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The Reelin receptors ApoER2 and VLDLR are direct target genes of HIC1 (Hypermethylated In Cancer 1)

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ABSTRACT

The tumor suppressor gene *HIC1* (*Hypermethylated In Cancer 1*) is located in 17p13.3 a region frequently hypermethylated or deleted in tumors and in a contiguous-gene syndrome, the Miller-Dieker syndrome which includes classical lissencephaly (smooth brain) and severe developmental defects. *HIC1* encodes a transcriptional repressor involved in the regulation of growth control, DNA damage response and cell migration properties. We previously demonstrated that the membrane-associated G-protein-coupled receptors *CXCR7*, *ADRB2* and the tyrosine kinase receptor *EphA2* are direct target genes of HIC1. Here we show that ectopic expression of HIC1 in U2OS and MDA-MB-231 cell lines decreases expression of the *ApoER2* and *VLDLR* genes, encoding two canonical tyrosine kinase receptors for Reelin. Conversely, knock-down of endogenous *HIC1* in BJ-Tert normal human fibroblasts through RNA interference results in the up-regulation of these two Reelin receptors. Finally, through chromatin immunoprecipitation (ChIP) in BJ-Tert fibroblasts, we demonstrate that HIC1 is a direct transcriptional repressor of *ApoER2* and *VLDLR*. These data provide evidence that HIC1 is a new regulator of the Reelin pathway which is essential for the proper migration of neuronal precursors during the normal development of the cerebral cortex, of Purkinje cells in the cerebellum and of mammary epithelial cells. Deregulation of this pathway through *HIC1* inactivation or deletion may contribute to its role in tumor promotion. Moreover, HIC1, through the direct transcriptional repression of *ATOH1* and the Reelin receptors *ApoER2* and *VLDLR*, could play an essential role in normal cerebellar development.

1. Introduction

The extracellular matrix protein Reelin mediates a key signaling pathway implicated in the regulation of neural progenitor cell migration and positioning during the early development of cortical structures in the brain [1,2]. Canonical Reelin signaling relies on two membrane bound receptors, the Apolipoprotein E Receptor 2 (ApoER2), also known as Low-density lipoprotein Receptor-related Protein 8 (LRP8), and the Very Low Density Lipoprotein Receptor (VLDLR); both of which are members of the LDL receptor family associated with cellular cholesterol homeostasis [3]. Binding of Reelin leads to clustering of the receptors and subsequent tyrosine phosphorylation of the cytoplasmic adaptator protein Disabled 1 (DAB1) associated with the intracellular domain of both receptors [4,5]. Phosphorylated DAB1 recruits several proteins to activate downstream signaling to promote neural migration and also interacts with LIS1 to remodel microtubules. Mice harboring null mutations in key components of the Reelin signaling including *Reelin* itself (*reeler* mice), *Dab1*, and double *VLDLR/ApoER2* homozygous mutants all manifest *reeler-like* phenotypes characterized by severe aberrations in cortical layering [5]. In humans, mutations in the Reelin pathway have been associated with lissencephaly, epilepsy and Alzheimer's disease [1].

Besides its crucial physiological function in the brain, Reelin is also expressed in several other non-neural tissues. Notably, Reelin is essential for cell migration and ductal patterning during normal mammary gland development, is expressed in the normal breast epithelium [6] and deregulation of this signaling pathway has been associated with tumorigenesis. However, contradictory results have been described since both increased and epigenetically silenced expression of Reelin has been observed in different cancer types. Its silencing, associated with promoter hypermethylation, is correlated with poor prognosis in breast cancer and ectopic expression of Reelin has been shown to suppress cell migration and metastatic properties of MDA-MB-231 breast cancer cells [7]. Strikingly, very few studies have investigated the expression levels and possible significance of the *ApoER2* and *VLDLR* receptors in tumors. Type I

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(full-length) *VLDLR* is overexpressed in well-differentiated intestinal carcinoma and in gastric adenocarcinoma as compared to adjacent normal tissues [8] whereas up-regulation of Type II *VLDLR*, a splice variant lacking exon 16, is correlated with a higher metastatic potential in gastric and breast cancers [9]. *VLDLR* and *ApoER2* are expressed at various levels in neuroblastoma cell lines and also in primary tumors [10].

HIC1 (*Hypermethylated In Cancer 1*) is a tumor suppressor gene located at 17p13.3, a region frequently hypermethylated or deleted in numerous cancers including those of breast, lung, ovary, liver, colon, kidney and brain [11]. This loss of heterozygosity is particularly frequent in childhood brain tumors including medulloblastoma, ependymona, and high grade glioma [12]. The tumor suppressor gene status of HIC1 has been confirmed by animal models; heterozygous *Hic1+/−* mice develop an age- and gender-dependent spectrum of spontaneous tumors [13]. In addition, homozygous loss of *Hic1* is marked by several abnormalities (perinatal death, small size, acrania, exencephaly, craniofacial abnormalities, limb defects and omphalocele) very similar to those found in a severe form of lissencephaly, the Miller–Dieker syndrome (MDS) [14]. MDS is a contiguous-gene syndrome marked by deletion of LIS1 and of multiple genes in a 17p13.3 critical region including HIC1 [15]. HIC1 encodes a sequence-specific transcriptional repressor consisting of three main functional domains: a BTB/POZ protein–protein interaction domain (Broad complex, Tramtrack and Bric à brac/POx viruses and Zinc finger) in the N-terminal part of the protein, a central region and a C-terminal domain containing five Krüppel-like C2H2 zinc fingers. These zinc fingers allow the specific binding of the protein to specific DNA sequences consisting of a 5′-(C/G)NG(C/G)G**GGCA**(C/A)CC-3′ centered on a GGCA motif and named HIC1 responsive elements (HiRE). HIC1 recruits several co-repressor complexes; CtBP, NuRD, SWI/SNF and Polycomb PRC2 [12]. Among the 12 HIC1 direct target genes described to date, half have been identified through four independent gene profiling experiments using its forced re-expression in HIC1-deficient tumor cell lines [12]. Notably, several validated target genes encode membrane-associated receptors implicated in cell migration such as the G-protein-coupled receptors (GPCR) CXCR7 and ADRB2 as well as the tyrosine kinase receptor, EphA2 and its cell-bound ligand ephrinA1 [16–19]. In our list of potential candidate HIC1 target genes generated from HIC1 re-expression in U2OS osteosarcoma cells, we decided to validate another membrane-bound tyrosine kinase receptor, ApoER2.

In this study, we demonstrate that the two genes encoding canonical Reelin receptors *ApoER2* and *VLDLR* are *bona fide* HIC1 target genes through overexpression of HIC1 in U2OS osteosarcoma cells and MDA-MB-231 breast cancer cells. Furthermore, HIC1 directly regulates *ApoER2* and *VLDLR* expression in normal BJ-Tert human fibroblasts as demonstrated by siRNA interference and by chromatin immunoprecipitation (ChIP) of endogenous *HIC1*.

As a whole, our results identify the two canonical receptors for Reelin as two new HIC1 direct target genes.

2. Material and methods

2.1. Cell lines and retroviral infection

U2OS, MDA-MB-231 and BJ-Tert cells were maintained in Dulbecco modified Eagle medium (Invitrogen) supplemented with 10% fetal calf serum, non-essential amino acids and gentamycin. Cells were cultured at 37 °C in water-saturated 5% CO^2 atmosphere.

Retroviral infection of U2OS osteosarcoma cells and MDA-MB-231 breast cancer cells with the pBABE-Puro-FLAG-HIC1 and the empty pBABE vector were performed as previously described [18].

2.2. Small interfering RNA

BJ-Tert fibroblasts were reverse-transfected with Lipofectamine RNAiMax (Invitrogen) according to manufacturer's instructions using 10 nM small interfering RNA targeting HIC1 (HIC1 siGENOME SMART Pool M-006532-01, Dharmacon) or a scrambled control sequence (si Ctrl; siGENOME RISC free control siRNA, Dharmacon) as previously described [20].

2.3. Quantitative RT-PCR

Total RNA was reverse transcribed using random primers and MultiScribeTM reverse transcriptase (Applied Biosystems). Real-time PCR analysis was performed by Power SYBR Green (Applied Biosystems) in a MX3005P fluorescence temperature cycler (Stratagene) according to the manufacturer's instructions. Results were normalized with respect to 18S RNA used as internal control [17,21]. The primers used for the qRT-PCR analyses reported in this study are summarized in Supplementary Table 1. *p*-values were calculated according to the Student test. *indicates $p < 0.1$; **$p < 0.01$; ***$p < 0.001$.

2.4. Chromatin immunoprecipitation

BJ-Tert cells were fixed by adding formaldehyde directly into the cell plate to a final concentration of 1% for 15 min at 37 °C. Adding glycine to a final concentration of 0.125 M stopped the cross-linking. After 5 min at 37 °C, cells were lysed directly in the plates by resuspension in cell lysis buffer (5 mM PIPES pH8, 85 mM KCl, 0.5% NP-40) for 5 min. Then, the samples were pelleted, resuspended in 100 µl of nuclei lysis buffer (50 mM Tris–HCl pH8, 10 mM EDTA, 0.2% SDS), and sonicated to chromatins with an average size of 250 bp using a BioRuptor (Diagenode, Liege, Belgium). 20 µg of chromatin was immunoprecipitated by anti-HIC1 antibody or IgG control [17] and classical or real-time PCR analyses were performed as already described [16,22]. The primers used are summarized in Supplementary Table 2.

2.5. Western blotting and antibodies

Proteins were separated by SDS–PAGE and transferred onto nitrocellulose membranes (GE healthcare). Western blot analyses were performed as previously described [21]. The anti-HIC1 antibody has been previously described [22] and anti-EphA2 and anti-actin antibodies were purchased from Santa Cruz Biotechnology.

3. Results

3.1. The two Reelin receptors ApoER2 and VLDLR are HIC1 target genes

Through gene profiling experiments, we previously generated a list of genes repressed in *HIC1*-null U2OS osteosarcoma cells following adenoviral infection and re-expression of HIC1 which allowed us to validate several membrane-bound receptors as direct HIC1 target genes [16–18]. In this list, *ApoER2*, coding for one of the two Reelin receptors, appears as another membrane-associated receptor which could be a new candidate HIC1 target gene strongly repressed at the earlier post-infection time points (Fig. 1A). To further test this hypothesis, we first investigated the effects of HIC1 ectopic expression in two HIC1-deficient cell lines; the osteosarcoma cells U2OS and the breast cancer cells, MDA-MB-231. After retroviral infection of these two cell lines with pBABE-FLAG-HIC1, we observed a significant increase in *HIC1* RNA and protein levels in comparison with the cells infected with the empty pBABE