglobulin domain containing 8

WGBS - whole genome bisulfite sequencing

WT - wild type

XCI - X-chromosome inactivation

Xist - X inactivation-specific transcript

XO.F - XO female mice

X^{Paf}Y.M - X^{Paf}Y male mice

XX.F - XX female mice

XX^{Paf}.F - XX^{Paf} female mice

XX^{Paf}Y.M - XX^{Paf}Y males

XY.F - XY^{TIR} sex-reversed female mice

XY.M - XY^{TIR} male mice

Yp - p (short) arm of Y chromosome

Yq - q (long) arm of Y-chromosome

Zmat1 - zinc finger matrin-type 1

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Format of the Thesis

This thesis follows a traditional thesis format. Chapter 1 is the Introduction, Chapter 2 is the Materials and Methods, Chapter 3 contains the Results, Chapter 4 is the Discussion, and Chapter 5 contains Conclusions and Future Directions. Chapter 6 is the references, and supplementary information is contained in the Appendices. This thesis includes work published in (Alogayil et al., 2021). These include sections 3.1 and 3.2.

Contribution of Authors

RNA was extracted by Klara Bauermeister for sequencing. RNA-seq was performed by the McGill Genome Centre, and the results were analysed by Klara Bauermeister. The colony of the ESR1KO mice was maintained by Dr. Anna Naumova. Mice were genotyped by Klara Bauermeister, Najla Alogayil for the ESR1KO mice, and members of Dr. Teruko Taketo's lab (Tir and Paf strains). Tissue collections were performed by Klara Bauermeister and Najla Alogayil. The study was designed and supervised by Dr. Anna Naumova. The abstract was translated into French by Jacqueline Bradbury-Jost

Chapter 1: Introduction

1.1 Sexual Dimorphism

Sexual dimorphism is defined as consistent differences between males and females of a species (reviewed in (Fairbairn, 2016)). Some of the most obvious examples of sexual dimorphism can be seen in differences in colouring in some bird species, such as ducks or peacocks, height in humans where males often tend to be taller than females, or genital anatomy. However, sexual dimorphism can also be seen in disease, metabolism, and drug response (Fuscoe et al., 2020; Greenblatt et al., 2004; Kato, 1974). For example, there are higher rates of autoimmune disease in females, but more severe infectious diseases occur more often in men (Beeson, 1994; Guerra-Silveira & Abad-Franch, 2013) (reviewed in (Ansar Ahmed et al., 1985; Markle & Fish, 2014; Mcclelland & Smith, 2011; Oertelt-Prigione, 2012; Whitacre et al., 1999; Zuk, 2009)).

1.2 Sexual Dimorphism in Gene Expression

One of the major differences between males and females is differences in gene expression, which ultimately results in phenotypic differences. Sexual dimorphism in gene expression has been observed in a variety of different tissues beyond reproductive organs, although the ovaries and testes remain the most sexually dimorphic tissues (Rinn et al., 2004) (reviewed in (Rinn & Snyder, 2005)). Sexual dimorphism in gene expression is often tissue specific (Yang, 2006). Somatic tissues that have shown sexual dimorphism in gene expression include the heart, liver, adipose tissues, skeletal muscle, brain, liver, kidney, hypothalamus (Amador-Noguez et al., 2005; Clodfelter et al., 2006; Isensee & Noppinger, 2007; Rinn et al., 2004; Yang, 2006) (reviewed in (Isensee et al., 2008)).

1.2.1 Sexual Dimorphism in the Liver

Approximately 72% of genes in the mouse liver are expressed at different levels in males and females (Yang, 2006). The first genes that were found to show sexual dimorphism in the liver were cytochrome p450 enzymes (Cyp450s) (Gustafsson et al., 1983; Roy & Chatterjee, 1983). These genes are particularly important for metabolizing steroids and drugs (Davatzikos & Resnick, 1998; Jarukamjorn et al., 2002; Rinn et al., 2004; Sakuma et al., 2002; Sundseth et al., 1992; Wiwi et al., 2004). However, differences in the liver have been implicated in sexual dimorphism in obesity susceptibility, non-alcoholic fatty liver disease (NAFLD), and metabolism (Arnold et al., 2017; Chikada et al., 2020; Della Torre et al., 2011; Meda et al., 2020) (reviewed in (Della Torre et al., 2017; Maggi & Torre, 2018; Palmisano et al., 2017)).

1.2.2 Sexual Dimorphism in the Brain

In contrast to the liver, only approximately 13.6% of genes in the brain have been shown to have sexually dimorphic expression levels (Yang, 2006). However, despite this, it is perhaps the most sexually dimorphic somatic organ in humans in terms of structure; male and female brains have differences in grey and white matter content, the sizes of different anatomical regions, and differences in hemispheric asymmetry (Coffey et al., 1998; Goldstein et al., 2001; Gur et al., 1999) (reviewed in (Sacher et al., 2013)).

The majority of studies that have looked at sexual dimorphism in whole brain gene expression have found relatively few differentially expressed genes (DEGs) in adults (Galfalvy et al., 2003; Rinn et al., 2004; Vawter et al., 2004). Moreover, most of DEGs found in adults were located on the Y chromosome or on the X chromosome (Vawter et al., 2004; Yang, 2006).

1.3 Sexual Dimorphism in DNA Methylation

One way through which sex-biased gene expression can be regulated is through DNA methylation (Compere & Palmiter, 1981; Holliday & Pugh, 1975). DNA methylation is important for gene regulation and cell differentiation (Aapola et al., 2000; Holliday & Pugh, 1975), and females tend to have higher levels of methylation compared to males (reviewed in (Moore et al., 2013)). It is also an important component of X-chromosome inactivation (Cotton et al., 2015; Sharp et al., 2011),

DNA methylation in mammals occurs when a methyl group is added to the fifth carbon of a cytosine residue, typically when the cytosine is located beside a guanine, known as CpG sites (Bird, 1980; Coulondre et al., 1978). Non-CpG methylation is also possible and has been observed in mammals (Xie et al., 2012), however, the majority of research has focused on CpG methylation (reviewed in (Moore et al., 2013)). CpGs concentrated in regions of approximately 1000 base pairs are known as CpG islands (Bird et al., 1985).

DNA methylation is catalyzed by DNA methyltransferases (DNMTs), which catalyze the reaction of adding a methyl group to a cytosine using *S*-adenyl methionine (SAM) as a source of methyl groups (Xie et al., 1999; Yen et al., 1992). There are three main DNMTs involved in the establishment and maintenance of DNA methylation; DNMT1, DNMT3A and DNMT3B. DNMT1's main role is copying methylation patterns from the parent strand of DNA to the daughter strands during DNA replication (Hermann et al., 2004). DNMT1 has also been shown to help in the repair of damaged DNA methylation (Mortusewicz et al., 2005). DNMT3A and DNMT3B on the other hand are mainly involved in *de novo* methylation (Okano et al., 1999). Sexual dimorphism in DNA methylation can be seen on both autosomes and sex chromosomes. and can contribute to sex bias in gene expression. Previous studies have shown a tendency for

higher global methylation levels in males (Burghardt et al., 2012; El-Maarri et al., 2007; El-Maarri et al., 2011; Sarter et al., 2005; Zhang et al., 2011), but sex-biased differentially methylated regions (sDMRs) in autosomal genes have higher methylation levels in females (Grimm et al., 2019).

1.4 Drivers of Sexual Dimorphism

There are two main molecular drivers of sexual dimorphism – sex phenotype and sex-chromosome complement. The first difference between males and females arises at fertilization, when the egg is fertilized with sperm carrying either an X or a Y sex chromosome, resulting in either XX or XY sex-chromosome pairs (Ford et al., 1959). The differences in the genes and their expression between the sex-chromosomes remains the only source of sexual dimorphism until gonadal differentiation begins around day 10.5 post conception (in mice), with the expression of the sex-determining region Y (*Sry*) in males (Gubbay et al., 1990; Hacker et al., 1995; Jeske et al., 1995; Sinclair et al., 1990; Swain et al., 1998). If *Sry* is not expressed, ovaries develop instead (Gubbay et al., 1990; Sinclair et al., 1990). Upon the development of gonads, sex-steroid hormones are also produced, which are the other potential drivers of sexual dimorphism (reviewed in (Swain, 2006)).

1.4.1 Sex-Chromosome Complement

Sex chromosomes originally evolved from a pair of homologous autosomes (Ohno, 1967). In placental mammals, females carry two X chromosomes, whereas males have one X and one Y chromosome. However, despite the fact that the X and Y chromosomes were initially homologous chromosomes, they have evolved to be very different, with only a small region in

most eutherians, and two regions in humans, known as the pseudo-autosomal region (PAR) remaining identical across the two today (reviewed in (Raudsepp & Chowdhary, 2015)). It is only in the PARs where meiotic pairing, synapsis and crossing over still occur between the X and Y chromosomes (Galtier, 2004; Skaletsky et al., 2003) (reviewed in (Blaschke & Rappold, 2006; Flaquer et al., 2008; Mangs & Morris, 2007; Raudsepp & Chowdhary, 2015; Raudsepp et al., 2012)).

1.4.1.1 X-Chromosome Dosage

The biggest genetic difference between males and females is that females have two X chromosomes and males only one. Because of this, a mechanism of dosage compensation has evolved in mammals. In 1961, Mary Lyon proposed the idea of X-chromosome inactivation (XCI), where only one X remains fully functional in female cells (Lyon, 1961a). However, since then, it has been shown that some genes on the silenced X chromosome maintain a degree of expression – this has since been termed escape from X-inactivation. It is estimated that approximately 12-20% of genes escape X-inactivation in humans (Balaton et al., 2015), and 3-7 % of genes escape X-inactivation in mice (Berletch et al., 2015).

In mice, there are two types of XCI at different stages of development. First, in female preimplantation embryos, the paternal X-chromosome is preferentially inactivated (Patrat et al., 2009; Takagi & Sasaki, 1975). Later, in the inner cell mass, this X-chromosome is reactivated in the blastula stage, and either the maternal or paternal X chromosome is randomly inactivated (Lyon, 1961b; Rastan, 1982; Takagi et al., 1982). An important player in the process of X-inactivation is the X inactive-specific transcript (*Xist)* (reviewed in (Augui et al., 2011)). This gene produces a long non-coding RNA (lncRNA) which coats the inactive X and recruits various

other cofactors, resulting in a series of events that work together to stably inactivate the X-chromosome (Brockdorff et al., 1991; Brown et al., 1991; Matarazzo et al., 2008; Penny et al., 1996) (reviewed in (Augui et al., 2011; Cerase et al., 2015; Lee & Bartolomei, 2013; Morey & Avner, 2011)).

Escape from X-inactivation varies across tissues and species (Berletch et al., 2015; Cotton et al., 2013). The genes that typically escape XCI are those located in the PAR (Berletch et al., 2010), or those with Y-linked paralogs (Bellott et al., 2014; Cortez et al., 2014). There are also genes that have variable escape – those that have different levels of escape depending on the tissue or cell type (Berletch et al., 2015; Berletch et al., 2011) (reviewed in (Balaton & Brown, 2016)). Furthermore, the escape is not necessarily complete. X-escape genes have been defined as those that have at least 10% of the expression from the inactive X compared to the active X (reviewed in (Balaton & Brown, 2016)).

In mice, fewer genes escape X-inactivation compared to humans (Raznahan et al., 2016) (reviewed in (Balaton & Brown, 2016)). However, it has been suggested that in mice, escape from XCI is mainly cell-type/tissue-specific (Berletch et al., 2015), although limited tissues have been studied. When examining the mouse brain, spleen, and ovaries, 12 genes have been shown to escape X inactivation in at least two of these tissues, but 26 were shown to only escape in one of the examined tissues (Berletch et al., 2015).

Genes that escape X-inactivation and encode expression regulators will have higher expression levels in individuals with two X chromosomes compared to those with one X and may contribute to sex bias in the expression of genes throughout the genome, as suggested by studies in humans and mice (Arnold, 2009; Arnold et al., 2017; Raznahan et al., 2018; Skakkebaek et al., 2018; Trolle et al., 2016).

1.4.1.2 Y Chromosome

While the X chromosome has largely retained the majority of the ancestral genes, the mouse Y chromosome only retained approximately 2% of the ancestral genes (Soh et al., 2014). The mouse Y chromosome is much bigger than the human and mainly consists of ampliconic euchromatin suggesting that it is gene dense, which is in contrast to the largely heterochromatic human Y-chromosome (Soh et al., 2014). Structurally, the short arm of the mouse Y chromosome (Yp) contains the majority of the ancestral genes, as well as *Sry*. The long arm (Yq) consists of highly repetitive regions of DNA including approximately 200 copies of a 500kb unit (Soh et al., 2014). The mouse Y chromosome contains approximately 700 protein coding genes (Soh et al., 2014). The caveat here that this count includes genes that are part of the repeat unit present in hundreds of copies. Moreover, due to the repetitive nature of much of the Y chromosome, sequencing has remained challenging and incomplete.

The Y chromosome harbors several genes that play a role in gene regulation. In mice, these include eukaryotic translation initiation factor 2 subunit 3, Y-linked (*Eif2s3y*), zinc finger protein 1, Y-linked (Zfy1), ubiquitin specific peptidase 9, Y chromosome (*Usp9y*), DEAD box helicase 3, Y-linked (*Ddx3y*), ubiquitously transcribed tetratricopeptide repeat containing, Y-linked (*Uty*), ubiquitin-activating enzyme, Y chromosome (*Uba1y*), lysine (K)-specific demethylase 5D (*Kdm5d*), RNA binding motif protein, Y chromosome (*Rbmy*), and *Sry*. These genes can be involved in different aspects of gene regulation, including translation, transcription, ubiquitination, splicing, and chromatin modifications (Bellott et al., 2014). Hence, expression of these genes in XY individuals may influence expression of autosomal genes in *trans*, through direct regulation of gene expression, regulatory cascades, or epigenetic remodeling.

1.4.2 Sex Phenotype

The other major driver of sexual dimorphism is sex phenotype. Sex phenotype encompasses the presence of sex-specific gonads – ovaries in females and testes in males, and the production of the associated sex-steroid hormones. The two best known sex-steroid hormones are testosterone and estradiol; however, progesterone is also considered to be a sex-steroid (reviewed in (Ruiz-Cortés, 2012)). Testosterone is typically seen as the 'male' sex hormone, whereas estradiol is seen as the 'female' sex hormone, however, both testosterone and estradiol are integral to males and females (Hamilton et al., 2014).

Steroid hormones, including testosterone, estradiol, progesterone, as well as other steroid hormones are all synthesised from cholesterol (Miller, 1988). These are mainly synthesized in the gonads; in males they are synthesized in Leydig cells in the testes, and in females, in grandulosa cells in the ovaries (Dorrington & Armstrong, 1975) (reviewed in (Hillier et al., 1994; Palmer & Wilhelm, 2013)). However, steroid hormones can also be synthesized in other tissues such as the brain, adrenal gland, and adipose tissue (Bruch et al., 1992; Sasano et al., 1999) (reviewed in (Barakat et al., 2016; Naftolin et al., 1975; Sanderson, 2006)).

The process of steroid synthesis begins with cholesterol, as shown in Figure 1. This is then converted into pregnenolone, then dehydroepiandrosterone (DHEA), and then androstenedione. From there, it can be converted into estrone or testosterone by 17 beta hydroxysteroid dehydrogenase (17βHSD). Both testosterone and estrone can then be converted into estradiol, or testosterone can also be converted into dihydrotestosterone. Other variations of this pathway can result in the production of aldosterone, or cortisol (Figure 1) (Hu et al., 2001; Miller, 1988).

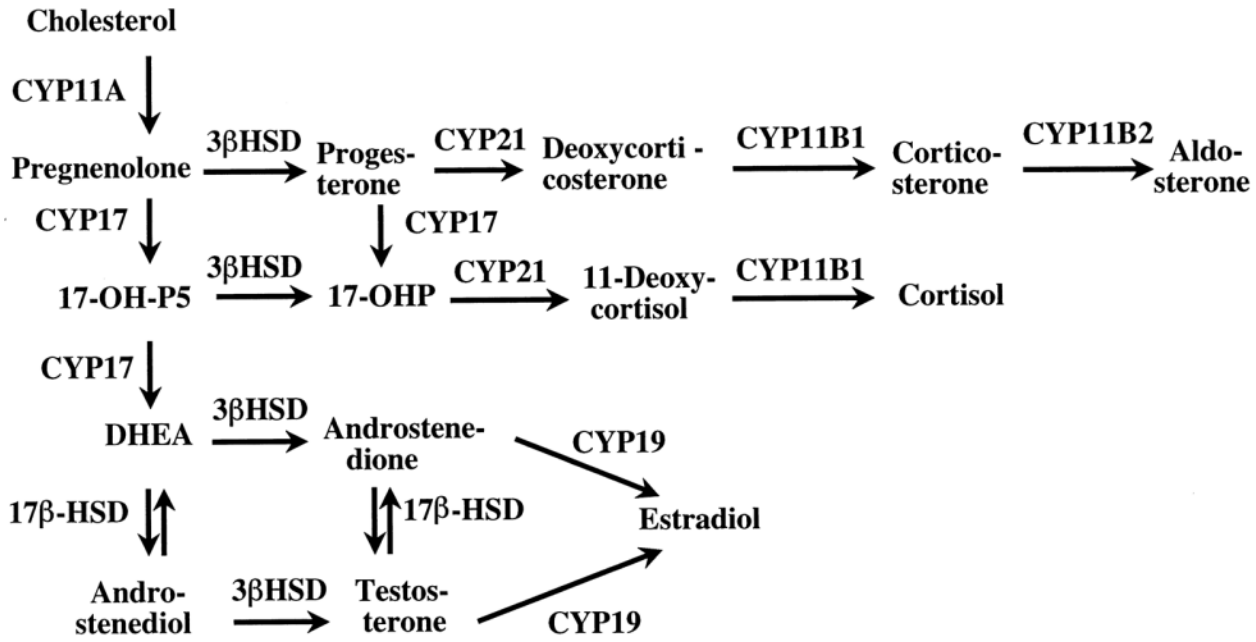

Figure 1: Steroid Synthesis Pathways

Steroids are synthesized from cholesterol. 17-OHP5: 17-hydroxy-pregnenolone, 17-OH-P4: 17-hydroxy-progesterone, DHEA: dehydroepiandrosterone, HSD: hydroxysteroid dehydrogenase. Important enzymes are shown above or next to arrows. Figure reproduced from (Hu et al., 2001)

1.4.2.1 Estrogens and Estradiol

The first estrogen, estrone, was first isolated in 1923 and was initially thought to be a female-specific hormone (Allen & Doisy, 1923). Since then, it has become clear the estrogens are also important in males (reviewed in (Hess & Cooke, 2018)). Estrogens are important in male and female reproduction and have been shown to be important in diseases such as obesity, cancers, and osteoporosis (reviewed in (Carreau & Hess, 2010; Hamilton et al., 2017; Vrtačnik et al., 2014)).

The term estrogen refers to a family of four steroids: estrone (E1), estradiol (E2), estriol (E3), and estetrol (E4) (reviewed in (Fuentes & Silveyra, 2019)). However, estradiol is the

predominant form in adults (Gruber et al., 2002). One of the main mechanisms through which estrogens act is through the binding of its receptors, which can in turn directly or indirectly regulate gene expression (Beato, 1989).

1.4.2.2 Estrogen Signalling Mechanisms

Estrogen signalling is mainly mediated through its receptors. There are three main estrogen receptors. The first, now known as estrogen receptor alpha (ERα or ESR1), was discovered in 1958 by Elwood Jensen (Jensen, 1958). Later, estrogen receptor beta (ERβ or ESR2) was discovered in 1996 (Kuiper et al., 1996). The third type of estrogen receptor is G Protein-Coupled Estrogen Receptor (GPER1), which is a membrane bound estrogen receptor (Levin, 2009) .

ESR1 is encoded by a gene located on chromosome 10 in mice and chromosome 6 in humans, and ESR2 is encoded for by a gene on chromosome 12 in mice, and chromosome 14 in humans (Enmark et al., 1997; Menasce et al., 1993). Both ESR1 and ESR2 are part of the nuclear hormone receptors superfamily, and thus have similar structures (reviewed in (Schwabe & Teichmann, 2004)). They consist of four main functional domains known as A/B, C, D, and E/F. The A/B region contains the amino-terminal domain (NTD), the C region the DNA binding domain (DBD), the D is a hinge region that allows the C and E domains to connect, and the E/F region is the ligand-binding domain (reviewed in (Beato, 1989; Beato et al., 1995)). Within the NTD and DBD, there are two regions now as activation function domains (AF), known as AF1 and AF2 respectively, which play important roles in regulation of transcription (reviewed in (Beato, 1989)). ESR1 and ESR2 can act by forming hetero- or homo-dimers, and then binding to DNA (reviewed in (Fuentes & Silveyra, 2019)). Typically, they bind to specific regions of DNA

known as estrogen response elements (EREs), however, it has been estimated that approximately 35% of genes targeted by estrogen lack EREs (reviewed in (Marino et al., 2006; O'Lone et al., 2004)).

ESR1 and ESR2 prevalence differs between various tissues. ESR1 is predominant in the pituitary gland, uterus, mammary gland, and in adipose tissue, whereas ESR2 is more common in the bladder, lungs, intestines, salivary glands, and prostate (Nishihara et al., 2000; Valimaa et al., 2004) (reviewed in (Kuiper & Gustafsson, 1997; Matthews & Gustafsson, 2003)

GPER on the other hand is a membrane bound estrogen receptor and encoded for by a gene on chromosome 7 in humans and chromosome 5 in mice. It is structurally unique relative to ESR1 and ESR2, and has a relatively low binding affinity for estradiol (reviewed in (Prossnitz & Barton, 2014)). However, it has been shown to be important for rapid response to estrogen (reviewed in (Filardo & Thomas, 2012)).

Estrogen can act through ESRs either through direct genomic action or indirect action. Direct action is when the receptors bind directly to DNA at EREs, enhancer regions, or the 3'-untranslated region of target genes (reviewed in (Klinge, 2001)). In this case, estradiol binds to the ligand binding domain (LBD), and the ESR dimerizes, allowing it to enter the nucleus of the cells, where it then binds to the DNA. However, indirect mechanisms of regulation have also been detected. In this case, estrogen receptor complexes can interact with other transcription factors and response elements, in which case they can impact activation or suppression of gene expression (reviewed in (Aranda & Pascual, 2001; Gottlicher et al., 1998)).

Estrogen receptor binding is most commonly associated with transcriptional activation; however, it is important to note that estrogen receptors have been shown to switch between

activation and repression through antagonist binding (Jackson et al., 1997; Smith et al., 1997) (reviewed in (Hu & Lazar, 2000))

1.4.2.3 Testosterone and Androgen Receptor

Testosterone is often considered to be the 'male' sex hormone as they are required for male reproductive development, as well as other sexual characteristics (reviewed in (Davey & Grossmann, 2016)); however, it is also important in females. Its action is often mediated through androgen receptor (AR), the gene for which is located on the X chromosome. While testosterone can bind directly to AR, it is more often converted into dihydrotestosterone first. AR action is similar to ESR1 and ESR2, where it dimerizes, then binds to specific regions of the DNA known as androgen response elements (reviewed in (Ansar Ahmed et al., 1985; Brinkmann, 2011; Brinkmann et al., 1999; Davey & Grossmann, 2016; Li & Al-Azzawi, 2009)).

1.5 Alternative Mechanisms of Regulation of Gene Expression

While ESRs and AR can act directly on DNA in target tissues to regulate transcription, and through this impact sexual dimorphism, other mechanisms are also important. For example, testosterone and estrogen can also interact with the hypothalamic-pituitary-gonadal (HPG) axis.

1.5.1 Hypothalamic-Pituitary-Gonadal Axis

The HPG axis refers to hormonal interactions between the hypothalamus, the pituitary gland, and the gonads. The hypothalamus releases gonadotrophin releasing hormone (GnRH), which stimulates the release of gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. These, in turn, act on the gonads to stimulate the

release of estradiol and testosterone, which in turn inhibit the release of GnRH and gonadotropins (reviewed in (Meethal & Atwood, 2005)) (Figure 2).

One of the important ways through which the HPG axis can impact sex-biased gene expression is though interactions between growth hormone (GH) and the Signal transducer and activator of transcription 5 signaling pathway (STAT5) pathway. GH secretion in mice is pulsatile in males, and more continuous in females (Xu et al., 2011) (reviewed in (Jansson et al., 1985; Shapiro et al., 1995)). STAT5 is a transcription factor that has been shown to be important in transcriptional response to GH, particularly in the liver (Choi & Waxman, 2000; Waxman et al., 1995). Both estrogen and testosterone have been shown to play a role in GH secretion and action (Avtanski et al., 2014) (reviewed in (Fernández-Pérez et al., 2020; Fernández-Pérez et al., 2013; Mode & Gustafsson, 2006; Waxman & Holloway, 2009)). There are also other genes that have been shown to respond to GH, including targets of STAT5. One of these is B cell leukemia/lymphoma 6 (BCL6), which is best known as a transcriptional repressor with higher expression in males (Chang et al., 1996; Chikada et al., 2018; Seyfert et al., 1996; Zhang et al., 2012).

BCL6 is a transcription factor that has been shown in previous studies to have sex-biased expression with higher expression in males, and be an important regulator of gene expression (Chikada et al., 2018; Chikada et al., 2020). A liver specific *Bcl6* knockout mouse model was recently developed, and male mice showed female-like expression of male biased cytochrome P450s (CYPs), whereas female biased-gene expression increased (Chikada et al., 2018). Our lab also demonstrated that this liver-specific knockout of *Bcl6* also shows loss of sex-bias in DNA methylation in the liver (Alogayil et al., 2021).

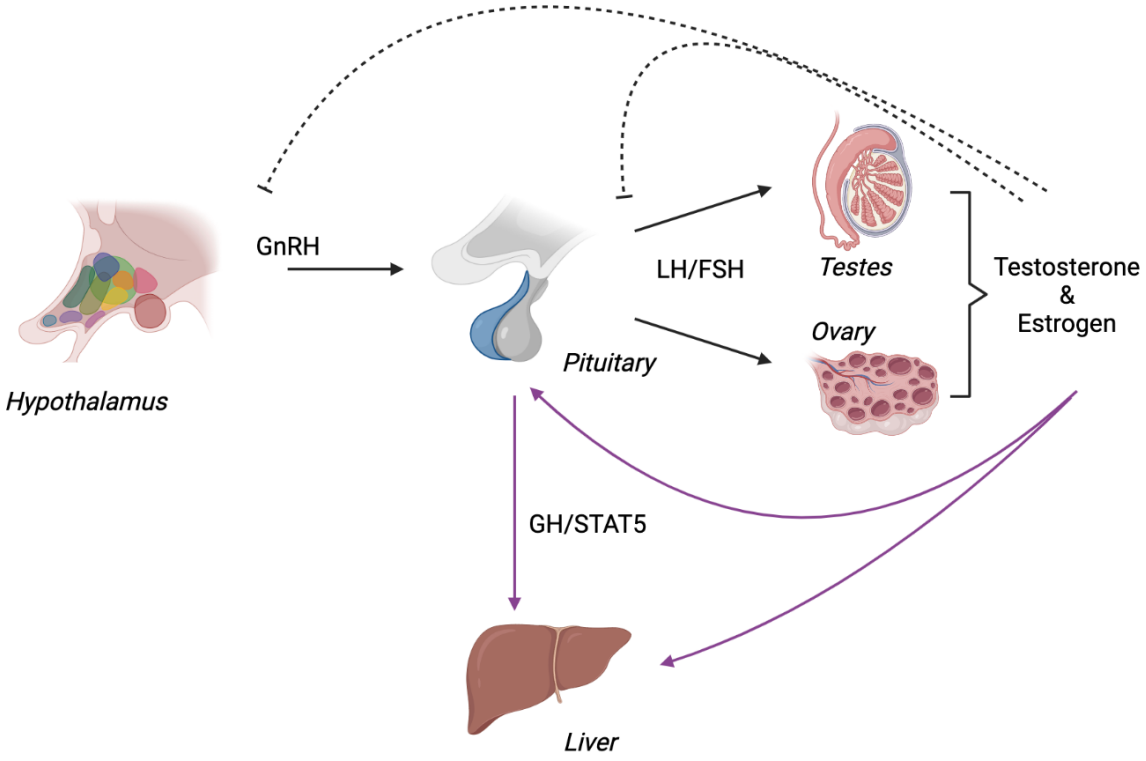

Figure 2: Schematic of the HPG Axis

1.6 Disentangling the roles of the sex-chromosome complement and sex phenotype in gene regulation in mammalian cells

Outlining the specific contributions of the sex-chromosome complement and the sex phenotype represents a challenge as in most studies of male to female comparisons the phenotypic males also carry the XY chromosome complement, and phenotypic females carry the XX complement. Several studies of blood transcriptomes in humans with sex chromosome aneuploidies, such as Turner (females with monosomy X) and Klinefelter (males with XXY karyotype) syndromes (Ho et al., 2018; Sharma et al., 2015; Skakkebaek et al., 2018; Trolle et al., 2016) provide a way to separate the effects of the sex-chromosome complement and gonadal sex hormones. These studies demonstrate global changes in methylation compared to

karyotypically normal males and females. Females with Turner syndrome have hypomethylated DNA (Trolle et al., 2016). On the other hand, Klinefelter syndrome is associated with global hypermethylation (Skakkebaek et al., 2018). They further demonstrate changes in gene expression relative to karyotypically normal controls in both individuals with Turner syndrome and those with Klinefelter syndrome (Skakkebaek et al., 2018; Trolle et al., 2016).

In mice, castration and ovariectomy at different ages were used to identify those sex-biased genes that depend on testosterone or estradiol signaling (Reizel et al., 2015). Castration at 3 weeks and at 20 weeks found that early exposure to testosterone, for some genes, results in long term activation of the genes and lasting demethylation (Reizel et al., 2015). On the other hand the same study found no significant impact of ovariectomy on DNA methylation, and thus did not examine it in terms of gene expression (Reizel et al., 2015).

A series of studies have been done to characterize the contribution of GH signaling by the mouse pituitary to gene regulation in mouse liver (Lau-Corona et al., 2017; Waxman & O'Connor, 2006; Zhang et al., 2012). The main findings are that persisting GH signalling results in feminization of gene expression in the mouse liver, and that GH signalling is an important regulator of sex-biased gene expression in the mouse liver (Lau-Corona et al., 2017; Waxman & O'Connor, 2006). Furthermore, it can impact other signalling pathways such as the STAT5 pathway, that in turn impact sex-biased gene expression (Zhang et al., 2012).

Recently, our lab has used mice with different combinations of sex-chromosome complement and sex phenotype, i.e. XX females, XY males, XY females, and XO females, to examine the contributions of sex-chromosome complement and sex phenotype on sex-biased gene expression and DNA methylation in the mouse liver (Zhuang et al., 2020). Our main findings were that sex phenotype is the main driver of sex-biased DNA methylation in the liver on autosomes, however,

X-dosage and the presence of the Y chromosome also impact it. Furthermore, sex bias in autosomal DNA methylation was found to be associated with sex-biased gene expression (Zhuang et al., 2020).

1.7 Research Questions and Study Design

For this study, we address the question of **How do sex phenotype or sex-chromosome complement impact sex-biased gene expression in the mouse brain and liver?**

Our main **hypotheses** are:

1) Estradiol signaling through ESR1 contributes to sex-phenotype dependent sex bias in gene expression in the mouse liver
2) ESR1, AR, and BCL6 influence sex-biased DNA methylation through the binding of sex-biased differentially methylated regions (sDMRs), or in the immediate proximity of them.
3) sex-chromosome complement and sex phenotype influence gene expression in the mouse brain;
4) X-dosage and escape from X-inactivation are the major contributors to sex-biased gene expression in mouse liver and brain.

For this, we have four main objectives: 1) examine the extent to which estradiol, mediated by ESR1, impacts gene expression in the mouse liver; 2) examine the likelihood of direct binding of the transcription factors AR, ESR1, and BCL6 being a major mechanism through which they regulate sex phenotype dependant DNA methylation in the mouse liver; 3) examine the influence of sex phenotype and sex-chromosome complement on sex-biased gene

expression levels in the mouse brain; 4) examine the impact of sex phenotype or sex-chromosome complement on the expression of X-linked genes in the mouse liver and brain.

For this study, we use a combination of different mouse strains and publicly available data to test our hypotheses. To test the role of ESR1-mediated estradiol signalling on sex-biased gene expression, we use an ESR1 knockout model and perform RNA-seq on male and female ESR1 knockouts and their wildtype littermates. By overlapping the differentially expressed genes found in the wildtype comparison with those in the knockout comparison, we are able to identify a) which genes maintain sex-biased expression despite the loss of ESR1; b) which genes loose sex-biased expression; and c) which genes, if any, gain sex-biased expression with the loss of ESR1.

For our second objective, we use publicly available CHIP-seq data for ESR1, AR, and BCL6 binding as well as whole genome bisulfite sequencing (WGBS) results for sex-phenotype dependant sex-biased differentially methylated regions in the mouse liver. We colocalize the binding sites from the CHIP-seq datasets with the locations of sDMRs to assess the possibility of direct binding of these transcription factors impacting sex-biased DNA methylation in the mouse liver.

For the third and fourth objectives, we extract RNA and performed RNA-seq on the brains and livers of XX female, XY male, and XY female mice, as well as XO female mice for the liver. Using these, we are able to differentiate the effects of sex-phenotype and sex-chromosome complement on sex-biased gene expression.

Chapter 2: Materials and Methods

2.1 Mouse Strains and Crosses

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

B6.Y^{TIR} (Tirano) mice have a Y chromosome derived from mice caught in Tirano, Italy. This Y chromosome on the C57BL/6J background results in insufficient upregulation of SRY-box transcription factor 9 (*Sox9*) by *Sry.* As this is required for testicular differentiation, this results in some mice that are phenotypically female with XY chromosomes (Coward et al., 1994; Nagamine et al., 1987; Park et al., 2011). These mice were maintained and bred by Dr. Teruko Taketo (McGill University, QC, Canada). B6.Y^{TIR} males were crossed with wild-type C57BL/6J females to generate XY^{TIR} sex-reversed females (XY.F), XX females (XX.F), XY^{TIR} (XY.M) males, as well as true hermaphrodites (Figure 3a). Genotyping was performed on females using PCR amplification of the zinc finger protein on the Y (*Zfy1*) sequence from ear punches, using the primers shown in Table 1 and the procedure described in (Amleh et al., 2000). Livers and brains from 8-week-old XX.F, XY.F, and XY.M mice were collected for use in DNA methylation analyses (retrieved from Zhuang et al., 2020) for the liver, and expression analyses (liver data retrieved from Zhuang et al., 2020).

C3H/HeSn-Paf/J (referred to as *Paf* from this point on) mice, that carry the patchy fur (*Paf*) mutation on their X chromosome were purchased from Jackson Laboratory (Bar Harbor, Maine, USA), and provided by Dr. Teruko Taketo (McGill University, QC, Canada). The Paf mutation on the X chromosome interferes with the segregation of the X and Y chromosomes, which results in the generation of both XO females, and rarely, XXY males (Burgoyne & Evans, 2000; Korobova et al., 1998; Lane & Davisson, 1990). Paf males were backcrossed to C57BL/6J

females for five to six generations and experiments were conducted on N5 and N6 mice. Genotyping was performed using reverse transcription PCR (RT-PCR) for the *Xist* gene, which is only expressed in XX^{Paf} females, and not XO females (Alton et al., 2008; Kay et al., 1993). The primers are shown in table 1. The cross between N4 or N5 *Paf* males and C57BL/6J females produced XX^{Paf} female mice (XX^{Paf}.F), X^{Paf}Y males (X^{Paf}Y.M), as well as XO females (XO.F) (Figure 3b). XX^{Paf}Y males (XX^{Paf}Y.M) are found extremely rarely and were not observed in our colony (Figure 3b). Livers were collected from 8-week-old XX^{Paf}.F and XO.F littermates for use in DNA methylation analyses and expression analyses (data retrieved from Zhuang et al., 2020).

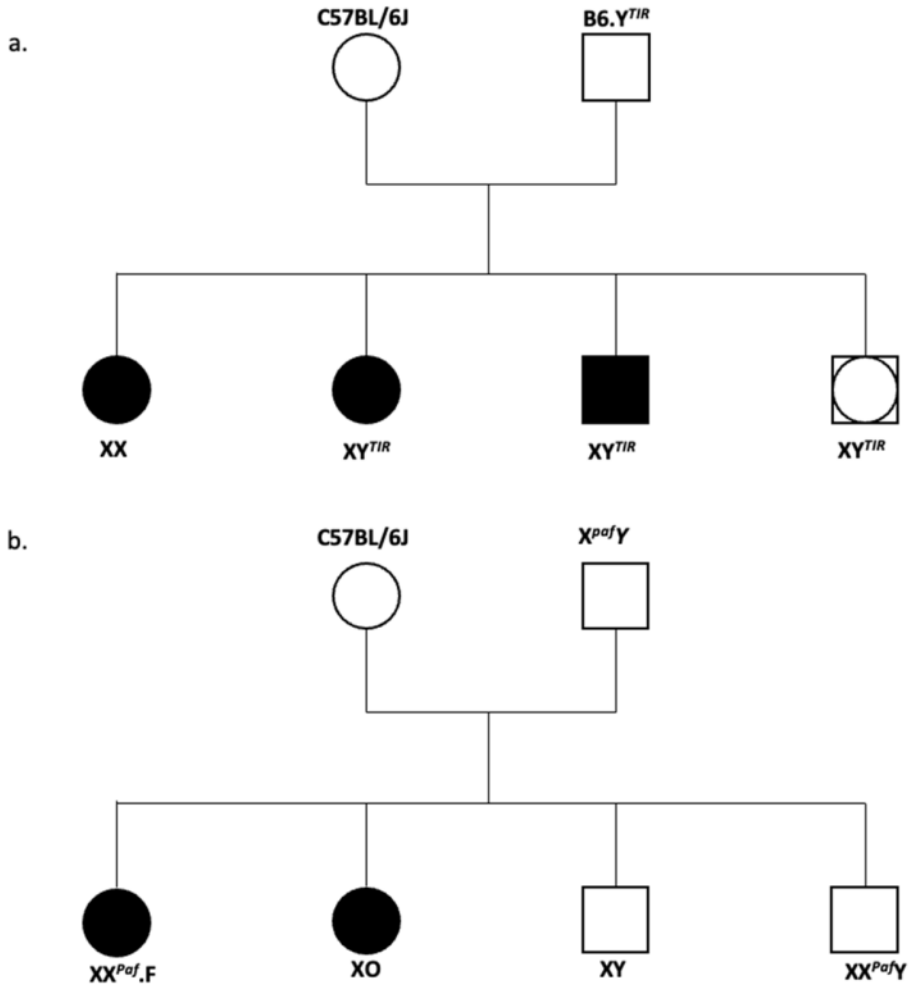

Figure 3: Mouse crosses used to generate sex-reversed XY females and females with monosomy X

a*. TIR cross* ***b****. Paf cross. Circles represent females, squares represent males, and a circle within a square represents hermaphrodites. Filled in shapes represent mice used.*

Table 1: Genotyping Primers Used

Gene	Mouse Cross	Sequencing Technique	Forward Primer	Reverse Primer
Zfy	B6.TIR	Standard PCR	AAGATAAGCTTACATAATCACATGGA	CCTATGAAATCCTTTGCTGCACATGT
Xist	*Paf*	RT-PCR	ACTGCCAGCAGCCTATACAG	GTTGATCCTCGGGTCATTTA
Esr1	ESR1KO	Standard PCR	ATCCCATGTGCTTGAGTGGT	CCACTTCTCCTGGGAGTCTG

B6N(Cg)-*Esr1*$^{tm4.2Ksk}$/J mice [28] (JAX stock #026176, henceforth referred to as ESR1KO) carrying a deletion of exon three of the estrogen receptor alpha (*Esr1*) gene were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). Homozygous ESR1KO mice were produced by mating heterozygous male and female mice. Genotype of the offspring was determined by standard PCR using the primers shown in Table 1, and *Esr1* expression (or loss of expression) in the liver was confirmed using RT-PCR for *Esr1* by Najla Alogayil (forward primer: CAGACACTTTGATCCACCTGA; reverse primer: CGTTCTTGCATTTCATGTTGTAG). Livers were collected from 8-week-old ESR1KO mice and their wild type (WT) littermates for expression analysis.

All procedures were conducted in accordance with the guidelines set by the Canadian Council on Animal Care (Ottawa, Ontario, Canada) and were approved by the Animal Care Committee of the McGill University Health Center (Montreal, Quebec, Canada).

2.2 RNA Extraction, RNA sequencing, and Analysis

Total RNA was extracted using Trizol Reagent (Thermo Fisher Scientific, MA, US) and purified using the RNeasy MinElute Cleanup Kit (Qiagen, NL). RNA was extracted from the livers of 8-week-old homozygous ESR1KO mice (five females and four males) and WT controls ((three females, three males), as well as from the brains of XY^{TIR} sex-reversed females (XY.F, n=5), XX females (XX.F, n=4), and XY^{TIR} males (XY.M, n=4).

RRNA-depleted library preparation and paired-end sequencing was performed using an Illumina NovaSeq6000 S4 sequencer in a single lane by the McGill Genome Centre (Montreal, QC, Canada), and a second top-up run was performed for any samples with low reads. Differential expression analysis was performed using the GenPipes RNA-seq pipeline (version 3.2.0) (Bourgey et al., 2019). Reads were first trimmed and filtered for quality, before being

aligned to the mouse reference genome (GRCm38) using STAR (version 2.7.3a) (Dobin et al., 2013). Transcript abundance was estimated using HT-Seq Count (version 0.11.0) (Anders et al., 2015). PC analysis was performed on the abundance data after doing a regularized log transformation using DESeq2, and differential gene expression was determined using the DESeq2 (version 1.20.0) (Anders & Huber, 2010) and EdgeR (version 3.22.5) (Robinson et al., 2010) packages. ESR1 liver data are deposited to the NCBI GEO repository as GSE174535.

Chromosomes were annotated in RStudio using biomaRt (version 2.48.3) (Durinck et al., 2005; Durinck et al., 2009) and separated to allow for the analysis of autosomal differentially expressed genes (DEGs) and those located on sex chromosomes separately.

Pathway analysis was performed using gprofiler2 (v.0.2.1) (Kolberg et al., 2020) in RStudio for genes with an absolute log fold change (log2FC) greater or equal to 1.5 and a DESeq2 adjusted p-value (padj) less than 0.05. Pathways in Kegg (KEGG), Reactome (REAC), and WikiPathways databases were considered.

2.3 Colocalization of ChIP sites with sex phenotype associated sDMRs

Lists of liver sex phenotype dependant sDMRs (generated by comparing XY.F to XY.M using WGBS) (Zhuang et al., 2020) were compared to ChIP-seq data for AR, ESR1, and BCL6. The list of sDMRs was filtered to remove any on sex-chromosomes, leaving 3847 autosomal sDMRs. This was then separated into sDMRs with lower methylation in males (male-biased, n= 3414), and those with lower methylation in females (female-biased, n = 433), and each set was analyzed separately. Processed ChIP-seq data in 20-week-old mouse liver for ESR1 in females, and AR in males were retrieved from Li et al. (2012). These were filtered to remove sites with a fold enrichment of -2 and converted to the mm10 assembly. 7850 autosomal AR ChIP peaks and

31,235 autosomal ESR1 ChIP peaks remained after filtering. Raw data for BCL6 ChIP peaks were retrieved from GSE31578 (Zhang et al., 2012), and processed by Matthew Chang. Female replicate three (GSM784032) and male replicate two (GSM784028) were used in the analysis, as they had the most similar number of peaks on autosomes (22,851 and 20,994, respectively).

The BEDOPs closest-features tool (version 2.4.35) (Neph et al., 2012) was then used to find the nearest ChIP peaks to each sDMR. The results were plotted as a density plot in RStudio using the function *density* of the package stats (version 4.0.3). To generate a random control, the sDMRs were shuffled 100 times using BEDtools shuffle (version 2.29.2) (Quinlan & Hall, 2010), and added to the density plot.

The number of direct overlaps between sDMRs and ChIP peaks, as well as sDMRs with ChIP peaks within 10kb were found in RStudio by filtering the BEDOPs results. A random control was generated by randomly shuffling sDMRs 500 times using BEDtools shuffle (version 2.29.2) (Quinlan & Hall, 2010), counting the number of direct overlaps or within 10kb between the ChIP peak and sDMR or shuffled control.

Genomic locations of sDMRs were annotated using annotatr (version 1.18.1) in RStudio (Cavalcante & Sartor, 2017).

2.4 Data from public databases

For analysis of the effects of the sex phenotype vs the sex-chromosome complement and X-linked gene expression in the mouse liver, RNA-seq results were extracted from Zhuang et al., 2020. Whole genome bisulfite sequencing (WGBS) results for sex-phenotype dependant sDMRs were also extracted from Zhuang et al., 2020.

CHIP-seq data for ESR1 and AR binding was extracted from Li et al., 2012, and for BCL6 from Zhang et al., 2012.

Chapter 3: Results

The purpose of this study was to examine how sex phenotype and sex-chromosome complement influenced sex-biased gene expression in the mouse brain and liver. To differentiate these effects, we used mouse crosses with different combinations of sex-chromosome complement and sex phenotype. We also looked further into the factors that mediate the impact of sex phenotype which was found in an earlier study to be the major driver of sex-biased gene expression in the liver (Zhuang et al., 2020). While AR has been shown to impact sex-biased gene expression in the mouse liver in previous studies (Delic et al., 2010; Gatti et al., 2010; Reizel et al., 2015; van Nas et al., 2009; Zheng et al., 2017), the role of ESR1 has not been as clearly shown. For this reason, we used an ESR1KO mouse model to see how the loss of this transcription factor influenced sex bias in gene expression. We also examined the binding of ESR1 as well as AR and BCL6, which have also been shown to impact sex-biased gene expression (Chikada et al., 2018; Chikada et al., 2020; Delic et al., 2010; Gatti et al., 2010; Reizel et al., 2015; van Nas et al., 2009; Zheng et al., 2017), relative to the location of sex-phenotype dependant differentially methylated regions (sDMRs) to test if direct binding of the transcription factors is one potential mechanism through which they impact DNA methylation, which can in turn impact gene expression.

3.1 **Loss of ESR1 results in loss of sex-bias in gene expression**

Our previous study showed that sex phenotype was the major driver of sex biased gene expression in the adult mouse liver (Zhuang et al., 2020). While in previous studies it has been shown that both testosterone and AR are important factors contributing to sex-bias in DNA methylation and gene expression in mice (Delic et al., 2010; Gatti et al., 2010; Reizel et al., 2015; van Nas et al., 2009; Zheng et al., 2017), the role of estradiol and its receptors, including ESR1, remains unclear. To assess the impact of estradiol signaling through ESR1 on sex-biased gene expression, we used an ESR1KO mouse model, which has a deletion in exon three of the ESR1 gene, which results in the ablation of ESR1 in the whole body. Loss of *Esr1* expression in the liver was verified in our lab and is shown in Figure 4. Loss of ESR1 results in higher body weight in females, and causes infertility in both males and females (Goulding et al., 2010; Hewitt et al., 2010) (reviewed in (Hamilton et al., 2014)). Our expectation was that genes that depend on the ESR1 pathway for sex-biased gene expression would lose sex bias in the ESR1 knockouts.

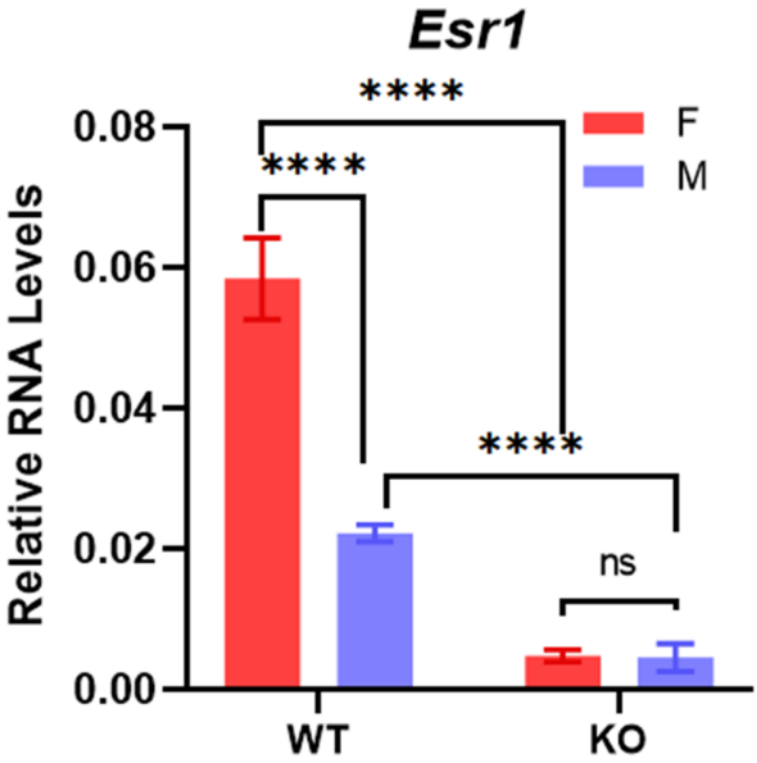

Figure 4: Loss of *Esr1* results in low expression in the livers of adult ESR1KO mice.

*Expression levels normalized to Rpl19. Error bars show standard deviation. Statistically significant differences are shown with asterisks **** P<0.0001, ns: non-significant (two-way ANOVA followed by multiple testing with Sidak's correction). Generated through RT-PCR by Najla Alogayil. (Figure reproduced from (Alogayil et al., 2021)).*

After performing RNA-seq analysis on the livers of male and female ESR1 knockout mice (MKO, FKO), and their wild type littermates (MWT, FWT), we conducted principal component analyses (PCAs) on the top 500 DEGs. We found that each of the sex/genotype groups formed unique clusters, however, both of the KO clusters were closer to the MWT than the FWT (Figure 5a).

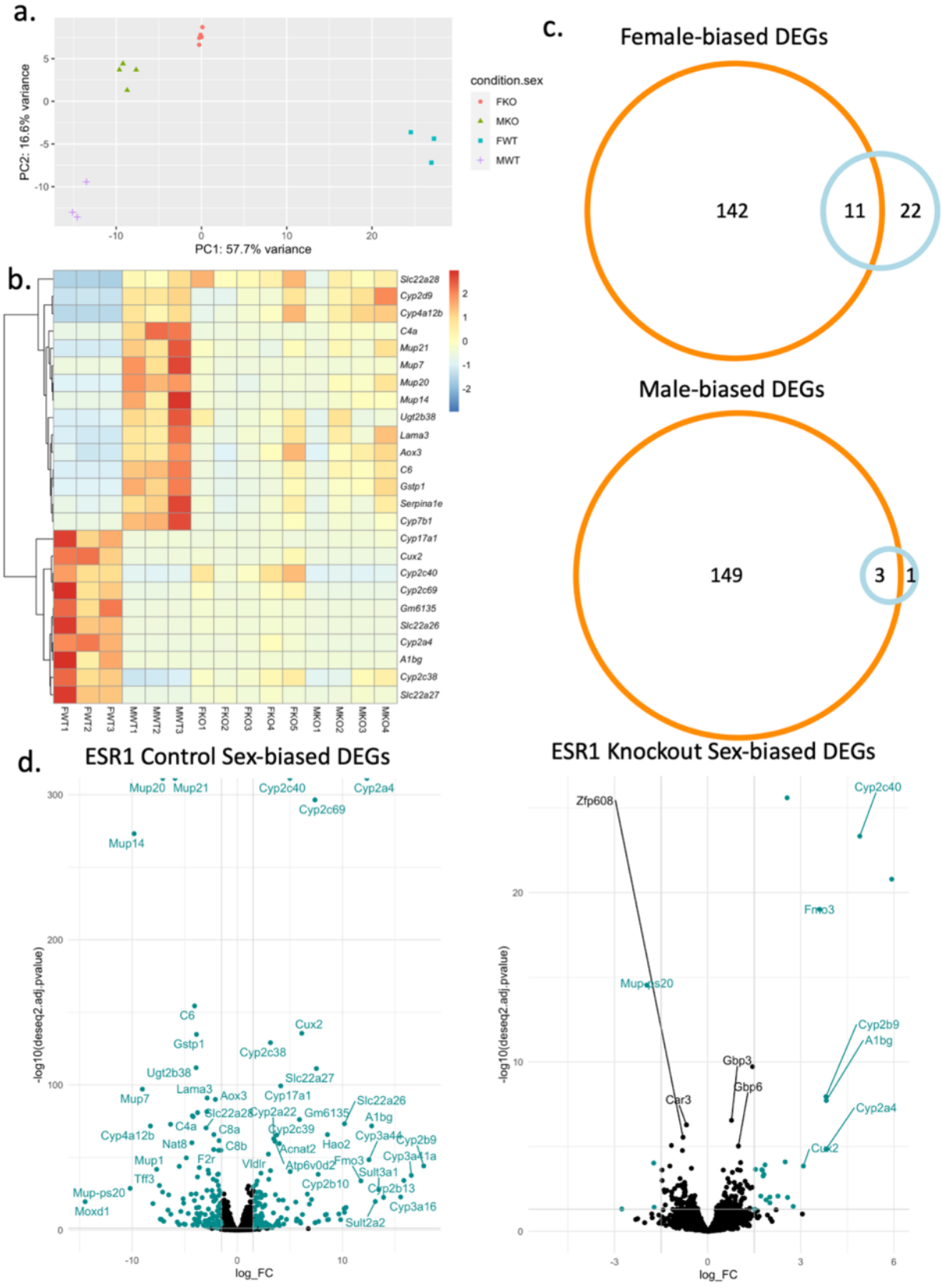
a.
PC2: 16.6% variance
PC1: 57.7% variance
condition.sex
FKO
MKO
FWT
MWT
b.
Slc22a28
Cyp2d9
Cyp4a12b
C4a
Mup21
Mup7
Mup20
Mup14
Ugt2b38
Lama3
Aox3
C6
Gstp1
Serpina1e
Cyp7b1
Cyp17a1
Cux2
Cyp2c40
Cyp2c69
Gm6135
Slc22a26
Cyp2a4
A1bg
Cyp2c38
Slc22a27
FWT1
FWT2
FWT3
MWT1
MWT2
MWT3
FKO1
FKO2
FKO3
FKO4
FKO5
MKO1
MKO2
MKO3
MKO4
c.
Female-biased DEGs
142
11
22
Male-biased DEGs
149
3
1
d.
ESR1 Control Sex-biased DEGs
-log10(deseq2.adj.pvalue)
log_FC
Mup20
Mup21
Cyp2c40
Cyp2a4
Cyp2c69
Mup14
C6
Cux2
Gstp1
Cyp2c38
Ugt2b38
Slc22a27
Lama3
Aox3
Cyp17a1
Mup7
Slc22a26
Slc22a28
Cyp2a22
Gm6135
A1bg
Cyp4a12b
C4a
C8a
Cyp2c39
Cyp3a44
Cyp2b9
Nat8
C8b
Hao2
Acnat2
Mup1
F2r
Vldlr
Fmo3
Cyp3a41a
Atp6v0d2
Tff3
Sult3a1
Cyp2b10
Cyp2b13
Mup-ps20
Cyp3a16
Moxd1
Sult2a2
ESR1 Knockout Sex-biased DEGs
-log10(deseq2.adj.pvalue)
log_FC
Zfp608
Cyp2c40
Fmo3
Mup-ps20
Cyp2b9
A1bg
Gbp3
Gbp6
Car3
Cyp2a4
Cux2

Figure 5: Loss of ESR1 results in reduced sex-biased differential gene expression

a. *Principal component analysis (PCA) for RNA-seq data from ESR1KO WT and KO mouse livers shows that samples cluster by genotype and sex, when comparing the first two principal components.* ***b.*** *Heatmap shows the top 25 significant DEGs in WT and KO mice.* ***c.*** *Overlapping DEGs between WT (orange) and KO (blue) samples: female-biased DEGs (top panel) and male-biased DEGs (bottom panel) are shown separately.* ***d.*** *Volcano plots for the differential gene expression (DGE) analysis using DESeq2. DEGs with an absolute log2 fold change ≥ 1.5 and a DESeq2 adjusted P-value < 0.05 are shown in green. DEGs found in WT comparison are shown in the left panel, and those found in the KO comparison are shown in the right panel.*

Using cut-offs of an absolute log2FC greater or equal to 1.5 and DESeq2 padj < 0.05, we compared autosomal sex-biased DEGs in the WT mice to those in the KO mice. We found 305 autosomal DEGs in the FWT vs MWT comparison, 14 of which maintained sex-biased expression in the KO comparison (three male-biased, 11 female-biased) (Figure 5 b-d). An additional 13 genes gained sex-biased expression in the KO mice. There were 291 autosomal genes that lost sex-bias in gene expression

Pathway analyses were performed for genes that maintain sex-biased expression in the absence of ESR1, and on those that lose it. For these analyses, we examined genes located on both autosomes and sex chromosomes but used the same significance criteria as above (absolute log2FC ≥1.5, padj < 0.05). Among the 14 genes that maintained sex-biased expression with the loss of ESR1, (ie. DEGs found in the FKO vs MKO comparison), we detected significant enrichment of genes from only one pathway, which was retinol metabolism from the KEGG database. For genes that lost sex-biased gene expression with the loss of ESR1, there was

significant enrichment of genes from 36 pathways (Table 2). The top three were retinol metabolism metabolic pathways, and arachidonic acid metabolism. Not all genes had an associated pathway, and some genes were associated with multiple pathways. Complete results can be accessed at https://biit.cs.ut.ee/gplink/l/L57aE2_CQq.

Table 2: Pathway analysis for genes that loose sex-biased expression with the loss of ESR1.

source	term_name	adjusted_p_value	term_size	query_size	intersection_size
KEGG	Retinol metabolism	1.73E-10	85	212	13
KEGG	Metabolic pathways	8.54E-10	1381	212	43
KEGG	Arachidonic acid metabolism	1.38E-09	61	212	11
KEGG	Steroid hormone biosynthesis	1.24E-08	74	212	11
WP	Eicosanoid metabolism via Cytochrome P450 Mono-Oxygenases (CYP) pathway	2.42E-07	16	212	6
REAC	Metabolism of lipids	2.20E-06	513	212	22
REAC	Fatty acids	5.88535E-06	19	212	6
REAC	Fatty acid metabolism	1.02159E-05	152	212	12
KEGG	Bile secretion	1.38914E-05	86	212	9
KEGG	Chemical carcinogenesis	1.87209E-05	89	212	9
REAC	Miscellaneous substrates	2.11926E-05	23	212	6
REAC	Cytochrome P450 - arranged by substrate type	2.7382E-05	58	212	8

REAC	Eicosanoids	6.99554E-05	15	212	5
KEGG	Inflammatory mediator regulation of TRP channels	7.12983E-05	104	212	9
REAC	Biological oxidations	7.85198E-05	183	212	12
REAC	Arachidonic acid metabolism	0.000160452	50	212	7
REAC	Terminal pathway of complement	0.000178276	8	212	4
KEGG	Ovarian steroidogenesis	0.000230531	41	212	6
REAC	Metabolism	0.000548761	1519	212	35
WP	Complement Activation, Classical Pathway	0.000592732	16	212	4
KEGG	Fatty acid degradation	0.000594289	48	212	6
REAC	Synthesis of Leukotrienes (LT) and Eoxins (EX)	0.000921538	24	212	5
REAC	Phase I - Functionalization of compounds	0.001085292	93	212	8
KEGG	Biosynthesis of unsaturated fatty acids	0.001158762	32	212	5
KEGG	Drug metabolism - other enzymes	0.001679031	85	212	7

KEGG	Drug metabolism - cytochrome P450	0.002419145	61	212	6
WP	PPAR signaling pathway	0.002766088	71	212	6
KEGG	Neuroactive ligand-receptor interaction	0.004954672	136	212	8
WP	Eicosanoid metabolism via Cyclo Oxygenases (COX)	0.005252769	27	212	4
KEGG	KEGG root term	0.006204788	6309	212	88
WP	WIKIPATHWAYS	0.008226465	3692	212	57
KEGG	PPAR signaling pathway	0.009071624	77	212	6
KEGG	Fatty acid elongation	0.01092506	27	212	4
KEGG	Complement and coagulation cascades	0.016723955	86	212	6
KEGG	Amoebiasis	0.017817937	87	212	6
KEGG	Metabolism of xenobiotics by cytochrome P450	0.039271861	66	212	5

Term size represents the total number or genes associated with the pathway; intersection size is the number of genes that lost sex biased gene expression that intersect with the pathway.

Finally, we tested the hypothesis that the genes that lose sex-biased expression with the loss of ESR1 would also be genes that we found to be sex-phenotype dependant in our previous study (Zhuang et al., 2020). 115 of the 291 autosomal genes that were found to lose sex-biased expression with the loss of ESR1 were also found to be sex-phenotype dependant in the previous study.

Ultimately, we found that loss of ESR1 resulted in the loss of sex bias in gene expression in the majority of sex-biased genes in mouse liver. Reduced sex-bias in gene expression impacted not only female-biased DEGs, but also male-biased DEGs.

3.2 Co-localization of sex-phenotype dependent DEGs and sDMRs with transcription factor binding sites.

After establishing that loss of ESR1 does result in reduced sex-biased gene expression, we wished to examine how it does this. One mechanism through which sex-biased gene expression may arise is through a sex bias in the binding of transcription factors (TFs). TFs may then act in conjunction with cofactors to either activate or repress expression. TF binding may occur in the immediate proximity of genes that they act on, or much further away (reviewed in (Waxman & O'Connor, 2006)). Studies have also suggested that DNA methylation is an important mechanism associated with transcription factor action, and DNA methylation has in turn been linked to gene expression (Compere & Palmiter, 1981; Holliday & Pugh, 1975). The presence of methyl marks on DNA is associated with repression of gene expression, and they act either by recruiting repressive proteins or by preventing the binding of TFs to the DNA (reviewed in (Moore et al., 2013)). In our previous study, we found significant enrichment of the transcription start site of DEGs by sDMRs (Zhuang et al., 2020). Thus, we hypothesized that if

TFs that were previously implicated in sex-biased gene regulation acted through direct binding to sDMRs located near sDEGs, we would be able to detect the enrichment of TFs in close proximity to sDMRs.

We selected three TFs that have been implicated in sex-biased gene regulation and for which ChIP-seq data from adult mouse livers were available: ESR1, AR, and BCL6 (Chikada et al., 2018; Zheng et al., 2017). ChIP-seq data for ESR1, AR, and BCL6 binding in the mouse liver (ESR1 and BCL6 in the female, and AR and BCL6 in the male) were extracted from (Li et al., 2012) for AR and ESR1, and (Zhang et al., 2012) for BCL6. The locations of these regions were compared to the genomic positions of liver sex-phenotype dependant sDMRs from our 2020 study (Zhuang et al., 2020).

As a first step, we found the nearest of each of the TF enriched regions to each sDMR and plotted the distance from the TF to the sDMR on density plots (Figure 6a). To generate a negative control, we randomly shuffled the sDMR coordinates, keeping the regions the same length, and repeated the analysis 100 times for the male-biased and female-biased sDMRs separately. We found that there was a tendency for male-biased sDMRs to be nearer to the ChIP enriched region for AR and BCL6 in the male liver, and ESR1 and BCL6 in the female liver compared to the random controls (Figure 6b). Female-biased sDMRs showed a tendency to be nearer to ESR1 and BCL6 ChIP enriched regions in the female livers relative to the random controls (Figure 6b).

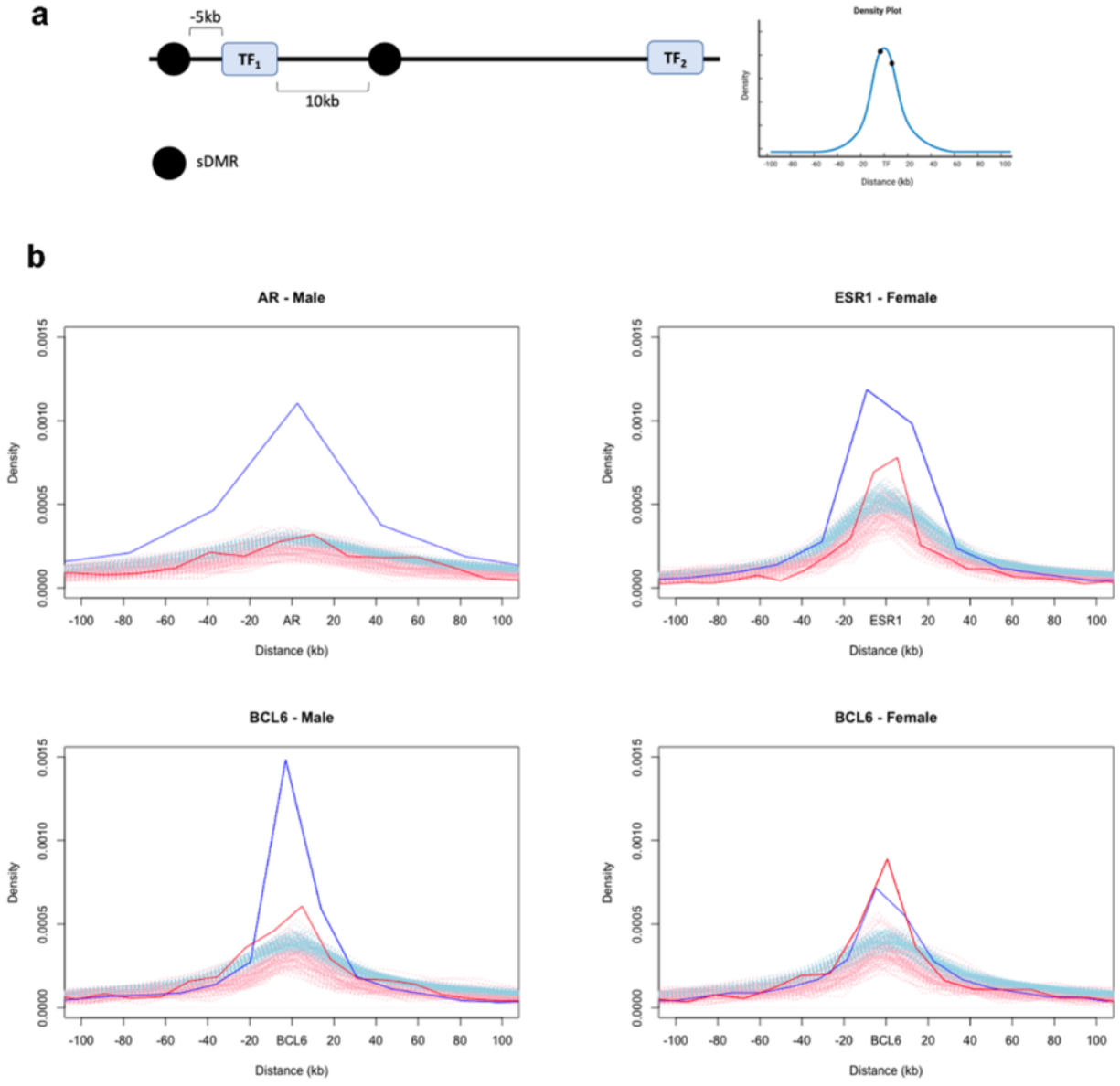

Figure 6: Colocalization of ChIP enriched regions for AR, ESR1, and BCL6 with sex-phenotype dependant sDMRs

a. Schematic shows how plots were generated. The distance between either end of the sDMR and the nearest ChIP-enriched regions was found, then a density plot was generated for each male-biased and female-biased sDMRs, relative to each AR-, ESR1-, or BCL6-enriched region in male or female liver. Density was calculated based on kernel density using a bandwidth of 100 in R,

using the density *function. b. Density of sDMRs relative to AR ChIP peaks in male liver (left top panel); BCL6 ChIP peaks in male liver (left bottom panel); ESR1 ChIP peaks in female liver (right top panel), and BCL6 ChIP peaks in female liver (right bottom panel). The x-axis shows distance from the TF-enriched site, the y-axis shows density of sDMRs. Male-biased sDMRs – solid blue line, female-biased sDMRs - solid red line, dashed lines show 100 permutations for the male-biased (blue) or female-biased (red) sDMRs. (Figure reproduced from (Alogayil et al., 2021)).*

In the female liver, we found that 12 (2.8%) of the 433 female-biased sDMRs overlapped with BCL6 ChIP-enriched regions, and 81 (2.4%) of the 3414 male-biased sDMRs overlapped with BCL6 ChIP-enriched regions (Table 3)

Within 10kb, there were 133 (30.7) female-biased sDMRs with ChIP enriched regions located nearby, and 932 (27.3%) male-biased (Table 3). When colocalized with ESR1 ChIP, we found there were 26 (6.0%) female-biased and 255 (7.5%) male-biased sDMRs directly overlapping (Table 3). There were 153 (35.3%) female-biased and 1449 (42.4%) male biased sDMRs with ChIP enriched regions within 10kb (Table 3). These were all found to be statistically significant when comparing 500 permutations to ChIP peaks.

Table 3: Colocalization of autosomal sDMRs autosomal sDMRs with BCL6, AR, or ESR1-enriched regions (reproduced from (Alogayil et al., 2021)).

	Sex-phenotype dependent sDMRs	Female Liver				Male Liver			
		Colocalized with BCL6-ChIP enriched regions** (% DMRs)		Colocalized with ESR1-ChIP enriched regions*** (% DMRs)		Colocalized with BCL6-ChIP enriched regions** (% DMRs)		Colocalized with AR-ChIP enriched regions**** (% DMRs)	
		Direct Overlap	Within 10kb	Direct Overlap	Within 10kb	Direct Overlap	Within 10kb	Direct Overlap	Within 10kb
Female-biased sDMRs	433	12 (2.8)*	133 (30.7)*	26 (6.0)*	153 (35.3)*	4 (0.9)	100 (23.1)*	1 (0.2)	46 (10.6)*
Male-biased sDMRs	3414	81 (2.4)*	932 (27.3)*	255 (7.5)*	1449 (42.4)*	413 (12.1)*	1573 (46.1)*	87 (2.5)*	721 (21.1)*
Total sDMRs	3847	103 (2.7)	1065 (27.7)	295 (7.7)	1602 (41.6)	477 (12.4)	1673 (43.5)	97 (2.5)	767 (19.9)
Total ChIP Peaks	n/a	22,851		31,235		20,994		7850	

* indicates statistical significance.

** BCL6-ChIP peaks in female and male livers extracted from GSE31578- GSM784032, GSM784028 replicates three and two, respectively

*** ESR1-ChIP peaks in female liver extracted from GSE32244

**** AR-ChIP peaks in male liver extracted from GSE32244

In the male liver, we found only four (0.9%) female-biased DMRs overlapped with ChIP-enriched regions for BCL6, which was not found to be statistically significant in the permutation analysis (**Table 3**). However, there were 413 (12.1%) male-biased sDMRs overlapping with BCL6 ChIP enriched regions, which was found to be statistically significant (Table 3). There were also 100 (23.1%) female-biased sDMRs and 1573 (46.1%) male-biased with BCL6 ChIP enriched regions within 10kb, both of which were found to be statistically significant (Table 3). When the sDMRs were colocalized with AR ChIP enriched regions, we found one (0.2%) female-biased sDMR and 87 (2.5%) male-biased sDMRs that directly overlapped with AR ChIP enriched regions (Table 3). While again, the female-biased sDMR overlap was not found to be statistically significant, the male-biased were (Table 3). We also found that 46 (10.6%) female-biased sDMRs and 721 (21.1%) male-biased sDMRs had AR ChIP enriched regions within 10kb, both of which were found to be statistically significant using 500 permutations (Table 3). These results show that direct binding of ESR1 and BCL6 in females, and AR and BCL6 in males, is a likely mechanism through which they regulate sex-biased DNA methylation.

We then used annotatR to see if there was a tendency for sDMRs with TF-enrichment nearby to be in either intronic, exonic, or intergenic regions. We found that both when comparing sDMRs with direct overlaps with transcription factor binding sites (Table 4), and when comparing sDMRs with TF-enrichment within 10kb (Table 5), the majority of the sDMRs were located in intronic regions. However, given that exons are much shorter than introns, in the female liver there is enrichement of the exons on a per base-pair basis.

Table 4: Annotation of sDMRs with ChIP enriched regions directly overlapping shows that the majority of these sDMRs are located in introns.

Sex-phenotype dependent sDMRs	Female liver						Male liver					
	Co-localized with BCL6-ChIP enriched regions*			Co-localized with ESR1-ChIP enriched regions **			Co-localized with BCL6-ChIP enriched regions *			Co-localized with AR-ChIP enriched regions ***		
	Intron (%)	Exon (%)	Intergenic (%)	Intron (%)	Exon (%)	Intergenic (%)	Intron (%)	Exon (%)	Intergenic (%)	Intron (%)	Exon (%)	Intergenic (%)
Female-biased sDMRs	9 (8.7)	5 (4.9)	0 (0.0)	19 (6.4)	6 (2.0)	6 (2.0)	2 (0.4)	0 (0.0)	1 (0.2)	1 (1.0)	0 (0.0)	0 (0.0)
Male-biased sDMRs	64 (62.1)	36 (35.0)	13 (12.6)	170 (57.6)	61 (20.7)	52 (17.6)	274 (57.4)	86 (18.0)	99 (20.8)	60 (61.9)	33 (34.0)	15 (15.5)
Total sDMRs	64 (62.1)	36 (35.0)	13 (12.6)	170 (57.6)	61 (20.7)	52 (17.6)	274 (57.4)	86 (18.0)	99 (20.8)	60 (61.9)	33 (34.0)	15 (15.5)

Percentage represents the percent of the total sDMRs located within each region that are overlapping with the TF in question

* BCL6-ChIP peaks in female and male livers extracted from GSE31578- GSM784032, GSM784028 replicates three and two, respectively

** ESR1-ChIP peaks in female liver extracted from GSE32244

*** AR-ChIP peaks in male liver extracted from GSE32244

Table 5: Annotation of sDMRs with ChIP enriched regions within 10kb shows that the majority of these sDMRs are located in introns.

Sex-phenotype dependent sDMRs	Female liver						Male liver					
	Co-localized with BCL6-ChIP enriched regions *			Co-localized with ESR1-ChIP enriched regions **			Co-localized with BCL6-ChIP enriched regions *			Co-localized with AR-ChIP enriched regions ***		
	Intron (%)	Exon (%)	Intergenic (%)	Intron (%)	Exon (%)	Intergenic (%)	Intron (%)	Exon (%)	Intergenic (%)	Intron (%)	Exon (%)	Intergenic (%)
Female-biased sDMRs	94 (8.8)	40 (3.8)	26 (2.4)	105 (6.6)	36 (2.2)	36 (2.2)	63 (3.8)	19 (1.1)	26 (1.6)	31 (4.0)	9 (1.2)	8 (1.0)
Male-biased sDMRs	656 (61.6)	201 (18.9)	169 (15.9)	1003 (62.6)	268 (16.7)	285 (17.8)	1036 (61.9)	248 (14.8)	382 (22.8)	498 (64.9)	151 (19.7)	133 (17.3)
Total sDMRs	750 (70.4)	241 (22.6)	195 (18.3)	1107 (69.1)	304 (19.0)	321 (20.0)	1099 (65.7)	267 (16.0)	408 (24.4)	529 (69.0)	160 (20.9)	141 (18.4)

Percentage represents the percent of the total sDMRs located within each region that are overlapping with the TF in question

* BCL6-ChIP peaks in female and male livers extracted from GSE31578- GSM784032, GSM784028 replicates three and two, respectively

** ESR1-ChIP peaks in female liver extracted from GSE32244

*** AR-ChIP peaks in male liver extracted from GSE32244

3.3 Few DEGs were found in the Mouse Brain

While in our previous study we addressed the impact of sex phenotype and sex-chromosome complement on sex-biased gene expression in the mouse liver, other tissues, including the brain, have also shown sexual dimorphism in gene expression (Yang, 2006) (reviewed in (Arnold, 2020; Pallayova et al., 2019)). The brain in particular is an especially important organ to study, as it may regulate sex-bias in other tissues (reviewed in (Brie et al., 2019)). To test what drives sex-biased gene expression in the mouse brain, we used B6.Y^{TIR} mice which have a Y chromosome derived from the wild mice caught in Tirano, Italy (Nagamine et al., 1987). The Y^{TIR} chromosome on the C57BL/6J background results in insufficient upregulation of SRY-box transcription factor 9 (*Sox9)* by *Sry*, which is necessary for testicular differentiation (Park et al., 2011). This results in approximately 40% of the XY progeny developing ovaries and a female phenotype (XY.F) (Coward et al., 1994). Forty percent of the XY progeny develop bilateral testis and a male phenotype (XY.M), and the remaining 20% are true hermaphrodites with a single ovary and single testis (Figure 3a) (Park et al., 2011). The hermaphrodites were excluded from this study.

We used RNA-seq data from XX.F, XY.M, and XY.F mice to differentiate the effects of sex phenotype and sex-chromosome complement on sex bias in gene expression in the mouse brain.

Using the RNA-seq results, we performed PCA on the expression levels of the top 500 DEGs across all genes, autosomes, and the X chromosome separately. In plotting the top genes two main clusters formed; XX females formed one cluster, and the XY males and XY females formed the second (Figure 7a). However, when plotting only autosomal genes, the clustering was lost (Figure 7b). The PCA for only X-linked genes showed 81.9% variance between the same

clusters (Figure 7c). These PCAs shows that sex-chromosome complement is the major driver of sex-biased gene expression in the mouse brain. However, when *Xist* was excluded and all remaining X-linked genes were plotted in a PCA, the clustering was lost once again (Figure 7d), suggesting that *Xist* is a major driver of the clustering.

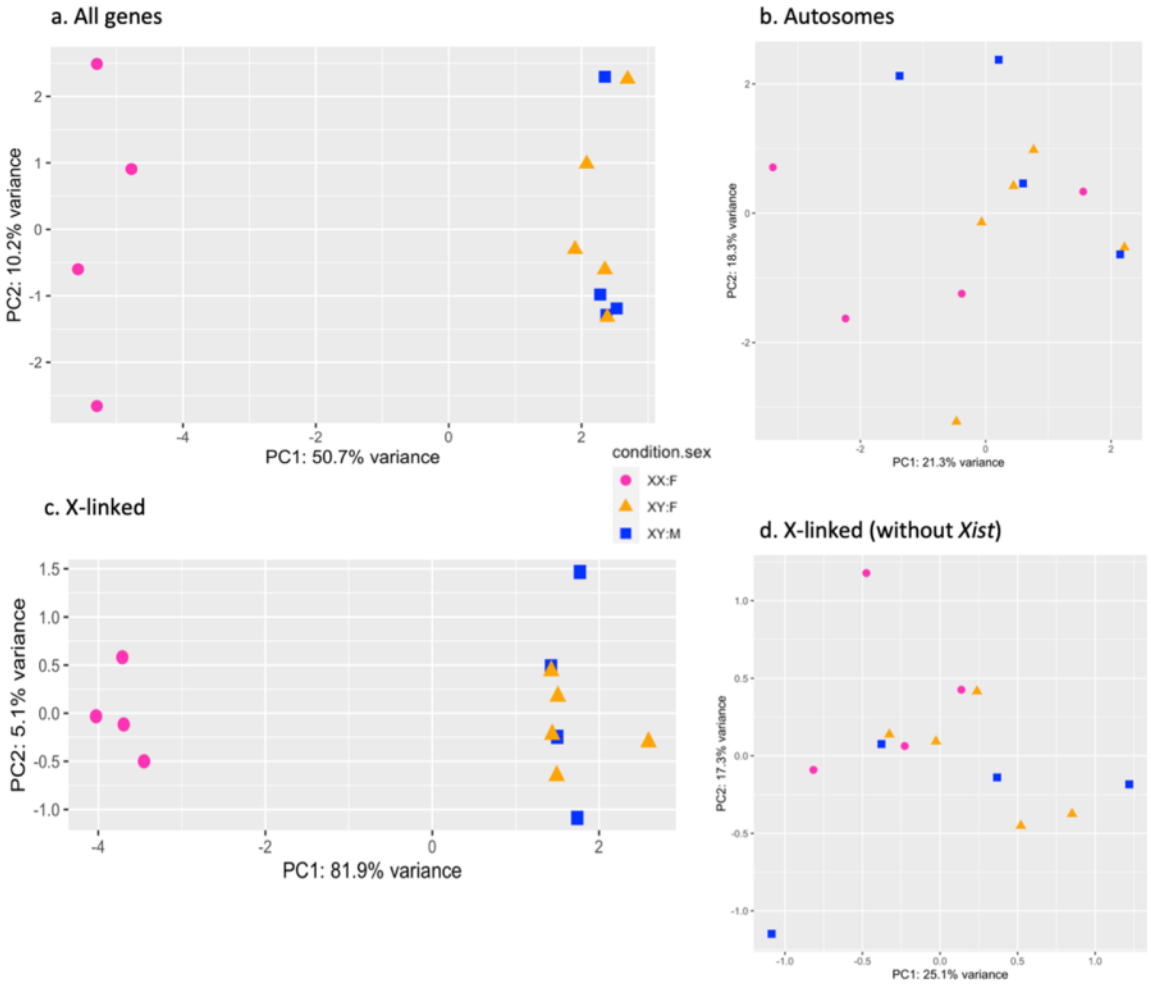

Figure 7. Sex-chromosome linked genes are the major driver of clustering in the mouse brain

a. *PCA for all genes shows that samples cluster by sex-chromosome complement, with the XY.F and XY.M clustering together, and the XX.F clustering alone.* ***b.*** *PCA for only autosomes shows*

that the clustering is lost. ***c.*** *PCA for only x-linked genes shows a strong clustering by sex-chromosomes again.* ***d.*** *PCA for X-linked genes excluding Xist shows that the clustering is again lost.*

To assess the impact of the Y-chromosome, we performed PCA on all genes except those located on the Y-chromosome. This PCA showed 39.7% variance, and the groups still clustered as expected (Figure 8a), however, the percent variance is slightly reduced compared to when examining all genes, suggesting that Y-linked genes are important drivers of sex-biased gene expression in the mouse brain. Furthermore, when plotting a PCA that excludes *Xist* as well genes on the Y chromosome, all of the clustering is lost (Figure 8b). This further shows that *Xist* and Y-linked genes are responsible for the sample clustering by sex-chromosome complement in the mouse brain.

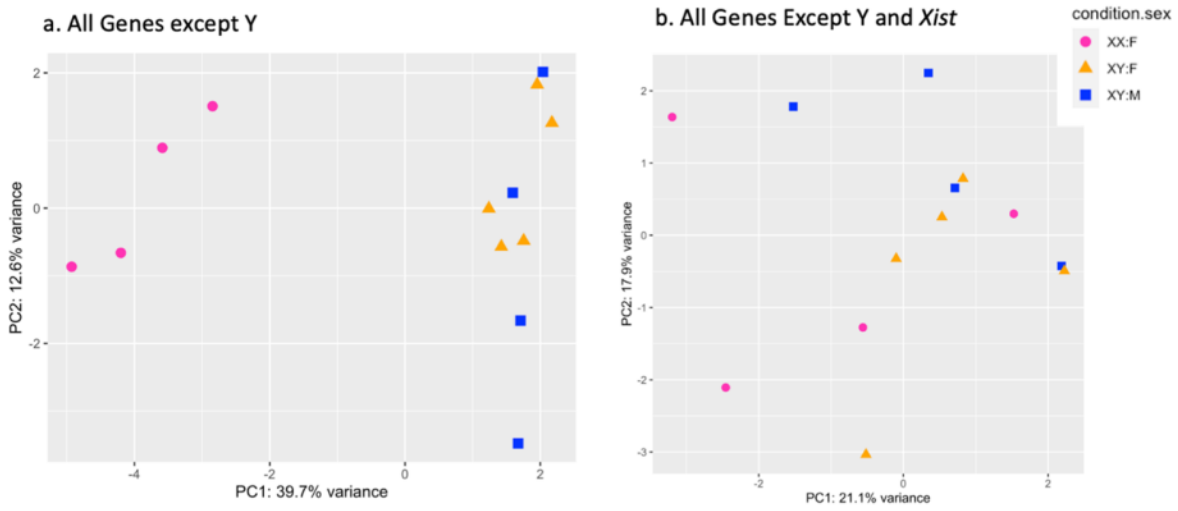

Figure 8: Y-linked genes contribute to sex-biased gene expression in the mouse brain.

a. *PCA of all genes excluding Y-linked genes maintains distinct clustering by sex-chromosome complement, but with reduced variance in PC1 compared to all genes.* ***b.*** *When both Y-linked genes and Xist are excluded, clustering is lost.*

We then looked at the DEGs found in three different comparisons; XX.F vs XY.M, XY.F vs XY.M, and XX.F vs XY.F. Our expectation was that DEGs found in the XX.F vs XY.M comparison would be those dependent on both sex phenotype and sex-chromosome complement, the XY.F vs XY.M comparison would be those dependent on sex phenotype, and the XX.F vs XY.F comparison would be those dependent on sex-chromosome complement. Using cut-offs of an absolute log2FC ≥1.5, and a DESeq2 padj < 0.05, we found very few DEGs (Figure 9). All the sex-chromosome complement dependent DEGs were located on sex-chromosomes (10 on the Y-chromosome, and *Xist*) (

Table 6).

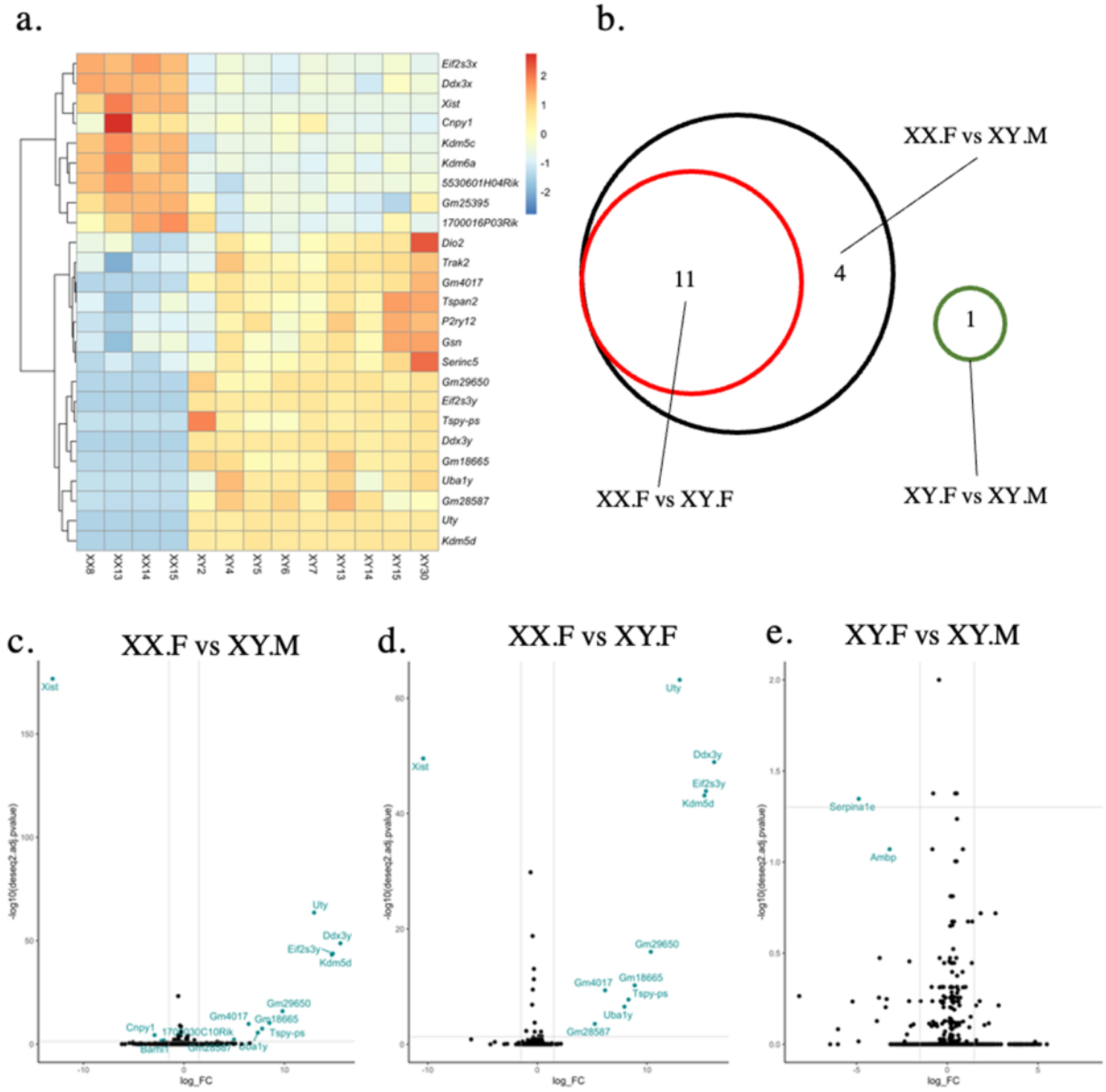

Figure 9: DEG analysis in the mouse brain shows that the majority of DEGs were found to be associated with sex-chromosome complement.

a. *Heatmap of top 25 most significant differentially expressed genes in the mouse brain.* ***b.*** *Venn diagram showing the overlaps between sex-phenotype dependant DEGs (XY.F vs XY.M), those dependant on both sex phenotype and sex-chromosome complement (XX.F vs XY.M), and those dependant on sex-chromosome complement (XX.F vs XY.F).* ***c-e****. Volcano plots showing most*

*significant DEGs in the XX.F vs XY.M (**c**), XX.F. vs XY.F (**d**), and XY.F vs XY.M (**e**) comparisons, respectively. Significant DEGs (those with an absolute log2FC ≥ 1.5, and an adjusted p-value < 0.05 are shown in green.*

Table 6: Number of DEGs in brain and liver in different comparisons.

Comparison	Brain			Liver			Brain & Liver Overlaps		
	Autosomes	X	Y	Autosomes	X	Y	Autosomes	X	Y
XX.F vs XY.M (sex-chromosome complement and sex phenotype)	3	1	10	254	5	9	0	1	8
XX.F vs XY.F (sex-chromosome complement)	0	1	10	4	1	8	0	1	7
XY.F vs XY.M (sex phenotype)	1	0	0	188	3	0	1	0	0

The number is the total, including those present on both autosomes and sex-chromosomes. Liver data extracted from Zhuang et al., 2020

Overall, in the brain very few sex-biased DEGs were observed and among these the majority were located on the Y chromosome, which is not due to sex-bias in transcriptional regulation but to different karyotypes. These results contrast with our findings in the liver from the previous study (Zhuang et al., 2020), which found that the majority of DEGs found in the liver were dependant on sex-phenotype (

Table 6). There were also many more DEGs found in the liver compared to the brain, however, of the Y-linked ones that were found in the brain, eight of the 10 were also found to be differentially expressed in the liver (

Table 6).

The Y-linked genes are listed in Table 7. Interestingly, the majority of Y-linked DEGs were detected in both the liver and the brain, with the exceptions of predicted gene 28587 (*Gm28587)* and ubiquitin-like modifier-activating enzyme 1 Y (*Uba1y)* which were brain specific, and predicted gene 21064 (*Gm21064)*, which was detected only in the liver. Furthermore, with the exception of *Gm21064*, all of the remaining Y-linked genes were located within a 500 kb region of the short arm of the Y-chromosome (Figure 10).

Table 7: Y-linked genes expressed in brain or liver and their functions

Gene Name	Organ	Functions
Ddx3y	Brain & Liver	ATP binding, hydrolysis, RNA binding, formation of intramolecular functions*
Eif2s3y	Brain & Liver	Protein synthesis**, spermatogenesis***
Gm18665	Brain & Liver	Predicted gene
Gm28587	Brain	Predicted gene
Gm29650	Brain & Liver	Predicted gene
Gm4017	Brain & Liver	Predicted gene
Kdm5d	Brain & Liver	Lysine demethylation (H3K4)
Tspy-ps	Brain & Liver	Pseudogene[⊥]
Uba1y	Brain	Activates ubiquitin [+]
Uty	Brain & Liver	Protein-protein interactions, lysine demethylation (H3K27me3)[#]
Gm21064	Liver	Predicted gene

*Retrieved from https://www-ncbi-nlm-nih-gov.proxy3.library.mcgill.ca/gene/8653

** https://www.uniprot.org/uniprot/Q9Z0N2

***** (Mazeyrat et al., 2001) (Matsubara et al., 2015) (Yamauchi et al.){Matsubara, 2015 #285}**

****** http://www.informatics.jax.org/marker/MGI:99780**

[⊥] http://www.informatics.jax.org/marker/MGI:1201688

[+] https://www.uniprot.org/uniprot/P31254

[#] **(Walport et al., 2014)**

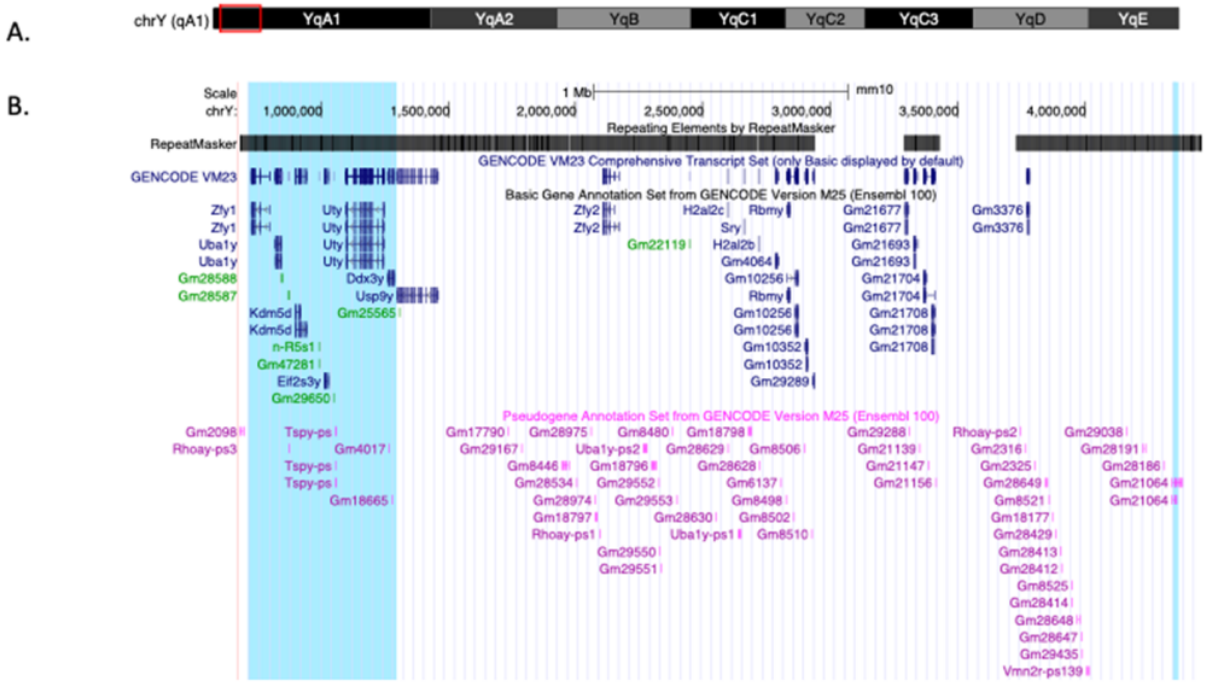

Figure 10: Nearly all Y-linked genes that were expressed in the mouse brain and liver were found within a 500kb region of the Y chromosome

A. region of chromosome represented in figure

B. Highlighted regions show areas where Y-linked genes are located. On the left, the large highlight represents the 500kb region containing the majority of Y-linked genes, and the smaller

highlight on the right shows the location of Gm21064, which was the only Y-linked gene in the brain or liver not found in this region.

While the majority of genes detected in the brain were located on the Y chromosome, there were also three autosomal DEGs found in the XX.F vs XY.M comparison, and one in the XY.F vs XY.M. The three found in the XX.F vs XY.M comparison were RIKEN cDNA 1700030C10 gene (*1700030C10Rik)*, BarH-like Homeobox 1 (*Barhl1)*, and Canopy FGF signaling regulator 1 (*Cnpy1*). Their genomic locations and functions are detailed in Table 8. The single significant DEGs found in the XY.F vs XY.M comparison, representing those dependant on sex-phenotype, was serine (or cysteine) peptidase inhibitor, clade E, member 1 (*Serpina1e*), which is located on chromosome 12. However, *Serpina1e* had low expression, with a logCPM of -1.3.

Table 8: Autosomal DEGs in Brain

Gene Name	Chromosome	ensembl gene id	log_FC	log_CPM	padj	Protein Function
1700030C10Rik	12	ENSMUSG00000099759	-1.993	-0.612	1.50E-02	Uncharacterized protein*
Barhl1	2	ENSMUSG00000026805	-2.265	-1.83	2.90E-02	Transcriptional activation**
Cnpy1	5	ENSMUSG00000044681	-2.928	-1.036	4.20E-05	FGF signalling regulator***

*https://www.uniprot.org/uniprot/Q8BYB8

**https://www.uniprot.org/uniprot/P63157

***http://www.informatics.jax.org/marker/MGI:2442451

3.4 The impact of sex phenotype or sex-chromosome complement on the expression of X-linked genes in the mouse liver and brain

For our final objective, we examined the expression of X-linked DEGs in the mouse brain and liver. We extracted all DEGs located on the X-chromosome from the XX.F vs XY.M, XX.F vs XY.F, XY.M vs XY.F lists of DEGs for both the brain and liver, as well as the liver XX^{Paf}.F vs XO.F. The XX.F vs XY.M comparison shows DEGs dependant on both sex-chromosome complement and sex phenotype, XY.F vs XY.M shows DEGs dependent on sex phenotype, XX.F vs XY.F shows those dependent on sex-chromosome complement. In the liver, we were able to differentiate the effects of X dosage and Y-chromosome presence, with the XX^{Paf}.F vs XO.F showing those dependant on X dosage, and the DEGs present in the XX.F vs XY.F comparison but not in the XX^{Paf}.F vs XO.F being those dependent on the presence of the Y chromosome. We also looked at X-linked DEGs in the ESR1KO comparisons to isolate the impact of loss of ESR1 on X-linked gene expression in the mouse liver. The lists were then filtered to look at only DEGs with a DESeq2 padj less than 0.1 and no cut-off for log2FC. This more relaxed cut-off was used because genes that escape X-inactivation do not necessarily show double the expression in XX mice (reviewed in (Balaton & Brown, 2016)).

In the brain, no X-linked DEGs that depended on the sex phenotype were found, whereas the few that were found depended on the sex-chromosome complement. In the liver, the majority of DEGs were dependant on the sex phenotype (Table 10). In the brain, all of the DEGs found, with the exception of glycoprotein m6b (*Gpm6b)*, had higher expression in females with two X chromosomes compared to males, which may be due to escape from X-inactivation. All of the DEGs found in the brain have previously been shown to escape X-inactivation (Berletch et al., 2015; Keown et al., 2017; Lopes et al., 2011).

In the liver, there were four female-biased X-linked DEGs (adhesion G protein-coupled receptor G2 (*Adgrg2*), rho GTPase-activating protein 6 (*Arhgap6*), predicted gene 37956 *(Gm37956),* and retinoic acid induced 2 (*Rai2)*) and one male-biased DEG (MAGE family member E1 (*Magee1*)) that were found only in the sex-phenotype comparison (Table 10). Interestingly, *Adgrg2* has been shown to escape X-inactivation (Andergassen et al., 2017). A total of nine DEGs found across all liver comparisons have been shown in literature to escape X-inactivation (Barros de Andrade et al., 2019; Berletch et al., 2015; Peeters et al., 2014; Reinius et al., 2012; Wang et al., 2016; Wang et al., 2010), and an additional eight have been predicted to escape based on the surrounding genomic features (Barros de Andrade et al., 2019). We also found seven X-linked DEGs with higher expression in males, two of which are known not to escape X-inactivation (*Gria3* and *Magee1*) (Yang et al., 2010) (reviewed in (Li & Carrel, 2008)). There were also two DEGs found in the XX.F vs XO.F comparison, (*Xist* and holocytochrome C synthase *(Hccs)*), both of which are also known to escape X-inactivation (Berletch et al., 2015).

Table 9: X-linked DEGs in the brain and liver across different comparisons.

DEG	XX.F vs XYM				XY.F vs XY.M				XX.F vs XY.F				XX.F vs XO.F	
	Brain		Liver		Brain		Liver		Brain		Liver		Liver	
	LogFC	LogCPM	LogFC	LogCPM	LogFC	LogCPM	LogFC	LogCPM	LogFC	LogCPM	LogFC	LogCPM	LogFC	LogCPM
5530601H04Rik	-0.333	4.175	-0.589	2.828					-0.415	4.12				
Adgrg2							0.776	0.803						
Alas2			2.628	6.012			-2.506	6.019						
Ar			-1.623	3.466			1.561	3.41						
Arhgap6							0.521	3.87						
Cdx4			-3.697	-1.149			3.317	-1.474						
Cfp			-0.938	4.854										
Chic1			-0.953	4.505										
Ddx3x	-0.288	8.413							-0.314	8.391				
Eif2s3x	-0.575	5.865	-0.544	5.115					-0.615	5.821	-0.563	5.123		
Ftx			-0.702	3.244										
Gm26652			-1.119	-0.7			1.596	-0.367						
Gm37956							0.847	3.241						
Gpm6b	0.215	9.746												
Gria3			1.134	2.512			-1.335	2.442						
Hccs													-0.808	4.174
Hprt			0.479	4.878			-0.443	4.882						
Igsf1			2.011	-2.816										
Ikbkg			0.546	6.525			-0.427	6.564						
Jpx									-0.433	2.423				
Kdm5c	-0.305	6.425							-0.32	6.407				
Kdm6a	-0.392	5.834	-0.632	5.615					-0.436	5.798	-0.699	5.605		
Klhl13			-0.795	3.525			0.742	3.482						
Magee1							-0.59	1.79						
Maob			-0.597	7.569			0.574	7.545						
Mid1									-1.008	2.788				
P2ry4			-0.558	5.522										
Pbdc1									-0.228	4.365				
Rai2							0.739	1.396						
Serpina7			1.273	3.994										
Slc6a8			0.766	2.729			-0.686	2.748						
Sytl5			-1.343	2.448										
Vsig4			-0.733	5.556										
Xist	-13.069	8.178	-9.461	10.372					-10.416	8.015	-12.851	10.386	-12.737	10.52
Zbtb33			0.467	5.093										
Zmat1			-0.746	2.516										
Total	7		24		0		15		9		3		2	
Total Overlaping	4				0				3				NA	

Pink indicates genes with higher expression in XX.F, XY.F, XX.F, and XX.F in each comparison (left to right).

We also tested if the loss of ESR1 also resulted in the loss of sex-bias in gene expression of X-linked genes in the mouse liver. We found a total of 68 X-linked sex-biased DEGs in the control mice, and 22 in the knockout mice. There were 10 that overlapped between the two comparisons (Table 10). There were 36 female-biased DEGs in the FWT vs MWT comparison, which was reduced to 17 female-biased DEGs in the FKO vs MKO comparison. Nine female-biased DEGs were present in both the FWT vs MWT and FKO vs MKO comparison, meaning that 27 female-biased genes lost sex-biased differential gene expression with the loss of ESR1. There were also 32 male-biased DEGs present in the control comparison, and five in the knockout. However, there were no overlaps between the male-biased genes in control comparison with those in the knockout, so all 32 male-biased X-linked DEGs lost sex-biased differential gene expression. In total, there were ten X-linked DEGs that maintained sex-biased gene expression with the loss of ESR1, however, one of those genes switched in its bias; in the FWT vs MWT comparison it was female-biased, whereas in the FKO vs MKO comparison it was male-biased.

Table 10: Loss of ESR1 results in reduced sex-biased gene expression in both autosomal and X-linked genes

	FWT vs MWT		FKO vs MKO		Overlap	
	Autosomes	**X**	**Autosomes**	**X**	**Autosomes**	**X**
FDEGs	153	36	23	17	11	9
MDEGs	152	32	4	5	3	0
Total	305	68	27	22	114	10*

For autosomes, a cut-off of absolute log2FC >= 1.5 and an adjusted p-value < 0.05 were used. For X-linked DEGs, a cut-off on an adjusted p-value < 0.1 was used. FDEGs indicates female-biased DEGs, MDEGs is male-biased DEGs. FWT vs MWT is the comparison between female and male WT, where FKO vs MKO is the comparison between the female and male ESR1KO. Overlap indicated the number of DEGs present in both the WT and ESR1KO comparisons.

Since in testing the effect of the loss of ESR1, we are looking at the impact of sex-phenotype, we hypothesized that the DEGs that lose sex-biased expression with the loss of ESR1 are the same as those that we determined to be sex-phenotype dependant above. Of the 58 X-linked DEGs that lost sex-biased expression in the ESR1 KOs, and the 15 present in the XY.M vs XY.F comparison, there were only seven that were present in both. This suggests that loss of ESR1 results in the loss of sex-biased gene expression of sex-phenotype dependent X-linked genes in the mouse liver, however, is likely not the only factor, and we must consider that certain differences could be due to different genetic backgrounds of the mice (C57BL/6J vs C57BL/6N) and different RNA-seq experiments.

Chapter 4: Discussion

In this study, we address the question of how sex phenotype or sex-chromosome complement impact sex-biased gene expression in the mouse brain and liver. We show that estradiol action mediated through ESR1 is an important regulator of sex-biased gene expression in mouse liver, and that the direct binding of ESR1, as well as AR and BCL6, is a potential mechanism through which sex-bias in DNA methylation in mouse liver may be regulated. We also show that there are relatively few DEGs in the brain compared to the liver, and that these are often Y-linked.

4.1 Loss of ESR1 causes loss of sex-biased gene expression in mouse liver

As we have previously shown, sex-phenotype is the major driver of sex-biased gene expression in the mouse liver (Zhuang et al., 2020). To follow up on this, here we tested how the estradiol signalling through ESR1 impacted sex-biased gene expression. Expression analysis for the livers of ESR1 knockout mice shows that loss of ESR1 results in the majority of DEGs losing sex bias in expression (Figure 7, Table 11). These results clearly show that ESR1 is an important factor for sex-biased gene expression in mouse liver, however, we found that loss of ESR1 does not result in completely male-like or female-like gene expression profiles. It is also important to consider that the effects seen in this study may not be all due to direct effect in the liver, as we are working with a whole-body knockout. Some of the loss of sex-biased gene expression may be due to downstream effects in other tissues, such as the hypothalamus or pituitary (Avtanski et al., 2014) (reviewed in (Brie et al., 2019)). For example, ESR1 has been shown to stimulate pituitary GH expression, which can have downstream impacts on the STAT5 pathway, which in

turn has previously been shown to be an important regulator of sex-biased gene expression (Avtanski et al., 2014; Clodfelter et al., 2006).

4.2 Co-localization between sDMRs and transcription factor binding regions

Our previous study of sex bias in gene expression and DNA methylation in mouse liver has demonstrated an association between sex-biased expression and sex-biased DNA methylation (Zhuang et al., 2020). More specifically, it has been shown that sex-biased DEGs and sDMRs tend to localize closer than one would expect if no association between the two groups existed. Therefore, it was reasonable to hypothesize that TFs that were implicated in regulation of sex-biased expression were also directly bound to the sDMRs. Thus, we tested the direct binding of TFs in the immediate proximity of sDMRs. To do this, we compared ChIP-seq data for ESR1 and BCL6 in females, and AR and BCL6 in males, to a list of sex-phenotype dependant sDMRs.

For ESR1, we found a significant colocalization between ESR1 enrichment and both male- and female-biased sDMRs, in the female liver. This suggests that the direct binding of ESR1 could be associated with both hyper and hypo-methylation in female liver and thereby regulation of sex-biased expression. Previous studies have shown that both estradiol and ESR1 can act as regulators of DNA methylation (Ariazi et al., 2017; Marques et al., 2013; Stone et al., 2012), which supports what is seen here. One potential way through which ESR1 may impact DNA methylation is through interactions with DNMTs. In breast cancer, it has been shown that ESR1 present in the promotor region of DNMT1 inhibits its expression (Jin et al., 2019). ESR1 action has also been linked to DNMT3B, suggesting that it may be important in *de novo* methylation (Zhao et al., 2012).

For BCL6, we found significant enrichment in the immediate proximity of male-biased and female-biased sDMRs in the female liver, but in the male liver, BCL6 binding was significantly enriched only with male-biased sDMRs when looking at direct overlaps. BCL6 is best known as a transcriptional repressor of female-biased genes, with higher expression in males (Chikada et al., 2018; Meyer et al., 2009; Zhang et al., 2012). Because of this, we expected to find enriched binding of BCl6 near female-biased sDMRs in the male liver. However, we found significant enrichment of BCL6 binding sites near male-biased sDMRs in the male liver, and enrichment of both male- and female-biased sDMRs in the female liver. This suggests the possibility that BCL6 may also play a role in methylation, and through this, gene expression. This idea is supported by pyrosequencing assays done in a liver-specific BCL6 knockout mouse model by our lab, which found that loss of BCL6 prevented the demethylation of five of the six tested sDMRs (Alogayil et al., 2021).

We also examined AR binding in the male liver. We found significant enrichment of AR near male-biased sDMRs, but when looking at direct overlaps between AR and sDMRs, there was no significant overlap with female-biased sDMRs. AR is best known as a transcriptional activator (Brinkmann et al., 1999), and the results we found support this, as they show that direct binding of AR in male mouse liver is associated with lower levels of DNA methylation.
We also found that the majority of sDMRs with ChIP binding sites located nearby, were located in introns. These results are consistent with our previous study, where we found in general, the majority of sDMRs were located in introns (Zhuang et al., 2020).

4.3 Whole brain transcriptome analysis shows very few sex-biased genes

In the whole brain, we found a very small number of sex-biased genes overall, a finding that suggests that there are not many genes that are regulated by sex phenotype or sex-chromosome complement and common between different brain regions. Previous studies have focused on the impact of sex-steroid hormones on the brain, which would suggest that sex phenotype would be a major driver of sex-biased gene expression in the mouse brain. For example, in humans, shorter CAG repeats in the encoding polyglutamine tracts in the AR genes have been associated with more effective functioning of the AR gene, and a more masculine adolescent development of the cortex (Raznahan et al., 2010). Furthermore, both prenatal and perinatal testosterone has been shown to impact behaviour in rodents and humans (reviewed in (Berenbaum, 2018)). On the other hand, sex-chromosome complement has also been suggested to be important in studies of humans with different combinations of sex chromosomes, and found that increasing numbers of X chromosomes was associated with a decrease in brain volume, cortical volume, and cortical surface area, suggesting that they are also important (Raznahan et al., 2016). However, in our study we found that the majority of genes that showed sex biased expression (ten out of 15) were Y-linked genes. It is possible that this is because sex-steroid hormones have been shown to act on particular parts of the brain (reviewed in (Marrocco & McEwen, 2016)), so perhaps because we used the whole brain, some sex-biased expression was not detected. Indeed, in other studies that have examined sex-biased gene expression in the mouse brain, few DEGs were also detected. Using a cut-off of a log fold change > 1.2, one study found only 34 significant DEGs in the brain, of which the X and Y chromosomes contained the majority of genes (Yang, 2006). However, unlike our study, they only used XX females and XY

males, which does not allow them to differentiate the impacts of sex phenotype from sex-chromosome complement.

If our hypotheses that the use of the whole brain obscures differential expression of some genes is true, one would assume that the genes that are detected are those more highly expressed in the largest parts of the brain. For the mice, this would be the cortex. However, this was not quite the case; for *1700030C10Rik,* it has a relatively low RPKM in the adult cortex, and highest expression in the testis and the adrenal glands (Yue et al., 2014). However, for *Barhl1* and *Cnpy1,* the highest expression is seen in the cerebellum (Yue et al., 2014), which is also a large part of the brain, which does somewhat support this hypothesis.

4.4 Both X-dosage and sex phenotype influence expression of X-linked genes

Transcriptional regulation of X-linked genes depends on general regulatory mechanisms shared with the rest of the genome as well as the X-inactivation status. Certain X-linked genes escape X inactivation in females, resulting in higher expression in females compared to males. Both mechanisms may impact X-linked genes differently in different tissues. For this reason, we wished to examine expression levels of X-linked genes in the mouse brain and liver to understand how X-dosage and sex-phenotype contribute to sex bias in the transcriptional regulation of X-linked genes. In the brain, we found seven X-linked DEGs dependant on both sex-chromosome complement and sex phenotype, and nine X-linked DEGs that were only dependant on the sex-chromosome complement (Table 10), suggesting that some of these DEGs may have higher expression in females due to escape from X-inactivation. Indeed, those with higher expression in XX.F were also known to escape X-inactivation (Berletch et al., 2015; Keown et al., 2017; Lopes et al., 2011). Interestingly, *Gpm6b,* was the only gene with male-

biased expression in the XX.F vs XY.M brain comparison (Table 10), but it has previously been shown to escape X-inactivation (Berletch et al., 2015).

In the liver, sex bias in the expression of X-linked genes was found to be mainly sex-phenotype dependant, similar to the finding for autosomal genes (Table 10; (Zhuang et al., 2020)) . Most of the DEGs that were found in the XX.F vs XY.M liver comparison have also been previously shown to escape X-inactivation (Barros de Andrade et al., 2019; Berletch et al., 2015; Peeters et al., 2014), however, some have had limited research done. Complement factor properdin *(Cfp)* and V-set and immunoglobulin domain containing 8 (*Vsig8)* have been shown to escape X-inactivation in the spleen in previous studies, but not other tissues (Berletch et al., 2015), so this study suggests that they may also escape in the liver. We found eight DEGs (*Arhgap6,* cysteine rich hydrophobic domain 1 *(Chic1),* predicted gene 26652 (*Gm26652), Hccs*, inhibitor of nuclear factor kappa B kinase regulatory subunit gamma *(Ikbkg)*, kelch like family member 13 *(Klhl13),* solute carrier family 6 member 8 *(Slc6a8),* and zinc finger matrin-Type 1 (*Zmat1*)) which have only been predicted to escape X-inactivation by computer models, but this has not been confirmed experimentally (Barros de Andrade et al., 2019). Interestingly, there was one gene (*Adgrg2*) that was only significantly differentially expressed in the XY.F vs XY.M comparison, suggesting that its expression is sex-phenotype dependant, however, in previous studies, it has been shown to escape X-inactivation (Andergassen et al., 2017).

There were also 11 X-linked DEGs which only had sex-biased expression in the XX.F vs XY.M comparison. This suggests the possibility that for these genes, neither sex phenotype nor sex-chromosome complement on its own is sufficient to establish sex-bias in gene expression, but rather, both are necessary.

There were also very few X-linked genes found in common between the mouse liver and brain. In the XX.F vs XY.M comparison, only four genes had sex biased gene expression in both tissues. This suggests that escape from X-inactivation may be tissue specific.

We also examined the impact of the loss of ESR1 on sex-biased gene expression of X-linked genes in the mouse liver. We found that with the loss of ESR1, ten X-linked genes maintained sex-biased gene expression, all of which showed female-biased expression in the FWT vs MWT comparison (Table 11). However, loss of ESR1 resulted in loss of sex-biased gene expression in 26 female-biased genes, and 32 male biased genes. This result suggests that loss of ESR1 may impact X-escape, but as of now, the interactions between estradiol and ESR1 with X escape and inactivation have not been well characterized.

4.5 Study limitations

There are some important limitations to consider when looking at the results of this study. First, with the sDMR co-localization analysis, we used data from mice of various different ages, as well as different strains. The AR and ESR1 ChIP-seq data were from 20-week old mice, while both the BCL6 ChIP-seq and the WGBS data were from 8-week old mice (Li et al., 2012; Zhang et al., 2012; Zhuang et al., 2020). Furthermore, while the sDMRs are from mice with a C57BL/6J genetic background, the BCL6 ChIP-seq experiments were done on mice with a CD-1 background (Zhang et al., 2012; Zhuang et al., 2020). The differences in strains and ages may impact both DNA methylation and transcription factor binding, and thus our colocalization analysis.

With the ESR1KO mice, we also need to consider the fact that we were using a whole-body ESR1 knockout model. This means that while we were looking at the liver, the results we

saw are not necessarily due to loss of ESR1 expression in the liver. Instead, they may be due to loss of ESR1 in other tissues, such as the pituitary gland, and interactions with the HPG axis, having a downstream impact on gene expression.

Another important caveat to consider is when we extracted RNA from the brain, we used the whole brain. As the brain is a very complex tissue, and it has been shown that sex-bias in gene expression is not the same across all parts of the brain (Pallayova et al., 2019), it is important to consider the fact that we may not be able to detect sex-bias in some genes because the signal is overwhelmed by expression in other parts of the brain.

Chapter 5: Conclusions and Future Directions

5.1 Conclusions

This study demonstrated that direct binding of AR, ESR1, and BCL6 remains a potential mechanism through which they regulate sex-biased DNA methylation, which may in turn impact gene expression. It also showed g g ggggg

. This study found very few DEGs located in the brain, but those that were found were mainly located on the Y chromosome. Sex-chromosome complement was also found to be the main driver of sex-biased gene expression of X-linked genes in the mouse brain. This is in contrast to our data from the liver, where both sex-phenotype and sex-chromosome complement impact X-linked sex-biased gene expression, and many more DEGs were found. Loss of ESR1 was also shown reduce the number of sex-biased X-linked genes, suggesting that it may interact with escape from X-inactivation.

5.2 Future Directions

In terms of next steps, there are a few different directions in which one could go with this study. With regard to the transcription factor co-localization, it would be particularly interesting to assess AR and ESR1 binding in female and male mice respectively, and to see if the binding sites differ when assessing mice of different age groups. In this study, the mice used for the AR and ESR1 ChIP-seq were 20-weeks old, whereas the WGBS results were from 8-week-old mice – it would be interesting to see if this significantly impacts the results. Furthermore, in the liver, it has been shown that AR expression is higher in females than males (Alogayil et al., 2021), however, AR is primarily seen as a transcriptional activator. By performing ChIP-seq in the female mouse liver, it would be possible to see if the binding corresponds with the increase in expression.

It would also be interesting to address how much of the loss of sex bias in gene expression in the ESR1KO mouse liver is due to loss of ESR1 in the liver and how much is due to interaction with other tissues and pathways. To do this, one could perform RNA-seq on the liver of a liver-specific ESR1KO mouse model, and compare those results with the ones generated in this study.

In the brain, one of the large issues we encounter in this study is the heterogeneity of the tissue and the possible impacts this may have on our ability to detect sex-bias in gene expression. While one solution to this would be to do single-cell RNA-seq, it may also be interesting to investigate if there are algorithms or packages that have been developed to computationally factor in the heterogeneity of the brain using our existing RNA-seq data to detect other differentially expressed genes.

Chapter 6: References

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Appendices:

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Appendix 2: Training Certificates

McGill University
Environmental Health and Safety

THIS IS TO CERTIFY THAT

Klara Bauermeister

SUCCESSFULLY COMPLETED CORE TRAINING IN

WHMIS 2015 Online Course & Exam

Hazardous products in Canada. Introduction to the 4 key elements: Classification, Training, Labeling and Safety Data Sheets (SDS)

ON 31-May-21

Valid Until: 31-May-24

EHS Officer & Instructor

Joseph Vincelli
E. H. S. Manager

Training Session ID: TW4392

Trainer Name:

McGill University
Environmental Health and Safety

THIS IS TO CERTIFY THAT

Klara Bauermeister

SUCCESSFULLY COMPLETED CORE TRAINING IN

Safe Use of Biological Safety Cabinet Online Course & Exam

HEPA filtration, cabinet airflow characteristics, types of BSCs, and safe work practices.

ON 18-Aug-21

Valid Until: 18-Aug-24

Biosafety Officer & Instructor

Joseph Vincelli
E. H. S. Manager

Training Session ID: TW4842
Trainer Name:

McGill University
Environmental Health and Safety

THIS IS TO CERTIFY THAT

Klara Bauermeister

SUCCESSFULLY COMPLETED CORE TRAINING IN

Introduction to Biosafety Online Course & Exam

Safe laboratory work practices, risk assessments, sterilization and disinfection principles, spill procedures.

ON 31-May-21

Valid Until: 31-May-24

RBlanchette
Biosafety Officer & Instructor

Joseph Vincelli
Joseph Vincelli
E. H. S. Manager

Training Session ID: TW4388
Trainer Name:

McGill University
Environmental Health and Safety

THIS IS TO CERTIFY THAT

Klara Bauermeister

SUCCESSFULLY COMPLETED CORE TRAINING IN

Hazardous Waste Management for Laboratory Online Course & Exam

Basic requirements necessary for the HWM to dispose of hazardous waste in a safe, efficient and environmentally friendly manner.

ON 12-Oct-20

Valid Until: 12-Oct-23

Christian Bouchard
HWM Manager

Training Session ID: TW213
Trainer Name: