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HIC1 (Hypermethylated in Cancer 1) epigenetic silencing in tumors

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Abstract

HIC1 (Hypermethylated In Cancer 1), as its name implied, was originally isolated as a new candidate tumor suppressor gene located at 17p13.3 because it resides in a CpG island that is hypermethylated in many types of human cancers. *HIC1* encodes a transcription factor associating an N-terminal BTB/POZ domain to five C-terminal Krüppel-like C₂H₂ zinc finger motifs. In this review, we will begin by providing an overview of the current knowledge on *HIC1* function, mainly gained from *in vitro* studies, as a sequence-specific transcriptional repressor interacting with a still growing range of HDAC-dependent and HDAC-independent corepressor complexes. We will then summarize the studies that have demonstrated frequent hypermethylation changes or losses of heterozygosity of the *HIC1* locus in human cancers. Next, we will review animal models which have firmly established *HIC1* as a *bona fide* tumor suppressor gene epigenetically silenced and functionally cooperating notably with p53 within a complex *HIC1*-p53-SIRT1 regulatory loop. Finally, we will discuss how this epigenetic inactivation of *HIC1* might “addict” cancer cells to altered survival and signaling pathways or to lineage-specific transcription factors during the early stages of tumorigenesis.

Keywords

HIC1; 17p13.3; transcriptional repression; p53; SIRT1

1. Introduction

For a long time cancer has been considered as a group of diseases driven by genetic modifications including chromosomal translocations, gene amplifications or point mutations in oncogenes or in tumor suppressor genes. However, it is now becoming increasingly clear that epigenetic changes, marked by DNA methylation and histone tail modifications, which convey heritable gene expression patterns, are also central to many human diseases including cancer.

DNA hypermethylation changes at the D17S5 locus in many cancers allowed the positional cloning of *HIC1 (Hypermethylated In Cancer 1)* as a candidate tumor suppressor located in

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17p13.3, a region frequently hypermethylated or deleted in many types of prevalent human tumors (Wales et al., 1995). *HIC1* encodes a transcription factor with five Krüppel-like C₂H₂ zinc finger motifs (Wales et al., 1995) (Deltour et al., 1999) (Deltour et al., 2002) (Pinte et al., 2004b).

In this review, we will first provide an overview of the current understanding of *HIC1* function as a sequence-specific transcriptional repressor. We will then describe the studies and animal models which unambiguously defined *HIC1* as a tumor suppressor gene epigenetically silenced in many prevalent human cancers.

2. *HIC1*: genomic organization

From sequencing analyses of genomic clones and from functional assays, the exon-intron structures of the human and murine *HIC1* genes appeared very similar with alternative first exons followed by a unique second exon, containing a coding region and a 3' untranslated region (Carter et al., 2000) (Guerardel et al., 2001). The human *HIC1* genomic region contains three promoters that give rise to different alternatively spliced transcripts. Exon **1a** is non-coding and associated with the major G-C rich promoter, P1 whereas exon **1b** is coding and associated with a minor TATA promoter called P0 (Figure 1) (Carter et al., 2000) (Guerardel et al., 2001). Exon **1c**, as well as internally spliced exons called **1d** and **1e** are transcribed from a third upstream GC rich promoter, P2 conserved in the murine and rat genomes (Pinte et al., 2004a). However, transcripts initiated from this P2 promoter can only be detected by sensitive RT-PCR analyses. By contrast, **1a** and **1b** transcripts are detected by Northern blot in various normal tissues with a predominance for exon **1a** transcripts and are upregulated by p53 and other p53 family members, notably TAp73β and ΔNp63α (Wales et al., 1995) (Guerardel et al., 2001) (Britschgi et al., 2006). The p53 responsive element in *HIC1* is highly conserved in position (in the intron between exons **1a** and **1b**) and in sequence among various species (Britschgi et al., 2006).

In addition, a novel, unspliced transcript variant, called **f**, has been recently identified in normal leukocytes and in two human glioma cell lines, U87MG (wild-type p53) and U373MG (mutant p53) (Mondal et al., 2006). This unspliced transcript initiates from an alternate transcription start site localized 164bp downstream of the **1b**-specific promoter and could possibly encode a 22-amino acid polypeptide. In addition, this **f** transcript is not under the direct control of p53 but is induced in p53 wild-type cells grown in adverse conditions with a kinetic in opposite phase with the spliced transcripts. However, the biological significance of this **f** transcript has still to be elucidated although it has been suggested to regulate the level of *HIC1* protein in cancer cells (Mondal et al., 2006).

3. *HIC1*: functional aspects

The *HIC1* protein is a sequence-specific transcriptional repressor containing three main functional domains (Figure 2): (i) the N-terminal BTB/POZ (which stands for *Broad complex*, *Tramtrack* and *Bric à brac*/Poxviruses and Zinc finger) domain of about 120 amino-acid is a dimerization domain known to play direct or indirect (through conformational effects) roles in protein-protein interactions. This domain is also an autonomous transcriptional repression domain (Albagli et al., 1995) (Stogios et al., 2005) (Kelly and Daniel, 2006); (ii) the C-terminal end contains five Krüppel-like C₂H₂ zinc fingers which bind a recently defined DNA sequence, termed the HiRE (*HIC1* responsive element) (Pinte et al., 2004b) and a tail that displays no obvious functional domain but has been phylogenetically conserved (Deltour et al., 1998); (iii) a central region which is a second autonomous transcriptional repression domain (Deltour et al., 2002) (Stankovic-Valentin et al., 2006) (Stankovic-Valentin et al., 2007).

Due to alternative splicing mechanisms, *HIC1* could encode two highly related proteins. The major **1a**-type transcripts direct the synthesis of a 714 amino acids protein encoded by the ORF in exon 2. Transcripts containing the conserved coding exon **1b** could encode an alternative putative protein containing 19 additional N-terminal amino acids (12 derived from exon **1b** and 7 derived from 5' sequences in exon **2** that are converted into coding sequences) (Figure 1) (Guerardel et al., 2001).

3.1 The BTB/POZ domain

The BTB/POZ domain is a highly conserved and widely distributed structural motif found mainly in transcription factors (Albagli et al., 1995). Crystal structures of several human BTB/POZ domains have demonstrated that this domain is a butterfly-shaped tightly intertwined obligate dimer with a conserved dimerization interface (Ahmad et al., 1998). BTB/POZ domains are protein-protein interaction domains and mediate homo-oligomerization, hetero-oligomerization as well as interactions with non-BTB/POZ proteins (Stogios et al., 2005). These properties are essential for the biological function of BTB/POZ-containing proteins. For example, the BCL6 BTB/POZ domain directly recruits nuclear corepressors SMRT, N-CoR or B-CoR/Histone deacetylase (HDACs) complexes in an exclusive manner. Co-crystal structure between the BCL6 BTB/POZ domain and a 17 amino-acid SMRT peptide have highlighted a lateral groove (Ahmad et al., 2003) that is bound competitively by B-CoR (Ghetu et al., 2008). Furthermore, the critical residues engaged in the interaction are not very well conserved, suggesting that distinct binding sites may exist and that protein-protein recognition modes could be specific to each BTB/POZ domain. The HIC1 BTB/POZ domain is also an autonomous transcriptional repression domains but is insensitive to trichostatin A (TSA), a specific inhibitor of class I and class II HDACs (Deltour et al., 1999; Deltour et al., 2001). It has been recently shown that HIC1 forms a transcriptional repression complex with the class III HDAC, SIRT1 and that this complex directly binds the *SIRT1* promoter to repress its transcription (Chen et al., 2005). SIRT1 is a NAD-dependent deacetylase which is insensitive to TSA but sensitive to nicotinamide (NIA) (Saunders and Verdin, 2007). The HIC1 BTB/POZ domain interacts with SIRT1 (Chen et al., 2005) but its repressive activity in the context of a Gal4 chimera is not inhibited by NIA (our unpublished results).

Thus, the repression mechanisms inherent to the HIC1 BTB/POZ domain have still to be deciphered.

3.2 The HIC1 Central Region

The HIC1 central region has not been subjected to a strong selection pressure except for 4 peptidic motifs perfectly conserved from human to zebrafish (Bertrand et al., 2004). One of them, GLDLSKK, is highly related to the canonical motif PxDSLxK/R found in proteins interacting with the co-repressor CtBP (C-terminal Binding Protein) (Chinnadurai, 2007). We have demonstrated that HIC1 interacts with the two related CtBP1 and CtBP2 corepressors through this conserved GLDLSKK motif thus extending the CtBP binding site (Deltour et al., 2002) (Stankovic-Valentin et al., 2006). Notably, mutation of the central leucine residue, Leu 225 in HIC1, which is the only invariant residue in CtBP-interaction motifs, abolished the interaction between HIC1 and CtBP (Figure 2) (Stankovic-Valentin et al., 2006). As would be expected from the corepressor activity of CtBP, the L225A point mutation or deletion of the GLDLSKK motif impairs the repression potential of the HIC1 central region, but does not fully abolish it (Stankovic-Valentin et al., 2006). Thus, the HIC1 central region appears to be a second repression domain exhibiting both CtBP-dependent and CtBP-independent repression mechanisms, both of which are sensitive to TSA.

The second conserved motif is an YRWM/VK³¹⁴xEP motif (Stankovic-Valentin et al., 2007) which contains a potential SUMOylation consensus site, ψ KxE. SUMOylation is a reversible

post-translational modification in which a member of the Small Ubiquitin-like Modifier (SUMO) family of proteins is covalently conjugated to lysine residues in target proteins (Verger et al., 2003) (Hay, 2005) (Geiss-Friedlander and Melchior, 2007). Unlike ubiquitination which generally marks proteins for rapid degradation, SUMOylation of nuclear proteins has very diverse effects on transcriptional activity ranging from regulation of DNA-binding activity, subcellular localization and assembly of multiprotein complexes. In the case of HIC1, SUMOylation of K314 does not affect its subnuclear localization and its interaction with CtBP, HDAC4 and SIRT1 but does positively regulate the transcriptional repression potential (Stankovic-Valentin et al., 2007).

Lysine residues can be targeted by several post-translational modifications including SUMOylation, acetylation, ubiquitination or methylation. The ψ KxEP motif in HIC1 with the Proline residue conserved from human to zebrafish is related to the G/SKxxP consensus motif for acetylation by CBP/P300. Indeed, we have shown that HIC1 is acetylated on various Lysine residues including K314. Thus, the ψ KxEP motif is an acetylation/SUMOylation switch which is related to the ψ KxEPxxSP, phosphorylation-regulated SUMOylation-acetylation switch motif (SAS) found in the major MEF2 isoforms (Shalizi et al., 2006). In both cases, the cross-talk between these two competitive post-translational modifications of Lysine residues is orchestrated by a new complex associating two distinct types of deacetylases, HDAC4 and SIRT1. Eventhough the precise mechanisms are not fully understood, these results identify HIC1 as a new target of the class III deacetylase SIRT1.

In conclusion, the HIC1 central region is essential for the transcriptional repression potential of HIC1. But, corepressors and complexes interacting with this region have still to be characterized.

3.3 The Zinc finger DNA-binding domain and the HIC1 target genes

The C-terminal end contains a cluster of four conserved C₂H₂ zinc fingers (ZF2-5) which are separated by the typical 7-8 amino acid conserved H/C links found in *Krüppel*-like Zinc fingers, making them likely to be involved in sequence-specific DNA binding. Using a combination of functional assays and *in silico* analyses, we have identified the sequence 5'-C₁/GNG^C/GGGGCA^C/A₁CC-3' as an optimal HIC1 binding site (HiRE for HIC1-responsive element) and validated by mutational analyses a GGCA core motif bound by Zinc fingers 3 and 4 (Figure 2) (Pinte et al., 2004b). In addition, HIC1 proteins contain a more distant and isolated upstream Zinc finger motif (ZF1) which is conserved but is unlikely to contribute to DNA-binding.

We have also shown that the full-length HIC1 protein is modified with O-linked *N*-acetylglucosamine (*O*-GlcNAc) (Lefebvre et al., 2004). This glycosylation is a dynamic modification of nuclear or cytoplasmic proteins by a single residue of *N*-acetylglucosamine on serine and threonine residues and can compete with phosphorylation (Guinez et al., 2005). Using deletion mutants, we have shown that most of the *O*-GlcNAc modifications of HIC1 occur in the DNA-binding domain although it does not affect its specific DNA-binding activity.

The full-length HIC1 protein is unable to bind to a single optimized site and this DNA-binding inhibition is clearly BTB/POZ-dependent. Whereas the HIC1 BTB/POZ domain impedes binding to a single site, it mediates strong cooperative binding to a probe containing multiple optimized sites (Pinte et al., 2004b).

The first *bona fide* HIC1 direct target gene, *SIRT1*, contains a cluster of two HiRE in the same orientation located at the 5' end of the promoter (Chen et al., 2005). ChIP and ChIP upon ChIP assays demonstrated that HIC1 forms a transcriptional repression complex with the deacetylase SIRT1 to directly bind the *SIRT1* promoter and repress its transcription. *SIRT1* can also be

regulated by HIC1 in response to acute metabolism changes by a complex between HIC1 and the redox sensor CtBP corepressor (Zhang et al., 2007).

The second HIC1 target gene, *FGF-BP1* (Fibroblast Growth Factor-Binding Protein 1) has been shown to play key roles in blood vessel development in the growing embryo as well as in tumor angiogenesis. This extracellular matrix bound protein enhances fibroblast growth factor (FGF) signaling which induces endothelial cell proliferation and smooth cell differentiation. TGF- β represses *FGF-BP1* mRNA expression through its intermediates, the transcription factors Smad2 and Smad3. However, functional analyses of the *FGF-BP1* promoter using deletion constructs identify a region containing a HIC1 binding site, which could participate to the repression of *FGF-BP1* by TGF- β (Briones et al., 2006). This potential link between HIC1 and two pathways involved in angiogenesis is particularly interesting given HIC1's strong association with many human tumors.

Very recently, *Atonal Homolog 1* (*Atoh1/Math1*), a basic helix-loop-helix (bHLH) transcription factor expressed in and required for the proper development of various neural progenitor cells, notably including cerebellar granule cells has been identified as a direct transcriptional target of HIC1 through genome-wide profiling analysis of *HIC1*-deficient medulloblastoma D425 cells infected with adenoviruses expressing HIC1 (Briggs et al., 2008). The proper expression of *Atoh1* in the developing central nervous system relies on a 1.4 Kbp downstream enhancer region that contains two adjacent HiRE in the same orientation, as observed in the *SIRT1* promoter (Chen et al., 2005). These two sites are bound by HIC1 as shown by chromatin immunoprecipitation experiments in normal mouse embryonic cerebella (Briggs et al., 2008).

Besides its direct target genes which remain largely to be identified, HIC1 could also bind to other transcription factors and “indirectly” repress transcription of target genes that do not contain HIC1 binding sites. HIC1 was shown to interact with TCF4, a major component of the Wnt signaling pathway, in a complex multidomain mode of interaction. As a consequence, TCF4 and β catenin are recruited to discrete nuclear structures called HIC1 bodies and diverted from the Wnt-responsive promoters (Valenta et al., 2006). Thus, the intracellular amounts of HIC1 protein can modulate the transcriptional stimulation of genes regulated by canonical Wnt/ β catenin signaling.

Further work will be required to determine if these HIC1 bodies can also sequester other transcription factors away from their DNA targets.

4. Inactivation of *HIC1* is frequent in cancers

From the beginning, the history of *HIC1* has been directly linked to *P53*. Indeed, loss of heterozygosity (LOH) on the short arm of chromosome 17 (17p) is one of the most common genetic alterations in human cancers and allelic losses most often coincide with mutations in the *TP53* gene at 17p13.1 (Ko and Prives, 1996). However, it became rapidly obvious that in breast and ovarian tumors, 17p allelic losses occur at high frequency in regions distal to *TP53* and even in the absence of *TP53* mutations. Consequently, these studies led to the proposal that one or more tumor suppressor genes whose loss of function is required for early tumorigenesis may reside in a region telomeric to *TP53* (Cornelis et al., 1994) (Sakamoto et al., 1996) (Liscia et al., 1999). A good candidate region was 17p13.3 containing the VNTR (Variable Number of Tandem Repeats) microsatellite marker YNZ22/D17S5/D17S30 that exhibits frequent DNA hypermethylation in lung and colon cancer (Makos et al., 1992) as well as in neural (Makos et al., 1993a) (Rood et al., 2002) and renal tumors (Makos et al., 1993b). Indeed, DNA hypermethylation changes of the NotI restriction sites at the D17S5 locus in many cancers allowed the positional cloning and characterization by nucleotide sequencing of a new gene called *HIC1* (*Hypermethylated In Cancer 1*) (Wales et al., 1995). *HIC1*'s role as a candidate tumor suppressor gene is supported by several observations: it is epigenetically

silenced by promoter hypermethylation in many tumor cell lines as well as in primary colon and brain tumors; its enforced expression by stable transfection in various cancer cell lines results in a significant decrease in their clonogenic survival and its expression can be upregulated by p53 (Wales et al., 1995).

4.1 HIC1 inactivation in solid tumors

After this pioneering work, several groups have confirmed these observations using the methylation-sensitive restriction enzyme NotI and Southern blotting with D17S5 or *HIC1* probe as well as more sensitive and informative assays such as methylation-specific PCR (MSP) and bisulfite sequencing. These studies have extended the epigenetic silencing of *HIC1* to prostate cancers (Morton et al., 1996); non-small cell lung cancers (Eguchi et al., 1997) (Hayashi et al., 2001); breast cancers (Fujii et al., 1998); gastric and liver cancers (Kanai et al., 1998) (Kanai et al., 1999); esophageal cancers (Huang et al., 2000) (Eads et al., 2001) and human male non-seminomatous germ cell tumors (Koul et al., 2002). Epigenetic silencing of *HIC1* has been also very frequently observed in the most common malignant brain tumor of childhood medulloblastoma (Rood et al., 2002) (Rathi et al., 2003) (Waha et al., 2003) and the glial malignancy, ependymoma (Waha et al., 2004).

HIC1 is thus epigenetically silenced in many types of common human cancers. However, some general conclusions can be drawn. First, loss of heterozygosity (LOH) is often associated and in fact preceded by DNA hypermethylation (Makos et al., 1993b) (Ahuja et al., 1997) (Kanai et al., 1999). For example, *HIC1* is virtually completely methylated in 27 out of 39 breast cancer tissues tested (67%) and LOH of the telomeric part of 17p where *HIC1* is located, is observed in 22 out of these 27 cases with *HIC1* hypermethylation (Fujii et al., 1998). Second, the level of *HIC1* promoter hypermethylation is variable and a high density of methylation is associated with aggressiveness of the tumor and poor overall survival (Wales et al., 1995) (Nicoll et al., 2001) (Hayashi et al., 2001) (Rood et al., 2002) (Waha et al., 2004). Finally, dense hypermethylation of one *HIC1* allele has also been detected in some normal tissues including kidney (Makos et al., 1993a), histologically normal prostate and benign hyperplastic (BPH) tissues (Morton et al., 1996), normal ductal breast tissues (Fujii et al., 1998) and in normal brain and cerebellum (Rood et al., 2002). This is in striking contrast with the lack of *HIC1* hypermethylation in most normal tissues (Wales et al., 1995) and suggests that these tissues might be at higher risk for developing cancer since one copy of *HIC1* is already constitutively inactivated.

There is some evidence from human tumor specimens to suggest that epigenetic *HIC1* silencing events predispose tissues to tumorigenesis. In paired tumorous and non-tumorous tissues from patients with non-small cell lung cancer (NSCLC), *HIC-1* was found to be methylated in 33% and 31%, respectively. Further, methylation occurred with a significantly greater incidence in tumorous and non-tumorous tissues from smokers compared to non-smokers (Eguchi et al., 1997). DNA methylation was inversely correlated with tumor cell differentiation and positively correlated with LOH at the same locus. These results support *HIC-1* methylation as a smoking-related pre-cancerous event involved in the tumorigenesis of less differentiated NSCLC. In addition, methylation in the region of the tumor suppressor gene *HIC-1* increases across the histologic spectrum extending from normal liver tissues to the pre-cancerous conditions of chronic hepatitis and cirrhosis to hepatocellular carcinoma (Kanai et al., 1999). In colon cancer, DNA on chromosome arm 17p13.3 exhibits increasing methylation density from normal colonic mucosa to pre-cancerous colonic polyps to colon cancer (Makos et al., 1992).

4.2 HIC1 inactivation in hematological malignancies

Besides its implication in solid tumors, *HIC1* is also a tumor suppressor gene epigenetically silenced in some leukemia. Indeed, *HIC1* is not methylated in normal peripheral blood cells,

in bone marrow cells or in purified CD34⁺ progenitor cells and only rarely methylated in acute leukemias at diagnosis (10%) and in the chronic-phase of chronic myelogenous leukemia CML (50%) (Issa et al., 1997a) (Issa et al., 1997b). By contrast, *HIC1* is found methylated in virtually all recurrent acute lymphocytic leukemia and in the blast-crisis of all CML. *HIC1* hypermethylation has thus been considered to be a late event in hematopoietic neoplasms (Issa et al., 1997a) (Issa et al., 1997b). Two others studies have detected a high level of methylation in acute myeloid leukemia by bisulfite sequencing, but in the central coding region of *HIC1* (Melki et al., 1999b) (Melki et al., 1999a). A recent study has demonstrated that *HIC1* expression is low in 128 patient samples of AML and in CD34⁺ progenitor cells as compared to terminally differentiated granulocytes (Britschgi et al., 2008). However, the *HIC1* promoters are not methylated as shown by bisulfite sequencing or treatment with the demethylating agent 5-Aza-dC which induces *HIC1* expression but less efficiently than the differentiation agent all-trans retinoic acid (ATRA) (Britschgi et al., 2008). Thus, these results suggest that *HIC1* might play a role in granulocytic differentiation and that low expression of *HIC1*, which is not due to promoter hypermethylation, could contribute to leukemogenesis.

Finally, *HIC1* has also been recently implicated as a tumor suppressor gene in a subset of diffuse large B-cell lymphomas (DLBCLs). Classically, hypermethylation of *HIC1* promoter is accompanied by simultaneous deletion of the second allele in ca 90% of the cases (Stocklein et al., 2007).

5. Mouse models of *Hic1* and cancer

The epigenetic silencing or deletion of *HIC1* in numerous primary tumors and cell lines strongly suggested that it could be a new tumor suppressor gene. Unquestionable clues to the tumor suppressor function of *HIC1* have come from the study of *Hic1* deficient mice developed in Dr Baylin's laboratory (Chen et al., 2003). Building on this work, two double heterozygote models have shown that *Hic1* can cooperate with *p53* and *Ptch1* in tumorigenesis (Chen et al., 2004) (Briggs et al., 2008).

5.1 *Hic1*^{+/-} heterozygotes

Whereas homozygous disruption of *Hic1* impairs development and results in embryonic and perinatal lethality (Carter et al., 2000), heterozygous *Hic1*^{+/-} mice develop an age- and gender-dependent spectrum of malignant tumors with a predominance of epithelial cancers in males (75%) and lymphomas and sarcomas (80%) in females (Chen et al., 2003). Overall, 44% of the malignant tumors were epithelial cancers in *Hic1*^{+/-} mice. Such a high percentage is unusual when compared with the preponderance of lymphomas and sarcomas observed in other knockout models of tumor suppressor genes or in aged mice but is reminiscent of the cancer types seen in adult humans (DePinho, 2000). So far, these genders effects seen in mice have not been reported in human cancer: a female predominance has not been observed among *HIC1* heterozygous diffuse large B-cell lymphoma patients (Stocklein et al., 2007). Another attribute is illustrated by the *Dmp1* heterozygous knockout model. *Dmp1* is haplo-insufficient for tumor suppression and the wild-type *Dmp1* allele is retained and expressed in spontaneous tumors arising in *Dmp1*^{+/-} heterozygous mice (Inoue et al., 2001). By contrast, no *Hic1* expression has been detected in the *Hic1*^{+/-} tumors (Chen et al., 2003). In fact, loss of function of the remaining *Hic1* allele in heterozygous mice is not due to gross chromosomal deletions but rather occurs only in association with hypermethylation of one of the two major alternative promoters of the gene as in human cancers. Bisulfite genomic sequencing of human tumor cell lines not expressing *HIC1* have detected in most cases a dense methylation only of the **1b** promoter although the most abundant *HIC1* transcripts in the corresponding normal tissues are derived from the **1a** promoter. In the *Hic1*^{+/-} heterozygous mice, a dense methylation of the **1a** but not the **1b** promoter on the remaining allele is observed in the lymphomas and sarcomas arising mainly in females. Conversely, a dense methylation of the **1b** promoter is found in the

carcinomas and adenomas arising mainly in males (Chen et al., 2003). The transcription factor networks and the mechanisms underlying this complex tissue-, age- and sex-dependant epigenetic regulation of *HIC1* are yet to be identified.

These results clearly demonstrate that loss of function of *Hic1* can lead to cancers. *HIC1* was originally identified by virtue of its frequent epigenetic inactivation in tumors and so far point mutations inactivating *HIC1* have not been reported. For example, a search for SSCP in the *HIC1* coding sequence in 68 medulloblastomas only detected a single case of an in-frame deletion of 4 GGC codons in a Glycine-rich sequences located after the BTB/POZ domain with no effect on the downstream reading frame (Waha et al., 2003).

Thus, the case of *HIC1* further illustrates that characterization of the “methylome”, the complete set of DNA methylation modifications of a cell, is a potent tool for discovery of new tumor suppressor genes (Jones and Baylin, 2007).

5.2 *Tp53*^{+/-} and *Hic1*^{+/-} cis or trans double heterozygotes

Hic1 and *p53* are located on the same chromosome in humans (chromosome 17) and mice (chromosome 11). Two models of double heterozygous knockout mice with disruption of both genes either on the same chromosome (*cis*) or on separate chromosomes (*trans*) have been generated (Chen et al., 2004). The *cis* double heterozygotes have an accelerated tumorigenesis as compared to *p53*^{+/-} mice and predominantly develop aggressive metastatic osteosarcomas, breast and ovarian tumors instead of soft tissues sarcomas. In these *cis* tumors, the remaining *p53* and *Hic1* wild-type alleles are simultaneously deleted due to loss of the entire chromosome. By contrast, the *trans* *Hic1*^{+/-} *p53*^{+/-} mice have no acceleration of tumorigenesis as compared to *p53*^{+/-} mice, albeit their tumors also develop in an age-dependant manner with a higher incidence of osteosarcomas. In the *trans* tumors, the remaining *Hic1* allele is retained and epigenetically silenced by hypermethylation while the *p53* allele undergoes interstitial deletion. The combination of these two events as compared to simultaneous deletion of the two genes could easily explain the earlier appearance and more severe phenotype observed in the *cis* animals (Chen et al., 2004). Thus, the epigenetically silenced gene *Hic1* cooperates with the mutated tumor suppressor gene *p53* in determining cancer prevalence, progression and spectrum.

5.3 *Ptch1*^{+/-} and *Hic1*^{+/-} double heterozygotes

Medulloblastoma, a childhood brain tumor arising from cerebellar granule cell precursors (GCPs) frequently displays inappropriate constitutive activation of the Hedgehog (Hh) signaling pathway as well as chromosome 17p deletion (Ferretti et al., 2005). The *PATCHED* (*PTCH*) transmembrane receptor, an inhibitor of the *SMOOTHENED* (*SMO*) co-receptor for Hedgehog (Hh) is a tumor suppressor gene inactivated in sporadic medulloblastoma and *Ptch1*^{+/-} heterozygotes spontaneously develop medulloblastomas at a frequency of 10-15% (Goodrich et al., 1997). In addition, germline mutations in *PATCHED* result in the nevoid basal cell carcinoma syndrome (Gorlin syndrome) which is often associated with medulloblastoma. Whereas mutations in elements of the Hedgehog signaling pathway are found in less than 25% of sporadic medulloblastomas, deletion of chromosome 17p occurs in up to 50% of the cases and is frequently restricted to the 17p13.1-13.3 region containing several tumor suppressor genes including *p53*, *REN*, *MNT* and *HIC1* (Ferretti et al., 2005). *P53* is not frequently mutated in medulloblastomas (less than 10%) but loss of function of *p53* significantly increases the frequency of medulloblastomas occurring in *Ptch1*^{+/-} *p53*^{+/-} animals as compared to *Ptch1*^{+/-} heterozygotes (Wetmore et al., 2001). Similarly, a recent study revealed a four-fold increase in medulloblastomas incidence in *Ptch1*^{+/-} *Hic1*^{+/-} (19/45) as compared to their *Ptch1*^{+/-} littermates (Briggs et al., 2008). Apart from this increased incidence, the time frame and the type of tumors did not vary between these two cohorts, in

striking contrast with the *p53*^{+/-} *Hic1*^{+/-} heterozygotes in which distinct tumors appear earlier than in the *p53*^{+/-} cohort (Chen et al., 2004).

Whereas *Hic1* is expressed during the differentiation of granule cell precursors, its expression (measured at mRNA and proteins levels) is markedly reduced in *Ptch1*^{+/-} *Hic1*^{+/-} and *Ptch1*^{+/-} medulloblastomas (Briggs et al., 2008). In both cases, this is due to the methylation of the **1b** promoter, as demonstrated by bisulfite sequencing. At the molecular level, *Hic1* does not play a key role in Hedgehog pathway regulation. Thus, its loss of function is not likely to directly participate in the activation of this pathway but could rather result in the overexpression of target genes ultimately leading to tumorigenesis. ATOH1, a transcription factor expressed in precursor granule cells and in medulloblastoma, activated by the Hh pathway and identified as a direct target of the HIC1-mediated transcriptional repression, nicely fulfills these criteria (Briggs et al., 2008). Moreover, siRNA-mediated knockdown of *ATOH1* dramatically reduces the viability of a cell line derived from a *Ptch1*^{+/-} *Hic1*^{+/-} medulloblastoma. According to these data, Briggs et al. proposed the following model (Briggs et al., 2008). In *Ptch1*^{+/-} heterozygotes, puffs of GCP-like progenitors persist in the external granule cell layer, forming a presumed precursor lesion. Since *Hic1* is hypermethylated in the *Ptch1*^{+/-} *Hic1*^{+/-} and *Ptch1*^{+/-} medulloblastomas, its epigenetic inactivation favored in the context of the *Ptch1*^{+/-} *Hic1*^{+/-} double heterozygotes could be one of the next deficiencies accumulated in the *Ptch1*^{+/-} precursor lesion contributing to tumorigenesis (Briggs et al., 2008). The cooperation between these two tumor suppressors, the *HIC1* transcriptional repressor and the Hh-receptor *PATCHED1*, is highly relevant to human medulloblastoma, in which deletion of the chromosomal 17p region would thus result in the loss of Hedgehog modulators such as *REN* (Di Marcotullio et al., 2004) and in the loss of regulators of lineage-specific transcription factors, as exemplified above by the *HIC1*-*ATOH1* connection.

6. *HIC1*: a cancer stem cell gene pre-marked for aberrant silencing in ES cells

According to the cancer stem cell hypothesis which is now widely accepted, tumors contain and are driven by a minority cellular population which has retained key stem-cell properties including self-renewal, pluripotency and the ability to reinitiate the tumor mass containing the cellular heterogeneity observed in the original tumor (Jones and Baylin, 2007). Numerous studies have highlighted the key roles of epigenetic signatures in stem-cell identity (reviewed in (Spivakov and Fisher, 2007)). In pluripotent embryonic stem cells (ES), whereas pluripotency-associated transcription factors such as Nanog or Oct4 are expressed, the promoters of some developmental transcription factors are in a “bivalent state” with both activating (H3K9Ac and H3K4me) and repressive (H3K27me) epigenetic marks. Thus, these promoters are “poised” in a transcription-ready state which, depending on the developmental cues, can be tipped toward activation of tissue-specific genes or reversible silencing of genes involved in other developmental pathways. Indeed, in differentiated cells, the promoters of many non-transcribed genes have lost the activating marks and are enriched with the H3K27 trimethylated mark which is deposited by the *Polycomb* repressor complex 2 (PRC2) (Spivakov and Fisher, 2007).

Besides these genome-wide analyses, three studies in ES cells have determined the epigenetic status of restricted lists of genes defined as tumor suppressor genes frequently hypermethylated in cancer. Two of these studies have demonstrated that a very high percentage of genes specifically methylated in colon, breast and ovarian cancers are pre-marked with trimethylated H3K27 and other PRC2 components in ES cells (Lee et al., 2006; Schlesinger et al., 2007) (Widschwendter et al., 2007). The third study analyzed the malignant counterpart of ES cells, the embryonal teratocarcinoma (EC) (Ohm et al., 2007). EC are neoplastic embryonic cells, but in contrast with adult cancer cells they can retain properties of spontaneous differentiation and of lineage commitment *in vivo* and *in vitro*, albeit to a lesser extent than their normal ES

counterparts. This study has further shown that embryonal carcinoma (EC) cells retain the bivalent marks typical of ES cells but display two additional repressive marks clearly linked to DNA-methylation, H3K9me2 and H3K9me3 (Ohm et al., 2007).

Collectively, these three studies strongly support the stem-cell origin of cancer and help to understand how normal stem cells with a transient transcription-ready state are converted into cancer progenitor cells with the permanent and heritable epigenetic silencing via DNA hypermethylation of some key genes probably due to aberrant crosstalk between the EZH2 component of PRC2 and DNA-methyl transferases (DNMTs). In fact, the loss of function of these genes could draw these progenitor cells from the normal differentiation pathway and lock them into a state of perpetual self renewal poised for tumorigenesis with the accumulation of further epigenetic or genetic events. Indeed, these cells could become dependent upon the pathways that are deregulated through the stable silencing of these genes. This dependency is termed “epigenetic sensitization” or “epigenetic cellular addiction” (Baylin and Ohm, 2006) and can favor the selection of cells that have additional cooperating mutations.

From these studies, *HIC1* has been defined as a “stem cell gene” (a gene marked with at least two out of the three components SUZ12, EED and H3K27me3 in human ES cells) in colorectal cancer, ovarian cancer and CD34 positive hematopoietic progenitor cells (Widschwendter et al., 2007) but not in breast cancer. This exception may be due to its hemi-methylation in normal breast epithelium (Fujii et al., 1998). In addition, *HIC1* is unmethylated in human ES cells and partially or fully methylated in two embryonal carcinomas cell lines, Tera-1 and Tera-2 respectively (Ohm et al., 2007).

Thus, in early tumorigenesis, epigenetic inactivation of *HIC1* could induce an increase in SIRT1 deacetylase activity. The resulting deacetylation of P53 impairs its function leading to a defective apoptotic response to DNA-damage and a reduced ability to transactivate its target genes including *HIC1* (Chen et al., 2005). This HIC1-SIRT1-P53 regulatory loop is an essential pathway through which *HIC1* may function as a tumor suppressor gene and cooperate with P53, as shown by the double heterozygote model. SIRT1 can deacetylate not only P53 but also many other target proteins essential for normal homeostasis. Thus, epigenetic silencing of *HIC1* can have both P53-dependent and P53-independent effects.

Moreover, *HIC1* can also interfere with several other essential signaling pathways. For example, in normal conditions, HIC1 sequesters TCF4, a key transcriptional factor to which converges Wnt signaling, into nuclear substructures called “HIC1 nuclear bodies” and thus divert TCF4 from its transcriptional targets (Valenta et al., 2006). Loss of *HIC1* expression in early tumorigenesis results in an increase of TCF4-mediated target gene expression and thus “addicts” the cells to an activated Wnt/ β catenin signaling pathway.

A similar situation occurs through the direct transcriptional regulation of ATOH1, a transcriptional relay of the Hedgehog signaling pathway as detailed above (Briggs et al., 2008).

8. Conclusions and future directions for research

Thirteen years after its discovery by Dr Baylin's laboratory, *HIC1* is now well-recognized as a tumor suppressor gene modulating p53-dependent apoptotic DNA damage responses through direct binding to the *SIRT1* promoter; cooperating with *Patched*, an inhibitor of the Hedgehog pathway to induce medulloblastomas and able to modulate the Wnt pathway.

Despite this significant progress, many basic questions remain unresolved. First, an incomplete picture exists for the mechanisms of transcriptional regulation by HIC1. Second and more importantly, the list of characterized target genes appears to be very short considering the large

numbers of potential physiological roles of *HIC1*. A better knowledge of these targets genes would also help to decipher the potentially numerous signaling pathways that could be deregulated through epigenetic inactivation of *HIC1*.

HIC1 is another example of a tumor suppressor gene silenced in cancer in association with promoter hypermethylation. As such, *HIC1* could by itself be a new target for epigenetic therapies through the combined use of pharmacologic DNA methyltransferase inhibitors and histone deacetylase inhibitors. Moreover, the identification of genes directly regulated by *HIC1* warrants a more in-depth investigation due to their potential use as therapeutic targets. For example, cambinol, a compound that inhibits the NAD-dependent deacetylase activity of SIRT1 induces apoptosis of xenograft tumors *in vivo* derived from Burkitt's lymphomas overexpressing BCL-6, a transcription factor essential for B-cell fate and differentiation and deacetylated by SIRT1 (Heltweg et al., 2006). It would be interesting to determine if cambinol could also trigger apoptosis in tumors with loss of *HIC1*.

Further characterizing *HIC1* function and its direct transcriptional targets would not only help to understand its role as a tumor suppressor gene and provide new insights into epigenetics and transcriptional repression in general but would also open new therapeutic approaches to major human cancers.

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Figure 1. Genomic organization of the human *HIC1* locus

The structure of the human *HIC1* locus as derived from several studies is schematically drawn (Wales et al., 1995) (Guerardel et al., 2001). A similar organization is found in mice (Guerardel et al., 2001) (Carter et al., 2000). The two major promoters, called P1 and P0 and the minor P2 promoter generating *HIC1* transcripts with heterogeneous 5' ends are represented (Guerardel et al., 2001; Pinte et al., 2004a). For clarity, only the major types **1a** and **1b**, but not the minor **1c**, **1d** and **1e** transcripts have been shown below the human *HIC1* genomic locus (Pinte et al., 2004a). In addition, the type **1a** transcripts are by far the most abundant *HIC1* transcripts. In human, only the **1b** exon contains an ATG codon in-frame with the ATG initiation codon located in exon 2. It should be noted however that the existence of this 733 amino acid protein deduced from computer analyses has never been firmly established, through immunodetection with specific antibodies.

Recently, RT-PCR analyses have identified an unspliced transcript, called **1f** initiated at an alternative promoter within exon **1b**. This transcript could potentially encode a short 22 amino-acid polypeptide initiated at the ATG codon of exon **1b** and terminated by a stop codon in the intron (Mondal et al., 2006). The p53 binding site involved in the direct regulation of *HIC1* transcription (Wales et al., 1995) (Guerardel et al., 2001) (Britschgi et al., 2006) is shown as a dark oval.

Figure 2. Domain organization of the human HIC1 protein

The two autonomous repression domains of HIC1, the BTB/POZ domain and the central region, are shown together with the known molecular mechanisms underlying their repression potential. Although HIC1 interacts with the class III deacetylase SIRT1 (Chen et al., 2005), its direct role in the BTB/POZ-mediated repression is not fully established. The co-repressor complex(es) involved in the TSA-insensitive repression potential of the BTB/POZ domain (Deltour et al., 1999) have still to be identified. The Central Region contains 4 short peptides motifs which have been phylogenetically conserved. The function of two of them has been deciphered. The GLDLSKK motif is a variant of the classical CtBP interacting domain (CID), the PLDLS motif, involved in the recruitment of the CtBP corepressor (Deltour et al., 2002) (Chinnadurai, 2007). The only invariant residue among CtBP interacting domains, Leucine 225 is highlighted since its mutation impairs the interaction between HIC1 and CtBP (Stankovic-Valentin et al., 2006). The Lysine K314 is contained within a MKHEP motif which is a SUMOylation/Acetylation switch motif (Stankovic-Valentin et al., 2007). The transcriptional repression potential of HIC1 is positively controlled by a complex between two types of deacetylases, SIRT1 and HDAC4. SIRT1 deacetylates HIC1 at lysine K314 whereas HDAC4 favors its SUMOylation through a mechanism still poorly understood (Stankovic-Valentin et al., 2007). The corepressor(s) whose recruitment is positively regulated by HIC1

SUMOylation as well as the CtBP-independent corepressors recruited by the central region are currently unknown (Stankovic-Valentin et al., 2006) (Stankovic-Valentin et al., 2007). HIC1 binds specifically to DNA through its 5 C-terminal Zinc fingers (ZF) domain that recognizes the consensus sequence 5'-^C/GNG^C/G GGGCA^C/A CC-3' centered on a GGCA core motif bound by Zinc fingers 3 and 4 (Pinte et al., 2004b). The HIC1 BTB/POZ domain negatively regulates the DNA-binding of the full-length protein to a single site whereas the oligomerization of the protein *via* this domain mediates cooperative DNA-binding to multiple sites (Pinte et al., 2004b).