Ehd3, a regulator of vesicular trafficking, is silenced in gliomas and functions as a tumor suppressor by controlling cell cycle arrest and apoptosis

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Ehd3 [Eps15 homology (EH) domain-containing protein 3] is a protein that resides in tubular and vesicular membrane structures and participates in endocytic recycling, although all its functions are unknown. Since Ehd3 is most abundantly expressed in brain tissues, we examined its role in brain cancer progression. Using immunohistochemistry, we report loss of EHD3 expression in gliomas, including low-grade astrocytomas, suggesting that this is an early event in gliomagenesis. EHD3 expression is also very low in most of glioma cell lines tested. In two cell lines, a bisulfite sequencing method identifies promoter hypermethylation as a mechanism of Ehd3 silencing, and its expression was restored by the demethylating agent 5-Aza-2′-deoxycytidine. Doxycycline-inducible restoration of EHD3 expression to glioma cells decreases their growth and invasiveness and induces cell cycle arrest and apoptosis. Furthermore, shRNA-mediated Ehd3 silencing increases cell growth. Using a xenograft model, we demonstrate Ehd3 growth inhibitory functions in glioma cells in vivo. We suggest that Ehd3 functions as a tumor suppressor gene and loss of its expression is a very common event in gliomas. This is the first study to highlight the importance of a member of the C-terminal EHD proteins in cancer and to link their functions to the cell cycle and apoptosis.

Introduction

Brain tumors represent the second most common cancer and the most common solid pediatric tumor. Among these, gliomas are the most frequent (86%) (1). There are four groups of gliomas with glioblastomas (also known as grade IV astrocytoma or glioblastoma multiforme) being the highest and most prevalent grade. Glioblastoma multiforme is a poorly differentiated astrocytic tumor of high-grade heterogeneity, extremely invasive and resistant to current therapeutics and showing complex signaling interactions (2). Despite the use of multimodality therapies, glioblastoma multiforme patients rarely survive long term.

Endocytosis and vesicular trafficking play an important role in the spatiotemporal integration of signaling pathways, a topic which was the subject of many excellent recent reviews (3–7). Therefore, it is not surprising that these essential biological processes are involved in cancer progression (8–10). The C-terminal Eps15 homology (EH) domain (EHD) is a relatively newly identified highly conserved family of proteins involved in endocytic trafficking. The EH domain is a motif of ~100 residues, which is typically found at the N-terminus of many proteins. However, in mammals, the EHD family of proteins has the EH domain at the C-terminus. This family of four paralogs (EHD1–EHD4) has been implicated in receptor intracellular trafficking, particularly in internalization and recycling to the plasma membrane (11,12). The so far restricted list of functions of EHD proteins is just starting to be populated. In particular, Eps15 homology domain-containing protein 3 (EHD3), which was shown to be involved in early endosome-to-recycling-endosome transport (13), has recently been assigned an additional role in the regulation of endosome-to-Golgi transport (14). Whether EHD3 regulates other functions and signaling pathways is not known. To the best of our knowledge, the role of EHD3 or any of the EHD family members in cancer has never been investigated so far.

In this study, we provide evidence that Ehd3 is a new glioma tumor suppressor gene. Immunohistochemistry (IHC) shows that EHD3 expression is lost in most of the cancer clinical specimens. We identified promoter hypermethylation and expression status of Ehd3 in a series of glioma cancer cell lines. Using a set of in vitro and in vivo experimental assays, we found that EHD3 displays tumor growth inhibitory effects through induction of cell cycle growth arrest and apoptosis and inhibits cell invasion.

Materials and methods

Data mining

The gene portal BioGPS (http://biogps.org/#goto=welcome) (15) was used to analyze the expression of Ehd family members in various organ tissues. The Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo) database (16) was mined for Ehd3 expression in brain cancers. The largest set of data (accession: GSE4290) contains 23 samples from epilepsy patients, considered as ‘normal’ non-tumor specimens, and 157 glioma samples (7 grade II astrocytomas, 19 grade III astrocytomas, 81 grade VI glioblastomas, 38 grade II oligodendrogliomas and 12 grade III oligodendrogliomas) analyzed on Affymetrix U133 Plus 2.0 microarrays. We reviewed Oncomine (https://www.oncomine.org/resource/login.html) (7,17) for independent human cancer microarray datasets comparing different normal tissues on one hand and brain normal versus cancer tumors on the other hand.

Data for the Kaplan–Meier survival plot were obtained from the Repository of Molecular Brain Neoplasia data (REMBRANDT) website (https://caintractor.nci.nih.gov/rembrandt/home.do) accessed on 28 September 2013. Only one sample showed upregulated expression and was not included in the analysis.

Glioma cell lines

The glioma cell lines U251 and U87MG were originally obtained from the American Type Culture Collection (ATCC). The SF126, SF188 and SF539 cell lines were originally from the Brain Tumor Research Center of San Francisco. It should be noted that according to the ATCC and upon sequencing, the authenticity of U-251 cells has been questioned and this cell line has been suggested to be similar to U-373MG cells. However, since U-373MG cells are also glioblastomas, the results of this work are not affected in any way by the controversy. The GM2493, GM133 and GM1600 cell lines were a kind gift from Dr Paul S. Mischel (School of Medicine at UCLA (University of California, Los Angeles), Los Angeles, CA). All cells were maintained in Dulbecco’s modified Eagle’s medium with 1-glutamine (Life Technologies, Invitrogen Corp.), 10% fetal bovine serum (Sigma–Aldrich) and 1% penicillin–streptomycin (Life Technologies, Invitrogen) and grown at 37°C.

Real-time reverse transcription–polymerase chain reaction

Total RNA was isolated from glioma cell lines using Trizol (Life Technologies, Invitrogen) or the RNAqueous–4 PCR Kit (Ambion) according to the manufacturers’ instructions. The TaqMan universal PCR master mix and predesigned

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TaqMan Gene Expression probe and primer sets (Hs00205916_m1 for *Ehd3* and 4326317E for *GAPDH*) were purchased from Applied Biosystems. Amplification data measured as an increase in reporter fluorescence were collected in real time with the Applied Biosystems 7500 Fast Real Time PCR System. The mRNA expression level of *Ehd3* was calculated by the comparative threshold cycle (CT) method. GAPDH was used as endogenous control.

Treatment with 5 μM 5-Azacytidine (Sigma–Aldrich) was carried out for 5 days. For the SYBR green protocol, we used the iTaq Fast SYBR Green Supermix with ROX (Bio-Rad) as directed by the manufacturer. The list and sequences of the primers designed by the authors are detailed in Table I. The primers not on this list were directly obtained from realtimeprimers.com. All experiments were carried out at least in triplicates.

### Transfection and colony formation assay

Colony formation assays were done in monolayer cultures. U251 cells were plated at 10^4 per well into six-well plates and transfected with the *Ehd3* cDNA plasmid kindly provided by Dr Steve Caplan (University of Nebraska) or the pcDNA3.0 control vector, using the lipofectamine LTX reagent (Invitrogen) following the manufacturer’s instructions. Twenty-four hours after the transfection, cells were harvested and plated onto 100 mm tissue culture dishes and put under selection with G418 (500 μg/ml), changing the medium every 3 days. After 3 weeks, colonies were stained with either the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent (Sigma) or with 0.1% trypan blue in 50% ethanol and were photographed. Similarly, for colony formation assays of doxycycline (DOX)-induced cells, 1–5000 cells were plated into 6-well tissue culture plates, incubated for 7–10 days, changing the medium and keeping under DOX treatment. Visible colonies were counted in three different field views and at least two independent experiments were performed.

### Map of *Ehd3* promoter CpG islands and bisulfite sequencing analysis

Bisulfite sequencing analysis was performed using the EZDNA Methylation Kit (Zymo Research, Orange, CA). First, genomic DNA was extracted from cell lines. Briefly, 1 mg of genomic DNA was denatured by NaOH and modified by sodium bisulfite, which converts unmethylated cytosines to uracils while leaving intact methylated cytosines (18). The modified DNA was purified using a Wizard DNA cleanup system (Promega, Madison, WI). Bisulfite modification of DNA was performed using the EZDNA Methylation Kit following the manufacturer’s instructions. Using the MethPrimer program (http://www.urogene.org/methprimer/index1.html), we identified three potential methylated regions in the *Ehd3* promoter region (NCBI Reference Sequence: NT_022184.15). The locations of the three CpG islands are shown in Figure 2c. The CpG1 region was amplified and PCR amplicons were cloned into pGEM-T vector (Promega) and sequenced using the following pair of primers: 5'-TTGGTTTTAGGAGTAGGGAG-3' and 5'-TCTTCCAAAAAACAAACAACCTAC-3'. To determine the methylation status of the *Ehd3* promoter, at least five individual clones from each sample were sequenced using forward and reverse M13 primers.

### Antibody preparation

A rabbit polyclonal antibody was generated against the sequence DFPNLKRMQDLQADDFS from the human EHD3 sequence (GenBank: AAF40471.1). The serum was affinity purified and titrated by ELISA before testing in different applications (Supplementary Figure 1, available at Carcinogenesis Online).

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**Table I. Sequences of the primers used in this study**

<table>
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<th>Name</th>
<th>Sequence</th>
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<td><em>Ehd3</em>-Forw</td>
<td>5'-ACGAATGACCCACCCAGTTA-3'</td>
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<tr>
<td><em>Ehd3</em>-Rev</td>
<td>5'-GGTGGAGGCTGTCGTTGTC-3'</td>
</tr>
<tr>
<td><em>Cdki</em>-Forw</td>
<td>5'-GCTGCGGTCAGCTGTTACTCA-3'</td>
</tr>
<tr>
<td><em>Cdki</em>-Rev</td>
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<tr>
<td><em>Cdki</em>-Rev</td>
<td>5'-GCGAGACGTGGTTCCTCCAC-3'</td>
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</tr>
<tr>
<td>β-Actin-Rev</td>
<td>5'-AGCATGTTGGCGTACG-3'</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Representative images of IHC staining of EHD3 in normal brain and samples of different grades of gliomas in the TMA, using a custom-made rabbit antibody. Shown are EHD3-positive (a) and EHD3-negative (b) non-tumor samples and negative grade I (c), grade II (d), grade III (e) and grade IV (f) gliomas. The proportion of EHD3-negative versus EHD3-positive specimens is shown as stacked columns (g). Grades I and II are combined. n = number of samples per category.
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vanadate) containing 1× protease inhibitor cocktail. Then, the cell lysates were solubilized by sonication and cleared by centrifugation at 14 000 r.p.m. for 10 min at 4°C. For immunoblotting detection of EHD3, we used either a mouse monoclonal antibody (Clone RR-L; Santa Cruz Biotechnologies) or our custom-made rabbit polyclonal antibody. Other antibodies against caspases 3, 7 and 9, PARP, BAX, BAK (Apoptosis sampler kit), actin and GAPDH were obtained from Cell Signaling.

Tissue microarray and IHC

Tissue microarray. A brain tumor tissue array (GL2083a), including TNM, clinical stage and pathology grade of 208 cases/208 cores, was obtained from Biomax.us.

IHC for EHD3. The paraffinized sections were dewaxed in xylene and then in alcohol. Heat-induced epitope recovery was done by microwaving the sections in a pressure cooker in a microwave oven in 0.01 M citrate buffer (pH 6.0) for 22 min. The slides were blocked in 0.3% H$_2$O$_2$ in methanol for 30 min, then with the Avidin/Biotin Blocking Kit (Cat# SP-2001; Vector Laboratories, Burlingame, CA) and then with a 1/50 dilution of horse serum (Cat# 008-000-001; Jackson ImmunoResearch, West Grove, PA) in PBS for 20 min. The sections were incubated overnight at 4°C in a 1/50 dilution of our custom-made rabbit polyclonal EHD3 primary antibody, then incubated for 30 min in a 1/200 dilution of biotinylated horse anti-rabbit secondary antibodies (Vector Laboratories) and then processed with the Vectastain ABC Kit (Cat# PK-4000; Vector Laboratories). They were then incubated in 3,3′-diaminobenzidine solution for 20 min and then counterstained with Mayer’s hematoxylin (Cat# MHS16; Sigma–Aldrich, Oakville, Ontario, Canada) for 4 min. Finally, they were dehydrated in alcohol and then xylene and mounted with Permount (Cat# SP15; Fisher Scientific, Hanover Park, IL).

TetON inducible expression system

The generation of U251 cells that inducibly express the EHD3 protein was performed using the Tet-On Advanced Inducible Gene Expression System and the Retro-X Universal Packaging System (Clontech). The human Ehd3 ORF was amplified from a constitutive expression construct (Generous gift from Dr Steve Caplan, University of Nebraska) and cloned in the BamHI and EcoRI sites in the retroviral pRetroX-Tight-Pur vector (Clontech), producing the pRetro-EHD3 construct. GP2-293 packaging cells were transfected with both the pTet-On-Advanced vector and the pRetro-EHD3 construct. GP2-293 packaging cells were transduced with both the pTet-On-Advanced vector and the pRetro-EHD3 vector and the viral envelope-producing pVSV-G construct. To collect viral particles, the cell culture

Fig. 2. (a) Immunoblotting of established human glioma cell lines for EHD3 expression, using a mouse antibody. (b) Real-time RT–PCR of Ehd3 expression in SF126, SF188, U251 and GM1600 glioma cell lines, treated (T) or untreated (UT) with the 5-Azacytidine demethylating agent (5 µmol/l for 5 days). **P < 0.01. (c) Immunoblotting of EHD3 expression in SF126 cells, treated (T) or untreated (UT) with 5-Azacytidine (5 µmol/l for 5 days), using a mouse monoclonal antibody. (d) A schematic of predicted CpG islands (shown in blue) within the Ehd3 promoter using the MethPrimer program. The black arrows indicate CpG islands that were analyzed and found to be differentially methylated in glioma cell lines. (e) Bisulfite sequencing analysis of the Ehd3 promoter. The left panel shows sequences protected from bisulfite-mediated C→T conversion due to hypermethylation, as opposed to unmethylated sequences (right panel). Only a short sequence of the cloned CpG island is shown. A representative result is shown for SF126 cells.
medium was collected 2–4 days after transfection, centrifuged and filtered. A single round of infection was performed with the viral particles. Double G418 and Puromycin-resistant pools and clones were selected and screened by DOX induction and immunoblotting analysis for EHD3 expression.

Gene silencing
Ehd3 silencing in GM2493 cells was done using a pGIPZshRNA sequence (OpenBiosystems) or an siRNA sequence (Sigma–Aldrich), with the respective non-silencing control sequences. Briefly, GM2493 cells were plated 16h before transfection to produce monolayers that were 60% confluent and these were transfected with 50 nM of either control siRNA (MISSION® siRNA, SIC001; Sigma–Aldrich) or Ehd3 siRNA (MISSION® siRNA, S101_00241701 and S101_00241702; Sigma–Aldrich) using Lipofectamine 2000 reagent according to the manufacturer’s instructions (Invitrogen). Transfection efficiency was monitored by measuring the level of Ehd3 mRNA using quantitative reverse transcription–polymerase chain reaction (RT–PCR) or by immunoblotting analysis.

MTT assay and cell cycle analysis
The MTT metabolic assay was used to assess cell growth. Briefly, exponentially growing cells were seeded in 96-well microplates for 24 h. For the inducible system, cells were induced by DOX at 1 µg/ml. Survival was evaluated every day by replacing the culture media with 50 µl of 2.5 mg/ml MTT (Sigma, St Louis, MO) in PBS pH 7.5. After 1 h of incubation at 37°C in the dark, MTT was replaced with 100 µl of solubilization solution (10% triton X100, and 1 N HCl in anhydrous isopropanol). Absorbance was determined at 570 nm with a microplate reader (Bio-Rad). In some experiments, cell survival was quantified using Alamar Blue by fluorimetry as indicated by the manufacturer (Invitrogen). Cell cycle analysis was performed using the propidium iodide (PI) staining and flow cytometry. Cells were harvested, fixed and stained with 5 µg/ml PI. Cells were subsequently analyzed by flow cytometry and the population of cells with G0/G1 or sub-G1 levels of DNA was determined based on the fluorescence. The numbers indicated are the mean result of three independent experiments (mean ± SD).

Fig. 3. Restoring EHD3 expression suppressed clonogenic growth of U251 cells. U251 glioblastoma cells were transfected with either the pcDNA3.0 empty vector or the pcDNA3.0-EHD3 vector. (a) Protein expression levels in stable cell lines 14 days after transfection and selection in 600 µg/ml of G418 were assessed by immunoblotting using a mouse anti-EHD3 antibody. (b) Colonies were stained with the MTT reagent for 4 h and the numbers of the colonies were counted. Experiments were done in triplicates and the results presented as mean ± SD. (c, d) Dox-inducible expression of EHD3; (d) suppressed growth of U251tetEHD3 cells as assessed using an Alamar Blue assay, over a period of 1–4 days (**P < 0.001). (e) Dox-inducible EHD3 expression decreases U251tetEHD3 cells’ invasiveness using the Boyden chamber-like assay. Quantification was performed by spectrophotometry at OD540. Experiments were done three times in triplicates and the results presented in a histogram as means with standard deviation bars (*P < 0.05).
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Modified Boyden chamber cell invasion assay

The invasion assay was performed on glioma cells after 3 days of EHD3 induction with Dox (1 μg/ml). Cell invasion assays were done in modified Boyden chambers fitted with Matrigel-coated 8 μm transwell filters (250 μg/ml Matrigel, Cat# 356231; BD Biosciences). Cells were seeded in the top of transwells of the 24-well plate at a density of (2 × 10^4) cells/0.5 ml of 1% bovine serum albumin in RPMI medium. Fetal bovine serum at 10% was used as a chemoattractant in the bottom chambers. After 18 h, the cells on the upper side of the membrane were removed and cells penetrating the filter were stained with a Cell Stain Solution (Chemicon) and imaged by bright field microscopy. Relative cell attachment was determined by spectrophotometry measurement at OD549 after dissolution of the stained cells.

In vivo experiments

The DOX-inducible U251tetEHD3 cells were stably transfected with the pGL4[hRlac/Hygro] Vector (Promega) to generate the U251tetEHD3-Luc cells, which were examined by luciferase assay (Promega) to ensure high luciferase activity levels. 4 × 10^4 U251tetEHD3-Luc cells were injected subcutaneously into athymic nude mice. Tumors were allowed to develop for 21 days, where tumors were visible, and Ehd3 expression was induced in vivo by 2 mg/ml DOX (Clontech) in 5% sucrose in drinking water. Tumor growth was measured and the tumor volume (cm^3) was calculated as length × width^2. At the final time point (80 days), the luciferase signal was monitored by injecting the luciferase substrate luciferin (150 mg/kg) in PBS intraperitoneally, after anesthesia with isoflurane. Illustrative images were taken with exposure of 2 min. using the Kodak IS4000MM in vivo imaging system. Institutional and national guidelines for the care and use of animals were followed and an animal use protocol was approved by the Wayne State University Institutional Animal and Care Use Committee (IACUC).

**Statistical analysis**

Statistics were conducted using GraphPad Prism 5.0 for Windows (GraphPad Software) and tests were done with a Student t-test (**P < 0.05, ***P < 0.01, ****P < 0.001). P values <0.05 were considered to be statistically significant. Results are displayed as averages with error bars indicating standard deviations.

**Results**

**Loss of EHD3 expression is a highly frequent event in gliomas**

The family of EHD proteins includes four members (EHD1–EHD4), whose identification is relatively recent. Their respective expression, distribution and functions in specific tissues are not well known. A data mining approach using BioGPS, a web-based cDNA microarray database (15), identifies the central nervous system as a main tissue where Ehd3 is highly expressed in the adult (Supplementary Figure 2, available at Carcinogenesis Online), whereas all other three family members seem to show less specific expression in these tissues (data not shown). This observation suggests a privileged role for Ehd3 in the central nervous system. We subsequently used the Oncomine database (19) and the GEO database from the NCBI (20) to assess Ehd3 expression in brain cancers. A cancer gene microarray meta-analysis shows that Ehd3 is mainly expressed in the non-tumorigenic brain and that it decreases significantly in brain tumors of different grades. For instance, a close analysis of data from the large study by Sun et al. (21), which includes 157 brain and central nervous system tumors and 23 normal brain samples, shows Ehd3 to be significantly lost in cancer tissues in comparison with non-tumor counterparts (Supplementary Figure 3, available at Carcinogenesis Online). Compared with the normal samples, there was a significant decrease in Ehd3 expression in malignant samples, ranging from −2.919-fold in anaplastic astrocytomas (P value: 1.55E-9), to −2.466-fold in oligodendrogliomas (P value: 4.73E-11) and −4.581-fold in glioblastomas (P value: 6.04E-20) (Supplementary Figure 3, available at Carcinogenesis Online). This result is further confirmed by the Oncomine analysis of microarray data from a second study by Liang et al. (22), which included 3 normal brain samples and 31 tumors, and by another study by Bredel et al. (23), which included 50 glial brain tumors and 4 normal brain specimens (23) (data not shown).

![Fig. 4. Inducible EHD3 expression induces a G_{2}/G_{1} cell cycle arrest and apoptotic cell death.](https://academic.oup.com/carcin/article-abstract/35/4/877/269357)
To validate the gene microarray data in brain cancers and specifically in gliomas, we performed an IHC analysis of the expression of EHD3 in a tissue microarray (TMA) of normal and malignant brain specimens. We used a large glioma progression TMA, containing 132 cases of brain astrocytomas, 31 brain glioblastomas, 7 oligoastrocytomas, 9 oligodendroglomas, 11 ependymomas and 8 each of normal tissues and tumor-adjacent normal tissues, in addition to single cases of ganglioglioma and gliosarcoma. Staining with our custom-made and characterized polyclonal anti-EHD3 antibody showed that although >68% of the normal tissues were positive for EHD3 expression, only 11.7% of grades I and II, 5.7% of grade III and 2.9% of grade IV showed positive staining (Figure 1), indicating a major loss of EHD3 expression in the early cancer progression stages that persists and even seems to be accentuated in the later and more aggressive stages.

Taken together, these data show that loss of the Ehd3 gene expression, in comparison with non-tumor tissues, is a very common event in gliomas of all grades. Importantly, we have queried the REMBRANDT database, for the analysis of Ehd3 gene expression in glioma specimens in correlation with patient’s survival (Supplementary Figure 4, available at Carcinogenesis Online). The Kaplan–Meier survival plot shows a lower survival in patients with downregulated Ehd3 expression in comparison with patients with intermediate levels (P = 0.0122543336 < 0.05).

Many glioma tumor suppressor genes have been shown to be subject to epigenetic silencing. To determine whether an epigenetic event such as promoter methylation might be involved in the observed loss of EHD3 expression, we treated four cell lines showing low EHD3 expression (SF126, SF188, U251 and GM1600) with the demethylating agent 5-AzaCytidine (5 µmol/l, 5 days), which is known to induce reexpression of genes whose promoters are heavily hypermethylated. Next, the Ehd3 levels were quantified by real-time quantitative RT–PCR. We found that 5-AzaCytidine treatment of SF126 and GM1600 cells resulted in an increase in the Ehd3 expression (Figure 2b). This increase in transcript levels translated into a significant increase at the protein level, as shown for SF126 cells (Figure 2c). The other two cell lines did not show any significant change, suggesting alternative modes of silencing.

To confirm that the Ehd3 promoter is subject to hypermethylation in SF126 and GM1600 cells, we used bisulphite sequencing analysis. The MethPrimer prediction program suggested the presence of three putative CpG islands (Figure 2d). Genomic DNA was extracted from the glioma cell lines, treated with bisulphite and PCR amplified to clone the region corresponding to the first CpG island. The sequencing results confirmed the hypermethylation of the Ehd3 promoter in the SF126 and GM1600 cell lines (Figure 2e, result shown for SF126), thus indicating a role for this mode of gene regulation in the loss of Ehd3 expression in gliomas. Following this initial characterization, we decided to use the EHD3-low U251 and U87MG cells and the EHD3-high GM2493 cells for subsequent experiments.

**EHD3 expression suppresses glioma cells’ growth and invasion**

We examined the effect of constitutively restoring EHD3 expression on the clonal growth of U251 glioma cells, by stable transfection with the EHD3-encoding vector. EHD3 overexpression was confirmed at the protein level by immunoblotting following transfection and selection of stable colonies (Figure 3a). The results show that EHD3 expression strongly suppressed clonal growth of U251 cells, as illustrated by a lower number of growing colonies (Figure 3b). Next, we developed a DOX-inducible system using U251 and U87MG cells, here referred to as U251tetEHD3 and U87tetEHD3 cells, respectively, to express EHD3 on demand. As shown in Figure 3c, DOX treatment efficiently induced EHD3 expression. To ensure that DOX does not elicit non-specific effects, we treated the parental U251 cells with DOX. The results of the MTT assay show that DOX has no effects in the absence of the inducible system (Supplementary Figure 5, available at Carcinogenesis Online). Using the U251tetEHD3 cells to perform an Alamar Blue growth assay over a period of 4 days, we found that inducible expression of EHD3 reduces dramatically the growth of U251 (Figure 3d) and U87MG cells (data not shown).

Next, we investigated the effect of EHD3 expression on glioma cell invasion in vitro. Using a modified Boyden chamber assay, we found that EHD3 expression inhibited the invasion capacity of U251tetEHD3 cells (Figure 3e).

EHD3 induces G0/G1 growth arrest and apoptosis in glioma cells.

To assess the mechanisms of cell growth inhibition by EHD3, we performed cell cycle analysis of the U251tetEHD3 cells using fluorescence-activated cell sorting/PI staining analysis. Induction of EHD3 expression significantly increased the percentage of cells in G0/G1 phases from 50% in the control non-induced cells to 70% after 5 days induction of EHD3 expression (Figure 4a), indicating
that EHD3 induces a G0/G1 growth arrest in these cells. Quantitative real-time RT–PCR showed significant decreases in the expression of various cell cycle regulatory genes including cyclin-dependent kinases CDK1 and CDK4, as well as cyclins D1 (CCND1) and D3 (CCND3) (Figure 4b). Therefore, EHD3’s growth inhibitory function involves an induction of cell cycle arrest. Also, we found that subsequently to this effect, an increase in the sub-G1 cell fraction was observed (Figure 4c) over time of induction of EHD3 expression, from ~5% at day 1 to 25% at day 5, whereas the untreated counterparts did not show any significant change in the sub-G1 fraction (Figure 4c), suggesting that EHD3 expression induces apoptotic cell death in glioma cells. This is further confirmed by the finding that caspases 3, 7 and 9 are activated and the apoptotic marker PARP is cleaved as a result of EHD3 expression (Figure 4d). These results indicate that EHD3 growth inhibition involves the arrest of cell cycle progression and the induction of apoptosis. It is noteworthy that the above-mentioned growth inhibitory and proapoptotic effects were reproduced in U87tetEHD3 cells (data not shown). To recapitulate the clinical observation of loss of EHD3 expression and assess its consequences in glioma cells, we used GM2493 cells, which naturally express high levels of endogenous EHD3 (Figure 2a) to silence the latter’s expression using a specific shRNA-based lentiviral system. The shRNA sequences efficiently knocked down the expression of EHD3 with a silencing rate of >90% (Figure 5a and c). An MTT growth assay performed after 5 days of gene knockdown showed significant (50%) growth induction after EHD3 silencing in comparison with the non-silenced control counterparts (Figure 5b), thus demonstrating the role of EHD3 in regulating glioma cells growth. In parallel, EHD3 silencing inhibited the expression of proapoptotic markers such as BAX and BAK (Figure 5c).

EHD3 inhibits tumor growth in vivo

We further analyzed the role of EHD3 in tumor suppression in vivo, by implanting U251tetEHD3-Luc cells subcutaneously in nude mice. These cells were obtained by stable transfection of U251tetEHD3 cells with a luciferase-encoding vector (pGL4[hRluc/Hygro]). Once the tumors were palpable (day 21 postinjection), the mice were randomized and separated into two groups with close tumor sizes, of which only one is given DOX in drinking water, to induce EHD3 expression. As shown in Figure 6a, DOX-mediated induction of EHD3 expression resulted in a dramatic growth inhibition in comparison with the control counterparts, maintained 57 days after cell injection. As an additional illustration of the extent of this effect, luciferin, a luciferase substrate, was administered intraperitoneally to the mice before their euthanasia and tumors were imaged using a Kodak IS-4000MM Live Animal Multimodal Imager, showing a much larger tumor formed by the control cells than the EHD3-expressing cells (Figure 6a, inset). A real-time RT–PCR performed on the tumors collected postmortem confirmed that the DOX-induced tumors express higher levels of EHD3 (Figure 6b). In parallel, we analyzed protein extracts from these tumor samples and performed immunoblotting analysis. We found that the activation of caspases 3, 7 and 9 in these tumors was globally increased, thus indicating the onset of apoptotic cell death after restoring EHD3 expression in vivo (Figure 6c). Therefore, as we previously observed in vitro, EHD3 also induces tumor suppression in vivo by increasing apoptosis.

Discussion

In the present work, we provide evidence suggesting that Ehd3 is a potential tumor suppressor gene in gliomas. This tumor suppressor gene regulates cell cycle progression and induces apoptosis in glioma cells.
role may be particular to brain cancers. In fact, according to many data mining analyzes, out of the four Ehd family members, which seem to be ubiquitously expressed, only Ehd3 shows a prominent differential distribution of expression, with a higher presence in brain tissues. This is consistent with the original description of these proteins, showing a differential histological expression pattern for EHD3 in the brain (24). The significance of this observation is not clear and very little if any information of possible functional complementation is available for this protein family. There are, however, indications that the four EHD proteins share some functions without being identical (25–27). By interrogating the Oncomine database (19), we performed a meta-analysis of microarray expression studies. Again, we found that Ehd3 transcripts are expressed mostly in brain tissues and we identified several expression data sets with Ehd3 data, all of which showed that Ehd3 levels are significantly down-regulated in glioma samples compared with normal brain tissue. Using IHC to examine the expression of EHD3 in a large number of samples of a TMA, we confirmed that loss of EHD3 expression is indeed a prominent event in most of the glioma tumor specimens versus normal tissue. Interestingly, the loss is observed even in the least advanced grades I and II, to almost the same degree as in the advanced grades III and IV, suggesting that EHD3 loss is an early event during gliomagenesis.

We analyzed EHD3 expression in a panel of glioma cell lines and found it to be silenced by promoter hypermethylation in at least two glioma cell lines. Interestingly, an earlier and single work reported that the Ehd3 promoter is methylated in 63% of acute myeloid leukemia patients examined, and the methylation was lost upon complete remission (28). However, beyond reporting this observation, the role of Ehd3 in acute myeloid leukemia has not been investigated. In other respect, the fact that 5-Azacytidine was not effective at inducing Ehd3 expression in two cell lines suggests that alternative modes of gene silencing might also be involved in loss of Ehd3 expression. Since the lower protein expression in these cells is preceded by lower transcript levels, one might speculate that the effects are transcriptional (e.g. chromatin acetylation) or posttranscriptional (e.g. transcript stability) rather than translational.

Moreover, using a Dox-inducible system, we found that when expressed in glioma cells, EHD3 has growth inhibitory functions and induces a G0/G1 cell cycle arrest and apoptotic death. This effect is observed in vitro as well as in vivo, using a xenograft model in nude mice. In addition, restoring EHD3 expression was able to inhibit glioma cell invasion in a modified Boyden chamber assay. Taken together, these results strongly support the hypothesis that EHD3 loss could be considered as a novel marker of tumor progression in gliomas. They suggest that EHD3 is a tumor suppressor in the brain. To the best of our knowledge, this is the first report of a member of the C-terminal EHD proteins being assigned a role in oncogenesis or tumor suppression. The mechanisms of action appear to implicate the recruitment of the apoptotic machinery, by activating caspases 3, 7 and 9, which are important for intrinsic and extrinsic apoptosis. EHD3, along with other EHD family members, regulates endocytic recycling (13,14,17). Whether EHD3 regulation of cell cycle arrest and apoptosis occurs or not by virtue of the reported role of EHD3 in endocytic recycling remains to be investigated. Although endocytic recycling has long been regarded as a trafficking route, it later became evident that this process is important in the control of multiple signaling pathways and biological functions (4,7). Many proteins involved in the endocytic machinery have been shown to be implicated in cancer (8) and may provide insight for future studies. The interaction between the endocytic recycling and apoptotic machineries is ill defined. However, important links have been established that argue for an essential connection between the two processes (29–35). Thus, it is our hypothesis that EHD3, like other endocytic recycling-related proteins (36–39), regulates apoptosis and survival. This could be achieved through modulating the compartmentalization and hence the signaling ability of the apoptotic machinery, a mechanism consistent with the acknowledged function of endocytosis in modulating cell signaling and tumorigenic progression. Nevertheless, because the knowledge about EHD3 functions is very limited so far, it is also possible that the proapoptotic role of EHD3 involves functions not related to its role in trafficking, but rather to its ATP/GTP-binding ability and possible impact on protein kinase signaling. These mechanistic considerations would be further enlightened by a better knowledge of EHD3 functions in normal cells. In this regard, the proliferative effect driven by loss of EHD3 expression in tumor cells poses the question of the impact of expression of EHD3 in normal brain tissues. It is possible that EHD3 contributes to the prevalent postmitotic state of normal brain cells. Alternatively, this state might make them less sensitive to the antiproliferative and proapoptotic effect of EHD3 in non-ex-malignant cells. EHD3 might also possess different functions in the brain biology. This is understandable by virtue of the role of EHD3 in protein trafficking, a process of utmost importance in the receptor/ligand-mediated communication between various brain cells.

Supplementary material
Supplementary Figures 1–5 can be found at http://carcin.oxfordjournals.org/

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References
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