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Inactivation of the *hypermethylated in cancer 1* tumour suppressor – not just a question of promoter hypermethylation?

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Summary

The chromosomal region 17p13.3 is frequently deleted or epigenetically silenced in a variety of human cancers. It includes the *hypermethylated in cancer 1 (HIC1)* gene placed telomerically to the p53 tumour suppressor gene. HIC1 encodes a transcriptional repressor, and its targets identified to date are genes involved in proliferation, tumour growth and angiogenesis. In addition, *HIC1* functionally cooperates with p53 to suppress cancer development.

Frequent allelic loss at position 17p13.1 in human cancers often points to mutations of the tumour suppressor p53. However, in a variety of cancer types, allelic loss of the short arm of chromosome 17 may hit regions distal to p53 and, interestingly, without leading to p53 mutations. Furthermore, the neighbouring region 17p13.3 often shows loss of heterozygosity or DNA hypermethylation in various types of solid tumours and leukaemias. In line with this concept, Wales et al. described a new potential tumour suppressor in this region and named it *hypermethylated in cancer 1 (HIC1)*. Further, it was shown that in the majority of cases hypermethylation of this chromosomal region leads to epigenetic inactivation of *HIC1*.

A role for *HIC1* in tumour development is further supported by a mouse model, since various spontaneous, age- and gender-specific malignant tumours occur in heterozygous *Hic1*$^{+/-}$ knockout mice. Furthermore, exogenously delivered *HIC1* leads to a significant decrease in clonogenic survival in cancer cell lines. This review highlights the role of *HIC1* inactivation in solid tumours and particularly in leukaemia development.

Key words: *HIC1; hypermethylation; solid cancers; leukaemia*

HIC1 gene structure and regulation

There are conflicting reports in the literature about the *HIC1* genomic nomenclature. Our review will confine itself to the most frequently used nomenclature proposed by Carter et al. [1]. Up to now six *HIC1* transcripts (1a-f) have been described which are generated from different promoters through alternative splicing events (fig. 1A). The *HIC1* transcripts 1a, 1b and 1c were detected in various normal tissues with strong predominance of the exon 1a transcript [2, 3]. For the *HIC1* transcripts 1d and 1e, which yield from an additional alternative splice event in exon 1c, no expression data are available. Recently, Mondal et al. discovered a further unspliced *HIC1* transcript 1f in human leukocytes. The authors stated that transcript 1f may play a role in modulating HIC1 protein levels in cancer cells [4].

The HIC1 protein is a sequence-specific 714 amino acid transcriptional repressor with five Krüppel-like zinc fingers in its C-terminal part, and an N-terminal broad complex, tramtrack, and bric à brac/poxviruses and zinc-finger (BTB/POZ) protein-protein interaction domain (fig. 1B) [5–7]. The zinc fingers are needed for DNA binding, whereas the BTB/POZ domain is important for dimerisation and transcriptional repression [5]. A second repression domain was found in the central region which may recruit the C-terminal binding proteins (CtBPs) for repression [8, 9]. Deltour et al. [8] demonstrated that HIC1 mediates transcriptional repression by both HDAC-dependent and -independent mechanisms.

At present there are two known transcriptional regulators of *HIC1*. A potential role has been suggested for the tumour suppressor p53 in activating transcription of *HIC1* [2, 7]. Later we identified a p53 responsive element (PRE) 500bp upstream of the TATA-box containing promoter P0 [10]. We demonstrated that this PRE is necessary and sufficient to mediate induction of transcription by p53. Moreover, knockdown of p53 prevented *HIC1* mRNA induction in response to UV-induced DNA damage. Inter-

estingly, other members of the p53 family, for example *TAp73α/β* and *ΔNp63α*, also induced *HIC1* transcription via PRE. We recently identified E2F1 as a novel, positive regulator of *HIC1* [11]. E2F1 is a transcripton factor involved in different cellular processes such as cell cycle progression, DNA replication, oncogenic transformation and apoptosis responses. E2F1 may promote cell growth or cell death depending on the amount of active E2F1 in the cell and the cellular context [12]. We showed that E2F1 binds the *HIC1* promoter *in vivo,* and identified two E2F-responsive elements in the proximal *HIC1* promoter which are necessary to mediate induction of *HIC1* transcription by E2F1. When we induced DNA damage by treating cells with etoposide, *HI***C***1* was upregulated. This effect was dependent on E2F1, since E2F1 knockdown by RNA interference diminished *HIC1* induction. We therefore propose a role for the E2F1-HIC1 pathway in the DNA damage response.

On the other hand, several posttranslational regulatory mechanisms have been described for *HIC1*: (a) glycosylation of the HIC1 protein preferentially occurs in the DNA-binding domain but does not affect its specific DNA-binding activity; (b) sumoylation of the conserved lysine K314 in the central region was found to reduce HIC1 repressive activity; and (c) acetylation of the same K314 residue affected HIC1 transcriptional activity (reviewed in [13]).

Possible HIC1 tumour suppressor pathways

The list of known HIC1 targets is steadily growing, and currently there are eight confirmed, transcriptionally repressed HIC1 targets as well as one target that is inactivated by HIC1 via sequestration to so-called "HIC1 bodies" (fig. 1C). In the following section we will discuss how inactivation of HIC1 and hence induction of the HIC1-repressed targets might lead to cancer.

The silent mating type information regulation 2 homolog 1 *(SIRT1)* deacetylase was the first transcriptional target of HIC1 to be identified [14]. SIRT1 belongs to the type III NAD^+-dependent histone/protein deacetylases family and is involved in regulating cellular senescence and longevity. In line with this concept, enhanced SIRT1 expression has been found in different human cancers [15]. An important, non-histone substrate of SIRT1 is the p53 tumour suppressor. Deacetylation of the p53 transcription factor attenuates its ability to activate downstream targets involved in regulation of apoptosis and/or proliferation. HIC1 directly interacts with the SIRT1 protein, forming a transcriptional repression complex which then binds and represses the *SIRT1* promoter [14]. Further, the authors suggested a model where under normal physiological conditions HIC1 represses *SIRT1* transcription and therefore inhibits p53 deacetylation. Indeed, acetylation is indispensable for p53 activation, to control growth arrest and apoptosis in response to stress such as DNA damage. Since p53 is a positive transcriptional regulator of *HIC1*, activated p53 would induce *HIC1* expression, which in turn represses *SIRT1,* thus representing a positive feedback loop. In tumour cells where *HIC1* is inactivated, *SIRT1* levels may increase, leading to deacetylation and therefore inactivation of p53 activity and allowing cells to bypass apoptosis and survive DNA damage. However, a recent publication did not find a correlation between *HIC1* and *SIRT1* expression in primary diffuse large B-cell lymphomas, arguing against direct regulation of *SIRT1* by functional HIC1 [16].

Briones et al. described an *HIC1* binding site in the fibroblast growth factor binding protein (*FGF-BP;FGFBP1*) promoter that is necessary to mediate repression of FGF-BP [17]. *FGF-BP* has been shown to enhance FGF-mediated biochemical and biological events specifically during blood vessel growth. For example, constitutive expression of *FGF-BP* resulted in highly angiogenic tumours in xenograft tumour assays. Additionally, blocking FGF-BP inhibited proliferation of prostate cancer cells [18]. In conclusion, inactivation of HIC1 in tumour cells may allow expression of FGF-BP, resulting in an increase in angiogenesis and/or proliferation.

Hic1 was recently described as a novel tumour suppressor in a murine medulloblastoma model [19], where it cooperates with the *Patched (Ptch) 1* tumour suppressor. *Ptch1*$^{+/-}$/*Hic1*$^{+/-}$ heterozygous knockout mice showed a

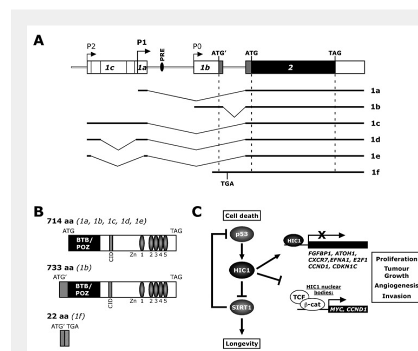

Figure 1

Gene structure of human *HIC1*. (**A**) Schema of the human *HIC1* gene: *HIC1* transcription can initiate at three separate promoters (P0, P1 and P2) that give rise to three alternative first exons 1a, 1b and 1c, followed by a second coding exon, which contains the 3' untranslated region. Exons 1a and 1c are noncoding and associated with the major GC-rich promoters P1 and P2 respectively, whereas exon 1b is partially coding and associated with the P0 TATA box promoter. Exon 1b contains an ATG' codon that is in frame with the ATG initiation codon located in exon 2. Additional alternative splice events in exon 1c yield two new transcripts named 1d and 1e, both transcribed from promoter P2. Translation of the unspliced transcript 1f starts at ATG' within exon 1b, but contains a TGA stop codon in the unspliced intron sequence. Possible translational starts (ATG; ATG', alternative start codon of 1b and f) and stops (TAG; TGA, stop codon of unspliced 1f) are indicated. Black and grey colouring represents translated exons. PRE, p53 responsive element. (**B**) Functional protein domains of HIC1 and possible HIC1 isoforms. Splicing variants leading to the respective protein isoform are indicated. Transcript 1b could encode an alternative protein containing additional 19 amino acids (12 derived from exon 1b and 7 from 5' sequences in exon 2) at the N-terminus. The unspliced transcript 1f might result in a 22 amino acid polypeptide due to a premature stop codon in the intron. BTB/BOZ, broad complex, tramtrack and bric à brac/poxviruses and zinc finger repression domain; CID, consensus motif of the CtBP-interacting domain; Zn, zinc fingers. (**C**) Putative *HIC1* tumour suppressor pathways. Please see text for details. *FGFBP1, Fibroblast growth factor binding protein; ATOH1, Atonal Homolog 1; CXCR7, chemokine (C-X-C motif) receptor 7; EFNA1, Ephrin-A1; CCND1, Cyclin D1; CDKN1C, cyclin-dependent kinase inhibitor 1C/p57*^{KIP2}.

markedly increased incidence of medulloblastoma as compared to *Ptch1*$^{+/-}$ heterozygous mice. Moreover, it was found that the proneural transcription factor *Atonal Homolog 1 (Atoh1)* is directly suppressed by *Hic1*. *Atoh1* is essential for cerebellar growth and development. The authors suggested a model where inactivation of *Hic1* leads to untimely *Atoh1* expression which then might support the malignant growth of medulloblastoma.

Another direct *HIC1* target is the scavenger chemokine receptor 7 (*CXCR7)*. It was identified as one of several down-regulated genes in a genome-wide expression profiling in *HIC1*-deficient U2OS osteosarcoma cells transduced with an adenoviral *HIC1*-expression vector. Further analyses, including confirmatory real-time PCR and promoter analyses using luciferase assays and chromatin immunoprecipitation, showed that HIC1 forms a complex with the co-repressor CtBP and binds to *HIC1*-responsive elements in the *CXCR7* promoter to repress its expression [20]. It is therefore conceivable that loss-of-*HIC1*-function in tumour cells leads to increased *CXCR7* expression. Indeed, CXCR7 is highly expressed in primary human breast and lung cancer [21]. In line with this concept, suppression of *CXCR7* by RNAi reduces *in vivo* tumour growth in nude mice [22]. Taken together, by regulating *CXCR7* expression, *HIC1* may be involved in regulation of the chemokine cross-talk between tumour cells and the surrounding stroma.

There is yet another mechanism whereby *HIC1* has been implicated in epithelial malignancy: HIC1 has recently been shown to be a direct transcriptional repressor of the gene encoding *ephrin-A1 (EFNA1)* [23]. Similarly to the above studies, HIC1 has been shown to bind the ephrin-A1 promoter directly and repress transcription. *Ephrin-A1* functions as a cell surface ligand for Eph receptors, which are a subfamily of receptor tyrosine kinases. Bidirectional ephrin/Eph-signalling has been implicated in many aspects of malignancy, such as tumour growth, invasion, metastasis and angiogenesis (reviewed in [24]). Consequently, in breast cancer cells restoration of HIC1 function reduced tumour growth *in vivo,* which could partly be reversed by ectopic expression of *ephrin-A1*.

In addition to being a transcriptional target of *E2F1* [11], *HIC1* in turn suppresses E2F-responsive genes, such as *E2F1,* by recruiting corepressors of the SWI/SNF family to the promoters [25]. This forms another feedback loop finetuning the actions of the E2F family members. E2Fs are crucial in regulating cell cycle progression, and inactivation of *HIC1* leads to undue activation of E2F signalling, thus favouring cell cycle progression and tumour growth.

Lastly, *HIC1* is involved in regulation of the T-cell-specific transcription factor 4 (*TCF4*), although by a different inhibitory mechanism. *TCF4* belongs to the T-cell receptor/lymphoid enhancer binding factor (TCR/LEF) protein family, and as its relatives it functions as a nuclear effector of the Wnt signalling pathway. *TCF4* interacts with β-catenin in active Wnt signalling and co-activates downstream target genes. This activity is important during normal development, but its deregulation plays a pivotal role in cancer progression, as evidenced by the frequent disruption of the negative Wnt regulator APC (adenomatous polyposis coli) in colorectal cancer. Earlier it was shown that *HIC1* sequestered C-terminal binding proteins (CtBPs) to nuclear dot-like structures called HIC1-bodies [8]. Valenta et al. [26] have now found that HIC1 also sequestered TCF4 as well as TCF4 bound β-catenin via CtBP to HIC1-bodies and thereby attenuated Wnt signalling. This may suppress tumour formation, since *TCF4* and β-*catenin* are prevented from activating TCF-responsive genes possibly involved in tumour development, such as *c-Myc (MYC)* or *Cyclin D1 (CCND1)*. Interestingly, *Cyclin D1,* a positive cell cycle regulator that is frequently amplified in tumours, was also identified as a direct *HIC1*-repressed gene [20, 27]. The same study revealed the cell cycle inhibitor *P57KIP2 (CDKN1C)* as a novel HIC1 target. This apparent contradiction of HIC1 repressing a cell cycle accelerator, Cyclin D1 and inhibitor, *P57KIP2,* may be explained by the observation that low levels of *P57KIP2* are able to promote cyclin/CDK complex formation and thus cell cycle progression [28].

HIC1 inactivation in solid cancers

HIC1 promoter hypermethylation was found in a wide variety of solid cancers, for example in breast, brain, liver, colorectal, cervical and lung tumours (see table 1 for a detailed list). Importantly, while few studies have tried to link *HIC1* promoter hypermethylation to *HIC1* mRNA expression, it is generally assumed that hypermethylation of the *HIC1* promoter region leads to silencing of *HIC1* gene expression [7, 29]. On the other hand, Fujii et al. found that in normal breast ductal tissue one *HIC1* allele is often hypermethylated, whereas both alleles were hypermethylated in the major portion of primary breast cancer tissue [30]. In line with this concept, two studies found increasing *HIC1* promoter hypermethylation from normal liver tissue, to precancerous liver tissue showing chronic hepatitis or cirrhosis, to primary hepatocellular carcinoma [29, 31]. Overall *HIC1* expression levels decreased during the development of cancer, but hypermethylation did not correlate significantly with *HIC1* expression levels in hepatocellular carcinoma cells or the corresponding noncancerous tissue, indicating that *HIC1* inhibitory mechanisms other than hypermethylation contribute to the low *HIC1* expression.

In addition, *HIC1* promoter hypermethylation was found in normal brain tissue of children [32], in adult brain [33] and in prostate epithelium [34], but since *HIC1* expression was not measured it cannot be concluded that these normal tissues are predisposed to tumour development due to low *HIC1* expression. In summary, these findings indicate that low *HIC1* levels contribute to cancer development, but also that inhibitory mechanisms other than hypermethylation of the *HIC1* promoter exist, e.g. mutations/inactivation of the positive *HIC1* regulator p53, or aberrant expression of not yet identified *HIC1* inhibitors.

HIC1 in normal and malignant haematopoiesis

A possible role for *HIC1* in leukaemogenesis is suggested by the fact that the distal arm of chromosome 17p is often altered in advanced stages of chronic myeloid leukaemia (CML) or acute lymphoid leukaemia (ALL). In a first pub-

lication, Issa et al. investigated whether *HIC1* is hypermethylated in a panel of different haematopoietic disorders. Using restriction enzyme methylation analysis of five *NotI* sites within the *HIC1* 5' untranslated region (promoter P0), they found that normal haematopoietic cells, such as peripheral blood leukocytes, CD34- or CD34+ cells, were not hypermethylated. In contrast, *HIC1* promoter methylation was seen in ALL, in chronic-phase CML (CML-CP) as well as in primary non-Hodgkin's lymphoma (NHL) [35]. Moreover, they found increased *HIC1* methylation in ALL patients who relapsed after a chemotherapy-induced complete remission, as well as in CML in blast crisis (CML-BC) as compared to CML-CP. These observations indicate that *HIC1* promoter hypermethylation may be a late event in ALL and CML [35].

In the most common type of NHL, diffuse large B-cell lymphoma (DLBCL), Stocklein et al. [16] identified *HIC1* as a novel tumour suppressor telomeric to p53. In most cases hypermethylation of one *HIC1* allele was accompanied by deletion of the second allele, resulting in $HIC1^{-/-}$ tumours. Moreover, DLBCL patients with complete inactivation of both p53 and *HIC1* genes presented a worse clinical course than patients with inactivation of p53 alone. This functional cooperation of the two proteins in tumour development is supported by data from $Hic1^{+/-}/p53^{+/-}$ double heterozygous mice, which show a higher incidence of osteosarcoma and lymphoma than *Hic1* altered mice [36].

Only 10% of primary acute myeloid leukaemia (AML) showed methylation of the *HIC1* P0 promoter at diagnosis, suggesting that *HIC1* promoter hypermethylation is an infrequent event in this disease [35]. Ekmekci et al. did not find *HIC1* promoter hypermethylation in a second cohort of AML [37], and recently low levels (12.5%) of HIC1 promoter hypermethylation were found in myelodysplastic syndromes or secondary AML from MDS [38]. Other studies found higher levels of *HIC1* hypermethylation in MDS (32%, [39]) and in 107 AML patients (overall 51%, [40]). It is worth noting that the second study explicitly excluded AML of the M3 subtype (acute promyelocytic leukaemia, APL). A different study found hypermethylation of exon 2 (referred to intron 2 and exon 3 in their publication) in 10 of 12 AML samples. Importantly, no correlation between *HIC1* mRNA levels and methylation of exon 2 was found in AML or bone marrow from healthy donors [41]. Taken together, there are a significant number of AML cases that do not exhibit *HIC1* promoter hypermethylation. In agreement with those results, we found that the P0 and P1 *HIC1* promoters are not hypermethylated in HL60 promyelocytic cells as measured by bisulphite genomic sequencing [42]. Nevertheless, primary AML samples showed significantly lower *HIC1* expression than granulocytes, and *HIC1* was upregulated in different models of myeloid differentiation. Hence the reasons behind the low expression of *HIC1* in AML remain unclear. A finding of note was a 10-fold higher induction of *HIC1* in the t(15;17)-positive APL cell line NB4 than in HL60 or U937 myeloid leukaemic cells (fig. 2A and [42]) upon all-*trans* retinoic acid (ATRA)-induced granulocytic differentiation. To rule out the possibility that enhanced *HIC1* induction in NB4 cells is due to a higher basal level of promoter methylation – ATRA effects include inhibition of methyltransferases – we analysed the *HIC1* promoter in NB4 cells. As seen with HL60 cells, no hypermethylation of the *HIC1* P0 promoter was found. As a control we used U2OS osteosarcoma cells that showed dense hypermethylation of P0 (fig. 2B). We only investigated the P0 promoter because tumours that do not express *HIC1* most often experience P0 promoter hypermethylation [43]. These findings indicate that other inhib-

Table 1
HIC1 promoter hypermethylation in primary solid tumours.

Tissue	Technique	Hyper-methylation	Expression	Reference
Colorectal cancer	MSED	38–80%	n.d.	[44]
Colorectal cancer	MSP	62%	n.d.	[45]
Colorectal cancer	ML	52%	n.d.	[46]
Non-small cell lung cancer	MSED	31–33%	n.d.	[47]
Hepatocellular carcinoma*	MSED	38–90%	+	[29]
Breast cancer*	MSED	67%	+	[30]
Breast cancer*	MSP	43–64%	n.d.	[48]
Cervical cancer	MSP	9–30%	n.d.	[49]
Cervical cancer	MSP	59%	n.d.	[50]
Cervical adenocarcinoma	MSP, BGS	32–64%	n.d.	[51]
Ovarian carcinoma	MSP, COBRA	11–39%	n.d.	[52]
Ovarian carcinoma	MSP	34%	n.d.	[53]
Bladder carcinomas	MSP	0%	n.d.	[54]
Prostate cancer	MSED	96%	n.d.	[34]
Renal tumours*	MSED	50–74%	n.d.	[55]
Glioblastoma*	MSED	60%	n.d.	[56]
Glioma*	ML	100%	n.d.	[33]
Medulloblastoma*	MSP	75–90%	+	[57]
Ependymonas*	MSP, BGS	83%	+	[58]
Germ cell tumour	MSP, BGS	32%	n.d.	[59]
Paediatric neoplasms*	MSP	0–100%	CL	[60]
Gastric tumours*	MSED	15–45%	n.d.	[61]

* = including normal tissue; CL = cell lines only; n.d. = not done; BGS = bisulphite genomic sequencing; BiPS = bisulphite treatment/PCR-single strand conformation polymorphism; COBRA = combined bisulphite restriction analysis; ML = MethyLight analysis; MSED = methylation sensitive enzymatic digestion; MSP = methylation specific PCR.

itory mechanisms than hypermethylation must be active in NB4 cells. Since the major molecular difference between HL60 and NB4 cells is the expression of promyelocyte leukaemia – retinoic acid receptor α (PML-RARα) fusion protein in NB4 cells, and because the *HIC1* promoter is not hypermethylated in these cells and because *HIC1* induction upon ATRA treatment is higher than in PML-RARa negative cells, we speculate that PML-RARa may repress *HIC1* transcription. In line with this concept, we identified a putative ATRA responsive element upstream of exon 2 (fig. 2C). Ongoing research aims to elucidate the role of RARa/PML-RARa in *HIC1* regulation. Obviously, a PML-RARα *HIC1* repression model would only explain low *HIC1* levels in APL and not in other AML subtypes, and therefore further studies are needed to identify novel *HIC1* inhibitory pathways in AML.

Conclusions

HIC1 is a central transcriptional regulator of key genes controlling cell growth as well as cell death in response to DNA damage. Alteration of the *HIC1* pathway may lead to abnormal cell proliferation and stress responses contributing to a cancerous phenotype. Accordingly, *HIC1* is frequently hypermethylated in a variety of solid tumours and leukaemias, making it an interesting therapeutic target for hypomethylating agents such as decitabine. Moreover, low *HIC1* expression in the absence of *HIC1* promoter hypermethylation, as seen in certain leukaemias, points to additional inhibitory pathways that may serve as starting points for new therapeutic approaches.

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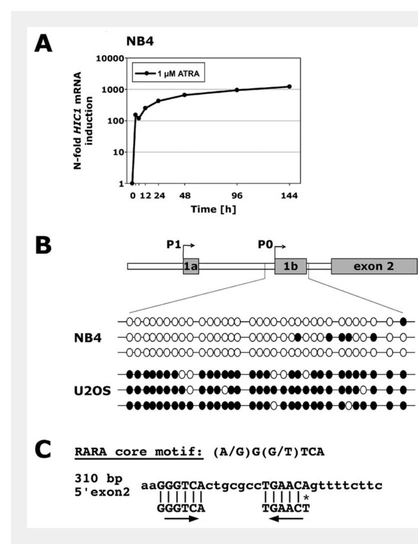

Figure 2

HIC1 regulation in acute promyelocytic leukaemia (APL). (**A**) NB4 APL cells were differentiated towards granulocytes in vitro for times indicated using 1 µM *all*-trans retinoic acid (ATRA). Successful differentiation was confirmed by morphology and FACS analysis of CD11b surface expression. Total RNA was extracted and levels of *HIC1* mRNA were assessed using real-time quantitative RT-PCR. mRNA levels are expressed as n-fold changes in regulation compared to untreated cells using *HMBS* mRNA expression as a reference gene. (**B**) Bisulphite sequencing of NB4 APL and U2OS osteosarcoma cells. Top: Genomic organisation of the *HIC1* promoter region highlighting the analysed CpG island. Methylation was assessed by sequencing of three individual clones derived by PCR on bisulphite-treated genomic DNA. Black circles represent methylated CpG sites, white circles unmethylated CpG sites, and each horizontal line indicates an individual allele. (**C**) Putative retinoic acid receptor binding element in the *HIC1* promoter. Homologies to a region 5' of exon 2 as well as orientation of the two core motifs are indicated.

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