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The sex chromosome complement is an important determinant in obesity and related diseases

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The Sex Chromosome Complement is an Important Determinant in Obesity and Related Diseases

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular Biology

by

Jenny Chen Link

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ABSTRACT OF THE DISSERTATION

The Sex Chromosome Complement
is an Important Determinant
in Obesity and Related Diseases

by

Jenny Chen Link
Doctor of Philosophy in Molecular Biology
University of California, Los Angeles, 2015
Professor Karen Reue, Chair

Obesity is associated with numerous metabolic disorders, including hypertension, hyperlipidemia, insulin resistance, and atherosclerosis. In addition, obesity is a risk factor for cardiovascular disease and cancer, the leading causes of mortality. It is thus critical to understand the environmental and genetic components that contribute to the development of obesity, one of which is sex.

The sexual dimorphism in fat accumulation and distribution has been well established for a few decades. Premenopausal women tend to gain fat near the hips and thighs, while men and postmenopausal women tend to accumulate fat in the abdominal cavity. These sex differences have often been attributed to sex hormones, but sex differences in metabolic traits remain long after gonadal hormones have declined. This suggests that factors other than sex hormones could contribute to sex differences in metabolism.

The sex chromosome complement is the fundamental difference between male and female cells. Because XX chromosomes often coexist with ovaries and XY chromosomes are coupled with testes, the distinct effects of gonadal type and sex chromosomes are difficult to distinguish. A unique mouse model called the Four Core Genotypes (FCG) generates XX female, XX male, XY female, and XY male mice, and is an innovative tool to study effects of the sex chromosome complement (XX male/female *vs.* XY male/female) independently from effects of gonadal hormones (XX/XY male *vs.* XX/XY female).

We used FCG mice to study sex differences in obesity and related metabolic disorders. We compared gonadally intact mice with gonadectomized mice to assess acute effects of gonadal hormones, and we nutritionally challenged mice with a high fat diet and a high cholesterol diet to identify diet-sex interactions. Male and female mice with two X chromosomes gain more weight, have increased adiposity, and accumulate more hepatic lipid compared to XY mice. In addition, XX mice have increased HDL cholesterol levels, regardless of the dietary or hormone milieu. Sex chromosome differences were also detected at the molecular level, modulating mRNA and microRNA expression.

Our studies demonstrate that the sex chromosome complement is a major determinant of body weight, adiposity, and associated metabolic traits such as hepatic lipid and plasma lipid levels. The key findings from this dissertation will shed light on sex differences in obesity and are important for improving prevention and treatment of metabolic disease.

The dissertation of Jenny Chen Link is approved.

Kathrin Plath

Stephen G. Young

Peter Tontonoz

Arthur P. Arnold

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2015

For Mom and Dad

and Natee

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Chapter 2 is a reprint of "Metabolic Impact of Sex Chromosomes" from *Adipocyte* 2013 Apr 1; 2(2):74-9, used with permission. Xuqi Chen, Arthur P. Arnold, and Karen Reue are co-authors.

Chapter 3 is a reprint of "The number of X chromosomes causes sex differences in adiposity in mice" from *PLoS Genet*. 2012 May; 8(5): e1002709 under the Creative Commons Attribution (CC BY) license. Xuqi Chen, Rebecca McClusky, Simon W. Beaven, Peter Tontonoz, Arthur P. Arnold, and Karen Reue are co-authors.

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PRESENTATIONS

1. Genetic sex differences in obesity. Invited talk for UCLA/Santa Monica College Science and Research Initiative. Santa Monica, CA. May 2014

2. Link JC, Ronquillo E, Chen X, Arnold AP, Reue K. The sex chromosome complement causes sex differences in diet-induced obesity. Poster abstract for the Organization for the Study of Sex Differences meeting. Minneapolis, MN. Apr 2014

3. Gonadal hormones and the sex chromosome complement mediate diet-induced obesity. Oral presentation for UCLA Lusis Program Project Grant Retreat. Lake Arrowhead, CA. Oct 2013

4. Link JC, Chen X, Prien C, Arnold AP, Reue K. The sex chromosome complement is a determinant of plasma lipid levels. Poster abstract for Tri-Campus NIGMS Training Program Symposium. Los Angeles, CA. Apr 2013

5. Chen J, Chen X, McClusky R, Prien C, Wang X, Lusis AJ, Arnold AP, Reue K. Sex chromosome complement and male/female sex have independent effects on lipid metabolism and atherosclerosis. Poster abstract for Kern Conference. Vail, CO. Jul 2012

6. Chen J, Chen X, Arnold AP, Reue K. The number of X chromosomes is a risk factor in obesity and diabetes. Poster abstract for UCLA Molecular Biology Institute annual retreat. Lake Arrowhead, CA. Oct 2011

7. The number of X chromosomes is a determinant in adiposity and metabolic dysregulation. Oral presentation for UCLA Lusis Program Project Grant Retreat. Lake Arrowhead, CA. Oct 2011

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CHAPTER 1

Introduction

The prevalence of obesity

Obesity is a worldwide epidemic, affecting both developed and developing countries. In 2013, the American Medical Association recognized obesity as a disease, bringing attention to the prevalence and health costs of excess weight. In the past three decades, the incidence of obesity nearly doubled in the United States (1). Over one-third of the population is reported to be obese, creating a multibillion-dollar burden on healthcare (1–3).

Obesity is defined as having a body mass index of at least 30 kg per squared meter of height. The excess weight in relation to height is typically due to expansion of fat tissue. Sedentary lifestyles and increased access to processed and fast food have contributed to bulging waistlines in recent decades. Mammals are genetically programmed to store excess energy when available in preparation for future starvation conditions, an adaptation that is detrimental in modern society (4). Economic globalization has led to fewer food shortages, allowing us to simultaneously consume more and move less. Thus, there is a critical need to understand the interplay of genetic and environmental factors on the development of obesity.

Increased body fat accumulation perturbs numerous physiological and cellular functions, leading to metabolic dysregulation. As the incidence of obesity rises, so does the incidence of its comorbidities, including heart disease, diabetes, fatty liver, infertility, sleep disorders, and cancer. It is crucial to understand the risk factors for obesity in order to improve prevention and remedial intervention.

Sex differences in obesity and related diseases

Sex is a key determinant in obesity and the associated diseases diabetes and cardiovascular disease, which are leading causes of morbidity and death in the United States (5,6). Major risk factors for these diseases include adiposity, insulin resistance, and elevated plasma lipid levels, all of which exhibit sex differences (7,8).

Sexual dimorphism in fat accumulation and distribution is well characterized. Premenopausal women tend to gain fat subcutaneously near the hips and thighs, a distribution that is often referred to as a “pear shape” (9,10). Men and postmenopausal women tend to gain fat in the abdominal cavity, leading to the “apple shape.” Expansion of the visceral fat depot is associated with increased risk for heart disease and type 2 diabetes (11–13).

Increased adiposity is closely tied to insulin resistance, a hallmark of type 2 diabetes that is characterized by the reduced ability of peripheral tissues to respond to insulin signaling and properly clear glucose from the bloodstream. Studies in rodents and humans suggest that females are more insulin sensitive than males (14). In females, glucose production is more readily suppressed in the liver, and glucose clearance from the plasma is more efficient (15). Despite this protective effect in females, diabetic women have a greater risk of mortality compared to diabetic men, but the contributing mechanisms are not understood (16).

Hyperlipidemia is a common co-morbidity of obesity. Increased levels of serum low density lipoprotein cholesterol (LDL-C) and triglyceride levels are risk factors for cardiovascular disease. Men tend to have higher LDL-C and lower high density lipoprotein cholesterol (HDL-C) compared to premenopausal women (8,17). After menopause, women have proatherogenic lipid levels that are similar to or worse than those in men (18).

Taken together, the observed male–female differences in adiposity, insulin resistance, and plasma lipid levels suggest that estrogen is metabolically protective. Studies in humans and animals have shown that estrogen administration can lead to decreased food intake, reduced adipose tissue, and overall cardioprotective effects. However, hormone replacement therapy in women does not always yield the same protection in women of various ages (19,20). In fact, hormone replacement therapy increases the risk of stroke (21,22). Overall, post-menopausal women have elevated risk of mortality from cardiovascular disease or diabetic complications (16,23). This suggests that factors other than sex hormones contribute to sex differences in metabolic disorders.

Identifying the impact of the sex chromosome complement

The sex chromosome complement defines the fundamental difference between female and male mammalian cells. Upon oocyte fertilization, the sperm cell dictates the sex of the zygote depending on the sex chromosome it carries. The presence of a Y chromosome triggers a cascade of molecular events leading to male development, while the presence of two X chromosomes leads to female development (24). Because XX chromosome complement is usually coupled with female gonadal hormones and XY chromosomes with male gonadal hormones, the independent effects of gonadal hormones and sex chromosomes are difficult to differentiate.

The Four Core Genotypes (FCG) mouse model is an innovative tool to distinguish effects of the sex chromosome complement from gonadal hormones (25). This model generates four mice roughly equivalent to XX female, XX male, XY female, and XY male. The Y chromosome in this model (denoted Y^{-}) harbors a mutation in the endogenous *Sry* gene, which determines testis development (Fig. 1A). Thus, XY^{-} mice develop as females with ovaries. In addition, FCG mice carry an *Sry* transgene on an autosome, so that the testis-determining gene independently segregates from the Y^{-} chromosome. By mating XX females with XY^{-}(*Sry*+) males, the four combinations of sex chromosome complement and gonad type are created: XX female, XX(*Sry*+) male, XY^{-} female, and XY^{-}(*Sry*+) male (Fig. 1B). Effects of gonadal hormones are identified when comparing XX and XY males to XX and XY females (Fig. 2). The comparison of XX females and males with XY females and males allows the detection of effects associated with the sex chromosome complement. This mouse model is a unique instrument for separating the etiologies of sex differences in disease.

The dissertation

This dissertation reports the analysis of roles played by gonadal hormones and the sex chromosome complement in modulating adiposity, dyslipidemia, and fatty liver. We have used

the FCG mouse model described in the preceding section to parse sex differences derived from gonadal hormones and sex chromosome complement. We demonstrate that the number of X chromosomes is a determinant of obesity and related metabolic dysregulation. Our results suggest that specific candidate genes on the X chromosome may be involved in driving these sex differences.

Chapter 2 is a reprint of "Metabolic impact of sex chromosomes," a review published in *Adipocyte*. It provides an overview of how hormones and the sex chromosome complement determine sex differences in obesity. In addition, the article touches upon the tight regulation of circadian rhythms and food intake, the disruption of which leads to metabolic disorders. Furthermore, we discuss metabolic disease in humans with sex chromosome aneuploidies, such as Turner syndrome (X0) and Klinefelter's syndrome (XXY). The review also summarizes key findings from our studies in gonadectomized FCG mice, which comprise Chapter 3 of the thesis.

Chapter 3 is a reprint of "The number of X chromosomes causes sex differences in adiposity in mice," originally published in *Public Library of Science (PLoS) Genetics*. This was the first study demonstrating that the sex chromosome complement influences adiposity and fatty liver in gonadectomized FCG mice. We studied body weight and body composition in FCG mice after the removal of acute effects of gonadal hormones by gonadectomy of adult mice. Under these conditions, the influence of sex chromosomes on body weight and adiposity was dramatic. When eating a mouse chow diet, XX mice had greater body weight than XY mice, regardless of the original presence of male or female gonads. The difference between XX and XY mice was enhanced by feeding a high fat diet for several weeks after their recovery from gonadectomy. Within 3 days of eating the high fat diet, XX mice weighed more than XY mice and after several weeks, XX mice had nearly twice the fat mass as XY mice. The XX mice, but not XY mice, also developed fatty liver in response to the high fat diet. The underlying mechanism of increased adiposity in XX mice is associated with increased food consumption

during the inactive phase of the circadian cycle; this might be roughly akin to “midnight snacking” in humans. We suspect that a few genes on the X chromosome, which are expressed at higher levels in XX compared to XY mice, drive the sex chromosome difference in food intake and adiposity.

As described in Chapter 3, XX *vs.* XY chromosome complement promotes adipose tissue accumulation in mice after removal of the gonads. The question remained, however, whether chromosome complement influences metabolism under conditions typically encountered in humans, *i.e*., in the presence of normal gonadal hormone levels. We addressed this question in Chapter 4, entitled “The sex chromosome complement is a determinant of sexual dimorphism in diet-induced obesity in mice.” In gonadally intact FCG mice fed a low-fat mouse chow diet, male mice have substantially higher body weight than female mice, in the presence of XX or XY chromosomes, indicating a role for gonadal hormones as has long been accepted. Based on our observations in Chapter 3, we hypothesized that a high fat diet might amplify sex chromosome effects on body weight. Indeed, when the gonadally intact FCG mice were fed a high fat diet, XX male and female mice gained more weight and body fat, accumulated more hepatic lipids, and consumed more food during the inactive phase of the circadian cycle. These results demonstrate that the sex chromosome complement is a major player in the development of obesity in response to a high fat diet, even in the presence of gonadal hormones.

XX mice exhibit higher expression levels of specific X chromosome genes as a result of their escape from X chromosome inactivation. These genes are our leading candidates for the effects observed in XX *vs*. XY mice. Chapter 4 describes the initial investigation of the role of genes that escape X chromosome inactivation on body weight and adiposity. We used genetically engineered mouse strains to test the effects of two candidate genes, and determined that dosage one of these, the histone demethylase *Kdm5c*, influences body weight, body fat and food consumption. Interestingly, these effects were enhanced when mice were fed a high fat

diet. This is the first report of X chromosome escapee gene dosage driving sex differences in adiposity.

As described in Chapter 4, we have identified candidate protein coding genes on the X chromosome that we suspect contribute to differences in adiposity between XX and XY mice. In addition to protein coding genes, small RNA molecules have been shown to play a role in the differentiation and maintenance of adipose tissue (26). The potential role of microRNAs (miRNAs) in sexual dimorphism in obesity has not been studied. As a first step, we sequenced the miRNA population in gonadal fat of FCG mice under gonadally intact and gonadectomized conditions, and in mice fed chow or high fat diets. We present the analysis in Chapter 5, entitled "The sex chromosome complement and gonadal hormones mediate sex differences in white adipose tissue miRNAs." We identified specific miRNA species that are regulated by gonadal hormones, sex chromosome complement, or diet. Through prediction of miRNA target genes, we identified pathways that are likely influenced by sexual dimorphism in miRNA levels. This study provides a first "catalog" of miRNAs that exhibit sexual dimorphism in adipose tissue, which warrant further study as potential contributors to sexual dimorphism in obesity.

Dyslipidemia is a common co-morbidity of obesity and a risk factor for diabetes and heart disease. Chapter 6 investigates the determinants of sexual dimorphism in serum lipid levels using the FCG mouse model. The resulting manuscript, "The presence of XX *versus* XY sex chromosomes is associated with increased HDL cholesterol levels in the mouse," will be published in the journal *Arteriosclerosis, Thrombosis, and Vascular Biology*. We identified some sex differences in lipid levels that were dependent on acute effects of gonadal hormones, and others that were associated with XX or XY chromosome complement. We also identified interactions between gonadal or chromosomal sex and diet. Notably, under all dietary and gonadal conditions, the levels of high density lipoproteins (HDL) were higher in mice with two X chromosomes compared to mice with an X and a Y chromosome, suggesting that the sex

chromosome complement is a strong regulator of HDL cholesterol levels. Our studies shed new light on the regulation of sex differences in plasma lipid levels.

Chapter 7 is a concluding summary of the dissertation and offers a discussion on current and future directions for studying the role of the sex chromosome complement in obesity and related diseases.

Figure 1

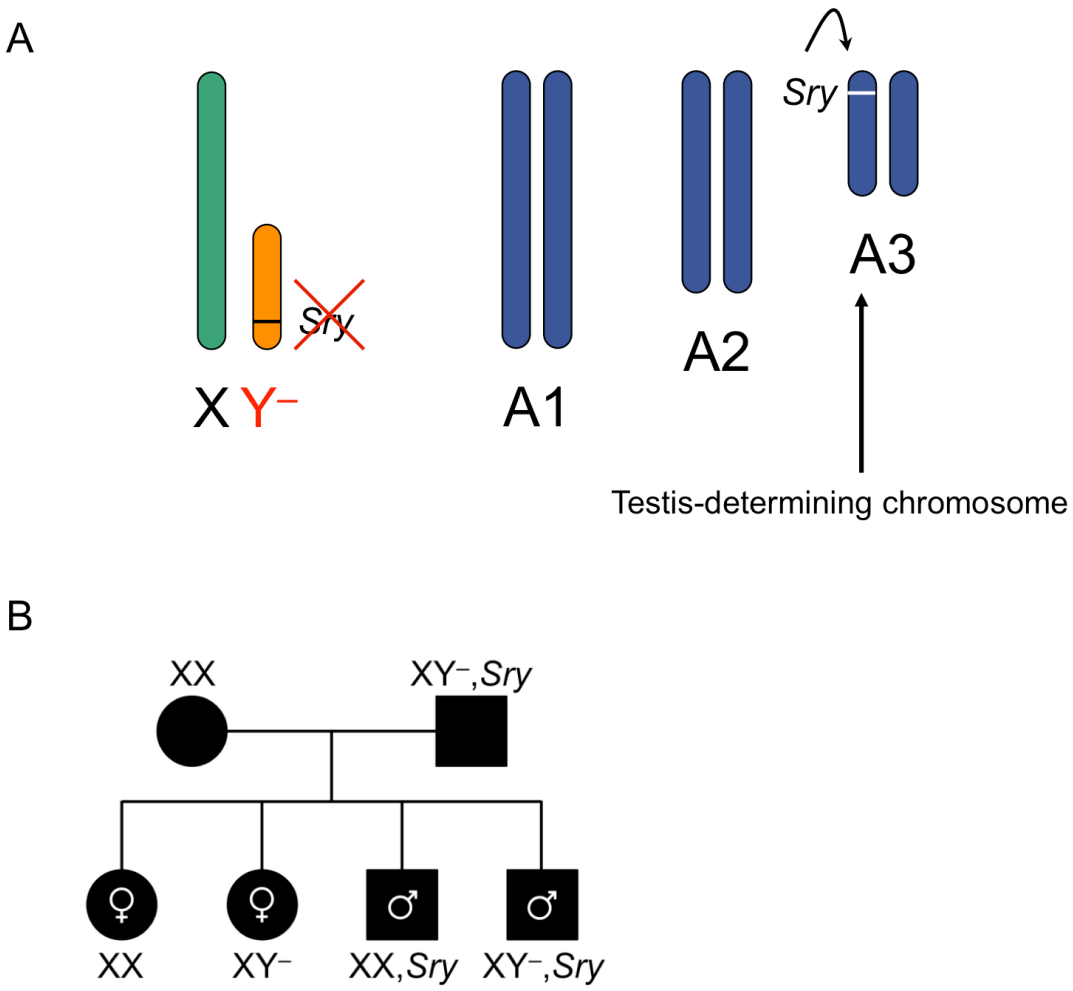

Figure 1. Genetic basis of the Four Core Genotypes. (A) The Y⁻ chromosome harbors a mutation in endogenous *Sry*. Without the presence of the testis-determining gene, XY⁻ animals develop as females. *Sry* is inserted as a transgene on an autosome, so *Sry* and the Y⁻ chromosome independently segregate during meiosis. (B) By crossing XY⁻,*Sry(+)* males with XX females, the four combinations of gonad type and sex chromosome complement are generated. A1, A2, A3: autosomes

Figure 2

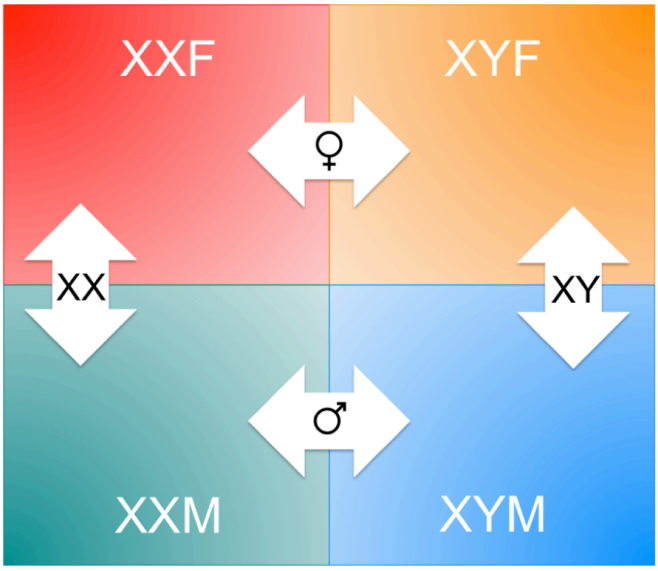

Figure 2. Using the FCG model to determine causes of sex differences. Effects of the sex chromosome complement can be distinguished from effects of gonadal hormones. The comparison of XX and XY females with XX and XY males allows detection of gonadal hormone effects. The effects of the sex chromosome complement can be identified by comparing XX males and females with XY males and females.

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CHAPTER 2

Metabolic Impact of Sex Chromosomes

Chapter Preface

The following chapter is a review of sex differences in obesity and related metabolic disorders. We discuss origins of sex differences and the methods of teasing apart the effects of gonadal hormones from the effects of the sex chromosome complement. We also review the current understanding of sexual dimorphism in adiposity and metabolic dysregulation, including fatty liver. Factors that affect obesity, such as food intake and circadian rhythms, are also discussed.

The article summarizes key findings from our studies in gonadectomized Four Core Genotype mice, which is reprinted in Chapter 3. When these studies were published, I was finishing my second year of graduate school. Writing the review afforded me an opportunity to critically assess the literature, which helped guide my future studies.

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Metabolic impact of sex chromosomes

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Keywords: metabolic disease, sex differences, obesity, food intake, fatty liver, circadian rhythm

Obesity and associated metabolic diseases are sexually dimorphic. To provide better diagnosis and treatment for both sexes, it is of interest to identify the factors that underlie male/female differences in obesity. Traditionally, sexual dimorphism has been attributed to effects of gonadal hormones, which influence numerous metabolic processes. However, the XX/XY sex chromosome complement is an additional factor that may play a role. Recent data using the four core genotypes mouse model have revealed that sex chromosome complement—independently from gonadal sex—plays a role in adiposity, feeding behavior, fatty liver and glucose homeostasis. Potential mechanisms for the effects of sex chromosome complement include differential gene dosage from X chromosome genes that escape inactivation, and distinct genomic imprints on X chromosomes inherited from maternal or paternal parents. Here we review recent data in mice and humans concerning the potential impact of sex chromosome complement on obesity and metabolic disease.

Hormonal and Genetic Factors Contribute to Sex Differences

Obesity and the metabolic syndrome are complex diseases regulated by many genetic and environmental factors. It is well known that risk, development and manifestations of obesity-related conditions such as diabetes and atherosclerosis are sexually dimorphic. Sex differences in obesity are strongly influenced by gonadal hormone effects.[1,2] However, an additional fundamental difference that may contribute to metabolic differences between females and males lies within the nucleus of each cell—the XX and XY sex chromosome complement.

The sex chromosome complement of female and male cells imposes several known genetic differences[3] (**Fig. 1**). For example, female cells with an XX chromosome complement never express any of the 78 protein-coding genes, nor an unknown number of noncoding RNAs, that are present on the Y chromosome. In addition, only XX cells undergo the process of transcriptional inactivation of one of the two X chromosomes during early development as a mechanism to balance gene dosage between males and females.[4-6] X chromosome inactivation involves the expression of X chromosome noncoding RNAs, production of high levels of histones, widespread chromatin remodeling and chromosome condensation, such that XX cells experience a distinct nuclear microenvironment during this process.[4] Furthermore, whereas XY cells inherit only a maternally imprinted X chromosome, XX cells carry one X chromosome with maternal imprints and another with paternal imprints.[5] The random inactivation of one X in XX cells therefore could lead to differential expression of imprinted genes compared with XY cells. Finally, although X-inactivation silences most genes on one X chromosome, some genes escape this process and are expressed at a higher level in tissues of females than males.[6-8]

In humans and standard animal models, it has been difficult to distinguish the contributions of gonadal hormones and sex chromosome complement as determinants of obesity, since female gonads are virtually always present in combination with XX chromosomes, and male gonads with XY chromosomes. To tease apart the influence of sex chromosome complement from gonadal hormones in obesity and related metabolic traits, we have used the four core genotypes mouse model.[9,10] In this model, the testis-determining gene, *Sry*, is deleted from the Y chromosome and an Sry transgene is inserted into an autosome. Thus, the Y chromosome segregates independently from the *Sry* gene and formation of male gonads. As a result, this model allows the generation of mice with four different "sexes" on a C57BL/6 background: XX mice with either male or female gonads, and XY mice with either male or female gonads (**Fig. 2**). In addition, we gonadectomized these mice to remove acute gonadal hormone action and uncover the effects of sex chromosome complement, as well as to detect long-lasting (organizational) effects of gonadal hormones.

In our recent study,[11] we observed dramatic effects of sex chromosome complement on obesity and metabolism (summarized in **Fig. 3**). Male and female mice with two X chromosomes had higher body weight and nearly twice as much body fat than mice with one X and one Y chromosome. XX mice also had increased food intake during the light (inactive) phase of the circadian cycle, potentially contributing to their higher body weight. When placed on a high fat diet, XX mice gained weight at an accelerated pace, and developed fatty liver and insulin resistance. A potential mechanism may be that genes escaping X-inactivation drive these differences observed between XX and XY mice. In support of this, we observed increased expression of X-inactivation escapees in key metabolic tissues (adipose and liver) of XX mice. In this article, we discuss these findings in the context of other reported

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sex differences in obesity, metabolic dysregulation, circadian rhythms and food intake.

Sex Chromosomes Influence Obesity and Associated Metabolic Dysregulation

The sexually dimorphic nature of human adipose tissue content and anatomical distribution has been widely documented and reviewed in recent excellent review articles.[12,13] It is well known that pre-menopausal women tend to store fat in subcutaneous depots, while men tend to store fat in visceral depots. After menopause, women begin to accumulate fat in the viscera, commonly associated with increased risk for metabolic diseases such as type 2 diabetes, non-alcoholic fatty liver disease and atherosclerosis. These differences in fat distribution have been attributed largely to the acute action of circulating sex hormones. Studies in the four core genotypes mouse model described above, however, revealed that sex chromosome complement also influences adiposity and metabolism.

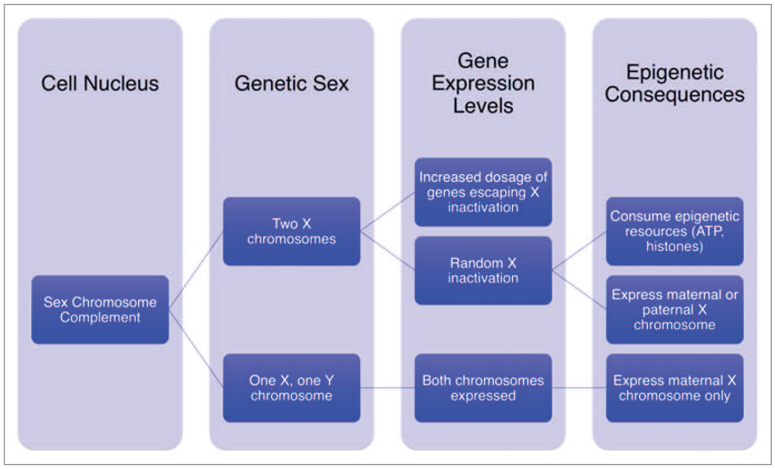

Figure 1. Genetic and epigenetic consequences of sex chromosome complement. XX cells undergo X inactivation, which requires cellular resources to produce histones and non-coding RNAs. Some genes escape X inactivation and are expressed at a higher level in XX compared with XY cells. Additionally, differential genomic imprinting (silencing) of genes on the X chromosome inherited from maternal and paternal parents can lead to gene dosage differences in XX cells and XY cells.

In gonadally intact mice of the four core genotypes, both gonadal sex and sex chromosome complement had effects on body weight.[11] Gonads were subsequently removed in adulthood, thus eliminating acute effects of gonadal hormones. XX mice fed a standard mouse chow diet (~5% fat) had almost double the fat mass of XY mice, regardless of their original gonadal sex. When the mice were fed a high fat diet after gonadectomy, the difference between XX and XY mice was amplified, with XX mice gaining weight more rapidly, and diverging from XY mice after only three days on the high fat diet. Analysis of specific fat depots revealed that XX mice had larger subcutaneous inguinal adipose tissue depots, whereas XY mice had larger gonadal fat pads. This result suggests that sex chromosome complement is a contributing factor to the differences in fat distribution that are observed between females and males.

Obesity causes many complications in tissues other than adipose, such as ectopic accumulation of fat in the liver. In addition to the increased adiposity in XX mice, a high fat diet promoted lipid droplet accumulation in livers of XX mice, whereas XY mice were virtually protected.[11] This was reflected in increased liver mass, increased hepatic triglyceride levels and reduced fatty acid oxidation gene expression in XX mice compared with XY mice. These results implicate sex chromosome complement as a risk factor for fatty liver. Epidemiological data in humans also show that sexual dimorphism exists in susceptibility to non-alcoholic steatohepatitis, but some populations exhibit increased incidence in women, while others report increased incidence in men.[14-16] Data in rodents are also contradictory. Male rats fed a methionine-choline-deficient diet develop more severe hepatic steatosis than females,[17] whereas C57BL/6J mice fed a high fructose diet showed a similar degree of steatosis in males and females, but greater inflammation in females.[18] The different diets used in each of these rodent studies make it difficult to make comparisons and firm conclusions. However, the results in Chen et al.[11] strongly suggest that sex chromosome complement may be an underlying determinant of hepatic steatosis that should be considered in studies of rodents and humans.

Another typical feature of obesity is insulin resistance. In the four core genotypes model, the high fat diet did not alter glucose levels, but induced a 2-fold elevation in fasting insulin levels in XX mice, but not XY mice.[11] Thus, XX chromosome complement appears to be a risk factor for insulin resistance in this model. Interestingly, studies in humans show that pre-menopausal women are more insulin sensitive than men.[19-21] This may be due to the positive effects of estrogen and/or the negative effects of androgens on insulin sensitivity.[22] Thus, while gonadal hormones clearly play a significant role in insulin signaling, our work shows that having two X chromosomes may be an additional genetic determinant, and may become most relevant in a hypogonadal state, such as after menopause.

Determinants of Sex Differences in Food Intake and Circadian Rhythms

To understand why XX mice accumulate more adipose tissue than XY mice, Chen et al.[11] assessed energy expenditure, physical activity, and food intake at a point when body weights among the four genotypes were the same (four weeks post-gonadectomy). No differences were detected in energy expenditure or activity among the genotypes, but XX mice exhibited increased food intake. This was notable because it occurred prior to their

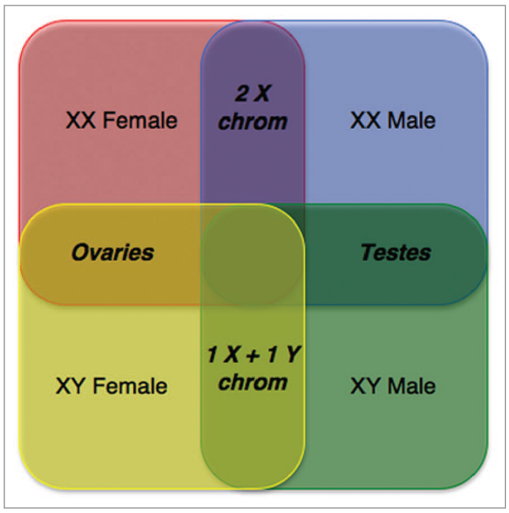

Figure 2. The four core genotypes mouse model. This mouse model generates four different "sexes": mice with female gonads that have either XX or XY sex chromosomes, and mice with male gonads that have either XX or XY sex chromosomes. Differences between gonadal females and gonadal males are attributed to acute or organizational gonadal hormone effects, while differences between XX and XY mice are attributed to the sex chromosome complement.

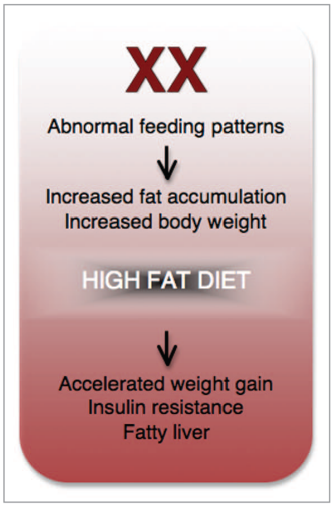

Figure 3. Metabolic impact of two X chromosomes. Mice with two X chromosomes have a higher food intake during the inactive phase, higher body weight and greater adipose tissue content. When placed on a high fat diet, these mice exhibit rapid weight gain and develop insulin resistance and fatty liver.

divergence from XY mice in body weight, and therefore may be a causative factor in the subsequent increased adiposity in XX mice. It was also notable that the increased food intake occurred exclusively during the light phase, during which mice (which are nocturnal) have reduced activity and typically consume only about 30% of their daily food. The increased food intake during the light phase raises the intriguing possibility that sex chromosome complement influences circadian regulation.

There is increasing evidence in mice and humans that disruption in the circadian cycle affects eating patterns and may contribute to obesity.[23,24] The distribution of caloric intake throughout the day is an important factor in human obesity.[25] In particular, eating during the inactive phase of the circadian cycle, such as midnight snacking, is associated with weight gain and greater risk of the metabolic syndrome.[26-28] Genetic disruption of the circadian cycle by mutation of the mouse *Clock* gene, a central transcriptional regulator of circadian rhythm, causes increased feeding during the light (inactive) period and obesity.[29] Furthermore, wild-type mice that eat on a circadian-shifted schedule are also prone to obesity. For example, when mice were fed equivalent amounts of a high fat diet exclusively during the dark phase or exclusively during the light phase, those who ate during the light phase gained double the weight, despite similar activity levels.[30] Additional studies of light-restricted feeding in mice have also shown increased weight gain, as well as altered metabolic gene expression in fat and liver, and higher respiratory exchange ratio, indicating reduced reliance on fatty acid oxidation.[31,32] Interestingly, the increased adiposity in XX mice in the study by Chen et al.[11] was associated with altered metabolic gene expression and higher respiratory exchange ratio. The mechanisms for the increased weight gain with disruption of circadian rhythm are not well understood, but may depend on a rhythmic profile of leptin levels that normally occurs throughout the circadian cycle.[33] It will be interesting to determine whether altered rhythmicity of leptin, or perhaps leptin resistance, is present in XX compared with XY mice, and whether limitation of feeding in XX mice to the dark period may alleviate the increased weight gain.

Sexual dimorphism in eating behavior of male and female rodents has been described, and gonadal hormones have been implicated. Several studies show that food intake and satiety are highly regulated by estradiol.[34] In gonadally intact female rats, a rise in estradiol secretion is followed by a reduction in food intake, whereas ovariectomy results in increased food intake. Furthermore, estradiol interacts with other satiety signals in the central nervous system and in the periphery.[34] It has also been shown that female rats that have been fasted for 48 h activate orexin neurons (which promote feeding) to a greater extent and consume more food than males.[35] Nevertheless, the data in Chen et al.[11] showing that gonadectomized mice with XX chromosomes eat more than XY mice reveals that additional genetic factors beyond gonadal hormones also contribute to sex differences in food intake and meal patterns. The four core genotypes mouse model provides a tool to further dissect the effects of gonadal hormones and sex chromosome effects on feeding behavior and metabolic disease.

Relevance to Humans

With the increasing longevity of humans and thus a longer time spent in a hypogonadal state, it is important to understand non-hormonal regulators of sexual dimorphism. Chen et al.[11] demonstrated that the sex chromosome complement is an important determinant of adiposity, fatty liver, and insulin levels in the absence of sex hormones. These results in the mouse raise the question of whether sex chromosome aneuploidies in humans might be instructive about the role of sex chromosomes in metabolic regulation. Human sex chromosome aneuploidies include 45,X0 females (1/2,500 live female births), 47,XXY males (1/500 live male births), 48,XXYY (1/17,000 live male births), 48,XXXY males (1/50,000 live male births), and 46,XX males (1/20,000 live male births, resulting from translocation of the *SRY* gene to the X chromosome).[36-38] The rarity of most sex chromosome aneuploidies has made it difficult to assess their effects on metabolism. However, some data available for XXY males and X0 females indicate higher incidence of metabolic disease than the general population, as described below.

Compared with XY males, individuals with Klinefelter syndrome (XXY) are more likely to have an atherogenic lipoprotein profile, with elevated low density lipoprotein levels, and reduced high density lipoprotein levels.[39] XXY men may also have increased incidence of type 2 diabetes and metabolic syndrome, defined as the co-occurrence of visceral obesity, insulin resistance and enhanced cardiovascular disease risk.[40,41] These findings in XXY men are consistent with mouse studies in which the presence of two X chromosomes compared with a single X was associated with obesity and metabolic dysregulation.[11] XXY men have lower levels of testicular androgens, which could contribute to the metabolic effects associated with the syndrome, so it is unclear which factors are the most important.

Turner syndrome women (X0) tend to have greater fat mass when adjusted for body weight than XX females.[42,43] X0 women may also experience increased risk for type 2 diabetes mellitus and heart disease.[44] These results appear to contradict the finding in mice that presence of one X chromosome leads to reduced adiposity compared with XX mice.[11] One factor that potentially contributes to increased metabolic dysregulation in Turner syndrome women is that they are hypogonadal throughout their lifetime. In contrast, in the mouse studies, animals had intact, normal gonads until adulthood and therefore may have experienced critical activational and organizational effects of gonadal hormones that are metabolically beneficial and not experienced by X0 humans. Interestingly, an analysis of body fat and lipid levels in a group of Turner syndrome patients showed differences depending on the parental origin of the single, normal X chromosome. Individuals that inherited the maternal X chromosome had 78% more visceral fat than those carrying an X chromosome of paternal origin, and higher triglyceride and low density lipoprotein cholesterol levels.[45] The maternal and paternal X chromosomes carry distinct genomic imprints, which silence the expression of specific genes and result in different expression levels. These results dovetail with those in the mouse showing that X chromosome dosage influences metabolic regulation, and highlight the mechanism of differential imprinting of the X chromosome from the two parents as a potential contributing mechanism.

Future Perspectives

Sexual dimorphism is a critical component that affects numerous aspects of metabolism and, hence, susceptibility to metabolic disease. The pervasive effect of sex on metabolism is strikingly illustrated by a recent metabolomic study of serum from more than 3,000 men and women.[46] An unbiased analysis of 131 blood metabolites (including amino acid, fat and sugar molecules) revealed significant concentration differences between males and females for 102 of the metabolites. Thus, widespread differences in metabolism clearly occur in males and females, and there is a need to better understand the nature and origin of these for effective treatment of metabolic diseases in both sexes.

Studies in humans are valuable, and every effort should be made to perform well-designed, sufficiently powered studies of both sexes. But such studies will always be limited in their ability to provide insight into the underlying mechanisms for sex differences, and it is in this realm that animal models are critical. The four core genotypes mouse model is one such model and is unique in that it allows the discrimination of effects due to gonadal sex or sex chromosome complement.[10] Based on the initial identification of sex chromosome effects on adiposity, feeding behavior, fatty liver and insulin resistance, we anticipate that this model will be useful to provide additional insight into the genetic factors and physiological mechanisms involved. As presented in Chen et al.,[11] genes that escape X chromosome inactivation exhibit increased expression levels in tissues such as adipose and liver, which may lead to phenotypic differences. In addition to the expression of protein coding genes examined thus far, additional potential players include long non-coding RNAs that are known to escape X-inactivation,[8] and potentially some of the 70 microRNAs that are present on the X chromosome, which have not been assessed for potential inactivation escape.

A valid question is whether results obtained in a model such as the four core genotypes mouse will translate to humans. Several factors suggest that they will. The genes on the human and mouse X chromosomes are highly conserved, as is the process of X chromosome inactivation. Furthermore, the genes that are known to escape inactivation in the mouse also escape inactivation in humans.[47] Additional genes have also been shown to escape X-inactivation in humans, suggesting that sex chromosome effects may potentially be amplified in humans compared with mice.[48] And finally, in the mouse studies, sex chromosome effects were most apparent in mice that had been gonadectomized as adults. Although humans are not typically gonadectomized, both women and men experience periods of hypogonadal hormone levels, typically beginning in middle age. It is likely that the sex chromosome effects observed in mice may become more influential in humans with advancing age, which is when the majority of individuals develop metabolic diseases such as type 2 diabetes and cardiovascular disease. It will be interesting to determine the effects on obesity and

dysregulated metabolism when gonadectomy is performed in four core genotype mice at advanced ages, to better parallel the onset of menopause in humans. Moreover, mouse models offer considerable advantages for identifying the X gene(s) responsible for the sex chromosome effect, which can then be tested in studies of humans. Ultimately, the identification of genes or factors that promote or protect against metabolic diseases in a sex-specific manner may suggest novel pathways as targets for therapeutic intervention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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CHAPTER 3

The Number of X Chromosomes Causes Sex Differences in Adiposity in Mice

Chapter Preface

Chapter 3 comprises our studies in gonadectomized Four Core Genotype mice. This publication was the first report of the effect of the sex chromosome complement in obesity.

The Four Core Genotype mouse model was pioneered by Dr. Art Arnold to examine the effects of gonadal sex independently from the effects of the sex chromosome complement. To fully understand sex differences in obesity and related metabolic disorders, Dr. Arnold began a collaboration with Dr. Karen Reue, an expert in identifying novel genes in lipid metabolism.

When I first joined the laboratory in 2010, the initial studies of gonadectomized Four Core Genotype mice had just commenced. Physical metabolic parameters such as body weight, body composition, energy expenditure, and hepatic lipid levels were already analyzed. We hypothesized that a few genes known to escape X chromosome inactivation may contribute to sex chromosomes differences in body weight and adiposity. However, the expression levels of these candidate genes in metabolic tissues were unknown. I measured the mRNA levels of several escapee genes in liver, gonadal fat, and inguinal fat, and found that some genes (*Ddx3x*, *Eif2s3x*, and *Kdm6a*) were expressed at higher levels in XX compared to XY mice. These results helped us narrow down our list of candidate genes. We postulated that the dosage of these escapee genes in relevant metabolic tissues could drive the sex differences observed in adiposity.

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The Number of X Chromosomes Causes Sex Differences in Adiposity in Mice

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Abstract

Sexual dimorphism in body weight, fat distribution, and metabolic disease has been attributed largely to differential effects of male and female gonadal hormones. Here, we report that the number of X chromosomes within cells also contributes to these sex differences. We employed a unique mouse model, known as the "four core genotypes," to distinguish between effects of gonadal sex (testes or ovaries) and sex chromosomes (XX or XY). With this model, we produced gonadal male and female mice carrying XX or XY sex chromosome complements. Mice were gonadectomized to remove the acute effects of gonadal hormones and to uncover effects of sex chromosome complement on obesity. Mice with XX sex chromosomes (relative to XY), regardless of their type of gonad, had up to 2-fold increased adiposity and greater food intake during daylight hours, when mice are normally inactive. Mice with two X chromosomes also had accelerated weight gain on a high fat diet and developed fatty liver and elevated lipid and insulin levels. Further genetic studies with mice carrying XO and XXY chromosome complements revealed that the differences between XX and XY mice are attributable to dosage of the X chromosome, rather than effects of the Y chromosome. A subset of genes that escape X chromosome inactivation exhibited higher expression levels in adipose tissue and liver of XX compared to XY mice, and may contribute to the sex differences in obesity. Overall, our study is the first to identify sex chromosome complement, a factor distinguishing all male and female cells, as a cause of sex differences in obesity and metabolism.

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Introduction

Obesity represents a risk factor for many types of metabolic disease, including diabetes, coronary heart disease, osteoarthritis, and even cancer. The study of rare mutations in humans and induced mutations in mouse models has identified numerous genetic factors that influence energy balance [1,2,3]. Less is known, however, about common genetic factors that may contribute to moderate differences in body fat storage among individuals in a population [4]. In humans and many other mammals, differences exist between males and females in the amounts and anatomical distribution of fat storage [5,6,7,8,9]. In general, males tend to have more visceral fat while females have more lower body and subcutaneous fat [10]. The two sexes also differ in the levels of adipose tissue-derived hormones leptin and adiponectin [11,12,13], and in the response of fat store depletion to caloric restriction [14]. These differences may contribute to differences between men and women in susceptibility to metabolic disease.

The genetic origins of sex differences in fat tissue accumulation are not well understood. Most studies have focused on the role of gonadal hormones (reviewed in [15,16]. It is well established that the reduction in levels of estrogens, progestins, and androgens occurring at menopause in women increases central fat accumulation and risk for diabetes, cardiovascular diseases and other disorders [17]. Further evidence that estrogens play an important role in fat metabolism comes from mouse studies. For example, both male and female mice lacking estrogen receptor α have increased white adipose tissue mass and insulin resistance [18]. In men, the accumulation of excess abdominal adipose tissue is associated with low levels of gonadal androgens [19]. Hyperandrogenism is also associated with increased abdominal obesity in women with polycystic ovarian syndrome [20]. Androgen receptor-deficient male mice develop late onset obesity, particularly affecting visceral fat [21,22]. In addition, the administration of dihydrotestosterone suppresses the development of subcutaneous adipose tissue in wild-type but not androgen receptor-deficient

Author Summary

Differences exist between men and women in the development of obesity and related metabolic diseases such as type 2 diabetes and cardiovascular disease. Previous studies have focused on the sex-biasing role of hormones produced by male and female gonads, but these cannot account fully for the sex differences in metabolism. We discovered that removal of the gonads uncovers an important genetic determinant of sex differences in obesity—the presence of XX or XY sex chromosomes. We used a novel mouse model to tease apart the effects of male and female gonads from the effects of XX or XY chromosomes. Mice with XX sex chromosomes (relative to XY), regardless of their type of gonad, had increased body fat and ate more food during the sleep period. Mice with two X chromosomes also had accelerated weight gain, fatty liver, and hyperinsulinemia on a high fat diet. The higher expression levels of a subset of genes on the X chromosome that escape inactivation may influence adiposity and metabolic disease. The effect of X chromosome genes is present throughout life, but may become particularly significant with increases in longevity and extension of the period spent with reduced gonadal hormone levels.

mice [22]. Thus, gonadally derived hormones from both males and females influence body fat, albeit in distinct ways.

Although gonadal hormones are a key determinant of sexual dimorphism in body fat and metabolism, it is notable that even prior to the differentiation of the gonads, human and mouse male embryos are larger than female embryos, suggesting that non-gonadal factors also contribute [23,24]. In addition to gonadal hormones, an additional fundamental genetic difference exists within every cell in the body of females compared to males (reviewed in [25,26,27,28]. This is the presence in female cells of two X chromosomes, and in male cells of an X and a Y chromosome. The Y chromosome, and specifically the *Sry* gene located there, initiates differentiation of the testes. Mice that have a Y chromosome from which *Sry* has been deleted develop ovaries rather than testes. Conversely, an *Sry* transgene inserted onto autosome is sufficient to convert XX female mice to gonadal males [29]. Inactivation of one X chromosome in each non-germline XX cell greatly reduces the sex difference in level of expression of X genes that is predicted based on the number of copies of X genes [30]. However, a finite set of genes on both mouse and human X chromosomes escape inactivation, and would therefore be expected to exhibit higher expression levels in XX compared to XY cells [31,32,33,34]. Recent studies indicate that genes escaping X chromosome inactivation exhibit elevated expression in metabolic tissues from XX compared to XO mice [35], and could potentially contribute to sex differences in metabolic phenotypes.

In the present study, we utilize the Four Core Genotypes (FCG) mouse model to distinguish between the effects of gonadal sex (testes or ovaries) and sex chromosomes (XX or XY) on adiposity and related metabolic traits [25,26,27,36]. The FCG model allows the generation of gonadal male and female mice carrying either XX or XY sex chromosome complements. Specifically, the FCG Y chromosome sustained a mutation deleting the *Sry* gene (yielding the "Y minus" chromosome, Y^-), which is complemented in some mice by an *Sry* transgene located on an autosome. Mice having both the Y^- chromosome and the *Sry* transgene will develop normally as fertile gonadal male mice. If these mice are bred to a normal female (XX), four types of progeny are produced: female mice with ovaries and XY or XX sex chromosomes (XYF and XXF, respectively), and male mice with testes and XY or XX sex chromosomes (XYM and XXM, respectively). If differences in a trait of interest occur between the gonadal male mice (XYM and XXM) and gonadal female mice (XYF and XXF), it is most likely related to differences in gonadal hormones, although the groups also potentially differ because of possible effects of *Sry* on non-gonadal tissues. By contrast, differences between XX and XY mice suggest a sex chromosome effect, likely directly caused by the difference in number of X or Y chromosomes.

In our study, FCG mice were gonadectomized as adults to remove the acute sex differences resulting from gonadal hormones, and thereby uncover the contribution of sex chromosome complement. We found that gonadectomized XX mice of both gonadal sexes have two-fold increased adiposity compared to XY mice of either gonadal sex. Further genetic studies with mice carrying XO and XXY chromosome complements revealed that the difference is attributable to dosage of the X chromosome, rather than effects of the Y chromosome. These results demonstrate a fundamental difference in adiposity and metabolism conferred by genes on the sex chromosomes, and specifically implicate X chromosome genes as the direct cause of these differences. These results further suggest that X chromosome genes whose expression levels are influenced by dosage or parental imprinting are candidates for metabolic disease differences in men and women.

Results

Sex chromosome complement influences body weight and fat mass independent of sex hormones

To determine whether sex chromosome effects contribute to sex differences in body weight and fat mass in adulthood, we examined these traits in C57BL/6 FCG mice (XX gonadal females, XX gonadal males, XY gonadal females, and XY gonadal males). Mice were maintained on a standard chow diet with low fat content (5% by weight). At the time of weaning at postnatal day 21, the four groups of FCG mice did not differ in body weight (Figure 1A). By 45 days of age, gonadal males of either sex chromosome complement were approximately 20% heavier than gonadal females. At 75 days of age the gonadal males were 25% (XX background) or 28% (XY background) heavier than corresponding gonadal females (Figure 1B, time 0). Importantly, however, in these gonadally intact mice there was also a significant influence of sex chromosomes on body weight. At 75 days of age, XX mice were heavier than XY mice by 6.3% (XX>XY gonadal males) and 8.8% (XX>XY gonadal females) ($p<0.0001$) (Figure 1B, time 0).

Differences observed between male and female gonadally intact FCG mice can be attributed to either activational effects of gonadal hormones (reversible effects caused by sex differences in on-going action of gonadal hormones) or organizational effects (long-lasting or permanent gonadal hormone effects exerted at an earlier stage of development). To distinguish between these alternatives, mice were gonadectomized at 75 days of age to remove activational effects of gonadal hormones. At the time of gonadectomy, male XY and XX mice had significantly higher body weight than female XX and XY mice, although XX mice of either gonadal type weighed more than XY mice, as described above (Figure 1B). In the 4 weeks following gonadectomy (GDX), the body weights of all genotypes converged, and differences between mice that were originally gonadal males and females disappeared (Figure 1B). By 7 weeks, there emerged significant

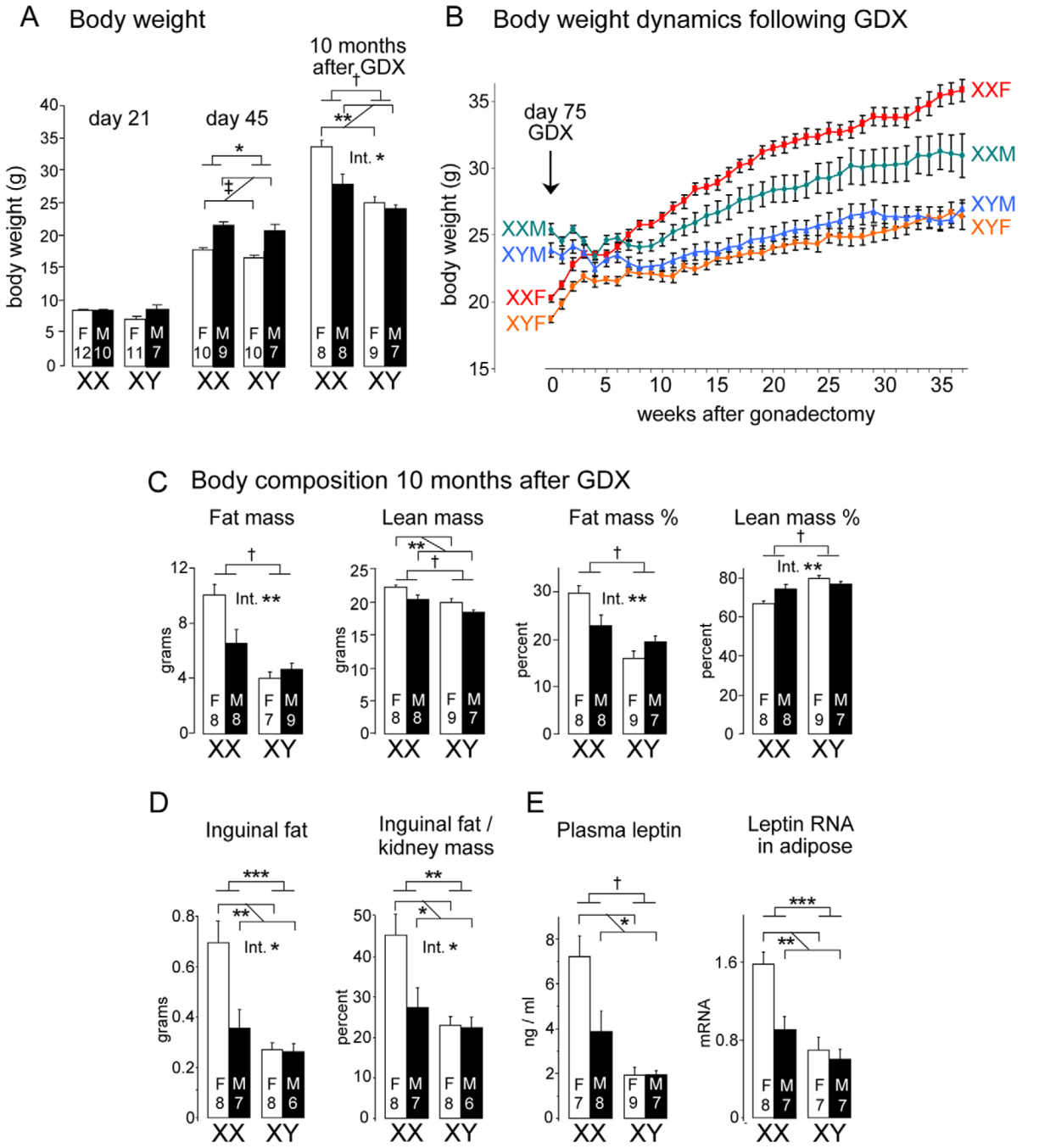

Figure 1. Increased body weight and fat mass in XX versus XY mice on a chow diet. (A) Body weight of four core genotype (FCG) mice at day 21 and day 45, prior to gonadectomy (GDX), and at 10 months after GDX. F, gonadal female; M, gonadal male. Values represent the mean ± SEM for the number of animals indicated in each bar. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement and gonadal sex is denoted by "Int.". The p values are as described at the end of the legend. (B) Body weight curve for FCG mice from the point of gonadectomy through 10 months following gonadectomy. Values represent mean ± SEM. Values are significantly different between mice with XX vs. XY genotypes beginning at week 7 following GDX and beyond that. (C) Body composition of mice in panel (B) determined by NMR in FCG mice at 10 months after GDX. (D) Fat pad mass in mice from panel (B) at 10 months following GDX expressed as absolute mass (grams) or relative to kidney weight, which is invariant among the genotypes. (E) Plasma leptin levels and leptin mRNA levels in inguinal adipose tissue. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<0.0001$; ‡, $p<0.000001$.
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differences based on sex chromosome complement, with XX mice weighing more than XY mice ($p<0.000005$; Figure 1). At 10 months after GDX, the XX mice weighed 24% more than XY mice ($p<0.0001$, Figure 1A). In addition, XX gonadal females continued to weigh more than XX gonadal males despite the absence of gonadal secretions for 10 months (Figure 1A, $p<0.01$ for XX female vs. XX male mice), suggesting an interaction between XX sex chromosome complement and long-acting (organizational) gonadal hormone effects (interaction $p<0.05$). Thus, the male-female difference in number of X chromosomes influences body weight in the opposite direction to the male-female difference in gonadal hormones.

The increased body weight in XX compared to XY mice reflects a near doubling of the absolute fat mass as ascertained by NMR analysis of whole mice, with 88% higher fat mass in XX compared to XY mice (Figure 1C). When expressed as a percent of body weight, XX mice had 50% higher proportional fat mass than XY mice ($p<0.00001$, Figure 1C). This dramatic difference in fat mass between XX and XY mice is particularly striking considering that the mice were fed a standard mouse chow diet with very low fat content. XX mice also had slightly higher lean body mass than XY mice (Figure 1C). The increased total body adiposity of XX compared to XY mice was reflected in isolated fat pad mass (Figure 1D; $p<0.0005$ for absolute fat pad mass, $p<0.005$ for mass relative to kidney; kidney weight did not differ among genotypes). Fat mass, percent lean mass, and fat pad mass all exhibited significant sex chromosome effects, and also significant interactions between sex chromosome and gonadal sex (indicated in Figure 1A, 1C and 1D by 'Int').

In parallel with the increased adiposity, plasma leptin levels were elevated 2–3-fold in XX compared to XY mice ($p<0.00005$) (Figure 1E). Plasma leptin was also higher in females ($p<0.05$), but only in XX mice (interaction $p<0.05$). This suggests that long lasting gonadal effects, as well as genetic factors conferred by sex chromosome complement, directly or indirectly influenced leptin levels. Leptin mRNA levels in adipose tissue mirrored plasma leptin levels, with highest levels in XX mice ($p<0.0005$ vs. XY mice), and significantly higher levels in mice that previously had ovaries rather than testes ($p<0.01$) (Figure 1E).

XX mice exhibit increased daytime food intake preceding increased body weight

To identify metabolic differences that could contribute to the increased adiposity of XX mice, we measured food intake, physical activity, and energy expenditure parameters while mice were housed in metabolic cages. We performed these studies at two ages: (1) at 4 weeks following gonadectomy, at which time the body weights for all four genotypes were similar and measurements were not complicated by differences in body weight or composition, and (2) at 10 months after gonadectomy, after body weight differences in XX vs. XY mice were pronounced (see Figure 1B).

At 4 weeks post-GDX, we detected a difference among the genotypes in food intake patterns monitored continuously throughout the circadian cycle. During the dark period when mice typically consume 70% of total calories [37], gonadal female mice of both XX and XY chromosome complements consumed more than gonadal males ($p<0.05$; Figure 2A). Since these measurements were made only 4 weeks after GDX, this may reflect lingering effects of gonadal secretions. However, during the daytime, food intake was significantly higher in XX females and males compared to XY mice ($p<0.01$; Figure 2A and 2C). Since this difference occurred at an age when no differences exist in body weight, the increased daytime food intake is likely to contribute to subsequent divergence of body weight between XX and XY mice. At 10 months after GDX, the average absolute food intake for all genotypes was reduced compared to values at 4 weeks post-GDX, but no significant differences in food intake were observed among the four genotypes (Figure 2B).

Using indirect calorimetry, we detected significant sex chromosome effects on respiratory quotient (RQ), a measure of the relative reliance on carbohydrate (RQ = 1) and fat substrates (RQ = 0.7) as metabolic fuel. At four weeks post-GDX, all mice exhibited the expected diurnal variation in RQ, with highest values in the dark phase. Notably, however, XX mice maintained a significantly higher RQ than XY mice during the light phase (Figure 2D), which may be related to the differential feeding pattern in XX mice (Figure 2A). In addition, compared to XY mice, XX mice exhibited a smaller amplitude change in RQ from dark to light periods (Δ RQ), suggesting reduced flexibility in fuel switching (Figure 2D). By contrast, at 10 months post-GDX, after XX mice had accumulated nearly twice as much adipose tissue as XY mice, the pattern of fuel utilization had changed. At this point, the XX mice had lower RQ than XY mice during the dark phase (Figure 2E), indicating increased fat utilization in the fed state, possibly an adaptive change in response to the excess fat storage.

Besides food intake and RQ, other energy balance parameters did not differ significantly among the four genotypes. These include oxygen consumption (which was assessed per mouse, per lean body mass [38], and via linear regression [39] to account for contributions of both lean and fat mass in energy metabolism), thermogenic gene expression, and physical activity in the horizontal and vertical planes (Figure S1). Thus, the key differences in energy metabolism between XX and XY mice were increased daytime food intake and reduced flexibility in RQ in XX mice. Both of these were apparent prior to the divergence in body weight.

Despite the greater adiposity, XX mice did not exhibit substantially impaired glucose homeostasis. At four weeks after GDX, fasting glucose levels were higher in gonadal female than gonadal male mice ($p<0.0001$), and slightly higher in XX than XY mice ($p<0.05$); there were no differences in fasting insulin levels among the genotypes (Figure S2A). At ten months after GDX when XX mice had considerably greater adiposity, glucose and insulin levels were similar among the four genotypes, and no differences were revealed by glucose tolerance test (Figure S2A). The ability to maintain glucose homeostasis despite excess fat storage in the XX mice may be related to adaptive changes in metabolism in these mice. For example, at ten months after GDX when XX mice had substantially higher fat mass, they exhibited increased expression of fatty acid oxidation genes encoding acyl CoA oxidase (*Aox1*) and carnitine palmitoyl transferase (*Cpt1*) in both muscle and liver (Figure S2B). Increased fatty acid oxidation may reduce the extent of lipid accumulation in liver and skeletal muscle, and prevent impaired glucose homeostasis.

Enhanced weight gain, dyslipidemia, and fatty liver in XX mice on a high fat diet

As described above, on a chow diet containing minimal fat, XX mice accumulate excess adipose tissue without impaired glucose homeostasis. Metabolic dysregulation in human obesity typically occurs in the presence of a more stressful nutritional environment. We hypothesized that a combination of sex chromosome complement and a high fat diet may make XX mice more vulnerable to metabolic dysregulation than XY mice. To test this, we placed FCG on a high fat, simple carbohydrate diet that promotes weight gain [40]. Mice were gonadectomized at 75 days of age, continued on a chow diet for 4 weeks, and then fed the high fat diet for 16 weeks. As shown in Figure 3A, the mice of all four

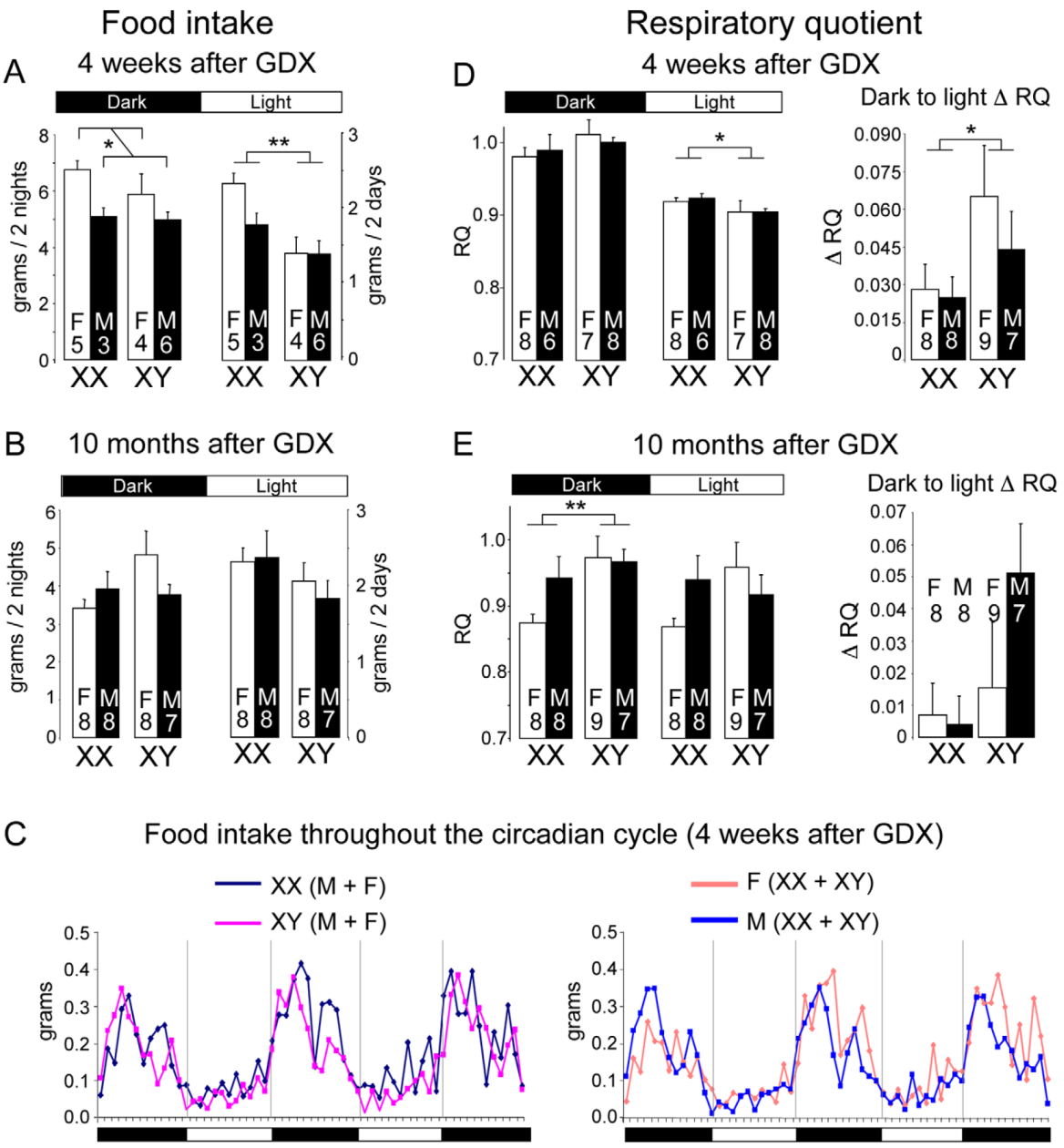

Figure 2. Altered food intake and RQ in XX versus XY mice. Mice were individually housed in metabolic cages to assess food take and energy balance parameters continually throughout the diurnal cycle. (A, B) Food intake determined at 4 weeks or 10 months following GDX. Values represent the mean ± SEM food intake summed over two dark or two light periods. Data shown represent raw values; normalization to body weight or to lean body mass gave the same outcome. (C) Food intake patterns throughout 3 nights and 2 days. At left, lines represent mean values for XX (n = 8) and XY (n = 10) mice; at right, lines represent mean values for gonadal females (n = 9) and gonadal males (n = 9). (D, E) Mean Respiratory Quotient (RQ) ± SEM for light and dark periods determined at 4 weeks or 10 months following GDX. At right, the change in RQ between dark and light periods (Δ RQ) is shown. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ‡, $p<0.000001$.
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genotypes had similar body weight at the beginning of the high fat diet. However, within just 3 days of beginning the high fat diet, the XX and XY mice diverged, with significantly higher body weight in XX gonadal males and females than in the corresponding XY mice ($p<0.005$). XX mice continued to gain weight at an accelerated pace throughout most of the 16 weeks, and weighed about 15% more than XY mice at the end of the diet ($p<0.000005$). The enhanced weight gain on the high fat diet appeared to obscure the male-female difference in XX mice that was observed on the chow diet (Figure 1B).

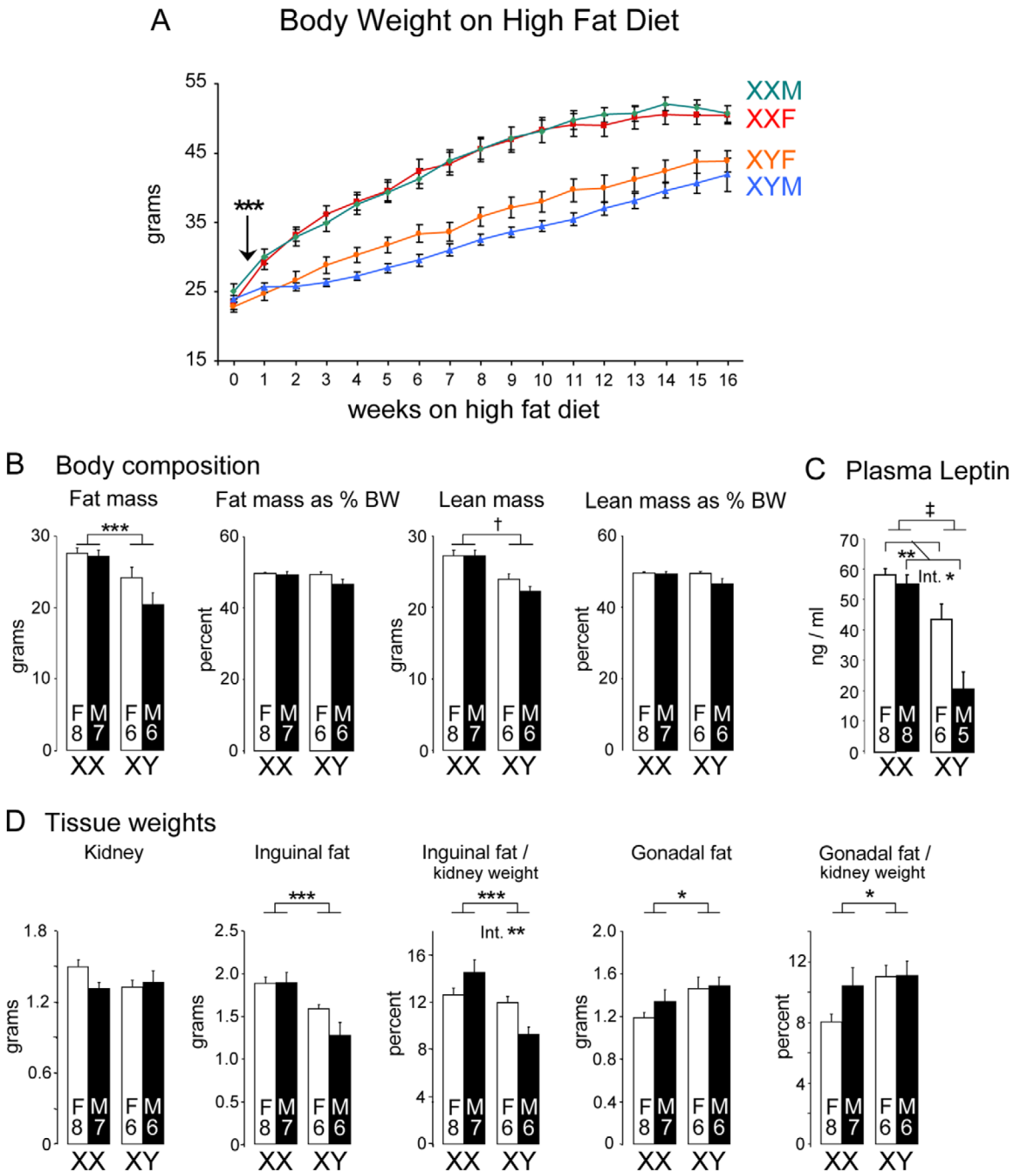

Figure 3. Enhanced weight gain and fat mass in XX compared to XY mice fed a high fat-high carbohydrate diet. (A) FCG mice were fed a high fat-high carbohydrate diet beginning at 4 weeks following GDX (week 0). XX mice first exhibited significantly higher body weight beginning at 3 days on the diet (arrow), and throughout the remainder of the study. (B) Body composition was determined by NMR at 16 weeks of the high fat-high carbohydrate diet. Fat and lean mass are shown as absolute mass and as percent of total body weight (BW). (C) Plasma leptin levels were higher in XX mice than XY mice at the end of the 16 week high fat diet period, and higher in gonadal females than gonadal males. (D) Tissue weights were determined by excision after 16 weeks on the high fat-high carbohydrate diet. Kidney weight did not differ among the four genotypes. Inguinal and gonadal fat pads are shown as absolute weights and normalized to kidney weight. Values shown for all bars represent mean ± SEM for the number of each genotype indicated. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<0.0001$; ‡, $p<0.000001$.
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NMR assessment of body composition showed that after 16 weeks on the high fat diet, XX mice had higher absolute fat mass than XY mice ($p<0.005$), but the increase in fat mass was not significant when expressed as a proportion of body weight (Figure 3B). Nevertheless, the increased fat mass was reflected in elevated plasma leptin levels in XX compared to XY mice ($p<0.000001$); leptin levels were also significantly higher in female vs. male mice ($p<0.01$; interaction of sex by sex chromosome complement $p<0.05$) (Figure 3C). Absolute lean mass was also increased in XX mice ($p<0.00005$), but not when expressed as a proportion of body weight (Figure 3B). Thus, it appears that the greater increase in body weight observed in XX compared to XY mice on a high fat diet is attributable to increases in both fat and lean mass, and that XX mice exhibit increased absolute fat mass and circulating leptin levels.

The analysis of tissue weights of mice after 16 weeks on the high fat diet revealed sex chromosome effects on the liver and adipose tissue depots. Absolute kidney weight did not differ among the four genotypes despite differences in body weight (Figure 3D), and was used to normalize the weights of other tissues. Inguinal subcutaneous fat pads weighed more in XX compared to XY mice (Figure 3D; absolute weight, $p<0.0005$; normalized to kidney, $p<0.001$). We also detected a sex chromosome by gonadal sex interaction in inguinal fat pad weight when normalized to kidney weight ($p = 0.006$), suggesting a role for organizational hormone action in combination with XX or XY status in determining subcutaneous fat pad expansion on a high fat diet. Unexpectedly, the gonadal fat depot showed the opposite pattern, with slightly higher values in XY mice expressed both as absolute weight ($p<0.05$) and normalized weight ($p<0.05$) (Figure 3D). These results indicate that distinct genetic and hormonal factors may influence the expansion of the gonadal and inguinal fat depots on a high fat diet.

The high fat diet elicited formation of a fatty liver specifically in XX mice. XX mice exhibited a significant increase in liver weight, an abundance of lipid droplets, and increased triglyceride content ($p<0.0005$, XX vs. XY mice) (Figure 4A, 4B). The XX mice also exhibited evidence of reduced insulin sensitivity, as fasting insulin levels and HOMA were elevated 2-fold compared to XY mice in the presence of similar glucose levels (Figure 4C). These metabolic disturbances were not associated with increased circulating triglyceride or free fatty acid levels in XX mice, which instead differed between gonadal males and females (Figure 4D). This suggests that triglyceride and fatty acid levels are influenced by organizational hormone effects rather than sex chromosome complement, and are not likely an underlying factor in the development of the fatty liver in XX vs. XY mice.

Gene expression in liver of mice fed the high fat diet showed enhanced expression of lipogenic factors, including the transcription factor peroxisome proliferator-activated receptor γ, and the triglyceride biosynthetic enzyme diacylglycerol acyltransferase 1 (Figure 4E). Hepatic expression of genes encoding proteins involved in fatty acid uptake (Cd36), fatty acid synthesis (fatty acid synthase), and fatty acid desaturation (stearoyl CoA desaturase) were not significantly different among the four genotypes (data not shown). Despite the increased triglyceride accumulation, fatty acid oxidation gene expression was also elevated in XX compared to XY liver (Figure 4F; $p<0.005$). This pattern was also observed in XX mice fed the chow diet (Figure S2B), and may represent an adaptive or compensatory response that prevents even more pronounced fat storage in XX mice. In contrast to liver, *Aox1* and *Cpt1b* mRNA levels in muscle correlated with gonadal sex rather than sex chromosome complement (Figure 4G). Metabolic gene expression is clearly under complex control, with sex chromosomes and gonadal sex effects having differing roles in specific tissues and conditions. Overall, our results reveal that the XX chromosome complement led to accelerated weight gain and less desirable metabolic profile than XY mice in response to a high fat diet.

The number of X chromosomes, not the presence of the Y chromosome, determines differences in body weight and adiposity

XX mice differ genetically from XY mice in both the dose of the X chromosome and in the absence of a Y chromosome. We analyzed body weight and fat mass in mouse strains with abnormal Y chromosomes that allow the dissection of effects of X and Y chromosome number. As described below, our results indicate that the XX vs. XY difference is caused by genes on the X chromosome and not the Y chromosome.

We took advantage of mice carrying an unusual Y chromosome, Y*, that undergoes abnormal recombination with the X chromosome, producing mice with aberrant numbers of X or Y chromosomes [25,41]. Thus, by breeding XY* fathers, we obtain progeny with the following genotypes: XX, XX^{Y*} (similar to XXY), XY* (similar to XY), and $XY*^{X}$ (similar to XO+an extra pseudoautosomal region, PAR) (see Table S1). After gonadectomy at day 75, mice with two X chromosomes (XX and XXY) had higher body weight ($p<0.000001$) and fat mass ($p<0.0005$) than mice with one X chromosome (XY and XO+PAR) (Figure 5A, 5B). The presence of the Y chromosome appeared to have no effect. We conclude that the inherent genetic difference conferred by presence of two X chromosomes is responsible for the effects on body weight and adiposity.

Elevated expression levels of genes escaping X chromosome inactivation in adipose tissue and liver

A potential mechanism underlying the observed effect of two X chromosomes on adiposity is the presence of a higher dose of X chromosome genes in XX compared to XY cells. Although X inactivation prevents most X genes from being expressed at higher levels in females, it is well established that a proportion of X chromosome genes in both mouse and human escape inactivation [31,32,33,34]. If genes that escape X chromosome inactivation are expressed at higher levels in metabolic tissues of XX than XY mice, they may contribute to the differences that we have observed between XX and XY mice. We evaluated the expression levels in adipose tissue depots and liver of the FCG mice for protein-coding genes that have been shown to escape X inactivation in an interspecific female mouse cell line, or are candidate ''escapees'' from X-inactivation because of higher expression in XX vs. XO mice, or XX vs. XY mice (listed in Figure 6A) [34,35,42,43].

We found that 8 of 11 genes in our panel exhibited higher expression levels in XX compared to XY mouse adipose tissues (gonadal and/or inguinal) (Figure 6A). These include four genes that are established X escapees in both mouse and human (*Eif2s3x*, *Kdm6a*, *Ddx3x*, *Kdm5c*), and these genes also show higher expression in gonadectomized XX liver as well as adipose tissue (Figure 6A–6D). Another gene that is also known to escape inactivation in mouse and human, *Mid1*, exhibited a unique expression pattern, with significantly lower expression in XX compared to XY inguinal fat and liver. The mechanism for this reduced expression in XX tissues is unclear, but nevertheless constitutes a difference that is determined by sex chromosome complement. Only a handful of genes (*Ddx3x*, *Uba1*, *Mid1*) showed significant differences in expression levels between gonadal female and male mice, which may reflect long-lasting effects of gonadal hormones on

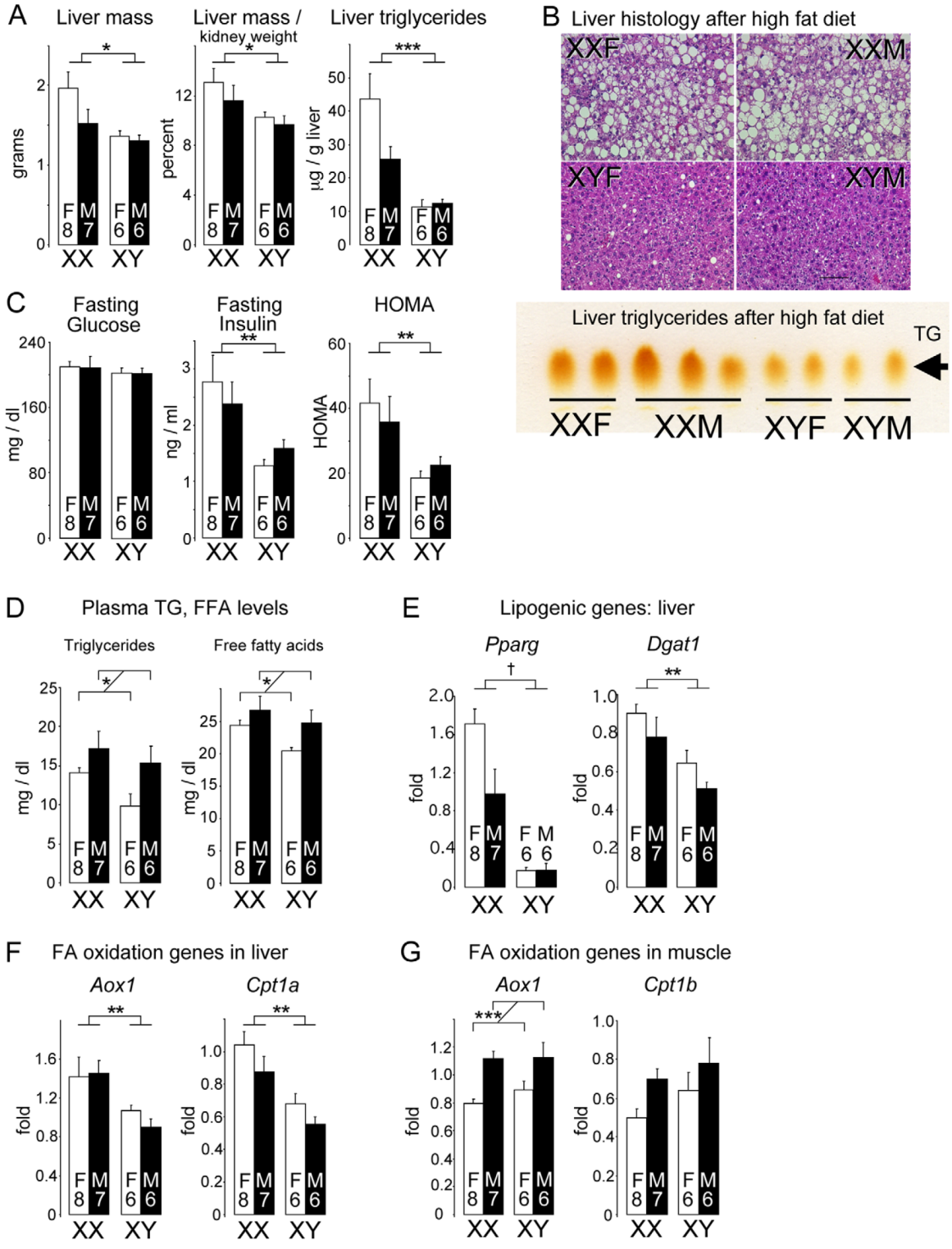

Figure 4. Diet-induced fatty liver and impaired glucose homeostasis are more pronounced in XX than XY mice. FCG mice were fed a high fat-high carbohydrate diet for 16 weeks. All values represent mean ± SEM. (A) XX mice had increased liver mass and hepatic triglyceride levels compared to XY mice. (B) Hematoxylin and eosin staining of liver sections shows hepatic lipid droplet accumulation in XX mice. In the lower panel,

thin layer chromatography demonstrates increased triglyceride levels in liver of XX mice relative to XY mice. (C) Fasting glucose and insulin levels, and homeostatic model assessment (HOMA) were determined. XX mice had significantly higher insulin levels than XY mice. (D) Fasting plasma triglyceride (TG) and free fatty acid (FFA) levels were higher in gonadal males than in gonadal females. (E) Increased triglyceride accumulation in XX liver is associated with increased peroxisome proliferator-activated receptor γ (*Pparg*) and diacylglycerol acyltransferase 1 (*Dgat1*) mRNA levels. (F) Increased expression levels for fatty acid oxidation genes acyl CoA oxidase 1 (*Aox*1) and carnitine palmitoyltransferase 1α (*Cpt1a*) in liver of XX mice. (G) Increased expression of Aox1 in skeletal muscle of gonadal male mice. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<0.0001$.
doi:10.1371/journal.pgen.1002709.g004

expression levels of these genes. These results reveal that a subset of genes escaping X inactivation are expressed at elevated levels in metabolic tissues of XX compared to XY mice. These genes represent candidates for future studies to identify the mechanism by which increased X chromosome dosage affects adiposity and metabolism.

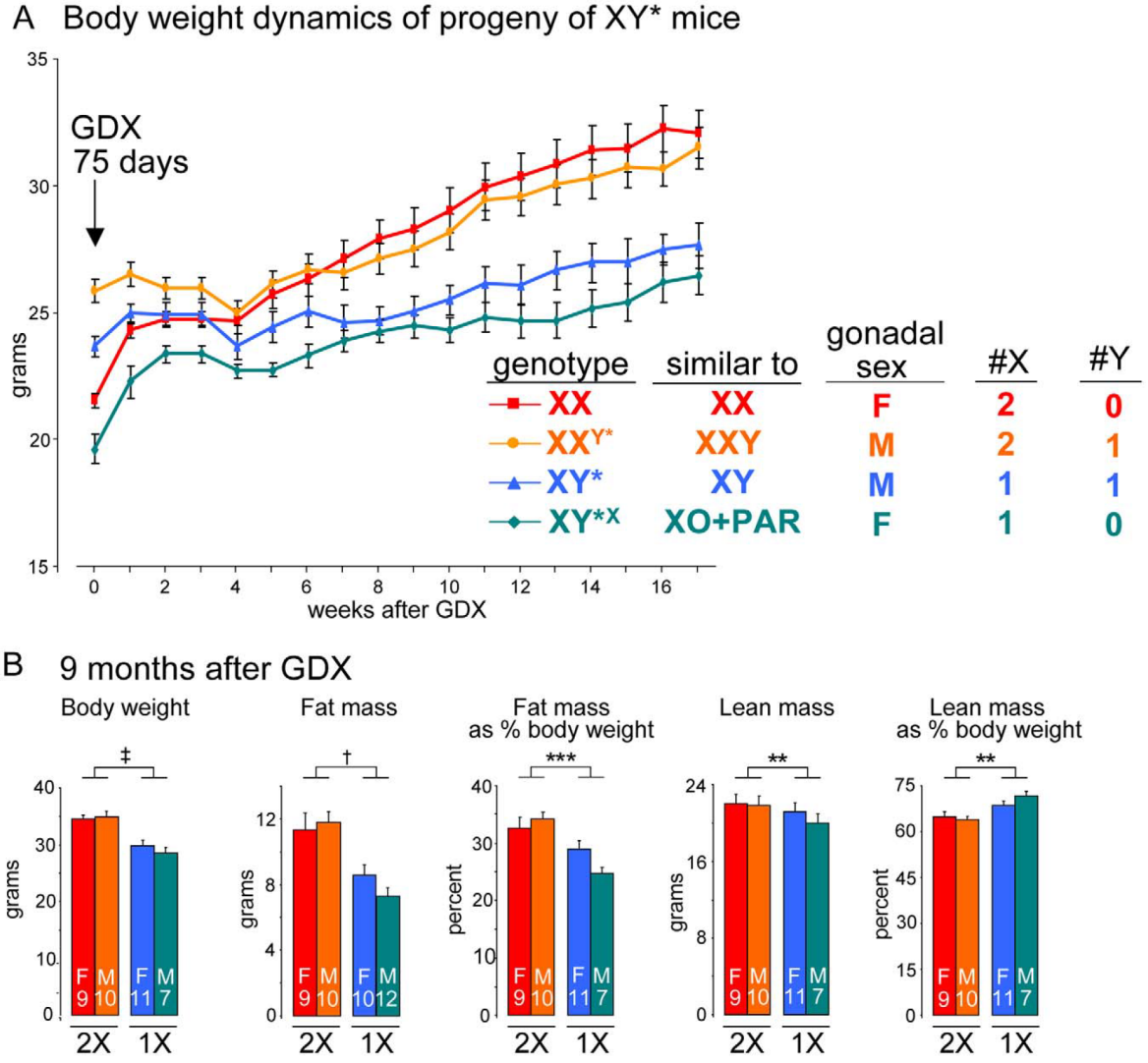

Figure 5. The number of X chromosomes determines differences in body weight and adiposity. (A) Body weight following gonadectomy at day 75 of mice having the indicated sex chromosome complements fed a chow diet. The Y* chromosome has been described [41], and the near equivalent genotype in terms of X and Y chromosome complement is shown. Two groups of mice with two X chromosomes had higher body weight, relative to mice with one X chromosome, beginning at 8 weeks after GDX and thereafter ($p<0.000001$). Mice with a Y chromosome did not differ from mice lacking a Y chromosome. (B) Body weight and body composition determined by NMR in mice shown in panel (A) at 9 months after GDX. Values shown for all bars represent mean ± SEM for the number of each genotype indicated. **, $p<0.01$; ***, $p<0.001$; †, $p<0.0001$; ‡, $p<0.000001$.
doi:10.1371/journal.pgen.1002709.g005

A

X chrom gene	Liver		Inguinal fat		Gonadal fat	
	XX > XY	F vs. M	XX > XY	F vs. M	XX > XY	F vs. M
Eif2s3x	0.000001	NS	0.002	NS	0.000002	NS
Kdm6a	0.002	NS	0.003	NS	0.003	NS
Ddx3x	0.002	NS	0.003	NS	0.000001	0.006
Kdm5c	0.02	NS	NS	NS	0.00008	NS
Usp9x	NS	NS	NS	NS	0.04	NS
Uba1	NS	NS	NS	NS	0.04	0.003
Rik	NS	NS	0.007	NS	na	na
Shroom4	NS	NS	0.04	NS	na	na
Carb5b	NS	NS	NS	NS	na	na
Bgn	NS	NS	NS	NS	na	na
	XX < XY	F vs. M	XX < XY	F vs. M	XX > XY	F vs. M
Mid1	0.009	NS	0.0001	0.00004	na	na

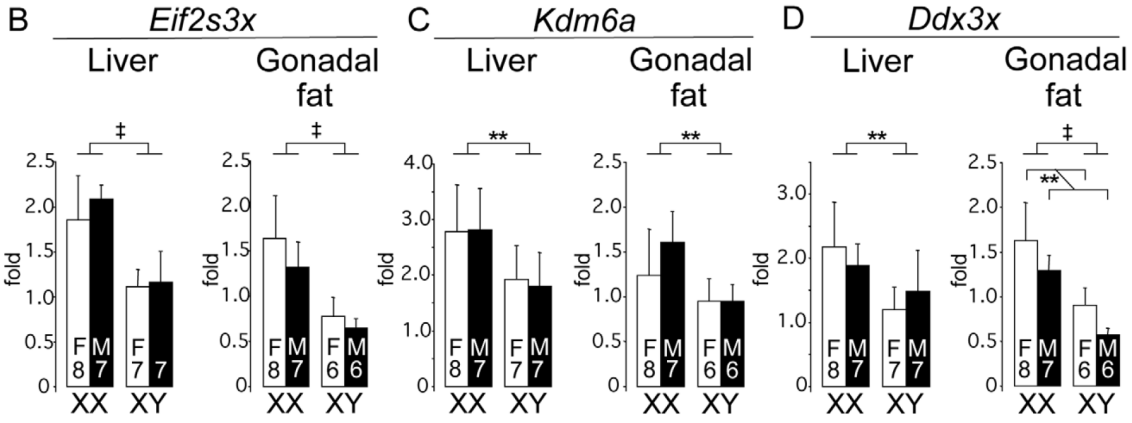

Figure 6. Differential gene expression in liver and fat tissues of X chromosome genes that escape inactivation. Genes previously shown to escape X chromosome inactivation (see text) were assessed for expression levels by quantitative PCR in liver and adipose tissue (subcutaneous inguinal and gonadal depots) of gonadectomized, chow fed FCG mice shown in Figure 1B (10 months post-GDX). (A) Statistical differences in gene expression levels among the FCG genotypes for genes escaping X chromosome inactivation. For each gene, the p value for differences between XX and XY, and female (F) vs. male (M), are shown. Several escapees exhibit increased expression in liver and/or adipose tissues of XX compared to XY mice; a few genes also exhibit differences between levels in gonadal females and gonadal males. *Mid1* shows a unique pattern, with lower expression levels in XX compared to XY tissues. The full name for *Rik* is 2610029G23Rik. NS, not significantly different. (B–D) mRNA levels are shown for liver and gonadal fat tissue of FCG mice for *Eif2s3x*, *Kdm6a*, and *Ddx3x*. Values shown for all bars represent mean ± SEM for the number of each genotype indicated. **, $p<0.01$; ‡, $p<0.000001$.
doi:10.1371/journal.pgen.1002709.g006

Discussion

Sexual dimorphism occurs in many fundamental metabolic processes, which likely influence the development of metabolic diseases. Understanding the sex-specific factors and pathways that promote or mitigate disease may lead to a better understanding of disease pathogenesis and useful interventions. The present results illustrate the complex interplay between several major classes of sex-specific factors that cause sexual dimorphism in obesity, and highlight the utility of the FCG model for investigating such interactions. For the first time, we demonstrate that sex chromosome complement, independent of gonadal sex, has substantial effects on body weight and adiposity in adult mice on a chow diet, and on the rate of weight gain in mice fed a high fat diet. We found that the increased adiposity observed in XX mice is attributable to the presence of two X chromosomes rather than to the lack of a Y chromosome. These results focus attention of future studies on a specific set of X chromosome genes that exhibit altered expression in metabolic tissues of XX compared to XY animals because of escape from X chromosome inactivation or sex chromosome-specific imprinting.

The role of sex hormones in the determination of body weight and adiposity has been documented in many studies in humans and rodent models. For example, gonadally intact male mice have higher body weight, and exhibit more pronounced diet-induced weight gain, than females; this sex difference is reversed partially

or completely by ovariectomy of female mice [44]. In humans, the loss of estrogens with menopause is associated with deposition of visceral body fat, and this effect can be ameliorated to some extent by hormone replacement therapy [45,46,47,48]. Modulating testosterone levels also affects adipose tissue storage in healthy young men, with testosterone levels inversely correlated with adipose tissue mass [49]. Thus, it is clear that gonadal hormones play a strong role in determining sex differences in adiposity in mice and humans. However, few models have allowed the interrogation of potential genetic effects underlying sex differences independent of gonadal hormones.

In our characterization of the FCG mice, body weight and/or metabolic traits were influenced by all three of the major classes of sex-biasing factors: activational (acute) hormonal effects, long-lasting (organizational) hormonal effects, and sex chromosome effects [25,28]. Several traits were influenced by interactions between two or more of these factors. At 75 days of age, gonadal males weighed 25–28% more than gonadal females, irrespective of their sex chromosome complement, suggesting that the sex difference is caused by gonadal secretions. That conclusion was confirmed because the sex difference disappeared by one month after gonadectomy. However, further analysis of the FCG model revealed that attributing sex differences in body weight solely to gonadal hormones would be a significant oversimplification. Prior to gonadectomy, XX mice weighed 6–9% more than XY mice, in both gonadal males and females. The XX vs. XY difference became dramatically larger after gonadectomy, with XX mice having up to 2-fold greater adiposity than XY mice. Layered on top of this was an effect of *Sry* (likely mediated by long-lasting effects of the original gonadal hormones), as without their gonads, gonadal female XX mice lacking *Sry* had higher body weight, fat pad mass, and plasma leptin levels than gonadal male XX mice possessing *Sry*. The results indicate that although sex chromosome effects act in both intact and gonadectomized mice, gonadal hormones blunt the influence of sex chromosome complement, suggesting that the hormones may have different effects depending on the chromosomal sex of cells. Thus, understanding how males and females differ from one another is not simply a matter of studying an apparently dominant factor that causes the sex difference, but requires disentangling the interactive effects of several sex-biasing factors.

The increased body weight of XX mice was preceded by increased food intake compared to XY mice; interestingly, this was restricted to the light portion of the diurnal cycle (see Figure 2A). After differences in adiposity were established between XX and XY mice, food intake was not distinguishable, but leptin levels were elevated in XX mice, suggesting relative leptin resistance in the XX mice. Since there were no detectable compensatory changes in energy expenditure or physical activity in XX mice, this increase in food intake likely contributes to the increased body weight. The increased consumption of carbohydrates during the light period was reflected in slightly elevated RQ during the same period. This difference was evident even before the GDX XX mice had increased body weight. A trend toward increased food intake during the light period continued after the XX mice were substantially heavier (at 10 months post-GDX), although it was no longer statistically significant. A recent study has shown that mouse food intake during the light period of the circadian cycle leads to greater weight gain than equivalent intake during the dark period, when mice typically consume the majority of their calories [50]. Many other studies have provided evidence that energy balance is tightly integrated with the circadian clock and that disruption of this cycle has detrimental effects on many aspects of metabolism [51,52]. Thus, a focus of future studies in the FCG model will be the investigation of whether sex chromosome complement influences regulation of the circadian clock and/or networks for food consumption and satiety.

Sex chromosome complement had a key effect on the response to a high fat diet, with XX mice having an almost immediate divergence in weight gain from XY mice. An interesting finding was the greater expansion in the subcutaneous fat depot in XX mice, and greater increase in the gonadal fat depot in XY mice. It has been shown that women store a greater percentage of dietary fatty acids in subcutaneous adipose tissue than men [53]. Our observations in mice raise the possibility that sex chromosome complement may be a factor in determining the propensity to store fat in various anatomical depots.

The high fat diet also led to the development of more pronounced metabolic dysregulation in XX mice, particularly fatty liver. Non-alcoholic fatty liver disease affects up to one-third of American adults, usually in association with obesity and insulin resistance [54,55]. The occurrence of fatty liver disease and its progression to cirrhosis, end-stage liver disease and hepatocellular carcinoma are influenced by many factors, including genetic factors. Our studies reveal that XX sex chromosome complement is one genetic factor that promotes development of fatty liver in mice. It is likely that the fatty liver in XX mice fed the high fat diet was influenced by risk factors such as increased adiposity and hyperinsulinemia. Interestingly, however, fatty liver did not parallel circulating triglyceride and fatty acid levels, which were more influenced by gonadal sex (likely influenced by organizational effects of gonadal hormones) rather than sex chromosome complement. In future studies, it will be interesting to determine whether sex chromosome complement also influences the propensity for progression of steatotic livers to cirrhosis, the basis of which is currently not understood.

The sex chromosome effects reported here indicate that inherent sex differences in expression of X chromosome genes, stemming from the difference in number or parental imprint of X genes in XX vs. XY mice, contribute to sex differences in adiposity and metabolic disease. The sex chromosome effects are unlike typical linkage of genes to phenotype, because they are not caused by differences in the genetic sequence of the X chromosome, which was identical in all mice studied. Because X-inactivation effectively reduces the inherent bias toward higher expression of X genes in XX mice relative to XY mice, prime candidates for the genes causing the adiposity are those that escape X inactivation, or those that receive a parental imprint, leading to differential expression in XX compared to XY mice [28].

A significant proportion of X chromosome genes (15–25%) are thought to escape X chromosome inactivation in humans [56], and most of the genes escaping X inactivation in mice also escape in humans [34]. We tested expression levels of candidate genes that are known to escape inactivation in both mouse and human (*Eif2s3x*, *Kdm6a*, *Kdm5c*, *Ddx3x*) or have a Y paralogue with some evidence for higher expression in XX than XY mice and humans (*Usp9x*, *Uba1*) [31,34,35,42,43,57]. Each of these genes was expressed at higher levels in XX than XY gonadal fat in gonadectomized mice, providing evidence that these genes escape inactivation in a metabolic tissue. Thus, these genes are candidates for those causing the XX–XY differences in physiology and adiposity reported here. Alternatively, differential expression of X chromosomes escapee genes could occur secondarily to differences in adiposity between XX and XY mice, in which case they may be downstream players in the observed metabolic differences. In addition to sex chromosome genes, autosomal genes that are differentially expressed in response to X chromosome gene dosage may contribute to the observed metabolic differences between XX

and XY mice. It is known, for example, that the dysregulation of genes involved in mitochondrial metabolism and protein translation occurs in tissues of XX compared to XO mice, but the metabolic consequences are not known [35].

A reasonable question is whether these studies in the mouse have relevance to obesity in humans. Unusual numbers of sex chromosomes in human conditions such as Klinefelter (XXY) and Turner (XO) syndromes are associated with metabolic disease and/or adiposity [58,59,60,61]. However, in these diseases, endocrine abnormalities may contribute and are difficult to distinguish from the sex chromosome effects. The utility of our model is that it is genetically tractable in a way that human studies are not. Since fundamental genetic and metabolic processes are shared between mice and humans, we propose that the identification of X-linked genes that have a large impact on obesity in the mouse could lead to the discovery of novel mechanisms that impact obesity in humans. The increasing longevity of the human population means that the hypogonadal period may extend for up to half of a persons' lifetime, and the inherent genetic sex differences uncovered here may have important ramifications. Furthermore, since the gene content of the X chromosome is conserved in mouse and human, and several of the same genes escape inactivation in both species, there is hope that characterizing the action of X gene(s) in mouse will advance our understanding of human metabolic disease.

Materials and Methods

Ethics statement

All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the appropriate committee. All experiments in this paper were carried out with UCLA IACUC approval.

Mice

"Four core genotypes" (FCG) mice were used. "Male" denotes a mouse with testes, and "female" denotes a mouse with ovaries. In these mice, the testis-determining *Sry* gene is deleted from the Y chromosome and inserted as a transgene onto an autosome [27,36]. Thus, gonadal type is no longer controlled by sex chromosome complement (XX vs. XY), and the effect of sex chromosome complement on traits can be studied independent of the gonadal type of the mouse. For the present study, the FCG model was transferred to a C57BL/6J (B6) genetic background by backcrossing male MF1 XY^{-}*Sry* (Y^{-} chromosome denotes deletion of *Sry*; *Sry* denotes presence of the autosomal *Sry* transgene) to B6 XX females for 13–14 generations. Four groups of mice are generated, XX and XY gonadal males (XXM and XYM, carrying the *Sry* transgene), and XX and XY gonadal females (XXF and XYF, without *Sry*). In all the FCG mice the Y^{-} chromosome derives from strain 129. Advantages and caveats in the use of FCG mice have been discussed [25,27].

Gonadectomy was performed at 75 days of age. Under isoflurane anesthesia, mice were given a subcutaneous injection of carprofen and the gonads were removed. Using aseptic procedures, gonads were exposed, clamped, ligated, and excised. Successful gonadectomy was confirmed at the time of euthanasia. Although no gonadal hormones are present in GDX mice, sex steroid hormones (*e.g.*, androgens or estrogens produced *de novo* in adrenal, adipose tissue, or brain) are probably present in mice after GDX.

In one study, we compared mice born of XY* fathers, which have an aberrant Y chromosome that recombines abnormally with the X chromosome. The XY* males from strain B6Ei.LT-Y*/EiJ from the Jackson Laboratories were crossed with B6/J females for 2–3 generations, so that the mice were a mixture of C57BL/6J and C57BL/EiJ strains. In all case littermates were compared, so that the percentage of the two B6 parental strains was comparable across groups. We studied four different types of progeny of XY*: XX, XX^{Y*}, XY*, and $XY*^{X}$. These mice are roughly similar to XX, XXY, XY, and XO+an extra pseudoautosomal region, respectively (see Table S1) [41].

Gonadal males and females were housed in separate cages and maintained at 23°C with a 12:12 light:dark cycle. For studies using chow fed mice, mice were fed Purina 5001 chow diet (approximately 5% fat, PMI Nutrition International, St. Louis, MO) throughout their lifetime. For high fat diet treatment, mice were gonadectomized at 75 days of age and 4 weeks later were switched from chow to a high fat diet containing 35% fat, 33% carbohydrate (Diet F3282, Bio-Serve, Frenchtown, New Jersey). Fresh diet was added to cages twice per week. Animal studies were performed under approval of the UCLA Institutional Animal Care and Use Committee.

Genotyping and karyotyping

DNA was extracted from tails using Chelex resin (Bio-Rad, Hercules, CA). The genotype of mice was determined by PCR based on the presence or absence of *Sry* and of the X/Y chromosome paralogues Jarid1d/Jarid1c [41]. Ear fibroblasts from offspring of XY* mice were cultured and metaphase spreads were used to determine the sex chromosome status based on karyotype [41].

Measurement of body weight and body composition

FCG and XY* mice were weighed on postnatal days 21, 45 and day 75 and then gonadectomized (GDX) on day 75. After GDX mice were weighed at weekly intervals. At various ages, body composition was determined with a Mouse Minispec apparatus (Bruker Woodlands, TX) with Echo Medical Systems (Houston, TX) software. This apparatus uses NMR spectroscopy for fat and lean mass measurements with coefficients of variation of $<3\%$ [62]. Correlation between NMR and gravimetric measurements is better than 0.99.

Energy balance measurements

Eight calibrated Oxymax metabolic cages (Columbus Instruments) were used to detect numerous variables related to energy balance: food and water intake, horizontal and vertical physical activity, heat production, oxygen consumption, CO_2 production, energy expenditure, and respiratory quotient (RQ). The room housing the metabolic cages was kept very quiet to avoid stress or other interference with the activity of the mice. Mice were housed individually in the Oxymax metabolic cages from midday Friday to midday Monday, during which parameters were monitored dynamically at 20 min intervals. Mice had free access to water and food resented from a food hopper attached to a scale. Data for 3 full nights and 2 full days were analyzed.

Glucose homeostasis

Baseline glucose and insulin levels were determined after a 4.5-hour fast (8:00AM–12:30PM). Glucose tolerance tests were performed after similar fast by injecting mice intraperitoneally with glucose (2 mg/g body weight) and determining glucose levels (using Lifescan OneTouch glucose meter) at 15, 30, 60 and 180 minutes after injection [63].

Quantitative RT–PCR

Liver, quadriceps skeletal muscle, gonadal fat, inguinal fat and brown fat were dissected out rapidly, flash frozen in liquid nitrogen, and stored at −80°C. RNA was isolated from tissues using Trizol (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNase (Promega, Madison, USA) to remove possible genomic DNA contamination. First-strand cDNA synthesis was generated by reverse transcription with SuperScript III RNase H-RT (Invitrogen). Quantitative real time PCR (n = 7–8 per genotype) was performed on an ABI 7300 Sequence Detection system (Applied Biosystems, Foster City, CA, USA) using the SensiMix*Plus*SYBR Green & Fluorescein Master Mix Kit (Quantace, USA). Two or three control genes were amplified as normalization controls: beta-2 microglobulin, TATA box-binding protein (TBP), and BC022960. Primer sequences for all genes assessed are listed in Table S2. Cycling conditions were: 95°C for 10 min; 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. assay contained a standard curve for the target gene and control genes with 4 serial dilution points of control cDNA: 50 ng, 10 ng, 2 ng and 0.4 ng. Dissociation curves were examined to eliminate the possibility of genomic DNA contamination.

Statistical analyses

Groups were compared using two-way ANOVA (NCSS 2001; Number Cruncher Statistical Systems, Kaysville, UT, USA) with main factors of sex (gonadal male vs. gonadal female, same as *Sry* present vs. absent) and sex chromosome complement (XX vs. XY). Sometimes a three-way repeated measures ANOVA was also applied with between factors of sex and sex chromosome complement, and within factors of gonadal status (before vs. after GDX) or age. Statistical analyses (main effects of each of the two factors, or interaction of the two) are presented if they were statistically significant, but usually not if they were not significant ($p>0.05$). Multiple regression analyses of energy metabolism data was performed with Stata Data Analysis and Statistical Software (StataCorp LP, College Station, TX).

Supporting Information

Figure S1 Energy metabolism measurements in FCG mice fed a chow diet. Oxygen consumption (VO_2) and physical activity along horizontal and vertical axes were determined in mice individually housed in metabolic cages at 4 weeks (A) and 10 months (B) following GDX. No significant differences were detected among the four genotypes in these parameters during dark or light cycles. (C) Thermogenic gene expression was assessed in brown adipose tissue at 4 weeks and 10 months following GDX. Uncoupling protein 1 (*Ucp1*) gene expression was higher in male than female mice at 4 weeks after GDX, but this difference was no longer apparent at 10 months. No significant differences in peroxisome proliferator-activated receptor γ coactivator α (*Pgc1*) mRNA levels were detected. Each bar represents mean ± SEM for the number of mice of each genotype indicated. **, $p<0.01$.
(DOC)

Figure S2 Glucose homeostasis and gene expression in FCG mice fed a chow diet. (A) Glucose and insulin levels were determined after a 4 hour fast (08:00–12:00). Glucose tolerance was assessed by intraperitoneal injection of glucose and blood collection at intervals over 3 hours. The GTT AUC represents the area under the curve of blood glucose levels plotted from time 0 to 3 hours post glucose injection. (B) Gene expression determined by qPCR for acyl CoA oxidase 1 (*Aox1*) and carnitine palmitoyltransferase (*Cpt*) in muscle and liver, as indicated. Each bar represents mean ± SEM for the number of mice of each genotype indicated. *, $p<0.05$; **, $p<0.01$; †, $p<0.0001$.
(DOC)

Table S1 Sex chromosome composition of offspring from XY* x XX mice. The copy number of specific regions of the X and Y chromosomes present in mice of each genotype is indicated. NPX, non-pseudoautosomal region of the X chromosome. MSY, male-specific region of the Y chromosome. Xm, maternal X imprint. Xp, paternal X imprint. Refer to [41] for illustrations of chromosome structures.
(DOC)

Table S2 Primer sequences for gene expression analyses by qPCR.
(DOC)

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Author Contributions

Conceived and designed the experiments: XC APA KR. Performed the experiments: XC RM JC SWB KR. Analyzed the data: XC JC APA KR. Contributed reagents/materials/analysis tools: PT. Wrote the paper: APA KR.

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SUPPLEMENTAL INFORMATION

FIGURE LEGENDS

Figure S1. Energy metabolism measurements in FCG mice fed a chow diet. Oxygen consumption (VO_2) and physical activity along horizontal and vertical axes were determined in mice individually housed in metabolic cages at 4 weeks (A) and 10 months (B) following GDX. No significant differences were detected among the four genotypes in these parameters during dark or light cycles. (C) Thermogenic gene expression was assessed in brown adipose tissue at 4 weeks and 10 months following GDX. Uncoupling protein 1 (*Ucp1*) gene expression was higher in male than female mice at 4 weeks after GDX, but this difference was no longer apparent at 10 months. No significant differences in peroxisome proliferator-activated receptor γ coactivator α (*Pgc1*) mRNA levels were detected. Each bar represents mean ± SEM for the number of mice of each genotype indicated. **, $p<0.01$.

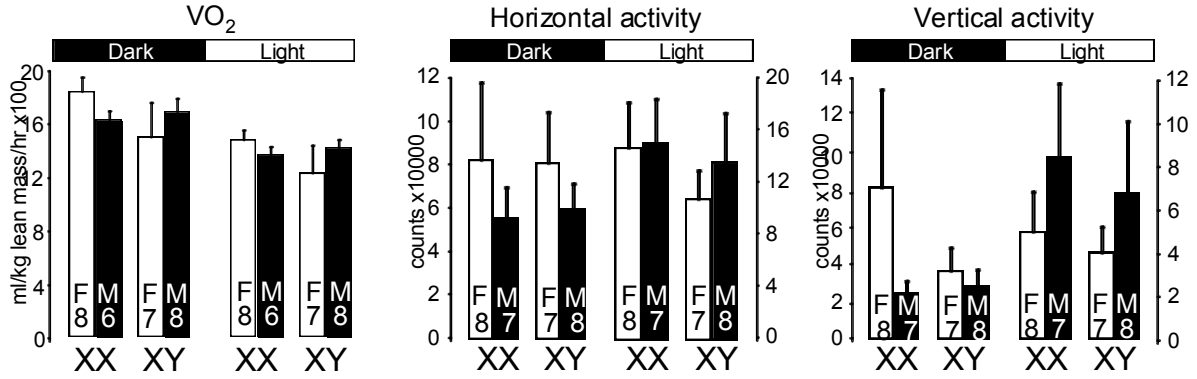
Figure S2. Glucose homeostasis and gene expression in FCG mice fed a chow diet. (A) Glucose and insulin levels were determined after a 4 hour fast (08:00–12:00). Glucose tolerance was assessed by intraperitoneal injection of glucose and blood collection at intervals over 3 hours. The GTT AUC represents the area under the curve of blood glucose levels plotted from time 0 to 3 hours post glucose injection. (B) Gene expression determined by qPCR for acyl CoA oxidase 1 (*Aox1*) and carnitine palmitoyltransferase (*Cpt*) in muscle and liver, as indicated. Each bar represents mean ± SEM for the number of mice of each genotype indicated. *, $p<0.05$; **, $p<0.01$; †, $p<0.0001$.

Table S1. Sex chromosome composition of offspring from XY* x XX mice. The copy number of specific regions of the X and Y chromosomes present in mice of each genotype is

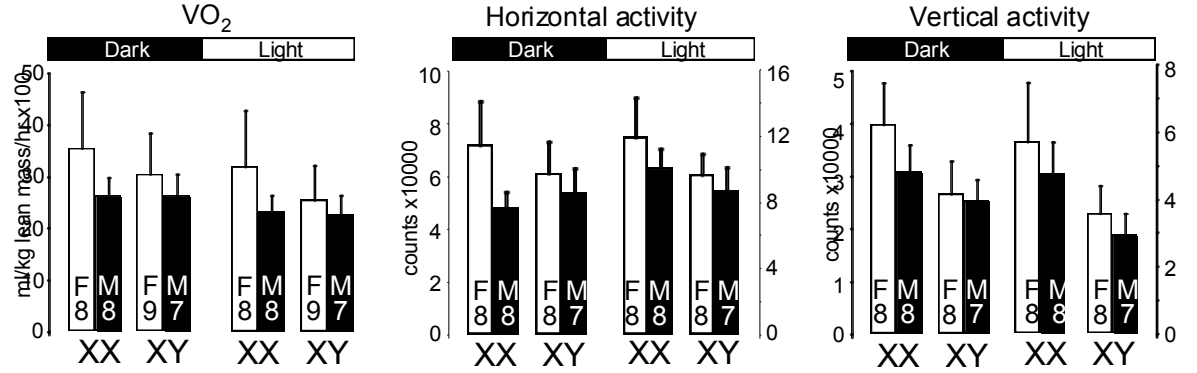
indicated. NPX, non-pseudoautosomal region of the X chromosome. MSY, male-specific region of the Y chromosome. Xm, maternal X imprint. Xp, paternal X imprint. Refer to [41] for illustrations of chromosome structures.

Table S2. Primer sequences for gene expression analyses by qPCR.

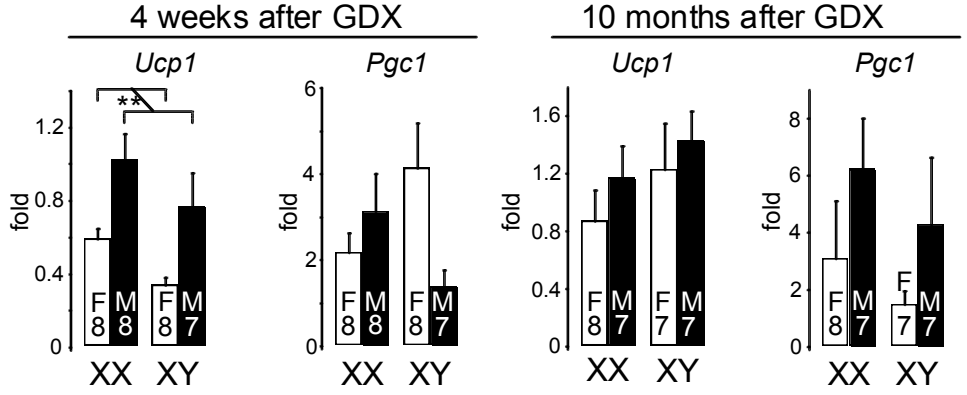
A 4 weeks after GDX



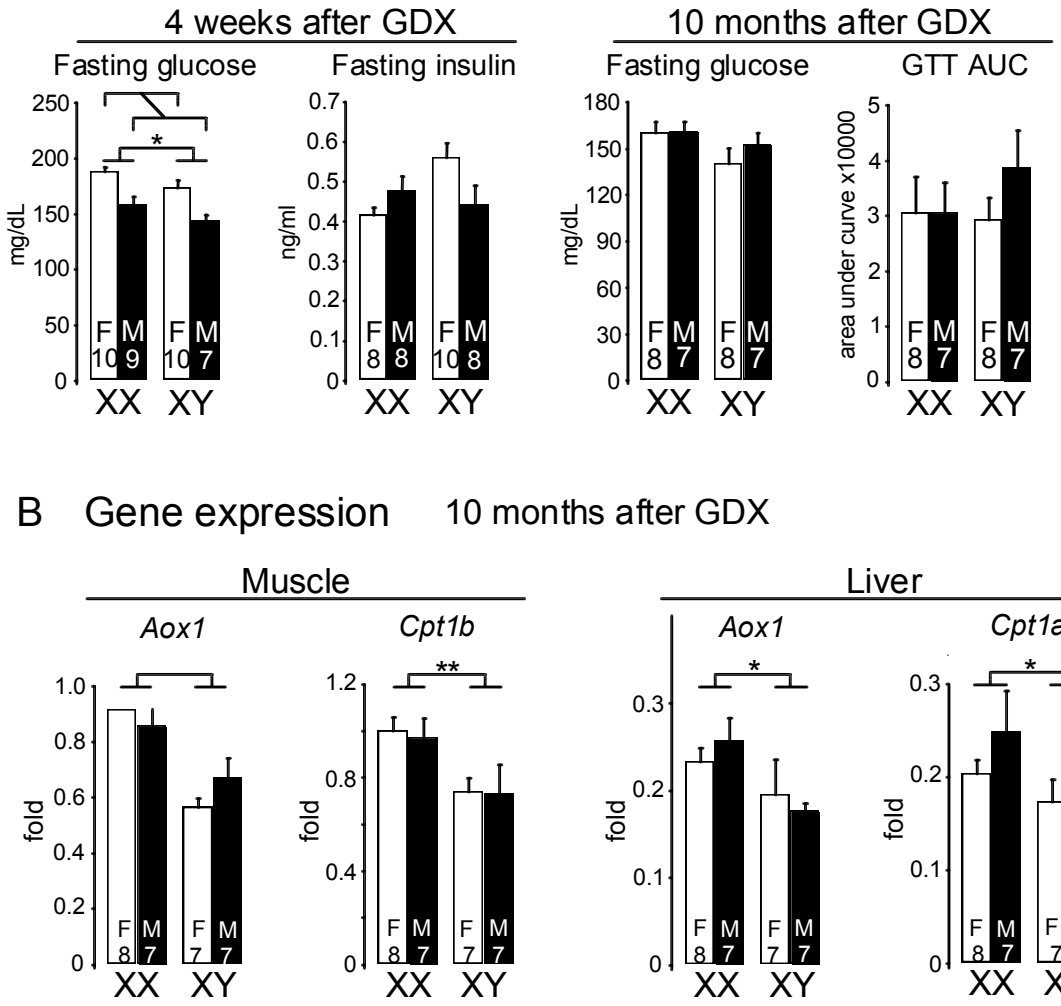
B 10 months after GDX



C Gene expression in brown adipose tissue



Suppl. Fig. 1


A Glucose homeostasis
Chow diet
4 weeks after GDX
10 months after GDX
Fasting glucose
Fasting insulin
Fasting glucose
GTT AUC
mg/dL
ng/ml
mg/dL
area under curve x10000
F 10
M 9
F 10
M 7
F 8
M 8
F 10
M 8
F 8
M 7
F 8
M 7
XX
XY
B Gene expression
10 months after GDX
Muscle
Liver
Aox1
Cpt1b
Aox1
Cpt1a
fold
F 8
M 7
F 7
M 7
XX
XY

Suppl. Fig. 2

SUPPLEMENTAL TABLE 1

Offspring from XY* x XX mice.

NPX, non-pseudoautosomal region of the X chromosome. MSY, male-specific region of the Y chromosome. Xm, maternal X imprint. Xp, paternal X imprint.

	similar to	Sex	*Sry*	NPX	MSY	Xm	Xp
XX	XX	F	0	2	0	1	1
XX^{Y*}	XXY	M	1	2	1	1	1
XY*	XY	M	1	1	1	1	0
XY^{*X}	XO+PAR	F	0	1	0	1	0

SUPPLEMENTAL TABLE 2. qPCR primers used for gene expression analysis

Genes	primers
β2m (beta-2 microglobulin)	forward: 5'-TGGTGCTTGTCTCACTGACC-3' reverse: 5'-GTATGTTCGGCTTCCCATTC-3'
Tbp (TATA box binding protein)	forward: 5'-ACCCTTCACCAATGACTCCTATG-3' reverse: 5' ATGATGACTGCAGCAAATCGC-3'
Bc022960 (Mus musculus cDNA BC022960)	forward: 5'-ATCCCCAAGATCATCAGCAG-3' reverse: 5'-TTCGTTAGCCCACTTGCTTT-3'
Aox1 (acyl CoA oxidase 1)	forward: 5'-CAGGAAGAGCAAGGAAGTGG-3' reverse: 5'-CCTTTCTGGCTGATCCCATA-3'
*Cpt1a (*carnitine palmitoyltransferase Ia)	forward: 5' AAACCCACCAGGCTACAGTG-3' reverse: 5'-TCCTTGTAATGTGCGAGCTG-3'
*Cpt1b (*carnitine palmitoyltransferase Ib)	forward: 5'-GTCGCTTCTTCAAGGTCTGG-3' reverse: 5'- AAGAAAGCAGCACGTTCGAT-3'
Dgat1(diacylglycerol O-acyltransferase 1)	forward 5'-TGCTACGACGAGTTCTTGAG-3' reverse: 5'-CTCTGCCACAGCATTGAGAC-3'
Leptin	forward 5'-GCTCCAGCAGCTGCAAGGTG-3' reverse: 5'-AAGTCCAAGCCAGTGACCCTC-3'
Adiponectin	forward: 5'-GGAACTTGTGCAGGTTGGAT-3' reverse: 5'-GCTTCTCCAGGCTCTCCTT-3'
Ucp1 (uncoupling protein1)	forward: 5'-GGGCCCTTGTAAACAACAAA-3' reverse: 5'-GTCGGTCCTTCCTTGGTGTA-3'
Pparγ (peroxisome proliferator activated receptor gamma)	forward: 5'-CCAGAGCATGGTGCCTTCGCT-3' reverse: 5'-CAGCAACCATTGGGTCAGCTC-3'
Pgc1α (Pparγ, coactivator 1 alpha)	forward: 5'-CTCACAGAGACACTGGACAGT-3' reverse: 5'-TGTAGCTGAGCTGAGTGTTGG-3'
Cd 36 (CD36 antigen)	forward: 5'-TGCAGCTGTTATTGGTGCAG-3' reverse: 5'-TGGGTTTTGCACATCAAAG-3'
Fasn (fatty acid synthase)	forward: 5'-CGTGTTGGCCTACACCCAGAG-3' reverse: 5'-GGCAGCAGGGCCTCCAGCAC-3'
Scd1 (stearoyl-Coenzyme A desaturase 1)	forward: 5'-GGT GATGTTCCAGAGGAGGTA-3' reverse: 5'-GGTGCTAACGAACAGGCT-3'
Ddx3x	forward:5'-GGATCACGGGGTGATTCAAGAGG-3' reverse:5'-CTATCTCCACGGCCACCAATGC-3'
Kdm5c	forward: 5'-ACCCACCTGGCAAAAACATTGG-3' reverse: 5'-ACTGTCGAAGGGGGATGCTGTG-3'
Kdm6a	forward: 5'-CCAATCCCCGCAGAGCTTACCT-3' reverse: 5'-TTGCTCGGAGCTGTTCCAAGTG-3'
Eif2s3x	forward: 5'-TTGTGCCGAGCTGACAGAATGG-3' reverse: 5'-CGACAGGGAGCCTATGTTGACCA-3'
Usp9x	forward: 5'-GCATGTCAGCGATTTTTCCGAGA-3' reverse: 5'-CACATAGCTCCACCAGGCGATG-3'
Uba1	forward: 5'-ACACTGGGCCTCTTGTCGAGGA-3' reverse: 5'-CAGGCCTCTTGTATCTGCCACCA-3'
Mid1	forward: 5'-GCGATCATCAGGTGGCAGCTT-3' reverse: 5'-TTTGGCTTCTTGACGGGATGC-3'
Car5b	forward: 5'-CATTGATGCTTGGGGCTCTGA-3' reverse: 5'-CAGCCAAGCCATTTTCTTCCA-3'
Shroom4	forward: 5'-CAGGAAGGCAGCCATACAGGA-3' reverse: 5'-TCTCAATGCAAGGTCGGAAGG-3'
Bgn	forward: 5'-GTATCCGCAAAGTGCCCAAGG-3' reverse: 5'-TTTGGGGATGCCAGTGAGCTT-3'
2610029G23Rik	forward: 5'-CTCTCTCACCCTCCGGAGTCATA-3' reverse: 5'-CTCACGGAACTCAGAGTAGATTTGG-3'

CHAPTER 4

The Sex Chromosome Complement is a Determinant of Sexual Dimorphism in Diet-Induced Obesity in Mice

Chapter Preface

In the previous chapter, we demonstrated that the sex chromosome complement is a determinant of adiposity. The XX–XY difference in body weight and fat mass became more apparent after mice were gonadectomized, and exacerbated when mice were fed a high fat diet. The conclusions from the studies in gonadectomized mice were relevant for humans in later stages of life, after gonadal hormone levels have waned.

Typically, studies are conducted in gonadectomized Four Core Genotype mice to remove the confounding effect of circulating gonadal hormones. However, this design left a large gap in our understanding of sex differences in obesity in the presence of gonadal hormones. Before this study, it was unknown whether there were sex chromosome effects in diet-induced obesity of gonadally intact mice.

The diet-induced amplification of sex chromosome differences in body weight piqued my interest. In gonadally intact Four Core Genotype mice, XX and XY males weighed more than XX and XY females, and the effect of the sex chromosome complement was small but significant. I wondered if we could enhance sex chromosome differences in body weight by inducing obesity in gonadally intact mice, a diet-sex chromosome effect observed in gonadectomized mice. In the summer of 2013, I began feeding gonadally intact Four Core Genotype mice a high fat diet, and over the course of 10 weeks, lo and behold, XX male and female mice gained more weight than XY mice. When we examined liver tissue for evidence of hepatic steatosis, I was surprised at the striking difference between XX and XY mice — large lipid droplets accumulated only in XX males and females.

Chapter 4 details these studies and offers preliminary data on a candidate gene, *Kdm5c*, which may contribute to the differences observed between XX and XY mice. We provide evidence that supports the role of sex chromosomes as a mediator of sex differences in obesity, independent of gonadal hormones.

The sex chromosome complement is a determinant of sexual dimorphism in diet-induced obesity in mice

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ABSTRACT

Body composition, fat distribution, and metabolic disorders are sexually dimorphic. Sex differences are often attributed to sex hormones, but the sex chromosome complement can also drive differences between males and females. Because ovaries typically co-exist with an XX genotype and testes co-exist with an XY genotype, the independent effects of gonadal hormones *versus* sex chromosomes are difficult to distinguish. Here we use the Four Core Genotypes mouse model (XX female, XX male, XY female, and XY male) to identify independent effects of gonadal hormones and sex chromosome complement in diet-induced obesity. When fed a chow diet, the predominant dimorphism in body weight is associated with male–female gonads. By contrast, the XX *vs*. XY chromosome complement influences response to a high fat diet. Thus, male and female mice with two X chromosomes gain more weight, have higher percent fat mass, consume more food, and have increased hepatic lipid accumulation compared to XY male and female mice. We hypothesize that the dosage of genes escaping X chromosome inactivation partly explains differences in body weight and adiposity between XX and XY mice. In support of this hypothesis, XX mice with two functional alleles of the X chromosome inactivation escapee *Kdm5c,* which encodes a histone demethylase, have greater body weight and food intake than XX mice with one functional *Kdm5c* allele (similar to XY mice). These studies demonstrate that the sex chromosome complement is a significant mediator of sex differences in obesity and metabolism, and implicate dosage of specific genes that escape X chromosome inactivation as likely contributors.

INTRODUCTION

Obesity is a prominent risk factor for some of the leading causes of death, including cancer, heart disease, and diabetes (1–4). Perturbations in numerous factors including genes, diet, physical activity, and circadian rhythm can impair energy homeostasis. An additional factor that has widely recognized effects on energy homeostasis and susceptibility to related diseases is sex.

Fat accumulation and distribution are sexually dimorphic. Women tend to store fat subcutaneously on the hips and thighs, whereas men and postmenopausal women tend to accumulate fat in the abdominal viscera (5). Because an increase in adiposity is seen after a decline in gonadal hormones, these sex differences in obesity have often been attributed to ovarian and testicular hormones. Consistent with this, estrogen replacement in postmenopausal women results in reduced adipose tissue in the central abdominal cavity (6,7). However, in some cases, estrogen hormone therapy does not reduce adipose tissue (8). These data suggest that sex hormones contribute to sex differences in adiposity, but raise the possibility that additional other sex-biasing factors may also be involved.

The defining genetic difference between male and female cells derives from the sex chromosome complement. Typically, effects of the sex chromosome complement are indistinguishable from effects of sex hormones because XX chromosomes are paired with the development of ovaries, and XY chromosomes occur in combination with testes. The Four Core Genotype (FCG) mouse model is an innovative tool to distinguish the effects of gonadal type from sex chromosome complement (9). In FCG mice, gonadal type and sex chromosome complement segregate independently to derive four genotypes that are roughly equivalent to XX female, XX male, XY female, and XY male mice. This is accomplished through the use of a Y chromosome that has an inactivating mutation in the endogenous *Sry* gene (denoted Y^{-}), preventing testes differentiation and causing XY^{-} mice to develop female gonads. In addition, FCG mice segregate an *Sry* transgene that is inserted into an autosome so that it is inherited

independently from the Y^- chromosome. By crossing XX females to XY^-*Sry* males, the four combinations of sex chromosome complement/gonadal type listed above can be obtained. The study of FCG mice allows discrimination between the effects of gonadal type (which manifest as differences between XX/XY male and XX/XY female mice) from effects of sex chromosome complement (which manifest as differences between XX male/female vs. XY male/female mice).

Using the FCG mouse model, we previously demonstrated that mice with two X chromosomes accumulate nearly twice as much fat as mice with XY sex chromosomes when gonadal secretions are removed by gonadectomy after mice reach adulthood (10). This condition may be viewed as analogous to the declining gonadal hormone levels in humans during middle age. However, with the prevalence of obesity increasing in younger populations, it is crucial to understand the effect of the sex chromosome complement on obesity in a gonadally intact state. In the present study, we demonstrate that sex chromosome complement is a determinant of diet-induced obesity and the development of fatty liver in mice with intact gonads. These results suggest that sex differences in obesity and related co-morbidities that are observed between young males and females are determined, in part, by sex chromosome complement. These findings have implications in understanding sex-specific mechanisms that influence development of obesity.

RESULTS

Two X chromosomes promote adipose tissue accumulation

In our previous study, we detected effects of sex chromosome complement on adiposity in mice that had been gonadectomized to remove effects of gonadal secretions. These effects were evident in mice fed a chow diet, but were exacerbated in response to feeding a high fat diet. We wondered whether sex chromosome effects on adiposity can be detected in gonadally intact mice, which would more closely relate to humans prior to middle age. To assess the contribution of the sex chromosome complement to body weight and adiposity in gonadally intact mice, we monitored the body weights of C57BL/6 FCG mice fed a standard chow diet. XX and XY males weighed significantly more than XX and XY females from 10 weeks of age to 32 weeks of age (Fig. 1A). We did not detect sex chromosome differences. We then induced obesity by feeding FCG mice a high fat diet (60% calories from fat) beginning at 14 weeks of age for 10 weeks (Fig. 1B). We chose to study mice at this age, when mice are sexually mature and variations in gonadal secretions that occur during puberty have been stabilized. At 14 weeks of age, before the start of the high fat diet, XX and XY male mice weighed on average 6.1 grams more than female mice (Fig. 1C). This was associated with 44% higher lean mass in males compared to females. On the other hand, females had proportionally higher fat mass (expressed as percent body weight) than males. Sex chromosome complement also influenced body weight. XX males and females weighed nearly 2 grams more than their XY counterparts (Fig. 1C). These results suggest that in young adult mice fed a chow diet, both gonadal hormones and sex chromosome complement influence body weight and composition.

To determine the contributions of gonadal secretions and sex chromosome complement to diet-induced obesity, FCG mice were fed a high fat diet. After 10 weeks on the high fat diet, the impact of the sex chromosome complement on body weight was pronounced. While male mice remained heavier than female mice (Fig. 1D), mice with two X chromosomes, regardless of male or female gonads, gained more weight throughout the diet compared to XY mice (Fig. 1A,

E). This increase in body weight was largely due to increased fat mass in XX mice (Fig. 1B). XX mice had proportionally more fat mass than XY mice; gonadal sex did not significantly influence fat mass, although male mice had higher proportional lean mass than female mice. Gonadal and inguinal fat depots were 34% and 64% larger, respectively, in XX mice compared to XY mice (Fig. 1F). Our results indicate that the sex chromosome complement is a major sex-biasing factor in determining adiposity. The effects of XX *vs*. XY genotype were detectable on a chow diet, and were enhanced by a high fat diet.

XX mice consume more food during the inactive phase of the circadian cycle

Expansion of fat mass is due to an imbalance in energy homeostasis, either by increasing food intake or decreasing energy expenditure. Studies in rodents and humans have shown that in addition to the amount of food consumed, the timing of food intake influences its effect on weight gain, with enhanced weight gain associated with food consumption during the inactive phase of the circadian cycle (11–13). To determine if there were sex differences in food intake, we measured food intake during the light and dark periods in FCG mice after 1 week or 8 weeks on the high fat diet. After 1 week, XX mice consumed more food than XY mice during both the inactive light period and the active dark period of the circadian cycle (Fig. 2A). In addition, males consumed more food during the dark period compared to females. After 8 weeks on the high fat diet, the four genotypes had similar food intake during the dark period. However, XX mice continued to consume more than XY mice during the light period, and males consumed more than females. These data suggest that the sex chromosome complement is a significant modulator of food intake during the circadian cycle. They further indicate that the effect of sex on food intake varies with length of time on the diet, but that XX chromosome complement was consistently associated with increased food intake during the inactive (light) period.

All four genotypes exhibited typical diurnal variations in energy expenditure and locomotor activity as assessed by monitoring in metabolic cages (Suppl Fig. 1A, B). No significant sex

differences were detected in respiratory quotient or energy expenditure at either 1 week or 10 weeks of high fat diet (Fig. 2B, Suppl. Figs. 1, 2). Interestingly, there was an effect of gonadal sex on physical activity, with females typically traveling greater distance in the horizontal plane than male mice (Suppl. Fig. 3). This difference in physical activity cannot account for the observed greater weight gain in XX compared to XY mice.

The gut microbiome has been identified as a significant contributor to energy homeostasis and body weight gain (14–17). In addition, the composition of the gut flora is responsive to sex hormones (18,19). To investigate the potential contribution of the gut microbiome to sex differences in energy balance, we sequenced 16S rRNA in fresh fecal samples collected from the same cohort of mice while eating a chow diet and after eating the high fat diet for one week. On the chow diet, 74-82% of the gut microbial species belonged to the Bacteroidetes phylum, 11-17% were Firmicutes, and members of Verricomicrobia and other phyla comprised smaller proportions (Fig. 2C). After a single week on the high fat diet, the gut microbiota exhibited a major shift, with Bacteroidetes reduced to 32-36% of the total species, and Firmicutes increased to 41-48%. These results are consistent with previous observations that a high fat diet leads to increased levels of the Firmicutes species, which are associated with efficient energy harvest from the diet (20,21). Our data demonstrate that the high fat diet-induced this transition in microbiome composition occurs very rapidly, within 1 week of introduction of a high fat diet. However, we observed no differences in microbiota composition that were associated with gonadal sex or sex chromosome complement (Fig. 2C).

XX mice develop fatty liver

A common co-morbidity of obesity is the development of fatty liver. After 10 weeks on a high fat diet, all four genotypes had gained at least 10 grams of body weight and body fat composition had increased by at least 15% (Fig. 1). We determined whether the increased adiposity was associated with hepatic lipid accumulation. Histological analysis revealed the

presence of lipid droplets in livers of all genotypes after eating the high fat diet, but livers from XX mice had substantially larger lipid droplets than those from XY mice (Fig. 3A). Consistent with this, biochemical analysis revealed that XX mice, regardless of male or female sex, accumulated more than twice the amount of hepatic triglyceride as XY mice (Fig. 3A). Expression of key fatty acid synthesis genes (*Srebp1c*, *Fasn*, *Elovl6*, and *Scd1*) were elevated in XX mice. In addition, *Srebp1c* and *Scd1* expression levels were increased in males compared to females. These data suggest that the sex chromosome complement is a risk factor for the development of fatty liver in response to a high fat diet.

We assessed whether the increased hepatic lipid accumulation in XX mice was associated with inflammation and fibrosis. Plasma alanine transaminase (ALT) levels were elevated in XX male mice compared to XX female, XY female, and XY male mice (Suppl. Fig. 4A). However, expression levels for genes encoding tumor necrosis factor α (*Tnfa*) and the inflammasome component NALP3 (*Nalp3*) were higher in XY compared to XX mice, despite the higher lipid accumulation in XX livers (Suppl. Fig. 4B). Staining for collagen accumulation and analysis of expression levels for several genes involved in fibrosis revealed no effects of sex on the minor levels of fibrosis elicited by 10 weeks of high fat diet (Suppl. Fig. 4C, D).

An additional co-morbidity of increased adiposity and hepatic lipid content is impaired glucose homeostasis. At 14 weeks of age, before the start of the high fat diet, there were no sex differences in fasting glucose levels or glucose tolerance (Suppl. Fig. 5A). After 10 weeks of high fat diet, male–female and sex chromosome differences emerged. Fasting glucose and insulin levels, and HOMA-IR index of insulin resistance, were higher in males than females (Fig. 4A). Insulin levels were also influenced by sex chromosome complement, with XX mice having higher insulin levels and HOMA-IR than XY mice. In glucose tolerance tests performed after 10 weeks on the high fat diet, XX and XY males had higher baseline glucose levels and did not clear the bolus of glucose as quickly as XX and XY females (Fig. 4B). We assessed the impact of diet-induced obesity on several hepatic gluconeogenesis genes. *Pdk4*, *Pcx*, and *G6p* showed

sex chromosome effects on expression levels, although *Pck1* was not different among the four genotypes (Fig. 4C). These results indicate significant and independent effects of gonadal sex

Kdm5c dosage is associated with adiposity

The results described above indicate that when fed a high fat diet, mice with XX compared to XY chromosomes have increased food intake, body weight, adiposity, and hepatic lipid accumulation. We previously used mouse models with various numbers of sex chromosomes to demonstrate that the number of X chromosomes—rather than the presence/absence of a Y chromosome—was a determinant of adiposity in gonadectomized mice (10,22). We hypothesize that a key determinant of differences between XX and XY mice is the higher dosage of specific genes on the X chromosome that escape transcriptional silencing by X chromosome inactivation. We focused on four genes (*Kdm5c*, *Kdm6a*, *Ddx3x* and *Eif2s3x)* that we and others previously identified as having robust evidence of escape from X chromosome inactivation in metabolic tissues in gonadectomized mice (adipose tissue, liver, muscle, hypothalamus) (10,23). The proteins encoded by these genes have the potential to influence cellular function through effects on chromatin modification (histone demethylases Kdm5c and Kdm6a) or RNA structure and translation (RNA helicase Ddx3x and translation initiation factor Eif2s3x). We assessed mRNA expression levels for X chromosome escapee genes in visceral and subcutaneous adipose tissue depots of gonadally intact FCG mice that had been fed high fat diet for 10 weeks. In both fat depots, *Kdm5c*, *Kdm6a*, *Ddx3x*, and *Eif2s3x* had significantly higher expression levels in XX compared to XY mice (Fig. 5A–D). In the subcutaneous inguinal depot, all four escapee genes also exhibited increased expression levels in male compared to female mice, indicating effects of both sex chromosome complement and gonadal secretions in the inguinal fat. The enhanced dosage of these X chromosome escapee genes in intact XX mice raises the possibility that one or more of these genes contribute to effects on adiposity and related traits.

Two of the X chromosome escapees encode histone demethylases: *Kdm5c* encodes histone 3 lysine 4 trimethyl (H3K4me3) demethylase and *Kdm6a* encodes histone 3 lysine 27 trimethyl (H3K27me3) demethylase. Histone methylation status has been shown to regulate transcription of numerous genes associated with adipogenesis and obesity (24–26). To determine whether *Kdm5c* or *Kdm6a* dosage differences in XX *vs*. XY mice may contribute to the X chromosome-mediated adiposity effects, we generated mice that mimic the gene dosage present on one *vs*. two X chromosomes. Specifically, using mouse strains carrying loss-of-function alleles, we generated female XX mice having one (+/–) or two (+/+) functional copies of either *Kdm5c* or *Kdm6a* and evaluated body weight and adiposity.

We initially assessed mice fed a chow diet. *Kdm6a* +/+ and +/– mice had similar body weight, and we did not characterize them further (Fig. 6A). By contrast, *Kdm5c* haploinsufficiency had an impact on body weight. Female XX mice with one functional *Kdm5c* allele had lower body weight than mice with two functional alleles from 3 weeks of age on (Fig. 6B). Body composition was assessed at 26 weeks of age and showed ~20% difference in body fat percentage between *Kdm5c* +/+ and +/– mice (Fig. 6C). The increased body weight and adiposity were associated with increased food intake by *Kdm5c* +/+ compared to +/– mice (Fig. 6D). The effect of *Kdm5c* dosage on body weight was amplified by feeding mice a high fat diet. In preliminary studies, over the course of 14 weeks on a high fat diet, *Kdm5c* +/+ mice gained 75% of their starting body weight, whereas *Kdm5c* –/– mice gained <30% of their initial body weight (Fig. 6E). After 14 weeks on the high fat diet, there was a trend to substantially higher body fat mass in *Kdm5c* +/+ compared to +/– mice (Fig. 6F). These data indicate that *Kdm5c* haploinsufficiency reduces food intake, body weight, and high fat diet-induced weight gain and may contribute to differences observed in XX (*Kdm5c* +/+) *vs*. XY (similar to *Kdm5c* +/–) mice.

DISCUSSION

Sex is a major factor in determining fat accumulation and distribution. Traditionally, sex differences in adiposity have been attributed to sex hormones. Evidence for the influence of gonadal hormones on adiposity comes from studies of pre- and postmenopausal women. The decline in circulating estrogen levels after menopause is associated with an increase in fat mass and a shift in fat distribution from the typical “female” subcutaneous fat in the hips and thighs to more closely resemble the typical “male” abdominal fat depots. In addition to hormones, other sex-biasing factors may contribute to sex differences in obesity. The sex chromosome complement is the fundamental genetic component that defines male (XY) from female (XX) mammals. Our previous studies identified sex chromosome complement as a major determinant of adiposity in gonadectomized mice (10), findings that may have particular relevance to the changes in adiposity that accompany reduced gonadal hormone levels in middle- to late-aged humans.

Obesity is rapidly becoming a significant health problem in juvenile and young adult populations (27), and sex differences in adiposity are evident in both early development and in adolescence (28). Sex differences in adolescent obesity have been attributed to hormonal, genetic, and social factors (29). To identify the relative roles of hormonal and genetic differences between males and females in the development of obesity we characterized adiposity and related traits in FCG mice with natural levels of circulating sex hormones. In chow-fed mice, males weighed more than females, and we did not detect significant sex chromosome differences in body weight from 10 weeks to 32 weeks of age. This suggests that when fed the standard chow diet containing <5% fat, gonadal hormones are the primary driver of sex differences in body weight in mice.

To induce obesity, 14-week-old FCG mice were fed a high fat diet for 10 weeks. Regardless of gonadal type, mice with two X chromosomes gained more weight than XY male and female mice. The weight gain was largely due to the expansion of fat mass in XX mice. Male sex and

XX chromosome complement each promoted higher body weight. Strikingly, sex chromosome complement was the exclusive determinant of total percent fat mass and mass of the inguinal subcutaneous fat pad. XX mice had ~10% higher percent fat mass and 30% larger inguinal fat depots than XY mice. These data identify sex chromosome complement as a key determinant of increases in adiposity that occur in mice in response to a high fat diet.

Fat accumulation is a result of energy imbalance — increased food intake, reduced energy expenditure, or a combination of both can lead to body weight gain. To determine the mechanism by which XX mice gain more body fat than XY mice, we measured energy expenditure through indirect calorimetry and food intake at multiple points during the diet. Energy expenditure (heat production, respiratory quotients) measured at 1 week or 10 weeks after the start of the high fat diet did not differ across the four mouse genotypes. Differences in physical activity were detected between males and females, but these could not account for the XX *vs*. XY differences in body weight. However, food intake was significantly different between XX and XY mice. XX mice consumed more food than XY mice during the light period (inactive phase) of the circadian cycle. This pattern was observed both early in the high fat diet (1 week of diet), before large changes in body weight had occurred, and near the end of the feeding period (8 weeks of diet). Multiple reports in humans and animal models have shown that feeding during the inactive phase leads to enhanced weight gain compared to isocaloric feeding during the active phase of the circadian cycle (11–13). It has been postulated that the timing of food consumption influences metabolism of dietary nutrients (30). These results provide impetus for further study of the effect of sex chromosome complement on the circadian regulation of appetite and satiety, which may in turn contribute to sex differences in weight gain on a high fat diet.

A common co-morbidity of obesity is fatty liver. Around 30% of the population is thought to have hepatic steatosis, the earliest stage in non-alcoholic fatty liver disease (NAFLD) (31). In human and rodent studies, sex is a factor in NAFLD, but different human ethnic populations

have varying sexual dimorphism (32–35). To determine the effect of sex hormones and the sex chromosome complement in fatty liver, we analyzed in the development of fatty liver in FCG mice fed a high fat diet. Regardless of sex, XX mice accumulated twice as much hepatic triglyceride, and much larger lipid droplets, than XY mice. It is notable that no differences were observed based on the presence of male or female gonads, and that lipid accumulation was associated solely with having two X chromosomes. Enhanced lipid accumulation in XX livers was correlated with higher mRNA levels of fatty acid synthesis genes (*Srebp-1c*, *Fasn*, *Elovl6*, and *Scd1*), suggesting that XX chromosome complement influences hepatic gene expression.

A small subset of patients with NAFLD progress to steatohepatitis, characterized by inflammation and/or collagen accumulation, also known as fibrosis (31). We observed sex differences in hepatic inflammation after 10 weeks of a high fat diet, with XY mice having substantially higher hepatic expression of genes encoding tumor necrosis factor α and inflammasome components. Unexpectedly, sex differences in inflammatory gene expression (XY > XX) were decoupled from sex differences in hepatic lipid accumulation (XX > XY). This reveals that lipid accumulation and inflammation are regulated by distinct factors and provides an interesting basis for further studies. None of the mice developed significant fibrosis; future studies in which mice are fed a high fat diet for longer than 10 weeks may be informative to determine whether sex differences exist in this process.

In addition to fatty liver, obesity is also correlated with insulin resistance and impaired glucose homeostasis. Recent studies analyzing a panel of 100 inbred mouse strains demonstrated that sex is a major determinant of insulin sensitivity (36). After 8 weeks of a high fat diet, females were generally more insulin sensitive than males (36). We asked whether sex differences in glucose homeostasis are influenced by gonadal sex or sex chromosome complement. No sex differences were detected in glucose tolerance in 14-week-old FCG mice fed a chow diet. After 10 weeks of a high fat diet, glucose and insulin levels were influenced by both gonadal type and sex chromosomes. XX and XY males had increased glucose and insulin

levels compared to XX and XY females, suggesting modulation by gonadal hormones. In addition, XX mice had higher levels of plasma insulin than XY mice. Taken together, our data indicate that both gonadal hormones and the sex chromosome complement modulate glucose homeostasis. XX and XY males showed a trend toward glucose intolerance. While our data demonstrate that the sex chromosome complement influences expression of hepatic genes involved in glucose homeostasis, the effect of the sex chromosomes on glucose- and insulin-sensitive pathways in other peripheral metabolic tissues, such as adipose and skeletal muscle, will require further investigation.

The sex chromosome effects that we observed in diet-induced obesity, fatty liver, and glucose homeostasis raise the question of how the sex chromosome complement influences these traits. One determinant of differences between XX and XY mice is gene dosage. Although one X chromosome is transcriptionally silenced in XX mice, a few genes escape inactivation and are expressed at higher levels in XX compared to XY mice. Genes classified as "X chromosome escapees" vary in expression levels from low (≤10% of active X chromosome levels) to robust (>50% of active X chromosome levels) (37,38). In addition, individual genes have varying tissue-specific levels of escape (37,39).

We examined the expression of some well-established X escapees (*Kdm5c*, *Kdm6a*, *Ddx3x*, and *Eif2s3x*) in gonadal and inguinal fat depots. *Kdm5c*, *Kdm6a*, *Ddx3x*, and *Eif2s3x* had higher expression in XX compared to XY mice, suggesting that they escape X inactivation in both visceral and subcutaneous fat depots. To determine if gene dosage of these escapee genes affects body weight, we generated mouse models that are haploinsufficient for *Kdm5c* and *Kdm6a*. *Kdm5c* haploinsufficiency had substantial effects on body weight. From weaning to 26 weeks of age, *Kdm5c* +/– mice consistently had reduced body weight compared to *Kdm5c* +/+ mice, and this was associated with a 20% reduction in fat mass. *Kdm5c* dosage was correlated with food intake levels, as we have observed for X chromosome dosage, suggesting that the same physiological mechanism is responsible for the effects on body weight in XX *vs*. XY mice

and *Kdm5c* +/+ *vs*. *Kdm5c* +/– mice. *Kdm5c* +/– mice had reduced food intake compared to mice with both copies. This suggests that dosage of this X-linked gene contributes directly to energy balance. We also determined that dosage of another X escapee gene, *Kdm6a*, does not influence body weight. These results are consistent with a hypothesis that dosage of specific genes — but not all genes — that escape X chromosome inactivation underlie the differences in body weight that we observed in XX compared to XY mice. This is the first reported evidence of an X escapee gene associated with sex differences in disease.

The physiological role of *Kdm5c* in metabolic tissues is not known. *Kdm5c* is a histone demethylase for trimethylated and dimethylated H3K4, yielding H3K4me2 and H3K4me1 (40). In cultured cells, histone methylation is important for regulating adipogenesis and obesity (24–26). It is likely that *Kdm5c* dosage influences chromatin modification and gene expression in metabolic tissues, with potential effects on adipocyte biology and other metabolic effects. It is known, for example, that the promoter region of the master regulator of adipogenesis, PPARγ, is regulated by H3K4 methylation (26). The *Kdm5c* +/– mouse model will be valuable for identifying genes that are influenced by altered dosage of this X chromosome escapee.

In conclusion, our studies demonstrate that the sex chromosome complement is a significant mediator of sex differences in diet-induced obesity, with key effects on food intake that likely lead to sex differences in adiposity and development of fatty liver. The identification of *Kdm5c* as a specific X chromosome escapee gene that has similar dosage effects as those seen for the entire X chromosome suggests that epigenetic modulation may be an important determinant of sexual dimorphism in response to diet-induced obesity.

METHODS

Animals

Four Core Genotype (FCG) C57BL/6J mice were used for the majority of this study. In these mice, the Y^{-}chromosome harbors a deletion of the testis-determining *Sry* gene. *Sry* is inserted as a transgene onto chromosome 3 (41) and confers testes development in *Sry*+ mice. XX female mice were mated with XY^{-}*(Sry+)* male mice to generate XX, XX(*Sry+)*, XY^{-}, and XY^{-} *(Sry+)* offspring. Genotyping was performed by PCR to detect presence of the *Sry* transgene (forward: AGCCCTACAGCCACATGATA; reverse: GTCTTGCCTGTATGTGATGG) and Y-chromosome–specific sequence (forward: CTGGAGCTCTACAGTGATGA; reverse: CAGTTACCAATCAACACATCAC).

Floxed *Kdm5c* C57BL/6J mice were obtained from Dr. Yang Shi from Harvard/Children's Hospital in Boston. Floxed *Kdm5c* mice were crossed with C57BL/6J Ella-Cre mice (Jackson Laboratories) to generate heterozygous knockouts of the floxed allele through Cre-Lox recombination. Subsequent generations were crossed with wild-type mice to eliminate Cre and the floxed allele. Because *Kdm5c* is X-linked, only female mice were studied.

The haploinsufficient mouse model for *Kdm6a* is on a mixed 129/C57BL6 background and carries a "knocked-out first" (KOF) allele, generated through promoterless targeting cassettes in C57BL/6N embryonic stem cells (Wellcome Trust Sanger Institute). Mice were crossed with C57BL/6J Gt(ROSA)26Sor–Flp mice (Jackson Laboratories) to generate floxed *Kdm6a*. Offspring were then crossed with C57BL/6J Ella-Cre mice to generate heterozygous knockouts of *Kdm6a*. Because *Kdm6a* is X-linked, only female mice were studied.

FCG mice were pair-housed with identical genotypes, and mice haploinsufficient for *Kdm5c* and *Kdm6a* were group-housed in 12:12 light:dark cycle. All mice were initially fed Purina mouse chow diet containing 13% fat from calories (Purina 5053). Where indicated, FCG and *Kdm5c* mice were fed a high fat diet containing 60% fat from calories (Bio-Serv).

Mouse studies were conducted in accordance with and approved by the Institutional Animal Research Committee of the University of California, Los Angeles.

Measurement of body composition and energy balance

All mice were weighed at weekly intervals. Body composition was measured on live FCG mice at 14 weeks of age, before the start of the high fat diet, and after 10 weeks of the high fat diet, using a Mouse Minispec apparatus with Echo Medical Systems software. Food intake was measured per cage over three 24-hour periods at 0800 and 1800. Due to logistical reasons, two separate FCG cohorts were used to measure energy balance. Six calibrated Oxymax metabolic chambers were used to detect oxygen consumption, CO_2 production, locomotor activity, and heat production over 72 hours at weeks 1 and 10 of the high fat diet (Oxymax, Columbus Instruments). Mice were housed individually in the chambers during the measurements. Parameters were monitored dynamically at 20-minute intervals. Mice had *ad libitum* access to food and water. Average RQ (Fig. 2B), cumulative heat production (Suppl. Fig. 2B), and cumulative locomotor activity (Suppl. Fig. 3A, B) were calculated from the second dark and second light intervals of the 72-hour measurement period.

Gut Microbiome Analyses

Microbial DNA was extracted from fresh fecal pellets obtained from mice before and one week after starting the high fat diet (PowerSoil DNA Isolation Kit, MoBio). Sample preparation and filtering was performed as previously described (42). Briefly, phyla composition was assessed by pyrosequencing 16S rRNA genes and using the software package Quantitative Insights Into Microbial Ecology (QIIME) to count and map sequences to phylogenetic clusters (43).

Liver analyses

At time of dissection, liver sections were either fixed in 10% formalin or snap-frozen in liquid nitrogen. Fixed tissue was mounted in paraffin, sliced, and stained with hemotoxylin and eosin or Picrosirius Red. Lipids were isolated from 15 mg frozen tissue using Folch extraction. After drying under nitrogen gas, lipids were resuspended in 200 uL isopropanol and used in subsequent colorimetric triglyceride assay (Wako Diagnostics).

Glucose Homeostasis

Blood samples were obtained by retro-orbital bleeding at time of dissection after fasting (0800-1300). Plasma was collected after centrifugation of whole blood at 3,400 x *g* for 10 minutes at 4°C, at which point glucose and insulin levels were measured. The HOMA ratio was calculated using the equation [(Glucose mg/dL x Insulin pg/mL)/405]. Plasma ALT levels were determined using an enyzmatic colorimetric assay (BQ Kits).

A separate FCG cohort was used to perform glucose tolerance tests at 15 weeks of age, before the start of the high fat diet, and at 10 weeks of the high fat diet. After fasting (0800-1300), mice were given a bolus (2 mg per gram body weight) of glucose through intraperitoneal injection. Blood was drawn through a clipped tail and glucose was measured through AlphaTrak glucometer before injection and 15, 30, 60, 120, and 180 minutes after injection. Area under the curve was calculated by setting the glucose levels of the first timepoint to zero and summing all positive increments.

Quantitative RT-PCR

Liver, gonadal fat, and inguinal fat tissues were dissected out rapidly, flash frozen in liquid nitrogen, and stored at –80°C. RNA was isolated from tissue using TRIzol (Life Technologies). First strand cDNA was generated by reverse transcription with iScript (Bio-Rad). Quantitative

RT-PCR was performed with a Bio-Rad CFX Connect Real-Time PCR Detection System using SsoAdvanced SYBR Green Supermix. 36B4 and β2 microglobulin mRNA were amplified in each liver sample as normalization controls. 36B4 mRNA served as a normalization control for gonadal and inguinal fat samples. All primer sequences are shown in Supplemental Table 1.

Statistical Analysis

Groups were compared using two-factor ANOVA (NCSS) with main factors of sex (gonadal male *vs*. gonadal female) and sex chromosome complement (XX *vs.* XY). For growth curves and the glucose tolerance test, repeated measures ANOVA was used to include time as a variable. Statistically significant comparisons or interactions are presented ($p<0.05$). All error bars represent one standard deviation.

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Floxed *Kdm5c* mice were a generous gift from Dr. Yang Shi from Harvard/Children's Hospital in Boston.

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FIGURE LEGENDS

Figure 1. XX mice gain more weight than XY mice on a high fat diet. (A) In gonadally intact FCG mice fed a chow diet, males weigh more than females from 10 weeks of age to 32 weeks of age ($p<0.000001$). (B) Body weight and weight gained in FCG mice over 10 weeks on high fat diet, starting at 14 weeks of age. Significant comparisons determined by repeated measures 2-way ANOVA are shown. Body composition was measured by MRI for FCG mice (C) before the start of high fat diet and (D) at the tenth week of the diet. (E) Appearance of FCG mice after 10 weeks on a high fat diet. (F) Gonadal and inguinal fat pad mass normalized to kidney mass. All values represent the mean ± standard deviation. Data were analyzed by 2-way ANOVA. Statistically significant differences for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement and gonadal sex is denoted by "Int." *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<0.0001$; ‡, $p<0.00001$. F, gonadal female; M, gonadal male; HFD, high fat diet.

Figure 2. XX mice consume more food than XY mice during inactive phase of the circadian cycle. (A) Food intake was measured per pair of co-housed mice over three 24-hour periods after 1 week and 8 weeks of the high fat diet. Cumulative food intake was divided into light and dark periods. (B) Average respiratory quotient during the first and tenth weeks on the high fat diet. (C) Gut microbiome composition was determined by sequencing microbial 18S RNA from fresh feces. Samples were collected from mice fed a chow diet, and from the same mice 1 week after eating a high fat diet. Percent reads of the three most populous gut microbial phyla are shown. Values represent the mean ± standard deviation. Data were analyzed by 2-way ANOVA. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement and gonadal sex

is denoted by “Int.” **, p<0.01; †, p<0.0001. White horizontal bar, light period; black horizontal bar, dark period; F, gonadal female; M, gonadal male; HFD, high fat diet

Figure 3. XX mice develop fatty liver. (A) Hematoxylin and eosin staining of fixed liver tissue shows an accumulation of large lipid droplets in XX mice. In right panel, hepatic triglyceride concentration was quantitated after Folch extraction of hepatic lipids. (B) Relative mRNA expression of hepatic genes involved in fatty acid synthesis determined by qPCR. Values represent the mean ± standard deviation. Data were analyzed by 2-way ANOVA. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. *, p<0.05; **, p<0.01; ***, p<0.001; †, p<0.0001. F, gonadal female; M, gonadal male; TG, triglyceride.

Figure 4. Sex chromosomes and gonadal hormones influence insulin sensitivity. (A) Fasting levels of glucose, insulin, and homeostatic model assessment (HOMA) were measured at 10 weeks on high fat diet. (B) Glucose tolerance tests after 10 weeks on the high fat diet. Asterisks denote statistical significance between males and females. XX–XY differences were not significant. (C) Relative mRNA expression levels in liver of key enzymes in gluconeogenesis determined by qPCR. Values represent the mean ± standard deviation. Data were analyzed by 2-way ANOVA. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement and gonadal sex is denoted by “Int.” *, p<0.05; **, p<0.01; ***, p<0.001; †, p<0.0001; ‡, p<0.00001. F, gonadal female; M, gonadal male.

Figure 5. Genes that escape X inactivation have elevated expression in adipose tissue of XX compared to XY mice. Relative mRNA expression levels of *Kdm5c* (A), *Kdm6a* (B), *Ddx3x* (C), and *Eif2s3x* (D) in gonadal fat and inguinal fat depots of FCG mice fed a high fat diet for 10

weeks were determined by qPCR (*n*=3-8). Data were analyzed by 2-way ANOVA. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. *, p<0.05; **, p<0.01; ***, p<0.001. F, gonadal female; M, gonadal male.

Figure 6. *Kdm5c* dosage affects body weight, food intake, and weight gain on a high fat diet. (A) Body weight is not affected by haploinsufficiency for *Kdm6a*. *Kdm6a* +/+ and *Kdm6a* +/– mice fed a chow diet and body weight tracked from 3-12 weeks of age. (B) Female mice haploinsufficient for *Kdm5c* (*n*=7-18) have a lower body weight compared to female control mice (*n*=13-45) when fed a chow diet (p<0.000001). (C) Body fat in *Kdm5c* +/+ and *Kdm5c* +/– mice at 26 weeks of age. (D) Kdm5c +/+ mice have higher food intake that Kdm5c +/– mice at 10 weeks of age. White horizontal bar denotes light period; black horizontal bar denotes dark period. (E) *Kdm5c* haploinsufficiency attenuates weight gain on a high fat diet (*n*=2-3, p<0.02). (F) Trend toward higher body fat composition in *Kdm5c* +/+ compared to *Kdm5c* +/– mice fed a high fat diet for 14 weeks.

Figure 1

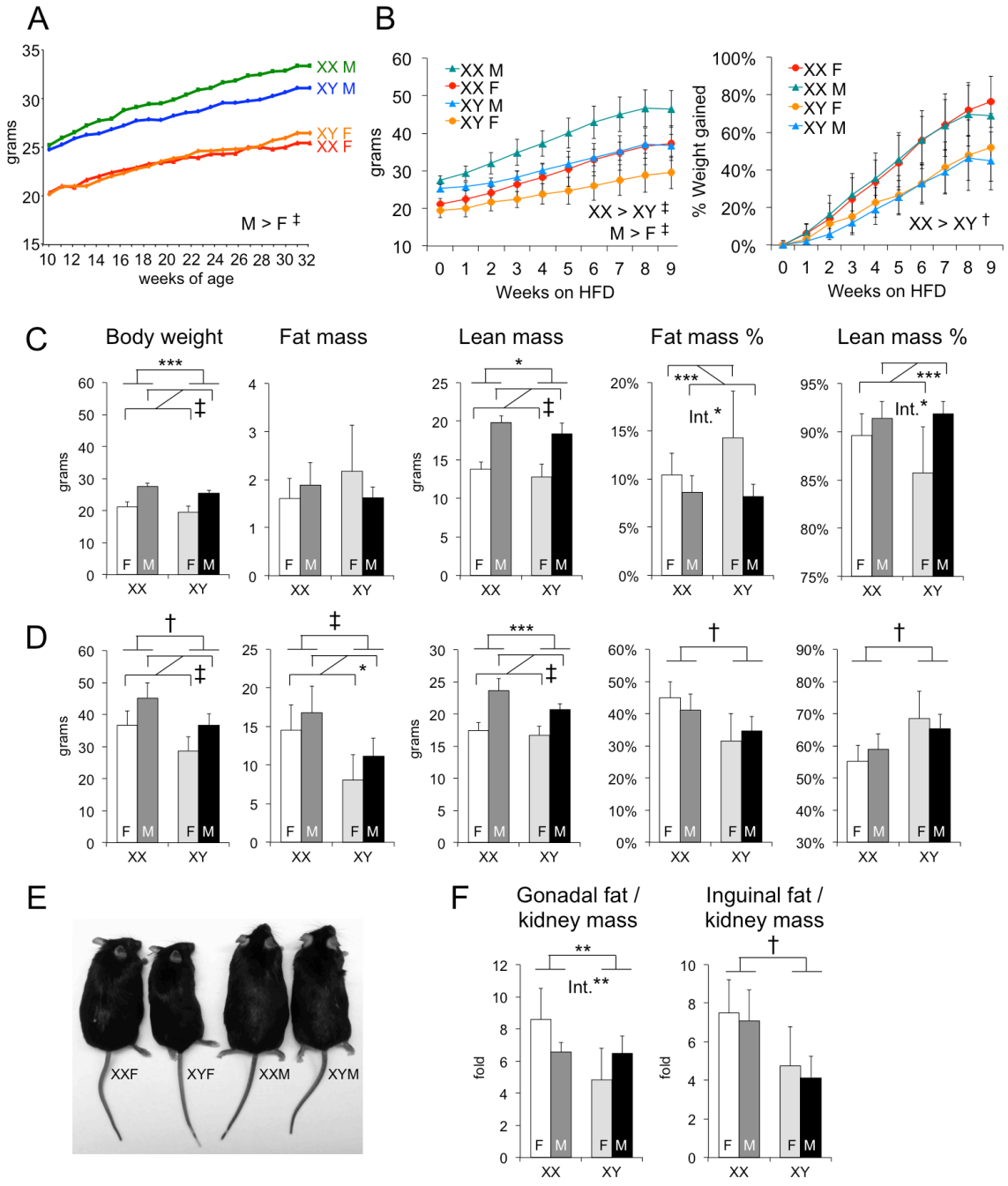
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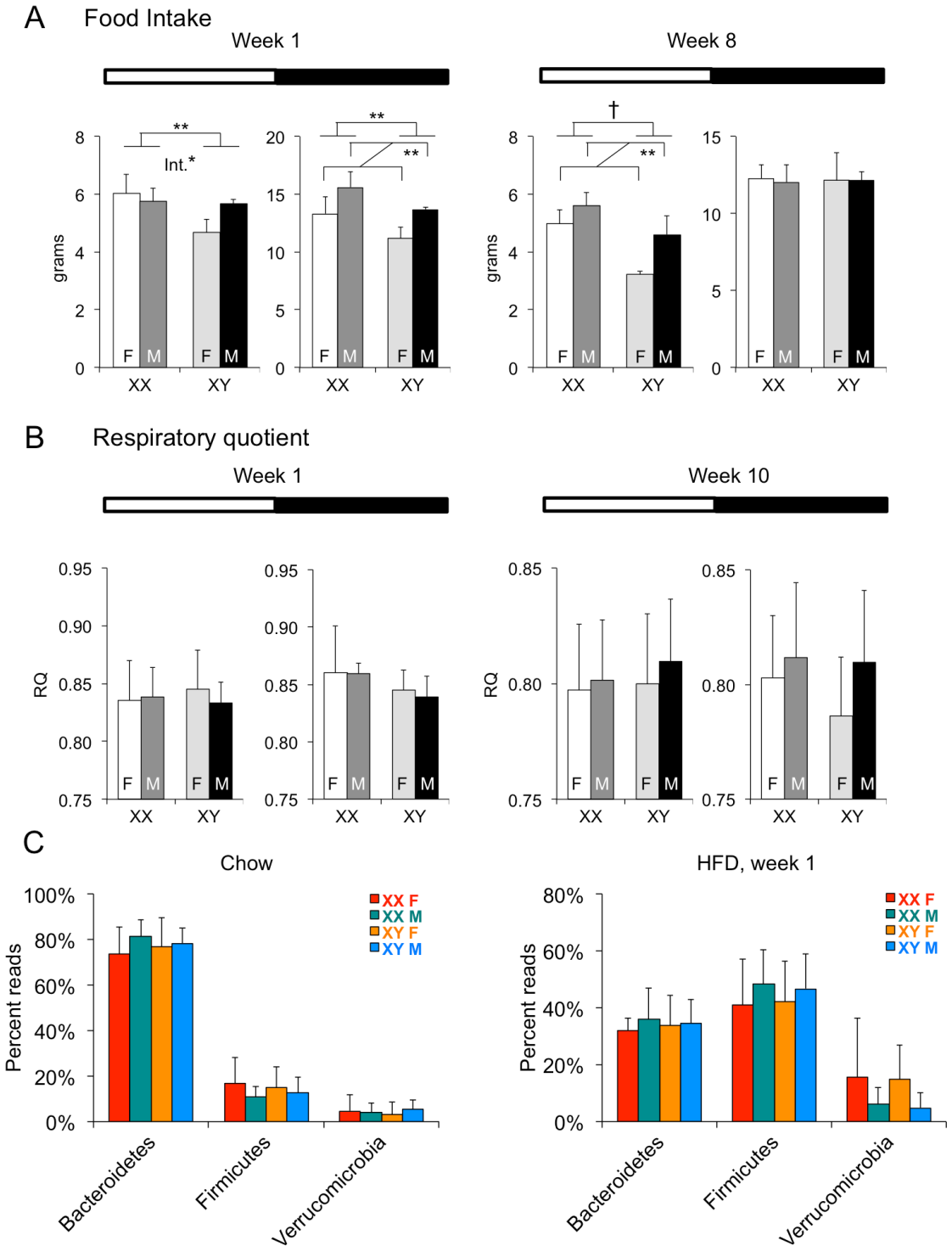
Figure 2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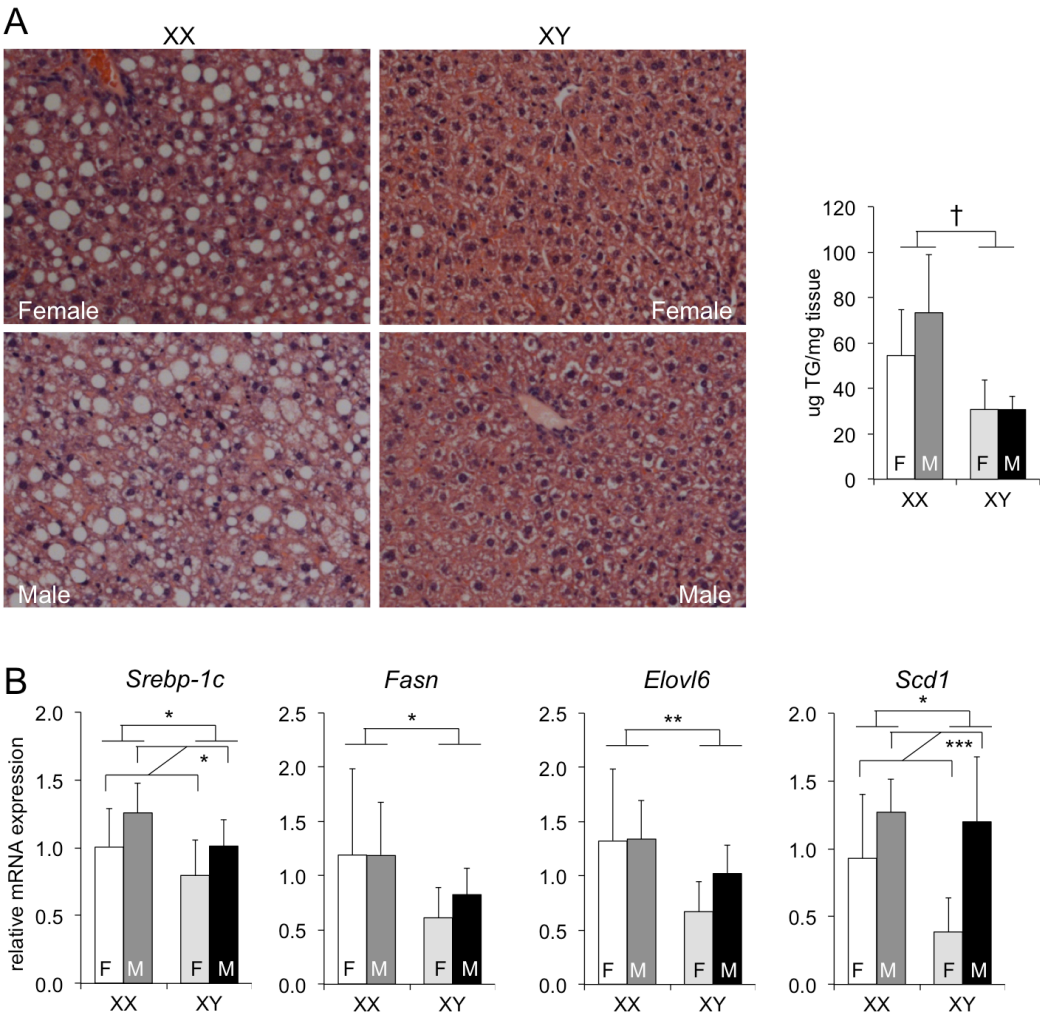
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Figure 4

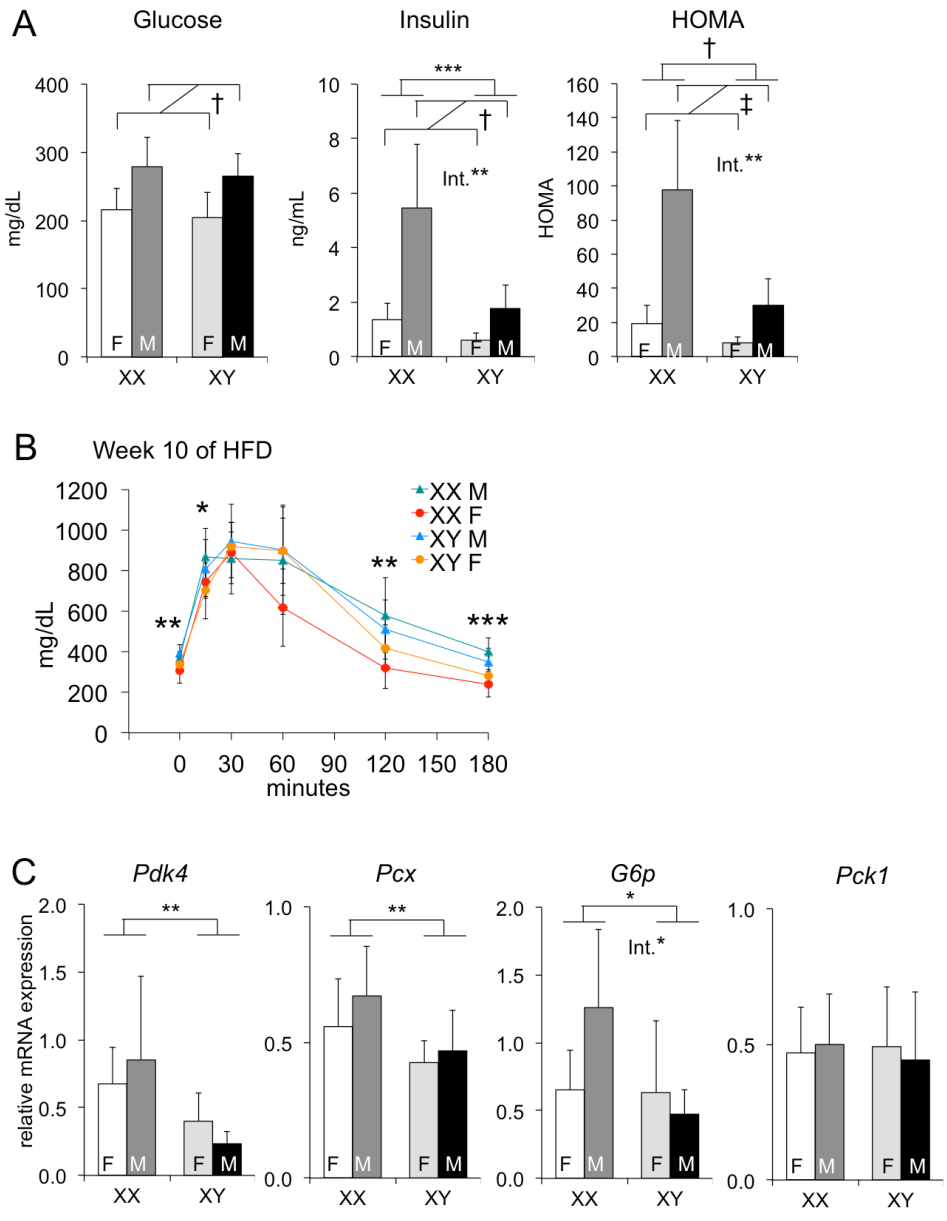
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Figure 5

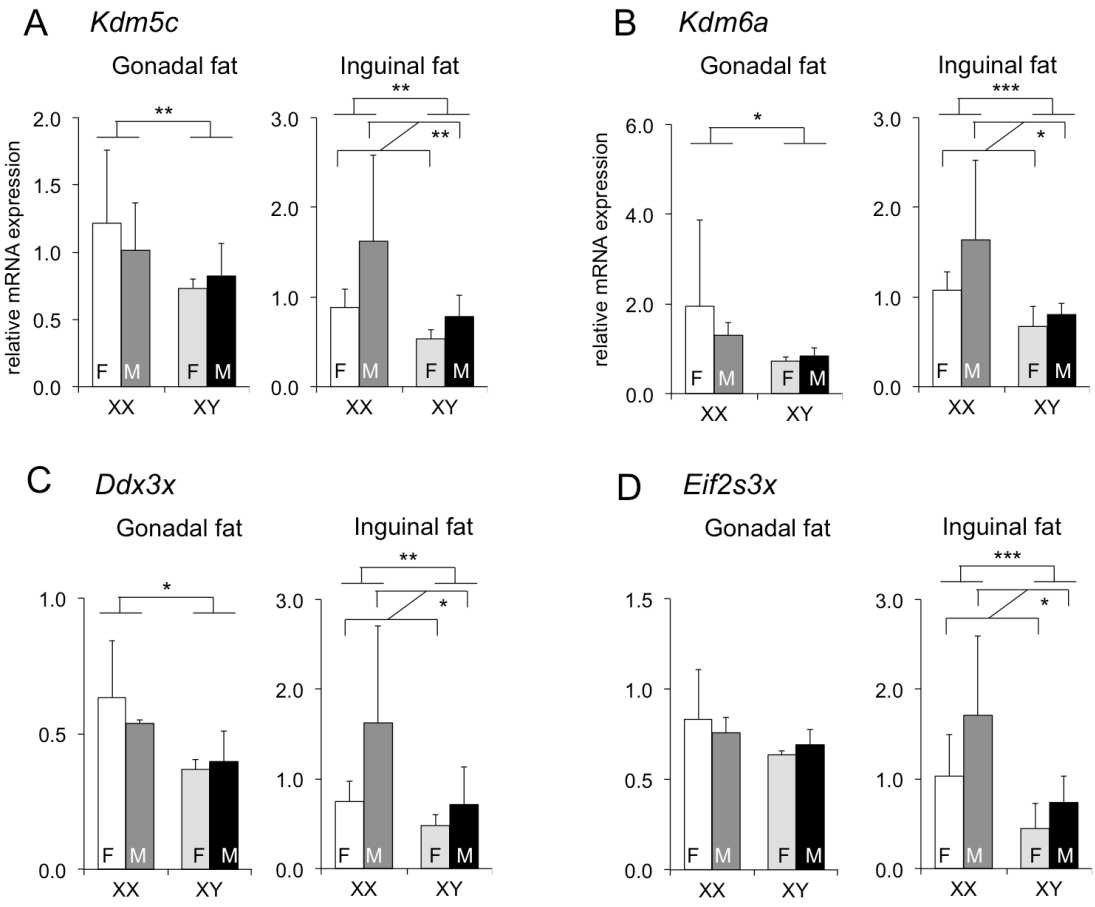
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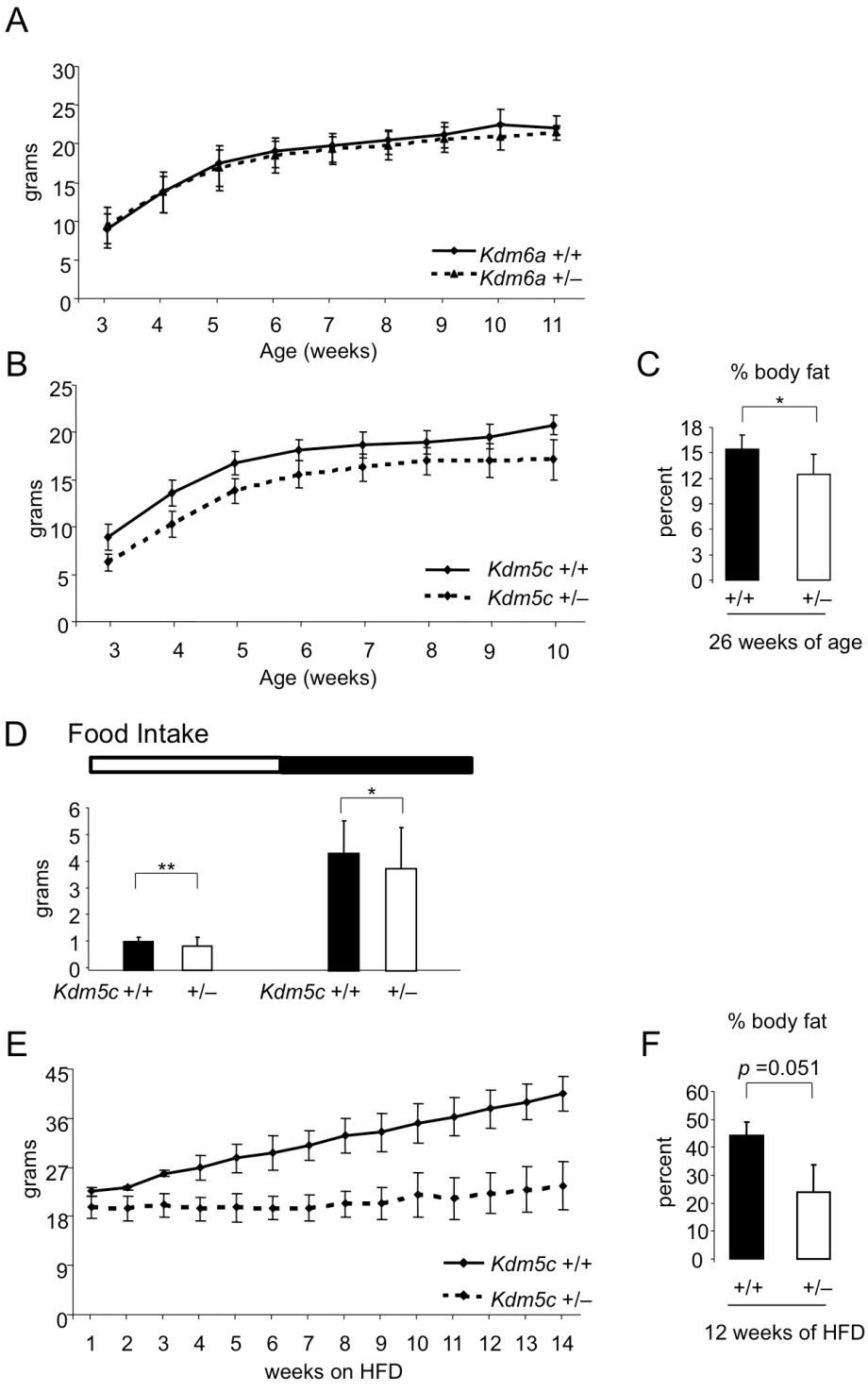
Figure 6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F
% body fat
p =0.051
percent
+/+
+/–
12 weeks of HFD

SUPPLEMENTAL FIGURES

Supplemental Figure 1. Respiratory quotient amplitude is suppressed on high fat diet. Related to Figure 2. Average respiratory quotient over three nights and two days during the first and tenth weeks of the high fat diet.

Supplemental Figure 2. Weight gain in XX mice is not associated with altered energy expenditure . (A) Energy expenditure over three nights and two days during the first and tenth weeks of the high fat diet. (B) Cumulative heat produced during the second dark and second light periods. F, gonadal female; M, gonadal male.

Supplemental Figure 3. Weight gain in XX mice is not associated with altered locomotor activity. Cumulative horizontal (A) and vertical (B) activity measured in 12-hour phase during the first and tenth weeks on the high fat diet. (C) Average horizontal locomotor activity over three nights and two days. Values represent the mean ± standard deviation. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement and gonadal sex is denoted by "Int." *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. F, gonadal female; M, gonadal male.

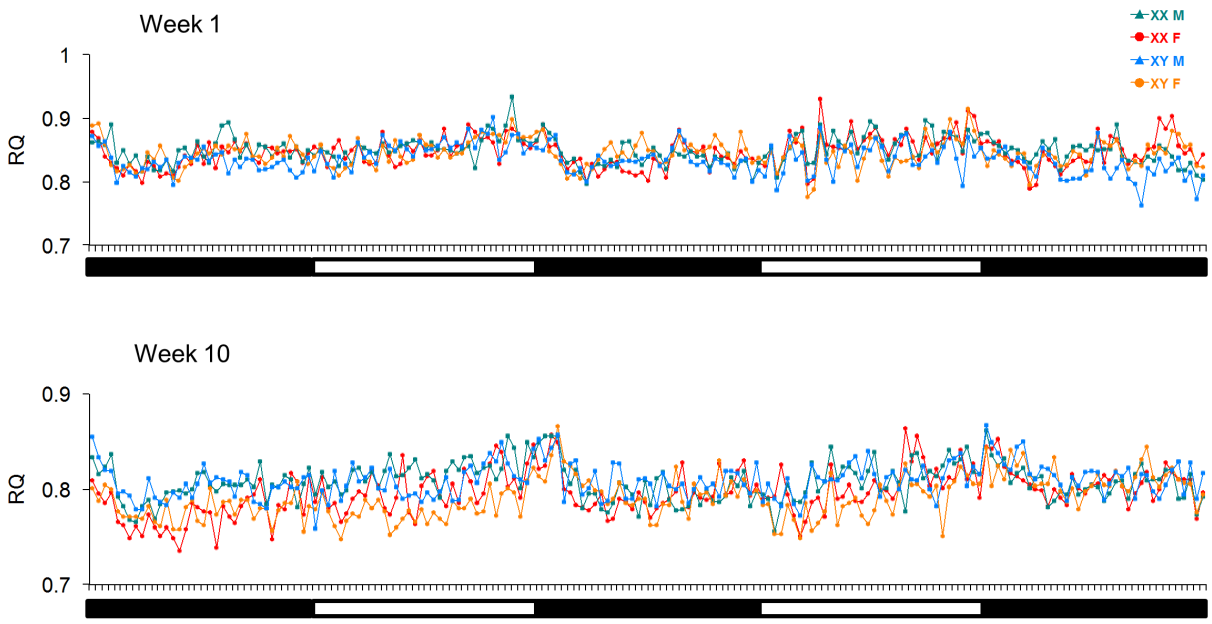
Supplemental Figure 4. Upregulation of inflammatory markers, but not fibrosis in XY livers. (A) Plasma alanine transaminase (ALT) levels, relative expression of hepatic inflammatory markers (B), Picrosirius red staining of fixed liver tissue (C), and relative expression of hepatic fibrotic markers (D) after 10 weeks on high fat diet. Values represent the mean ± standard deviation. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement

and gonadal sex is denoted by “Int.” *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ‡, $p<0.00001$. F, gonadal female; M, gonadal male.

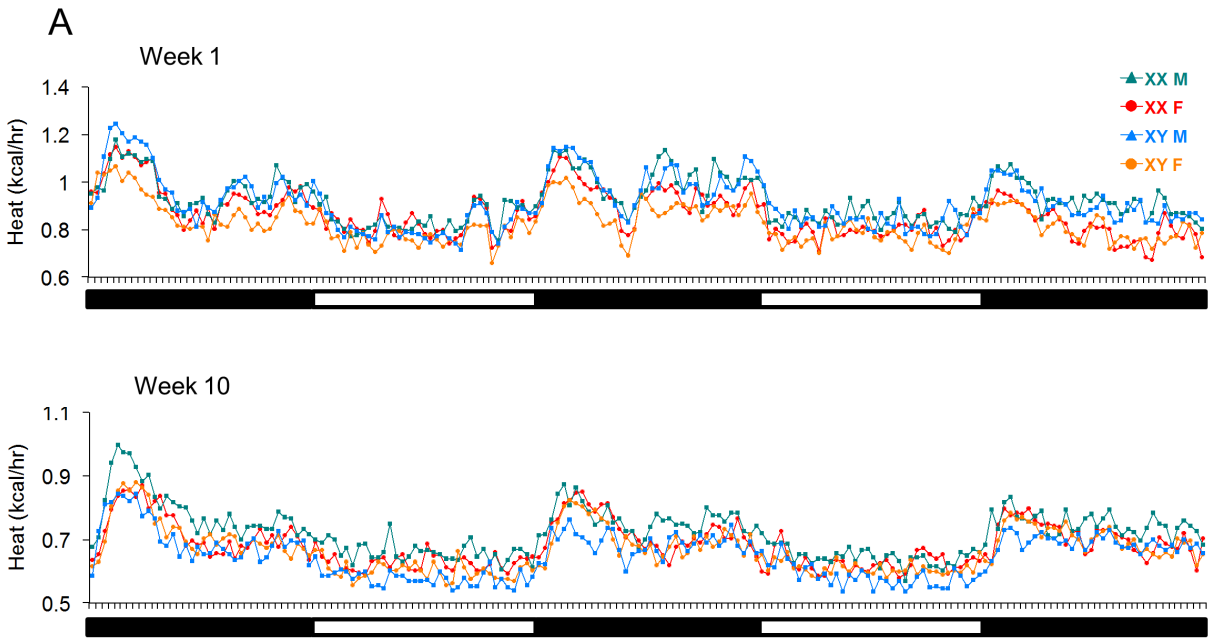
Supplemental Figure 5. Glucose tolerance tests. Related to Figure 4. (A) Glucose tolerance tests for 14-week-old FCG mice fed a chow fed diet. Area under the curve for glucose tolerance tests at (B) the start of the high fat diet (15 weeks of age), and (C) 10 weeks after the high fat diet. There were no significant male–female or XX–XY sex differences. F, gonadal female; M, gonadal male.

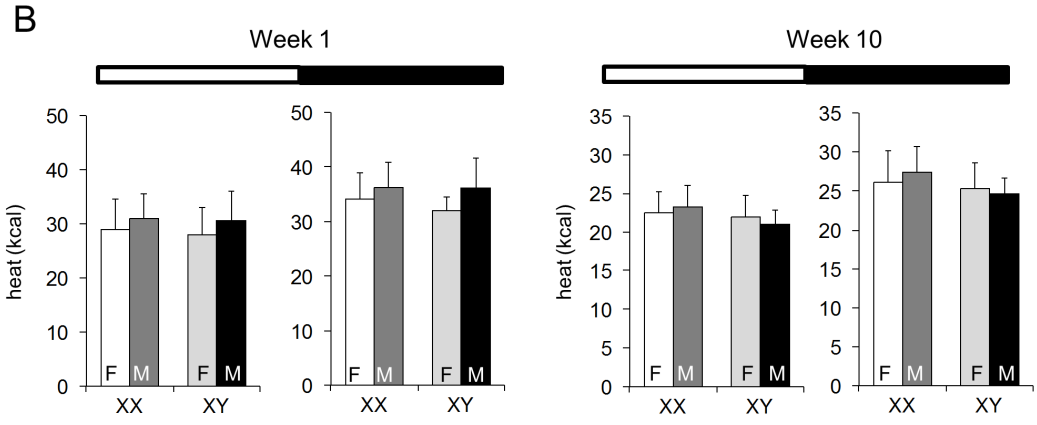
Supplemental Table 1. Mouse primer sequences for qPCR

Supplemental Figure 1

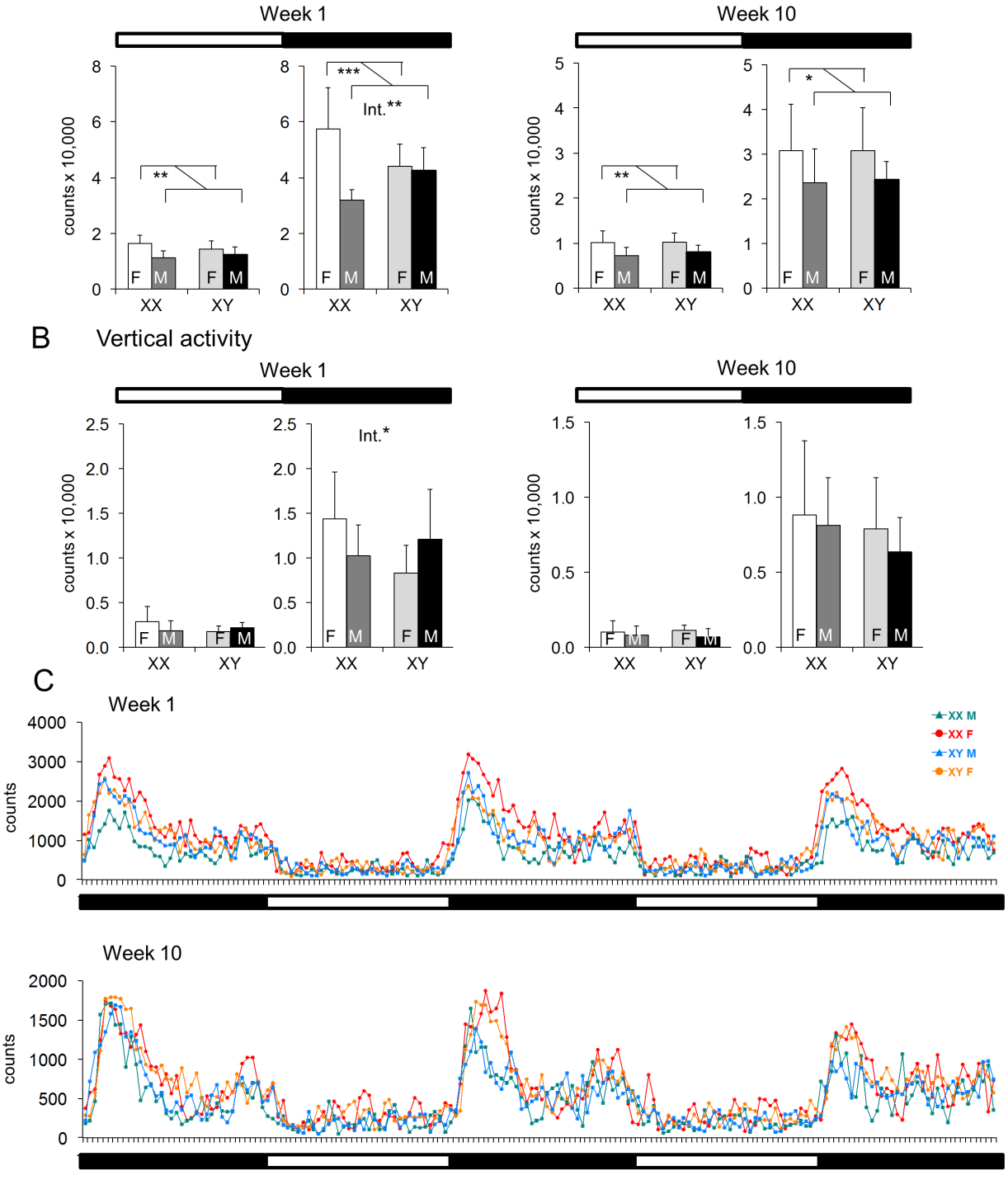


Supplemental Figure 2





Supplemental Figure 3



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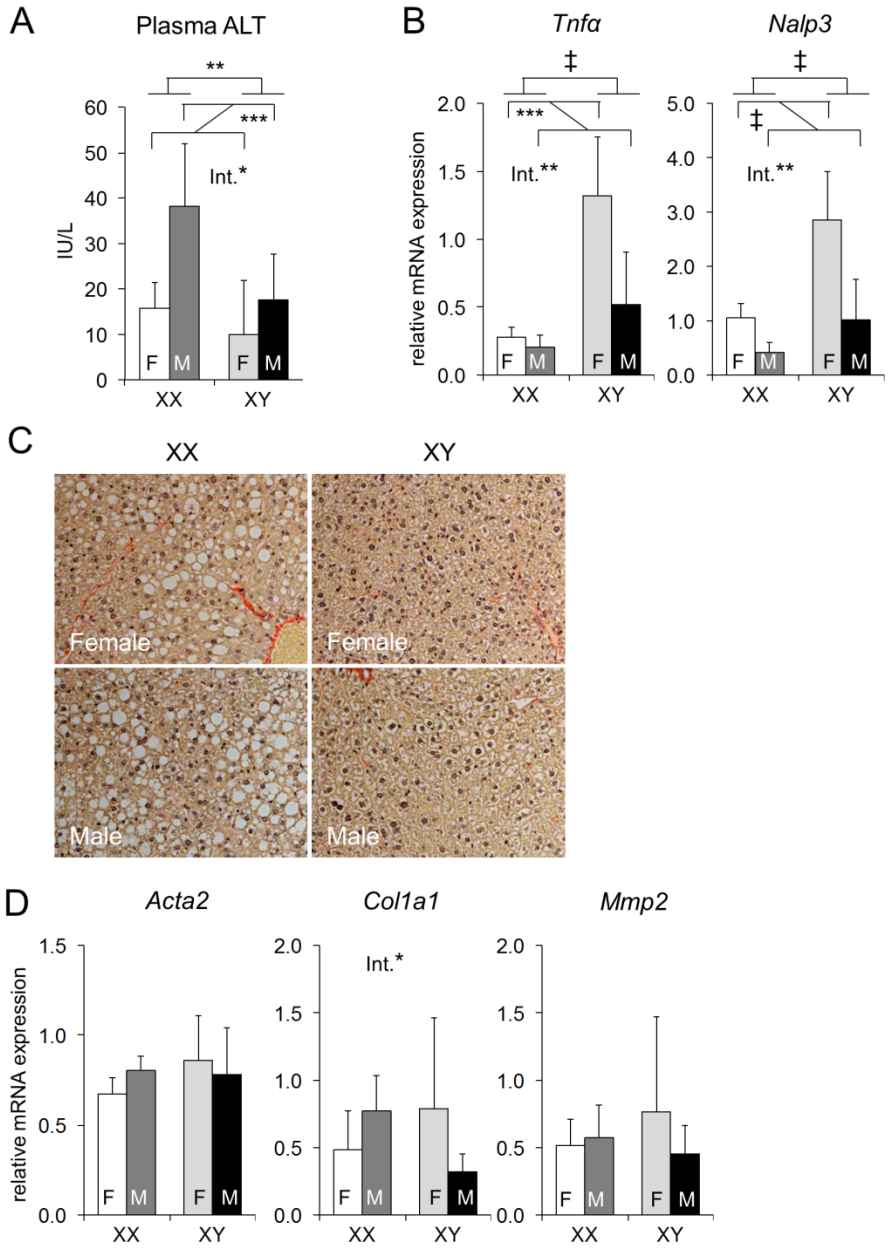
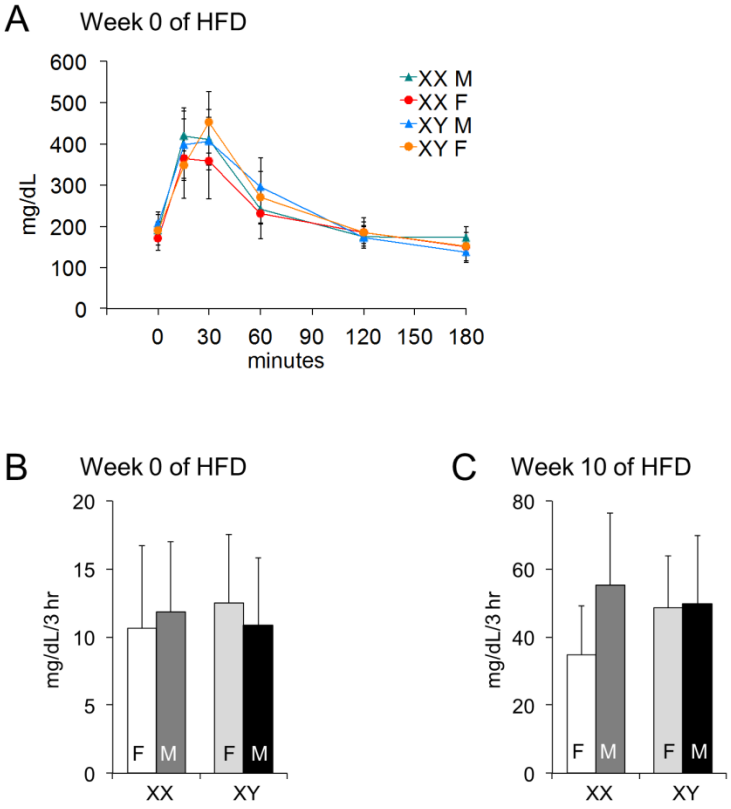


Supplemental Figure 5



Supplemental Table 1

Mouse primer sequences for qPCR

Genes	Forward primer	Reverse primer
β2m	CAGCATGGCTCGCTCGGTGAC	CGTAGCAGTTCAGTATGTTCG
36b4	AGATGCAGCAGATCCGCAT	GTTCTTGCCCATCAGCACC
Acta2	GCTGACAGGATGCAGAAGGAG	GTAGACAGCGAAGCCAGGATG
Col1a1	GCTTCACCTACAGCACCCTTG	ATGTCCAAGGGAGCCACATC
Elovl6	GATGACCAAAGGCCTGAAGC	GTGGTGGTACCAGTGCAGGA
Fasn	TGTGGCTATGCAGATGGCTGT	GGCAGCGCTGTTTACATTCCT
G6p	ACTTGTTCCCTGGCCCTGCTGC	CTCTGCAAATCAGCCGAGGC
Mmp2	TGGAATGCCATCCCTGATAAC	TGCTTCCAAACTTCACGCTCT
Nalp3	GCCAGGAGGACAGCCTTGAAGA	CTAGACGCGCGTTCCTGTCCTT
Pck1	CATCCCAACTCGAGATTCTGC	CCAGCTGAGGGCTTCATAGAC
Pcx	CCGTTCCAGATGCTACTGAGG	CAGCAGCATGTTTGGCAAGTA
Pdk4	TGTCAGGTTATGGGACAGACG	CCTGCTTGGGATACACCAGTC
Scd1	GCGATACACTCTGGTGCTCA	TAGTCGAAGGGGAAGGTGTG
Srebp-1c	GTGGTCTTCCAGAGGGTGAG	AGGTGCCTACAGAGCAAGAG
Tnfa	CTCCAGCTGCTCCTCCACTTG	ACCACGCTCTTCTGTCTACTG
Ddx3x	GGATCACGGGGTGATTCAAGAGG	CTATCTCCACGGCCACCAATGC
Eif2s3x	TTGTGCCGAGCTGACAGAATGG	CGACAGGGAGCCTATGTTGACCA
Kdm5c	ACCCACCTGGCAAAAACATTGG	ACTGTCGAAGGGGGATGCTGTG
Kdm6a	CCAATCCCCGCAGAGCTTACCT	TTGCTCGGAGCTGTTCCAAGTG

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CHAPTER 5

The Sex Chromosome Complement and Gonadal Hormones Mediate Sex Differences in White Adipose Tissue miRNAs

Chapter Preface

In the previous two chapters, we focused on protein-coding genes that were good candidates for contributing to differences between XX and XY mice. It is possible that noncoding genes, such as microRNAs, could also play a role in sex chromosome differences in obesity. To answer this question, we sequenced miRNAs from gonadal fat of Four Core Genotype mice that were gonadally intact or gonadectomized. We also sequenced miRNAs from gonadal fat of gonadectomized mice fed a high fat diet, the same mice from Chapter 3. Using these hormonal and dietary conditions, we compared the effects of gonadectomy and high fat diet on sex differences in adipose tissue miRNAs.

I worked with Dr. Xuqi Chen from the laboratory of Dr. Art Arnold to obtain the gonadal fat samples needed for this study. Because this was our laboratory's first venture in next-generation sequencing, I needed the expertise of others to guide me through the preparation of libraries and analysis of sequencing results. Dr. Yehudit Hasin-Brumshtein from the laboratory of Dr. A. Jake Lusis was instrumental in teaching me to prepare miRNA libraries. She processed all the raw sequencing reads and distilled the information down to a simple table of counts so that I could analyze expression levels of miRNAs. Although she had no obligation to teach me, she patiently explained all the steps she took to process the sequencing data.

Chapter 5 is the first reported study of sex differences in miRNAs from adipose tissue. The study of Four Core Genotype mice enables analysis of the effects of gonadal hormones in addition to sex chromosomes. When Dr. Karen Reue and I first designed this study, we also aimed to identify miRNAs that may escape X chromosome inactivation in adipose tissue. While we did not find X-linked miRNAs that were expressed higher in XX compared to XY mice, we did find a number of autosomal miRNAs that were differentially expressed between XX and XY mice, and between male and female mice. Understanding the sexual dimorphism in adipose tissue miRNAs will be a critical step toward understanding regulatory pathways in adipocyte development and function.

The sex chromosome complement and gonadal hormones mediate sex differences in white adipose tissue miRNAs

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ABSTRACT

MicroRNAs are key regulators of adipogenesis, and have differential expression between lean and obese animals. Fat accumulation and distribution of white adipose tissue are sexually dimorphic, and it is unknown whether adipose-specific miRNAs show sex differences in expression. Sex hormones, in addition to the sex chromosome complement, may regulate sex differences in gene expression in adipocytes. In this study, we use the Four Core Genotype mouse model (XX female, XX male, XY female, and XY male) to assess independent effects of the sex chromosome complement *versus* gonadal hormones in gonadal fat miRNAs. Dozens of miRNAs have differential expression between XX/XY males and XX/XY females, while others show XX–XY differences. In addition, sex differences in miRNA expression change after gonadectomy, suggesting that circulating levels of gonadal hormones modulate gonadal fat miRNAs. Furthermore, sex differences in miRNA expression change after 16 weeks of high fat diet. Our results demonstrate that miRNAs are regulated by gonadal hormones and the sex chromosome complement, and expression levels change in response to gonadectomy and diet-induced obesity.

INTRODUCTION

MicroRNAs (miRNAs) are small noncoding RNA molecules that modulate gene expression by targeting mRNA transcripts for degradation or interfering with mRNA translation (1,2). These 21- to 22-nucleotide molecules are derived from the processing of longer mRNAs transcribed from intergenic regions or introns of protein-coding genes, subject to co-transcription under the same promoter (3,4). There are around 2000 miRNAs in the human and mouse genomes, many of which are expressed in a tissue-dependent manner (5,6). Bioinformatic predictions estimate that 30–80% of mammalian mRNAs are targeted by miRNAs, and a given mRNA may be targeted by multiple miRNAs (7,8). In addition, a single miRNA can regulate multiple mRNA transcripts, potentially affecting several genes within a metabolic pathway (9,10). It is generally thought that miRNAs act to fine-tune mRNA levels, but there is also evidence of full repression of protein production, allowing miRNAs to act as a molecular switch (11–13).

miRNAs have important roles in the regulation of metabolic homeostasis. For example, miR-33a and miR-33b, which are embedded within genes for sterol regulatory element-binding proteins 2 and 1, respectively, have key roles in the modulation of cholesterol homeostasis (14,15). The discovery of the cellular roles of miR-33a/b in the repression of genes involved in cellular cholesterol export has revealed a new therapeutic target. Indeed, studies in mice and non-human primates have shown that antagonism of miR-33a/b reduces plasma lipid levels and atherosclerosis (16,17). Many other examples of important roles for miRNAs in metabolism have emerged recently (reviewed in 18). Among these are roles for miRNAs in the regulation of adipogenesis (18–20). For example, miR-30c, miR-143, miR-146b, and miR-378 have been shown to enhance adipocyte differentiation in mouse and human cells (21–24). Other miRNAs, such as miR-27, miR-130, and miR-138, inhibit adipogenesis (25–27). Some miRNAs that are induced during adipocyte development are dysregulated in obese mice, possibly due to chronic inflammation (28,29). While these few miRNAs have been well characterized, the roles of the majority of miRNAs expressed in adipose tissue are unknown.

Many properties of adipose tissue accumulation, distribution, and metabolism differ in a sexually dimorphic manner (30). To understand the basis for sex differences in adipose tissue and obesity, previous studies have focused on identifying mRNA species that differ between male and females in specific fat depots (31,32). It is likely that sex differences also occur in the expression levels of miRNAs in adipose tissue, and that these differences may, in turn, contribute to sex differences in mRNA levels. Consistent with this, sex differences in miRNA expression have been reported in brain (33), lung (34), and liver (35). However, the effect of sex on miRNAs in adipose tissue has not been investigated.

The mechanisms underlying sex differences in miRNA levels have not been studied. Sex differences can be attributed to both hormonal and genetic factors. Gonadal hormones have been considered to be the primary drivers of sex differences, and some miRNA levels in a variety of tissues may be responsive to estradiols (36). Importantly, however, the sex chromosome complement also plays a major role in determining adiposity (37,38), and it is possible that this is mediated in part by effects on miRNA levels.

In traditional human and animal studies, individuals with two X chromosomes also possess ovaries, and XY sex chromosomes coexist with testes. This makes it difficult to distinguish the effects of gonadal hormones from sex chromosome components. To overcome this limitation, we have used the Four Core Genotype (FCG) mouse model, which generates four combinations of gonads and sex chromosomes to create XX female, XX male, XY female, and XY male mice (39). FCG mice have a Y chromosome that carries a mutation in the testis-determining gene, *Sry* (denoted Y^-), such that XY^- mice develop ovaries rather than testes. In addition, FCG mice carry an autosomal *Sry* transgene that independently segregates from the Y^- chromosome. Mice that inherit the *Sry* transgene develop testes. By crossing XX females to XY^-*Sry* males, the four combinations of gonads and sex chromosomes can be obtained. Using this model, we previously demonstrated that the presence of two X chromosomes leads to increased adiposity compared to XY mice in the presence of either ovaries or testes (37).

Here, we analyze the genetic determinants of sex differences in miRNA expression levels in adipose tissue. We performed RNA sequencing (RNA-seq) of small RNAs present in gonadal fat of FCG mice, and determined that sex hormones and sex chromosomes both influence miRNA levels in fat. In addition, comparison of lean and obese mice revealed sex-specific changes in miRNA profiles in response to diet induced-obesity. These data raise the possibility that sexually dimorphic miRNA levels in adipose tissue contribute to the widely observed differences between males and females in adipose tissue biology and related diseases.

RESULTS

Study design to identify sex differences in adipose tissue miRNAs under three physiological conditions

To assess the effect of gonadal hormones and sex chromosomes on miRNA levels in gonadal adipose tissue, we sequenced miRNAs in FCG mice. Comparisons were made between XX/XY males and XX/XY females to reveal sex differences due to gonadal hormones, and comparisons between XX male/female mice and XY male/female mice were made to reveal sex chromosome effects. Additionally, we analyzed the effect of circulating gonadal hormones and dietary stress on sex differences in miRNAs by the analysis of three cohorts of mice: (1) a baseline group—FCG mice fed a chow diet; (2) a gonadectomized group—chow-fed FCG mice that were gonadectomized at 75 days of age to remove the acute actions of gonadal hormones; and (3) a high fat diet group—FCG mice that were gonadectomized and then fed a high fat diet for 16 weeks to identify changes related to diet and obesity. Adipose tissue samples from the gonadal fat depot were collected from all cohorts at the same age (7.5 months) and miRNA was sequenced. The resulting miRNA counts were used to establish a miRNA profile for adipose tissue in the basal state (chow fed, gonadally intact), and then to identify miRNAs that are responsive to the acute effects of gonadal hormones (comparison of the baseline group to the chow fed gonadectomized group), and miRNAs that are responsive to high fat feeding/obesity (by comparing the chow and high fat diet groups).

Sexual dimorphism in adipose tissue miRNA profile

To establish a baseline miRNA profile in adipose tissue, we sequenced and mapped 1,841 mature and precursor miRNAs in gonadal adipose tissue of FCG mice fed a standard laboratory chow diet. Of these, 183 miRNAs (10%) had at least 100 counts per million reads, indicating substantial expression levels in gonadal fat. Three miRNA species—miR-10b-5p, miR-143-3p, and miR-22-3p—accounted for nearly 50% of all miRNA reads (Figure 1A). 21 additional miRNA

species had greater than 0.5% relative abundance in gonadal fat (Figure 1A). Many of the most prevalent miRNAs in mouse adipose tissue have previously been shown to be abundant in human subcutaneous white adipose tissue (40).

To investigate the effects of sex on adipose tissue miRNA levels in the basal state, miRNAs were clustered based on expression pattern among the four genotypes. This revealed groups of miRNAs with sex-specific patterns (Supplemental Figure 1). To identify effects of male/female gonads, we searched for miRNA species that differ between mice with male *vs*. female gonads (male XX and male XY^- *vs*. female XX and female XY^-). To identify effects related to sex chromosome complement, we searched for miRNA species that differ between mice with XX *vs*. XY chromosomes (XX female and XX male *vs*. XY female and XY male). 10 miRNAs were upregulated in gonadal females compared to males, and 19 miRNAs were upregulated in gonadal males (Figure 1B). These miRNAs are likely responsive to gonadal hormones. Two miRNAs upregulated in females, miR-196a and miR-133a, are known to influence browning of white adipose tissue (41,42). It is possible that these miRNAs play a role in the previously reported sexual dimorphism of beige/brown fat. Studies in humans showed that females have increased brown adipose tissue and increased activity in brown fat (43–45). 19 miRNAs exhibited differences in expression levels based on sex chromosome complement, with 13 miRNAs expressed at higher levels in XX mice, and 6 miRNAs at higher levels in XY mice (Figure 1C). Thus, even in the presence of circulating gonadal hormones, the sex chromosome complement is a determinant of miRNA expression levels.

To identify potential target genes of the miRNAs that exhibit differences in expression levels dependent on gonadal or sex chromosome effects, we queried a miRNA target prediction database (mirdb.org) (46). Predicted targets were then clustered on the basis of gene ontology (GO) terms using DAVID (47,48). The predicted targets of the 19 miRNAs showing differences between XX and XY mice were associated with transcriptional regulation, chromatin organization, and cytoskeleton organization (Table 1). All of these processes have known

effects on adipocyte metabolism. For example, genes in the transcription regulation GO term include *E2f1, Ppargc1b*, and *Clock,* all of which have been implicated in oxidative metabolism (49–51). In addition, cytoskeleton remodeling is essential for the occurrence of adipocyte differentiation and expansion of fat cells (52).

Adipose tissue miRNAs regulated by gonadal hormones

Gonadal hormones cause sex differences in two primary ways. First, they impose permanent, or "organizational," sex-specific effects early in development, which remain throughout life. Second, male and female gonadal hormones in the circulation have acute effects on processes such as gene expression. In this case, when the gonadal hormone levels are reduced, the sex difference is also attenuated. To distinguish between organizational and acute gonadal hormone effects, we gonadectomized FCG mice as adults to remove the circulating gonadal hormones. Any remaining male–female differences can be attributed to organizational hormone effects.

To identify miRNA species that are influenced by male–female gonadal secretions, we performed miRNA-seq in the gonadal fat of FCG mice 5 months after gonadectomy. We hypothesized that gonadectomy would diminish male–female differences in miRNA levels. Unexpectedly, in gonadectomized mice, 61 adipose tissue miRNAs showed differences between males and females, which were twice as many as in gonadally intact mice (compare Figure 2B and Figure 1B). Because the gonadectomized mice were without circulating gonadal secretions for several months, the male–female differences are likely due to organizational effects of gonadal hormones that were suppressed by circulating hormones and became more evident when acute gonadal effects were removed. The predicted target genes for miRNAs that exhibit male–female dimorphism in the absence of gonads are enriched for pathways such as transcription regulation and chromatin organization (Table 2).

A comparison of miRNA species that exhibit male–female dimorphism in gonadectomized mice and gonadally intact mice showed little overlap between the two groups (Figure 2C). Both groups of miRNAs are responsive to gonadal secretions. In gonadally intact mice, miRNA species that are regulated differentially by circulating male or female gonadally-derived hormones are shown in Figure 1B. On the other hand, miRNAs that show male–female differences after gonadectomy (Figure 2_) may represent genes that are regulated by circulating hormones to normalize the levels between males and females, such that when acute gonadal effects are removed, male–female differences are uncovered. Taken together, our data identify several miRNA species in fat tissue that are highly responsive to gonadal hormone levels.

After gonadectomy, XX mice gain more weight and adiposity compared to XY mice, regardless of being male or female (37). We postulated that sex chromosome differences in miRNAs may contribute to the XX *vs*. XY differences in body weight. Of the miRNAs that are present above our defined threshold level in gonadal fat, 19 miRNAs showed an XX–XY difference in gonadectomized mice (Figure 2B). The predicted targets of these miRNAs are enriched for genes involved in macromolecule catabolism and transcription regulation (Table 2). Some of these targets, such as *Ppargc1a* and *Hoxa3,* have been implicated in obesity (53,54). In sum, these data provide insight into miRNAs that are influenced by the sex chromosome complement and by gonadal hormones. Further work is needed to determine whether these miRNAs are determinants of sex differences in adiposity.

Adipose tissue miRNA sex differences influenced by high fat diet or obesity

Several studies have shown that miRNA expression levels are regulated during adipogenesis or are altered in obesity (28,55). However, it is unknown whether sex influences miRNA expression levels in obese adipose tissue. We identified miRNAs that exhibit sexual dimorphism in obese adipose tissue by performing miRNA-seq on gonadal fat from gonadectomized FCG mice fed a high fat diet (60% calories from fat) for 16 weeks. 19

abundantly expressed miRNAs showed male–female differences in gonadal fat, and 43 miRNAs showed differences between XX and XY mice. Interestingly, there were more miRNA species that differed based on sex chromosome content in mice fed a high fat diet than in mice maintained on chow (Figure 3A). This raises the possibility that differential miRNA expression levels may contribute to the observed dramatic difference in adiposity of XX compared to XY mice in response to a high fat diet.

The targets of miRNAs that show sex differences in response to a high fat diet could be categorized into two functional groups. Targets of miRNAs showing XX–XY differences were enriched for phosphorus metabolic processes, while targets of miRNAs showing male–female differences were enriched for transcription regulation. miRNAs that showed sex chromosome differences also had predicted targets that were enriched for multiple KEGG pathways (Table 4). Among these were several pathways that are linked to adipogenesis and fat tissue expansion, including pathways associated with focal adhesion, MAPK signaling, and endocytosis (56,57). Our data show that the sex chromosome complement is a key regulator of miRNA levels, especially in mice fed a high fat diet.

DISCUSSION

miRNAs are key regulators of gene expression through their effects on the degradation or translation of mRNA transcripts. In metabolic tissues, miRNAs are essential for regulating processes that include adipogenesis, cholesterol homeostasis, and glucose homeostasis (18). These metabolic processes are also affected by sex. While there is evidence for sexual dimorphism in miRNA expression in brain, skeletal muscle, and immune cells, sex effects on miRNAs in adipose tissue have not been characterized (36). In this study, we used the FCG mouse model to identify sex chromosome complement and gonadal hormone effects on in miRNA abundance in gonadal fat. We sequenced miRNAs in three distinct cohorts of FCG mice in different hormonal and dietary conditions to determine the effects of circulating gonadal hormones and high fat diet on sex differences in gonadal fat miRNAs. Our analyses focused on the most abundant miRNAs, as we suspect that sex differences in these miRNAs are the most likely to have significant physiological effects. In gonadally intact mice, we identified a number of miRNAs for which levels differed between males and females, and other miRNAs that differed between XX and XY mouse adipose tissue. As expected, male–female differences in the levels of some miRNAs were abolished by gonadectomy. Unexpectedly, gonadectomy also induced male–female differences in a few dozen additional miRNA species. Because these male–female differences were revealed after gonadectomy, this suggests that organizational effects of gonadal hormones establish sex differences in miRNA expression early in development, but that circulating gonadal hormones suppress the differences between males and females.

Sex differences observed in miRNA levels may contribute to sex differences in adipogenesis and adipocyte maintenance and function. For example, miR-196a is much more highly expressed in females compared to males in both gonadally intact and gonadectomized FCG mice, and is a key regulator in brown fat cell development from white fat progenitor cells (41). Consistent with this, females have increased brown fat content as well as increased thermogenesis (43,44). Predicted targets of sex-specific miRNAs in both gonadally intact and

gonadectomized mice are associated with transcription regulation, chromatin organization, and catabolic processes. It is possible that these miRNAs fine-tune the expression of transcription factors and chromatin modifiers, which in turn modulate expression of their respective targets. Further studies are needed to explore the role of miRNAs in sex-specific adipocyte function.

After 16 weeks of a high fat diet, sex differences in miRNA levels change, suggesting a diet–sex interaction in miRNA expression. We identified 19 miRNAs with male–female differences, and 43 miRNAs with XX–XY differences. Compared to chow-fed mice, mice fed a high fat diet show twice as many XX–XY differences in miRNA expression. These miRNAs may contribute to the XX–XY difference observed in adiposity, in which mice with two X chromosomes gain more fat compared to mice with X and Y chromosomes (37).

It is known that the mammalian X chromosome encodes at least 100 miRNAs (5) and is thought to have to have a higher density distribution of miRNAs compared to autosomes (58). By contrast, there are no known miRNA genes on the Y chromosome. This raised the possibility that sex chromosome differences in miRNA levels could derive from miRNAs encoded by the X chromosome. However, only two of the miRNAs with levels that differed between XX and XY mice were encoded on the X chromosome (miR-221-5p, miR-322-5p), suggesting that factors other than X chromosome dosage drive the differences in miRNA levels related to sex chromosome complement.

Our study is the first report of sex differences in white adipose miRNAs. We identify specific miRNAs that are regulated by the sex chromosome complement and others that are responsive to circulating gonadal hormones. The sexually dimorphic miRNAs could potentially affect multiple cellular processes, including chromatin modification, cytoskeleton remodeling, and transcription regulation, thus altering adipocyte development and function. Future studies will be aimed at understanding the physiological roles of these sex-specific miRNAs in adipogenesis and diet-induced obesity.

METHODS

Animals

Four Core Genotypes (FCG) C57BL/6 mice were bred and genotyped as described previously (Chen 2012, Link *in press*). Briefly, XX female mice were mated with XY^{-}*(Sry+)* male mice to generate XX, XX(*Sry+)*, XY^{-}, and XY^{-}*(Sry+)* offspring, and genotyping was performed by PCR to detect the *Sry* transgene and Y-chromosome–specific sequence. Where indicated, gonadectomy was performed at 75 days of age.

Gonadal males and females were housed in separate cages and maintained at 23°C with a 12:12 hour light:dark cycle. All mice were initially fed Purina mouse chow diet containing 5% fat (Purina 5001; PMI Nutrition International, St. Louis, MO). Where specified, mice were fed a chow diet until 3.5 months of age (4 weeks after gonadectomy), and then fed a high fat diet for 16 weeks (diet #S3282 containing 60% calories from fat, Bio-Serv, Flemington, NJ). Adipose tissue was harvested from all mice at 7.5 months of age.

Mouse studies were conducted in accordance with approval by the Institutional Animal Research Committee of the University of California, Los Angeles.

RNA extraction and quality control

At the time of dissection, gonadal fat tissue was flash frozen in liquid nitrogen and stored at –80°C. Small RNAs were isolated from 100 mg tissue using QIAzol and Qiagen's miRNeasy Mini kit (Cat. 217004, Qiagen, Valencia, CA). After homogenization, samples were centrifuged at 12,000 x *g* for 10 minutes to separate the transparent lipid layer from the pink organic layer. Only the organic layer was used in chloroform extraction. All subsequent steps followed the Qiagen protocol. RNA samples were submitted to Agilent BioAnalyzer Eukaryote Total Nano-RNA chip analysis, yielding RNA integrity numbers of 7.5 or greater.

miRNA library preparation

Three samples of each genotype were pooled into equimolar amounts for library preparation. In total, 12 miRNA libraries were made: 4 libraries for each of the genotypes in chow-fed, gonadally intact mice, 4 libraries for chow-fed, gonadectomized mice, and 4 libraries for gonadectomized mice fed a high fat diet.

miRNA libraries were processed individually using standard protocol from Illumina TruSeq Small RNA kit, with indexes 1-12 and gel purified according to Illumina protocol instructions. Final sequencing library concentration (19.07 nM) was determined using KAPA library quantification qPCR kit (KK4854). Sequencing was performed by the Broad Stem Cell Research Center core facility at UCLA, on Illumina HiSeq 2000.

Reference preparation

miRNA gene expression is typically quantified by counting reads that map to the miRNA genes. However, in some families of miRNAs, several genes give rise to identical mature sequence, thus it is impossible to distinguish which miRNA gene gave rise to the mature sequence based on sequencing only. The purpose of reference preprocessing was to compile a list of unique expressed mouse miRNA sequences available, regardless of their gene of origin, so that quantification is done on the level of mature miRNA sequence, rather than on the gene level.

Reference sequence was compiled based on all mature and precursor miRNA sequences available from the main repository of miRNA studies, miRBase, version 18 (http://www.mirbase.org) and included all mature and precursor sequences. The respective mature sequences within precursor sequences were masked to "N", to prevent mature sequences mapping both to mature and precursor. Thus reads mapping to precursor sequences mapped to precursor exclusively.

In cases where several genes gave rise to the same mature sequence, that sequence was represented only once in the reference under one name, with additional column listing all matching genes as potential targets in the results. In total 1007 mature miRNA sequences were unique (listed in Ref_Uq.txt file), while 256 miRNAs grouped into 82 unique sequences (listed in Ref_Eq.txt file). Furthermore, in 7 cases the miRNAs were not completely equivalent, rather one miRNA was 1 base shorter than the other. In such cases the shorter sequence would map to both mature sequences, while the longer one would map uniquely to the longer sequence. For these pairs the counts of the shorter sequence was determined as the total count mapping to longer sequence minus the unique mappings to longer sequence (listed in Ref_NEq.txt file).

Read processing

Reads were demultiplexed in .qseq format based on perfect match to barcode sequence, and converted to .fastq format using in-house Perl scripts (available upon request). Read quality was assessed with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and adaptor sequence was clipped using cutadat tool (https://code.google.com/p/cutadapt/). Clean reads 18-30 bp long were retained for subsequent mapping and analysis. Identical reads were collapsed using colapse.pl script from mirDeep package (https://www.mdc-berlin.de/8551903/en/). Reads were then mapped to reference with BWA aligner allowing for up to 1 mismatch and no gaps (80-90% of the reads mapped).

Read counting and result table

Reads that uniquely mapped to one target were counted towards that target. Reads that had both perfect and imperfect (1 mismatch) matches, were counted only towards the perfect match target. Results were summarized in a count table that listed the counts for each target in each sample. Precursor miRNA counts are based on reads mapping exclusively to precursor sequences, and not to any mature miRNA sequence in the reference. For each reference

sequence total count of reads, and the number of reads that originate from 1-mismatched reads were presented separately. The number of mismatched reads is a subset of total reads mapping to the reference.

Percent abundance of miRNAs was calculated by normalizing the reads from one miRNA to the total number of reads mapped for all miRNAs. The mean of the four genotypes is represented in Figure 1A and Supplemental Figure 2.

All mapped miRNA reads will be deposited online.

Hierarchical clustering

1,841 miRNAs were mapped to the mouse genome. 183 miRNAs having ≥100 counts per million reads in at least one of the 12 libraries were identified as “highly expressed” in gonadal fat. These miRNAs were submitted to the Hierarchical Clustering module in the Broad Institute’s software package GenePattern (59,60). Within the module, we used Pearson correlation for the distance measure and pairwise complete-linkage for the clustering method. The color scheme for heat map representations was normalized within each miRNA species, such that red represents the maximum value, blue represents the minimum value, and white represents the mean across four genotypes. miRNAs were selected for further analysis based on clusters that showed differences between gonadal male and gonadal female, or differences between XX and XY mice. Counts for these miRNAs were transformed to $\log_2$ fold-change over the mean of each miRNA, and then resubmitted to the Hierarchical Clustering module. The color scheme was normalized globally, such that yellow represents upregulated changes greater than 2-fold ($\log_2$ value of 1), magenta represents downregulated changes greater than 2-fold ($\log_2$ value of -1), and black represents no change.

Target prediction

Targets for miRNAs showing sex differences were predicted by mirdb.org (46). All targets with a prediction score of 95 or above were included in subsequent functional analysis using DAVID (47,48). The functional annotation tool was used to identify enriched annotation terms in the gene ontology (GO) category GOTERM_BP_FAT or in the KEGG Pathway. Unique GO terms and KEGG pathways with a Bonferroni-corrected p-value < 0.05 were included in Tables 1-4.

Table 1. Predicted targets of miRNAs with sex differences in gonadally intact mice

	GO Term	Genes	Fold Enrichment	Bonferroni-corrected *P*-value
Intact SCC difference	GO:0045449~ regulation of transcription	*E2f1, Cops2, E2f5, Thrb, Tsg101, Znfx1, Ezh1, Arid4b, Nr6a1, Bbx, Foxo3, Cnot7, Gli2, Cbx7, Epc2, Zfp781, Hif1an, Wdr77, Mier3, Eed, Kdm5a, Kdm5c, Rarg, Sox13, Foxn2, Nfam1, Topors, Rbbp7, Prox1, Ube2b, Dmrtb1, Flna, Ppargc1b, Ell2, Myt1l, Kdm2b, Ep300, Rab18, Trim33, Zfp462, Zfp120, Tcfl5, Zfp280d, Hipk3, Foxg1, Zfpm2, Wasl, Kdm6b, Clock, Med1, Camta1, Mtdh, Ell, Taf9b, Gon4l, Elk3, Sec14l2, Nr2c2, Med12l, Npas2, Tcerg1, Cdyl, Rb1cc1, Ovol1, Hoxa9, Kdm3a, Hbp1, Lhx8, Bcor, Runx2, Klf6, Rfx6, Maml1, Creb1, Klf10, Rfx7, Zbtb41, Smad1, Klf15, Sfmbt1, Rps6ka5, Sall1, Rnf2, Jazf1, Zfp800, Setd7, Irf4, Tmpo*	1.58	0.01
	GO:0006325~ chromatin organization	*Shprh, Arid4a, Ezh1, Arid4b, Bnip3, Rbbp7, Ube2b, Cbx7, Epc2, Cdyl, Kdm2b, Ep300, Rnf2, Smarcd1, Eed, Setd7, H2afy, Kdm3a, Irf4, Kdm5a, Bcor, Kdm6b, Kdm5c*	2.93	0.03
	GO:0030036~ actin cytoskeleton organization	*Fmnl2, Mtss1, Trpm7, Capza1, Rictor, Itgb1, Prox1, Flna, Elmo1, Coro1c, Pfn2, Arhgap6, Ezr, Sorbs1, Limch1, Wasl*	3.89	0.03
Intact Sex difference	GO:0019941~ modification-dependent protein catabolic process	*Usp2, Ube2g1, Uba6, Rnf216, Ube2v2, Anapc10, Edem3, Znrf1, Cd2ap, Senp7, Arih1, Cul5, Fbxw7, Ppp2cb, Klhl20, Usp32, Fbxo45, Tbl1xr1, Shprh, Usp1, Hace1, Ube2q2, Wsb1, Cblb, Trim33, Uba2, Fbxo30, Det1, Rnf139, Usp46, Fbxo33, Usp42, Fbxl3*	2.51	0.006

Table 2. Predicted targets of miRNAs with sex differences in gonadectomized mice.

	GO Term	Genes	Fold Enrichment	Bonferroni -corrected *P*-value
GDX SCC difference	GO:0044265~ cellular macromolecule catabolic process	*Ube2z, Ube3a, Ube2g1, Usp9x, Uba6, Anapc10, Fem1b, Otub2, Lin28b, Mycbp2, Auh, Cul3, Fbxl19, Fbxw7, Fbxo42, Shprh, Adam10, Pan3, Usp1, Hace1, Spsb4, Ubr1, Usp28, Prkcq, Rnf6, Trim33, Asb1, Fbxo32, Fbxl7, Chfr, Spopl, Usp25, Fbxo11*	2.55	0.004
	GO:0045449~ regulation of transcription	*Mef2c, E2f5, Elf5, Nr6a1, Stat5b, Hira, Zeb2, Ctcf, Cbfb, Pnn, Bzw1, Epc1, Epc2, Pcgf3, Hsf1, Hoxc4, Mier3, Sertad3, Kdm5a, Satb1, Cdk8, Med12, Foxn2, Grhl3, Ccnc, Six4, Grhl1, Pkia, Ppargc1a, Ell2, Prkcq, Dcaf6, Adrb2, Zfp462, Trim33, Btg2, Ereg, Hipk3, Nab1, Wasl, Suv420h1, Camta1, Zfp395, Ube3a, Zfp398, Sox5, Lin28b, Nr2c2, Mycbp2, Hic2, Hoxa3, Dmd, Tef, Hbp1, Baz2a, Mllt3, Erf, Rex2, Zfp600, Arid3b, Mafk, Foxp4, Foxp2, Rnf6, Zfhx4, Meox2, Csrnp2, Ebf3, Atxn7, Trps1, Pbx1, Setd8, Hivep1, Tmpo, Pbx3, Ncor1*	1.58	0.05
GDX Sex difference	GO:0045449~ regulation of transcription	*Thrb, Morf4l2, Naa15, Bbx, Rest, Rora, Foxo3, Nfxl1, Cbx7, Cbfb, Zfp781, Sin3b, Epc2, S1pr1, Mier1, Hoxc4, Mier3, Phtf2, Rara, Sap30l, Rreb1, Emx2, Eomes, Foxn2, Hmg20a, Prox1, Ell2, Sltm, Suz12, Kdm2b, Zfp120, Kdm2a, Rab18, Trim33, Zfp280d, Jun, Vegfa, Tgif1, St18, Smarca2, Tshz3, Hmgb1, Camta1, Ell, Taf9b, Mybl1, Lin28b, Vdr, Hoxa3, Tcerg1, Dmd, Ovol1, Hoxa9, Pbrm1, Asf1a, Ikzf5, Asxl2, Klf6, Dnmt3a, Yeats4, Nrbf2, Ehmt1, Klf12, Crebzf, Smad7, Klf10, Rfx7, Zfp775, Arid3b, Klf15, Smad1, Abcg4, Ubp1, Sfmbt1, Foxp4, Sfmbt2, Foxp2, Hdac4, Notch1, Zfhx4, Hoxb7, Dmtf1, Trps1, Jazf1, Zfp800, Hivep1, Rfx3, Tmpo, Tcf12, Ncor1, Rere, Cops2, Hlf, Elf2, E2f5, Ezh2, Pparg, E2f8, Nr6a1, Arid4b, Zeb2, Zeb1, Gli2, Cbfa2t3, Bzw1, Pcgf3, Hif1an, Hsf1, Pou4f1, Kdm5a, Zfp423, Tbl1xr1, Pogz, Cdk8, Lef1, Ncoa7, Nfam1, Rbbp7, Ube2b, Mycn, Adrb2, Brwd1, Hipk1, Ereg, Hipk3, Cand1, Wasl, Lin54, Vopp1, Clock, Zfp711, Zmynd11, Lcorl, Ube3a, Zfp398, Ctnnd2, Nr3c1, Arid2, Sec14l2, Hic2, Tnfrsf1a, Npas2, Tsc22d2, Cdyl, Hand1, Nr1d2, Cenpb, Rb1cc1, Gm608, Hbp1, Zfp521, Baz2a, Mllt3, Ssrp1, Erf, Jarid2, Nlk, Hmbox1, Aff3, Phf19, Id2, Yaf2, Dr1, Setd7, Zbtb5, Kdm4c, Pbx1, Setd8, Irf4, Tceb1, Pbx3, Nfia, Nfib*	1.63	2.9E-08

Table 2, continued

	GO Term	Genes	Fold Enrichment	Bonferroni-corrected *P*-value
GDX Sex difference (continued)	GO:0010629~ negative regulation of gene expression	*Cops2, Thrb, Pparg, Nr6a1, Bnip3, Zeb2, Rest, Zeb1, Gli2, Lin28b, Vdr, Sin3b, Hand1, Ovol1, Gm608, Rara, Baz2a, Tbl1xr1, Dnmt3a, Ehmt1, Klf12, Jarid2, Lef1, Rbbp7, Ube2b, Foxp4, Foxp2, Suz12, Hdac4, Ereg, Hipk1, Id2, Yaf2, Hipk3, Trps1, Bnip3l, Jazf1, Tgif1, Hivep1, Rfx3, St18, Ncor1*	2.14	0.02
	GO:0006325~ chromatin organization	*Ezh2, Morf4l2, Arid4b, Ino80, Bnip3, Nr3c1, Cbx7, Epc2, Cdyl, Pbrm1, Chd6, Kdm5a, Baz2a, Asf1a, Yeats4, Tbl1xr1, Dnmt3a, Shprh, Ehmt1, Hmg20a, Arid1a, Rbbp7, Ube2b, Suz12, Hdac4, Kdm2b, Kdm2a, Kdm4c, Setd7, Setd8, Irf4, Smarca2, Ncor1, Map3k12, Rere*	2.33	0.02
	GO:0010605~ negative regulation of macromolecule metabolic process	*Cops2, Thrb, Pparg, Nr6a1, Bnip3, Zeb2, Fkbp1a, Rest, Zeb1, Gli2, Lin28b, Vdr, Sin3b, Hand1, Ovol1, Gm608, Rara, Baz2a, Tbl1xr1, Dnmt3a, Ehmt1, Klf12, Jarid2, Paip2, Lef1, Rbbp7, Ube2b, Prkcd, Foxp4, Foxp2, Suz12, Hdac4, Ereg, Hipk1, Id2, Yaf2, Hipk3, Trps1, Jun, Psen2, Bnip3l, Jazf1, Tgif1, Hivep1, Rfx3, St18, Igfbp3, Ncor1*	1.99	0.02

Table 3. Predicted targets of miRNAs with sex differences in gonadectomized mice fed a high fat diet.

	GO Term	Genes	Fold Enrichment	Bonferroni-corrected *P*-value
HFD SCC difference	GO:0006793~ phosphorus metabolic process	*Cdk19, Tbk1, Synj1, Prkg2, Acvr1c, Pak6, Pak4, Synj2, Stk39, Tlk2, Sik1, Cdk13, Ret, Sgk1, Pan3, Pik3cb, Trpm7, Ptprg, Pik3cd, Ppp1cc, Pdik1l, Acvr2a, Styk1, Eya1, Mapk6, Hipk3, Pdgfra, Mapk8, Nrk, Reln, Map3k12, Fgfr3, Mknk2, Bmpr2, Pxk, Kit, Ppm1b, Atp5g3, Uhmk1, Ercc6, Stk40, Ptplb, Snrk, Lmtk2, Camk2d, Prkaa2, Mtmr4, Nlk, Ptpn13, Ick, Plk2, Ulk2, Ptp4a1, Tgfbr3, Ptpn1*	1.82	0.04
HFD Sex difference	GO:0045449~ regulation of transcription	*E2f1, Cops2, Elf2, E2f5, Thrb, Tsg101, Znfx1, Ezh1, Arid4b, Morf4l2, Nr6a1, Bbx, Hira, Rest, Cnot7, Cbx7, Cbfb, Pnn, Epc1, Bzw1, Epc2, Zfp781, Pcgf3, Mier3, Kdm5a, Zfp275, Eomes, Cdk8, Topors, Rbbp7, Prox1, Ube2b, Prkcq, Myt1l, Eya1, Adrb2, Zfp120, Zfp462, Trim33, Zfp280d, Vegfa, Nab1, Zfpm2, Clock, Taf9b, Sox5, Gon4l, Elk3, Lin28b, Nr2c2, Mycbp2, Med12l, Vdr, Hic2, Tnfrsf1a, Npas2, Tcerg1, Cdyl, Rb1cc1, Dmd, Cenpb, Hbp1, Plagl2, Dnmt3a, Klf6, Rex2, Creb1, Zfp600, Klf15, Sfmbt1, Abcg4, Foxp2, Rps6ka5, Hdac4, Rnf6, Hoxb7, Meox2, Sall1, Zfp800, Zbtb5, Pbx1, Nfia*	1.63	0.009

Table 4. Enriched KEGG pathways of predicted miRNA target genes

	KEGG Pathway	Genes	Fold Enrichment	Bonferroni -corrected *P*-value
Intact Sex difference	mmu05211: Renal cell carcinoma	*Hif1a, Ets1, Sos1, Sos2, Pik3cd, Gab1, Vegfa, Rap1b, Akt3*	5.31	0.03
GDX Sex difference	mmu04512: ECM-receptor interaction	*Col4a2, Npnt, Col3a1, Itga2, Reln, Col5a3, Col11a1, Itgb1, Thbs2, Col5a2, Col5a1, Sdc2*	3.62	0.05
HFD SCC difference	mmu04510: Focal adhesion	*Col4a2, Pik3cb, Col3a1, Pik3cd, Itga3, Ppp1cc, Col5a3, Col5a2, Flna, Col5a1, Pak6, Sos1, Pak4, Vegfa, Sos2, Pdgfra, Rap1a, Mapk8, Reln, Rap1b, Col11a1*	3.40	3.4E-04
	mmu04010: MAPK signaling pathway	*Mef2c, Cacna2d1, Fgfr3, Nlk, Ppp3r1, Mknk2, Fgf21, Ppm1b, Flna, Acvr1c, Tnfrsf1a, Sos1, Sos2, Pdgfra, Rap1a, Mapk8, Rap1b, Fasl, Rapgef2, Gadd45a, Pla2g4e, Map3k12, Rasa1*	2.78	0.003
	mmu04144: Endocytosis	*Ret, Fgfr3, Vps37a, Psd3, Kit, Psd2, Acvr1c, Eps15, Adrb3, Adrb2, Cblb, Pikfyve, Rab11b, Vps4b, Pdgfra, Stam, Arap2, Epn2, Ap2m1*	3.02	0.006

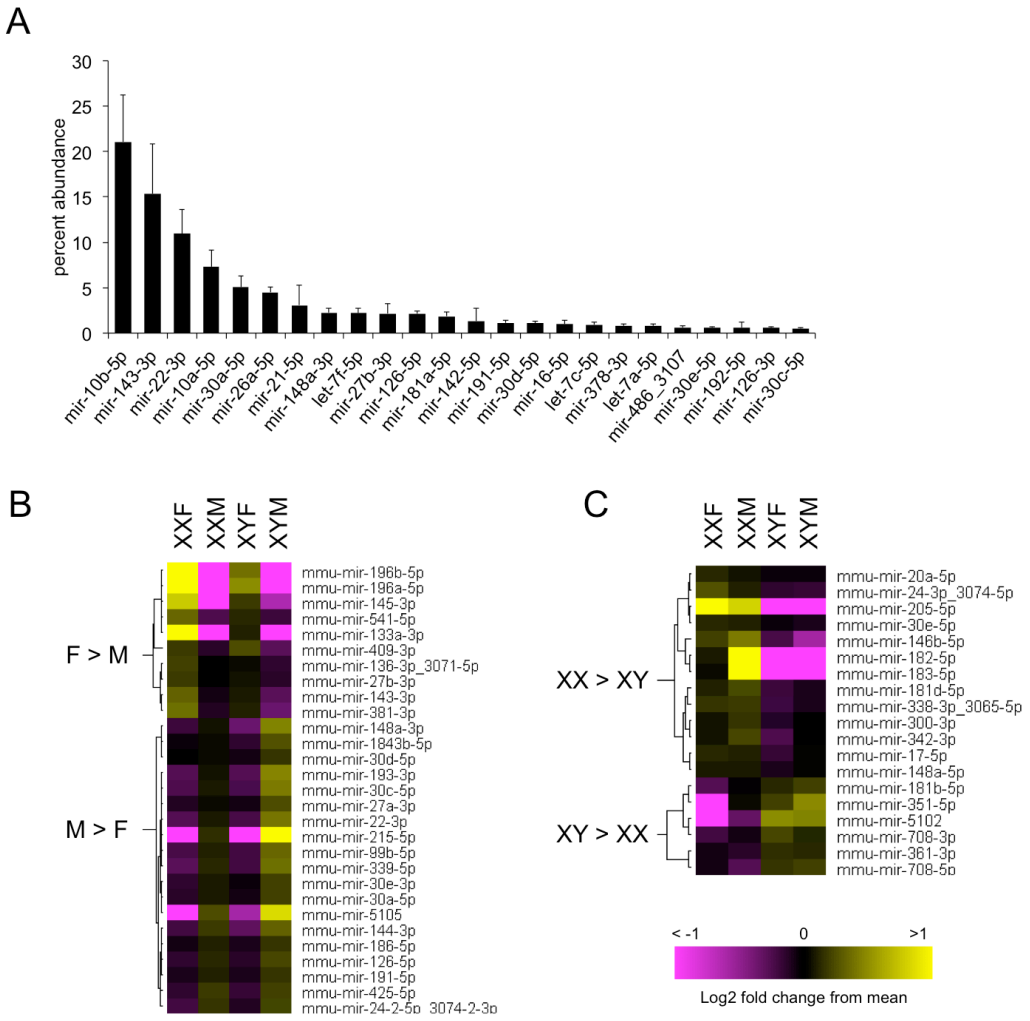
FIGURE LEGENDS

Figure 1. miRNA sequencing of gonadal fat reveals sexual dimorphism. (A) Percent abundance of miRNAs in gonadal fat of FCG mice. Gonadal fat miRNAs are expressed differently between males and females (B) and between XX and XY (C) gonadally intact mice fed a standard lab chow diet.

Figure 2. Gonadectomy reveals long-term organizational hormone regulation of miRNAs. (A, B) Sex differences in chow-fed gonadectomized FCG mice. Gonadal fat miRNAs show differences between males and females (A) and between XX and XY mice (B). (C) Number of gonadal fat miRNAs that show male–female differences in gonadally intact and gonadectomized (GDX) mice.

Figure 3. High fat diet brings out sex chromosome differences in miRNAs. (A) Number of miRNAs that show XX–XY differences in chow-fed gonadally intact and gonadectomized (GDX) mice, and gonadectomized mice fed a high fat diet (HFD). Sex differences between male and female (B) and between XX and XY (C) gonadectomized mice fed the high fat diet.

Figure 1


A
percent abundance
30
25
20
15
10
5
0
mir-10b-5p
mir-143-3p
mir-22-3p
mir-10a-5p
mir-30a-5p
mir-26a-5p
mir-21-5p
mir-148a-3p
let-7f-5p
mir-27b-3p
mir-126-5p
mir-181a-5p
mir-142-5p
mir-191-5p
mir-30d-5p
mir-16-5p
let-7c-5p
mir-378-3p
let-7a-5p
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mir-192-5p
mir-126-3p
mir-30c-5p
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XYF
XYM
F > M
M > F
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mmu-mir-196a-5p
mmu-mir-145-3p
mmu-mir-541-5p
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mmu-mir-27b-3p
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XYF
XYM
XX > XY
XY > XX
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mmu-mir-351-5p
mmu-mir-5102
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mmu-mir-361-3p
mmu-mir-708-5p
< -1
0
>1
Log2 fold change from mean

Figure 2

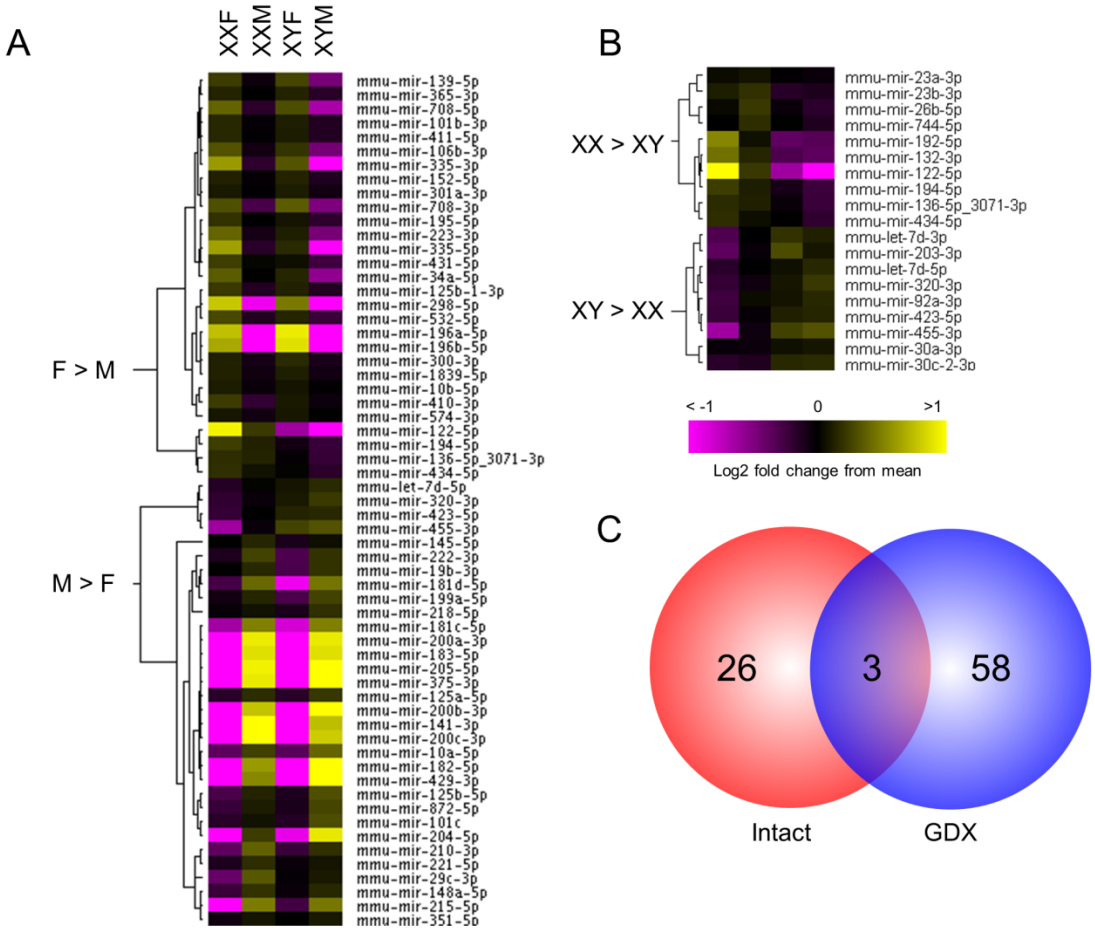
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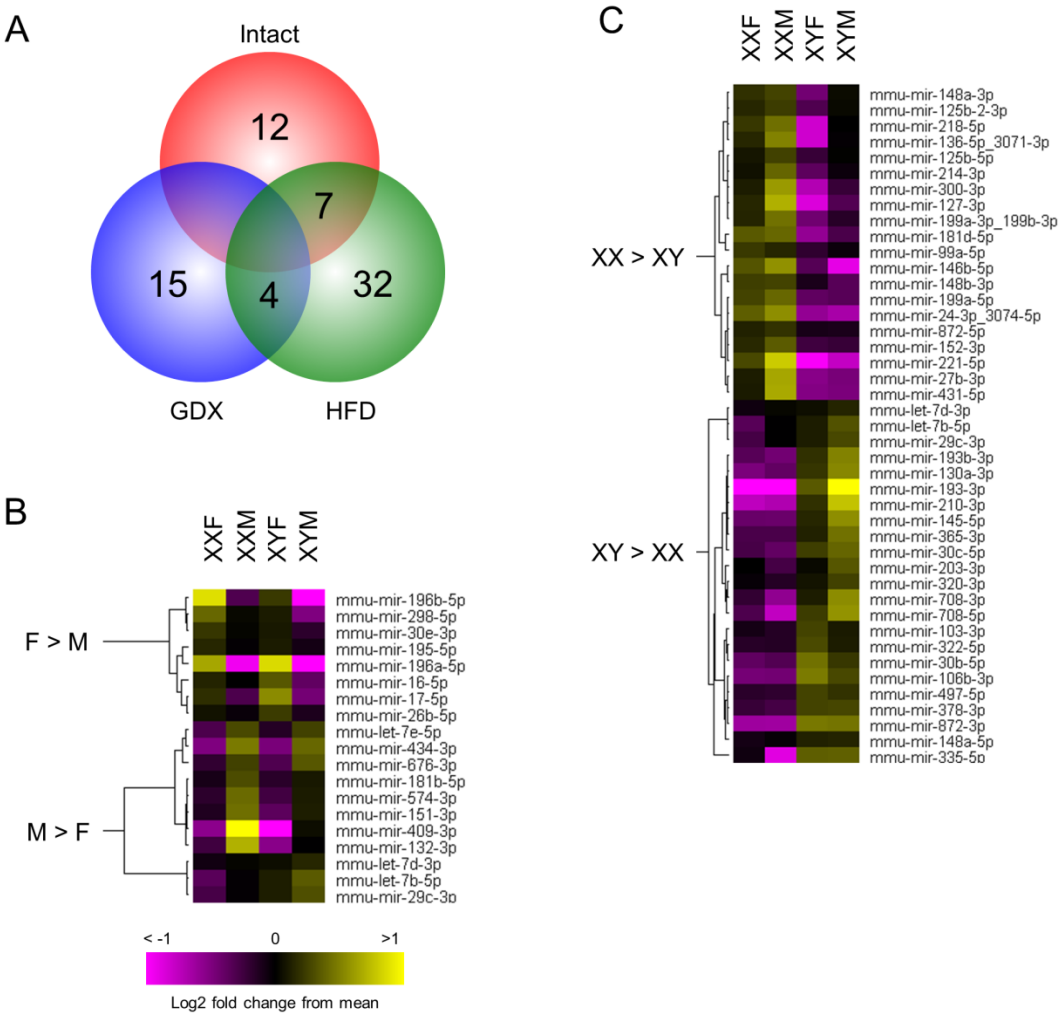
Figure 3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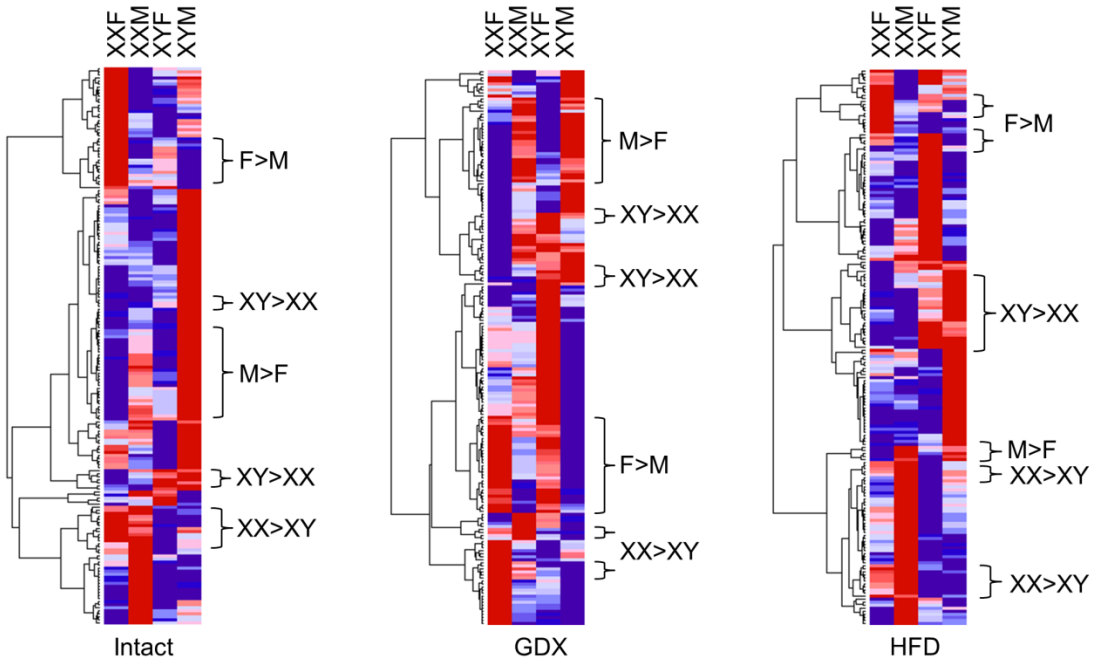
Supplemental Figure 1



Supplemental Figure 1. Clustering of miRNAs abundant in gonadal fat. miRNAs expressed higher than 100 per million reads in at least one library.

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CHAPTER 6

The Presence of XX versus XY Sex Chromosomes is Associated with Increased HDL Cholesterol Levels in the Mouse

Chapter Preface

Dyslipidemia is a co-morbidity of obesity. Plasma cholesterol and triglyceride levels are associated with the metabolic syndrome and are risk factors for cardiovascular disease. Published human studies indicate that sexual dimorphism exists in plasma LDL and HDL cholesterol levels, but this is dependent on ethnicity. This chapter examines the effect of gonadal hormones and the sex chromosome complement on plasma lipid levels in Four Core Genotype mice.

The Four Core Genotype model is unique in allowing the independent detection of sex chromosome effects from gonadal hormone effects. In addition, we can use this model under different dietary and hormonal conditions to assess the change in sex differences in metabolic parameters. In this chapter, we compare plasma lipid levels of gonadally intact with gonadectomized mice, as well as chow-fed mice with mice fed a high cholesterol diet. I worked with Dr. Xuqi Chen from the laboratory of Dr. Art Arnold to obtain plasma from these mice and measure different lipid species using colorimetric assays and fast protein liquid chromatography (FPLC). We noticed that no matter the diet or levels of circulating gonadal hormones, XX mice had higher levels of HDL cholesterol than XY mice. We collaborated with Dr. Mark Borja and Dr. Michael Oda to determine HDL-apoA-I exchange activity, which is an assessment of HDL function. The exchange activity reflected HDL concentrations.

I sought to determine the molecular mechanisms underlying observed sex differences in plasma lipid levels. A dedicated undergraduate student, Christopher Prien, and I measured hepatic expression of genes involved in cholesterol synthesis and transport and bile acid synthesis. Although we identified several genes that showed sex differences in mRNA expression, there was no single canonical pathway associated with the increased HDL cholesterol in XX mice *versus* XY mice.

However, the hepatic expression of a few X escapee genes (*Ddx3x*, *Eif2s3x*, *Kdm5c*, and *Kdm6a*) was positively correlated with HDL levels. This was consistent across all dietary and

hormonal conditions. These results reinforced our hypothesis that these candidate genes have key roles in mediating sex differences in lipid metabolism.

Dr. Karen Reue and I worked closely to design these experiments and critically analyze the results. Our studies demonstrate that both gonadal hormones and the sex chromosome complement influence plasma lipid levels. In addition, HDL cholesterol levels are increased in XX *versus* XY mice irrespective of diet and gonadal hormone levels. These studies have been accepted for publication in the journal *Arteriosclerosis, Thrombosis*, *and Vascular Biology*.

The presence of XX versus XY sex chromosomes is associated with increased HDL cholesterol levels in the mouse

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Running Title: Increased HDL-C in mice with two X chromosomes

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Keywords: sex differences, sex chromosome complement, plasma lipids, HDL cholesterol

ABSTRACT

Objective—The molecular mechanisms underlying sex differences in dyslipidemia are poorly understood. We aimed to distinguish genetic and hormonal regulators of sex differences in plasma lipid levels.

Approach and Results—We assessed the role of gonadal hormones and sex chromosome complement on lipid levels using the Four Core Genotypes mouse model (XX females, XX males, XY females, and XY males). In gonadally intact mice fed a chow diet, lipid levels were influenced by both male–female gonadal sex and XX–XY chromosome complement. Gonadectomy of adult mice revealed that the male–female differences are dependent on acute effects of gonadal hormones. In both intact and gonadectomized animals, XX mice had higher HDL cholesterol (HDL-C) levels than XY mice, regardless of male–female sex. Feeding a cholesterol-rich diet produced distinct patterns of sex differences in lipid levels compared to a chow diet, revealing the interaction of gonadal and chromosomal sex with diet. Notably, under all dietary and gonadal conditions, HDL-C levels were higher in mice with two X chromosomes compared to mice with an X and Y chromosome. By generating mice with XX, XY and XXY chromosome complements, we determined that the presence of two X chromosomes, and not the absence of the Y chromosome, influences HDL-C concentration.

Conclusions—We demonstrate that having two X chromosomes *versus* an X and Y chromosome complement drives sex differences in HDL-C. It is conceivable that increased expression of genes escaping X-inactivation in XX mice regulates downstream processes to establish sexual dimorphism in plasma lipid levels.

ABBREVIATIONS

HDL — high density lipoprotein

HDL-C — high density lipoprotein cholesterol

LDL — low density lipoprotein

LDL-C — low density lipoprotein cholesterol

TG — triglyceride

FFA — free fatty acid

FCG — four core genotypes

INTRODUCTION

Plasma lipid levels are used as both clinical predictors and as therapeutic targets for cardiovascular disease. As such, substantial effort has been expended to identify genetic and environmental factors that influence plasma lipid levels.[1–4] A key genetic determinant of plasma lipid levels is male–female sex. Inherent sex differences in lipid levels have led to distinct standards for the diagnosis of hyperlipidemia in men and women, but the underlying mechanisms that contribute to differences in lipid levels are not well understood. Men tend to have higher low density lipoprotein (LDL) and triglyceride (TG) levels, and lower high density lipoprotein (HDL) levels, than premenopausal women.[5,6] After menopause, women often have proatherogenic lipid levels that reach or exceed those in men.[5–7] These observations support a role for gonadal hormones as a key determinant of sexual dimorphism in lipid levels.[7] However, it is unlikely that differences in lipid levels between men and women can be explained exclusively by gonadal hormone levels, since hormone replacement in post-menopausal women does not provide an overall cardioprotective effect.[8,9] Furthermore, estrogen therapy does not benefit both sexes equally.[10]

The findings described above suggest that additional factors besides gonadal hormones may influence sex differences in lipid levels. Besides gonadal secretions, another fundamental

difference between males and females is the presence of an XX or XY sex chromosome complement. Sex differences due to gonadal sex *vs*. sex chromosome complement have been difficult to discriminate because, typically, female gonads occur together with XX chromosomes, and male gonads with XY chromosomes. In the current study, we used the Four Core Genotypes (FCG) mouse model to identify independent effects on plasma lipid levels of gonadal sex (testes *vs.* ovaries) and sex chromosome complement (XX *vs.* XY). The FCG model consists of four types—or "sexes"—of mice: gonadal male mice with either XX or XY sex chromosomes, and gonadal female mice with XX or XY sex chromosomes.[11–13] In the FCG model, the Y chromosome is deleted for the testis-determining *Sry* gene, which is provided instead by an *Sry* transgene inserted into an autosome. As a result, gonadal sex segregates independently from the sex chromosome complement. Sex differences observed between gonadal males and females can be attributed to the action of gonadal hormones, whereas differences between XX and XY mice can be ascribed to the number of X or Y chromosomes. Additionally, by comparing intact and gonadectomized mice, further distinction can be made between the effects of gonadal hormones during development and those resulting from acute effects of hormones in adulthood.

We recently used the C57BL/6 FCG mouse model to determine how sex chromosome complement contributes to sex differences in metabolic traits, such as body weight, adiposity, and hepatic lipid content. Specifically, when gonadectomized as adults to remove acute gonadal effects, XX mice have increased obesity and fatty liver compared to XY mice, regardless of whether they originally had male or female gonads.[14] We hypothesized that sex chromosome complement may also contribute to sex differences in plasma lipid profiles. Using FCG mice, here we identify independent effects of gonadal hormones and sex chromosome complement on plasma lipoprotein levels. These results have implications for understanding the basis for sex differences in men and women, and may inform about key risk factors in the metabolic syndrome.

MATERIALS AND METHODS

Materials and Methods are available in Supplemental Material.

RESULTS

Acute gonadal hormones and the sex chromosome complement influence plasma lipid levels

To analyze sex differences, we measured fasting lipid levels (total cholesterol, HDL cholesterol (HDL-C), TG, and free fatty acids (FFA)) in the four genotypes of FCG mice. We defined HDL-C levels as the cholesterol present in particles that lack apoB, and LDL/VLDL cholesterol levels as that from all non-HDL particles (see Materials and Methods). Statistical analyses were performed by two-way ANOVA, with gonadal sex (male or female) and sex chromosome complement (XX or XY) as covariates.

We first assessed plasma lipid levels in gonadally intact mice fed a chow diet. Total cholesterol levels were similar to those reported previously for C57BL/6 mice, with HDL-C accounting for the majority of plasma cholesterol, as is typical in mice.[15,16] Compared to females, male mice had higher levels of total and HDL-C, as well as TG and FFA (Fig. 1A). Males also had slightly higher amounts of unesterified cholesterol (UC in Fig. 1). Notably, however, animals of both gonadal sexes with XX chromosomes had 20% higher HDL-C levels than XY mice ($p<0.02$). These results indicate that male–female gonads are a determinant of sex differences in plasma lipid levels, but also reveal that the sex chromosome complement influences HDL-C levels, even in the presence of normal gonadal hormone levels.

The sex differences in lipid levels that were observed between males and females could result from either long-term or short-term effects of gonadal secretions.[17] To distinguish between these, we gonadectomized mice after they reached adulthood (75 days of age) and determined lipid levels 5 months later, at which point acute effects of gonadal hormones should be absent,

but long-term effects might persist. Gonadectomized mice did not exhibit the male–female differences in lipid levels that were present in gonadally intact mice indicating that much of the male–female dimorphism in plasma lipid levels is related to acute effects of gonadal secretions. However, as observed in intact mice, HDL and unesterified cholesterol levels were higher in XX compared to XY mice ($p<0.0003$ and $p<0.04$), regardless of original gonad type (Fig. 1B). After gonadectomy, total cholesterol levels and FFA levels were also higher in XX than XY mice ($p<0.007$ and $p<0.003$). Thus, the effects of XX chromosome complement on HDL and unesterified cholesterol levels are robust, occurring in both the presence and absence of gonadal secretions, and gonadectomy exposes underlying effects of chromosome complement on total cholesterol and FFA levels.

Lipoprotein composition differs in XX and XY mice

As described above, HDL-C values (determined after fractionation of apoB-containing lipids) are higher in mice with XX compared to XY chromosome complement. We wondered if sex chromosome complement influences HDL characteristics such as particle size, apolipoprotein content, or HDL-apolipoprotein (apo)A-I exchange activity (a measure of HDL function). To assess whether HDL particle size differs among the FCG genotypes, we fractionated plasma samples by fast protein liquid chromatography (FPLC) and quantified cholesterol content of the resulting fractions. HDL-C peaks directly mirrored the results of biochemical fractionation, with highest HDL-C levels in the XX mice within each sex (XX males > XY males and XX females > XY females), and higher levels in males than females in gonadally intact mice (Fig. 2A). In mice that had been gonadectomized as adults, the HDL-sized particles were more abundant in XX compared to XY mice, and male–female differences were not detectable (Fig. 2B). Minor sex differences were observed in cholesterol levels in LDL-sized particles, with slightly higher levels in females than males in both intact and gonadectomized mice (Fig. 2A and B).

We assessed the relative plasma apolipoprotein levels in plasma from the FCG mice. Consistent with the higher HDL-C levels in intact male mice, levels of the major HDL apolipoprotein, apoA-I, were slightly higher in males than females ($p<0.02$; Fig. 2C; Suppl. Table I; Suppl. Fig. I). A similar male–female difference was observed for apoE, a component of multiple lipoprotein classes ($p<0.0005$). Apolipoprotein levels were also influenced by sex chromosome complement. The levels of apoA-I, apoA-IV and apoE were higher in XY compared to XX mice of both gonadal sexes ($p<0.02$, $p<0.03$ and $p<0.01$, respectively). This was unexpected, given the higher HDL-C levels in XX compared to XY mice. Gonadectomy abolished the male–female differences in apolipoprotein levels, but maintained the higher apoA-I levels in XY compared to XX mice ($p<0.006$). Removal of the gonads also uncovered interactions between sex chromosome complement and the original gonadal sex (Fig. 2C; Suppl. Table 1). ApoB levels were very low in all chow-fed mice, and neither apoB nor apoA-II levels differed among the four genotypes (Suppl. Table I and Suppl Fig. I). Overall, our results reveal complex effects of sex chromosome complement on plasma lipoprotein composition, with XX chromosome complement favoring higher HDL-C content, but lower total levels of apolipoproteins that are often associated with HDL including apoA-I and apoA-IV.

The lower apolipoprotein-to-cholesterol ratio of HDL from XX compared to XY mice could influence HDL function. One assessment of HDL function is the degree to which apoA-I present on HDL can be dissociated from the lipoprotein particle (HDL–apoA-I exchange).[18] A reduced HDL–apoA-I exchange rate correlates with metabolic syndrome and acute coronary syndrome in humans, and with increased atherosclerotic plaque burden in rabbits.[19] We assessed the HDL–apoA-I exchange rate using site-directed spin-label electron paramagnetic resonance.[19] In both intact and gonadectomized mice, the HDL-apoA-I exchange activity in plasma mirrored HDL-C concentrations, with male > female in intact mice ($p=0.001$) and XX > XY in gonadectomized mice ($p<0.003$) (Fig. 2D and E). Thus, sex differences in HDL–apoA-I

exchange rates parallel those in HDL-C levels, and are influenced by both gonadal and chromosomal sex determinants.

Sex chromosome–diet interactions influence cholesterol levels and HDL activity

Lipid levels are highly responsive to diet. We investigated the factors underlying sexual dimorphism in lipid levels in response to dietary cholesterol by feeding FCG mice a diet containing 1.25% cholesterol (in contrast to 0.02% in chow). As expected, the cholesterol-enriched diet caused substantial increases in the absolute levels of total and LDL cholesterol (LDL-C) in all genotypes compared to levels in mice fed the chow diet. While both sex chromosomes and gonadal sex influenced lipid levels on a high cholesterol diet, specific effects differed from chow diet. As we observed on chow diet, sex chromosome complement remained an important determinant of HDL-C levels, with XX > XY (Fig. 3A). Unlike chow diet, however, sex chromosome complement also influenced TG and FFA levels, with XY > XX. HDL and UC levels were both influenced by gonadal sex in intact mice fed a cholesterol-enriched diet, with female > male. Thus, in some cases, the determinants of sexual dimorphism in lipid traits are responsive to diet.

Removal of the acute effects of gonadal secretions by gonadectomy of adult mice produced unique patterns of lipid levels among the four genotypes compared to chow diet or intact mice fed cholesterol diet. HDL-C and UC levels were higher in XX compared to XY mice (Fig. 3B). Thus, HDL and UC cholesterol levels were consistently influenced by sex chromosome complement across diets (chow and high cholesterol) and gonadal state (intact and gonadectomized). Unexpectedly, in gonadectomized mice, the cholesterol diet uncovered male–female differences in several lipid traits that were not apparent in gonadally intact mice. Thus, males had higher levels than females of total cholesterol, LDL-C, UC, TG and FFA (Fig. 3B). Interestingly, the only condition examined in which LDL-C levels exhibited sexual dimorphism was in mice gonadectomized and fed a cholesterol-enriched diet. Detection of male–female lipid

level differences in gonadectomized mice suggests that long-lasting (organizational) effects of gonadal hormones present in early life are responsible, or an effect of the *Sry* acting outside of the gonads. Furthermore, the emergence of male–female dimorphism in cholesterol traits exclusively in gonadectomized animals suggests that acute effects of gonadal secretions in intact mice may counteract these organizational hormone effects.

As described above, the presence of XX sex chromosomes was associated with higher HDL-C levels than XY chromosome complement. On the cholesterol diet, HDL-C levels were ~60% higher in XX than XY mice in both intact and gonadectomized mice fed the high cholesterol diet (Fig. 3). In the intact mice on both diets, the sex chromosome effect was overlaid with male–female sex differences. The XX > XY differences in HDL-C levels of mice fed a cholesterol-enriched diet were recapitulated when HDL particles were defined by size *via* FPLC fractionation (Fig. 4A, B). Analysis of apolipoprotein content on the cholesterol-enriched diet showed XX > XY for several HDL apolipoproteins (apoA-I, apoA-II, and apoA-IV) in intact mice; female > male effects were also evident for apoA-IV and apoE (Fig. 4C; Suppl. Table I; Suppl. Fig. I). In gonadectomized mice fed a cholesterol-enriched diet, the sex chromosome effects on apolipoprotein content were less pronounced, but still apparent for apoA-II and apoE.

Assessment of HDL–apoA-I exchange activity revealed a strong effect of diet. On a chow diet, HDL activity in gonadally intact mice was higher in males than females (Fig. 2D); after cholesterol feeding, XX mice had higher HDL–apoA-I exchange activity than XY mice, and female mice had higher activity than males (Fig. 4D). Gonadectomy in combination with dietary cholesterol reduced the absolute levels of HDL–apoA-I exchange activity compared to all other dietary–gonadal hormone conditions, particularly in females (Fig. 4E); in chow fed mice, gonadectomy reduced HDL–apoA-I exchange activity only in males (Fig. 2E). These results suggest that acute effects of gonadal hormones are a determinant of HDL–apoA-I exchange capacity, with distinct sexually dimorphic effects that respond to diet.

Gene expression levels for components of cholesterol synthesis and metabolism do not explain sex differences in plasma cholesterol levels

In all dietary and gonadal conditions examined here, HDL-C levels were higher in mice with XX compared to XY chromosome complement. To investigate potential mechanisms, we examined hepatic gene expression levels for key players in cholesterol synthesis and metabolism. These included determinants of cholesterol biosynthesis (*Hmgcr, Mvk*), cellular lipoprotein uptake (*Ldlr*, *Scarb1*), cholesterol conversion to bile acids (*Cyp7a1*, *Cyp8b1*, *Cyp27a1*), and HDL lipid accumulation (*Lcat, Pltp, Abca1, Abcg1*). We searched for patterns of gene expression that mirrored the elevated HDL-C levels in XX compared to XY genotypes across the four cohorts of mice, all of which had higher HDL-C levels in XX compared to XY mice. Although we identified some instances of XX *vs*. XY differences in gene expression, we did not detect patterns that are consistent with the sex differences in HDL or other lipid levels (Table 1 and Suppl. Figs. II-IV).

Differences in X chromosome gene dosage associate with plasma HDL-C levels

The association of HDL-C levels with XX chromosome complement suggests a mechanism that is directly related to the presence of a second X chromosome or the absence of a Y chromosome. To distinguish between these two possibilities, we measured plasma lipid levels in a mouse model differing in the number of sex chromosomes.[12,20] The abnormal Y* chromosome undergoes recombination with the X chromosome to produce XX, XX^{Y*} (similar to XXY), and XY* (similar to XY) mice. Chow-fed, gonadectomized mice with two X chromosomes (XX and XXY) had higher levels of total, unesterified, and HDL-C than mice with a single X chromosome (XY); $p<0.007$, $p<0.006$ and $p<0.04$, respectively). The presence of the Y chromosome did not affect HDL-C levels (compare XX with XXY; Fig. 5). By contrast, mice with a Y chromosome (XXY and XY) had higher LDL-C and TG levels than mice without a Y ($p<0.03$ and $p<0.02$, respectively), regardless of the number of X chromosomes. These data indicate that the

presence of X and Y chromosomes have distinct effects on lipid species, with HDL-C influenced by the number of X chromosomes, and LDL-C influenced by the presence of the Y chromosome.

In general, dosage of X chromosome gene expression is normalized between XX and XY cells through inactivation of one X chromosome in XX cells. However, a small subset of genes escape X chromosome inactivation and exhibit higher expression levels in XX compared to XY cells.[21] Genes that are well established to escape inactivation in both mice and humans include *Ddx3x*, *Eif2s3x*, *Kdm5c*, and *Kdm6a*. The expression levels of these genes have the potential to influence numerous downstream cellular processes through their roles as histone methylases (*Kdm5c*, *Kdm6a*), a DNA helicase (*Ddx3x*), and a translation initiation factor (*Eif2s3x*). To assess whether the X-inactivation escapee genes have enhanced expression levels in the four cohorts of mice studied here in a relevant metabolic tissue, we quantitated gene expression in liver of both intact and gonadectomized FCG mice on chow and high cholesterol diets. These genes were expressed at higher levels in XX mice compared to XY mice in nearly all cohorts (Table 1 and Suppl. Fig. V). In some cases, male–female dimorphism was also observed. The higher hepatic expression levels of X chromosome escapee genes in XX compared to XY liver raise the possibility that altered X chromosome gene dosage may contribute to sexual dimorphism in HDL-C levels, and likely other metabolic traits.

DISCUSSION

Sexual dimorphism in plasma lipid levels has been observed for decades,[22] and the lower lipid levels in premenopausal women compared to men has traditionally been attributed to effects of estrogens.[23] However, the reduction in estrogens during menopause does not fully account for the subsequent unfavorable lipid profile associated with metabolic syndrome.[23,24] In fact, postmenopausal HDL-C levels are only slightly decreased or similar to premenopausal HDL-C levels, and these are both elevated compared to HDL-C levels observed in men.[25–27] These observations suggest that additional mechanisms beyond gonadal hormones influence sexual dimorphism in lipid levels. We used the FCG mouse model to investigate the relative contributions of gonadal secretions and sex chromosome complement to lipid levels. Using this model, we were able to detect sex chromosome complement as a determinant of sexual dimorphism in plasma lipids and lipoproteins, particularly HDL-C.

In gonadally intact mice fed a chow diet, total and HDL-C, as well as TG and FFA levels, were higher in male mice (XX and XY) compared to female mice (XX and XY). In addition, HDL-C levels were influenced by sex chromosome complement, with higher levels in XX compared to XY mice. Thus, even in the presence of normal levels of gonadal hormones, the effect of sex chromosomes on HDL-C levels was apparent. To further explore the influence of sex chromosome complement on lipid levels, we reduced hormone levels by gonadectomy, which eliminated male–female differences observed in the intact mice, and amplified the XX *vs*. XY effects on HDL-C levels. Gonadectomy also revealed that XX chromosome complement promotes higher total cholesterol and FFA levels. These results suggest that sex chromosome complement may become a particularly important determinant of lipid levels under conditions characterized by reduced gonadal hormones, such as middle age and post-menopause in humans.

Using a high cholesterol diet to increase lipid levels, we detected interactions between sex chromosome complement and diet. As with the chow diet, gonadally intact XX mice fed a diet

enriched in cholesterol had higher HDL-C levels than XY mice. Additionally, the high cholesterol diet brought out a novel sex chromosome effect on TG and FFA levels, with higher levels in XY compared to XX mice. Analysis of gonadectomized mice that were fed a high cholesterol diet unexpectedly exhibited prominent effects of original gonadal sex. Thus, whereas intact mice did not show significant male–female differences for any trait except HDL-C levels (female > male), following gonadectomy, male mice had significantly higher levels than females for total cholesterol, LDL-C, TG and FFA. Since mice were gonadectomized 5 months prior to lipid measurements, the observed male–female dimorphism must be a result of long-lasting (*i.e.*, organizational) effects of gonadal hormones, or non-gonadal effects of S*ry* that are confounded with gonadal sex in this model. Together, our results indicate that sex differences in lipid levels are determined by a combination of hormonal and sex chromosome effects, and further, these difference are dependent on hormonal (high or low gonadal hormone concentrations) and diet (chow or high cholesterol) context (Fig. 6A).

Regardless of diet or sex hormone milieu, HDL-C levels were elevated in mice with two X chromosomes. We assessed the efficiency of apoA-I exchange from HDL particles, which has been correlated with metabolic syndrome in humans and atherosclerosis in rabbits.[19] Total HDL-apoA-I exchange activity was greater in XX mice, concordant with the increased HDL-C concentration. We measured hepatic gene expression of key enzymes in plasma cholesterol homeostasis, including *Hmgcr*, *Scarb1*, and *Cyp7a1*, but did not identify sex chromosome effects on gene expression that accounted for the XX–XY difference observed in HDL-C levels and HDL-apoA-I exchange activity. We cannot rule out that post-transcriptional effects on the hepatic pathways investigated exist between XX and XY tissues. Future studies will be necessary to assess this possibility, as well as to evaluate extrahepatic pathways that modulate HDL-C levels, such as cholesterol efflux from macrophages.

By altering the number of X and Y chromosomes using the Y* mouse model, we determined that the presence of two X chromosomes is associated with increased HDL-C. We previously

determined that the presence of two X chromosomes also leads to increased adiposity and fatty liver development on a high fat diet.[14] Thus, a future goal of broad importance is to understand how increased X chromosome dosage impacts metabolism. Here we demonstrate that hepatic expression of genes that escape X chromosome inactivation is higher in intact and gonadectomized mice fed chow and high cholesterol diets. Proteins encoded by the X chromosome escapee genes *Ddx3x*, *Kdm5c*, *Kdm6a*, and *Eif2s3x* have roles in transcriptional regulation, RNA processing, and protein translation.[28–35] These proteins, acting alone or in combination, could conceivably modulate the levels of mRNAs and proteins that influence lipid homeostasis (Fig. 6B). The specific targets of X chromosome escapee gene activity are not well characterized at present, but ongoing studies are focused on their identification using large-scale transcriptional and epigenomic profiling.

Our data using the Y* mouse model revealed that in addition to effects of two X chromosomes on HDL-C levels, the presence of the Y chromosome may influence LDL-C levels. The Y chromosome has traditionally been viewed to harbor genes restricted to male gonad development and spermatogenesis. However, a recent study using consomic mouse strains with Y chromosomes derived from distinct inbred strains suggests that genetic variation in Y chromosome genes influences plasma lipoprotein levels.[36] The Y chromosome carries a set of genes that encode Y-specific proteins that are similar to paralogous genes on the X chromosome. These include the Y chromosome counterparts of the X chromosome escapee genes that exhibit increased dosage in mice with two *vs*. a single X chromosome (*Eif2s3x*/*Eif2s3y*, *Kdm5c*/*Kdm5d*, *Ddx3x*/*Ddx3y*, *Kdm6a*/*Uty*). In our study, all X and Y chromosomes were genetically identical, derived from the C57BL/6 strain, so that dosage alone was manipulated. Nevertheless, it is interesting to speculate that both dosage and genetic variation in these X and Y chromosome genes may influence lipid levels in the general human population.

In conclusion, our studies demonstrate that sexual dimorphism in lipid levels is a result of interactions between gonadal hormones, sex chromosome complement, and diet. The results further indicate that XX chromosome complement has a major influence on HDL-C levels, irrespective of diet or gonadal status. Future studies with FCG mice will facilitate the identification of sex-dependent biomarkers of disease associated with altered lipid levels, such as atherosclerosis. Such studies are crucial for improving the assessment and treatment of cardiovascular disease risk in men and women.

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DISCLOSURES

Michael N. Oda is founder an holds an ownership stake in Seer BioLogics, Inc. This did not influence his or his laboratory's interpretation or presentation of results.

SIGNIFICANCE

Lipid profiles are an important indicator of the metabolic syndrome. Reports of sexually dimorphic LDL and HDL-C levels suggest regulation by sex hormones. Here, we show that the sex chromosome complement is also a key factor in modulating plasma lipid levels. HDL-C is consistently elevated in mice with two X chromosomes compared to mice with XY sex chromosomes, regardless of diet or circulating sex hormone levels. These findings are important for understanding cardiovascular disease risk in both men and women.

TABLES

Table 1. Sex differences in hepatic gene expression.

Hepatic gene expression was measured by quantitative PCR. *P*-values are represented by direction of sex difference and magnitude of significance. *, $P \leq 0.05$; **, $P \leq 0.01$; ***,$P \leq 0.001$; †, $P \leq 0.0001$; ‡, $P \leq 0.000001$; n.s., not significant. F, gonadal female; M, gonadal male; HCD, high cholesterol diet.

Table 1. Sex differences in hepatic gene expression.

	Chow Intact	Chow GDX	HCD Intact	HCD GDX
Cholesterol synthesis and transport				
Abca1	M > F**	n.s.	F > M**	XY > XX* Int.**
Abcg1	F > M† XY > XX*** Int.**	XY > XX† Int.*	F > M** XY > XX**	XY > XX**
ApoB	XX > XY**	M > F*	M > F†	n.s.
Hmgcr	n.s.	n.s.	M > F** XY > XX**	n.s.
Lcat	F > M*	M > F*	M > F†	F > M* XX > XY***
Ldlr	XX > XY*	n.s.	M > F†	XX > XY*
Lipc	F > M* Int.**	XX > XY**	M > F†	n.s.
Mvk	n.s.	n.s.	Int.*	n.s.
Pltp	F > M*** XY > XX*	n.s.	F > M† XY > XX**	XY > XX**
Scarb1	n.s.	n.s.	M > F**	XX > XY*
Bile acid synthesis				
Cyp7a1	F > M*** XX > XY**	n.s.	F > M* Int.*	n.s.
Cyp8b1	M > F** XX > XY**	F > M**	n.s.	n.s.
Cyp27a1	XX > XY***	XX > XY* Int.*	M > F**	n.s.
X-inactivation escape				
Ddx3x	XX > XY***	M > F*	M > F** XX > XY** Int.***	XX > XY***
Eif2s3x	M > F** XX > XY ‡ Int.**	M > F* XX > XY**	XX > XY†	XX > XY† Int.*
Kdm5c	M > F** XX > XY ‡	M > F*	M > F** XX > XY ‡ Int.*	XX > XY† Int.**
Kdm6a	XX > XY**	F > M* XX > XY***	XX > XY ‡	M > F** XX > XY**

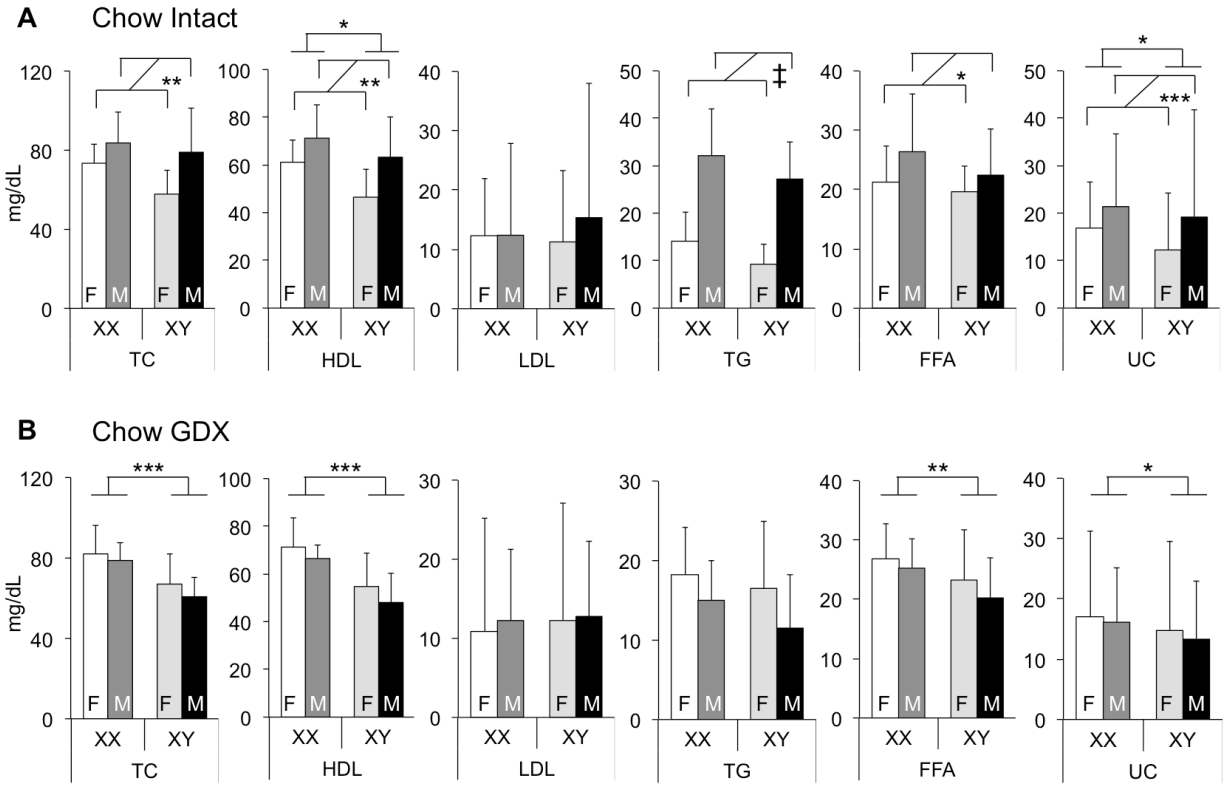
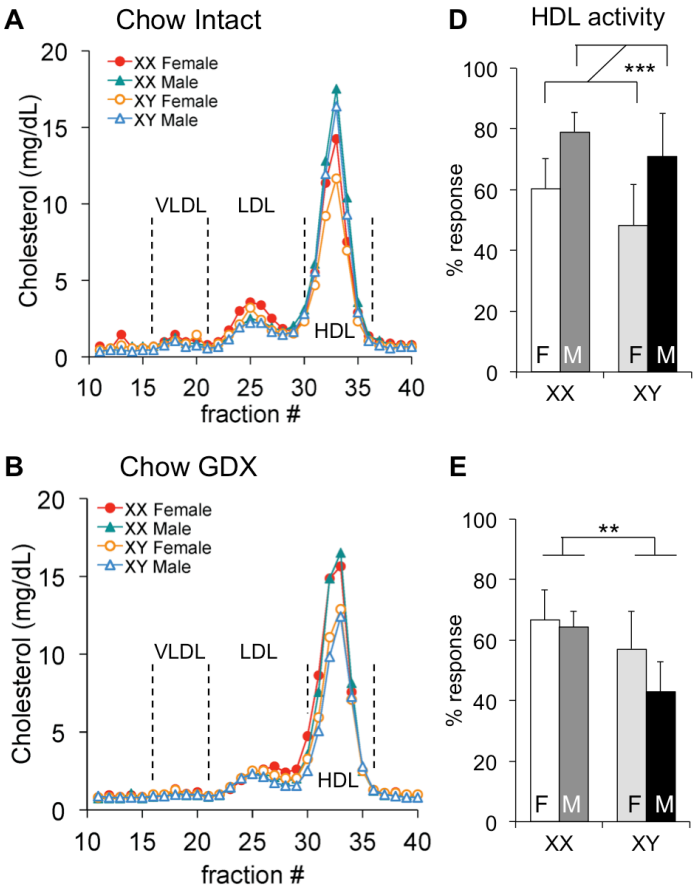


Figure 1. Plasma lipid levels are regulated by both gonadal sex and sex chromosome complement. **A** and **B**, Concentrations of total cholesterol (TC), unesterified cholesterol (UC), high-density lipoprotein (HDL) cholesterol, triglyceride (TG), and free fatty acids (FFA) were measured in 7.5-month-old gonadally intact (**A**) and gonadectomized (GDX, **B**) Four Core Genotypes mice fed a standard chow diet (n = 8). Low-density lipoprotein (LDL) cholesterol values were calculated by subtracting HDL from TC. Values represent the mean ± standard deviation. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement and gonadal sex is denoted by "Int." *, $P \leq 0.05$; **, $P \leq 0.01$; ***,$P \leq 0.001$; ‡, $P \leq 0.000001$. F, gonadal female; M, gonadal male.



C

	ApoAI	ApoAII	ApoAIV	ApoE
Chow Intact	M > F* XY > XX*	n.s.	XY > XX**	M > F*** XY > XX*
Chow GDX	XY > XX** Interaction**	Interaction**	n.s.	Interaction*

Figure 2. Composition of HDL lipoproteins differs between sexes. Plasma was collected from 7.5-month-old gonadally intact (**A**, **D**) and gonadectomized (GDX, **B**, **E**) chow-fed mice. **A** and **B**, Three representative plasma samples from each genotype were pooled and assayed using fast protein liquid chromatography. **C**, Plasma levels of apolipoproteins were quantified by immunoblot densitometry. Direction of statistically significant comparisons for gonadal sex and for sex chromosomes are shown. A significant interaction of sex chromosome complement and gonadal sex is denoted by "Interaction." **D** and **E**, HDL apoAI dissociation activity was measured by electron paramagnetic resonance and represented as % response and as activity per unit of HDL cholesterol. Values represent the mean ± standard deviation. *, $P \leq 0.05$; **, $P \leq 0.01$; ***,$P \leq 0.001$; n.s., not significant. F, gonadal female; M, gonadal male.

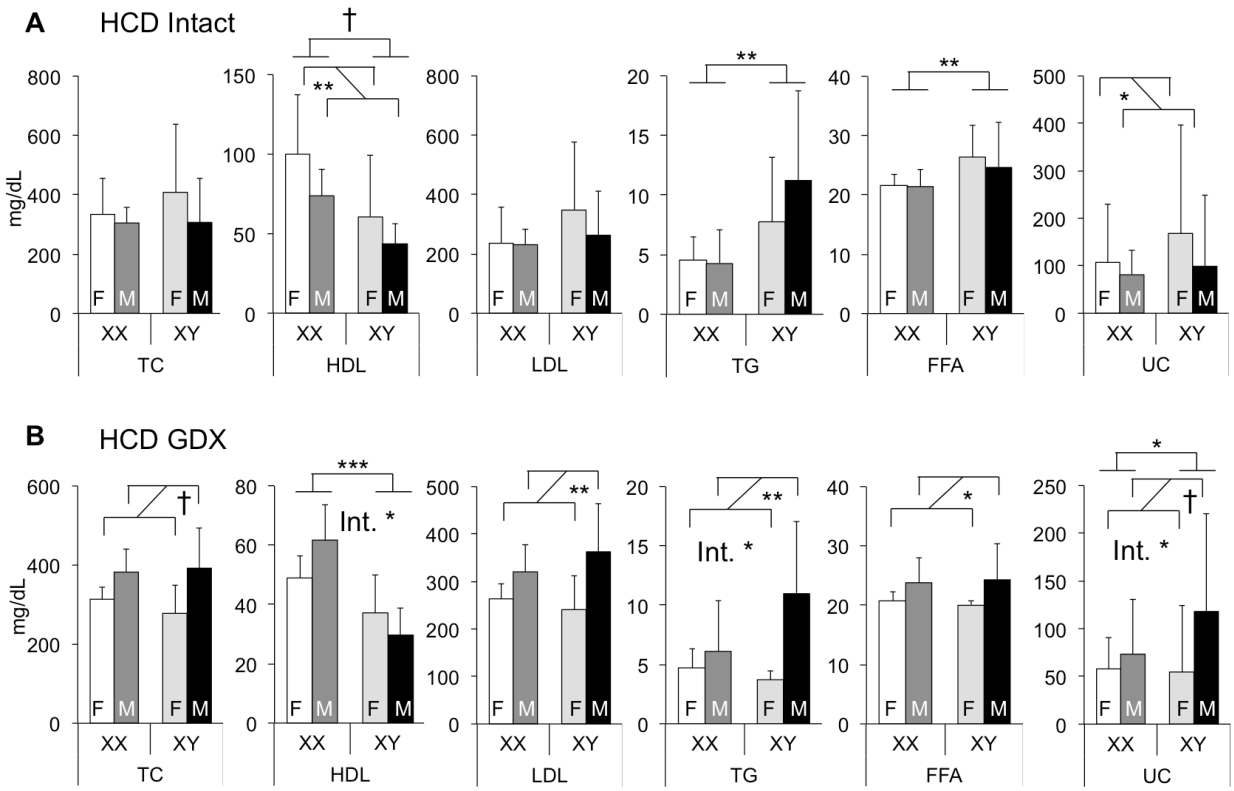
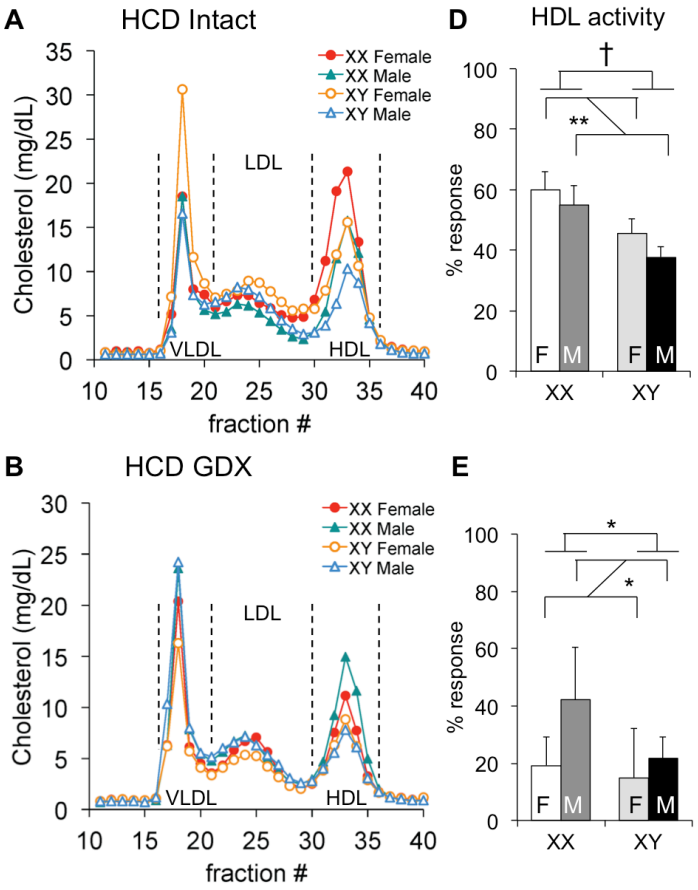


Figure 3. High cholesterol diet interacts with gonadal sex and sex chromosome complement to modulate plasma lipid levels. **A** and **B**, Concentrations of total cholesterol (TC), unesterified cholesterol (UC), high-density lipoprotein (HDL) cholesterol, triglyceride (TG), and free fatty acids (FFA) were measured in 7.5-month-old gonadally intact (**A**) and GDX (**B**) FCG mice fed a high cholesterol diet (HCD, *n* = 4-10). Low-density lipoprotein (LDL) cholesterol values were calculated by subtracting HDL from TC. Values represent the mean ± standard deviation. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement and gonadal sex is denoted by "Int." *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; †, $P \leq 0.0001$. F, gonadal female; M, gonadal male.



C

	ApoAI	ApoAII	ApoAIV	ApoB	ApoE
HCD Intact	XX > XY* Interaction*	XX > XY**	F > M* XX > XY*	n.s.	F > M**
HCD GDX	n.s.	XX > XY**	F > M**	Interaction**	M > F** XY > XX*

Figure 4. High cholesterol diet increases VLDL/LDL levels and suppresses HDL activity. Plasma was collected from 7.5-month-old gonadally intact (**A**, **D**) and gonadectomized (GDX, **B**, **E**) mice fed the high cholesterol diet (HCD). **A** and **B**, Three representative plasma samples from each genotype were pooled and assayed using fast protein liquid chromatography. **C**, Plasma levels of apolipoproteins were quantified by immunoblot densitometry. Direction of statistically significant comparisons for gonadal sex and for sex chromosomes are shown. A significant interaction of sex chromosome complement and gonadal sex is denoted by "Interaction." **D** and **E**, HDL apoAI dissociation activity was measured by electron paramagnetic resonance and represented as % response and as activity per unit of HDL cholesterol. Values represent the mean ± standard deviation. *, $P \leq 0.05$; **, $P \leq 0.01$; †, $P \leq 0.0001$; n.s., not significant. F, gonadal female; M, gonadal male.

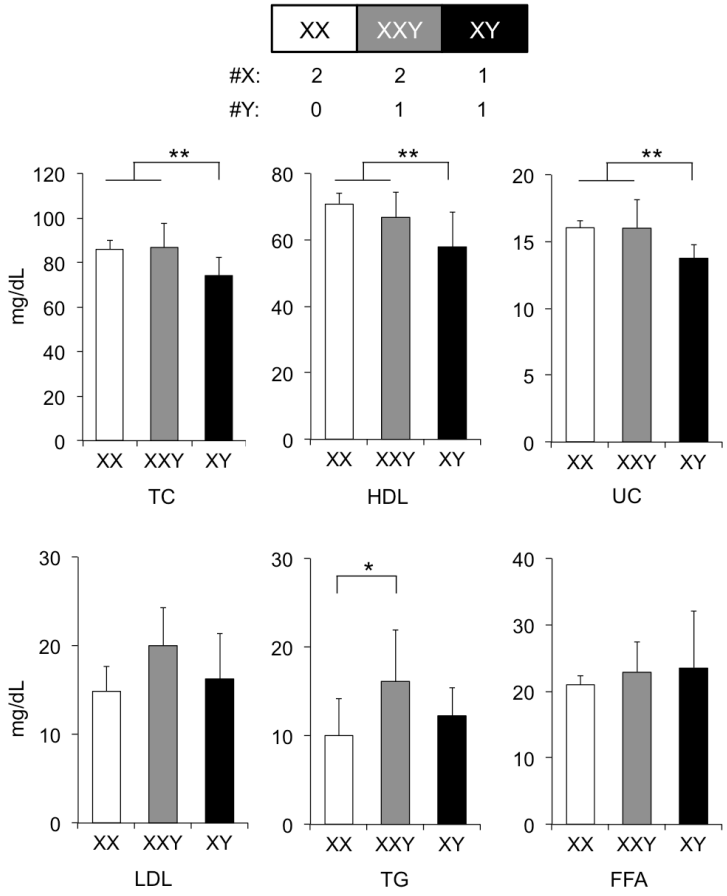


Figure 5. Plasma cholesterol levels are increased in gonadectomized mice with two X chromosomes. Concentrations of total cholesterol (TC), unesterified cholesterol (UC), high-density lipoprotein (HDL) cholesterol, triglyceride (TG), and free fatty acids (FFA) were measured in gonadectomized XY* mice fed a standard chow diet (n = 7-8). Low-density lipoprotein (LDL) cholesterol values were calculated by subtracting HDL from TC. Values represent the mean ± standard deviation. Significant comparisons by one-way ANOVA with Duncan's multiple-comparison test are denoted by brackets. *, $P \leq 0.05$; **, $P \leq 0.01$

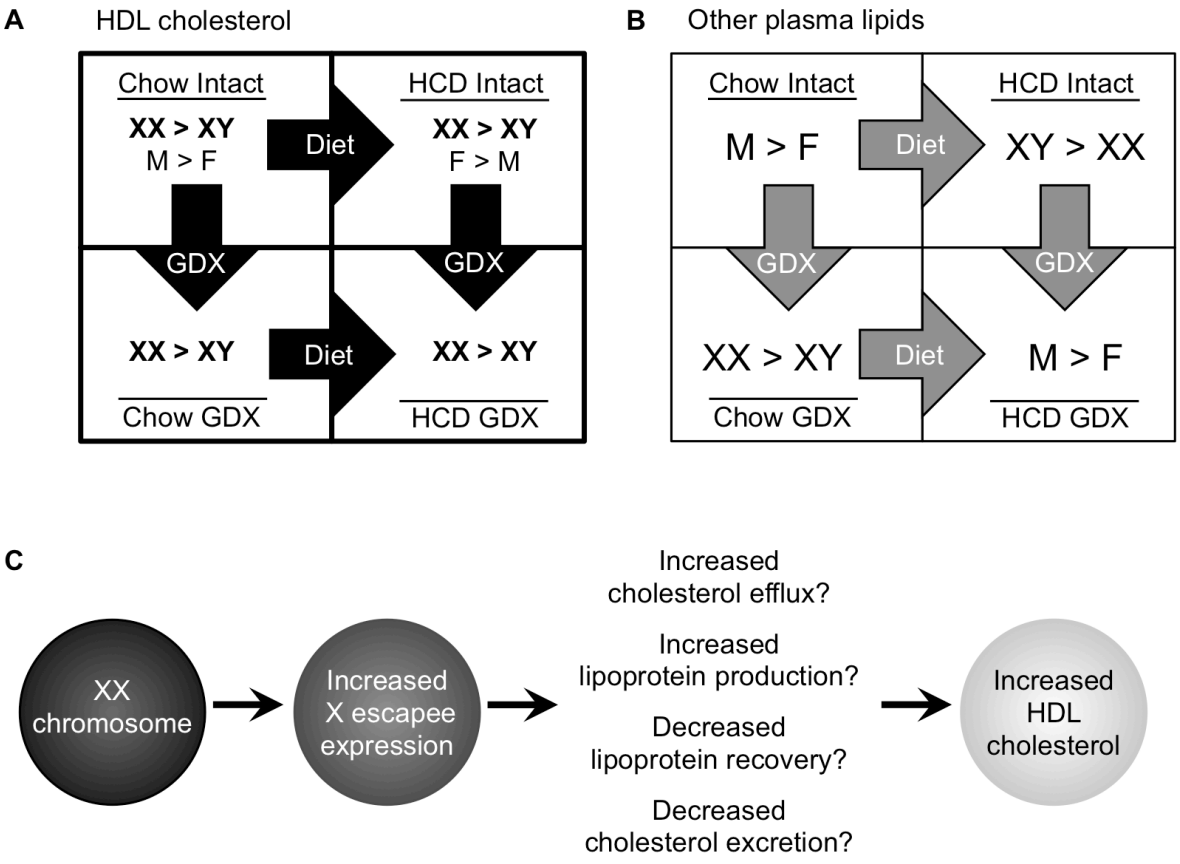


Figure 6. Factors influencing circulating HDL cholesterol. **A**, Regardless of diet or sex hormone presence, HDL cholesterol is increased in XX mice compared to XY mice. **B**, Overall sex differences in other plasma lipid levels change in response to a high cholesterol diet (HCD) or gonadectomy (GDX). F, gonadal female; M, gonadal male. **C**, Mice with two X chromosomes have increased X escapee expression in liver. These genes may influence several metabolic pathways to increase circulating HDL cholesterol.

SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

Mice

Four Core Genotypes (FCG) C57BL/6 mice were bred and genotyped as described previously.[1] Briefly, XX female mice were mated with XY^{-}*(Sry+)* male mice to generate XX, XX(*Sry+)*, XY^{-}, and XY^{-}*(Sry+)* offspring, and genotyping was performed by PCR to detect presence of the *Sry* transgene (forward: AGCCCTACAGCCACATGATA; reverse: GTCTTGCCTGTATGTGATGG) and Y-chromosome–specific sequence (forward: CTGGAGCTCTACAGTGATGA; reverse: CAGTTACCAATCAACACATCAC).[1] Where indicated, gonadectomy was performed at 75 days of age, as previously described.[1]

XY* mice, backcrossed to strain C57BL/6EiJ for >10 generations, were bred as described previously.[1,2] XY* males have the Y* chromosome that recombines aberrantly with the X chromosome. Mating XY* males with XX females generates the three genotypes included in this study: XX, XX^{Y}*, and XY*, which are similar to XX, XXY, and XY mice, respectively. Progeny of XY* mice were gonadectomized at 75 days of age, as previously described.[1]

Gonadal males and females were housed in separate cages and maintained at 23°C with a 12:12 hour light:dark cycle. All mice were initially fed Purina mouse chow diet containing 5% fat (Purina 5001; PMI Nutrition International, St. Louis, MO). Where specified, mice were fed a chow diet until 3.5 months of age (4 weeks after gonadectomy), and then fed an atherogenic diet for 16 weeks (diet TG90221 containing 7.5% cocoa butter, 1.25% cholesterol, 0.5% sodium cholate; Teklad Research Diets, Madison, WI).

For all studies, blood samples were obtained at 7.5 months of age (FCG mice) or 14 months of age (XY* mice) after fasting 0800–1300. Plasma was collected after centrifugation of whole blood at 3,400 x *g* for 10 minutes at 4°C. Mouse studies were conducted in accordance with and

approved by the Institutional Animal Research Committee of the University of California, Los Angeles.

Measurement of plasma lipid

Total cholesterol, HDL cholesterol, free cholesterol, triglycerides, and free fatty acid levels were determined by enzymatic colorimetric assays.[3] Combined LDL cholesterol and VLDL cholesterol concentration was determined by subtracting HDL cholesterol values from total cholesterol values. Lipoproteins were fractionated from 150 µL of plasma pooled from 3 mice of each genotype by fast protein liquid chromatography at the Mouse Metabolic Phenotyping Center (Vanderbilt University, Nashville, TN).

HDL-ApoA-I Exchange Assay

The HDL-ApoA-I exchange assay was performed on freshly thawed plasma using site-directed spin-label electron paramagnetic resonance (EPR) as described by Borja et al.[4] Briefly, plasma samples (in triplicate) were diluted by a factor of 4 in PBS and PEG 6000 was added to a final concentration of 4%. ApoB-containing lipoproteins were removed by centrifugation (13,000 rpm, 10 min, 4°C), and clarified plasma was combined with spin-labeled apoA-I. EPR measurements were performed on each sample at 6°C and again after 15 min at 37°C using a Bruker eScan EPR spectrometer with temperature controller (Noxygen). HDL-apoA-I exchange activity was defined as the value obtained at 6°C (normalized to an internal standard) from value obtained at 37°C (normalized to same internal standard) followed by subtracting the baseline spectra for spin-labeled apoA-I in PBS. Additional calculations were performed as described.[4]

Immunoblotting

Plasma aliquots (0.5 uL) were fractionated by SDS-PAGE in a 4-20% Tris-glycine gel and transferred onto a nitrocellulose membrane. Rabbit anti-mouse antibodies against ApoA-I,

ApoA-II, and ApoA-IV were described previously[5,6] and used at 1:4000 dilution. Rabbit anti-mouse antibody against ApoE (Cat. K23100R, Meridian Life Science, Memphis, TN) was used at 1:2000. A mouse monoclonal antibody against ApoB[7] was used at 1:1000. HRP-conjugated rabbit anti-mouse antibody against IgG or HRP-conjugated goat anti-rabbit antibody against IgG was used at 1:10,000 (Cat. Sc-2030, Santa Cruz Biotechnology, Santa Cruz, CA). Chemiluminescence (ECL2, Cat. 80196, Thermo Fisher, Rockford, IL) was detected using ChemiDoc XRS+ and quantified by ImageLab 4.0.1 (Bio-Rad, Hercules, CA).

Quantitative RT-PCR

Mouse livers were dissected, flash frozen in liquid nitrogen, and stored at –80°C. RNA was isolated from tissues using Ribozol (Cat. N580, Amresco, Solon, OH). First-strand cDNA was generated by reverse transcription with iScript (Cat. 170-8840, Bio-Rad). Quantitative RT-PCR was performed with a Bio-Rad CFX Connect Real-Time PCR Detection System using SsoAdvanced SYBR Green Supermix (Bio-Rad). b2 microglobulin and TATA box-binding protein mRNA were amplified in each sample as normalization controls. All primer sequences are shown in Supplemental Table II.

Statistical Analysis

Groups were compared using two-way ANOVA (NCSS 2001; Number Cruncher Statistical Systems, Kaysville, UT) with main factors of sex (gonadal male *vs*. gonadal female) and sex chromosome complement (XX *vs*. XY). In the XY* study, the three groups were compared using one-way ANOVA with Duncan's multiple comparison test. Statistically significant comparisons or interactions are presented ($p<0.05$). All error bars represent one standard deviation.

Methods References

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Supplemental Table I. Apolipoprotein quantification

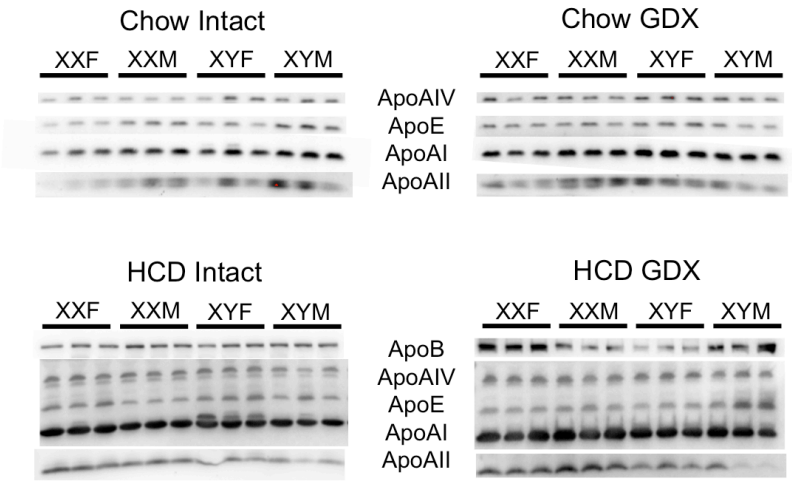
		ApoAI	ApoAII	ApoAIV	ApoB	ApoE
Chow Intact	XXF	1.10 (0.32)	0.61 (0.39)	0.63 (0.19)	not detected	0.62 (0.14)
	XXM	1.70 (0.19)	1.98 (0.58)	0.59 (0.07)		1.39 (0.19)
	XYF	1.72 (0.37)	1.85 (1.23)	1.09 (0.41)		1.01 (0.24)
	XYM	2.11 (0.12)	3.14 (1.60)	1.07 (0.14)		1.79 (0.34)
Chow GDX	XXF	1.77 (0.28)	1.80 (0.42)	1.03 (0.37)	not detected	1.07 (0.12)
	XXM	2.30 (0.07)	3.24 (0.64)	1.06 (0.06)		1.09 (0.28)
	XYF	3.10 (0.40)	3.18 (0.76)	1.53 (0.14)		1.61 (0.27)
	XYM	2.12 (0.19)	1.78 (0.80)	1.19 (0.27)		0.99 (0.25)
HCD Intact	XXF	1.27 (0.13)	7.14 (1.76)	3.20 (0.35)	4.79 (0.44)	3.41 (0.33)
	XXM	1.12 (0.08)	5.78 (0.39)	2.76 (0.16)	5.29 (0.88)	2.08 (0.16)
	XYF	0.91 (0.07)	3.39 (0.84)	2.83 (0.10)	4.66 (0.83)	3.16 (0.37)
	XYM	1.15 (0.17)	3.28 (1.29)	2.15 (0.49)	5.08 (0.06)	2.66 (0.66)
HCD GDX	XXF	3.18 (0.46)	2.62 (0.21)	0.75 (0.02)	6.52 (2.76)	0.69 (0.03)
	XXM	3.59 (0.55)	3.07 (0.66)	0.65 (0.05)	2.16 (0.52)	1.12 (0.19)
	XYF	3.64 (0.20)	1.63 (0.54)	0.78 (0.03)	3.31 (0.68)	0.76 (0.14)
	XYM	3.46 (0.63)	0.97 (1.08)	0.68 (0.07)	7.47 (0.49)	1.78 (0.63)

Supplemental Table I. Quantification of apolipoproteins. Three representative plasma samples from each genotype and from each cohort were separated by gel electrophoresis. Protein levels of apolipoproteins were quantified by densitometry and given as mean values with standard deviation in parentheses. F, gonadal female; M, gonadal male; HCD, high cholesterol diet.

Supplemental Table II. Mouse primer sequences for qPCR

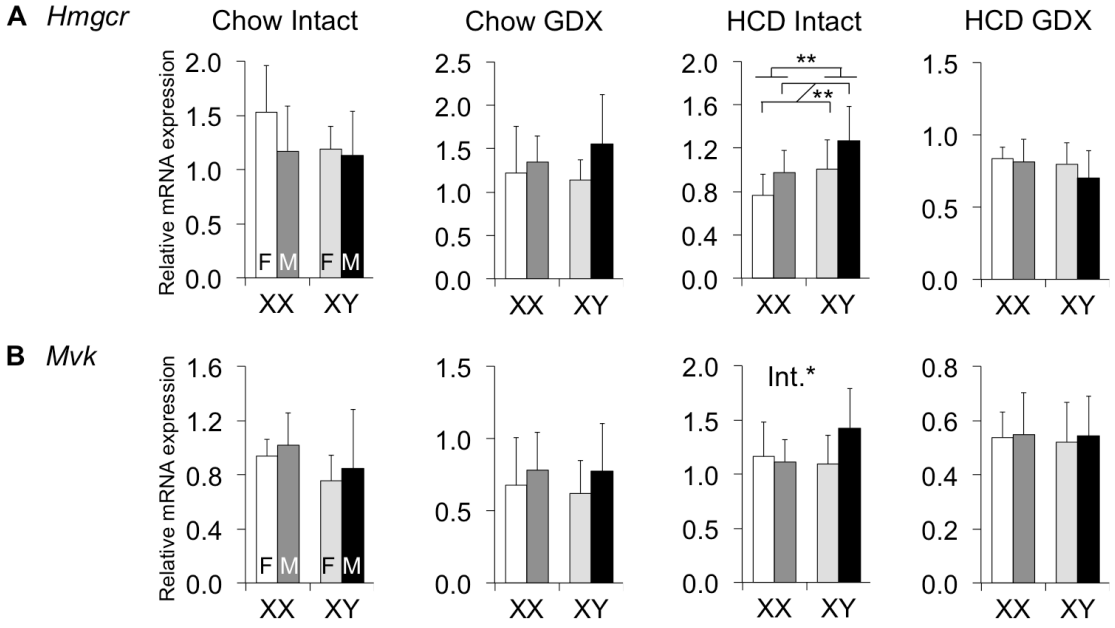
Genes	Forward primer	Reverse primer
β2m	CAGCATGGCTCGCTCGGTGAC	CGTAGCAGTTCAGTATGTTCG
Tbp	ACCCTTCACCAATGACTCCTATG	ATGATGACTGCAGCAAATCGC
Abca1	ACCAGCTTCCATCCTCCTTGT	TTGGTCCTTGGCAAAGTTCAC
Abcg1	CCTGCTCTTCTCCGGATTCTT	ATGTCGCAGTGCAGGTCTTCT
ApoB	CAGTATTCTGCCACTGCAACC	AGGACTTCACTAGATAAGGTCC
Hmgcr	ATGCCTTGTGATTGGAGTTGG	TGGACGACCCTCACGGCTTTC
Lcat	CCCACCAGCAGGATGAATACTAC	AGGCTATGCCCAATGAGGAA
Ldlr	CTTCTCCTTGGCCATCTATGAGG	CATTGGGGAGGAGGGCTGTTGT
Lipc	TGGAACACAGTGCAGACCATC	TGGAGGTCATCCAGATTTTCG
Mvk	TGACCAAGTTCCCTGAGATTG	CTTGCTCTAGACCTGGCTTC
Pltp	GGCCGTCTCAGTGCTAAGTTG	ATCACTCCGATTTGCAGCAGT
Scarb1	CGTACCTCCCAGACATGCTTC	TCTTGCTGAGTCCGTTCCATT
Cyp7a1	CAATGAAAGCAGCCTCTGAAG	AGCCTCCTTGATGATGCTATC
Cyp8b1	AAGGCTGGCTTCCTGAGCTT	AACAGCTCATCGGCCTCATC
Cyp27a1	CCACAAGGGCCTCACCTATG	GCACCTGGTCCAGCCGGGTG
Ddx3x	GGATCACGGGGTGATTCAAGAGG	CTATCTCCACGGCCACCAATGC
Eif2s3x	TTGTGCCGAGCTGACAGAATGG	CGACAGGGAGCCTATGTTGACCA
Kdm5c	ACCCACCTGGCAAAAACATTGG	ACTGTCGAAGGGGGATGCTGTG
Kdm6a	CCAATCCCCGCAGAGCTTACCT	TTGCTCGGAGCTGTTCCAAGTG

Supplemental Figure I



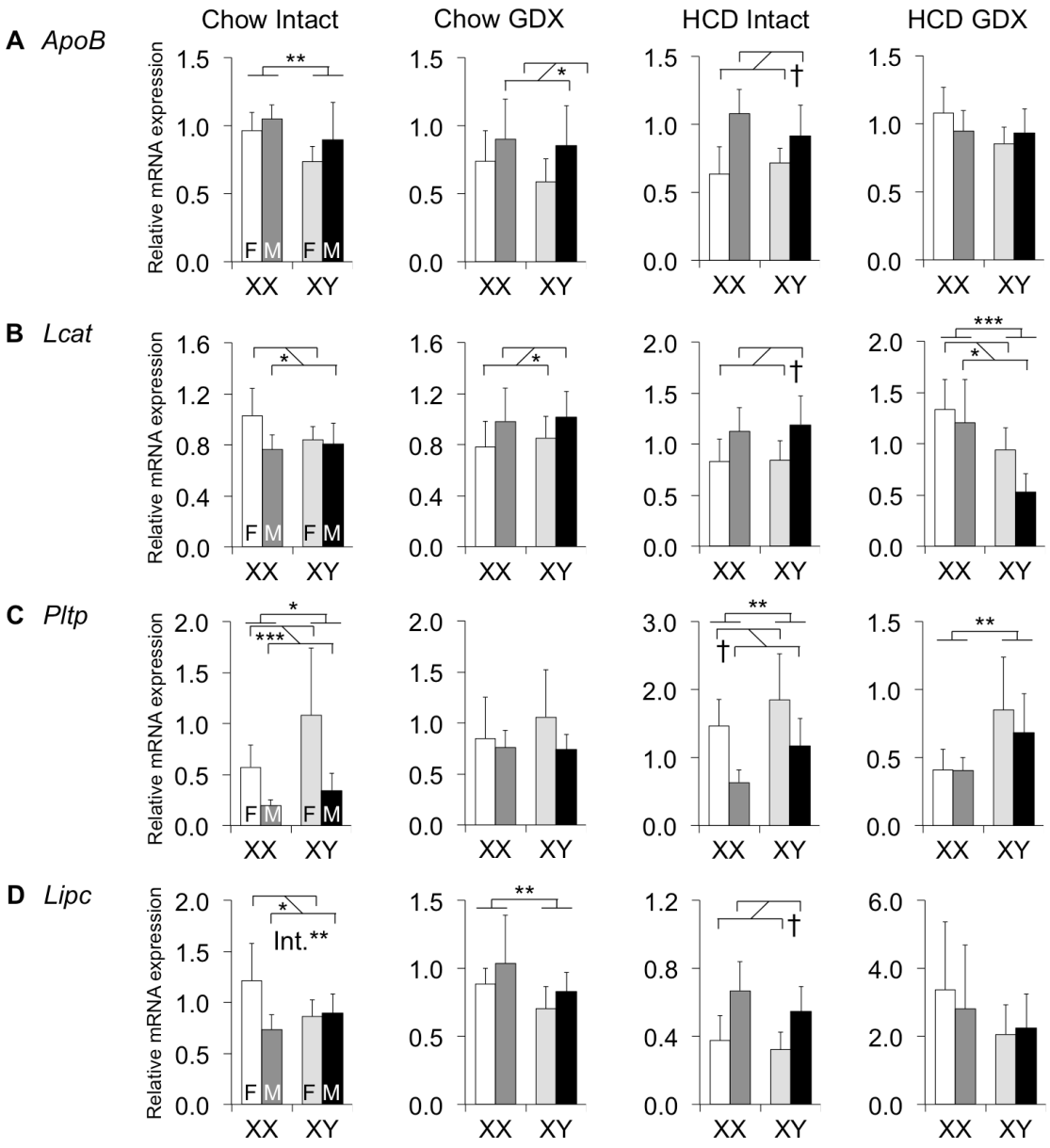
Supplemental Figure I. Plasma levels of apolipoproteins. Three representative plasma samples from each genotype and from each cohort were separated by gel electrophoresis. F, gonadal female; M, gonadal male; HCD, high cholesterol diet.

Supplemental Figure II

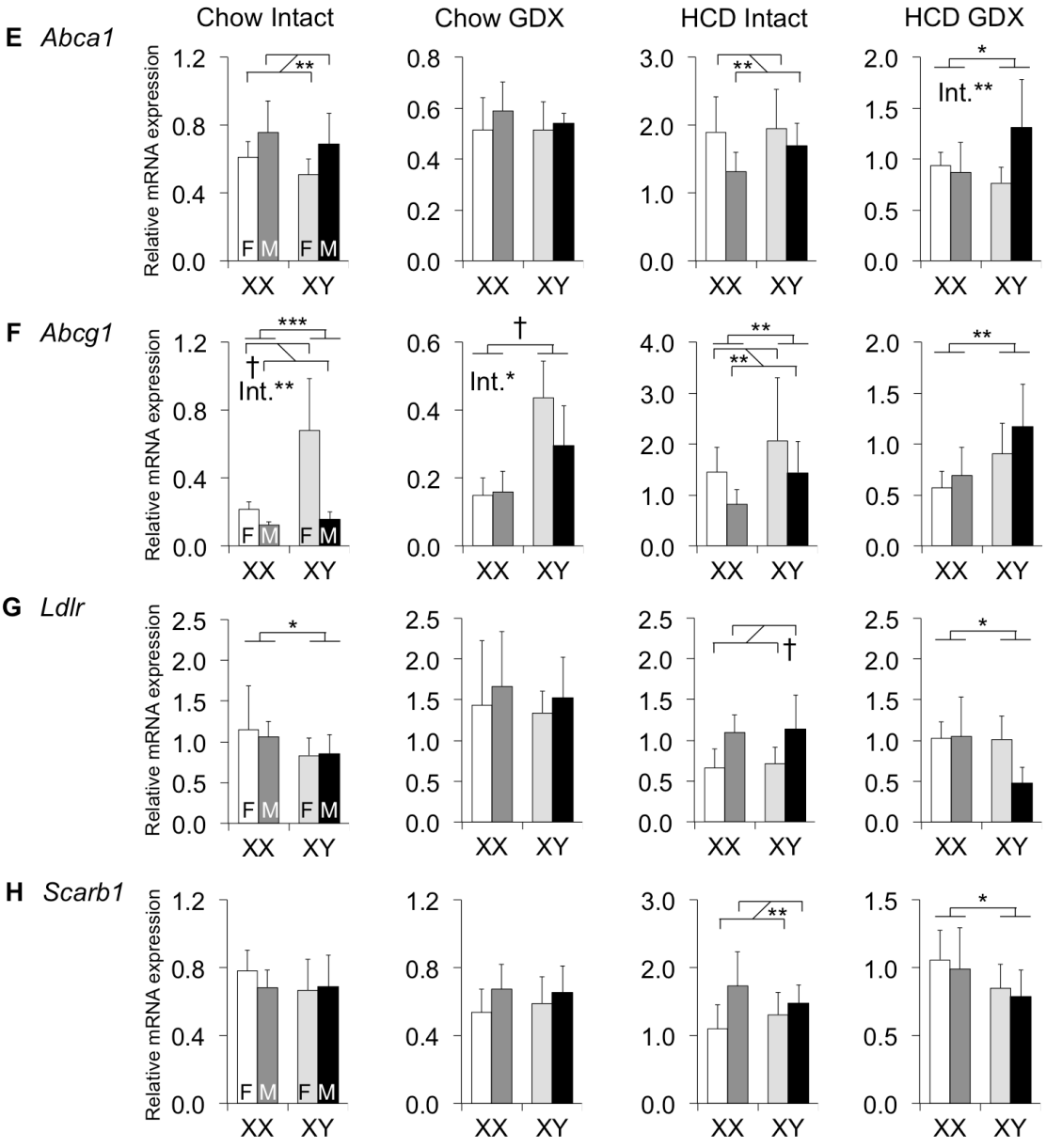


Supplemental Figure II. Key enzymes of cholesterol synthesis are not associated with sex differences in plasma cholesterol levels. Hepatic levels of *Hmgcr* (**A**) and *Mvk* (**B**) were measured by quantitative PCR. All values represent the mean ± standard deviation. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement and gonadal sex is denoted by "Int." *, $P \leq 0.05$; **, $P \leq 0.01$. F, gonadal female; M, gonadal male; HCD, high cholesterol diet.

Supplemental Figure III

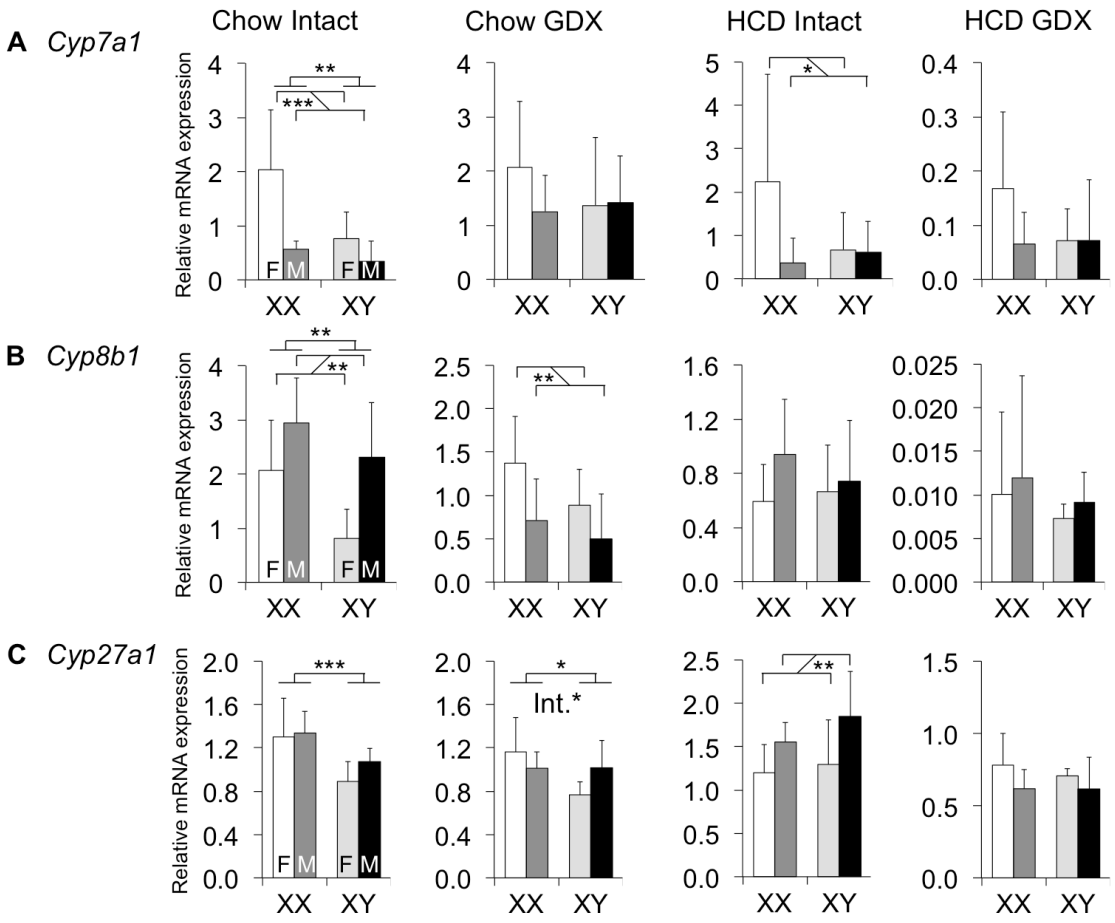


Supplemental Figure III, continued



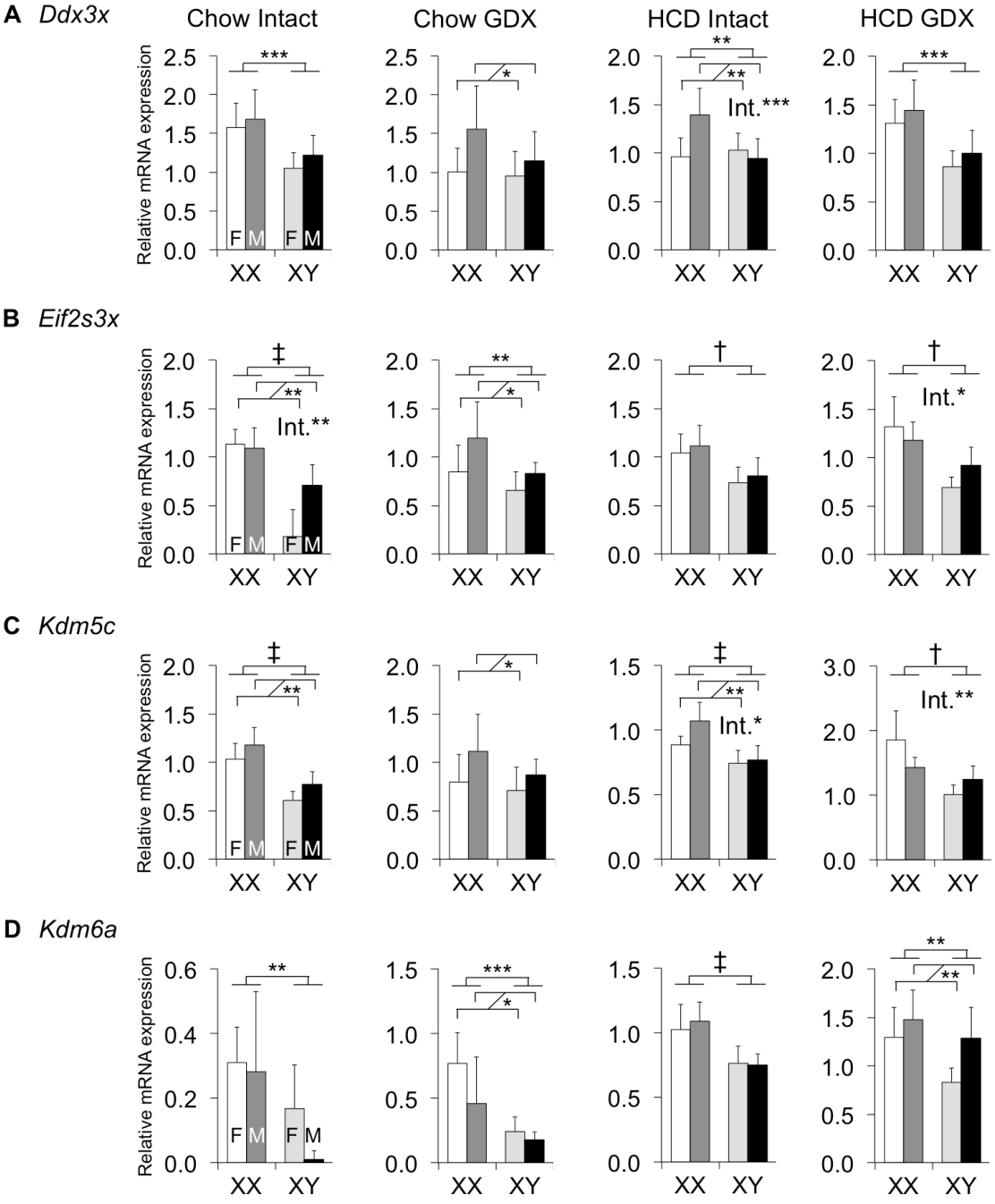
Supplemental Figure III. Components of lipoprotein synthesis, remodeling, and uptake are not associated with plasma HDL cholesterol levels. Relative mRNA expression was measured by quantitative PCR. All values represent the mean ± standard deviation. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement and gonadal sex is denoted by "Int." *, $P \leq 0.05$; **, $P \leq 0.01$; ***,$P \leq 0.001$; †, $P \leq 0.0001$. F, gonadal female; M, gonadal male; HCD, high cholesterol diet.

Supplemental Figure IV



Supplemental Figure IV. Key enzymes of bile acid synthesis do not explain sex differences in plasma cholesterol levels. All values represent the mean ± standard deviation. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement and gonadal sex is denoted by "Int." *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. F, gonadal female; M, gonadal male; HCD, high cholesterol diet.

Supplemental Figure V



Supplemental Figure V. Genes escaping X-inactivation are consistent with XX–XY differences in HDL cholesterol levels. All values represent the mean ± standard deviation. Significant comparisons for sex chromosome complement and for gonadal sex are denoted by brackets. A significant interaction of sex chromosome complement and gonadal sex is denoted by "Int." *, $P \leq 0.05$; **, $P \leq 0.01$; ***,$P \leq 0.001$; †, $P \leq 0.0001$; ‡, $P \leq 0.000001$. F, gonadal female; M, gonadal male; HCD, high cholesterol diet.

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CHAPTER 7

Conclusion

The study of sex differences in obesity is important for human health

Obesity is a worldwide epidemic, affecting people of all ages, ethnicities, and socioeconomic backgrounds. It is a major risk factor for the leading causes of death, including cardiovascular disease and cancer. The development and morbidity of obesity is highly influenced by many environmental and genetic factors, one of which is sex. Fat accumulation and fat distribution are highly sexually dimorphic. For example, both premenopausal and postmenopausal women have higher percent body fat compared to men (1). Premenopausal women tend to accumulate fat below the waist, while men and postmenopausal women tend to accumulate fat in the central abdominal cavity. These sex differences are often attributed to sex hormones. While sex hormones are no doubt important in differentiating males from females, the sex chromosome complement is the fundamental genetic source from which all sex-biasing factors are derived. Before the effects of gonadal hormones organize sex-specific development in mammals, the presence of two X chromosomes or the presence of X and Y chromosomes distinguish female from male cells. It is crucial to understand the effects of sex-biasing factors in the development and progression of obesity and related metabolic disorders.

The study of sex differences in human health and disease is a relatively small field (2). Historically, researchers preferred to use only male human subjects and animal models, in an effort to reduce variability caused by the female estrous cycle and assuming that findings in males can be generally applied to females (3). Interestingly, a meta-analysis of studies published between 2009 and 2012 demonstrated that variation in measurements among individual females is no greater than that among individual males (4). The National Institutes of Health recently released a statement recognizing the negative impact of the lack of research in sex differences, especially on women's health (5). The grant-funding organization plans to implement policies requiring inclusion of both sexes, and will actively encourage endeavors in sex-related research. This is a critical step forward in understanding how sex influences all aspects of biology, from organ and tissue development to pathophysiological disease.

The sex chromosome complement drives sex differences in metabolic traits

Our studies used the Four Core Genotypes (FCG) mouse model, which consists of XX female, XX male, XY female, and XY male mice, to investigate the effects of the sex chromosome complement independently from the effects of gonadal hormones (6). The comparison of XX and XY male mice with XX and XY female mice allows detection of gonadal hormone effects. In addition, the comparison of XX males and females with XY males and females reveals effects associated with the sex chromosome complement. Using this model, we demonstrated that the sex chromosome complement is a key determinant in obesity and related diseases. The sex-biasing impact of the sex chromosome complement was detected for multiple metabolic traits, from molecular transcript levels and cellular lipid accumulation to circulating plasma lipid levels and whole-body adiposity.

In FCG mice fed a standard chow diet, XX/XY male mice weighed more than XX/XY females, consistent with the role of gonadal hormones as a determinant of sex differences in body weight. The male–female sexual dimorphism is maintained from the time of weaning (3 weeks of age) to adulthood and old age. There was also a detectable difference between XX and XY mice, indicating a role for the sex chromosome complement. We hypothesized that removal of gonadal hormones could accentuate effects of the sex chromosome complement on body weight and adiposity. Indeed, after gonadectomy, the effects of the sex chromosome complement were very evident — XX mice had nearly twice the adiposity of XY mice (7). This difference was amplified in mice fed a high fat diet for 16 weeks. These data demonstrate that the sex chromosome complement is an important factor in driving sex differences in obesity.

The findings described above are particularly relevant to modern society. As human lifespan increases, more time is spent in middle to late life stages with low levels of gonadal hormones. The decline of estrogens during menopause is associated with increased visceral fat in the abdominal cavity, which can be partly attenuated by hormone replacement therapy (8). However, hormone therapy is not always effective, and sex differences remain in other metabolic traits

associated with obesity, such as atherosclerosis (9–12). It is possible that the sex chromosome complement mediates sex differences in human obesity and other risk factors for cardiovascular disease.

Because the prevalence of obesity is increasing in younger populations, it is crucial to understand how the sex chromosome complement impacts sex differences in the presence of gonadal hormones. To investigate this, we induced obesity in gonadally intact FCG mice with a high fat diet. After 10 weeks on the diet, there were striking differences between XX and XY mice, regardless of gonadal type. Male and female mice with two X chromosomes gained more weight, had increased adiposity, and accumulated more hepatic lipid compared to XY males and females. Male–female differences were not detected in these metabolic traits. These results revealed a previously unappreciated role for the sex chromosome complement as an important determinant in obesity, even in the presence of gonadal hormones.

Our studies of obesity in gonadectomized and gonadally intact mice demonstrated that effects of the sex chromosome complement are sensitive to circulating levels of gonadal hormones and a high fat diet. Gonadectomy revealed sex chromosome differences in adiposity, and high fat diet enhanced sex chromosome differences in body weight and fatty liver, suggesting that sex chromosome-specific effects are responsive to the levels of circulating gonadal hormones and to nutritional stressors (Fig. 1). This interaction also occurred in plasma lipid levels, a trait associated with obesity and a risk factor for cardiovascular disease. In gonadally intact FCG mice fed a standard chow diet, male mice had higher levels of plasma triglyceride and cholesterol compared to female mice (13). Consistent with the body weight phenotype, gonadectomy eliminated effects of the gonadal hormones and revealed effects of the sex chromosome complement. XX mice had elevated cholesterol and fatty acid levels compared to XY mice. To analyze sex differences in plasma lipid levels of mice under dietary stress, we used a cholesterol-enriched diet to increase plasma lipid levels in both gonadally intact and gonadectomized FCG mice. Interestingly, the cholesterol-enriched diet eliminated

nearly all male–female differences in plasma lipids that were initially observed in gonadally intact mice. In addition, the diet eliminated nearly all XX–XY differences and revealed male–female differences in gonadectomized mice. These results suggest a complex interaction between the sex chromosome complement, gonadal hormones, and the high cholesterol diet. Our studies also demonstrated that irrespective of the diet or gonadal status, high-density lipoprotein (HDL) cholesterol levels were consistently increased in XX compared to XY mice. These data identify the sex chromosome complement as a novel factor for plasma lipid levels.

Together, our studies in obesity and plasma lipid levels demonstrated that the sex chromosome complement mediates sex differences in multiple metabolic traits, including fat accumulation, fatty liver, and HDL cholesterol levels. The effects of the sex chromosome complement are important to consider when assessing the risk and improving treatment for metabolic disease in men and women.

Future directions — molecular mechanisms of sex chromosome-mediated differences

By modulating the number of X and Y chromosomes, we showed that the effect of the sex chromosome complement on obesity and related metabolic traits is derived from the number of X chromosomes, and not the presence or absence of the Y chromosome (7,13). Thus, we surmised that genes on the X chromosome could play a critical role in observed XX–XY differences. Nearly 1,000 protein-coding genes and over 500 noncoding genes reside on the mouse X chromosome (14). In general, gene dosage between XX and XY animals is equalized through X chromosome inactivation, in which all but one X chromosome is transcriptionally active (15). However, a small number of genes escape inactivation and have elevated expression in XX compared to XY cells (16). We reasoned that these genes are good candidates for mediating XX–XY differences in metabolic traits. We focused on a few genes (*Ddx3x, Eif2s3x, Kdm5c, Kdm6a*) that were known to escape inactivation. Prior to our studies, it was unknown whether these genes escape X-inactivation in metabolic tissues. To answer this

question, we measured mRNA expression of these genes in liver and white adipose tissue. Regardless of diet or gonadal status, *Ddx3x*, *Eif2s3x*, *Kdm5c*, and *Kdm6a* were expressed at higher levels in liver and adipose tissue of XX male and female mice compared to XY mice. These data suggest that dosage of these specific genes could contribute to differences in metabolic traits observed in XX *versus* XY mice.

To assess the influence of gene dosage, we generated two mouse models: one strain that was haploinsufficient for *Kdm5c*, and one strain that was haploinsufficient for *Kdm6a*. Body weights did not differ between female mice with one or two copies of *Kdm6a*, suggesting that dosage of this gene does not contribute to body weight. Mice with two copies of *Kdm5c* weighed more and had increased adiposity compared to mice with only one copy of *Kdm5c*. Preliminary data suggest that mice with two copies of *Kdm5c* consumed more food. This is consistent with the increased food consumption observed in XX *versus* XY FCG mice. It is likely, then, that the dosage of *Kdm5c* contributes to the difference observed in body weight and adiposity between XX and XY mice.

Kdm5c encodes a histone lysine demethylase, specific for di- and trimethylated H3K4 (17). The chromatin modifier has the potential to affect transcription of numerous genes, but the specific gene targets in metabolic tissues are currently unknown. We are engineering a cell line to identify the targets of Kdm5c in 3T3-L1 preadipocytes. By inducing the expression of *Kdm5c* in vitro, we can assess the dosage effects of *Kdm5c* in a controlled environment. We plan to use an assay for transposase-accessible chromatin using sequencing (ATAC-seq) to profile transcriptional changes in 3T3-L1 cells with and without induction of *Kdm5c* (18). The data from this analysis will provide putative targets of Kdm5c in preadipocytes and differentiating adipocytes.

The assay described above is merely one step in characterizing the metabolic effect of genes that escape X-inactivation. The dosage of Kdm5c may also influence metabolic pathways in other tissues, such as brain, liver, and gut. The haploinsufficient *Kdm5c* mouse model will be

an indispensable tool for exploring these questions. Future studies will include haploinsufficient mouse models for other X escapee genes, *Ddx3x* and *Eif2s3x*, as well as engineered cell lines for these two genes and *Kdm6a*.

In addition to protein-coding genes, noncoding genes such as microRNAs (miRNAs) and long noncoding RNAs could certainly contribute to sex differences in obesity and related metabolic traits. We explored this hypothesis by sequencing miRNAs in gonadal fat tissue of FCG mice under three conditions: chow-fed gonadally intact, chow-fed gonadectomized, and high fat diet-fed gonadectomized mice. In addition to detecting effects of gonadal hormones and the sex chromosome complement on miRNA expression, this study design allowed identification of sex-specific miRNAs sensitive to circulating hormones and high fat diet. For example, the comparison of gonadally intact mice with gonadectomized mice revealed alterations of miRNA expression associated with the removal of circulating gonadal hormones. Likewise, the comparison of chow-fed mice with mice fed a high fat diet enabled identification of miRNAs responsive to diet-induced obesity. This unique approach allowed analysis of multiple factors that influence miRNA expression. Our study is the first report of sex differences in miRNAs in adipose tissue. We demonstrate that the sex chromosome complement and gonadal hormones influence the expression of miRNAs in gonadal fat.

Some sex-specific miRNAs had been previously implicated in adipogenesis or obesity. For example, miR-196a is essential for brown fat differentiation, a process thought to be increased in females compared to males (19–21). No matter the diet or sex hormone milieu, miR-196a was upregulated in females compared to males. It is possible that sex differences in miR-196a influence the differentiation of brown fat. Likewise, sex differences in other adipose tissue miRNAs could potentially affect adipose development and function. Because multiple miRNAs can target a single mRNA transcript, and each miRNA can target multiple mRNA transcripts, it is conceivable that differential expression of miRNAs influences entire networks of gene regulation (22,23).

Another area of interest is long noncoding RNAs. A recent study found that long noncoding RNAs can regulate adipogenesis (24). One of these RNAs, lnc-RAP-1, also known as *Firre*, is located on the X chromosome and is thought to escape X-inactivation (25). It is possible that the dosage of *Firre* could influence sex differences in obesity and related metabolic traits. To assess the expression of *Firre* in FCG mice, we measured relative mRNA levels in white adipose tissue. Our preliminary data suggest that *Firre* is indeed expressed at higher levels in certain adipose depots of XX compared to XY mice, but its function in adipose tisse is unknown.

In embryonic stem cells, *Firre* acts as a *trans*-chromosomal scaffold and may function in compartmentalization of nuclear DNA (25). Interchromosomal associations have been shown to facilitate active gene transcription (26,27). It is conceivable that the dosage of *Firre* affects gene expression, and this in turn could have important ramifications for sex differences. Indeed, knockdown of *Firre* in a mouse fibroblast cell line was associated with mRNA expression changes in hundreds of genes (28). To understand its role in metabolic tissues, we plan to engineer a mouse model for the whole-body and tissue-specific knockout of *Firre*. Using this mouse model, we plan to analyze the effects of *Firre* dosage on body weight and adiposity. The results from this study will shed light on the role of long noncoding RNAs in sex differences in metabolism.

Concluding remarks

This dissertation demonstrated the role of the sex chromosome complement in obesity and related metabolic diseases. While gonadal hormones are undoubtedly drivers of sex differences, we showed that the sex chromosome complement is a significant, independent factor of sex differences in fat accumulation, fatty liver, plasma lipid levels, and miRNA expression. The genetic mechanisms underlying these sex differences will be an exciting area to explore. Considering the prevalence of obesity, it is of paramount importance that we understand the factors that drive obesity and associated metabolic disorders.

Figure 1

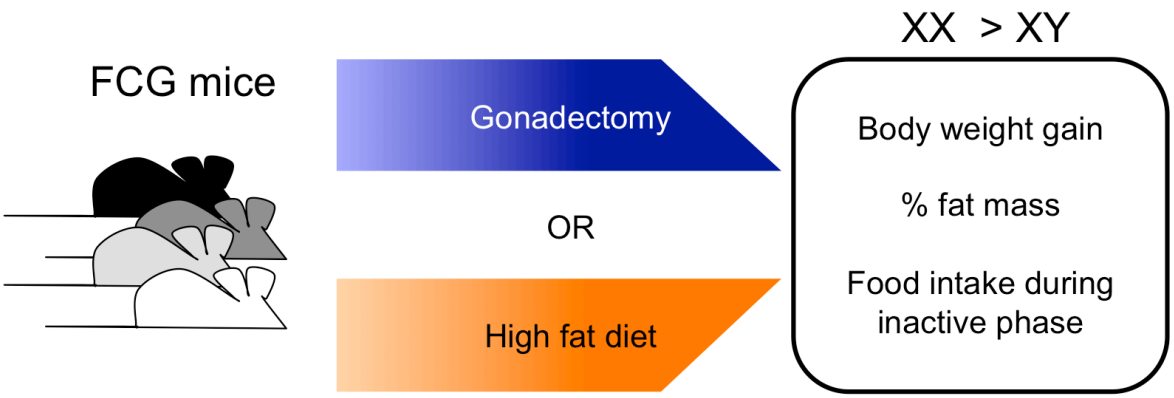

Figure 1. Sex chromosome differences are enhanced with gonadectomy or high fat diet. After gonadectomy or a high fat diet, XX mice gain more weight, have increased fat mass, and consume more food during the inactive phase of the circadian cycle compared to XY mice. FCG, Four Core Genotypes.

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