Molecular and Clinical Delineation of Rare Disorders of Stature

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Abstract

There are more than 7000 described rare genetic disorders; however, the molecular basis underlying approximately half of these disorders is unknown, and the majority are currently untreatable. Stature and growth abnormalities are a common clinical feature of many rare disorders including: Floating-Harbor syndrome (FHS), a short stature syndrome characterized by delayed osseous maturation, language deficits, and unique dysmorphic facial features; Weaver syndrome, an overgrowth syndrome characterized by advanced osseous maturation, developmental delay, and macrocephaly; and Sotos syndrome with cutis laxa, an overgrowth syndrome with marked tissue laxity in addition to the typical Sotos characteristics of developmental delay, macrocephaly, and a unique facial gestalt. The genetic basis underlying these three rare stature conditions were unknown at the outset of this study. We utilized high-throughput exome sequencing approaches to investigate the molecular etiology of these rare disorders and identified truncating mutations in the final exon of *SRCAP* as the genetic cause underlying FHS, missense mutations in *EZH2* in Weaver syndrome, and novel mutations in the Sotos syndrome gene *NSD1* in Sotos syndrome with cutis laxa. Next, we investigated the spectrum of *SRCAP* mutations in FHS and established the clustering of truncating *SRCAP* mutations in the final exon as being highly suggestive of a non-haploinsufficiency mutational mechanism in FHS. Finally, global methylation array analysis identified a unique methylation ‗epi-signature' in FHS individuals, providing further insight into FHS disease mechanism and a diagnostic signature. These studies have delineated the molecular etiology of these three rare stature/growth disorders, furthered our understanding of the associated clinical spectrum, and provided biological insight into disease pathogenesis.

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List of Abbreviations

ATPase	Adenosine triphosphatase
ATRX	X-linked alpha thalassemia/mental retardation
CpG	Cytosine-guanine dinucleotide
CREB	cAMP response element-binding protein
CREBBP/CBP	CREB-binding protein
CSGE	Conformational sensitive gel electrophoresis
DNA	Deoxyribonucleic acid
DMRs	Differentially methylated regions
DNMT	DNA methyltransferase
dsDNA	Double-stranded DNA
EED	Embryonic ectoderm development protein
EVS	Exome variant server
EZH2	Enhancer of zeste homolog 2
FBN1	Fibrillin 1
FGFR	Fibroblast growth factor receptor
FHS	Floating-Harbor syndrome
FMR1 / FMRP	Fragile X mental retardation gene / Fragile X mental retardation protein
FXS	Fragile X syndrome
GH	Growth hormone
HELLS	Lymphoid specific helicase
HSA	Helicase-SANT-associated domain

IMAGe syndrome	Intrauterine growth retardations, metaphyseal dysplasias, adrenal hypoplasia congenital, and genital anomalies syndrome
iPSC	Induced pluripotent stem cell
MBD	Methyl-CpG-binding domain
MOPD	Majewski/microcephalic osteoplastic primordial dwarfism
MPD	Microcephalic primordial dwarfism
mRNA	Messenger ribonucleic acid
NSD1	Nuclear receptor-binding SET domain containing protein1
OFC	Occipito-frontal circumference
OMIM/MIM	Online/Mendelian Inheritance in Man
PCR	Polymerase chain reaction
PRC2	Polycomb repressive complex
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RTS	Rubinstein-Taybi syndrome
SANT	Switching-defective protein 3 [Swi3]), adaptor 2 [Ada2], nuclear receptor corepressor [N-CoR], transcription factor [TF]IIIB′)
SD	Standard deviation
SET	Su(var)3,9, enhancer of zeste, trithorax domain
SkD	Skeletal dysplasia
SNP	Single nucleotide polymorphism
SWI/SNF	Switch/sucrose nonfermentable
SRCAP	SNF2-related CREBBP activator protein

TGFβ	Transforming growth factor β
VPI	Velopharyngeal insufficiency
WES	Whole exome sequencing
WGS	Whole genome sequencing

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Preface

Research ethics approval was obtained in advance of the research work undertaken to complete this thesis. All research ethics approvals for the various published manuscripts are stated in their respective chapters. A copy of the formal letters of approval can be found in Appendix 1.

Chapter 1: General Introduction

Rare Genetic Disorders

Approximately one in 12, or three million, Canadians are affected by a rare disorder (www.raredisorders.ca). The Online Mendelian Inheritance in Man database (OMIM) estimates that there are more than 7000 single-gene disorders (McKusick, 2007), which while individually rare, are collectively frequent. This subset of disorders, which typically result from mutations having severe effects on gene function, are of particular importance in pediatrics, as a significant fraction of pediatric hospital admissions involve genetic conditions (McCandless et al., 2004; Scriver et al., 1973; Yoon et al., 1997). Most recently data from Western Australia demonstrated the marked disparity between the number of individuals in the population with a rare disorder and their impact on combined health-care costs (Walker et al., 2016). While the rare disease cohort in this study constituted only approximately 2% of the state population, on average they had a higher length of hospital stay and a higher number of hospital discharges than the general population, and contributed up to 10.5% of the state in-patient hospital costs. These findings emphasize the need for rare disease research and the development of strategies aimed at improving the health of individuals living with rare conditions.

At the onset of this thesis the causative genes underlying more than half of described rare genetic disorders remained unknown (McKusick, 2007). Moreover, the majority of rare disorders are currently untreatable and, as such, they represent one of the great unmet medical challenges of the 21st century. Therefore, the identification of the genetic variant(s) underlying these disorders, followed by downstream functional characterization of disease

mechanism, will make it possible to both accurately diagnose and manage children and, for a subset, provide insight which might lead to the development of a therapeutic strategy.

Three rare disorders are examined in this thesis that share clinical features of abnormal stature and growth: Floating-Harbor syndrome (short stature); Weaver syndrome (overgrowth); and, Sotos syndrome (overgrowth) with concomitant insights into molecular mechanism, syndrome delineation, and disease mechanism.

Skeletal Development and Endochondral Ossification

Skeletal development is a complex and fluid process that continues into early adulthood. Skeletal bones are formed via intramembranous or endochondral ossification however, both initiate with condensation of mesenchymal cells. Intramembranous ossification, which is responsible for the generation of the cranium, parts of the clavicle and the pubic bone initiates with the condensation of neural crest-derived mesenchymal cells, which directly transdifferentiate into osteoblasts (reviewed in (Geister and Camper, 2015; Karsenty et al., 2009)). Conversely, endochondral ossification, which is responsible for the generation of the axial and appendicular skeleton, and thus contribute to stature, originates with the condensation of paraxial and lateral mesoderm respectively, which differentiate into intermediary chondrocytes (reviewed in (Geister and Camper, 2015; Hojo et al., 2010)). As the chondrocytes undergo hypertrophic maturation into bone tissue, the cartilage template matures and mineralizes and undifferentiated chondrocytes become sealed within the epiphyseal/growth plate of the developing bone (Geister and Camper, 2015). The growth plate is made up of three distinct zones: resting, proliferative, and hypertrophic, followed by

an ossification region. In general, undifferentiated chondrocyte cells residing in the resting zone begin dividing longitudinally in the proliferative zone until cell signals trigger their differentiation into hypertrophic chondrocytes, resulting in a large cell volume. Recent studies have suggested that the final addition of bone matrix is generated by the terminal transdifferentiation of hypertrophic chondrocytes into osteoblasts (Yang et al., 2014; Zhou et al., 2014). The transition of chondrocytes through the growth plate is a dynamic process regulated by a number of cell signalling pathways including: Indian Hedgehog (IHH), fibroblast growth factor (FGF), bone morphogenetic protein (BMP), Notch, WNT, parathyroid hormone-related peptide (PTHrP), transforming growth factor β (TGFβ), C-type natriuretic peptide (CNP) (reviewed in (Geister and Camper, 2015)). The number of signalling pathways and molecules involved in bone development emphasize how complex this process is, and how genetic mutations impacting even one of these pathways can profoundly impact organization and function at the growth plate and result in stature aberrations.

Rare Disorders Affecting Stature and Growth

Abnormalities in stature are a frequent clinical feature of many rare genetic disorders and can involve either short or tall stature. A patient is considered to be short statured when their height is more than two standard deviations below the mean for their age and sex (Cheetham and Davies, 2014). A patient is considered tall when their height is more than two standard deviations above the mean for their age and sex (Davies and Cheetham, 2014). Tall stature can also be associated with a large head circumference and body weight; when these clinical phenotypes occur together they are collectively referred to as overgrowth.

Disorders with Short Stature

Short stature disorders are relatively common and include hundreds of genetic disorders in OMIM. For example, microcephalic primordial dwarfism (MPD) disorders are autosomal recessive conditions characterized by microcephaly and severe pre- and post-natal growth retardation (Khetarpal et al., 2016). Four subtypes, Seckel syndrome, Majewski/microcephalic osteodyplastic primordial dwarfism (MOPD) types I/III, MOPD type II and Meier-Gorlin syndrome have been characterized and are shown to be the result of genetic mutations in: *ATR*, *ATRIP*, *CENJP*, *CEP152*, and *RBBP8*; *U4ATAC*; *PCNT*, *IGF1R*; and *ORC1*, *ORC4*, *ORC6*, and *CDC6* respectively (reviewed in (Khetarpal et al., 2016). Several of these genes function in multiple stages of the cell cycle, while others are involved in cell cycle progression. *U4ATAC*, however, encodes a component of the minor spliceosome complex, and mutations in this gene are anticipated to impact splicing for a number of genes including some involved in cell cycle. As such, MPD-causing mutations can result in the disruption of multiple functional pathways involved in cell cycle regulation and cell division including: DNA replication, centrosome duplication and/or maturation, DNA repair, and mitotic spindle formation (reviewed in (Geister and Camper, 2015; Khetarpal et al., 2016)); highlighting the broad range of biological processes that can be dysregulated yet lead to the same recognizable short stature syndrome.

Another example, skeletal dysplasias (SkD), constitute a large group of individually rare cartilage and bone disorders which typically cause disproportionate short stature, but also some instances of overgrowth. More than 400 cases of skeletal dysplasia have been reported in the literature, with the genetic basis of less than 60 having been described in the 2011 report of the Nosology and Classification of Genetic Skeletal Disorders (reviewed in (Geister

and Camper, 2015; Warman et al., 2011). Mutations in many of the causative genes have been shown to disrupt the organization and function at various zones of the growth plate, thus affecting growth. There are many forms of SkDs and can range from mild anomalies to life-limiting conditions. One example is the most common nonlethal form of SkD, achondroplasia, a disproportionate short-limb dwarfism, caused by autosomal dominant mutations in the fibroblast growth factor receptor, *FGFR3* (Shiang et al., 1994). FGFR3 is expressed in the resting cartilage of the growth plate and functions as a negative regulator of chondrocyte proliferation and differentiation; *FGFR3* mutations in achondroplasia lead to constitutive FGFR3 expression thus resulting in growth inhibition (Deng et al., 1996; Horton, 2006). Another example is IMAGe (intrauterine growth retardations, metaphyseal dysplasia, adrenal hypoplasia congenital, and genital anomalies) syndrome, a multisystem growth restricted condition caused by autosomal dominant (maternally imprinted) mutations in the cyclin dependent kinase inhibitor gene *CDKN1C*, a negative regulator of cell proliferation (Lee et al., 1995). CDKN1C has been shown to function as a negative regulator of cell proliferation and it is thought to be involved in the transition from proliferation to hypertrophic differentiation at the growth plate (Hirata et al., 2009). It is anticipated that IMAGe causing mutations of CDKN1C likely result in premature inhibition of chondrocyte differentiation thus impeding growth.

Disorders with Tall Stature and/or Overgrowth

Similarly, tall stature and overgrowth can be caused by dysregulation of a wide variety of biological processes. For example, Marfan syndrome is an autosomal dominant condition characterized by tall stature, long limbs, and connective tissue abnormalities including serious

aortic and mitral valve heart complications shown to be caused by mutations in fibrillin 1 (*FBN1*) (Dietz et al., 1991). Fibrillins combine to form microfibril macromolecules, which provide extracellular matrix support thereby contributing to the integrity and function of elastic and non-elastic connective tissues (Sakai et al., 2016; Tsang et al., 2013). These microfibrils also store transforming growth factor β (TGFβ), which is involved in cell growth and division (von Kodolitsch and Robinson, 2007). Therefore, by regulating the availability of TGFβ these microfibrils function as a mediator of TGFβ signalling (von Kodolitsch and Robinson, 2007). A deficiency of fibrillin, such as *FBN1* mutations in Marfan syndrome, has been shown to result in an excess of TGFβ, leading to enlargement and cardiac valve disease in mice (Neptune et al., 2003; Ng et al., 2004). While there is currently no cure for Marfan syndrome, early diagnosis by confirmation of *FBN1* mutation, followed by careful clinical management including: prescribing beta-receptor blockers, regular echocardiograms (every 1-2 years), and prophylactic surgery in patients with an aortic root diameter >50 mm, dramatically improve the survival rates and quality of life of these patients (Pepe et al., 2016).

Fragile X syndrome (FXS), is an X-linked condition characterized by intellectual disability, distinct facial features, which often presents with overgrowth during childhood (Cohen, 2003; de Vries et al., 1995). FXS is most often caused by >200 trinucleotide CGG repeats in the 5‘ untranslated region of the fragile X mental retardation gene (*FMR1*), which encodes fragile X mental retardation protein (FMRP) (Kremer et al., 1991; Mor-Shaked and Eiges, 2016) and results in the loss of FMRP, an RNA binding protein, which regulates translation of many mRNAs involved in synaptic plasticity (Darnell and Richter, 2012). The large CGG trinucleotide repeat expansion results in aberrant hypermethylation of this region, which leads

to the epigenetic silencing of *FMR1* transcription (as reviewed in (Mor-Shaked and Eiges, 2016).

A final example is Proteus syndrome, an asymmetric and disproportionate overgrowth condition caused by a specific (c.49G>A; p.Glu17Lys) autosomal dominant (somatic mosaic) mutations in the protein kinase encoding gene *AKT1*, resulting in overactivation of the P13K-AKT signalling pathway (Lindhurst et al., 2011). While there is some debate about the role of the P13K-AKT signalling pathway in bone development, the large majority of the literature agrees that this pathway is required to promote hypertrophic cell differentiation and endochondral ossification (Beier and Loeser, 2010). Therefore, overactivation of this pathway by mutations of AKT1 in Proteus syndrome may therefore lead to the disproportionately accelerated bone growth evident in this condition.

What each of these examples of short and tall/overgrowth syndromes highlights is the complexity of the biological pathways impacted that result in the same or similar clinical feature in these rare disorders.

Understanding of the Rare Stature Disorders in this Thesis at the Outset of the Study

Floating-Harbor Syndrome

Floating-Harbor syndrome (FHS) [MIM 136140] is a rare genetic disorder characterized by short stature, delayed osseous maturation, and language deficit (Leisti et al., 1975; Pelletier, 1973; Robinson et al., 1988; White et al., 2010). There is a unique facial dysmorphology associated with this disorder where FHS patients tend to have a triangular shaped face,

prominent nose: long with a broad base, full tip and low-hanging columnella, short philtrum, and a wide flat mouth with a thin upper lip (Leisti et al., 1975; Pelletier, 1973; Robinson et al., 1988; White et al., 2010) (Chapter 2, Figure 1). FHS patients typically exhibit receptive and expressive language deficits and some level of learning or intellectual disability. The name ‗Floating-Harbor' is a portmanteau of the locations where the first two patients were described: the Boston Floating Hospital (Pelletier, 1973) and the Harbor General Hospital in Torrence, California (Leisti et al., 1975). Typically FHS occurs sporadically in families with an affected child being born to unaffected parents; however a few parent-child transmissions have been reported which is consistent with FHS being an autosomal dominant disorder (Arpin et al., 2012; Lacombe et al., 1995; Penaloza et al., 2003; Robinson et al., 1988). Despite the initial cases of FHS being described in the early 70's, the genetic basis of this disorder was unknown at the outset of this study.

Weaver Syndrome

Weaver syndrome [MIM 277500], which was first described in 1974 by Weaver *et al.*, is characterized by overgrowth, advanced osseous maturation, developmental delay, and macrocephaly (Cole et al., 1992; Weaver et al., 1974). Weaver syndrome displays a high degree of phenotypic overlap with another overgrowth condition, Sotos syndrome; however it is occurs less frequently than Sotos syndrome, with approximately 70 cases having been reported in the literature (Coulter et al., 2008; Huffman et al., 2001; Weaver et al., 1974). The facial appearance of Weaver and Sotos syndrome patients are subtly different, enabling experienced clinical dysmorphologists to distinguish the two conditions. The unique facial features of Weaver syndrome include a broad forehead, widely spaced eyes, large and low set

ears, and a prominent chin crease (Chapter 3, Figure 1) (Weaver et al., 1974). There are a variety of other clinical features that Weaver individuals may have including: intellectual disability, speech delay, behavioural problems, campodactyly, and epilepsy (Cole et al., 1992; Weaver et al., 1974). The vast majority of reported cases of Weaver syndrome have occurred sporadically; however some instances of parent-child transmission have been reported, which is consistent with an autosomal dominant mode of inheritance (Fryer et al., 1997; Proud et al., 1998). Despite the initial cases of Weaver syndrome being described in the early 70's, the genetic basis of this disorder was unknown at the outset of this study.

Sotos Syndrome

Sotos syndrome [MIM 117550], which was first described in 1964 (Sotos et al., 1964), is considered to be a well characterized and „relatively' common overgrowth syndrome, with >400 reported cases in the literature (Baujat and Cormier-Daire, 2007; Tatton-Brown et al., 2009) The key features of Sotos syndrome include pre- and postnatal overgrowth, developmental delay, macrocephaly and a unique facial gestalt (which includes a prominent forehead with a high hairline, pointed chin, down-slanting palpebral fissures, and facial flushing) (Cole and Hughes, 1990, 1994; Douglas et al., 2003; Rio et al., 2003; Sotos et al., 1964). In addition to these cardinal features, patients with Sotos syndrome may also have a variety of other symptoms including advanced bone age, cardiac anomalies, scoliosis, hypothyroidism, cataracts, conductive hearing loss, nystagmus, and umbilical hernia etc. (Tatton-Brown et al., 2005). In 2002, Kurotaki et al. identified heterozygous mutations of the nuclear receptor-binding SET domain containing protein 1 (*NSD1*) gene, resulting in haploinsufficiency, as the genetic cause of Sotos syndrome (Kurotaki et al., 2002). Since this

initial report, hundreds of *NSD1* mutation positive Sotos syndrome patients, with genetic alterations including whole-gene deletions, partial-gene deletions, frameshift, nonsense, splice site and missense mutations, have been reported in the literature (Douglas et al., 2003; Tatton-Brown et al., 2005; Turkmen et al., 2003). NSD1 encodes a histone methyltransferase, which has been shown to be the catalytic force behind methylation of lysine residue 36 of histone 3 (H3K36) and lysine residue 20 of histone 4 (H4K20) (Huang et al., 1998; Qiao et al., 2011; Rayasam et al., 2003).

Sotos-like Syndrome with Cutis Laxa

In 1999, Robertson and Bankier reported three patients as having a Sotos-like phenotype with marked cutis laxa, joint hyperextensibility, vesicoureteric reflux, and aortic dilatation: ‗Sotos syndrome with cutis laxa' (Chapter 4, Figure 1A) (Robertson and Bankier, 1999). Despite mild connective tissue dysfunction being described in >15% of Sotos syndrome cases in a review of 266 Sotos individuals with pathogenic *NSD1* mutations (Tatton-Brown et al., 2005), the pronounced nature of connective tissue dysfunction in these Sotos-like with cutis laxa patients suggested a more profound phenotype that what has been previously described. The authors therefore speculated that a second gene might be responsible for the disease in this subset of patients. The genetic basis of Sotos-like syndrome with cutis laxa was unknown at the outset of this study.

The Impact of High-Throughput Sequencing

Prior to the development of high-throughput sequencing technologies, studies utilized genetic linkage information and positional cloning strategies to identify the candidate genes

underlying rare genetic disorders. These approaches were work intensive and required robust familial information: preferably several multi-generational families with many affected individuals. As such, most of these studies took many years to complete; however, they led to the identification of genetic causes for many of the more ‗common' disorders including the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene as the cause underlying Cystic Fibrosis (CF) (Rommens et al., 1989), and expanded CAG trinucleotide repeats in the huntingtin (*HTT*) gene as the cause underlying Huntington's disease (1993). Studies of genetic disorders with increasing rarity have typically been more difficult to solve, especially in cases where researchers are studying families with few affected family members or instances where there is only a single affected individual (sporadic occurrences of disease) (Boycott et al., 2013).

The emergence of high-throughput sequencing strategies has revolutionized modern genetic studies by enabling researchers to obtain DNA sequence information and elucidate novel biological interactions at an unprecedented rate. These sequencing technologies have dramatically increased the success rate of identifying causative genetic variant(s) underlying rare genetic disorders (Boycott et al., 2013). Whole genome sequencing is an unbiased high-throughput sequencing approach which enables researchers to examine variations throughout the entire coding and non-coding regions of the genome. This sequencing strategy provides users with a uniform level of sequence coverage, but is quite cost- and data-intensive. Conversely, whole exome sequencing is a high-throughput sequencing strategy which involves the selective capture and sequencing of only protein-coding regions of the genome (Choi et al., 2009; Ng et al., 2010b). This approach provides users with a high-depth of sequence coverage for the majority of protein coding genes; constituting approximately 1%

(30 Mb) of the human genome. As it is anticipated that more than 85% of disease-causing mutations are contained within protein-coding regions of the genome, exome sequencing is a cost-, data-, and time-efficient alternative to whole-genome sequencing for the identification of causative genetic variants. (Choi et al., 2009; Teer and Mullikin, 2010). The success of exome sequencing since the emergence of high-throughput sequencing technologies is apparent in Figure 1. Following the identification of novel genetic variants, functional approaches can be used to better understand impact of the mutation on biological processes (e.g. methylation) and, ultimately, provide insight into disease mechanism.

DNA Methylation Abnormalities in Disorders of Stature

DNA methylation involves the 5‘ covalent addition of a methyl group (-CH3) to the cytosine nucleotide of a cytosine-guanine dinucleotide (CpG) in a DNA sequence by a DNA methyltransferase (DNMT) enzyme (Mazzio and Soliman, 2012). It is a form of epigenetic modification, as the functional alteration occurs without changing the DNA sequence itself. CpG methylation is spread unevenly across the genome: with the majority of individual CpGs being methylated (~60-80%) (Jones and Takai, 2001), and regions with dense CpGs clustering together in high-frequency stretches called ‗CpG islands‘, most often being unmethylated (Deaton and Bird, 2011). CpG islands are associated with the promoter/regulatory regions of genes, and it is anticipated that approximately 1/2 to 2/3 of mammalian genes have a CpG island in their promoter (Hernando-Herraez et al., 2015). The methylation status of CpG islands within promoter regions can dramatically impact gene expression: in an unmethylated state they are in a transcriptionally-permissive or ‗open‘ state, whereas methylated promoter regions tend to render their corresponding genes

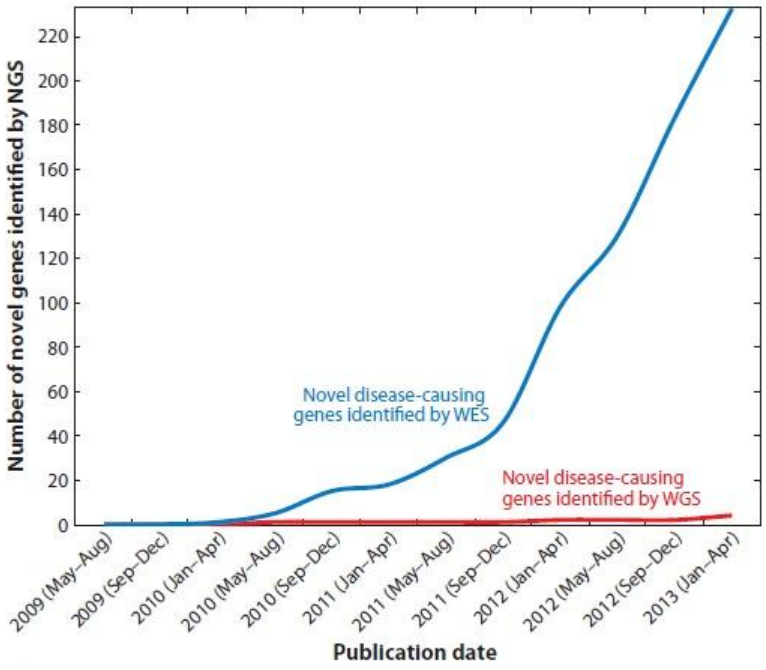

Figure 1. Cumulative number of novel gene discoveries made by WES and WGS since the emergence of high-throughput sequencing technologies between 2010 and 2013.
Abbreviations: WES: whole exome sequencing; WGS: whole genome sequencing.
Figure reprinted with permission from Annual Reviews (see Rights and Permissions; Chapter 1, Figure 1) (Boycott et al., 2013).

transcriptionally inactive (a ‗closed' state) (Inbar-Feigenberg et al., 2013). This transcriptional inactivity can occur by one of two mechanisms. First, the attached methyl groups may themselves physically impede transcriptional activators from binding to the promoter region (Mazzio and Soliman, 2012). Second, proteins which contain a methyl-CpG binding domain (MBD) symmetrically bind methylated CpGs, and recruit histone deacetylase complexes (HDAC) and additional chromatin remodeling proteins, which results in the formation of compact, inactive chromatin and thus transcriptional repression (Roloff et al., 2003).

The link between abnormal methylation in cancer and tumour progression has been extensively studied (Baylin et al., 1998; Jones and Takai, 2001); however, far less is known about how methylation is impacted in rare genetic disorders. It is a logical assumption that mutations in genes involved in methylation regulatory pathways will impact global methylation status. For example, mutations in *DNMT3b* which causes the rare disease immunodeficiency, centromeric instability, facial anomalies syndrome (ICF) (Xu et al., 1999), and mutations in *MECP2*, a MBD protein which causes Rett syndrome (Amir et al., 1999), are both believed to be caused by alterations in the methylation machinery (Amir et al., 1999; Okano et al., 1999). Similarly, mutations in two chromatin remodeling proteins, lymphoid specific helicase (*HELLS*) and X-linked alpha thalassemia/mental retardation (*ATRX*), have also been shown to impact methylation status (Dennis et al., 2001; Gibbons et al., 2000; Zhu et al., 2006). Additionally, as mentioned previously, CGG tri-nucleotide repeat expansion alterations in the 5'UTR of *FMR1*, in Fragile X syndrome (FXS), has been shown to result in aberrant hypermethylation of the *FMR1* promoter, and transcriptional repression of the gene (Mor-Shaked and Eiges, 2016).

As described earlier, a characteristics of FXS is childhood overgrowth (Cohen, 2003; de Vries et al., 1995). Interestingly, two of the other disorders associated with altered methylation described above, ATRX and Rett syndromes, have also been associated with growth aberrations. Multiple studies have identified growth failure as a common characteristic of Rett syndrome, demonstrating that by 7-8 years affected children have an average height of approximately two standard deviations below the mean (Hagberg et al., 2001; Oddy et al., 2007). Additionally, normal pubertal height increases appear to be absent in individuals with Rett syndrome (Tarquinio et al., 2012). Similarly, short stature is a relatively common feature present in individuals with ATRX syndrome (Basehore et al., 2015). While not all ATRX patients have short stature, it has been a notable feature in a number of reported cases of ATRX. In fact, the original report which identified mutations in *ATRX* as the genetic cause underlying this syndrome reported short stature in half of the patient cohort (Gibbons et al., 1995). Based on this information, it is apparent that some rare disorders with stature abnormalities are associated with methylation alterations.

The recent cost-effective development of high resolution methylation array technologies, such as the Infinium HumanMethylation450 Beadchip methylation array, have now made it possible to examine how other monogenic conditions impact methylation status. Recently, a unique methylation ‗epi-signature' was reported for the overgrowth condition, Sotos syndrome (Choufani et al., 2015). This study was able to distinguish between individuals with Sotos syndrome, *NSD1* mutation positive, and those with the clinically similar Weaver overgrowth syndrome (Choufani et al., 2015). It may therefore be possible that mutations causing other rare stature disorders will also have an impact on global methylation status.

Thesis Rationale

High-throughput exome sequencing has emerged as a disruptive tool for investigating the genetic etiology of rare disorders, which were intractable with previous approaches. Genetic insight into rare genetic disorders involving stature provides an opportunity to delineate the phenotypic spectrum of these conditions and begin to understand the complex biological mechanism involved in disease pathogenesis.

Hypothesis and Specific Aims

My hypothesis is that high-throughput exome sequencing can be used to facilitate our understanding of the molecular etiology, clinical spectrum, and disease biology associated with rare disorders impacting stature. To test my hypothesis, I set out to accomplish the following Specific Aims:

Aim 1: Identify the genetic cause underlying these rare stature disorders

a) Floating-Harbor Syndrome (FHS)

We set out to use a high-throughput exome sequencing approach to identify the causative gene and mutation(s) underlying the short stature syndrome, FHS. To do so, we assembled an international patient cohort and employed a multi-proband exome sequencing strategy using DNA from five unrelated ‗classic' FHS patients. This strategy was aimed at first identifying rare variant(s) shared amongst the patients which may account for the disorder. Next, we would validate our findings by identifying mutations in the same gene in a second FHS cohort, and confirm that these mutations were *de novo* by screening parental DNA when possible. This analysis is presented in Chapter 2.

b) Weaver Syndrome

We set out to use a high-throughput exome sequencing approach to identify the causative gene and mutation(s) underlying the overgrowth condition Weaver syndrome. To do so, we employed a trio-sequencing strategy, whereby we sequenced DNA from three Weaver patients and their parents. This strategy was aimed at first identifying the rare variant(s) in each Weaver patient that were not in their parents (*de novo*) and then comparing these *de novo* variants among the exome data from all the Weaver patients. Finally, we would validate these findings by Sanger sequencing additional Weaver patients to identify additional mutations. This analysis is presented in Chapter 3.

c) Sotos-like Syndrome with Cutis Laxa

We set out to use a high-throughput exome sequencing approach to determine the causative gene and mutation(s) underlying Sotos-like syndrome with cutis laxa. The currently accepted phenotypic spectrum of Sotos syndrome symptoms does not include cutis laxa and sequencing of one of the probands from the initial publication by Robertson and Bankier (1999) for changes in *NSD1* was negative, suggesting a novel gene might be identified. To examine this, we exome sequenced DNA from this patient and their parents, to identify candidate *de novo* variants. Next, we would validate identified variants by Sanger sequencing and perform experiments to examine how candidate variants may impact gene function. These findings would be further validated by the study of three additional Sotos-like with cutis laxa patients (including the two other patients originally described by Robertson and Bankier (1999)) to determine if they have variants in the same gene. This analysis is presented in Chapter 4.

Aim 2: Establish the FHS mutation spectrum

The initial gene identification manuscript (Chapter 2) included 13 mutation-positive FHS patients. However, despite FHS being a rare disorder, approximately 50 clinical cases had been reported in the literature (Arpin et al., 2012; Lacombe et al., 1995; Penaloza et al., 2003; White et al., 2010). We gathered additional patients world-wide and I focussed on the mutational spectrum while my clinical collaborators defined the phenotypic characteristics of the syndrome. This analysis is presented in Chapter 5. The intention was to use this information to facilitate a working hypothesis based on how these mutations might contribute to the FHS phenotype. In addition, as this disorder is quite difficult to diagnose, a more in-depth examination into the phenotypic features of FHS would improve the diagnostic criteria for this disorder; ie. determine which symptoms are common to all patients and mandatory for diagnosis, versus those symptoms that occur in some patients, or are not a part of the FHS clinical presentation at all.

Aim 3: Determine if mutations in FHS impact global methylation status

The final aim of this study was to use high resolution methylation array approach to examine whether FHS-causing mutations impact global methylation status. To do so, we assembled a FHS patient cohort (18 individuals) for methylation analysis and methylation changes were compared across this group to determine a common methylation profile. Next, we would perform subsequent clonal bisulfite sequencing of four target regions to confirm methylation array results. This analysis is presented in Chapter 6.

In summary, three syndromes that impact growth and stature are examined in this thesis to gain insight into this subset of rare diseases. The genetic basis of FHS (novel gene, *SRCAP*, Chapter 2), Weaver syndrome (novel gene, *EZH2*, Chapter 3), and Sotos-like syndrome with cutis laxa (novel mutations, *NSD1*, Chapter 4) were identified. The phenotypic spectrum of Sotos syndrome was expanded (Chapter 4), and the clinical spectrum of molecularly confirmed FHS was defined (Chapter 5). Finally, the abnormal methylation profile of FHS with concomitant insight into disease mechanism was elucidated (Chapter 6).

Chapter 2: Mutations in *SRCAP*, Encoding SNF2-Related CREBBP Activator Protein, Cause Floating-Harbor Syndrome

Preface

The following chapter consists of data previously published in the American Journal of Human Genetics under the title "Mutations in SRCAP, Encoding SNF2-Related CREBBP Activator Protein, Cause Floating-Harbor Syndrome" (PMID: 22265015) by Hood RL, Lines MA, Nikkel SM, Schwartzentruber J, Beaulieu C, Nowaczyk MJ, Allanson J, Kim CA, Wieczorek D, Moilanen JS, Lacombe D, Gillessen-Kaesbach G, Whiteford ML, Quaio CR, Gomy I, Bertola DR, Albrecht B, Platzer K, McGillivray G, Zou R, McLeod DR, Chudley AE, Chodirker BN, Marcadier J; FORGE Canada Consortium., Majewski J, Bulman DE, White SM, Boycott KM.

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Along with Dr. Sarah Nikkel, we determined the initial cohort to be sent for massively parallel (Illumina) sequencing. After the sequence was aligned and rare variants were computationally flagged by our McGill collaborators, I analyzed the variant files for all of the patients. I also performed all of the PCRs and Sanger sequencing validation of these variants to confirm the mutations identified in *SRCAP*. I performed all subsequent PCR and Sanger sequencing reactions in a secondary FHS cohort and analyzed all Sanger output files. I wrote the manuscript and generated figures. I responded to reviewers comments.

The specific contributions of each author to this paper are listed below.

Hood RL: (see above)

Lines MA: Co-wrote the manuscript. Generated figures for the manuscript. Responded to reviewers comments.

Nikkel SM: Contributed to initial cohort to be sent for Illumina sequencing. Compared patient phenotypes and generated the patient symptom table in the manuscript.

Schwarzentruber J: Performed bioinformatics analysis of the Illumina sequencing data. Wrote bioinformatics analysis methods section of the manuscript. Generated bioinformatics table for the manuscript.

Beaulieu C: Project Manager of the FORGE Canada Consortium.

Nowaczyk MJ, Allanson J, Kim CA, Wieczorek D, Moilanen JS, Lacombe D, Gillessen-Kaesbach G, Whiteford ML, Quaio CR, Gomy I, Bertola DR, Albrecht B, Platzer K, McGillivray G, McLeod DR, Chudley AE, Chodirker BN: Collected and sent patient samples for sequencing analysis.

Zou R: Performed the Sanger sequencing reactions.

Marcadier J: Clinical Coordinator of the FORGE Canada Consortium

FORGE Canada Consortium: Provided funding for this project.

Majewski J: Oversaw bioinformatics analysis of the Illumina sequencing data.

Bulman DE: Contributed to initial cohort to be sent for Illumina sequencing. Oversaw Sanger sequencing validation experiments. Contributed to writing/editing the manuscript. Responded to reviewers comments.

White SM: Contributed to initial cohort to be sent for Illumina sequencing. Collected and sent patient samples for sequencing analysis.

Boycott KM: Oversaw the project. Contributed to initial cohort to be sent for Illumina sequencing. Contributed to writing/editing the manuscript. Oversaw the response to reviewers comments.

Abstract

Floating-Harbor syndrome (FHS) is a rare condition characterized by short stature, delayed osseous maturation, expressive-language deficits, and a distinctive facial appearance. Occurrence is generally sporadic, although parent-to-child transmission has been reported on occasion. Employing whole-exome sequencing, we identified heterozygous truncating mutations in *SRCAP* in five unrelated individuals with sporadic FHS. Sanger sequencing identified mutations in *SRCAP* in eight more affected persons. Mutations were de novo in all six instances in which parental DNA was available. SRCAP is an SNF2-related chromatin-remodeling factor that serves as a coactivator for CREB-binding protein (CREBBP, better known as CBP, the major cause of Rubinstein-Taybi syndrome [RTS]). Five *SRCAP* mutations, two of which are recurrent, were identified; all are tightly clustered within a small (111 codon) region of the final exon. These mutations are predicted to abolish three C-terminal AT-hook DNA-binding motifs while leaving the CBP-binding and ATPase domains intact. Our findings show that *SRCAP* mutations are the major cause of FHS and offer an explanation for the clinical overlap between FHS and RTS.

Main Text

Floating-Harbor syndrome (FHS [MIM 136140]) is a rare condition characterized by short stature, delayed osseous maturation, language deficits, and a distinctive facial appearance. The dysmorphic features typical of this disorder include a triangular face, short philtrum, wide mouth with a thin vermilion border of the upper lip, and long nose with a narrow bridge, broad base, full tip, and low-hanging columella (Leisti et al., 1975; Pelletier, 1973; Robinson et al., 1988; White et al., 2010). Some degree of intellectual or learning disability is present in most individuals, and language (both receptive and expressive) is typically more severely affected. The name "Floating Harbor" is a portmanteau of Boston Floating Hospital and Harbor General Hospital (Torrance, CA), the two institutions from which the initial case reports originated (Leisti et al., 1975; Pelletier, 1973). Of the 50 or so cases of FHS in the literature, the majority are sporadic, although four reported instances of parent-to-child transmission suggest that this is an autosomal-dominant disorder in at least some instances (Arpin et al., 2012; Lacombe et al., 1995; Penaloza et al., 2003; White et al., 2010). Some authors have highlighted the clinical overlap between FHS and Rubinstein-Taybi syndrome (RTS [MIM 180849]), which shares several key features (short stature, a long nose with low-hanging columella, a thin vermilion border of the upper lip, and anomalous thumbs) (Arpin et al., 2012; Robinson et al., 1988). Despite the recognition of FHS as a distinct clinical entity more than 25 years ago, no causative mutations have been identified to date.

To identify the genetic basis of FHS, we assembled a cohort of 13 unrelated probands, three of whom were previously reported (White et al., 2010). The clinical details of these individuals are presented in Table 1 and Figure 1. To identify FHS-causing mutations, we

Table 1. Phenotype of Floating-Harbor syndrome probands with SRCAP mutations[a].

Proband	1	2	3	4	5	6	7	8	9	10	11	12	13
Mutation (cDNA)	c.7330C>T	c.7330C>T	c.7330C>T	c.7303C>T	c.7549delC	c.7330C>T	c.7330C>T	c.7330C>T	c.7303C>T	c.7303C>T	c.7303C>T	c.7218_7219 delTC	c.7316dupC
Alteration (protein)	p.Arg2444*	p.Arg2444*	p.Arg2444*	p.Arg2435*	p.Gln2517fs*5	p.Arg2444*	p.Arg2444*	p.Arg2444*	p.Arg2435*	p.Arg2435*	p.Arg2435*	p.Gln2407fs*35	p.Ala2440fs
Inheritance	unknown	unknown	unknown	*de novo*	*de novo*	*de novo*	*de novo*	unknown	unknown	unknown	unknown	*de novo*	*de novo*
Sex	M	F	M	M	M	M	M	F	M	M	M	M	M
Ethnicity	French	mixed European	mixed European	Finnish	German and Mexican	Brazilian	German	Caucasian	Caucasian	Brazilian	Brazilian	Chinese	Polish
Paternal age (year)	28	43	29	35	39	32	44	40	34	41	40	40	35
Gestation (weeks)	40	40	38	37	39	40	39	31	41	39	40	40	41
Birth weight (g)	3040 (-0.7 SD)	3060 (-0.6 SD)	2400 (-1.7 SD)	2620 (-0.5 SD)	2515 (-2.2 SD)	2555 (-1.8 SD)	2430 (-2.4 SD)	1655 (0 SD)	2900 (-1 SD)	2550 (-1.8 SD)	2030 (-3.1 SD)	2800 (-1.1 SD)	2730 (-1.5 SD)
Age at diagnosis	3 years	10 years	15 months	11 years	4 years, 3 months	3 years, 3 months	4 years, 4 months	4 years	11 months	7 years, 5 months	8 years	10 years	35 months
ALA	8 years	12 years	12 years	11 years	4 years, 3 months	4 years	4 years, 4 months	10 years, 5 months	11 years	19 years	19 years, 7 months	11 years	7 years, 5 months
Head circumference (cm) ALA)	54 (+1 SD)	0 SD	53 (-1 SD)	53.5 (0 SD)	48.5 (-1.7 SD)	48 (-2 SD)	50 (+0.7 SD)	49.5 (-1 SD)	50.5 (-2 SD)	52 (-2.5 SD)	56 (0 SD)	53 (0 SD)	51.5 (-0.5 SD)
Weight (kg) ALA	25.5 (0 SD)	35.6 (-0.8 SD)	N/R	22.4 (-3.2 SD)	11 (-3.4 SD)	12 (-2.5 SD)	12.5 (-2.4 SD)	19.3 (-3.3 SD)	20 (-4.2 SD)	37.6 (-5.3 SD)	62.1 (-0.7 SD)	35 (0 SD)	20 (-1.4 SD)
Height (cm) ALA	123 (-0.8 SD)	133.5 (-2.2 SD)	134.8 (-2.0 SD)	122 (-3.1 SD)	86.5 (-4.3 SD)	89.8 (-3.2 SD)	90 (-3.6 SD)	118.5 (-3.5 SD)	116.8 (-3.9 SD)	145.5 (-4.1 SD)	148 (-3.8 SD)	139 (-0.6 SD)	111 (-2.5 SD)
Age at puberty	N/A	12 years	N/A	N/A	N/A	N/A	N/A	N/A	Pubertal age 14 years at CA 11 years	N/R	N/R	10 years	N/A
Prepubertal height	-0.8 SD	-2.2 SD	-2.0 SD	-3.1 SD	-4.4 SD	-3.2 SD	-3.6 SD	-3.5 SD	N/R	N/R	N/R	-3 SD	-2.5 SD
BA vs. CA	BA 2.5 years at CA 7.5 years	BA 2 years, 6 months at CA 5 years, 7 months; BA 11 years at CA 9 years, 9 months	BA 8 years at CA 11 years	BA 2 years at CA 4 years, 8 months; BA 9 years at CA 10 years, 8 months	BA 1 year at CA 2 years, 11 months	BA 1 year at CA 3 years	BA 3 years at 4 years	BA 1 year at CA 5 years	BA 3-6 months at CA 1 year	BA 2 years, 8 months at CA 7 years	BA 3 years at CA7 years	BA 10 years, 6 months at CA 11 years, 4 months	BA 8 months at CA 2 years, 8 months

Table 1. Phenotype of Floating-Harbor syndrome probands with SRCAP mutations[a] (continued).

Proband	1	2	3	4	5	6	7	8	9	10	11	12	13
Triangular face	+	+	+	+	+	N/R	-	+	+	+	+	+	+
Distinctive nose	+	+	+	+	+	+	+	+	+	+	+	+	+
Low-hanging columella	+	+	+	+	+	+	+	+	+	+	+	+	+
Short philtrum	+	+	+	+	+	+	+	+	+	-	+	-	+
Thin upper vermilion border	+	+	+	+	-	-	+	+	+	-	-	+	+
Wide mouth	+	-	+	+	+/-	+	+	+	+	+	+	+	+
Low-set ears	+	+	-	+	+	-	+	+	+	-	-	+	+
Broad thumbs	-	+	-	+	+	+	-	N/R	N/R	N/R	+	+	+
Broad fingertips	-	+	+	+	N/R	N/R	-	N/R	+	N/R	+	+	+
Brachydactyly	-	+	+	+	-	fifth toes	-	+	-	N/R	+	+	+
Clinodactyly	-	N/R	radial deviation fifth distal phalanx	+	+	-	-	+	+	N/R	+	+	+
Other skeletal	N/R	N/R	dislocated radial head, 11 rib pairs	N/R	short fifth metacarpal	N/R	clavicular hypoplasia	kyphoscoliosis	dysplastic hips	11 rib pairs, ivory epiphyses in distal phalanges	short middle phalanges of second and fifth fingers	short first metacarpal	short fifth metacarpal
Dental issues	N/R	N/R	maxillary retrusion, underbite	caries, microdontia	N/R	N/R	N/R	caries, delayed loss of primary teeth	N/R	N/R	normal	caries, microdontia, underbite	N/R
Other health issues	hypospadias, celiac disease	hydro-nephrosis, nephro-calcinosis, recurrent otitis media	aortic coaretation (mild)	cryptorchidism, hyperopia, conductive hearing loss	hyperopia	ASD, hyperopia, unilateral renal pelviectasis	mesocardia, persistent left superior vena cava, conductive hearing loss	constipation	bilateral inguinal hernia, cryptorchidism, VPI, hearing loss	posterior urethral valves,umbilical hernia	strabismus	bilateral epididymal cysts, left varicocele	unilateral cleft lip, cryptorchidism

Table 1. Phenotype of Floating-Harbor syndrome probands with SRCAP mutations[a] (continued).

Proband	1	2	3	4	5	6	7	8	9	10	11	12	13
Intellectual development	borderline normal	normal	borderline normal	borderline normal	normal	moderate delay	borderline normal	mild intellectual disability	moderately severe learning disability	significant intellectual disability	intellectual disability	borderline normal	mild intellectual disability
Expressive language delay	delay	moderate delay	moderate delay	impairment	borderline normal, bilingual	moderate delay	moderate delay	severe delay	moderate delay	some words	moderate delay	moderate delay	moderate delay
Education	mainstream with support	mainstream with support	mainstream with support	special school	mainstream	N/A	mainstream with support	modified classroom	special school	special school	special school	modified classroom	mainstream with support
Microarray findings and (type)	normal (44k)	normal (Affymetrix 2.7M)	normal (180K Agilent)	normal (105K)	7q31 dup (paternally inherited)	Xp22.31 dup (maternally inherited)	N/A	N/A	N/A	N/A	normal 22q11	normal (Agilent 6.1)	normal (244K Agilent)
Reference	White et al. (2010) person 10	White et al. (2010) person 9	this report	this report	this report	this report	this report	this report	White et al. (2010) person 8	this report	this report	this report	this report

Numbering of mutations is relative to NM_006662.2 (gene) and NP_006653 (protein). Abbreviations are as follows: ALA, at last assessment; N/A, Not Applicable; N/R, Not Reported; +, Feature Present; -, Feature Absent; SD, standard deviations; BA, bone age; CA, chronological age; ASD, atrial septal defect; and VPI, velopharyngeal incompetence.

[a] This table summarizes the clinical findings in all study participants. Five participants (discovery cohort; individuals 1-5) underwent exome sequencing. Mutations in individuals 6-13 (validaton cohort) were identified with Sanger sequencing.

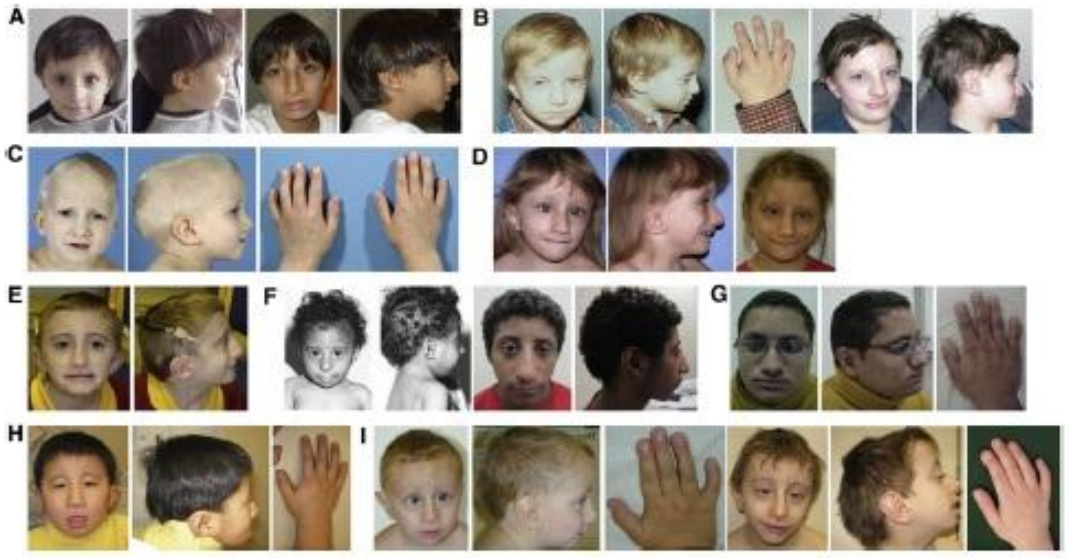

Figure 1. Floating-Harbor syndrome due to SRCAP mutations.
Clinical photos depicting 9 of 13 unrelated FHS probands with a confirmed SRCAP mutation are shown with the characteristically triangular face, long eyelashes, typical nose (long and narrow nasal bridge, broad base, full tip, and low-hanging columella), short philtrum, wide mouth, thin vermilion border of the upper lip, short chin, and low-set, posteriorly rotated ears. Clinical details concerning all study participants are presented in Table 1.
(A) Individual 1. Age 3 years; age 8 years.
(B) Individual 3. Age 3 years, 7 months; age 11 years, 5 months.
(C) Individual 4. Age 4 years, 6 months.
(D) Individual 8. Age 4 years; age 10 years, 5 months.
(E) Individual 9. Age 11 years.
(F) Individual 10. Age 15 months; age 19 years.
(G) Individual 11. Age 19 years, 7 months.
(H) Individual 12. Age 4 years.
(I) Individual 13. Age 3 years; age 7 years, 5 months.

performed exome capture and high-throughput sequencing of five unrelated affected persons (probands 1–5). Approval of the study design was obtained from the institutional research ethics board (Children's Hospital of Eastern Ontario), and free and informed consent was obtained from each study subject (or parent, if appropriate) prior to enrollment. We performed exome target enrichment by using the Agilent SureSelect 50 Mb All Exon Kit, and sequencing (Illumina HiSeq) generated 35–40 Gbp of 100 bp paired-end reads per sample. Reads were preprocessed (trimmed) and aligned to hg19 (see Web Resources for list of tools). We used an in-house annotation pipeline to identify coding and splice-site variants that met a minimum quality threshold (i.e., ≥20% of reads supported the variant). Next, we filtered the variants to exclude common polymorphisms (>1% minor-allele frequency) represented in dbSNP131, in the 1000 Genomes pilot release, or in 270 exomes sequenced for individuals with unrelated disorders at our center.

Presuming FHS to be an autosomal-dominant condition, we identified genes containing a single rare variant in each of several probands in a combinatorial fashion. Table 2 lists the numbers of potential candidate genes containing rare variants in any *n* probands as *n* is increased. Of five sequenced individuals with classic FHS, we noted that all contained heterozygous truncating variants clustered in the final (34^{th}) exon of a single gene, *SRCAP* (encoding SNF2-related CREBBP activator protein). To confirm *SRCAP* as the gene mutated in FHS, we identified *SRCAP* exon 34 mutations with Sanger sequencing in a validation cohort of eight more unrelated probands (Table 1 and Figure 2; Figure S1). All of these mutations are truncating (nonsense or frameshift) alleles, tightly clustered between codons 2,407 and 2,517; none are represented in dbSNP131, 1000 Genomes, or the National

Table 2. Variant analysis in Floating-Harbor syndrome probands.

Any X of 5 individuals	1	2	3	4	5
Number of genes containing missense, nonsense, insertion, deletion, or splice-site variants	2375	307	48	8	2
Allele frequency ≤1% in dbSNP131, and 1000 Genomes; not represented in and 270 local exomes	1178	70	3	*SRCAP*	*SRCAP*

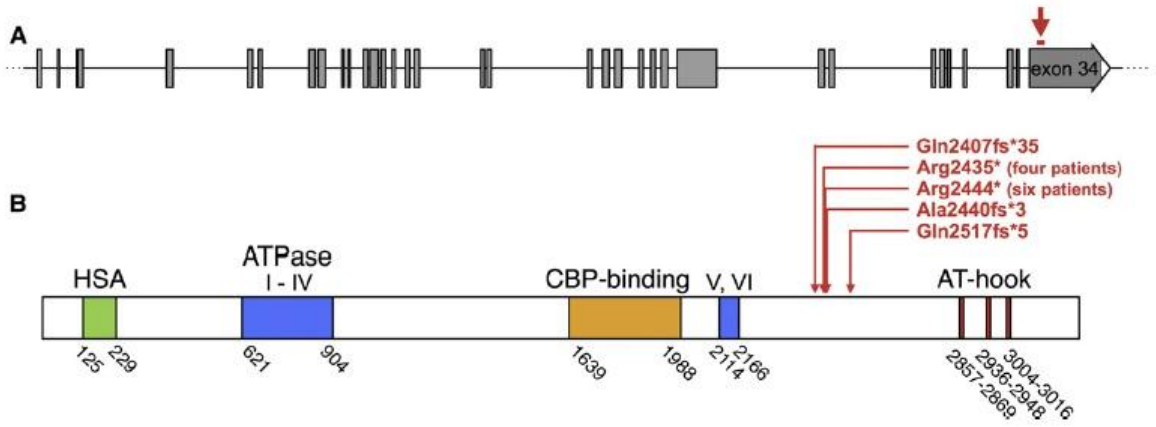

Figure 2. Locations of FHS-causing mutations within SRCAP.
(A) Intron-exon structure of SRCAP. Exon 34 mutation cluster is indicated by a red bar. (B) Domain architecture of SRCAP8–10 indicates amino acid positions of recognized domains and FHS-causing mutations. All probands are heterozygous for truncating mutations at the positions shown. The ATPase domain of SRCAP is divided into two sections, one containing conserved motifs I-IV and one containing V-VI. The following abbreviation is used: HSA, Helicase-SANT-associated domain.

Heart, Lung, and Blood Institute (NHLBI) Exome Variant Server (see Web Resources). Two mutations in particular, c.7330C>T (NM_006662.2) (p.Arg2444* [NP_006653]) in six individuals and c.7303C>T (p.Arg2435*) in four individuals, accounted for the large majority of mutations. FHS-causing mutations were shown to be de novo in all six instances in which parental DNA samples were available.

SRCAP encodes a switch/sucrose nonfermentable (SWI/SNF)-type chromatin-remodeling ATPase identified in a two-hybrid screen for interacting partners of CREB-binding protein (CREBBP, hereafter called CBP) (Johnston et al., 1999). In reporter assays, SRCAP is a potent coactivator for CREB and CBP-mediated transcription (Johnston et al., 1999; Monroy et al., 2001). In transgenic *Drosophila*, exogenous SRCAP colocalizes with transcriptionally active chromatin and augments CBP's presence at these sites (Johnston et al., 1999; Monroy et al., 2001). Affinity-purified SRCAP precipitates as a large complex that catalyzes ATP-dependent substitution of the variant histone H2A.Z into nucleosomes (Eissenberg et al., 2005). This activity has been confirmed by knockdown experiments with natural promoters, and it is correlated with in vivo target-gene expression (Wong et al., 2007). Separately, SRCAP has also been shown to transduce signals belonging to the nuclear (steroid) hormone receptor and Notch pathways, indicating that it has diverse roles in gene expression (Eissenberg et al., 2005; Monroy et al., 2003).

In keeping with its multiple coactivator roles, SRCAP contains several discrete functional domains (Eissenberg et al., 2005; Johnston et al., 1999; Monroy et al., 2001). These domains include an SNF2-like ATPase, an N-terminal HSA (Helicase-SANT-associated) domain, and three C-terminal AT-hook DNA-binding motifs; the CBP interaction domain of SRCAP is

located centrally (Figure 2). Given the structure of SRCAP, the nonrandom clustering of truncating mutations seen in our study participants is strongly suggestive of a dominant-negative disease mechanism due to loss of one or more critical domain(s), for instance the three C-terminal AT-hook motifs. Several more arguments support this. First, in reporter assays, the major transactivation function of SRCAP is located in a 655 residue C-terminal fragment abolished by FHS-causing truncations (Monroy et al., 2001). Second, expression of a construct solely consisting of the CBP interaction domain of SRCAP strongly inhibits CREB-mediated transactivation in a dominant-negative fashion (Monroy et al., 2001). Third, the Database of Genomic Variants (see Web Resources) contains two HapMap control individuals who bear a 208 kb deletion copy-number variation (#2,209) containing *SRCAP* and nine adjacent genes and who have no reported phenotype.

In general, the phenotype of persons with *SRCAP* mutations is concordant with earlier clinical descriptions of FHS (Table 1 and Figure 1), and nearly all individuals have short stature and expressive-language impairment. Despite the remarkable similarity among mutations seen in our study subjects, cognitive outcomes ranging from ~~–n~~ormal" to ~~–s~~ignificant intellectual disability" were reported. It is unclear whether genetic modifier(s) and/or currently unidentified environmental factors are responsible. Many of our study subjects had additional systemic malformations, particularly genitourinary (eight individuals) and cardiac (three individuals) malformations. Again, genotype-phenotype correlations explaining these features are lacking. Given that FHS is a dominant condition exhibiting a high de novo mutation rate, one might expect a paternal age effect to be present, and indeed the mean paternal age of the affected individuals was advanced (36.9 years; range: 29–44 years).

Importantly, our findings suggest a basis for the long-recognized phenotypic overlap between FHS and RTS, the latter of which is caused by alterations in CBP or its homolog, p300 (Petrij et al., 1995; Roelfsema et al., 2005; Stevens, 2002). Because alterations in both CBP and SRCAP are expected to produce widespread target-gene dysregulation, further studies are needed before we can determine which transcriptional targets are primarily responsible for each phenotype and whether any of these might be valid therapeutic targets. The availability of a molecular test for FHS will greatly improve the reliability of a formerly challenging clinical diagnosis. Historically, a diagnosis of FHS has relied upon the presence of typical facial features because many other key findings (e.g., short stature and language impairment) are nonspecific. The advent of molecular diagnosis for this condition will allow us to gain a better appreciation of the true prevalence and phenotypic spectrum of FHS.

Acknowledgements

The authors would first like to thank the study participants and their families, without whose participation and cooperation this work would not have been possible. This work was funded by the government of Canada through Genome Canada, the Canadian Institutes of Health Research (CIHR), and the Ontario Genomics Institute (OGI-049). Additional funding was provided by Genome Québec and Genome British Columbia. K.M.B. is supported by a Clinical Investigatorship Award from the CIHR Institute of Genetics. This work was selected for study by the FORGE Canada Steering Committee, consisting of K. Boycott (University of Ottawa), J. Friedman (University of British Columbia), J. Michaud (University of Montreal), F. Bernier (University of Calgary), M. Brudno (University of Toronto), B. Fernandez (Memorial University), B. Knoppers (McGill University), M. Samuels (Université de Montreal), and S. Scherer (University of Toronto).

Supplemental Data

Supplemental data includes one figure and can be found in Appendix 3.

Web Resources

The URLs for data presented herein are as follows:

Database of Genomic Variants, http://projects.tcag.ca/variation/

FASTX-Toolkit, http://hannonlab.cshl.edu/fastx_toolkit/

NHLBI Exome Variant Server, http://evs.gs.washington.edu/EVS/

Online Mendelian Inheritance in Man (OMIM), http://www.omim.org

Picard, http://picard.sourceforge.net/

SAMtools, http://samtools.sourceforge.net/

Accession Numbers

The NCBI accession number for the SRCAP sequence reported in this paper is NM_006662.2 and local identifiers are as follows:

NM_006662.2: c.7330C>T (NCBI ss477606270)

NM_006662.2: c.7303C>T (NCBI ss477606271)

NM_006662.2: c.7549delC (NCBI ss477606272)

NM_006662.2: c.7218_7219delTC (NCBI ss477606273)

NM_006662.2: c.7316dupC (NCBI ss477606274)

Chapter 3: Mutations in *EZH2* Cause Weaver Syndrome

Preface

The following chapter consists of data previously published in the American Journal of Human Genetics under the title "Mutations in *EZH2* Cause Weaver Syndrome" (PMID: 22177091) by Gibson WT, Hood RL, Zhan SH, Bulman DE, Fejes AP, Moore R, Mungall AJ, Eydoux P, Babul-Hirji R, An J, Marra MA; FORGE Canada Consortium., Chitayat D, Boycott KM, Weaver DD, Jones SJ.

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I performed the initial Sanger sequencing of the patient cohort to screen for *NSD1* mutations (which cause the phenotypically similar Sotos syndrome). I was involved in analyzing the computed variant sequencing data and performed all of the PCR and Sanger sequencing of *EZH2* which confirmed the Illumina sequencing results. I performed all subsequent Sanger sequencing of the entire *EZH2* gene in a secondary Weaver cohort. I performed PCR and Sanger sequencing to confirm that inheritance was *de novo* in all 3 instances. I analyzed all Sanger output files and generated the Sanger sequencing figure for the manuscript. I also contributed writing/editing of the manuscript.

The specific contributions of each author to this paper are listed below.

Gibson WT: Oversaw the project. Determined the initial cohort to be sent for Illumina sequencing. Analyzed the computed Illumina sequencing data. Wrote the manuscript. Responded to reviewers comments.

Hood RL: (see above)

Zhan SH: Performed 3D modeling of human EZH2. Generated figures for the manuscript.

Bulman DE: Oversaw Sanger sequencing validation experiments. Contributed to writing/editing the manuscript.

Fejes AP, Moore R, Mungall AJ, Eydoux P, Babul-Hirji R, An J, Marra MA: Performed bioinformatics analysis of the Illumina sequencing data to generate the variant files. Wrote bioinformatics analysis methods section and tables of the manuscript.

FORGE Canada Consortium: Provided funding for this project.

Chitayat D: Determined the initial cohort to be sent for Illumina sequencing. Contributed to writing/editing the manuscript.

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Abstract

We used trio-based whole-exome sequencing to analyze two families affected by Weaver syndrome, including one of the original families reported in 1974. Filtering of rare variants in the affected probands against the parental variants identified two different de novo mutations in the enhancer of zeste homolog 2 (*EZH2*). Sanger sequencing of *EZH2* in a third classically-affected proband identified a third de novo mutation in this gene. These data show that mutations in *EZH2* cause Weaver syndrome.

Main Text

Weaver syndrome [MIM 277590] is a rare congenital anomaly syndrome first described in two families in 1974 (Weaver et al., 1974). It consists of generalized overgrowth, advanced bone age, marked macrocephaly, hypertelorism, and characteristic facial features. Intellectual disability is common. Approximately 40 cases are known from the literature. Typically, Weaver syndrome occurs as a sporadic condition, though cases of parent-to-child transmission have been documented (Fryer et al., 1997; Proud et al., 1998). Some patients thought to have Weaver syndrome have mutations in *NSD1*, which is mutated or deleted in most patients with classic Sotos syndrome [MIM 117550] (Baujat et al., 2005; Douglas et al., 2003; Rio et al., 2003). This molecular finding has fuelled debate among clinical geneticists regarding whether the Sotos and Weaver syndromes represent variable expressivity of a single locus with allelic heterogeneity or whether they represent distinct disorders caused by mutations in different genes. Clinical features shared by both syndromes include developmental delay, overgrowth, and prominent macrocephaly, and features distinguishing Weaver syndrome from Sotos syndrome are retrognathia with a prominent chin crease (sometimes described as a stuck-on chin), increased prenatal growth, and a carpal bone age that is greatly advanced compared to metacarpal and phalangeal bone age.

Whole-exome sequencing (WES) facilitates the identification of sequence changes in the protein-coding genome in small families and has enabled identification of rare and ultrarare Mendelian disorders that have hitherto been refractory to linkage mapping (Bamshad et al., 2011; Iafrate et al., 2004). To search for disease-causing alleles in Weaver syndrome, we collected saliva-derived DNA by using kits (Oragene) from one of the probands included in

the original report describing Weaver syndrome (Weaver et al., 1974), from two other unrelated probands with classical features of the condition, and from all six unaffected parents. We obtained informed consent from the parents, assent from the affected individuals, and ethical review and approval according to the Finding of Rare Disease Genes (FORGE) Canada Consortium. Probands 1 and 2 were personally examined by D.D.W., who also reviewed photos and clinical details on proband 3 (examined in childhood; records of examination and referral provided by D.C.; proband was reexamined in adulthood by W.T.G.); probands were confirmed in childhood to have classical features of Weaver syndrome and not Sotos syndrome (Figure 1 and Table 1), and parents were confirmed to be unaffected. None of the three probands had submicroscopic abnormalities detectable by microarray analysis (Illumina Human Omni2.5-Quad chip, analyzed with CNVPartition from GenomeStudio V2010.3). None of the probands had expanded *FMR1* alleles [MIM 309550] or abnormalities on clinical karyotyping. We also ruled out rare variants in *NSD1* [MIM 606681] by using Sanger sequencing on saliva-derived DNA in all three probands (data not shown, primers available on request).

We performed exome sequencing on samples from six individuals (probands 1 and 2 and the parents of both), and quantified the DNA concentration by using a Quant-iT dsDNA HS assay kit and a Qubit fluorometer (Invitrogen). We sheared approximately 500 ng DNA for 75 s at a duty cycle of 20% and an intensity of 5 with a Covaris E210 and size fractionated the DNA on an 8% polyacrylamide gel. We excised the 200–250 bp size fraction, eluted it from the gel slice, and ligated it to Illumina paired-end adapters following a standard protocol as previously described (Morin et al., 2010). Adaptor-ligated DNA was amplified for 10 cycles with the PE primer set (Illumina) and purified. The pre-exome capture library DNA

Figure 1. Proband from Weaver et al. (1974) and two additional probands described in this study.
Proband 1 is shown at 18 months (A), 6 years (B), 11 years (C), 17 years (D), 21 years (E), and 30 years (F, G, and H). Proband 2 is shown at age 7 years (I) and 13 years (J, K, and L). Proband 3 is shown at birth (M), 12 months (N), 24 months (O), 42 months (P), 6 years (Q),10 years (R), 11 years (S), 16 years (T), and 19 years (U and V). Proband 3 at 8 years in a stance that shows elbow and knee contractures (W).
Photos are published with the proxy consent of the parents and assent of the probands.

Table 1. Phenotypic manifestations of Weaver syndrome in patients with *EZH2* mutations.

Phenotypic Manifestation	Proband 1 c.457_459del (p.Tyr153del)	Proband 2 c.2080C>T (p.His694Tyr)	Proband 3 c.394C>T (p.Pro132Ser)
Gestational age at delivery (weeks)	~ 36.5	32	42
Birth weight (kg)	4.82	3.26	4.5
Birth length (cm)	55	50.8	55
Birth head circumference (cm)	36.5	~ 34	Nk
Recent weight (kg) [age measured]	118 [30 years]	64.1 [10 years, 11 months]	103.3 [19 years, 9 months]
Recent height (cm) [age measured]	190.5 [30 years]	179 [10 years, 11 months]	177.7 [19 years, 9 months]
Excessive growth of prenatal onset	+++	+++	+++
Accelerated osseous maturation	++++	++++	++++
Neurological features			
Hypertonia	++	-	-
Hypotonia	-	++	++
Hoarse low-pitched cry	++	++	++
Intellectual disability	mild	borderline-mild	mild
Excessive appetite	++	++	-
Ventriculomegaly	++	Nk	-
Delayed myelination	Nk	Nk	+
Cerebellar hypoplasia (mild)	-	Nk	+
Seizures [age of onset]	tonic-clonic [13 years]	-	brief absence [15 years]
Poor fine motor coordination	++	+	++
Poor balance and gravitational insecurity	++	++	++
Fatty filum terminale	-	-	++
Craniofacial			
Macrocephaly	+++	+++	+++
Large bifrontal diameter	+++	+++	+++
Flat occiput	+	-	+
Large ears	+++	+	+
Ocular hypertelorism	++	++	-
Downslanting palpebral fissures	+	+	+
Long philtrum	++	++	++
Micrognathia	+	+	
Cardiovascular			
Patent ductus arteriosus	-	-	+
Hands			
Prominent digit pads	++	++	++
Singular transverse palmar crease	-	-	-
Camptodactyly	++	+	-
Broad thumbs	++	++	
Thin, deep-set nails	++	++	++

Table 1. Phenotypic manifestations of Weaver syndrome in patients with *EZH2* mutations (continued).

Phenotypic Manifestation	Proband 1 c.457_459del (p.Tyr153del)	Proband 2 c.2080C>T (p.His694Tyr)	Proband 3 c.394C>T (p.Pro132Ser)
Feet			
Clinodactyly, toes	+	+	-
Talipes equinovarus	++	-	-
Short fourth metatarsals	+	-	-
Hind foot valgus	-	-	+
Limited elbow and knee extension in early life	+	-	+
Limited elbow and knee extension after puberty	+	-	+
Widened distal femurs and ulnas	++	Nk	-
Skin			
Excessive loose skin	++	++	-
Inverted nipples	+	-	-
Thin hair	+	-	-
Increased pigmented nevi	-	++	++
Connective tissue			
Umbilical hernia	++	+	+
Inguinal hernia	++	-	-
Scoliosis (degrees)	20	10	16
Endocrine			
Hypothyroidism [age of onset]	~25 years	-	-
Growth hormone deficiency [age of onset]	~27 years	-	-

Key: + = minimally present, ++ = obviously present, +++ = very prominent, ++++ = severe, - = assessed and found to be absent, Nk = not known.

was assessed with an Agilent DNA 1000 Series II assay and subsequently hybridized 500 ng to the 50 Mb exon probe with the Human All Exon Kit (G3370) following Agilent's SureSelect Target Enrichment protocol. The captured DNA was purified with a QIAGEN MiniElute column, and amplified for 12 cycles with the standard Illumina PE primer set. PCR products were separated by size on an 8% PAGE gel before gel extraction at the desired size range (320–370 bp). The samples were then assessed with an Agilent DNA 1000 series II assay. The final library was diluted to a concentration of 10 nM, which was confirmed via a Quant-iT dsDNA HS assay kit and a Qubit fluorometer as above, prior to cluster generation and exome sequencing.

We performed paired-end tag (PE100) sequencing with an Illumina HiSeq2000 machine. Sequencing reads that failed chastity filtering were removed with Illumina's GA Pipeline (1.12.0 RTA 1.12.4.2), and the remaining reads were mapped to the reference genome sequence (hg18) with BWA 0.5.7 (Li and Durbin, 2009); duplicate reads and reads with a mapping score of 0 were removed. The aligned reads were exported to pileup format and called with SAMtools 0.1.13 (Li et al., 2009). We filtered single nucleotide variants and retained those with a minimum SNP quality of 20 at varFilter parameter −D 1000. Small insertions and deletions (indels) were processed similarly with varFilter parameters −D 1000, −d 2 and −l 30. We then imported all the variants into a local PostgreSQL database used to store and process human variation data (Fejes et al., 2011). We annotated the filtered variants as known or novel depending on whether they had been previously reported in a public database such as dbSNP (Sherry et al., 2001) or the 1000 Genomes Project (Marth et al., 2011) or previously observed in the in-house database of normal germline genomes sequenced at the British Columbia Cancer Agency, Genome Sciences Centre (BCGSC).

Currently, this database contains over 1.47 billion observed sequence variants mapping to 63.9 million unique base substitutions derived from over 1,360 individuals. Specifically, we sought to identify variations that cause nonsynonymous changes in protein-coding regions and those that fell within two bases of exon boundaries (such that they might interfere with intron splicing; Table S1).

In proband 1, we identified a heterozygous c.457_459del (p.Tyr153del) variant in isoform A of *EZH2* [MIM 601573] (RefSeq NM_004456.4). This was not seen in either of his parents, indicating that this was a de novo mutation. We also identified a heterozygous de novo missense variant c.2080C>T (p.His694Tyr) of the same gene in proband 2. These variants were seen at high coverage in both probands (in 121 out of 239 reads and in 153 out of 304 reads, respectively) but were not seen in the parental reads. In our hands, coverage at this level has a positive predictive value of 100% for subsequent Sanger verification. We went on to validate the presence of both of these mutations and their de novo status by using Sanger sequencing (Figure 2, primers available on request). After filtering out parental variants, no other gene demonstrated private mutations in both of the probands (Table S1), where ―private" is defined as not found in dbSNP, 1000 Genomes Project data, or among normal genomes (including the parents of the probands) sequenced in-house at the BCGSC. We then analyzed *EZH2* by Sanger sequencing in a third trio (proband 3 and her parents) in whom we had not performed exome sequencing. We identified a c.394C>T (p.Pro132Ser) mutation in Proband 3 and confirmed that it was *de novo* (i.e., absent in both parents).

The p.Tyr153 del mutation in Proband 1 lies six amino acid residues from the N terminus of the Simple Modular Architecture Research Tool (SMART) (Letunic et al., 2012) predicted

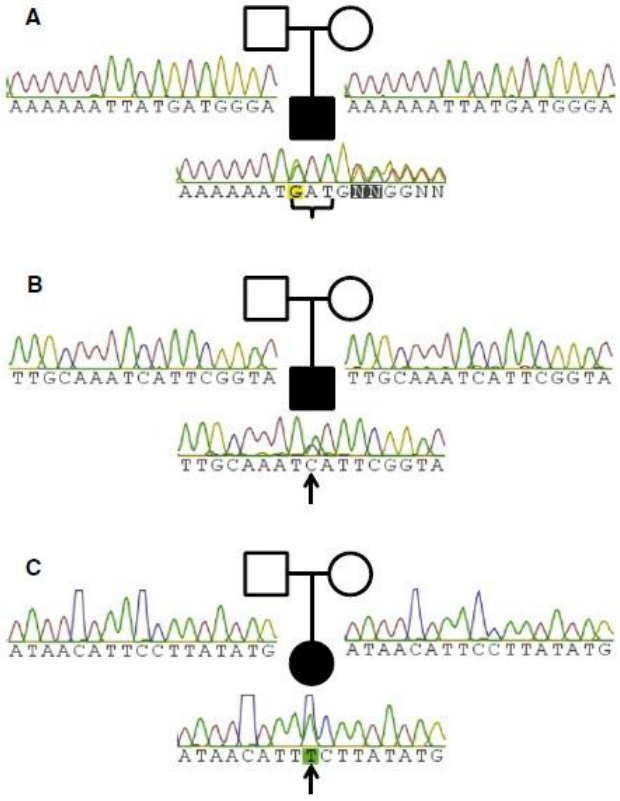

Figure 2. Sanger confirmation of sequence variants.
(A) The c.457_459del mutation in Proband 1 (curly bracket) is de novo.
(B) The c.2080C>T mutation in Proband 2 (arrow) is de novo.
(C) The c.394C>T mutation in Proband 3 (arrow) is de novo.

SANT (switching-defective protein 3 [Swi3]), adaptor 2 [Ada2], nuclear receptor corepressor [N-CoR], transcription factor [TF]IIIB′) domain (Figure 3, annotated with InterPro (Hunter et al., 2009)). The deletion of an entire amino acid (p.Tyr153del) in EZH2 removes a bulky polar residue near the putative SANT DNA-binding domain, which is suggestive of functional consequences for the protein. Also, the deleted codon is evolutionarily conserved according to phyloP (Pollard et al., 2010). The placental mammalian genome-wide alignment-based phyloP score averaged across the three codon sites (chromosome 7:148,157,778–148,157,780) is 1.59 (taken from the UCSC Genome Browser (Fujita et al., 2011) Conservation track for the hg18 assembly). A positive phyloP score is interpreted as a signature of evolutionary conservation, which is consistent with functional importance.

The Sorting Intolerant From Tolerant (SIFT) (Kumar et al., 2009; Ng and Henikoff, 2003) scores for p.Pro132Ser and p.His694Tyr were 0.00 (with values $\leq$ 0.05 interpreted as damaging). With PolyPhen2 (Adzhubei et al., 2010) trained on the HumDiv data set, p.Pro132Ser and p.His694Tyr were both predicted to be probably damaging. Additionally, the nucleotide site (chromosome 7:148,157,843) where Pro132 occurs has a placental mammalian phyloP score of 3.17, and the nucleotide site (chromosome 7:148,137,365) where His694 occurs has a score of 2.90. The positive phyloP scores suggest that these nucleotide sites are evolutionarily conserved, whereas a score near 0 would have suggested neutral selection. Perhaps the most suggestive evidence for the pathogenicity of p.His694Tyr comes from the specific location of this histidine residue—it is located in the Su(var)3,9, Enhancer of zeste, Trithorax (SET) domain, within the knot substructure of the active site, and is predicted to form part of the binding domain for the enzymatic cofactor *S*-adenosyl-L-methionine (AdoMet) (Jacobs et al., 2002). We made a 3D model of human EZH2

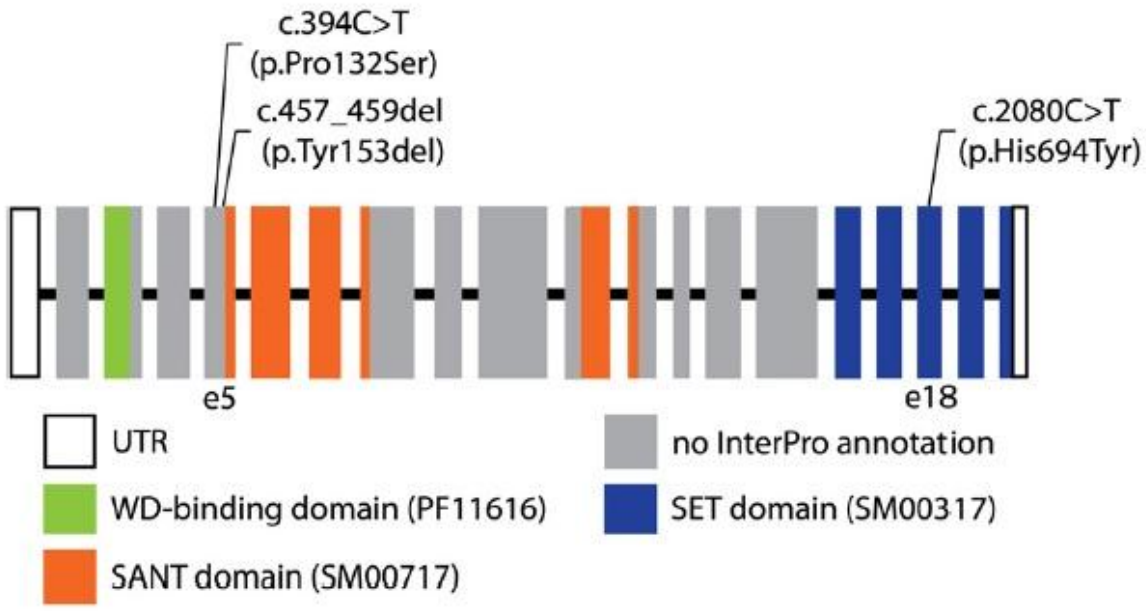

Figure 3. Schematic of human *EZH2*.
Coding exons are indicated by rectangles and noncoding exons by open rectangles. The exons are numbered starting from the exon containing the 50 untranslated region (UTR). The putative SANT DNA binding domain is shown in orange, the SET domain in blue, and the WD-binding domain in green. The SMART or Pfam domain identifier is presented in parentheses. Exons with no InterPro annotation are indicated in gray.

p.His694Tyr by using SWISS-MODEL (Arnold et al., 2006) and ICM software (Abagyan, 1994) (Molsoft) based on the structure of the related protein euchromatic histone-lysine N-methyltransferase 1 ([MIM 607001] selected because of its lack of gaps and high-resolution crystal structure [1.6 Å]; see Figure 4) (Wu et al., 2010). The conformation of this conserved histidine is highly similar across known crystal structures for the SET domain. Because of its proximity to the AdoMet-binding site, replacement of this histidine with a bulkier tyrosine side chain could well interfere with cofactor binding and methyltransferase activity of the mutant molecule. Mutation of the histidine residue that occupies a similar position in SUV39H1 abolished its methyltransferase activity in an in vitro assay (Rea et al., 2000), and a mutation of this specific histidine residue to arginine (with functional effects) has been reported in a 41-year-old male with chronic myelomonocytic leukemia (Makishima et al., 2010). Furthermore, mutations in nearby residues at positions 690 and 693 were also reported in other hematological malignancies (Makishima et al., 2010).

The EZH2 protein partners with SUZ12 and EED to form the polycomb repressive complex (PRC2). This complex catalyzes the trimethylation of lysine 27 of histone H3 (H3K27), and EZH2 itself forms the catalytic subunit for this reaction. Thus, EZH2 forms a key component of molecular machinery that shuts off transcription of loci to which trimethylated H3K27 is bound. *EZH2* is known to be mutated somatically in lymphoid and myeloid cancers (Makishima et al., 2010; Morin et al., 2010). Mutation of arginine 690 to histidine or histidine 694 to arginine appears to block EZH2's ability to facilitate trimethylation of H3K27 (Makishima et al., 2010), and mutation of tyrosine at position 641 also alters the affinity of EZH2 for H3K27 (Yap et al., 2011). Residues other than tyrosine at position 641 reduce the preference for unmethylated and monomethylated lysine and favor trimethylation of lysine

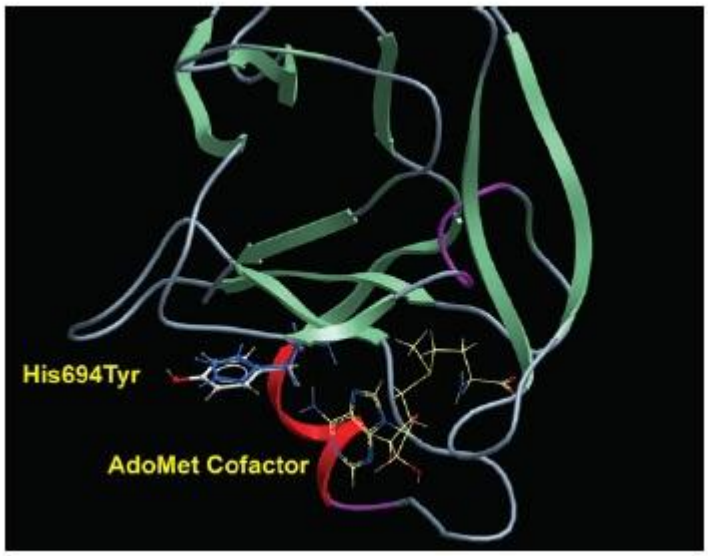

Figure 4. Ribbon model of the EZH2 SET domain.
The wild-type histidine residue is shown in blue and the bulkier tyrosine in white and red. The nearby binding site of the S-Adenosylmethionine cofactor is also indicated.

(Yap et al., 2011). It is worth noting that some patients with Weaver syndrome have been reported to develop tumors or malignancies, including acute lymphoblastic leukemia (Basel-Vanagaite, 2010). The lifetime risk of malignancy in Weaver syndrome patients has been estimated at 11%, though this is likely an overestimate because of reporting bias. Nevertheless, constitutive EZH2 mutations might confer a mild predisposition to malignancy. Mutations that conferred a more profound effect on histone methylation might also confer a stronger selective advantage for cell growth. Such mutations might not be viable in the fully heterozygous state and would be observed in nature only as postzygotic somatic mutations. In vitro studies will be required to determine whether the p.His694Tyr mutation we have observed in this study affects the affinity of EZH2 for AdoMet and whether any of the three mutations affect H3K27 trimethylation.

It is clear from the resulting phenotypes that *EZH2* mutations and structural variants also affect developmentally important pathways. Several patients with deletions and duplications encompassing *EZH2* are reported in the DECIPHER database (Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources) (Firth et al., 2009), and one patient with a duplication (patient 250841) does manifest macrocephaly. The fact that there is little other concordance between the DECIPHER phenotypes (apart from intellectual disability) and those of our patients is likely attributable to the multiple other genes affected by these structural variants. It is also possible that the EZH2 protein variants expressed in our patients act through molecular mechanisms other than haploinsufficiency; subtle but important changes in specific subfunctions of EZH2 are known to occur in association with specific protein variants (Yap et al., 2011).

Animal studies are yielding insights into the specific role of mouse *Ezh2* in organ systems other than the hemopoietic system. *Ezh2* regulates proximodistal axis elongation and anteroposterior axis specification in the developing mouse limb (Wyngaarden et al., 2011). Limb anomalies in humans with Weaver syndrome are relatively mild, though deep-set nails, joint contractures, and dysharmonic bone age might be consequences of aberrant EZH2 signaling in human limb patterning. Mice with targeted knockout of *Ezh2* in beta cells had reduced beta cell proliferation and beta cell mass (Chen et al., 2009), and mice with targeted knockout of *Ezh2* in satellite cells had impaired regeneration of muscle (Juan et al., 2011). None of our three probands had elevated fasting glucose, though probands 2 and 3 continue to manifest hypotonia (Table 1).

Several patients with Weaver syndrome are reported to have mutations in *NSD1*, a gene first associated with overgrowth in the Sotos syndrome (Baujat et al., 2005; Douglas et al., 2003; Rio et al., 2003). *NSD1* mutations in Weaver syndrome appear to cluster toward the C terminus of the molecule, 5′ of the SET domain, though one frameshift mutation in exon 5 and one mutation within the SET domain itself have been reported (Rio et al., 2003). Direct protein-protein interactions between *EZH2* and *NSD1* are not yet known, but the similarity of the human phenotypes caused by rare mutations in these genes suggests interactive links between gene networks containing these two SET-domain-containing proteins.

Our data demonstrate de novo mutations in *EZH2* in three families, including one of the original families that led to the definition of the disorder. These results illustrate the power of next-generation sequencing methods to identify rare disease-causing variants with a small number of samples (six individuals in this case, only two of whom are affected), provided

that detailed clinical studies first identify phenotypic concordance and additional families are available as a replication set. Because Weaver syndrome is a genetically heterogeneous condition, other genes associated with the condition might yet be identified.

Finally, it is interesting to note the involvement of SET-domain proteins in molecular networks that, when perturbed, cause intellectual disability syndromes and/or cancer. Mutations in *NSD1* cause Sotos syndrome and Weaver syndrome (Baujat et al., 2005; Douglas et al., 2003; Rio et al., 2003), and mutations in *MLL2* (which also bears a SET domain) cause Kabuki syndrome (Ng et al., 2010a). Histone-modifying proteins such as NSD1, EZH2, and MLL2 appear repeatedly as targets of somatic mutation in hematological malignancies (Morin et al., 2011) and are emerging as a cause of neurodevelopmental disorders. Detailed studies of larger cohorts of well-phenotyped probands will assist in determination of the prevalence of mutations in EZH2 and other SET-domain proteins in Weaver and other syndromes and of their consequences on metabolism and cancer risk. At this time, the evidence from animal models is insufficient to recommend routine surveillance in Weaver syndrome for diabetes or myopathy, beyond what would ordinarily be performed in pediatric and adult practice. Data from long-term follow-up of adult individuals with Weaver syndrome will assist physicians in deciding the optimal time to screen for potential metabolic and neoplastic complications of this rare disorder. The possibility that dietary supplementation with methionine (or with other methyl donors such as betaine and choline) might improve the activity of certain EZH2 variants is attractive but awaits further investigation.

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Supplemental Data

Supplemental data includes one figure and one table and can be found in Appendix 4.

Web Resources

The URLs for data presented herein are as follows:

1000 Genomes Project, http://www.1000genomes.org/

Burrows-Wheeler Aligner BWA, http://bio-bwa.sourceforge.net/

dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/

Database of Genomic Variants, http://projects.tcag.ca/variation/

DECIPHER database, http://decipher.sanger.ac.uk/

Human Variation Database (Vancouver version), http://vancouvershortr.sourceforge.net/

Online Mendelian Inheritance in man (OMIM), http://www.omim.org

Phred, http://www.phrap.com/phred/

phyloP, http://compgen.bscb.cornell.edu/phast/

PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/

PostgreSQL, http://www.postgresql.org/

RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq/

SAMtools, http://samtools.sourceforge.net/

SIFT, http://sift.jcvi.org/

Simple Modular Architecture Research Tool, http://smart.embl-heidelberg.de/

SWISS-Model Server, http://swissmodel.expasy.org/

Accession Numbers

Reference sequences reported in this study are available from GenBank under the following accession codes: *EZH2* longest isoform, NM_004456.4. Local identifiers are as follows: NM_004456.4:c.457_459del (NCBI ss 472336142), NM_004456.4:c.2080C>T (NCBI ss 472336143) and NM_004456.4:c.394C>T (NCBI ss 472336144).

Chapter 4: Severe Connective Tissue Laxity Including Aortic Dilatation in Sotos Syndrome

Preface

The following chapter consists of data previously published in the American Journal of Medical Genetics Part A under the title ―Severe Connective Tissue Laxity Including Aortic Dilatation in Sotos Syndrome" (PMID: 26613968) by Hood RL, McGillivray G, Hunter MF, Roberston SP, Bulman DE, Boycott KM, Stark Z; Care4Rare Canada Consortium.

Approval for this article to be reused in this thesis was granted by John Wiley and Sons (see Rights and Permissions; Chapter 4 Manuscript)

I performed the initial Sanger sequencing of the full *EZH2* gene in Patient 3 to rule out the gene which causes phenotypically similar Weaver syndrome. I helped determined the trio cohort to be sent for Illumina sequencing and analyzed the variant data. I performed the PCR and Sanger sequencing of *NSD1* to confirm the Illumina sequencing results. I cultured a lymphoblastoid cell line derived from Patient 3, isolated RNA from this cell line, and performed RT-PCR experiments to determine the impact of the identified mutation on the *NSD1* transcript. I also performed Sanger sequencing of the RT-PCR transcripts and analyzed the Sanger output files. I wrote the manuscript and generated the manuscript figure.

The specific contributions of each author to this paper are listed below.

Hood RL: (see above)

McGillivray G, Hunter MF, Roberston SP: Collected and sent patient samples for analysis. Performed Conformational sensitive gel electrophoresis (CSGE) analysis of *NSD1* in all

patients. Sent Patient 3 sample for sequencing of *TGFBR1* and *TGFBR2* genes, and SNP microarray analysis.

Bulman DE: Oversaw Sanger sequencing and cell culture validation experiments. Contributed to writing/editing the manuscript.

Boycott KM: Oversaw the project. Determined the initial cohort to be sent for Illumina sequencing. Oversaw Sanger sequencing validation experiments. Contributed to writing/editing the manuscript.

Stark Z: Oversaw the project. Sent Patient 3 sample for sequencing of *TGFBR1* and *TGFBR2* genes, and SNP microarray analysis. Determined the initial cohort to be sent for Illumina sequencing. Co-wrote the manuscript. Contributed to editing the manuscript. Responded to reviewers comments.

Care4Rare Canada Consortium: Provided funding for this project.

To The Editor

Sotos syndrome (MIM 117550) is characterized by prenatal and postnatal overgrowth, macrocephaly, distinctive facial features, learning difficulties of variable severity and advanced bone age. It is caused by heterozygous mutations in *NSD1* predicted to result in haploinsufficiency (Kurotaki et al., 2002). Over 15% of patients with Sotos syndrome display features suggestive of mild connective tissue dysfunction, including joint hyperextensibility and pes planus (Tatton-Brown et al., 2005). Herein, we report four patients with novel truncating mutations in *NSD1* and severe connective tissue laxity, manifesting as redundant skin, joint hypermobility, vesicoureteric reflux and aortic dilatation.

Three of these patients were originally reported by Robertson and Bankier in 1999 as ‗Sotos syndrome and cutis laxa' (Robertson and Bankier, 1999) and presented a diagnostic dilemma in the era before highly sensitive molecular testing was available. None of them had advanced bone age, a major feature of Sotos syndrome present in 75–80% of prepubertal individuals (Tatton-Brown et al., 2005). Two of the patients underwent bilateral uretric reimplantation for severe vesico-ureteric reflux, with one demonstrating megacystis and hypoplastic abdominal musculature. Given the extreme connective tissue laxity, echocardiograms were performed in all three patients, and two were identified as having diffuse dilatation of the ascending aorta, a previously unreported feature of Sotos syndrome. The diffuse ascending aortic dilatation has persisted on 5-yearly echocardiograms in adulthood, and one of the patients has been treated with β-blockers. The diagnostic considerations for these patients included the possibility of a novel phenotype, extreme manifestations of Sotos syndrome, or that they may have two co-existing conditions. In the absence of molecular confirmation of the diagnosis

and further reported cases, skepticism has remained regarding whether these Sotos-like patients lie within the Sotos syndrome phenotypic spectrum (Hennekam, 2010).

Conformational sensitive gel electrophoresis (CSGE) of *NSD1* was performed in patients 1–3 in 2004, and revealed pathogenic mutations in Patient 1 (exon 5, c.2894C>G, p. (Ser965*)) and Patient 2 (exon 5, c.2859dupT, p. (Lys954*)), but did not yield a disease-causing mutation in Patient 3.

Adult photographs of Patient 3 are presented in Figure 1A, demonstrating a long narrow face, prominent forehead and chin, downslanting palpebral fissures, arched eyebrows, deep set nails and statural disproportion. He remained under active investigation, and subsequent testing included SNP microarray analysis (Illumina HumanCytoSNP-12v2.1) and Sanger sequencing of the *TGFBR1*, *TGFBR2* and *EZH2* genes, all of which returned normal results. The possibility that Patient 3 represented a novel syndrome was considered so we performed whole-exome sequencing using peripherally derived blood DNA from the patient and his parents as described previously (Srour et al., 2014). In brief, genomic DNA was captured with the Agilent SureSelect v5 exome capture oligonucleotide library and sequenced with paired-end 100 bp reads on the Illumina HiSeq 2000. Sequencing analysis identified a *de novo* splice mutation in the 7th intron of *NSD1* (c.4192+1G>A), which was confirmed by Sanger sequencing (Figure 1B). The mutation, which is expected to alter splicing of the *NSD1*, is located in the donor site of intron 7 and was not detected by CSGE.

To determine how the mutation in Patient 3 impacts *NSD1* splicing, RNA was isolated from a lymphoblastoid cell line derived from the patient and RT-PCR was performed using forward and reverse primers specific to *NSD1* exons 6 and 8, respectively. Three PCR

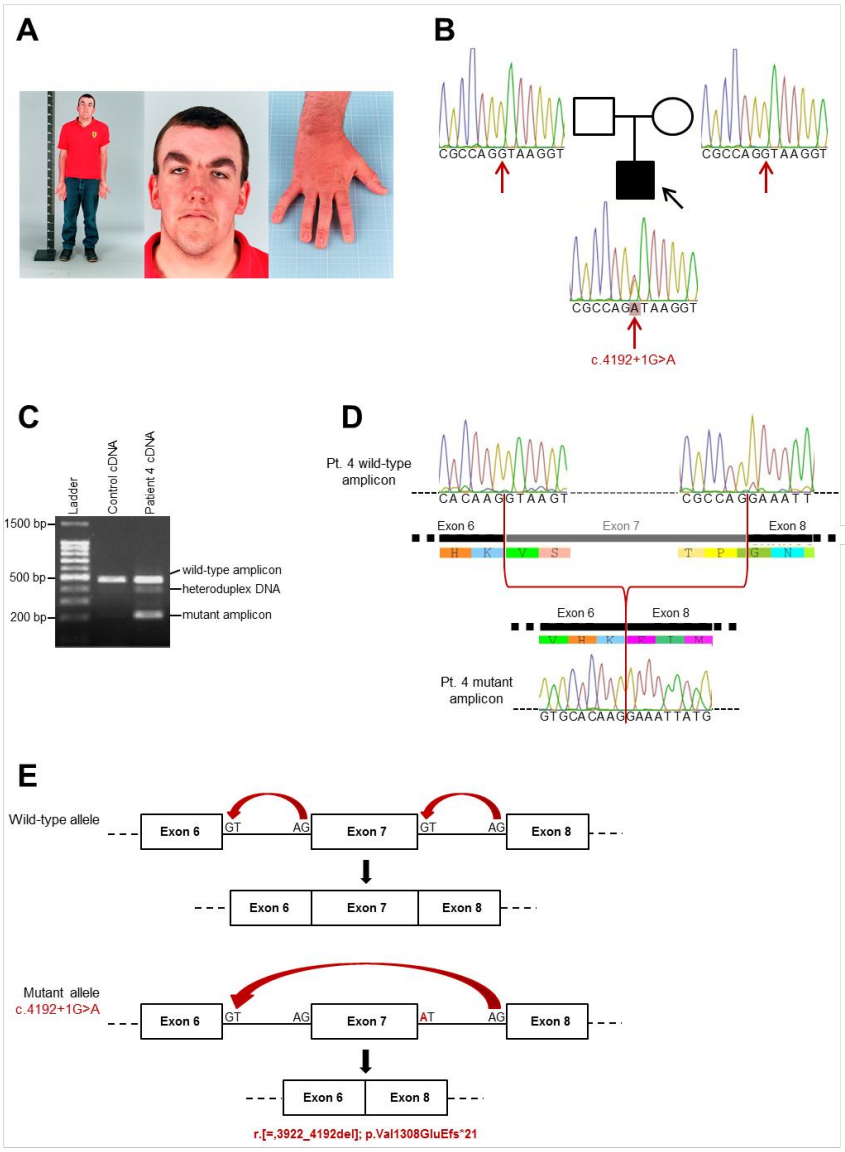

Figure 1. Identification and characterization of the NSD1 mutation in patient 3.
(A) Clinical photographs of Patient 3 at age 32 years of age, demonstrating long narrow face, prominent forehead and chin, downslanting palpebral fissures, arched eyebrows, deep set nails and statural disproportion.
(B) Pedigree with sequencing chromatogram illustrating the de novo NSD1 mutation (c.4192þ1G>A) Patient 3.
(C) Reverse transcriptase PCR amplification of NSD1 exons 6–8 using an unaffected control (single band 478 bp) and Patient 3 cDNA (wild-type 478 bp, heteroduplex, and alternatively spliced product 207 bp)
(D) Comparison of the sequencing results of the wild-type 478 bp PCR amplicon with that of the alternatively spliced 207 bp PCR amplicon.
(E) Schematic illustrating the effect of the mutation on the splicing of exons 6–8 of NSD1.

products were identified (Figure 1C). Sanger sequencing of the 207 bp band demonstrated a deletion of exon 7 from the transcript (r.[=,3922_4192del]); Figure 1D), leading to a premature

truncation of the NSD1 protein after exon 7 (p.Val1308Glufs*21). The larger 478 bp band represented normal splicing of exons 6–8, while the middle band was a heteroduplex product (Figure 1C). A schematic illustrating the effect of the mutation on splicing is shown in Figure 1E.

We recently identified a fourth patient with phenotypic features of Sotos syndrome including overgrowth, advanced bone age and typical facial gestalt, who also demonstrated extreme connective tissue laxity including loose redundant skin, bilateral hydronephrosis requiring ureterostomy, mildly dilated aortic root and moderate diffuse dilatation of the ascending aorta. Sanger sequencing of *NSD1* (NM_022455.4) revealed a novel truncating mutation in exon 5, c.3618_3619insGAGTT, p. (Arg1207Glufs*14). The clinical features and results of *NSD1* sequencing of the four patients are summarized in Table I.

Sotos syndrome is caused by haploinsufficiency of *NSD1* (Kurotaki et al., 2002). Hundreds of patients with Sotos syndrome have been reported in the literature with *NSD1* alterations including whole-gene deletions, partial-gene deletions and frameshift, nonsense, splice site and missense mutations (Douglas et al., 2003; Tatton-Brown et al., 2005; Turkmen et al., 2003). Truncating mutations of *NSD1* have been reported throughout the gene sequence and mutation hotspots have not been observed. The phenotype of *NSD1* mutation-positive individuals has been shown to be extremely variable with a broad range of clinical features.

Table I. Clinical features of the four Sotos syndrome with cutis laxa patients in our study.

Clinical Features	Patient 1	Patient 2	Patient 4	Patient 1
Birth weight	4030 g (90th)	4300 g (>97th)	2760 g (50th)	4580 g (>97th)
Birth length	53 cm (90th)	57 cm (>97th)	47.5 cm (50th)	54.9 cm (>97th)
Birth OFC	40 cm (>97th)	37.5 cm (>97th)	n/a	38.2 kg (>97th)
Current age	38 years	32 years	32 years	17 months
Weight	66 kg (50th)	71 kg (90th)	97 kg (97th)	17.15 kg (>97th)
Height	178.3 cm (50-75th)	168 cm (75th)	181.5 cm (75th)	85 cm (90-97th)
OFC	61 cm (>97th)	57 cm (75th)	59 cm (>97th)	51 cm (>97th)
Bone age	Not advanced at 21 months	Not advanced at 9 months, 6, 8 and 14 years	Not advanced at 4 months, 3, 5 and 9 years	Advanced bone age at 10 months
Intellectual function	Mild intellectual disability	Moderate intellectual disability	Moderate intellectual disability	Moderate global developmental delay,non-verbal
Skin	Redundant loose skin, improved with age	Redundant loose skin, improved with age	Redundant loose skin, improved with age	Redundant loose skin, normal wound healing
Skeletal	Pectus carinatum, Joint hypermobility	Pectus carinatum, Joint hypermobility, Mild thoraric kyphosis, Severe coxa valga, Plantar valgus deformities requiring orthoses	Pectus carinatum, Joint hypermobility, Statural disproportion (arm span:height ratio 1.07), Bilateral pes planus requiring orthoses	No pectus deformity, Joint hypermobility, High palate
Renal	None	Vesicoureteric reflux, Bilateral pelvicalyceal dilatation, Bilateral ureteric reimplantations	Megacystis, hydroureters and hydronephrosis, Bilateral ureteric reimplantation	Hydronephrosis, ureterostomy
Cardiac	Diffuse dilatation of the ascending aorta	Trivial PDA (no surgery)	Diffuse dilatation of the ascending aorta	Mildly dilated aortic root, moderately dilated ascending aorta
Other	Left talipes, High arched palate	High arched palate, 2,3 toe syndactyly	Bilateral inguinal herniae, Hypermetropia, Severe gastro-esophageal reflux, Redundant duodenal mucosal folds	Infantile spasms, Dilated cerebral ventricles, Constipation
NSD1 mutation	Exon 5 c.2894C>G p.(Ser965*)	Exon 5 c.2859dupT p.(Lys954*)	Intron 7 c.4192+1G>A r.[=,3922_4192del] p.(Val1308Glufs*21)	Exon 5 c.3618_3619insGAGTT p.(Arg1207Glufs*14)

In addition to the key diagnostic features (characteristic facial gestalt, learning disability and childhood overgrowth), which are present in >90% of patients, there are also major features such as cardiac anomalies, scoliosis, and advanced bone age which occur in >15% of patients (Tatton-Brown et al., 2005). There are many additional features (>30) which occur less frequently such as hypothyroidism, cataracts, conductive hearing loss, nystagmus and umbilical hernia (Tatton-Brown et al., 2005)

Given the variety of *NSD1* mutations which cause Sotos syndrome and the broad phenotypic variability of the disorder, analyses have been performed to search for genotype-phenotype relationships (Tatton-Brown et al., 2005; Turkmen et al., 2003). It has been suggested that patients with the 5q35 microdeletion have more pronounced intellectual disability and less overgrowth than patients with intragenic mutations (Douglas et al., 2003; Fickie et al., 2011; Tatton-Brown et al., 2005). However, no other significant genotype-phenotype correlations are evident and specific mutations have not been linked to either disease severity or particular clinical features (Tatton-Brown et al., 2005; Turkmen et al., 2003).

The four patients reported here with Sotos syndrome have significant features of connective tissue laxity and novel truncating mutations occurring in exon 5 (3 patients) or 8 (1 patient) (Table I). There is one other report in the literature describing a patient with ‗Sotos syndrome and cutis laxa' and this patient has a novel truncating mutation in exon 11 of *NSD1* (c.4558G>T; p.Glu1520*) (Cortes-Saladelafont et al., 2011). There are many previously reported truncating mutations in patients with Sotos syndrome across all functional domains of NSD1 and thus a genotype-phenotype correlation with connective tissue laxity is not readily apparent. In addition, there are reported patients without a marked

connective tissue phenotype and with mutations in close proximity to the novel exon 5 and 8 mutations identified in this study, as well as to the exon 11 mutation reported previously, which further supports the absence of a genotype–phenotype correlation for marked connective tissue laxity.

The identification of pathogenic *NSD1* mutations in all four patients reported herein highlights that marked connective tissue laxity can be part of the clinical presentation of Sotos syndrome and resolves the diagnostic dilemma presented by Robertson and Bankier (Robertson and Bankier, 1999), providing the molecular explanation of the three originally reported cases of ‗Sotos syndrome and cutis laxa'. Although structural cardiac abnormalities are a recognized association of Sotos syndrome, to the best of our knowledge aortic dilatation has not previously been reported and was observed in three of the patients reported here, the youngest being 17 months of age at the time of diagnosis. The prevalence of aortic dilatation may be underestimated as screening echocardiograms may not be performed in children with Sotos syndrome in the absence of cardiac signs or symptoms. Although the aortic dilatation has not been progressive to date in any of our patients, the long-term natural history is unknown, making screening and management recommendations challenging.

Acknowledgements

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University), Michael Brudno (University of Toronto), Dennis Bulman (University of Ottawa), and David Dyment (University of Ottawa). We would also like to thank Dr Katrina Tatton-Brown and the Childhood Overgrowth Collaboration. This work was funded in part by Genome Canada, Canadian Institutes of Health Research, the Ontario Genomics Institute, Ontario Research Fund, Genome Quebec and Children's Hospital of Eastern Ontario Foundation. The authors wish to acknowledge the contribution of the high-throughput sequencing platform of the McGill University and Genome Quebec Innovation Centre in Montreal. RLH is supported by an Ontario Graduate Student Scholarship.

Chapter 5: The Phenotype of Floating-Harbor Syndrome: Clinical Characterization of 52 Individuals with Mutations in Exon 34 of SRCAP

Preface

The following chapter consists of data previously published in the Orphanet Journal of Rare Diseases under the title "The Phenotype of Floating-Harbor Syndrome: Clinical Characterization of 52 Individuals with Mutations in Exon 34 of SRCAP" (PMID: 23621943) by Nikkel SM, Dauber A, de Munnik S, Connolly M, Hood RL, Caluseriu O, Hurst J, Kini U, Nowaczyk MJ, Afenjar A, Albrecht B, Allanson JE, Balestri P, Ben-Omran T, Brancati F, Cordeiro I, da Cunha BS, Delaney LA, Destrée A, Fitzpatrick D, Forzano F, Ghali N, Gillies G, Harwood K, Hendriks YM, Héron D, Hoischen A, Honey EM, Hoefsloot LH, Ibrahim J, Jacob CM, Kant SG, Kim CA, Kirk EP, Knoers NV, Lacombe D, Lee C, Lo IF, Lucas LS, Mari F, Mericq V, Moilanen JS, Møller ST, Moortgat S, Pilz DT, Pope K, Price S, Renieri A, Sá J, Schoots J, Silveira EL, Simon ME, Slavotinek A, Temple IK, van der Burgt I, de Vries BB, Weisfeld-Adams JD, Whiteford ML, Wierczorek D, Wit JM, Yee CF, Beaulieu CL; FORGE Canada Consortium., White SM, Bulman DE, Bongers E, Brunner H, Feingold M, Boycott KM.

My specific contribution to this paper was in performing the majority of molecular analysis on the patient cohort. I performed targeted PCR and Sanger sequencing of *SRCAP,* exons 31-34, for 83 individuals suspected to have FHS. Within this cohort, I identified *SRCAP* mutations in 46 instances, which corresponded to 46 of the 52

mutation positive cases reported in this paper (13 of these were also reported in the Chapter 2 manuscript). Amongst these, I identified two cases of parent-child transmission. I also performed PCR and Sanger sequencing to confirm that inheritance was *de novo* in 22 instances where parental DNA was available. I performed PCR and Sanger sequencing for the full *SRCAP* gene sequence in three individuals who were negative for *SRCAP* mutations in the targeted region, but who most closely resembled the FHS phenotype. In addition to contributing data to this paper I was involved in writing portions of the manuscript pertaining to my work and editing the final manuscript.

The specific contributions of each author to this paper are listed below.

Nikkel SM: Oversaw the project. Organized the collection of samples and survey data from clinicians and patients. Collected and sent patient samples for sequencing analysis. Wrote the manuscript. Generated the figures and tables. Responded to reviewers comments.

Dauber A: Assisted with organizing the collection of samples and survey data from clinicians and patients. Collected and sent patient samples for sequencing analysis. Co-wrote the manuscript. Assisted generating figures and tables.

de Munnik S: Performed PCR and Sanger sequencing which identified mutations in 6 of the 52 patients reported in this paper. Contributed to writing/editing the manuscript.

Connolly M: Assisted with organizing the collection of samples and survey data from clinicians and patients. Collected and sent patient samples for sequencing analysis. Co-wrote the manuscript. Assisted generating figures and tables. Contributed to writing/editing the manuscript.

Hood RL: (see above)

Caluseriu O, Hurst J, Kini U, Nowaczyk MJ, Afenjar A, Albrecht B, Allanson JE, Balestri P, Ben-Omran T, Brancati F, Cordeiro I, da Cunha BS, Delaney LA, Destrée A, Fitzpatrick D, Forzano F, Ghali N, Gillies G, Harwood K, Hendriks YM, Héron D, Hoischen A, Honey EM, Hoefsloot LH, Ibrahim J, Jacob CM, Kant SG, Kim CA, Kirk EP, Knoers NV, Lacombe D, Lee C, Lo IF, Lucas LS, Mari F, Mericq V, Moilanen JS, Møller ST, Moortgat S, Pilz DT, Pope K, Price S, Renieri A, Sá J, Schoots J, Silveira EL, Simon ME, Slavotinek A, Temple IK, van der Burgt I, de Vries BB, Weisfeld-Adams JD, Whiteford ML, Wierczorek D, Wit JM, Yee CF: Collected and sent patient samples for sequencing analysis.

Beaulieu CL: Project Manager of the FORGE Canada Consortium.

FORGE Canada Consortium: Provided funding for this project.

White SM: Assisted with organizing the collection of samples and survey data from clinicians and patients. Collected and sent patient samples for sequencing analysis. Contributed to writing/editing the manuscript.

Bulman DE: Oversaw Sanger sequencing validation experiments. Contributed to writing/editing the manuscript.

Bongers E: Oversaw Sanger sequencing validation experiments. Contributed to writing/editing the manuscript.

Brunner H: Oversaw Sanger sequencing validation experiments. Contributed to writing/editing the manuscript.

Feingold M: Assisted with organizing the collection of samples and survey data from clinicians and patients. Collected and sent patient samples for sequencing analysis. Contributed to writing/editing the manuscript.

Boycott KM: Oversaw the project. Contributed to writing/editing the manuscript. Responded to reviewers comments.

Abstract

Background: Floating-Harbor syndrome (FHS) is a rare condition characterized by short stature, delays in expressive language, and a distinctive facial appearance. Recently, heterozygous truncating mutations in *SRCAP* were determined to be disease-causing. With the availability of a DNA based confirmatory test, we set forth to define the clinical features of this syndrome.

Methods and Results: Clinical information on fifty-two individuals with *SRCAP* mutations was collected using standardized questionnaires. Twenty-four males and twenty-eight females were studied with ages ranging from 2 to 52 years. The facial phenotype and expressive language impairments were defining features within the group. Height measurements were typically between minus two and minus four standard deviations, with occipitofrontal circumferences usually within the average range. Thirty-three of the subjects (63%) had at least one major anomaly requiring medical intervention. We did not observe any specific phenotype-genotype correlations.

Conclusions: This large cohort of individuals with molecularly confirmed FHS has allowed us to better delineate the clinical features of this rare but classic genetic syndrome, thereby facilitating the development of management protocols.

Keywords: *SRCAP*, Floating Harbor syndrome, Phenotype, Short stature

Background

Floating-Harbor syndrome (FHS [MIM 136140]) is a rare disorder characterized by short stature with delayed bone age, deficits in expressive language and a distinctive facial appearance. The name of the syndrome is derived from the two hospitals where the first patients were reported over 35 years ago (Leisti et al., 1975; Pelletier, 1973). Recently, we used exome sequencing to investigate a cohort of 13 unrelated individuals with classic features of FHS and identified heterozygous mutations in *SRCAP* [MIM 611421] as causative of this disorder (Hood et al., 2012). All reported mutations were truncating and occurred between codons 2,407 and 2,517 in exon 34 resulting in loss of three C-terminal AT-hook motifs. *SRCAP* encodes a SNF2-related chromatin-remodeling ATPase that serves as a coactivator for CREB-binding protein, better known as CBP, the major cause of Rubinstein-Taybi syndrome (RTS). The disrupted interaction between these two proteins likely explains some of the clinical overlap between FHS and RTS (Robinson et al., 1988). The mechanism of disease in FHS is suspected to be dominant-negative (Hood et al., 2012) due to the non-random clustering of truncating mutations in the final exon that result in the loss of the major transactivation function of SRCAP located in a 655 residue C-terminal fragment, evidence that expression of a construct solely consisting of the CBP interaction domain of SRCAP strongly inhibits CREB-mediated transactivation in a dominant-negative fashion (Monroy et al., 2001), and the existence of patients with haploinsufficiency of *SRCAP* who do not have features of FHS (Hood et al., 2012; Leisti et al., 1975; Pelletier, 1973).

Many of the features of FHS are non-specific (short stature, delayed bone age, and language delays) and if the distinctive facial features are not recognized, this diagnosis can be difficult. Several years ago, Feingold (Feingold, 2006) provided a thirty-two year follow-up on the first reported patient accompanied by a review of the literature. He suggested that some of the patients reported to have FHS did not fit the classical description and likely had a different condition. With the availability of a molecular test, we are now able to further delineate the distinctive and recognizable features of this syndrome.

Methods

Subjects and Clinical Data

Individuals with a presumptive clinical diagnosis of FHS were invited to be part of this study. Clinical data was collated from three sources: FORGE Canada Consortium (Finding of Rare Disease Genes in Canada), based at the Children‘s Hospital of Eastern Ontario, the Manton Center for Orphan Disease Research at Boston Children‘s Hospital, and the Radboud University Nijmegen Medical Centre. All samples that were referred for analysis were accepted for the study. Approval of the study design was in compliance with the Helsinki Declaration and was obtained from each of the participating institutions‘ research boards. Free and informed consent was obtained from each study subject (or guardian, if appropriate) prior to enrollment. Recruitment e-mails were sent to all members of the Floating-Harbor syndrome support group. Interested families or physicians contacted the genetic counselor at the Manton Center. A medical history questionnaire was administered to the family or physician via telephone, which reviewed all pertinent medical and developmental history, as well as FHS-specific questions (see Additional file 1). Referring providers who submitted

cases directly to the above institutions completed the same questionnaire. In most cases, clinical photographs were available prior to molecular testing and the likelihood of finding a mutation was noted. Due to the diversity of the sample sources, there was wide pre-test probability of referred individuals actually having FHS, as this is a rare condition and most clinicians do not have familiarity with it. The clinical information from the first 13 subjects described by Hood et al. (Hood et al., 2012) was also included.

Molecular Analysis

Sanger sequencing of exons 31–34 of *SRCAP* was performed using DNA samples from individuals with suspected FHS (see Additional file 2). When available, parental studies were performed to determine *de novo* or inherited status. The clinical information, from twenty-seven individuals who did not carry a mutation in exons 31–34 of *SRCAP*, was used to help clarify key diagnostic features. For three individuals, who most closely resembled the FHS phenotype and for which no mutations were identified in exon 34, complete sequencing of the *SRCAP* gene was performed (primer sequences available on request).

Results and Discussion

Molecular

In total, 24 males and 28 females were identified with mutations in *SRCAP*; 39 new individuals and 13 previously reported (Hood et al., 2012). Ages at time of data collection ranged from two years to 52 years of age. The average age of diagnosis was 8 years. Two mother/daughter pairs (Arpin et al., 2012; White et al., 2010) and a number of the other subjects have been previously reported in the literature (Feingold, 2006; Garcia et al., 2012;

Hood et al., 2012; Pelletier, 1973; Reschen et al., 2012; White et al., 2010; Wieczorek et al., 2001). All the mutations identified in our cohort were truncating (nonsense or frameshift) alleles (Table 1). Two mutations are recurrent; the Arg2444* mutation was observed in about half (24/52) (including the original patient described by Pelletier and Feingold (Pelletier, 1973), while the Arg2435* mutation was present in approximately one quarter (13/52) of the individuals with FHS. In our original cohort of 13 patients with FHS we delineated the boundaries of the critical region to between codons 2407 and 2517. The extended cohort of molecularly-defined patients we present here extends the critical region to between codons 2389 and 2748, a further 249 amino acids in exon 34. Interestingly, the boundaries of this critical region are delineated by mutations observed in our two mother-daughter pairs (Table 1), however, the significance of this finding is unclear.

Facial Gestalt

The face of FHS is the most distinctive aspect of this syndrome (Figures 1, 2 and 3) and although there are changes with age, the cardinal features, as originally described (Leisti et al., 1975; Pelletier, 1973; Robinson et al., 1988), remain constant. The overall facial shape is triangular. The nose is narrow at the root and broadens to the tip. The columella is low hanging, nares are large and the philtrum is often short. The upper vermillion is typically thin and the lower lip is often everted. The lips tend to be in a horizontal plane at rest or when smiling. The eyes are frequently deep set and the eyelashes tend to be long. The ears can be low set and large in appearance. As seen in the photos, the FHS phenotype is more difficult to recognize in infancy.

Table 1. Mutations detected in exon 34 of *SRCAP* in individuals with FHS.

	c.DNA	Frequency (52)	Comments
Glu2389*	c.7165G>T	2	Mother/Daughter
Gln2407*	c.7219C>T	1	
Gln2407fs*35	c.7218_7219delTC	1	
Asn2410fs*32	c.7230insA	1	
Thr2425fs*17	c.7274insC	1	
Arg2435*	c.7303C>T	13	2nd Recurrent mutation
Ala2440fs*3	c.7316dupC	1	
Arg2444*	c.7330C>T	24	Most frequent Recurrent mutation
Pro2459fs*125	c.7374dupT	2	
Pro2459fs*16	c.7376delC	1	
Thr2512fs*5	c.7533_7534insAA	1	
Gln2517fs*11	c.7549delC	1	
Asn2618fs*11	c.7852insC	1	
Arg2748*	c.8242C>T	2	Mother/Daughter

Figure 1. Facial photographs of 6 females with FHS with the common Arg2444* mutation.

Figure 2. Facial photographs of 4 individuals with FHS of varying ages with the Arg2435* mutation.

Figure 3. Facial photographs of 7 individuals with FHS as examples of the other mutations.
A. A female with the Gln2407* mutation.
B. A male with the Ala2440fs*3 mutation.
C. A female with the Asn2618fs*11 mutation.
D. A female and male with the Pro2459fs*125 mutation.
E. A mother and daughter with the Arg2748* mutation.

Skeletal

Of the 17 individuals where thumb morphology was formally assessed, broad thumbs were only seen in 10 individuals indicating that they are a frequent but not mandatory finding in FHS. The differential diagnosis of broad thumbs includes Rubinstein-Taybi syndrome, where they are a cardinal feature. FHS is also in the differential, which is logical as SRCAP interacts with CBP. Other skeletal findings include broad first toes and brachydactyly. Broad fingertips are seen frequently, and the fingers are often described as being clubbed, although would be more accurately classified as having broad fingertips (Figure 4). Leisti et al. (Leisti et al., 1975) reported a right-sided pseudoarthrosis-type anomaly of the clavicle noted at age two in one of their patients. Four individuals in our series have uni- or bilateral clavicular anomalies including pseudoarthroses or hypoplasia. Two individuals have 11 pairs of ribs and four have hip dysplasia.

Growth

Where available, growth parameters were plotted on aggregate graphs. Thirteen of 49 individuals had birth weights less than the third percentile (Figure 5). For females, the maximum height was at the 20th percentile, with most data points between minus two and minus four standard deviations (SDs) (Figure 6). For the males, the height measurements varied more widely, with maximum height at the 25th percentile and two adult heights below four SDs (Figure 7). Occipito-frontal circumferences (OFC) were more variable, with most being well within the average range (Figures 8 and 9). Seven individuals had OFCs less than two standard deviations, and only one measurement was less than minus 3 SDs. This suggests relative sparing of head size in relation to stature. Body weights were not consistent to

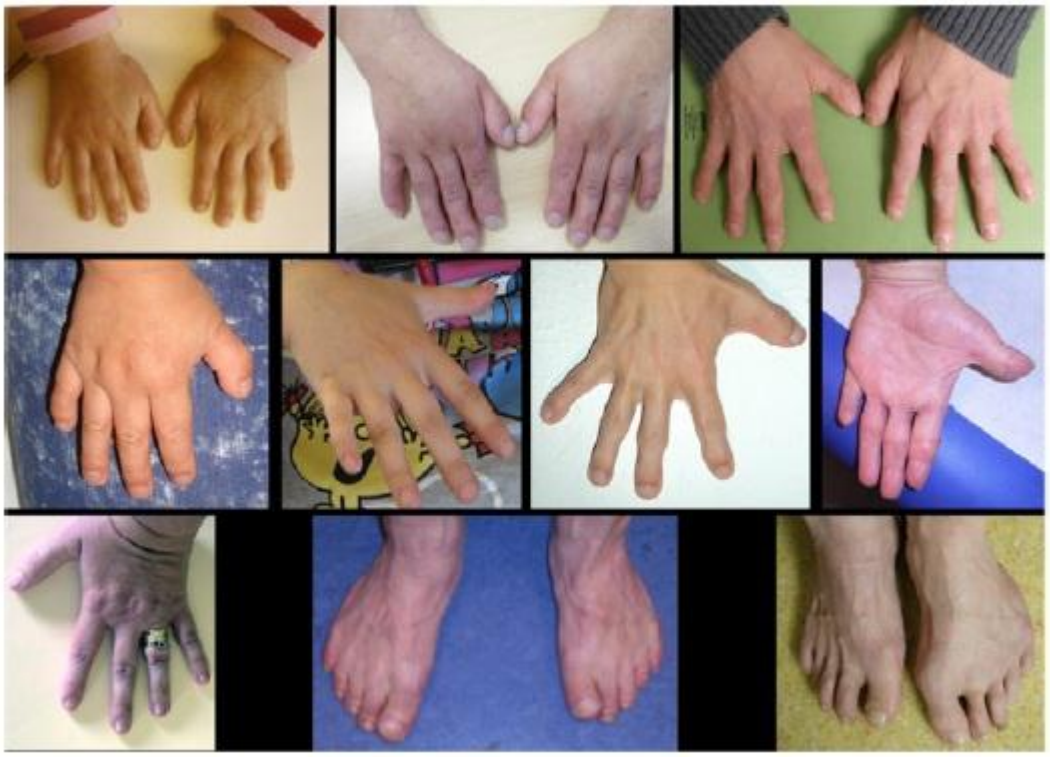

Figure 4. Hands and feet of individuals with FHS.
Clinical photos demonstrating the variability of features ranging from unremarkable to brachydactyly, short broad thumbs and big toes, broad fingertips.

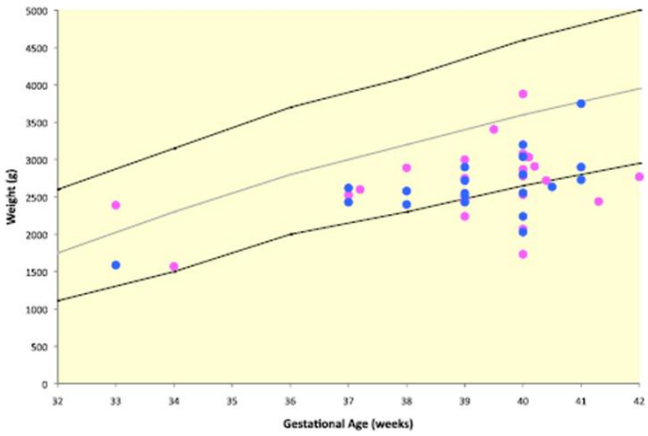

Figure 5. Birth weights of individuals with FHS.
Male birth weights - blue dots; Female birth weights – pink dots. The mean, 5th and 95th confidence intervals are indicated.

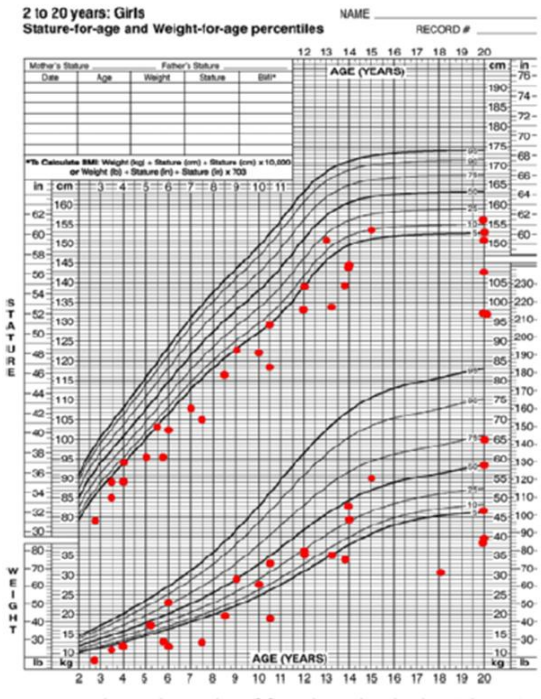

Figure 6. Height and weight of female individuals with FHS.
Each point represents a single individual‘s measurements at the time of data collection

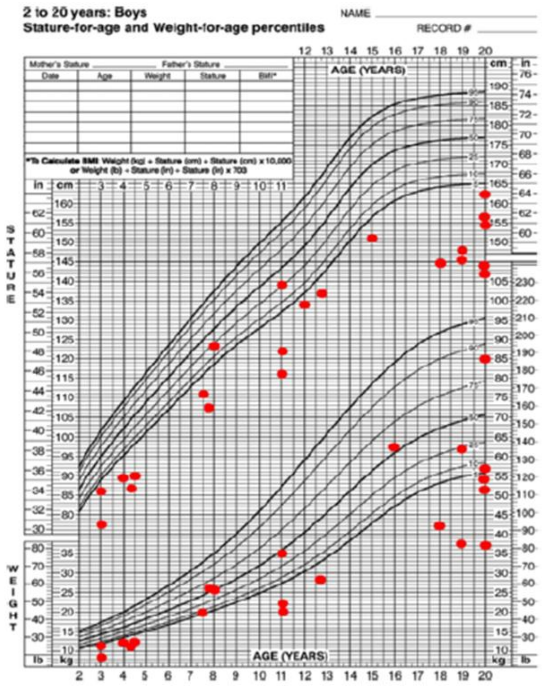

Figure 7. Height and weight of male individuals with FHS.
Each point represents a single individual‘s measurements at the time of data collection.

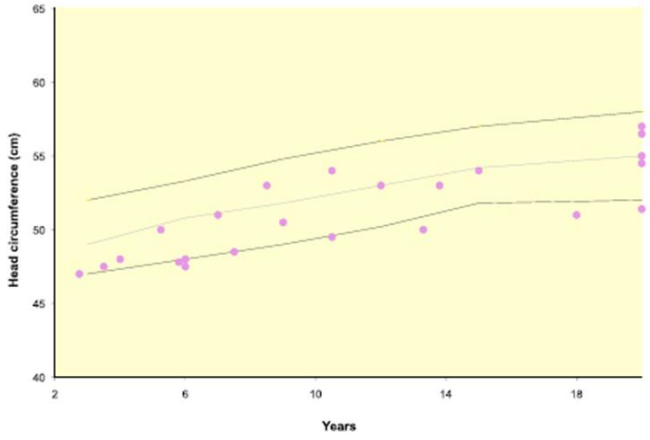

Figure 8. OFCs of females with FHS.
Each point represents a single individual‘s measurements at the time of data collection. The mean, 5th and 95th confidence intervals are indicated.

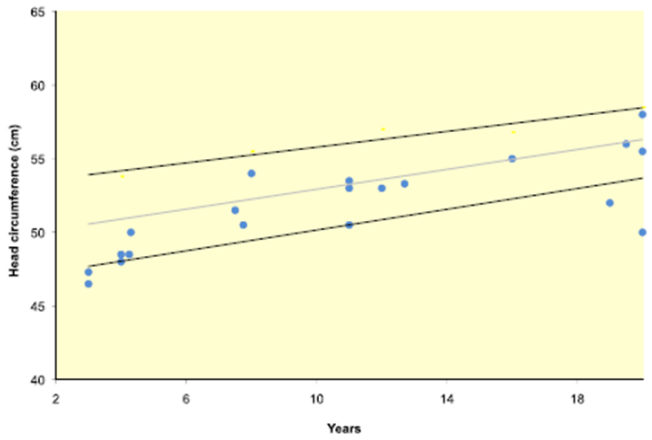

Figure 9. OFCs of males with FHS.
Each point represents a single individual‘s measurements at the time of data collection. The mean, 5th and 95th confidence intervals are indicated.

suggest a particular body habitus for this syndrome, and probably reflect the variability seen in the general population.

Bone Age and Endocrine

Bone age values were plotted against chronological age (Figure 10) and all values in subjects less than 8 years old showed significant delays. There were no data values between ages 8–10 years, however, the bone ages approached the chronological age or became advanced after age 10 years. A number of participants in this study have been on growth hormone (GH) therapy, which may alter the natural history of growth in this population. Two of our subjects have been assessed in more detail regarding this issue (Garcia et al., 2012; Wieczorek et al., 2001). Some GH treated individuals with FSH had documented GH deficiency, while others had modest responses to treatment despite normal levels of GH (Galli-Tsinopoulou et al., 2011; Garcia et al., 2012; Wieczorek et al., 2001). Early puberty has previously been reported (Stagi et al., 2007) in FHS and was documented in four individuals in our study. Some of our subjects are currently pre-pubertal, while others could not accurately report pubertal timing, rendering the data incomplete. However, early puberty could explain the advanced bone age seen in teenage individuals with FHS as well as contributing to shorter adult heights.

Structural Anomalies

A number of structural anomalies were detected in our cohort (Table 2), but no particular finding was seen with enough frequency to consider it a distinguishing feature of this syndrome. However, as some anomalies may affect clinical management, comprehensive screening is necessary in this population.

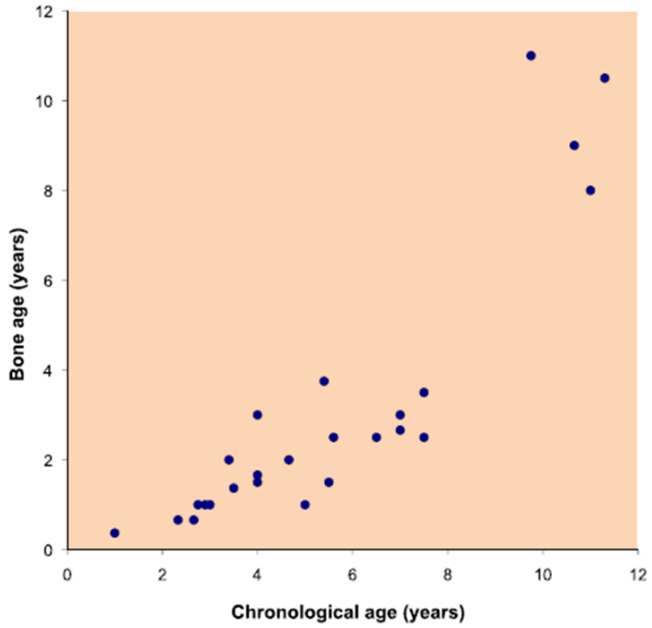

Figure 10. Bone age values plotted against chronological age for 25 individuals with FHS.

Table 2. Frequency of different clinical features in individuals with FHS.

Clinical feature	Frequency reported
Eyes	
-Strabismus	7/43
-Hyperopia	5/43
-Nystagmus	1/43
Ears	
-Recurrent otitis media/T-tube placement	6/52
-Hearing loss	9/52
-Cochlear anomaly	1/U
Other ENT	
-Cleft lip and pseudocleft lip	2/52
-Velopharyngeal insufficiency	2/U
-Choanal atresia	1/U
Dental Issues	
-Small teeth/increased spacing	13/38
-Cavities	6/38
-Malocclusion/underbite	3/38
Cardiac Malformation*	3/52
Gastrointestinal	
-Motility issues (reflux/constipation)	13/52
-Colonic stricture	1/U
-Celiac disease	2/52
Genitourinary	
-Cryptorchidism	5/24
-Renal/collecting system anomalies	7/U
Seizures	6/52
Hypothyroidism	2/52

U- denominator unknown

* Mild aortic coarctation, atrial septal defect, Tetrology of Fallot.

Voice Quality and Language

A high-pitched voice is often commented upon in individuals with FHS and was reported in 8/11 individuals. Others noted a nasal quality to the voice. An additional individual had documented velopharyngeal insufficiency (VPI), which may indicate that VPI is under-recognized. Expressive language delay is a cardinal feature of this syndrome, and was reported in all subjects. There was significant variability in severity with one individual who was bilingual, while another could only speak a few words as an adult. However, language development could potentially be hampered by the high frequency of recurrent otitis media and conductive hearing loss found in our cohort.

Cognition

The cognitive abilities in individuals with FHS range from average (IQ of 104) to significant intellectual impairment in a few instances. Most individuals had some modifications of their schooling (37/41). Obtaining full psychoeducational assessments on this cohort was beyond the scope of this study. However, when assessing global cognition in an individual with FHS, one must consider the language impairments, and in some instances sensory impairments, and adjust accordingly.

Behaviour

The caregivers, in comparison to physicians, who filled out the questionnaires, often commented upon behavioral issues for their children (5/25). It is likely that these issues are under-recognized in this population. Rigid mannerisms were observed (7/25), as were some

obsessive tendencies (e.g. skin picking). Parents often described their children as anxious individuals and attention deficit hyperactivity disorder (ADD or ADHD) was common (9/32). We acknowledge that the data collection in our study was incomplete as data was obtained from a number of sources without a centralized clinical assessment. We also recognize that the ethnic backgrounds of the study subjects were mostly Caucasian and that FHS may be more difficult to diagnosis in other populations. However, three individuals of Chinese origin were clinically diagnosed and identified to have mutations in *SRCAP*. In addition to growth and developmental issues, all of these subjects had classical FHS facial features, which were distinct from those of their family members.

Lastly, we evaluated for the presence of a genotype-phenotype correlation in FHS. Upon review of the clinical data, no clinical features were identified which discriminated between the different mutations. Given that all mutations cause truncation in a very defined area of the gene, this observation was not entirely unexpected.

Development of Diagnostic Criteria

The indication for analysis of the *SRCAP* gene was a presumptive diagnosis of FHS. The majority of those who underwent testing had short stature, delayed bone age, language delays and a distinctive facial appearance, usually with a prominent nose. Clinicians very familiar with FHS were able to distinguish those who ultimately carried a mutation in *SRCAP*, by his/her clinical information and facial photographs, from those who did not have a mutation. Those individuals who were referred who did not have a mutation detected often had dysmorphic facial features, but these were distinct from the classical FHS gestalt, making

facial features the defining characteristic of FHS. The nose is quite distinctive in FHS with its overall triangular appearance, the orientation and size of the nares and the low hanging columella. The linear orientation of the mouth, at rest or when smiling, is also an important defining feature. Additional consistent features of those who tested negative were a formal diagnosis of autism or head circumferences at a comparatively smaller OFC percentile than that for height. Russell-Silver syndrome and 3-M syndrome are included in the differential diagnosis for FHS, but we do not believe any of the patients in our negative group had either of these diagnoses.

Three individuals, whose phenotype most closely resembled FHS, had sequencing of the entire *SRCAP* gene to explore the possibility of mutations outside of exons 31–34. However, no mutations were detected. It is plausible that their phenotypes could be due to a mutation in another gene that codes for a protein, which interacts with SRCAP and CBP**.** Further research is needed to elucidate this possibility. Given that we have no evidence of genetic heterogeneity within our cohort, we conclude that the detection of a truncating mutation in exon 34 of *SRCAP* is a mandatory feature for a diagnosis of FHS. This is contrary to the report put forth by Le Goff et al. (Le Goff et al., 2013). Six of their nine subjects were found to have mutations in exon 34 within the boundaries we describe, and they proposed that their three mutation-negative individuals indicate genetic heterogeneity for FHS. However, we reviewed the two photographs of their *SRCAP*-negative patients and did not believe their facial features were consistent with a diagnosis of FHS.

A high frequency of associated anomalies was seen in this study (33/52 had at least one major anomaly requiring medical intervention); however, none are pathognomonic for FHS. This

large cohort of FHS individuals clarifies which clinical features are observed frequently and informs patient management guideline development. For example, celiac disease was initially thought to be more common in FHS, however, only 2 of 52 subjects had this finding. Although this is more than expected in comparison to the general population, the numbers are not such to suggest generalized screening. In comparison, genitourinary, ocular and dental issues were seen often enough to warrant investigations.

Suggestions for Management

Based on our clinical data, we suggest the following guidelines for the care of individuals with FHS:

1. Sequencing of *SRCAP* exons 31–34 in all suspected cases to confirm the diagnosis
2. Complete assessments of auditory and visual systems
3. Renal and urinary tract ultrasound
4. Neurologic assessment if there is a suspicion of seizures
5. Dental hygiene to prevent cavities and to monitor for malocclusion
6. Evaluation for growth hormone deficiency at baseline, to be repeated if loss of growth velocity occurs
7. Monitoring of bone age and pubertal timing. In cases of precocious puberty, referral to a pediatric endocrinologist
8. Psychoeducational assessments corrected for deficiencies in expressive language and sensory issues
9. Monitoring of behavioral disturbances and provision of early intervention

10. Counseling for families regarding recurrence risk (extremely low) and to offspring of individuals with FHS (50% chance).

Conclusions

We have assembled the largest cohort of individuals with Floating-Harbor syndrome; documenting pathogenic mutations in *SRCAP* in 52 affected individuals. Characteristic clinical findings include short stature, delayed bone age, distinctive facial features, expressive language delay, and broad thumbs. If the characteristic facial gestalt is not present, the likelihood of finding a mutation in *SRCAP* is very low. It is not uncommon for an individual with FHS to have additional anomalies and health complications that require medical intervention and thus comprehensive baseline screening and surveillance is warranted. In general, individuals with FHS are healthy and despite some impairments, enjoy a good quality of life.

Abbreviations

FHS: Floating-harbor syndrome; SRCAP: SNF2-related CBP activator protein; CBP: CREB-binding protein; SD: Standard deviation; OFC: Occipitofrontal circumference; GH: Growth hormone.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

SMN and AD were involved in design, acquisition and analysis of data, and drafting of the manuscript. SM was involved in design, acquisition and analysis of data, and made contributions to the draft of the manuscript. EB, HB, MC, OC, JH, UK, NM, SMW and MF were involved acquisition and analysis of data, and made contributions to the draft of the manuscript. RLH and DEB were involved in analysis of the data and made contributions to the draft of the manuscript. AA, BA, JEA, PB, TBO, FB, IC, BSC, LAD, AD, DF, FF, NG, GG, KH, YMCH, DH, AH, EMH, LHH, JI, CMJ, SGK, CAK, EPK, NVAM, DL, CL, IFML, LSL, FM, VM, JSM, STM, SM, DTP, KP, SP, AR, JS, JS, ELS, MEHS, AS, IKT, IvdB, BBAdV, JDWA, MLW, DW, JMW and CFOY were involved in acquisition of the data and made contributions to the draft of the manuscript. CMB and FORGE were involved in acquisition and co-ordination of the data. KMB was involved in design, analysis of data and critical revision of the manuscript. All authors read and approved the final manuscript.

Supplemental Material

Supplemental material includes two additional files: the questionnaire used to collect the clinical data, and the primer pairs used for sequencing exon 34 of *SRCAP*. These files can be found in Appendix 5.

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Chapter 6: The Defining DNA Methylation Signature of Floating-Harbor Syndrome

Preface

The following chapter consists of data previously published in Scientific Reports under the title "The Defining DNA Methylation Signature of Floating-Harbor Syndrome" (PMID: 27934915) by Hood RL, Schenkel LC, Nikkel SM, Ainsworth PJ, Pare G, Boycott KM, Bulman DE, Sadikovic B.

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I determined the initial FHS patient cohort to be used for the methylation array. Based on results of methylation array data analysis, I determined the four regions (two hyper- and two hypo-methylated regions) for clonal bisulfite sequencing. I determined the FHS patient and gender matched control cohorts to be used for bisulfite confirmation experiments and performed the clonal bisulfite sequencing and analysis which included the design of the bisulfite primers. I cloned the PCR amplicons for each region into the pGEM-T Easy Vector, transformed them into Top10 cells, picked >20 colonies for each sample and region, and subsequently sequenced the clones. I analyzed the Sanger output files and analyzed this data. I wrote and generated figures for the manuscript and responded to reviewers comments.

The specific contributions of each author to this paper are listed below.

Hood RL: (see above)

Schenkel LC: Analyzed the methylation array data. Generated figures corresponding to methylation array data. Wrote portions of the manuscript pertaining to methylation array analysis. Responded to reviewers comments.

Nikkel SM: Assisted with the collection of patient DNA samples.

Ainsworth PJ, and Pare G: Oversaw performance of the methylation array experiments. Contributed to writing/editing the manuscript.

Boycott KM: Oversaw the project. Determined the initial cohort to be used for methylation array. Contributed to writing/editing the manuscript.

Bulman DE: Oversaw the project. Oversaw Sanger sequencing validation experiments. Contributed to writing/editing the manuscript.

Sadikovic B: Oversaw the project. Based on the methylation array data, helped determine regions to use for clonal bisulfite sequencing confirmation analysis. Wrote portions of the manuscript pertaining to methylation array analysis and overall conclusions. Responded to reviewers comments.

Abstract

Floating-Harbor syndrome (FHS) is an autosomal dominant genetic condition characterized by short stature, delayed osseous maturation, expressive language impairment, and unique facial dysmorphology. We previously identified mutations in the chromatin remodeling protein SRCAP (SNF2-related CBP Activator Protein) as the cause of FHS. SRCAP has multiple roles in chromatin and transcriptional regulation; however, specific epigenetic consequences of SRCAP mutations remain to be described. Using high resolution genome-wide DNA methylation analysis, we identified a unique and highly specific DNA methylation ~~"e~~pi-signature" in the peripheral blood of individuals with FHS. Both hyper and hypomethylated loci are distributed across the genome, preferentially occurring in CpG islands. Clonal bisulfite sequencing of two hypermethylated (*FIGN* and *STPG2*) and two hypomethylated (*MYO1F* and *RASIP1*) genes confirmed these findings. The identification of a unique methylation signature in FHS provides further insight into the biological function of SRCAP and provides a unique biomarker for this disorder.

Main Text

Floating-Harbor syndrome (FHS; MIM 136140) is a rare autosomal dominant genetic disorder characterized by short stature, delayed osseous maturation, expressive language impairment, and facial dysmorphology (Leisti et al., 1975; Pelletier, 1973; Robinson et al., 1988; White et al., 2010). The facial features characteristic of FHS include: a triangular-shaped face, prominent nose, short philtrum, and a wide flat mouth with a thin upper lip. Individuals with FHS typically exhibit language deficits and some level of learning or intellectual disability. FHS usually occurs sporadically; however, a few autosomal dominant parent-child transmissions have been reported (Arpin et al., 2012; Lacombe et al., 1995; Penaloza et al., 2003; White et al., 2010). In 2012, we identified heterozygous truncating mutations in the final exon of *SRCAP* (SNF2-related CBP Activator Protein) as the genetic cause underlying FHS (Hood et al., 2012). This report was followed by a more in-depth clinical analysis of a large cohort of 52 affected individuals, which better defined both the mutation and the clinical spectrum of FHS (Nikkel et al., 2013). *SRCAP* encodes a large SWI/SNF-type chromatin remodeling ATPase, which was first identified in a yeast two-hybrid screen for CREB-binding protein (CREBBP) interaction partners (Johnston et al., 1999). Mutations in *CREBBP*, or its homolog, *p300*, are known to cause Rubinstein-Taybi syndrome, another short stature disorder that shares some features with FHS (Petrij et al., 1995; Roelfsema et al., 2005). Multiple coactivator roles have been described for SRCAP, in CREB and CREBBP-mediated, nuclear (steroid) hormone receptor, and Notch signaling pathways (Eissenberg et al., 2005; Johnston et al., 1999; Monroy et al., 2003). SRCAP has also been shown to immuno-precipitate as part of a large chromatin remodeling complex involved in the ATP-dependent displacement of the histone variant H2A by H2A.Z (Ruhl et

al., 2006; Wong et al., 2007). Additionally, the SRCAP-complex is known to function as a regulator of DNA damage and double strand break repair (Dong et al., 2014). While many roles for SRCAP have been described, the downstream impact of disease-causing mutations remains largely unknown.

DNA methylation, the addition of a methyl (CH3) group typically to a cytosine residue within CpG dinucleotides, is the most comprehensively described form of epigenetic modification. These epigenetic changes have an essential role in many nuclear functions, and in particular, transcriptional regulation and regulation of chromatin structure. In general, promoter regions with unmethylated CpGs are associated with a transcriptionally permissive chromatin state, whereas methylated CpG islands (high density CpG regions observed at approximately 50% of gene promoters) are associated with transcriptional repression. As such, the fine regulation of methylation constitutes an extra layer of control over gene expression.

Several genes, such as the DNA methyltransferases *DNMT1*, *DNMT3A*, and *DNMT3B*, have been linked with the regulation of methylation status through interaction with histone deacetylases (Bai et al., 2005; Fuks et al., 2001). Chromatin remodeling proteins, including two members of the same SNF2-ATPase chromatin family as SRCAP, Lymphoid Specific Helicase (HELLS) and X-linked alpha thalassemia/mental retardation (ATRX), have also been shown to impact methylation status (Dennis et al., 2001; Gibbons et al., 2000; Zhu et al., 2006). It is therefore possible that other chromatin remodeling proteins, such as SRCAP, may also impact methylation status. In this case, we hypothesized that the truncating mutations of *SRCAP* seen in FHS could cause differential methylation, and that these differences may

provide insight into the pathogenesis of this disorder. We therefore set out to determine if individuals with FHS have a unique DNA methylation epi-signature in their peripheral blood.

Results

Differential Methylation Attributed to Mutations in SRCAP

The methylation array data identified a unique methylation profile specific to FHS individuals. Within the FHS cohort of 18 affected individuals, methylation differences with respect to the particular *SRCAP* mutation were not observed. Additionally, there were no gender-specific global methylation differences or sex chromosome methylation changes found within the FHS cohort. Hierarchical clustering of significant probes ($p<0.01$, $F>50$, Estimate>15%) clearly demonstrated a unique methylation profile and sub-clustering for these patients compared with our large laboratory reference cohort (Figure 1). Overall, a higher frequency of hypermethylation was observed in individuals with FHS, regardless of genomic location and CpG island proximity (Supplementary Figure 1). A comprehensive list of differentially methylated regions shows 116 loci, 31 of which are hypomethylated and 85 are hypermethylated, with 73 of the 116 loci overlapping CpG islands and 8 overlapping CpG shore (Supplementary Table 1 and 2). The 116 DMRs represent regions with decreased cut-off criteria (methylation difference > 15%), and include 28 regions (Table 1) where the more restrictive criteria were used (methylation difference > 20%). Of the 28 identified FHS-specific methylation regions: 19 regions were found to have significantly increased methylation (20.01-32.23% higher methylation estimates) in the FHS samples compared to controls, and 9 regions were found to have significantly decreased methylation (20.17-

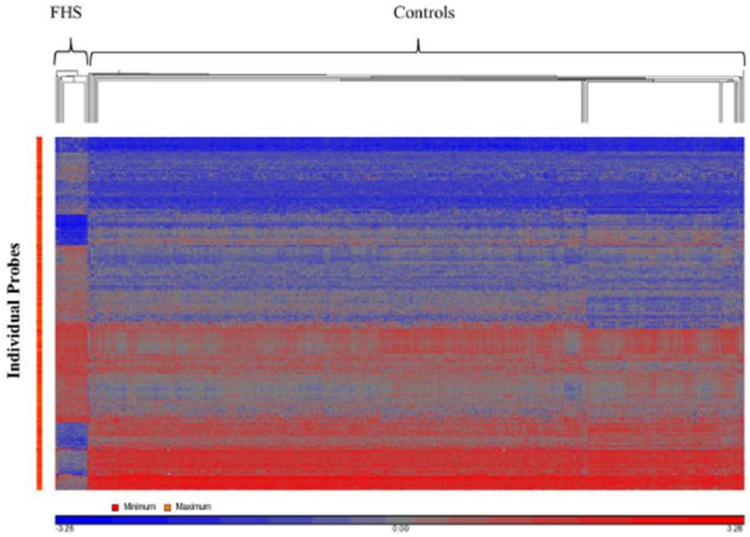

Figure 1. Euclidean hierarchical cluster analysis.
Hierarchical clustering of probes differentially methylated between FHS and controls demonstrating marked asymmetry of the 2 groups. Cases are represented in the columns and significant probes ($p < 0.01$) in the rows.

Table 1. Regions with significantly altered methylation (>20%) in FHS individuals identified by methylation array.

Location	Region Start [a]	Region Stop [a]	Region Length (bp)	# Probes	Methylation Estimate [b]	Nearest Gene	Overlapping CpG Island
chr1	174843744	174843981	238	3	0.2428	RABGAP1L (+)	No
chr1	27676195	27676662	468	3	-0.2197	SYTL1 (+)	Yes
chr1	1003116	1003539	424	4	-0.2773	RNF223 (-)	Yes
chr2	164204618	164205353	736	7	0.3223	FIGN (-)**	Yes
chr3	159557542	159558041	500	4	0.2235	SCHIP1 (+)	No
chr4	99064092	99064914	823	9	0.2394	STPG2 (-)**	Yes
chr4	46126056	46126458	403	7	0.2392	GABRG1 (-)	No
chr4	62382922	62383250	329	4	0.2065	LPHN3 (+)	Yes
chr4	11370304	11370882	579	5	0.2028	MIR572 (+)	Yes
chr5	110062333	110062847	515	7	0.2514	TMEM232 (-)	No
chr5	42944020	42944504	485	4	0.2232	FLJ32255 (-)	Yes
chr7	32358054	32358550	497	3	0.2215	LOC100130673 (-)	No
chr7	92672802	92673186	385	5	0.2094	SAMD9 (-)	Yes
chr8	81478162	81478344	183	3	0.2572	ZBTB10 (+)	No
chr8	39172010	39172130	121	6	0.2537	ADAM5 (+)	No
chr8	102235917	102236841	925	6	0.2057	ZNF706 (-)	Yes
chr9	139258514	139259084	571	3	-0.2055	CARD9 (-)	Yes
chr10	89167447	89167981	535	4	0.2216	LINC00864 (-)	No
chr10	50649656	50650258	603	5	0.2001	ERCC6 (-)	No
chr12	75784531	75785305	775	11	0.2007	GLIPR1L2 (+)	Yes
chr13	23412240	23412632	393	4	0.2263	BASP1P1 (-)	Yes
chr19	49222477	49224464	1988	12	-0.2017	RASIP1 (-)**	Yes
chr19	1063614	1064228	615	3	-0.2126	ABCA7 (+)	Yes
chr19	523290	523652	363	3	-0.2162	TPGS1 (+)	Yes
chr19	49133411	49133855	445	4	-0.2469	DBP (-)	Yes
chr19	8591354	8591786	433	4	-0.3725	MYO1F (-)**	Yes
chr20	62679245	62679723	479	3	0.2034	SOX18 (-)	Yes
chr22	50737968	50738900	933	4	-0.254	PLXNB2 (-)	Yes

Significantly methylated regions met the following criteria: Estimate value >20%, F value>50, and $p<0.01$.

a. hg19 Location; bp

b. Positive methylation estimate values indicate hypermethylation whereas negative values indicate hypomethylation in FHS subjects compared to controls.

** indicates regions used for bisulfite sequencing confirmation analysis.

Abbreviations: chr = chromosome; bp = base pair; (+) = sense strand; (-) = anti-sense strand.

37.25% lower methylation estimates). The majority of these regions were located within genes (n=17), including 7 in promoters, 6 intragenic and 4 in intronic regions (Table 2). Although only 1/3 of the array probes map to CpG islands, approximately 2/3 (19 of the 28) of the identified regions with differential methylation correspond to locations within CpG islands. Specifically, of these 19 regions, 10 were hypermethylated while 9 were hypomethylated, the latter representing all of the hypomethylated regions identified in the set of 28 and demonstrating a relative increase in the proportion of hypomethylated regions within CpG islands as compared to non-CpG islands for FHS individuals (Supplementary Figure 1).

DNA Methylation Age and Cell Counts Estimation

Average age acceleration was used to determine whether the DNA methylation age of a FHS individuals is consistently higher (or lower) than expected (as in controls). We observed that the average age acceleration did not significantly differ between FHS patients and controls (1.48 ±12 and 2.66 ±3.4, respectively). In addition, the cell type estimation showed no significant differences of blood cell composition between FHS patients and controls (Supplementary Figure 2). Taken together these results indicate that changes in DNA methylation observed in the FHS cohort cannot be attributed to differences in DNA methylation age and/or blood cell type.

Table 2. Genomic region distribution of the 28 differentially methylated regions (>20%) in FHS individuals.

	Within CpG island	Outside CpG island
Within gene		
Gene body	9	1
Promoter	3	4
Intergenic	7	4

No regions detected in CpG shores and shelves

Validation of Methylation Assay

Amongst the 28 regions found to be differentially methylated in FHS, two hypermethylated (*FIGN* and *STPG2*) and two hypomethylated (*MYO1F* and *RASIP1*) regions were selected based on robust methylation differences and statistical significance for comparative analysis of array data to bisulfite sequencing. Methylation array showed a mean of 32.23% and 23.49% hypermethylation in FHS individuals at the *FIGN* and *STPG2* gene loci, respectively (Table 1). Conversely, *MYO1F* and *RASIP1* genes loci showed a mean of 37.25% and 20.17% hypomethylation in the FHS cohort relative to the control cohort (Table 1). These loci were well represented on the array, with multiple probes spanning each of the respective differentially methylated regions. Methylation profiles showed consistent hypermethylation in FHS individuals as compared to controls for both *FIGN* and *STPG2* regions across 7 and 9 probes respectively, and consistent hypomethylation for *MYO1F* and *RASIP1* regions across 4 and 12 probes, respectively (Figure 2). In addition, samples from the individuals with FHS had a higher average methylation level over the *FIGN* and *STPG2* regions and a lower average methylation level over the *MYO1F* and *RASIP1* regions as compared with controls (Supplementary Figure 3).

Bisulfite sequencing analyses were performed to technically confirm the FHS-specific methylation profile by examining methylation status across *FIGN*, *STPG2*, *MYO1F,* and *RASIP1* regions (Figure 3). Bisulfite sequencing analysis across the *MYO1F* and *RASIP1* loci included 45 and 25 CpG sites, respectively. Consistent with the array findings, the average degree of methylation for FHS individuals across the *MYO1F* and *RASIP1* regions determined by bisulfite sequencing was correspondingly lower than for controls (Figure 2A,B). Additionally, the average percent methylation for *MYO1F* in FHS individuals was

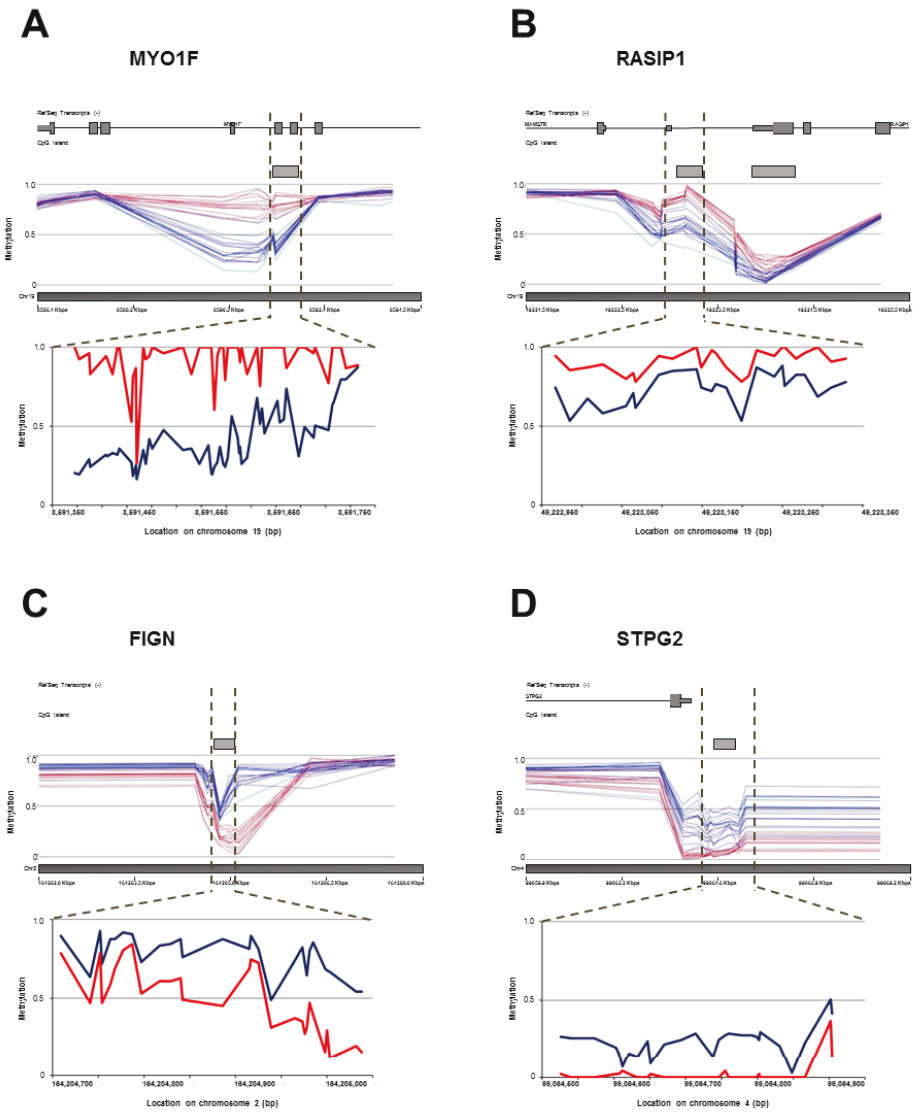

Figure 2. DNA methylation profiles in FHS.
Methylation level from 0 (not methylated) to 1 (100% methylated) is shown across regions with significantly altered methylation in FHS: hypermethylated regions (A) MYO1F and (B) RASIP1; and hypomethylated regions (C) FIGN and (D) STPG2. RefSeq genes and CpG islands tracks are annotated on top of the figures. The top image corresponds to methylation array data visualized using Genomic Browser Viewer (Partek). Red lines correspond to representative control sample data. Blue lines correspond to FHS individual data. The bottom image corresponds to average methylation based on bisulfite sequencing data. The red and blue lines indicate the average methylation for control and FHS patient sample, respectively. Dotted lines correlate chromosome location between top (array generated) and bottom (bisulfite sequence generated) images

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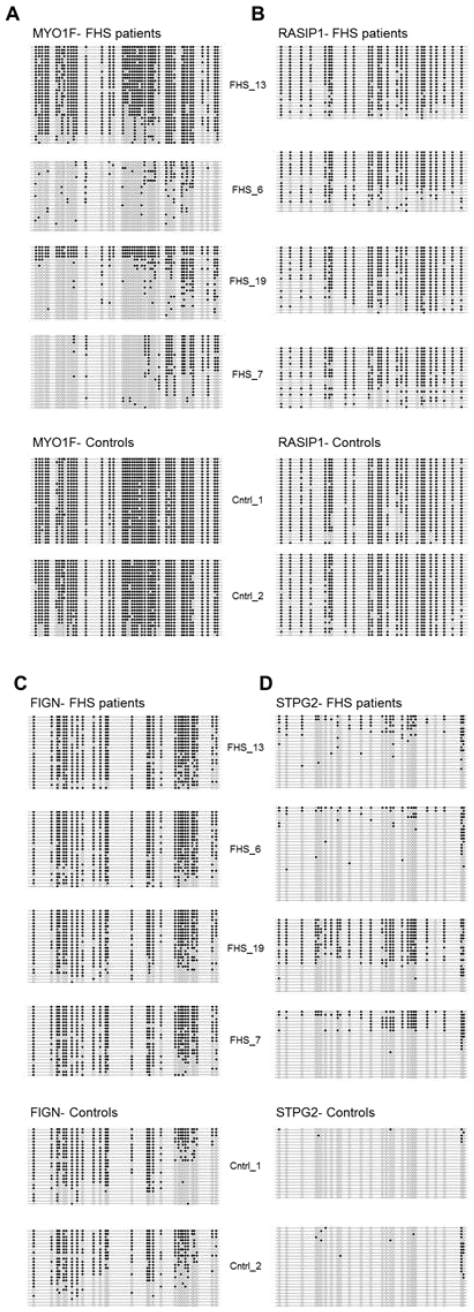

Figure 3. Methylation string diagrams of significantly altered regions in FHS individuals compared to controls.
String diagrams indicating methylation status across regions with significantly altered methylation in FHS: hypermethylated regions (A) MYO1F and (B) RASIP1, and hypomethylated regions (C) FIGN and (D) STPG2; for four FHS individuals (top) and two gender matched control samples (bottom). Each dot on the string indicates a CpG sequence, and potential site for methylation. Black dots indicate the CpG is methylated. Open dots indicate the CpG is un-methylated.

37% compared to 92% in controls (Figure 3A). For *RASIP1* the average percent methylation in FHS individuals versus controls was 73% and 91%, respectively (Figure 3B). The bisulfite data for these two regions supported the methylation array results, confirming that these two regions are hypomethylated in FHS individuals. For *FIGN* and *STPG2* regions, 29 and 27 CpG sites were examined, respectively. The bisulfite data demonstrated hypermethylation in FHS individuals versus controls (77% versus 49% for *FIGN*, and 24% versus 3% for *STPG2*; Figure 3C,D). The bisulfite data for the *FIGN* and *STPG2* regions also confirm similar levels of hypermethylation in FHS patients relative to the microarray findings (Figure 2C,D).

The results of the bisulfite data, which included one independent FHS patient that was not included in the microarray discovery cohort, corroborate the methylation data, confirm the existence of an epigenetic signature associated with FHS, and support the diagnostic utility of such an array-based approach.

Pathway Analysis

Pathway analysis, performed using the list of 116 differentially methylated genes (>15%; Supplementary Table 1) identified significantly enriched gene groups involved in a number of biological processes. More specifically, an over-representation of genes was found to be involved in synaptic transmission and the neurological system process (Supplementary Table 3). Additionally, an overall enrichment was found for genes associated with developmental processes. These findings suggest that altered methylation status may disrupt the expression of neurodevelopmental genes and may play a role in the pathophysiology of FHS.

Discussion

The identification of a unique methylation profile associated with FHS suggests that truncating mutations of SRCAP, the genetic cause underlying FHS, result in recurrent, locus specific, DNA methylation alterations. These may be the direct result of altered function of the truncated SRCAP protein; alternatively, they may be the result of a secondary compensatory mechanism in response to altered SRCAP function. Regardless of the underlying molecular mechanism, these findings suggest a role for SRCAP in the regulation of genomic DNA methylation, which in turn may regulate the expression of specific genes.

Rare genetic diseases caused by mutations in genes involved in the regulation of methylation have been recognized for more than 15 years. For example, mutations in the epigenetic regulatory gene *ATRX* were shown to cause X-linked mental retardation with α-thalassemia (ATRX syndrome) in 1995 (Gibbons et al., 1995) and, in early studies, disease-causing mutations were shown to alter the methylation patterns of several repetitive genomic regions including Y-specific satellite and subtelomeric repeats (Gibbons et al., 2000). Mutations in the histone H3 lysine 4 demethylase, KDM5C, were identified to cause an X-linked form of intellectual disability syndrome in 2005 (Jensen et al., 2005). More recently, patients with mutations in KDM5C were studied using a DNA methylation array approach, providing evidence of recurrent global DNA methylation defects in the peripheral blood as well as post-mortem brain tissue samples of these individuals (Grafodatskaya et al., 2013). Most recently, a unique methylation epi-signature was reported for Sotos syndrome, a rare overgrowth disorder caused by mutations in the *NSD1* gene, encoding histone H3 lysine 36 methyltransferase (Choufani et al., 2015). This study utilized the same high resolution

methylation array used here and was able to distinguish individuals with Sotos syndrome (secondary to pathogenic NSD1 mutations) from individuals with non-pathogenic mutations of NSD1, as well as from cases of the clinically similar disorder Weaver syndrome, caused by mutations in the histone methyltransferase *EZH2* (Enhancer of Zeste, Drosophila, Homolog 2). This field is currently in its infancy and we anticipate that similar genome-wide epi-signatures will be characterized for this emerging group of rare diseases in the years to come. How the epigenetic consequences of these disease-causing mutations actually result in the rare disease itself is not well understood. It may be anticipated that the methylation alterations could result in differences in transcriptional regulation. For example, hypomethylation in a gene promoter CpG island may result in increased transcription, whereas hypermethylation may result in decreased transcriptional activity. Our pathway analysis for FHS, and that recently reported for Sotos syndrome (Choufani et al., 2015), suggest impact on genes that might be relevant to the cardinal developmental processes disrupted in these rare diseases; however, further research is necessary to fully understand the downstream consequences of these methylation alterations, particularly given that their impact is predicted to be cell-, tissue- and developmental timing- specific. Such data will provide new insight into the pathogenesis of these developmental disorders.

Here we presented a pipeline to sensitively and specifically detect regional methylation differences in a cohort of patients. Our analysis employs a region detection algorithm that controls for the most common limitations of the array. Probes containing SNPs may affect the assessment of DNA methylation when analyzing single-probe methylation, as described in the study by Price *et al*. (Price et al., 2013). One way to address this is to employ a region detection algorithm, which relies in multiple adjacent probes (at least 3) to meet significant

stringent p-value, F-value and mean methylation cut off criteria. A single polymorphic probe would not be expected to significantly affect the methylation results across a multi-probe region. Furthermore, the difference in performance of Infinium I and Infinium II was demonstrated to be minimum and so do not significantly affect differential methylation detection (Bibikova et al., 2011).The authors found an average shift on beta values of 2 to 8% on Infinium II assay probe sets. In fact, similarly to the SNP related effects, a region detection algorithm is used in part to address this feature. Considering that our analysis include a cut off of at least 15% methylation difference, and employs a multi-probe region detection algorithm, the effect of the probe chemistry is expected to be negligible. Age-related and cell composition-related changes in the DNA methylation have been observed including our recent description of global DNA methylation changes with age using the Illumina 450K array (Schenkel et al., 2016a). Some possible explanations for this include differences in composition of nucleated cell subtypes in premature births versus newborns and children (ie. increased levels of nucleated red blood cells; differences in the levels of leukocyte subtypes), or alternatively global methylation differences associated with maturation of leukocytes. For this reason our methylation analysis includes a large reference cohort with a broad range of ages (from 1 month to 62 years). This allows the exclusion of sites with methylation changes related to age, and consequently cell type, as such genomic regions present with hyper variable DNA methylation profiles in the reference cohort, and would not meet the statistical cut off criteria. Finally, the ability of our algorithm to sensitively and specifically detect methylation differences across this patient cohort was demonstrated by a confirmatory analysis on a subset of these regions using the ―gold standard" Clonal Bisulfite Sequencing.

In conclusion, our findings demonstrate the existence of a unique DNA methylation epi-signature in the peripheral blood of individuals with FHS. The epi-signature provides further insight into the FHS disease etiology, and represents a potential diagnostic biomarker for this disorder. Identification of similar types of epi-signatures in constitutional genetic syndromes will further expand our understanding of these diseases and facilitate the efficient diagnosis of these individuals.

Methods

Methylation Array and Analysis

Global methylation status of 18 individuals with mutation-confirmed FHS was performed using the Infinium HumanMethylation450 Beadchip (Illumina) methylation arrays at the Genetic and Molecular Epidemiology Laboratory at McMaster University, according to manufacturer‘s instructions. Blood-derived DNA samples were obtained from 9 FHS individuals with the most common FHS-causing *SRCAP* mutation (c.7330C>T; p.Arg2444*) and 9 additional FHS individuals with distinct mutations in SRCAP (c.7165G>T; p.Glu2389*, c.7218_7219delTC; p.Gln2407fs*35, c.7219C>T; p.Gln2407*, c.7282dupC; p.Arg2428fs*15, c.7303C>T; p.Arg2435*, c.7316dupC; p.Ala2440fs*3, c.7549delC; p.Gln2517fs*5, c.8117C>G; p.Ser2706*, and c.8242C>T; p.Arg2748*). FHS cohort included 6 males and 12 females with mixed ethnicity, which ranged in age from 2 to 42 years (Supplementary Figure 4). The FHS individual cohort was compared to an unmatched reference control cohort of 361 individuals (151 females and 210 males) of mixed ethnicity, with average age of 8.5 years (0-62 years; Supplementary Figure 4). Our reference cohort included individuals that were previously preselected from a larger cohort of about 1000

individuals across the broad range of age, sex and ethnicity distribution. The methylation analysis of these individuals was performed in the same facility as patients and same data processing pipeline was used. Based on the individual analysis (1 sample vs cohort) these reference controls showed no significant changes in DNA methylation relative to the entire reference cohort. This analysis takes into account the fact that significant portion of genomic DNA methylation is hyper-variable across individuals (including age-related hyper-variable regions). Such regions with the normal inter-individual and/or age-related methylation variability would not produce significant p-values when comparing an individual or a patient cohort to a reference. Therefore, this analytical approach is designed to take into consideration methylation variability including sex and age, while taking advantage of analytical power of a large reference control database and focuses on identification of unique, non-age/sex variable DNA methylation changes in individual patients.

Methylation array coverage included >485,000 individual methylation sites, 99% of RefSeq genes and 96% of annotated CpG islands. Methylation values were generated using the Illumina Genome Studio Software, and data (.idat files) containing β-values were imported in the Partek Genomic Suite (PGS) software. Data (.idat files) were normalized using the PGS Genome Studio Normalization Algorithm that includes background subtraction and control normalization (normalized value = original value * target mean/control mean), while removing individual probes with poor signal intensity. An ANOVA test was performed to determine the methylation estimate (net methylation difference in FHS individuals as compared to controls) and generate probe-level statistics, including p-value (t-test), and F value (signal to noise ratio). Genomic regions with significant DNA methylation patterns were identified that met the following statistical criteria: (1) minimum of 3 consecutive

probes with significant methylation change $p<0.01$; (2) mean F-value across the region >50; and, (3) methylation estimate value differing by more than 20%. Probes on the X chromosome were further analyzed by comparing sex matched FHS individuals and controls. Significant regions were mapped against the CpG islands and gene promoter regions using Hg19 as reference genome. Data was visualized using PGS genomic browser. Lastly, regions with the most significant methylation changes were annotated in reference to the location of the CpG islands and distance to gene promoters. This analytical methods have been developed and validated previously (Kernohan et al., 2016; Schenkel et al., 2016b) and were performed in accordance with the relevant REB guidelines and regulations.

Age and Cell Composition Prediction

DNA methylation age and blood cell composition measures derived from the DNA methylation data were performed using the Epigenetic Clock software developed by Horvath, 2013 (Horvath, 2013). Briefly DNA methylation age, defined as predicted age, was calculated based on 21,369 CpG probes that were present both on the Illumina 450K and 27K platform and had fewer than 10 missing values. Age acceleration was measured by the difference between predicted age and chronological age. Blood cell proportions of CD8 T cells, CD4 T cells, natural killer cells, B cells, monocytes and granulocytes were estimated as described by Houseman *et al.* (Houseman et al., 2012).

Clonal Bisulfite Sequencing and Analysis

Blood-derived DNA samples from four FHS probands (three from the original array and an independent FHS sample) and two control subjects were bisulfite converted using the EZ

DNA Methylation-Direct Kit according to manufacturer‘s instructions (Zymo Research). Methylation-specific primers were designed for each region using the software program MethPrimer (Li and Dahiya, 2002). Converted DNA was PCR amplified using methylation primer pairs specific to each gene region and the resulting PCR amplicons were subcloned into the pGEM-T Easy Vector (Promega). For *MYO1F*, a nested-primer PCR strategy was used for amplification. Recombinant vectors were transformed into Top10 chemically competent *E. coli* cells (ThermoFisher). Transformant colonies were picked for clonal PCR amplification and subsequently Sanger sequenced. Chromatogram sequencing results for ≥20 clones were manually analyzed and string diagrams were generated for each affected individual and region.

Methylation Array Pathway Analysis

The 116 differentially methylated genes identified by methylation array (Supplementary Table 1) were assessed using the pathway analysis tool in the Partek Genomics Suite software. Briefly, statistical analysis included Fisher Exact test and Chi Square test, and was restricted to functional groups at least two genes. Results show the Enrichment p-value (*p*-value of the Fisher Exact test and Chi Square test reflective of the number of the genes in vs. not in the list or functional group) and the Enrichment score (negative log of the enrichment *p*-value; a high score indicates that the genes in the functional group are overrepresented in the gene list).

Ethical Approval and Consent to Participate

The CHEO Research Ethics Board approved the project. All analytical methods were performed in accordance with the relevant REB guidelines and regulations. All patients provided a written informed consent before their inclusion in the study, in accordance with the Declaration of Helsinki.

Supplementary Material

Supplementary material includes three tables and four figures and can be found in Appendix 6.

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Author Contributions

R.L.H. designed and performed bisulfite validation experiments and wrote the manuscript. L.C.S. analyzed the methylation data and co-wrote the manuscript. S.M.N. performed clinical evaluation of patients. G.P and P.J.A oversaw DNA methylation analysis. K.M.B. performed clinical evaluation of patients and oversaw study. D.E.B. oversaw bisulfite validation experiments. B.S. oversaw methylation array analysis and co-wrote the manuscript and oversaw the study. All authors gave final approval of the manuscript.

Competing Financial Interests Statement

The authors declare no competing financial interests

Chapter 7: Discussion

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The advent of high-throughput sequencing technologies represents one of the greatest medical advancements of the 21st century in the field of rare disorders. These technologies have revolutionized and advanced genetic research and molecular diagnostics for these conditions in ways that only other disruptive techniques including, Southern blotting, PCR amplification and Sanger sequencing, have done. The identification of disease-causing mutations by high-throughput sequencing approaches have not only accelerated our ability to identify novel disease genes, but it has also opened up a realm of research focussed at gaining a better understanding of the clinical spectrum and biology underlying each respective genetic disorder.

In this study, three rare syndromes impacting stature/growth were examined to gain insight into this subset of rare disorders. The molecular mechanism of FHS, Weaver syndrome, and Sotos-like syndrome with cutis laxa were delineated using exome sequencing. Next, the clinical spectrum of two of these disorders, FHS and Sotos syndrome, was further refined. Finally, a combined high resolution methylation array/clonal bisulfite sequencing approach was used to determine how FHS-causing mutations impact global methylation patterns.

While the exome sequencing approaches we utilized in these studies were ultimately successful in identifying the underlying genetic cause of the three rare disorders of stature studied, it should be noted that not all exome sequencing analyses succeed in this pursuit. As previously mentioned, exome sequencing involves the selective capture and sequencing of only the protein-coding regions of the genome, and while the vast majority of disease-causing variants are anticipated to be contained in these regions, not all disease-causing mutations are. Additionally, exome sequencing involves a hybridization-based capture and PCR

amplification steps, which can result in sequencing bias. As such, a whole genome sequencing approach may prove successful in determining the genetic basis underlying a disorder in instances where exome sequencing is not.

Molecular Insights into Rare Disorders Impacting Stature and Growth

Floating Harbor Syndrome is Caused by Mutations in the Final Exon of *SRCAP*

The results of this study identified for the first time that heterozygous truncating mutations in *SRCAP* is the genetic cause of Floating-Harbor syndrome (FHS) (Hood et al., 2012). All five of the patients that underwent exome sequencing were found to have heterozygous truncating mutations clustering in the final (34^{th}) exon of *SRCAP*. Subsequent Sanger sequencing of the final exon of *SRCAP* in an expanded cohort identified mutations in eight additional unrelated FHS patients. Following identification of *SRCAP* as responsible for FHS, we then focused on establishing a more extensive spectrum of mutations by screening additional patients from national and international collaborators. We collected clinical information and samples from 83 referred patients. Truncating mutations of *SRCAP* were identified in an additional 39 individuals (52 total), including 2 instances of mother-daughter transmission (Nikkel et al., 2013). For all 52 individuals, the mutations disrupted the translational reading frame and were restricted to a small 359 amino acid coding region within the final exon of *SRCAP* (Figure 1).

Thirty-seven patients from this cohort did not have a mutation in exon 34 of *SRCAP*; this finding was supported by detailed review of clinical photographs by our collaborator Dr. Sarah Nikkel who was blinded to molecular results and scored these patients as not

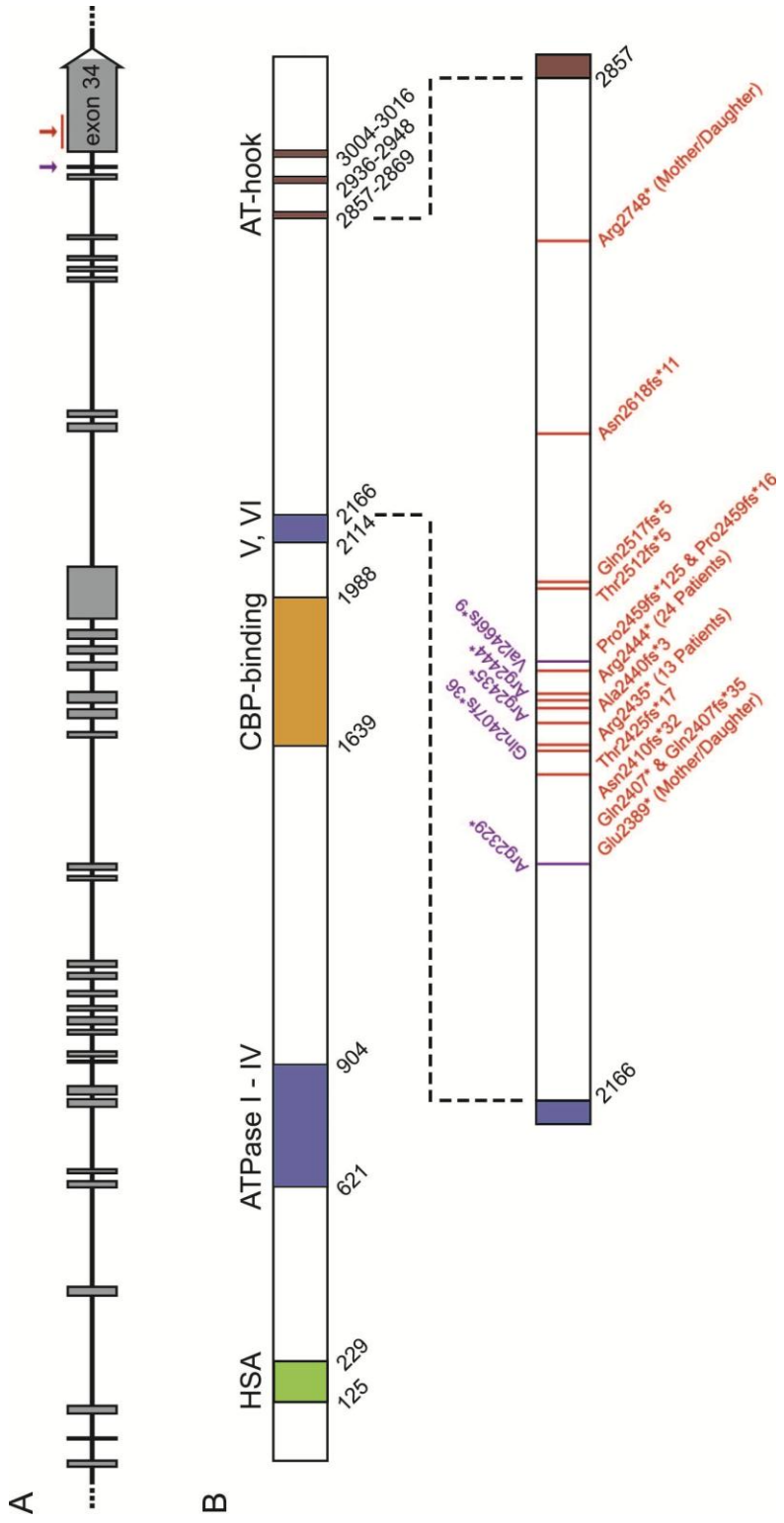

Figure 1. FHS-causing mutations in SRCAP.
(A) Intron-exon structure of SRCAP. Exon 34 mutation cluster is indicated by a red bar and arrow. Exon 33 mutation is indicated by a purple arrow.
(B) Domain architecture of SRCAP indicates amino acid positions of recognized domains and FHS-causing mutations. All probands are heterozygous for truncating mutations at the positions shown. Mutations indicated in red (52) were identified in Hood *et al.* (2012) and Nikkel *et al.* (2014) studies. Mutations indicated in purple (5) were identified by Seifert *et al.* (2014).
The ATPase domain of SRCAP is divided into two sections, one containing conserved motifs I-IV and one containing V-VI. The following abbreviation is used: HSA, Helicase-SANT-associated domain.
Figure modified from (Hood et al. 2012).

completely typical for FHS. We selected three whom were thought to be phenotypically most FHS-like and sequenced the entire coding region and flanking intron-exon boundaries of *SRCAP* and no mutations were detected. These results supported the hypothesis that FHS-causing mutations are clustered in the 3‘ end of *SRCAP*. Subsequently, a study by Seifert *et al.* (2014) identified five additional cases of FHS caused by truncating *SRCAP* mutations (Seifert et al., 2014). Interestingly, while their findings supported our conclusion that FHS-causing mutations are clustered, they identified one case of FHS caused by a mutation in exon 33 of *SRCAP*. These findings were consistent with the idea that FHS-causing mutations may be exclusively clustered between the second ATPase domain and the AT-hook DNA binding motifs of SRCAP, rather than exclusively within *SRCAP* exon 34 (Figure 1).

SRCAP is composed of several functional domains including an N-terminal Helicase, SANT-associated (HSA), a bi-partite SNF2-like ATPase, and three C-terminal AT-hook motifs (Figure 1B; Chapter 2 Figure 2). Additionally, it contains a central CBP interaction domain (Johnston et al., 1999). The mutations of *SRCAP* shown to cause FHS are all clustered within a 419 amino acid residue region, from amino acids 2329-2748, which occur between the second ATPase domain and the AT-hook DNA binding motifs (Figure 1) (Hood et al., 2012; Nikkel et al., 2013; Seifert et al., 2014).

SRCAP encodes a 3230 amino acid SWI/SNF-type chromatin remodeling ATPase, with a predicted molecular mass of 344 kDa, which was first identified in a yeast two-hybrid screen for CREB-binding protein (CREBBP/CBP) interaction partners (Johnston et al., 1999). As previously described, mutations in *CREBBP* or its homolog, *p300* were identified as the genetic cause underlying the phenotypically very similar Rubinstein-Taybi syndrome (Petrij

et al., 1995; Roelfsema et al., 2005). The interaction of CBP and SRCAP may provide a basis for the long-recognized phenotypic overlap between these two disorders.

SRCAP has been described as having multiple coactivator roles in CREB and CBP-mediated, nuclear (steroid) hormone receptor, and Notch signaling pathways (Eissenberg et al., 2005; Johnston et al., 1999; Monroy et al., 2003). SRCAP has also been shown to co-immunoprecipitate in a large complex that catalyzes ATP-dependent displacement of the histone variant H2A by H2A.Z (Ruhl et al., 2006; Wong et al., 2007). More recently, in 2014, a novel role for the SRCAP complex as a regulator of DNA damage and double strand break repair was described (Dong et al., 2014). How mutations in *SRCAP* impact any of these biological functions and result in the clinical presentation of FHS is not yet well understood.

Further insight into how the mutations in SRCAP cause FHS can be garnered from population and disease datasets. The Database of Genomic Variants lists one entry of a large deletion encompassing *SRCAP* in a participant of the HapMap project who had no obvious disease (Locke et al., 2006) and a second entry of another healthy middle-aged male who has an in-frame deletion encompassing the first coding exon of *SRCAP* (de Smith et al., 2007). Exome Variant Server (EVS) currently reports DNA sequence data from a total of 6497 genomes from normal individuals (without congenital disease) enrolled in the National Heart, Lung, and Blood Institute (NHLBI) project. This data includes 189 missense variants within the coding region of *SRCAP*. These variants occur indiscriminately throughout *SRCAP*, including 20 located between amino acids 2329-2748 which is within the ‗FHS mutation region' (Table 1). Additionally, a known frameshift mutation (c.2896_2900delCGGCA; p.Arg966fs*15), located between sequences encoding the first ATPase domain and the CBP-binding domain does not result in FHS. This finding suggests that the disease-mechanism in

Table 1. Missense variants located within the 'FHS mutation region' of *SRCAP* as reported in Exome Variant Server.

EVS reported variant	Genotypic frequency of variant
c.7121C>A; p.Thr2374Asn	AA=0/AC=11/CC=6486
c.7286G>A; p.Cys2429Tyr	AA=0/AG=1/GG=6496
c.7334C>T; p.Pro2445Leu	TT=0/TC=1/CC=6496
c.7354G>A; p.Ala2452Thr	AA=0/AG=3/GG=6491
c.7378G>A; p.Val2460Ile	AA=0/AG=1/GG=6486
c.7414C>G; p.Pro2473Ala	GG=0/GC=1/CC=6481
c.7451C>T; p.Ser2484Phe	TT=0/TC=1/CC=6492
c.7571C>G; p.Ser2524Cys	GG=0/GC=90/CC=6407
c.7723T>A; p.Ser2575Thr	AA=0/AT=2/TT=6495
c.7765G>A; p.Ala2589Thr	AA=0/AG=1/GG=6496
c.7850C>T; p.Pro2617Leu	TT=0/TC=1/CC=6496
c.7853A>G; p.Asn2618Ser	GG=0/GA=6/AA=6491
c.7870G>A; p. Ala2624Thr	AA=0/AG=1/GG=6496
c.7892C>T; p.Thr2631Ile	TT=0/TC=1/CC=6496
c.7910C>G; p. Thr2634Arg	GG=0/GC=1/CC=6496
c.8000C>T; p.Asp2667Leu	TT=0/TC=1/CC=6496
c.8021C>T; p.Ser2674Leu	TT=0/TC=1/CC=6496
c.8074G>C; p.Glu2692Gln	CC=0/CG=1/GG=6496
c.8089G>A; p.Glu2697Lys	AA=0/AG=1/GG=6496
c.8141C>A; p.Pro2714His	AA=0/AC=1/CC=6496

Only missense variants located within the 419bp 'FHS mutation region' of *SRCAP,* corresponds to amino acids 2329-2748, are listed.

FHS is very specific, with only truncating mutations in *SRCAP* located between sequences encoding the second ATPase domain and the C-terminal AT-hooks causing disease. Formally, I would postulate that the FHS phenotype is at least partially due to the loss of the AT-hook DNA-binding motifs.

Further data informing the possible mutational mechanism of FHS comes from allele-specific qPCR results of RNA isolated from FHS patient fibroblast cell lines which have shown that both wild-type and mutant *SRCAP* are expressed in these individuals at comparable levels (data not shown). Therefore, based on the genetic and molecular data available, it is likely that the loss of one allele, haploinsufficiency, is not the mechanism of FHS. Rather, the clustering of the truncating mutations of *SRCAP* in FHS suggest that a stable truncated SRCAP protein may be produced which functions in an alternative manner to the wild type protein (such as a gain-of-function or by a dominant negative effect).

Weaver Syndrome is Caused by Mutations in *EZH2*

The results of this study identified for the first time that heterozygous mutations in *EZH2* cause Weaver syndrome (Gibson et al., 2012) These findings were corroborated by an independent group who later reported *EZH2* mutations as the genetic cause underlying 19 cases of Weaver syndrome (Tatton-Brown et al., 2011). Similar to *NSD1* (Sotos syndrome), *EZH2* encodes a histone methyltransferase, which when combined with the core components EED (embryonic ectoderm development protein) and SUZ12 (suppressor of Zeste 12, drosophila homolog) of the polycomb repressor complex 2 (PCR2), is the catalytic force behind the tri-methylation of lysine residue 27 of histone 3 (H3K27me3) (Cao et al., 2002).

As NSD1 and EZH2 are both histone methyltransferases, with similar functions and overlapping expression patterns, it is not surprising that mutations in these genes result in patients with phenotypic overlap. More recently, two groups have identified missense mutations in *EED* (an interaction partner of EZH2 in the PCR2 complex) in EZH2-negative Weaver syndrome patients (Cohen et al., 2015; Cooney et al., 2017). These findings suggest that Weaver syndrome is due to pathogenic mutations in either *EZH2* or *EED*, and that dysregulation of the PCR2 complex results in overgrowth. Additionally, a recent study identified aberrations in growth plate proliferation and hypertrophy in mice with a combined *Ezh1/Ezh2* loss in cartilage (Lui et al., 2016). While this study was not designed to be a model of Weaver syndrome, their findings suggest roles for Ezh1 and Ezh2 in growth at the epiphyseal plate.

Sotos-like Syndrome with Cutis Laxa is Caused by Mutations in *NSD1*

Novel truncating mutations in *NSD1* in four patients presenting with Sotos syndrome and cutis laxa identified that this clinical presentation is a phenotypic expansion of Sotos syndrome and not a novel syndrome or, alternatively, patients presenting with two different rare disorders (Hood et al., 2016a). These findings are consistent with two other reports which each describe an individual patient with Sotos syndrome and cutis laxa and *NSD1* mutations in exon 11 (Cortes-Saladelafont et al., 2011) and exon 15 (Bou-Assi et al., 2016). Mutations in Sotos syndrome have been reported across all functional domains of *NSD1*. The position of the *NSD1* mutations identified for patients presenting with Sotos syndrome and cutis laxa are all in close proximity to previously reported Sotos syndrome mutations, which

suggests there is no obvious genotype-phenotype correlation for marked connective tissue laxity and that other factors (e.g genetic modifiers) are contributing to the clinical spectrum.

Clinical Spectrum Delineation of Rare Disorders Impacting Stature and Growth

The identification of disease-causing mutations for rare genetic disorders has a dramatic impact on our understanding of these syndromes. Not only do these discoveries provide much needed answers to affected individuals and families, but they also result in a critical examination and expansion of the phenotypic spectrum of the disorder. For example, in this study we identified mutations in the Sotos syndrome gene, *NSD1*, in a cohort of Sotos syndrome with cutis laxa patients (Hood et al., 2016a). It was initially presumed that mutation in novel gene would be responsible for this condition (Robertson and Bankier, 1999); however the un-biased exome sequencing approach we utilized in this study lead to the finding that the marked tissue laxity seen in these patients is a phenotypic expansion of the Sotos syndrome clinical spectrum.

As we come to understand the true clinical spectrum of rare disorders they will be progressively easier to diagnose, ultimately leading to better clinical management. Prior to this study, the clinical recognition of FHS was extremely difficult, with approximately 50 FHS clinical cases in the literature and disagreement in the clinical community that all cases actually had FHS. The identification of *SRCAP* as responsible for FHS (Hood et al., 2012), followed by the phenotypic characterization of 52 mutation-positive FHS patients (Nikkel et al., 2013) in this study provides the most comprehensive phenotypic and diagnostic analysis of FHS patients to date.

The advent of high-throughput sequencing approaches have resulted in unprecedented growth in our understanding of human disease. To truly understand the large amounts of genetic data generated by these technologies there is a need for parallel phenotypic annotation (Boycott et al., 2013). Such a global phenome initiative would provide clinicians with rapid access to the complete clinical spectrum of rare disorders, leading to improved clinical diagnosis and patient management.

Global Methylation Abnormalities in Rare Stature Disorders Give Insight into Disease Mechanism

As previously mentioned mutations in several genes have been shown to impact methylation status including: the DNA methyltransferases *DNMT1* (Kernohan et al., 2016), *DNMT3A* (Fuks et al., 2001), and *DNMT3B* (Bai et al., 2005); two chromatin remodelling proteins, *HELLS* (Dennis et al., 2001; Zhu et al., 2006) and *ATRX* (Gibbons et al., 2000); the histone H3 lysine 4 demethylase, *KDM5C* (Grafodatskaya et al., 2013); and *FMR1* (Kremer et al., 1991; Schenkel et al., 2016b). Interestingly, mutations in three of these genes result in disorders associated with growth/stature aberrations: *ATRX* in ATRX syndrome (Basehore et al., 2015; Gibbons et al., 1995); *FMR1* in FXS (Cohen, 2003; de Vries et al., 1995), and *KDM5C* in an X-linked form of intellectual disability syndrome (Jensen et al., 2005). Most recently, Choufani *et al.* (2015) identified a unique methylation ‗epi-signature' caused by Sotos syndrome mutations in *NSD1* (Choufani et al., 2015). These studies suggest a possible link between methylation abnormalities and stature.

In this study we identified a methylation signature unique to FHS individuals, regardless of their respective *SRCAP* mutation (Hood et al., 2016b). Amongst the 28 regions found to be at

least 20% differentially methylated as compared to controls, 19 were found to be hypermethylated and 9 found to be hypomethylated in FHS patients. Therefore, truncating mutations within *SRCAP* result in locus specific DNA methylation alterations. These alterations may either be a direct consequence of altered function of the truncated SRCAP protein, or the result of a secondary compensatory mechanism in response to altered SRCAP function. Further molecular investigation is required to determine the specific underlying molecular mechanism; however, these findings suggest a role for SRCAP in the regulation of genomic DNA methylation, which may in turn impact the expression of specific genes and thus contribute to the clinical features of FHS, including stature.

Future Studies in FHS

Based on the work presented here, which demonstrate that clustered truncating mutations of SRCAP cause FHS and that these mutations result in global methylation alterations, it is hypothesized that truncating mutations of SRCAP will result in widespread gene dysregulation; however the mechanisms and biological pathways underlying the FHS phenotype are currently unknown. As only patient lymphoblast and fibroblast cell lines are available for analysis, neither of which is implicated in the FHS phenotype, the best way to examine how mutations in *SRCAP* impact downstream gene expression is to utilize an induced pluripotent stem cell (iPSC) disease modeling approach. This approach would involve first reprogramming FHS patient (and control) fibroblasts into iPSCs and then differentiating these iPSCs into different cell lineages impacting in FHS (such as: chondroprogenitor, neural and neural crest lineaged cells). These generated cells could then be used to molecularly characterize how mutations of *SRCAP* in FHS contribute to particular

symptoms of the disorder. Such experiments would provide a better understanding of the precise mechanisms and biological pathways involved in various aspects of the FHS phenotype.

Conclusion

The development of high-throughput sequencing strategies has significantly advanced our knowledge of the genetics underlying many conditions. Utilizing this technology, along with downstream techniques, we were able to identify: mutations in *EZH2* as the genetic cause of Weaver syndrome; *NSD1* mutations in a cohort of Sotos with cutis laxa patients, thus expanding the phenotypic spectrum of Sotos syndrome; and truncating mutations in the chromatin remodelling protein *SRCAP* as the genetic cause of Floating-Harbor syndrome. Choosing to further our analysis of FHS, we expanded on our initial patient cohort and identified *SRCAP* mutations in 52 FHS individuals thus enabling us to better appreciate the full clinical spectrum of FHS mutations. Lastly, we have demonstrated that truncating mutations of *SRCAP* in FHS cause global methylation changes, which suggests a role for SRCAP in the regulation of genomic DNA methylation. These alterations may in turn alter downstream gene expression thus contributing to clinical manifestations of FHS.

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Chapter 1, Figure 1

For Chapter 1 Figure 1 from Boycott et al. (2014) "Identification of Genes for Childhood Heritable Diseases" in Annual Review of Medicine (PMID: 24422568):

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Chapter 5 Manuscript

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Chapter 6 Manuscript

For Hood et al. (2016) "The Defining DNA Methylation Signature of Floating-Harbor Syndrome" in Scientific Reports (PMID: 27934915):

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Appendix 2 Manuscript

For Reschen M, Kini U, Hood RL et al. (2012) "Floating-Harbor Syndrome and Polycystic Kidneys Associated with SRCAP Mutation" (PMID: 23165645):

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Joaquim Sá				X		
Jeroen Schoots				X		
Elizabeth L Silveira				X		
Marleen E H Simon				X		
Anne Slavotinek				X		
I Karen Temple				X		
Ineke van der Burgt				X		
Bert B A de Vries				X		
James D Weisfeld-Adams				X		
Jan M Wit				X		
Connie Fung On Yee				X		
Ernie Bongers				X		
Han Brunner				X		
Murray Feingold				X		
Laila C. Schenkel					X	
Peter J. Ainsworth					X	
Guillaume Pare					X	
Bekim Sadikovic					X	
Michael Reschen						X
Christopher A. O'Callaghan						X

Appendices

Appendix 1: Ethics Approval Forms

Children's Hospital of Eastern Ontario
Centre hospitalier pour enfants de l'est de l'Ontario

2011 FINAL APPROVAL LETTER

CHEO Site Investigator: Dr. Kym Boycott	**Department/PSU** Genetics
Date of Final Approval Letter March 08, 2011	
Protocol Title: Finding of Rare Disease Genes in Canada (FORGE Canada)	**REB #:** 11/04E **Other Study #:**
Original Approval Date and REB Meeting Date January 05, 2011	**Valid until** January 04, 2012
c.c.: Janet Marcedier ,CHEO RI Administration	
Protocol Date February 23, 2011	**Protocol Version** #1
Consent Form Version and Date Version #1, Dated March 1, 2011	
Date of Health Canada Non-Objection Letter, and Control # Not Applicable	**Assent Form Version and Date** Not Applicable
Investigators Brochure/Drug Monograoph Version and Date Not Applicable	**Recruitment Poster Version and Date** Not Applicable
SITE Specific Restrictions Not Applicable	**Recruitment Pamphlet Version and Date** Not Applicable
	Other Study Documentation Received by REB Not Applicable

This protocol was approved at a meeting of the CHEO Research Ethics Board in which the quorum rules were met and only those REB members who were independent of the investigator(s) conducting the study voted on the final decision.

In fulfilling its mandate, the CHEO REB is guided by: Tri-Council Policy Statement; ICH Good Clinical Practice Practices: Consolidated Guideline; Applicable laws and regulations of Ontario and Canada (e.g., Health Canada Division 5 of the Food and Drug Regulations & the Food and Drugs Act - Medical Devices Regulations).

Final approval is granted with the understanding that the investigator agrees to comply with the following requirements:

- The investigator must conduct the study in compliance with the protocol and any additional conditions set out by the Board.
- The investigator must not implement any deviation from, or changes to, the protocol without the approval of the REB except where necessary to eliminate an immediate hazard to the research subject, or when the change involves only logistical or administrative aspects of the study (e. g., change of telephone number or research staff). As soon as possible, however, the protocol deviation form and, if appropriate, the proposed protocol amendment(s) should be submitted to the Board for review.
- The investigator must, prior to use, submit to the Board changes to the study documentation, e.g., changes to the informed consent letters, recruitment materials. Should major revisions to the consent form be made, the investigator agrees to re-consent those subjects who have originally consented to the study and who wish to continue on the study.
- For clinical drug or device trials, investigators must promptly report to the REB all adverse events that are both serious and unexpected (SAEs). For SAE reports on CHEO patients, the investigator must also comply with the hospital-wide Policy regarding, Procedures For Considering Medical Error In The Differential Diagnosis of Severe Adverse Events (SAE) Associated with the Drugs Administered in a Clinical Trial (see http://cheonet/data/1/rec_docs/3792_Medical%20Error%20Policy%20revised%20january%2020061.doc).
- For all other research studies, investigators must promptly report to the REB all unexpected and untoward occurrences (including the loss or theft of study data and other such privacy breaches).
- Investigators must promptly report to the REB any new information regarding the safety of research subjects (e.g., changes to the product monograph or investigator's brochure for drug trials). Where available, any reports produced by Data Safety Monitoring Board should be submitted to the REB.
- Investigators must notify the REB of any study closures (temporary, premature or permanent), in writing along with an explanation of the rationale for such action.
- Investigators must submit an annual renewal report to the REB 30 days prior to the expiration date stated above.
- Investigators must submit a final report at the conclusion of the study.
- Investigators must provide the Board with French versions of the consent form, unless a waiver has been granted.

For complete procedures relating to these modifications, please refer to the REB website at http://www.cheori.org/about_ethics.html or contact Sharon Haig, Ethics Coordinator

Regards,

Dr. Carole Gentile, C.Psych.
Chair, Research Ethics Board
CG/smeh

Children's Hospital of Eastern Ontario
Centre hospitalier pour enfants de l'est de l'Ontario

RECEIVED FEB 0 1 2012

KP

KB

CHEO Research Ethics Board ANNUAL RE-APPROVAL NOTICE

Principal Investigator	Dr. Kym Boycott
REB Protocol Number	11/04E
Protocol Title	Finding of Rare Disease Genes in Canada (FORGE Canada)
Department or PSU	Genetics
Approval Date	January 30, 2012
Approval Valid Until	**January 15, 2013**
Date for Submission for Next Annual Renewal Report	**December 18, 2012**
Documents Reviewed & Approved	▪ Reporting Form – Annual Renewal (January 20, 2012)

This is to notify you that the CHEO REB has granted approval to the renewal for the above named research study for a period of one year. The renewal was reviewed and approved by the Chair only. Decisions made by the Chair under delegated review are ratified by the full Board at its subsequent meeting.

In fulfilling its mandate, the CHEO REB is guided by: Tri-Council Policy Statement; ICH Good Clinical Practice Practices: Consolidated Guideline; Applicable laws and regulations of Ontario and Canada (e.g., Health Canada Division 5 of the Food and Drug Regulations & the Food and Drugs Act - Medical Devices Regulations).

Approval is granted with the understanding that the investigator agrees to comply with the following requirements:

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- Investigators must notify the REB of any study closures (temporary, premature or permanent), in writing along with an explanation of the rationale for such action.

401 Smyth Road, Ottawa, ON K1H 8L1, Canada
Tel (613) 737-7600 www.cheo.on.ca

401, chemin Smyth, Ottawa (ON) K1H 8L1, Canada
Tél. (613) 737-7600 www.cheo.on.ca

Making a difference in the lives of children, youth and families

Faire la différence dans la vie des enfants, des adolescents et des familles

- Investigators must submit an annual renewal report to the REB 30 days prior to the expiration date stated on the final approval letter.
- Investigators must submit a final report at the conclusion of the study.
- Investigators must provide the Board with French version of the consent form, unless a waiver has been granted.

Regards,

Dr. Carole Gentile, C.Psych.
Chair, Research Ethics Board

Children's Hospital of Eastern Ontario
Centre hospitalier pour enfants de l'est de l'Ontario

Research Ethics Board
ANNUAL RENEWAL (DELEGATED)

Principal Investigator	Dr. Kym Boycott
REB Protocol Number	#11/04E
Protocol Title	Finding of Rare Disease Genes in Canada (FORGE Canada)
Department or PSU	Genetics
Date reviewed by the REB	March 18, 2013
Date Approved	March 18, 2013 ☒ No Modifications required. ☐ With required Modifications.
Approval Valid Until	January 15, 2014
Submission Deadline	December 15, 2013
Contingencies	☒ Not Applicable ☐ Applicable ~ Date contingencies were met: _____
Documents Reviewed & Approved	▪ Continuing Review Form – Annual Renewal for Delegated Review received March 1, 2013

This is to notify you that the CHEO REB has granted approval to the renewal for the above named research study for a period of one year. The renewal was reviewed and approved by the Chair only. Decisions made by the Chair under delegated review are ratified by the full Board at its subsequent meeting.

In fulfilling its mandate, the CHEO REB is guided by: Tri-Council Policy Statement; ICH Good Clinical Practice Practices: Consolidated Guideline; Applicable laws and regulations of Ontario and Canada (e.g., Health Canada Division 5 of the Food and Drug Regulations & the Food and Drugs Act - Medical Devices Regulations).

Approval is granted with the understanding that the investigator agrees to comply with the following requirements:

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- Investigators must notify the REB of any study closures (temporary, premature or permanent), in writing along with an explanation of the rationale for such action.

- Investigators must submit an annual renewal report to the REB 30 days prior to the expiration date stated on the final approval letter.
- Investigators must submit a final report at the conclusion of the study.
- Investigators must provide the Board with French version of the consent form, unless a waiver has been granted.

Regards,

Dr. Carole Gentile, C.Psych.
Chair, Research Ethics Board
CG/smeh

c.c. Megan Vanstone, Research Coordinator

RESEARCH INSTITUTE
INSTITUT DE RECHERCHE

Research Ethics Board
2014 Annual Renewal (Delegated)

Principal Investigator: Dr. Kym Boycott
REB Protocol No: 11/04E
Romeo File No: 10000236
Project Title: Enhanced Care for Rare Genetic Diseases in Canada
Primary Affiliation: Biomedicine\Genetics
Contingencies: None
Protocol Status: Active
Approval Date: January 13, 2014
Approval Valid Until: January 15, 2015
Annual Renewal Submission Deadline: December 15, 2014

This is to notify you that the CHEO REB has granted approval to the renewal for the above named research study for a period of one year. The renewal was reviewed and approved by the Chair only. Decisions made by the Chair under delegated review are ratified by the full Board at its subsequent meeting.

In fulfilling its mandate, the CHEO REB is guided by: Tri-Council Policy Statement; ICH Good Clinical Practice Practices: Consolidated Guideline; Applicable laws and regulations of Ontario and Canada (e.g., Health Canada Division 5 of the Food and Drug Regulations & the Food and Drugs Act - Medical Devices Regulations).

Approval is granted with the understanding that the investigator agrees to comply with the following requirements:

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3. The investigator must, prior to use, submit to the Board changes to the study documentation, e.g., changes to the informed consent letters, recruitment materials. Should major revisions to the consent form be made, the investigator agrees to re-consent

those subjects who have originally consented to the study and who wish to continue on the study.

4. For clinical drug or device trials, investigators must promptly report to the REB all adverse events that are both serious and unexpected (SAEs). For SAE reports on CHEO patients, the investigator must also comply with the hospital-wide Policy regarding, Procedures For Considering Medical Error In The Differential Diagnosis of Severe Adverse Events (SAE) Associated with the Drugs Administered in a Clinical Trial (see http://cheonet/data/1/rec_docs/3792_Medical%20Error%20Policy%20revised%20january%2020061.doc).
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8. Investigators must submit an annual renewal report to the REB 30 days prior to the expiration date stated on the final approval letter.
9. Investigators must submit a final report at the conclusion of the study.
10. Investigators must provide the Board with French version of the consent form, unless a waiver has been granted.

If you have any questions, pertaining to this letter, please contact Natalie Anderson, Research Ethics Board office

Regards,

Dr. Carole Gentile
Chair, Research Ethics Board
Présidente, Comité d'éthique de la recherche

RESEARCH INSTITUTE
INSTITUT DE RECHERCHE

Research Ethics Board
2015 Annual Renewal (Delegated)

Principal Investigator: Dr. Kym Boycott
REB Protocol No: 11/04E
Romeo File No: 10000236
Project Title: CHEOREB#11/04E - Enhanced Care for Rare Genetic Diseases in Canada

11/04E (a) - Emerging Team to Identify and Characterize Novel and Existing Hereditary Spastic Paraplegia (HSP) Diseases in Genes – Adjunct study to CARE for RARE

11/04E (b) - High Throughput Diagnostic Sequencing for Newborns with Rare Diseases - Adjunct Study to CARE for RARE

11/04E (c) - Evaluating Exome Sequencing for the Diagnosis of Limb Girdle Muscular Dystrophy - Adjunct Study to CARE for RARE

11/04E (d) - Gene Identification and Functional Characterization of Mitochondrial Disorders

Primary Affiliation: Biomedicine\Genetics
Protocol Status: Active
Approval Date: January 14, 2015
Approval Valid Until: January 15, 2016
Annual Renewal Submission Deadline: December 15, 2015

This is to notify you that the CHEO REB has granted approval to the renewal for the above named research study for a period of one year. The renewal was reviewed and approved by the Chair only. Decisions made by the Chair under delegated review are ratified by the full Board at its subsequent meeting.

In fulfilling its mandate, the CHEO REB is guided by: Tri-Council Policy Statement; ICH Good Clinical Practice Practices: Consolidated Guideline; Applicable laws and regulations of Ontario and Canada (e.g., Health Canada Division 5 of the Food and Drug Regulations & the Food and Drugs Act - Medical Devices Regulations).

Approval is granted with the understanding that the investigator agrees to comply with the following requirements:

1. The investigator must conduct the study in compliance with the protocol and any additional conditions set out by the Board.

2. The investigator must not implement any deviation from, or changes to, the protocol without the appval of the REB except where necessary to eliminate an immediate hazard to the research subject, or when the change involves only logistical or administrative aspects of the study (e.g., change of telephone number or research staff). As soon as possible, however, the implemented deviation or change, the reasons for it and, if appropriate, the proposed protocal amendment(s) should be submitted to the Board for review.
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5. For all other research studies, investigators must promptly report to the REB all unexpected and untoward occurrences (invluding the loss or theft of study data and other such privacy breaches).
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If you have any questions, pertaining to this letter, please contact Natalie Anderson, Research Ethics Board office

Regards,

Dr. Carole Gentile
Chair, Research Ethics Board
Présidente, Comité d'éthique de la recherche

RESEARCH INSTITUTE
INSTITUT DE RECHERCHE

Research Ethics Board
2016 Annual Renewal (Delegated)

Principal Investigator: Dr. Kym Boycott
REB Protocol No: 11/04E
Romeo File No: 10000236
Project Title: CHEOREB#11/04E - Enhanced Care for Rare Genetic Diseases in Canada

11/04E (a) - Emerging Team to Identify and Characterize Novel and Existing Hereditary Spastic Paraplegia (HSP) Diseases in Genes – Adjunct study to CARE for RARE

11/04E (b) - High Throughput Diagnostic Sequencing for Newborns with Rare Diseases -Adjunct Study to CARE for RARE

11/04E (c) - Evaluating Exome Sequencing for the Diagnosis of Limb Girdle Muscular Dystrophy - Adjunct Study to CARE for RARE

11/04E (d) - Gene Identification and Functional Characterization of Mitochondrial Disorders

Primary Affiliation: Biomedicine\Genetics
Protocol Status: Active
Approval Date: January 8, 2016
Approval Valid Until: January 15, 2017
Annual Renewal Submission Deadline: December 15, 2016

Documents Reviewed & Approved:

Document Name	Comments	Version Date
French Consent Form	Current French adult consent form	2013/02/01
Consent Form	Current English adult consent form	2013/02/01
French Consent Form	Current French pediatric consent form	2013/02/01
Consent Form	Current English pediatric consent form	2013/02/01
Protocol	Current protocol	2014/09/01

This is to notify you that the CHEO REB has granted approval to the renewal for the above named research study for a period of one year. The renewal was reviewed and approved by the Chair or a delegate of the Chair. Decisions made by the Chair under delegated review are ratified by the full Board at its subsequent meeting.

In fulfilling its mandate, the CHEO REB is guided by: Tri-Council Policy Statement; ICH Good Clinical Practice Practices: Consolidated Guideline; Applicable laws and regulations of Ontario and Canada (e.g., Health Canada Division 5 of the Food and Drug Regulations & the Food and Drugs Act - Medical Devices Regulations).

Approval is granted with the understanding that the investigator agrees to comply with the following requirements:

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9. Investigators must notify the REB of any study closures (closed to accrual, temporary, premature or permanent).
10. Investigators must submit a final report at the conclusion of the study.

If you have any questions, pertaining to this letter, please contact the Research Ethics Board Office

Regards,

Dr. Carole Gentile

Appendix1: Ethics Approval Forms

Chair, Research Ethics Board
Présidente, Comité d'éthique de la recherche

RESEARCH INSTITUTE
INSTITUT DE RECHERCHE

Research Ethics Board
2017 Annual Renewal (Delegated)

Principal Investigator: Dr. Kym Boycott
REB Protocol No: 11/04E
Romeo File No: 10000236
Project Title(s):

CHEOREB#11/04E - Enhanced Care for Rare Genetic Diseases in Canada

11/04E (a) - Emerging Team to Identify and Characterize Novel and Existing Hereditary Spastic Paraplegia (HSP) Diseases in Genes – Adjunct study to CARE for RARE

11/04E (b) - High Throughput Diagnostic Sequencing for Newborns with Rare Diseases - Adjunct Study to CARE for RARE changed to 'Rapid and comprehensive diagnostic sequencing for rare, genetic disease in the critically ill newborn'

11/04E (c) - Evaluating Exome Sequencing for the Diagnosis of Limb Girdle Muscular Dystrophy - Adjunct Study to CARE for RARE

11/04E (d) - Gene Identification and Functional Characterization of Mitochondrial Disorders

11/04E (e) - Personalized therapies for improved seizure control in Dravet syndrome: an assessment of novel in vivo and in vitro model systems

Primary Affiliation: Biomedicine\Genetics
Protocol Status: Active
Approval Date: January 11, 2017
Approval Valid Until: January 15, 2018
Annual Renewal Submission Deadline: December 15, 2017

This is to notify you that the CHEO REB has granted approval to the renewal for the above named research study for a period of one year. The renewal was reviewed and approved by the Chair or a delegate of the Chair. Decisions made by the Chair under delegated review are ratified by the full Board at its subsequent meeting.

Approval is granted with the understanding that the investigator agrees to comply with the following requirements:

1. The investigator must conduct the study in compliance with the protocol and any additional conditions set out by the Board.
2. Investigators must submit an annual renewal report to the REB 30 days pror to the expiration date stated on the final approval letter.
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9. Investigators must notify the REB of any study closures (closed to accrual, temporary, premature or permanent).
10. Investigators must submit a final report at the conclusion of the study.

If you have any questions, pertaining to this letter, please contact the Research Ethics Board Office

Regards,

Richard Carpentier, PhD
Chair, Research Ethics Board
Président, Comité d'éthique de la recherche

Appendix 2: Floating-Harbor Syndrome and Polycystic Kidneys Associated with *SRCAP* mutation

Preface

The following appendix consists of data previously published in the American Journal of Medical Genetics A under the title "Floating-Harbor Syndrome and Polycystic Kidneys Associated with *SRCAP* Mutation" (PMID: 23165645) by Reschen M, Kini U, Hood RL, Boycott KM, Hurst J, O'Callaghan CA.

Approval for this article to be reused in this thesis was granted by John Wiley and Sons (see Rights and Permissions; Appendix 1 Manuscript)

I performed targeted PCR and Sanger sequencing of *SRCAP,* exons 31-34, for the FHS individual in this case report. I generated a figure for this paper. I was involved in writing the portion of the manuscript pertaining to my work and editing the final manuscript.

The specific contributions of each author to this paper are listed below.

Reschen M: Oversaw the project. Provided patient nephrology clinical data. Sent patient sample for *PKD1* and *PKD2* testing. Wrote the manuscript. Generated figures and tables for the manuscript. Responded to reviewers comments.

Kini U: Provided patient genetic clinical data. Sent patient sample for clinical microarray and cytogenetic analysis. Collected and sent patient DNA sample for sequencing analysis. Co-wrote the manuscript. Responded to reviewers comments.

Hood RL: (see above)

Boycott KM: Oversaw Sanger sequencing. Contributed to editing the manuscript.

Hurst J: Provided patient genetic clinical data. Sent patient sample for clinical microarray and cytogenetic analysis. Contributed to writing and editing the manuscript.

O'Callaghan CA: Oversaw the project. Wrote the manuscript. Responded to reviewers comments.

Appendix 2: Floating-Harbor syndrome and polycystic kidneys

Abstract

Floating–Harbor syndrome (FHS) is a rare genetic disorder recently shown to be caused by mutations in the Snf2-related CREB-binding protein activator protein gene (*SRCAP*). It comprises three key clinical features of characteristic facies, expressive and receptive speech impairment and short stature. We report on a patient with this syndrome associated with early adult-onset hypertension and bilateral polycystic kidneys. Family screening for polycystic kidney disease was negative and mutations in polycystic kidney disease 1 and 2 genes (*PKD1* and *PKD2*) were absent. Sequencing of the SRCAP gene demonstrated a de novo mutation matching one of the known FHS-associated mutations. The patient required treatment with anti-hypertensives and will require lifelong renal monitoring. We suggest this patient's presentation may be due to the pleiotropic effects of SRCAP mutations. Further, the protein encoded by SRCAP is known to interact with CREB-binding protein, the product of the gene mutated in Rubinstein–Taybi syndrome, which is associated with renal abnormalities. A literature review of the renal findings in patients with Floating–Harbor syndrome identified another patient with possible polycystic kidneys, two patients with early onset hypertension, and a young patient with a ruptured intracranial aneurysm, which can be a feature of classic adult polycystic kidney disease. Collectively, these findings suggest that all patients with Floating–Harbor syndrome should undergo regular blood pressure monitoring and screening for polycystic kidneys by ultrasound at the time of the FHS diagnosis with imaging to be repeated during adulthood if a childhood ultrasound was negative.

Appendix 2: Floating-Harbor syndrome and polycystic kidneys

Introduction

Floating–Harbor syndrome (FHS) is a rare disorder, which comprises three key clinical features of characteristic facies, expressive and receptive language delay, and short stature. It is named after the Boston Floating Hospital in Massachusetts and the Harbor General Hospital in Torrance, California from where the first two cases were reported (Leisti et al., 1975; Pelletier, 1973). In a recent report of 13 patients, the cause of FHS was attributed to dominant mutations in the *SRCAP* gene encoding the SNF2-related cAMP-response element binding protein binding protein (CREBBP) activator protein (Hood et al., 2012). SRCAP is an SNF2-related chromatin remodeling factor, so mutations are likely to exert pleiotropic effects (Ruhl et al., 2006). The exact mechanism by which the clinical features develop remains unknown.

About 87 cases of FHS have been reported over the last 29 years and the phenotype delineated further. Reliable features such as a short neck, finger clubbing, brachydactyly, mild to moderate intellectual impairment, and a broad chest can aid diagnostic precision (Stagi et al., 2007; White et al., 2010). Various additional associations have been described including middle ear abnormalities (Hendrickx et al., 2010), ocular abnormalities (Asseidat and Kaufman, 2009), celiac disease (Chudley and Moroz, 1991), ruptured cerebral aneurysm (Paluzzi et al., 2008), precocious puberty (Stagi et al., 2007), spinal cord ganglioma (Nelson et al., 2009), cardiac septal defects (Lazebnik et al., 1996) and growth hormone deficiency (Femiano et al., 2000). It remains unclear whether these have arisen by chance or reflect genetic pleiotropism. We report on a patient with hypertension and polycystic kidneys in association with Floating–Harbor syndrome and discuss the implications for other patients.

Clinical Report

A 25-year-old man diagnosed with FHS was referred to our nephrology clinic for assessment of recently diagnosed hypertension. The patient was born to nonconsanguineous white Caucasian parents and had two unaffected siblings. He was delivered at 40 weeks gestation with a birth weight of 3.57 kg. At the age of 2 months he was admitted to hospital with *Haemophilus influenza* meningitis, but made a good recovery. However, his post-natal and early development was characterized by markedly poor growth, expressive language delay and hearing problems. He was noted to have dysmorphic features, including a prominent nose and ears, which ultimately led to a clinical diagnosis of Floating–Harbor syndrome being made at the age of 7 years by a clinical geneticist (see Figure 1). Karyotype analysis was normal and wrist radiographs were consistent with a two and a half year bone age delay. Velo-cardio-facial syndrome was subsequently excluded by normal 22q11 FISH analysis. He was reported as Patient 5 of a series by White et al. (White et al., 2010).

He was treated with growth hormone therapy with a good initial response. He attended a normal school, but his progress was slow and he required intensive speech therapy. At the age of 15 years his height remained below the 3rd centile and he still displayed paucity of speech, relying more on signing to communicate. He progressed through puberty and growth hormone therapy was discontinued. He currently lives in supervised accommodation and is employed in the retail sector.

Two months prior to referral to the nephrology clinic his blood pressure was found to be elevated at 167/111 mmHg during a routine check for enrollment at a gymnasium. A history

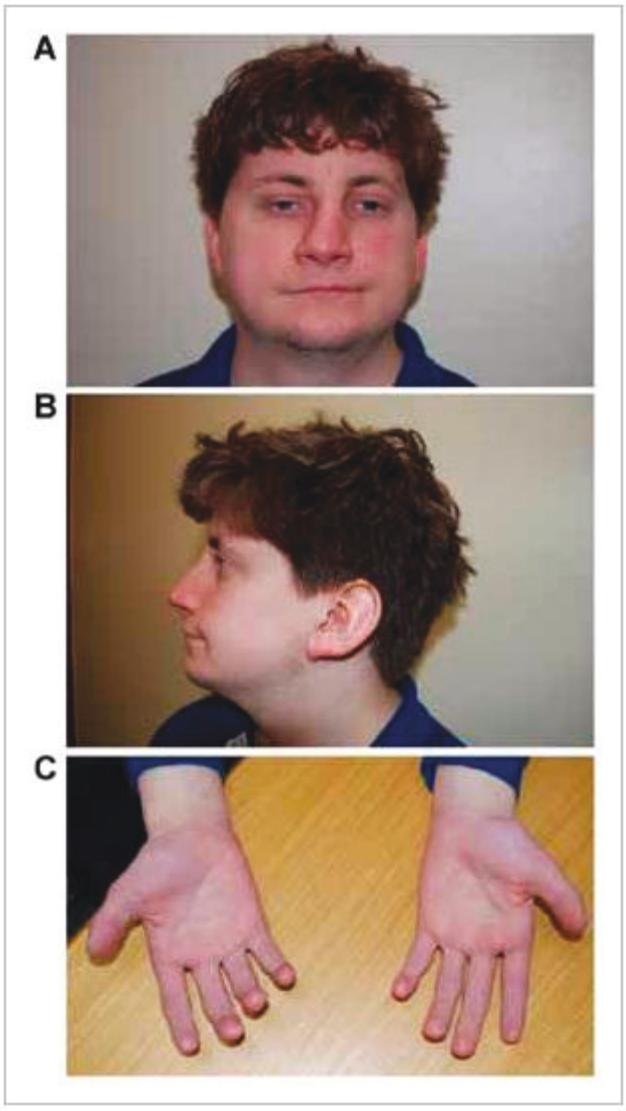


Figure 1. Photographs of the patient's face and hands.
The nose becomes bulbous at its tip and the mouth is wide with thin lip vermilion (panel A). The ears are slightly low-set and posteriorly rotated (panel B). There is brachydactyly and clubbing of the fingers (panel C).

revealed an episode of macroscopic hematuria and left flank pain 6 months previously which was treated empirically as a urinary tract infection by his primary care physician. There was no family history of renal problems or sudden death/intracranial haemorrhage. Repeated blood pressure measurements by his primary care physician confirmed stage 2 hypertension (Williams et al., 2004). Cardiovascular examination including fundoscopy was otherwise normal. Ambulatory blood pressure monitoring demonstrated an average of 147/106 mmHg and an echocardiogram demonstrated mild left ventricular hypertrophy. Urinary catecholamines were normal. An ultrasonographic examination of his kidneys revealed multiple bilateral cysts (see Figure 2), which were considered typical of adult polycystic kidney disease by a senior radiologist with a specialist interest in renal disease (an abdominal ultrasound at the age of 6 had noted normal kidneys). Laboratory tests revealed a creatinine of 87 micromol/L (1.14 mg/dl), commensurate with an estimated glomerular filtration rate (eGFR) of 99 ml/min/1.73 m2 with the MDRD equation or 106 ml/min/1.73 m2 with the more accurate CKD-EPI equation (normal range eGFR > 90) (Levey et al., 2009). Urinalysis revealed no blood and 30 mg/dl proteinuria. Magnetic resonance angiography of the brain demonstrated a left fetal posterior cerebral artery with a hypoplastic segment of the pre-communicating part of the posterior cerebral artery and co-dominant vertebral arteries but intracranial aneurysms were not identified.

Good blood pressure control was achieved with the angiotensin converting enzyme inhibitor lisinopril. The patient's parents and one of his two brothers were screened for polycystic kidneys by ultrasound, which revealed no abnormalities and all were demonstrated to have normal renal function.

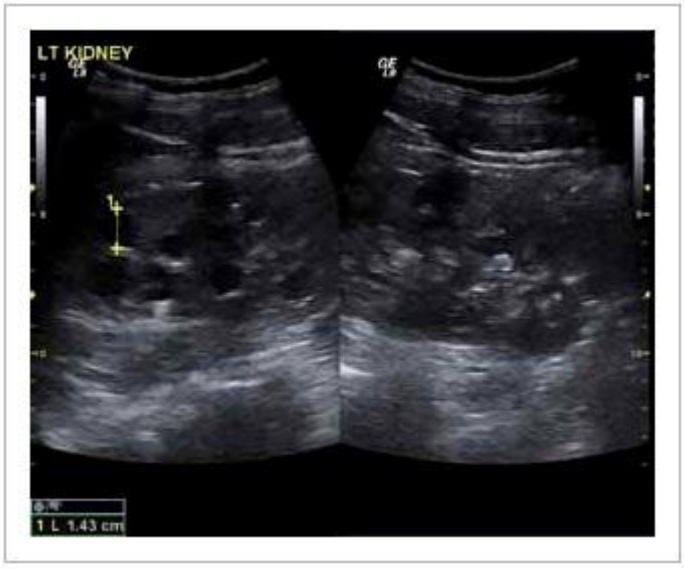


Figure 2. Ultrasound image of the left kidney demonstrating multiple cysts.

Results of Genetic Testing

Sequencing of the *PKD1* and *PKD2* genes [including multiplex ligation-dependent probe amplification (MLPA)] was unremarkable. An Agilent Custom 105K microarray test did not reveal any sub-microscopic chromosomal abnormalities. Sequencing of exon 34 of the *SRCAP* gene revealed a heterozygous truncating mutation (c.7303C > T;p.Arg2435*) which has been previously identified in other patients with FHS (see Figure 3) (Hood et al., 2012). The mutation was not present in either of his parents.

Discussion

Recently, whole-exome sequencing in a cohort of FHS patients revealed dominant negative mutations in the *SRCAP* gene as the major cause of FHS (Hood et al., 2012). All mutations were shown to be de novo where parental DNA was available. Our patient has the typical clinical features and a de novo *SRCAP* mutation matching one of the common FHS-associated *SRCAP* mutations. The non-synonymous coding mutation in exon 34 is likely to cause truncation of the encoded protein, resulting in the loss of terminal AT-hook DNA-binding motif domains. SRCAP is known to interact with CREBBP, which affects chromatin remodeling (Johnston et al., 1999). Mutations in *CREBBP* cause Rubinstein–Taybi syndrome, which has some phenotypic overlap with FHS and is associated with malformations of the renal tract (Melekos et al., 1987).

This is the first report of bilateral polycystic kidneys in a patient with Floating–Harbor syndrome. A variety of genetic defects can give rise to diseases characterized by polycystic kidneys, but the commonest by far are mutation of the *PKD1* and *PKD2* genes in autosomal

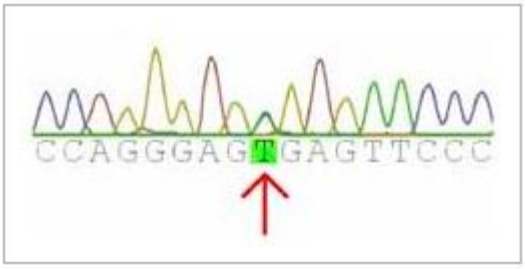


Figure 3. Chromatogram of *SRCAP* mutation (c.7303C > T;p.Arg2435*) in the patient.
Mutation denoted by arrow.

dominant polycystic kidney disease. These mutations lead to extensive renal cyst formation over many years resulting in obliteration of the normal renal parenchyma and variable degrees of hypertension, episodic haematuria, nephrolithiasis, and reduced GFR (Torres et al., 2007) as well as susceptibility to intracranial aneurysms. We sequenced our patient's *PKD1* and *PKD2* genes but did not find a mutation. An Agilent Custom 105K microarray test did not reveal any sub-microscopic chromosomal abnormalities. Other rarer causes of polycystic kidneys include mutation in the *PKHD1*, *SEC63*, *TSC1*, *TSC2*, *VHL*, *MCKD1*, *MCKD2*, *NPHP (1,2,3,4,5,6)*, *JBTS2*, *BBS (1-11)*, *MKS1/3*, *OFD1*, and *HNF1β* genes. Diseases arising from these mutations typically have other characteristic phenotypic features lacking in this patient.

To establish whether Floating–Harbor syndrome might be associated with renal abnormalities we scrutinized all previous case reports for evidence of renal problems, including hypertension. In the previous absence of a genetic test, an authoritative study by White et al. (White et al., 2010) divided the published cases into those for whom the diagnosis is certain, based on photographic and clinical evidence, and those for whom it cannot be definitely validated (see Supplementary Information). Several additional reports were not included in that review and we graded these using similar criteria (Supplementary eTable II). It should be noted that this second group may indeed have Floating–Harbor syndrome and are discussed separately in the supplementary materials in the online Supporting Information. Genetic testing for mutations in the *SRCAP* gene is likely to be highly useful in the future but has only recently become available on a research basis, thus it will be some time before long-term follow up data based on the *SRCAP* genotype will become available.

Thirty-five reports were identified in which the diagnosis of Floating–Harbor syndrome seemed certain (Supplementary eTable I). These included 71 patients (37 females, 34 males) with a mean age of 11 years (our patient was previously included in a case series as Patient 5 (White et al., 2010) prior to the diagnosis of polycystic kidneys and hypertension).

Renal Findings in Patients with a Clinical Diagnosis of FHS

In a follow-up report on the original patient with FHS, then aged 37, Feingold reported that the patient had developed hypertension requiring medication for 20 years (Feingold, 2006). Hypertension was also reported in a 23-year-old male (White et al., 2010). In the other 68 cases, blood pressure measurements were not mentioned. Notably the blood pressure was not reported for a patient who suffered a subarachnoid hemorrhage from a cerebral aneurysm at the age of 22 (Paluzzi et al., 2008). Therefore, it is difficult to assess the prevalence of hypertension from the literature. Furthermore, most patients were reported in childhood when their blood pressure may not have become elevated as with our patient whose blood pressure was 110/70 at aged 16 years. The finding of hypertension at a relatively young age in our patient, coupled with that reported by Feingold and White raises the possibility that this is a significant unrecognized feature of Floating–Harbor syndrome, which manifests in early adult life.

Imaging of the urinary tract was specifically reported in 10 other patients and was abnormal in six of these. In one case, an ultrasound scan demonstrated a right polycystic kidney in a 7-year-old girl—whether the left kidney was also polycystic is unclear (Stagi et al., 2007). Zabransky (Zabransky, 1984) reported a left duplex ureter and calyceal cyst demonstrated by

intravenous pyelography in a 14-year-old male. White et al. (White et al., 2010) reported that a 12-year-old girl had nephrocalcinosis and hydronephrosis. Hood et al. (Hood et al., 2012) reported on unilateral renal pelviectasis and posterior urethral valves in 4 and 19-year-old males, respectively. Normal ultrasound findings in the other four patients cannot exclude polycystic kidneys because the patients were aged 6 months to 5 years old and our patient had a normal renal ultrasound at 6 years of age. Cyst development can be a slow process and in adult polycystic kidney disease the cysts are typically not visible using routinely available ultrasound scanning techniques until early adulthood.

Although our patient does not have a *PKD1/2* mutation, given the similarity in presentation to classic adult polycystic kidney disease we scrutinized the literature for reports of intracranial aneurysms. There is a single report of a ruptured intracranial aneurysm in a 22-year-old patient with FHS although neither blood pressure nor renal ultrasound findings were reported (Paluzzi et al., 2008). A further 10 patients had reportedly normal CT or MRI brain imaging, although the use of intravenous contrast was mostly not commented upon, so small aneurysms cannot be excluded.

The serum creatinine was not reported in any of the patients, but normal renal function was indicated in 14 patients from statements such as ~~"b~~iochemical screening negative". Our patient had a normal serum creatinine at presentation. The onset of renal failure in patients with polycystic kidneys is variable and for patients with typical adult polycystic kidney disease caused by *PKD1/2* mutations is often not apparent until beyond the fourth decade of life (Torres et al., 2007). Thus normal renal function cannot exclude the presence of polycystic kidneys in the reported Floating–Harbor syndrome patients. In addition, a

creatinine value in the normal range for adults of all ages can be associated with a reduced glomerular filtration rate in a young adult patient. Urinalysis results were not specifically reported in the literature apart from one case in which there was no proteinuria. See Table I for a summary of the renal findings in patients with FHS.

Conclusions

Our patient is the first patient with FHS and *SRCAP* mutation to be formally diagnosed with hypertension and polycystic kidneys. We have sequenced his *PKD1/2* and excluded mutations in the two genes commonly associated with polycystic kidney disease. He is the third FHS patient with early adult onset hypertension requiring medication. There is a significant paucity of data regarding renal problems and hypertension in the literature for this condition. Various renal tract structural abnormalities were reported in five other cases, one of which was suggestive of polycystic kidneys. Interestingly a case of ruptured cerebral aneurysm has been reported which would be consistent with polycystic kidney disease, but renal imaging and blood pressure were not reported. Together, these data suggest that hypertension and/or polycystic kidneys are important associations with FHS. Given that blood pressure measurement and ultrasound examination are non-invasive tests, we recommend screening for these conditions in patients with FHS. On the basis of the current data, we suggest annual blood pressure measurement and a baseline ultrasound scan at diagnosis with a repeat scan in adulthood if the first scan occurred during childhood. Further, if the presence of polycystic kidneys is confirmed we would recommend that consideration is given to screening for intracranial aneurysms in the light of the report of a ruptured intracranial aneurysm in a 22-year-old patient with Floating–Harbor syndrome. Long-term

Table I. Summary of renal findings in patients with FHS.

Feature	Number reported	Number abnormal
Blood pressure	3/71	3/3
Renal impairment	14/71	0/14
Proteinuria	1/71	0/1
Haematuria (history or dipstick)	2/71	1/2
Renal tract structural abnormality by imaging (excluding testicular/penile)	10/71	6/10
Intracranial imaging	11/71	1/11

follow-up data on patients with Floating–Harbor syndrome will expand on our observations and future genetic studies may shed light on the effect of chromatin remodeling on the development and health of the renal system.

Acknowledgements

We are grateful to Professor Albert Ong for helpful discussions and for oversight of genetic testing for PKD1 and PKD2. C.A. O'C and U.K. acknowledge research support from the National Institute for Health Research Oxford Comprehensive Biomedical Research Centre (BRC) Program. K.M.B is supported by a Clinical Investigatorship Award from the Canadian Institutes of Health Research (CIHR) – Institute of Genetics and acknowledges the contribution of the FORGE Canada Consortium funded by the Government of Canada through Genome Canada, CIHR and the Ontario Genomics Institute (OGI-049).

Supporting Information

Supporting information includes two text documents entitled: _Criteria Used to Establish the Diagnosis of FHS‘ and _Renal Findings in Reports where the Clinical Diagnosis of FHS Cannot be Definitively Established Based on Reported Information‘ and two tables. These are located on the subsequent pages.

Supporting Information

Criteria Used to Establish the Diagnosis of FHS

We divided the published reports into two sets. Firstly, those where the clinical diagnosis of FHS was secure based on the same criteria used by White et al., or on the basis of genetic testing and secondly, those for which it was difficult to definitively establish the diagnosis from the published information (White et al., 2010). The criteria consisted of photographic evidence and detailed reported clinical features comprising: marked speech delay, short stature, characteristic facies (including at least three of these features - distinctive nose, low hanging columella, triangular face, low set ears, wide mouth, short philtrum) and an absence of features suggestive of an alternative diagnosis. These features have proven robust predictors of the recently identified FHS-associated genetic mutation (Hood et al., 2012). Those cases in which it was difficult to establish a definitive diagnosis may indeed represent FHS and for completion we include a separate, but equally detailed analysis of these cases in Supplementary Table II.

Renal Findings in Reports where the Clinical Diagnosis of FHS Cannot be Definitively Established Based on Reported Information

The clinical diagnosis of Floating-Harbor syndrome was difficult to definitively establish from the information or images available in 14 reports (Ala-Mello and Peippo, 1999; Cannavo et al., 2002; Davalos et al., 1996; De Benedetto et al., 2004; Fryns et al., 1996; Innis et al., 2000; Karaer et al., 2006; Lazebnik et al., 1996; Morel et al., 2003; Nagai, 2001; Penaloza et al., 2003; Rosen et al., 1998; Selimoglu et al., 2004; Smeets et al., 1996). These reports included 16 patients (8 females, 6 males, 2 undocumented). The mean age was 8.5 years at the time of reporting. Hypertension could not be determined as no specific BP measurements were reported and hypertension was not mentioned in the reports. Renal tract imaging was only reported in one case and was normal. There were no reports of abnormal renal function. Normal renal function was reported in 7 cases, although serum creatinine values were not provided. There were no reports of aneurysms in cases where the brain was imaged, but use of intravascular contrast was not documented. Urinalysis was normal in the two cases in which it was mentioned. In no case was a family history of intracranial aneurysm/sudden death reported.

Supplementary Table I. Renal analysis of reports where the diagnosis of FHS is secure on the basis of genetic testing or the reported information.

Case report	Age at time of report(Years.months)/Gender (M=male, F=Female)	Hypertension (NR = not reported)	Renal impairment	Renal tract imaging or structural abnormality	Intracranial imaging (iv = intravenous)	Proteinuria	Haematuria	Family history of sudden death/intracranial haemorrhage
[Hood et al., 2012]	1. Person 10 in White et al. 2010 2. Person 9 in White et al. 2010 3. 12M 4. 11M 5.4.3 M 6. 4M 7. 4.4M 8. 10.5F 9. Person 8 in White et al. 2010. 10. 19M 11. 19.7M 12. 11M 13. 7.5M	NR	NR	4.Cryptorchism 6. unilateral renal pelviectasis 9. cryptorchism 10. posterior urethral valves 12. epididymal cysts, left varicocele 13. cryptorchism Others NR as to negative imaging	NR	NR	NR	NR
[Pouliquen et al., 2012]	27F	NR	NR	NR	"Cerebral tomodensitometry was normal"	NR	NR	no
[Lopez et al., 2012]	1. 4F 2. 13M 3. 10M 4. 30F 5. 37F 6. 7F 7. 8M 8. 9M 9. 8F	NR	NR	1 - Normal renal USS 3 - Hypospadias Others not reported imaging	1 - Normal MRI brain Others not reported imaging	NR	NR	1 – no, others NR
[Galli-Tsinopoulou et al., 2011]	5.4F	NR	No	Normal	Normal MRI brain	No	No	No
[Arpin et al., 2012]	4.5F	NR	NR	NR	NR	NR	NR	NR

Supplementary Table I. Renal analysis of reports where the diagnosis of FHS is secure on the basis of genetic testing or the reported information (continued).

Case report	Age at time of report(Years.months)/Gender (M=male, F=Female)	Hypertension (NR = not reported)	Renal impairment	Renal tract imaging or structural abnormality	Intracranial imaging (iv = intravenous)	Proteinuria	Haematuria	Family history of sudden death/intracranial haemorrhage
[White et al., 2010]	(includes our patient – number 5) 1. 11M 2. 12M 3. 7F 4. 23M 5. 20M 6. 34F 7. 15M 8. 11M 9. 12 F 10.8M	4. has hypertension Others not specifically reported blood pressure.	NR	9. Nephrocalcinosis, hydronephrosis	NR	NR	NR	No
[Angelillo et al.,]	7.4M	NR	NR	NR	MRI/CT normal (iv contrast - NR)	NR	NR	No
[Hendrickx et al., 2010]	5M	NR	NR	NR	NR	NR	NR	No
[Asseidat and Kaufman 2009]	5M	NR	NR	NR	NR	NR	NR	No
[Nelson et al., 2009]	5M	NR	NR	NR	NR	NR	NR	NR
[Paluzzi et al., 2008]	22F	NR	NR	NR	Subarachnoid haemorrhage. Left internal carotid bifurcation aneurysm	NR	NR	NR
[Genc et al., 2008] (good evidence with photos)	5M	NR	No	Abdominal USS normal	NR	NR	NR	No
[Genc et al., 2008]	0.5 F	NR	no	Abdominal USS normal	NR	NR	NR	No
[Stagi et al., 2007]	7+7monthsF	NR	no	Abdominal USS – right polycystic kidney, Left kidney - NR	MR with gadolinium normal	NR	NR	No
[Bastaki et al., 2007]	8+5 months F	NR	NR	NR	CT/MR normal (iv contrast -NR)	NR	NR	No
[Feingold 2006]	32M	Yes	NR	NR	NR	NR	NR	No
[Wiltshire et al., 2005]	7F	NR	NR	NR	NR	NR	NR	NR
[Ala-Mello and Peippo 2004]	F/U of their 1996 case 14M	NR	NR	NR	NR	NR	NR	NR

Supplementary Table I. Renal analysis of reports where the diagnosis of FHS is secure on the basis of genetic testing or the reported information (continued).

Case report	Age at time of report(Years.months)/Gender (M=male, F=Female)	Hypertension (NR = not reported)	Renal impairment	Renal tract imaging or structural abnormality	Intracranial imaging (iv = intravenous)	Proteinuria	Haematuria	Family history of sudden death/intracranial haemorrhage
[Ioan and Fryns 2003]	20F (sisters)	NR	NR	NR	NR	NR	NR	NR
[Ioan and Fryns 2003]	17F (sister)	NR	No	NR	NR	NR	NR	NR
[Wieczorek et al., 2001]	11F	NR	NR	NR	NR	NR	NR	NR
[Wieczorek et al., 2001]	8F	NR	NR	NR	NR	NR	NR	NR
[Femiano et al., 2000]	9year 8month M	NR	NR	NR	CT head normal (iv contrast - NR)	NR	NR	No
[Muir et al., 1999]	5.5F	NR	NR	NR	NR	NR	NR	No
[Hersh et al., 1998]	12F	NR	NR	NR	NR	NR	NR	NR
[Midro et al., 1997]	12F	NR	'laboratory tests normal'	NR	NR	NR	NR	No
[Lacombe et al., 1995]	5.5F	NR	'metabolic screening negative'	NR	CT head normal (iv contrast – NR)	NR	NR	NR
[Houlston et al., 1994]	2F	NR	NR	NR	NR	NR	NR	NR
[Houlston et al., 1994]	15F	NR	NR	NR	NR	NR	NR	NR
[Patton et al., 1991]	1. 5F 2. 5M 3. 8F 4. 8M 5. 7F 6. 8F 7. 5F	NR	NR	NR	NR	NR	NR	NR
[Majewski and Lenard 1991]	6yr 11month F	NR	'metabolic screen negative'	NR	CT head normal (iv contrast – NR))	NR	NR	NR
[Chudley and Moroz 1991]	17F	NR	NR	NR	NR	NR	NR	No
[Robinson et al., 1988]	1. 15M 2. 16M 3. 12F 4. 15F 5. F(patient later reported in White et al., 2010) 6. F ?age	NR	NR – but many metabolic conditions screened e.g. thyroid thus probable renal function also screened	NR	NR	NR	NR	NR
[Zabransky 1984]	14 M	NR	NR	IV pyelogram – duplicated ureter with left calyceal cyst	CT brain normal (iv contrast NR)	NR	NR	No
[Leisti et al., 1975]	9yr 9mo M	NR	'normal laboratory data'	Penile meatal stenosis	NR	NR	NR	NR

Supplementary Table II. Reports where the clinical diagnosis of FHS cannot be fully validated on the basis of the reported information.

Case report	Age/gender (Years.months.M=male, F=female)	Hypertension (NR – not reported)	Renal impairment	Renal tract imaging	Intracranial imaging (iv – intravenous)	Proteinuria	Haematuria	FH-SD/intracranial haemorrhage
[Karaer et al., 2006]	8F	NR	'Normal renal function'	NR	MRI brain 'abnormal white matter signal'	No	No	No
[Selimoglu et al., 2004])	12M	NR	Normality inferred	No	NR	No	No	NR
[De Benedetto et al., 2004]	11M	NR	NR	NR	NR	NR	NR	NR
[Penaloza et al., 2003]	2.11M	NR	Normality inferred from 'normal routine tests'	NR	NR	NR	NR	NR
[Morel et al., 2003]	6M	NR	NR	NR	MRI brain normal (iv contrast – NR)	NR	NR	NR
[Cannavo et al., 2002]	9.1F	NR	'routine chemistry normal'	USS normal	MRI brain normal (iv contrast NR)	NR	NR	NR
[Nagai 2001]	NR	NR	NR	NR	NR	NR	NR	NR
[Innis et al., 2000])	2 months	NR	NR	NR	NR	NR	NR	NR
Ala-Mello 1999	6F	NR	NR	NR	NR	NR	NR	NR
[Rosen et al., 1998]	5.3F	NR	NR	NR	CT head at 4.9yr = normal (iv contrast NR)	NR	NR	No
[Rosen et al., 1998]	30F	'extensive med exam normal'	NR	NR	CT head aged 19, normal (iv contrast NR)	NR	NR	No
[Smeets et al., 1996]	4F	NR	'biochemical screening normal'	NR	NR	NR	NR	No
[Lazebnik et al., 1996]	10M	NR	NR	NR	NR	NR	NR	NR
[Fryns et al., 1996]	6 months F	NR	'extensive biochemical tests normal'	NR	NR	NR	NR	NR
[Fryns et al., 1996]	16M	NR	'routine chemical screening negative'	NR	NR	NR	NR	NR
[Davalos et al., 1996]	6.11F	NR	'screening for metabolic defects normal'	NR	NR	NR	NR	'no FH of similar disorder'
Total	16 patients							

Appendix 3: Supplemental Data for Chapter 2

The following is supplemental data (1 figure) previously published in the American Journal of Human Genetics under the title –Mutations in SRCAP, Encoding SNF2-Related CREBBP Activator Protein, Cause Floating-Harbor Syndrome" (PMID: 22265015) by Hood RL, Lines MA, Nikkel SM, Schwartzentruber J, Beaulieu C, Nowaczyk MJ, Allanson J, Kim CA, Wieczorek D, Moilanen JS, Lacombe D, Gillessen-Kaesbach G, Whiteford ML, Quaio CR, Gomy I, Bertola DR, Albrecht B, Platzer K, McGillivray G, Zou R, McLeod DR, Chudley AE, Chodirker BN, Marcadier J; FORGE Canada Consortium., Majewski J, Bulman DE, White SM, Boycott KM.

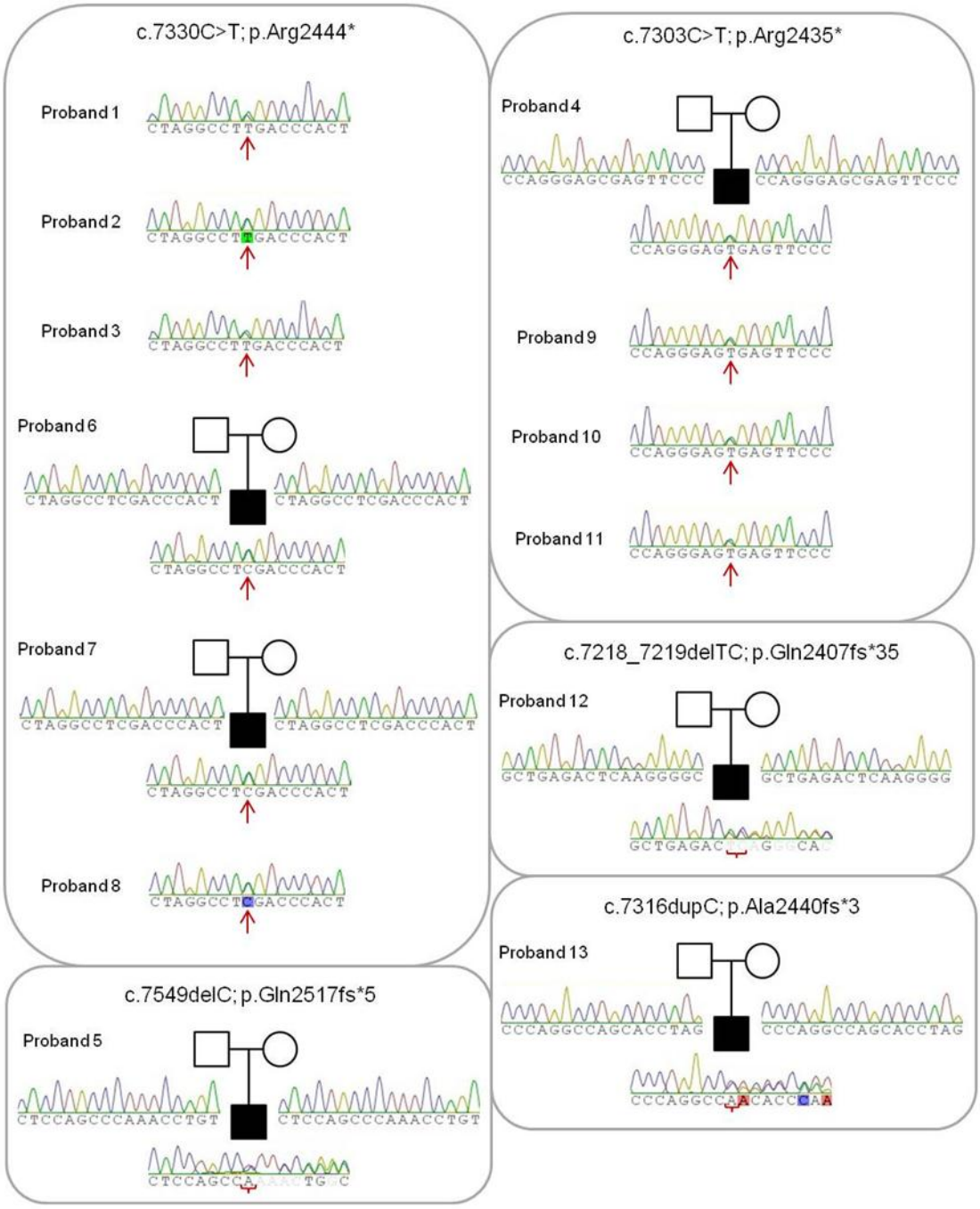


Figure S1. Chromatograms demonstrating SRCAP mutations in FHS patients. Nonsense (red arrows) and frameshift (red brackets) mutations are indicated at the positions shown.

Appendix 4: Supplemental Data for Chapter 3

The following is supplemental data (1 figure and 1 table) previously published in the American Journal of Human Genetics under the title "Mutations in *EZH2* Cause Weaver Syndrome" (PMID: 22177091) by Gibson WT, Hood RL, Zhan SH, Bulman DE, Fejes AP, Moore R, Mungall AJ, Eydoux P, Babul-Hirji R, An J, Marra MA; FORGE Canada Consortium., Chitayat D, Boycott KM, Weaver DD, Jones SJ.

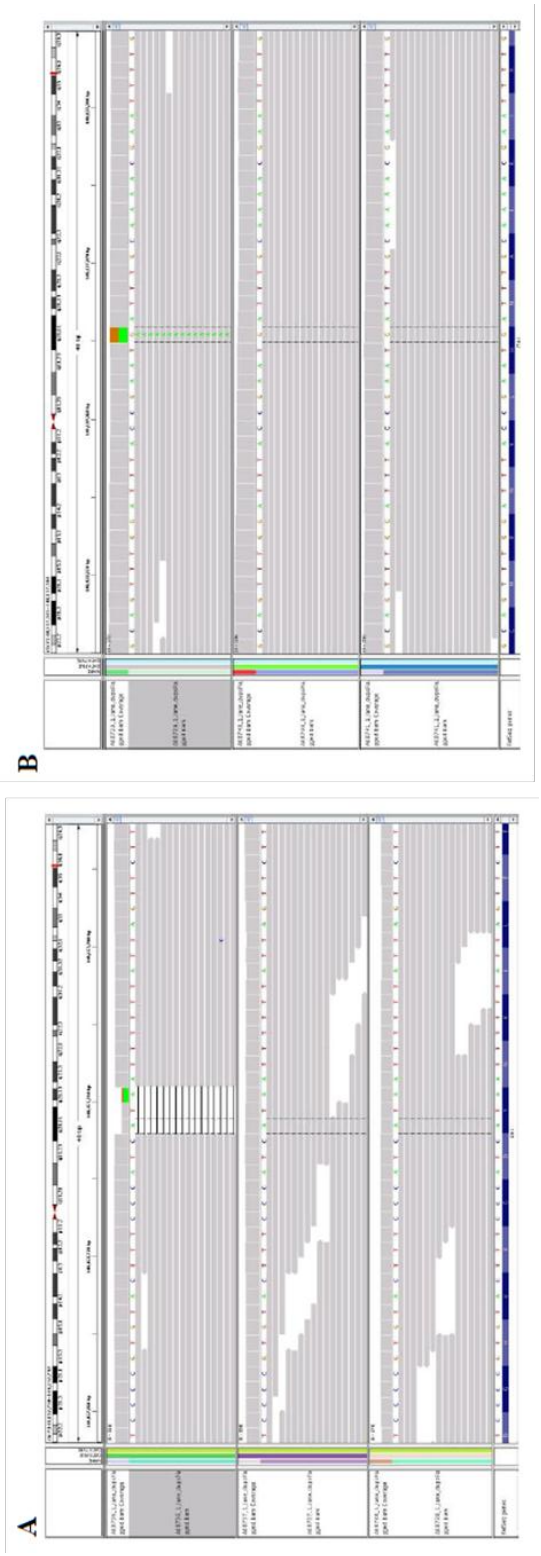


Figure S1. Integrative Genomics Viewer (Robinson et al., 2011) generated images of BAM file read alignments to hg18 for probands 1 and 2 and their parents.
(A) EZH2 mutation c.457_459del (p.Tyr153del) in Proband 1 (top), his mother (middle) and father (bottom). (B) EZH2 mutation c.2080C>T (p.His694Tyr) in Proband 2 (top), her mother (middle) and father (bottom).

Table S1. Summary statistics of exome resequencing.

Subject	Proband 1 (A08736)	Mother 1 (A08737)	Father 1 (A08738)	Proband 2 (A08739)	Mother2 (A08740)	Father 2 (A08741)
Total sequence yield (Gb) [a]	1.86	1.97	2.02	2.08	1.9	1.98
Total reads	429,667,422	424,496,474	463,366,406	506,490,420	463,258,182	409,296,116
Chastity-passed reads	348,012,586	386,608,086	414,972,084	441,242,928	415,420,736	372,566,906
Reads aligned to hg18	343,664,632	382,044,390	409,662,802	435,193,786	410,538,142	368,077,609
Reads aligned with mapping quality>=10	314,547,518	350,236,460	374,396,369	400,054,154	376,045,604	336,881,973
Reads used for variant calling [b]	143,593,996	141,579,235	187,165,231	202,315,301	176,426,879	182,790,043
Mean exome coverage [c]	213	236	253	274	260	228
Total exonic variants [d]	24,287	24,396	25,207	25,127	24,914	24,688
Exonic synonymous SNVs	11,355	11,420	11,777	11,782	11,704	11,676
Exonic non-synonymous (ns) variants [e]	12,932	12,976	13,430	13,345	13,210	13,012
nsSNVs	11,091	11,079	11,396	11,306	11,207	11,081
Splice-site SNVs [also nsSNVs]	889 [273]	905 [265]	964 [274]	985 [288]	935 [276]	912 [267]
Insertions/deletions [f]	585/640	583/674	636/708	637/705	618/726	602/684
ns variants not in dbSNP129/130	2,885	2,839	3,173	3,093	3,046	2,977
Novel ns variants [g]	2 (17)	117	196	2 (16)	148	113
Rare variants in compound heterozygous state	None	Not Analyzed	Not Analyzed	None	Not Analyzed	Not Analyzed
de novo candidate variants	c.457_459del (p.Tyr153del)	None	None	c.2080C>T (p.His694Tyr)	None	None

[a] - span of the human genome (hg18) covered by ≥1 read aligned with Phred-scaled mapping quality of ≥10

[b]- reads having Phred-scaled mapping quality of ≥10 and after duplicates are removed

[c]- average read depth of exons annotated in Ensembl 54; (sum of the number of reads aligned per site for all exonic sites) / (total number of exonic sites)

[d]- variants as output from [samtools.pl varFilter - D 1000 | awk '$6>=20'], exclusing those in 5'UTR, 3'UTR, introns and intergenic regions

[e]- variants include nsSNVs, splice-site SNVs within 2bp of exon boundaries, and small indels

[f]- coding region insertions/deletions supported by ≥6 aligned reads

[g]- not previously reported in dbSNP129/130, 1000 Genomes Project, or other non-cancer genomes collected in the Genome Sciences Centre local database. For probands, the total of "novel" variants excludes those seen in their unaffected parents and represents de novo variants.
Numbers in parentheses include apparently *de novo* variants seen at low coverage (ie. Fewer than 100 reads per variant)

Appendix 5: Supplementary Material for Chapter 5

The following is supplementary material (the questionnaire used to collect the clinical data, and the primer pairs used for sequencing exon 34 of *SRCAP*) previously published in the Orphanet Journal of Rare Diseases under the title "The Phenotype of Floating-Harbor Syndrome: Clinical Characterization of 52 Individuals with Mutations in Exon 34 of SRCAP" (PMID: 23621943) by Nikkel SM, Dauber A, de Munnik S, Connolly M, Hood RL, Caluseriu O, Hurst J, Kini U, Nowaczyk MJ, Afenjar A, Albrecht B, Allanson JE, Balestri P, Ben-Omran T, Brancati F, Cordeiro I, da Cunha BS, Delaney LA, Destrée A, Fitzpatrick D, Forzano F, Ghali N, Gillies G, Harwood K, Hendriks YM, Héron D, Hoischen A, Honey EM, Hoefsloot LH, Ibrahim J, Jacob CM, Kant SG, Kim CA, Kirk EP, Knoers NV, Lacombe D, Lee C, Lo IF, Lucas LS, Mari F, Mericq V, Moilanen JS, Møller ST, Moortgat S, Pilz DT, Pope K, Price S, Renieri A, Sá J, Schoots J, Silveira EL, Simon ME, Slavotinek A, Temple IK, van der Burgt I, de Vries BB, Weisfeld-Adams JD, Whiteford ML, Wierczorek D, Wit JM, Yee CF, Beaulieu CL; FORGE Canada Consortium., White SM, Bulman DE, Bongers E, Brunner H, Feingold M, Boycott KM.

Additional File 1:

FLOATING-HARBOR QUESTIONNAIRE

Patient
Sex
Ethnicity
Paternal age (years)
Gestation (weeks)
Birth weight (g)
Age at diagnosis
Age at last assessment (ALA)
Head circumference (cm) ALA
Weight (kg) ALA
Height (cm) ALA
Age at puberty
Pre-pubertal height
Bone age years versus chronological age
Triangular face
Distinctive nose
Low-hanging columella
Short philtrum
Thin upper vermilion
Wide mouth
Low set ears
Broad thumbs
Broad fingertips
Brachydactyly
Clinodactyly
Other skeletal
Dental issues
Gastrointestinal problems
Seizures
Other health issues/ /serious illnesses/hospitalizations
Behaviour issues
Attention Deficit/Hyperactivity
Psychiatric issues/Anxiety/Depression
High pitched voice
Speech delay
Intellectual development/Developmental Delay/Mental Retardation

*ALA – at last assessment

Additional file 2:

Primer pairs used to sequence exon 34 of *SRCAP*

	primer name	sequence	amplicon size
1	SRCAP.exon34_A.F	TGACCTGCTGGCCTTAACTT	381
2	SRCAP.exon34_A.R	GGCGGATATGACAGGAGTGT	
3	SRCAP.exon34_B.F	CACCGGCGCAGTAAAAAG	400
4	SRCAP.exon34_B.R	TATGAGCTGGTGGAGGGGTA	
5	SRCAP.exon34_C.F	TCAGCCCCAAATCCAATAAC	374
6	SRCAP.exon34_C.R	AGGATCTCCACAGCAACTGG	
7	SRCAP.exon34_D.F	GCATTGGCATCTCCAGAGTC	368
8	SRCAP.exon34_D.R	TCTGTCAGCTCTTCCGAGGT	
9	SRCAP.exon34_E.F	GTGAGGAGTTGCCCCTGT	499
10	SRCAP.exon34_E.R	ATGGGAGGGGAGGATTCTG	
11	SRCAP.exon34_F.F	GGCTGGTAACTGTGGTAGAGGA	488
12	SRCAP.exon34_F.R	CCCAGGAATAAGCTGTGGTTC	
13	SRCAP.exon34_G.F	ACCTTGAAGGGAAAAACCAATG	457
14	SRCAP.exon34_G.R	AGCTCTCGAGAACAGAAGTGCT	
15	SRCAP.exon34_H.F	CCTCCCAAGAATCCTCCATC	500
16	SRCAP.exon34_H.R	GCTTCAGCCTCAGACTCCTCTA	
17	SRCAP.exon34_I.F	TGCGTCCAGGGTCTCTAGTC	494
18	SRCAP.exon34_I.R	TGACAACCAGTTGCCTACCC	

Appendix 6: Supplementary Material for Chapter 6

The following data is supplemental information (3 tables and 4 figures) previously published in Scientific Reports under the title "The Defining DNA Methylation Signature of Floating-Harbor Syndrome" (PMID: 27934915) by Hood RL, Schenkel LC, Nikkel SM, Ainsworth PJ, Pare G, Boycott KM, Bulman DE, Sadikovic B.

Supplementary Table 1. Regions with significantly altered methylation (>15%) in FHS individuals identified by methylation array.

Location	Region Start (hg19 location; bp)	Region Stop (hg19 location; bp)	Region Length (bp)	Number of Probes in Region	Methylation Estimate[a]	p-value[b]	Nearest gene	Overlapping CpG Island
chr1	1003116	1003539	424	4	-0.27727	4.46E-05	RNF223 (-)	Yes
chr1	45278961	45279694	734	6	-0.15405	4.46E-05	BTBD19 (+)	Yes
chr1	174843744	174843981	238	3	0.242846	4.46E-05	RABGAP1L (+)	No
chr1	169429594	169429982	389	3	0.193606	4.46E-05	CCDC181 (-)	No
chr1	224363439	224363585	147	3	0.160763	0.00766933	DEGS1 (+)	No
chr1	95698817	95699323	507	6	0.151871	4.46E-05	LOC101928118 (-)	No
chr1	2979301	2980947	1647	10	-0.18146	4.46E-05	LINC00982 (-)	Yes
chr1	27676195	27676662	468	3	-0.21966	0.0061533	SYTL1 (+)	Yes
chr1	178455597	178456280	684	5	0.166236	4.46E-05	RASAL2 (+)	Yes
chr1	211652266	211652751	486	4	0.152705	4.46E-05	RD3 (-)	Yes
chr1	143663852	143664141	290	3	0.173754	4.46E-05	MIR6077 (+)	Yes
chr1	228890791	228891316	526	6	0.160177	0.00124849	RHOU (+)	Yes
chr1	43814159	43815475	1317	7	-0.17638	4.46E-05	MPL (+)	Yes
chr1	1229095	1229800	706	6	-0.16002	0.000178356	ACAP3 (-)	Yes
chr1	108022757	108023492	736	6	0.155427	0.00209569	NTNG1 (+)	Yes
chr1	2120975	2121734	760	6	-0.19186	4.46E-05	C1orf86 (-)	Yes
chr1	149162209	149162528	320	4	0.154546	4.46E-05	LOC388692 (+)	Yes
chr1	38461530	38461906	377	4	-0.17179	4.46E-05	FHL3 (-)	Yes
chr1	55246857	55247418	562	5	0.154141	0.00133767	TTC22 (-)	Yes
chr10	89167447	89167981	535	4	0.221604	4.46E-05	LINC00864 (-)	No
chr10	50649656	50650258	603	5	0.200051	0.000445891	ERCC6 (-)	No
chr10	81743118	81743556	439	4	0.157609	4.46E-05	SFTPD (-)	Yes
chr11	106698552	106698704	153	3	0.154062	0.000445891	GUCY1A2 (-)	No
chr11	85393683	85393905	223	3	0.153076	0.00222946	CCDC89 (-)	No
chr11	65360113	65360519	407	5	-0.1999	4.46E-05	KCNK7 (-)	Yes
chr11	58830181	58830867	687	5	0.151247	4.46E-05	FAM111B (+)	Yes
chr12	75784531	75785305	775	11	0.200664	4.46E-05	GLIPR1L2 (+)	Yes
chr12	21926437	21926534	98	3	0.151756	0.00120391	KCNJ8 (-)	No
chr12	95840303	95840905	603	5	0.176415	4.46E-05	METAP2 (+)	No
chr12	58011754	58011885	132	4	0.167664	4.46E-05	LOC101927583 (-)	No
chr12	64215601	64215907	307	3	0.188272	0.000445891	TMEM5 (+)	Yes
chr13	23309764	23310685	922	9	0.184989	4.46E-05	BASP1P1 (-)	No
chr13	31506675	31507149	475	8	0.16312	4.46E-05	TEX26 (+)	No
chr13	23412240	23412632	393	4	0.226345	4.46E-05	BASP1P1 (-)	Yes
chr13	23270675	23270860	186	3	0.158339	4.46E-05	BASP1P1 (-)	Yes
chr13	110521946	110522307	362	5	0.15779	0.000401302	IRS2 (-)	Yes
chr15	23157717	23158348	632	4	0.161761	4.46E-05	WHAMMP3 (-)	Yes
chr15	40583217	40583432	216	3	-0.18611	4.46E-05	PLCB2 (-)	Yes
chr15	69222582	69223378	797	4	0.151475	4.46E-05	SPESP1 (+)	Yes
chr15	29034659	29034960	302	3	0.158286	0.000178356	PDCD6IPP2 (+)	Yes
chr16	30907236	30907689	454	3	-0.15839	4.46E-05	CTF1 (+)	No
chr16	279736	280056	321	3	-0.19244	4.46E-05	LUC7L (-)	No
chr16	57562442	57563405	964	5	-0.15829	0.000178356	CCDC102A (-)	Yes
chr17	33842171	33842311	141	3	0.151089	4.46E-05	SLFN12L (-)	No
chr17	9550127	9550555	429	5	0.158825	0.00120391	USP43 (+)	No
chr17	17603521	17604194	674	6	-0.16929	4.46E-05	RAI1 (+)	Yes
chr17	40835839	40836135	297	4	0.173002	4.46E-05	CNTNAP1 (+)	Yes
chr17	46641494	46642114	621	5	-0.15273	4.46E-05	HOXB3 (-)	Yes
chr18	32173074	32173237	164	4	0.171691	4.46E-05	DTNA (+)	No
chr18	19476854	19477069	216	4	0.196499	4.46E-05	MIR1-2 (-)	Yes
chr19	49222477	49224464	1988	12	-0.20169	4.46E-05	RASIP1 (-)	Yes
chr19	8591354	8591786	433	4	-0.37247	4.46E-05	MYO1F (-)	Yes
chr19	1063614	1064228	615	3	-0.21259	4.46E-05	ABCA7 (+)	Yes
chr19	18543819	18544429	611	3	-0.17983	4.46E-05	SSBP4 (+)	Yes
chr19	3480353	3480682	330	5	-0.17453	0.00931912	SMIM24 (-)	No
chr19	46801547	46801682	136	3	-0.15352	4.46E-05	HIF3A (+)	No
chr19	2428112	2429219	1108	7	-0.15526	4.46E-05	LMNB2 (-)	Yes
chr19	49133411	49133855	445	4	-0.24687	4.46E-05	DBP (-)	Yes
chr19	523290	523652	363	3	-0.21615	0.000312124	TPGS1 (+)	Yes
chr2	164204618	164205353	736	7	0.322347	4.46E-05	FIGN (-)	Yes
chr2	50201372	50201521	150	5	0.189996	4.46E-05	NRXN1 (-)	No

Supplementary Table 1. Regions with significantly altered methylation (>15%) in FHS individuals identified by methylation array (continued),

Location	Region Start (hg19 location; bp)	Region Stop (hg19 location; bp)	Region Length (bp)	Number of Probes in Region	Methylation Estimate[a]	p-value[b]	Nearest gene	Overlapping CpG Island
chr2	173539081	173539631	551	4	0.158672	4.46E-05	RAPGEF4-AS1 (-)	No
chr2	105735607	105735766	160	3	0.171228	4.46E-05	LOC101927492 (-)	No
chr2	129659306	129659956	651	6	0.196434	4.46E-05	LOC101927881 (+)	Yes
chr2	87036616	87037048	433	4	0.190747	4.46E-05	CD8B (-)	Yes
chr2	96191005	96191364	360	3	0.163192	0.000312124	TRIM43B (-)	Yes
chr20	62693647	62694015	369	7	-0.16163	4.46E-05	TCEA2 (+)	Yes
chr20	62679245	62679723	479	3	0.203354	4.46E-05	SOX18 (-)	Yes
chr22	50737968	50738900	933	4	-0.25404	4.46E-05	PLXNB2 (-)	Yes
chr3	87137923	87138710	788	7	0.168147	4.46E-05	LINC00506 (+)	Yes
chr3	109056339	109056907	569	4	0.155988	4.46E-05	DPPA4 (-)	No
chr3	159557542	159558041	500	4	0.223463	0.000445891	SCHIP1 (+)	No
chr3	156323942	156324128	187	3	0.152503	4.46E-05	SSR3 (-)	No
chr3	113160061	113160647	587	9	0.16965	4.46E-05	CFAP44 (-)	Yes
chr3	105072527	105073087	561	3	0.196409	4.46E-05	ALCAM (+)	Yes
chr3	350493	351013	521	6	0.173252	4.46E-05	CHL1 (+)	Yes
chr4	99064092	99064914	823	9	0.239439	4.46E-05	STPG2 (-)	Yes
chr4	46126056	46126458	403	7	0.239231	4.46E-05	GABRG1 (-)	No
chr4	165898656	165898977	322	8	0.193923	4.46E-05	TRIM61 (-)	No
chr4	1512860	1513269	410	5	-0.16639	4.46E-05	NKX1-1 (-)	No
chr4	62382922	62383250	329	4	0.206485	4.46E-05	LPHN3 (+)	Yes
chr4	14864533	14864873	341	3	0.151104	4.46E-05	CPEB2-AS1 (-)	Yes
chr4	11370304	11370882	579	5	0.202768	4.46E-05	MIR572 (+)	Yes
chr4	155702399	155703148	750	6	0.191892	4.46E-05	RBM46 (+)	Yes
chr5	11588961	11589059	99	3	0.18154	4.46E-05	CTNND2 (-)	No
chr5	42756776	42757181	406	5	0.189881	4.46E-05	CCDC152 (+)	No
chr5	78985415	78985910	496	10	0.154164	4.46E-05	CMYA5 (+)	No
chr5	110062333	110062847	515	7	0.251366	4.46E-05	TMEM232 (-)	No
chr5	42944020	42944504	485	4	0.2232	4.46E-05	FLJ32255 (-)	Yes
chr5	145758576	145758891	316	5	0.15963	4.46E-05	POU4F3 (+)	Yes
chr5	78365245	78366086	842	7	0.162276	4.46E-05	BHMT2 (+)	Yes
chr5	8457538	8458402	865	7	0.171172	0.000668837	MIR4458HG (+)	Yes
chr6	49681168	49681784	617	9	0.174187	4.46E-05	CRISP2 (-)	No
chr6	17016216	17016494	279	3	0.159429	4.46E-05	STMND1 (+)	No
chr6	32847367	32847855	489	22	0.178924	0.000668837	LOC100294145 (+)	Yes
chr7	39170487	39171123	637	6	0.186442	4.46E-05	POU6F2 (+)	No
chr7	32358054	32358550	497	3	0.221514	4.46E-05	LOC100130673 (-)	No
chr7	143582136	143582640	505	4	0.176392	4.46E-05	FAM115A (-)	Yes
chr7	92672802	92673186	385	5	0.209416	4.46E-05	SAMD9 (-)	Yes
chr7	16890421	16891089	669	6	0.151387	0.000222946	AGR3 (-)	Yes
chr8	81478162	81478344	183	3	0.2572	4.46E-05	ZBTB10 (+)	No
chr8	39172010	39172130	121	6	0.253711	4.46E-05	ADAM5 (+)	No
chr8	2585656	2586235	580	3	0.18257	0.00111473	LOC101927815 (-)	No
chr8	74282802	74282941	140	3	0.194527	4.46E-05	LOC101926926 (-)	No
chr8	119086570	119086772	203	3	0.164421	0.000133767	EXT1 (-)	No
chr8	54605556	54605798	243	3	0.161386	4.46E-05	ATP6V1H (-)	No
chr8	145730808	145731419	612	3	-0.1794	0.000178356	GPT (+)	Yes
chr8	102235917	102236841	925	6	0.20572	4.46E-05	ZNF706 (-)	Yes
chr8	99984524	99985059	536	4	0.172668	0.00552905	OSR2 (+)	Yes
chr8	67454536	67454902	367	5	0.191337	4.46E-05	MYBL1 (-)	Yes
chr9	139258514	139259084	571	3	-0.20546	4.46E-05	CARD9 (-)	Yes
chrX	111623890	111624333	444	4	0.173589	4.46E-05	ZCCHC16 (+)	Yes
chrX	11157132	11157620	489	5	0.184296	0.00129308	ARHGAP6 (-)	Yes
chrY	6114245	6114414	170	3	-0.1983	0.000178356	TSPY2 (+)	Yes
chrY	9385529	9385871	343	3	-0.18799	0.00374549	TSPY10 (+)	Yes
chrY	21239338	21240014	677	4	0.162264	0.00124849	CD24 (-)	Yes

Significantly altered regions listed in this table have: methylation estimate value>15%, F value >50, and p<0.01.

[a] Positive Estimate values indicate hypermethylation and negative Estimate values indicate hypomethylation in FHS subjects compared to controls.

[b] p-value shows the significance for the region (within minimum of 3 probes).

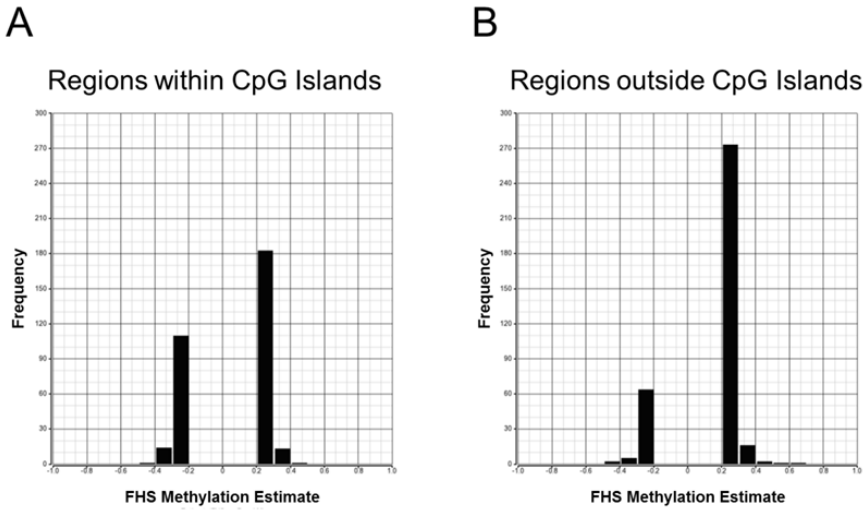
Abbreviations: chr = chromosome; bp = base pair; (+) = sense strand; (-) = anti-sense strand.

Supplementary Table 2. Genomic region distribution of the 116 differentially methylated regions (>15%) in FHS individuals.

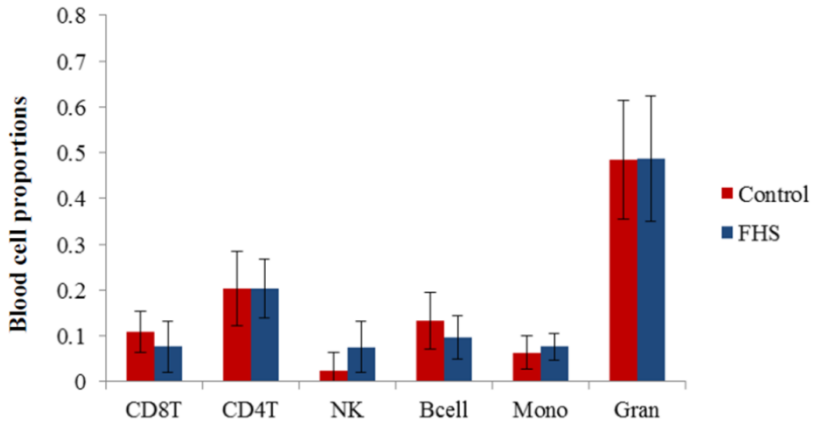
	Within CpG island	Within CpG shores	Within CpG shelves	Outside CpG site
Within gene body	42	4	0	20
Outside gene body	31	4	0	19

Supplementary Table 3. Pathway analysis of the differentially methylated genes in FHS.

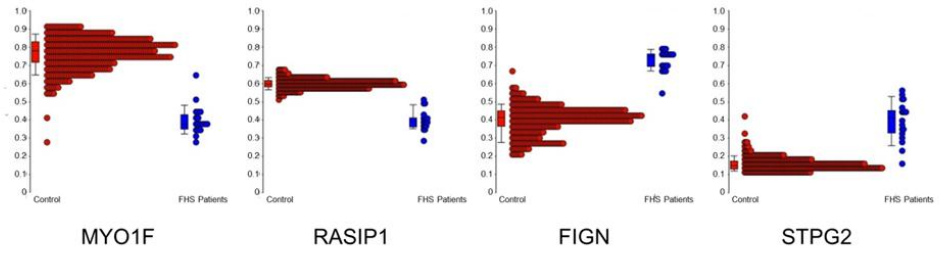
Biological Pathway	Neurological System Process	Synaptic Transmission	Developmental Process	
Number of genes in group	10	8	29	
Fisher Exact Enrichment Score - ln(p-value)	4.967	7.048	3.973	
Fisher Exact right-tail p-value	0.0069	0.00087	0.0188	
Chi Square Enrichment Score - ln(p-value)	6.06099	10.9996	3.7652	
Chi Square p-value	0.00233	1.67E-05	0.02316	
Genes	ABCA7 CHL1 CNTNAP1 CTNND2 NKX1-1 NRXN1 PLCB2 POU4F3 POU6F2 RD3	CD24 DTNA GABRG1 KCNJ8 KCNK7 NRXN1 PLCB2 TPGS1	CD24 CHL1 CNTNAP1 CTF1 CTNND2 DBP DPPA4 ERCC6 EXT1 FHL3 HIF3A HOXB3 IRS2 KCNJ8 NRXN1	NTNG1 OSR2 PLXNB2 POU4F3 POU6F2 RAI1 RASIP1 RD3 SFTPD SOX18 SPESP1 TPGS1 TSPY2 TSPY10



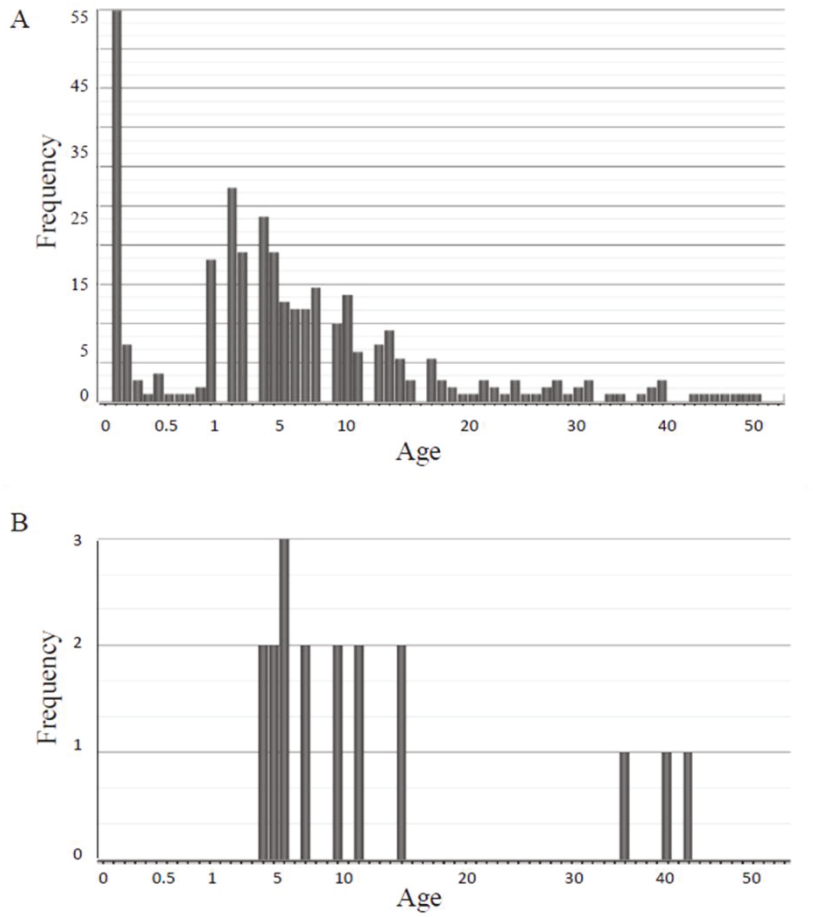
Supplementary Figure 1. Histogram comparison of differential FHS methylation estimates (a) within CpG islands and (b) outside of CpG islands.
The frequency of individual probes with significant methylation differences (Y-axis) with methylation estimates >20% (X-axis) in FHS individuals compared to controls. Positive estimate values show hypermethylation in FHS, negative values show hypomethylation in FHS. (a) Methylation of statistically significant regions within CpG Islands. (b) Methylation of statistically significant regions outside CpG Islands.



Supplementary Figure 2. Blood cell measures.
Estimated proportions of CD8 T cells (CD8T), CD4T cells (CD4T), natural killer cells (NK), B cells, monocytes (Mono) and granulocytes (Gran) in FHS patients and controls based on Illumina 450K methylation data.



Supplementary Figure 3. Methylation of significantly altered regions in FHS individuals compared to controls based on methylation array data.
Methylation array data: level from 0 (not methylated) to 1 (100% methylated) is plotted for individual FHS individual and control samples for regions with significantly altered methylation in FHS: hypermethylated regions (MYO1F and RASIP1) and hypomethylated regions (FIGN and STPG2). Each individual in the control (red) and FHS individual (blue) groups are represented as a circle. Corresponding box and whisker plots illustrate the methylation median, upper and lower quartiles, and standard deviation for FHS individuals compared to controls.



Supplementary Figure 4. Age distribution of (A) 361 controls and (B) 18 patients with FHS included in this study.
X axis shows the age in years and Y axis shows the frequency observed.

Curriculum Vitae

REBECCA HOOD

EDUCATION

2011-present **PhD (Biochemistry)**
Specialization in Human and Molecular Genetics
Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON

2008 **Master of Science**
Specialization in Cellular and Molecular Biology
Department of Biology, University of Western Ontario, London, ON

2005 **Bachelor of Science (Honours Genetics)**
Department of Biology, University of Western Ontario, London, ON

TECHNICAL EXPERIENCE

2011-present **Doctoral Thesis**: Molecular and Clinical Delineation of Rare Disorders of Stature
Supervisors: Dr. Dennis Bulman & Dr. Kym Boycott
CHEO Research Institute; Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON
Using an exome sequencing approach, we identified mutations in the chromatin remodeling protein *SRCAP* as the genetic cause underlying Floating-Harbor syndrome (FHS), mutations in *EZH2* as the genetic cause underlying Weaver syndrome, and mutations in *NSD1* as being associated with Sotos with cutis laxa. We then followed up on FHS, first expanding our patient cohort to examine a more comprehensive spectrum of FHS-mutations and resulting phenotypes in 52 FHS individuals. Second, we discovered a unique methylation signature in FHS patients as a result of SRCAP mutations.

2009-2010 **Research Assistant**
Supervisors: Dr. Aikaterini Dounavi, Dr. Heinz Rennenberg, & Dr. Albert Reif
Forestry Research Institute (FVA), Freiburg, GERMANY
Institute of Silviculture, University of Freiburg, Freiburg, GERMANY
Institute of Forest Botany and Tree Physiology, University of Freiburg, Freiburg, GERMANY
Examined RNA expression profiles of various genes in different provenances of European Beech in response to drought stress. This work was also focussed on setting up qPCR protocols and teaching this technique to other researchers in the form of lab tutorials and seminars.

2005-2008 **Master of Science Thesis**: RNA Expression of Six *AROGENATE DEHYDRATASE* Genes in *Arabidopsis thaliana*
Supervisor: Dr. Susanne Kohalmi
Department of Biology, University of Western Ontario, London, ON
Determined the RNA expression patterns of a six-member gene family in the five main plant tissues using RT-PCR and qPCR. Characterized the expression profiles of these genes in response to heat and cold stress treatments over a 24 hour period and following a 24 hour post-stress recovery using qPCR.

2004-2005 **Honours Undergraduate Thesis**: Expression Pattern Analysis of Select *PREPHENATE DEHYDRATASE-LIKE* (*PDL*) Genes in *Arabidopsis thaliana*.
Supervisor: Dr. Susanne Kohalmi
Department of Biology, University of Western Ontario, London, ON
Determined the tissue-specific expression profile of *PDL1*, and identified which *PDL*s are expressed in developing floral tissue of *Arabidopsis* using RT-PCR.

PUBLICATIONS

Hood, R.L., Schenkel, L.C., Nikkel, S.M., Ainsworth, P.J., Pare, G., Bulman, D.E, Boycott, K.M., and Sadikovic, B. (2016) The defining DNA methylation signature of Floating-Harbor Syndrome. *Sci Rep.* 6: 38803.

Hood, R.L., McGillivray, G, Hunter, M.F., Robertson, S.P., Bulman, D.E., Boycott, K.M., Stark, Z, and Care4Rare Consortium. (2016) Severe connective tissue laxity including aortic dilatation in Sotos syndrome. *Am J Med Genet A.* 170: 531-535.

Nikkel, S.M., Dauber, A., de Munnik, S., Connolly, M., **Hood, R.L.**, Caluseriu, O., Hurst, J., Kini, U., Nowaczyk, M.J., Afenjar, A., Albrecht, B., Allanson, J.E., Balestri, P., Ben-Omran, T., Brancati, F., Cordeiro, I., da Cunha, B.S., Delaney, L.A., Destrée, A., Fitzpatrick, D., Forzano, F., Ghali, N., Gillies, G., Harwood, K., Hendriks, Y.M., Héron, D., Hoischen, A., Honey, E.M., Hoefsloot, L.H., Ibrahim, J., Jacob, C.M., Kant, S.G., Kim, C.A., Kirk, E.P., Knoers, N.V., Lacombe, D., Lee, C., Lo, I.F., Lucas, L.S., Mari, F., Mericq, V., Moilanen, J.S., Møller, S.T., Moortgat, S., Pilz, D.T., Pope, K., Price, S., Renieri, A., Sá, J., Schoots, J., Silveira, E.L., Simon, M.E., Slavotinek, A., Temple, I.K., van der Burgt, I., de Vries, B.B., Weisfeld-Adams, J.D., Whiteford, M.L., Wierczorek, D., Wit, J.M., Yee, C.F., Beaulieu, C.L., White, S.M., Bulman, D.E., Bongers, E., Brunner, H., Feingold, M., and Boycott, K.M. (2013) The Phenotype of Floating-Harbor Syndrome: Clinical Characterization of 52 Individuals with Mutations in Exon 34 of SRCAP. *Orphanet J Rare Dis.* 8:63.

Reschen, M., Kini, U., **Hood, R.L.**, Boycott, K.M., Hurst, J., and O'Callaghan, C.A. (2012) Floating-Harbor syndrome and polycystic kidneys associated with SRCAP mutation. *Am J Med Genet.* 158A:3196-3200.

Hood, R.L., Lines, M.A., Nikkel, S.M., Schwartzentruber, J., Beaulieu, C., Nowaczyk, M.J.M., Allanson, J., Kim, C.A., Wieczorek, D., Moilanen, J.S., Lacombe, D., Gillessen-Kaesbach, G., Whiteford, M.L., Quaio, C.R.D.C., Gomy, I., Bertola, D.R., Albrecht, B., Platzer, K., McGillivray, G., Zou, R., McLeod, D.R., Chudley, A.E., Chodirker, B.N., Marcadier, J.,

FORGE Canada Consortium,Majewski, J., Bulman, D.E., White, S.M., and Boycott, K.M. (2012) Mutations in SRCAP, Encoding SNF2-Related CREBBP Activator Protein, Cause Floating-Harbor Syndrome. *Am J Hum Genet.* 90:308-313.

Gibson, W.T., **Hood, R.L.**, Zhan, S.H., Bulman, D.E., Fejes, A.P., Moore, R., Mungall, A.J., Eydoux, P., Babul-Hirji, R., An, J., Marra, M.A., FORGE Canada Consortium, Chitayat, D., Boycott, K.M., Weaver, D.D., and Jones S.J. (2012) Mutations in EZH2 Cause Weaver Syndrome. *Am J Hum Genet.* 90, 110–118.

Cho, M-H., Corea, O.R., Yang, H., Bedgar, D.L., Laskar, D.D., Anterola, A. M., Moog-Anterola, F.A.,**Hood, R.L.**, Kohalmi, S.E., Bernards, M.A., Kang, C., Davin, L.B., and Lewis, N.G. (2007) Phenylalanine biosynthesis in *Arabidopsis thaliana*. Identification and characterization of arogenate dehydratases. *J. Biol. Chem.* 282, 30827-30835.

SEMINARS

Hood, R.L.* (2016) Characterization of SRCAP Mutations in Floating-Harbor Syndrome (FHS). Biochemistry, Microbiology and Immunology Seminar Day. Ottawa, Ontario, March 8.

Hood, R.L.* (2014) Mutations in the Chromatin Remodeling Protein SRCAP Cause Floating-Harbor Syndrome (FHS). Biochemistry, Microbiology and Immunology Seminar Day. Ottawa, Ontario, March 7.

Nikkel, S.M.*, Dauber, A., **Hood, R.L.,** Feingold, M., Connolly, M., Nowaczyk, M.J.M., White, S.M., Afenjar, A., Brancati, F., Cordeiro, I., Destree, A., Forzano, F., Honey, E.M., Heron, D., Jacob, C.M., Kant, S.G., Kini, U., Kirk, E., Lemos Silveira-Lucas, E., Silveira Lucas, L., Audi Delaney, L., Santos da Cunha, B., Mericq, V., Pope, K., Price, S., Wit, J.M., Bulman, D.E., Boycott, K.M., and FORGE Canada Consortium (2012) Clinical features of individuals with Floating-Harbor syndrome due to mutations in *SRCAP*. *American Society of Human Genetic Annual Meeting.* San Francisco, USA, November 6-10.

Nikkel, S.M.*, Dauber, A., **Hood, R.L.,** Feingold, M., Nowaczyk, M.J.M., White, S.M., FORGE Canada Consortium, Bulman, D.E., and Boycott, K.M. (2012) Clinical Features of Individuals with Floating-Harbor syndrome due to Mutations in *SRCAP*. *David W. Smith Workshop on Malformations and Morphogenesis*, Georgia USA, August 8-12.

Hood, R.L.* (2012) Use of Next Generation Sequencing to Determine the Genetic Cause of Floating-Harbor Syndrome. Biochemistry, Microbiology and Immunology Seminar Day. Ottawa, Ontario, February 22.

Hood, R.L.* (2010) Introduction to quantitative (q)-PCR. Forest Botany and Tree Physiology Seminar Series. Freiburg, Germany, May 10.

Hood, R.L.* (2010) qPCR: Practical application, theory and methodology. Forestry Research Institute and Forest Botany and Tree Physiology Joint Seminar. Freiburg, Germany, May 10.

Armstrong, Z.B., Corea, O.R.A., **Hood, R.L.,** Bernards, M.A., and Kohalmi, S.E.* (2008) Regulation and localization of arogenate dehydratases in *Arabidopsis Thaliana*. Plant Development Workshop and the CSPP Eastern Regional Meeting. Toronto, Ontario, December 5-6.

Hood, R.L.* (2007) RNA Expression of Six *AROGENATE DEHYDRATASE*s (*ADT*s) in *Arabidopsis thaliana*. Molecular and Cell Biology Graduate Seminar Series. London, Ontario, January 31.

Hood, R.L., Crawley, C.D., Bernards, M.A., and Kohalmi, S.E.* (2005) Expression Analysis of Select *PREPHENATE DEHYDRATASE-LIKE* (*PDL*) Genes in *Arabidopsis thaliana*. Plant Canada 2005. Edmonton, Alberta, June 15-18.

* presenting author

POSTER PRESENTATIONS

Hood, R.L.*, Boycott, K.M., Stanford, W.L., and Bulman, D.E. (2014) Examining the Molecular Mechanisms Underlying SRCAP Mutations in Floating-Harbor Syndrome. Biochemistry, Microbiology & Immunology Poster Day. Ottawa, Ontario, May 15.

Hood, R.L.*, Boycott, K.M., Stanford, W.L., and Bulman, D.E. (2014) Utilization of an Induced Pluripotent Stem Cell Approach to Examine SRCAP Mutations in Floating-Harbor Syndrome. Till & McCulloch Meeting. Ottawa, Ontario, October 27-29.

Hood, R.L.*, Boycott, K.M., Stanford, W.L., and Bulman, D.E. (2014) Examining the Molecular Mechanisms Underlying SRCAP Mutations in Floating-Harbor Syndrome. American Society of Human Genetics 64th Annual Meeting. San Diego, California, USA, October 18-22.

Hood, R.L.*, Boycott, K.M., Stanford, W.L., and Bulman, D.E. (2014) Utilization of an Induced Pluripotent Stem Cell Approach to Examine SRCAP Mutations in Floating-Harbor Syndrome. International Society for Stem Cell Research 12th Annual Meeting. Vancouver, British Columbia, June 18-21.

Hood, R.L.*, Boycott, K.M., and Bulman D.E. (2013) SRCAP Mutations Cause Floating-Harbor Syndrome. CHEO Research Day. Ottawa, Ontario, October 23.

Hood, R.L.*, Boycott, K.M., and Bulman D.E. (2013) Mutations in the Chromatin Remodeling Protein SRCAP cause Floating-Harbor Syndrome. IRIC Symposium 2013: Epigenetics, Genome Integrity and Stem Cell Biology. Montreal, Quebec, June 29-30.

Hood, R.L.*, Boycott, K.M., and Bulman D.E. (2013) SRCAP Mutations Cause Floating-Harbor Syndrome. Biochemistry, Microbiology & Immunology Poster Day. Ottawa, Ontario, May 16.

Hood, R.L.*, Lines, M.A., Nikkel, S.M., Schwartzentruber, J., Beaulieu, C., FORGE Canada Corsortium, Majewski, J., Bulman, D.E., White, S.M., and Boycott, K.M. (2012)

Mutations in the Chromatin Remodeling Protein SRCAP Cause Floating-Harbor Syndrome. CHEO Research Day. Ottawa, Ontario, October 24.

Hood, R.L.*, Lines, M.A., Nikkel, S.M., Schwartzentruber, J., Beaulieu, C., FORGE Canada Corsortium, Majewski, J., Bulman, D.E., White, S.M., and Boycott, K.M. (2012) Mutations in the Chromatin Remodeling Protein SRCAP Cause Floating-Harbor Syndrome. 55th Annual CSMB Conference: Epigenetics and Genomic Stability. Whistler, British Columbia, March 14-18.

Hood, R.L.*, Douglas, S., Goldsmith, C., Bulman, D.E., and Boycott, K.M. (2011) Determining the mutation underlying a novel 46, XY Disorder of Sexual Development (DSD) using next-generation sequencing strategies. 11th Annual OHRI Research Day. Ottawa, Ontario, November 10.

Hood, R.L.*, Douglas, S., Goldsmith, C., Bulman, D.E., and Boycott, K.M. (2011) Determining the mutation underlying a novel 46, XY Disorder of Sexual Development (DSD) using next-generation sequencing strategies. 12th International Congress of Human Genetics and the American Society of Human Genetics 61st Annual Meeting. Montreal, Quebec, October 11-15.

Hood, R.*, Douglas, S., Goldsmith, C., Bulman, D., and Boycott, K. (2011) Using Next-Generation Sequencing to Determine the Mutation Underlying a 46,XY Disorder of Sexual Development (DSD). CHEO Genetics Research Day. Ottawa, Ontario, September 26.

Hood, R.*, Douglas, S., Goldsmith, C., Bulman, D., and Boycott, K. (2011) Using Next-Generation Sequencing to Determine the Mutation Underlying a 46,XY Disorder of Sexual Development (DSD). Biochemistry, Microbiology & Immunology Poster Day. Ottawa, Ontario, May 19.

Kohalmi S.E.*, Armstrong Z.B, Bernards M.A., Corea, O.R.A. **Hood, R.L.** (2009) Molecular characterization of an Arabidopsis enzyme family required for the synthesis of phenylalanine. WORLDiscoveries Research Showcase. London, Ontario, January 30.

Hood, R.L., Bernards, M.A., and Kohalmi, S.E.* (2008) Quantitative expression analysis for six *Arabidopsis AROGENATE DEHYDRATASE*s in response to heat and cold stress. Plant Development Workshop and the CSPP Eastern Regional Meeting. Toronto, Ontario, December 5-6.

Corea, O.R.A.*, Yang, H., Cho, M-H., **Hood, R.L.**, Bernards, M.A., Kohalmi, S.E., Davin, L.B., Lewis, N.G. (2008) Towards Determining the Individual Physiological Roles of Arogenate Dehydratase Isoforms in Phenylalanine Biosynthesis and Downstream Metabolism. Phytochemical Society of North America Annual Meeting. Pullman, Washington, June 25-30.

Hood, R.L.*, Bernards, M.A., and Kohalmi, S.E. (2007) Relative *ADT* expression patterns in response to heat and cold treatments in *Arabidopsis thaliana*. CSPP Eastern Regional Meeting. London, Ontario, December 1.

Hood, R.L.*, and Kohalmi, S.E. (2006) Analysis of *AROGENATE DEHYDRATASE (ADT)* expression patterns in *Arabidopsis thaliana* using RT-PCR. CSPP Eastern Regional Meeting and 40th Plant Development Workshop. Hamilton, Ontario, December 2.

Hood, R.L.*, and Kohalmi, S.E. (2006) RNA Expression Analysis of six *AROGENATE DEHYDRATASE*s (*ADT*s) in *Arabidopsis thaliana*. 56th Annual. Meeting Canadian Society of Microbiologists and 49th Annual Meeting of the Genetics Society of Canada. London, Ontario, June 18-21.

SCHOLARSHIPS

2014-2015 **Queen Elizabeth II graduate scholarships in science and technology (QEII - GSST**) -accepted

2013-2014 **Ontario Graduate Scholarship (OGS)**- accepted

2010-2013 **National Sciences and Engineering Research Council of Canada Postgraduate Scholarship (NSERC PGS (D3))**- accepted

2010-2015 **Excellence Scholarship**
Faculty of Medicine, University of Ottawa, Ottawa, ON

2008, 2009 **Ontario Graduate Scholarship (OGS)**- declined

2005-2007 **Western Graduate Research Scholarship**
University of Western Ontario, London, ON

2001-2002 **Western Scholarship of Distinction**
University of Western Ontario, London, ON

OTHER AWARDS

2016 **Graduate Studies Biochemistry PhD Award of Excellence**
Faculty of Medicine, University of Ottawa, ON

2016 **Department of Biochemistry, Microbiology and Immunology Syed Sattar PhD Student Award**
Faculty of Medicine, University of Ottawa, ON

2016 **BMI Seminar Award: 3rd place, PhD in Biochemistry**
Department of Biochemistry, Microbiology and Immunology, University of Ottawa, ON

2015 **BMI Poster Award: 1st place, PhD in Biochemistry**
Department of Biochemistry, Microbiology and Immunology, University of Ottawa, ON

2014 **BMI Seminar Award: 2rd place, PhD in Biochemistry**
Department of Biochemistry, Microbiology and Immunology, University of Ottawa, ON

2014 **Graduate Studies Leadership Award**
Faculty of Medicine, University of Ottawa, ON

2012 **Travel Award: 55th Annual CSMB Conference: Epigenetics and Genomic Stability**
Whistler, British Columbia

2012 **BMI Seminar Award: 3rd place, PhD in Biochemistry**
Department of Biochemistry, Microbiology and Immunology, University of Ottawa, ON

2007 **Graduate Thesis Research Award**
University of Western Ontario, London, ON

2006, 2007 **Graduate Student Teaching Award Nominee**
University of Western Ontario, London, ON

TEACHING EXPERIENCE

2011/12/13/14/15/16 **Molecular Biology Laboratory Teaching Assistant, Biochemistry 3356**
University of Ottawa, Ottawa, Ontario
Demonstrated and taught basic biochemistry lab techniques and principles, and marked weekly lab assignments and exams.

2012/13/14/15/16/17 **Introduction to Biochemistry Laboratory Teaching Assistant, Biochemistry 2333**
University of Ottawa, Ottawa, Ontario
Taught and supervised basic nucleic acid and amino acid lab protocols and marked quizzes and formal lab reports.

2007 **Head Teaching Assistant and Tutorial Coordinator for Introduction to Genetics, Biology 281b**
University of Western Ontario, London, Ontario
Developed and implemented teaching tutorials: established new tutorial material, gave tutorial-related lectures within class, and prepared student quizzes and examinations. Organized and managed eight teaching assistants to ensure that the tutorial curriculum was effectively taught to the 1200 enrolled students.

2006/08 **Introduction to Genetics Teaching Assistant, Biology 281b**
University of Western Ontario, London, Ontario
Taught new course material in tutorial format, administered and marked weekly quizzes, and led exam review sessions.

2007 **Scientific Methods in Biology Teaching Assistant, Biology 290a**
University of Western Ontario, London, Ontario
Helped undergraduate students develop scientific writing skills, marked and edited student scientific papers, and met one-on-one with students to discuss their papers.

2005/06 **Human Genetics Teaching Assistant, Biology 392a**
University of Western Ontario, London, Ontario
Led weekly tutorials to support the learning of lecture material, marked weekly assignments, and led exam review sessions.

VOLUNTEER EXPERIENCE

2014-current **Faculty of Medicine Student Representative, Faculty of Medicine Council Appeals Committee**
University of Ottawa, Ottawa, Ontario
Part of a committee that is responsible for hearing and deciding upon appeals (including the decision to put someone on remediation, probation, or of dismissal from the program) of trainees in the Faculty of Medicine.

2015-2016 **CHEO Representative and General Member, Biochemistry, Microbiology and Immunology Graduate Students Association (BMIGSA)**
University of Ottawa, Ottawa, Ontario
Responsible for the organization of the 2015-16 BMI student-invited seminar speakers (as part of the 'Advances in Biomedical Research Seminar Series'). Assisted the committee with other events including social events and BBQs.

2012-2015 **Vice-President Academics, Biochemistry, Microbiology and Immunology Graduate Students Association (BMIGSA)**
University of Ottawa, Ottawa, Ontario
Responsible for the organization of the BMI student-invited seminars speakers (as part of the 'BMI Seminar Series' (2012-13 and 2013-14) and the 'Advances in Biomedical Research Seminar Series' (2014-15)). Organized other academic events throughout the year, of particular importance was the 'The Faculty of Medicine Career Day'. This is a day-long symposium where speakers employed in various scientific-oriented careers are invited to give seminars to talk about their jobs and the pathways which lead to their current positions: the overall goal being to help graduate students and post-doctoral fellows in thinking about potential job areas and connections. Also served as the BMI student representative at Faculty of Medicine meetings.

2011-2016 **Stem Cell Talks volunteer**
University of Ottawa, Ottawa, Ontario
Introduce high-school students to stem cell technologies in an interactive format and assist at annual 'Stem Cell Talks' symposium.

2011-2013 **Let's Talk Science volunteer**
University of Ottawa, Ottawa, Ontario
Create and deliver science programs to school-aged children to engage them in science learning. Was a volunteer advisor in the 2011-12 academic year.

2011-2013 **BMI student representative on Ottawa Hospital Research Institute (OHRI) Committee**
University of Ottawa, Ottawa, Ontario
Provided a student perspective on institute issues. The committee addressed such issues as trainee space allocation and minimal stipend policies.

2011-2013 **BMI student representative on Ethics, Diversity and Gender Equality Committee**
Faculty of Medicine, University of Ottawa, Ottawa, Ontario
Discussed ethical issues and policies in the Faculty of Medicine including harassment, racial discrimination, and gender equity. Assisted in medical student harassment workshops.

2011-2012 **OHRI Representative and General Member, Biochemistry, Microbiology and Immunology Graduate Students Association (BMIGSA)**
University of Ottawa, Ottawa, Ontario
Helped organize and assist committee with student events such as pub nights and

barbeques etc.

2006-2008 **Social Representative, Society of Biology Graduate Students (SOBGS)**
University of Western Ontario, London, Ontario
Organized various social events for faculty and students including pub nights, Christmas parties, movie nights, Halloween parties etc.

2007-2008 **Member of Operations Committee, The Grad Club**
University of Western Ontario, London, Ontario
Contributed to budgeting, staffing, and operations planning for a non-profit restaurant owned by the Society of Graduate Students (SOGS).

2005-2006 **SOBGS student representative, SOBGS**
University of Western Ontario, London, Ontario
Biology department representative for the Society of Graduate Students (SOGS).