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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

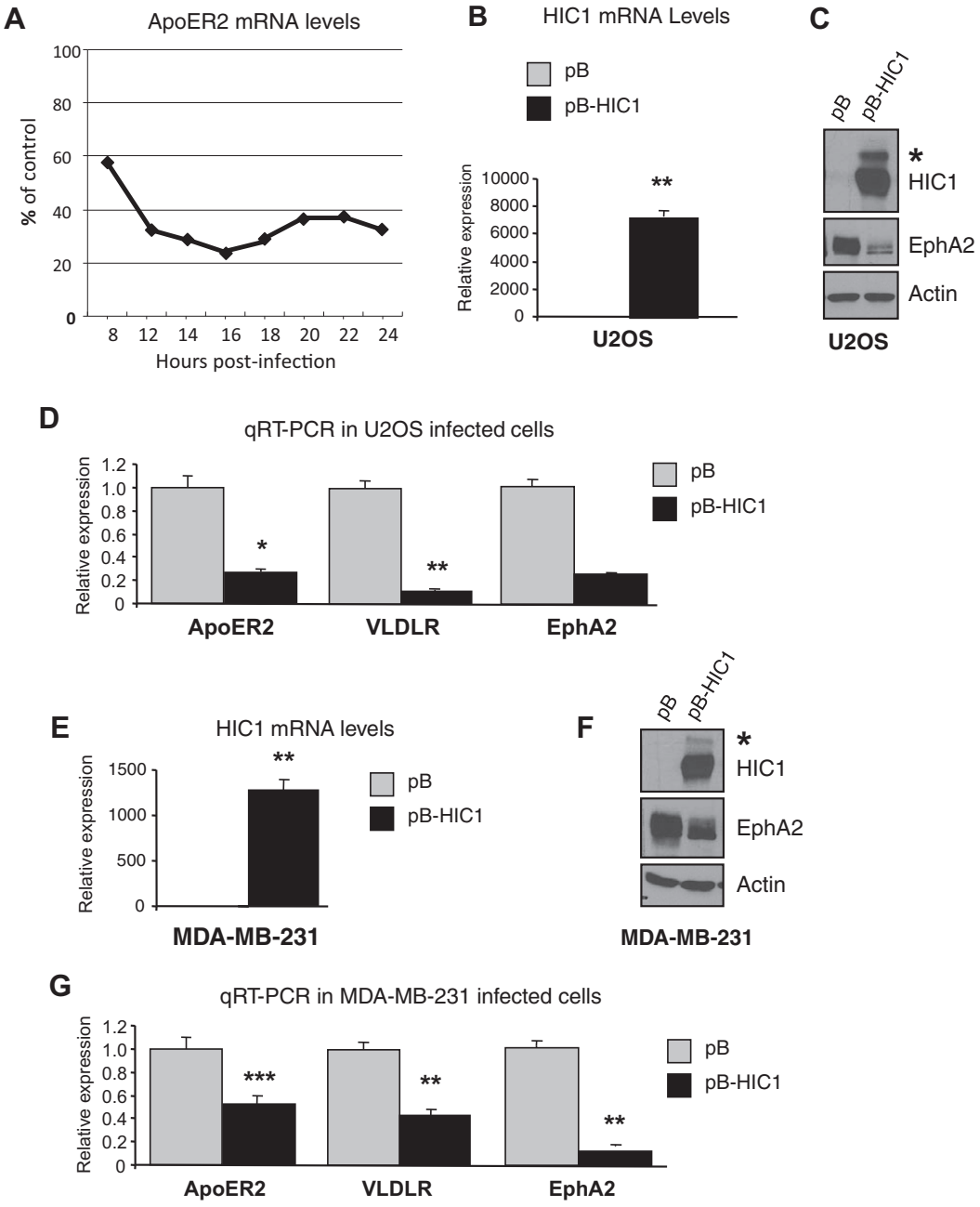

Fig. 1. *ApoER2* and *VLDLR* are down-regulated in pBabe-HIC1 infected U2OS and MDA-MB-231 cells. (A) Effects of HIC1 overexpression on *ApoER2* mRNAs level in infected cells. Total RNAs from U2OS cells (HIC1 null) infected with Ad-FLAG-HIC1 and Ad-GFP were prepared at the indicated times (from 8 to 24 h) and Affymetrix HG U133A chips were used to measure the gene expression. Expression values were normalized to Ad-GFP infected control cells at the same time points. "% of control" corresponds to the ratio between the expression levels of *ApoER2* measured in Ad-GFP and Ad-FLAG-HIC1 infected cells at each time point. (B) Quantitative Real Time PCR (qRT-PCR) analyses of *HIC1* and (C) Western blot (WB) analyses of HIC1 and of EphA2 used as positive control in U2OS cells infected with pBABE-FLAG-HIC1 or pBABE-FLAG (pBABE) as control, abbreviated as pB-HIC1 and pB respectively, in all figures. Actin protein levels were used as a loading control. (D) qRT-PCR analyses of *ApoER2* and *VLDLR*, in U2OS cells infected by pB-HIC1 or by pB, as control. *EphA2* was used as positive control. Values were normalized to *18S*. (E) qRT-PCR analyses of *HIC1* and (F) Western blot (WB) analyses of HIC1 in infected MDA-MB-231 cells. * corresponds to SUMOylated form of HIC1. (G) qRT-PCR analyses of *ApoER2* and *VLDLR* in MDA-MB-231 cells.

vector. This *HIC1* expression correlated with a strong repression of *EphA2*, used as a control HIC1-target gene (Fig. 1B–F) [18]. As measured by qRT-PCR analyses, re-expression of HIC1 resulted in a significant decrease in mRNAs levels of *ApoER2* in both cell types (Fig. 1D and G). Interestingly, we obtained similar results for the second related Reelin receptor *VLDLR* which was absent in our microarray analyses (absent call). Conversely, to confirm the role of HIC1 in the regulation of the *ApoER2* and *VLDLR* genes, we inactivated endogenous *HIC1* expression in normal BJ-Tert fibroblasts by RNA interference. Transfection of these cells with a pool of four siRNAs targeting HIC1 results in the efficient inhibition of endogenous *HIC1* expression and leads to a robust concomitant increase of *VLDLR* mRNA levels as well as a moderate increase in *ApoER2* and *EphA2* (used as a positive control) mRNA levels (Fig. 2). These observed effects were not owing to off target effects, since similar results were obtained after transfection of individual siRNAs targeting HIC1, as previously described [20] (data not shown). Therefore, our results strongly suggest that the two genes coding

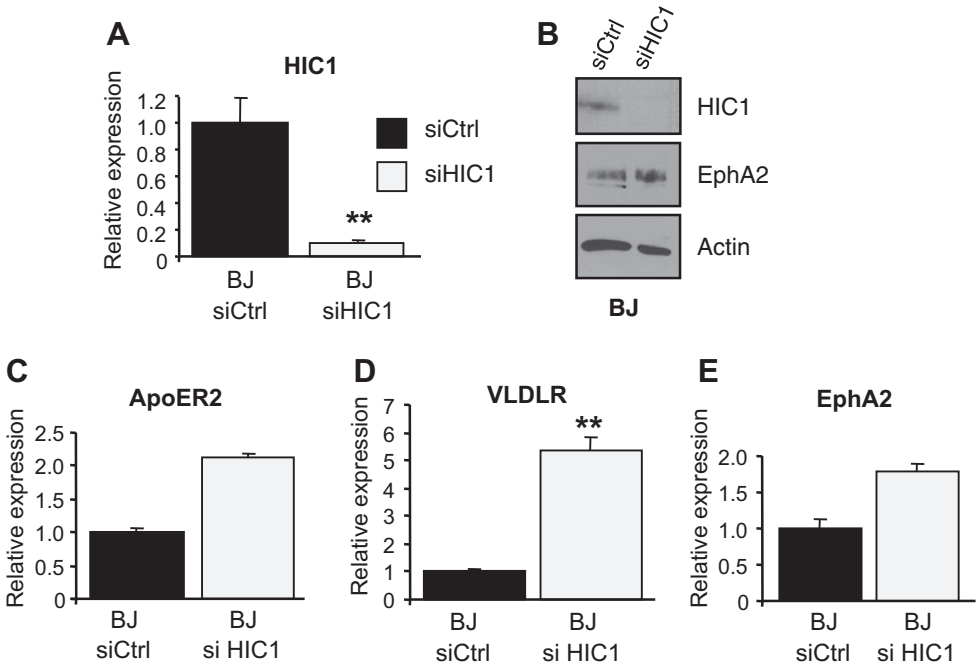

Fig. 2. Knock-down of endogenous *HIC1* in normal BJ-Tert fibroblasts up-regulates *ApoER2* and *VLDLR* expression. BJ-Tert fibroblasts were transfected with non-target siRNA control (siCtrl) or with HIC1 siRNA pool (siHIC1). 72 h later, cells were harvested for RNA/protein extraction. Total RNAs were extracted and the mRNA expression levels of relevant genes were assessed by qRT-PCR. Values were normalized to *18S*. (A) and (B) qRT-PCR and Western blot showing siRNA knockdown of HIC1 and a concomitant increase of EphA2 protein as a positive control. (C) and (D) qRT-PCR analyses of *ApoER2* and *VLDLR* in human BJ-Tert fibroblasts transfected with a non-targeting control siRNA (siCtrl) or with a pool of siRNAs targeting HIC1 (siHIC1). (E) *EphA2* was used as positive control.

for functionally distinct but related receptors for Reelin, *ApoER2* and *VLDLR* are both target genes of HIC1 mediated transcriptional repression.

3.2. HIC1 directly represses the expression of ApoER2 and VLDLR

To determine whether *ApoER2* and *VLDLR* are HIC1 direct target genes, we first performed CLUSTAL alignments of the upstream promoter region and first coding exons of the human and murine loci and searched for the presence of consensus HIC1-responsive elements (HiREs) to which HIC1 could bind [23]. Based only on the GGCA core motif, we identified a cluster of three phylogenetically conserved and adjacent putative HiREs in the same orientation downstream of the *ApoER2* transcription start site and two isolated HiREs in the opposite orientation in the 5′untranslated region of *VLDLR* (Fig. 3A). Next, we attempted to design a set of oligonucleotides to PCR amplify the regions containing the putative HiRE in each gene (arrows in Fig. 3A) in DNA samples obtained from Chromatin immunoprecipitation (ChIP) of endogenous HIC1 in BJ-Tert fibroblasts. Despite numerous attempts, we were unable to design a pair of primers able to efficiently amplify in quantitative ChIP-PCR analyses, only one PCR product according to the melting curve in the relevant region of the *ApoER2* 5′untranslated region, perhaps due to its high GC content (data not shown). Nevertheless, as shown in Fig. 3B, we were able to specifically amplify the relevant regions of *ApoER2* and *SIRT1* (used as a control HIC1 target gene), by performing classical PCR followed by agarose gel electrophoresis [24], in chromatin immunoprecipitated with anti-HIC1 antibody but not with non-relevant rabbit IgG. Primers designed to amplify the *GAPDH* promoter were used as a negative control. By contrast, for *VLDLR*, we obtained a set of oligonucleotides that was suitable for ChIP-qPCR analyses and clearly demonstrated specific binding of HIC1 on this site (Fig. 3C). *EphA2* and *GAPDH*, used for a control HIC1 target gene and a non-relevant gene respectively, were also tested. Taken together these results demonstrate that *ApoER2* and *VLDLR* are two new HIC1 direct target genes and that HIC1 could regulate the Reelin-Dab1 pathway through direct transcriptional repression of the two canonical Reelin receptors.

4. Discussion

In this study, we characterized the two canonical Reelin Receptors *ApoER2* and *VLDLR* as two new target genes of HIC1, thus demonstrating that HIC1 could directly regulate the Reelin-DAB1 signaling pathway which plays an essential role during the development of the brain by guiding the migration and correct positioning of neural cells (Fig. 4) [1,5].

HIC1 is a tumor suppressor gene central to complex regulatory loops, involving the tumor suppressors genes *P53* and *E2F1* as well as the deacetylase *SIRT1*, that regulate growth and cell survival in response to stresses such as DNA damage. In line with these functions, HIC1 regulates transcription of *SIRT1* itself and of several cell cycle regulatory genes such as *cyclin D1* and the cell cycle inhibitors $P57^{Kip1}$ and $P21^{Waf1}$ [12,21].

The lack of complete genome-wide screens for HIC1 target genes from comparisons of HIC1 knock-out versus normal cells or from CHIP-seq analyses preclude gene ontology analyses. Nevertheless, one category of genes that is clearly over-represented among the very few HIC1 target genes identified to date is membrane bound receptors involved in cell migration and homing properties, including *CXCR7*, *ADRB2*, *EphA2* and its cell surface ligand *ephrin A1* [12]. The present study has added two other members to this group, *ApoER2* and *VLDLR*, encoding the two canonical receptors for Reelin (Fig. 4). As the Reelin pathways is essential for mammary gland development, these results strongly suggests that the loss of *HIC1* expression by promoter hypermethylation or loss of heterozygosity could contribute to early tumorigenesis in part through deregulation of the EphA2/ephrinA1 and Reelin pathways in breast cancers and perhaps more generally in epithelial cancers.

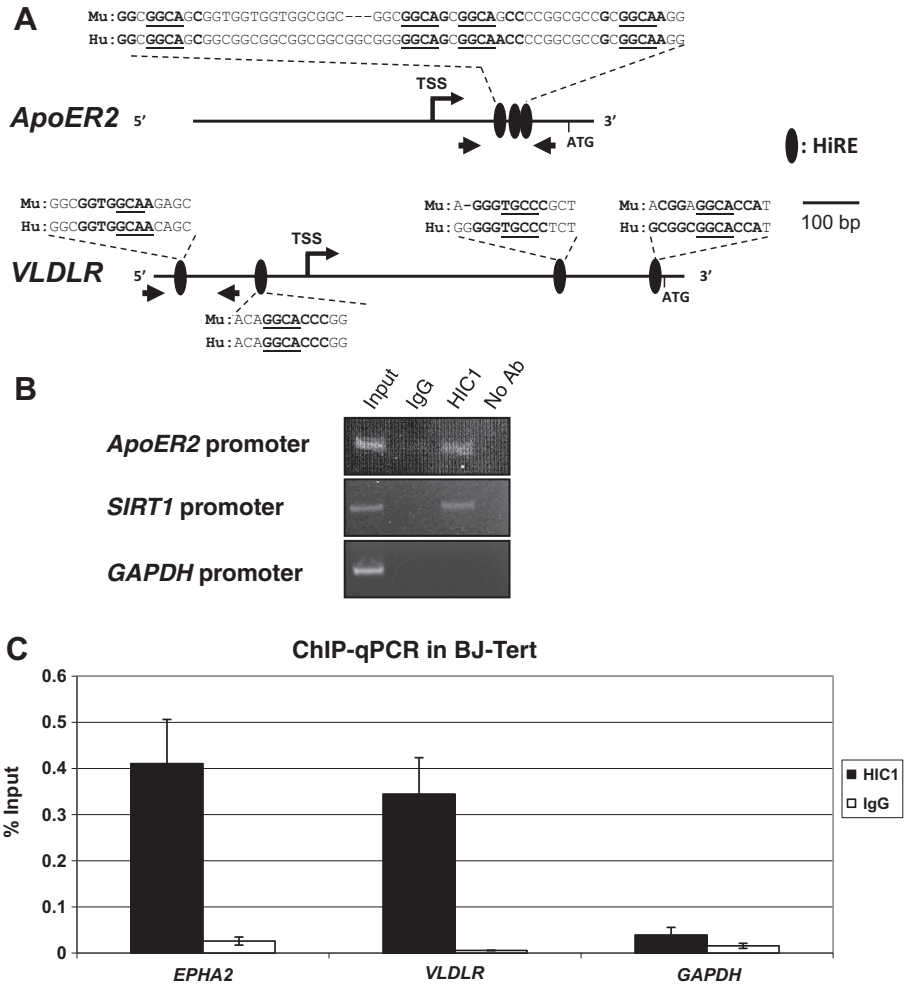

Fig. 3. HIC1 is a direct transcriptional repressor of *ApoER2* and *VLDLR*. (A) Schematic drawing of a part of the 5′ region and first coding exon of the human and mouse *ApoER2* and *VLDLR* genes. CLUSTAL analyses identified phylogenetically conserved potential HIC1 binding sites, HiREs. Arrows indicate the position of the primers used in the ChIP experiment. (B) BJ-Tert fibroblast chromatin was immunoprecipitated with anti-HIC1 antibody, as previously described [22]. The bound material was eluted and analyzed by classical PCR using primers flanking the putative HiREs in *ApoER2*. The PCR products were analyzed by agarose gel-electrophoresis. *SIRT1* was used as a positive control and *GAPDH* as an internal non-binding control. (C) Similarly, samples were analyzed by quantitative PCR using primers flanking a putative HiRE in *VLDLR*. *EphA2* was used as positive control and *GAPDH* as an internal non-binding control.

However, the Reelin signaling pathway, and hence these two receptors, is of paramount importance for correct neural cell migration and positioning during early development as demonstrated by the severe phenotypes of knock-out mice and the mutations in these genes associated with human diseases such as lissencephaly and Alzheimer's disease. *HIC1* is located at 17p13.3 within the critical deletion chromosomal region for the Miller–Dieker syndrome combining severe lissencephaly and mental retardation with developmental abnormalities. Lissencephaly is attributed to *LIS1* haploinsufficiency and mouse models have confirmed that *HIC1* is responsible for the severe developmental defects associated with MDS [14]. Indeed, *in situ* hybridization studies have detected *Hic1* expression in the embryonic anlagen of many tissues affected in MDS [25]. Moreover, *Hic1*−/− homozygous mice, obtained through homologous recombination, display various combinations of acrania, exencephaly, omphalocele and limb defects found in MDS patients. By contrast, none of these *Hic1*−/− mice analyzed displayed significant neuronal migration defects or disorganization of the cerebral cortex similar to those observed in graded *Lis1* mutant mice [14]. In addition, *HIC1* is essential for normal cerebellar development through the direct transcriptional repression of the *ATOH1* gene coding for a pro-neuronal transcription factor which controls the migration and differentiation of the proliferative, *ATOH1+/HIC1*− neuronal precursor cells of the external granule cell layer (EGL) into non-proliferative, fully differentiated *ATOH1*−/*HIC1+* cells of the internal granule cell layer (IGL) [26]. As a consequence, loss of function of *HIC1* is a common feature found in two-thirds of human medulloblastomas and, in mice, inactivation of *Hic1* in the *Ptch1*−/+ *Hic1*−/+ double heterozygote model increases the incidence of medulloblastoma over that found in the *Ptch1*−/+ model [27] [26]. Whereas this EGL differentiation process is mainly controlled by the *Sonic hedgehog* pathway through ATOH1, a direct target gene of HIC1, Reelin is also expressed in EGL cells which are otherwise negative for HIC1.

Besides its well-established function as a direct transcriptional repressor of *ATOH1*, HIC1 could have another role in the cerebellum outside of neuronal development. Indeed, in addition to strong *Hic1* expression in the IGL and no expression in EGL, a pattern inversely mirroring that of *ATOH1*, immunohistochemistry analyses demonstrate robust *Hic1* expression in the Purkinje cell layer of P15 mouse embryos [26]. Interestingly, each of the Reelin receptors, *ApoER2* and *VLDLR*, direct the dispersal and correct positioning of distinct populations of Purkinje cells (expressing one or both receptors) in the developing cerebellum [28]. Our study demonstrating that *ApoER2* and *VLDLR* are both direct transcriptional targets of HIC1 could nicely tie together these two set of observations since it suggests that HIC1 could participate to the regulation of these two receptors in Purkinje cells expression.

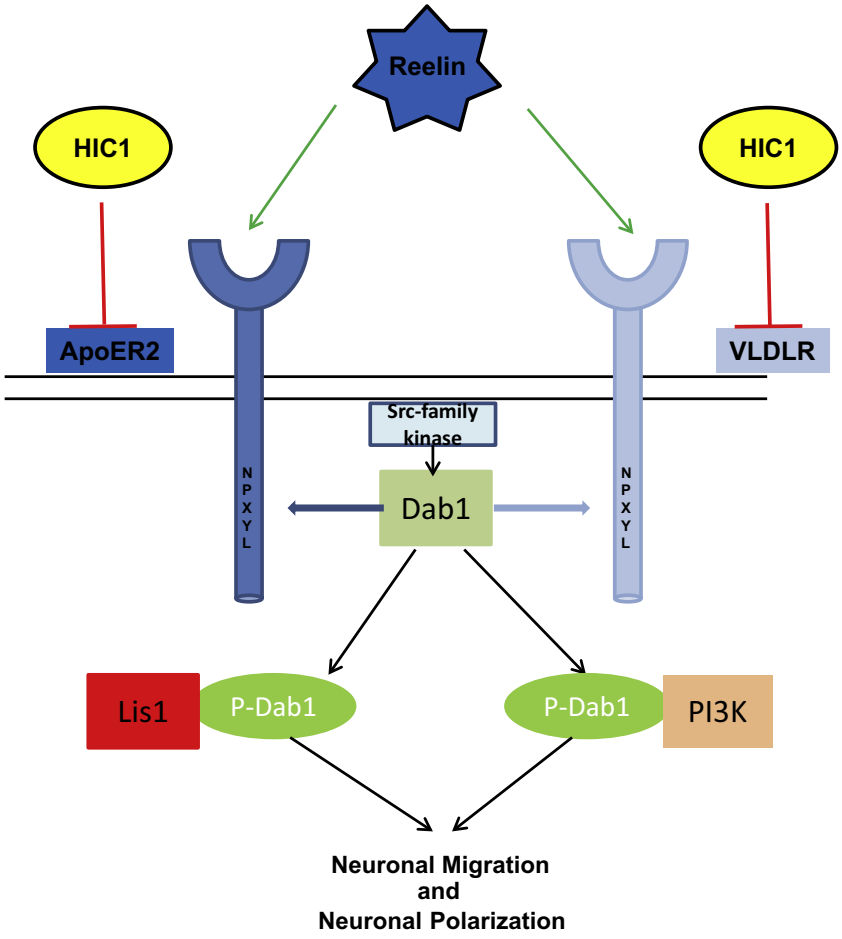

Fig. 4. HIC1 regulates the Reelin pathway through direct transcriptional repression of its canonical receptors ApoER2 and VLDLR. This simplified and schematic drawing of the Reelin pathway is adapted from references [5] and [29].

In conclusion, HIC1 through the direct transcriptional repression of *ATOH1* [26] and of the Reelin receptors *ApoER2* and *VLDLR*, as shown in this study, could be involved in various essential processes of normal cerebellum development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.091.

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