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Beckwith–Wiedemann syndrome: multiple molecular mechanisms

Thorsten Enklaar, Bernhard U. Zabel and Dirk Prawitt*

Beckwith–Wiedemann syndrome (BWS) is a congenital overgrowth condition with an increased risk of developing embryonic tumours, such as Wilms' tumour. The cardinal features are abdominal wall defects, macroglossia and gigantism. BWS is generally sporadic; only 10–15% of cases are familial. A variety of molecular aberrations have been associated with BWS. The only mutations within a gene are loss-of-function mutations in the *CDKN1C* gene, which codes for an imprinted cell-cycle regulator. *CDKN1C* mutations appear to be particularly associated with umbilical abnormalities, but not with increased predisposition to Wilms' tumour. In the remaining BWS subgroups, a disturbance of the tight epigenetic regulation of gene expression (patUPD 11p, microdeletions or epimutations) seems to be the cause of the syndrome. Here we describe the clinical presentation of BWS and its dissociation from phenotypically overlapping overgrowth syndromes. We then review the current concepts of causative molecular genetic and epigenetic mechanisms, and discuss future directions of research.

Beckwith–Wiedemann syndrome (BWS) was initially called EMG syndrome (Ref. 1), from its three main features of exomphalos (umbilical hernia, abdominal wall defects), macroglossia (enlarged tongue) and neonatal gigantism (overgrowth). However, the phenotypic parameters of BWS vary and can include other diagnostic findings. In particular, BWS patients are at increased risk (approximately 5%) of developing specific embryonic tumours, mainly Wilms' tumours and hepatoblastomas, in early childhood (Ref. 2). BWS occurs in all ethnic groups at a more or less constant frequency of approximately 1 in 13 000 newborns (Refs 3, 4), with equal representation in males and females. Most BWS cases are sporadic. The chance of recurrence is dependent on the molecular defect. Most BWS pedigrees resemble that of autosomal dominant inheritance with incomplete penetrance.

Although genetic alterations in various genomic regions are reported in patients with BWS (Refs 5, 6, 7), the current understanding of the molecular basis of the syndrome is that aberrations in the chromosomal region 11p15.5 involving imprinted genes have a pivotal role in disease formation. Genomic imprinting is usually accompanied by CpG methylation of

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regulatory regions during gametogenesis, resulting in silencing of one specific parental copy (maternal or paternal) of an affected gene. Five imprinted genes in two chromosomal clusters seem to be causative for BWS: *IGF2* (insulin-like growth factor 2) and *H19* in the distal cluster (imprinting cluster 1; IC1); and *CDKN1C* (cyclin-dependent kinase inhibitor 1C), *KCNQ1* ('potassium voltage-gated channel, KQT-like subfamily, member 1') and *KCNQ1OT1* (KCNQ1 overlapping transcript 1) in the proximal cluster (imprinting cluster 2; IC2) (Fig. 1). The genetic and epigenetic findings identified so far in BWS are summarised in Box 1. To date, these findings allow the molecular diagnosis of approximately 85% of BWS patients.

Clinical description of BWS

The cardinal features of BWS – abdominal wall defects (exomphalos, umbilical hernia or diastasis recti), macroglossia and pre-/post-natal gigantism – were initially set as the criteria to diagnose the syndrome (Refs 18, 19). Over time, however, it became clear that the phenotypic appearance and symptoms of BWS patients showed considerable variation, and could include additional features (see for example Ref. 20) such as neonatal hypoglycaemia (as a result of pancreas cell hyperplasia), hemihypertrophy, organomegaly (e.g. spleen, liver, kidney, pancreas), earlobe creases, polyhydramnios, haemangiomata (mostly facial nevus flammeus), cardiac defects (cardiomyopathy) and embryonal tumours. Most of the tumours observed in BWS patients are Wilms' tumours (nephroblastoma), liver tumours (hepatoblastoma), neuroblastomas, tumours of the adrenals (adrenocortical carcinomas), soft-tissue tumours (e.g. rhabdomyosarcomas), and tumours of the bladder. As a result of this clinical variability, the consensus for diagnosis of BWS became the presence of all three main features or at least two of them and three of the additional symptoms (Ref. 21).

Increased infant mortality rates seem to be mainly due to cardiovascular abnormalities or complications associated with macroglossia, hypoglycaemia or prematurity (Refs 20, 22, 23). The majority of BWS cases encounter no serious problems in later childhood. The main issues that have to be addressed when caring for BWS patients are related to the macroglossia, abdominal wall defects, possible neonatal hypoglycaemia, renal dysplasia, and increased tumour risk. Macroglossia can lead to feeding and respiratory complications, and abdominal wall defects may need to be addressed surgically if severe. Blood glucose should be controlled to prevent brain damage from possible neonatal hypoglycaemia. These aspects of BWS are monitored by standard paediatric protocols as they also occur in other, common conditions. This is different for the increased tumour risk: the recommended tumour surveillance currently suggests the monitoring of serum alpha fetoprotein (AFP) as a blood tumour marker up to the age of 5 years (Ref. 24) and abdominal ultrasound examination every three months to the age of 8 years (Refs 25, 26). Differential diagnosis of BWS should be verified carefully as some conditions presenting with BWS-like symptoms are easy to handle and are not burdened with tumour risk. Macrosomia might also be due to maternal diabetes mellitus during pregnancy, or inborn errors of metabolism might mimic overgrowth symptoms.

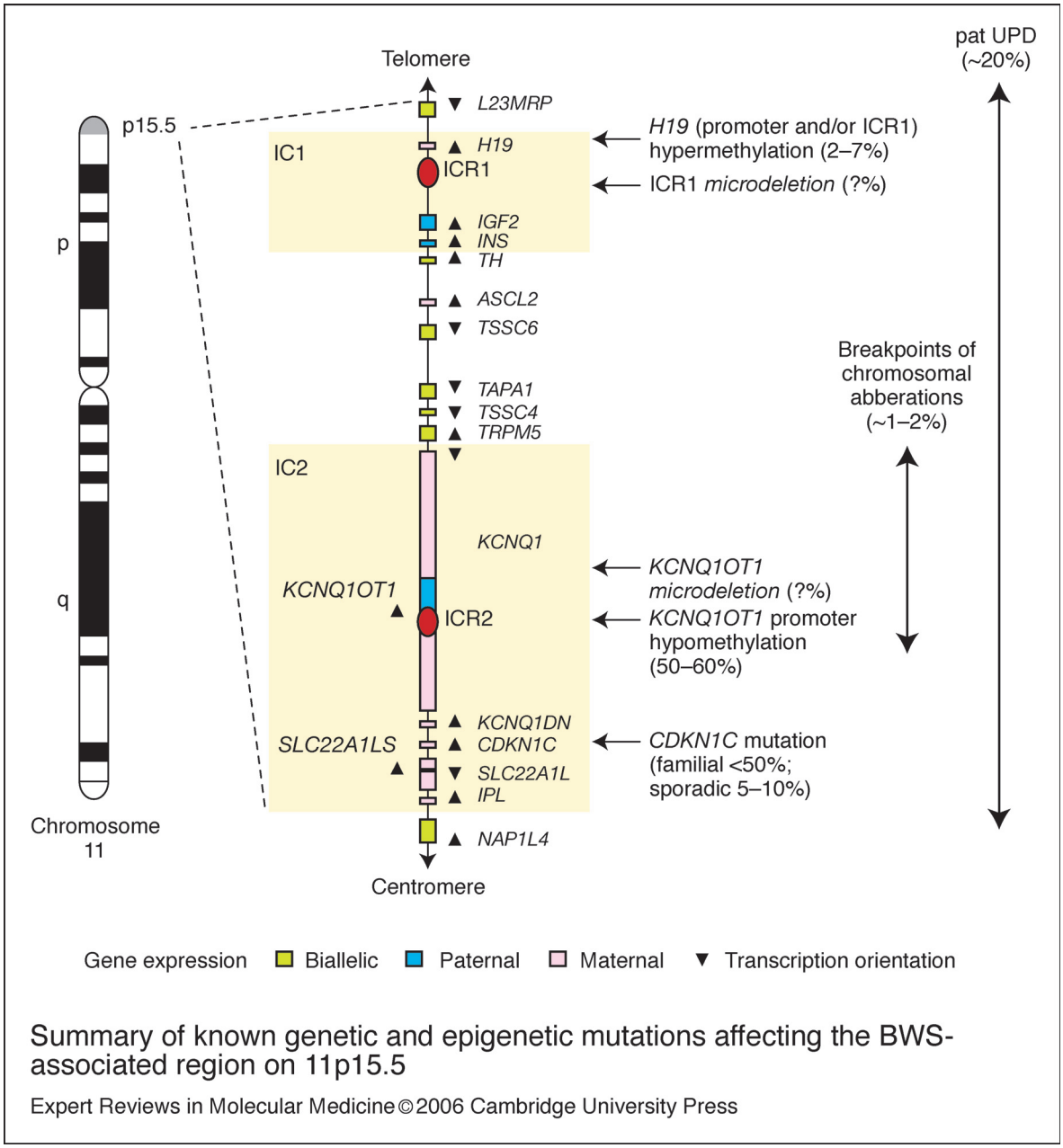
Other overgrowth syndromes

Diagnosis of BWS is made difficult not only by its variable presentation, but also by phenotypic overlap with other overgrowth syndromes, including Sotos, Simpson–Golabi–Behmel, Weaver and Perlman syndromes (Table 1).

Sotos syndrome patients present with characteristic facial features (high and broad forehead, sparse frontotemporal hair, long narrow face, down-slanting palpebral fissures, malar flushing, prominent chin) and macrocephaly. These major diagnostic criteria are often associated with advanced bone age and variable mental retardation (Refs 27, 28). The majority of patients (60%; Ref. 29) show a partial deletion or mutation of the *NSD1* gene (encoding nuclear receptor SET-domain-containing protein 1) on chromosome 5q35. As for BWS, Sotos syndrome patients (confirmed by *NSD1* mutations) can have a variable phenotype (Ref. 30). A study of Sotos syndrome patients with *NSD1* gene alterations reported tumours in 3.4% of patients (Ref. 30). In contrast to the monitoring of BWS cases, no routine tumour screening protocol for children with Sotos syndrome exists because a significant

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Summary of known genetic and epigenetic mutations affecting the BWS-associated region on 11p15.5

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Figure 1. Summary of known genetic and epigenetic mutations affecting the BWS-associated region on 11p15.5. Schematic representation of the Beckwith–Wiedemann syndrome critical region 1 (BWSCR1) on chromosome 11p15.5, with the imprinting status of determined genes. Two clusters of imprinted genes (IC1, 1C2) are separated by a group of biallelically expressed genes. Aberrant findings in BWS and their frequency are indicated to the right in parentheses. Abbreviations: ASCL2, achaete-scute complex-like 2; BWS, Beckwith–Wiedemann syndrome; CDKN1C, cyclin-dependent kinase inhibitor 1C; IC, imprinting cluster; ICR, imprinting control region; IGF2, insulin-like growth factor 2; INS, insulin; IPL, imprinted in placenta and liver; KCNQ1, potassium channel, voltage-gated, KQT-like subfamily, member 1; L23MRP, L23 mitochondrial-related protein; NAP1L4, nucleosome assembly protein 1-like 4; patUPD, paternal uniparental disomy; SLC22A1L, solute carrier family 22 (organic cation transporter), member 1-like; TAPA1, target of antiproliferative antibody 1; TH, tyrosine hydroxylase; TRPM5, transient receptor potential cation channel, subfamily M, member 5; TSSC4, tumour-suppressing subchromosomal transferable fragment candidate gene 4.

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Box 1. Summary of genetic and epigenetic features of Beckwith–Wiedemann syndrome

Rare balanced chromosomal translocations/inversions involving chromosomal region 11p15, with the chromosome 11p15 breakpoints clustering in three regions of 11p15.3-5 (Refs 8, 9) (1%).

Loss-of-function alterations in the *CDKN1C* gene (reviewed in Ref. 10) (5–10% in sporadic cases; up to 50% in familial cases).

Duplication of parts of paternal 11p15.5, in most cases associated with loss of the respective maternal material (resulting in patUPD), affecting the distal imprinting cluster or both imprinting clusters (Refs 11, 12) (trisomy 1%; patUPD 20%).

Epigenetic (methylation) alterations smaller than patUPDs that affect the transcription of epigenetically regulated genes in 11p15.5 – i.e. *H19* hypermethylation (2–7%) or *KCNQ1OT1* hypomethylation (50–60%) (e.g. Refs 13, 14).

Microdeletions of imprinting control regions at *H19* (Refs 15,16) and *KCNQ1OT1* (Ref. 17) that disturb the tight epigenetic regulation of 11p15 gene expression (no statistics available to date).

Abbreviations: CDKN1C, cyclin-dependent kinase inhibitor 1C; KCNQ1OT1, KCNQ1 overlapping transcript 1; patUPD, paternal uniparental disomy.

number of the tumours are benign and the observed tumour types as well as their anatomic locations vary greatly. Interestingly, Baujat and colleagues (Ref. 5) identified two cases with features of BWS and mental retardation that had *NSD1* mutations, as well as two cases of Sotos syndrome with BWS-characteristic 11p15 uniparental disomy. This illustrates that these two overlapping syndromes may be indistinguishable on a mere phenotypical level, yet differential diagnosis may become feasible with the identification of syndrome-specific molecular defects.

Weaver syndrome patients have a very similar appearance to Sotos syndrome cases. Differential diagnosis may be enabled by specific facial features or contractures. There is considerable debate about whether Sotos and Weaver syndromes constitute distinctive entities or are representative of locus or allelic heterogeneity (Ref. 31). The latter view is supported by a study of Douglas and co-workers (Ref. 32), who found that three out of seven patients with Weaver syndrome had mutations in *NSD1*, the gene mutated in the majority of Sotos syndrome patients.

Simpson–Golabi–Behmel syndrome (SGBS), an X-linked recessive disorder, was initially described as a condition resulting in a 'bulldog-like' appearance (Ref. 33). Like BWS patients, patients with SGBS present with macroglossia, overgrowth, visceromegaly, renal cysts and an increased risk for developing embryonal tumours (Ref. 34). However, additional symptoms not found in BWS can be observed in SGBS cases, such as cleft lip, supernumerary nipples, skeletal anomalies like polydactyly, and a high frequency of cardiac defects (Ref. 35). SGBS is caused by defects involving the *GPC3* (glypican 3) gene, located on Xq26, which encodes an extracellular proteoglycan believed to function in growth regulation (Ref. 36). The need for exact molecular diagnostics for the various overgrowth syndromes is demonstrated by the fact that the cell line used to identify the *GPC3* gene by a positional cloning approach originated from a woman diagnosed with BWS and showing a karyotype with a de novo X/1 translocation (Ref. 37). Also, in a *GPC3* deletion screening on 80 male patients with somatic overgrowth, one *GPC3* deletion was identified in a patient from a family diagnosed with Sotos syndrome and another in a family diagnosed with Perlman syndrome with nephroblastomatosis (Ref. 38).

Perlman syndrome (Ref. 39) is a very rare disorder [21 live-born patients described to date (Ref. 40)] with a high risk of neonatal mortality, and presents with polyhydramnios, fetal overgrowth, neonatal macrosomia, visceromegaly, nephromegaly, and predisposition for (bilateral) Wilms' tumour

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Table 1. Clinical characteristics of Beckwith–Wiedemann syndrome and related overgrowth syndromes

Characteristic	Beckwith–Wiedemann syndrome	Sotos syndrome	Weaver syndrome	Simpson–Golabi–Behmel syndrome	Perlman syndrome
Genetic alterations	*CDKN1C* mutation	*NSD1* mutation/deletion	*NSD1* mutation	*GPC3* mutation/deletion	?
Epigenetic alterations	UPD 11p15 Hypomethylation of *KCNQ1OT1* Hypermethylation of ICR1/*H19* LOI *IGF2*				
Clinical findings					
Pre-/post-natal overgrowth		+	+	+	+
Hypoglycaemia/hyperinsulinism	+	+	+	+	+
Macroglossy	+	−	−	+	−
Hemihypertrophy	+	+	+	−	−
Umbilical wall defects	+	−	−	−	−
Neoplasms	+	+	−	+	+
Ear creases	+	−	−	+	+
Congenital renal anomalies	+	−	−	Renal cysts	Nephromegaly
Facial naevus flammeus	+	−	−	−	−
Macrocephaly	−	+	+	+	+
Seizures	−	+	+	−	−
Mental retardation	−	+	+	(+)	−
Coarse facies	−	−	−	+	−
Supernumerary nipples	−	−	−	+	−

Abbreviations: CDKN1C, cyclin-dependent kinase inhibitor 1C; GPC, glypican 3; ICR, imprinting control region; IGF2, insulin-like growth factor 2; KCNQ1, potassium voltage-gated channel, KQT-like subfamily, member 1; LOI, loss of imprinting; NSD1, nuclear receptor SET-domain-containing protein 1; UPD, uniparental disomy.

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(28%). Other features are macrocephaly, dysmorphic facial features (micrognathia, round face, broad flat nasal bridge), and mental retardation. Perlman syndrome shows autosomal recessive inheritance (Ref. 41), suggesting it is a distinct genetic entity; however, molecular findings such as the *GPC3* deletion in one Perlman syndrome patient as well as the de novo extra G-positive band on the tip of the short arm of chromosome 11 (the BWS critical region) (Ref. 42) indicate it might represent a heterogeneous group of conditions overlapping with other overgrowth syndromes.

The above description of the various overgrowth syndromes shows that there is no developmental anomaly unique to a specific syndrome. Although appearing to be different entities, the syndromes may involve overlapping signalling pathways. Molecular diagnosis will facilitate classification of the patients, thus allowing an adequate estimation of syndrome-associated risks. The differential diagnosis of BWS should consider another overgrowth condition to be possible when features not commonly associated with BWS (e.g. developmental delay) are present or molecular alterations characterising other syndromes (e.g. *NSD1* mutations) are found.

Possible molecular defects underlying BWS

Diagnosis of BWS is currently still made by clinical evaluation and not by genetic testing because of the heterogeneity of molecular defects. Patients can be divided into subgroups with particular cytogenetic, molecular genetic or epigenetic alterations. Nevertheless, these categories are not yet congruent with the phenotypic subgroups. The only common finding of BWS-associated aberrations is the involvement of the chromosomal region 11p15.

Cytogenetic aberrations

Rare translocation breakpoints in BWS patients (1–2%) can be grouped into three different genomic 11p15 regions, named BWS critical regions 1–3 (BWSCR1–3). BWSCR1 encompasses an approximately 300 kb interval 200–300kb proximal of *IGF2* (Ref. 8). All breakpoints in BWSCR1 disrupt the *KCNQ1* gene (Ref. 43), but the involvement of *KCNQ1* itself in BWS is not yet fully understood. BWSCR2 and BWSCR3 map 5 Mb and 7 Mb, respectively, more proximal to BWSCR1 (Ref. 6). BWSCR2 is defined by breakpoints of two patients with some BWS features (hemihypertrophy, ear pits, hypoglycaemia, cardiac abnormalities, Wilms' tumour). Alders and colleagues (Ref. 7) cloned and sequenced BWSCR2 and detected a zinc-finger gene, *ZNF215.* They proved that two of its five alternatively spliced transcripts were disrupted by both BWSCR2 breakpoints. Although they found some sequence alterations in *ZNF215* in other BWS patients, no evidence for functional mutations in this gene exists. It has been suggested that the translocation event itself may alter gene transcription of distal 11p15 (e.g. 11p15.5). BWSCR3 is defined by only one cell line, with a balanced germline chromosomal rearrangement. As for BWSCR2, it is suggested that there is a gene(s)/regulatory region at BWSCR3 that interacts with some components of the *IGF2*/BWSCR1 region and influences the BWS phenotype via this mechanism rather than BWS arising directly from a gene disruption at the translocation breakpoints (Ref. 7). The analyses of the breakpoint cluster regions helped to refocus the search for molecular mechanisms that are causative for BWS on the telomeric part of 11p15, namely the already mentioned region including the BWSCR1 that ranges from distal *H19* to proximal *SLC22A1L* ['solute carrier family 22 (organic cation transporter), member 1-like] (Fig. 1).

Imprinting in the genomic BWS regions

A remarkable observation when analysing genes in the 11p15.5 region was the finding that transcription is significantly regulated by imprinting, an epigenetic phenomenon.

Imprinted genes are transcribed from only one specific parental allele – either the maternally derived copy or the paternally derived copy. This monoallelic expression of imprinted genes is associated with epigenetic modifications – DNA methylation – in the gametes that is parent-of-origin specific, which means it differs between oocyte and sperm. This parent-specific modification pattern observed in the germline leads to differentially methylated genomic regions (DMRs) after fertilisation. It stays more or less intact after fertilisation and escapes the genome-wide demethylation ('reprogramming') that occurs in the zygote as well as the de novo methylation

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during differentiation of the somatic cells through as yet mostly unknown mechanisms (Ref. 44). Only during gametogenesis is this parental modification erased and specifically set anew (Ref. 45). The DMRs originating in the germline are also called primary DMRs, to distinguish them from secondary DMRs that arise only after the de novo methylation in the postimplantation embryo, as a result either of spreading of methylation from primary DMRs or of differential gene expression. Secondary DMRs can be stage- and cell-type-specific, whereas primary DMRs in general show allele-specific methylation. As a result, if paternal genes are silenced due to a DMR, maternal genes are predominantly or exclusively expressed or vice versa.

To date, approximately 50 imprinted human genes are known (see 'Further reading' box), many of them influencing the growth and development of the embryo, and a total number of up to 100 is postulated based on data obtained from animals. As a rule of thumb, paternally expressed imprinted genes support and enhance growth while maternally active genes at least partly suppress growth (Ref. 46). It seems obvious that for the correct development of an embryo a well-balanced dosage of such imprinted genes is required and that deregulation would lead to disease. In the 11p15.5 BWS-associated region, two domains of imprinted genes exist, which are referred to as imprinting clusters IC1 (distal/telomeric) and IC2 (proximal). The imprinting of genes within these domains is regulated by so-called imprinting centres, which present as DMRs and control in cis the epigenetic modification and transcription of imprinted genes contained in their domain. The first hints that imprinting defects are one possible cause of BWS came from the preferentially maternal transmission of BWS in autosomal dominant pedigrees and the observation of the high number of paternal uniparental disomies (patUPDs) for chromosome 11p15.5, arguing for a misregulation of the affected imprinted genes.

Genes and regulatory regions implicated in BWS

The distal group of significant genes and regulatory elements of 11p15.5 implicated in BWS contains the imprinting control region 1 (ICR1) and the *H19* and *IGF2* genes, whereas the proximal cluster contains the ICR2 and the *CDKN1C* gene (Fig. 1). Both clusters include additional genes, which are (with the exception of the proinsulin gene) either biallelically expressed (e.g. tyrosine hydroxylase gene) or paternally imprinted (e.g. the *IPL* gene). (The proinsulin gene in the distal imprinting domain is maternally imprinted.) These additional genes may also contribute to some aspects of BWS, but in this review we focus on the three genes and associated regulatory regions (*H19*, *IGF2* and *CDKN1C*; ICR1 and ICR2) that have a proven causative effect on the syndrome.

CDKN1C mutations

The proximal cluster gene *CDKN1C* exhibits a paternal imprint. However, it is not expressed strictly monoallelically, but rather preferentially transcribed from the maternal allele (Ref. 47). In most tissues the paternal allele is also expressed at low levels, and in fetal brain and some embryonal tumours both alleles seem to be transcribed equally. *CDKN1C* codes for a 316 amino acid protein ($p57^{KIP2}$) with four structurally distinct areas: a p21/p27-related cyclin-dependent kinase (CDK) inhibitory domain, a proline-rich domain, an acidic domain, and a C-terminal nuclear targeting domain that contains a putative CDK phosphorylation site and has sequence similarity to $p27^{KIP1}$ but not to p21 (Ref. 48). In mammalian cells it is located in the nucleus and associates with G1 CDK components, and its overexpression causes a complete cell cycle arrest in the G1 phase. *CDKN1C* is expressed in a tissue-specific manner, as a 1.5 kb mRNA transcript in placenta and at lower levels in various other tissues, and as a 7 kb mRNA in skeletal muscle and heart, suggesting distinct roles in cell cycle control (Ref. 48).

Several *CDKN1C* mutations have been described in BWS patients, and are thought to be causative for 5–10% of sporadic cases and for up to 40% of the familial cases (Refs 49, 50, 51). Carriers of *CDKN1C* mutations do have a higher recurrence risk for BWS (if the mother is the transmitting parent, the risk is 50%). All known mutations were heterozygous loss-of-function mutations that resulted in a BWS phenotype. Patients with these mutations seem to have a higher risk of abdominal wall defects, but the lowest tumour risk when compared with the remaining groups of BWS (Ref. 52). Although

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Wilms' tumours have so far not been reported in patients with *CDKN1C* mutations, it is still assumed that they have a higher risk than normal for embryonal tumours, including hepatoblastomas, rhabdomyosarcomas and gonadoblastomas (Refs 4, 52, 53). The mutations seem to be effective when inherited from the mother or when appearing de novo on the maternal allele, but there were a minimum of two cases with a paternally transmitted *CDKN1C* mutation that was associated with BWS, suggesting a complex situation involving at least incomplete imprinting and haploinsufficiency resulting in some features of the syndrome (Refs 54, 55). The mutations all affect the CDK inhibitory domain and result in an inactive protein that does not function as a cell cycle inhibitor and does not localise in the nucleus.

A mouse model with targeted disruption of *CDKN1C* was generated by Zhang and co-workers (Ref. 56) to reveal the function of the gene product in the organism. The resulting *Cdkn1c*-deficient mice present with altered cell proliferation and differentiation, leading to abdominal muscle defects, renal medullary dysplasia, adrenal cortical hyperplasia, cleft palate and cytomegaly – features also observed in BWS patients. The mice also suffered from defects not seen in BWS patients: endochondral bone ossification defects with incomplete differentiation of hypertrophic chondrocytes, and hyperproliferation and apoptosis of lens cells. This suggested that loss of *CDKN1C* expression/function observed in BWS cases accounts directly for at least some phenotypic aspects of the disorder and may influence other phenotypic alterations in a concerted action with other genes. Transgenic mice carrying *Cdkn1c* on BAC clones that spanned 315 kb around the gene locus showed no allele-specific expression of the transgenic *Cdkn1c*, suggesting that imprinting elements for this gene may lie outside this area and that disruption of appropriate expression of *CDKN1C* in a subset of BWS cases might be caused by mutations that occur at a substantial distance from the gene itself (Ref. 57). In addition, another regulatory factor with a known role in imprinting, the CTCF (CCCTC-binding factor) protein (which is discussed later in this article), also binds in vitro in the *CDKN1C* gene locus with a silencing effect on gene transcription (Ref. 58).

UPD 11p15

Initial reports of increased loss of heterozygosity (LOH) of 11p15.5 polymorphic markers in tumours of BWS patients (Ref. 59) suggested a causative link between BWS and imprinted genes on the distal arm of chromosome 11p. Following the discovery of loss of maternal alleles in BWS-associated tumours, Henry and co-workers (Ref. 11) found a significant proportion of BWS patients had patUPD for region 11p15.5 (with two paternally derived copies of chromosome 11p15 and no maternal contribution to this region). Further analysis of BWS patients with 11p and 11q markers revealed variable duplication of the paternal genomic region (isodisomy) in the UPD cases (Ref. 60).

Although the region of UPD varies, it always involves IC1 and IC2, thus resulting in overexpression of paternally active genes like *IGF2* and silencing of maternally active genes like *CDKN1C*, which may both contribute to the abnormal phenotype. Consistent with this is the finding that the risk of tumour development seems to be higher (50%) for patients with patUPD 11p15.5 than for BWS patients in general (7.5%) (Ref. 60). The most likely explanation would be the deregulation of tumour-relevant imprinted genes in at least one of the ICs. In addition, known maternal 11p15.5 trisomic cases are not associated with BWS or Wilms' tumour, but rather with growth retardation (Ref. 61).

The cases presented by Henry and co-workers (Ref. 60) also demonstrate that somatic mosaicism for UPD must have occurred after fertilisation. This view is supported by the report of a specific loss of maternal 11p15 alleles in cells of focal hyperplastic pancreatic areas, resulting in isolated persistent hyperinsulinaemic hypoglycaemia not observed in normal pancreatic cells (Ref. 62). Although in cases of patUPD all imprinted genes in both clusters should be deregulated, a variable BWS(-like) phenotype ranging from isolated hemihypertrophy up to a full EMG spectrum (Ref. 63) can result, depending on the population of cells affected by this postzygotic event. In cases with inherited or de novo *CDKN1C* defects, where the mutations are present in all cells, different phenotypes might emerge from variable penetrance of the genetic defect.

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Hypomethylation or microdeletion at KCNQ1OT1

In addition to the *CDKN1C* gene, the proximal gene cluster IC2 contains *KCNQ1*, a maternally expressed gene encoding the subunit of a voltage-gated potassium channel, together with its antisense transcript *KCNQ1OT1* (formerly called *LIT1* for 'long QT intronic transcript 1'). Mutations in the *KCNQ1* gene (formerly called *KvLQT1*) have not been described in BWS patients, but rather in persons suffering from cardiac arrhythmia syndromes like the Long-QT syndrome 1 (Romano–Ward syndrome) (Ref. 64) or the Jervell–Lange–Nielsen syndrome (Ref. 65). Although the BWS-associated chromosomal breakpoints cluster mainly within *KCNQ1*, functional defects of this gene cannot be associated with any phenotypic finding in BWS patients. Rather, detailed analysis has implicated the antisense gene *KCNQ1OT1* in epigenetic control.

KCNQ1OT1 lies within the *KCNQ1* locus, overlapping with intron 10 (containing the putative promoter), exon 10 and intron 9 (Refs 66, 67). It has no obvious coding potential and is maternally silent. The putative promoter contains two CpG islands that are maternally methylated (transcript silenced) and paternally unmethylated (*KCNQ1OT1* transcribed) (Refs 67, 68). This DMR, called *KvDMR* (for '*KvLQT1 DMR*'), is regarded as an in cis effective ICR (ICR2) for genes in IC2, because Horike and co-workers (Ref. 69) showed reactivation of the normally silent paternal alleles of *KCNQ1* and *CDKN1C* after deletion of *KvDMR* in the cell line DT40. Loss of maternal methylation of *KvDMR* is seen in over 50% of patients with sporadic BWS without UPD 11p15 (Ref. 68), thus representing the most common aberration in this syndrome. The effect of this hypomethylation regarding the aetiology of BWS is probably the reduction in *CDKN1C* expression, as similar phenotypic aspects can be observed in cases with *CDKN1C* loss-of-function mutations. As for *CDKN1C*, a CTCF-binding site has been described in KvDMR, which mediates a unidirectional enhancer-blocking activity that is reduced when the CpG islands are methylated (Ref. 70). In BWS patients, a maternal loss of methylation of these CpG islands is associated with hypermethylation at flanking sites, thus acquiring a patUPD IC2 epigenotype (Ref. 71).

ICR2 hypomethylation is the main BWS-related observation seen in female monozygotic twins (Ref. 72). They usually show discordance for the condition (only one is affected). This could be explained by a mosaicism of cells in the inner cell mass and an unequal splitting of these during twinning, thereby causing differential maintenance of imprinting in IC2, as already discussed (Ref. 72). The pre-implantation phase of embryonic development seems to be critical for imprint maintenance in IC2, not only in cases of monozygotic twinning but also in offspring born after assisted reproductive technology (ART). This is supported by a retrospective case–control study (Ref. 73) that found an approximately threefold increased risk of BWS development (with most cases showing abnormalities in IC2) in a population conceived by in vitro fertilisation compared with the general population. On the basis of these observations it is intriguing to speculate that the procedures of ART (e.g. the gamete stimulation protocol and maturation state, media used for embryo culture) may influence imprinting, although this remains controversial (Refs 74, 75, 76).

Recently, microdeletions have been reported encompassing the entire *KCNQ1OT1* gene locus that, when inherited maternally, cause BWS with silencing of *CDKN1C*. This observation supports the previously discussed model that the *KCNQ1OT1/KvDMR1* domain contains regulatory elements that play a mechanistic role in the regulation of imprinting in IC2. Interestingly, the deletion does not cause a phenotype when inherited paternally, suggesting that the *KCNQ1OT1* transcript itself is not essential for normal human development. The loss of *CDKN1C* expression as a result of the maternal *KCNQ1OT1/KvDMR1* deletion is probably due to loss of an enhancer within or telomeric to *KCNQ1OT1/KvDMR1* (Ref. 77). The specific IC2 aberrations found in BWS patients are illustrated in Fig. 2.

Hypermethylation in the H19 ICR, ICR microdeletions and loss of imprinting of IGF2

Although aberrations within the IC2 other than the patUPD (*CDKN1C* mutations, *KvDMR* hypomethylation, *KvDMR* microdeletion) account for most of the associated defects in BWS patients, the distal imprinting cluster IC1

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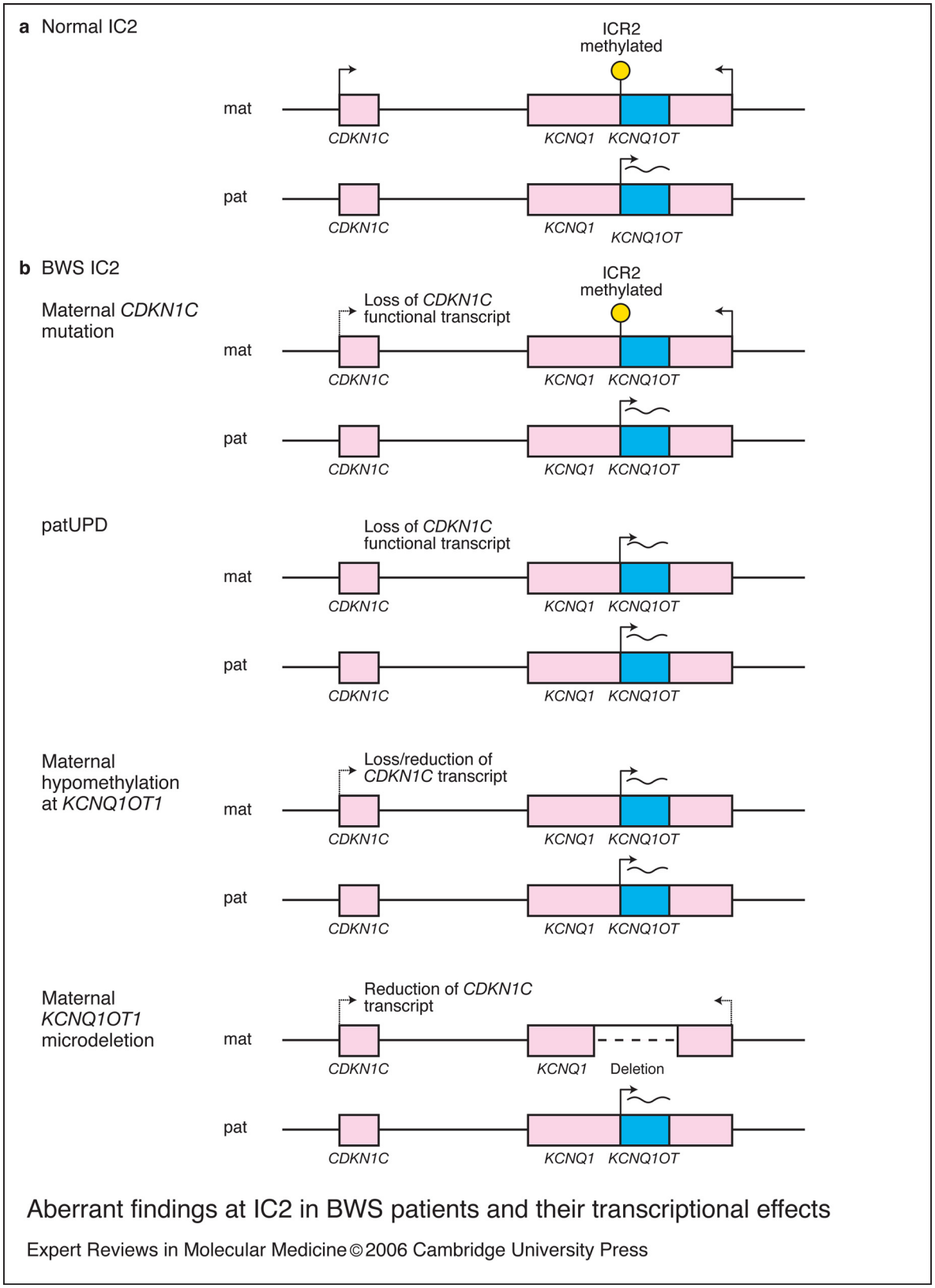


Figure 2. Aberrant findings at IC2 in BWS patients and their transcriptional effects. (*See next page for legend.*)

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enables a far better insight into epigenetic gene (dys)regulation in BWS. Several epigenetic IC1 alterations can be observed in BWS patients as well as in isolated tumours (e.g. Wilms' tumours), suggesting an association of these defects with tumourigenesis in general. In support of this idea is the finding that BWS patients with IC1 alterations have an increased risk for tumour development.

In IC1, *H19* and *IGF2* are the essential two genes. *H19* is maternally expressed and encodes an mRNA that, although without obvious protein-coding potential, may function as a tumour suppressor (Ref. 78). *IGF2*, in contrast, is paternally expressed in most cells. It encodes an embryonic growth factor that appears to be an autocrine regulator of cell proliferation (Ref. 79). Tight dosage and developmental-stage-specific regulation of IGF2 seems to be very crucial for regular tissue growth. *IGF2* (as well as *H19*) is widely expressed during prenatal development and downregulated in most adult tissues.

Over 20 years ago, Reeve and colleagues (Ref. 80) observed that in several Wilms' tumours *IGF2* transcripts were highly elevated as compared with the adjacent normal kidney tissues. The reason for this enhanced gene dosage was later reported to be due to loss of *IGF2* imprinting leading to biallelic expression, and this has been observed in multiple tumours including Wilms' tumour (Refs 12, 81) as well as in some patients with BWS (Ref. 82). In addition, hypermethylation of the *H19* promoter was reported in a significant number of BWS patients (Refs 13, 14) and Wilms' tumours (Ref. 81). In BWS, a hypermethylation at the maternal *H19* promoter is associated with loss of *H19* expression and biallelic *IGF2* expression. This finding is classified as an *H19*-dependent *IGF2* loss of imprinting (LOI), in contrast to the *H19*-independent biallelic expression of *IGF2*, where the monoallelic *H19* expression is maintained with normal methylation of *H19* (Ref. 83).

IGF2 is transcribed from four alternative promoters, encoding mRNAs with alternative 5′ untranslated regions but identical coding regions. Two differentially methylated regions are known within the human *IGF2* gene locus:

Figure 2. Aberrant findings at IC2 in BWS patients and their transcriptional effects. (*Legend; see previous page for figure.*) The *KCNQ1* gene, its antisense transcript *KCNQ1OT1* and the *CDKN1C* gene in the proximal imprinting cluster IC2 are affected by (epi-)mutations in Beckwith–Wiedemann syndrome (BWS). Imprinted genes are represented schematically, curved lines illustrate functional mRNA and the yellow lollipop denotes a known CpG methylation. (a) At a normal IC2, the promoter of the *KCNQ1OT1* gene is maternally methylated, resulting in silencing of the *KCNQ1OT1* gene but active transcription of the *KCNQ1* and *CDKN1C* genes. On the paternal allele, the promoter is unmethylated, resulting in active transcription of the *KCNQ1OT1* gene, while the two other genes in cis are silent. Note that the silencing on the paternal allele is not associated with a methylation in the vicinity of *CDKN1C* or *KCNQ1*. (b) At IC2 in BWS patients a variety of alterations can be found. First, mutations in *CDKN1C* result in loss of function of this gene, with a normal persisting epigenotype at IC2. Second, in cases of paternal uniparental disomy (patUPD), patients have two copies of the paternal epigenotype for IC2, resulting in a loss of maternal *CDKN1C* and *KCNQ1* transcription and a biallelic *KCNQ1OT1* transcription. (Loss of maternal *KCNQ1* transcription would be expected to lead to heart defects, as observed in the Long-QT syndrome; however, this is thought not to occur in BWS because *KCNQ1* is not imprinted in heart and therefore is biallelically expressed in this tissue. Thus normal function would be retained despite the epigenetic defect.) The known UPDs also extend into IC1, resulting in a biallelic *IGF2* expression and a loss of *H19* expression (not shown). Third, maternal hypomethylation at the *KCNQ1OT1* promoter also results in two copies of the paternal epigenotype for IC2, similar to the patUPD, with at least a reduction of *CDKN1C* transcription and loss of the maternal *KCNQ1* transcription. Fourth, maternal microdeletions encompassing the *KCNQ1OT1* promoter also result in two copies of the paternal epigenotype for IC2, similar to the patUPD, with at least a reduction of the *CDKN1C* transcription. Interestingly, the deletion does not cause a phenotype when inherited paternally, although the *KCNQ1OT1* transcript itself is lost. Therefore, it is thought that *KCNQ1OT1* itself is not necessary for normal development in humans. In fact, the microdeletion is thought to delete an enhancer within or telomeric to the *KCNQ1OT1* locus. Rare paternal duplications (<1%) and translocations/inversions (<1%) of maternal origin also affect IC2, but their epigenotypes are not yet well characterised; for that reason they are not listed here. Abbreviations: CDKN1C, cyclin-dependent kinase inhibitor 1C; IC, imprinting cluster; IGF2, insulin-like growth factor 2; KCNQ1, potassium channel, voltage-gated, KQT-like subfamily, member 1; *KCNQ1OT1*, KCNQ1 overlapping transcript 1.

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one in between the first and second promoter (DMR0), and a second that is maternally unmethylated at the 3′ end of the gene (DMR2). In Wilms' tumours and colorectal carcinomas with relaxed imprinting or 11p15.5 LOH, DMR0 was hypomethylated (Refs 84, 85). Sullivan and co-workers showed that the exon 9 region (DMR2) was biallelically unmethylated in the normal kidney and in Wilms' tumours regardless of intact *IGF2* imprinting. However, biallelic *IGF2* expression could be linked to epigenetic alterations at DMR2 by the group of Reik (Ref. 86), who found a hypermethylation of this DMR in BWS patients with LOI of *IGF2*.

However, single methylation aberrations in the *IGF2* or *H19* gene region cannot explain the coordinated regulation of the two genes. Significant progress in understanding the regulation of the two genes was made when it became clear that both genes compete for the same mesodermal and endodermal enhancers. Interaction of the enhancers with *IGF2* on the paternal allele and *H19* on the maternal allele is regulated by the paternally methylated ICR1 located between the two genes, approximately 5 kb upstream of *H19*. The human ICR1 consists of two clusters each containing one 450 bp A-repeat followed by three to four 400 bp B-repeats. The two A-repeats contain an evolutionary conserved duplicated octamer motif (Dyad-oct) that on the maternal allele seems to support the maintenance of the unmethylated status of the neighbouring B-repeats (Ref. 87). Each B-repeat, with the exception of the shortened B4-repeat, contains one binding site for the insulator zinc-finger protein CTCF (Ref. 88). The binding affinity of CTCF to the DNA is significantly reduced by CpG methylation (Ref. 89). At least two human CTCF-binding sites (in B1 and B7) are paternally methylated (Refs 90, 91) and there are observations suggesting that the CTCF binding sites in B2, B3 and B6 might also be allele-specifically methylated (Ref. 15). It has been shown that CTCF binds to the unmethylated ICR1 on the maternal chromosome, inhibiting the interaction of the maternal *IGF2* allele with mesodermal and endodermal enhancers downstream of *H19* and thereby resulting in maternal *H19* expression and *IGF2* silencing in cis. The resulting model is called the enhancer insulation model (Fig. 3) (Ref. 89). Insulation is thought to occur by chromatin looping (Fig. 3), based on interaction of DMRs at *H19* (ICR1) and *IGF2* in cis, thus creating a functional chromatin boundary and blocking or releasing the interaction of *IGF2* promoters and enhancers. On the maternal allele this DMR interaction is enabled by CTCF binding to the unmethylated maternal ICR1 (Ref. 92). Loss of paternal methylation at the ICR1 is observed in a proportion of human

Figure 3. Epigenetic IC1 findings and current regulatory models. (*Legend; see next page for figure.*) *IGF2* and *H19* in IC1 are reciprocally imprinted. Current models suggest that the reciprocal monoallelic expression is regulated by the ICR1, with specific factor binding at ICR1 affecting the function of nearby enhancers that are shared by *IGF2* and *H19*. (a) Enhancer-insulation model. The human ICR1 consists of two clusters, each containing one A-repeat followed by three or four B-repeats. Each B-repeat, with the exception of the shortened B4-repeat, contains one binding site for the insulator zinc-finger protein CTCF (CCCTC-binding factor), which binds to this site when the target sequence is unmethylated. The two human CTCF-binding sites in B1 and B7 are known to be paternally methylated. On the maternal chromosome CTCF binds to the unmethylated ICR1 and thereby inhibits the interaction of the maternal *IGF2* allele with enhancers downstream of *H19*, resulting in maternal *H19* expression and *IGF2* silencing The contribution of the mesodermal enhancer upstream of *H19* to the imprinted gene expression is still not resolved. Interaction with the *H19* promoter might be inhibited by CTCF binding at ICR1. (b) Chromatin-loop model. This proposed model transfers murine findings to the human orthologous chromosomal region. Enhancer insulation is thought to occur by chromatin looping, based on specific protein interaction of DMRs at ICR1 and *IGF2* in cis, thus creating a functional chromatin boundary. The resulting chromatin loop represents a transcriptionally inactive domain by blocking or releasing the interaction of *IGF2* promoters and enhancers. At *IGF2*, two DMRs have been described to date: one in intron 2, the other (which is paternally methylated) in intron 8/exon 9. Placement of the maternal *IGF2* promoters within the inactive chromatin domain by this looping (possibly as a result of interaction of ICR1 with the DMR in intron 2, involving CTCF) inhibits *IGF2* transcription. Positioning of paternal *IGF2* outside the chromatin domain requires interaction of ICR1 with the DMR in intron 8/exon 9 and makes the promoters accessible to transcription factors. Abbreviations: DMR, differentially methylated region; IGF2, insulin-like growth factor 2.

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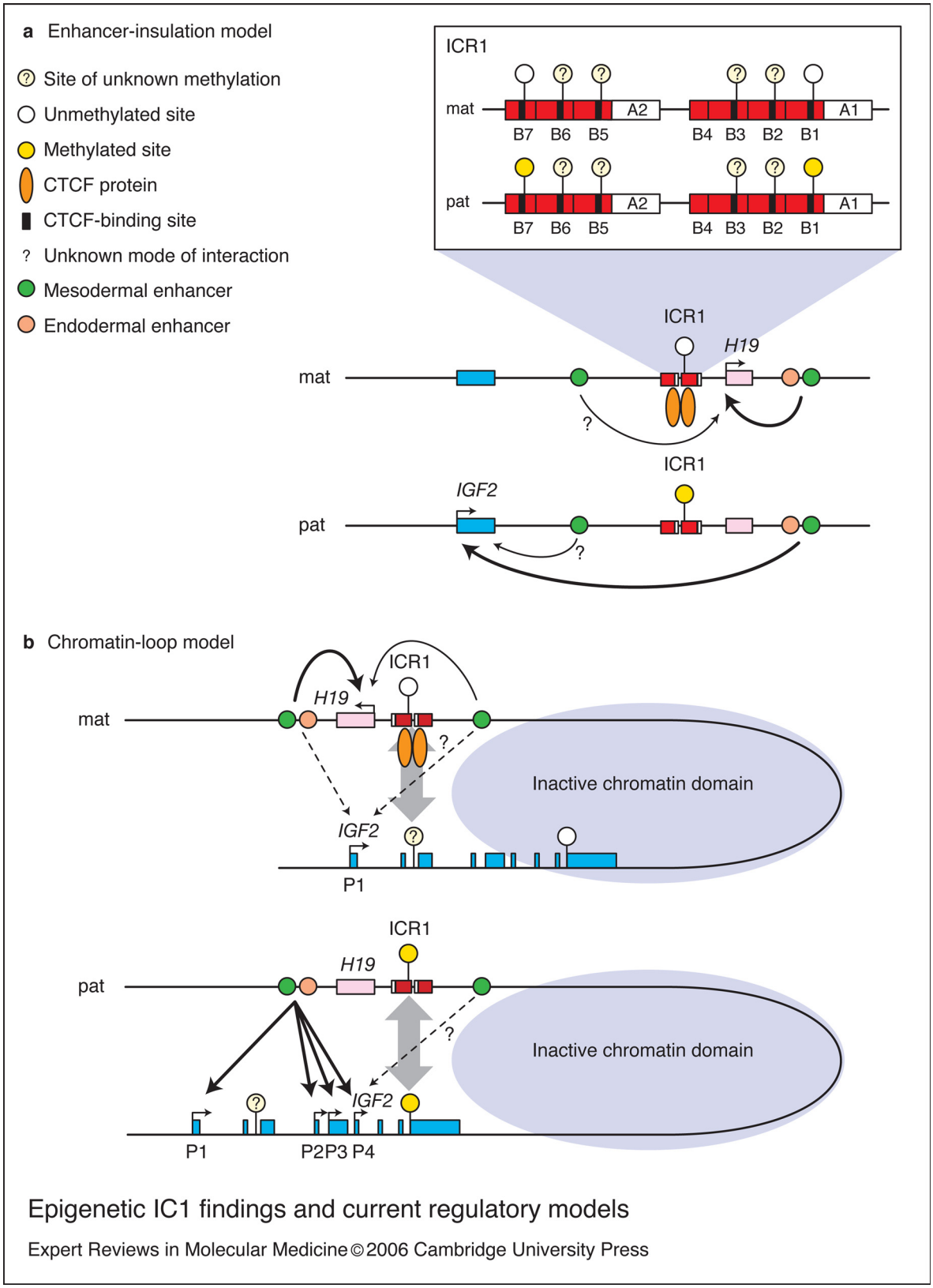


Figure 3. Epigenetic IC1 findings and current regulatory models. (*See previous page for legend.*)

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bladder cancer and results in LOI of *H19* with biallelic *H19* expression (Ref. 93). Recently, maternal ICR1 microdeletions have been reported in familial BWS cases, where fusion of B-repeats abolishes the A2-repeat and a variable number of B-repeats, and therefore CTCF-binding sites (Refs 15, 16). In all reported cases the individual microdeletions result in LOI of *IGF2*, although they were only partly accompanied by hypermethylation of the remaining maternal ICR1 (Ref. 94).

Disruption of imprint regulation in IC1 seems to have LOI of *IGF2* as a driving force for the phenotype observed in BWS and associated tumours as well as in other tumour entities. Mouse models with transgenic *Igf2* overexpression develop features of BWS dependent on the *Igf2* mRNA dosage, including prenatal and neonatal overgrowth, polyhydramnios, organ overgrowth, and macroglossia (Ref. 95). Although in mice a twofold increase in Igf2 dosage is sufficient to generate some of the somatic overgrowth features of BWS, biallelic *IGF2* expression in humans does not necessarily give the same result as significant biallelic expression was observed in approximately 10% of normal individuals (Ref. 96). Similarly, some members of one BWS family with the reported ICR1 microdeletion exhibit LOI of *IGF2* but do not develop BWS (Ref. 16). Since in mice a combination of inactivation of *Cdkn1c* and LOI of *Igf2* (resembling the patUPD situation in BWS patients) is necessary for a BWS-like phenotype, including dysplasic placentomegaly, kidney dysplasia, macroglossia and omphalocele (Ref. 97), it has been proposed that genes of both clusters have to be involved in BWS. The observation of enhanced Igf2 protein levels leading stage- and tissue-specifically to decreased levels of Cdkn1c (Ref. 98), suggests a common BWS causative pathway based on a concerted action of IGF2 and CDKN1C.

Concluding remarks

Although several molecular mechanisms have been revealed in the past decade that link genetic and epigenetic aberrations to the development of BWS, it is still not understood how the phenotypic spectrum originates. While precise definition of BWS symptoms and the dissociation from overlapping conditions that represent other syndrome entities have improved the clinical management of individuals with BWS, no simple (epi-)genotype–phenotype correlation can be established. One central question still remains unanswered: why do some BWS patients develop tumours whereas others do not? Large registry studies revealed that both IC1 and IC2 are involved in BWS pathogenesis, but ICR1 defects have not yet been implemented in BWS diagnostic strategies. Hypermethylation of ICR1 is likely to be observed not only in isolated tumours, but also in BWS patients.

Future studies on the nature and function of insulation by the ICR1 should reveal to what extent its repetitive structure is necessary to achieve monoallelic gene expression. The current ICR1 functional models are primarily based on murine findings. Although the mouse has a conserved syntheny of the BWS-relevant 11p15.5 region on chromosome 7, the main regulatory elements, namely the ICR1 and additional DMRs, display essential structural differences. In this context, identification of the human genomic regions interacting with ICR1 that are most likely differentially methylated should refine the chromatin looping model and verify this mode of action in human cells. A detailed understanding of human ICR function will also facilitate identification of molecular downstream signalling cascades associated with BWS and tumour development when disturbed.

Another point of interest of future BWS research will be the analysis of the IC1–IC2 interconnection at a genomic as well as at a single-gene level. The most relevant factors for the BWS phenotype, *IGF2* and *CDKN1C*, are regulated by two independent ICRs, yet seem to be part of a common pathway. Uncovering of such cascades will aid understanding of how specific BWS symptoms evolve even when only one or the other locus is affected by an (epi-)mutation. Based on the findings in familial cases discussed above it is likely that not only the gene sequence but also the level of expression can trigger causative effector cascades for BWS. Definition of critical transcription thresholds at critical developmental stages will facilitate the identification of conditions that make one patient prone to neoplasms and leave others without tumours. The hope would be to use these findings to provide accurate risk assessments for the individual BWS patient.

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Further reading, resources and contacts

The Online Mendelian Inheritance in Man (OMIM) website provides a brief overview of Beckwith–Wiedemann syndrome with references to the significant primary literature:

http://www.ncbi.nlm.nih.gov/Omim

The genetests site provides a description and review of Beckwith–Wiedemann syndrome:

http://www.GeneTests.org

A catalogue of imprinted genes and parent-of-origin effects in humans and animals can be found at:

http://igc.otago.ac.nz/home.html

A more general website for several imprinting resources can be found at:

http://www.geneimprint.com/

Authors' institute URL: http://molgen.medizin.uni-mainz.de

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Features associated with this article

Figures

Figure 1. Summary of known genetic and epigenetic mutations affecting the BWS-associated region on 11p15.5.
Figure 2. Aberrant findings at IC2 in BWS patients and their transcriptional effects.
Figure 3. Epigenetic IC1 findings and current regulatory models.

Table

Table 1. Clinical characteristics of Beckwith–Wiedemann syndrome and related overgrowth syndromes.

Box

Box 1. Summary of genetic and epigenetic features of Beckwith–Wiedemann syndrome.

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