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 C2H2 zinc fingers motifs (1, 11–13). Via these zinc fingers motifs, HIC1 represses transcription of its target genes as recruitment of corepressors such as CtBP and MTA1 (11, 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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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 CXCRT7 (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 over-expressed 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 gentamicin. The MCF10A human mammary epithelial cells, grown in minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen) and gentamicin. The MCF10A human mammary epithelial cells, grown in minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen) and gentamicin. The MCF10A human mammary epithelial cells, grown in minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen) and gentamicin. The MCF10A human mammary epithelial cells, grown in minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen) and gentamicin. The MCF10A human mammary epithelial cells, grown in minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen) and gentamicin. The MCF10A human mammary epithelial cells, grown in minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen) and gentamicin. The MCF10A human mammary epithelial cells, grown in minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen) and gentamicin. The MCF10A human mammary epithelial cells, grown in minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen) and gentamicin. The MCF10A human 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 BgIII and BamHI restriction sites was cloned in the correct orientation into the BamHI-digested pBabe vector to yield pBabe-FLAG. Then a BamHI-Xhol fragment containing the full-length HIC1 coding sequence in-frame with the FLAG epitope was cloned in the BamHI-Sall 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 Polybren (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).
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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 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’-CGACGACTCAAAAGAGCAGC-AGC-3’ and antisense 5’-CAGGTGTTCACCGAAGCTCTC-3’; ephrinA1 sense 5’-ATCGCACACGTCCTCTG-3’ and antisense 5’-CACGATGTTCACGTGCACAT-3’; and EphA2 sense 5’-TGTGCCAGGCAGGTACG-C3’ and antisense 5’-CTCCAAGCAGGGGTCTC-3’ as well as control primers for 18 S sense 5’-GGGCCCCTCCTAGTGCCT-3’ and antisense 5’-GCTGGGCGCTGGTGGAA-CACCT-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 chromatin 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’-CCCTCGCTTTCTACGAGGAC-3’; EphA2 sense 5’-CAGTGAGAGAGAGATTGAC-3’ and antisense 5’-GCTGGGCGGCTGGTGGAA-CACCT-3’.

Migration and Invasion—For the cell migration assay, 5 × 104 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 Colony.1® software. For the invasion assay, 10 × 104 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 × 105 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 × 105 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.
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. 1A). 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. 1A). Anchorage-independent growth was also evaluated by measuring tumor cell colonization of soft agar (Fig. 1B). 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. 1C). 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. 1D). 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. 1E). Together, these results clearly demonstrate an inhibitory effect of HIC1 on the proliferation, migration, and invasion of tumor cells.

**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).
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. 2A). 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. 2B). 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. 2C). 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.
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) (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. 3A). 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 in mammary epithelial cells.

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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. 3B). 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 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
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. 5A). 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. 5A, 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. 5A). 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. 5B, 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).
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To test EphA2, we performed PCR experiments with the same W138 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 W138 chromatin but not from chromatin immunoprecipitated by the control IgG, demonstrating that EphA2 is a new direct target gene of endogenous HIC1 proteins in W138 cells (Fig. 5B).

Infection of MCF10A cells with a pBabe-FLAG-HIC1 retrovirus results in a significant decrease of EphA2 mRNAs (Fig. 3A). 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. 5C).

Taken together, these results demonstrate that endogenous or ectopically expressed HIC1 proteins are present at the EphA2 promoters in both W138- 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). Chromatin from these infected cells and performed ChIP assays. In close agreement with all our results, HIC1 was found at the EphA2 promoter (Fig. 5C).

**Effects of HIC1 and EphA2 Down-regulation in Normal Mammary Epithelial Cells**

Using transwell assays, it appeared that down-regulation of EphA2 mRNAs (Fig. 7A). 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. 7B). 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. 7A), 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. 8A) favored cell-cell contact (Fig. 8B) and significantly impaired their migration properties (Fig. 8C). These results are in close agreement with the destabilization of adherens junctions observed in MCF10A cells overexpressing EphA2 (34) and with 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.
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
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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