2020

# An investigation of regulation of MKRN3 monoallelic expression

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

**AN INVESTIGATION OF REGULATION OF *MKRN3* MONOALLELIC EXPRESSION**

by

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B.S., St. Bonaventure University, 2016

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2020

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## ACKNOWLEDGMENTS

I would like to thank Dr. Ana Paula Abreu and Dr. Mina Moussavi for their critical reading and guidance throughout this project. I express my gratitude to Monica Reyes for her expertise with methylation analysis techniques and assistance with bisulfite-treated PCRs. I would show my appreciation for Dr. Ursula Kaiser and Dr. Simon Levy for their support. Additionally, I would like to thank Melissa Magnuson, Dr. Rona Carroll, Dr. Delanie Macedo, Dr. Yongbhum Song, Han Kyeol Kim, Dr. Rajae Talbi, Dr. Victor Navarro, Dr. Lydie Naule, Dr. Elizabeth McCarthy, and Dr. Stephanie Roberts for their help and support in the lab.

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ABSTRACT

Age at pubertal initiation is associated with the risk of many severe health outcomes later in life. "Reactivation" of the hypothalamic-pituitary-gonadal axis, signified via an increase in the amplitude and frequency of gonadotropin-releasing hormone, (GnRH) marks the commencement of puberty. For decades, physicians and scientists have attempted to unravel the mysterious mechanisms underlying the onset of this transitional period of reproductive maturation. In 2013, loss-of-function mutations in maternally imprinted Makorin RING Finger 3 (*MKRN3*) were determined to be the one of the causes of central precocious puberty (CPP), establishing *MKRN3* as the first inhibitory gene with mutations in humans indicated in the upstream regulation of GnRH. *MKRN3* accomplishes this suppression through its function as an E3 ligase, marking components involved in the transcription of kisspeptin and neurokinin B, two stimulatory regulators of GnRH, for degradation by the proteasome. *MKRN3* remains the most common genetic cause of CPP to date. As a maternally imprinted gene, loss-of-function mutations in *MKRN3* have only been shown to produce a phenotype in patients when inherited paternally. However, the mechanisms conferring monoallelic expression of *MKRN3* are not well understood – nor are the downstream implications of *MKRN3* imprinting abnormalities on the development, or even simply, association, with pubertal disorders. It has been determined that differential methylation – a common

epigenetic modification involved in the demarcation of imprinted genes – plays a role in the imprinting mechanism of *MKRN3*. The purpose of this study is to characterize the differentially methylated region (DMR) and to expand understanding of how this region may serve to mark the maternal allele for repression. This will set the foundation for subsequent studies into the potential contribution of methylation defects in the *MKRN3* DMR toward the onset of pubertal disorders. Two potential regulatory sites decorated with CpG islands were identified: the proximal-promoter and a potential enhancer. In classical imprinting studies, differential methylation of the proximal-promoter has been shown to mark alleles on a parent-specific basis for transcriptional inhibition – subsequently silencing expression dependent on parental origin. Additionally, many studies describe a similar process with an upstream or downstream enhancer region being the site influencing transcriptional activity of the alleles on a parent-of-origin basis; preliminary studies indicate a region 3.7 kb upstream of the *MKRN3* transcription start site which may act as an enhancer for *MKRN3*. In this study, DNA was extracted from whole, peripheral blood of individuals with normal pubertal development, followed by bisulfite conversion and PCR amplification, with clean-up and submission for next generation sequencing. Sequencing analysis allowed for the quantification of the methylation profile of each sample at its respective regulatory site. The proximal-promoter was found to be almost entirely methylated while the enhancer exhibited differential methylation, but with notable variability between samples. We identified this enhancer region as the presumptive DMR of *MKRN3*.

Additional research is required to confirm the status of the proposed enhancer as the DMR regulating the paternal-specific expression of *MKRN3*.

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## LIST OF ABBREVIATIONS

ARC ........ Arcuate Nucleus

AP ........ Anterior Pituitary

AS ........ Angelman Syndrome

AVPV ........ Anteroventral Periventricular Nucleus

BAM ........ Binary Alignment Map

BCL ........ Binary Base Call file

bsDNA ........ Bisulfite Treated DNA

BWA ........ Burrow-Wheeler Aligner algorithm

CDP ........ Constitutional Delay of Puberty

CNS ........ Central Nervous System

CPP ........ Central Precocious Puberty

CRISPR ........ Clustered Regularly Interspaced Short Palindromic Repeats

DNA ........ Deoxyribonucleic Acid

DMR ........ Differentially Methylated Region

DYN ........ Dynorphin A

FSH ........ Follicle-Stimulating Hormone

GnRH ........ Gonadotropin-Releasing Hormone

GSNAP ........ Genomic Short-read Nucleotide Alignment Program

GWAS ........ Genome-Wide Association Study

GWLA ........ Genome-Wide Linkage Analysis

HH ........................................ Hypogonadotropic Hypogonadism

HPG Axis ........................................ Hypothalamic-Pituitary-Gonadal Axis

IC ........................................ Imprinting Center

KISS1 ........................................ Kisspeptin

KISS1R ........................................ Kisspeptin Receptor

KOR ........................................ κ Opioid Receptor

LH ........................................ Luteinizing Hormone

MKRN3 ........................................ Makorin Ring Finger Protein 3

ME ........................................ Median Eminence

ncRNA ........................................ Non-Coding RNA

NGS ........................................ Next Generation Sequencing

NKB ........................................ Neurokinin B

NK3R ........................................ Neurokinin B Receptor

NRF-1 ........................................ Nuclear Respiratory Factor 1

PCR ........................................ Polymerase Chain Reaction

POA ........................................ Preoptic Area

PWS ........................................ Prader-Willi Syndrome

RING ........................................ Really Interesting New Gene

RNA ........................................ Ribonucleic Acid

SNP ........................................ Single Nucleotide Polymorphism

TAC3 ........................................ Tachykinin Precursor 3 (Neurokinin B)

TACR3 ........................................ Tachykinin Precursor 3 Receptor (Neurokinin B Receptor)

TSS ........ Transcription Start Site

TZF ........ Tandem Zinc FInger

UPD ........ Uniparental Disomy

UTR ........ Untranslated region

WES ........ Whole Exome Sequencing

# INTRODUCTION

## Puberty and the Hypothalamic-Pituitary-Gonadal Axis

Puberty is the developmental period between childhood and adulthood. It is during this transitionary period that secondary sexual characteristics and reproductive capacity are acquired. The reproductive system is under the control of the hypothalamic-pituitary-gonadal (HPG) axis, which exerts its control via the pulsatile release of gonadotropin-releasing hormone (GnRH) (Teresawa & Fernandez, 2001). GnRH neurons are located in the preoptic area with projections to the median eminence of the hypothalamus (Plant, 2015; Herbison, 2015). Following secretion into the median eminence, GnRH travels through the hypophyseal portal system to stimulate the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the gonadotropes of the anterior pituitary. LH and FSH in turn travel through the blood stream to the gonads, stimulating gametogenesis and sex steroid production (Abreu & Kaiser, 2016). These steroid hormones are then dispersed systemically, allowing for the downstream activation of receptors on target organs and ultimately leading to the development of secondary sexual characteristics (Wood et al., 2019).

The output of the HPG axis changes throughout the course of the human lifespan (Figure 1). It is activated during embryonic life, aiding in sexual differentiation. It is then inhibited for a short interval by placental hormones. After birth, and no longer under the influence of these hormones, the hypothalamus once again secretes GnRH within the first couple of days until about 6 months in boys and 3-4 years in girls. This postnatal period of activity is known as minipuberty and is associated with gonadal activation (Kuiri-Hänninen et al., 2014). From this point until the onset of puberty, the HPG axis is mostly inactive, still releasing GnRH in a pulsatile manner but at a significantly reduced amplitude and frequency (Abreu & Kaiser, 2016). This relatively quiescent period defines most of childhood and normally lasts until from about 8 to 12 years in girls and 9 to 14 years in boys (Rosenfield et al., 2009).

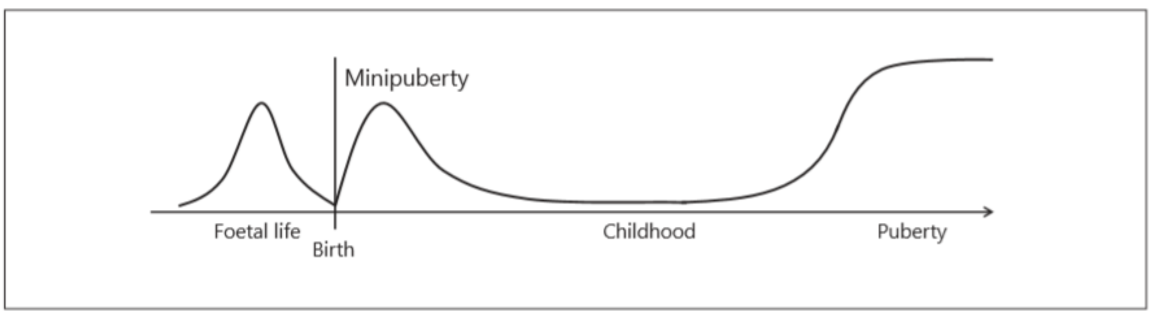



**Figure 1. Activity of Hypothalamic-Pituitary-Gonadal Axis from Fetal Life to Puberty.** In fetal life, GnRH levels peak during mid-gestation and begin to decline prior to birth as a result of inhibition from placental hormones. About a week after birth, activity begins to pick up again and peaks around 1-3 months: a period referred to as minipuberty. Following the decline in activity after minipuberty, gonadotropin secretion does not stop, but remains low throughout childhood until "reactivation" of the axis at the initiation of puberty. (Kuiri-Hänninen et al., 2014)

It has been shown that this period of relative dormancy between minipuberty and puberty is the result of the active suppression of GnRH secretion (Conte et al., 1980). Removal of this restraint causes an increase in pulsatory GnRH secretion, hailing the onset of puberty. However, the exact mechanism underlying this phenomenon is not yet entirely known. It is clear that the HPG axis is influenced by many factors within a complex, hierarchical regulatory network (Ojeda & Lomniczi, 2013). Furthermore, it has been postulated for many years that the onset of puberty is the result of suppression of inhibitory regulation in conjunction with an increase in stimulatory regulation of GnRH secretion (Lomniczi et al., 2015).

One major regulator within this hierarchy is kisspeptin (KISS1). KISS1, an active secretagogue of GnRH, binds the kisspeptin receptor (KISS1R) expressed on GnRH neurons (Herbison, 2015). KISS1 neurons, located in the arcuate nucleus (ARC) (Plant et al., 1978), and additionally, in the preoptic area (POA) of the hypothalamus (Lehman et al., 2013), project to GnRH cell bodies as well as GnRH fibers (Herbison, 2015). Like GnRH, kisspeptin is secreted in a pulsatile manner (Keen et al., 2008). Additionally, co-expressed in kisspeptin neurons are neurokinin B (NKB) and its receptor (NK3R) as well as dynorphin A (DYN) and its receptor, κ opioid receptor (KOR) (Goodman et al., 2007). NKB/NK3R signaling has been described to play a stimulatory role in neuronal activity (Zhang et al., 2008) while DYN/KOR signaling has been indicated in the suppression of neuronal activity (Sajda et al., 2003). It has been hypothesized that the pacemaker capacity of KISS1 neurons could be the result of an oscillatory feedback loop between NKB/NK3R and DYN/KOR signaling, the output of each oscillation being a pulse of kisspeptin release (Navarro et al., 2009).

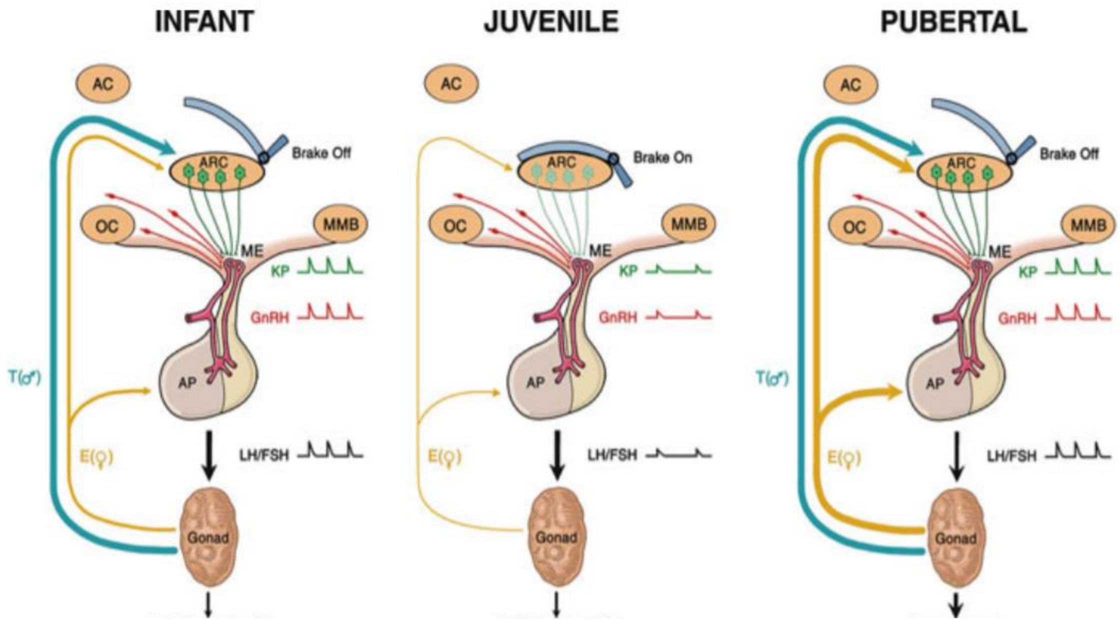



**Figure 2. Model for Regulation of Pubertal Initiation.** In this model, kisspeptin (KP) plays an integral role in the generation of the pulsatile secretion of GnRH into the median eminence (ME). In the infant stage, inputs of kisspeptin allow for robust pulsatile secretion of GnRH. GnRH then stimulates pulsatile secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), allowing for production and release of testosterone (T) or estradiol (E). In the juvenile stage, a neurobiological "brake" is applied to KISS1 neurons within the arcuate nucleus (ARC) and the pulsatile release of the stimulatory neuroregulator kisspeptin is suppressed. With the reduction in secretion of this key secretagogue, GnRH secretion markedly declines. Although the HPG axis is commonly described to be "off" during this period, there is still intermittent GnRH secretion throughout the juvenile stage. However, the frequency and amplitude of the GnRH secretion is not significant enough to stimulate gametogenesis. At the initiation of puberty, the "brake" is released. No longer under the influence of this brake, pulsatile secretion of kisspeptin begins to increase in both frequency and amplitude, with a resultant increase in frequency and amplitude of GnRH secretion. GnRH secretion stimulates the gonadotropes of the anterior pituitary (AP) to secrete LH and FSH, which in turn stimulate the gonads to initiate gametogenesis and sex steroid production. (Teresawa et al., 2013)

However, while this oscillating feedback loop is applicable to the pulse generator mechanism underlying GnRH secretion patterns, it does not establish kisspeptin neurons as the gatekeepers to pubertal initiation; kisspeptin neurons play a crucial role in GnRH pulse generation, but are subject to upstream regulatory mechanisms that lay reign over the timing of puberty (Teresawa et al., 2013). As LH and FSH levels during infancy are dependent upon GnRH stimulation (Mann et al., 1984) and gonadotropin levels are elevated in minipuberty (Plant et al., 2015), it can be ascertained that the GnRH pulse generator mechanism is already functional prior to the initiation of puberty (Plant, 2015). In alignment with this, during the hypogonadotropic state characterizing the juvenile stage, dampened gonadotropin levels indicate that the neuronal network maintaining GnRH pulsatile secretion is still intact during this period (Plant et al., 2015). Taken together, this data is suggestive of an upstream mechanism suppressing the kisspeptin neuronal network of the arcuate nucleus which is ultimately responsible for the initiation of puberty (Figure 2).

**Disorders of Puberty**

In normal individuals, there is 4-5-year variation in the timeframe under which an individual begins pubertal development; the initiation of puberty outside of these limits is considered pathological (Abreu & Kaiser, 2016).

Precocious puberty is clinically defined as initiation of puberty before the age of 8 years in girls and 9 years in boys. Central precocious puberty (CPP) is GnRH-dependent: early activation of the HPG axis causes secretion of GnRH, and, consequently, activation of the gonads prior to the typical parameters (Abreu & Kaiser, 2016).

Delayed puberty is defined as the absence or incomplete development of secondary sexual characteristics by the age of 14 years in boys and 13 years in girls. Late activation of the HPG axis results in constitutional delay of puberty (CDP) and can be a variant of normal development. Complete absence of pubertal onset or incomplete development of secondary sexual characteristics by the age of 18 years old in girls and boys is considered hypogonadism. If this absence is a result of a defect in the central nervous system that prevents the reactivation of the HPG axis, it is classified as hypogonadotropic hypogonadism (HH) (Abreu & Kaiser, 2016).

**Genetics and Pubertal Timing**

Population studies indicate that up to 50-80% of variation in pubertal onset is genetically determined (Palmert & Hirschhorn, 2003). Family studies have demonstrated the impact of genetics in the timing of the initiation of puberty (Fischbein, 1977; Sklad, 1977). Additionally, environmental and nutritional factors are significant elements affecting the progression of pubertal development but even these influences are secondary to genetic mechanisms (Palmert & Hirschhorn, 2003).

Much of the recent developments regarding the regulation in the timing of the onset of puberty, and thus the re-activation of the HPG axis following the juvenile development stage, have been elucidated through genetic studies such as whole-exome sequencing (WES), genome-wide linkage analysis (GWLA), and genome wide association studies (GWAS).

In 2003, one of the major excitatory contributors involved in this network was discovered through genetic studies; loss-of-function mutations in the kisspeptin receptor were identified as a cause of hypogonadotropic hypogonadism (HH) and delayed or absent puberty by two separate studies (Seminara et al., 2003; de Roux et al., 2003). Another positive regulator in the onset of puberty was identified through genome-wide single nucleotide polymorphism (SNP) analysis in families with HH and found to be expressed in KISS1 neurons: tachykinin precursor 3 (TAC3) and tachykinin precursor 3 receptor (TAC3R), also known as neurokinin B and neurokinin B receptor (Topaloglu et al., 2009).

In 2013, Abreu et al. discovered through whole-exome sequencing that loss-of-function mutations in the maternally imprinted gene *Makorin RING Finger 3* (*MKRN3*) were associated with CPP. High levels of *Mkrn3* mRNA are present during the juvenile period in the ARC of mice, rats and monkeys, followed by a decline prior to the onset of puberty (Abreu et al., 2013; Abreu et al., 2020). This decrease in *Mkrn3* expression corresponds with the augmentation of *Tac3* and *Kiss1* hypothalamic expression at pubertal initiation (Gill et al., 2010; Gill et al., 2012). In conjunction with genome-wide and custom-genotyping arrays associating parent-of-origin SNPs in *MKRN3* with age of menarche, these revelations have established *MKRN3* as the first identified gene with an inhibitory effect on GnRH release (Abreu

& Kaiser, 2016). To date, *MKRN3* mutations remain the most common genetic cause of CPP (Valadares et al., 2019).

**Makorin RING Finger 3**

Makorin RING Finger 3 is a member of the Makorin ribonucleoprotein family (Gray et al., 2000). This family is highly conserved across species, hailing from ancestral vertebrate *MKRN1* (Gray et al., 2000; Bohne et al., 2010). They are ubiquitously expressed, with highest expression in the gonads (Jong et al., 1999, 1; Jong et al., 1999, 2; Gray et al., 2000; Bohne et al., 2010). Given these factors, it has been hypothesized that this family is important for sexual development or gonadal function (Gray et al., 2000; Bohne et al., 2010).

As a member of the Makorin protein family, MKRN3 shares the familial profile of highly conserved zinc finger domains: tandem $C_3H$ zinc finger (TZF) domains at the N-terminal end, followed by a Makorin-specific cysteine-histidine arrangement, a $C_3HC_4$ Really Interesting New Gene (RING) finger domain, and another $C_3H$ zinc finger domain at the C-terminal end, as depicted in Figure 3 (Jong et al., 1999, 1; Jong et al., 1999, 2; Gray et al., 2000). The $C_3H$ zinc fingers are likely to confer RNA binding functionality to MKRN3 (Jong et al., 1999, 1; Jong et al., 1999, 2; Barabino et al., 1997; Carballo et al., 1998; Lai et al., 1999); notably, TZF domains, like that at the N-terminus of MKRN3, have been shown to provide high specificity RNA binding (Hudson et al., 2004). The RING finger domain employs interwoven cysteine and histidine residues to coordinate two zinc atoms (Saurin et al., 1996;

Schwabe & Klug, 1994). This conformation results in a robust, globular platform ideal for the protein-protein interactions MKRN3 mediates as an E3 ligase (Barlow et al., 1994; Borden et al., 1995; Abreu et al., 2020).

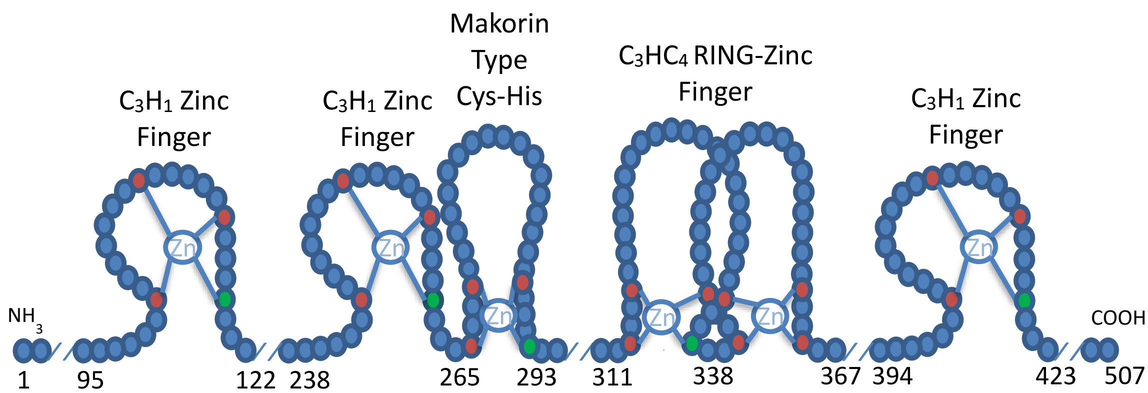



**Figure 3. Schematic Representation of Zinc Finger Motifs in Makorin RING Finger 3 Protein.** Characteristic of the Makorin protein family, MKRN3 has a central Makorin type cysteine-histidine zinc finger. There are also three C3H zinc finger motifs: two at the N-terminus and one at the C-terminus. Additionally, there is a central RING Zinc Finger motif. Each circle represents an amino acid residue. Orange indicates cysteine residues within a zinc finger and green indicates histidine residues within a zinc finger. Zn represents a zinc finger and numbers denote the location of the amino acid within the protein sequence (Modified from Abreu et al., 2015).

E3 ubiquitin ligases work to mark proteins for degradation via the proteasome. E3 ligases accomplish this by a) recognizing target proteins, b) recruiting and activating E2 ubiquitin conjugating enzymes, and c) facilitating the transfer of the ubiquitin from the E2 enzyme to the target protein. E3 ligases are the proteins that provide specificity to the ubiquitination process and this activity is typically conferred via a RING domain (Deshaies & Joazeiro, 2009).

Two recent studies confirmed the E3 activity of MKRN3 (Li et al., 2020; Abreu et al., 2020). It is likely that this function is involved in MKRN3's inhibition of the *KISS1* and *TAC3* promoters (Abreu et al., 2020). These findings help to further characterize the mechanism with which MKRN3 functions to repress the HPG axis prior to the initiation of puberty: transcriptional repression of *KISS1* and *TAC3* would result in decreased kisspeptin and NKB, thereby decreasing stimulatory input to GnRH (Abreu et al., 2020). In the years since the determination of *MKRN3* loss-of-function mutations as a cause of CPP, *MKRN3* has been established as a prime candidate for a critical role in the neurobiological "brake" that regulates the timing of puberty.

**Genomic Imprinting**

Genomic imprinting is a non-Mendelian inheritance phenomenon. Typically, in mammals, offspring are conferred two chromosomes and thus receive two alleles of each gene: one from the maternal parent and one from the paternal. In canonical Mendelian patterns of inheritance, both of these alleles would be equally expressed. In cases of genomic imprinting, however, one allele becomes silenced on a parent-of-origin basis (Barlow et al., 1991; Bartolomei et al., 1991; DeChiara et al., 1991). Imprinting can be conceptualized as a mode of preferential expression of one parental allele over the other. There are many characterized mechanisms that have been implicated in this unequal parent-of-origin specific gene expression such as DNA methylation, histone modification/chromosome condensation and noncoding

RNAs (Bartolomei & Tilghman, 1997; Barlow, 1995; Nicholls et al., 1998; Sleutels et al., 2002; Mancini et al., 2006; Williamson et al., 2011).

In order to achieve parent-of-origin specific gene expression, there are some qualifications that must be met. The establishment of an epigenetic mark or "imprint" is necessary to ensure that the cell is able to differentiate between the two parental chromosomes. The imprint needs to provide a mechanism – either inhibitory or stimulatory – with which the transcriptional machinery will transcribe one parental allele over another within the same nucleus. It must be heritable through somatic lineages: able to remain intact through mitotic division, as to allow for perpetuation of the differential expression in daughter cells. Contrastingly, the imprint must also be erasable as to be reset in the embryonic gonads prior to sexual differentiation; the acquisition of the imprint will be dependent on the sex of the fetus (Barlow & Bartolomei, 2014; Pfiefer, 2000). Thus, imprinting calls for a mechanism that enables the parental imprint to switch in the germline of opposite-sex progeny (Nicholls, 2000). This imprint switch mechanism allows for the production of haploid gametes all either carrying the imprint or without it, corresponding with parental origin and must thus be established during gametogenesis or directly after fertilization, when the parental chromosomes are isolated from one another (Ferguson-Smith, 2011).

**Differential Methylation**

One well-characterized mechanism of imprinting is through the differential methylation of CpG islands on the promoter or enhancer region of an imprinted gene, dependent on parental origin (Li et al., 1994; Tilghman, 1999; Constancia et al., 1998; Pfiefer, 2000). Parent-of-origin specific, differential methylation of cytosine nucleotides in CpG dinucleotide rich regions, referred to as CpG islands, has been recognized as a mechanism of setting the parental imprint during gametogenesis of oocytes and spermatogonia (Nicholls, 2000; Paulsen & Ferguson-Smith, 2001).

Methylation of CpG islands is able to influence transcription via direct and indirect methods; the binding of transcriptional regulatory factors/complexes can be blocked by the methyl groups themselves, or DNA methylation can recruit factors that promote the formation of heterochromatin: condensing the region and thus making it inaccessible to transcriptional machinery (Ng & Bird, 1999; Saitoh & Wada, 2000; Paulsen & Ferguson-Smith, 2001). Parent-of-origin specific, differential methylation results in discordant transcription of alleles and is typically associated with silencing of a particular parental allele (Schubeler, 2015). Differentially methylated regions (DMRs) evidently serve as important regulatory regions for the monoallelic expression of imprinted genes (Pfeifer, 2000).

**Prader-Willi/Angelman Syndrome Critical Region**

Imprinted genes are often clustered within the genome (Bartolomei & Ferguson-Smith, 2011). *MKRN3* is a paternally imprinted gene located on one such cluster called the Prader-Willi/Angelman syndrome critical region on chromosome 15q11-13 (Jong et al., 1999). This region is comprised of two clusters of imprinted genes: the maternally imprinted, paternally expressed Prader-Willi syndrome (PWS) region and the paternally imprinted, maternally expressed Angelman syndrome (AS) region (Figure 4) (Nicholls, 1998).

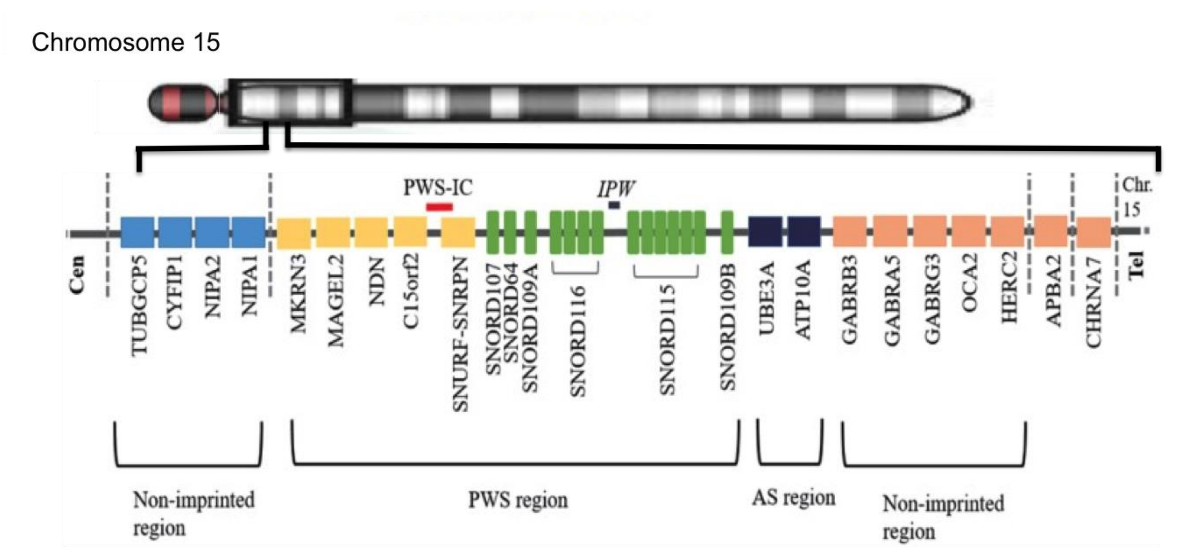



**Figure 4. Genetic Map of Prader-Willi/Angelman Syndrome Critical Region.** Ideogram of region on the long arm of chromosome 15 containing clusters of imprinted genes associated with Prader-Willi and Angelman syndromes. Shown in yellow are maternally imprinted/paternally expressed coding genes; in green, are paternally expressed noncoding RNA; and, in dark blue, are maternally expressed, paternally imprinted coding genes of the Angelman syndrome region (Jong et al., 1999).

Coordinated imprinting in this region is regulated in *cis* through a bipartite imprinting center (IC) integral to the imprint switching mechanism during gametogenesis: the 3.4 kb PWS-IC spanning the *SNURF/SNRPN* promotor and exon 1, and the 880 bp AS-IC situated 35 kb upstream of the PWS-IC (Paulsen & Ferguson-Smith, 2001; Buiting et al., 1999; Ohta et al., 1999). The exact mechanism by which this coordinated imprinting is accomplished has yet to be elucidated. It has been hypothesized that the PWS-IC serves as a positive regulator, activating the paternal-specific gene expression in the male germline (Bielinska et al., 2000; Bressler et al., 2001), while the AS-IC acts in the maternal imprint to repress the PWS-IC, precluding the activation of paternal imprinting (Ohta et al., 1999; Shemer et al., 2000; Rodriguez-Jato et al., 2005).

Loss of the proper parent-of-origin gene expression in either of these regions results in the titular syndromes. This can occur through multiple mechanisms, such as large chromosomal deletions, uniparental disomy (UPD), genetic mutation, imprinting mutations – such as aberrant DNA methylation – and balanced translocations (Nicholls et al., 1998).

Although *MKRN3* is located within the PWS region, the extent to which it may or may not contribute to the syndrome has yet to be determined. Reports of PWS patients without a paternal deletion of *MKRN3,* in combination with a patient that has a chromosomal deletion including *MKRN3* in which PWS has not developed, suggest that deficiency in paternal expression of *MKRN3* is not a critical component in the progression of PWS (Kanber et al., 2009).

In accordance with the other genes found within this region, *MKRN3* is a maternally imprinted, paternally expressed gene – and follows the pattern of expression expected of other genes within the region, as is illustrated in Figure 5 (Abreu et al., 2013; Nicholls et al., 1998). It is under the regulatory influence of the PWS-IC (Nicholls et al., 1998). However, the local regulatory mechanism by which *MKRN3* receives and responds to the IC signal is yet to be determined (Jong et al., 1999, 2).

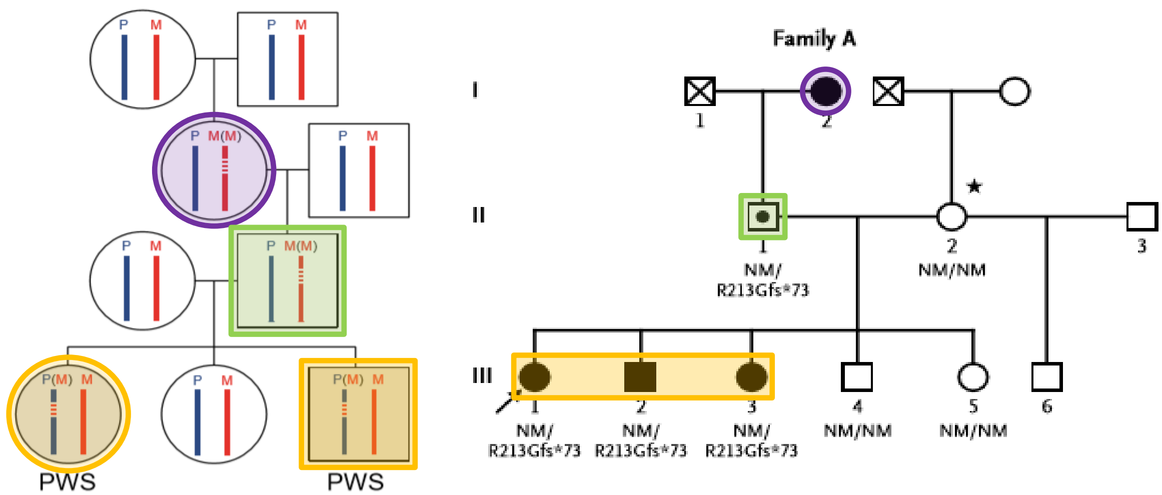



**Figure 5. Pedigree of Family with MKRN3 Mutation as Compared to that of Family with PWS.** Just as in families with PWS (left – Nicholls et al., 1998), the mutation only manifests phenotypically two generations after the silent transmission from the affected patient's grandmother (purple) to the carrier father (green). The mutated allele was imprinted or silenced in the father due to its maternal inheritance. However, the mutation will manifest in about 50% this male's offspring because the initially maternally inherited paternal genes were passed onto the subsequent generation by the indicated parent. On the left, blue chromosomes represent the paternally inherited chromosome while red represents the maternally inherited chromosome; mutations are indicated via the breakpoints in the depicted chromosomes. On the right (Abreu et al., 2013), black symbols indicate clinically affected individuals; symbols with black circles represent asymptomatic carriers. Squares indicate male family members, circles female family members, yellow shading clinically affected family members (Modified from Nicholls et al., 1998 and Abreu et al., 2013).

## Monoallelic Expression of *MKRN3*

The precise mechanism that regulates the monoallelic expression of *MKRN3* has not been well characterized. Studies in mice have proposed that the DMR responsible for the silencing of the maternal allele of *Mkrn3* resides in the *Mkrn3* promoter (Jong et al., 1999, 2; Hershko et al., 1999). In humans, there have been conflicting and unspecific reports as to the location of the *MKRN3* DMR (Jong et al., 1999, 1; Jong et al., 1999, 2; Monk et al., 2018; Suzuki et al., 2019). However, these studies do reach one, significant consensus: paternal-specific expression of *MKRN3* is likely regulated on some level by differential methylation (Jong et al., 1999, 1; Jong et al., 1999, 2; Monk et al., 2018; Suzuki et al., 2019). Interestingly, early characterization of *MKRN3* methylation implicates a tissue-specific methylation pattern (Figure 6) (Jong et al., 1999, 1).

Preliminary data collected in PWS studies by collaborator Robert Nicholls through plasmid-based *in vitro* assays identifies a prospective DMR CpG islet 3.7 kb upstream of the *MKRN3* transcription start site (TSS), suggestive of an enhancer region that may regulate *MKRN3* monoallelic expression in humans (Nicholls, unpublished data). Further investigation into regions with the potential to regulate *MKRN3* maternal imprinting in humans is needed for greater understanding of *MKRN3* and its role in the complex network associated with the re-activation of the HPG axis.

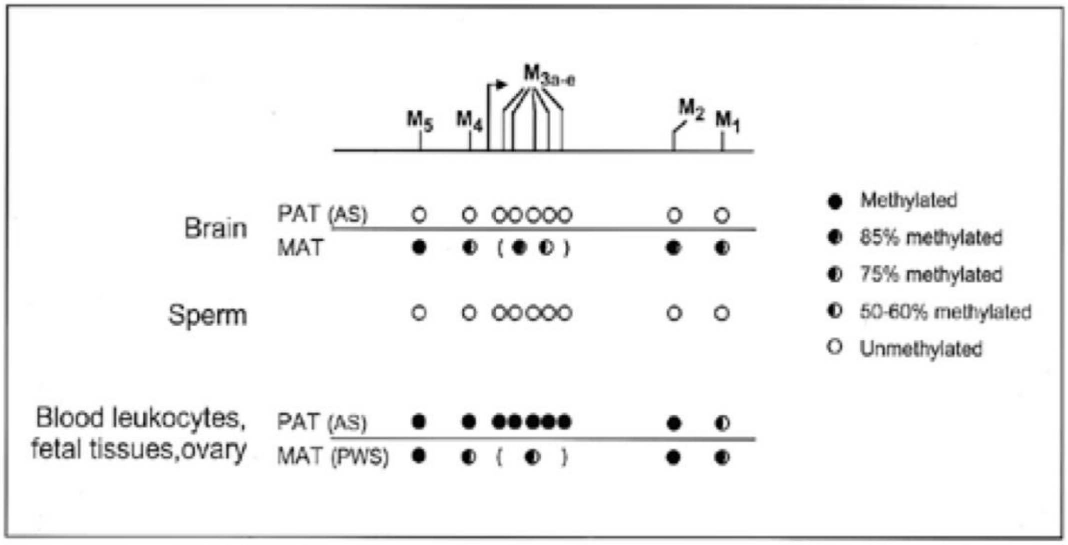



**Figure 6. Tissue-specific DNA methylation of *MKRN3* alleles.** In blood leukocytes, fetal tissues, and ovary tissues, it was found that both parental alleles were hypermethylated, with CpG island methylation ranging from 50/60% -100% methylated. In sperm, the DNA is unmethylated. And in the brain, the maternal allele is hypermethylated while the paternal allele remains unmethylated (Jong et al., 1999, 1).

As roles for imprinted genes have been implicated in the timing of multiple developmental stages in humans as well as in the regulation of growth and development (Howard, 2019; Peters, 2014), there is reason to speculate that imprinting – and deviations in imprinting mechanisms – may influence the progression of pubertal development. The association of genetic mutations in *MKRN3* with the timing of puberty has been well defined (Valadares et al., 2019). It would be valuable to gain insight into the effects of *MKRN3* methylation defects in *MKRN3* expression and their consequent influence over the timing of pubertal onset or contribution to the development of pubertal disorders.

This study aims to identify the location of the DMR carrying the maternal imprint of *MKRN3* in humans and to characterize the methylation pattern of this region. The characterization of the *MKRN3* DMR will allow us greater understanding of the maternal imprinting process which governs the monoallelic expression of *MKRN3*. Understanding of this mechanism can allow us to glean further knowledge surrounding the ways in which epigenetic modifications and regulation interact with and impact the HPG axis. This will be accomplished via bisulfite treatment, with subsequent amplification by polymerase chain reaction, clean up and submission for next generation sequencing. Once the region conferring repression of maternal allelic expression in humans is determined, the influence of aberrant methylation of the *MKRN3* DMR on the timing of puberty can be assessed by screening CPP and CDP patients for variants and methylation abnormalities in this region.

## SPECIFIC AIMS

The purpose of this preliminary study is to characterize the regulatory mechanisms that establish the monoallelic expression of *MKRN3*. The determination of the regulatory site of the MKRN3 imprinting mechanism will allow for investigation into whether imprinting defects, specifically abnormal methylation, in the region regulating the expression of *MKRN3* are associated with pubertal disorders. Greater understanding of the imprinting regulation of *MKRN3* will provide better insight into the complex network regulating pubertal onset and the epigenetic regulation of the HPG axis. This could provide more avenues for the development of treatments for pubertal disorders, genetic counseling and research techniques.

# METHODS

## Sample Collection

The Institutional Review Board of Brigham and Women's Hospital approved this study: "The Molecular Basis of Developmental Disorders of the Reproductive System", IRB number: 2005P001440.

Peripheral blood was collected from individuals with normal pubertal onset and without personal or family history of pubertal disorders. Informed consent was obtained according to HIPAA guidelines.

## DNA Extraction

DNA extraction of up to 5 mL whole blood was performed using Qiagen QIAamp Blood Maxi Kit (catalogue no. 51194) Spin Protocol.

500 µL Qiagen Protease K was added to 50 mL centrifuge tube, followed by 3-5 mL fresh, peripheral blood. If less than 5 mL blood was available, PBS was added to adjust to the necessary volume. 6 mL Buffer AL was added and solution was mixed via inversion 15 times followed by vigorous shaking for 1 minute in order to ensure a homogenous solution. Sample was incubated at 70 $^{o}$C for 10 minutes for lysis. 5 mL 100% ethanol was added to the tube, mixed by inversion 10 times and vigorously shaken for 1 minute to ensure efficient binding and a homogenous solution.

Solution was transferred onto the QIAamp Maxi column placed in a 50 mL centrifuge tube, being cautious not to moisten the rim. Tube was sealed and centrifuged at 3000 rpm for 3 minutes or until the solution passed through the membrane fully. QIAamp Maxi column was temporarily removed, the filtrate was discarded, and centrifuge tube was wiped down.

QIAamp Maxi column was placed back into the centrifuge tube. 5 mL Buffer AW1 was added to the QIAamp Maxi column without wetting the rim. Tube was sealed and centrifuged at 5000 rpm for 1 minute. 5 mL Buffer AW2 was added to the QIAamp Maxi column without wetting the rim. Tube was sealed and centrifuged at 5000 rpm for 15 minutes, Sample was incubated for 10 min at 70 $^{o}$C to evaporate residual ethanol.

QIAamp Maxi column was wiped dry and placed in a clean 50 mL centrifuge tube – old collection tube was discarded. 600 μL distilled water at room temperature was applied directly to the membrane of the column and cap was closed. Column was incubated at room temperature for 5 minutes and centrifuged at 5000 rpm for 2 minutes. Eluate was reloaded onto the column and cap was closed. Incubation for 5 minutes was followed by centrifugation at 5000 rpm for 2 minutes.

Samples were aliquoted into 2 mL microcentrifuge tubes and stored at -20 $^{o}$C.

## DNA Spectrophotometry

The concentration of DNA in each sample was determined using Nanodrop machine. 1 μL of each sample was pipetted onto pedestal of the Nanodrop machine and was measured for sample concentration (ng/μL).

## Bisulfite Conversion

DNA was bisulfite treated using EZ DNA Methylation Gold Kit (Zymo Research, catalogue no. D5005). 260 μL CT Conversion Reagent and 40 μL sample (1200ng DNA – if less than 40 μL, volume was adjusted with water) were added to 1.5 mL microcentrifuge tube. Sample was mixed by pipetting up and down, then centrifuged (Sorvall Biofuge Pico) to the bottom of the tube.

Sample was separated into 6 PCR tubes, 50 μL each. Sample incubated in the thermal cycler (Bio-Rad S1000) with the following cycle: 98 $^{o}$C for 10 min, 64 $^{o}$C for 2.5 hours, 4 $^{o}$C optional storage up to 20 hours.

Following incubation, samples were combined back into one tube and pipetted up and down. 600 μL M-Binding Buffer was added into two Zymo-Spin Columns and columns were placed in a collection tube. 150 μL sample was loaded into each spin column. Cap was closed and solution was mixed by inversion several times. Spin columns centrifuged at full speed for 30 seconds and flow through was discarded.

100 μL M-Wash Buffer was added to column and spin column was centrifuged at full speed for 30 seconds. Flow-through was discarded.

200 µL M-Desulphonation Buffer was added to the column and allowed to incubated at room temperature for 15 minutes. Following incubation, samples were centrifuged at full speed for 30 seconds. Flow-through was discarded.

200 µL M-Wash Buffer was added to the column, followed by centrifugation for 30 seconds. Flow-through was discarded. 200 µL M-Wash Buffer was added to the column again, followed by centrifugation for 30 seconds.

Column was placed into labeled 1.5 mL microcentrifuge tube and 12 µL M-Elution Buffer was loaded directly to the center of the column matrix. Sample was incubated at room temperature for 1 min, then centrifuged at full speed for 30 seconds. Eluate was reloaded directly to the center of the column matrix. Sample was incubated at room temperature for 1 min, then centrifuged at full speed for 30 seconds.

DNA concentration was measured on Nanodrop machine and samples were stored at -80 °C.

| | |
|---|---|
| Template: | 5′-GGATT**C**GAGGATTTAAA**CCC**GA**C**AAT-3′<br>3′-**CC**TAAG**C**T**CC**TAAATTTGGG**C**TGTTA-5′ |
| Bisulfite Converted: | 5′-GGATT**C**GAGGATTTAAA**TTC**GA**T**AAT-3′<br>3′-**TT**TAAG**C**T**TT**TAAATTTGGG**C**TGTTA-5′ |

**Figure 7. Bisulfite Conversion of Double Stranded DNA Templates.** Example of activity that occurs on a DNA template during bisulfite conversion. All the cytosines on the original DNA template are bolded. Methylated cytosines are underlined. Treatment of DNA with sodium bisulfite converts cytosine to uracil, but leaves 5-methylcytosine unaffected. Thus, DNA that has been treated with bisulfite retains

only the originally methylated cytosines. Following amplification via polymerase chain reaction, the uracil nucleotides are replaced with thymine nucleotides (as Taq is a DNA polymerase). This provides a mechanism with which to determine the methylation pattern of the template.

## Primer Design

Potential CpG islands at both the proximal promoter and proposed enhancer of *MKRN3* were identified through the GRCh38/hg38 reference sequence on the University of California Santa Clara Genomics Institute genome database. Primers were designed using MethPrimer software (Li & Dahiya, 2002) – a bisulfite-conversion-based methylation PCR primer designing program – following conversion of all cytosine bases to thymine on the reference sequence. A list of the primers used is provided in Table 1.

**Table 1. DNA Primers Used for PCR of Bisulfite Treated DNA.** The primers targeting the MKRN3 promotor and enhancer CpG islets are listed below, as well as the name of the primer, the sequence, the primer ID and product size. Primers were designed using MethPrimer (Li & Dahiya, 2002).

| PRIMER NAME | REGION | SEQUENCE | PRIMER ID | PRODUCT SIZE |
|---|---|---|---|---|
| bsMKRN3-F1 | **Promoter** | **GGGAAGGAAAAAGAGATGTATATTTTT** | **352017** | **201 BP** |
| bsMKRN3-R1 | **Promoter** | **CCCTTACTACCTCAACACCTACCTA** | **352018** | **201 BP** |
| bsMKRN3-F5 | **Enhancer** | **TGTTGTTAGATGGGATAAAAGAAGG** | **357193** | **522 BP** |
| bsMKRN3-R4 | **Enhancer** | **ATAAAATCCCCTAACCAATCAACAT** | **357194** | **522 BP** |

**Polymerase Chain Reaction**

Bisulfite treated DNA samples were amplified by polymerase chain reaction using reagents from the Qiagen Taq PCR Core Kit (catalogue no. 201223).

*The following protocol was used for amplification of the promoter:*

Each PCR tube was loaded with PCR buffer 10x (5 μL), Q-Solution (10 μL), Q-$MgCl_2$ (3.5 μL), Q-dNTP (3 μL), Qiagen Taq (0.5 μL), bsMKRN3-F2 100 uM (0.5 μL), bsMKRN3-R2 (0.5 μL), bisulfited DNA (2 μL), and nuclease free water (to 50 μL) (25 μL) for a total volume of 50 μL per tube. The samples were loaded into the thermal cycler (MiniAMP) with the following specifications: 95 $^{o}$C for 5 minutes; 95 $^{o}$C for 30 seconds, 58 $^{o}$C for 30 seconds, 72 $^{o}$C for 1 minute x40 cycles; 72 $^{o}$C for 10 minutes.

*The following protocol was used for amplification of the enhancer:*

Each PCR tube was loaded with PCR buffer 10x (5 μL), Q-Solution (10 μL), Q-$MgCl_2$ (3.5 μL), Q-dNTP (3 μL), Qiagen Taq (0.5 μL), bsMKRN3-F2 100 uM (0.5 μL), bsMKRN3-R2 (0.5 μL), bisulfited DNA (2 μL), and nuclease free water (to 50 μL) (25 μL) for a total volume of 50 μL per tube. The samples were loaded into the thermal cycler (MiniAMP) with the following specifications: 95 $^{o}$C for 5 minutes; 95 $^{o}$C for 30 seconds, 58 $^{o}$C for 30 seconds, 72 $^{o}$C for 30 seconds x40 cycles; 72 $^{o}$C for 10 minutes.

Samples subjected to both protocols were either immediately assessed by gel electrophoresis or stored at -80 $^{o}$C for later use.

## Gel Electrophoresis

To assess the quality of the PCR amplification, 12 µL of the sample, 12 µL of a negative control, and 15 µL 100 bp ladder were loaded on a 1% agarose gel. The gel was run at 100 V (Bio-Rad PowerPac 300 Electrophoresis Power Supply) for 35-40 minutes, after which it was evaluated under ultraviolet light for bands indicating amplification of the target sequence: a 201 bp band for the promoter region and a 522 bp band for the enhancer.

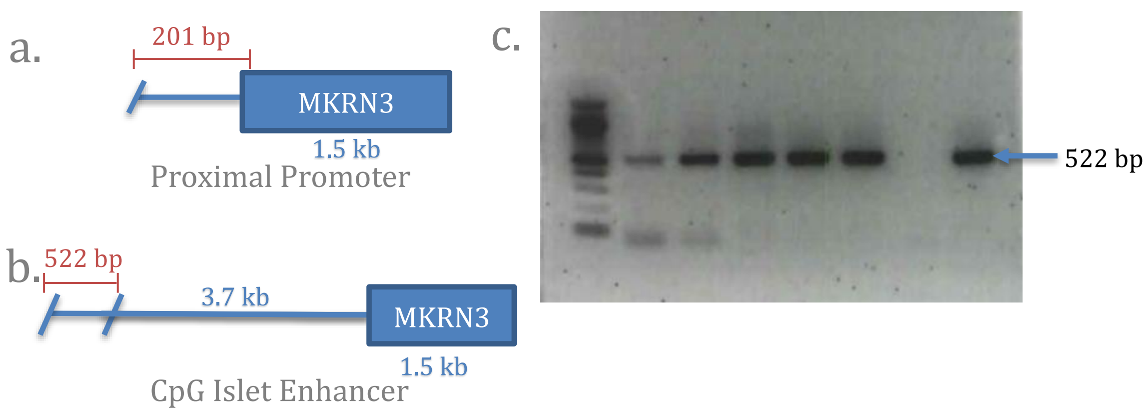



**Figure 8. Locations of Target Regions in Context and Amplification of Enhancer Region.** Red regions present the targeted regions spanning the primers. (a) Primer encompassing the *MKRN3* proximal promotor. Differential methylation of the promoter is the classical model of imprinting. As such, this was the first region screened for differentially methylated CpG islands. (b) CpG island rich region 3.7 kb upstream of *MKRN3* TSS which has been proposed as an enhancer regulating *MKRN3* in PWS studies. (c) Example of the bands produced via the successful amplification of the proposed enhancer region.

## DNA Purification

Samples were cleaned using Qiagen QIAquick PCR Purification Kit (catalogue no. 28104). 30 µL sample was first diluted with 150 µL PB Buffer in a 1.5 mL microcentrifuge tube, then pipetted up and down to mix. Sample was then loaded into QIAquick column in a collection tube and centrifuged for 30-60 seconds at 13,000 rpm. Flow through was discarded and column was placed back into the collection tube.

To wash, 750 µL Buffer PE was added to the QIAquick column. Column was centrifuged for 30-60 seconds at 13,000 rpm. Flow through was discarded and column was placed back into the collection tube. The column was centrifuged for 1 minute at 13,000 rpm.

The column was then placed in a clean 1.5 mL microcentrifuge tube and 30 µL water was loaded directly to the center of the matrix. This was allowed to incubate at room temperature for 1 minute, then centrifuged for 1 minute to elute the DNA.

**Next Generation Sequencing**

Samples were submitted for Next Generation Sequencing (NGS) using Illumina MiSeq sequencer with Illumina Reagent Kit V2 Chemistry at the DNA Core under the Center for Computational & Integrative Biology at Massachusetts General Hospital (Figure 9). The data from the NGS sequencing was exported into a Binary Base Call (BCL) file. Data from this file was converted by the MiSeq sequencer into a .sit file for the submitted sequence containing a FASTQ file and SEQ file for qualitative analysis as well as a Microsoft Excel file for quantitative analysis.

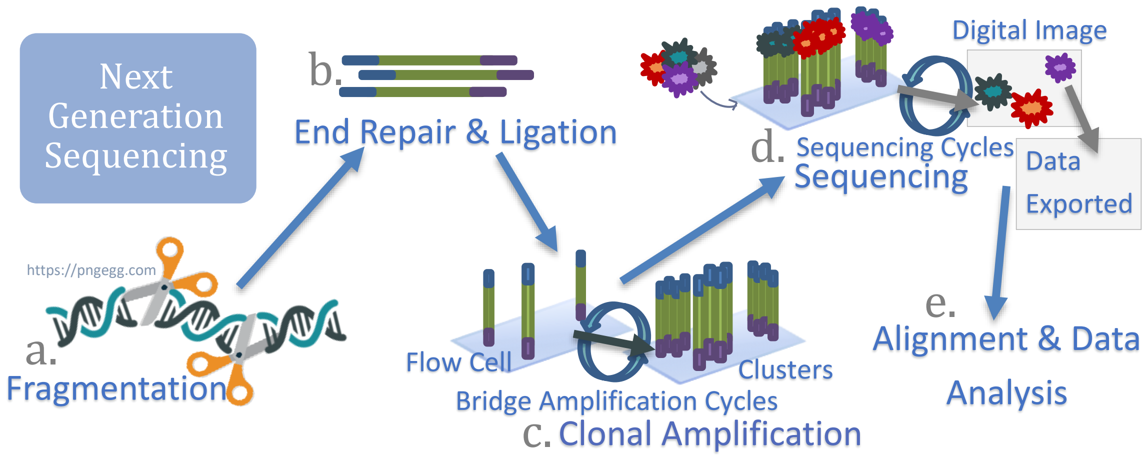



**Figure 9. Steps in Next Generation Sequencing.** (a) Random fragmentation of the DNA (or cDNA) is the first step in NGS library preparation. (b) The second step in NGS library preparation involves ligation of 5' and 3' adapters to the fragmented DNA. The adapter-ligated DNA fragments are then amplified through PCR and gel purified. (c) The adapter-ligated DNA fragments are loaded onto the flow cell for "capture" via oligos complementary to the adapters. Each fragment is then amplified in parallel through a bridge amplification, in which the adapters on each end of the fragment bind to complementary oligos on the flow cell, resulting in clusters of clonal amplification. (d) The clones are sequenced with the addition of one fluorescent-tagged nucleotide at a time, followed by imaging and removal of the bases. This data is exported to an output file. (e) During the alignment and data analysis phase, sequence reads are aligned to a reference with bioinformatics software. The aligned sequences can then be analyzed for genomic applications (Illumina Inc., 2017).

The qualitative analysis was performed using Sequencher version 5.4.6 DNA sequence analysis software (Gene Codes Corporation). The FASTQ file is a text-based file with the raw data on the sequence reads in combination with their respective quality scores. This file was aligned to a bisulfite-converted GRCh38/hg38 reference sequence from the University of California Santa Clara Genomics Institute genome database in Sequencher using a Burrow-Wheeler Aligner algorithm (BWA-MEM) (Li, 2013) or the Genomic Short-read Nucleotide

Alignment Program (GSNAP) algorithm (Wu & Nacu, 2010). The BWA-MEM and GSNAP algorithms generated a Binary Alignment Map (BAM) file, which was visualized using the Tablet integrative genomic viewer (The James Hutton Institute) (Milne et al., 2009) on Sequencher.

Quantitative analysis was performed using the Microsoft Excel files provided in the .sit file from the Massachusetts General Hospital DNA Core. The sequences were aligned manually. The methylation status at each CpG island was determined by compiling the percentage of cytosine nucleotides remaining at each of the pre-bisulfite-converted CpG dinucleotide locations along the region from all the sequence reads of each sample (or percentage of guanine nucleotides remaining on complementary strands at the equivalent position).

# RESULTS

## Subjects

The final cohort consisted of 3 female individuals with normal pubertal timing and without reproductive disorders to characterize the wildtype *MKRN3* differentially methylated region (DMR). Spontaneous, normal pubertal onset and development was established on the basis of verbal report of personal and family reproductive history to clinical investigators.

## Methylation of *MKRN3* Proximal Promoter

*MKRN3* is a maternally imprinted gene, expressed from the paternal allele (Jong et al., 1999, 1; Jong et al., 1999, 2; Hershko et al., 1999). DMRs regulating the monoallelic expression of imprinted genes have been well-characterized in the proximal promoter of the genes (Ferguson-Smith et al., 1993; Vu & Hoffman et al., 1994; Tremblay et al., 1995; Varrault et al., 2001; Valleley et al., 2007). With this in mind, we first decided to look at the proximal promoter of *MKRN3* as a potential DMR in the regulation of monoallelic expression.

Much of the classical literature describes a 50% methylation pattern at each of the CpG islands within the promoter region. This is indicative of half of the chromosomes being entirely methylated at the CpG islets of the proximal promoter, correlating with the half of the genetic information inherited from the maternal line, and thus differential methylation on the basis of parental origin at the CpG islands.

However, in line with *MKRN3*-specific studies, we did not expect a precise 50% methylation pattern as it has been shown that *MKRN3* methylation on the maternal allele in peripheral tissues is not 100% (Figure 6) (Jong et al, 1999, 1).

We did not identify differentially methylated CpG islands at the locations identified through the GRCh38/hg38 reference sequence on the University of California Santa Clara Genomics Institute genome database within the analyzed region of the *MKRN3* proximal promoter; methylation analysis revealed that all the CpG islands analyzed within the 201 bp region of the promoter were either approximately 0% or 100% methylated.  This can be seen in the BAM of the *MKRN3* promoter region – comprised of the raw data produced from the NGS aligned to the bisulfite-converted reference (Figure 10). Exact methylation percentages at each island represented in the samples are listed in Table 2.

In total, 13 CpG islands were analyzed and found to be localized to a region starting 128 bp upstream of the *MKRN3* exon and extending 20 bp into it – none of which were differentially methylated. Locations of the islands relative to the start of the *MKRN3* exon are depicted in Figure 11. The first seven were 100% methylated, followed by two which were unmethylated, another pair of fully methylated sites, and one last set of unmethylated islets. This data led us to pursue analysis of the other potential DMR regulating *MKRN3* monoallelic repression: the CpG islands 3.7 kb upstream of the *MKRN3* transcription start site.

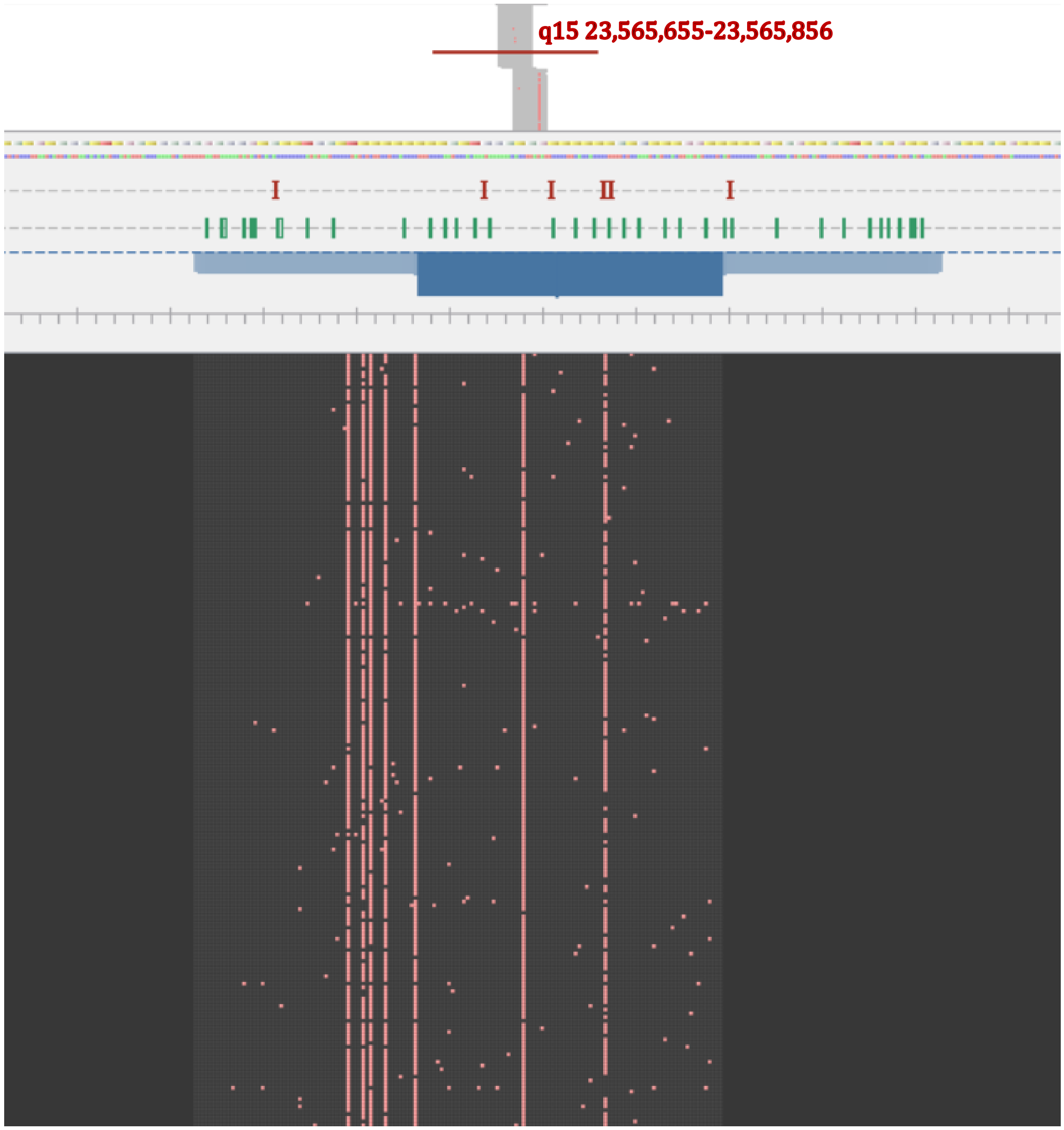



**Figure 10. Binary Alignment Map of *MKRN3* Proximal Promoter Region.** Binary Alignment Map (BAM) of *MKRN3* promoter CpG islands visualized on Tablet integrative genomic (The James Hutton Institute) (Milne et al., 2009) viewer. Pink boxes represent methylated cytosine nucleotides remaining at CpG islands in the MKRN3 proximal promoter region after bisulfite conversion treatment. Bisulfite conversion treatment converts unmethylated cytosine nucleotides into uracil nucleotides – replaced with thymine nucleotides during PCR. The resulting pink "columns" as seen in the aligned sequences show almost complete methylation at the CpG islands throughout the reads.

**Table 2. Percent Methylation of CpG Islands within the Proximal Promoter Region of *MKRN3*.** Percentage of cytosine nucleotides remaining at CpG dinucleotides in the proximal promoter region of *MKRN3* after bisulfite treated Next Generation Sequencing. Positions of CpG islands as depicted in Figure 11.

***Proximal Promoter***

| *CpG Island* | Percent Methylation |
|---|---|
| *1* | 93.97% |
| *2* | 93.98% |
| *3* | 94.74% |
| *4* | 92.72% |
| *5* | 98.59% |
| *6* | 94.26% |
| *7* | 93.36% |
| *8* | 3.91% |
| *9* | 3.97% |
| *10* | 97.02% |
| *11* | 96.75% |
| *12* | 5.54% |
| *13* | 5.54% |

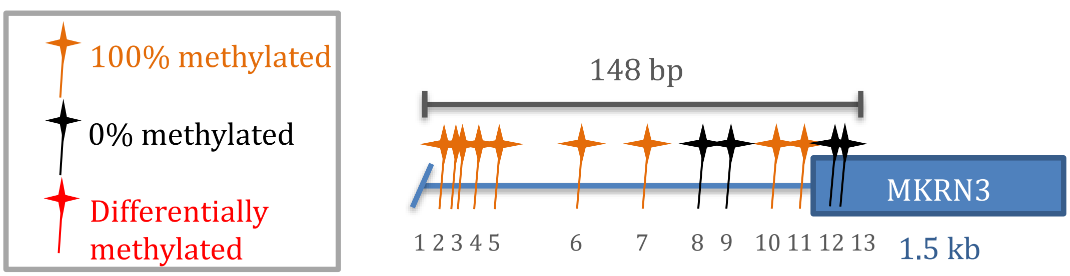



**Figure 11. Methylation Status of CpG Islands in the Proximal Promoter of *MKRN3*.** Of the 201 bp area that we analyzed in the proximal promoter region of *MKRN3*, we identified islands within a 148 bp region starting 128 bp upstream of the *MKRN3* exon and all islands were either 100% or 0% methylated. The CpG islands numbers coordinate with the methylation percentages in Table 2. Orange represents CpG Islands that are 100% methylated; black represents islands that are 0% methylated; red represents CpG Islands that are differentially methylated.

**Methylation of *MKRN3* Potential Enhancer**

Studies indicate that DMRs mediating downstream regulation of monoallelic expression can also be located at enhancer regions (Nickol & Felsenfeld, 1988; Ferguson-Smith et al., 1993; Bartlomei & Tilghman, 1997; Hark et al., 2000). Our collaborator, Robert Nicholls, has *in vitro* data from studies of PWS that a CpG island-containing region 3.7 kb upstream of the *MKRN3* TSS may be an enhancer of *MKRN3* (Robert Nicholls, unpublished data). The absence of differential methylation of the CpG islands at the proximal promoter led us to investigate this potential regulatory site of *MKRN3* for differentially methylated CpG islands.

We identified differentially methylated CpG islands at the locations identified through the GRCh38/hg38 reference sequence on the University of California Santa Clara Genomics Institute genome database within the analyzed region of the presumptive *MKRN3* enhancer; methylation analysis revealed that the majority of the CpG islands analyzed within the 522 bp region 3.7 kb upstream of the *MKRN3* TSS were found to be differentially methylated in two individuals. Unmethylated CpG islands at the beginning and the end of the region also coordinated between the two individuals. The visualization of the BAMs of the sequence data illustrate the differential methylation patterns apparent at the CpG islands of each subject well (Figure 12). Exact percentages at each island are listed in Table 3.

On average, the methylation percentage at each CpG island was higher in subject 2 than in subject 1 (Table 3). This is evident from the visibly more complete "columns" in the binary alignment map of subject 2: these pink boxes representing cytosine nucleotides that resisted bisulfite conversion to uracil nucleotides (Figure 12). This resistance indicates the methylated status of the carbon-5 position on the pyrimidine ring of these cytosine nucleotides.

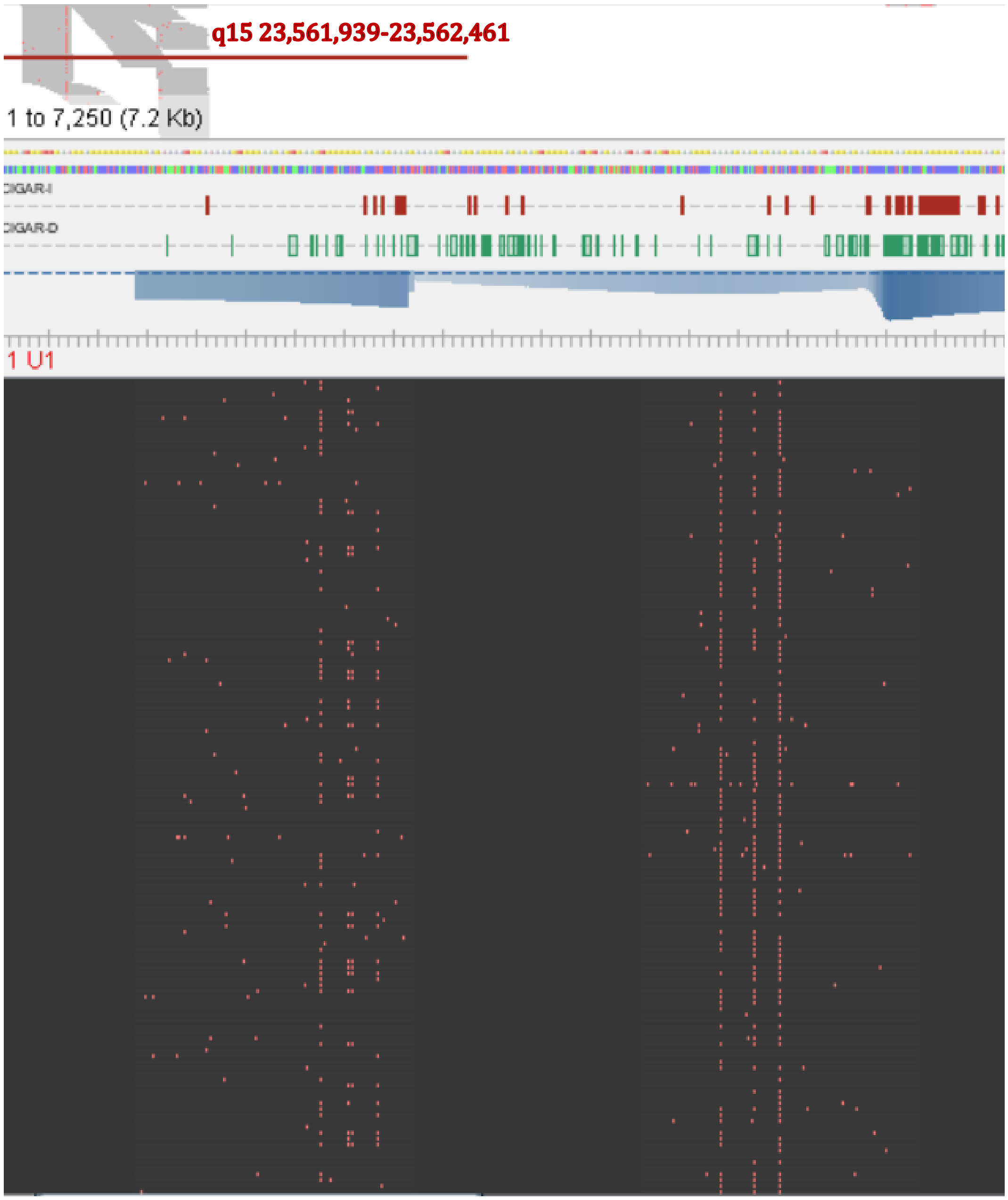
Overall, 23 CpG islands were analyzed and found to be localized to a 506 bp region positioned 3.7 kb upstream of the *MKRN3* TSS – 91% (21/23) of which were differentially methylated; the differentially methylated CpG islands were localized to a 393 bp region. As anticipated, the islands do not follow the 50% methylation pattern associated with classical reports of methylation imprints conferring monoallelic expression of genes (Figure 6; Table 3). However, the percent methylation of the CpG islands in the proposed enhancer of subject 2 were higher on average than that of subject 1. Locations of the islands relative to the *MKRN3* TSS are depicted in Figure 13.

**Table 3. Percent Methylation of CpG Islands within the Enhancer Region of *MKRN3*.** Percentage of cytosine nucleotides remaining at CpG dinucleotides in the proposed enhancer of MKRN3 after bisulfite treated Next Generation Sequencing in both normal puberty controls. Positions of CpG islands as depicted in Figure 13.

***Enhancer***

| *CpG Island* | Percent Methylation | | |
|---|---|---|---|
| | Subject 1 | Subject 2 | Average |
| *1* | 2.93% | 0.24% | 1.59% |
| *2* | 35.96% | 47.85% | 41.91% |
| *3* | 28.79% | 31.03% | 29.91% |
| *4* | 32.46% | 20.86% | 26.66% |
| *5* | 47.14% | 63.80% | 55.47% |
| *6* | 35.51% | 34.19% | 34.85% |
| *7* | 46.84% | 47.16% | 47.00% |
| *8* | 27.66% | 38.09% | 32.88% |
| *9* | 31.30% | 40.55% | 35.93% |
| *10* | 27.88% | 54.23% | 41.06% |
| *11* | 26.86% | 36.79% | 31.83% |
| *12* | 39.91% | 33.90% | 36.91% |
| *13* | 16.07% | 45.10% | 30.59% |
| *14* | 16.94% | 42.71% | 29.83% |
| *15* | 20.83% | 66.04% | 43.44% |
| *16* | 17.43% | 35.05% | 26.24% |
| *17* | 25.44% | 57.62% | 41.53% |
| *18* | 17.32% | 70.68% | 44.00% |
| *19* | 38.69% | 79.52% | 59.11% |
| *20* | 30.45% | 73.76% | 52.11% |
| *21* | 25.72% | 66.54% | 46.13% |
| *22* | 25.36% | 68.66% | 47.01% |
| *23* | 0.00% | 17.48% | 8.74% |

a.



b.

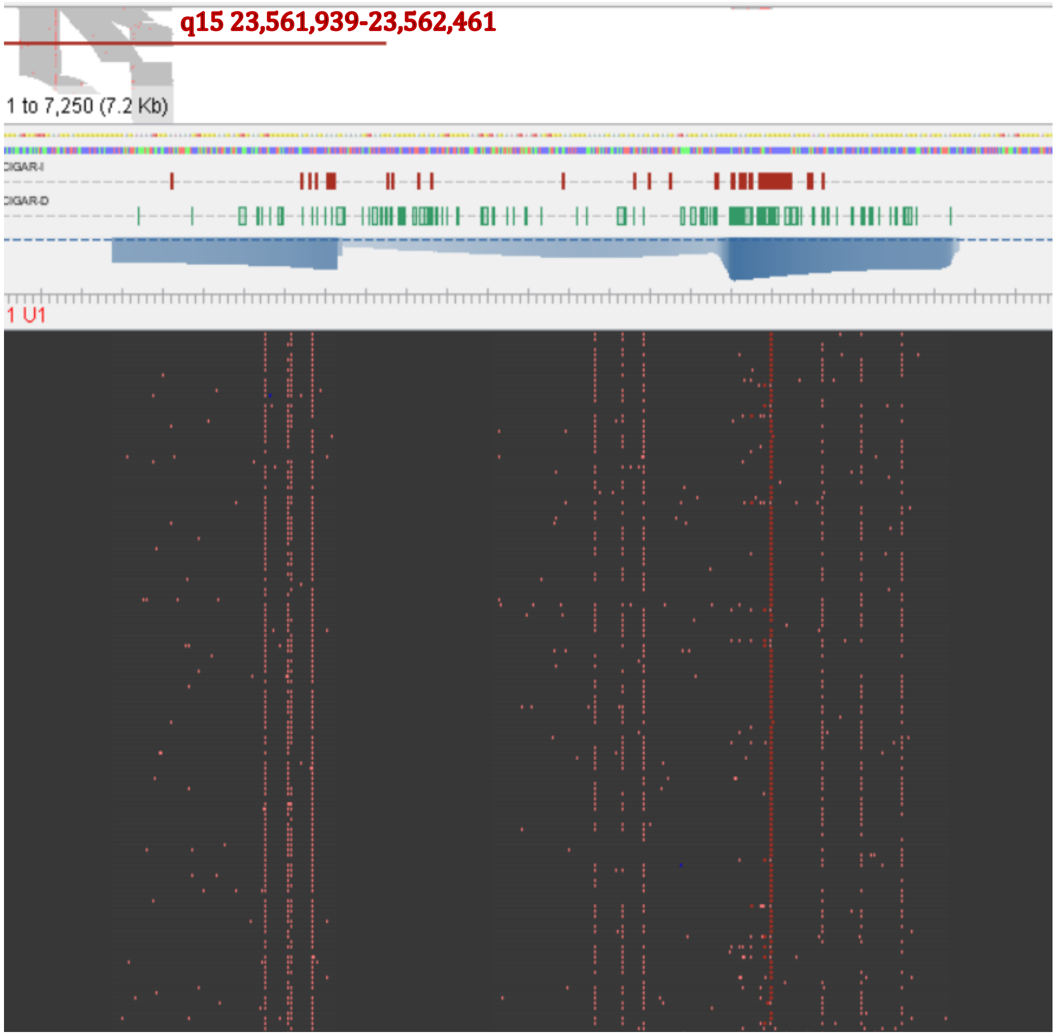



**Figure 12. Binary Alignment Map of *MKRN3* Enhancer Region.** Binary Alignment Map (BAM) of *MKRN3* proposed enhancer visualized on Tablet integrative genomic (The James Hutton Institute) (Milne et al., 2009) viewer. Pink boxes represent methylated cytosine nucleotides remaining at CpG islands in the *MKRN3* potential enhancer region after bisulfite conversion treatment. Bisulfite conversion treatment converts unmethylated cytosine nucleotides into uracil – which are subsequently replaced with thymine nucleotides during PCR amplification. The resulting pink "columns" as seen in the aligned sequences show differential methylation at the CpG islands throughout the reads. (a) BAM of subject 1's *MKRN3* enhancer region showing differential methylation at CpG islands with lower average percentage methylation at each site than subject 2. (b) BAM of subject 2's *MKRN3* enhancer region showing differential methylation at CpG islands with higher average percentage methylation across islands as compared to subject 1.

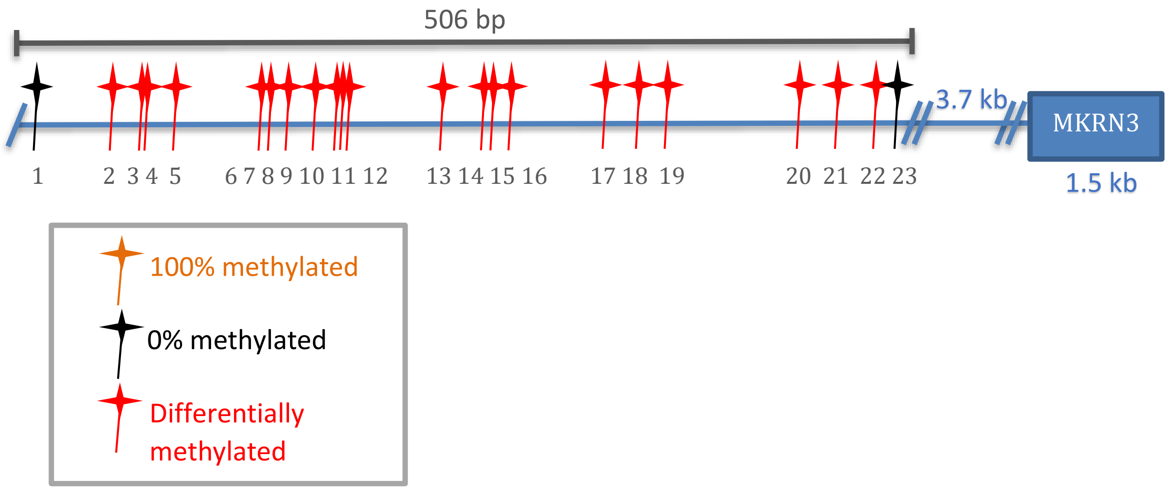



**Figure 13. Methylation Status of CpG Islands in the Enhancer Region of *MKRN3*.** Of the 522 bp area that we analyzed in the potential enhancer region of *MKRN3*, we identified islands within a 506 bp region 3.6 kb upstream of the *MKRN3* TSS. Except for the beginning and ending islands which were 0% methylated in the control samples we tested, all islands within the region were differentially methylated. The CpG islands are numbered coordinating with the methylation percentages in Table 3. Orange represents CpG Islands that are 100% methylated; black represents islands that are 0% methylated; red represents CpG Islands that are differentially methylated.

# DISCUSSION

Age at pubertal onset is associated with elevated risk for a variety of adverse, adult-onset, health outcomes, including, but not limited to, cancer, obesity, type II diabetes and cardiovascular disease (Kvale, 1992; Prentice & Viner, 2012; He et al., 2009; Lakshman et al., 2009). Imprinting has been deeply linked with the regulation of human development and reproduction (Plasschaert & Bartolomei, 2014; Mackay & Temple, 2017; Elhamamsy, 2017). Additionally, it has been postulated that there is a significant contribution of epigenetic regulation interweaved within the complex network controlling the reproductive axis (Lomniczi et al., 2015). Functional genomic imprinting and differential methylation of *MKRN3* has been demonstrated (Jong et al., 1999, 1; Jong et al., 1999, 2; Hershko et al., 1999). However, the exact mechanism underlying the imprinting of *MKRN3* has yet to be elucidated. A more thorough understanding of this process could provide greater context as to the ways in which epigenetic modifications and regulation impact and interact with the HPG axis.

In order to investigate the imprinting mechanism of *MKRN3*, we first sought to determine the location of the DMR directing *MKRN3* monoallelic expression and to characterize the methylation profile of the region. The cohort of this study was limited; while a differentially methylated region was identified, there was considerable variation between samples. These results should be interpreted with caution and necessitate further validation. The results from this study serve as preliminary data and will contribute toward control cohorts in future studies.

Many early studies identifying individual imprinted genes exhibit a mechanism including hypermethylation of CpG islands located on a parent-specific allelic promoter conferring repression of the corresponding parental allele (Ferguson-Smith et al., 1993; Stoger, 1993; Tremblay et al., 1995). In alignment with these classical models in which the DMR was situated at a CpG-rich promoter, we initially investigated the proximal promoter of *MKRN3* as a potential DMR in the regulation of its paternal monoallelic expression.

As demonstrated in these studies of other genes, we expected hypermethylation of the maternal MKRN3 proximal promoter juxtaposed with hypomethylation of the paternal promoter. Since next generation sequencing does not provide a means of differentiating between maternal and paternal sequence contributions, the clearest suggestion of this profile would be by 50% methylation of the islands. This 50/50 pattern would represent the equal distribution of sequences derived from a fully methylated maternal promoter and an unmethylated paternal promoter. The methylation would serve to inhibit the transcription of the maternal allele, yielding solely paternal expression.

Contrastingly, we found that the thirteen characterized CpG islands within this region were either fully methylated or entirely unmethylated (Figure 11). Such a methylation pattern correlates with identical methylation at the CpG islands within the promoter region on each parental allele; this is incompatible with the requirements of a site directing monoallelic expression.

DNA methylation has been established as a means of marking the parental origin of the chromosomes (Nicholls, 2000). In order for the transcriptional machinery to discriminate between the origins of the chromosomes, there must be distinctive imprints as to indicate the preferred parental allele (Ferguson-Smith, 2011). If DNA methylation is the imprint conferring repression on the maternal allele, the two chromosomes must then have separate, distinguishable methylation profiles. Thus, our findings do not support the CpG islands of the *MKRN3* proximal promoter as the CpG islands that comprise the DMR regulating the paternal expression of *MKRN3*.

From preliminary studies in PWS, a collaborator involved in the initial cloning and characterization of *MKRN3* identified a promising CpG islet positioned 3.7 kb upstream of the *MKRN3* transcription start site (Jong et al., 1999, 1; Jong et al., 1999, 2; Robert Nicholls, unpublished data). Through plasmid-based *in vitro* assays, he discovered a differentially methylated region which may regulate *MKRN3* monoallelic expression (Robert Nicholls, unpublished data).

Twenty-three CpG islands were identified within the region, twenty-one of which were differentially methylated at this site amongst the two subjects screened (Figure 13). Data from samples were in congruence that the CpG islands at the outermost portions of the region analyzed were unmethylated. However, the percent methylation of subject 2 was consistently higher than that of subject 1 (Table 3).

Classical literature has often described a 50% methylation pattern at each of the CpG islands populating a site regulating monoallelic expression. This methylation profile is suggestive of half of the chromosomes in the sample being (hyper)methylated at the CpG islets of the regulatory site – such as an enhancer, corresponding with the half of the genetic information inherited from the parental-specific, silenced line.  Given this, differential methylation on the basis of parental origin at the CpG islands of the enhancer could be inferred to influence transcription in a way that is preferential to one allele over the other – in a manner consistent with the dependence on the parent from which it originated. However, deviation from this traditional pattern does not exclude this prospective enhancer of *MKRN3* monoallelic expression from the differentially methylated label.

Differentially methylated regions can be stratified into two categories: somatic (secondary) and germline. Germline DMRs are set during gametogenesis and comprise the imprinting centers (IC) coordinating imprinting across clusters. Somatic or secondary DMRs – like that of *MKRN3* (Hershko et al., 1999)– are set following fertilization but before the fusion of the pronuclei (Ferguson-Smith, 2011). Maternally-imprinted, secondary DMRs can be tissue-specific (Clayton-Smith, 2003; Edwards & Ferguson-Smith, 2007; Vu & Hoffman, 1994).

In previous reports investigating *MKRN3* imprinting, *MKRN3* was described to be ubiquitously expressed throughout all tested tissues with highest expression in the testis; *MKRN3* methylation was also found to be tissue-specific (Figure 6) (Jong et al., 1999, 1; Jong et al., 1999, 2). Our ongoing mice studies also indicate maternally-imprinted, tissue-specific expression of *Mkrn3*, with particularly high expression in the hypothalamus (Ana Abreu, unpublished data). Additionally, our mice studies suggest that the methylation profile of peripheral tissues does not include 100% methylation of the maternal allele (Ana Abreu, unpublished data). Presumably, as *MKRN3* functions in the regulation of the HPG axis, such findings are as expected.

As a maternally-imprinted, secondary DMR regulating a tissue-specific gene, it is conceivable that the methylation profile of the *MKRN3* DMR may indeed be tissue-specific. If this is the case, the deviation from the classical 50% methylation of the CpG islands at the prospective enhancer may reflect the tissue-specific methylation profile of the *MKRN3* proposed enhancer in leukocytes. Furthermore, as whole blood is a mixed population, some of the variation reflected in the methylation percentages of the CpG islands within the potential enhancer of *MKRN3* amongst the subjects may be a function of the differences in methylation profiles of the many cells comprising each sample in combination with the lack of control for the differing percentages of specific cells in each sample.

While more data needs to be collected to confirm our results and properly characterize the normal methylation profile of the region, our findings are suggestive of a DMR enhancer located 3.7 kb upstream of the *MKRN3* TSS.

Considering the tissue-type we sampled and our data, we hypothesize that this is the DMR regulating the monoallelic expression of *MKRN3*.

In future studies, given the tissue-specific expression of *MKRN3*, we will cultivate wildtype and *MKRN3* knockout hypothalamic cells from induced pluripotent stem cells. The knockouts will be produced using CRISPR/Cas9 to delete CpG islands of the enhancer and promoter. This will provide a means to characterize the methylation profile of *MKRN3* in the tissue of which it carries out its main function. This will also give data regarding the effect of disruption to these regulatory sites and help to confirm the DMR. We will additionally use CRISPR/Cas9 to delete CpG islands in the enhancer and promoter of wildtype and $Mkrn3^{m-/p+}$ mice. *Mkrn3* will be measured to confirm the DMR *in vivo*.

Once we have validated our results and the DMR has been determined, we will expand the current study and screen more normal pubertal onset patients to establish a well-characterized baseline methylation profile. We will screen PWS patient DNA with paternal deletions including *MKRN3* as controls; without the contribution of the paternal allele, the regulatory sites are expected to be 100% methylated in these patients. We can then screen CPP and CDP patients for variants in the regulatory region as well as for methylation abnormalities.

These studies will help determine if and how the imprinting regulation of *MKRN3* may contribute to the development of pubertal disorders. Further studies will need to be performed to piece together the precise mechanism by which monoallelic expression of *MKRN3* is achieved and how this factors into the regulation of pubertal development; *MKRN3* is associated with non-coding RNAs (ncRNAs) and it is unclear exactly how all these elements might factor into the maternal imprinting mechanism acting on *MKRN3* (Jong et al., 1999, 1; Castellano et al., 2019). Elucidation of the mechanisms conferring monoallelic expression of *MKRN3* will provide insight into the complex network influencing pubertal onset and the epigenetic regulation of the HPG axis. More functional understanding of the intricacies of the reproductive axis has the potential to impact speed and efficacy in the development of treatments for pubertal disorders, genetic counseling and research techniques.

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**CURRICULUM VITAE**

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