Imprinting of *RBI* (the new kid on the block)

Karin Buiting, Deniz Kanber, Bernhard Horsthemke and Dietmar Lohmann

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Abstract

Recent data have revealed that the paradigmatic tumour suppressor gene *RBI* on chromosome 13 is preferentially expressed from the maternal allele. Imprinted expression of *RBI* is linked to a differentially methylated CpG island in intron 2 of this gene (CpG 85). On the paternal chromosome, CpG 85 is unmethylated and acts as a weak promoter of an alternative *RBI* transcript. Paternal mRNA levels are probably reduced as the result of transcriptional interference of the regular promoter and the alternative promoter on this chromosome. CpG 85 is part of a truncated processed pseudogene (*KIAA0649P*) that integrated into the *RBI* gene prior to the speciation of extant primates. It is plausible that differential penetrance and variation of age at diagnosis, which have been observed in patients with hereditary and non-hereditary retinoblastoma, respectively, are a consequence of imprinted expression of the *RBI* gene. Interestingly, *RBI* is imprinted in the same direction as *CDKNIC*, which operates upstream of *RBI*. The imprinting of two components of the same pathway indicates that there has been strong evolutionary selection for maternal inhibition of cell proliferation.

Keywords: *RBI; imprinting; DNA methylation*

DISCOVERY OF AN IMPRINTED CPG ISLAND IN THE *RB1* GENE

In diploid organisms, usually both alleles of a gene are active or inactive. In placental mammals, however, a small subset of genes is imprinted and expressed in a parent-of-origin dependent manner. Imprinting is an epigenetic process leading to parent-of-origin specific DNA methylation and gene expression. Because of experimental limitations, the identification of imprinted genes in humans is challenging. Kanber *et al.* [1] have identified a novel imprinted locus by genome wide CpG methylation analysis of DNA from blood in a patient who was hypomethylated at all known imprinted loci. By this, a 1.2 kb CpG island inside intron 2 of the human retinoblastoma gene (*RB1*) was found to show parent-of-origin specific methylation—it is methylated on the maternal chromosome 13 and unmethylated on the paternal chromosome 13. This CpG island (CpG 85) serves as a promoter for an alternative transcript of the human retinoblastoma gene (*RB1*), which is expressed from the unmethylated paternal chromosome only (Figure 1). The first exon of this alternative transcript is E2B, which is spliced onto exon E3. This feature distinguishes CpG 85 from two other CpG islands associated with the *RB1* gene: CpG 42, which is located a few kilobasepairs upstream of CpG 85, is biallelically methylated, whereas CpG 106, which overlaps the *RB1* promoter and exon E1, is biallelically unmethylated. Greger *et al.* [2] were the first to show that CpG 106 is methylated in some retinoblastomas. This was the first hint that promoter methylation of a tumour suppressor gene plays a role in tumourigenesis.

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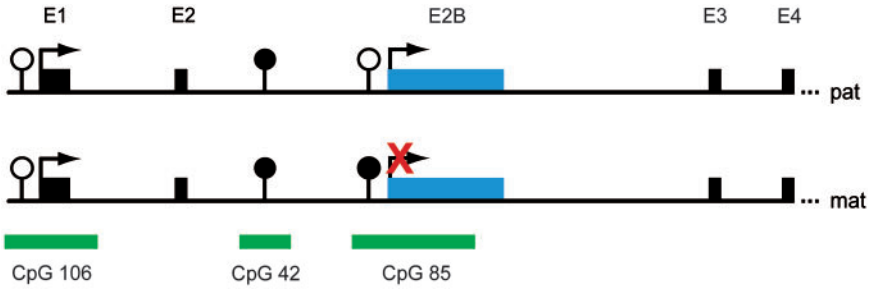

Figure 1: Physical map. Schematic representation of the 5′-region of the *RBI* locus (not drawn to scale) and location of CpG islands (CpG 106, CpG 42, CpG 85). EI, E2, E3 and E4 are regular exons whereas E2B is the newly discovered exon, which is the start exon of the alternative *RBI*-2B transcript. Open lollipops, unmethylated CpGs; filled lollipops, methylated CpGs; arrows, transcription start sites; pat, paternal allele; mat, maternal allele.

EVOLUTION OF CPG 85

CpG 85 is part of a 4.5 kb 5′ truncated processed pseudogene derived from the protein coding gene *KIAA0649* on chromosome 9 that was integrated in the *RB1* locus in reverse orientation (Figure 2). An independent retrotransposition event has lead to the existence of four additional truncated processed pseudogenes of *KIAA0649* located in close proximity on chromosome 22q11.21. The open reading frame, which is located in exon 4 of the ancestral gene, is lost in all five processed copies. In contrast to the chromosome 13 copy, no evidence for genomic imprinting was found for the chromosome 22 integrated sequences.

The four small (<300 bp) CpG islands (CpG 19, CpG 17, CpG 26 and CpG 19) present in exon 4 of *KIAA0649* are not present in the pseudogene copies on chromosome 22. In the chromosome 13 pseudogene copy (*KIAA0649P*), however, these CpG islands correspond to two big CpG islands CpG 85 and CpG 42 (Figure 2). Thus, it appears that the human CpG 85 has evolved from two small CpG islands in the ORF of *KIAA0649*. However, it is also possible that an originally big CpG island in the ancestral locus was maintained in *KIAA0649P*, but deteriorated in the ancestral gene as well as in the chromosome 22 pseudogene copies after the retrotransposition events.

In contrast to CpG 42, which is completely methylated, CpG 85 has acquired differential methylation after retrotransposition leading to allele specific methylation and monoallelic expression of the alternative *RB1* transcript. *KIAA0649P* and the CpG island are also present in the *RB1* gene of other primates (chimp, orangutan, macaque) and New World monkeys (marmoset), but not in the *Rb1* gene of mice and rat (for details see [1]).

ALLELIC EXPRESSION IMBALANCE OF *RB1* TRANSCRIPTS

If the alternative transcript (*RB1*) were expressed independently of and in addition to the regular paternal *RB1* transcript, then the total level of paternal transcripts should be higher than that of the maternal transcripts. However, analysis of parent-of-origin dependent expression of *RB1* transcripts revealed an ~3-fold excess of maternal *RB1* mRNA [1]. In mice, which do not have the intronic CpG island, no parent-of-origin specific expression imbalance was found, indicating that skewed allelic expression of the human *RB1* is linked to the differentially methylated CpG 85. This notion was further substantiated by the finding that demethylation of CpG 85 in lymphoblastoid cell lines by 5-aza-2′-deoxycytidine treatment resulted in reduced skewing of the allelic *RB1* transcripts, which is to be expected because after loss of CpG 85 methylation the maternal allele resembles the paternal allele [1]. These results show that allele-specific methylation of CpG 85 affects expression of *RB1*, probably by transcriptional interference.

TRANSCRIPTIONAL INTERFERENCE

Transcriptional interference refers to a mechanism in which the transcription of one gene has a suppressive influence on the transcription of another gene [3]. This suppressive influence is due to the interference of the RNA polymerase II machinery transcribing one gene with transcriptional initiation, elongation or termination at a neighbouring gene. Thus the act of transcription itself rather than the sequence of the transcribed RNA is important.

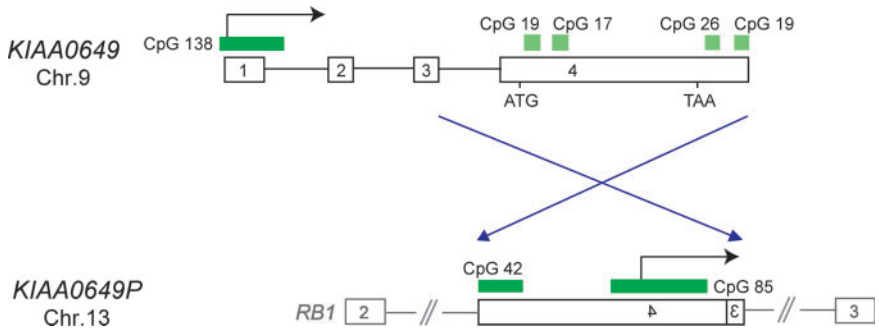

Figure 2: Structure of *KIAA0649* and the processed pseudogene *KIAA0649P* on human chromosome 13. The two small CpG islands in exon 4 of *KIAA0649* (CpG 19/CpG 17 and CpG 26/CpG 19) correspond to CpG 85 and CpG 42 in the chromosome 13 copy, respectively. The open reading frame of *KIAA0649* in exon 4 is indicated by the start and stop codons (ATG and TAA) and is lost in *KIAA0649P*. CpG 85 serves as a promoter for an alternative *RB1* transcript (transcript *RB1-2B*). Arrow at CpG 138 and CpG 85, orientation of transcription; arrows between *KIAA0649* and *KIAA0649P* indicate integration of the processed *KIAA0649* gene in intron 2 of the *RB1* gene.

Transcriptional interference is the most likely mechanism underlying skewed *RB1* expression depending on differential CpG 85 methylation. Possibly, the transcription complex binding to the unmethylated 2B-promoter acts as a roadblock for the regular transcript on the same (paternal) allele resulting in reduced abundance of paternal *RB1* transcripts (Figure 3) [3].

BIOLOGICAL EFFECTS OF *RB1* IMPRINTING

The findings reported here extend the observations on epigenetically controlled transcriptional interference by retrotransposons [4–6] to include truncated processed pseudogenes and support the notion that genomic imprinting builds on host defence mechanisms [7–10]. Imprinting of *RB1* may explain certain parent-of-origin effects in human phenotypes caused by mutations in the *RB1* gene.

Mutations of the *RB1* gene cause predisposition to retinoblastoma and to other tumours

According to current knowledge, the only phenotypic consequences of *RB1* gene mutations in the human are tumour predisposition and tumour development. Specifically, mutations in this gene are a prerequisite for development of retinoblastoma (Rb).

Rb is a malignant tumour of the eye that is almost exclusively diagnosed in young children. In 60% of patients, the tumour affects one eye only (unilateral Rb) while the remaining 40% of patients have tumours in both eyes (bilateral Rb). Most children with bilateral Rb show multiple tumour foci in both eyes. Moreover, patients with bilateral Rb have a high risk to develop other tumours (second cancer) later in life. Almost all patients with sporadic bilateral Rb and all patients with familial Rb have a hereditary tumour predisposition that is transmitted as an autosomal dominant trait (hereditary Rb). Hereditary Rb is caused by heterozygous mutations in the *RB1* gene. Tumour development is initiated by a second mutation that inactivates the other allele of the *RB1* gene. Most patients (>85%) with sporadic unilateral Rb have non-hereditary Rb. In these patients, the first and second *RB1* gene mutations are detected in the tumour only.

Genotype–phenotype correlations

Hereditary Rb shows variable phenotypic expression. The main parameters of variation are (i) the number of eyes affected (no Rb, unilateral Rb and bilateral Rb), (ii) age at diagnosis and (iii) the development of second tumours later in life. Because of the stochastic nature of the mutational events that are required for tumour development (notably second mutations), phenotypic expression in individual patients is influenced by chance. In addition, phenotypic expression in hereditary Rb varies depending on the functional type of the first (i.e. predisposing) mutation in the *RB1* gene:

- *loss-of-function:* most germline *RB1* gene mutations are point mutations that result in premature termination codons (nonsense, frameshift, splice

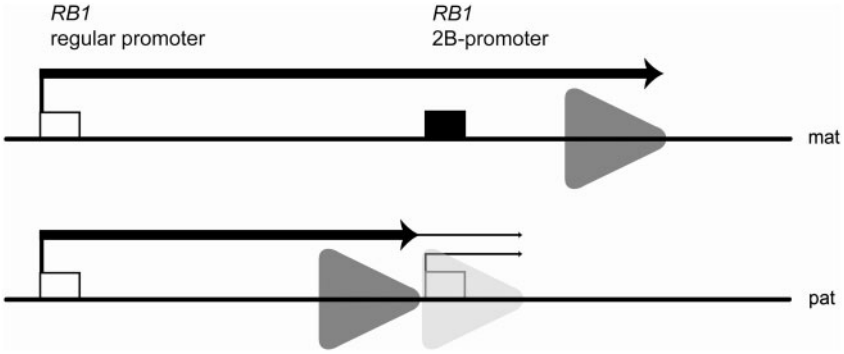

Figure 3: The road block model of transcriptional interference. The transcription complex binding to the paternally unmethylated 2B-promoter acts as a roadblock for the transcription complex of the regular transcript on the same allele. Open rectangle, unmethylated promoter; filled rectangle, methylated promoter; dark grey triangle, transcription complex of the regular *RBI* transcript; light grey triangle, transcription complex of the *RBI*-2B transcript.

mutations causing out-of-frame exon skipping) and trigger nonsense mediated decay. Families segregating a loss-of-function mutation almost invariably show complete penetrance and bilateral Rb.

- *partial loss-of-function:* patients heterozygous for mutations that do not result in premature termination (regulatory, missense, in-frame) develop fewer Rb foci and families segregating partial loss-of-function mutations often show incomplete penetrance (low-penetrance retinoblastoma).

In non-hereditary Rb the spectrum of *first* somatic *RB1* gene mutations is much the same as the spectrum of *RB1* germline mutations. The spectrum of *second* somatic mutations, however, is distinct in two respects. First, in about 70% of Rb tumours chromosomal mechanisms such as mitotic recombination have led to loss of the normal and thus demasking of the mutant allele (loss of heterozygosity). Second, about 10% of Rb tumours show hypermethylation of the CpG island associated with the regular promoter of the *RB1* gene.

Parent-of-origin effects associated with germline mutations in the *RB1* gene

In order to detect parent-of-origin effects one might wish to compare phenotypic expression between patients who have a new germline mutation on the paternal allele to those patients who have the same functional type of new germline mutation on the maternal allele. However, for reasons that are most likely not associated with imprinting (most notably: differential mutation rate), almost all new *RB1* gene mutations arise in the paternal germline (the only known exception being gross deletions). Given this situation, the best source for an analysis of parent-of-origin effects are extended families with several transmissions of the same mutant allele via both sexes.

Survival of patients with retinoblastoma improved only a few generations ago. Therefore, reports of families with retinoblastoma reaching back several generations are rare. In 1960, Macklin [11] reported a retinoblastoma family (pedigree 60 and 63) with remarkable variation of phenotypic expression and penetrance. When analysing this family under the aspect of parental origin, which was not done in the paper by Macklin [11], it appears that phenotypic expression in sibships is more severe if the predisposing allele was transmitted via the father (five bilateral and four unilateral patients among 29 children of fathers at risk and no patient in any of 22 offspring of mothers at risk, Fisher's exact test two-tailed $P = 0.00007$). When analysing large Rb pedigrees published since then it becomes clear that the parent-of-origin effect seen in Macklin's Rb family is unusual but not without parallel.

A parent-of-origin effect in two families segregating the same splice site mutation in the *RB1* gene, IVS6+1G>T, that causes skipping of exon 6, was reported by Klutz *et al.* [12]. We found that variation of phenotypic expression was associated with the relative abundance of mutant transcript and with the sex of the parent that transmitted the mutant allele. The regular promoter of the *RB1* gene showed no abnormal methylation status in mutation carriers of these families. The direction of the parent-of-origin effect is the same as in the family reported by Macklin: paternally transmitted mutant alleles are associated with a more severe phenotype.

Since then, additional families segregating the IVS6+1G>T mutation have been identified. In all, nine families are known. In these families, a total of 38 individuals have inherited the mutant allele from their fathers and 22 individuals have inherited the mutant allele from their mothers. In summary, of the 38 individuals with mutant alleles of paternal origin 18 (47%) developed bilateral Rb and 9 (24%) showed unilateral Rb. The remaining 11 (29%) stayed free of Rb. By contrast, no one of the 22 individuals with mutant alleles of maternal origin developed bilateral Rb and only two (9%) had unilateral Rb. Most family members heterozygous for a IVS6+1G>T allele of maternal origin did not develop Rb (20/22, 91%). To address the possibility that all these families share a single founder in whom the IVS6+1G>T mutation has occurred on the background of a genetic variation that is required to bring about the parent-of-origin effect we determined the haplotype background in phase with the IVS6+1G>T allele in those families available to us (Lohmann *et al.*, unpublished data). We genotyped a set of sparsely correlated SNPs from the *RB1* gene region (rs1981434, rs2227311, rs2854345, rs3092904, rs4151540, rs4151551, rs4151636) [13] in four families. In three families the mutant allele was in phase with the most frequent (66%) European haplotype. However, the haplotype background was distinct in the fourth family (other alleles at rs1981434, rs3092904 and rs4151540). Therefore, common ancestry of the mutant allele is very unlikely and we conclude that the parent-of-origin effect in these families is associated with the IVS6+1G>T base substitution proper. It is to be noted that all known patients with other base substitutions that result in exon 6 skipping (i.e. IVS6+1G>A or >C) have non-familial retinoblastoma and—if parental DNA was available for testing—new germline mutations. Therefore it is unlikely, that the aberrantly spliced *RB1* mRNA that lacks exon 6 *per se* is the cause of the parent-of-origin effect.

Parent-of-origin effects associated with somatic mutations in the *RB1* gene

Genetic alterations in sporadic osteosarcoma can involve somatic mutations of the *RB1* gene, including chromosomal mechanisms that result in loss of heterozygosity at loci in the region of the *RB1* gene. In a series of sporadic osteosarcomas, Toguchida *et al.* [14] identified 13 tumours with allele loss at the *RB1* locus and found that in 12 of them the initial mutation was on the paternal gene ($P = 0.03$ Fisher's exact test). Such a bias suggests that the oncogenic effect of mutations on the paternal *RB1* allele is higher compared to that of mutations on the maternal allele. To date, no other reported study has addressed *RB1* parent-of-origin effects in osteosarcoma.

In a study designed to identify clinical parameters discriminating patients with hereditary Rb we found that patients with the initial mutation on the paternal *RB1* allele had an earlier age at diagnosis than patients with an initial mutation on the maternal allele (median age at diagnosis 482 versus 865 days [15]). When comparing the distribution of age at diagnosis (Figure 2 in [15]) it appears that this difference might be caused by a subgroup of patients with early diagnosis and initial mutation on the paternal allele.

The above-mentioned study [15] included 111 tumours from patients with non-hereditary Rb and known parental origin of the first mutation (excluding whole *RB1* gene deletions). In these data a small excess of paternal first mutations is observed (61/111, 55%). We now know the parental origin of allele loss in 171 tumours from patients with sporadic unilateral Rb (Lohmann *et al.*, unpublished data). Of these, 104 (61%) have retained the paternal allele which is a significant departure from an expected 50:50 ratio (likelihood-ratio Chi^2 $P < 0.0045$) in favour of first somatic mutations on the paternal *RB1* allele. This bias suggests that in some Rbs the oncogenic effect of mutations on the paternal *RB1* allele is higher compared to that of mutations on the maternal allele.

THE GREATER PICTURE: IMPRINTING AND CELL-CYCLE REGULATION

The *RB1* gene is not the only cell-cycle regulatory gene known to be imprinted. Specifically, the *CDKN1C* gene, which encodes a cyclin-dependent kinase inhibitor operating upstream of the RB1 protein, is maternally expressed [16]. Intriguingly, the imprint imposed on the *RB1* gene acts in the same direction. A rough sketch of the pathway is shown in Figure 4. Unphosphorylated RB1 prevents the transcription factor E2F from binding to its target genes, recruits the histone deacetylase HDAC1 to silence them and thus blocks cell-cycle progression. Phosphorylation of RB1 by a cyclin-dependent kinase (CDK) relieves this block by allowing E2F to activate its target genes. CDK is inhibited by

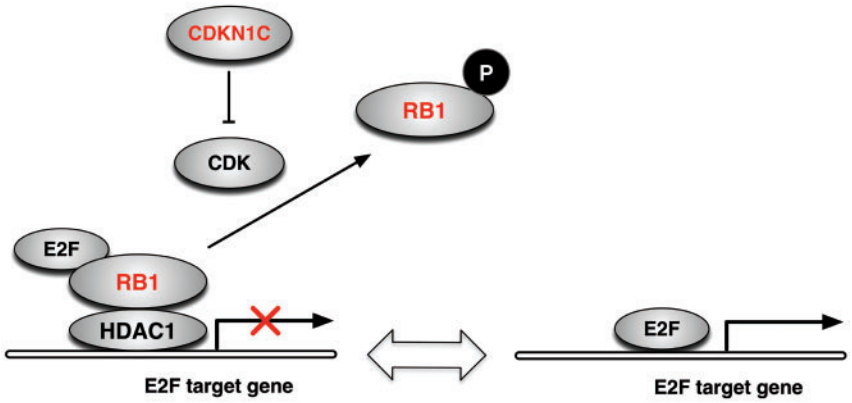

Figure 4: Overview of the E2F/RBI pathway. When unphosphorylated, the product of the *RBI* gene binds the transcription factor E2F thus preventing it from binding to its target genes. In addition, RBI recruits the histone deacetylase HDACI to silence E2F dependent genes and thereby blocks cell-cycle progression. Upon phosphorylation of RBI by cyclin-dependent kinase (CDK) E2F is released and activates its target genes thus deblocking cell-cycle progression. CDKNIC can block cycle progression by inhibiting CDK.

CDKN1C. Thus, both RB1 and CDKN1C block cell-cycle progression. The *CDKN1C* gene maps to 11p15.5 and is expressed preferentially from the maternal allele. Mutations of the maternal allele account for ~5% of patients with Beckwith–Wiedemann syndrome, which is an overgrowth syndrome. Imprinted expression of *CDKN1C* is regulated by a differentially methylated region (DMR) within the *KCNQ1* gene. The DMR is methylated on the maternal chromosome and unmethylated on the paternal chromosome. It serves as a promoter for a long non-coding RNA (*LIT1* or *KCNQ1OT1*), which is expressed from the paternal allele only. The unmethylated DMR and/or transcription of *LIT1* appears to downregulate the paternal allele of *CDKN1C* by a not yet completely understood mechanism. Similar to *RB1*, *CDKN1C* expression is not strictly monoallelic, probably because complete imprinting would make an individual vulnerable to childhood cancer and would thus have been selected against. On the other hand, imprinting of two components of the same pathway (*CDKN1C* and *RB1*) indicates that there has been evolutionary selection for maternal inhibition of cell proliferation.

Key Points

- The human retinoblastoma gene (*RBI*) is imprinted.
- Imprinted expression of *RBI* is linked to a differentially methylated CpG island within a truncated processed pseudogene that integrated into the *RBI* locus.
- Imprinting of *RBI* probably explains parent-of-origin effects in patients with retinoblastoma.
- Imprinting of *RBI* indicates that there has been strong evolutionary selection for maternal inhibition of cell proliferation.

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