Sveriges lantbruksuniversitet
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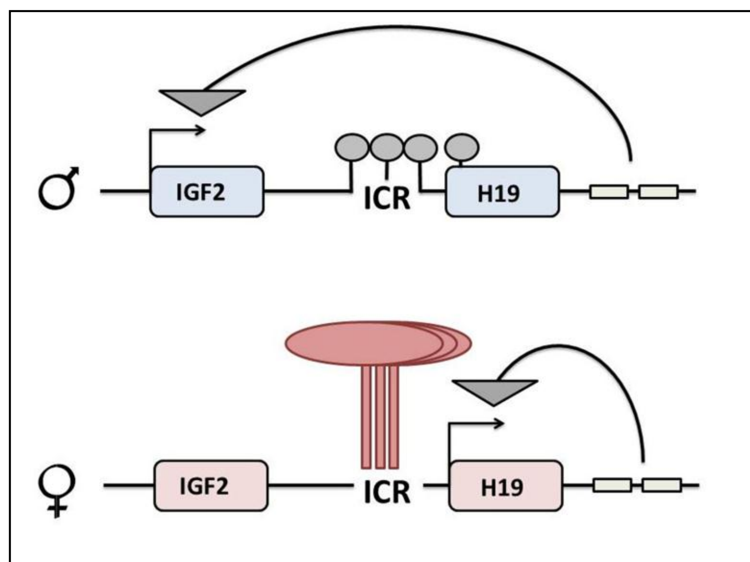
*Matilda Nordin*

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# Epigenetic regulation of the Igf2/H19 gene cluster

Den epigenetiska regleringen av genklustret innehållande Igf2 och H19

*Matilda Nordin*

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## SAMMANFATTNING

Igf2 (insulin-lik tillväxtfaktor 2) och H19 är präglade gener hos däggdjur. Präglade gener uttrycks ojämnt; den allel som ärvts från den ena föräldern uttrycks mer än den från den andra. Igf2-genen kodar för en viktig embryonal tillväxtfaktor och uttrycks till protein bara från den paternella allelen. H19 transkriberas bara från den maternella allelen, men translateras inte till protein. Igf2 spelar en viktig roll under dräktigheten, då den bidrar till tillväxten av både placenta och foster och reglerar såväl näringstillgång och tillväxthastighet för embryot.

Enligt en teori kallad ’the genetic conflict theory’ (~konflikten mellan genomen) så ligger det i honans intresse att fördela resurserna jämnt mellan alla syskon. Eftersom en kull kan bestå av embryon med olika fäder, så ligger det i faderns intresse att gynna sin egen avkomma. Därför kommer den paternellt nedärvda delen av embryo-genomet vilja tävla med de andra syskonen om näringstillgången i livmodern. Det embryot som utrustats med den starkaste paternellt nedärvda igf2-genen utvecklar störst placenta och får mest näring. Det finns dock en risk med detta eftersom modern löper större risk om fostren växer sig alltför stora och dessutom har ett eget intresse att propagera sina egna gener i så många fullt friska foster som möjligt. Därför finns en antagonistisk mekanism i form av Igf2-receptorn som har till uppgift att binda, internalisera och bryta ned Igf2. Genen för Igf2-receptorn är omvänt präglad, dvs uttrycks bara från den maternella allelen. Dessa fynd har lett fram till en populär teori som kallas kriget mellan genomen. Det här arbetet innefattar en modifiering av teorin, som beskriver hur det också skulle kunna ligga i moderns intresse att gynna avkommor med stark Igf2 eftersom hanungar med stark Igf2 kommer vara mer effektiva spridare av hennes gener.

Arbetet innefattar en översikt av artiklar som beskriver den epigenetiska regleringen av Igf2/H19-klustret som leder till ett präglat uttryck av generna. Studier rörande viktiga DNA-sekvenser och epigenetiska modifieringar är utvalda för att förklara betydelsen för präglingen. Metylerings- och kromatin- mönster skiljer sig åt beroende på om allelen är maternellt eller paternellt nedärvd och dessa mönster beskrivs. Isolerande protein (CTCF-proteiner) binder till specifika DNA-sekvenser och har betydels för präglingen. Den tredimensionella uppdelningen av kromosomerna kan också vara avgörande. Det är klarlagt att vissa DNA-sekvenser är oundgängliga för präglingen, och hela klustrets organisation är väl bevarat genom lång tid av evolution. Präglingen fungerar inte utan korrekt ordning av gener och viktiga DNA-element såsom gemensamma ’enhancers’ (DNA-sekvenser med transkriberings-förstärkande effekt) och DNA-sekvenser som binder viktiga isolerande proteiner (CTCF-proteiner). Utan korrekt ordning av de olika DNA-komponenterna fungerar inte präglingen.

I “The enhancer competition’-modellen tävlar generna om tillgång till de gemensamma DNA-sekvenser (enhancers) som behövs för genuttryck. ‘The boundary’-modellen stipulerar att bindning av CTCF-proteiner isolerar den maternella Igf2-allelen från enhancer-sekvenserna. ‘The chromatin-loop’-modellen beskriver en tredimentionell olikhet som förklarar varför enhancer-sekvenser verkar på respektive gen på de två allelerna. Alla nämda teorier vill förklara präglingen, men trots att de är relativt olika så bygger alla på skillnader i metylering

som springande punkt som bygger på antagandet att enbart den paternella H19-promotorn och en viktig sekvens belägen ~2-4kb uppströms om H19, är metylerad vid korrekt prägling. En nyligen publicerad studie har dock funnit motsägande data; bialleliskt uttryck av Igf2 men korrekt metylering enbart på den paternella allelen. Olikheter i metyleringen är dock hitintills den oftast använda förklaringsmodellen, och flera tänkbara sätt på vilket dessa olikheter kan upprättas finns kortfattat förklarade.


## ABSTRACT

The Igf2- (insulin-like growth factor 2) and H19-genes are imprinted in mammals; they are expressed unevenly from the two parental alleles. The Igf2 is a growth factor expressed in normal tissues solely from the paternal allele. The H19-gene is transcribed (but not translated to a protein) from the maternal allele. The Igf2-protein is a growth factor particularly important during pregnancy, where its growth inducing effect via the placenta affect its embryo-carrier.

'The genetic conflict theory' postulated that it is in the maternal interest to evenly supply all individuals in a litter, while the paternally derived genome in an embryo compete with its siblings for the resources. The paternal-specific expression of the growth factor Igf2 is in accordance with this. The embryo with the most resource-demanding Igf2 gets the most nutrients via the placenta. The down-regulation of the maternally derived Igf2 and the maternal-specific expression of a receptor (the IGF2-receptor) whose only reaction to ligand-binding is neutralization of the IGF2 is the maternal answer in what has been described as 'the war of the genomes'. This article presents a modification of this theory that propose maternal interest in the unequally distribution of resources to offspring, since male offspring with effective recource-demanding Igf2 will be more effective in spreading her genes.

This article review articles concerning the epigenetic regulation of the Igf2/ H19 gene-cluster that leads to parent-specific expression. Selected studies concerning the DNA-sequences and epigenetic modifications that contribute to the imprint is included. Parental-specific methylation and chromatin patterns, DNA-binding of insulator-proteins (CTCFs) and the three-dimensional partitioning of DNA in the nucleus are epigenetic features described in included models. Certain DNA-sites are essential for the imprint. The strong conservation of the organization of the genes and other DNA-sequences like the shared common enhancers and CTCF- binding-sites is necessary; without the correct order the imprint is lost.

'The enhancer competition model', 'the boundary model', 'the chromatin-loop model' are three models based on differential methylation as the epigenetic mark responsible for the imprinted expression pattern. A recent study contradicts the up until now accepted fact that biallelic expression is accompanied with loss of the differential methylation-pattern. Still, the methylation is the common explanatory event that leads to the parental differences in gene expression. A few possible pathways, ending up in the methylation differences, are included in a summarized fashion.

## INTRODUCTION

The aim of this article is to review and discuss the imprinted gene complex Igf2/H19. An imprinted gene differs from unimprinted counterparts since it is not expressed equally from the parentally inherited alleles; one allele is transcribed more than the other. The Igf2 and H19-genes are imprinted in a reciprocal way; paternal-exclusive expression of Igf2 and maternal-exclusive transcription of H19. Studies that clarify how the cluster is operating are reviewed, partly conflicting theories will be presented as well as areas where further research is necessary.

### Basics about genetics and epigenetics

Genes are sequences on the DNA-molecules (chromosomes) coding for functional RNA-sequences or, via RNA, expressing proteins. An allele is a gene that exists in different forms in a population. Alleles and genes, exist in eukaryotic cells as doublets, where one allele is inherited from the mother and the other from the father. A promoter is the DNA-sequence that can bind the transcription factors necessary for transcription. The basic heredity is accompanied by epigenetic changes that do not affect the DNA-sequence itself, but by otherwise modify the genome change the way the DNA is transcribed and expressed. Epigenetic modifications of the DNA and nearby structures turn genes on and off, and thus provides the basic prerequisite for the differentiation of a pluripotent stem-cell into the different cells in a multicellular creature.

### Imprinted genes and ‘the genetic conflict theory’

In the 70s a theory appeared that proposed a conflict between the different interests amongst ‘fathers’, ‘mothers’ and ‘siblings’ within the endosperm of flowering plants, a tissue analogous to mammalian placenta. The theory postulated that ‘siblings’, especially when derived from different ‘fathers’, compete with each other about the ‘mothers’ resources, while the ‘mother’ wants to give equally to all ‘siblings’ since they are equally related to her.
(Smith & Fretwell, 1974)

In 1984 the discovery that mammalian one-cell-embryo needs one pro-nucleus from each parent made it clear that there is some functional distinction between paternal and maternal chromosomes. The zygote cannot function with two maternal or two paternal sets of chromosomes. This was contradictory to the Mendelian law stating that genes are passed on independently of each other. (Surani *et al*, 1984).

Haig & Westoby (1989) summarized these ideas about the conflict of different interests in flowers and presented a model describing how evolution could favour a new, hypothetical allele that when derived from the paternal half of the genome acquires extra resources to its embryo-carrier. They also hypothesized that the evolutionary response in the maternal allele would be to silence it, in an attempt to forestall fetal overgrowth. It is applicable to mammals since both flowering plants and mammals start life as ‘parasites’ on their mother. Avian

embryos, on the other hand, cannot count on extra resources from the mother, since the energy-content in the egg is predetermined.

DeChiara *et al* (1991) studied mice with Igf2-gene-deletions. The Igf2-protein (insulin-like growth factor 2) was well characterized and known to induce growth and proliferation *in vitro*. DeChiara *et al* found that if the gene-deletion was inherited via the egg the offspring was phenotypically normal, but when the deletion came via the sperm the offspring was growth deficient with a birth-weight that was ~60% of the normal newborn mice. The genetic conflict theory was at least in theory confirmed and subsequent data accumulation has substantiated the validity of this theory. The Igf2 was the first discovered imprinted gene; it was unequally expressed depending on parental inheritance. Since then many more imprinted genes are detected, in mouse 150 imprinted genes are detected.

It is now known that Igf2 affects the size of the placenta, the embryo-uptake of nutrients and the birth-weight. In a litter with more than one embryo with different fathers, the individuals compete on getting the most resources from the mother's body. The embryo that inherited an extra demanding Igf2 will get a bigger placenta and get more energy than the others. The maternal genome has a strategy that counteracts the still present risk of fetal overgrowth: a receptor whose only answer upon binding Igf2 is to neutralize it: the Igf2-receptor. The Igf2-receptor is also imprinted, expressed only on the maternal allele. The interaction between paternally derived Igf2 and maternally derived Igf2-receptor has been described as "the war of the genomes" (Chao & D'Amore, 2008).

## LITERATURE REVIEW

### Igf2-gene structure

The Igf2-gene is comprised by a varying number of exons and promoters in experimental mammals. It is transcribed and translated into a precursor-hormone. After a number of processing steps the end result is Igf2, in most species a 67 amino acid protein with a certain sequence homologous to Igf1, relaxin and insulin, but many alternatively spliced variants occur as well. The Igf2-proteins show tissue and developmental specific patterns, with particular fetal and adult promoters and alternate splicing sites. The different promoters and splicing patterns contribute to the complex regulation of Igf2s effects in both fetal and adult tissues.

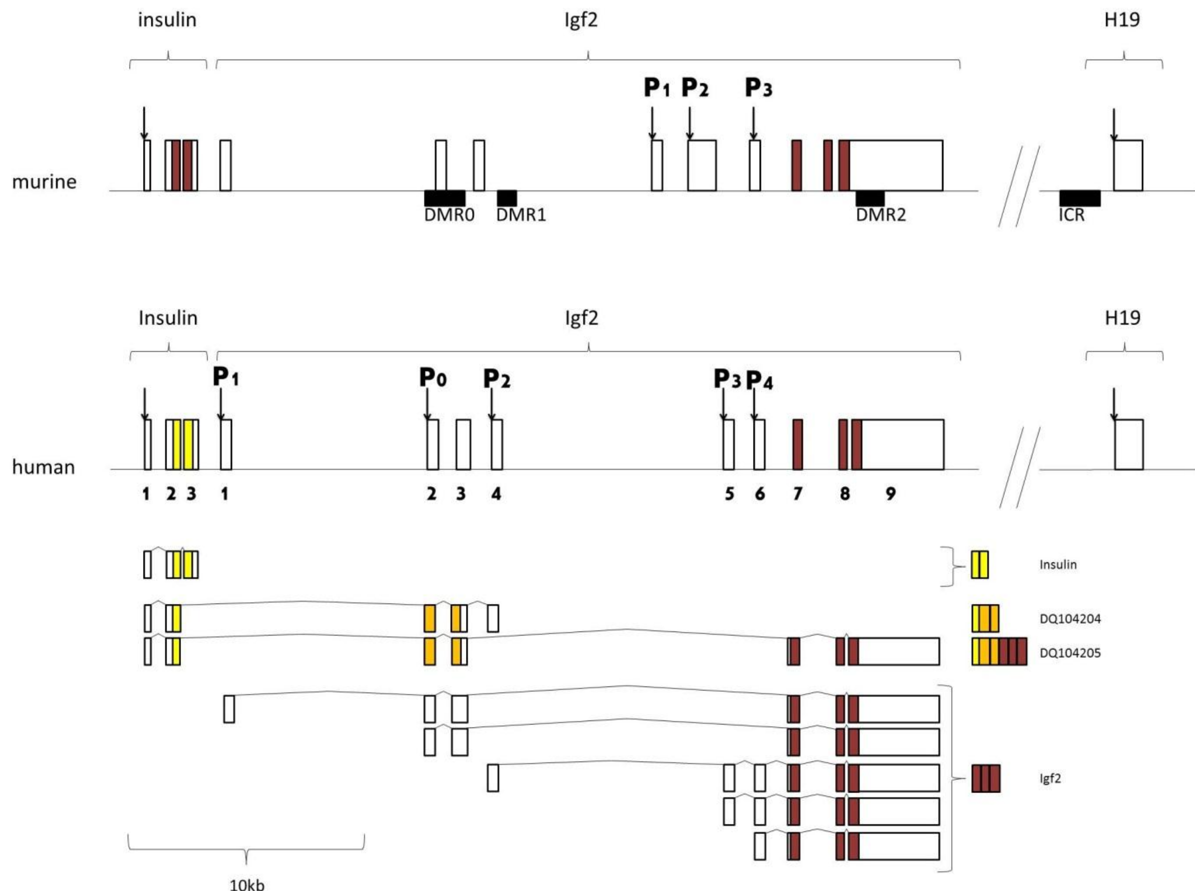



*Fig1 Schematic illustration of the murine and human Igf2/H19-domain. Boxes above the line indicate exons; colored exons compose the end-result-proteins. Small, black boxes below the line represent differently methylated regions (DMRs) and H19 ICR. Below the schematic murine and humane Igf2/H19 domains, differently spliced human transcripts are presented.*
*Adapted from Monk et al ( 2006).*

## The Igf2-protein; its function and receptors

The Igf2 is a growth factor important particularly in placental and embryonic growth. In mice the expression terminates in almost all tissues after birth, making it fetal-specific, since mice have no adult promoters. In other species, like humans, pigs and horses, the Igf2-gene is expressed in adults as well, but their gene contains a fetal-specific promoter and placental and embryonic development is dependent on correct Igf2 expression. (Braunschweig *et al*, 2011)

The Igf1-receptor mediates Igf2s proliferative and growth-inducing effects, while binding to the Igf2-receptor has no intracellular effect apart from degradation. As mentioned before, the Igf2-receptor-gene is also imprinted, expressed only on the maternal chromosome, but located on another chromosome than Igf2. (Chao & D’Amore, 2008)

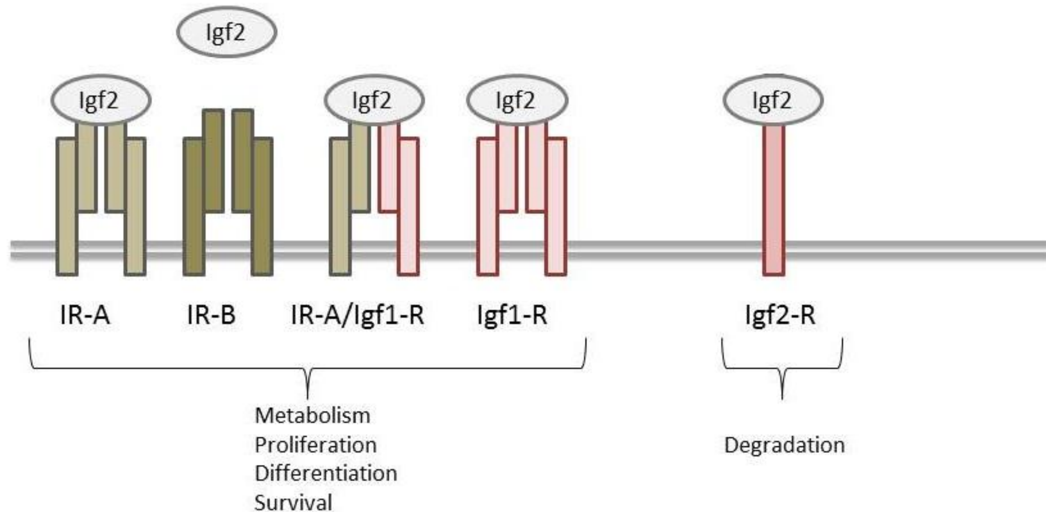



*Fig2 Igf2 binds to both isoforms of the insulin-receptor (IR-A and IR-B), although with lower affinity to the IR-B, and to the IR-A/Igf1-R dimer. Binding to mentioned receptors induce various cellular responses, while binding to Igf2-R mediates internalization and degradation. Adapted from Chao & D'Amore (2001)*

Igf2 is produced locally in tissues in an autocrine or paracrine fashion and in the liver from where it is distributed via the blood as a typical endocrine hormone. *In vitro* studies have shown that IGF1-receptors are often expressed by the same cells that express IGF2, making an autocrine loop possible. Human corneal-cells that gets exposed to IGF2 enter S-phase, prepare to divide, but do not express Igf2 themselves. Cells in the posterior eye do express the Igf2 that induces growth in the cornea, illustrating a paracrine mode of function. (Hyldahl *et al*, 1986)

### The Igf2/H19-cluster

Upstreams of the Igf2-gene is the H19-gene. The H19 is transcribed but never translated to a protein. Both the Igf2-gene and the H19-gene are imprinted in a reciprocal matter in most somatic cells, where the paternal chromosome express IGF2 but not H19, and the maternal transcribe H19 but not IGF2 The conservation of the close vicinity indicates some common regulating factor, but the cluster as a whole resides on different chromosomes in different mammals. (Fig 4)(Chao & D'Amore, 2008).

### The H19 express non-coding RNA

Numerous non-coding H19-mRNAs are found in many fetal tissues but are generally down-regulated after birth. More recent work have found H19-derived miRNAs (micro-RNA). MiRNAs are 19 to 25 kb of non-coding RNA, shown to have the ability to repress translation or promote RNA degradation. (Tsang *et al*, 2010).

### Epigenetic modifications control gene expression

DNA-methylation means the addition of a methyl group ($CH_3$) to certain residues on the DNA, usually to a cytosine in a CpG (a DNA sequence with a cytosine nucleotide next to a

guanine nucleotide with one phosphate in between). CpGs often occur in 'islands', with many CpGs in the DNA-sequence. Dnmts (DNA-methyltransferases) are the enzymes that carry out the DNA-methylation that can affect the epigenetic expression of genes. A gene with a methylated promoter cannot be expressed. The H19-promoter is methylated on the paternal chromosome, inhibiting transcription of paternal H19. (Bartolomei *et al*, 1993)

Chromatin is comprised by the DNA-molecule rolled up onto histonoctamers and can thus be packed as dense heterochromatin or more loosely in euchromatin, depending on the modifications that alters the local structures and as a consequense gene expression. These modifications are exerted by enzymes that acetylate, de-acetylate, methylate or de-methylate different amino-acid residues on histones. (Fig 6a)

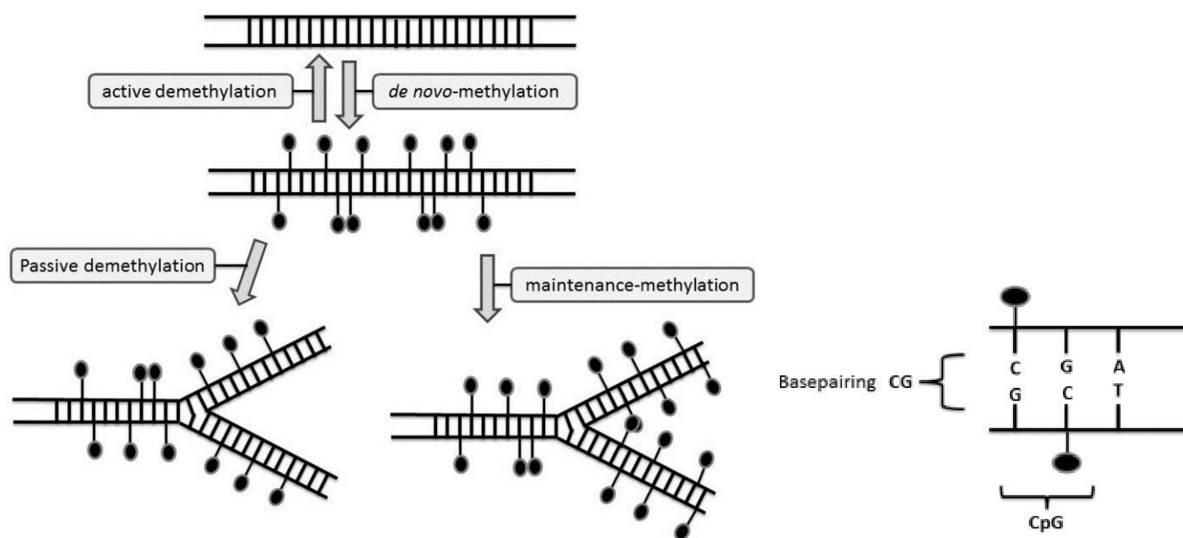


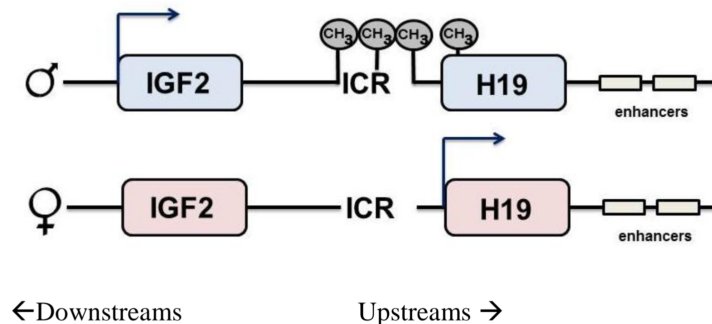

*Fig3 Some DNA-methyltransferases (Dnmts) uses hemi-methylated DNA (newly replicated DNA where only one strand is methylated) as a substrate, adding a methyl group to C-residues. Other Dnmts are responsible for de novo-methylation. Adapted from Reik & Walter (2001)*

## Igf2 and H19 share enhancers

During development, Igf2 and H19 are expressed identically; in tissues where Igf2 is transcribed, so is H19. This has led to the idea of common regulatory elements that mechanically link the genes together, for example common enhancers. Enhancers are small sequences of DNA that can bind transcription factors and enhance the transcription level of genes. Two endodermal H19-enhancers were previously found downstreams of H19. Leighton *et al* (1995) made a targeted deletion of the H19-enhancers in mice and found that a maternally inherited enhancer-deletion resulted in a dramatic decline of H19 in endodermal derived tissues (like the liver), and identical enhancer deletion on the paternally inherited allele resulted in equivalent decline of Igf2 and growth-impaired newborn, with about 80% of normal birth-weight, reflecting the partial loss of Igf2. This elegant deletion showed that these enhancers work on both alleles.

## The Imprint Control Region

The sequence ~2~4 kb up-streams of the H19 transcription start site has been shown to be important to the imprinting state of both H19 and Igf2 and normally referred to as the (the imprint control region). ICR corresponds to the H19-DMD (differently methylated domain) since it is rich in CpG-islands that differ in degree of methylation in the alleles; the paternal is methylated. (Fig 4)




←Downstreams Upstreams →

*Fig 4 Big boxes represent genes on DNA and arrows indicate gene expression. CH3-lollipops represent methylated cytosin-residues on DNA. The small boxes show the downstream position of the enhancers. Adapted from Chao & D'Amore.*

## The enhancer-competition model

This dissimilarity in methylation was discovered early on and Bartolomei *et al* (1993) reveled important facts when experimenting on transgenic mice. The lack of methylation on the maternal promoter and ICR is independent of the degree of expression, showing that it is not the expression that caused the pattern. ICR-methylation degree correlates with H19-expression; high methylation in tissues with low expression. They realized that the parental-specific methylated ICR didn't prove that this or other methylation was the imprinting mark, but suggested that the methylation of the paternal H19 inhibits H19-expression. They suggested that this gave the downstream enhancers a chance to work on the Igf2-gene. This model called the 'enhancer-competition model' could explain the reciprocal imprint of both Igf2 and H19-gene. A H19-transgene, including the enhancers parental-depending methylation-pattern when the ICR was included *in vitro*. But the H19-trangene without the ICR lost the imprint; it was hypo-methylated and thus expressed from both alleles. Thorvaldsen *et al* (1998) made it clear that the ICR is essential for the imprint of H19 *in vivo* as well. Mice with the ICR replaced with a gene-casette lost the imprint; both H19-alleles were expressed and the methylation pattern was lost.

## CTCFs and the boundary model

The ICR-sequence also contains direct and indirect repeats, including several CAGCCC. CTCF's are specific zinc-finger proteins that bind to CAGCCC-sequences.

Bell & Felsenfeld (2000) and Hark *et al* (2000) both suggested that CTCF's binding on the maternal ICR blocks the enhancer and thereby silences the maternal Igf2, and that paternal-

specific methylation prevents CTCF-binding. This enhancer-blocking ICR-function was confirmed by different approaches but both made clear that ICR-bound CTCF's functioned as a mechanical insulator and that the ICR-position between H19 and Igf2 made this possible. The enhancer-competition model was ruled out, since it couldn't explain why the ICR position between the two genes was essential. The conservation of the cluster was to some part explained since the cluster couldn't be regulated if the genes, the insulator-binding domains or the enhancers were not in correct order . Hark *et al* (2000) were a bit more specific when analyzing CTCF-binding on methylated DNA; they analysed hemi-methylated DNA and found that only hemi-methylation on the top strand of DNA inhibits CTCF-binding, while the bottom-strand methylation was unimportant. In replicating cells this means that the paternal allele transiently have one hemi-methylated bottom strand were CTCF's could in theory bind, but (in at least normal cells) do not. The CTCF's and the boundary model added components to the imprinting-puzzle, but it was based on the methylation as the dictating element, and is also called 'the insulator-inhibiting model'. (Fig 5)

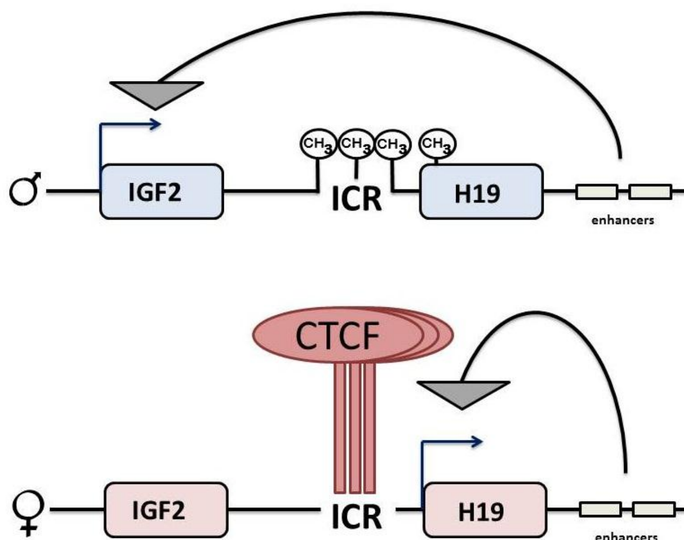



*Fig 5 The boundary model states that the binding of CTCF-proteins on the maternal ICR works as an insulator that prevents the enhancer elements from acting on the maternal Igf2. Adapted from Chao & D'Amore (2008)*

### Histone acetylation and DNA-methylation affects the -expression

Pedone *et al* (1999) immunoprecipitated cells with antibodies against acetylated H3 (histone 3) and H4 tails to detect hypoacetylated histone-tails, commonly found in dense chromatin and hyperacetylated tails, associated with chromatin open for transcription. The silent paternal H19-allele was hypoacetylated compared to the maternal, but the Igf2-alleles were equally acetylated. Pedone *et al* (1999) also cultivated cells with either added inhibitors of DNA methylation (leading to less DNA-methylation) or inhibitors of histone deacetylatos (leading to more open chromatin) or in a medium with both, and it did affect the imprints. The H19 imprint was lost only when both inhibitors were added, whilst Igf2 was biallelical expressed

when either or both components were present. This finding suggested that both DNA-methylation and histone modification is important for the maintenance of the Igf-2 imprint.

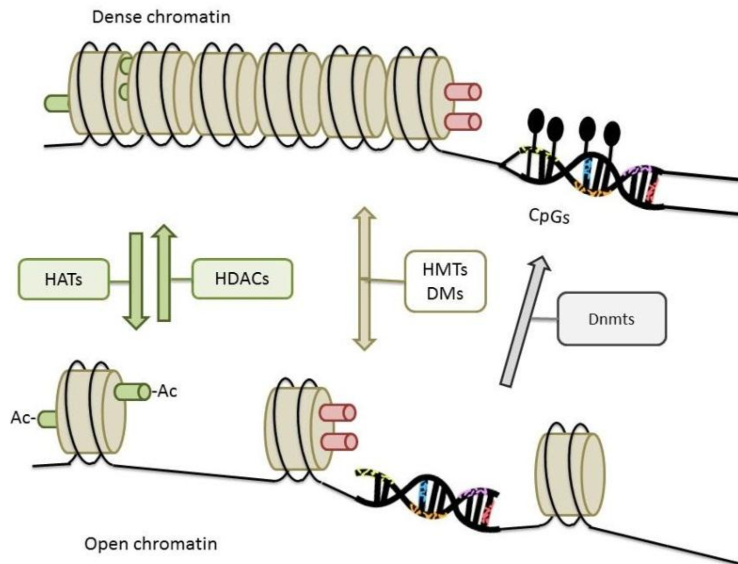



*Fig 6a HATs (histone acetyltransferares),HDACs (histone deacetyltransferases), HMTs (histonemethyltransferases), DMs (demethylases) are enzymes involved in forming open or dense chromatin. Model created with from data from Verona et al (2007)*

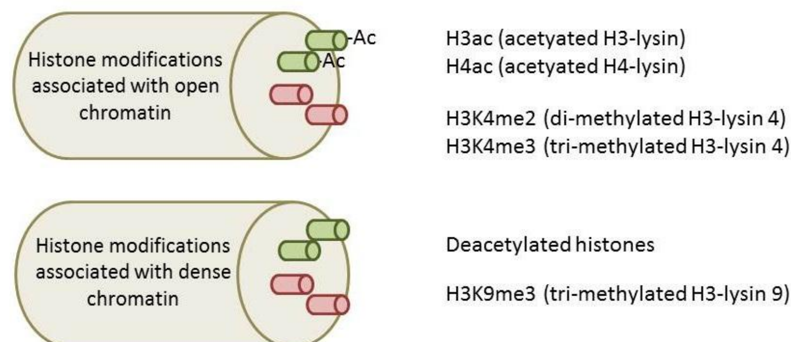



*Fig 6b(Chromatin differences) Model created with data from Verona et al (2007)*

Verona *et al* (2007) characterized the histone modification in imprinted regions, including the H19 region with the ICR on both parental alleles. They found allele-specific acetylation and methylation (of the histones) in the ICR, H19-promoter and H19-gene. They studied specific modifications and found specific differences of active versus dense chromatin, summarized in figure b. The highest level of "active histones" was found in the H19 promoter. The differences in the ICR raised a question; is specific chromatin modifications in the ICR allowing transcription or is it a consequence of transcription? To answer that, they compared a H19-gene with deleted ICR in tissues where it doesn't express, to neonatal liver where it was expressed (even without the ICR). There was "active" chromatin in the neonatal liver but not in other tissues, suggesting that it is the transcription level that gives the allele-specific chromatin-pattern. Thus the differences in chromatin it is not an effect of some demanding imprinted mark. They didn't rule out that the allele-specific methylation and the found chromatin differences could be interconnected and reinforcing each other.

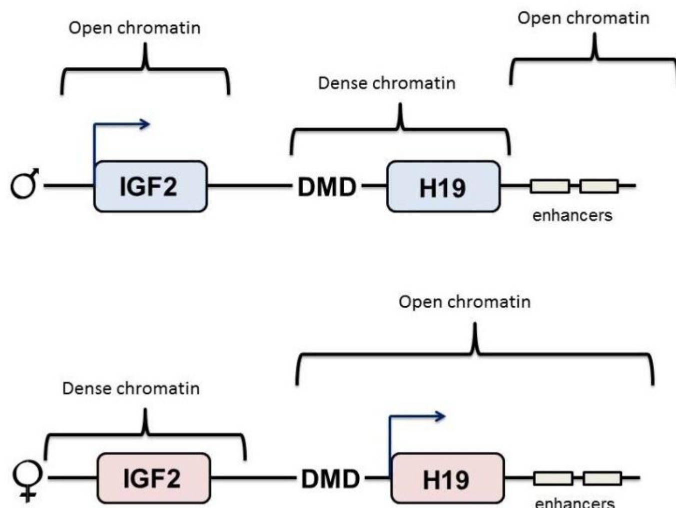



*Fig 7 According to Verona et al (2007) the differences in chromatine on the parental alleles are effects of the uneven degree of transcription and are not generating them.*

## Parent-specific loops

Murrell *et al* (2004) made a targeted insertion of the Igf/H19-genes to generate a mouse where the genes and promoters behave as in a normal cell. They used a chromosome conformation capture technique that enables analysis of the physical organization of the chromosomes in the nucleus, which is utterly important for epigenetic regulation of gene expression. If, for an example, an enhancer cannot physically reach a gene, it cannot enhance it. Murell *et al* (2004) reported gender-specific methylation in DMR1 (differently methylated region 1) and DMR2 (Fig 8). Gender-specific partitions were detected; the maternal ICR interacts with DMR1 on Igf2, and the paternal ICR meshes with DMR2 on Igf2. This generated a three-dimensional model that provided a simple epigenetic explanation to the gender-specific expression of the genes: the DMRs and ICR contain insulators, silencers and activators and are turned on with differential methylation that enables or inhibits expression of the genes.

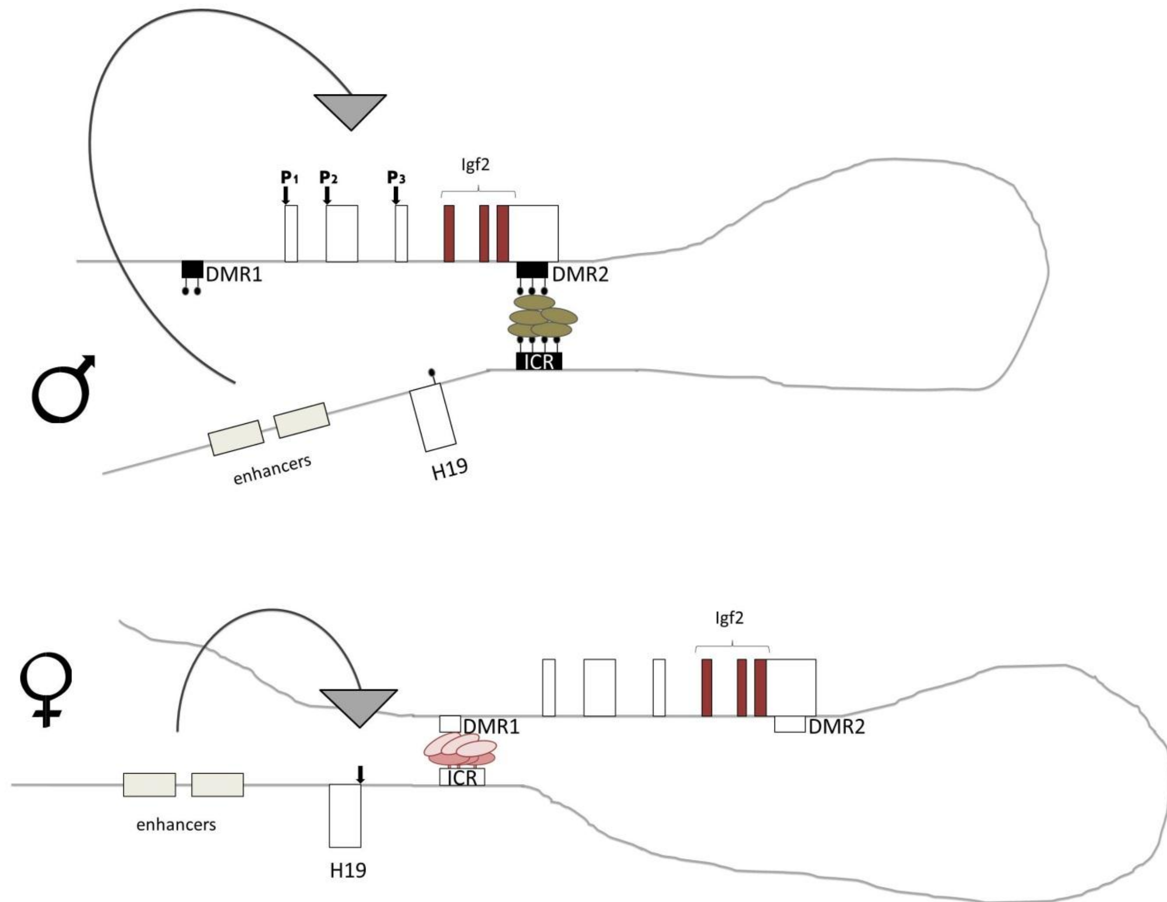



*Fig 8 DMR1, DMR2and ICR are methylated on the paternal chromosome, and putative proteins bind the methylated ICR to DMR2. The enhancers can work on the paternal Igf2. On the maternal chromosome the ICR binds CTCFs (and possibly other proteins)and they interact with DMR1, creating an inactive domain where the maternal Igf2 has no access to enhancers. Adapted from Murrell et al (2004)*

## Stretches of unique direct repeats

The Igf2/H19-cluster have a neighboring imprinted gene-cluster, located on the same chromosome. The neighboring cluster including the imprinted Kcnq1-gene (coding for a voltage-gated potassium channel) includes a CpG-island proposed to be ICR2 on the chromosome and the two clusters are to some degree are co-regulated. (Smilinich *et al*, 1999). Engemann *et al* (2000) investigated the distribution of interspersed repetitive elements in different imprinted clusters. They found that they were not at all as common as in the X-chromosome, where they are thought to contribute to the silencing of one of the chromosomes, but remarkably unevenly distributed with specific classes in specific regions. The most pronounced enrichment in LINEelements was found next to the ICR2, and this region was strongly conserved in murine and human-genome. Tandemly repeated gene arrays, like interspersed repetitive elements can lead to formation of dense chromatin and this could be an important mechanism in these two clusters as well. Methylation is an epigenetic mechanism that could heterochromatize the repeats and spread to nearby CpG’s in the ICR2

and from there affect the epigenetic state of H19-ICR. These repeats remain to be fully elucidated (Engemann *et al*, 2000).

### Germ cells carry parental information

In a developing embryo some cells turns into PGC's (primordial germ cells), still diploid but after adjacent signals destined to migrate to the gonads, proliferate and differentiate into haploid sperms and eggs. The epigenetic status of these cells changes dramatically before meiosis. The PGC's undergo de-methylation (~embryo day 10,5 in mice) where most of the DNA-methylation is erased, including imprinted areas. Parental differences in modification of histones and chromatin are also removed before meiosis. So far no definite statement explaining the de-methylation and removing of chromatin differences is at hand. It is not known if the de-methylation causes the removal of the chromatin, the other way around, or other possible scenarios. It is known that around embryo day 12,5 *de novo*-methylation takes place and that meiosis is not possible without it The de-methylation of the haploid PGC is a necessary step, since all old parental methylation pattern needs to be erased, so that all alleles get the new correct gender-specific methylation-pattern. *De novo*-methylation is the methylation of un-methylated DNA, as in demethylated PGCs.
(Kota & Feil, 2010)

### Methylation- how important is it for the imprint?

Since parental differences in methylation was found and since it was already known that methylation could act as an epigenetic silencer, it was a small step to suggest that it was the methylation-pattern that formed the imprint. Bartolomei *et al* (1993) and Thorvaldsen et al (1998) both came to the conclusion that it is the methylation in the ICR that silences the paternal H19. It was easy to describe how the pattern could get inherited in dividing cells, but not as easy to explain how these differences are established in germ-line cells. Bartolomei *et al* (1993) proposed that the epigenetic mark could be parental-specific methylation in the germ-line, with specific testis- and ovary- DNA methylases as the functioning unit, carrying out d*e-novo* methylation. More recent work has shown that the de-methylation and re-methylation occurs in the gametogenes (Kota & Feil, 2010), but gender-specific methylation is still a possibility.

The methylation pattern in the Igf2/H19-ICR could be the true imprinting mark. If that is the case there are three hypothetical ways by which the imprinting mechanism can be exerted;

1. There are gender-specific methylating enzymes in PGC's
2. Some enzymes can methylate specific alleles
3. Only one of the alleles is protected against (or open for) methylation .

(basic idea from: Reik & Walter (2001))

Engemann *et al* (2000) found that ICR2 (the suggested ICR in the cluster containing the Kcnq1-gene) was highly methylated in oocytes, and since the H19-ICR is methylated in

sperms (Bartolomei *et al*, 1993) this ruled out the simplest, gender-specific Dnmt as an explanation. Other DMRs in numerous more recent researched imprinted genes are also methylated on the parental alleles in an interspersed manner (Reik & Walter, 2001). But enzymes could methylate gender-specifically in both PGCs, in meiotic and replicating cells. This gender-specific recognition could emanate from various other factors, like parental specific expression of Dnmts or factors allowing or repressing the DNA-methylation. The parental-specific expression of Dnmts could also be due to different gender-specific transcription factors, thus opening up a lot of different pathways, all leading to parental-specific methylation in various regions, in the interspersed way now known to be the case.

### But is the methylation really important for the imprinting of Igf2?

Braunschweig *et al* (2011) compared fetal and adult pig tissues. They found bi-allelic Igf2-expression in increasing amounts in aging tissues like muscle and liver, along with the typical parental-specific methylation pattern of one examined CTCF-site in ICR.. Braunschweig *et al* (2011) found no significant parental-specific methylation variation in DMR1 and DMR2 that could explain the finding. This finding challenges both the boundary model and the chromatin loop model, while chromatin differences as an important factor are not completely ruled out.

Even if the methylation is crucial or not, there could be another epigenetic or genetic modification making gender-specific *de novo*-methylation possible in and transgenes and normal mammal cells. An interesting difference in the two parental alleles is asynchronous DNA-replication in several imprinted genes, found by Kitesberg *et al* (1993). The paternal allele is often (but not always) (Reik & Walter, 2001), replicated earlier than the maternal, and this could permit uneven accessibility for the *de novo*-methylation or other modifications done in replicating cells.

Studies that compared frequencies of recombinations in imprinted clusters during meiosis have revealed some interesting differences. During male meiosis there was many more recombinations in the H19/Igf2-region, than in female meiosis. This is an effect of more open chromatin during male meiosis and could also contribute to the establishment of the parent-specific mark. (Chao & D'Amore, 2008)

## DISCUSSION

The operational role of IGF2 is only partly known. Even though we have gathered a lot of information about the structure and function of the gene, the time is ripe to link this info to how the peptide participate in growth development and pathogenecis of key diseases. There is obviously some (one or more) epigenetic mark on one (or both) of the parental alleles, a key element(s) that makes parental-specific expression possible. The imprinting of the H19/Igf2 gene cluster is important in many different cancers and syndromes that include growth-disorders, and deeper knowledge about the imprint could reveal both mechanisms and possible treatment

Many possible factors contributing to the imprint are at hand. In some cases one possible explanation rules out another, but others could co-exist. It is a complex field, since so many possibilities are present and many data contradict each other. Both genetic and epigenetic factors could be crucial as well as events in meiosis, replication and embryonic development. There is by no means consensus, or an adopted comprehensive overview explaining how the imprint is established and maintained.

Bartolomei *et al* (1993) suggested that the methylation of the paternal H19 inhibits H19-expression, but they realized that the parental-specific methylated ICR didn't prove that this or other methylation was the imprinting mark. The CTCFs gave rise to the boundary model, based on findings that methylation inhibited the insulating CTCFs and that this was the important step in the gene regulation (Bell & Felsenfeld, 2000; Hark *et al*, 2000). The chromatin loop included the CTCFs as insulator but suggested that a more complicated three-dimensional structure allowed or stopped expression of Igf2.

Data from Bartolomei *et al* (1993) suggest equally open chromatin on maternal and paternal promoter, while Pedone *et al* (1999) found unequal acetylation in the H19-promoter but equal acetylated Igf2s. Verona *et al* (2007) found differences throughout the locus, and they included more numerous modifications than the two previous studies. Data from Verona et al (2007) proposes that the chromatin differences are only effects of differential transcription, but is it not in line with the chromatin loop model.

If the very recent data from Braunschweig *et al* (2011) is confirmed, a cornerstone of many theories can be removed. They found bi-allelic Igf2-expression in tissues with intact methylation-differences, suggesting that the methylation might not be important for the imprint of Igf2.

H19-derived micro-RNAs play an obscure  role in cell proliferation and different types of cancer and might also play some part in maintenance and establishment of the imprint as well. (Tsang *et al*, 2010; Cai & Cullen, 2007).

### Evolution has preserved the imprint of Igf2-but why?

The mothers IGF2 has no effect on the fetuses, but her IGF2R could be described as a anti-IGF2 since there is no response when the ligand binds other than destruction. The mother wants a healthy litter, but she does not aim to provide more growth stimulus than necessary since she will probably have more litters in the future, giving her the chance to get more off-springs. The male always wants most energy to his off-springs, while the mother will keep the overall litter-size low. This theory was controversial when first presented but is now well established and called the genetic conflict theory. (Haig & Westoby, 1989) There are some possibly harmful aspects of an imprinted gene. If the paternal gene is faulty, there is zero protein expression from the imprinted gene, with negative effects for the offspring and where the maternal genome can do nothing to compensate. Another plausible negative effect from a

supposed imprinted gene could be that less protein can be expressed from only one copy, due to the limited amount of transcription factors.

### *The benefits of an extra demanding Igf-2 from the maternal perspective*

The benefits for a male carrying a new mutant allele that demands extra nutrition from the mother is obvious: his offspring gets favoured in the womb. The model by Smith and Fretwell (1974) suggested that females do not want to favourise some offspring, since she is equally related to all of them. This statement is perhaps not applicable in an imprinted context.

Imagine a pregnant cat genome in a context where imprinting in igf2-region is established. The offspring have different fathers with different Igf2s, and one Igf2 is far more efficient in demanding resources. If the female could choose Igf2 for her offspring, it is obvious that she would choose a strong Igf2 for her sons, since that would make them more effective in giving her grand-off-springs. The fact that a son with a strong Igf2 demand extra resources might then be beneficial for her to. Extra-demanding daughters would not have the same obvious advantage, although her daughters son would have an increased chance to inherit the extra demanding Igf2. An equation could probably be made show to that the imprinted concept is beneficial for the female genome as well, but this remains to be done

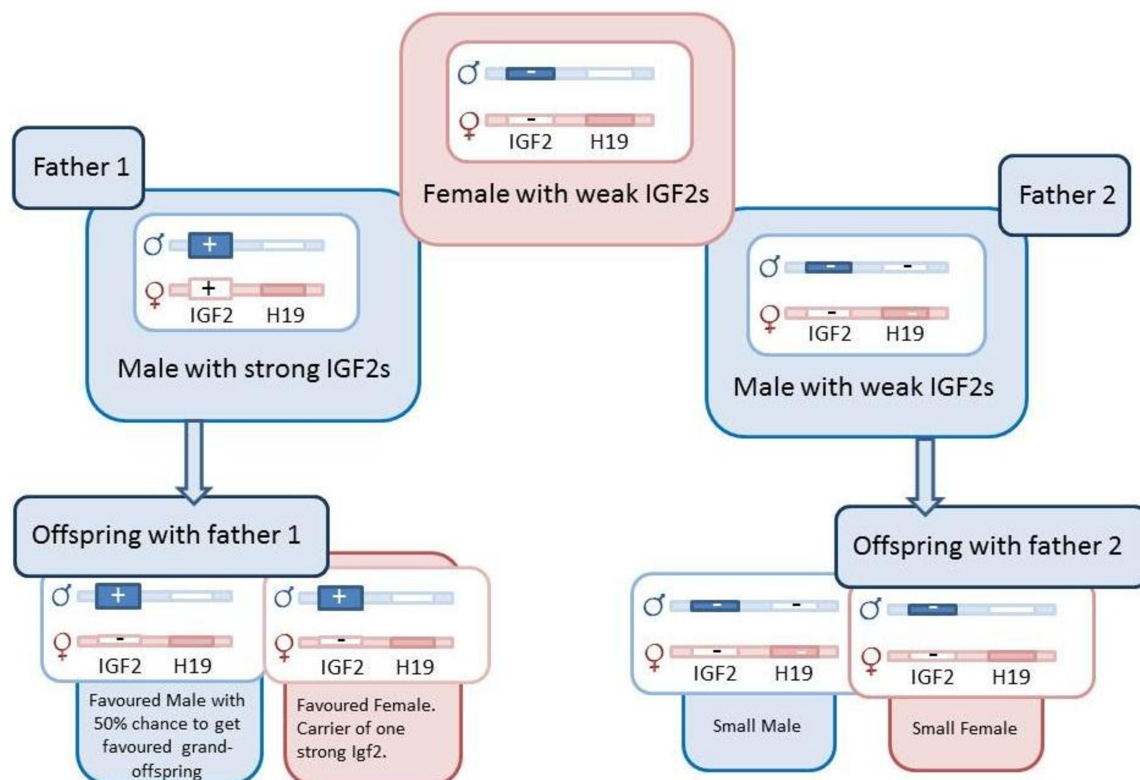



*Fig 9. A model attempting to illustrate why a female would choose a male with more resource-demanding Igf2s rather than a male with less demanding counterpart. Since the offsprings with father 1 are more likely to get favoured offsprings, and thereby spreading her genes, it lies in her interest aswell to favour the offspring with father 1.*

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