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The Receptor Tyrosine Kinase EphA2 Is a Direct Target Gene of Hypermethylated in Cancer 1 (*HIC1*)*

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Background: The tumor suppressor gene *HIC1* epigenetically silenced in many human cancers encodes a transcriptional repressor.
Results: We identified the receptor tyrosine kinase *EphA2* as a new HIC1 direct target gene.
Conclusion: HIC1 directly represses a gene implicated in cell adhesion and migration.
Significance: Loss of *HIC1* contributes to epithelial tumorigenesis through deregulation of the *EphA2* signaling pathway.

The tumor suppressor gene hypermethylated in cancer 1 (*HIC1*), which encodes a transcriptional repressor, is epigenetically silenced in many human tumors. Here, we show that ectopic expression of HIC1 in the highly malignant MDA-MB-231 breast cancer cell line severely impairs cell proliferation, migration, and invasion *in vitro*. In parallel, infection of breast cancer cell lines with a retrovirus expressing HIC1 also induces decreased mRNA and protein expression of the tyrosine kinase receptor EphA2. Moreover, chromatin immunoprecipitation (ChIP) and sequential ChIP experiments demonstrate that endogenous HIC1 proteins are bound, together with the MTA1 corepressor, to the *EphA2* promoter in WI38 cells. Taken together, our results identify EphA2 as a new direct target gene of HIC1. Finally, we observe that inactivation of endogenous HIC1 through RNA interference in normal breast epithelial cells results in the up-regulation of *EphA2* and is correlated with increased cellular migration. To conclude, our results involve the tumor suppressor HIC1 in the transcriptional regulation of the tyrosine kinase receptor *EphA2*, whose ligand *ephrin-A1* is also a HIC1 target gene. Thus, loss of the regulation of this Eph pathway through *HIC1* epigenetic silencing could be an important mechanism in the pathogenesis of epithelial cancers.

Hypermethylated in cancer 1 (*HIC1*) is a tumor suppressor gene located at 17p13.3 on the short arm of human chromosome 17, a region frequently affected by genetic alterations such as deletion or hypermethylation in human cancers, including the p53 tumor suppressor gene at 17p13.1 (1). Moreover, *HIC1* is epigenetically silenced in many types of common human cancers such as prostate cancers (2), non-small cell lung carcinomas (3, 4), and breast cancers (5). *HIC1* promoter methylation is variable, but dense methylation is associated with tumor aggressiveness and poor survival (1, 4, 6–8). Treatment of MDA-MB-231 with a demethylating agent increased expression of p53 and the proto-oncogene *ErbB2* as well as causing re-expression of HIC1 by reversing *HIC1* promoter hypermethylation (6). Recently, it has been shown that demethylation treatment restored *HIC1* expression and impaired aggressiveness of head and neck squamous cell carcinoma (9). Furthermore, dense hypermethylation of one *HIC1* allele has been detected in some normal tissues, notably normal ductal breast tissues (5), and heterozygous *HIC1* mice spontaneously develop age-dependent and gender-determined tumors associated with promoter hypermethylation and gene silencing of the remaining wild-type allele (10). Taken together, these data suggest that epigenetic *HIC1* silencing predisposes tissues to tumorigenesis.

HIC1 encodes a transcriptional repressor containing an N-terminal BTB/POZ (Broad complex Tramtrack and Bric à brac/POxviruses and Zinc finger) domain and five C-terminal Krüppel-like C_2H_2 zinc fingers motifs (1, 11–13). Via these zinc fingers motifs, HIC1 represses transcription of its target genes by binding to a specific DNA sequence consisting of a 5′-(C/G)NG(C/G)G**GGCA**(C/A)CC-3′ sequence centered on a GGCA motif named HIC1-responsive element (HIRE)[5] (12, 14). The transcriptional repressor activity of HIC1 comes from its N-terminal BTB-POZ domain and from its central region capable of both autonomous transcriptional repression as well as recruitment of corepressors such as CtBP and MTA1 (11, 15–17). To date, about 10 genes have been identified as direct

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[5] The abbreviations used are: HIRE, HIC1-responsive element; qPCR, quantitative PCR; hTERT, human TERT; siCt, control siRNA.

target genes of HIC1 as follows: the class III histone deacetylase silent information regulator 2a homologue 1 (Sirt1) (14); the fibroblast growth factor-binding protein FGF-BP1 involved notably in blood vessel growth (18); the proneural transcription factor atonal homolog 1 (Atoh1) essential for cerebellar growth and development (19); the G-protein-coupled receptor CXCR7 (20), which could involve HIC1 in regulation of the chemokine cross-talk between tumor cells and the surrounding stroma; *Cyclin D1* and *P57KIP2 (CDKN1C)* (17); ΔNp73, a truncated isoform of p73 up-regulated in various tumors, which lacks the N-terminal transactivating domain (21); *Sox9* (22), and finally *ephrin-A1*, encoding a cell surface ligand for Eph receptor tyrosine kinases (23). Ephrins and Eph receptors are key regulators of physiological and pathological processes in development and disease (24–31).

Even if several target genes that could be responsible for the tumor suppressor function of HIC1 have already been identified, no single gene can fully account for the decrease of proliferation, migration, and invasion observed after HIC1 overexpression in MDA-MB-231 cells. Drawing from our previous results of genome-wide expression profiling of *HIC1*-deficient cells transduced with an adenoviral HIC1 expression vector, we decided to confirm the EphA2 tyrosine kinase receptor, a receptor of ephrin-A1, as a new putative target gene of HIC1, which could explain these biological effects. Indeed, EphA2 is expressed at low levels in normal breast epithelium and overexpressed in about 70% of breast cancers. More generally, expression of many of the Eph receptors is often elevated in a wide variety of tumors, including breast cancer, yet their precise roles in cancer are not well understood (24, 32). In this study, we have used EphA2 expression studies such as real time quantitative RT-PCR and immunoblot analyses and characterization of the *EphA2* promoter using chromatin immunoprecipitation to demonstrate that *EphA2* is a *bona fide* direct target gene of HIC1. Loss of *EphA2* regulation through HIC1 silencing could be an important mechanism contributing to the progression of breast cancer.

EXPERIMENTAL PROCEDURES

Cell Culture—U2OS, the packaging cell line HEK293 GP, and human mammary adenocarcinoma cells MDA-MB-231 were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen) and gentamicin (Invitrogen). WI38 cells were grown in minimal essential medium (Invitrogen) supplemented with sodium pyruvate, nonessential amino acids, 10% FCS, and gentamicin. The MCF10A human mammary epithelial cells, spontaneously immortalized, were cultured in DMEM and Ham's F-12 (Invitrogen) (v/v) supplemented with 5% horse serum (Invitrogen), 0.5 μg/ml hydrocortisone (Sigma), 20 ng/ml epidermal growth factor (PeproTech), 10 μg/ml insulin (Sigma), 100 ng/ml cholera toxin (Sigma), and antibiotics. Cells were cultured at 37 °C in water-saturated 5% CO_2 atmosphere. The normal mammary cells hTERT-HMEC were cultured in mammary epithelial cell growth medium (C-21010, PromoCell, Heidelberg, Germany) supplemented with gentamicin and a mix (C-3911S) to obtain a final concentration of 0.004 ml/ml bovine pituitary extract, 10 ng/ml epidermal growth factor (human recombinant), 5 μg/ml insulin (human recombinant), and 0.5 μg/ml hydrocortisone.

Western Blotting and Antibodies—After treatments, cells were washed twice with PBS and suspended in lysis buffer, and protein concentration was determined by Bio-Rad protein assay. Western blotting was performed as described previously (17). Results are representative of at least two experiments. Except for the anti-HIC1 2563 or anti-HIC1 325 polyclonal antibodies (15), commercial antibodies of the following specificities were used: FLAG from Sigma (M2 mouse monoclonal antibody F3165); EphA2 (C-20) from Santa Cruz Biotechnology (rabbit polyclonal antibodies sc-924), and actin (I-19) from Santa Cruz Biotechnology (rabbit polyclonal antibodies sc-1616-R); MCM6 (C-20) from Santa Cruz Biotechnology (goat polyclonal antibody sc-9843); MTA1 from Santa Cruz Biotechnology (mouse monoclonal antibody sc-17773X), and CtBP2 from BD Biosciences (mouse monoclonal antibody 612044).

Vectors and Retroviral Infection—The pBabe-Puro vector was used to effect retrovirus-driven FLAG-HIC1 expression. First, a double strand oligonucleotide encoding the FLAG epitope and flanked 5′ and 3′, respectively, by BglII and BamHI restriction sites was cloned in the correct orientation into the BamHI-digested pBabe vector to yield pBabe-FLAG. Then a BamHI-XhoI fragment containing the full-length HIC1 coding sequence in-frame with the FLAG epitope was cloned in the BamHI-SalI digested pBabe-FLAG vector to yield pBabe-FLAG-HIC1. These two constructs were verified by sequencing analyses. For the production of retroviruses, HEK293 GP cells were transfected with the pVSVG vector (expressing envelope) and with HIC1 expressing pBabe retroviral vector using the polyethyleneimine Exgen 500 procedure (Euromedex). After 48 h, culture supernatants were collected, passed through 0.45-μm filters, and mixed with fresh medium (1:2) and Polybrene (for U2OS, MCF-10A, and MDA-MB-231, 6 μg/ml) to infect target cells. The infected cells were then selected by puromycin treatment at 1 μg/ml for U2OS and MCF-10A and 2 μg/ml for MDA-MB-231. The retroviral plasmids LZRS, LZRS EphA2 WT, and two mutants of conserved juxtamembrane tyrosine residues required for optimal kinase activity, LZRS EphA2 Y587F/Y593F and Y587E/Y593E, were the kind gifts from Prof. Jin Chen (Vanderbilt University) and have been described previously (33, 34).

EphA2 Promoter Cloning and Luciferase Assay—The *EphA2* promoter region was PCR-amplified from normal human genomic DNA (Clontech) using primers containing XhoI and HindIII restriction sites, respectively (forward primer, GG<u>CTCGAG</u>CCTGGACTGGTCAAATGGTGATT; reverse primer, GG<u>AAGCTT</u>TTCTCGCTCTCGGTCCGAT). The PCR product was cloned in the PCR-TOPO-Blunt vector (Invitrogen) and verified by sequencing. After restriction digestion, the XhoI-HindIII fragment was cloned in the pGL3 basic reporter to generate the *EphA2* promoter construct, pGL3 EphA2 −1139/−8. The −433/−8 and −217/−8 promoter constructs were prepared from this construct by PCR using the same reverse primer and the following forward primers: GG<u>CTCGAG</u>GAATGGGTATGGCTCAGCGC for the −433/−8 construct and TTAAAGACATTCCTGAGGGCGGGC for the −217/−8 construct. U2OS cells were maintained in Dulbecco's medium supplemented with 10% fetal calf serum and transfected in OptiMEM (Invitrogen) by the PEI (Euromedex) method in 12-well plates with 500 ng of DNA (20). Cells were transfected for 6 h and then were incubated in fresh complete medium. They were rinsed in cold phosphate-buffered saline (PBS) 48 h after transfection and lysed with the luciferase assay buffer. Luciferase and β-galactosidase activities were measured by using, respectively, beetle luciferin (Promega) and the Galacto-light kit (Tropix) with a Berthold chemiluminometer. After normalization to β-galactosidase activity, the data were expressed as fold activation relative to the empty pGL3 basic control vector. Results represented are the means ± S.D. from at least two independent transfections in triplicate.

Quantitative RT-PCR—Total RNA was reverse-transcribed using random primers and MultiScribe™ reverse transcriptase (Applied Biosystems). Real time PCR analysis was performed by Power SYBR Green (Applied Biosystems) in an MX3005P fluorescence temperature cycler (Stratagene) according to the manufacturer's instructions. Results were normalized with respect to 18 S RNAs used as internal control. The primers used are as follows: *HIC1* sense 5′-CGACGACTACAAGAGCAGCAGC-3′ and antisense 5′-CAGGTTGTCACCGAAGCTCTC-3′; *ephrinA1* sense 5′-ATCGCCACACCGTCTTCTG-3′ and antisense 5′-CACGTAGTCATTCAGCTGCACAT-3′; and *EphA2* sense 5′-TGTGCCAGGCAGGCTACG-3′ and antisense 5′-CTCCAAGCAGGGGCTCTCA-3′ as well as control primers for 18 S sense 5′-GGCGCCCCCTCGATGCTCTTAG-3′ and antisense 5′-GCTCGGGCCTGCTTTGAACACTCT-3′.

Primers were used at a concentration of 0.5 μM. According to a melting point analysis, only one PCR product was amplified under these conditions. RNAs extracted from pBabe-infected cells were used to generate a standard curve for each gene. Results were normalized with respect to the internal controls and are expressed relative to the levels found in pBabe-infected cells.

Small Interfering RNA—WI38 cells were reverse-transfected with INTERFERin™ according to manufacturer's instructions using 50 nM small interfering RNA targeting the HIC1 (HIC1 siGENOME SMART Pool M-006532–01, Dharmacon) or a scrambled control sequence (si Ct). 72 h later cells were lysed for RNA or protein extraction.

hTERT-HMEC were reverse-transfected with RNAiMax according to the manufacturer's instructions using 10 nM small interfering RNA targeting the HIC1 (HIC1 siGENOME SMART Pool M-006532-01, Dharmacon) or a control siRNA (si Ct). For the "double" siRNA experiment, 20 nM control siRNA or 10 nM EphA2 (EphA2 SiGENOME SMART pool D-003116-22, Dharmacon) in combination with 10 nM control siRNA or 10 nM HIC1 SiRNA were used. 72 h later, the cells were lysed for RNA or protein extraction or used in migration assays.

Chromatin Immunoprecipitation—ChIP was performed according to published protocols with slight modifications. Briefly, formaldehyde was added directly to the cultured cells to a final concentration of 1% for 10 min at 37 °C. The cross-linking was stopped by adding glycine to a final concentration of 0.125 M. After 5 min at 37 °C, cells were lysed directly in the plates by resuspension in cell lysis buffer for 5 min. Then the samples were pelleted, resuspended in nuclei lysis buffer, and sonicated to chromatins with an average size of 500 bp using a BioRuptor (Diagenode, Liege, Belgium). After preclearing with a 50% slurry of protein A-G beads preincubated with salmon sperm DNA and BSA for 4 h at 4 °C, the chromatins were incubated with the anti-HIC1 antibodies, normal rabbit IgG, or with no antibodies overnight. The antibody-bound chromatin was then pooled down for 30 min with protein A-G beads, washed extensively, and eluted two times by 250 μl of elution buffer. After addition of 20 μl of 5 M NaCl, the cross-linking was reversed by overnight incubation at 65 °C. The immunoprecipitated DNAs as well as whole cell extract DNAs (input) were purified by treatment with RNase A and then proteinase K followed by purification on Nucleobond Extract II (Macherey-Nagel). Alternatively, we used the protocol previously described by Dahl and Collas (35). The purified DNAs were used for PCR analyses using the following relevant primers: *SIRT1* sense 5′-GATAGAAACGCTGTGCTCCA-3′ and antisense 5′-CCTTCCTTTCTAGCGTGAGC-3′; *EphA2* sense 5′-CTGTCAGGAAGGGAGATGAGCT-3′ and antisense 5′-CTGGGAGGCAGGGGTCAT-3′, and *GAPDH* sense 5′-TCCTCCTGTTTCATCCAAGC-3′ and antisense 5′-TAGTAGCCGGGCCCTACTTT-3′.

Migration and Invasion—For the cell migration assay, 5 × 10^4 cells were seeded in triplicate on polycarbonate membrane inserts in a Transwell apparatus (Transwell, BD Biosciences). Cells were then cultured for 16 h (MDA-MB-231) or 24 h (hTERT-HMEC). The filters were removed, and the cells on the lower surface of the filter were stained with Hoechst. The number of cells that had migrated was analyzed on each filter using an Axioplan 2 (Zeiss, Germany) microscope. Ten images of randomly chosen optical fields were captured on each migration filter using AxioVision® software for microscopy image analysis (Zeiss), and migrating cells were counted with the Colony1.1® software. For the invasion assay, 10 × 10^4 cells were seeded on polycarbonate membrane inserts previously coated with Type 1 collagen in a Transwell apparatus. After 16 h, the filters were removed, and the cells on the lower surface of the filter were stained with Hoechst and counted as described above for the migration assays.

Anchorage-dependent Cell Growth—To measure anchorage-dependent cell growth, 5 × 10^5 cells were seeded into a 60-mm tissue culture-treated dish. At indicated time points, cells were trypsinized and counted using a hemocytometer. Each experiment was repeated at least three times, and representative results are reported.

Cell Behavior in Matrigel—The behavior of cells on Matrigel was analyzed as described (36). Briefly, tissue culture dishes were coated with Matrigel (Collaborative Biomedical Products, Bedford, MA) at 37 °C before adding 1 × 10^5 MDA-MB-231 cells. Cells were incubated on Matrigel for 24 h at 37 °C, and cellular phenotype was assessed using an inverted light microscope.

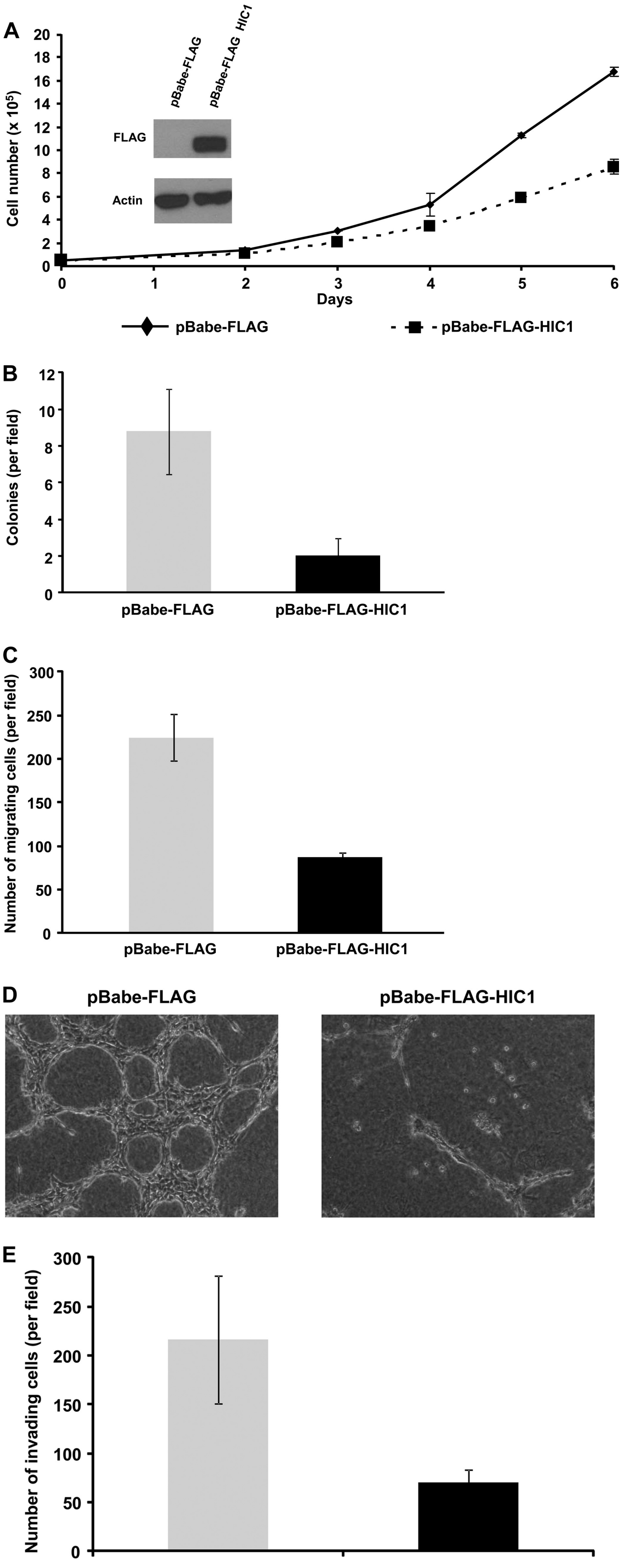

FIGURE 1. **Global effects of *HIC1* overexpression in MDA-MB-231 breast cancer cell line.** *A,* monolayer growth of 5 × 10^4 MDA-MB-231 pBabe-FLAG and pBabe-FLAG-HIC1 cells was evaluated microscopically every day for 6 days of incubation at 37 °C; *bars*, ±S.D. The *inset* corresponds to the FLAG-HIC1 protein detection by Western blot after 6 days (*B*). To measure anchorage-independent cell growth and survival, MDA-MB-231 cells were incubated for 7 days at 37 °C in soft agar. After 7 days, colony formation was scored microscopically, and clusters containing at least three cells were defined as a colony; *bars*, ±S.D. *C,* migration assay (6 h) using Transwells. *D,* phenotype of control and HIC1-infected MDA-MB-231 cells was evaluated after incubation on top of polymerized Matrigel. Whereas control MDA-MB-231 cells displayed a stellate growth pattern in Matrigel typical of the behavior of aggressive breast cancer cells, cells overexpressing HIC1 were organized into spherical colonies. *E,* collagen Transwell invasion assay (16 h).

RESULTS

Biological Effects of HIC1 Overexpression into Breast Tumor Cells—Enforced HIC1 expression results in growth arrest, reduced survival, and differentiation of glioblastoma, breast cancer, and adenocarcinoma cell lines (1). To further decipher its role as a tumor suppressor gene, we overexpressed HIC1 in the MDA-MB231 breast cancer cell line. These cells, which are tumorigenic and highly invasive, are known to have lost expression of *HIC1* through marked hypermethylation of its promoter (1). Furthermore, the demethylating agent 5-aza-2′-deoxycytidine restores HIC1 expression in the MDA-MB-231 cells (6). In our study, we used a pBabe retroviral infection system to reintroduce expression of HIC1 in these cells (Fig. 1*A*). We first confirmed that HIC1 re-expression leads to a growth arrest of these MDA-MB-231 cells. Indeed, after only 3 days in culture, HIC1 expression altered the anchorage-dependent (monolayer) growth of MDA-MB-231 cells. After 6 days in culture, the number of control cells was about twice that of HIC1-overexpressing cells (Fig. 1*A*). Anchorage-independent growth was also evaluated by measuring tumor cell colonization of soft agar (Fig. 1*B*). Microscopic evaluation revealed that HIC1 inhibited at least 75% of soft agar colony formation as compared with mock-infected cells. We also observed in Transwell migration assays that the empty vector-infected cells migrate twice as much as did the HIC1-overexpressing cells (Fig. 1*C*). Similar differences were obtained using a wound healing assay (data not shown and Ref. 66). Numerous studies have shown that tumor cell behavior within a three-dimensional microenvironment, such as Matrigel, can reliably predict the differentiation state and aggressiveness of mammary cells (37, 38). Thus, we investigated whether HIC1 re-expression would alter the behavior of these mammary epithelial cells in three-dimensional culture. MDA-MB-231 cells expressing empty pBabe vector or pBabe-FLAG-HIC1 were incubated on Matrigel (Fig. 1*D*). Consistent with their aggressiveness, mock MDA-MB-231 cells quickly assembled into tubular networks, with progressive invasion throughout the Matrigel. By contrast, HIC1 expression prevented tubular network organization and led cells to assemble into spheres as do normal epithelial cells suggesting that HIC1 re-expression could reverse, at least partially, the transformed phenotype of MDA-MB-231 cells. To test this hypothesis, the invasiveness of the cells was assessed using Boyden chambers coated with collagen. As expected, we observed an inhibition of invasion of the MDA-MB-231 cells overexpressing HIC1 (Fig. 1*E*). Together, these results clearly demonstrate an inhibitory effect of HIC1 on the proliferation, migration, and invasion of tumor cells.

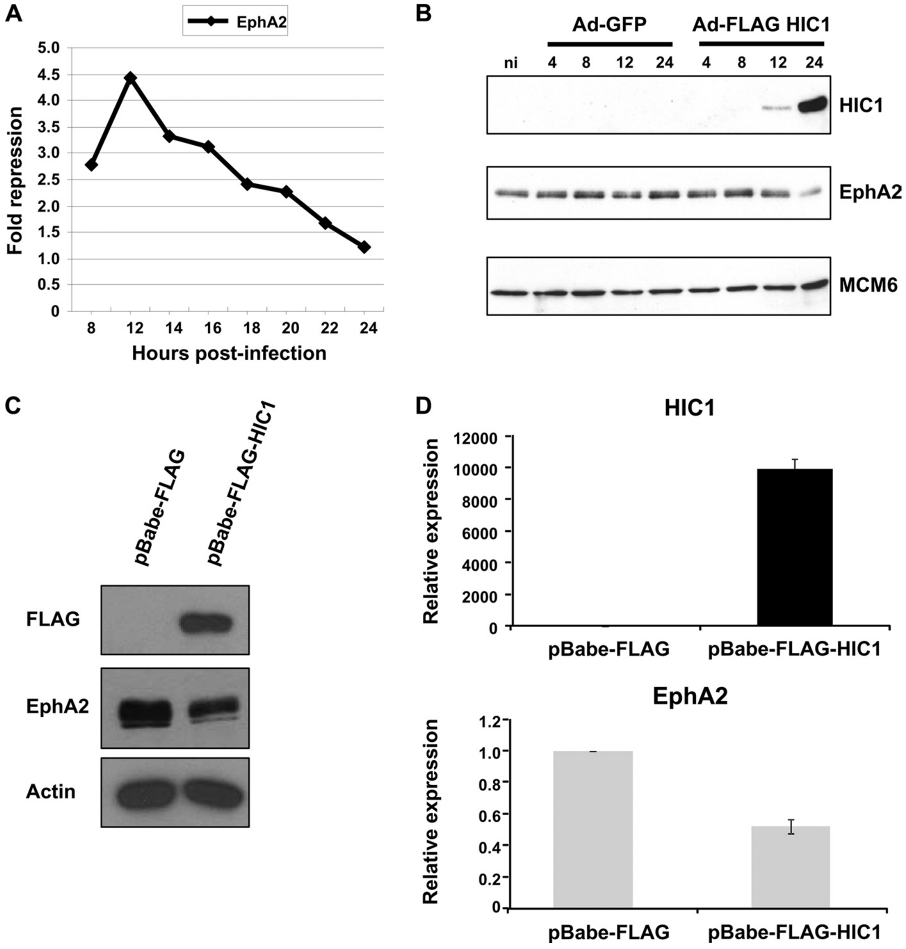

FIGURE 2. **Effects of *HIC1* overexpression on expression of *EphA2* in infected U2OS.** *A,* total RNAs from U2OS cells (HIC1 null) infected with Ad-FLAG-HIC1 and Ad-GFP were prepared at the indicated times after infection (8–24 h), and Affymetrix HG U133A chips were used to measure the gene expression. Expression values were normalized to Ad-GFP-infected control cells at the same time points. The *fold repression* corresponds to the ratio between the expression level of *EphA2* measured in Ad-GFP- and in Ad-FLAG-HIC1-infected cells at each time point. *B,* Western blot analysis of HIC1 and EphA2 in lysates obtained from U2OS cells infected with AdGFP or AdHIC1 adenoviruses for 0–24 h post-infection. Equal loading was confirmed by immunoblotting for MCM6. *Lane ni*, lysates from noninfected U2OS cells. *C,* U2OS cells were infected with the pBabe-FLAG or pBabe-FLAG-HIC1 retroviral vector. After 3 days of puromycin selection, cell extracts were prepared and resolved using SDS-PAGE. Constitutive expression of FLAG-tagged HIC1 levels was checked using immunoblot analysis with anti-FLAG tag antibody. Actin protein levels were used as a loading control. *D,* quantitative real-time RT-PCR assays for the *HIC1* and *EphA2/18S* mRNA ratio. qRT-PCR was performed using total RNAs isolated from U2OS infected with pBabe-FLAG or pBabe-FLAG-HIC1 for *HIC1* and *EphA2*. Values were normalized to 18 S as indicated.

Identification of EphA2 as a HIC1-repressed Gene in Infected U2OS Cells—To identify transcriptional targets of HIC1 that could potentially be implicated in these effects, we based this on our previous gene expression profiling of U2OS osteosarcoma cells infected by a control adenoviral vector Ad-GFP or by an adenoviral vector expressing HIC1, AdHIC1 (20). In brief, total RNAs from these U2OS cells were used to interrogate the Affymetrix Human Genome U133A chip containing 14,500 transcripts. 81 genes were found to be down-regulated more than 3-fold after 16 h of infection. Among them, *CXCR7*, *Cyclin D1,* and *Sox9* have been characterized as direct HIC1 target genes (17, 20, 22). Another gene repressed in U2OS cells infected with Ad-HIC1 was *EphA2*, a receptor for *ephrin-A1* recently described as a direct target gene of HIC1 through similar analyses in MCF-7 cells (23). Indeed, the receptor tyrosine kinase EphA2 is up-regulated in many cancers and notably in many highly aggressive breast cancers (36, 39–41). Furthermore, the biological effects on malignant MDA-MB-231 cells resulting from antibody therapy targeting EphA2 are similar to those that we observed with HIC1 overexpression (Fig. 1) such as inhibition of tubular network formation and soft agar colonization (36, 42). Our results show that the *EphA2* gene is rapidly repressed up to 4.5-fold 12 h post-Ad-HIC1 infection (Fig. 2*A*). In addition, Western blot analysis of AdHIC-infected cell lysates revealed a marked down-regulation of endogenous EphA2 protein inversely correlated with a high level of HIC1 protein expression over a 24-h period (Fig. 2*B*). For safety and convenience, we used retroviral infections to perform our biological assays in MDA-MB-231 (Fig. 1). To compare these results with our micro-array data obtained in U2OS using adenoviral expression, we used the pBabe retroviral system to overexpress HIC1 in U2OS cells (Fig. 2*C*). As expected, we obtained a high level expression of HIC1 with the pBabe vector that correlated with a decrease of RNA and protein expression of endogenous EphA2 (Fig. 2, *C* and *D*). Taken together, these data demonstrate that *EphA2* is a HIC1-repressed gene in U2OS osteosarcoma cells, regardless of the overexpression system used.

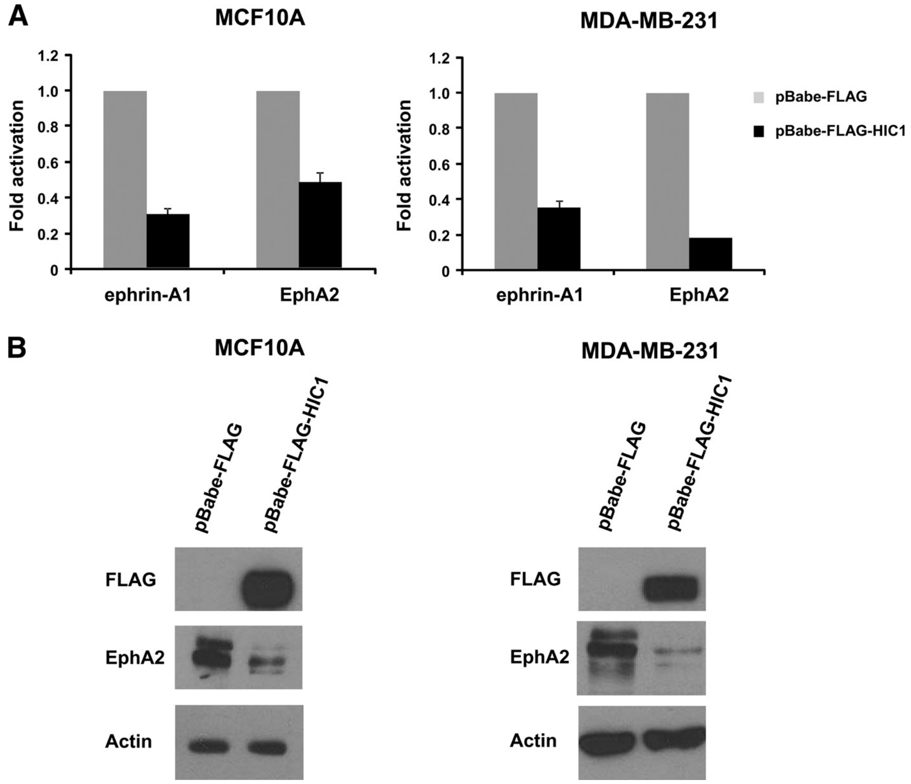

FIGURE 3. **EphA2 is down-regulated in pBabe-FLAG-HIC1-infected breast cells.** *A*, quantitative real time RT-PCR assays for the *EphA2*/18 S mRNA ratio. qRT-PCRs were performed using total RNAs isolated from MCF10A and MDA-MB-231 infected with pBabe-FLAG (*gray boxes*) or pBabe-FLAG-HIC1 (*black boxes*) for *EphA2* and *ephrin-A1*. Values were normalized to 18 S. *B,* Western blot analyses of FLAG-HIC1 and EphA2 in lysates obtained from MCF10A and MDA-MB-231 infected with pBabe-FLAG or pBabe-FLAG-HIC1. Equal loading was confirmed by immunoblotting for actin.

HIC1 Can Down-regulate the Expression of EphA2 in Breast Epithelial Cells—It has been shown that higher levels of EphA2 are present in MDA-MB-231 than in the non-neoplastic MCF10A breast epithelial cells, supporting its role in promoting tumorigenesis and invasiveness (43–45). Therefore, we decided to overexpress HIC1 in these two cell lines to establish a functional link between EphA2 and HIC1 in mammary epithelial cells.

Through retroviral infection, we obtained high level expression of HIC1 in MCF10A and MDA-MB-231 cells as confirmed by real time quantitative reverse transcriptase-PCR (RT-qPCR)[5] (data not shown). As measured by RT-qPCR, re-expression of HIC1 leads to a 2.2- and 5.5-fold reduction of *EphA2* mRNA in MCF10A and MDA-MB-231, respectively (Fig. 3*A*). As a control, we confirmed the down-regulation of the low expression levels of *ephrin-A1* (45), encoding a ligand of EphA2, by overexpression of HIC1 (Fig. 3*A*), as described previously (23).

We then investigated whether EphA2 was also down-regulated at the protein level following HIC1 overexpression. Western blot analyses of MCF10A and MDA-MB-231 lysates revealed high level protein expression of HIC1 in response to pBabe-FLAG-HIC1 retroviral infection (Fig. 3*B*). In the same blot using an EphA2-specific antibody, we observed a significant down-regulation of endogenous EphA2 protein in MCF10A and MDA-MB-231 cells overexpressing HIC1 (Fig. 3*B*). The down-regulation of *ephrin-A1* at the RNA level (Fig. 3*A*) could not be confirmed at the protein level because ephrin-A1 proteins are expressed at very low levels in MCF10A and MDA-MB-231 cells in line with the mutually exclusive expression of EphA2 and ephrin-A1 proteins in different breast cell lines (data not shown) (45). Collectively, these data suggested that *EphA2* is a target gene of HIC1 in normal and transformed breast cells infected by pBabe-FLAG-HIC1.

HIC1 mRNA Knockdown Increases EphA2 Expression in Normal WI38 Fibroblasts—All of the above-described results were obtained by ectopic HIC1 expression through adenoviral or retroviral infection. To confirm *EphA2* as a new HIC1 transcriptional target in a more physiological context, we used small interfering RNA (siRNA) strategy in normal human WI38 fibroblasts. These cells, which express endogenous HIC1, have been previously used to validate other direct HIC1 target genes, including *ephrin-A1* (14, 20, 23). They have the advantage of high level endogenous HIC1 expression correlated with low level endogenous EphA2 expression making them a good model for a *HIC1* mRNA knockdown strategy. Quantitative RT-PCR analyses demonstrated an ~60% knockdown of *HIC1* in WI38 transfected with siHIC1 in comparison with the cells transfected with the control siRNA (Fig. 4). In the same RNA samples, we detected a 2-fold increase in *EphA2* expression following knockdown of endogenous *HIC1* (Fig. 4). In addition, we also detected a similar increase of *ephrin-A1* expression, as shown previously (23). These results demonstrate that knockdown of endogenous *HIC1* in normal human WI38 fibroblasts results in the up-regulation of *EphA2* confirming its status as a target gene of HIC1.

HIC1 Is a Transcriptional Repressor of EphA2—To determine whether *EphA2* is a direct target gene of HIC1, we first

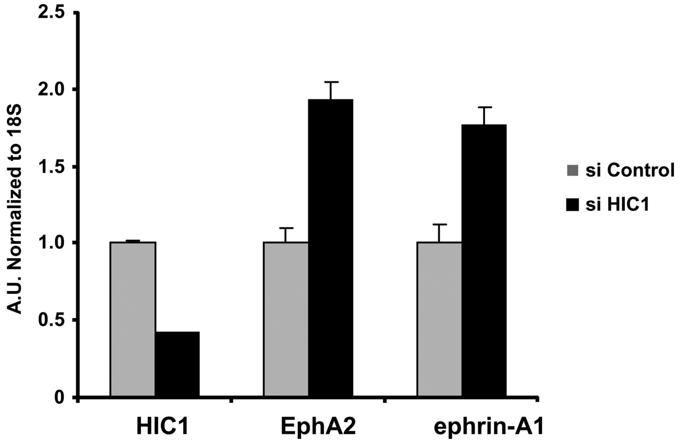

FIGURE 4. **Inactivation of endogenous *HIC1* in the WI38 human fibroblast cells up-regulated *EphA2* expression.** WI38 cells were transfected with non-target siRNA control or with HIC1 siRNA. Total RNA was extracted, and the mRNA expression levels of *HIC1*, *EphA2,* and *ephrin-A1* mRNAs were assessed by real time quantitative RT-PCR. Values were normalized to 18 S.

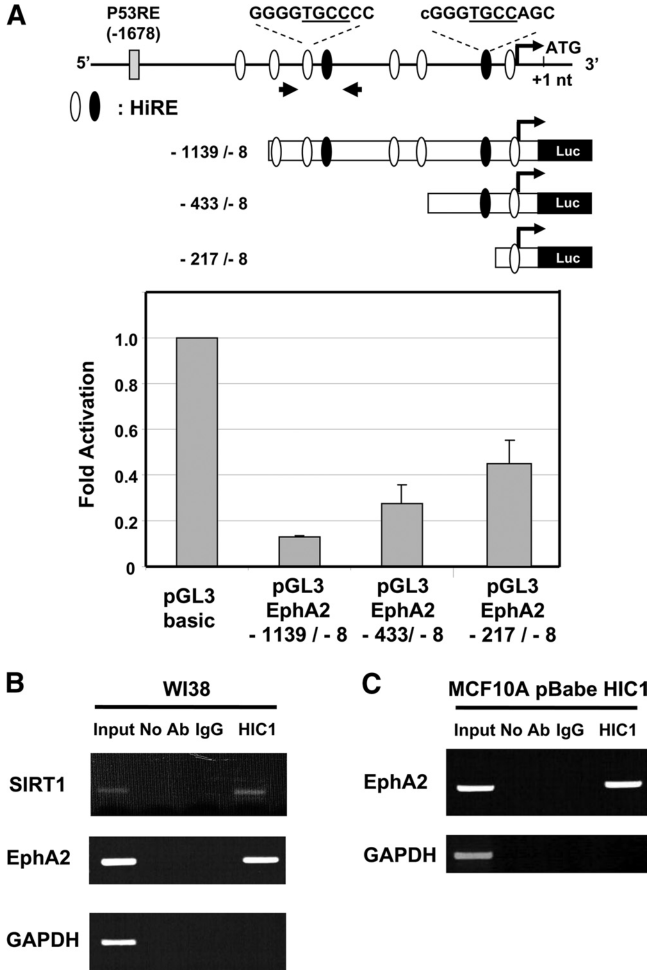

FIGURE 5. **EphA2 is a direct target gene of HIC1.** *A*, *EphA2* is down-regulated by HIC1. From *top to bottom*, a schematic drawing of the 5′ promoter region and the first coding exon of *EphA2* is shown. The transcription start site (*bent arrow*) as well as a portion of the first coding exon are described in GenBank™ under accession number AY 052403; gi 22820011. Numbering is relative to the *EphA2* translational start codon (ATG, nucleotide (*nt*) +1) as in Ref. 58. The potential HiREs are shown as *white ovals* and as *black ovals* for those that are highly homologous to the consensus (12). The *two small arrows* indicate the position of the primers used to amplify the relevant region of *EphA2* in the ChIP and ChIP upon ChIP experiments. *Below* is shown a schematic drawing of the various fragments of the human *EphA2* first coding exon subcloned in the luciferase reporter plasmid pGL3 basic. Reporter constructs were transfected in triplicate into U2OS cells and assayed for luciferase activity. Luciferase activity was normalized for transfection efficiency using a cotransfected β-Gal reporter. Repression of transcription of each construct by HIC1 was calculated by first dividing luciferase (*Luc*) activity in the absence of HIC1 by the activity in the presence of HIC1 (normalized for transfection efficiency using a cotransfected β-Gal reporter). The value obtained for each construct was then divided by the repressive effect elicited by HIC1 on the empty pGL3 basic vector to obtain the final fold of activation. Results, expressed relative to a value of 1.0 for cells transfected with the pGL3 empty vector, are expressed as the means of two different experiments, and *error bars* represent standard deviations. *B* and *C,* ChIP analyses of HIC1 on *EphA2* and *SIRT1* promoters. Normal human WI38 fibroblasts (*B*) expressing endogenous HIC1 proteins and MCF10A cells overexpressing HIC1 (*C*) were cross-linked with 1% formaldehyde. Cross-linked chromatin immunoprecipitated with polyclonal anti-HIC1 antibody (325), with rabbit IgG or no antibody, were used in PCR amplification with primers flanking the functional HiREs identified by sequence analysis in the *EphA2* promoter. The region previously identified in the *SIRT1* promoter in WI38 cells (14) was used as a positive control, whereas PCR with the 5′ promoter of *GAPDH* was used as an internal nonbinding control (14). An H_2O control corresponding to PCR without DNA yielded no amplified products (not shown).

scanned its promoter region for the presence of consensus HIC1-responsive elements (HiRE) centered on a GGCA (reverse TGCC) core motif to which HIC1 could directly bind (12). We identified several putative HiRE. Among them, two were highly homologous to the 10-nucleotide consensus sequence that we defined (12) (*black ovals* in Fig. 5*A*). To directly assess the ability of HIC1 to repress transcription of *EphA2* through these sites, we cloned ~1.1 kbp of genomic DNA upstream of the ATG codon in the first coding exon of *EphA2* and including the transcription initiation site as defined in GenBank™ (AY052403; gi 22820011) and performed luciferase promoter-reporter assays in U2OS cells, the cell line that we have used for the gene-profiling experiments. To this end, the −1139/−8 promoter region of *EphA2* was cloned in the pGL3 basic reporter vector as were two deletion constructs, −433/−8 and −217/−8, that gradually eliminate the HiREs were made. These constructs were then transfected alone or with the pcDNA3 FLAG-HIC1 expression vector in U2OS cells, and promoter activities were measured in the absence or presence of ectopic HIC1. As shown in Fig. 5*A*, transient transfection of HIC1 strongly repressed the *EphA2* promoter activity. This repression was gradually lost in the deletion constructs but still remained significant with the −217/−8 construct. This construct contains an HiRE site located very close to the transcription start site, which could contribute to the remaining HIC1-mediated repression, as observed previously for the *CXCR7* gene (20).

Next, we designed sets of oligonucleotides to PCR amplify the upstream cluster of HiRE in DNA samples obtained from chromatin immunoprecipitation (ChIP) of HIC1 (Fig. 5*A*). We first performed ChIP assays in normal human WI38 fibroblasts that express endogenous HIC1 proteins. As positive and negative controls for specificity, we used primers flanking the previously identified HiRE in the *SIRT1* promoter and primers located in the *GAPDH* promoter, respectively, as described previously (14, 17). As shown in Fig. 5*B*, we were able to specifically amplify the region, including the HiRE in the *SIRT1* promoter from WI38 chromatin immunoprecipitated with anti-HIC1 antibody but not with normal IgG. Primers designed to amplify the 5′ region upstream of the *GAPDH* promoter did not yield a product, validating the specificity of this ChIP assay (Fig. 5, *B* and *C*).

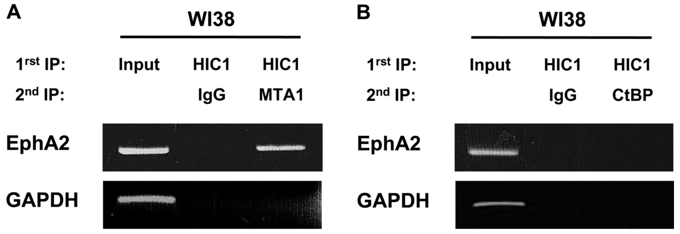

FIGURE 6. **ChIP upon ChIP assays demonstrate that HIC1 and MTA1 might form a stable complex on the *EphA2* promoter.** *A* and *B,* normal human WI38 fibroblasts were cross-linked with 1% formaldehyde. Cross-linked chromatin was sonicated and immunoprecipitated with polyclonal anti-HIC1 antibody (325) (*1^{rst} IP HIC1*). The bound material was eluted, divided in two, and subjected to a second round of immunoprecipitation with anti-MTA1 antibodies (*2^{nd} IP MTA1*), anti-CtBP (*2^{nd} IP CtBP1*), or with normal rabbit IgG (*2^{nd} IP IgG*) (17). PCR amplifications were performed using primers flanking the functional HiREs previously identified in *EphA2* (Fig. 5). PCR with the 5′ promoter of *GAPDH* was used as an internal nonbinding control. Representative gels of one ChIP upon ChIP among two experiments are shown.

To test *EphA2*, we performed PCR experiments with the same WI38 chromatin samples and primers designed to amplify the region containing the putative HiRE and thus potentially mediating the repressing effects of HIC1 on this promoter. *EphA2* was amplified from the HIC1-immunoprecipitated WI38 chromatin but not from chromatin immunoprecipitated by the control IgG, demonstrating that *EphA2* is a new direct target gene of endogenous HIC1 proteins in WI38 cells (Fig. 5*B*).

Infection of MCF10A cells with a pBabe-FLAG-HIC1 retrovirus results in a significant decrease of *EphA2* mRNAs (Fig. 3*A*). To demonstrate that this effect relied on a direct transcriptional repression of *EphA2* by exogenous HIC1, we prepared chromatin from these infected cells and performed ChIP assays. In close agreement with all our results, HIC1 was found at the *EphA2* promoter (Fig. 5*C*).

Taken together, these results demonstrate that endogenous or ectopically expressed HIC1 proteins are present at the *EphA2* promoters in both WI38- and in HIC1-infected MCF10A cells, thus validating *EphA2* as a new direct target gene of HIC1.

HIC1-MTA1 Complex Is Recruited on the EphA2 Promoter—To refine our understanding of the regulation of *EphA2* by HIC1, we investigated the potential role of its corepressors MTA1 and CtBP (15, 17). Chromatins prepared from WI38 fibroblasts were analyzed by sequential ChIP experiments as described previously (17) with HIC1 antibodies followed by MTA1, CtBP, or rabbit IgG as a negative control (Fig. 6). A band corresponding to the relevant region of the *EphA2* promoter was detected in the HIC1/MTA1 but not in the HIC1/CtBP or HIC1/IgG ChIP (Fig. 6). Primers designed to amplify the 5′ region upstream of the *GAPDH* promoter did not yield a product, further demonstrating the specificity of our assay. In conclusion, HIC1 recruits MTA1, a member of the NuRD repressing complex, to the *EphA2* promoter in normal WI38 fibroblasts, likely leading to its direct transcriptional repression as observed in our qRT-PCR analyses.

Effects of HIC1 and EphA2 Down-regulation in Normal Mammary Epithelial Cells, hTERT-HMEC—Ephrin-A1-EphA2 signaling is very complex and highly cell type-dependent (24, 30). EphA2 can have ligand-dependent tumor suppressor functions as well as ligand-independent pro-oncogenic functions notably through cross-talk with the Akt pathway (46). MDA-MB-231 cells used in our assays (Figs. 1 and 3) are negative for HIC1 (6) and ephrin-A1 (45) but wild-type for PTEN (47). Thus, in these cells, a lack of *ephrin-A1* is not associated with activation of the PI3K/Akt pathway. In agreement with previous results (46), coinfection of MDA-MB-231 with a retrovirus expressing wild-type EphA2 (34) or with two mutants of the conserved juxtamembrane tyrosine residues required for optimal kinase activity (33) was unable to rescue, even partially, the negative effects on growth and on cell migration induced by HIC1 overexpression (data not shown).

By contrast, in normal epithelium, EphA2 and ephrin-A1 are properly expressed and engaged in interactions with each other to maintain epithelial homeostasis (30). We next tested whether *EphA2* is a direct target gene of endogenous HIC1 in normal hTERT-HMEC mammary epithelial cells with the following biological consequences. HMEC cells that are not transformed but simply immortalized by exogenous hTERT transduction expressed higher levels of *HIC1* than MCF10A cells (data not shown), making them a better model to modulate endogenous HIC1 and biological features.

First, we used the siRNA strategy in hTERT-HMEC cells as described previously in WI38 fibroblasts (Fig. 4). In close agreement with those results, we observed a 50% up-regulation of *EphA2* mRNA levels correlated with a 3-fold down-regulation of *HIC1* mRNA (Fig. 7*A*). Moreover, careful examination of these transfected hTERT-HMEC cells revealed some salient differences. Indeed, cells transfected with the siRNA targeting *HIC1* seem more scattered and rounded up than cells transfected with the control siRNA (si Ct) (Fig. 7*B*). This observation, which could reflect a change in biological properties of these normal mammary cells, prompted us to investigate the migratory capacity of these transfected hTERT-HMEC cells. Using transwell assays, it appeared that down-regulation of HIC1 in hTERT-HMEC leads to an increase in cell migration. Thus, a decrease of *HIC1* expression correlates with an increase in the migration of normal mammary epithelial cells. This observation could be, at least partially, caused by the increase of *EphA2* mRNA expression observed in these cells (Fig. 7*A*), because *EphA2* has already been implicated in the migration and invasiveness of cancer cells. To address this point more directly, we inhibited *EphA2* by RNA interference in hTERT-HMEC cells. The resulting strong inhibition of *EphA2* expression (Fig. 8*A*) favored cell-cell contact (Fig. 8*B*) and significantly impaired their migration properties (Fig. 8*C*). These results are in close agreement with the destabilization of adherens junctions observed in MCF10A cells overexpressing EphA2 (34) and the promotion of tight junction formation in human brain endothelial cells treated with EphA2 siRNA (48). Finally, when both *HIC1* and *EphA2* were inhibited, the phenotypes were reverted and the cells reacquired their migratory properties. In conclusion, the direct repression of *EphA2* and of other target genes by HIC1 (20, 66) is involved in the control of migration properties of normal mammary epithelial cells.

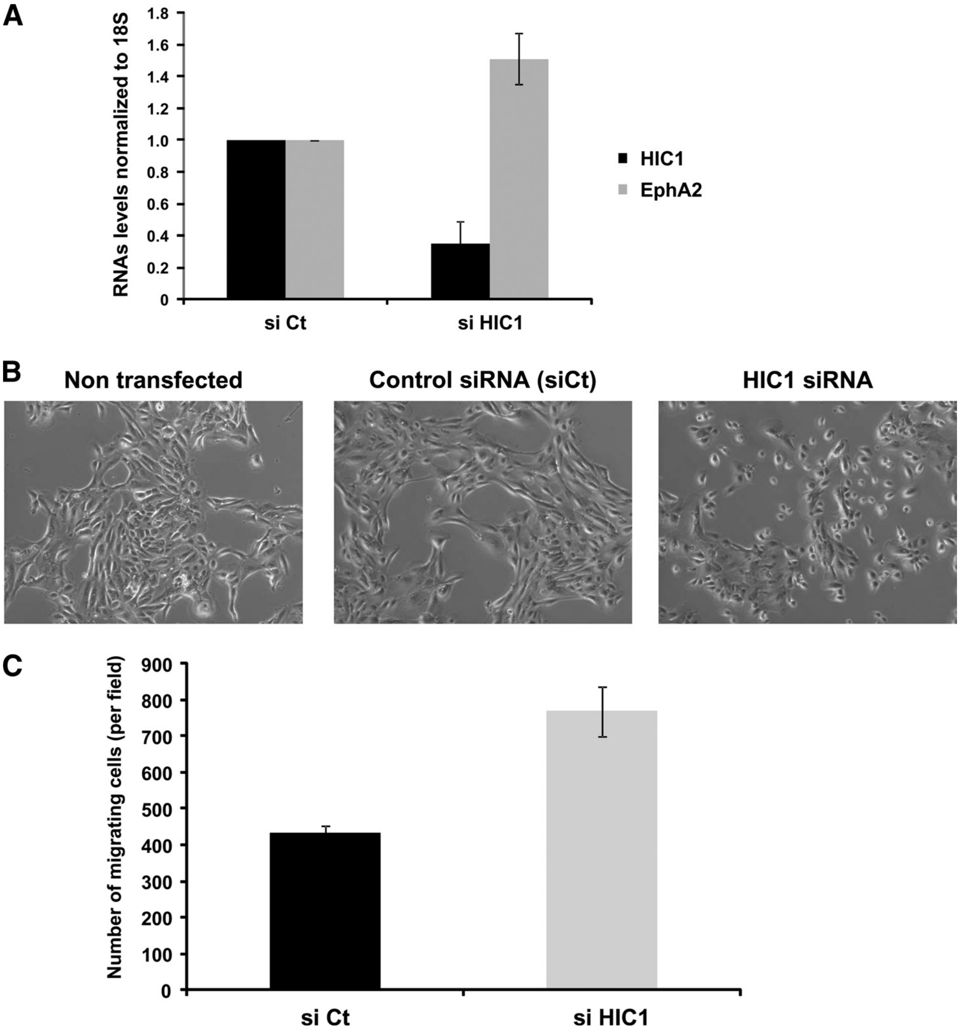

FIGURE 7. **Inactivation of endogenous *HIC1* enhances the migration properties of normal mammary cells hTERT-HMEC.** *A,* hTERT-HMEC cells were transfected with nontarget siRNA control and with HIC1 siRNA. Total RNA was extracted, and expression levels of *HIC1* and *EphA2* mRNAs were assessed by real time quantitative RT-PCR. Values were normalized to 18 S. *B,* phenotype of nontransfected hTERT-HMEC and hTERT-HMEC transfected with nontarget, si Ct, or with HIC1 siRNA. *C,* migration assay of hTERT-HMEC cells transfected with si Ct or HIC1 siRNA.

DISCUSSION

In this study, we characterized the tyrosine kinase receptor *EphA2* as a new direct target gene of HIC1-mediated transcriptional repression. We demonstrated that EphA2 mRNA and protein levels are strongly decreased upon infection of breast cancer cell lines with a retrovirus expressing HIC1. Furthermore, and in good agreement with the tumor suppressor function of HIC1, we observed that inactivation of HIC1 through RNA interference in normal breast epithelial cells results in the up-regulation of *EphA2* and is correlated with an increased migratory phenotype.

The *HIC1* promoter has been found to be hypermethylated in a wide variety of solid cancers, such as breast, brain, liver, colorectal, cervical, and lung tumors. As demonstrated for many tumor suppressor genes, hypermethylation of the *HIC1* promoter region leads to the epigenetic silencing of its gene expression (49). Moreover 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 (1, 4, 6–8). In addition, it now appears that other inhibitory mechanisms besides epigenetic silencing contribute to the low HIC1 expression because low HIC1 expression is observed in some leukemias in the absence of HIC1 promoter hypermethylation. In any case, *HIC1* expression levels decreased during the development of cancer.

To determine the incidence of HIC1 in breast cancer biology, we stably infected malignant MDA-MB-231 breast cancer cells with a pBabe-FLAG-HIC1 retroviral construct. We showed that HIC1-overexpressing cells (pooled) exhibited decreased cell proliferation but also decreased migration and invasion *in vitro*, both characteristic of tumor metastasis (Fig. 1). In our various experiments modulating the expression of *HIC1*, the biological effects were correlated with the level of *EphA2* expression leading us to hypothesize that in tumors with loss of *HIC1* expression the resulting increase in *EphA2* expression could participate in tumor progression.

The cell membrane-bound EphA2 receptor belongs to the largest subfamily of tyrosine kinase receptors. They are involved in different biological processes such as angiogenesis, cell migration, axon guidance, and synaptic plasticity (31). *EphA2* overexpression has been detected in pre-clinical

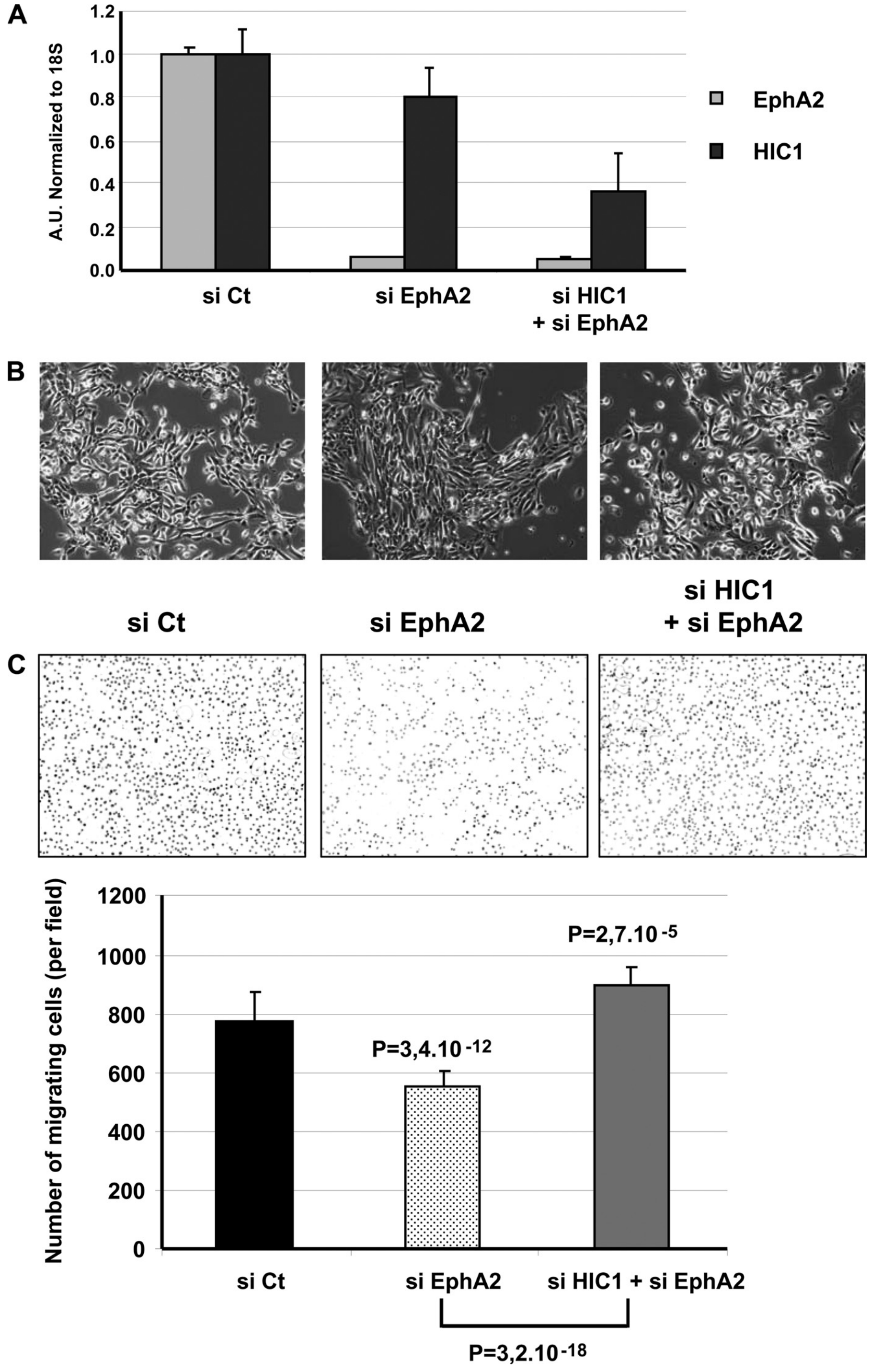

FIGURE 8. **Inactivation of endogenous *EphA2* reduces the migration properties of normal mammary cells hTERT-HMEC.** *A,* hTERT-HMEC cells were transfected with nontarget siRNA control, with EphA2 siRNA, or with a combination of HIC1 and EphA2 si RNAs. Total RNA was extracted, and expression levels of *HIC1* and *EphA2* mRNAs were assessed by real time quantitative RT-PCR. Values were normalized to 18 S. *B,* phenotype of hTERT-HMEC transfected with non-control siRNA (*si Ct*), with EphA2 siRNA or with a combination of HIC1 and EphA2 siRNAs. *C,* migration assay of hTERT-HMEC cells transfected with control siRNA, EphA2 siRNA, or a combination of HIC1 and EphA2 siRNAs. *Dark spots* correspond to nuclei of cells that had migrated to the other pole of the membrane. A representative field is shown for each condition. Each condition was performed in triplicate. 20 fields for each condition were counted. Statistical analyses were performed by Student's *t* test, and the *p* values are indicated.

cancer models and clinical specimens of many different types of cancer originating from the brain, breast, colon, esophagus, head and neck, liver, lungs, ovaries, prostate, and skin (50). Frequently, the overexpression of EphA2 in human cancers correlates with poor prognosis and increased metastatic potential (40, 51). Moreover, ectopic overexpression of EphA2 is sufficient to confer malignant transformation and tumorigenic potential as defined both *in vitro* and *in vivo* on nontransformed mammary epithelial cells (36), demonstrating that EphA2 contributes to malignant cancer phenotypes.

The mechanisms by which overexpressed EphA2 contributes to cancer are not entirely clear. Both kinase-dependent and

-independent functions of EphA2 have been reported to be involved in aggressive cancer phenotypes (44, 52). Overall, although EphA2 and its ligand ephrin-A1 are no doubt of major importance in both development and cancer, the exact contribution of these proteins to the specific processes involved in tumor formation, maintenance, and progression is extremely complex and dependent on many factors (31, 41). Briefly, EphA2 is present on the surface of normal cells and interacts with its ligand ephrin-A1 that is present on the surface of adjacent cells (53). Upon interaction, EphA2 becomes phosphorylated, which is important for the normal signaling through the MAPK and Akt pathways, and is subsequently degraded. In cancer cells, because of the loss of cell contacts, EphA2 fails to efficiently interact with its ligand on adjacent cells, leading to the accumulation of the unphosphorylated form of EphA2 on the cell resulting in constitutive oncogenic signaling (31). So the data suggest an oncogenic, ligand-independent role for EphA2 in tumor cells and a tumor-suppressing role for ephrin-A1 as a result of receptor phosphorylation and subsequent receptor degradation preventing oncogenic signaling.

In light of the understanding of the role of *EphA2* in cancers, it is particularly important to know how its regulation is dysregulated during tumorigenesis. *EphA2* is reported to be a direct transcriptional target of the Ras-MAPK and of the Akt pathways (45, 46, 54). Moreover, *EphA2* is overexpressed in Ras-transformed cells as well as in Ras-overexpressing transgenic mice (55). Thus, these results strongly suggest that *EphA2* is transcriptionally up-regulated during the process of malignant transformation, potentially as a result of aberrant growth factor signaling originating through other receptor tyrosine kinases such as EGF receptors and *ErbB2* (39, 56, 57). Here, we have shown that HIC1 is a direct transcriptional repressor of *EphA2* in U2OS (20) and in normal as well as transformed breast epithelial cells (Figs. 4 and 7). Moreover, a recent study using a similar overexpression approach in MCF7 has demonstrated that *ephrin-A1,* encoding the ligand of EphA2, is also a direct target gene of HIC1 (23). The expression of *EphA2* and *ephrin-A1* is mutually exclusive in a panel of 28 breast cancer cell lines, including MCF7 and MDA-MB-231 (45). Notably, *ephrin-A1* expression is restricted to cells that retain epithelial cell markers, whereas *EphA2* is expressed in cells with mesenchymal characteristics; MDA-MB-231 is the best characterized of this latter type (45). Thus, HIC1 is a key regulator of the EphA2 signaling pathway by repressing both the receptor tyrosine kinase and its cell-bound ligand on adjacent cells. *EphA2* was also reported to be regulated by the p53 family of proteins through a p53-response element located at −1678 in its promoter (58). In the same study, the authors showed that *ephrin-A1* is also up-regulated by p53 in a p53-inducible cell line. Therefore, *EphA2* and *ephrin-A1* are both activated by p53 and repressed by HIC1, a property also shared by another target gene, *SIRT1* (14). It would be interesting to determine whether these complex regulatory loops could be generalized to other p53 and HIC1 target genes.

Furthermore, another recent study has shown that EphA2 expression is induced by UV radiation in human melanocytes, keratinocytes, and fibroblasts in a p53-independent, MAPK-regulated manner (59). A regulatory mechanism has been proposed whereby cell adhesion induces EphA2 expression by induction of promoter activity increasing the *EphA2* transcription level (60). Finally, *EphA2* gene transcription is repressed by a variety of stimuli that are often lost in the most advanced stages of aggressive cancers, such as estrogen receptor signaling and c-Myc (61) in addition to the loss of *HIC1* expression.

In addition to these direct transcriptional regulatory events, data comparing EphA2 protein and mRNAs levels in malignant cell models suggest that high levels of EphA2 can arise in tumor cells as a result of increased protein stability (36, 62, 63). Recently, it has been shown that the molecular chaperone heat shock protein 90 (HSP90), also overexpressed in cancers, is implicated in this enhanced stability of EphA2 (64, 65).

In conclusion, we have identified the *EphA2* receptor as an additional *bona fide* HIC1 target gene. Our results demonstrate that HIC1 is a direct repressor of the *EphA2* gene in breast epithelial cells and more generally suggest that in epithelial tumors with loss of HIC1 expression the resulting increase in *EphA2* expression could participate in tumor progression.

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