

RESEARCH PAPER



# DNA hypermethylation/boundary control loss identified in retinoblastomas associated with genetic and epigenetic inactivation of the *RB1* gene promoter

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## ABSTRACT

DNA hypermethylation events occur frequently in human cancers, but less is known of the mechanisms leading to their initiation. Retinoblastoma, an intraocular cancer affecting young children, involves bi-allelic inactivation of the *RB1* gene (*RB1*<sup>-/-</sup>). *RB1* encodes a tumour suppressing, cell cycle regulating transcription factor (*pRB*) that binds and regulates the *RB1* core and other *E2F* responsive promoters with epigenetic functions that include recruitment of histone deacetylases (*HDACs*). Evidence suggests that bi-allelic epigenetic inactivation/hypermethylation of the *RB1* core promoter (*Pr*<sup>E-/E-</sup>), is specific to sporadic retinoblastomas (frequency~10%), whereas heritable *RB1* promoter variants (*Pr*<sup>-/+</sup>, frequency~1-2%) are not associated with known epigenetic phenomena. We report heritable *Pr*<sup>-/-</sup> retinoblastomas with the expected loss of *pRB* expression, in which hypermethylation consistent with distal boundary displacement (*BD*) relative to normal peripheral blood DNAs was detected in 4/4 cases. In contrast, proximal *BD* was identified in 16/16 *RB1*<sup>-/-</sup> retinoblastomas while multiple boundaries distal of the core promoter was further identified in *Pr*<sup>E-/E-</sup> and *Pr*<sup>E-/E+</sup> retinoblastomas. However, weak or no DNA hypermethylation/*BD* in peripheral blood DNA was detected in 8/9 *Pr*<sup>-/+</sup> patients, with the exception, a carrier of a microdeletion encompassing several *RB1* promoter elements. These findings suggest that loss of boundary control may be a critical step leading to epigenetic inactivation of the *RB1* gene and that novel DNA methylation boundaries/profiles identified in the *RB1* promoter of *Pr*<sup>-/-</sup> retinoblastomas, may be the result of epigenetic phenomena associated with epimutation in conjunction with loss of *pRB* expression/binding and/or *RB1* promoter interactions with boundary control elements.

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## Introduction

DNA hypermethylation in human cancers has been widely reported, but relatively little is known regarding the molecular signals that initiate epigenetic changes such as CpG hypermethylation, histone modification, and nucleosome packaging.

Retinoblastoma is an autosomal dominant retinal malignant tumour of the eye affecting young children with an estimated global incidence of ~1:18,000 live births. Of these, ~44% are heritable including ~27% bilateral disease and ~17% unilateral [1]. Retinoblastomas involve the tumour suppressor gene *RB1* where tumour initiation requires pathogenic variants affecting both *RB1* alleles.

The second allele most commonly involves somatic loss of heterozygosity (LOH), which on average affects ~70% of retinoblastomas.

Pathogenic variants affecting the *RB1* gene vary from deleterious high penetrance null variants including deletions and nonsense variants, to relatively low penetrance missense variants, cryptic splice variants and/or *RB1* core promoter variants. The *RB1* core promoter is a regulatory region containing elements sufficient for *RB1* transcriptional expression (between nucleotides c.-220 to c.-170 upstream of the *RB1* gene start codon) [2]. Known regulatory variants that occur within the *RB1* core promoter are expected to affect *RB1* gene expression by

disrupting the binding of transcription factor(s). An alternative mechanism of *RB1* gene regulatory inactivation observed in ~10% of retinoblastomas, involves bi-allelic epigenetic silencing of the *RB1* promoter ( $Pr^{E-/E-}$ ) [3]. Epigenetic silencing of the *RB1* gene in retinoblastoma is generally accepted as a sporadic phenomenon [3] and is thought to involve DNA hypermethylation. In such cases, the primary DNA sequence of the *RB1* gene and its promoter is otherwise unaffected.

While there has been generally no known association between promoter variants and DNA hypermethylation, a previous study identified hypermethylation of the *RB1* core promoter in peripheral blood DNA and melanoma of a patient with a germline *RB1* promoter variant [c.-188\_-187delinsGG][4], an observation considered by the authors to be consistent with an ‘epimutation event’, where a genetic variant gives rise to epigenetic changes of adjacent DNA. No other previous studies have identified *RB1* promoter variants in association with DNA hypermethylation while the requirements for epimutation(s) to exert epigenetic effects have not been explored.

*RB1* gene transcription is ubiquitous with minimal expression variability between tissues (<http://www.genecards.org/cgi-bin/carddisp.pl?gene=RB1>). The retinoblastoma gene protein product (*pRB*) controls cell cycle G1/S transition by binding and inactivating *E2F* transcription factor. One of the several pocket proteins, *pRB* complexes directly with *E2F*-docking protein heterodimers (*E2F-DP*) where binding and masking the *E2F* transactivation domain can lead to transcriptional repression. Some promoters and transcription factors are inhibited by recruitment of histone deacetylase [5], a function related to possible epigenetic effects of *pRB* [6,7]. Other promoters, however, are resistant and are repressed by *pRB* binding directly to transcription factors [6]. The self-regulation of the *RB1* gene may also involve activated expression of long non-coding RNAs, e.g., *RB1-DT* (aka LINC00441) which is transcribed in the opposite direction to the *RB1*

gene and which may repress *RB1* gene expression by recruiting DNA methyltransferase [8,9] or alternative formation of a triple helix as previously described in other genes [10] might also be possible.

Evidence suggests that the organization of chromatin into distinct epigenetically defined regions involves cohesin, a protein which extrudes chromosomal DNA in a manner thought to involve the ‘loop extrusion’ model [11]. In this model, DNA is extruded through a handcuff-structured cohesin molecule generating loops which become anchored to cellular structures such as the nuclear lamina [12]. Certain DNA sequence motifs bind to CCCTC-Binding factors [13] (*CTCF*) which are needed to anchor looped DNA in order to regulate the 3D structure of chromatin [14] potentially forming boundaries between an active A domain (euchromatin) and an inactive B domain (heterochromatin). A single *CTCF* binding site has been recently experimentally verified immediately upstream of the *RB1* gene core promoter with high homology to known *CTCF* binding sites [15]. The *RB1* *CTCF* binding site consists of four separate DNA contact regions which are assumed to correspond to previously identified regions detected by DNase I footprinting [16]. Interestingly, the *CTCF* binding site overlaps with the start of a long non-coding RNA transcript *LINC00441* which evidence suggests down-regulates *RB1* gene transcription in human cancers [8,9]. As the *LINC00441* transcript overlaps with the *CTCF* binding site it is possible that *CTCF* binding and *LINC00441* transcription are competing processes.

While much is known regarding *CTCF* and the biology of loop extrusion, the important subtle effects of how transcription factors and other factors modulate chromosomal boundary locations are less well characterized. In this study, we examine *RB1* promoter methylation status of retinoblastomas and peripheral blood DNA affected by *RB1* promoter variants to determine whether there may be a relationship between genetic/epigenetic changes and DNA methylation boundary control.

## Materials and methods

### Tissue specimens and bisulphite modification of DNA

All retinoblastomas (Germany, England and Toronto) were analysed anonymously following the guidelines of local Ethics committees. DNA was isolated by Roche High Pure DNA extraction kit. Genomic DNA (100 ng) extracted from frozen tumour specimens was treated with sodium bisulphite as previously described [17]. A summary of the DNA samples studied and their *RB1* gene, LOH status, etc., is shown in Table 1.

### Primer design and PCR

Bisulphite treated DNA specific primers were designed for four amplicons spanning the *RB1* promoter (summary of consensus sequences and CpG site locations in Figure 1, Amplicons in Fig. S1, Table S1) which were analysed by manual DNA sequencing and/or nextgen sequencing (BisNGS). Amplification primers for BisNGS (Amplicons 3 and 4 only) included a ten nucleotide sample-specific tag (barcode) which was added to the 5' end of each forward and reverse primer sequence (Table S1). Nested PCR was performed for 30 cycles in a 25 µl reaction.

### Nextgen sequencing of PCR products

For a single 454 sequencing run, equimolar amounts of 46 amplicons were pooled in a single tube. The emulsion PCR and subsequent sequencing reaction was performed as described in the GS FLX emPCR Method Manual (USM-00033.A, Roche, Mannheim, Germany). Using this configuration the expected yield was approx. ~1000 reads per amplicon and 17.5 Mbp sequence content in total.

A total of ~89,000 target reads were obtained from a single run (Summary of reads per tested sample can be seen in (Table S2)). As each single read corresponds to an individual DNA molecule, the fraction methylation was determined as the ratio  $C_n/(C + T)_n$  at each CpG. To confirm the overall results of BisNGS analysis, Sanger sequencing (T-tracking) analysis was carried out for all amplicons obtained from amplification of bisulphite

treated DNA as previously described [18]. In our hands, T-tracking reproducibility of replicate samples was within  $\pm 10\%$  margin of error of BisNGS results (not shown). Partial DNA methylation levels of amplicons 3 and 4 (Fig. S1), were corrected for bisulphite PCR amplification bias by the polynomial method (Fig. S2) as previously described [19]. Partially methylated CpGs were rare for Amplicons 1 and 2 (Figure S1). CpGs were either fully methylated or unmethylated and so no correction for amplification bias was carried out for these amplicons.

In addition to BisNGS, some peripheral blood DNA samples were analysed by DNA methylation microarray of bisulphite treated DNA (see Supplementary Figures for method summary). Statistical comparisons between methylation profiles in some cases were carried out by the standard Student's t-test.

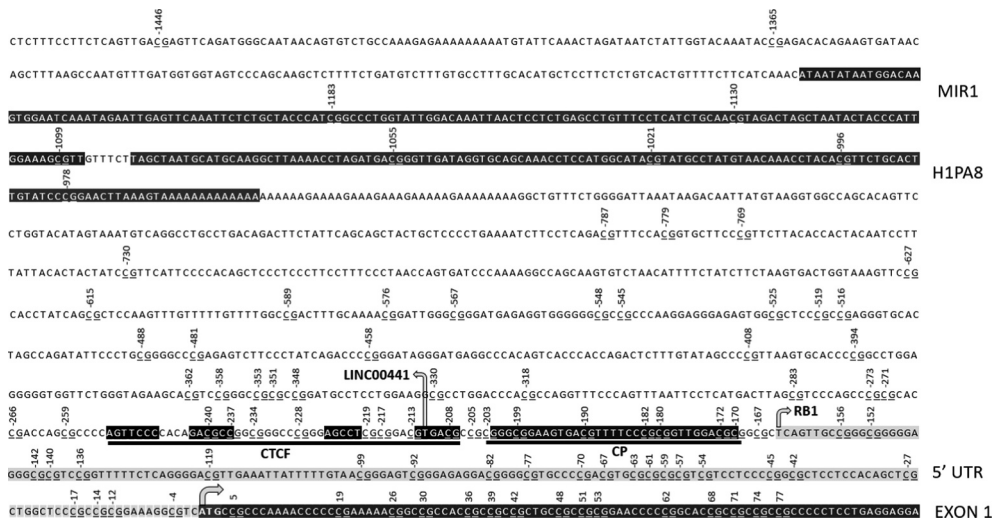
## Results

### *RB1* Promoter DNA methylation of *Pr*<sup>-/-</sup> retinoblastomas

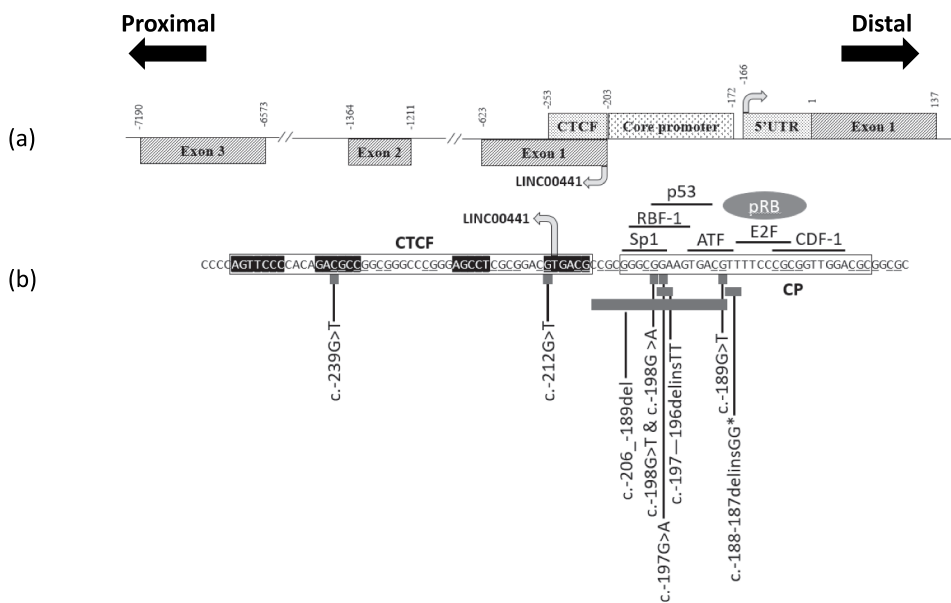
To determine whether DNA hypermethylation might be a recurrent feature of *Pr*<sup>-/-</sup> retinoblastomas, four available retinoblastomas homozygous for *RB1* promoter variants (1706 3208, 3252, and M22507) were analysed. The first (1706) contained a variant c.-212 G > T affecting the most distal one of the four elements of *CTCF*. The remaining three were found to have variants within putative overlapping *Sp1*, *RBF-1* and *p53* transcription factor binding sites (SpRp53) within the *RB1* core promoter (variants and promoter element motifs summarized in Figure 2). DNA methylation analysis was performed by BisNGS and the four retinoblastomas were initially compared to DNA extracted from normal peripheral blood and *RB*<sup>-/-</sup> retinoblastomas. Of the four *Pr*<sup>-/-</sup> retinoblastomas, 1706 was found to be the most extensively methylated with 64/94 at least partially methylated CpGs (within *RB1* promoter region extending from c.-1183 to c.+77) including 4/7 affecting the core promoter and 7/8 affecting the *CTCF* binding site region (Figure 3(a,b)) and summarized in Table 1). In contrast, peripheral blood DNA was not methylated in this region (Figure 3(c), Table 1). One of the eight core promoter CpGs of sample 1706 was

**Table 1.** Summary characteristics of retinoblastoma and peripheral blood DNA samples tested in this study. CpGs counted as methylated includes any CpG with at least partial methylation (>10% per allele). (PB=peripheral blood). 1011 (homo c.1363C>T), 1012 (homo c.1814+1291T>G), 1013 (homo c.844G>T), 1014 (homo c.1215+1G>A), 1015 (c.2663+1G>A/c.-?1814+?del). \*DNA methylation boundary location where CpGs are methylated up to and including boundary site with weak or no methylation downstream. Specimens PB=peripheral blood, T=tumour.

Sample ID	DNA sample origin	13q14 locus zygosity	RB1 promoter status	CpGs (n=38) methylated (c.-1183-c.-259)	CTCF CpGs (n=8) methylated (c.-240-c.-208)	CP CpGs (n=7) methylated (c.-203-c.-170)	CpGs (n=40) methylated (c.-167-c.+77)	RB1 coding exon status	DNA Methylation boundary CpGs*	Heritable Status
M22508	T	homo	c.-197_-196delinsTT	21/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-516	germline
3208	T	homo	c.-198G>A	21/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-516	germline
3532	T	homo	c.-198G>A	23/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-481	germline
1706	T	homo	c.-212G>T	36/38	7/8	4/7	4/40	RB <sup>+/+</sup>	c-190	germline
1323	PB	hetero	Pr <sup>+/+</sup>	7/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-978	normal
1456	PB	hetero	Pr <sup>+/+</sup>	7/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-978	normal
1789	PB	hetero	Pr <sup>+/+</sup>	7/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-978	normal
1876	PB	hetero	Pr <sup>+/+</sup>	7/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-978	normal
1987	PB	hetero	Pr <sup>+/+</sup>	7/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-978	normal
1011	T	Homo	Pr <sup>+/+</sup>	0/38	0/8	0/8	0/40	RB <sup>-/-</sup>	c-1365	sporadic
1012	T	homo	Pr <sup>+/+</sup>	0/38	0/8	0/8	0/40	RB <sup>-/-</sup>	c-1365	germline
1013	T	homo	Pr <sup>+/+</sup>	0/38	0/8	0/8	0/40	RB <sup>-/-</sup>	c-1365	sporadic
1014	T	homo	Pr <sup>+/+</sup>	0/38	0/8	0/8	0/40	RB <sup>-/-</sup>	c-1365	sporadic
1015	T	hetero	Pr <sup>+/+</sup>	0/38	0/8	0/8	0/40	RB <sup>-/-</sup>	c-1365	sporadic
M22507	PB	hetero	Pr <sup>+/+</sup> /c.-197_-196delinsTT	7/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-978	germline
3208	PB	hetero	Pr <sup>+/+</sup> /c.-198G>A	7/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-978	germline
3532	PB	hetero	Pr <sup>+/+</sup> /c.-198G>A	7/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-978	germline
1706	PB	hetero	Pr <sup>+/+</sup> /c.-212G>T	7/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-978	germline
664	PB	hetero	Pr <sup>+/+</sup> /c.-189G>T	8/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-978	germline
2149	PB	hetero	Pr <sup>+/+</sup> /c.-197G>A	10/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-978	germline
M5616	PB	hetero	Pr <sup>+/+</sup> /c.-198G>A	8/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-978	germline
M25851	PB	hetero	Pr <sup>+/+</sup> /c.-239G>T	8/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-978	germline
D16.31062	PB	hetero	Pr <sup>+/+</sup> /c.-198G>T	7/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-978	germline
1342	PB	hetero	Pr <sup>+/+</sup> /c.-206_-189del	23/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-481	germline
4220	PB	hetero	Pr <sup>+/+</sup> /c.-212G>T	7/38	0/8	0/8	0/40	RB <sup>+/+</sup>	c-978	germline



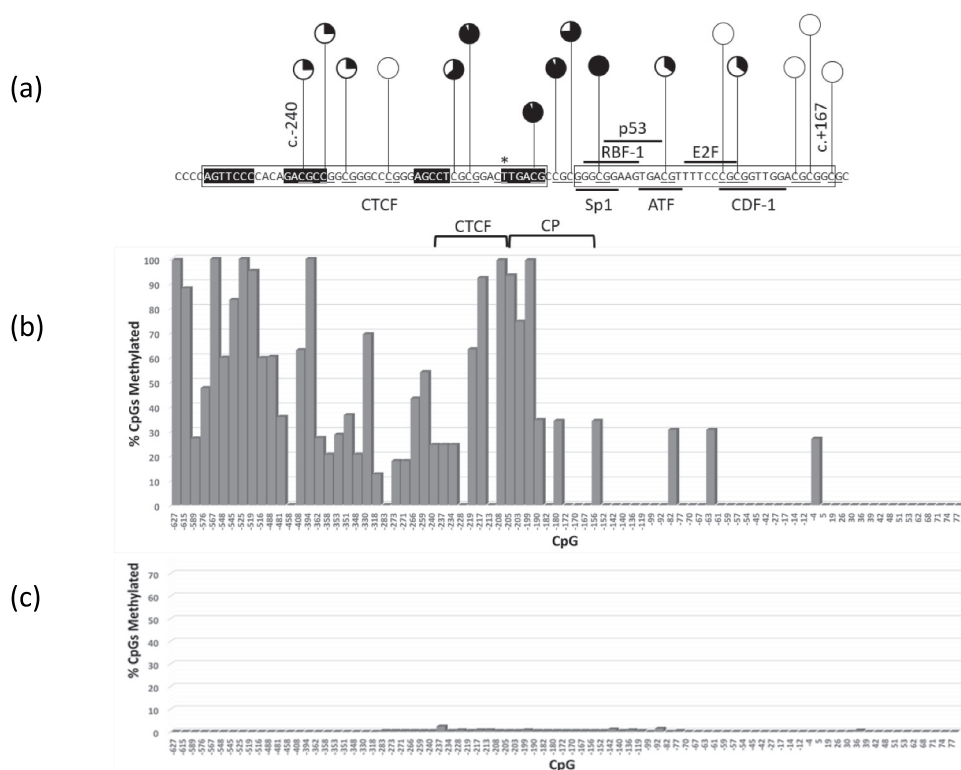
**Figure 1.** Known promoter elements and *CTCF* binding motif with DNA contact regions in black. Numbers indicate CpG location relative to translation start site (arrowed). *RB1* gene and *LINC00441* transcription start sites also arrowed. *RB1* 5' untranslated region in grey. Core promoter (CP) and ALU repeat sequences (*MIR1* and *H1PA8*) highlighted in black.



**Figure 2.** (a) Exons, 5'UTR, *CTCF* binding site, Core promoter region and long non-coding RNA exons. (b) *CTCF* binding site (boxed, contact regions in black), *RB1* gene core promoter (CP) (boxed) with variants reported here (spanning nucleotides -239 to -187, see text) and elsewhere (\*) [4], *CTCF* (boxed), and putative transcription factor binding elements. *RB1* and *LINC00441* transcription start sites (arrowed). Transcription factor *pRB* (grey ellipse) known to bind *E2F* directly.

abolished by the c.-212 G > T variant hence the core promoter of this sample, unlike other samples, contains 7 instead of 8 CpGs. In the remaining three *SpRp53<sup>-/-</sup>* retinoblastomas, however, DNA methylation was detected in only 20–29/94 CpGs which occur at a location proximal of the *CTCF* binding site (c.-1183 to ~c.-400-500, Figure 4(a–c) and

Table 1) including four upstream CpGs (c.-787, c.-779, c.-768, and c.-730) (Fig. S3(a)). The methylation profile of the four *Pr<sup>-/-</sup>* retinoblastomas contrasted with all analysed normal peripheral blood DNAs and *RB1<sup>-/-</sup>* retinoblastomas (Figure 3(c) and Table 1). In the former, a single boundary where CpGs proximal and including c.-978 were fully



**Figure 3.** *RB1* promoter CpG methylation profile of a *Pr*<sup>-/-</sup> retinoblastoma (1706) homozygous for the promoter variant c.-212 G > T spanning CpGs -627+77 (underlined). (a) pie chart summary of *RB1* promoter DNA methylation of CpGs that include putative CTCF binding site, core promoter (CP), coding and untranslated exons; (b) bar chart summary of 1706 CpG methylation of *RB1* upstream region -627 to +77; (c) bar chart summary of normal peripheral blood DNA.

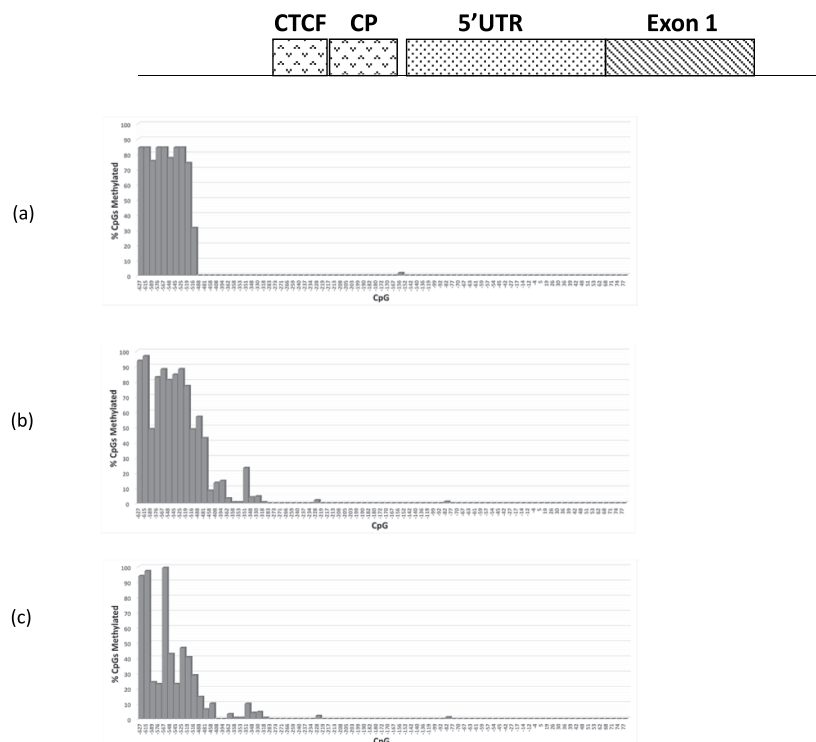
methylated in the peripheral blood DNA of analysed normal individuals (summarized Table 1), a result which was further confirmed by DNA methylation microarray analysis (see below). The DNA methylation profiles of five *RB1*<sup>-/-</sup> retinoblastomas analysed by manual Sanger sequencing only were found to have weak or no methylation of CpGs proximal to c.978 such that CpGs distal to c.-1365 were unmethylated (example shown in Fig. S3(b)), consistent with a boundary more proximal to that identified in normal peripheral blood DNA (summarized Table 1). A further 11 *RB1*<sup>-/-</sup> retinoblastomas showed similar results (not shown). Taken together these results suggest that relative to peripheral blood DNA, DNA hypermethylation/distal boundary displacement may be a recurrent feature of *Pr*<sup>-/-</sup> retinoblastomas, whereas methylation profiles suggestive of proximal boundary displacement were consistently observed in *RB1*<sup>-/-</sup> retinoblastomas.

As the *RB1* promoter hypermethylation identified in *Pr*<sup>-/-</sup> retinoblastomas was associated with homozygous alleles, it was unclear whether

heterozygous *RB1* promoter variants in isolation might be sufficient to initiate hypermethylation in cis. If so then DNA hypermethylation of the *RB1* promoter region in the peripheral blood DNA of patients carrying heterozygous promoter variants would also be expected.

### ***RB1* promoter DNA methylation in heterozygous *Pr*<sup>-/+</sup> peripheral blood DNA**

To determine whether heterozygous promoter variants (*Pr*<sup>-/+</sup>) might be sufficient to initiate *RB1* promoter DNA hypermethylation, we analysed corresponding peripheral blood DNAs of retinoblastomas M22507, 3208, 3532, and 1706 as well as five other patients carrying *RB1* promoter variants for whom no tumour was available. The *RB1* promoter variants of these patients are shown in Table 1. Relative to normal peripheral blood DNA (Fig. S4 (a)) and *RB*<sup>-/-</sup> retinoblastomas (Fig. S4(b)), weak or no hypermethylation was detected in the peripheral blood DNA of 8/9 carriers of *RB1* core promoter



**Figure 4.** Bar chart summary of *RB1* promoter CpG methylation profiles (CpGs c.-627–+77) of (a) 3208 (b) 3532 and (c) M22507; (a) and (b) are retinoblastomas homozygous for the *RB1* variant c.-198 G > A, (c) retinoblastoma homozygous for c.-197\_–196delinsTT variant.

variants (Fig. S4(c)-(i), Table 1). To further confirm this result, we performed in-depth microarray DNA methylation analysis of the *RB1* promoter of peripheral blood DNA (Region: hg19 13:48,876,603–13:48,892,244, Fig. S5). DNA of sufficient quantity and quality was available from three patients (664, D16.31062, and 3531, Table 1) which were analysed by methylation microarray, and compared with 20 controls 19 of which were derived from the Gene Expression Omnibus (GEO) database. No significant hypermethylation throughout the *RB1* promoter region downstream of CpG at c.-978 was detected. These findings further support the results obtained by manual sequencing and *BisNGS* of heterozygous carriers of *RB1* promoter variants indicating that there is little or no hypermethylation/boundary displacement in the peripheral blood DNA of carriers heterozygous which was comparable to normal peripheral blood DNA.

Only 1/9 carriers of heterozygous *RB1* promoter variants (patient 1342, c.-206\_–189del) were identified with significant *RB1* promoter DNA hypermethylation (CpGs between c.-627 to c.-458, Fig. S4(j)) consistent with distal *BD* of a single allele.

The variant identified in patient 1342 was an 18 bp deletion resulting in the loss of several putative *RB1* core promoter transcription factor binding sites including *Sp1*, *RB1*, *p53*, and *ATF* while adjacent *CTCF* binding site and putative *E2F* element were unaffected. It is not clear why monoallelic DNA hypermethylation/boundary displacement was observed in 1342 but not other *Pr*<sup>-/+</sup> carriers of promoter variants. One possibility is that the deletion of multiple transcription factor binding sites might affect the loop extrusion process and/or influence the location of DNA anchoring points.

The *RB1* promoter DNA hypermethylation detected in retinoblastomas 3208, 3532, M22507, and peripheral blood DNA of 1342 (~c.-500) (Figs. S3, S4(a)-(c), and S4(j)) are each consistent with distal *BD* to a new location at ~c.-500. A DNA methylation boundary at this location occurs ~250 base pairs upstream from a *CTCF* binding element as shown in Figure 1. This result suggests that the variants affecting core promoter transcription factors are able to exert epigenetic changes ~300 bp upstream despite a single *CTCF* insulating barrier separating the two regions.

## DNA methylation analysis of *RB1* promoter in $Pr^{E-/E-}$ and $Pr^{E-/E+}$ retinoblastomas

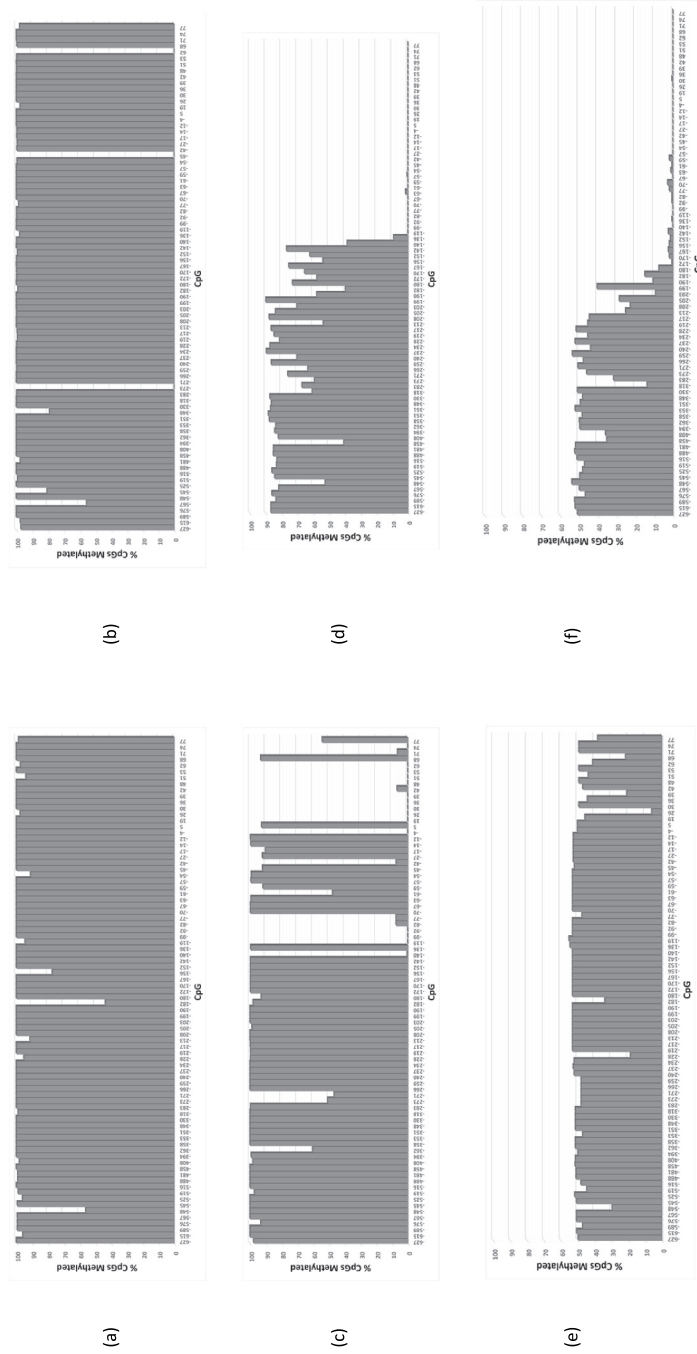
One explanation for the distal *BD* observed in  $Pr^{-/-}$  retinoblastomas, is the possibility of coincidental epigenetic inactivation as a separate somatic event. If DNA hypermethylation was coincidental and in addition to the presence of promoter variants then the obtained methylation profiles might be expected to resemble typical  $Pr^{E-/E-}$  and/or  $Pr^{E-/E+}$  retinoblastomas. This possibility was assessed by comparing the DNA methylation profiles across 94 CpGs (c.-1183 to c.+77) of  $Pr^{-/-}$  retinoblastomas and comparing with epigenetically silenced retinoblastomas including 22 available  $Pr^{E-/E-}$  and 8  $Pr^{E-/E+}$  sporadic retinoblastomas (examples shown in Figure 5(a–f), and Table S3). In all 22  $Pr^{E-/E-}$  and 8  $Pr^{E-/E+}$  retinoblastomas examined, hypermethylated CpGs were detected in most or all upstream CpGs including the core promoter (c.-1183–c.-180) and in some cases further distal (e.g. Figure 5(a,b,e)). Interestingly, hypermethylation extended to a relatively concise location just beyond the core promoter (~c.-100) in 4/22  $Pr^{E-/E-}$  and 4/8  $Pr^{E-/E+}$  retinoblastomas (Figure 5(d,f), Table S3). In some  $Pr^{E-/E-}$  and  $Pr^{E-/E+}$  cases, however, boundary displacement was not a clear mechanism, e.g., the retinoblastoma DNA of patient 4927 (Figure 5(c)), where significant regions of hypomethylation were observed mainly distal of the core promoter; however, a clear methylation boundary was not identified despite significant regions of hypomethylation. A distinct boundary for 4927 could not be ruled out as CpGs further downstream of the *RB1* promoter region were not analysed. Two other  $Pr^{E-/E-}$  retinoblastomas were found with DNA hypermethylation beginning to subside at CpGs approaching c.+77 (Fig. S6), suggesting a possible further distally placed boundary, but we did not confirm this result by further analysis of downstream CpGs. Collectively these results indicate that of 22  $Pr^{E-/E-}$  and 8  $Pr^{E-/E+}$  retinoblastomas, a variety of displaced methylation boundaries distal of the core promoter were detected that included 6/30 retinoblastomas consistent with a common boundary location at ~c.-100. Also observed was that the boundaries of  $Pr^{E-/E-}$  and  $Pr^{E-/E+}$  retinoblastomas were often associated with hypervariable CpGs

methylation and weakly methylated CpGs frequently detected at or near the new boundary, but no clear consensus pattern observed between samples (e.g. Figure 5(d,f)).

To determine whether the *RB1* promoter DNA methylation profile of  $Pr^{-/-}$  retinoblastomas was comparable to  $Pr^{E-/E-}$  and/or  $Pr^{E-/E+}$  retinoblastomas, the CpG methylation profiles were assessed and compared on the basis of three criteria, (a) boundary location, (b) the CpG methylation profile of putative functional elements within *CTCF* and core promoter elements (c.-240 to c.-170) and (c) the DNA methylation profile of 38 CpGs upstream of the core promoter (c.-627 to c.-259). Of 3/4  $Pr^{-/-}$  retinoblastomas (3532, 3208 and M22507), the CpG methylation profiles did not match  $Pr^{E-/E-}$  and/or  $Pr^{E-/E+}$  retinoblastomas in any of the above three criteria, since no methylation affecting putative functional elements were detected and the displaced boundary was clearly considerably 5' of the core promoter. As the *RB1* promoter DNA methylation profile of retinoblastoma 1706 was distinct from the other three  $Pr^{-/-}$  retinoblastomas, DNA from this tumour sample was evaluated separately.

To determine whether the *RB1* promoter methylation profile of retinoblastoma 1706 (c.-212 G > T) might be consistent with coincidental hypermethylation matching  $Pr^{E-/E-}$  and  $Pr^{E-/E+}$  retinoblastomas, CpG methylation was again compared in relation to the three criteria outlined above. While the DNA methylation boundary of 1706, which was the most extensively hypermethylated  $Pr^{-/-}$  retinoblastoma, was not sharply defined, DNA hypermethylation had clearly ceased at a location distal of the core promoter (Figure 3) and was hence potentially similar to some  $Pr^{E-/E-}$  and/or  $Pr^{E-/E+}$  retinoblastomas (Fig. S7, S8). With respect to the second criteria, CpGs spanning *CTCF* and putative *RB1* core promoter functional elements (CpGs in the region c.-240 to c.-170, n = 16) were quantitatively compared based on the difference of their mean and standard deviation between and individual  $Pr^{E-/E-}$  (~40.5% per allele) and  $Pr^{E-/E+}$  (~39.3% per allele) retinoblastomas where all the former (Fig.S7 (a)) reached statistical significance (p < 0.001) whereas 2/7 of the latter (Fig.S7(b)) did not reach significance. In addition, CpGs in the *CTCF* region were significantly undermethylated compared to all





**Figure 5.** *RB1* promoter CpG methylation profiles (CpGs –627 – +77) of  $Pf^{E/+}$  retinoblastomas, patients (a) 5492, (b) 3093, (c) 4927, and (d) 2504; and  $Pf^{E/+}$  retinoblastomas, patients (e) 17,921 and (f) 2958.

$Pr^{E-/E-}$  and  $Pr^{E-/E+}$  retinoblastomas whereas the core promoter was only marginally less methylated (Fig. S8). These results suggest that while the 1706 methylation profile appears unusual, a subset of  $Pr^{E-/E+}$  retinoblastomas was identified which were relatively closely matched at least with respect to CpGs spanning *CTCF* and the core promoter.

A comparison between 1706 and  $Pr^{E-/E-}$  as well as  $Pr^{E-/E+}$  retinoblastomas regarding the third of three criteria was carried out in which CpGs upstream of the *RB1* core promoter ( $n = 38$  CpGs c.-627 to -259) was analysed. The relative mean and overall variance of CpG methylation across these upstream CpGs was compared. The mean CpG methylation was significantly reduced in comparison to all  $Pr^{E-/E-}$  as well as  $Pr^{E-/E+}$  (Box plot and p value summaries of the CpG DNA methylation status shown in (Fig. S9(a) and 9 (b)). These results indicate that while core promoter CpG methylation and the boundary location identified in 1706 were potentially similar to two  $Pr^{E-/E+}$  retinoblastomas, there was a significant difference ( $p < 0.001$ ) in methylation of the 38 CpGs upstream of the *RB1* core promoter (c.-627 to c.-259), indicating that the overall DNA methylation profile of 1706 was not matched to any of our cohort of  $Pr^{E-/E-}$  or  $Pr^{E-/E+}$  retinoblastomas and most likely has a different mechanism of origin. It is possible that the c.-212 G > T variant may either weaken the binding of *CTCF* or alternatively distort its conformation at a critical region immediately upstream of core promoter transcription factors. With the expected loss of *pRB* expression after homozygosity of the *CTCF* variant, a cooperative effect of these two events may be sufficient for the observed DNA hypermethylation/*BD* affecting the *RB1* promoter.

Finally, the critical nature of *CTCF*-DNA contact regions is suggested by the placement of known *SNP* variants (summarized Fig.S10). Variants identified in this *CTCF* binding region are rare, particularly within the *CTCF* DNA contact regions (Figure 1). Variants associated with retinoblastoma are not otherwise known to *dbSNP* (c.-212 G > T c.-198 G > A and c.-189 G > T, Fig.S10). The c.-239 G > T variant reported here (patient M25851, Table 1) has not been seen in *dbSNP* although c.-239 G > A has been seen multiple times (Fig.S10). Unfortunately,

no retinoblastoma tissue is available for M25851 which would have been of considerable interest to determine whether this variant might also lead to hypermethylation/boundary displacement given that a DNA contact region of the *CTCF* binding site is potentially affected. In the absence of such data c.-239 G > T remains a variant of uncertain significance.

## Discussion

We have identified novel and unique DNA methylation profiles in retinoblastomas homozygous for *RB1* promoter variants and in the peripheral blood DNA of one patient heterozygous for a deleterious germline 18 bp deletion. The DNA methylation profiles observed in  $Pr^{-/-}$  retinoblastomas were clearly distinguishable from all other  $Pr^{E-/E-}$ ,  $Pr^{E-/E+}$ , and  $RB^{-/-}$  retinoblastomas that we have analysed. One trivial possibility that might have explained the hypermethylation detected in  $Pr^{-/-}$  retinoblastomas was coincidental iso-allelic epigenetic silencing. Independent epigenetic inactivation as a separate coincidental sporadic event might only have been expected if the promoter variants *per se* were insufficient to initiate a clonal tumour expansion and therefore requiring a more damaging event in *cis* for the allele to be pathogenic. If true, then an independent promoter hypermethylation event would be no more effective or probable than a nonsense/missense variant or deletion affecting the *RB1* gene in *cis*. Given that  $Pr^{E-/E-}$  retinoblastomas account for ~10% of retinoblastomas, then coincidental iso-allelic DNA methylation, and not other pathogenic variants in 6/6  $Pr^{-/-}$  retinoblastomas (five here and one reported elsewhere [4]) is extremely unlikely ( $\chi^2 \sim 48.6$ ,  $p < 0.00001$ , 5 degrees of freedom). However, all available molecular and familial segregation evidence suggests that promoter variants affecting transcription factor binding sites are pathogenic and sufficient to cause disease, but with no evidence of additional iso-allelic damaging events necessary to inactivate *RB1*. In addition, as discussed below the DNA methylation profiles of all four  $Pr^{-/-}$  retinoblastomas were dissimilar to all other analysed retinoblastomas, suggesting a novel mechanism giving rise to DNA hypermethylation/boundary displacement.

Of the four  $Pr^{-/-}$  retinoblastomas, the most distinctly unique *RB1* promoter methylation profiles were found in 3/3  $SpRp53^{-/-}$  retinoblastomas where limited *RB1* promoter DNA methylation/distal *BD* was identified. In this subset of  $Pr^{-/-}$  retinoblastomas, CpG hypermethylation/boundary displacement (CpGs  $\sim$ c.-627 to  $\sim$ c.-450) did not affect *CTCF* or the *RB1* core promoter itself. To our knowledge, no known functional elements or pathogenic variants or deletions affecting this upstream hypermethylated region have been reported, which suggests that methylation of these upstream CpGs in isolation is unlikely to lead to inactivation of *RB1* gene expression. Moreover, if functional elements critical for gene expression were indeed present in the c.-627 and  $\sim$ c.-450 region and/or methylation of this region alone was sufficient to disrupt *RB1* gene expression, then a subset of  $Pr^{E-/E-}$  and/or  $Pr^{E-/E+}$  retinoblastomas with matching hypermethylation profiles not affecting the core promoter would be expected. Among our cohort of retinoblastomas without *RB1* promoter variants none matched such a methylation profile, and to our knowledge, none have been previously reported. These observations suggest, therefore, that the unique hypermethylation profiles of 3/3  $SpRp53^{-/-}$  retinoblastomas are not coincidental events but rather epimutation driven phenomena.

Other evidence suggesting that the hypermethylation of upstream CpGs ( $\sim$ c.-627 to  $\sim$ c.-450, [Figure 4](#)) in  $Pr^{-/-}$  retinoblastomas is not a coincidental somatic event, can be inferred from the analysis of the peripheral blood DNA of 1342 (heterozygous for c.-206\_-189del, no tumour available), where a germline deletion encompassing multiple transcription factor binding sites including putative *Sp1*, *RBF-1*, *p53*, and *ATF* but not *CTCF* and putative *E2F-DP* elements ([Figure 2](#)) was identified. Comparable to the three homozygous  $SpRp53^{-/-}$  retinoblastomas, the single allele DNA hypermethylation profile of 1342 ([Figure S4 \(j\)](#)) is consistent with a significant distal displacement of the 5' DNA methylation boundary of the affected allele but not extending as far as the *RB1* core promoter. However, in contrast to the bi-allelic *RB1* gene inactivation of clonal retinoblastoma cells, the presence of methylation in peripheral blood lymphocytes indicates a germline and

not a somatically acquired event. To our knowledge, the only other known example of germline hypermethylation at the *RB1* locus was a previously reported translocation of chromosome 13 to an inactive X chromosome [20]. We have identified one similar case (not shown) but found that the entire *RB1* locus was hypermethylated, which was in sharp contrast to 1342 in which detected hypermethylation was restricted to a relatively small region  $\sim$ 2-400 bases upstream of the *RB1* core promoter. These observations support hypermethylation observed in 1342 occurring in response to a deleterious germline promoter epimutation rather than a coincidental independent somatic event.

Only 1/4  $Pr^{-/-}$  retinoblastomas analysed was found to have extensive DNA methylation that included the *RB1* core promoter region (1706), where hypermethylation of 11/15 *CTCF*/core promoter CpGs ([Figure 3\(a\)](#)) was identified. While the *RB1* core promoter methylation profile, and overall boundary displacement of 1706 was potentially similar to  $Pr^{E-/E-}$  and/or  $Pr^{E-/E+}$  retinoblastomas, the overall DNA methylation profile, in particular, CpGs upstream of the core promoter (c.-627 to -259) was qualitatively and quantitatively different to all other retinoblastomas that we have analysed. Specifically, we observed a reduced mean and increased variance of DNA methylation across the 38 CpGs in this region (further discussion below) when compared with our cohort of  $Pr^{E-/E-}$  and  $Pr^{E-/E+}$  retinoblastomas. We interpret this result to support a novel mechanism of hypermethylation/*BD* which is most likely epimutation driven.

The mechanism of *RB1* gene inactivation caused by core promoter or *CTCF* variants might be due to one of the two possibilities. One is that the variants simply inactivate the *RB1* core promoter by the disruption of transcription factor binding. The second possibility is that the variants tip the balance favouring activation of transcription of *LINC00441* transcription a negative feedback process which may down-regulate *RB1* gene transcriptional repression. Evidence suggests that *LINC00441* transcription may be linked to the recruitment of DNA methyltransferase [8,9]. While we found no significant hypermethylation in the peripheral blood DNA of promoter variant

carriers, activation of *LINC00441* leading to transcriptional inactivation of the *RB1* gene in retinoblastomas via a negative feedback pathway involving triple helix formation as previously described [10], remains a possibility.

As the transcription start site of *LINC00441* is immediately upstream (1 bp) of the c.-212 G > T variant identified in 1706, the non-coding transcript needs to be considered as a possible factor in DNA hypermethylation identified in this retinoblastoma. Our DNA methylation data, however, suggest that an involvement of *LINC00441* in *RB1* gene inactivation is less likely in case 1706. The core promoter in 1706 was clearly hypermethylated, a result strongly suggesting that it is no longer transcriptionally active either forwards or backwards rendering any effect of *LINC00441* unlikely. Furthermore, in the peripheral blood DNA of this patient significant hypermethylation of the core promoter region was not detected suggesting an initial non-epigenetic mechanism of *RB1* gene inactivation of the heterozygous allele. Such single allele gene inactivation is possible if *CTCF* is indeed critical for a fully functional *RB1* promoter as previously suggested [15]. However, after c.-212 G > T homozygosity in retinoblastoma, hypermethylation is best explained by both alleles initially being non-epigenetically inactivated and only after the expected loss of *pRB* expression does hypermethylation/boundary displacement in 1706 become manifest.

One of *CTCF*'s essential functions is as an insulator and to anchor DNA to nuclear lamina in order to separate gene domains; hence, it is of considerable interest as to how variants affecting the *SpRrp53* region, which occur on the 3' side of the *RB1* *CTCF* binding site might influence a shift in the chromatin/heterochromatin boundary several hundred bases away on the 5' side (Figure 4). One possibility is that the cohesin associated loop extrusion process is partially disrupted by genetic changes affecting the *RB1* promoter. Alternatively, loop extrusion may occur normally, but the location of DNA anchoring points may be affected, for example, by the combination of promoter variants and/or expected loss of *pRB* expression.

Hypomethylation of CpGs upstream of the *RB1* core promoter (~c.-1300 and c.-900) found in all *RB*<sup>-/-</sup> retinoblastomas with wild type *RB1*

promoter that we have analysed, is consistent with a proximal relocation of the DNA methylation boundary relative to normal peripheral blood DNA. This result may be explained by the functional loss or absent expression of *pRB* leading to reduced binding of *HDACs* to the *RB1* promoter. Reduced *HDAC* binding is expected to increase histone acetylation and reduce the binding affinity of adjacent nucleosomes followed by CpG hypomethylation. Alternatively, it is possible that the presence of *pRB* may be involved in limiting loop extrusion, a process which albeit may still be dependent on *pRB*'s *HDAC* recruiting function. We have observed similar hypomethylation in the DNA of mainly primordial *RB*<sup>+/+</sup> cells (e.g. embryonic stem cells and germ cells) (not shown) where although the *RB1* gene is intact, a role associated with loss of *pRB* function is still possible since cell cycle-dependent inactivation of *pRB* is known to occur indirectly by other mechanisms such as phosphorylation [21].

Although the loss of *pRB* binding in conjunction with other transcription factors offers the simplest explanation for the observed hypermethylation/*BD* of *Pr*<sup>-/-</sup> retinoblastomas, given that *pRB* is a transcription factor involved in the regulation of a multitude of other *E2F* responsive genes, we cannot rule out that loss or activation of other transcription factors regulated by *pRB* might also have a role. Furthermore, it is possible that there are other expressed factors specific to retinoblastomas but not peripheral blood that might contribute to an epimutation effect of *RB1* promoter variants; however, available evidence suggests that this is unlikely. Firstly, *RB1* transcription/expression is ubiquitous with relatively little variation between cell types (<http://www.genecards.org/cgi-bin/carddisp.pl?gene=RB1>). Secondly, the DNA methylation boundary identified in peripheral blood DNA is highly conserved since it is also found in at least several other adult cell types that we have analysed, e.g., cell lines *HCT116* (colon), *RK0* (colon), and *IPSc* (foreskin fibroblasts) (not shown). As *RB1* expression levels and DNA methylation boundaries in different adult tissue types are both highly conserved, any observed methylation differences between peripheral blood and retinoblastoma DNA are unlikely due to tissue-specific differences.

To our knowledge, the intriguing finding of the relocation of DNA methylation boundaries to a relatively concise location identified in 4/22  $Pr^{E-/E-}$  and 4/8  $Pr^{E-/E+}$  retinoblastomas ( $\sim$ c.+5 to -c.-140) (Fig. S5(d), S5(f) respectively, and Table S3) has not been previously reported. It is currently not known what factors might lead to such a defined and relatively stable location just distal of the *RB1* core promoter in some  $Pr^{E-/E-}$  and/or  $Pr^{E-/E+}$  retinoblastomas but not others (Figs.6(a) and 6 (b), and Table S3). Of the remaining  $Pr^{E-/E-}$  and  $Pr^{E-/E+}$  retinoblastomas, we did not explore the full extent of methylation boundary displacement as we have analysed CpGs only as far as c.+77. The displacement of the DNA methylation boundary to variable locations in epigenetically silenced retinoblastomas despite an intact *CTCF* binding site, is perhaps an indication of a malfunctioning loop extrusion and/or anchoring process.

A recurrent feature of  $Pr^{E-/E-}$ ,  $Pr^{E-/E+}$  and  $Pr^{-/-}$  retinoblastomas was the high degree of CpG hypermethylation variation surrounding the new boundary location, whereas little variation was detected in normal cells such as peripheral blood DNA. In the latter, the boundary was consistently very sharply defined (Fig. S5) with CpG methylation at or near 100% for CpGs upstream of c.-978 and  $\sim$ 0% for CpGs downstream of this boundary location. It is currently not clear what determines the relatively high degree of CpG methylation variation at or near the displaced boundaries identified in epimutation and epigenetically silenced retinoblastomas.

The displacement of DNA methylation boundaries reported here is consistent with the compaction and/or relaxation of nucleosomes. For example, the most 5' DNA methylation boundary was observed in  $RB^{-/-}$  retinoblastomas (CpGs up to and including c.-1365) potentially  $\sim$ 2-3 nucleosomes from the methylation boundary of normal peripheral blood DNA (CpGs at c.-978). Also, the DNA methylation boundary of  $SpRp53^{-/-}$  retinoblastomas (ca.  $\sim$ c.-500) corresponds to a distal displacement  $\sim$ 4-500 bp from the DNA methylation boundary found in normal peripheral blood DNA a genetic distance equivalent to  $\sim$ 3 nucleosomes. Furthermore, the DNA boundaries at  $\sim$ c.140 in four  $Pr^{E-/E-}$  and four  $Pr^{E-/E+}$  retinoblastomas (Table S3) correspond to a possible further

compaction of 1-2 nucleosomes relative to  $SpRp53^{-/-}$  retinoblastomas. Of at least four different DNA methylation boundaries ( $RB^{-/-}$  retinoblastomas, normal peripheral blood, three  $SpRp53^{-/-}$ , four  $Pr^{E-/E-}$  and four  $Pr^{E-/E+}$  retinoblastomas), the relative distance between boundaries is consistent with defined nucleosomal units in varying degrees of compaction.

Interestingly, the only other known example of promoter epimutation driven single allele DNA hypermethylation in peripheral blood DNA was the previously identified +/c.-188\_-187delinsGG variant [4] a deleterious variant affecting both the last and first base of putative *ATF* and *E2F* elements, respectively. Compared to heterozygous *RB1* promoter variants reported here, the previously reported epimutation [4] (Figure 2) is expected to be highly deleterious resulting in the disruption of *pRB* binding, since *pRB* directly interacts the *E2F-DP* heterodimer [22]. Of interest is that the epimutation leads to significant single allele hypermethylation/boundary displacement even though the *CTCF* binding sequence remains intact and *pRB* is still produced from the unaffected allele. This previously reported case [4] in conjunction with 1342 represent the only known examples of epimutation induced single allele hypermethylation associated with *RB1*. One explanation for these observed cases is that *RB1* core promoter variants, particularly those expected to affect the binding of the transcription factor *pRB* directly may be more likely to result in significant single allele hypermethylation/*BD* of the *RB1* promoter. In contrast, other variants such as c.-198 G > A having less expected direct effect with regard *pRB* binding to the *RB1* promoter may be more dependent on loss of *pRB* expression as found in  $Pr^{-/-}$  retinoblastomas, before more significant hypermethylation/*BD* is observed.

A question arises from the methylation profiles of analysed  $Pr^{E-/E-}$  and  $Pr^{E-/E+}$  retinoblastomas as to whether *RB1* core promoter and/or putative *CTCF* binding site hypermethylation or as yet some other unidentified process in some way perhaps disrupts loop extrusion/anchoring leading to the variable and erratic DNA methylation boundaries observed in these retinoblastomas. Of interest was that the boundaries of 4/8 (50%) of available  $Pr^{E-/E+}$  retinoblastomas were just 3' of the core

promoter, whereas only 3/22 (14%) of  $Pr^{E-/E-}$  retinoblastomas had such a relatively upstream boundary ( $p < 0.001$ ) (Table S3), while the remainder (86%) were post c.+77. The significance of these results in explaining the hypervariable boundary locations of epigenetically silenced retinoblastomas is not known but may suggest that boundary displacement is not a static event but rather may be associated with gradual deterioration of the loop extrusion process occurring during tumour evolution.

Obtaining a more detailed understanding of the molecular mechanisms associated with hypermethylation/*BD* in retinoblastoma has proven extremely difficult given that there are no known methods of stably hypermethylating small chromosomal regions. Studies involving *RB1* promoter epimutations, however, are feasible and could potentially allow a useful approach towards further delineating factors influencing *RB1* gene boundary control.

As shown in Fig.S10, rare SNPs are prevalent throughout the *CTCF* binding site, but very few are known to be pathogenic, other than those which have been reported associated with retinoblastoma although c.-239 G > A remains a variant of uncertain significance. Our findings suggest that the functional effect of *RB1* promoter variants such as c.-239 G > A could potentially be explored through their analysis in a cell line model preferably by introducing CrispR/cas9 knock in gene edits. Ideally, homozygous  $Pr^{-/-}$  or  $Pr^{-}/RB^{-}$  compound heterozygous variants at the *RB1* locus would be introduced such that *pRB* inactivation in combination with variants of interest could be analysed. However, an alternative simpler approach might be to introduce single allele variant edits, and silence *pRB* expression by the addition of antisense oligos targeting *RB1* gene mRNA. Through experimental analyses such as these genuine pathogenic variants affecting the *RB1* promoter may result in DNA hypermethylation/boundary displacement of the affected allele potentially offering important functional evidence of their pathogenicity. Introduction of such edits in plasmids for the purpose of stable transfection assays may not be as informative due to unpredictable position effects. Transient transfection assays would not be informative since free plasmid DNA would not be subject to the epigenetic forces present in chromosomal DNA.

In summary, the detection of hypermethylation and/or the displacement of DNA methylation boundaries in retinoblastomas suggests that components of the *RB1* promoter complex including *pRB* may be directly or indirectly interacting with boundary control/loop extrusion elements. Further study of epimutation events potentially offers a powerful approach to help refine our understanding of the early molecular events leading to promoter hypermethylation in cancer cells and in future facilitate the identification of novel factors involved in epigenetic silencing that may serve as more specific targets for future drug therapies.

## Acronyms

$Pr^{-/+}$  =genetic silencing of single allele;  $Pr^{E-/E+}$  = epigenetic silencing of single allele; SpRp53 = overlapping Sp1, RBF-1 and p53 binding motifs; CTCF = CCCTC-Binding factor; BisNGS = nextgen sequencing of bisulphite treated DNA; BD = Boundary displacement; HDACS=histone deacetylases SNPs=single nucleotide polymorphisms.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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