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GENOMIC AND EPIGENETIC INVESTIGATIONS OF SILVER-RUSSELL SYNDROME AND GROWTH RESTRICTION

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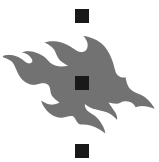

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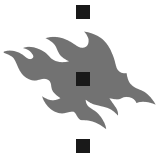

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ABSTRACT

A combination of genes, their epigenetic regulation, and the environment control the phenotypes of an individual, such as how short or tall one grows. Epigenetics refers to chemical modifications of DNA and histones that regulate gene activity and genome stability, and can take the form of, for example, addition of a methyl group to DNA. A rare but illustrative example of growth restriction is Silver-Russel syndrome (SRS), which also features a relatively large, triangular head and asymmetry between body halves. Molecular studies have demonstrated that SRS is an interesting model for how growth is controlled by both genetic architecture and epigenetics, and recurring findings include DNA hypomethylation at an imprinted region on chromosome 11 (*H19* ICR) in 20-65% of cases, maternal uniparental disomy of chromosome 7 (matUPD7) in 5-15%, and rare maternal duplications of chromosomes 7p and 11p. Imprinting is a rare but remarkable epigenetic phenomenon that describes parent-of-origin dependent gene activity, such that some genes are only expressed if they were inherited from, for example, the father. Differential DNA methylation (for example, maternal but not paternal methylation) is thought to regulate imprinting. All of the above mentioned molecular findings in SRS can cause dysregulation of imprinted genes. Interestingly, a large proportion of SRS patients remains molecularly unexplained.

In this thesis we applied genome-wide genotyping and targeted epigenetic studies of imprinted genes to investigate the genetic nature of SRS and to disentangle the epi(genotype) and phenotype correlations in SRS and growth restriction. We devised a new approach to confirm UPD by the use of genotyping arrays and demonstrated a much increased resolution compared to the commonly used microsatellite markers. We further demonstrated the power of using genome-wide genotyping arrays in rare disorders such as SRS where UPD, copy number variants, or shared homozygosity might occur. We identified pathogenic submicroscopic events on chromosomes 15, 22, and X in molecularly unexplained SRS patients. A simple method for quantification of locus-specific DNA methylation is described and its accuracy and quantitative nature are demonstrated. In addition, reference distributions of DNA methylation at imprinted genes in controls are defined. This method was used to evaluate *H19* ICR DNA methylation in SRS and isolated growth restriction, and 62% of SRS patients were hypomethylated. We further found a dose-response relationship between the degree of *H19* ICR hypomethylation and phenotype severity in SRS and reported for the first time the association of specific anomalies of the spine, elbows, hands and feet, and genital defects in SRS with severe hypomethylation.

In conclusion, we showed the utility of genotyping arrays to identify both UPD and submicroscopic genomic aberrations, and demonstrated that this genome-wide approach also enables the identification of important but unexpected events. Importantly, screens using genotyping arrays have the potential to detect the majority of genomic events in SRS. Through targeted epigenetic analysis we could conclude that *H19* ICR methylation is clinically important as demonstrated by a strong correlation between the degree of hypomethylation and SRS phenotype severity and specifically associated clinical findings.

LIST OF PUBLICATIONS

I. **Bruce S***, Leinonen R*, Lindgren CM, Kivinen K, Dahlman-Wright K, Lipsanen-Nyman M, Hannula-Jouppi K and Kere J.
Global analysis of uniparental disomy using high density genotyping arrays.
Journal of Medical Genetics 2005; **42**: 847-851.

II. **Bruce S**, Hannula-Jouppi K, Puoskari M, Fransson I, Simola K, Lipsanen-Nyman M and Kere J.
Heterogeneous etiology of Silver-Russell syndrome and growth restriction revealed by genomic screening.
Manuscript

III. **Bruce S**, Hannula-Jouppi K, Lindgren CM, Lipsanen-Nyman M, and Kere J.
Restriction site-specific methylation studies of imprinted genes with quantitative real-time PCR.
Clinical Chemistry 2008; **54**: 491-499.

IV. **Bruce S***, Hannula-Jouppi K*, Peltonen J, Kere J, and Lipsanen-Nyman M.
Clinically distinct epigenetic subgroups in Silver-Russell syndrome; the degree of *H19* hypomethylation associates with SRS phenotype severity and genital and skeletal anomalies.
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PUBLICATIONS NOT INCLUDED IN THIS THESIS

I. Melén E*, **Bruce S***, Doekes G, Kabesch M, Laitinen T, Lauener R, Lindgren CM, Riedler J, Scheynius A, van Hage-Hamsten M, Kere J, Pershagen G, Wickman M, Nyberg F; PARSIFAL Genetics Study Group.
Haplotypes of G protein-coupled receptor 154 are associated with childhood allergy and asthma.
American Journal of Respiratory and Critical Care Medicine 2005; **171**:1089-1095.
*Authors contributed equally

II. Vendelin J, **Bruce S**, Holopainen P, Pulkkinen V, Rytilä P, Pirskanen A, Rehn M, Laitinen T, Laitinen LA, Haahtela T, Saarialho-Kere U, Laitinen A, Kere J.
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III. D'Amato M, **Bruce S**, Bresso F, Zucchelli M, Ezer S, Pulkkinen V, Lindgren C, Astegiano M, Rizzetto M, Gionchetti P, Riegler G, Sostegni R, Daperno M, D'Alfonso S, Momigliano-Richiardi P, Torkvist L, Puolakkainen P, Lappalainen M, Paavola-Sakki P, Halme L, Farkkila M, Turunen U, Kontula K, Lofberg R, Pettersson S, Kere J.
Neuropeptide s receptor 1 gene polymorphism is associated with susceptibility to inflammatory bowel disease.
Gastroenterology 2007; **133**: 808-817.

IV. **Bruce S**, Nyberg F, Melén E, James A, Pulkkinen V, Orsmark-Pietras C, Bergström A, Dahlén B, Wickman M, von Mutius E, Doekes G, Lauener R, Riedler J, Eder W, van Hage M, Pershagen G, Scheynius A, Kere J.
The protective effect of farm animal exposure on childhood allergy is modified by *NPSR1* polymorphisms.
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CONTENTS

LIST OF ABBREVIATIONS

1p32.3	chromosome 1, short arm, cytoband 32.2 (example)
1q24.1	chromosome 1, long arm, cytoband 24.1 (example)
AS	Angelman syndrome
bp	base pairs
BWS	Beckwith-Wiedemann syndrome
CGI	CpG-island
CNV	Copy number variant
CpG	Cytosine connected to guanine by a phosphodiester bond
C_t	Threshold cycle
CTCF	CCCTC-binding factor (zinc finger protein)
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
Gb	Gigabases, one billion base pairs
GH	Growth hormone
H3K4	Histone 3 lysine 4 (example)
ICR	Imprinting control region
IGF	Insulin growth factor
IUGR	Intrauterine growth retardation
kb	Kilobases, 1000 base pairs
LD	Linkage disequilibrium
LOH	Loss-of-heterozygosity
Mb	Megabases, one million base pairs
MBD	Methyl-binding domain
mCpG	Methylated CpG
MLPA	Multiplex ligation-dependent probe amplification
MRKH	Mayer-Rokitansky-Küster-Hauser syndrome
NAHR	Non-allelic homologous recombination
NHEJ	Non-homologous end joining
nt	Nucleotides
OMIM	Online Mendelian inheritance in man
PCR	Polymerase chain reaction
PHP-1b	Pseudohypoparathyroidism, type 1b
PWS	Prader-Willi syndrome
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
SD	Standard deviation
SDS	Standard deviation score
SGA	Small for gestational age
SRS	Silver-Russell syndrome
SSR	Simple sequence repeat
TND1	Transient neonatal diabetes, type 1
UPD	Uniparental disomy
XCI	X-chromosome inactivation

1 ARCHITECTURE OF THE HUMAN GENOME

A major event in human genetics occurred when two drafts of the around 3 billion base pairs of human genome sequence were published in 2001 [Lander, et al., 2001; Venter, et al., 2001]. The subsequent eight years have seen finished sequences of all human chromosomes and several model organisms. The analysis and annotation of finished genome sequences have proved that genome research has only started and will be an important and puzzling topic for years to come.

The inheritance of traits or characteristics across generations is a well-appreciated phenomenon that was studied in a systematic fashion as early as 1850's by the monk Gregor Mendel [Strachan and Read, 2004]. Important advances for genetics came from studying *Drosophila melanogaster*, where some traits were observed to be co-inherited at higher frequencies than others [Sturtevant, 1913]. This was the origin of genetic maps and led to the proposition that traits are inherited on chromosomes [Strachan and Read, 2004]. Through studying bacterial transformation events Avery and colleagues [Avery, et al., 1944] made the unexpected suggestion that the biological material that induces heredity is deoxyribonucleic acid (DNA). The anti-parallel double-helical structure of DNA was described in 1953 by James Watson and Francis Crick [Watson and Crick, 1953].

1.1 DNA

Deoxyribonucleic acid (DNA) consists of the four nucleic bases adenine (A), guanine (G), cytosine (C) and thymine (T), attached to deoxyribose molecules, which are sequentially connected through phosphodiester bonds. The bases are subdivided into purines (A & G) and pyrimidines (T & C). The basic linear sequence of A, G, C, and T nucleotides constitutes the genetic code. Portions of the DNA serve as templates for transcription from which RNA molecules are created by the RNA polymerase machinery. The transcribed RNA serves as a template for the protein production of a cell. Importantly, DNA is virtually always in a double-stranded state and the bases have specific affinities for each other: A pairs with T and C pairs with G. This gives rise to the anti-parallel, double-helical structure of DNA, which is stabilized by the hydrogen bonds between the paired bases. In each cell division, the double-helix is unwound and DNA is replicated (copied) by the DNA polymerase machinery so that all genetic material is doubled and subsequently divided between two new cells [Strachan and Read, 2004].

1.2 CHROMOSOMES

DNA is organized into chromosomes, which constitute discrete units of DNA sequence that helps the cell in processes such as cell division and aids in densely packing the genetic material for storage. Already in the 1950's it was established that humans have 46 chromosomes [Tjio and Puck, 1958]. These include 22 autosomal chromosomes that we inherit in duplicate, one from our mother and one from our father. The sex chromosomes are inherited asymmetrically since the mother always passes on an X chromosome to her offspring while the father can pass on either an X or a Y chromosome. Starting from the double-helix, the basic packaging unit is the

nucleosome, constituting the DNA double-helix wrapped one turn around a core of small basic proteins called histones. Nucleosomes are organized into a 30 nm fiber through the addition of more histones and finally the metaphase shape of a chromosome is created with the help of scaffold proteins. Chromatin describes the three-dimensional packing of DNA, a property that influences its accessibility and function.

1.3 MEIOSIS AND RECOMBINATION

During the formation of germ cells a two-step process called meiosis takes place. Meiosis I includes DNA replication, homologous chromosome pairing, exchange of genetic material and separation of homologues, while meiosis II involves creation of haploid germ cells (Figure 1). During meiosis I the parental homologous chromosomes (two chromatids each) pair up and exchange material in a process called recombination. Recombination is enabled by close proximity between parental chromosomes (chiasma) formed with the help of protein complexes. Double-stranded breaks occur and the two chromosomes are ligated together. This results in sequential portions originating from different grandparents on the new physical chromosome.

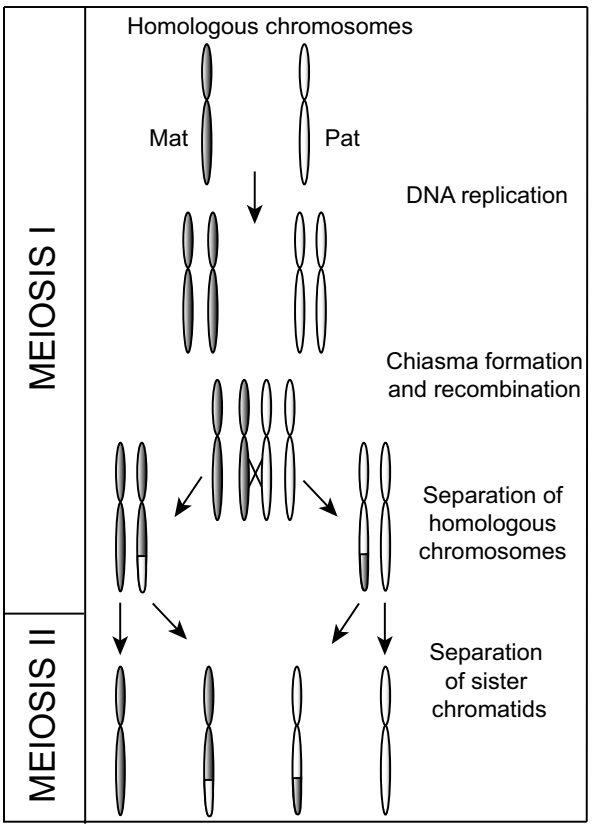

Figure 1 – Meiosis. During meiosis the homologous chromosomes are replicated, recombined and separated. In meiosis I, the homologous chromosomes are separated, while at meiosis II the sister chromatids are separated to form haploid germ cells.

Besides mixing genetic material, recombination is thought to be important for maintaining genome stability and at least one recombination on each chromosome arm must take place to ensure proper chromosome disjunction in meiosis I [Coop and Przeworski, 2007]. The number of recombinations in meiosis is limited by positive interference (the physical inability of chromosomes to form chiasmas in close proximity to each other) and the detrimental effect of being too permissive in allowing chiasmata to form [Coop and Przeworski, 2007]. Pairing is mediated through homology between chromosomes and thus inappropriate pairing between highly homologous regions in non-corresponding genomic positions is possible and might result in non-allelic exchange (see 1.7.3 Mechanisms creating structural variants). The human genome consists to a large extent of repetitive regions and there are consequently counteracting selective forces that on one hand require a minimum number of recombinations per meiosis for genome integrity and on the other hand the need to avoid incorrect pairing [Coop and Przeworski, 2007]. Interestingly, studies of individuals with compromised DNA methylation machinery have suggested a relation between DNA methylation, recombination, and genome integrity [Chen, et al., 1998].

It has been observed that recombination does not occur uniformly, but the human genome consists of blocks of recombination hot- and coldspots, with approximately 80% of all recombination occurring in 10-20% of the genome sequence [Myers, et al., 2005]. This has been demonstrated by sperm-typing approaches, studies of large pedigrees, and through pair-wise allelic association studies of unrelated samples [Broman, et al., 1998; Cullen, et al., 2002; Gabriel, et al., 2002; Kong, et al., 2002]. Interestingly, male and female recombination patterns differ (on average 1.6 times more recombination in females), suggesting that gender-specific factors regulate recombination [Coop and Przeworski, 2007].

1.4 GENES, EXONS, AND RNA SPECIES

Genes are functional units of the genome that are copied from the DNA and transcribed into the closely related nucleic acid species RNA. Intense research in recent years has revealed that RNAs can have diverse functions, besides coding for proteins, and that the future is likely to reveal new functions of the human transcriptome (all transcribed RNAs in a cell).

1.4.1 Protein-coding genes

A protein-coding gene contains the basic units exons and introns, which alternate sequentially. Both units are transcribed into a messenger RNA (mRNA), while only exons will remain in the mature mRNA, resulting from excision of introns (splicing). To protect the mRNA from exonucleases, mRNAs usually contain a protective 5' cap structure, and in the 3' end a protective poly-adenosine tail. An important functional unit in the 5' end of the gene is the promoter, where regulatory factors can bind and regulate recruitment of the RNA polymerase II transcription complex. It is now well appreciated that transcription does not initiate in one exact location, but the major transcription start site can be defined based on empirical likelihood estimates, with a gradient of transcription initiating before and after [Carninci, et al., 2006]. An mRNA will contain nuclear export signals, which will enable translocation into the cytoplasm and endoplasmic reticulum, where the protein-synthesizing ribosomes are located.

1.4.2 Non-coding RNAs

Not all RNAs encode proteins but some are functional as RNA (ncRNAs) and can exert regulatory effects through catalyzing biological reactions, binding to and modulating the activity of a protein, or base-pairing with a target nucleic acid [Goodrich and Kugel, 2006]. Interestingly, X-chromosome silencing is regulated by a ncRNA called *XIST* that associates with and epigenetically silences the X-chromosome from which it originated [Goodrich and Kugel, 2006]. Ribosomal RNA (rRNA) and transfer RNA (tRNA) are involved in protein synthesis of the cell and are thus functional in the cytoplasm, while small nuclear RNA (snRNA) are important units of the spliceosome. MicroRNAs are small RNAs that form secondary hairpin structures, which are extensively processed by protein complexes into their mature 22-nt shape that can regulate the bioavailability of target proteins [Goodrich and Kugel, 2006].

1.5 THE HUMAN GENOME PROJECT

As the drafts of the human genome sequence were published in February 2001, extensive annotation analyses were made possible [Lander, et al., 2001; Venter, et al., 2001]. The authors could investigate what types of DNA that make up our genome and how it related to other genomes that had already been sequenced. The drafts consisted of roughly 2.9 billion base pairs, of which only 1.1% constituted exons for protein coding genes, while 24% corresponded to intronic sequences [Venter, et al., 2001]. The draft genomes contained 30,000-40,000 genes [Lander, et al., 2001; Venter, et al., 2001], which was considerably less than the previously estimated 60,000-100,000 genes [Strachan and Read, 2004]. In later estimates, the number of human protein-coding genes has been reduced to 20,000-25,000 [International Human Genome Sequencing Consortium, 2004]. Genes were not evenly distributed over the genome, but about 20% of the genome constituted gene deserts (>500 kb regions without genes) [Lander, et al., 2001; Venter, et al., 2001].

1.5.1 Human repeat elements

Interestingly, up to 45% of the human genome was reported to consist of repetitive sequences derived from transposable elements or so-called mobile genetic elements, of which the majority is ancient and has the lost the ability to transpose [Lander, et al., 2001]. This was a much higher proportion compared to all fly (*Drosophila melanogaster*), worm (*Caenorhabditis elegans*), and mustard weed (*Arabidopsis thaliana*) genomes [Lander, et al., 2001; Venter, et al., 2001]. Another abundant repeat type in the human genome is tandem repeats of simple di- or trinucleotides, called simple sequence repeats (SSRs), which constitute about 3% of the human genome [Lander, et al., 2001]. The repeat lengths of SSRs are highly polymorphic between individuals, probably resulting from slippage during the replication process (see 1.7.3 Mechanisms creating structural variants). It was also confirmed that the human genome consists of close to identical sequences (1-200 kb in size) that have arisen from duplication events. The segmental duplications can be both intrachromosomal and interchromosomal, in which case they tend to cluster near centromeric and telomeric regions [Lander, et al., 2001]. The regions are very homologous (90-99% identity) suggesting their relative recent duplication origin. Segmental duplications were estimated to cover at least 3% of the human genome.

These regions are candidates for inappropriate recombination events and might also pose problems in genetic marker analysis [Lander, et al., 2001; Venter, et al., 2001].

1.5.2 Phylogenetic conservation

Large-scale sequence comparison between human and other species was made possible by the draft genome sequences [Lander, et al., 2001; Venter, et al., 2001]. The completion of genome sequences for several model organisms such as mouse (*Mus musculus*) [Waterston, et al., 2002], rat (*Rattus norvegicus*) [Gibbs, et al., 2004], monkeys (*Macaca mulatta* and *Pan troglodytes*) [Gibbs, et al., 2007; The Chimpanzee Sequencing and Analysis Consortium, 2005], and many others have resulted in comparative sequence analyses now being routinely applied in search for functional elements. In general, exons are more conserved than intronic sequences, while recent studies have identified non-coding (either intergenic or intronic) regions of extreme conservation between human and mouse [Bejerano, et al., 2004; Sandelin, et al., 2004]. Phylogenetic analyses have also been used to identify imprinting regulatory regions in humans (see 3.1 Principle and general characteristics).

1.5.3 A sequencing revolution

The sequences of human chromosomes 21 and 22 were published already before the whole genome drafts [Dunham, et al., 1999; Hattori, et al., 2000]. More complete and further annotated sequences for all human chromosomes (including the sex chromosomes) have been reported in *Nature* between 2001 and 2006. Importantly, all reported sequences were based on sequencing several individuals and determining a reference sequence. However, recently the first reports were published of individual genomes, sequenced through the traditional Sanger method [Levy, et al., 2007] and massively parallel sequencing [Wheeler, et al., 2008]. Complete sequences of individual genomes have followed and include different ethnicities [Bentley, et al., 2008; Wang, et al., 2008] and further, the healthy and cancerous genomes of a leukemic individual [Ley, et al., 2008]. Continued development of the new sequencing methods along with cost reduction are likely to result in more individual genomes being sequenced (see 6.1.5 Sanger sequencing and massively parallel sequencing).

1.6 GENETIC MARKERS

Genetic markers are defined as regions in the genome that differ on the nucleotide level between any two humans. There are different types of sequence variation, be it on the nucleotide level where bases have been replaced (e.g., A → G), or on the structural level where genetic material is present or absent in comparison between individuals (can comprise both repetitive and non-repetitive portions). Since the majority of common genetic markers are ancient and thus inherited, these can be used for investigating heritable traits. The earliest genetic markers used to study human disease were restriction fragment length polymorphism [Botstein, et al., 1980], which were screened through restriction enzyme digestion followed by fragment separation and visualization with radio-labeled probes (Southern blotting) [Southern, 1975].

1.6.1 Microsatellites

Microsatellites are tandemly repeated short sequences (1-13 bp), where the number of consecutive repeats is highly polymorphic between individuals (see 1.5.1, SSR). By designing polymerase chain reaction (PCR) primers in unique regions flanking the repeats, the number of repeats in an individual can be resolved by measuring the length(s) of the amplified fragment(s) through gel or capillary-based electrophoresis. Studies involving microsatellites have been important for disease-gene mapping in pedigrees, where their high information content, possible identification even before the completion of the human genome project, and automatability made them popular.

1.6.2 Single nucleotide polymorphisms

Single nucleotide polymorphisms (SNP) are sites in the genome where base-composition differs between individuals. This type of genetic marker comprises base-substitutions (A/G) or insertion/deletion polymorphisms (A/AG, A/-). Typically, SNPs are bi-allelic, which means that only two different bases are observed at a particular locus. Close to 15 million human SNPs have been reported to the database of SNPs (dbSNP, Table 1). It has been determined through individual genome sequencing that any human carries about 3 million SNPs, 1 SNP every kb on average, constituting 75% of all genetic variation events in an individual [Levy, et al., 2007; Wheeler, et al., 2008]. SNPs are currently a very popular genetic marker because of their abundance in the genome and the existence of large-scale genotyping methods (see 6.1 Methods to study human genome architecture). SNPs affect only one nucleotide base but can still have functional consequences: if occurring in a protein-coding region, a SNP can cause an amino acid change, a premature stop codon, or a frame shift in the protein code. Further, SNPs could affect splicing of genes, stability of mRNA and regulatory factor binding affinities in promoters, enhancers, or silencers.

The HapMap consortium (Table 1) took on the challenge of studying SNPs on a population level, including allele frequency and pair-wise linkage disequilibrium (LD) [International HapMap Consortium, 2005], and at present includes information for over 5 million SNPs [Frazer, et al., 2007]. The correlation (or LD) between any two common SNPs on a chromosome will be controlled by the recombination history since the mutation rate of SNPs is very low (estimated $2.5*10^{-8}$ mutations per base and generation) [Nachman and Crowell, 2000]. The sequential combination of SNPs on the same chromosome constitutes a haplotype. The LD and derived haplotype patterns across the human genome have important implications for studies of common diseases and have been utilized for the design of genome-wide association studies and other purposes [Manolio, et al., 2008].

1.7 GENETIC ARCHITECTURE

1.7.1 Copy number variants

Copy Number Variants (CNVs) constitute gain or loss of genetic material in comparison between individuals. The size can theoretically range from a handful of base pairs up to hundreds of thousands, even million base pairs of DNA. In the case where a whole chromosome is lost or gained, this is referred to as aneuploidy.

Although it has long been appreciated that structural variation occurs in the genome, it was not until recently that systematic, large-scale investigation of CNVs were initiated [Iafrate, et al., 2004; Redon, et al., 2006; Sebat, et al., 2004]. These studies suggested that CNVs were common in the genome and estimated them to affect 4-24 Mb in each individual [Sebat, 2007]. However, recent studies suggested that CNV sizes had been overestimated due to inherent biases of the platforms and design [Kidd, et al., 2008; McCarroll, et al., 2008]. Individual genome sequencing provides the ultimate resolution of CNV size, and a recent study suggested CNVs as the major source of inter-individual genetic variation (9 Mb, >70% of variant bases), while they are fewer in each genome than SNPs (only ~20% of all variation events) [Levy, et al., 2007].

Important characteristics of CNVs include an increased *de novo* germline mutation rate compared to SNPs (100- to 10,000-fold greater) [Lupski, 2007]. Further, mutation rates of CNVs are far from uniformly distributed, but the specific genomic context is highly correlated to CNV rate, with repetitive elements of retrotransposon or segmental duplication origin acting in a predisposing manner [Shaw and Lupski, 2004]. However, it should be noted that the majority of CNVs in an unselected population are inherited and show extensive LD with surrounding SNPs [McCarroll, et al., 2008]. The increasing number of studies reporting CNVs encouraged the generation of the Database of genomic variants (DGV, Table 1), which harbors the most complete and curated collection of reported CNVs [Iafrate, et al., 2004]. The most obvious effect of CNVs is gene-dosage effects, provided there are genes or important regulatory elements located within the deleted/duplicated segment [Beckmann, et al., 2007]. CNVs can also disrupt genes leading to deleterious gene products and importantly, heterozygous deletions might unmask recessive disease alleles, in the case where the normal copy is deleted [Beckmann, et al., 2007].

1.7.2 Copy number neutral genomic variants

Genomic rearrangements that cause loss or gains are not the only important structural variants, on the contrary it has been suggested that 1-20% of events are copy number neutral [Khaja, et al., 2006; Kidd, et al., 2008; Tuzun, et al., 2005]. Examples of balanced events include inversions, translocations, and uniparental disomy, all of which will be described below.

1.7.2.1 Inversions

Inversions constitute genomic regions that have been excised and then re-inserted in the opposite orientation. In a recent individual sequencing effort, inversions constituted only 0.2% of variant events, while they involved 16% of variant bases, with an average inversion size of 21 kb [Levy, et al., 2007]. This suggests that inversions contribute appreciably to genetic diversity. Inversions can disrupt genes, cause position effects (e.g., separation of a gene from its native promoter), and predispose carriers to deleterious recombination events.

1.7.2.2 Translocations

Translocation refers to the situation where non-homologous chromosomes have been joined and occur at a frequency of about 0.1-0.5% in populations [Pasternak, 2005; Warburton, 1991]. Translocations can be balanced, for example a portion of

chromosome 2 is translocated to chromosome 4 and vice versa, or unbalanced in which case one of the translocated portions has been lost or gained. Putatively balanced translocations can lack genetic material at the break-points. Translocations commonly cause clinical problems such as infertility and spontaneous abortions because they interfere with normal disjunction of chromosomes during meiosis.

1.7.2.3 Uniparental disomy

Uniparental disomy (UPD) is a copy number neutral event that results from the inheritance of a chromosome pair from one parent only. Most uniparental disomies are thought to arise from trisomy rescue events, while gametic complementation, monosomy rescue or somatic events are possible (see 5.2.2 Mechanisms of UPD formation) [Robinson, 2000]. Uniparental disomy can be either isodisomic where one grandparental chromosome is inherited in duplicate (i.e., homozygous across region) or heterodisomic where both grandparental copies have been transmitted (heterozygosity possible). Isodisomic UPD can cause penetrance of recessive disease alleles, while imprinted genes (see Chapter 3 - Imprinting) can be affected by both heterodisomic and isodisomic UPD [Robinson, 2000]. UPD in the general population has been estimated to be very rare (<0.001%) through using the frequency of UPD with known phenotypes (see 3.6.1 Human imprinting disorders) [Engel, 1998].

1.7.3 Mechanisms creating structural variants

Structural variation is created through errors in the normal house-keeping mechanisms of the cell such as recombination, replication or double-stranded break repair. Non-allelic homologous recombination (NAHR) describes the process of recombination as a consequence of mispairing between non-allelic homologous regions (Figure 2A). Typically these regions have very high homology (>90%) and are often repetitive in nature or constitute genome duplication events [Shaw and Lupski, 2004]. Since NAHR is guided by homology, breakpoints will tend to cluster between individuals. In the case of intrachromosomal NAHR when the homologous regions occur on the same chromosome, recombination will result in reciprocal loss and gain (Figure 2A). Intrachromosomal NAHR is most likely, since homologous chromosomes have the best affinity for each other. Inter-chromosomal NAHR will result in translocations, where chromosomes are reciprocally fused. A recent study that performed paired-end sequencing of eight human genomes suggested that NAHR was the most common mechanism contributing to 48% of all structural variation [Kidd, et al., 2008].

Replication slippage is typical of repetitive regions that form secondary structures. In the simplest example, a tandem repeat forms secondary structure and the replication machinery does not appreciate the true number of repeats resulting in a deletion or insertion on the replicated strand (Figure 2B). It was recently proposed that a similar mechanism termed FoSTES (replication *Fo*rk *S*talling and *Te*mplate *S*witching) is responsible for rearrangements in more complex copy number variant regions [Lee, et al., 2007]. Backwards and forwards slipping of the replication fork machinery, where re-initiations are guided by microhomologies within a region, are thought to cause complex rearrangement patterns of consecutive gains and losses within a region [Lee, et al., 2007].

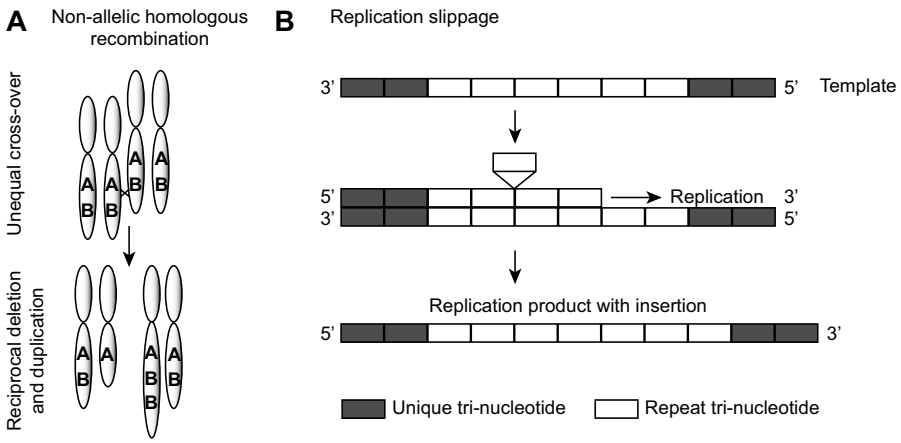

Figure 2 - Mechanisms creating genomic diversity. A) Non-allelic homologous recombination (NAHR): highly homologous regions A and B mediate illegitimate recombination resulting in one duplicated and one deleted chromosome. B) Replication-slippage: in this example the replicated strand forms a secondary structure that results in a tri-nucleotide insertion.

Non-homologous end joining (NHEJ) occurs at genomic regions that are prone to double-stranded breaks, such as palindromes or repeats that form susceptible secondary structures. Deletions caused by NHEJ typically have insertion of a few novel bases at the repair junction [Shaw and Lupski, 2004]. A rare event that causes variation in genomic structure is insertion of active transposable elements. Importantly, non-disjunction at meiosis is a rather common and age-dependent event in human oocytes that causes aneuploidy [Robinson, 2000]. The most common aneuploidies in human live births are 47, XXY, 47, XYY, 47, XXX, and 47, +21 and these occur at frequencies of about 1% [Pasternak, 2005]. As discussed above (see 1.3 Meiosis and recombination), decreased recombination rate is a predisposing factor to meiotic non-disjunction [Coop and Przeworski, 2007].

1.8 MAPPING DISEASE VARIANTS

How can disease-causing genetic variants be discovered? The approaches have differed based on the available methodology and the type of disease studied. Three types of genetic disorders can be recognized: Mendelian (single-gene) disorders, complex disorders, and genomic (gene-dose or chromosomal) disorders. Mendelian disorders are often rare and found clustered in families, with transmissions following dominant or recessive models. Complex disorders are typically common, with a lesser degree of familiar clustering, which is thought to be the consequence of several contributing genes in combination with environmental factors. A genomic disorder does not necessitate extensive familial clustering and most cases are sporadic, however on the genomic level patients share disruptions of a common region, often caused by *de novo* deletion or duplication events or aneuploidy.

Linkage mapping refers to the study of co-segregation of genetic markers with disease phenotypes within a pedigree and has been most successful for Mendelian traits [Altshuler, et al., 2008]. This approach typically gives a limited resolution, due

to the limited number of recombinations that occur within pedigrees, to narrow down the disease-haplotype that co-segregates with disease. Typically, highly penetrant disease alleles can be mapped using relatively few genetic markers (increased marker density does not always increase the chance of narrowing down disease haplotype) [Altshuler, et al., 2008]. Online Mendelian inheritance in man (OMIM) is a genotype-phenotype oriented database (see Table 1) that contains information on all known Mendelian disorders and over 12,000 genes [McKusick, 2007].

In association studies, frequencies of genetic marker alleles are compared in population-based cases and controls to find alleles that confer risk or protection. Complex diseases are often studied in this way and the underlying assumption is the "common disease-common variant" hypothesis, stating that individuals with complex disease often share an ancient and common mutation with limited risk effect [Reich and Lander, 2001]. Most association studies are indirect and take advantage of the LD between SNPs and the assumed disease allele. Since unrelated cases are used, but a common ancestral allele is assumed, the resolution will be increased as compared to linkage, since many recombinations have narrowed down the haplotypes over numerous generations. In association studies, increased marker resolution can greatly benefit the approach, and examples of genome-wide association studies have successfully identified novel loci contributing to risk of complex disease [McCarthy, et al., 2008]. Interestingly, common, inherited CNVs can be predisposing to complex disease in the same way as SNPs [Estivill and Armengol, 2007].

When genomic disorders are studied, direct approaches are applied and thus the aim is to find structural genomic variants that are shared between individuals that share a distinct phenotype. The penetrance is assumed to be relatively high and no particular inheritance models are assumed. Since the size of the contributing variant cannot be predicted, the marker resolution is very important. Usually, array-based comparative genome hybridization or array-based genotyping approaches are applied, although some genomic aberrations can be identified by studying karyotypes.

Table 1 – Web addresses for Chapter 1.

Database	Web address
Database of SNPs (dbSNP)	www.ncbi.nlm.nih.gov/projects/SNP/
Internationl HapMap project	www.hapmap.org
Database of genomic variants (DGV)	projects.tcag.ca/variation/
Online Mendelian inheritance in man (OMIM)	www.ncbi.nlm.nih.gov/omim/

2 EPIGENETICS – DNA METHYLATION

Epigenetics is a term derived from combining the words "epigenesis" (describing the development and differentiation process of the multicellular organism) and "genetics", introduced by C.H. Waddington in 1942 [Waddington, 1942]. Nowadays we often think of epigenetics as the extra-level of information "on top" of DNA, which takes the form of chemical modifications of DNA itself and its related proteins, and exerts regulatory effects on DNA usage. However, epigenetics is also related to the theory of epigenesis, in the sense that chemical modifications of DNA could explain the differentiation process of organisms, whereby cell specialization is acquired and maintained despite identical genetic content. The chemical modification of DNA involves the covalent addition of a methyl group to the fifth carbon of the cytosine ring (Figure 3A, see 2.1 DNA methylation mechanisms). The other type of epigenetic signal includes modifications at residues of protruding histone tails, where both methyl and acetyl groups can be added in a quantitative manner. Extensive research efforts have started to reveal how the modifications are set, how they are recognized, and their functional consequences for the cell. Since epigenetic studies in relation to imprinting have focused on DNA methylation, and imprinting is the main focus of this thesis, I will mostly discuss DNA methylation in this chapter. In the context of epigenetic regulation of transcription, chromatin modifications will be highlighted.

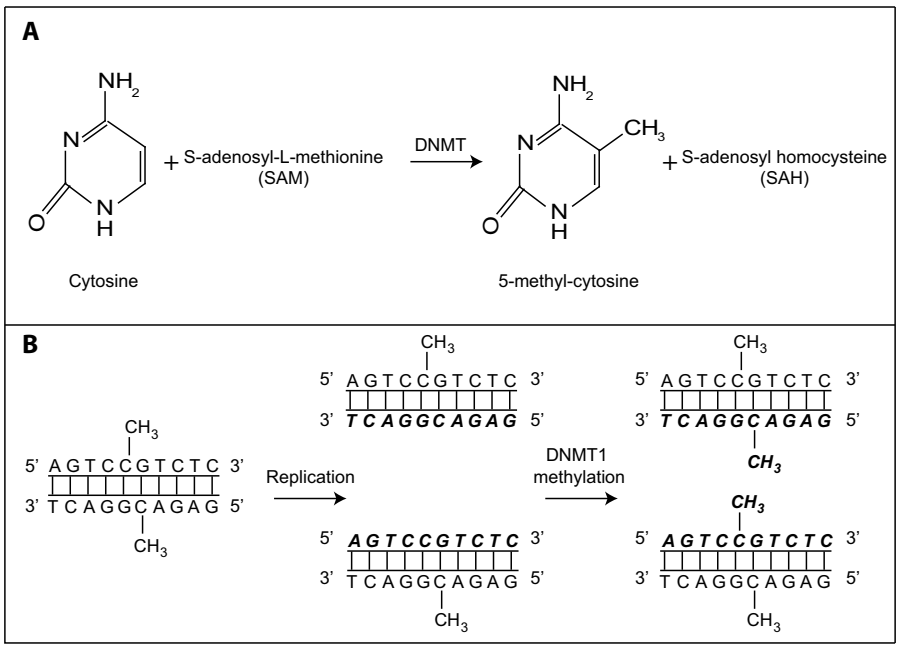

Figure 3 - CpG methylation. A) Methyl group addition to a cytosine by a methyltransferase using SAM as the methyl group donor. B) DNMT1 recognizes hemi-methylated CpGs (resulting from replication) and adds a methyl group to the CpG on the replicated strand.

2.1 DNA METHYLATION MECHANISMS

Methylation in mammals predominantly occurs at the CpG dinucleotide (cytosine connected to guanine by a phosphodiester bond) (Figure 3A). It is not fully appreciated why methylation is not found at other CpN dinucleotides; although one important property of the CpG is that it can be symmetrically methylated, meaning that the complementary C of the double-helix also acquires methylation (Figure 3B). In mammals, symmetric methylation seems to be the rule rather than the exception [Sulewska, et al., 2007]. Besides being a stable, covalent modification, once established, methylation tends to be inherited in a clonal fashion by all daughter cells [Schubeler, et al., 2000].

2.1.1 Methyl transferase enzymes

Three DNA methyl transferase enzymes have been recognized in human: the maintenance methyltransferase DNMT1, and the two *de novo* methyltransferases DNMT3A, and DNMT3B [Bestor, et al., 1988; Okano, et al., 1998]. DNMT1, 3A, and 3B all share the catalytic domains and function through adding a methyl group at the fifth carbon of the cytosine ring, using S-adenosyl-L-methionine (SAM) as the methyl donor (Figure 3A). The DNMTs are well conserved between mammals, with DNMT1 and DNMT3A showing over 90% amino acid identicality between human and mouse (HomoloGene, Table 3). DNMT1 does not show much homology to the DNMT3 family outside the catalytic domains [Okano, et al., 1998]. DNA methylation seems to be essential for life since murine *Dnmt1* and *Dnmt3b* knockouts are embryonic lethal, while *Dnmt3a*-/- embryos develop to term but die within 4 weeks after birth [Klose and Bird, 2006]. DNMT3L shares sequence homology with DNMT3A and DNMT3B but lacks the catalytic domain and has been suggested to be a co-factor, stimulating *de novo* methyltransferase activity (see 3.2.2 Dnmt3 family and genomic imprinting) [Suetake, et al., 2004].

2.1.2 Substrates for DNA methylation

None of the methyltransferases have been shown to have precise DNA sequence recognition, but some overlapping and specific target preferences have been described [Costello and Plass, 2001]. Alternative target identification approaches that have been proposed include: **1)** recognition of characteristic features of DNA or chromatin, **2)** recognition through protein-protein interactions where the other protein guides specificity [Klose and Bird, 2006]. Importantly, DNMT1 is considered the maintenance methyltransferase since it has higher affinity for hemi-methylated DNA, which is the result of replicating a symmetrically methylated target (Figure 3B) [Bestor, 1992]. DNMT3A and B are considered *de novo* methyltransferases, since no preference for hemi-methylated DNA has been demonstrated. Examples of specific substrate recognition that have been experimentally suggested in mammals are described in Table 2 [Klose and Bird, 2006].

Although CpG-methylation is by far the predominant methylated dinucleotide, CpA methylation has been demonstrated in mouse embryonic stem cells [Ramsahoye, et al., 2000]. Dnmt3a was suggested to be responsible for this specific methylation, underlining the complexity of methyltransferase action and specificity [Ramsahoye,

et al., 2000]. Some evidence has been presented for non-CpG methylation in human lung carcinoma, suggesting that it exists also in humans [Kouidou, et al., 2005].

Table 2 - DNMT substrates

	DNA/Chromatin recognition	Protein-protein guidance
DNMT1	Hemimethylated DNA [Bestor, 1992]	Association with replication machinery *in vivo* and *in vitro* [Chuang, et al., 1997; Leonhardt, et al., 1992]
DNMT3A	SINEs and LINEs in spermatogonia [Kato, et al., 2007]	*De novo* methylation more efficient *in vivo*; co-factors and chromatin context important [Lei, et al., 1996; Okano, et al., 1998]
DNMT3B	Satellite repeats and LINEs in spermatogonia [Kato, et al., 2007]	*De novo* methylation more efficient *in vivo*; co-factors and chromatin context important [Lei, et al., 1996; Okano, et al., 1998]

2.2 RULES OF EPIGENETIC INHERITANCE

Once established, the methyl marks are typically maintained through cell divisions in a clonal manner. This enables a cellular memory and the methylation pattern is often specific for distinct cell types, and a range of tissue-specific differentially methylated regions have been described [Eckhardt, et al., 2006]. DNA methylation does not follow Mendelian laws of inheritance, instead global demethylation happens twice during development: first at gamete formation and the second time immediately following conception, where the paternal genome is rapidly demethylated while the maternal genome is demethylated at a slower rate (Figure 4) [Reik, et al., 2001]. The wave of demethylation after conception has been observed on a global scale and it is known that some genomic regions, such as imprinted domains (see Chapter 3 - Imprinting) can escape demethylation. Interestingly, the paternal genome has been shown demethylated in a replication independent manner, rendering active demethylation as the only possibility [Oswald, et al., 2000]. Various proteins have been suggested to have DNA demethylase activity, including 5-methylcytosine DNA glycosylase (requiring RNA), MBD4, MBD2 (see 2.4.2, methyl-binding proteins), and DNMT3 family members themselves [Ooi and Bestor, 2008; Richardson, 2003]. However, the demethylase activity of these proteins is still debated [Ooi and Bestor, 2008].

It should be noted that trans-generational epigenetic inheritance, although rare, has been observed in mammals. Such heritable methylation is referred to as epialleles. The most cited example is the *Agouti* viable yellow allele in an inbred mice strain, where failure to methylate and silence a retrotransposon upstream of the *Agouti* gene results in yellow fur color and predisposition to develop diabetes [Rakyan, et al., 2002]. Once the epigenetic state of this allele is established (methylation vs. no methylation) both epigenotype and phenotype are stably propagated to offspring in controlled environments [Rakyan, et al., 2002].

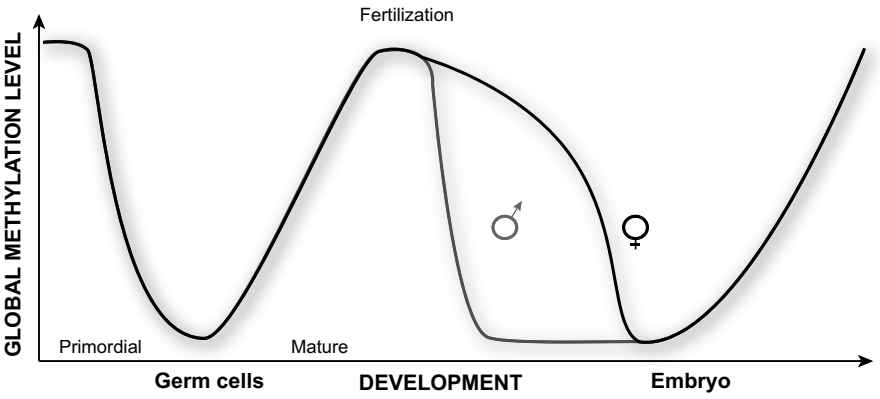

Figure 4 - Developmental reprogramming. Global methylation levels decrease during early germ cell development and later increase and peak in mature germ cells. After fertilization, the paternal (♂) and maternal (♀) genomes are demethylated at different rates. The global methylation level in somatic cells is relatively high. Adapted from [Reik, *et al*., 2001].

2.3 CpG REPRESSION AND CpG-ISLANDS

The CpG dinucleotide occurs much less frequently in the genome than would be expected from its nucleotide composition: 0.9% (observed) versus 4% (expected) [Saxonov, et al., 2006]. It is assumed that this is a consequence of the tendency of the methylated C to spontaneously deaminate into a uracil, causing a mutation from methylated CpG to TpG in the next round of replication [Pfeifer, 2006]. Despite the global CpG-repression, CpGs tend to co-occur in clusters at a rate close to the expected, referred to as CpG-islands (CGI) [Gardiner-Garden and Frommer, 1987]. CGIs tend to co-localize with 5' promoter regions in around 30-70% of human genes [Costello and Plass, 2001; Cross and Bird, 1995; Klose and Bird, 2006; Larsen, et al., 1992]. Typically, CGIs are unmethylated and associated with a transcriptionally active state. Lander and colleagues recently suggested a further sub division of CGIs based on murine data, where some are relatively CpG-poor, associated with tissue-specific genes and undergo extensive and dynamic methylation, while other CGIs are CpG-rich, associate with house-keeping genes and remain unmethylated in most somatic cell types [Meissner, et al., 2008]. *In silico* approaches strictly based on sequence composition are used to define CGIs (e.g., region >500 bp, GC-content >55%, and CpG observed/expected >0.6) and thus their function has to be proven experimentally [Takai and Jones, 2002].

2.4 FUNCTION OF THE METHYLATED CpG

2.4.1 Genomic localization of DNA methylation

The majority of CpG dinucleotides throughout the mammalian genome are methylated and do not occur in CGIs [Suzuki and Bird, 2008]. Functional groups of DNA that can acquire methylation are repetitive sequences, CGIs, genes and imprinted regions (see Chapter 3 – imprinting). As described above, CGIs are often unmethylated and thus diverge from other sequence types where methylation is the default state. The global methylation pattern of human is different from, e.g., fungi where a mosaic pattern is observed with only repetitive DNA sequences being

methylated [Alves, et al., 1996; Florl, et al., 1999; Suzuki and Bird, 2008; Thayer, et al., 1993]. Importantly, CpG methylation seems to be regulated in units, since CpG methylation within a window of 1 kb is typically concordant to 70-90%, while it deteriorates rapidly over larger distances [Eckhardt, et al., 2006].

2.4.2 Promoter methylation - Gene silencing

There are two major ways in which the methylated CpG (mCpG) could have specific functional consequences for transcriptional activity **1)** through abolishing regulatory factor binding (methyl-sensitive transcription factors) or **2)** through factors that specifically recognize mCpGs, activating specific processes in the presence of the modification [Klose and Bird, 2006]. Several examples of methyl-sensitive transcription factors have been described, including AP-2, cMyc/Myn, CREB, E2F, and NFκB [Sulewska, et al., 2007]. The CCCTC-binding factor (CTCF) is another interesting example of a methyl-sensitive DNA binding factor, which will be thoroughly discussed in the context of imprinting (see Chapter 3 - Imprinting).

An important function of DNA methylation is the crosstalk with chromatin modifying complexes and a recent study on mouse epigenetics suggested that DNA methylation is better correlated to histone modifications than sequence context [Meissner, et al., 2008]. The cross-talk could work through direct interactions with chromatin modifying complexes and both DNMTs (adding the mark) or methyl-binding factors (reading the mark). Important factors in chromatin complexes that work to condense chromatin and thus silence transcription are histone deacetylases and histone methyltransferases. DNMT1 and DMT3A have both been found to associate with histone deacetylases, suggesting a direct co-operation between the DNA methylation and chromatin-condensation machineries to inactivate transcription [Fuks, et al., 2001; Rountree, et al., 2000].

A family of highly conserved proteins with methyl-binding domains (MBDs) has been identified in mammals, consisting of MBD1-4, and MeCP2 [Hendrich and Bird, 1998; Meehan, et al., 1989]. MBDs have been found to specifically recognize mCpG through a highly conserved motif of 16 amino acids and have the potential to act as gene silencing adaptors through recruitment of chromatin modifying factors [Hendrich and Bird, 1998]. The MBD proteins recognize different targets, with MBD4 having affinity for mCpG:TpG mismatches, implicating a possible role in DNA-repair mechanisms [Bellacosa, et al., 1999; Hendrich, et al., 1999] and MeCP2 preferentially binding mCpG surrounded by adjacent AT-stretches [Klose, et al., 2005]. Further, MBD2 and MeCP2 typically associate with histone deacetylases, while MBD1 associates with histone methyltransferases [Clouaire and Stancheva, 2008]. The hierarchy and chronology of the events resulting in DNA methylation, histone modifications, and a lowered transcriptional activity are yet to be established. One important feature of histone modifications is that both histone acetylators (contributing to transcriptional activity) and histone deacetylators clearly exist, thus providing a mechanism for the reversible nature of histone modifications.

2.4.2.1 Histone modifications and transcription

The mammalian core histones can acquire all acetylation, methylation, phosphorylation and ubiquitination at their protruding tails. Acetylation of lysines of histone tails is associated with chromatin accessibility and transcription [Bernstein, et al., 2007]. Methylation at histone tails can be a sign of both an active and a repressed chromatin state with histone 3 lysine 4 methylation (H3K4) and H3K36 associating with active chromatin and, e.g., H3K9, H3K27, and H4K20 correlating with repressive chromatin [Bernstein, et al., 2007]. Histones segregate randomly at cell division and each daughter cell should thus inherit some modified histones [Bernstein, et al., 2007].

2.4.3 DNA methylation as a sign of active transcription

New results have shown that DNA methylation commonly occur within transcribed mammalian genes, with the 5´ extremities being significantly less methylated [Suzuki and Bird, 2008]. The function of DNA methylation within genes is yet to be explored, while theories include prevention of spurious initiation of transcription within the unit or antisense transcription [Suzuki and Bird, 2008].

2.4.4 Parasitic elements and genome stability

It has long been recognized that repetitive elements such as Alu-sequences, satellite repeats, centromeric repeats, etc., are extensively methylated throughout the genome [Costello and Plass, 2001]. The methylation is thought to stabilize genome structure through preventing promiscuous recombination, which has been noted in animal models and human diseases where the DNA methylation machinery is compromised [Chen, et al., 1998]. Since DNA methylation is generally thought to prevent transcription it has also been hypothesized that the methylation prevents transposon transcription and thus their ability to transposition in the genome [Alves, et al., 1996; Florl, et al., 1999; Thayer, et al., 1993].

2.5 EPIGENETICS: INTERACTION BETWEEN GENOME AND ENVIRONMENT

Several external nutrients such as folate, vitamin B12, choline and methionine are important methyl group donors for synthesizing the methyl-donor S-adenosyl-L-methionine, and these can be found in our diet, with liver being one of the richest sources [Beck and Olek, 2003]. On the global level, individuals with low dietary intake of folate show global hypomethylation [Jacob, et al., 1998]. In this sense, epigenetics can be environmentally regulated. Interestingly, the maternal diet of the *Agouti* yellow mice (see 2.2 Rules of epigenetic inheritance) has been demonstrated to affect the epigenotype in offspring and subsequent generations [Rakyan, et al., 2002]. Further, toxic environmental compounds are known to affect DNA methylation, through, e.g., methyltransferase inhibition or affecting methyl-donor nutrient uptake [Vaissiere, et al., 2008].

Table 3 – Web addresses for Chapter 2.

Database	Web address
HomoloGene	http://www.ncbi.nlm.nih.gov/homologene

3 IMPRINTING – FUNCTIONAL INEQUALITY BETWEEN THE PARENTAL GENOMES

Imprinting describes the rare but remarkable situation where genes are expressed in a parent-of-origin dependent manner. This means that some genes are only expressed if they were inherited from the father and likewise some are only expressed if they were inherited from the mother. The first evidence for this phenomenon dates back to the mid 1980's, when it was discovered through murine nuclear transfer experiments that parthenogenetic (maternal only) and androgenetic (paternal only) embryos fail to develop [McGrath and Solter, 1984; Surani, et al., 1984]. Further studies of UPD (uniparental contribution of one chromosome pair) in mice homed in on which chromosomes carry these imprints, since UPD for some chromosomes were lethal or with severe growth and developmental defects while others were without phenotype [Cattanach and Kirk, 1985]. In this chapter I will describe what is known about the complex phenomenon of imprinting, including mechanisms, functions, and associated human diseases. Many detailed studies of imprinting can only be performed in animals (such as knockouts) and consequently several of the studies reviewed below were performed in mice.

3.1 PRINCIPLE AND GENERAL CHARACTERISTICS

The insulin growth factor 2 (*Igf2*), the insulin growth factor receptor 2 (*Igfr2*) and the *H19* gene were the first mammalian imprinted genes to be identified [Barlow, et al., 1991; Bartolomei, et al., 1991; DeChiara, et al., 1991] and today about 55 imprinted human genes have been described (Geneimprint, Table 7). These have been identified in various ways including positional cloning approaches, cDNA screens of partheno- and androgenetic embryos and UPDs, phylogeny-based approaches, allele-specific DNA methylation and expression [Bartolomei and Tilghman, 1997], and recently some success has been gained through *in silico* (computational) approaches [Luedi, et al., 2007]. Imprinting is specific to placental mammals, and importantly most imprinted genes are conserved between human and mouse [Tycko and Morison, 2002]. Exceptions include the mouse genes *Zrsr1* (*U2afbp-rs*), *Rasgrf1*, *Igf2r*, and *Ascl2* that are imprinted in mice but bi-allelically expressed in human [Kalscheuer, et al., 1993; Miyamoto, et al., 2002; Pearsall, et al., 1996]. Human chromosomes 7, 11, and 15 harbor the largest number of imprinted genes, while some chromosomes contain no imprinted genes (Table 4). The mouse chromosome 7, which is syntenic to imprinted regions on both human chromosomes 11 and 15, contains a total of 27 imprinted genes. Roughly half of the imprinted genes are maternally expressed in both human and mice (Table 4).

Some typical characteristics of imprinted genes have been described, including a tendency to occur in clusters, where imprinting of several genes is controlled by a shared regulatory element [Edwards and Ferguson-Smith, 2007]. The regulatory element is often a differentially methylated region between the parental chromosomes, which is associated with tandem-repeat type elements [Constancia, et al., 1998; Neumann, et al., 1995]. Further, most imprinted regions contain at least one non-coding RNA-species, which is transcribed in the antisense direction (see 3.3

Mechanisms of imprinting). Asynchronous replication of the parental alleles has also been reported as a characteristic feature of imprinted genes [Wood and Oakey, 2006]. It should be noted that most imprinted regions include both maternally and paternally expressed genes that are regulated by the same control region. Interestingly, a clear majority of imprinting control regions are established (methylated) in the maternal germ line [Edwards and Ferguson-Smith, 2007].

Table 4 - Human imprinted genes, adapted from Geneimprint website (see Table 7).

Gene	Gene name	Cyto-band	Expressed Allele	Imprinting Syndromes*
TP73	tumor protein p73	1p36.3	Maternal	
DIRAS3	DIRAS family, GTP-binding RAS-like 3	1p31	Paternal	
PLAGL1	pleiomorphic adenoma gene-like 1	6q24.2	Paternal	TND1
HYMAI	hydatidiform mole associated and imprinted (non-protein coding)	6q24.2	Paternal	TND1
SLC22A2	solute carrier family 22 (organic cation transporter), member 2	6q25.3	Maternal	
SLC22A3	solute carrier family 22 (extraneuronal monoamine transporter), member 3	6q25.3	Maternal	
GRB10	growth factor receptor-bound protein 10	7p12.2	Isoform	SRS?
TFPI2	tissue factor pathway inhibitor 2	7q21.3	Maternal	
SGCE	sarcoglycan, epsilon	7q21.3	Paternal	
PEG10	paternally expressed 10	7q21.3	Paternal	
PPP1R9A	protein phosphatase 1, regulatory (inhibitor) subunit 9A	7q21.3	Maternal	
CPA4	carboxypeptidase A4	7q32	Maternal	SRS?
MEST	mesoderm specific transcript homolog (mouse)	7q32	Paternal	SRS?
MESTIT1	MEST intronic transcript 1 (non-protein coding)	7q32	Paternal	SRS?
COPG2IT1	coatomer protein complex, subunit gamma 2, intronic transcript 1	7q32	Paternal	SRS?
KLF14	Kruppel-like factor 14	7q32.2	Maternal	SRS?
DLGAP2	discs, large (Drosophila) homolog-associated protein 2	8p23	Paternal	
KCNK9	potassium channel subfamily K member 9	8q24.3	Maternal	
H19	H19, imprinted maternally expressed transcript (non-protein coding)	11p15.5	Maternal	BWS & SRS
IGF2	insulin-like growth factor 2	11p15.5	Paternal	BWS & SRS
IGF2AS	insulin-like growth factor 2 antisense	11p15.5	Paternal	BWS & SRS
INS	insulin	11p15.5	Paternal	BWS & SRS
KCNQ1	potassium voltage-gated channel, subfamily Q, member 1	11p15.5	Maternal	BWS (SRS)
KCNQ1OT1	KCNQ1 overlapping transcript 1 (non-protein coding)	11p15.5	Paternal	BWS (SRS)
KCNQ1DN	KCNQ1 downstream neighbor	11p15.4	Maternal	BWS (SRS)
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	11p15.4	Maternal	BWS (SRS)
SLC22A18	solute carrier family 22, member 18	11p15.4	Maternal	BWS (SRS)
PHLDA2	pleckstrin homology-like domain, family A, member 2	11p15.4	Maternal	BWS (SRS)
OSBPL5	oxysterol binding protein-like 5	11p15.4	Maternal	BWS (SRS)
WT1-AS	Wilms tumor 1 antisense	11p13	Paternal	

Continued on next page.

Table 4 continued.

Gene	Names	Cyto-band	Expressed Allele	Imprinting syndrome
DLK1	delta-like 1 homolog (Drosophila)	14q32	Paternal	Mat and patUPD14
MEG3	maternally expressed 3 (non-protein coding)	14q32	Maternal	Mat and patUPD14
MKRN3	makorin ring finger protein 3	15q11.2	Paternal	AS, PWS
MAGEL2	MAGE-like 2	15q11.2	Paternal	AS, PWS
NDN	necdin homolog (mouse)	15q11.2	Paternal	AS, PWS
SNRPN	small nuclear ribonucleoprotein polypeptide N	15q11.2	Paternal	AS, PWS
SNURF	SNRPN upstream reading frame	15q11.2	Paternal	AS, PWS
SNORD107	small nucleolar RNA, C/D box 107	15q11.2	Paternal	AS, PWS
SNORD64	small nucleolar RNA, C/D box 64	15q11.2	Paternal	AS, PWS
SNORD108	small nucleolar RNA, C/D box 108	15q11.2	Paternal	AS, PWS
SNORD116@	small nucleolar RNA, C/D box 116 cluster	15q11.2	Paternal	PWS
SNORD115@	small nucleolar RNA, C/D box 115 cluster	15q11.2	Paternal	AS, PWS
SNORD109A	small nucleolar RNA, C/D box 109A	15q11.2	Paternal	AS, PWS
SNORD109B	small nucleolar RNA, C/D box 109B	15q11.2	Paternal	AS, PWS
UBE3A	ubiquitin protein ligase E3A	15q11.2	Maternal	AS
ATP10A	ATPase, class V, type 10A	15q12	Maternal	AS
TCEB3C	transcription elongation factor B polypeptide 3C (elongin A3)	18q21.1	Maternal	
ZIM2	zinc finger, imprinted 2	19q13.4	Paternal	
PEG3	paternally expressed 3	19q13.4	Paternal	
ZNF264	zinc finger protein 264	19q13.4	Maternal	
NNAT	neuronatin	20q11.23	Paternal	
L3MBTL	l(3)mbt-like (Drosophila)	20q13.11-12	Paternal	
GNASAS	GNAS antisense RNA (non-protein coding)	20q13.32	Paternal	
GNAS	GNAS complex locus	20q13.32	Maternal	PHP-1b

*Imprinting syndromes are described in Table 6.

3.2 DNA METHYLATION AND IMPRINTING

A role for DNA methylation in imprinting was first demonstrated by defective imprinting observed in the *Dnmt1*-/- mice, where imprinting of the *H19, Igf2*, and *Igf2r* genes was disrupted [Li, et al., 1993]. This study showed that methylated CpG maintenance was mandatory for imprinted expression. The DNMT3 family of proteins have also been demonstrated to be vital for imprinting, which I will review below (3.2.2 Dnmt3 family and genomic imprinting). As more and more imprinted genes have been identified, the effect of DNA methylation on imprinting has developed into a more complex picture, where some murine genes can maintain placental parent-of-origin specific expression in a DNA methylation independent manner [Lewis, et al., 2004; Tanaka, et al., 1999].

3.2.1 Resetting imprints

The two major waves of genome-wide demethylation during development, first at the germ cell stage and later after fertilization, are important for the propagation of

imprinted methylation. At the first genome-wide demethylation, imprints should be erased, in order for the new parent-of-origin dependent methylation to be set, while at the second wave of demethylation after fertilization, the established methylation pattern must be protected, in order for imprinting to be propagated to somatic cells (Figure 5). Further, Dnmt3a *de novo* methylation of imprinted regions should take place at germ cell development, while not during embryo development, where extensive *de novo* methylation occurs. Interestingly, the mechanism of epigenetic DNA methylation machinery seems to differ between male and female germ cells, both in terms of establishment dynamics [Davis, et al., 2000] and specific regulatory factors (3.2.2 Dnmt3 family and genomic imprinting and Table 5).

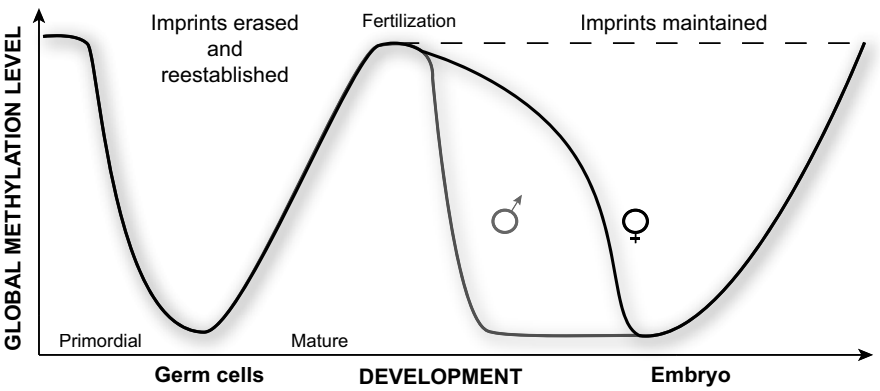

Figure 5 - Imprinted genes in developmental reprogramming. DNA methylation is erased and reestablished (in a gender-dependent manner) during germ cell formation. After fertilization, imprints are resistant to the global waves of de- and remethylation. Adapted from [Reik, *et al*., 2001].

3.2.2 Dnmt3 family and genomic imprinting

The Dnmt3 family of proteins includes the *de novo* methyltransferases Dnmt3a and Dnmt3b, and the co-factor Dnmt3l (see 2.1.1 Methyl transferase enzymes). Germline conditional knockout experiments of the murine Dnmt3 protein family enabled the exploration of *de novo* methyltransferases in relation to imprinting. Dnmt3a was found to be mainly responsible for imprinted DNA methylation, since offspring of mutant females lacked methylation and had lost allele-specific expression at all maternally imprinted loci. *Dnmt3a*-/- males showed impaired spermatogenesis and lacked methylation at two out of three imprinted loci (*H19*-ICR and *Dlk1*/*Meg3* ICR) [Kaneda, et al., 2004]. In contrast, the *Dnmt3b* germline knockouts showed no apparent genotype [Kaneda, et al., 2004]. The *Dnmt3l* gene, found in placental animals only, is highly expressed in germ cells at the time when imprints are established, and *Dnmt3l*-/- mice revealed demethylation and aberrant expression of maternally imprinted genes in oocytes, but unaffected global methylation levels [Bourc'his, et al., 2001; Hata, et al., 2002]. Male germ cells failed to develop in *Dnmt3l* knockouts and germ line specific target disruption showed that this was the consequence of meiotic errors, accompanied by lack of methylation at repetitive sequences [Bourc'his and Bestor, 2004]. The effect on male imprinting was more

complex, with aberrant methylation at two out of three paternally methylated loci [Bourc'his and Bestor, 2004; Webster, et al., 2005].

3.2.3 Imprinting control regions and differentially methylated regions

Differentially methylated CpG-rich regions with methylation specific to maternal or paternal chromosomes are typically found in the vicinity of imprinted genes. Some differentially methylated regions are called imprinting control regions (ICR) and by definition are established already in germ cells and are resistant to early embryonic epigenetic reprogramming (Figure 5, see 3.2.1 Resetting imprints). ICRs further have to be proven experimentally, through, e.g., knockout experiments in animal models, where the loss of the ICR disrupts the imprinting [Fitzpatrick, et al., 2002; Lin, et al., 2003; Thorvaldsen, et al., 1998; Wutz, et al., 1997; Yang, et al., 1998]. The ICR can function in different ways as reviewed below, but direct tandem repeats containing highly conserved core transcription factor binding sites are typical [Kim, 2008]. ICRs are relatively long (murine average 3.2 kb) compared to normal regulatory elements such as promoters and enhancers that rarely are longer than 500 bp [Kobayashi, et al., 2006]. Additional differentially methylated regions (DMRs) within the same imprinted region can exist and these undergo significant reprogramming through development, and are thought to be hierarchically regulated by the ICR [Lopes, et al., 2003]. DMRs are thought to aid the stabilization of allele-specific expression at the level of individual genes and can constitute functional units such as promoters (see 3.4 Complexity of imprinting). It is noteworthy that all ICRs/DMRs would not formally be defined as CGIs and differences in GC and CpG content have been reported between maternal and paternal ICRs [Kobayashi, et al., 2006]. There is no standardized nomenclature when referring to ICRs and therefore the literature can be confusing; examples include the intergenic ICR between *DLK1* and *MEG3* imprinted genes referred to as IG-DMR or *DLK1*/*MEG3* ICR [Kagami, et al., 2008] or the ICR on 11p15 that regulates *CDKN1C*, *KCNQ1* etc., which is referred to as KvDMR1, *KCNQ1OT1* ICR, or simply DMR2 [Weksberg, et al., 2003].

3.3 MECHANISMS OF IMPRINTING

Imprinting is a complex phenomenon, which we are still only beginning to appreciate. Fundamental questions concerning the mechanism of imprinting include **1)** how do the differential methylation patterns result in parent-of-origin specific expression, **2)** where and how are the imprints established, and **3)** how are the imprints maintained, both through the wave of demethylation after conception and throughout our life-span? For the last two questions the answer is very incomplete and researchers have only begun to identify individual factors of this machinery. I will mention some identified factors, which represent small pieces in this puzzle. However, in terms of reading the epigenetic mark, two major mechanisms (insulation and antisense transcription) have emerged as the result of intense research, and I will start by describing these.

3.3.1 Insulator model

In the insulator model, the ICR acts as a methylation-dependent insulator that directs allele-specific expression. The *H19* ICR, located between the *IGF2* protein coding gene and the RNA gene *H19*, is thought to regulate access to shared endo- and

mesodermal enhancer elements, and is the only clear example of this model (Figure 6A) [Ideraabdullah, et al., 2008]. The ICR, which contains CTCF protein binding sites, is methylated on the paternal chromosome, preventing CTCF binding (Figure 6A). The unmethylated maternal chromosome is bound by CTCF, which induces chromatin looping that inhibits *IGF2* contact with its enhancers, thus repressing expression (Figure 6A). In this model, paternal methylation is hypothesized to spread into the promoter region of *H19*, mediating its repression (Figure 6A) [Ideraabdullah, et al., 2008]. The exact nature of the chromatin looping induced through CTCF is still debated, although there is a consensus that the ICR acts as an insulator that directs long-range allele-specific interactions [Ideraabdullah, et al., 2008]. Secondary differentially methylated regions (DMR0 and DMR2, see 3.2.3 Imprinting control regions and differentially methylated regions) exist within the *IGF2* gene and these show concordant methylation patterns with the ICR (Figure 6A), possibly mediated through interactions with the ICR [Lopes, et al., 2003; Murrell, et al., 2008].

3.3.2 Antisense transcription of RNA genes

Most imprinted domains seem to utilize antisense transcription of a non-coding RNA to regulate parent-of-origin specific expression, including *Igf2r*, *Gnas*, *Dlk1/Meg3*, *Snrpn*, and *Kcnq1ot1* ICRs [Ideraabdullah, et al., 2008]. Here, the ICR typically constitutes the promoter of an RNA gene, which is transcribed in the antisense direction to silence protein-coding genes (Figure 6B). Methylation of the ICR prevents antisense transcription and allows sense transcription (Figure 6B). For several imprinted regions it has been proven that it is the expression of the full transcript and not the mere initiation of antisense transcription that maintains imprinting [Mancini-Dinardo, et al., 2006; Sleutels, et al., 2002]. The repressive mechanisms remain elusive, for further review see [Ideraabdullah, et al., 2008].

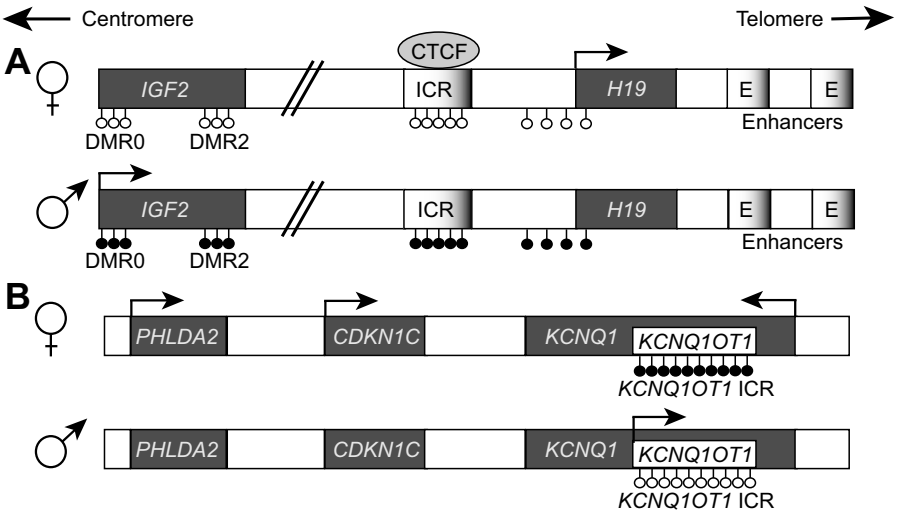

Figure 6 - Imprinting mechanisms. A) The insulator model: *H19* ICR and B) the antisense transcription model: *KCNQ1OT1* ICR. For explanations, see 3.3 Mechanisms of imprinting.

3.3.3 Imprinting regulatory factors

Several specific regulatory factors have been identified through murine and human studies of imprinting, and these are summarized in Table 5. Importantly, some factors regulate establishment of imprints such as CCCTC-binding factor (zinc finger protein)-like (Ctcfl), while Dppa3, Mbd3, ZFP57, and Yy1 have all been described to protect established imprints during preimplantation development either at individual or several imprinted regions (see Table 5). CTCF that was introduced in section 3.3.1 appears to be a multifunctional regulator of imprinting (see Table 5). Further studies are likely to reveal new regulatory factors, since imprinting requires intricate networks of several proteins in a developmentally controlled manner.

Table 5 - Imprinting regulatory factors

Gene Symbol	Gene Name	Function	Reference
Imprint establishment			
CTCFL	CCTC-binding factor-like	*Ctcfl* is expressed exclusively in testis and has been suggested to regulate *H19* ICR imprinting.	[Jelinic, et al., 2006; Loukinov, et al., 2002]
Protection of established imprints			
DPPA3	Developmental pluripotency associated-3	Murine knockout experiment revealed that Dppa3 specifically maintains established imprints of *Peg1*, *Peg3*, *Peg10*, *H19*, and *Rasgrf1* during preimplantation development.	[Nakamura, et al., 2007]
MBD3	methyl-CpG binding domain protein 3	Murine blastocyst knockdown of *Mbd3* revealed loss of methylation at the *H19* ICR (specifically) and *H19* paternal expression.	[Reese, et al., 2007]
YY1	YY1 transcription factor	*Yy1* knockdown in a neuronal cell line revealed loss-of-methylation and dysregulation of *Peg3* and *Gnas*, while *in vivo* experiments verified imprinting regulation but with a more complex pattern.	[Kim and Kim, 2008; Kim, et al., 2007]
ZFP57	Zinc-finger protein 57 homolog (mouse)	*ZFP57* is mutated in patients with loss of methylation at *HYMAI/PLAGL1*, *PEG3*, and *GRB10* ICRs. A murine knockdown study confirmed imprinting protection (*Snprn*, *Peg1*, *Peg3*, *Peg5*, and *Dlk1*/*Meg3* ICRs).	[Li, et al., 2008; Mackay, et al., 2008]
Multi-tasking			
CTCF	CCTC-binding factor	CTCF binds the unmethylated *H19* ICR to both mediate insulator function, and protect it from methylation. CTCF knockdown experiments in oocytes revealed dysregulation of several imprinted genes.	[Engel, et al., 2006; Wan, et al., 2008]

3.4 COMPLEXITY OF IMPRINTING: ALTERNATIVE PROMOTER USAGE AND TISSUE-SPECIFIC IMPRINTING

Reports on parent-of-origin specific expression usually lead to the definition of a gene as imprinted. However, many imprinted genes show complex regulatory patterns, including alternative splicing and tissue-specificity. Examples of tissue-specific imprinting include *UBE3A* that is only imprinted in brain, and *INS* that is exclusively imprinted in the yolk sac [Moore, et al., 2001; Vu and Hoffman, 1997]. Further, *IGF2*

is known to have five different promoters in human (P0-P4) and generally exhibits imprinted expression when promoters P0 and P2-P4 are used, while expression from the P1 promoter (typically active in adult liver and brain) escapes imprinting, enabling developmental stage-regulated imprinting of the same tissue [Ekstrom, et al., 1995; Monk, et al., 2006; Vu and Hoffman, 1994]. However, the reciprocally imprinted gene *H19* maintains imprinting in tissues where *IGF2* is bi-allelically expressed [Ekstrom, et al., 1995]. Isoform-dependent imprinting has been observed for *GRB10* on chromosome 7 and for *GNAS* on chromosome 20, where maternal, paternal as well as bi-allelic expression has been observed for distinct isoforms utilizing different first exons [Blagitko, et al., 2000; Hayward, et al., 1998].

3.5 FUNCTION OF IMPRINTED GENES

The nuclear transfer experiments creating parthenogenetic and androgenetic embryos were not only incompatible with development, but striking differences in the phenotype between the two types of conceptions were reported, with parthenogenetic embryos showing clear underdevelopment of extra-embryonic tissues and the opposite phenotype in androgenetic embryos. Further studies of chimeric mice created from normal cells in combination with parthenogenetic or androgenetic cells, revealed embryonal growth and skeletal defects and biased cell type contributions, underlining the functional inequality [Barton, et al., 1991; Fundele, et al., 1990; Mann, et al., 1990]. Subsequent knockout studies of imprinted genes have revealed different specific functions, with the general conclusion that imprinted genes foremost affect mammalian growth, metabolism, and behavioral traits [Bartolomei and Tilghman, 1997]. The parent-offspring model (kinship theory) for the evolution of imprinting relates to the fact that imprinting is specific to placental animals, and that paternally expressed genes tend to be growth-promoting. The placenta enables a continuous maternal-fetal nutrition exchange throughout gestation, where in the model the mother strives to preserve resources for future pregnancies (potentially with a different father), while the interest of the paternal genome is to maximize nutrient allocation to his offspring [Moore and Haig, 1991]. Clearly, this is just a theory and all imprinted genes do not support this model in an obvious way, and other models for the evolution of imprinting have been proposed including protection against parasitic DNA and maternal protection from invasive trophoblasts [Bartolomei and Tilghman, 1997].

3.6 HOW GENETICS CAN INFLUENCE IMPRINTED GENES

Since effects of imprinted genes by definition are dose-dependent, they are extra sensitive to structural variation that affects copy number. The biological effect will also differ depending on whether the duplicated or deleted segment is maternally or paternally derived, leading to relative gene-dose distortions or even complete lack of expression as the consequence of deleting the active allele (Figure 7). Imprinted genes also have an enhanced sensitivity to copy number neutral events such as UPD, which results in maternal-only or paternal-only gene expression profiles (Figure 7). Further, when mutations arise in imprinted genes these will create non-Mendelian inheritance patterns, with each mutation either being fully penetrant or silent, depending on the parental allele from which it was inherited, in relation to the imprinting status of the gene.

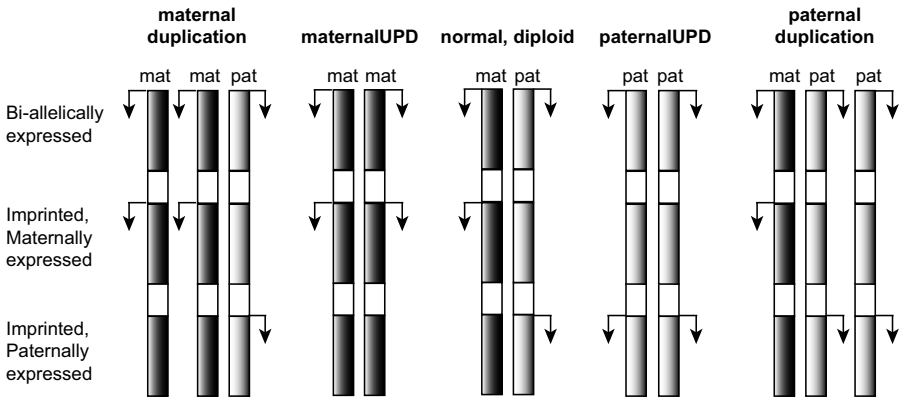

Figure 7 - Genetic effects on imprinted genes. In the normal diploid individual (middle), imprinted genes are expressed from one parental chromosome only. When a genomic region or chromosome is duplicated or uniparentally inherited, imprinted expression (gene-dose) is distorted.

3.6.1 Human imprinting disorders

What happens when imprinting fails? Although complete ablation of imprinting is incompatible with development, several human congenital syndromes that result from disruption of specific imprinted genes exist (Table 6). In general, these syndromes cause growth and behavioral dysfunction, as expected from imprinted gene function in mice (see 3.5 Function of imprinted genes). The congenital imprinting syndromes are heterogeneous in genetic nature and can result from maternal and paternal duplications and deletions (depending on imprinting status), UPD, mutations in imprinted genes, or aberrant imprinting (loss or gain of methylation). Interestingly, several of the imprinting disorders map to the same imprinted region, but show opposite parental effects and sometimes opposite phenotypes. One example is Prader-Willi syndrome (PWS) and Angelman syndrome (AS) that present with opposite growth phenotypes (obesity versus leanness), and with opposite parent-of-origin effects (maternal UPD and paternal deletions versus paternal UPD and maternal deletions) (Table 6). The phenotypes of maternal and paternal uniparental disomy of chromosome 14 (mat- and patUPD14) are also thought to be caused by opposite dysregulation of imprinted genes on 14q32, since patients with isolated methylation defects at the *DLK1/MEG3* ICR have the same phenotypes as UPDs [Kagami, et al., 2008]. Most cases of aberrant imprinting (i.e., hypo- or hypermethylation at an ICR) are unexplained and are referred to as "epimutations", while in the case of transient neonatal diabetes (see Table 6), a mutation in a gene that regulates imprinting has been identified (*ZFP57*) [Mackay, et al., 2008]. Further, imprinting can fail to be maintained during the lifespan of an individual that was born normal, and several types of human cancers are associated with loss of imprinting at, e.g., the *IGF2* gene [Cui, et al., 2003; Ito, et al., 2008]. Of the congenital imprinting syndromes, only Beckwith-Wiedemann syndrome, which involves disruption of the *IGF2* and related imprinted genes, is associated with an increased risk of developing cancer. Silver-Russell syndrome, which is the topic of this thesis, is considered an imprinting syndrome and the associated symptoms and molecular defects will be discussed in Chapters 4 and 5.

Table 6 - Disorders of imprinting.

Syndrome	Symptoms	Imprinted region(s)	Reference
Transient neonatal diabetes-type 1 (TND1) OMIM# 601410	Fetal growth restriction and neonatal insulin dependence.	*HYMAI*/ *PLAGL1* (6q24) ICR	[Temple and Shield, 2002]
Molecular findings:	Paternal UPD6, paternal dup6q24, and *HYMAI/ PLAGL1* ICR hypomethylation.		
Silver-Russell syndrome (SRS) OMIM# 180860	Growth restriction, skeletal defects, large, triangular face and asymmetry.	*GRB10* (7p12), *MEST* (7q32), and *H19* (11p15) ICRs	[Abu-Amero, et al., 2008]
Molecular findings:	Maternal UPD7, maternal dup7p, maternal UPD11, maternal dup11p, *H19* ICR hypomethylation.		
Beckwith-Wiedemann syndrome (BWS) OMIM# 130650	Overgrowth, asymmetry, large tongue and increased tumor risk.	*H19* and *KCNQ1OT1* (11p15) ICR	[Weksberg, et al., 2003]
Molecular findings:	Mosaic paternal UPD11, paternal dup11p, *KCNQ1OT1* ICR hypomethylation, *H19* ICR hypermethylation, *CDKN1C* mutations.		
Maternal UPD14 syndrome (matUPD14)	Growth restriction, skeletal deformities and early onset of puberty.	*DLK1/ MEG3* (14q32) ICR	[Sanlaville, et al., 2000]
Molecular findings:	Maternal UPD14 and *DLK1/ MEG3* ICR hypomethylation.		
Paternal UPD14 syndrome (patUPD14) OMIM# 608149	Joint contractures, narrow rib cage, dysmorphic facies and developmental delay.	*DLK1/ MEG3* (14q32) ICR	[Sutton, et al., 2003]
Molecular findings:	Paternal UPD14 and *DLK1/ MEG3* ICR hypermethylation.		
Prader-Willi syndrome (PWS) OMIM# 176270	Obesity, mild mental retardation, short stature and behavioral problems.	15q11.2 ICR	[Nicholls, et al., 1998; Sahoo, et al., 2008]
Molecular findings:	Maternal UPD15, paternal del(15q11-q13), 15q11.2 ICR hypermethylation, and minimal paternal del(*SNORD116@*).		
Angelman syndrome (AS) OMIM# 105830	Severe mental retardation, seizures, leanness and absent speech.	15q11.2 ICR	[Nicholls, et al., 1998]
Molecular findings:	Paternal UPD15, maternal del(15q11-q13), 15q11.2 ICR hypomethylation, and *UBE3A* mutations.		
Pseudohypoparathyroidism 1b (PHP-1b) OMIM# 603233	Hypocalcemia and hyperphosphatemia.	*GNAS* (20q13) ICR	[Bastepe, 2008]
Molecular findings:	*GNAS* ICR hypomethylation and paternal UPD20.		

ICR-imprinting control region, dup-duplication, del-deletion.

Table 7 – Web addresses for Chapter 3.

Database	Description	Web address
Geneimprint	Exhaustive lists of mouse and human imprinted genes.	www.geneimprint.com/site/home
MRC Harwell - Genomic imprinting	Murine imprinting maps, also include UPD effects and human homology.	www.har.mrc.ac.uk/ research/genomic_imprinting/
University of Otago imprinting database	Parent-of-origin effects across species. Can be searched for disorders with imprinting effects.	igc.otago.ac.nz/home.html

4 SILVER-RUSSELL SYNDROME

In 1953, Silver and colleagues described a syndrome of congenital short stature and asymmetry, in combination with effects on sexual development [Silver, et al., 1953]. A year later, Russell described a set of five patients with intrauterine onset growth retardation, short arms, distinctive facial features and pregnancy complications [Russell, 1954]. Later it was agreed that Silver and Russell described the same syndrome, although emphasizing distinct features, and the combined name Silver-Russell syndrome is currently used [Black, 1961]. Silver-Russell syndrome (SRS, OMIM# 180860) features severe intrauterine growth restriction, persisting in childhood and distinctive symptoms including a large, triangular head, fifth finger clinodactyly and body asymmetry (Figure 8). However, the features are relatively mild and it should be emphasized that the diagnosis of SRS relates to a range of problems and their accumulation in a patient. The majority of patients are diagnosed in early childhood due to their small presentation and failure to grow. Overall, the patients have good prognosis although growth restriction persists into adulthood (see 4.1.2 Childhood growth and final height). The incidence of SRS has been estimated to 1/3000-1/100,000 births [Abu-Amero, et al., 2008] and it has been reported in various ethnicities [Patton, 1988]. In Sweden, only 40 diagnosed patients are known, suggesting that the syndrome might be underdiagnosed (source: Socialstyrelsen, see Table 8).

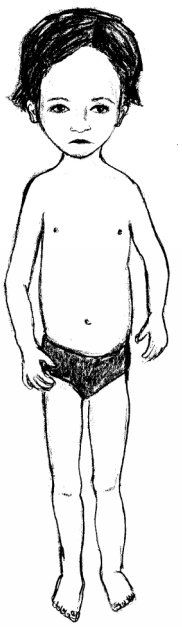

Figure 8 - Silver-Russell syndrome patient. The characteristic phenotype including a triangular, relatively large head, small chin, down-turned mouth corners and body asymmetry are shown.

4.1 GROWTH IN SRS

4.1.1 Intrauterine growth

Prenatal growth is reduced in SRS, with average birth weights and lengths of 1900 g and 43 cm [Wollmann, et al., 1995], corresponding to approximately -3 standard deviations (SD) from the mean [Abraham, et al., 2004; Price, et al., 1999; Tanner, et al., 1975]. Different terminology is in use to describe the nature of fetal growth restriction. Intrauterine growth retardation (IUGR) is the more dynamic term and refers to the process where fetal growth slows down during development, and it requires monitoring during pregnancy [Wollmann, 1998]. The term small for gestational age (SGA) refers to children that are born smaller than others born at the same week of gestation (birth weight < -2SD from the mean) [Wollmann, 1998]. SGA children can be naturally small, while IUGR often relates to a pathogenic mechanism such as placental insufficiency, infections, or underlying genetic disease of the fetus [Wollmann, 1998]. Provided mean and standard deviations (SD) are known for a population, the standard deviation score (SDS) can be calculated for an individual by subtracting, e.g., birth weight by mean birth weight and dividing this by the standard deviation. Most diagnostic criteria of SRS require the children to have birth weights below -2 SDS, and they are thus by definition SGA. Most SRS children are born at term [Abraham, et al., 2004; Tanner, et al., 1975; Wollmann, et al., 1995]. However, SRS can be diagnosed prenatally [Falkert, et al., 2005] and the children seem to experience growth retardation during gestation since premature SRS are relatively less growth restricted (length SDS -3.3, boys) than term SRS (length SDS -4.3, boys) [Wollmann, et al., 1995]. Further, the growth restriction appears to initiate early *in utero* since SRS children are proportionally growth-retarded in that both birth weight and length are affected [Wollmann, et al., 1995]. Importantly, the head seems less affected by the growth restriction in SRS (can even be appropriate for gestational age), which makes the children appear to have a large head [Tanner, et al., 1975; Wollmann, et al., 1995].

4.1.2 Childhood growth and final height

Two studies have carefully monitored neonatal and childhood growth in cohorts of SRS patients (39 and 386 patients, respectively) and these will be reviewed below [Tanner, et al., 1975; Wollmann, et al., 1995]. During infancy and early childhood, SRS children exhibit some further loss in growth, often related to episodes of hypoglycemia, feeding problems and failure to thrive [Wollmann, et al., 1995]. Between infancy and puberty, SRS children grow at a steady rate that parallels the 3rd percentile of a normal growth curve [Tanner, et al., 1975; Wollmann, et al., 1995]. The children are growth-retarded both in terms of length and weight-for-height measurements, although the relative deficit in height is more pronounced [Wollmann, et al., 1995]. The few cases that have been studied longitudinally were shown to have normal, although small, pubertal growth spurts [Tanner, et al., 1975]. Wollmann and colleagues reported the final heights of 151.2±7.8 cm in male SRS patients and 139.7±7.4 cm in female SRS patients [Wollmann, et al., 1995], which is clearly below population averages. It is important to use the most appropriate population references, for example, in Sweden the average height is 180 cm in males and 168 cm

in females [Albertsson-Wikland and Karlberg, 1994] and in Finland, 178 cm in males and 165 cm in females [Helakorpi, et al., 2004].

4.2 PHENOTYPE AND SYMPTOMS

4.2.1 Face

As reviewed by Patton, the typical SRS face is triangular in shape, as the result of a large skull and a hypoplastic chin/mandible (Figure 8) [Patton, 1988]. The face is small in relation to the skull, featuring a large, prominent forehead, the eyes are considered large and the ears are often low-set, meaning that they are positioned below the maximum circumference of the skull (Figure 8). Further, the mouth has an inverted V-shape (shark mouth) and the lips are typically thin [Patton, 1988]. The small chin results in crowded and irregularly placed teeth and in addition, congenital malformations of the mouth are observed, such as a high-arched palate and occasional cleft palates [Escobar, et al., 1978; Price, et al., 1999]. The patients are often reported to have a high-pitched, nasal voice [Wollmann, et al., 1995].

4.2.2 Musculo-skeletal findings

SRS children are born very thin, with lack of subcutaneous fat and hypoplastic muscles [Wollmann, et al., 1995]. The lean build with low muscle mass typically persist in childhood, and there are frequent reports of low muscle tone [Wollmann, et al., 1995]. A prominent feature of SRS is asymmetry (Figure 8), often relating to differences in limb lengths, but also hemihypoplasia, which refers to not only leg length differences but, e.g., the circumference of a leg (muscle mass) [Escobar, et al., 1978; Silver, et al., 1953; Wollmann, et al., 1995]. The asymmetry in SRS can range from a moderate leg length discrepancy of 0.5 cm up to 7 cm, contributing to a rather wide distribution [Abraham, et al., 2004; Price, et al., 1999]. Scoliosis (curving of the spine), which is also found in SRS is potentially related to the asymmetry [Abraham, et al., 2004; Price, et al., 1999]. SRS hands typically feature an incurving of the fifth finger, often in combination with shortened fingers, especially the fifth [Patton, 1988]. A less frequent finding is camptodactyly, or joint defects of the fingers [Price, et al., 1999]. The most frequent finding of the feet is second to third toe syndactyly [Marks and Bergeson, 1977; Wollmann, et al., 1995].

4.2.3 Sexual development

In his first description of the syndrome, Silver emphasized elevated urinary gonadotropins in SRS infants that were at the level expected during puberty [Silver, et al., 1953]. There have been reports on precocious pubertal development in SRS, but in general puberty is normal [Tanner, et al., 1975; Wollmann, et al., 1995]. Instead, malformation of genitalia are frequently reported, with undescended testes (uni- or bilateral cryptorchidism) and malformation of the urethral opening (hypospadia) being found in 20-40% of SRS males [Marks and Bergeson, 1977; Price, et al., 1999]. Less frequently, hypoplastic testes and ambiguous genitalia in combination with a male karyotype have been reported [Falkert, et al., 2005; Marks and Bergeson, 1977; Price, et al., 1999]. Genital malformations in females are seldom diagnosed at birth or early childhood, from which most SRS reports originate. However, there have been descriptions of SRS in combination with Mayer-

Rokistansky-Küster-Hauser syndrome (MRKH, OMIM# 277000), characterized by utero-vaginal aplasia [Abraham, et al., 2004; Bellver-Pradas, et al., 2001] and bicornuate uterus [Price, et al., 1999]. Despite defects in genital development, no comprehensive studies of fertility in SRS patients have been performed.

4.3 METABOLISM AND FEEDING

4.3.1 Hypoglycemia

Episodes of fasting hypoglycemia occur in a significant portion of SRS children, typically in association with over-night fasting [Azcona and Stanhope, 2005; Price, et al., 1999]. The hypoglycemia is thought to relate to the excessive sweating that is also observed in SRS [Patton, 1988].

4.3.2 Feeding problems

Feeding problems are reported by many parents of SRS children [Saal, et al., 1985]. The problem comprises different stages of development, where as newborns SRS patients typically fail to thrive and often require periodic tube feeding, while in childhood SRS children have poor appetite and demonstrate food fussiness [Blissett, et al., 2001]. Oromotor dysfunction was suggested as the cause of feeding problems, especially since many SRS patients have problems with solid foods and specific textures [Blissett, et al., 2001].

4.3.3 Internal organs

Although specific problems with internal organs are not a prominent feature of SRS, some interesting findings will be mentioned here. Cardiac defects identified in newborn patients, including atrial and ventricular septal defects, have been described [Abraham, et al., 2004; Marks and Bergeson, 1977]. Further, some early reports emphasized congenital renal malformations in SRS, which resulted in a predisposition for urinary tract infections [Arai, et al., 1988; Haslam, et al., 1973; Spirer, et al., 1974]. Their associations to frequent genital defects in SRS (see 4.2.3 Sexual development) suggest that full genitourinary tract evaluations would be beneficial for patients [Arai, et al., 1988].

4.4 COGNITIVE AND MOTOR DEVELOPMENT

Early motor development is often retarded in SRS [Marks and Bergeson, 1977; Wollmann, et al., 1995]. This probably relates to underdeveloped muscles and the relatively large head observed in SRS patients. Cognitive development in SRS is most often referred to as normal [Marks and Bergeson, 1977], while detailed studies have reported a moderate but significant impairment of cognitive abilities [Lai, et al., 1994; Noeker and Wollmann, 2004]. SRS children typically attend mainstream schools so their cognitive impairment is in the manageable range [Price, et al., 1999; Tanner, et al., 1975]. A considerable portion of SRS children receive speech therapy [Blissett, et al., 2001; Lai, et al., 1994; Price, et al., 1999]. The underlying causes of speech problems are likely to be multifactorial, where the mouth anatomy including a narrow mandible, crowded teeth, and a high-arched palate could cause dysfunction in speech [Blissett, et al., 2001; Lai, et al., 1994]. Further, the motor development in terms of muscular coordination could be impaired, which might influence speech. For a

discussion on speech problems in relation to genetic subgroups of SRS see 5.2.1, Distinct phenotype of matUPD7.

4.5 TREATMENT

Since SRS children have a relatively mild phenotype, they usually do not require extensive hospitalization and therapies are often targeted to specific symptoms. Growth hormone treatment is administered to SRS patients when deemed relevant and is importantly not only considered for patients who have a deficiency in growth hormone production [Azcona, et al., 1998]. The response to the treatment is rather heterogeneous, but an average improvement is well documented, although SRS children rarely reach their target height [Stanhope, et al., 1998]. Growth hormone treatment does not significantly improve asymmetry [Rizzo, et al., 2001], but this is usually adjusted with a shoe lift or in severe cases with leg-extending surgery [Abraham, et al., 2004]. Children who exhibit frequent hypoglycemic episodes in combination with feeding difficulties need tube feeding in order to improve their nutritional status during the neonatal period [Price, et al., 1999]. Urogenital defects in SRS such as undescended testes are often surgically corrected [Price, et al., 1999]. Speech therapy (see 4.4 Cognitive and motor development) and special education is common amongst SRS patients [Price, et al., 1999].

4.6 OBSTETRIC FINDINGS

In the initial report, Russell emphasized the contribution of teratogenic factors in SRS pathology, since 3 out of 5 cases had reported imminent abortion, vaginal bleeding and abnormal placentas [Russell, 1954]. However, there is no current evidence that such difficulty during pregnancy is pathogenically important, and reports on obstetric complications are not more common in SRS [Patton, 1988; Tanner, et al., 1975].

4.7 DIFFERENTIAL DIAGNOSES

Since SRS symptoms are relatively mild, the diagnosis can be uncertain. The most important differential diagnoses are muscle-liver-brain-eye (MULIBREY) nanism (OMIM# 253250), THREE-M syndrome (OMIM# 273750), and mosaic trisomy 18 syndrome [Karlberg, et al., 2004; Patton, 1988], but several other differential diagnoses are listed in various sources [Jones and Smith, 2006; Rimoin, et al., 1996]. For the mentioned syndromes genetic testing can be used to resolve the pathogenesis and the correct diagnosis [Huber, et al., 2005; Karlberg, et al., 2004].

Table 8 – Web addresses for Chapter 4.

Database	Web address
Swedish database of rare diagnoses (Socialstyrelsen)	www.socialstyrelsen.se/ovanligadiagnoser/Silver-Russells+syndrom.htm

5 MOLECULAR GENETICS OF SILVER-RUSSELL SYNDROME

The molecular findings in SRS patients (summarized below) demonstrate that this syndrome is a rare but illustrative example of how human growth is controlled by both genetic architecture and epigenetics, and further highlights the importance of the insulin growth factor (IGF) pathway and imprinting in human growth and development [Abu-Amero, et al., 2008].

5.1 A GENETIC BASIS OF SRS

The majority of SRS patients are sporadic, meaning that they occur as isolated cases within families [Wakeling, et al., 1998a]. This sporadic nature was used as an argument by Tanner and colleagues to support the idea that SRS is a distinct syndrome and not just the severe end of the SGA spectrum, since SGA children typically tend to have an excess of SGA sibs [Tanner, et al., 1975]. However, occasional familiar clustering of SRS has been reported [Duncan, et al., 1990]. There is no clear consensus regarding the mode of inheritance, but all autosomal dominant, autosomal recessive and X-linked dominant patterns have been suggested from pedigrees [Abu-Amero, et al., 2008]. Overall, the reports on familiar SRS have brought little success to understanding the specific etiology, since no linkage analyses have been performed and no causal variants reported. Further, the observation that familial SRS seems milder [Duncan, et al., 1990] highlights the issue of whether the appropriate diagnosis was made in some families.

Instead, there has been considerable success in finding molecular genetic causes of sporadic SRS, and the widely recognized associated anomalies are maternal UPD of chromosome 7 (matUPD7), found in about 5-15% of SRS patients, and hypomethylation of the *H19* ICR on chromosome 11p15.5, found in 20-65% of patients [Abu-Amero, et al., 2008]. Beyond these major molecular subgroups (matUPD7 and *H19* ICR hypomethylation), some recurring and some exceedingly rare genomic aberrations have been described in SRS patients. The recurring aberrations have helped to pinpoint candidate regions and highlight the importance of imprinting in SRS (segmental matUPD7 and maternal duplications of 7p and 11p, discussed in this chapter). More rare genetic findings in SRS will be mentioned (see 5.6 Additional genomic regions implicated in SRS), although their clinical relevance remains elusive. Importantly, DNA re-sequencing of coding exons in SRS candidate genes have so far failed to reveal mutations as a common cause of SRS (see 5.6.3 Excluded candidate genes), but instead most genetic findings have related to genome structure, either copy number neutral, such as in the case of matUPD7 or relating to gain or loss of submicroscopic genomic regions.

5.2 UNIPARENTAL DISOMY OF CHROMOSOME 7

Uniparental disomy (or uniparental contribution of a chromosome pair) as a genetic phenomenon in human was hypothesized already in 1980 based on the relatively high aneuploidy rate observed in human gametes [Engel, 1980]. Almost ten years later the first two UPDs of chromosome 7 (maternal in origin, matUPD7) were found through

homozygosity at the cystic fibrosis (CF, OMIM# 219700) locus on chromosome 7 [Spence, et al., 1988; Voss, et al., 1989]. The third matUPD7 was found through homozygosity at another recessive disease locus on chromosome 7, and it could be established that although carriers of different mutations, the three cases shared a phenotype of symmetrical, prenatal onset growth restriction, specific to the matUPD7 [Spotila, et al., 1992]. In 1995, Kotzot and colleagues reported four matUPD7s in a cohort of growth-retarded patients that were ascertained on the basis of a SRS diagnosis or a SRS-like phenotype [Kotzot, et al., 1995]. Three of the patients were isodisomic (one maternal chromosome in duplicate) and one was heterodisomic (both maternal chromosomes), which led the authors to suggest that dysregulation of an imprinted gene on chromosome 7 causes the observed growth restriction [Kotzot, et al., 1995]. Subsequent studies confirmed that matUPD7 is found in around 5-15% of SRS patients [Hannula, et al., 2002; Kotzot, 2008; Preece, et al., 1997; Price, et al., 1999] and that matUPD7 is not a common cause of growth restriction without SRS stigmata [Hannula, et al., 2002]. Today, over 60 cases of matUPD7 have been described in the literature [Kotzot, 2008]. Interestingly, only four cases of patUPD7 are known, and these do not appear to show the expected overgrowth [Fares, et al., 2006; Hoglund, et al., 1994; Le Caignec, et al., 2007; Pan, et al., 1998].

5.2.1 Distinct phenotype of matUPD7

The first three identified cases of matUPD7 were not diagnosed with SRS, while this diagnosis has been made for the majority of subsequently identified cases [Kotzot, 2008]. Indeed, in a review of matUPD7 patients, Price and colleagues noted a milder phenotype both in terms of dysmorphisms and intrauterine growth [Price, et al., 1999]. Later, a distinct and milder phenotype of matUPD7 was delineated through a detailed study of four matUPD7 patients, all presenting with pre- and postnatal growth restriction, a milder facial phenotype, and an observed elevated frequency of speech delay, feeding problems, excessive sweating, and hypoglycemia, as compared to non-matUPD7 SRS [Hannula, et al., 2001a]. The milder facial appearance related to a less pronounced triangularity, lack of micrognathia, and lack of down-turned mouth corners [Hannula, et al., 2001a]. Low-set or abnormal ears have also been reported at a higher frequency in matUPD7, compared to SRS [Hannula, et al., 2001a; Kotzot, 2008]. A candidate gene for the speech delay in matUPD7 has recently been proposed, since the *FOXP2* gene (7q31) shows preferential paternal expression [Feuk, et al., 2006]. Interestingly, the dysfunction in speech caused by aberrant *FOXP2* expression relates mostly to oromotor dysfunction and can thus also be related to the feeding problems [Feuk, et al., 2006]. A recent study suggested that the growth dynamics in matUPD7 is distinct from SRS, since matUPD7 are born relatively larger but exhibit more severe growth retardation during early childhood [Binder, et al., 2008].

5.2.2 Mechanisms of UPD formation

An overall elevated age has been reported for mothers of matUPD7 patients [Hannula, et al., 2001a; Kotzot, 2008]. There are several ways in which UPD could form, including trisomic rescue, monosomic rescue, gamete complementation, or mitotic recombination [Robinson, 2000]. Trisomic rescue occurs when one diploid and one haploid gamete are combined, with random exclusion of one chromosome for

compatibility with fetal development, resulting in uniparental disomic zygotes one-third of the times (Figure 9A). Monosomic rescue would arise from the combination of haploidy and monosomy for a chromosome, with a subsequent mitotic duplication event to restore diploidy (Figure 9B). Gamete complementation refers to the situation where diploid and nullisomic gametes are combined (combination of two aneuploidies) (Figure 9C). An early somatic recombination event with the subsequent loss of the reciprocal product would cause an isodisomic, segmental UPD (Figure 9D). Combinations of the above mechanisms could result in segmental heterodisomic UPD. The type of UPD (whether isodisomic, heterodisomic or a sequential combination) can be used to estimate the most likely mechanism of formation. Whole chromosome isodisomy often results from monosomy rescue, often a post-fertilization event, while regions of heterodisomy often indicate trisomy rescue as the result of a meiosis non-disjunction [Robinson, 2000]. The specific association between heterodisomy and increased maternal age further supports trisomic rescue as its primary mechanism of formation [Kotzot, 2008]. Notably, UPDs formed by trisomic rescue can be associated with confined placental mosaicism for trisomy 7, in the case where the third chromosome exclusion only occurred in embryonal cells [Robinson, 2000].

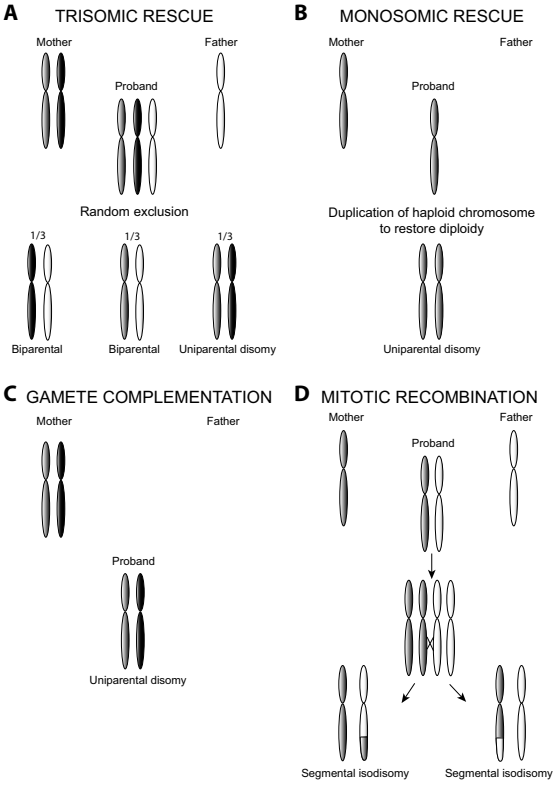

Figure 9 - Mechanisms creating uniparental disomy. A) Trisomic rescue, B) monosomic rescue, C) gamete complementation and D) mitotic recombination. See 5.2.2 Mechanisms of UPD formation for detailed explanations.

5.2.3 Pathogenic mechanism of matUPD7

UPD could be pathogenic in different ways including recessive allele penetrance, underlying trisomy mosaicism, or dysregulation of imprinted genes on the UPD chromosome [Robinson, 2000]. The penetrance of a recessive disease allele would require a shared isodisomic (homozygous) region between all patients. Importantly, both heterodisomic and isodisomic matUPD7 are observed, and one study failed to identify shared isodisomic regions in five matUPD7 patients, using microsatellite analyses along chromosome 7 [Preece, et al., 1999]. Of all described matUPD7 cases, 34 have been reported heterodisomic and 21 isodisomic, suggesting that the formation mechanism varies [Kotzot, 2008]. Although there have been isolated reports of matUPD7 in association with fetal mosaicism for trisomy 7 [Bilimoria and Rothenberg, 2003; Flori, et al., 2005], this has been excluded as a common cause of SRS [Monk, et al., 2001]. Further, confined placental mosaicism for trisomy 7 without matUPD7 is compatible with normal intrauterine growth [Kalousek, et al., 1996].

Several imprinted genes exist on chromosome 7 and their syntenic regions in mice have been shown to impose abnormal growth patterns when inherited uniparentally, thus supporting the idea that dysregulation of an imprinted gene causes the matUPD7 phenotype [Abu-Amero, et al., 2008]. Rare genetic events in SRS have homed in on two candidate imprinted regions on 7p12 and 7q32, which will be reviewed below.

5.3 7p13-p11.2 AND SRS

Part of the short arm of human chromosome 7 is syntenic with a region on mouse chromosome 11, which contains the imprinted gene *Grb10*, and which imposes opposite growth phenotypes when inherited as maternal UPD (growth restriction) and paternal UPD (overgrowth) [Cattanach and Kirk, 1985]. Other interesting candidate genes for SRS in this region include the insulin growth factor binding proteins *IGFBP1* and *IGFBP3*, and the epidermal growth factor receptor *EGFR* [Abu-Amero, et al., 2008].

5.3.1 Structural rearrangements of chromosome 7p

Four patients with duplications on 7p in combination with growth restriction and SRS-like phenotypes have been described (3 out of 4 maternal in origin) [Joyce, et al., 1999; Monk, et al., 2002b; Monk, et al., 2000]. Three of the duplications spanned *GRB10* (7p12.2), *IGFBP1*, and *IGFBP3* (both 7p13), while excluding the *EGFR* gene [Joyce, et al., 1999; Monk, et al., 2000]. A recent report of maternal duplication in 7p11.2-p12 without features of SRS strengthens the notion of the 7p12-7p13 region as a candidate for SRS [Leach, et al., 2007]. Taken together, the hypothesis is that the increased dosage of an imprinted gene on chromosome 7, either resulting from matUPD7 or a maternal duplication, contributes to growth restriction and SRS.

5.3.2 Imprinting status and mutational analyses of SRS candidate genes

To conform to the matUPD7 and 7p maternal duplication findings, the candidate gene for SRS on chromosome 7 should be imprinted and mutations or aberrant imprinting would be expected in molecularly undefined SRS patients. GRB10 is a cytoplasmic

adaptor protein that has been proposed as a negative regulator of tyrosine kinase receptor signaling (e.g., IGF1R) [Blagitko, et al., 2000]. Murine *Grb10* is imprinted and maternally expressed in all tissues apart from the brain where it shows paternal expression [Hitchins, et al., 2002b; Miyoshi, et al., 1998]. Targeted deletions of *Grb10* show an increased growth upon maternal transmissions, emphasizing its role as a negative regulator of growth [Charalambous, et al., 2003]. Human *GRB10* imprinting is complex, with paternal expression in brain and spinal cord, isoform specific maternal expression in skeletal muscle and bi-allelic expression in all other fetal tissues examined [Blagitko, et al., 2000; Hitchins, et al., 2001]. Interestingly, although the parent-of-origin specific expression differs between human and mouse, they share a well-conserved ICR that is maternally methylated in both species [Arnaud, et al., 2003].

Sequence analysis of *GRB10* exons in over 120 SRS patients has excluded coding mutations as a common cause of SRS [Mergenthaler, et al., 2001]. While a maternally inherited mutation was initially described in two Japanese SRS patients [Yoshihashi, et al., 2000], further screening in a larger Japanese control cohort determined its frequency to 0.5% in the normal population [Yoshihashi, et al., 2001]. The *GRB10* ICR has been screened for methylation defects in altogether 46 SRS patients, and no aberrant methylation was observed [Arnaud, et al., 2003; Monk, et al., 2003]. *GRB10* is still considered a candidate gene for SRS, although its complex imprinting pattern makes inference of its potential genetic and epigenetic role in SRS very difficult.

IGFBP1 and *IGFBP3*, which are located in the SRS candidate region, encode regulatory proteins that bind IGF-I and IGF-II in blood and control their bioavailability. Both genes have been shown bi-allelically expressed in all tissues examined [Eggermann, et al., 1999a; Wakeling, et al., 2000], and furthermore no mutations were revealed in a screen of 49 SRS patients [Eggermann, et al., 1999b]. *EGFR* has been considered a candidate gene for SRS, however Wakeling and colleagues demonstrated its bi-allelic expression in all fetal tissues examined [Wakeling, et al., 1998b]. No further imprinted genes have been reliably identified in the vicinity of *GRB10*, contrary to the observation that imprinted genes tend to occur in clusters [Hitchins, et al., 2002b] [Menheniott, et al., 2008]. This renders *GRB10* the only imprinted SRS and matUPD7 candidate gene on chromosome 7p.

5.4 7q32 AND SRS

Chromosome 7q32.2 contains five imprinted genes, including the paternally expressed *MEST*, *COPG2IT1*, *MESTIT1*, and the maternally expressed *CPA4* and *KLF14*.

5.4.1 Structural rearrangements of chromosome 7q

Reports on maternal 7q isochromosomes in SRS-like patients lended support for the involvement of the 7q region in growth restriction in SRS [Eggerding, et al., 1994; Kotzot, et al., 2001]. The finding of a segmental matUPD7q31-qter in a SRS patient pinpointed a narrow 35 Mb SRS candidate region on chromosome 7 [Hannula, et al., 2001b]. Segmental matUPD7 is a rare finding in SRS [Riegel, et al., 2003], but one

additional mosaic segmental matUPD7q21-qter and a recent report of two segmental matUPD7q11.2-qter have confirmed the association to growth restriction and SRS-like phenotypes [Eggermann, et al., 2008d; Reboul, et al., 2006].

5.4.2 Imprinting status and mutational analyses of SRS candidate genes

MEST is an isoform-specific imprinted gene with the isoform 1 showing paternal expression and the isoform 2 (utilizing a different first exon) showing biparental expression in all tissues but the placenta [Kosaki, et al., 2000; McMinn, et al., 2006]. The use of shared exons in the analysis of *MEST* imprinting led to some initial confusion as to its imprinted status [Kosaki, et al., 2000]. Despite its excellent candidate gene status on the basis of the growth restriction in the murine knockout model [Lefebvre, et al., 1998], several studies have failed to demonstrate *MEST* mutations or aberrant methylation at the *MEST* ICR [Kobayashi, et al., 2001; Riesewijk, et al., 1998; Schoherr, et al., 2008]. A girl with SRS born after *in vitro* fertilization was recently reported partially hypermethylated (8 out of 31 investigated CpG sites) for the *MEST* ICR [Kagami, et al., 2007]. However, the finding of a similar hypermethylation in the phenotypically normal father renders the pathogenic relevance elusive [Kagami, et al., 2007]. The imprinted antisense RNA gene *MESTIT1* [Nakabayashi, et al., 2002] has also been evaluated as a SRS candidate gene through exon re-sequencing, but no mutations were identified [Meyer, et al., 2003]. Mutation analysis of the maternally expressed *KLF14* and *CPA4* genes have failed to identify any mutations in SRS patients [Kayashima, et al., 2003; Parker-Katiraee, et al., 2007].

One additional imprinted locus is found on chromosome 7q21.3, which contains the paternally expressed genes *PEG10* [Ono, et al., 2001] and *SGCE* [Grabowski, et al., 2003], the *PPP1R9A* gene that is maternally expressed in embryonic skeletal muscle and extra-embryonic tissues [Nakabayashi, et al., 2004] and the *TFPI2* gene that is maternally expressed in the placenta [Monk, et al., 2008]. Mutations in *SGCE* have been reported to cause myoclonus dystonia (OMIM# 159900) [Zimprich, et al., 2001]. A targeted deletion of the mouse *Peg10* was reported to cause an early embryonic lethal phenotype [Ono, et al., 2006]. These genes have not been extensively evaluated in SRS since no structural variation on chromosome 7 has specifically involved this region. In conclusion, the jury is still out as to what gene or genes cause the SRS growth restriction phenotype in matUPD7 patients and other patients carrying chromosome 7 specific rearrangements.

5.5 IMPRINTING ERRORS OF CHROMOSOME 11p15.5 IN SRS

The 11p15.5 region contains two imprinted regions, the *H19* ICR that regulates the maternally expressed *H19* RNA gene and the paternally expressed growth factor *IGF2*, and the *KCNQ1OT1* ICR, which regulates several imprinted genes including the maternally expressed growth inhibiting gene *CDKN1C* [Abu-Amero, et al., 2008]. Both imprinted regions had been implicated in BWS through mutations, methylation errors, and genomic rearrangements (Table 6) [Weksberg, et al., 2003], but little attention had been paid to this region in SRS until two maternal duplications of 11p15.5 were reported in a screen of 46 SRS patients [Eggermann, et al., 2005].

Three patients with maternal duplications of the region had been described previously, all with pre- and postnatal growth restriction, and although several dysmorphisms were suggestive, none had been diagnosed with SRS [Fisher, et al., 2002].

5.5.1 The *H19* ICR is frequently hypomethylated in SRS

These reports prompted Gicquel and colleagues to investigate the methylation status of the two imprinted regions in SRS patients and remarkably, five out of nine showed variable hypomethylation at the *H19* ICR [Gicquel, et al., 2005]. The hypomethylation represents a transition to a maternal (non-methylated) epigenetic state of the region, where the methylation sensitive factor CTCF can recognize the ICR, also on the paternal allele, and induce biparental *H19* expression and repression of *IGF2* (Figure 10). In the initial study, it was shown that the hypomethylation was concordant also at the *IGF2* secondary differentially methylated region 2 (DMR2) and the *H19* promoter (Figure 10) [Gicquel, et al., 2005]. Further the authors demonstrated a corresponding down-regulation of *IGF2* in fibroblast RNA using quantitative real-time PCR, and the expected bi-allelic *H19* expression was confirmed in leukocyte RNA using reverse transcription and PCR [Gicquel, et al., 2005]. Since the initial study, over seven studies have confirmed the association between *H19* ICR hypomethylation and SRS, and the frequency of the methylation defect is 20-65%, making it the most common molecular finding in SRS [Binder, et al., 2006; Bliek, et al., 2006; Eggermann, et al., 2006; Netchine, et al., 2007; Schonherr, et al., 2006; Yamazawa, et al., 2008b; Zeschnigk, et al., 2008]. An early study suggested that *H19* ICR hypomethylation was found in isolated asymmetry patients without SRS [Bliek, et al., 2006]. This has only been confirmed by one study [Zeschnigk, et al., 2008], while other studies have failed to identify *H19* ICR methylation defects in asymmetric SGA patients [Netchine, et al., 2007; Schonherr, et al., 2006]. Recently, it was shown that also the DMR0 was concordantly hypomethylated in SRS (Figure 10) [Murrell, et al., 2008].

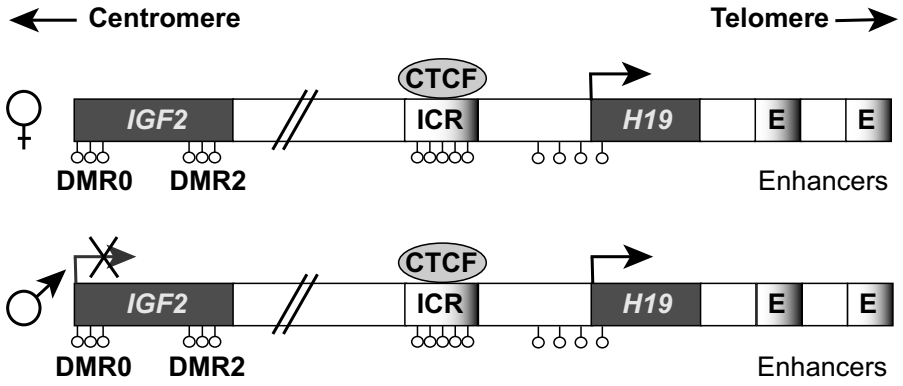

Figure 10 - *H19* ICR hypomethylation in SRS. In hypomethylated SRS patients, paternal methylation is lost at the *H19* ICR, resulting in a maternal expression profile (*IGF2* repressed and *H19* expressed).

5.5.2 No methylation defects at the *KCNQ1OT1* ICR but possible involvement in SRS pathogenesis

The methylation defects in SRS patients have only involved the *H19* ICR, while all six studies that have investigated *KCNQ1OT1* ICR found normal methylation levels [Binder, et al., 2006; Bliek, et al., 2006; Eggermann, et al., 2008b; Gicquel, et al., 2005; Yamazawa, et al., 2008b; Zeschnigk, et al., 2008]. However, the maternal duplications of 11p15.5 all spanned both imprinted regions, and recently a maternal duplication that specifically spans the *KCNQ1OT1* ICR was reported in SRS [Schonherr, et al., 2007b].

5.5.3 SRS and BWS

As the association between SRS and the 11p15.5 imprinted region has grown strong, its opposite nature to BWS is emerging. SRS and BWS are opposite in terms of phenotypes, where SRS presents with severe prenatal growth restriction and BWS with prenatal overgrowth (Table 6). Remarkably, the molecular findings are also opposite, 11p15.5 maternal duplications in SRS and 11p15.5 paternal duplications in BWS, hypomethylation of the *H19* ICR in SRS and hypermethylation in BWS [Weksberg, et al., 2003]. Recently, the first maternal UPD of chromosome 11 was described in an SRS patient [Bullman, et al., 2008]. This matUPD11 was segmental and mosaic (present in ~18% of cells), which is exactly opposite to the mosaic segmental patUPD11s that are found in around 20% of BWS patients [Weksberg, et al., 2003]. So far, the *KCNQ1OT1* ICR hypomethylation and *CDKN1C* mutations in BWS have not found counterparts in SRS [Obermann, et al., 2004]. Several monozygotic twin pairs that are discordant for BWS and consequently the *KCNQ1OT1* methylation defect have been described, with a majority of female twins [Weksberg, et al., 2003]. It has been proposed that the twinning and the methylation defect are causally related [Weksberg, et al., 2003]. Interestingly, two monozygotic twin pairs (both female) discordant for SRS and the *H19* ICR methylation defect have been described [Gicquel, et al., 2005; Yamazawa, et al., 2008a]. The opposite nature of the association between SRS and BWS is similar to that of Angelman and Prader-Willi syndromes (see 3.6.1 Human imprinting disorders).

5.5.4 A post-zygotic origin of the *H19* ICR methylation error

The abnormal *H19* ICR methylation percentages have ranged from 0 to 35 (depending on the cut-off for hypomethylation) in all studies, with relatively few patients showing complete lack of methylation. This means that only a proportion of the investigated cells have lost the paternal methylation mark and are unmethylated for both chromosomes. The mosaic nature of the imprinting defect suggests that it occurred post-zygotically, because if it was inherited from the father, all cells would show the defect. One study investigated *H19* ICR methylation in sperm from a father of a hypomethylated patient and demonstrated normal methylation [Zeschnigk, et al., 2008]. This is further supported by the observation that most SRS cases are sporadic, and there have as yet not been any reports of pedigrees with *H19* hypomethylation. The finding of monozygotic twins that are disconcordant for the methylation defects also speaks in favor of a post-zygotic error [Gicquel, et al., 2005; Yamazawa, et al., 2008a]. In BWS, an obscure association between haplotypes of the *IGF2* gene and *KCNQ1OT1* hypermethylation suggested that genetic background can influence either

BWS expressivity or predispose to methylation errors [Murrell, et al., 2004]. Since *IGF2* alleles have been reported to deviate in SRS as compared to controls [Obermann, et al., 2004], it would be interesting to correlate this to hypomethylation status.

5.5.5 Growth restriction and *H19* ICR hypomethylation

H19 ICR hypomethylation is the most precise molecular defect that is found in SRS but it is still unclear how the hypomethylation contributes to growth restriction. Different observations concerning fetal and postnatal growth and the *H19* ICR region will be discussed below.

5.5.5.1 Fetal growth restriction

SRS *H19* ICR hypomethylated patients have been reported more growth restricted at birth than those with normal methylation, but with a relative sparing of head growth [Binder, et al., 2008; Netchine, et al., 2007; Yamazawa, et al., 2008b]. This is in accordance with the bi-allelic expression of *IGF2* in human brain [Vu and Hoffman, 1994]. Interestingly, regression analysis has demonstrated a correlation between the degree of hypomethylation and the degree of growth restriction at birth and placental weight [Yamazawa, et al., 2008b]. This study also investigated *H19* ICR methylation status of the placentas of hypomethylated patients, and demonstrated a concordant placental hypomethylation [Yamazawa, et al., 2008b]. They further demonstrated the expected down-regulation of placental *IGF2*, while maintained mono-allelic expression of *H19* was unexpectedly observed in one hypomethylated placenta [Yamazawa, et al., 2008b]. Mice that carry a deletion of the placental-specific *Igf2* P0 transcript show marked intrauterine growth restriction [Constancia, et al., 2002]. Taken together, down-regulation of fetal and placental IGF-II expression is a likely candidate for causing prenatal growth restriction in hypomethylated SRS.

5.5.5.2 Postnatal growth restriction

An important feature of SRS is the absence of catch-up growth after birth, which is typical of other children born small for gestational age (SGA). Initial studies of hypomethylated SRS patients failed to show a distorted endocrine axis, with serum IGF-II levels in the high to normal range [Binder, et al., 2006; Netchine, et al., 2007; Yamazawa, et al., 2008b]. *IGF2* is known to be bi-allelically expressed from adult human liver, which is considered the major organ for production of IGF-II, and this finding was thus expected [Vu and Hoffman, 1994]. However, a recent study reported elevated serum IGF-I and IGFBP3 levels, suggestive of a compensatory mechanism for IGF-I insensitivity in hypomethylated patients, specifically [Binder, et al., 2008]. It is unclear how *H19* ICR hypomethylation would cause IGF-I insensitivity, but interestingly a murine study of *IGF2* loss of imprinting (over-expression) demonstrated enhanced sensitivity to IGF-II signaling [Kaneda, et al., 2007].

It is possible that IGF-II has autocrine or paracrine effects on postnatal growth, which could explain the persisting growth restriction in SRS. Interestingly, the human *IGF2* P0 transcript was recently described, and it both maintains imprinting and shows relatively abundant expression in adult tissues such as heart, muscle, and kidney [Monk, et al., 2006].

Little attention has been given to the possible involvement of the *H19* gene or the *H19* ICR itself in causing growth restriction. *H19* is a 2.5 kb RNA gene that consists of 5 exons and it is processed as an mRNA with capping, splicing and a polyA-tail [Gabory, et al., 2006]. The gene shows overlapping expression patterns with *IGF2* in fetal tissues, due to shared endo- and mesodermal enhancers (Figure 10) [Ohlsson, et al., 1994]. The overlapping expression patterns have prompted people to study potential regulatory effects of *H19* on *Igf2*, and a deletion of the *H19* gene affected *Igf2* imprinted expression, and further *H19* mRNA can bind IMP1, which normally binds *Igf2* mRNA [Gabory, et al., 2006]. *H19* was recently reported to contain a miRNA of unknown function, miR-675, which is well conserved between human and mouse [Cai and Cullen, 2007]. Furthermore, the *H19* ICR itself has been reported to function not only as an insulator element but also a mediator of contact with a plethora of other genomic regions, many of them imprinted [Zhao, et al., 2006]. A recent *in vitro* study reported expression of non-coding transcripts originating from the mouse *H19* ICR, which were required for *H19* silencing on the paternal allele [Schoenfelder, et al., 2007].

5.5.6 No global imprinting defect in SRS

One study has addressed DNA methylation in imprinted regions other than chromosome 11, where the authors studied the 6q24 transient neonatal diabetes imprinting control region (*HYMAI*/*PLAGL1* ICR) and the 14q32 *DLK1*/*MEG3* ICR in SRS patients with either matUPD7 or *H19* ICR methylation defects [Schonherr, et al., 2007a]. No additional imprinting defects were found. Isolated imprinting defects in the *MEST* ICR have also been excluded in 54 SRS patients [Schoherr, et al., 2008]. The *H19* ICR methylation status has also been evaluated in several matUPD7s patients and found to be normal [Binder, et al., 2006; Eggermann, et al., 2008b; Netchine, et al., 2007]. This supports the notion that matUPD7 and *H19* ICR hypomethylation are independently related to SRS.

5.5.7 Potential causes of hypomethylation

Although *H19* ICR hypomethylation seems to be caused after fertilization, no mechanisms for how this can happen have been proposed. Importantly, the *H19* ICR is rare in that it carries a paternal methylation mark, a feature only shared with the 14q32 *DLK1*/*MEG3* ICR [Edwards and Ferguson-Smith, 2007]. Below, potential genetic and environmental causes of the *H19* ICR hypomethylation are hypothesized and discussed.

5.5.7.1 A genetic cause of hypomethylation

Microdeletions of the *H19* ICR have been shown to cause hypermethylation in BWS patients upon maternal transmission [Sparago, et al., 2007]. The deletions remove important CTCF sites, which are required for keeping the maternal ICR in a non-methylated state (Figure 10). A similar mouse model has been created through deleting CTCF sites of the ICR, causing hypermethylation upon maternal transmission [Engel, et al., 2006]. An opposite model was created through introducing CpG mutations of the ICR while leaving the CTCF binding sites intact. This generated a model where maintenance of paternal methylation marks was disrupted and *H19* bi-allelic expression induced [Engel, et al., 2004]. Two studies

have screened for the reported microdeletions of the *H19* ICR in hypomethylated SRS patients without success [Bliek, et al., 2006; Gicquel, et al., 2005]. Further, *H19* ICR CTCF binding sites have been sequenced in a selection of hypomethylated SRS patients and all were normal [Bliek, et al., 2006; Yamazawa, et al., 2008b].

5.5.7.2 An environmental cause of hypomethylation

It is conceivable that the *H19* ICR methylation is environmentally caused since influences on DNA methylation are well established. If an increased rate of SRS and *H19* ICR hypomethylation following *in vitro* fertilization is established, environmental effects should be investigated. So far, there has been one report of a SRS patient who was conceived through *in vitro* fertilization and who also presented with *H19* ICR hypomethylation [Bliek, et al., 2006]. This possibility also throw new light upon the initial observation of complicated pregnancies in SRS [Russell, 1954]. Interestingly, exposure of mouse embryos to the carcinogen dioxin (2,3,7,8-TCDD, a byproduct of incomplete combustion) affected *H19* ICR methylation [Wu, et al., 2004], and furthermore administration of the estrogen receptor modulator tamoxifen to male mice caused *H19* ICR loss of methylation in spermatozoa [Pathak, et al., 2008]. A recent human study reported decreased methylation levels at the *IGF2* DMR2 (see Figure 10) in persons exposed to episodes of caloric restriction during early gestation [Heijmans, et al., 2008]. However, it would be difficult to conceive how relatively common environmental factors such as malnutrition might causally associate with a remarkably rare condition such as SRS. More rare adverse environmental effects, such as prenatal exposures to toxic compounds are very difficult to address in human and not even a well-designed prospective study would be feasible due to the rareness of SRS.

5.5.8 *H19* ICR hypomethylation an emerging clinical entity?

Several studies of *H19* ICR hypomethylation have suggested that it associates with a more classic/severe SRS phenotype. In terms of specific clinical findings there is agreement that hypomethylated children tend to be born smaller, but with relatively large heads, and that asymmetry is typical [Binder, et al., 2008; Gicquel, et al., 2005; Netchine, et al., 2007; Yamazawa, et al., 2008b]. Further detailed studies on larger materials and potential re-evaluations can aid in defining specific dysmorphisms and stigmata that are typical of *H19* ICR hypomethylation.

5.6 ADDITIONAL GENOMIC REGIONS IMPLICATED IN SRS

5.6.1 Chromosomes 15 and 17

Genomic rearrangements in SRS have been described for other regions of the genome, including primarily chromosomes 15 and 17. These findings in addition to other sporadic findings emphasize the genetically heterogeneous nature of SRS. Ring chromosomes 15 have been described in two patients with SRS, for one of whom a detailed molecular analysis revealed a hemizygous deletion of the *IGF1R* gene (15q26.3) [Tamura, et al., 1993; Wilson, et al., 1985]. Furthermore, a SRS patient with monosomy 15q26.3-qter in combination with trisomy 7q34-qter has been described [Kato, et al., 2001]. Several heterozygous deletions of the *IGF1R* gene have been described to date and these have all associated with severe pre- and postnatal growth restriction [Pinson, et al., 2005]. Further symptoms include a triangular face,

fifth finger clinodactyly, and micrognathia that are concordant with SRS, while atypical symptoms such as microcephaly and mental retardation are often observed [Pinson, et al., 2005]. Patients carrying heterozygous mutations in the *IGF1R* gene show phenotypes that are similar to the *IGF1R* deletion patients [Walenkamp, et al., 2008]. IGF1R is the receptor that binds and mediates the actions of IGF-I and IGF-II proteins and is thus an interesting candidate gene for SRS. However, *IGF1R* deletion screens and mutational analyses in SRS patients have failed to confirm *IGF1R* aberrations as a common cause of SRS [Abu-Amero, et al., 1997; Binder, et al., 2002; Rogan, et al., 1996].

Two balanced translocations with breakpoints mapping to 17q24-q25 have been described in SRS patients [Midro, et al., 1993; Ramirez-Duenas, et al., 1992]. The growth hormone cluster including *GH1*, and the placental growth hormone genes *GH2*, *CSH1*, and *CSH2* locate within this region, and the finding of *CSH1* deletions in three SRS patients suggested this as a candidate gene [Eggermann, et al., 1998; Prager, et al., 2003]. However, this deletion is also found in control populations, and the finding that the original *CSH1*-deletion carrier was also hypomethylated at the *H19* ICR has suggested that this gene is not casually related to SRS [Eggermann, et al., 2007b]. Other chromosome 17 genes that have been excluded as candidates for SRS include *GRB2*, *GRB7*, and *KPNA2* [Dorr, et al., 2001; Eggermann, et al., 2001; Hitchins, et al., 2002a].

5.6.2 Isolated chromosomal aberrations

Chromosomal aberrations that have occurred in isolated SRS cases include trisomy of 1q32.1-q42.1 [van Haelst, et al., 2002], duplication of 1q42-qter [Kennerknecht, et al., 1993], deletion of 8q11-q12 [Schinzel, et al., 1994], deletion of 13q22-q32 [Wahlstrom, et al., 1993], deletion of 18p [Christensen and Nielsen, 1978], 45, X mosaicism [Li, et al., 2004], and 47, XXY karyotype in two male SRS patients [Arico, et al., 1987]. Also patients with mosaic trisomy 18 have been diagnosed with SRS, although trisomy 18 is a distinct syndrome [Chauvel, et al., 1975; Punnett, et al., 1973].

5.6.3 Excluded candidate genes

Many single genes have been considered candidates for SRS, either based on their involvement in the IGF-pathway or skeletal growth (*IMP3*, *IRS1*, *PAX4*) or their imprinted status (*GNAS*, *IGF2*, *CDKN1C*, *KCNQIOT1*), but all sequencing efforts have failed to identify any causal mutations [Eggermann, et al., 2001; Eggermann, et al., 2007a; Kloos, et al., 2000; Mergenthaler, et al., 2000; Meyer, et al., 2005; Monk, et al., 2002a; Obermann, et al., 2004].

5.7 GENOMIC AND EPIGENETIC SCREENS OF SRS

Targeted screens have been primarily performed in SRS, and there have been no reports on genome-wide screens for submicroscopic events. Here I will describe the more large-scale efforts that have been performed. UPD for several chromosomes with frequent aneuploidies had been associated with intrauterine growth retardation. This prompted Kotzot and colleagues to screen for UPDs of chromosome 2, 6, 14, 16, 20, and 22 in ten SRS patients. No UPDs were found, thus underlining the specific

association between SRS and matUPD7 [Kotzot, et al., 2000]. Submicroscopic chromosomal aberrations in subtelomeric regions are a common cause of idiopathic mental retardation, often in combination with growth restriction and additional dysmorphisms. In order to evaluate whether growth restriction in SRS could be related to such subtelomeric aberrations, Eggermann and colleagues screened a cohort of 45 SRS patients and concluded that they all had normal subtelomeric regions [Eggermann, et al., 2008a].

X-chromosome inactivation (XCI) in females is a process that takes place in the inner cell mass of early embryo development at a stage where the cells are numerous enough for the process to be roughly random. Therefore, the expected proportion of inactivation of the maternal to paternal X is 50%, although some skewing is commonly observed [Bretherick, et al., 2005]. There has been one report of an increased frequency of skewed XCI in 29 non-matUPD7 SRS patients [Sharp, et al., 2001]. The authors argued that this might be evidence of trisomy 7 mosaicism in SRS, since fetuses with confined placental mosaicism (see 5.2.3 Pathogenic mechanisms of matUPD7) often show increased frequencies of skewed XCI [Lau, et al., 1997]. However, this is thought to relate to a reduced cell pool at the time of XCI, which could also be caused by slowly dividing cells [Lau, et al., 1997]. This might be the case in SRS. Another report demonstrated skewed XCI in mothers of non-matUPD7 SRS children, rather than their daughters [Beever, et al., 2003]. It is unclear what the skewed XCI in SRS mothers means, and further studies will be needed to evaluate possible underlying mechanisms, such as germ cell mosaicism, altered methylation patterns, or X-chromosomal mutations [Bretherick, et al., 2005].

6 METHODS TO STUDY THE HUMAN GENOME AND EPIGENOMES

6.1 METHODS TO STUDY HUMAN GENOME ARCHITECTURE

Studying the human genome and its chemical modifications is a daunting task. However, the completion of the human genome project [Lander, et al., 2001; Venter, et al., 2001], making the complete sequence readily available, and the HapMap [International HapMap Consortium, 2005] and ENCODE [Birney, et al., 2007] projects that have documented the majority of sequence variation between humans, have boosted development of new high-throughput technologies. Below I will discuss the nature of some technologies that are relevant to the topic of my thesis.

6.1.1 Methods to identify UPD

The inheritance of a chromosome pair from one parent only instead of one chromosome from each parent has been proven important in SRS through the discovery of matUPD7, discussed in Chapter 5. A UPD screen is usually performed in trios of two parents and a child, and employs PCR amplification of highly polymorphic microsatellites. Several microsatellites on the chromosome of interest in addition to control microsatellites on other chromosomes (to confirm maternity and paternity) are required. Fluorescently labeled PCR products are separated through capillary electrophoresis, and the microsatellite genotypes are used to determine whether the observed transmissions from parents to child follow Mendelian inheritance. Several concordant microsatellite results are needed to determine UPD, since microsatellites are known to have a higher mutation rate than SNPs [Banchs, et al., 1994]. In the case where parents are unavailable and UPD is suspected, DNA methylation based approaches can be used (see 6.2 Methods to study DNA methylation), provided that the relevant chromosome contains imprinted genes [Moore, et al., 2003]. UPD is suggested if the methylation patterns conform to a maternal-only or paternal-only methylation pattern. This approach cannot distinguish between germ line methylation defects and UPD. For some chromosomes, mosaic UPD patterns are observed, and this is a diagnostic challenge. The mosaic segmental matUPD11 recently described in SRS was identified through a slightly aberrant methylation pattern for both the *H19* and *KCNQ1OT1* ICRs, followed by failure to demonstrate any copy number variation for the region [Bullman, et al., 2008].

6.1.2 Methods to identify submicroscopic duplications or deletions

6.1.2.1 Genome-wide array-based hybridization methods

Array-based genomic methods often enable large-scale investigations of the genome and are thus typically used as screening methods. The technology makes use of the ability of single stranded nucleic acids to hybridize (pair) to their complementary sequence through Watson-Crick base-pairing. In brief, oligonucleotides of known sequences have been immobilized to a surface at a known position and dye-labeled, single-stranded target sequence will hybridize (Figure 11). The surface is interrogated by a laser exciting the hybridized dyes and the intensity of emitted light is recorded. The hybridization can be competitive, where two differently labeled targets are

hybridized to the same sequence and differences in dye-intensities determine the relative abundance of the targets (Figure 11B). The immobilization technique differs and oligonucleotides can be synthesized directly on the surface using photolithography, they can be attached to a bead which in turn is immobilized, or nucleic acids can be robotically spotted onto a slide and immobilized [Gresham, et al., 2008]. Array-based genomic methods can be further subdivided into those that target SNPs and those that only measure copy number or expression levels.

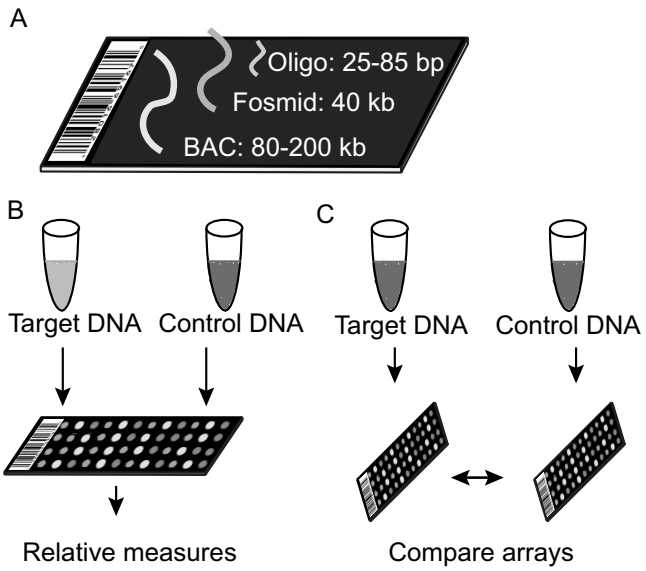

Figure 11 - Genome-wide array-based methods. A) Different types of probes (length and synthesis method) are immobilized on a solid surface. Shorter oligonucleotides are typically used for expression and genotyping arrays (C), while clones of fosmids or BACs are typically used in array-CGH (B). B) Array-comparative genome hybridization (array-CGH) with differential labeling of samples and hybridization to the same array. C) For genotyping arrays, one sample is hybridized per array, and relative intensity differences are subsequently inferred.

6.1.2.2 Array-based genotyping

The genotyping arrays have the advantage of producing combined results of genotypes and copy number estimates since also copy-number neutral variation such as UPD and extended homozygosity can be detected. A drawback with this technology is that it is limited to the sites where SNPs occur, although up to 1 million SNPs can be simultaneously assayed. Affymetrix offers an array-based genotyping technology with hybridization of whole-genome amplified and dye-labeled target DNA to oligonucleotides corresponding to the respective SNP alleles [Matsuzaki, et al., 2004]. Signal intensities of the oligonucleotides are compared with values from controls (often a group of individuals) to infer copy number (Figure 11C). Illumina offers a bead-based approach to genome-wide SNP genotyping, where the latest technologies include immobilized beads with 50-bp oligonucleotides that map adjacent to SNPs. The beads are used for hybridization capture of fragmented whole-genome amplified products, which can be assayed using a single-base extension

reaction followed by immunohistochemical fluorescence detection [Steemers, et al., 2006]. The intensity difference between the dyes can be used to infer both genotype and copy number [Peiffer, et al., 2006].

6.1.2.3 Array-comparative genome hybridization

Array-comparative genome hybridization (Array-CGH) can infer copy-number for all non-repetitive portions of the genome. The interrogating sequences are either oligonucleotides synthesized on a slide or printed bacterial artificial chromosome or fosmid clones with known sequences [Emanuel and Saitta, 2007]. A labeled target competes with a differentially labeled control sequence for hybridization and the relative difference in dye intensities is used to infer copy number (Figure 11B). The oligonucleotides are often in the range of 25-85 bp and can be used in a tiling manner to enable maximum coverage [Emanuel and Saitta, 2007]. The size of the bacterial artificial chromosome and fosmid clones is often much larger (80-200 kb and 40 kb, respectively), and thus their resolution is more limited, and they will tend to overestimate the size of the detected variants [Emanuel and Saitta, 2007; Kidd, et al., 2008]. Custom-made CGH arrays can be designed to meet the needs of specific research interests such as fine-mapping of deletion break-points in a genomic disorder.

6.1.3 Multiplex Ligation-dependent Probe Amplification

Multiplex ligation-dependent probe amplification (MLPA) can be used to interrogate up to 45 individual sequences for copy number variation in the same reaction [Slater, et al., 2003]. Two adjacent probes of a determined size are designed for each unique region. Universal primers (X and Y) are added to the probes and further a stuffer sequence (with unique length) is designed for each unique region and combined with primer Y. Following hybridization and ligation of the adjacent probes, a PCR step amplifying all ligated sequences is performed and the products (of different sizes due to the stuffer sequence) can be separated using capillary electrophoresis [Slater, et al., 2003]. Patients are compared to control samples, and missing or relatively larger peaks suggest a copy number variation. Additional probes are designed outside the region of interest to function as copy number controls. The method can be sensitive to sequence variation and thus the probes should be designed in regions without SNPs. A variant of this method can be used for measuring the extent of CpG methylation at specific loci (see 6.2.1 Methyl-sensitive restriction enzymes).

6.1.4 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) using the SYBR green method monitors the amount of double-stranded PCR product during each cycle of PCR extension [Giulietti, et al., 2001]. Alternatively, an amplicon-specific fluorescently labeled probe can be used to measure PCR product abundance [Giulietti, et al., 2001]. qRT-PCR is based on the notion that during the stages of exponential amplification, the amount of PCR product is proportional to the input amount of DNA into the reaction (Figure 12). The real-time monitoring enables an empirical estimate of the threshold where all targets are exponentially amplifying and the PCR-cycle at which this threshold is reached (C_t) can be compared in a relative fashion between targets (Figure 12). To control for differences in relative input, control amplicons, designed

in regions where copy number variants are not expected, can be used. This is known as the $\Delta\Delta C_t$-method [Livak and Schmittgen, 2001]. Although care should be taken in primer design and careful optimization of reaction efficiencies should be performed, this approach has been reported to successfully and reproducibly detect copy number variants [Weksberg, et al., 2005].

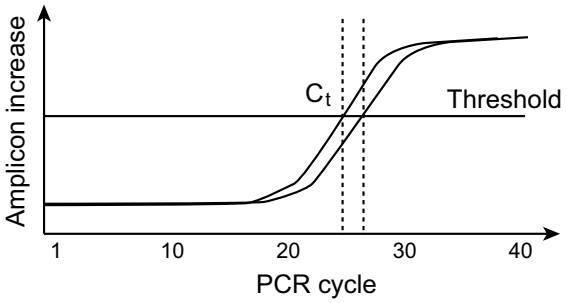

Figure 12 - Quantitative real-time PCR output. The amplicon increase rate during a PCR reaction is visualized for two input DNA concentrations (black and gray curves). The black solid line corresponds to the threshold where all reactions are simultaneously showing an exponential increase. The dashed lines correspond to the C_t-values of the respective input concentrations (i.e., cycles 26 and 27 of the PCR).

6.1.5 Sanger sequencing and massively parallel sequencing

The most widely used DNA sequencing approach is based on dideoxy terminated, primer directed, *in vitro* replication of single stranded DNA [Sanger, et al., 1977]. The method was further improved through the use of fluorescently labeled dideoxy nucleotides, enabling parallel termination reactions for all four nucleotides, followed by electrophoretic separation of products and determination of light intensities [Strauss, et al., 1986]. The main advantage is the long read lengths (up to 1000 bp), however the multiplex level is relatively low and specific amplicons are needed for each sequence. Recently, a new generation of massively parallel sequencing methods have become commercially available [Shendure and Ji, 2008]. These methods are based on universal PCR, mediated by ligated adaptor sequences to fragmented genomic DNA. The amplification products are clonal and either attached to a bead (454, SOLID) [Margulies, et al., 2005] or to a solid surface (Solexa) [Bentley, et al., 2008]. In the case where bead PCR is used, the beads are immobilized following amplification. Subsequently, sequencing-by-synthesis methods are applied to determine the underlying sequence at each position. The sequencing can be performed either with pyrosequencing (454) [Margulies, et al., 2005; Ronaghi, et al., 1996], sequential incorporation of fluorescently labeled nucleotides (Solexa) [Bentley, et al., 2008] or ligation of labeled oligonucleotides (SOLID) [Shendure, et al., 2005]. The available sequencing methods allow sequencing of up to gigabases in a single run and reduce the cost compared to Sanger sequencing by at least 10-fold [Shendure and Ji, 2008]. The disadvantages are shorter read lengths (35-400 bp, depending on method) and a lower accuracy per base, due to technical artifacts [Shendure and Ji, 2008]. Importantly, all the above-mentioned sequencing approaches are compatible with paired-end libraries, which can efficiently detect structural variation in the genome [Korbel, et al., 2007]. The development of new massively

parallel sequencing methods that do not require the initial amplification step are underway [Eid, et al., 2008; Harris, et al., 2008].

6.2 METHODS TO STUDY DNA METHYLATION

While the human genome is regarded as static in that all cells are expected to contain the same genetic material, the study of epigenetics should embrace the notion that there are as many epigenomes as there are cells. Since single-cell analysis is currently not feasible, a mix of different cells will be analyzed and therefore, epigenetic methods need to be highly quantitative. Epigenetic methods can be global, large-scale (but specific), or site-specific, all depending on the scope of the study. When imprinted genes are studied the regions are well-defined (ICRs, DMRs, and promoters), and site-specific methods are thus often sufficient. In general, human ICRs have been determined from bisulfite sequencing (see below) and within the defined region all CpG sites show a reproducible methylation pattern, with the methylation at one site predicting the state of the next site with high accuracy [Frevel, et al., 1999]. The study of DNA methylation is generally based on either of two basic phenomena; methyl-sensitive restriction endonuclease digestion or bisulfite conversion of DNA.

6.2.1 Methyl-sensitive restriction enzymes

Restriction endonucleases (RE) recognize and cut specific base combinations within a DNA sequence. Some REs are methyl sensitive in that they will not recognize the native sequence when it contains a methylated CpG. Examples are *Hpa*II that cuts its recognition sequence CCGG only if the internal CpG is unmethylated, and *Not*I that similarly only digests the first CpG of its recognition sequence GCGGCCGC if it is unmethylated. *Hpa*II has an isoschizomere, *Msp*I, which recognizes the same sequence but is methylation insensitive. A limitation with methyl-sensitive restriction is that only CpG-sites that occur within restriction sites can be interrogated. Further, if the digestion is not complete, the interpretation of the results will be biased. Classically, Southern blotting was used to visualize the restriction fragments of methylated versus non-methylated DNA, using a radiolabeled probe to determine relative methylation. MLPA (see 6.1.3 Multiplex ligation-dependent probe amplification) can be used in combination with methyl-sensitive restriction enzymes if probes are designed around a CpG-containing restriction site [Nygren, et al., 2005]. If the CpG-site is methylated it will remain intact and ligation and amplification will successfully occur, while an unmethylated site will be cut and no ligation and amplification will occur. Parallel MLPA reactions with and without methyl-sensitive restriction are compared to derive a quantitative estimate of the methylation percent [Nygren, et al., 2005]. Methylation-specific MLPA has been used by several groups to study methylation at the *H19* ICR, and has been suggested to have superior detection rates to Southern blotting [Eggermann, et al., 2008c; Zeschnigk, et al., 2008].

6.2.2 Bisulfite conversion

Sodium bisulfite has the property of inducing sulfonation and subsequent deamination of unmethylated cytosines in single-stranded DNA (Figure 13). Deamination will convert cytosine into a uracil, which will be interpreted as a

thymine in a subsequent PCR. The reaction takes place at high temperature and high pH and can consequently induce degradation of DNA [Fraga and Esteller, 2002]. Besides degradation, the main concern is completeness of conversion and therefore many methods include steps to control for this. The major advantage of bisulfite treatment is that it enables the study of all cytosines in the genome, which will only be limited by the downstream methods used [Fraga and Esteller, 2002].

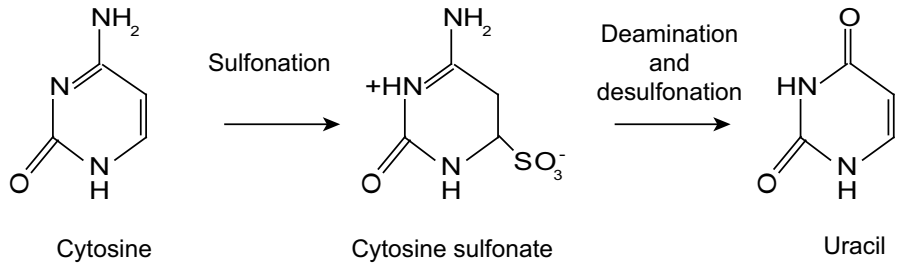

Figure 13 - Bisulfite conversion of unmethylated cytosines. Bisulfite induces sulfonation and subsequent deamination of unmethylated cytosines, resulting in a conversion to uracil.

6.2.2.1 Methylation-specific PCR

Since bisulfite treatment will introduce a mixed pool of sequences depending on the original methylation state of a CpG, PCR primers can be designed to detect specifically methylated or unmethylated CpGs, and the relative abundance of PCR products can be used to obtain an estimate of the methylation percent. This approach has been used for matUPD7 detection [Moore, et al., 2003]. However, most bisulfite-based methods employ PCR with primers that amplify both methylated and non-methylated targets. Often, the primers contain non-CpG cytosines to ensure that only bisulfite-converted DNA strands are amplified. Below I will describe some methods that are relevant to the study of imprinted genes.

6.2.2.2 Bisulfite sequencing

Bisulfite sequencing remains the most common method to study DNA methylation and it employs cloning of bisulfite converted DNA, followed by PCR amplification and Sanger sequencing [Fraga and Esteller, 2002]. The method enables a strand-specific analysis of methylation status at all CpGs within the amplicon. Several clones are sequenced to retrieve information from both chromosomes and different cells, however this method cannot be regarded as quantitative and biases involved in both cloning and PCR can affect its accuracy [Fraga and Esteller, 2002]. Pyrosequencing, employing sequencing by synthesis, has also been used on bisulfite treated DNA, where the relative abundance of C to T can be used to infer methylation percent [Tost and Gut, 2007].

6.2.2.3 Combined bisulfite restriction analysis

Bisulfite conversion can induce different restriction endonuclease patterns between methylated and unmethylated targets. In the Combined Bisulfite Restriction Analysis (COBRA) method, bisulfite-modified DNA is PCR amplified (non-CpG primers), followed by restriction digestion with *Bst*UI (RE recognition of methylated product only), gel separation, and quantification of the ratio of digested to undigested product

[Xiong and Laird, 1997]. This method is quantitative and has also been used to study *H19* ICR methylation in SRS [Yamazawa, et al., 2008b].

6.2.2.4 EPITYPER - mass-spectrometry-based methylation

Another quantitative method with a larger scope in terms of number of measured CpG-sites is EPITYPER. This method employs a PCR step after bisulfite treatment (minimum of 4 non-CpG Cs in primers) during which a T7 promoter sequence is incorporated into the PCR product. *In vitro* transcription follows the PCR and the generated RNAs are cleaved at non CpG-sites. The C to T difference has been converted to a G to A difference in the RNA, and the resulting fragments are analyzed using mass-spectrometry where the G to A sequence difference is detected due to a fragment mass difference and the relative abundance quantified [Ehrich, et al., 2005].

7 PRESENT INVESTIGATIONS

7.1 AIMS OF THE THESIS

The overall aim of this thesis was to study Silver-Russell syndrome (SRS) patients in order to gain an understanding of the genetic and epigenetic regulation of growth restriction. The aims were more specifically:

I To explore the use of genotyping arrays to diagnose uniparental disomy, maximize the potential resolution, and perform a detailed comparison of isodisomic and heterodisomic regions (**Paper I**).

II To perform a genome-wide screen in a set of SRS and growth restricted patients and their parents, in order to search for causal genomic variation, including sub-microscopic copy-number events and uniparental disomy (**Paper II**).

III To develop a method for detection of DNA methylation at CpGs in imprinted regions (**Paper III**).

IV To study CpG methylation at the *H19* ICR in SRS patients and growth restricted patients, with focus on epigenotype and phenotype correlations (**Paper IV**).

7.2 MATERIALS AND METHODS

7.2.1 Patients (Papers I-IV)

The patients included in this thesis were recruited from the endocrinological outpatient clinic at the Hospital for children and adolescents, Helsinki University, Finland. All patients have been evaluated by a pediatric endocrinologist. Moreover, all SRS patients have visited a geneticist and nearly all of them have had one or several follow-up examinations. Written consent was provided to take blood samples for extraction of genomic DNA from patients and from parents (when available). All patients included in the study have been screened for maternal uniparental disomy 7 (matUPD7), as previously described [Hannula, et al., 2002]. The study has been approved by the appropriate Ethical Review Boards at the University of Helsinki, Finland and Karolinska Institutet, Sweden (2005/750-31/1-4).

The diagnosis of SRS was based on the following criteria: **1)** Small for gestational age (SGA, birth length and/or weight $\leq$ 2.0 SDS for gestational age), **2)** postnatal growth retardation (height SDS below -2.5 at the age of 2 years), **3)** relative macrocephaly (head circumference at least > 1.5 SDS above the length SDS), **4)** a typical SRS face with at least three of the following facial characteristics: a triangular face, micrognathia (leading to a shark mouth and irregular teeth), prominent forehead, craniofacial dysproportion in early life, and **5)** at least one of the following relative criteria: asymmetry (limb length discrepancy and/or hemihypoplasia of skull, trunk, limbs), fifth finger clinodactyly and/or brachydactyly, low set/dysmorphic ears, syndactyly of the 2nd and 3rd toes, cryptorchidism, feeding difficulties, speech delay/difficulties, and excessive sweating.

SGA patients were included by the following criteria: **1)** SGA (birth length and/or weight ≤ 2.0 SDS for gestational age)**, 2)** postnatal short stature (height ≤ 2.5 SDS below the mean or expected midparental height), and **3)** no evidence of chronic illnesses known to stunt growth. Small groups of patients with familial short stature and subnormal growth hormone-secretion were accepted. Patients with an abnormal karyotype, metabolic disorders, and other growth disorders were excluded.

Paper I included 6 trios of matUPD7 patients and parents, **Paper II** included 28 trios of patients and parents (22 SRS and 6 SGA), **Paper III** included 40 normal-height parents of SGA children and 20 SRS patients, and **Paper IV** included 42 SRS and 90 SGA patients.

7.2.2 Affymetrix Genotyping Arrays (Papers I and II)

In **Paper I**, we used the Affymetrix 10K genotyping array (>10,000 SNPs) and in **Paper II**, the Affymetrix 250K *Sty* genotyping array (>250,000 SNPs). Both are based on the same principle as described below [Matsuzaki, et al., 2004]. Genome complexity is reduced through a restriction endonuclease digestion step (10K-*Xba* and 250K-*Sty*), followed by adapter ligation and universal PCR amplification of fragments in the 200-1000 bp range. PCR products are purified on columns, fragmented using DNase I, and 3' end-labeled with biotin. The samples are hybridized to the oligo-arrays together with human Cot-1 and herring sperm DNA (to reduce unspecific hybridization), followed by amplification of the signal through two rounds of biotin-streptavidin binding, and finally visualization through phycoerytrin conjugated streptavidin. The arrays include 20 matched (PM) and mismatched (MM) 25-mers for each SNP allele, which have been synthesized in quartets (PM and MM for each allele). The 250K *Sty* array has an average SNP spacing of 12.3 kb (median 5.1 kb), while the 10K array has an average and median SNP spacing of 104.0 and 209.8 kb, respectively [Matsuzaki, et al., 2004]. The relatively large differences between average and median SNP spacing reflects the uneven distribution of SNPs across the genome, resulting from the restriction fragments, gaps in the human genome sequence, repeat-rich regions, etc [Matsuzaki, et al., 2004].

7.2.3 Quantitative real-time PCR (Papers II, III, and IV)

Quantitative real-time PCR was used for both copy number variant confirmation in **Paper II** and for methylation percentage estimation in **Papers III-IV**. SYBR green technology (Applied Biosystems) was used in all studies, but since the Fast SYBR mix was used in **Paper II** and the Power SYBR mix used in **Papers III-IV**, the specific PCR amplification conditions differed (see papers). Dissociation curves were routinely performed to ensure specific amplification products and runs were manually inspected in the 7500 Fast System SDS software 1.3.1 (Applied Biosystems). The amount of products doubles in each cycle (assuming 100% PCR efficiency), which means that an obtained relative difference in C_t-values is on $\log_2$-scale. The $\Delta\Delta C_t$-method employs an initial subtraction of the target amplicon C_t with the control amplicon C_t, followed by a normalization step where the target-to-control difference is normalized between individuals [Livak and Schmittgen, 2001]. In order to achieve a relative copy number estimate, the $\log_2$ relative copy number is raised by the power

of 2. In **Papers III-IV** where methylation percent were measured, the target and control amplicons were the same apart from methyl-sensitive *Hpa*II digestion, and therefore the methylation percent was achieved through subtracting the non-digested C_t with the digested C_t, and converting from $\log_2$ to normal scale.

7.2.4 UPD analysis (Paper I)

In **Paper I** we used genotyping arrays to diagnose UPD through two alternative approaches. First we used PedCheck 1.1 to detect inconsistencies in Mendelian inheritance in the genotyped trios [O'Connell and Weeks, 1998]. We further evaluated enrichment of inconsistencies in Mendelian inheritance in chromosomal regions, assuming a random genome-wide distribution. Second, we searched for stretches of the genome that correspond to an isodisomic or heterodisomic genotype pattern in the trios. We defined potential isodisomic regions as consecutive homozygous genotypes in the child and heterodisomic regions as consecutive identical genotypes in child and parent. Only markers with a successful genotype for all individuals of the trio were included in the analysis. The enrichment of disomic regions was compared to the genomic background, i.e., all individual genotypes that would meet the disomy criteria and further binomial probabilities were used to assess significance.

7.2.5 Copy Number detection – data processing (Paper II)

When **Paper II** (Affymetrix 250K *Sty* genotyping) was initiated, excellent publicly available analysis tools had been developed as complement to those provided by Affymetrix. Several pre-processing steps are typically used when analyzing Affymetrix expression arrays, including subtraction of background signals and normalization of differences in the overall (and sometimes local) intensity of arrays [Gautier, et al., 2004]. Since the oligonucleotides for the genotyping arrays are of similar length and design, there is no reason why such analysis should not be performed, especially when relative copy number is inferred. Therefore, we used pre-processing steps as provided by the Aroma Affymetrix analysis routine (see Table 9) [Bengtsson, et al., 2008], implemented in the R statistical computing environment (see Table 9). These included allelic cross-talk hybridization (correcting for cross-hybridization between SNP alleles), quantile normalization (between all arrays), probe-level summarization (combining all the probes per SNP), and PCR fragment length normalization (corrects for differences in probe behavior originating from universal PCR length difference and probe GC-content) [Bengtsson, et al., 2008]. After this, normalized raw intensity values were obtained and the robust average across all samples was used to derive a relative $\log_2$ copy number for each individual [Venkatraman and Olshen, 2007].

7.2.6 Clinical score (Paper IV)

In **Paper IV**, we had performed group-based epigenotype and phenotype analysis, but wanted to develop this further to look at the sum of phenotypes in a specific patient and correlate this to the epigenotype (methylation percent at the *H19* ICR). Therefore, we created a clinical severity score, which corresponded to the percent of SRS phenotypes (derived from Table I, Paper IV) that was present in each patient. This gives a theoretical range between 0 and 100%, with 100% corresponding to the most severe phenotype.

7.3 RESULTS AND DISCUSSION

7.3.1 Paper I – UPD-screen

We devised a new approach to confirm UPD and to localize segments where transitions of UPD status occur.

Paper I was initiated to evaluate the use of the new SNP array genotyping technology for diagnosing UPD. At the time, UPD was typically detected through focused (specific chromosomes) microsatellite typing (see 6.1.1 Methods to identify UPD), and it was not obvious what a genome-wide analysis might reveal and if the lower information content of SNPs would suffice even if the marker density was increased on the genotyping arrays (chromosome 7: 585 SNPs on Affy10K versus typically 10-20 microsatellite markers). The observed inconsistencies in Mendelian inheritance derived with the Affy10K array reliably detected UPD, with 77% of all observed errors occurring on chromosome 7 and a significant enrichment to chromosome 7 in all six matUPD7 patients. However, the identification of Mendelian inheritance inconsistencies depends on SNP minor allele frequency and information content, and we observed an uneven distribution of errors across chromosome 7 that impaired the resolution (Figure 1 of the paper). Instead, by considering the expected genotype pattern of an isodisomic and heterodisomic UPD region, each SNP with genotypes available for the complete trio became informative and through statistical inference we could define the UPD regions at an increased resolution (Figure 1 of the paper).

With this increased resolution, we could look for common regions of isodisomy in the matUPD7 patients to evaluate the possibility of recessive alleles, as done previously at lower resolution [Preece, et al., 1999]. We confirmed that no isodisomic regions were shared between patients. Using both PedCheck and the genotype approach we reliably detected the segmental matUPD7, previously described by Hannula and colleagues [Hannula, et al., 2001b]. We further mapped the breakpoint to a 783 kb region, increasing the precision by 10-fold compared to the 8.7 Mb breakpoint region previously mapped by microsatellites [Hannula, et al., 2001b]. The genome-wide screening of matUPD7 patients also allowed us to conclude that all other chromosomes showed normal, biparental inheritance.

A drawback of genotyping array detection of UPD is that it is likely to miss mosaic UPDs, as was recently described for chromosome 11p in a SRS patient [Bullman, et al., 2008]. This segmental UPD was present in only 18% of the patient's blood cells and was first detected through a methylation assay and confirmed with microsatellite markers. At higher levels of mosaicism it is still possible that a mosaic UPD could be detected through the discrepancy between detection rate and call rate as has been observed for mixed samples [Matsuzaki, et al., 2004]. We further noted that the identified isodisomic regions are indistinguishable from heterozygous deletions. However, with more advanced genotyping arrays such as Affymetrix 250K *Sty* used in **Paper II**, copy number can be inferred, overcoming this issue.

7.3.2 Paper II – Genomic screening of SRS

We demonstrated the power of using genome-wide genotyping arrays in rare disorders such as SRS where UPD, copy number variants, or shared homozygosity might occur.

Paper II was designed to enable a genome-wide assessment of the genome architecture in SRS and primordial growth restriction. In more detail, we wanted to see if molecularly undiagnosed SRS and SGA patients (normal *H19* ICR and no matUPD7) carried submicroscopic copy number variants or potential segmental UPDs. By including 10 SRS patients with confirmed *H19* ICR hypomethylation (**Paper IV**) we wanted to evaluate a genetic cause of the methylation defect.

Taken together, we found large genomic events with pathogenic potential in twenty-five percent of molecularly unexplained SRS patients (3 out of 12). These included a heterozygous deletion of the *IGF1R* gene (2.6 Mb), an atypical distal 22q11.2 deletion (1.1 Mb), and a pseudoautosomal Xp22.33 duplication (2.7 Mb) in a male patient (Table 3 and Figure 1 of the paper). The *IGF1R* deletion (15q26.3) did not include the telomere and is the smallest deletion described to date that spans *IGF1R*. [Pinson, et al., 2005; Poot, et al., 2007; Walenkamp, et al., 2008]. Our patient shared the most typical symptoms of other patients with 15q26 deletions that include the *IGF1R*, such as intrauterine and postnatal growth restriction, a triangular face, and micrognathia, while she did not have microcephaly and mental retardation, which has been described in most *IGF1R* deletion patients [Pinson, et al., 2005]. In a female SRS patient we further identified a 22q11.21-q11.22 deletion (1.1 Mb) that overlaps with the 22q11.2 distal deletion syndrome (OMIM# 611867) with symptoms distinct from DiGeorge (OMIM# 192430) [Ben-Shachar, et al., 2008]. Interestingly, many symptoms are shared between our patient and those described by Ben-Shachar *et al.*, including pre- and postnatal growth restriction, clino- and brachydactyly, cox valga, and congenital bilateral hip displacement [Ben-Shachar, et al., 2008]. However, the skeletal phenotypes were only described in single 22q11.1 deletion patients, while all clino- and brachydactyly, a triangular face, micrognathia, and low set ears were noted in our patient. The finding of an Xp22.33 duplication in a male SRS patient with growth restriction, hemihypoplasia, and a typical SRS face was unexpected. However, two male SRS patients with a 47, XXY karyotype have been described previously [Arico, et al., 1987]. We failed to identify any major genomic events in *H19* ICR hypomethylated SRS patients or unexplained SGA patients. Our findings suggest that the stigmata of SRS, including facial and skeletal dysmorphisms are suggestive of an underlying genomic cause, which does not seem to be the case for isolated short stature.

By using genotyping arrays, we could further search for regions of extended homozygosity (by searching for loss-of-heterozygosity, LOH) that could suggest UPD or regions inherited identical-by-descent, revealing possible recessive disease alleles. We identified seven LOH regions in five different patients, of which three did not span a centromere (Table 4 of the paper). Two patients carried two independent LOH regions, suggesting potential cryptic relatedness between parents. None of the LOH regions contained an increase in Mendel errors, and the sizes are not

appreciably larger than has been reported in e.g., the HapMap population [Gibson, et al., 2006]. The LOH-region on chromosome 8q21-q22 (19 Mb, in a SGA patient) that has previously been implicated in regulation of stature [Perola, et al., 2007] and the LOH on 13q31-13q32 (6 Mb, in a molecularly unexplained SRS patient) that spans the interesting candidate genes *GPC5* and *GPC6* [Saunders, et al., 1997], warrant further study in larger sample sets.

Importantly, we did not identify any duplications or UPD of chromosomes 7 or 11 in our patient cohort, in contrast to results reported previously for SRS [Abu-Amero, et al., 2008]. Our findings of aberrations in genomic regions that were unexpected or rarely associated with SRS emphasize that SRS is surprisingly heterogeneous in molecular etiology beyond the major groups of *H19* ICR hypomethylation and matUPD7. All the identified aberrations were submicroscopic in size and thus genomic screens using array-based methods should be undertaken also in patients with normal karyotypes. Taken together, our results suggest that targeted screening of previous candidate regions in unexplained SRS is insufficient, and instead genome-wide screens should be undertaken to understand the underlying cause of idiopathic SRS.

7.3.3 Paper III – Methylation detection at imprinted genes

We proposed a simple method for quantification of methylation and demonstrated its accuracy and quantitative nature.

In **Paper III** we developed a site-specific methylation analysis approach that combines methylation-sensitive restriction enzyme digestion with subsequent quantitative real-time PCR (qRT-PCR) analysis of four previously characterized ICRs on chromosomes 7 (*GRB10* and *MEST*) and 11 (*H19* and *KCNQ1OT1*). Southern blotting had been used in the initial reports of *H19* ICR hypomethylation in SRS [Bliek, et al., 2006; Eggermann, et al., 2006; Gicquel, et al., 2005; Schonherr, et al., 2006], which prompted us to develop a method to screen for hypomethylation that was quicker, more flexible, and required less DNA. Quantitative real-time PCR is a frequently used method with a wide linear range, and we could demonstrate quantitative recovery across the entire methylation range using linear regression analysis (Figure 1 of the paper). We ensured digestion efficiency by using excess amounts of enzyme and overnight digestion and monitored efficiency by adding unmethylated λ DNA in the digestion and control reactions to measure dosage differences. High precision of replicate measurements was demonstrated with mean SDs ranging between 0.08-0.15 C_t (Figure 2 of the paper).

To demonstrate the extremes of the methylation range, we studied matUPD7 and patUPD7 individuals at imprinted loci on chromosome 7, which were distinct from the controls and consistent with the expected methylation patterns (Table 2 of the paper). These results suggest that the method can also be used for quick matUPD7 screening in SRS patients.

We found methylation percentage of control individuals to be normally distributed at imprinted loci (Table 2 and Figure 3 of the paper). The normal distribution of the data and the magnitudes of the SDs show that methylation can vary between individuals,

but only to a certain extent, as is suggested by the range of the measurements (Figure 3 of the paper). The normality further supports the use of mean±SD as cut-offs for abnormal methylation values, as was demonstrated in SRS patients for the *H19* ICR (Figure 4 of the paper).

We investigated loci of particular importance for the congenital syndromes SRS and BWS, but the method could potentially be applied to other imprinted regions through the design of additional amplicons. A limitation is the frequency of restriction sites, the high GC-content, and potential repeat regions associated with imprinting control regions. These obstacles can however be overcome by considering different methyl-sensitive restriction enzymes and careful design and optimization of the PCR assays. In summary, we showed that our method is a simple, efficient, and quantitative means for studying methylation, with the potential for parallel investigations of several imprinted loci.

7.3.4 Paper IV – *H19* ICR methylation correlates to SRS clinical phenotypes

We found a dose-response relationship between the degree of *H19* ICR hypomethylation and phenotype severity in SRS and report for the first time the association of specific anomalies of the spine, elbows, hands and feet, and genital defects in SRS with severe *H19* ICR hypomethylation.

Several of the studies of *H19* ICR hypomethylation had suggested that it associates with a more classic/severe SRS phenotype including intrauterine growth restriction with sparing of head size and asymmetry [Binder, et al., 2008; Gicquel, et al., 2005]. In **Paper IV** we used the method described in **Paper III** to investigate methylation status of the *H19* and *KCNQ1OT1* ICRs in 42 SRS patients (including seven matUPD7 patients) and 90 SGA children. We performed a detailed (epi)genotype-phenotype analysis with special attention paid to skeletal and genital defects, dysmorphic features, and growth failure.

H19 ICR hypomethylation was found in 62% of SRS patients but in no SGA children. We confirmed the results from other groups that hypomethylation related to a more severe SRS phenotype, where especially asymmetry and micrognathia were significantly more common (Table 1 of the paper). Interestingly, we could demonstrate a dose-response relationship between the degree of *H19* ICR hypomethylation and phenotype severity in SRS (Figure 1 of the paper).

Re-examination of hypomethylated SRS patients revealed specific skeletal anomalies including abnormally high lumbar vertebrae, lumbar hypomobility, elbow subluxations and distinct hand and foot anomalies (Table 2 of the paper). Interestingly, the skeletal anomalies grew milder as the methylation level increased. The extremely hypomethylated patients (<9%) presented with congenital aplasia of the uterus and upper vagina, equivalent to the Mayer-Rokitansky-Küster-Hauser syndrome (MRKH, OMIM# 277000) in two out of three females and cryptorchidism and testicular agenesis in four out of five males. Interestingly, MRKH has previously been reported in SRS [Abraham, et al., 2004; Bellver-Pradas, et al., 2001] and the spectrum of shared genital, skeletal, and renal malformations encountered in both

syndromes suggests a developmental field defect of closely related mesodermal derivatives [Oppelt, et al., 2006].

None of the matUPD7 patients carried additional methylation defects. Classic SRS features were associated with *H19* hypomethylation and a milder phenotype, with short stature and SRS-like features, was associated with matUPD7, distinguishing two separate clinical and etiological SRS subgroups (Table 3 of the paper). We conclude that *H19* ICR methylation is clinically important as demonstrated by a strong correlation between the degree of hypomethylation and SRS phenotype severity. The described dose-response relationship suggests that the degree of hypomethylation in blood is likely a reflection of how early in development the imprinting error occurred, contributing to varying presentation in distinct tissues.

7.4 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

7.4.1 Diagnosing molecularly unexplained SRS through genomic screens

In **Papers I and II** of this thesis we demonstrated the utility of genotyping arrays for understanding the molecular etiology of unexplained SRS, allowing identification of all UPD, copy number, and potential recessive disease alleles. Our findings in **Paper II** of genomic aberrations on chromosomes 15, 22, and X in SRS emphasize that unbiased, genome-wide screens are warranted. Remarkably, a diagnosis of SRS was compatible with a submicroscopic genomic cause in 25% of molecularly unexplained cases.

It is important to emphasize that genomic screens are limited by their inherent resolution. Copy number aberrations can occur through many different processes (see 1.7.3 Mechanisms creating structural variants) and some of them do not have a minimum size, but it is simply the functional units that will determine the minimum size of a disease-causing submicroscopic deletion or duplication. Therefore, it is not unlikely that genomic causes will be identified for most unexplained SRS patients if more detailed genomic screens are performed. In terms of UPD, the genotyping arrays used in this thesis are likely to be sufficient since segmental UPDs are caused by recombination and have limited minimal sizes. Massively parallel sequencing [Bentley, et al., 2008; Margulies, et al., 2005] and emerging single molecule sequencing approaches [Harris, et al., 2008] have great potential for exhaustive evaluation of genome copy number in SRS (also in relation to mosaicism).

Provided that the number of bases per experiment will increase alongside with a decrease in cost per base [Shendure and Ji, 2008], whole genome re-sequencing (with sufficient coverage) of SRS patients could be performed on a routine basis in the future. This would also provide the possibility of identifying causal mutations in SRS. It is feasible that previous mutational screens of SRS candidate genes have missed pathogenic mutations, for example, if they were located in alternative exons or important non-coding regions. Single gene mutations are unlikely to be a common cause of SRS, considering the high combined frequency of matUPD7 and *H19* ICR

hypomethylation, but mutations in single patients could still give important new insight into SRS etiology.

7.4.2 How will the cause of *H19* ICR hypomethylation be identified?

Clearly, the identification of a cause of the *H19* ICR hypomethylation in SRS is a teasing topic for SRS researchers. In **Paper II**, we studied 10 hypomethylated SRS patients in order to investigate potential causative genomic aberrations. We failed to identify any such at the resolution of the array. However, the argued post-zygotic origin of the methylation defect (see 5.5.4 A post-zygotic origin of the *H19* ICR methylation error) would be compatible with a somatic genomic rearrangement, which would also be mosaic (like the methylation defect). The 10-fold higher mutation rate of copy number variants over single-base mutations [Lupski, 2007] and the given notion that some genomic regions are prone to recurring rearrangement [Lupski, 2007] would speak in favor of a gene-dose effect as the cause.

Again, it is possible that genomic screens with increased resolution or a targeted candidate region approach might reveal a genetic basis of *H19* ICR hypomethylation. By focusing investigations on those patients with the most extreme hypomethylation (lowest level of mosaicism), the risk of missing important findings due to their mosaic nature will be minimized. Interesting regions to target would be the 11p15.5 imprinted region itself, but also methylation machinery members with a documented involvement in imprinting at the *H19* ICR. One such example is *MBD3*, since targeted knockdown in murine blastocysts revealed a specific loss of methylation at the *H19* ICR [Reese, et al., 2007]. If this gene is disrupted through genomic rearrangement during early development, and its function is conserved between human and mice, this could cause the hypomethylation observed in SRS.

7.4.3 Careful molecular and clinical evaluation of SRS to understand etiology

In **Paper IV** we demonstrated the utility of clinical re-evaluation in SRS, and report specific skeletal and genital defects associated with *H19* ICR hypomethylation. Our data emphasized that reproductive organ development might be affected in SRS, especially in combination with *H19* ICR hypomethylation. Bliek and colleagues also reported female reproductive organ defects in two hypomethylated patients [Bliek, et al., 2006], while all other studies have failed to mention such associations. The male versus female anatomy introduces a bias, where cryptorchidism in male SRS is a phenotype typically identified at birth or early infancy, while female reproductive organ defects might go unnoticed until investigations for amenorrhea.

Our detailed analysis delineated *H19* ICR hypomethylated SRS and matUPD7 patients as distinct clinical subgroups, with more severe intrauterine growth restriction, a classic SRS face, and asymmetry in *H19* ICR hypomethylation while the matUPD7 phenotype appeared milder but with increased frequency of feeding difficulties and speech delay. Interestingly, Binder and colleagues studied the endocrine and auxological phenotype of SRS in relation to the molecular subgroups and found this to differ substantially between *H19* ICR hypomethylation and matUPD7 [Binder, et al., 2008]. The authors found that the hypomethylated patients

presented with abnormally high levels of serum IGF-I and IGFBP3, indicative of IGF-I resistance, while the matUPD7 patients showed similar levels to SGA children of corresponding ages [Binder, et al., 2008]. Further, matUPD7 patients were reported to show less intrauterine growth restriction, while a marked catch-down growth was demonstrated during infancy, so that the growth restriction was comparable to *H19* ICR hypomethylation in childhood [Binder, et al., 2008].

Taken together, these results suggest that the molecular subgroups of SRS differ in presentation and etiology. The different clinical features and endocrine and auxological profiles related to *H19* ICR hypomethylation and matUPD7 complicate the etiological picture of SRS further, and efforts to identify a causal gene on chromosome 7 for the matUPD7 phenotype do not necessarily have to focus on the IGF pathway. Interestingly, the molecularly unexplained SRS patients showed an intermediate endocrine profile with indications of IGF-I resistance [Binder, et al., 2008]. This suggested that they were different from SGA and that the IGF pathway is possibly involved in the etiology and therefore molecular causes should be sought.

In **Paper II** we identified three SRS patients with pathogenic genomic aberrations in chromosomes other than 7 and 11. Two of these aberrations are not typically associated with a diagnosis of SRS, with *IGF1R* deletions forming a distinct clinical entity [Pinson, et al., 2005] and 22q11.2 distal deletions constituting a recognized syndrome (OMIM# 192430) [Ben-Shachar, et al., 2008]. It might be argued that our patients should not be regarded as SRS anymore when unexpected molecular causes have been identified. Logically, any disease entity with a different genetic cause should then be delineated as a different disease. However, matUPD7 patients are diagnosed with SRS although they constitute a distinct entity both in terms of clinical symptoms and endocrinological profile [Binder, et al., 2008; Hannula, et al., 2001a]. Furthermore, making a diagnosis based on molecular findings might also be an oversimplification, since a genomic screen is seldom exhaustive, and we do not know whether these patients carry mutations or genetic variants in unknown modifying genes that make their symptoms more compatible with SRS. Careful evaluation of the endocrinological profile of SRS patients with atypical genomic aberrations as performed by Binder and colleagues [Binder, et al., 2008] might give new insights. As more molecular aberrations in SRS patients will be found, similarities in both genetic etiology and phenotypes with other syndromes that were previously regarded as unrelated will likely emerge.

Table 9 – Web addresses for Chapter 7.

Database	Web address
Aroma Affymetrix	groups.google.com/group/aroma-affymetrix/
R statistical computing environment	www.r-project.org

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