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Differential expression of the tumor suppressor A-kinase anchor protein 12 in human diffuse and pilocytic astrocytomas is regulated by promoter methylation

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Abstract: The scaffold protein A-kinase anchor protein 12 (AKAP12) exerts tumor suppressor activity and is downregulated in several tumor entities. We characterized AKAP12 expression and regulation in astrocytomas, including pilocytic and diffusely infiltrating astrocytomas. We examined 194 human gliomas and 23 normal brain white matter samples by immunohistochemistry or immunoblotting for AKAP12 expression. We further performed quantitative methylation analysis of the AKAP12 promoter by MassARRAY® of normal brain, World Health Organization (WHO) grade I to IV astrocytomas, and glioma cell lines. Our results show that AKAP12 is expressed in a perivascular distribution in normal CNS, strongly upregulated in tumor cells in pilocytic astrocytomas, and weakly expressed in diffuse astrocytomas of WHO grade II to IV. Methylation analyses revealed specific hypermethylation of AKAP12 promoter in WHO grade II to IV astrocytomas. Restoration experiments using 5-aza-2'-deoxycytidine in primary glioblastoma cells decreased AKAP12 promoter methylation and markedly increased AKAP12 mRNA levels. In summary, we demonstrate that AKAP12 is differentially expressed in human astrocytomas showing high expression in pilocytic but low expression in diffuse astrocytomas of all WHO-grades. Our results further indicate that epigenetic mechanisms are involved in silencing AKAP12 in diffuse astrocytomas; however, a tumor suppressive role of AKAP12 in distinct astrocytoma subtypes remains to be determined.

Differential expression of the tumor suppressor A kinase anchor protein 12 (AKAP12) in human diffuse and pilocytic astrocytomas is regulated by promoter methylation.

<u>Running title:</u> AKAP12 promoter methylation in gliomas

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ABSTRACT

The scaffold protein A kinase anchor protein 12 (AKAP12) exerts tumor suppressor activity and is downregulated in several tumor entities. We aimed to characterize AKAP12 expression and regulation in astrocytomas including pilocytic and diffusely infiltrating astrocytomas. We examined 194 human gliomas and 23 white matter normal brain samples by immunohistochemistry or immunoblotting for AKAP12 expression. Quantitative methylation analysis of the *AKAP12* promoter by MassARRAY® of normal brain, astrocytomas of WHO-grade I-IV and glioma cell lines was performed followed by restoration experiments using 5-aza-2'deoxycytidine. Our results show that AKAP12 is expressed in a perivascular distribution in normal CNS, strongly upregulated in pilocytic astrocytomas and weakly expressed in diffuse astrocytomas of WHO grade II-IV. Methylation analyses revealed specific hypermethylation of *AKAP12α* promoter in astrocytomas of WHO grade II-IV. Consequently, restoration experiments decreased *AKAP12α* promoter methylation and drastically increased AKAP12α mRNA levels in primary glioblastoma cells. In summary, we could show that AKAP12 is differentially expressed in human astrocytomas showing high expression in pilocytic but low expression in diffuse astrocytomas of all WHO-grades. Our results further indicate that epigenetic mechanisms are involved in silencing *AKAP12* in diffuse astrocytomas, however a differential tumor suppressive role of AKAP12 in distinct astrocytoma subtypes remains to be determined.

Keywords: astrocytoma, AKAP12, Gravin, SSeCKS, promoter methylation.

INTRODUCTION

The scaffold protein A kinase anchor protein 12 (AKAP12; synonymous to AKAP250 and Gravin or to its rodent ortholog SSeCKS) is a central mediator of protein kinase A and protein kinase C signalling (1, 2). AKAP12 acts as a tumor suppressor and its expression is frequently downregulated in human malignancies. However, the molecular mechanisms responsible for reduced AKAP12 expression are poorly understood (3). To date, AKAP12 related tumor suppressive properties in neoplasms including gastric, liver and prostate cancer were linked to (i) inhibition of myosin light chain kinase (MLCK) mediated cytokinesis, (ii) suppression of the migratory potential by inhibiting the Raf/MEK/Erk pathway and matrix metalloproteinase-2 and -9 (MMP-2/-9), (iii) induction of apoptosis by regulating CDKI-cyclin D1 and caspase-3 and (iv) reduction of the angiogenic and metastatic capacity by inhibiting VEGF (vascular endothelial growth factor) (4-10). Recently, we demonstrated that AKAP12 is downregulated in hepatocarcinogenesis by hypermethylation of the AKAP12 promoter pointing to a primary epigenetic alteration being involved in AKAP12 mediated tumorigenesis (6). Under non-neoplastic conditions, the VEGF opposing effects of AKAP12 are involved in development and maintenance of the blood-brain barrier (BBB) and CNS angiogenesis (11, 12). Due to its anti-angiogenic and anti-migratory properties, we hypothesized that deregulation of AKAP12 may be involved in the development of malignant gliomas characterized by diffuse infiltration and high angiogenic potential. Malignant gliomas account for approximately 70% of adult malignant primary brain tumors and survival is closely related to WHO grade (13). Patients suffering from high grade astrocytomas show short survival times being as low as 1.6 years in anaplastic astrocytoma and 0.4 years in glioblastoma in population-based studies (14). High grade gliomas are treatment refractory despite multimodal treatment including neurosurgical resection followed by combined radio-

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/chemotherapy, a feature which is closely related to VEGF-mediated neovascularization and MMP-mediated glioma cell migration (15-17). Therefore, new targeted therapeutic options focusing on anti-angiogenesis entered clinical trials frequently in neurooncology (18). However, at least half of patients fail to respond to anti-angiogenic treatment and time to progression is short (19). Adverse effects of anti-angiogenic therapy such as formation of microsatellite metastases were identified and linked to increased migratory-invasive potential of glioma cells (20, 21). Although neovascularization is a hallmark of malignant progression in diffuse gliomas, non-diffuse pilocytic astrocytomas also frequently exhibit proliferating blood vessels and show a good clinical prognosis indicating distinct ways of neo-angiogenesis (22). Since AKAP12 is a potent VEGF-antagonist, we aimed at defining its expression pattern, regulation, and clinical impact in astrocytomas of different WHO grades to determine its potential involvement in malignant transformation and progression in human astrocytomas.

MATERIALS AND METHODS

Patient characteristics and tissue specimens

194 brain tumor samples obtained from the tissue bank of the Institute of Brain Research, University of Tuebingen, including 26 WHO grade I (pilocytic), 37 WHO grade II, 52 grade III astrocytomas and 79 glioblastomas (WHO grade IV) were investigated (for summary see **Table 1**). For WHO grade IV tumors, 25 infiltration zones and 31 relapses were included as well as 23 autopsy cases without any CNS pathologies serving as normal controls. Utilization of specimens was approved by the University's ethics commission. Neuropathological diagnoses were confirmed by two neuropathologists (RM, MM). The histopathological typing and grading was performed according to the WHO criteria for tumors of the nervous system (23). The same samples which were analysed in the neuropathological diagnostical setting were also used for immunohistochemical analyses. Median follow-up for each astrocytoma subtype is given in **Table 1**. Edema levels were analyzed in T2 MRI or CCT scans. The association of AKAP12 and edema (no=0/low=1/high=2) was semi-quantitatively assessed by ordinal regression analysis. Edema scores were analyzed in cooperation with the Department of Neuroradiology (University of Tuebingen).

Immunohistochemistry

Specimens were fixed in buffered formalin (4% formaldehyde; pH 7.4) and embedded in paraffin. Immunohistochemical analysis was performed according to routine protocols using the ABC method and diaminobenzidine as chromogen. In order to detect AKAP12, a polyclonal goat IgG antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA; dilution 1:200) was applied. Sections were counterstained with hemalaun. Antibody staining was initially established on testis and prostate tissue. Specificity was controlled by omission of the AKAP12-specific antibody and addition

of non-immunized goat IgG sera. Double immunohistochemical stainings were performed using an automated immunostaining system with standard protocols (Ventana, Strasbourg, France). The following antibodies were used: (i) AKAP12 (clone 1C5; mouse monoclonal; 1:10000; Sigma, Hamburg, Germany); (ii) CD31 (clone JC70A; mouse monoclonal; 1:200; DAKO, Hamburg, Germany); (iii) collagen IV (clone CIV 22; mouse monoclonal; 1:50; DAKO), (iv) GFAP (polyclonal; rabbit; 1:10000; DAKO) and α-SMA (clone 1A4; mouse monoclonal; 1:500; DAKO. A 28 min incubation with one drop of Universal Secondary Antibody was added (Ventana). For diaminobenzidine (DAB) visualization, the sections were incubated with one drop of I-View SA-HRP for 16 min and then with DAB/H2O2 for 8 min. The sections were incubated with a copper enhancer (Ventana) for 4 min. The application of one drop of Activator R CM and one drop of Naphtol CM (Ventana) for 4 min was followed by the visualization with one drop of Fast Red CM (Ventana) twice for 8 min. Finally, all sections were washed, counterstained with hematoxylin and mounted.

Kommentar [MM1]: IDH1 analyses ?

Immunofluorescence

To define the main source of AKAP12 protein expression in human astrocytic tumours and normal CNS tissue, immunofluorescent stainings were performed using the following antibodies: (i) for endothelia: CD31 (clone JC70A; mouse monoclonal; 1:200; DAKO), (ii) for basement membrane structures: collagen IV (clone CIV 22; mouse monoclonal; 1:50; DAKO) and (iii) for AKAP12 (abcam; JP74; mouse monoclonal; 1:10000). Subsequently, the following secondary ABs were used: Alexa Fluor488 (goat-anti-mouse IgG2a, Invitrogen, Darmstadt, Germany; dilution 1:500) and Alexa Fluor568 (goat-anti-mouse IgG1, Invitrogen; dilution 1:500). Nuclear counterstaining was performed using Topro-3 (dilution: 1:1000; Invitrogen). Fluorescence images were analyzed and recorded on a Nikon C1si (Nikon, Japan)

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confocal microscope, using the EZ-C1 software. After recording, digital images were further processed and adjusted for brightness, contrast and color balance.

Light microscopy and counting

To quantify AKAP12 immunoreactivity of a single sample, 200 cells per tumor were counted; a clear cytoplasmic or membranous stain was rated as positive. Blood vessels, intraluminal cells and cells which unequivocally could be referred to neurons or inflammatory cells by means of morphology were excluded. Immunohistochemical stainings were evaluated and photographically documented using an Olympus BX50 light microscope.

Immunoblotting

The fresh frozen tissues used for immunoblotting were also evaluated by 2 neuropathologists (PNH and MM) before preparing the lysates. Only samples showing a tumor cell amount of > 70% as well as none or only a minimum amount of necroses were selected for immunoblotting. Immunoblotting was performed using the following primary antibodies: a goat polyclonal anti-AKAP12 (dilution 1:1000; Santa Cruz Biotechnology) and a mouse monoclonal anti-AKAP12 antibody (dilution 1:1000; Abcam, Cambridge, MA). For detailed information see supplemental material.

Cell culture and reagents

The human malignant glioma cell lines LN-18, U138MG, U87MG, LN-428, D247MG, T98G, LNT-229, A172, U251MG and U373MG were kindly provided by N. de Tribolet (University Hospital, Lausanne, Switzerland). As LN-229 cells used in different laboratories differ by their p53 status, the p53 wild-type LN-229 cells used in our

experiments were renamed LNT-229 for clarification (T for Tuebingen). Cells were cultured in 75 cm^2 Falcon plastic flasks using DMEM supplemented with 1% glutamine (Life Technologies, Paisley, United Kingdom), 10% FCS (Biochrom, Berlin, Germany), and penicillin (100 IU/ml)/streptomycin (100 µg/ml). For immunocytochemical analyses, cells were centrifuged, fixed in buffered formalin (4% formaldehyde; pH 7.4) and embedded in paraffin. Subsequent immunohistochemical staining procedures were performed as described for human tissue. Primary human glioblastoma cell (NCH82, NCH89 and NCH440, provided by B. Campos) were cultured in high glucose DMEM (Life Technologies) supplemented with 10% FCS (24). DNA demethylation treatment was performed with 2 µmol/l 5-aza-2'-deoxycytidine (Sigma-Aldrich, St.Louis, MO). Cells were treated with 5-aza-2'-deoxycytidine for 72 hours, refreshing the media every 24 hours.

DNA and RNA isolation

DNA/RNA from fresh frozen tumor tissues or cell lines was isolated using the AllPrep DNA/RNA Mini kit (Qiagen, Hilden, Germany).

MassARRAY® methylation analysis

Quantitative DNA methylation analysis was performed by MassARRAY® technique. Genomic DNA from patients, healthy controls, or cell lines was chemically modified with sodium bisulfite using the EZ methylation kit (Zymo Research, Orange, CA, USA) and polymerase chain reaction (PCR) amplified. Regions for quantitative DNA methylation analysis covered the CpG islands around the respective transcription start sites of the two *AKAP12* isoforms *α* and *β* (6). The PCR product was transcribed *in vitro*, cleaved by RNase A, and subjected to MALDI-TOF mass spectrometry analysis to determine methylation patterns (25). Methylation levels of long

interspersed nucleotide elements (LINE-1) were analyzed to obtain an estimate for global DNA methylation changes after 5-aza-2'-deoxycytidine treatment (26, 27). DNA methylation standards (0% to100% (in 20% steps) methylated genomic DNA) were used to control for potential PCR bias. The methylation results are displayed as a heat map using the Multiple Experimental Viewer software (Version 4.3) (28). Primer sequences are listed in **Supplemental Table 1**.

cDNA synthesis and quantitative real-time PCR

1 µg of purified DNase-treated RNA was transcribed to cDNA with Superscript™ II Reverse Transcriptase (Invitrogen, Darmstadt, Germany) using oligo-dT primers. Quantitative Real-Time PCR expression analyses were performed on the Roche Lightcycler© 480 system using the LightCycler$^{®}$ 480 Probes Master (both Roche Diagnostics, Mannheim, Germany). Expression of target genes (see **Supplemental Table 2**) was normalized to three housekeeping genes (*ACTB*, *GAPDH* and *HPRT1).*

Statistical analysis

To stabilize variances, fractions of AKAP12-positive cells were transformed by taking the arcsine of the square-root. Means were compared by one-way analysis of variance (ANOVA). For post-hoc testing Tukey-Kramer's HSD test was used with a global significance level of 5%. For individual pairwise comparisons we used a one sample t-test. Back-transformed means are given together with their 95% confidence intervals (CI). To describe patient survival, univariate and multivariate survival analyses for censored data were performed. Expression and methylation differences in cell line experiments were statistically assessed by a two-sided t-test. Statistical analyses were performed using JMP 8.0 software (SAS, Cary, NC, USA).

RESULTS

AKAP12 is selectively overexpressed in human pilocytic astrocytomas as compared to diffusely infiltrating astrocytomas.

In normal human CNS areas **(Fig. 1A)**, AKAP12 expression is almost exclusively found in a perivascular distribution whereas glial cells and neurons remain AKAP12-negative **(Fig. 1A)**. In WHO grade I pilocytic astrocytomas, AKAP12 is also strongly expressed at small residual capillaries and activated or proliferating blood vessels. However, most neoplastic cells display a strong cytoplasmic AKAP12 expression as well **(Fig. 1B)**. In human WHO grade II diffuse astrocytomas, AKAP12 is again predominantly found at blood vessels **(Fig. 1C)**. Single neoplastic cells within WHO grade II gliomas show a faint AKAP12 expression, to a far lower extent as compared to their pilocytic counterparts. In WHO grade III anaplastic astrocytomas **(Fig. 1D, E)** and glioblastomas **(Fig. 1F-H)** AKAP12 is heterogeneously expressed at blood vessels while being partly absent in glioma cells **(Fig. 1D, G, H)**. Apart from astrocytes, which were predicted to be a major source of AKAP12 expression in the brain, we identified blood vessel associated mural cells (pericytes/smooth muscle cells) as a source of AKAP12 while endothelial cells mainly remain AKAP12-negative **(supplemental Fig. 1-3).**

AKAP12 is significantly upregulated in pilocytic astrocytomas as compared to diffuse gliomas.

For semiquantitative analyses, ratios of AKAP12-positive astrocytoma cells in relation to all cells were calculated for each glioma specimen **(Fig. 2)**. WHO grade I pilocytic astrocytomas showed AKAP12 expression in 57.2 % (CI 45.7-68.3 %) of tumor cells, which was significantly elevated as compared to all diffuse astrocytoma variants ($p<0.0001$ for all comparisons). AKAP12 expression showed a slight non-significant

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increase from grade II to IV gliomas. While only 16.7 % (CI 10.5-24.1 %) of cells in WHO grade II astrocytomas displayed an AKAP12-positivity, 21.6 % (CI 15.5-28.4 %) of WHO grade III astrocytoma cells and 25.3 % (CI 20-31.1 %) of WHO grade IV glioblastoma cells showed AKAP12 immunoreactivity. In glioblastomas, no significant changes of AKAP12 expression in recurrences were seen as compared to their primary counterpart (data not shown). To corroborate our quantification of the immunohistochemical analyses, lysates from WHO grade I to IV astrocytoma cryo specimens as well as normal control samples were analyzed for AKAP12 expression by immunoblotting. In line with the immunohistochemical findings, AKAP12 levels were strongly upregulated only in WHO grade I pilocytic astrocytoma samples, whereas samples of WHO grade II-IV diffuse gliomas and normal CNS control tissues showed similarly low AKAP12 expression levels **(Fig. 3 and supplemental Fig. 4)**.

AKAP12 expression is not associated with patient survival or edema formation in astrocytomas

We correlated AKAP12 expression for a potential association with patient survival in both univariate and multivariate analyses, taking patient age, gender, extent of resection, tumor localization, Karnofsky index, WHO grade and AKAP12 levels in the latter without obtaining a significant association for AKAP12 expression and patient survival (data not shown). No differences in AKAP12 expression levels between IDH1 mutated and wild-type tumors were observed (data not shown). As AKAP12 is supposed to be involved in the regulation of the BBB under non-neoplastic conditions, we further analyzed our cohort for a potential association of AKAP12 expression and edema formation assessed by MRI. However, no significant changes were observed (data not shown).

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AKAP12 expression is regulated by DNA promoter methylation.

In a cohort of 424 glioblastomas (publicly available platform at the US National Institute of Health: The Cancer Genome Atlas Data Portal (http://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp, assessed July 14th 2012) recently analyzed by genome wide human SNP array the *AKAP12* gene locus on chromosome 6q24-25.2 showed chromosomal losses (log2 ratio < -0.5) in 9.7 % (29). Strikingly, none of the cases showed chromosomal gains. Furthermore, no *AKAP12* mutations were found in the TCGA data portal. Therefore, these data do not sufficiently explain the low AKAP12 expression levels in glioblastomas. As a possible suppressive mechanism, we investigated the DNA methylation of promoter related CpG islands of both *AKAP12* isoforms in normal human CNS specimens, WHO grade I to IV astrocytomas as well as in various glioma cell lines and primary glioma cells by quantitative MassARRAY® analysis. In diffuse astrocytomas samples, hypermethylation was detected in the *AKAP12α* promoter **(Fig. 4A, B)**, and less pronounced in the *AKAP12β* promoter **(supplemental Fig. 5)**. For the *AKAP12α* promoter, a mean methylation of 77% for diffuse astrocytomas WHO grade II, 80% for anaplastic astrocytomas WHO grade III, 56% glioblastomas was recorded, while pilocytic astrocytomas showed a mean methylation of 19%. The level of *AKAP12α* promoter methylation was not associated with the mutational status of the *IDH1/2* gene within the group of diffusely infiltrating astrocytomas **(supplemental Fig. 6)**. We additionally analyzed histologically normal brain tissue, observing a mean methylation of 35%. Most glioma cell lines (n=11) showed highly elevated (>80%) DNA methylation levels of the *AKAP12α* promoter **(Fig. 4)**. Only two glioma cell lines showed a methylation status of below 80% (NCH89: 70%, U87MG: 41%). Low levels of *AKAP12α* promoter methylation were associated with higher AKAP12 protein expression levels **(supplemental Fig. 7)**. Most of the GBM cell lines showed low or

not detectable methylation values concerning the *AKAP12β* promoter. **(supplemental Fig. 5)**.

AKAP12 is re-expressed in glioma cells upon treatment with demethylating agents
To verify the functional relationship between promoter hypermethylation and loss of *AKAP12* gene expression, methylation and mRNA expression levels of both isoforms were compared before and after treatment with 5-aza-dC in three primary GBM cell lines (NCH82, NCH89 and NCH440). The 5-aza-dC treatment resulted in a significantly decreased *AKAP12α* promoter methylation in the highly methylated GBM cell lines **(Fig. 5A)** accompanied by a significant increase in AKAP12α mRNA **(Fig. 5B)** and protein **(supplemental Fig. 8)** expression, strongly suggesting a direct relationship between AKAP12α expression and methylation of its promoter. Treatment with 5-aza-dC also induced expression of AKAP12β in primary glioma cells **(supplemental Fig. 9)**. Furthermore, we determined average methylation levels of LINE-1 repeat elements before and after 5-aza-dC treatment **(Fig. 5C)**. The significant decrease in global LINE-1 methylation showed positive treatment response to the demethylating agent in GBM cell lines, as LINE-1 methylation was shown to be representative of genome-wide methylation status (27).

DISCUSSION

Tumor suppressive activity of AKAP12 and its down-regulation associated with tumor progression and increased malignancy have been demonstrated in several malignancies (4, 6, 9), but in gliomas, the role of AKAP12 is almost completely unknown. On the one hand, it has been shown that AKAP12 controls maintenance of the BBB and CNS angiogenesis in a VEGF-opposing manner under non-neoplastic conditions (11, 12). On the other hand, *AKAP12* constitutes a candidate gene potentially involved in the development of malignant gliomas harbouring a highly angiogenic potential. In this study, we show that AKAP12 is also localized in the perivascular space of normal brain vessels **(Fig. 1)**. These findings are in line with previous reports from animal experiments that SSeCKS (the rodent AKAP12 orthologue)-expressing astrocytes closely interact with ZO-1 expressing blood vessels by inhibiting VEGF expression via reduction of AP-1 and induction of the antipermeability factor Ang-1 leading to vessel quiescence (12). In our large cohort of 194 human astrocytomas, we still observed AKAP12 expression in the perivascular distribution in most astrocytomas of all WHO grades **(Fig. 1)**. In contrast, in WHO grade III anaplastic astrocytomas and WHO grade IV glioblastomas, AKAP12-negative blood vessels were also observed. In addition to astrocytic end-feet, which have been previously reported to be responsible for AKAP12 expression under non-neoplastic conditions, we could define vessel associated mural cells as another cellular source of AKAP12 **(supplemental Fig. 1-3)**. The finding of AKAP12-negative blood vessels in higher grade astrocytomas might be related to immature BBB properties partially lacking mature mural cells and/or properly functioning astrocytic endfeet (30, 31). In addition to its differential expression in the perivascular space, AKAP12 expression was inhomogeneously distributed in astrocytoma cells. While in the group of diffusely growing astrocytomas, only a low percentage of approximately

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20% of all cells exhibited only weak to moderate AKAP12 levels, more than 50% of all tumor cells in WHO grade I pilocytic astrocytomas displayed a strong AKAP12 expression **(Fig. 1 and 2)**. Since expression characteristics and functional data obtained in prostate, gastric, and hepatic cancer suggest a tumor suppressive function of AKAP12, its significant downregulation in the majority of diffuse astrocytomas as compared to WHO grade I pilocytic astrocytomas may correspond to malignant transformation which is in line with the clinical course of diffuse astrocytomas (4, 6, 9). The constantly high AKAP12 expression in slowly growing, curable pilocytic astrocytomas may reflect a preserved AKAP12 anti-tumoral activity. The similar AKAP12 protein expression levels in all diffuse astrocytomas and normal CNS tissue samples obtained from immunoblotting analyses is likely to result from a dilution effect related to the fact that normal CNS shows strong AKAP12 expression at the glial-vascular junctions while diffuse astrocytomas display much lower AKAP12 expression levels, however on a slightly higher absolute number of tumor cells **(Fig. 1-3 and supplemental Fig. 4)**. Since TCGA analysis showed chromosomal losses of the AKAP12 locus in only 9.7% of human glioblastomas and no AKAP12 mutations are known for pilocytic astrocytomas, these genetic data do not explain the distinct AKAP12 expression differences in the different astrocytoma subgroups (32, 33). To further analyze the potentially underlying mechanism of differential AKAP12 expression in astrocytoma subtypes, we analyzed their promoter methylation pattern since the AKAP12 gene is a target for epigenetic silencing in gastric, hepatocellular and lung cancer (4, 6, 34). Our study demonstrates that hypermethylation of *AKAP12α* promoter is specific for human diffuse astrocytoma tissue samples and various GBM cell lines **(Fig. 4)**. Thus, gene silencing by promoter hypermethylation may be the cause for the significant decrease of AKAP12 protein levels in GBM cells. Although existing antibodies fail to distinguish between AKAP12 isoforms, data on

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AKAP12α and β transcripts suggest that hypermethylation of the *AKAP12α* promoter is responsible for epigenetic silencing of AKAP12 (6). This is supported by the fact that highly methylated primary GBM cell lines with decreased *AKAP12α* promoter methylation showed increased expression of AKAP12α mRNA and protein expression after 5-aza-dC treatment **(Fig. 5 and supplemental Fig. 7)**. Interestingly, we determined that even a small decrease in average AKAP12α methylation can result in a strong increase in mRNA expression levels in the GBM cell lines. This finding may suggest that only a fraction of primary GBM cells in the treated pool show AKAP12α reactivation due to promoter demethylation, resulting in an exponential increase in transcript abundance, further emphasizing the importance of epigenetic regulation in AKAP12 expression control.

In contrast to the distinct hypermethylation of only the *AKAP12α* promoter in hepatocellular carcinoma, this finding was not observed in human astrocytoma (6). However, the *AKAP12α* and *β* promoter region was not hypermethylated to the same extent in our cohort, as seen e.g. in gastric cancer (4). Moreover, since the observed methylation pattern shows hypermethylation in all WHO grades of diffuse astrocytomas but not in pilocytic astrocytoma as compared to normal CNS tissue, this is likely to reflect a tumor-relevant epigenetic regulation in the development of astrocytoma subtype, although pilocytic astrocytomas share most transcriptional network changes with high grade gliomas except for single repressed pathways and specific genetic alterations (35, 36). Taken together, the findings of aberrant AKAP12 expression and its functionally relevant epigenetic modulation in human astrocytomas constitute a novel differential epigenetic methylation pattern showing lower methylation rates in pilocytic but higher methylation rates in the group of diffuse astrocytomas WHO grade II-IV. Finally, the presented data indicate a coordinate control between the *AKAP12* promoters in association with the development of

distinct astrocytoma subtypes. Further functional studies are needed to elucidate the putative tumor suppressive role of AKAP12 in human astrocytomas.

List of abbreviations:

AKAP12: A Kinase Anchor Protein 12

5-aza-dC: 5-aza-2'deoxycytidine

BBB: Blood-brain barrier

CNS: Central nervous system

FFPE: Formalin-Fixed Paraffin-Embedded Material

GBM: Glioblastoma

MRI: magnetic resonance imaging

PA: Pilocytic astrocytoma

SSeCKS: Src-Suppressed C Kinase Substrate

WHO: World Health Organisation

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FIGURE LEGENDS

Figure 1: *AKAP12 is selectively overexpressed in human pilocytic astrocytomas as compared to the normal CNS and diffusely infiltrating astrocytomas.*

(A-F) Immunohistochemical analysis in normal human CNS tissue and human astrocytomas of different WHO grades reveals AKAP12 expression in **(A)** normal brain samples in a perivascular localization (arrow) while the surrounding CNS tissue remains negative. **(B)** In WHO grade I pilocytic astrocytomas, apart from the still perivascular expression (arrow), most tumor cells strongly exhibit AKAP12 expression (inset: isotype control). **(C)** In WHO grade II diffuse astrocytomas, AKAP12 is predominantly localized in the perivascular space (black arrows) while most tumor cells (white arrows) display only a very subtle AKAP12 expression. In WHO grade III anaplastic astrocytomas **(D, E)** and glioblastomas **(F-H)**, absent to moderate AKAP12 expression levels are seen on tumor cells while blood vessel were either AKAP12-positive **(D, G, H,** arrows**)**, while AKAP12-negative blood vessels were also observed **(E, F,** arrows**)**. (scale bar = 50 μm for all images).

Figure 2: *AKAP12 is significantly upregulated in human WHO grade I pilocytic astrocytomas as compared to the group of diffuse astrocytomas WHO grade II-IV.* Quantification of relative proportions of AKAP12-positive tumor cells as determined by means of immunohistochemical analyses. The means of ratios of AKAP12 cell populations in each WHO grade were compared by a one-way analysis of variance (ANOVA) and for the post-hoc testing Tukey-Kramer's HSD test was used with a global significance level of 5%. (*** = $p < 0.0001$ as compared to WHO grade I pilocytic astrocytomas).

Figure 3: *Immunoblotting of AKAP12 in WHO grade I-IV human astrocytoma and normal brain.*

Immunoblotting of AKAP12 in whole tissue lysates from randomly selected cases of WHO grade I-IV human astrocytomas and normal brain showing a selective upregulation in WHO grade I astrocytomas. β-actin served as a loading control.

Figure 4: *The AKAP12α promoter is significantly hypermethylated in diffuse human astrocytomas of WHO grade II-IV as compared to WHO grade I pilocytic astrocytomas.*

(A) Quantitative DNA methylation analysis of the AKAP12α promoter region in normal brain (NB), pilocytic astrocytoma (PA), astrocytoma WHO grade II/III (II/III), primary glioblastoma samples (GBM) and GBM cell lines (CL). Each column represents the CpGs island analyzed; each row represents one patient sample. Percentages of methylation span from 0% to 100%. **(B)** The average percentage of methylation is blotted for each sample represented in (A). Horizontal bars represent average methylation values for each group. WHO grade II-III diffuse astrocytomas and primary WHO grade IV glioblastoma samples show statistically significant hypermethylation as compared to WHO grade I pilocytic astrocytoma (*** = p<0.001; ** = p=0.0034 in Mann-Whitney U test).

Figure 5: *AKAP12α is re-expressed in primary glioblastoma cells upon treatment with the demethylating agent 5-aza-2'-deoxycytidine.*

(A) Primary glioblastoma cells (NCH82, NCH89, and NCH440) were treated with 0 µmol/l (-DAC) or 2 µmol/l (+DAC) of the demethylating agent 5-aza-2'-deoxycytidine (DAC) for 72 hours. *AKAP12α* promoter methylation was determined before and after

treatment (n=3). **(B)** Quantitative real-time PCR showing upregulation of isoform *AKAP12α* transcript relative to *ACTB*, *GAPDH* and *HPRT1* after 72h 5-aza-2'-deoxycytidine treatment (n=3). **(C)** LINE-1 serving as internal positive control for demethylation experiments (n=3).

Supplementary data to

Differential expression of the tumor suppressor A kinase anchor protein 12 (AKAP12) in human diffuse and pilocytic astrocytomas is regulated by promoter methylation.

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Supplementary Tables:

Supplementary Table 1: Primers used for methylation experiments.

Isoform	Amplicon	Amplicon size (bp)	Left primer	Right primer	Number of cycles	Annealing temperature range (°C)
AKAP12-α	A1	196	GTAGAGGTGGTTTGGATGGGTA	CAAACCACRAATAAAATAAACCAC	10+37	61→56
AKAP12-β	B1	460	AAGTTGAGATATAGAAGTATTAGT	CCTAATCTCCTACCTACCAAC	10+37	61→56

Touch-down PCRs were performed (10 cycles with a range of temperatures decreasing of -0.5°C at each cycle, and 37 or 40 cycles at the final low temperature).

Supplementary Table 2: Primers used for mRNA expression determination.

Isoform	Amplicon size (bp)	Forward primer 5'-->3'	Reverse primer 5'-->3'	Number of cycles	Annealing temperature (°C)
AKAP12α	79	AACGGTCAAGGAGCCCTAAA	CATCTTCAGAGTCTCTCTGTCCAA	45	60
AKAP12β	89	CCGCTAAGCTGATCTCCTGT	CATCTTCAGAGTCTCTCTGTCCAA	45	60
ActinB	76	ATTGGCAATGAGCGGTTC	GGATGCCACAGGACTCCAT	45	60
HPRT1	102	TGACCTTGATTTATTTTGCATACC	CGAGCAAGACGTTCAGTCCT	45	60
TBP	119	AGCCAAGAGTGAAGAACAGTCC	CACAGCTCCCCACCATATTC	45	60

AKAP12α and AKAP12β expression values were normalized against the average expression of the three house-keeping genes ActinB, HPRT1 and TBP for the mRNAs from cell lines and ActinB and HPRT1 for the mRNAs from CNS/glioma tissues.

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Supplementary Information to Material and Methods:

Immunoblotting.

The preparation of total protein lysates, subsequent SDS-PAGE and Western immunoblotting were performed using the following antibodies: goat polyclonal anti-AKAP12 (dilution 1:1000; Santa Cruz Biotechnology) and a mouse monoclonal anti-AKAP12 antibody (dilution 1:1000; Abcam). Primary antibodies were diluted in TBST (5 % milk powder) and incubated at 4°C overnight. Equal loading of protein samples was confirmed via β-actin detection using a mouse monoclonal anti-actin antibody (dilution 1:10000, MP Biomedicals, Illkirch, France). Membranes were then incubated with a peroxidase (HRP) conjugated corresponding secondary antibody (dilution 1:2000; all from Cell Signaling Technology, Boston, MA) at room temperature for 1 h. Detection was performed using the chemiluminescence reagent Western Lightning® (Perkin Elmer, Rodgau, Germany). Blots were visualized and densitometrical analyses were performed using the FluorChem® SP Imaging System and the AlphaEase® FC Software (Alpha Innotech, San Leandro).

Genetic analyses for IDH-1/2 mutation

The IDH1 mutational status was assessed in all tumors samples by a mutation-specific monoclonal antibody for the IDH1 R132H mutation in tumor samples as described previously (1). Cases lacking the R132H mutation were assessed by direct sanger sequencing of the relevant exon for other rare IDH1 and IDH2 mutations using primer pairs as described in (2). DNA was extracted from GBM tissue sections using a BlackPREP FFPE kit (Analytik Jena, Germany), according to the manufacturer's instructions, from paraffin blocks microscopically controlled for at least 70% tumor content.

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Supplemental Figure legends:

Supplemental Fig. 1: *Immunofluorescent analyses of AKAP12 in the normal human brain.*
Immunofluorescent stainings of normal brain tissue show a similar expression pattern of collagen IV and AKAP12 indicating that AKAP12 is expressed in perivascular areas where basement membranes are present. In contrast, no co-expression of CD31-positive endothelial cells and AKAP12 was observed indicating a lack of AKAP12 expression in the respective cell type.

Supplemental Fig. 2: *Immunofluorescent analyses of AKAP12 in glioblastomas.*
In line with the findings in normal human brain samples, AKAP12 - if present in glioblastomas - was also most strongly expressed in areas where vessel associated collagen IV-positive basement membranes were present surrounding CD31-positive endothelial cells.

Supplemental Fig. 3: *Mural cells of blood vessels in glioblastomas are a source of AKAP12 expression.*
AKAP12 is co-expressed in α-SMA-positive cells in glioblastoma-associated vessels indicating that mural cells (pericytes/smooth muscle cells) are a major source of AKAP12 expression between areas where collagen IV-positive basement membranes are present. In contrast, GFAP-positive glioblastoma cells and CD31-positive endothelial cells are mainly AKAP12-negative.

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Supplemental Fig. 4: *AKAP12 protein expression is strongly upregulated in pilocytic astrocytomas.*

Immunoblotting of AKAP12 in whole tissue lysates from randomly selected cases of WHO grade I-IV human astrocytomas showing a selective upregulation in WHO grade I astrocytomas. β-actin served as loading control.

Supplemental Fig. 5: *AKAP12β promoter methylation status in human astrocytomas.*

There is a trend for *AKAP12β* promoter hypermethylation in diffuse human astrocytomas of WHO grade II-IV as compared to WHO grade I pilocytic astrocytomas although not reaching statistical significance. The average percentage of methylation is blotted for each sample of the AKAP12β promoter region. Horizontal bars represent average methylation values for each group. Normal brain (NB), pilocytic astrocytoma (PA), astrocytomas (WHO II-III) / primary GBM samples (GBM) and GBM cell lines (CL).

Supplemental Fig. 6: *IDH1/2 mutations are not associated with AKAP12α promoter methylation levels.*

Methylation levels of human astrocytomas of WHO grade I-IV are depicted according to the IDH1/2 mutation status. First, astrocytomas were assessed for expression of the mutant IDH1 (R132H) protein. For negative cases, sanger sequencing for mutation in the IDH1/2 gene was performed.

Supplemental Fig. 7: *AKAP12 protein expression correlates with the AKAP12 promoter methylation status in glioma cell lines.*

AKAP12 immunocytochemistry of FFPE glioma cell lines is depicted. Glioma cell lines with high AKAP12α promoter methylation levels (LN-18 is shown as an example of this group)

present with marked lower AKAP12 protein expression levels as compared to a strong AKAP12 protein expression seen in glioma cells with considerably lower AKAP12α promoter methylation status (U87MG is shown as an example of this group).

Supplemental Fig. 8: *Reactivation of AKAP12 protein expression in primary glioblastoma cells upon demethylation treatment.*
Primary glioblastoma cell lines NCH82 and NCH440 were treated with 0µmol/l (-DAC) or 2µmol/l (+DAC) 5-aza-2'-deoxycytidine for 72h. Immunoblotting for AKAP12 protein is depicted. β-actin served as loading control.

Supplemental Fig. 9: *Reactivation of AKAP12β in primary glioblastoma cells by demethylating drug treatment.* Primary glioblastoma cell lines NCH82, NCH89 and NCH440 were treated with 0µmol/l (-DAC) or 2µmol/l (+DAC) 5-aza-2'-deoxycytidine for 72h (see also Fig. 5). Re-expression of *AKAP12β* transcript relative to *ACTB*, *GAPDH* and *HPRT1* after 72h 5-aza-2'-deoxycytidine treatment.

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Table 1: Summary of tissue specimens and patient data

	Normal CNS tissue	Pilocytic astrocytoma WHO°I	Diffuse astrocytoma WHO°II	Anaplastic astrocytoma WHO°III	Glioblastoma WHO°IV	Recurrence of glioblastoma	Infiltration zone of glioblastoma
Male/female	13/10	17/9	21/16	32/20	46/33	20/11	14/11
Median age (range)	57 (1-92)	19.0 (3-56)	38.0 (23-77)	42.5 (19-76)	59 (23-88)	57 (27-69)	64 (23-77)
Specimens (n)	23	26	37	52	79	31	25
Karnofsky median (range)		100 (90-100)	90 (60-100)	90 (40-90)	90 (50-100)		
Tumor localisation (supratentorial/infratentorial)		11/15	35/2	52/0	79/0	31/0	25/0
Complete resection (% of cases)		46.0	32.4	32.7	38.0		
Edema score (no/moderate/strong)		10/1/1	20/11/3	10/25/15	1/15/50		
median follow-up (range) in months		50.3 (2.4-113.1)	41.1 (1.1-150.9)	24.4 (0.5-125.7)	9.8 (0.5-70.3)		
mIDH1 (R132H) (mut/wt)		0/26	16/19	24/26	2/74		