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A potential tumor suppressor role for *Hic1* in breast cancer through transcriptional repression of *ephrin-A1*

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Abstract

The tumor suppressor gene *HYPERMETHYLATED IN CANCER 1 (HIC1)*, which encodes a transcriptional repressor, is epigenetically inactivated in various human cancers. Here, we show that *HIC1* is a direct transcriptional repressor of the gene encoding *ephrin-A1*, a cell surface ligand implicated in the pathogenesis of epithelial cancers. We also show that mouse embryos lacking both *Hic1* alleles manifest developmental defects spatially associated with misexpression of *ephrin-A1*, and that overexpression of *ephrin-A1* is a feature of tumors arising in *Hic1* heterozygous mice in which the remaining wild-type allele is epigenetically silenced. In breast cancer, we find that *ephrin-A1* expression is common *in vivo*, but that in cell culture, expression of the EphA receptors is predominant. Restoration of *HIC1* function in breast cancer cells leads to a reduction in tumor growth *in vivo*, an effect that can be partially rescued by co-overexpression of *ephrin-A1*. Interestingly, overexpression of *ephrin-A1 in vitro* triggers downregulation of EphA2 and EphA4 levels, resulting in an expression pattern similar to that seen *in vivo*. We conclude that *Hic1* spatially restricts *ephrin-A1* expression in development, and that upregulated expression of *ephrin-A1* resulting from epigenetic silencing of *HIC1* in cancer cells may be an important mechanism in epithelial malignancy.

Keywords

Hic1; *Ephrin-A1*; EphA2; EphA4

Introduction

We first identified the tumor suppressor *HYPERMETHYLATED IN CANCER 1 (HIC1)* in a region of chromosome 17p13 frequently targeted for allelic loss and CpG island hypermethylation in human cancer (Wales *et al.*, 1995). *HIC1* resides within the critical deletion region associated with the Miller-Dieker Syndrome (MDS), a congenital disorder characterized by severe facial and neurologic deficits (Chong *et al.*, 1997; Hirotsune *et al.*, 1997). Homozygous deletion of *Hic1* in mice results in embryonic lethality with severe

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craniofacial and somatic defects similar to many of the abnormalities in MDS (Carter *et al.*, 2000), whereas *Hic1* heterozygotes develop age- and gender-dependent tumors associated with promoter hypermethylation and gene silencing of the remaining wild-type allele (Chen *et al.*, 2003). These studies suggest that *Hic1* has important roles in development and tumor suppression. However, the mechanisms behind this assumption have not been fully elucidated.

Hic1 is a sequence-specific transcriptional repressor with an N-terminal POZ domain, and five C2H2 zinc fingers at the C-terminus (Deltour *et al.*, 1998; Deltour *et al.*, 1999; Deltour *et al.*, 2002). Recent studies have demonstrated four transcriptional targets of Hic1, the class III histone deacetylase *Sirt1* (Chen *et al.*, 2005), the proneural bHLH transcription factor *Atoh1* (Briggs *et al.*, 2008), the cell cycle and apoptosis regulator *E2F1* (Jenal *et al.*, 2009), and a G-protein coupled receptor *CXCR7* (van Rechem *et al.*, 2009). Importantly, deregulation of *Sirt1* and *Atoh1* in the absence of Hic1 function is associated with widespread effects in both development and tumor growth (Chen *et al.*, 2005; Briggs *et al.*, 2008). Furthermore, these data suggest that the identification of other genes regulated by Hic1 might better define the functional importance of Hic1 in development and epithelial tumor suppression.

Here, we define a role for HIC1 in the regulation of *ephrin-A1*, which encodes a cell surface ligand for Eph receptors. Eph receptors form the largest subfamily of receptor tyrosine kinases (RTKs). Bidirectional ephrin/Eph signaling occurs in the context of cell-cell interactions and during the establishment of morphogenic boundaries in vertebrate development (Kullander and Klein, 2002; Pasquale, 2005; Pasquale, 2008). Ephrin-A1 is a glycosylphosphatidylinositol (GPI) linked EphA ligand that is frequently overexpressed in epithelial malignancies (Easty *et al.*, 1999; Ogawa *et al.*, 2000; Nakamura *et al.*, 2005; Herath *et al.*, 2006). Recent studies show that overexpression of ephrin-A1 can promote tumor growth *in vivo* (Brantley-Sieders *et al.*, 2006; Shi *et al.*, 2008). Using a combination of cDNA array analysis, *ephrin-A1* expression studies, and characterization of the *ephrin-A1* promoter, we show here that Hic1 is a direct transcriptional repressor of *ephrin-A1*. In *Hic1* deficient mouse embryos, widespread overexpression of ephrin-A1 is seen in epithelial tissues and the central nervous system. In breast cancer cells in which *HIC1* is epigenetically silenced, *in vivo* growth can be blocked by restoration of HIC1 function, and then partially rescued by co-expression of ephrin-A1. Based on our findings, we propose that the misexpression of ephrin-A1 may be important in many of the morphological abnormalities seen in mice lacking *Hic1*, and that epigenetic silencing of *HIC1* in cancer cells may constitute an important step in promoting the growth of breast cancer cells through upregulation of *ephrin-A1 in vivo*.

Results

Hic1 downregulates expression of ephrin-A1

To search for potential transcriptional targets of HIC1 in carcinomas, we used an adenoviral vector AdHIC1 (Chen *et al.*, 2005; Briggs *et al.*, 2008) to overexpress HIC1 protein in MCF-7 cells, a cell line in which *HIC1* expression is epigenetically silenced by promoter hypermethylation (Wales *et al.*, 1995). Western blot analysis of cell lysates with a HIC1 specific antibody revealed sustained, high level protein expression in response to AdHIC1 adenoviral infection over a 10-day period (Fig. 1a). Immunofluorescence analysis of infected cells revealed that overexpressed HIC1 protein was readily detectable in the nucleus (Supplementary Fig. S1). RNA from MCF-7 cells 9 hours after infection with a control virus, or AdHIC1, was analyzed using the Affymetrix platform. Highly significant expression changes of >2 fold were seen in 70 genes, 58 of which were downregulated. Genes repressed by 3 fold or more are shown in Supplementary Table S1. Although a

number of important genes involved in cell growth and differentiation are represented, we pursued *ephrin-A1* as the leading candidate based on its marked 23.4 fold repression, and the potential functional consequences of ephrin-A1 overexpression in both development and cancer.

Repression of *ephrin-A1* steady-state mRNA expression by HIC1 was confirmed in separate experiments by real-time quantitative RT-PCR in MCF-7 and MDA-MB231, both of which are known to have marked hypermethylation of *HIC1* (Wales *et al.*, 1995). As shown in Fig. 1b, re-expression of HIC1 leads to 3.8- and 3.5-fold reduction of *ephrin-A1* mRNA in MCF-7 and MDA-MB231 cells respectively. In cell culture, previous studies show that ephrin-A1 protein was expressed at extremely low levels in both MCF-7 and MDA-MB231 cells (Macrae *et al.*, 2005). To investigate whether HIC1 can repress ephrin-A1 expression at the protein level, we overexpressed HIC1 in two breast cancer cell lines, ZR-75-1 and MDA-MB453, in which *HIC1* is epigenetically silenced whereas ephrin-A1 is robustly expressed (Supplementary Fig. S2). In both cell lines, overexpression of HIC1 leads to marked downregulation of endogenous ephrin-A1 protein within 48 hrs (Fig. 1c). To validate *ephrin-A1* as a Hic1 transcriptional target in a more physiological condition, we examined *ephrin-A1* expression in mouse embryonic fibroblasts (MEFs) derived from *Hic1*^{+/+}, *Hic1*^{+/-}, and *Hic1*^{-/-} mice. Knockout of *Hic1* leads to 1.32- and 4.15-fold increase of *ephrin-A1* mRNA in *Hic1*^{+/-} and *Hic1*^{-/-} MEF cells respectively (Fig. 1d). In addition, we used a siRNA-based strategy to knockdown HIC1 in human WI38 cells as previously described (Chen *et al.*, 2005). We found that the mRNA levels of *ephrin-A1* increased by nearly 3-fold upon HIC1 knockdown, compared to a non-target siRNA control (Fig. 2). Since aging *Hic1*^{+/-} heterozygote mice develop carcinomas, sarcomas and lymphomas in which the remaining wild-type allele is silenced by promoter DNA hypermethylation (Chen *et al.*, 2003), we asked whether loss of Hic1 in these tumors was associated with increased expression of ephrin-A1. Of 8 tumors examined, 6 demonstrated membrane and cytoplasmic expression of ephrin-A1 in the absence of Hic1 expression (Supplementary Fig. S3). By contrast, a hemangiosarcoma (Tumor 8), which was not hypermethylated at the wild-type *Hic1* allele and which expressed Hic1 protein (Chen *et al.*, 2003; Chen *et al.*, 2004; Chen *et al.*, 2005), lacked ephrin-A1 protein expression (Supplementary Fig. S3). Only 1 of the 8 tumors lacked both proteins. These results indicate that ephrin-A1 expression is downregulated by Hic1.

Hic1 is a sequence-specific transcriptional repressor of ephrin-A1

We next analyzed 7.9 kb from transcription start site of *ephrin-A1* gene for consensus DNA binding sites using the TRANSFAC search engine (Wingender *et al.*, 2000). Numerous *trans* activation sequences were identified within 2 kb of the transcription start site, including consensus binding sites for NFkB and basic-helix-loop-helix transcription factors (Fig. 3a). Analysis of the HIC1 consensus sequence (Chen *et al.*, 2005) demonstrated a total of 8 binding sites, all between -4 kb to -8 kb from the transcription start site, and they are listed in Supplementary Table S2 and shown graphically in Fig. 3a. No potential HIC1 binding sites were identified within the first 4.0 kb upstream of the transcription start site. Functional correlates of these findings were investigated using luciferase reporter assays. Four human *ephrin-A1* promoter constructs (Fig. 3a) were created by subcloning the appropriate fragments into the pGL3-basic vector. Basal activity of these constructs, assessed by transient transfection in MCF-7 cells, showed that the *ephrin-A1* promoter is highly active, with a 2-fold increase in activity observed with the inclusion of sequence containing the putative NFkB binding sites (Fig. 3a).

Transient co-transfection experiments using an expression vector encoding wild-type HIC1 (pRcHIC1), and the ephrin-A1 reporter construct pEFNA1P8.3, showed that transfection of increasing amounts of pRcHIC1 in ng quantities resulted in repression of reporter gene

activity (Fig. 3b). Interestingly, this effect is lost with increases in the amount of HIC1 expression vector above 8ng/well. This result suggests quenching of the repressive effect above this dose. We next compared the effects of wild-type HIC1 with mutant expression vectors lacking the N-terminal POZ domain (pRcDPOZ) or the C-terminal zinc fingers (pRcDZFM). As shown in Fig. 3c, both mutant vectors failed to repress luciferase activity. These data suggest that both the C-terminal DNA binding domain and the N-terminal POZ domain are required for the biological function of HIC1 in transcriptional repression of *ephrin-A1*. In order to confirm that the HIC1 binding sites identified in the *ephrin-A1* promoter were necessary for repression in this assay, we compared the ability of exogenous HIC1 to repress luciferase activity in the four promoter constructs depicted in Fig. 3a. Our results demonstrate that the 8 HIC1 binding sites are required for full repression, and that the two reporter constructs that do not contain HIC1 binding sites do not respond to exogenous HIC1 (Fig. 3d).

To establish that endogenous Hic1 protein is present at the *ephrin-A1* promoter, we performed chromatin immunoprecipitation (ChIP) assays in NIH3T3 cells, which robustly express Hic1 protein that can be easily immunoprecipitated (Chen *et al.*, 2005; Briggs *et al.*, 2008). Importantly, there is high similarity between the human and mouse *ephrin-A1* promoters for positions of the Hic1 binding sites as shown in Fig. 3a. The mouse promoter has 9 sites from -4.0 kb to -8.0 kb and no sites within -2.0 kb from the transcription start site (Supplementary Table S2). Primers spanning 200–300bp regions of the mouse *ephrin-A1* promoter (Supplementary Table S3) were optimized for PCR using NIH3T3 genomic DNA (data not shown). PCR amplification at the predicted size occurred in several regions of the mouse *ephrin-A1* promoter containing predicted Hic1 binding sites (primers C, D, E, F, G) (Fig. 4), demonstrating sequence specific occupancy of the mouse *ephrin-A1* promoter by endogenous Hic1 protein. Thus, the functional Hic1 binding sites identified in the mouse promoter are located approximately between -4 kb and -8 kb upstream of the transcription start site, which is consistent with the location of HIC1 binding sites in the human *ephrin-A1* promoter identified by the promoter deletion analysis. Taken together, all of the above data in this section show that Hic1 is a direct sequence specific transcriptional repressor of *ephrin-A1*.

Misexpression of ephrin-A1 in Hic1 deficient mouse embryos

Having identified and verified *ephrin-A1* as a novel direct target for transcriptional repression by the tumor suppressor Hic1, we next examined *in vivo* correlates between Hic1 and ephrin-A1 in our *Hic1* knockout mouse model. Homozygous embryos show a variable phenotype, ranging from severe craniofacial, neural and somatic disorders to minimal macroscopic defects (Carter *et al.*, 2000). We analyzed *Hic1* knockout mice at E14.5, and compared them with wild-type littermates. In these embryos, we observed an overall increase in ephrin-A1 staining in *Hic1* knockout embryos. An example of this overall increase in staining can be seen in mid gestation embryos throughout the central nervous system (CNS) (Fig. 5a). Some increased ephrin-A1 staining sites in the *Hic1* knockout embryo, such as forebrain, midbrain, and skin, are correlated very well with the localization of Hic1 staining in a *Hic1*^{+/-} embryo (Supplementary Fig. S4). In the developing parietal cortex of a *Hic1*^{-/-} embryo (Fig. 5b), abnormal expression of ephrin-A1 was observed in the cortical plate cells staining positive for Tuj1, a marker of early cortical neurons. As can be seen by direct comparison, this domain of increased ephrin-A1 expression correlates with the region of normal Hic1 expression in wild-type embryos (Fig. 5b). In the E17.5 embryonic liver, we also observed an increase in the intensity of ephrin-A1 expression in *Hic1*^{+/-} and *Hic1*^{-/-} embryos (Fig. 5c). Interestingly, *Hic1*^{+/-} embryos manifest an intermediate increase when compared with *Hic1*^{+/+} and *Hic1*^{-/-} littermates. In summary,

these data show that ephrin-A1 expression domains in mouse development are markedly expanded in mice lacking *Hic1*.

HIC1 inhibits tumorigenicity of breast Cancer Cells partly through downregulation of ephrin-A1

Transcriptional regulation of *ephrin-A1* has direct implications for *HIC1* as a tumor suppressor. In cell culture, Western blot analysis of lysates from MCF-7 and MDA-MB231 cells showed that ephrin-A1 protein was expressed at extremely low levels, whereas EphA2 and EphA4 proteins were readily detectable (Fig. 6a). Strikingly, when both cell lines are grown as xenografts in nude mice, we observed a marked decrease in EphA2 and EphA4 expression with a concomitant upregulation of ephrin-A1 expression (Fig. 6a). Expression of EphA2 and EphA4 was not influenced by adenoviral infection or overexpression of HIC1 in cell culture (data not shown).

In initial studies, we did not observe any changes in breast cancer cell growth or viability in response to HIC1 overexpression *in vitro* (Supplementary Fig. S5), suggesting that an appropriate tumor-stromal/endothelial cell interaction may be required for the tumor suppressor function of HIC1. In fact, in breast cancer, ephrin expression by epithelial cells is associated with reciprocal Eph receptor expression in adjacent vascular endothelial cells (Ogawa *et al.*, 2000; Brantley-Sieders *et al.*, 2006). We hypothesized that this tumor-microenvironment signaling might be affected by HIC1 expression leading to downregulation of ephrin-A1 in cancer cells and suppression of tumor growth. Most studies assessing the effects of ephrin-A1 overexpression have employed exogenous ephrin-A1-Fc. In contrast, we generated a recombinant adenovirus expressing human ephrin-A1 (AdEphrin-A1) (Supplementary Fig. S6), which allowed us to express ephrin-A1 in breast cancer cells. MDA-MB231 cells infected with AdEphrin-A1 virus showed robust expression of ephrin-A1, which led to 8.4- and 9.3-fold reduction of EphA2 proteins at 24 or 48 hrs post viral treatment respectively. By contrast, overexpression of ephrin-A1 alone resulted in 3.2- and 3.9-fold decrease in the EphA4 protein level for the same time points (Fig. 6b). Cells infected with AdHic1, Ad β -gal or AdEphrin-A1 were then injected subcutaneously into nude mice. After 5 weeks, the tumor volume analysis revealed a marked decrease in engraftment efficiency induced by overexpression of HIC1 ($p < 0.05$), and a modest increase in tumor growth in response to ephrin-A1 overexpression ($p < 0.05$; Fig. 6c). To test the contribution of ephrin-A1 downregulation to the tumor suppressor effect of HIC1 overexpression, we then co-infected cells with equal amounts of AdHIC1/Ad β -gal or AdHIC1/AdEphrin-A1. As shown in Fig. 6d, co-overexpression of ephrin-A1 partially restores the ability of MDA-MB231 cells to engraft nude mice, consistent with a positive role for ephrin-A1 as an important protein in engraftment and growth of breast cancer (Ogawa *et al.*, 2000), and with observations demonstrating that ephrin-A1 siRNA can block angiogenesis and metastasis in an *in vivo* model of breast cancer (Brantley-Sieders *et al.*, 2006). Together, these data support the notion that ephrin-A1 can be overexpressed in breast cancer *in vivo* when there is a loss of the transcriptional repression of HIC1 on the *ephrin-A1* promoter.

Discussion

Using animal models, we have previously demonstrated that the transcriptional repressor *Hic1* is not only a critical developmental regulator but also an epigenetically regulated tumor suppressor (Carter *et al.*, 2000; Chen *et al.*, 2003; Chen *et al.*, 2004; Chen *et al.*, 2005; Briggs *et al.*, 2008). In this study, we identified and characterized *ephrin-A1* as another transcriptional target of *Hic1*. This finding has two important implications. First, we have previously shown that homozygous deletion of *Hic1* in mice results in embryonic lethality with severe craniofacial and somatic defects. Since the molecular mechanism underlying the

developmental disorders has not been fully characterized, identification of *ephrin-A1* as a new direct transcriptional target of *Hic1* provides another explanation for the functional importance of *Hic1* in development. Second, cancer has been traditionally viewed as a genetic disease that is driven by the sequential accumulation of mutations, resulting in the loss of function of tumor suppressor genes or the constitutive activation of proto-oncogenes (Kinzler and Vogelstein, 1996; Hanahan and Weinberg, 2000). It is now apparent, however, that epigenetic gene silencing and associated promoter CpG island DNA hypermethylation constitute an alternative mechanism to mutations that inactivate tumor suppressor genes (Jones and Baylin, 2007; Esteller, 2008). Given the high frequency of epigenetic silencing of *HIC1* in most common human tumor types, and the high incidence of malignancies developed in *Hic1* heterozygous mice, our data presented here reveals a potentially crucial role for *HIC1* as tumor suppressor through the transcriptional regulation of ephrin-A1 signaling.

Ephrins and their receptors play a critical role in tissue patterning, morphogenesis, angiogenesis, and neural development (Poliakov *et al.*, 2004; Pasquale, 2005; Arvanitis and Davy, 2008). Our data show that ephrin-A1 is misexpressed in *Hic1* deficient mouse embryos, especially in the CNS, where abnormalities are often severe in this knockout model (Carter *et al.*, 2000). In light of the normally restricted expression pattern of ephrin-A1, the misexpression of this ligand seen in *Hic1* knockout embryos is a striking finding, and suggests that *Hic1* plays an important role in coordinating development through transcriptional regulation of *ephrin-A1*. In addition, the broad specificity of ephrin-A1, which can bind to a wide range of EphA receptors, suggests that its misexpression could lead directly to the dramatic abnormalities in development in *Hic1* deficient embryos through aberrant ephrin-A1 signaling.

Perhaps the most important direct implication of our current data regards the role for *HIC1* as a tumor suppressor gene, and a potential functional link to overexpression of ephrin-A1 in epithelial malignancies such as breast cancer. In fact, previous studies have shown that *HIC1* is frequently hypermethylated and transcriptionally inactivated in breast cancer cell lines and primary tumors (Wales *et al.*, 1995; Fujii *et al.*, 1998). Meanwhile, overexpression of both ephrins and Eph receptors has been observed in a variety of human malignancies (Easty *et al.*, 1999; Tang *et al.*, 1999; Takai *et al.*, 2001) including breast cancer (Ogawa *et al.*, 2000). Decreased protein expression of ephrin-A1 by an expression vector-based antisense RNA inhibited 3-dimensional growth of HT29 colon adenocarcinoma cells (Potla *et al.*, 2002). Importantly, a positive role for epithelial-specific ephrin-A1 in tumor initiation *in vivo* has also been demonstrated in the *Apc^{min}* mouse model, in which overexpression of ephrin-A1 in enterocytes markedly enhanced the number of invasive tumors in both intestine and colon (Shi *et al.*, 2008). Furthermore, disruption of the ephrin-A1 receptor, EphA2, leads to increased susceptibility to chemically induced carcinogenesis in mouse skin, suggesting that EphA2 has tumor suppressor functions (Guo *et al.*, 2006). Together, these data suggest that ephrin-A1 exerts a positive role on tumorigenesis and ephrin-A1 receptor, EphA2, acts as a negative regulator of tumorigenesis. Our studies of breast cancer cells reveal a critical role of *HIC1* in the regulation of tumorigenesis, at least partly through transcriptional repression of ephrin-A1. In a xenograft model, we demonstrate that restoration of *HIC1* function in breast cancer cells leads to a significant reduction in tumor growth in nude mice, and importantly, co-overexpression of ephrin-A1 with *HIC1* partially restores the ability of breast cancer cells to engraft nude mice, supporting a positive role of ephrin-A1 in promoting malignant cell growth *in vivo*. In contrast to these above data, other studies in breast cancer and prostate cancer cell lines have shown that EphA2 expression is a prominent feature in many of these systems, and that activation of EphA2 by an ephrin-A1-Fc fusion protein results in inhibition of the oncogenic Ras/MAPK signaling in several different cell types (Miao *et al.*, 2001; Macrae *et al.*, 2005; Miao *et al.*, 2009).

Three important issues might explain these contradictory findings. First, data supporting a positive role for ephrin-A1, including data presented here, have been performed *in vivo*, suggesting that cell lines in tissue culture may not represent the potential for non-cell autonomous signaling mediated by ephrin-A1 expressed on tumor cells. It has been well established that vascular recruitment by tumors is a critical step in tumorigenesis. A growing body of evidence demonstrates that upregulation of ephrin-A1 in breast cancer cells has been directly implicated in tumor angiogenesis, neovascularization and metastasis through cancer-specific expression of ephrin-A1, and reciprocal expression of EphA receptors in tumor stromal and endothelial cells (Brantley-Sieders *et al.*, 2004; Brantley-Sieders *et al.*, 2006). For example, endothelial cells isolated from EphA2-deficient mice displayed defective ephrin-A1 induced migration *in vitro* and *in vivo*, and EphA2-deficient mice showed a diminished angiogenic response to ephrin-A1 *in vivo* (Brantley-Sieders *et al.*, 2004). Furthermore, siRNA-mediated ephrin-A1 knockdown in metastatic mammary cancer cells leads to decreased lung metastasis *in vivo* (Brantley-Sieders *et al.*, 2006). Interestingly, a recent study showed that a functional and soluble form of ephrin-A1 is released from ectopic ephrin-A1-expressing glioblastoma multiforme (GBM) cells and endogenous ephrin-A1 expressing breast cancer cells. The soluble ephrin-A1 may interact with EphA2 receptors on endothelial cells, and therefore provide a paracrine role for ephrin-A1 in stimulating angiogenesis (Wykosky *et al.*, 2008). Here, we clearly show that *in vivo*, the ratio of ephrin-A1 to EphA2/4 expression is dramatically altered in favor of ephrin-A1 expression, supporting the notion that tumor-expressed ephrin-A1 interacts with EphA2 on endothelial cell to promote tumorigenesis via angiogenesis. Second, when ephrin-A1 is overexpressed by tumor cells either endogenously or ectopically, these cells exhibit downregulation of EphA2 (Macrae *et al.*, 2005; Wykosky *et al.*, 2008; Miao *et al.*, 2009). We have shown that overexpression of ephrin-A1 results in reduction of EphA2 and EphA4 expression in breast cancer cells, thus reducing the capacity for these receptors to signal. Downregulation of EphA2/4 in response to ephrin-A1 overexpression in tumor cells could provide a mechanism by which the tumor cells can circumvent quenching the released ephrin-A1 before it has a chance to exert a paracrine function on the endothelial cells within the tumor environment (Wykosky and DeBinski, 2008). Finally, ephrin-A1 can bind to all Eph receptors of the A subclass, and the receptor EphA2 binds ephrin-A1 with the highest affinity (Holder and Klein, 1999). Therefore, the biological function of ephrin-A1 signaling during tumorigenesis is not limited to inhibition of Ras/MAPK signaling. For example, ephrin-A1-specific siRNA treatment can increase p21 expression in a hepatocellular carcinoma cell line (Iida *et al.*, 2005), suggesting that the balance between ephrin-A1 and EphA expression may have cell-type specific effects.

In summary, our studies support the concept that ephrin-A1 plays a positive role in tumor growth *in vivo*, and are consistent with previous reports of ephrin-A1 acting through EphA receptors in the tumor microenvironment *in vivo*. Our data suggest a mechanism to explain how loss of Hic1 function may be a potentially important event in carcinogenesis through the activation of aberrant tumor-endothelial cell interactions mediated by ephrin-Eph signaling.

Materials and methods

Antibodies

Antibodies for immunofluorescence: ephrin-A1 (Santa Cruz, sc-911) 1:1000; Tuj1 (Covance, MMS-435P) 1:100. For immunohistochemistry: HIC1 (Rabbit polyclonal) 1: 100 as previously described (12, 13); ephrin-A1 (Santa Cruz, sc-911) 1:200. For Western blot: HIC1 1:100; ephrin-A1 (Santa Cruz, sc-20719) 1:200; EphA2 (Santa Cruz, sc-924) 1:2000; EphA4 (Upstate, 07-309) 1: 1000; and GAPDH (Upstate, MAB374) 1: 10,000.

cDNA Array Analysis

Adenoviruses were prepared as described (He *et al.*, 1998). Total RNA was extracted from MCF-7 cells infected with either wild-type AdHIC1 or mutant HIC1 lacking POZ domain (Ad Δ POZ) as a control. The transduction efficiency in MCF-7 cells was 75–80% as determined by GFP expression. Analysis of gene expression was performed according to standard protocols on an Affymetrix HGU95A chip.

RT-PCR

All RNA samples were quality controlled using a Biorad Experion Bioanalyzer, then subjected to DNase I treatment prior to reverse transcription. Real time quantitative PCR studies were performed using QuantiTect SYBR Green Kit (QIAGEN). All quantitative calculations were performed using the $\Delta\Delta_{C-t}$ method. Primer sequences are listed in Supplementary Table S4.

Luciferase Reporter Assays

Reporter gene assays were performed as previously described (Zhang *et al.*, 2008). We measured luciferase activity in a luminometer (BD Biosciences) and transfection efficiency was normalized using the paired Renilla luciferase activity by using the Dual Luciferase Reporter Assay system (Promega) according to the manufacturer's instruction. Further experimental details can be found in Supplementary Information (SI) Methods.

Chromatin Immunoprecipitation

This was performed essentially as described (Briggs *et al.*, 2008) using a rabbit polyclonal antibody to Hic1 to detect the presence of endogenous Hic1 at the mouse *ephrin-A1* promoter. Details are provided in SI Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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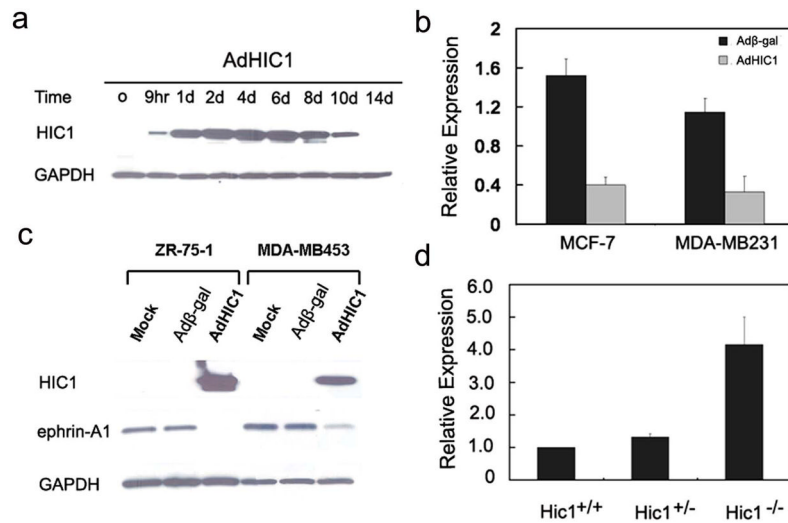


Fig. 1. Effects of HIC1 overexpression on expression of *ephrin-A1*. (a) Western blot analysis of MCF-7 cell lysates treated with AdHIC1 for 0–14 days. The filter was probed with HIC1 antibody. GAPDH was used as loading control. (b) Quantitative real time RT-PCR assays for the *ephrin-A1*/ β -actin mRNA ratio from cell lines treated with control (Ad β -Gal) or HIC1 expressing (AdHIC1) adenoviruses. Total RNA was prepared at 9 hrs post viral infection, followed by cDNA synthesis and expression analysis. The graph shows mean values and standard deviations of three independent experiments. (c) Western blot analysis of HIC1 and ephrin-A1 in lysates obtained from ZR-75-1 and MDA-MB453 cells infected with Ad β -gal or AdHIC1 adenoviruses. Equal loading was confirmed by immunoblotting for GAPDH. (d) Quantitative real time RT-PCR assays for the *ephrin-A1*/ β -actin mRNA ratio from *Hic1* wild-type and knockout MEF cells.

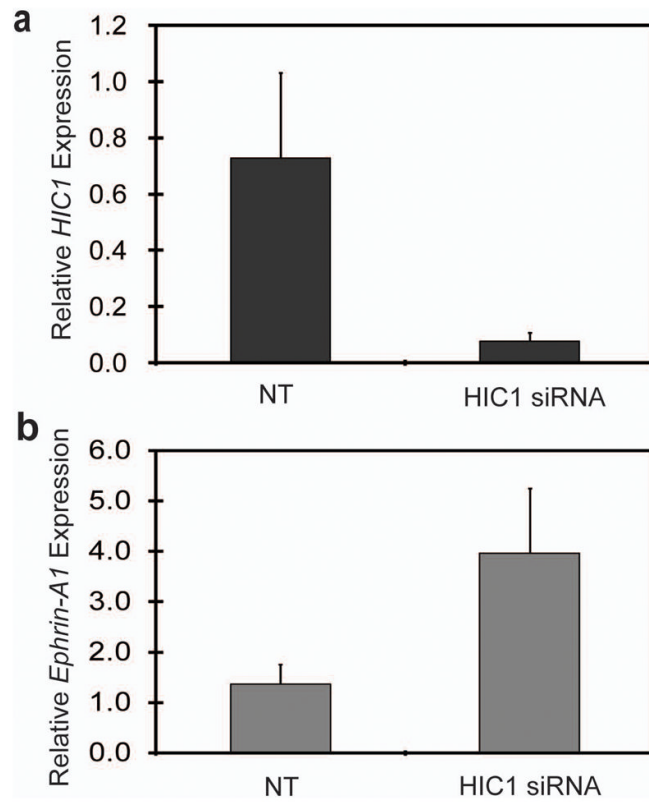
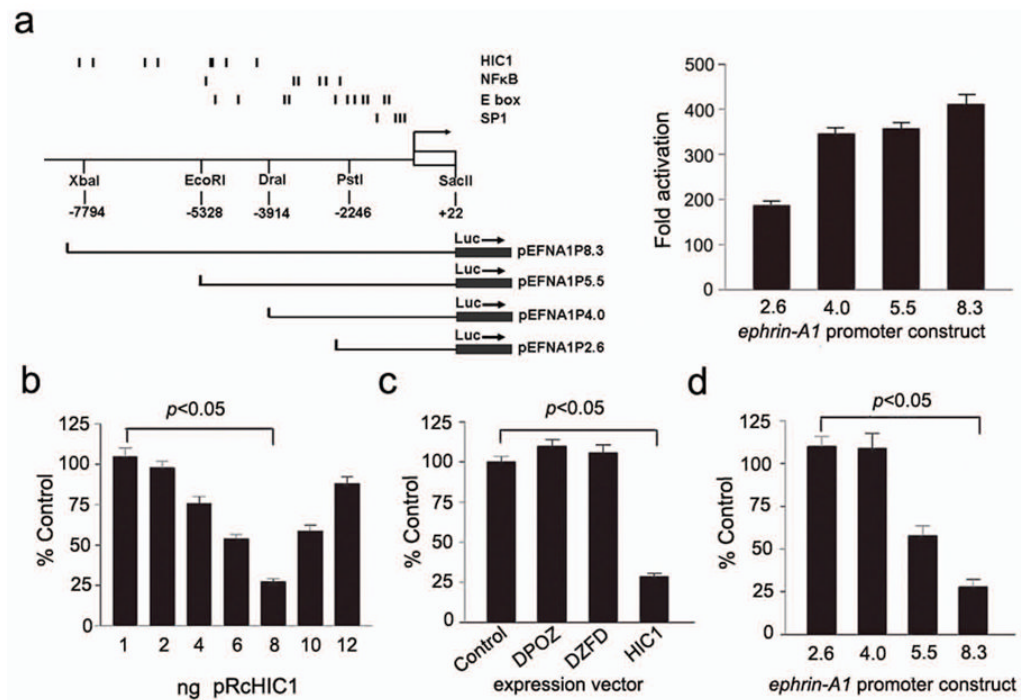


Fig. 2. Knockdown of endogenous HIC1 in the WI38 human fibroblast cells up-regulates *ephrin-A1* expression. WI38 cells were either mock-transfected, transfected with *HIC1* siRNA or transfected with non-target siRNA control (NT) as previously described. Total RNA was extracted and expression levels of *HIC1* (a) and *ephrin-A1* (b) mRNA were assessed by real-time quantitative RT-PCR. Values were normalized to human β -actin.

**Fig. 3.**

HIC1 is a direct transcriptional repressor of the *ephrin-A1* promoter. (a) *Left*, map of the *ephrin-A1* promoter region, with the positions of selected consensus binding sites indicated above. The length of the different promoter constructs used in reporter assays is shown below. *Right*, reporter activity in MCF-7 cells, transiently transfected with the four pEFNA1P promoter constructs as listed in left panel. Activity of each promoter construct is shown as fold activation compared with a pGL3-basic empty vector control. (b) Reporter activity in MCF-7 cells transiently co-transfected with the pEFNA1P8.3 reporter and increasing amount of the pRcHIC1 expression vector. The results are normalized to those for empty control vectors and are expressed as a relative ratio of firefly luciferase to Renilla luciferase. (c) Reporter activity in MCF-7 cells transiently co-transfected with the pEFNA1P8.3 reporter and 8ng of each of the expression constructs: pCDNA3.1 (control), a HIC1 mutant lacking the POZ domain pRcΔPOZ (DPOZ), a mutant lacking the zinc finger domain pRcΔZFM (DZFD), and pRcHIC1 (HIC1). (d) Reporter activity in MCF-7 cells transiently co-transfected with 8ng of pRcHIC1, and each of the four pEFNA1P reporter constructs listed in Fig. 3a. Results are shown as mean, SEM, N=6.

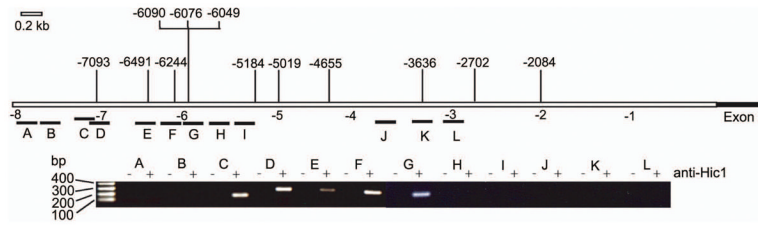


Fig. 4. ChIP analysis of Hic1 at the mouse *ephrin-A1* promoter in NIH3T3 cells. The mouse *ephrin-A1* promoter is depicted with the predicted Hic1 binding sites shown above, and the distance in kb from the start of exon 1 is shown below. The area of the promoter covered by each primer pair (A-L) is shown below this scale. Amplification products for each primer pair, with (+) and without (-) anti-Hic1 antibody in the immunoprecipitation are shown in the lower panel.

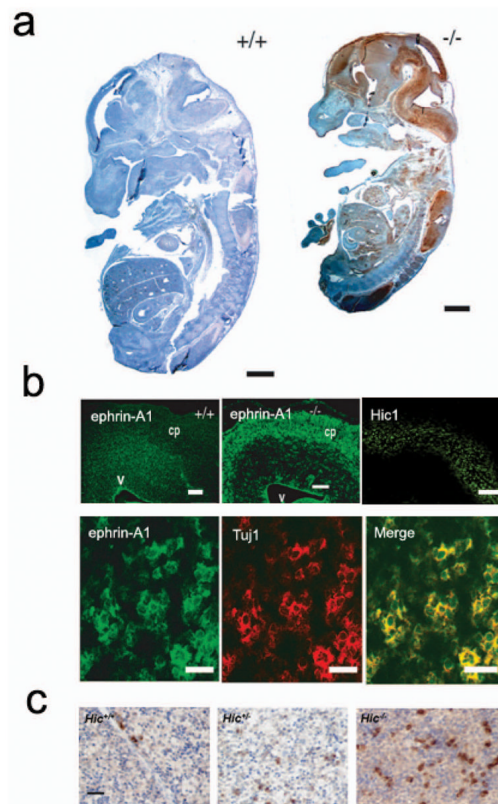


Fig. 5. Abnormal expression of ephrin-A1 in *Hic1* deficient mice. (a) Sagittal sections of E14.5 *Hic1* wild-type (+/+) and knockout (-/-) embryos stained for ephrin-A1 by immunoperoxidase, detected by DAB and counterstained with hematoxylin. Scale bar = 500µm. (b) Dual label confocal immunofluorescence analysis of ephrin-A1 expression in axial sections of the developing telencephalon from E14.5 embryos. cp = cortical plate, v = ventricle. *Upper panel*, ephrin-A1 is abnormally expressed in the cortical plate of a *Hic1*^{-/-} embryo. Immunolocalization of Hic1 (Green) in cortical plate (cp) neurons in an E14.5 *Hic1*^{+/+} embryo. Scale bar = 200µm. *Lower panel*, cortical plate neurons from a *Hic1*^{-/-} embryo stained with ephrin-A1 (green) and Tuj1 (red). Scale bar = 20µm. (c) Immunostaining of ephrin-A1 expression in E17.5 livers taken from *Hic1* wild-type (+/+), heterozygous (+/-), and null (-/-) mouse embryos. Scale bar = 50µm.

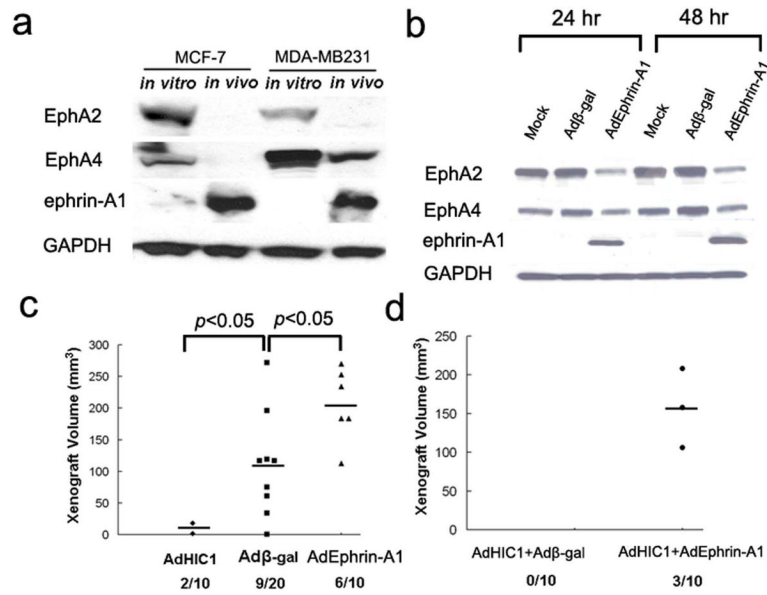


Fig. 6. HIC1 inhibits tumorigenic behavior of human breast cancer cells *in vivo*. (a) Western blot analysis on ephrin-A1, EphA2 and EphA4 expression in lysates from MCF-7 and MDA-MB231 cells grown *in vitro* or *in vivo*. (b) Western blot analysis of ephrin-A1, EphA2 and EphA4 in lysates obtained from MDA-MB231 cells infected with Adβ-gal or AdEphrin-A1 adenoviruses. Equal loading was confirmed by immunoblotting for GAPDH. (c) Tumor volume of flank xenografts in athymic nude mice injected with MDA-MB231 cells infected with AdHIC1, Adβ-gal or AdEphrin-A1 adenoviruses. The black bars represent the mean values of tumor volume. Numbers below the x-axis indicate the number of mice that developed tumors. (d) Tumor volume of flank xenografts in athymic nude mice injected with MDA-MB231 cells infected with a combination of with AdHIC1 and Adβ-gal, or AdHIC1 and AdEphrin-A1 adenoviruses. The mean values of tumor volume and the number of mice that developed tumors are also indicated as in C.