Tumor and Stem Cell Biology

CAMTA1, a 1p36 Tumor Suppressor Candidate, Inhibits Growth and Activates Differentiation Programs in Neuroblastoma Cells

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Abstract

A distal portion of human chromosome 1p is often deleted in neuroblastomas and other cancers and it is generally assumed that this region harbors one or more tumor suppressor genes. In neuroblastoma, a 261 kb region at 1p36.3 that encompasses the smallest region of consistent deletion pinpoints the locus for calmodulin binding transcription activator 1 (CAMTA1). Low CAMTA1 expression is an independent predictor of poor outcome in multivariate survival analysis, but its potential functionality in neuroblastoma has not been explored. In this study, we used inducible cell models to analyze the impact of CAMTA1 on neuroblastoma biology. In neuroblastoma cells that expressed little endogenous CAMTA1, its ectopic expression slowed cell proliferation, increasing the relative proportion of cells in G1/G0 phases of the cell cycle, inhibited anchorage-independent colony formation, and suppressed the growth of tumor xenografts. CAMTA1 also induced neurite-like processes and markers of neuronal differentiation in neuroblastoma cells. Further, retinoic acid and other differentiation-inducing stimuli upregulated CAMTA1 expression in neuroblastoma cells. Transcriptome analysis revealed 683 genes regulated on CAMTA1 induction and gene ontology analysis identified genes consistent with CAMTA1-induced phenotypes, with a significant enrichment for genes involved in neuronal function and differentiation. Our findings define properties of CAMTA1 in growth suppression and neuronal differentiation that support its assignment as a 1p36 tumor suppressor gene in neuroblastoma. Cancer Res; 71(8); 3142–51. ©2011 AACR.

Introduction

Neuroblastoma is a childhood tumor derived from precursor cells of the sympathetic nervous system. The clinical and biological behavior of this tumor is remarkably heterogeneous, encompassing fatal tumor progression, as well as spontaneous regression and differentiation into mature ganglioneuroma. Deletion within distal 1p characterizes about 30% of neuroblastomas and also frequently occurs in a broad range of other human malignancies including colorectal cancer, glioma, breast cancer, and melanoma. Further, 1p36 deletion is an independent predictor of neuroblastoma progression (1). Thus, it is widely assumed that distal 1p harbors genetic information mediating tumor suppression. The combination of recent fine mapping studies (2, 3) defines a 1p36.3 smallest region of consistent deletion shared by virtually all 1p-deleted neuroblastomas that spans 261 kb between D1S214 and D1S2731, encompassing the CAMTA1 locus (4). CAMTA1 encodes a calmodulin-binding transcription activator (5, 6) that is predominantly expressed in neuronal tissues (7). There is no evidence for CAMTA1 mutations in neuroblastoma (8), however, low CAMTA1 expression is significantly associated with markers of unfavorable tumor biology and poor outcome. Intriguingly, the prognostic value of CAMTA1 expression is independent of established risk markers, including 1p deletion, in multivariate survival analysis (4). Additional evidence supporting CAMTA1 involvement in tumor development comes from glioma and colon cancer. CAMTA1 is homozygously deleted in a subset of gliomas (9) and is the only gene mapping to the 1p36 smallest region of overlapping heterozygous deletion in this entity (10). In colorectal cancer, genome-wide copy number analysis revealed that loss of a 2 Mb region encompassing CAMTA1 has the strongest impact on survival among all identified genomic alterations (11). Further, as in neuroblastoma, low expression of CAMTA1 is an independent predictor of poor outcome in colorectal cancer (11).
In this study, we explore the effect of CAMTA1 on neuroblastoma biology using inducible cell models. Our data imply that CAMTA1 is a 1p36 tumor suppressor candidate that inhibits features of malignant cells and is involved in neuroblastoma cell differentiation.

Materials and Methods

Cell culture

Culture of the neuroblastoma cell lines SH-EP, IMR5-75, and Be(2)-C, was described previously (12). All lines were kindly provided by Dr. Larissa Saveljeva (German Cancer Research Center) and authenticated by multiplex-FISH karyotyping at the start of the project. Cells were tested for mycoplasma, viral, and foreign cell contamination using the Multiplex cell Contamination Testing (McCT) Service (13). Drugs were added at the following concentrations: all-trans retinoic acid (Sigma), 10 μmol/L (in ethanol, end concentration did not exceed 0.1%); valproic acid (Sigma), 1 mmol/L (in Dulbecco’s PBS); Helminthosporium carbonum-toxin (HC-toxin; Sigma, Lot #654K121), 15 mmol/L (in methanol, end concentration did not exceed 0.02%).

Polyclonal antibody production and Western blotting

A custom polyclonal CAMTA1 antibody was raised in rabbits against the epitope peptide NH2-CHRLYKRSER-IKEGQGT-COOH, representing the COOH-terminal CAMTA1 region (Pineda Antikörper-Service). Final bleeds were affinity purified, and antibody specificity was confirmed via Western blotting (Qiagen) were used for amplification of genes were described previously (4). QuantiTect Primer Assays (Qiagen) were used for amplification of tubulin, beta 3 (TUBB3, Hs_TUBB3_1_SG); neurofilament, light polypeptide (NEFL, Hs_NEFL_1_SG); microtubule-associated protein 2 (MAP2, Hs_MAP2_1_SG), cyclin-dependent kinase inhibitor 1C (p57Kip2) (CDKN1C, Hs_CDKN1C_1_SG); tropomodulin 2 (neuronal) (TMOD2, Hs_TMOD2_1_SG); sodium channel, voltage gated, type VIII, alpha subunit (SCN8A, HsSCN8A_1_SG); Si100 calcium binding protein B (Si100B, Hs_SI00B_1_SG); and stathmin-like 3 (STMN3, Hs_STMN3_1_SG).

Microarray analysis

Total RNA was isolated using Trizol (Invitrogen) from CAMTA1 expressing SH-EP cells at 0, 3, 6, 12, and 24 hours after CAMTA1 induction and at 12 hours from noninduced controls. Two biological replicates were carried out for time-series experiments. RNA was converted to cRNA, labeled, and hybridized to Agilent whole human genome 4 × 44 K (G1112F) microarrays according to the Two-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technologies). Raw data were background-corrected using the “normexp”-method and quantile-normalized employing the “limma” package included in the Bioconductor release 2.4 (www.bioconductor.org) for R statistical software v2.9.0 (www.r-project.org). Unspecific filtering was applied to the normalized data as implemented in the “genefilter” R package (Bioconductor release 2.4) for each biological replicate separately (15). Probes were selected for which expression values were greater than or equal to the first quartile of the expression range for at least 2 time points (rather than selecting an absolute expression

growth assays, cells were seeded in triplicate onto 6-well plates (1,500 per well), and growth rates were determined by Alamar Blue assay (AbD Serotec) on days 0, 2, 4, and 6 according to the manufacturer’s instructions. Cells were formaldehyde-fixed and stained with 10% Giemsa solution to visualize colonies 2 weeks after seeding.

For soft agar assays, 6-well plates were precoated with 0.7% agarose in full medium (RPMI-1640 supplemented with 10% FCS), and 4,000 cells were seeded into 0.35% agarose in full medium per well in triplicate. Cells were fed weekly and stained with crystal violet 4 weeks after seeding.

Growth of xenograft tumors in nude mice

IMR5-75 cells were cultured to 80% confluency, harvested, and suspended in Matrigel (BD Bioscience). Eight-week-old athymic NCR (nu/nu) mice were inoculated s.c. in the flank with 2 × 10^6 cells in 200 μL Matrigel (sample size: 8 mice inoculated with IMR5-75-CAMTA1 and 6 mice inoculated with IMR5-75-LacZ control cells). Doxycycline was administered via drinking water (2 mg/ml) and oro gastric lavage (2 mg/mouse) when all tumors were progressive and reached a volume of at least 100 mm^3. Tumor size was measured with a digital calliper to calculate tumor volume. Mice were sacrificed at day 4 after induction.

Quantitative real-time RT-PCR

The quantitative real-time reverse transcriptase PCR (QPCR) protocol and primers for CAMTA1 and housekeeping genes were described previously (4). QuantiTect Primer Assays (Qiagen) were used for amplification of tubulin, beta 3 (TUBB3, Hs_TUBB3_1_SG); neurofilament, light polypeptide (NEFL, Hs_NEFL_1_SG); microtubule-associated protein 2 (MAP2, Hs_MAP2_1_SG), cyclin-dependent kinase inhibitor 1C (p57Kip2) (CDKN1C, Hs_CDKN1C_1_SG); tropomodulin 2 (neuronal) (TMOD2, Hs_TMOD2_1_SG); sodium channel, voltage gated, type VIII, alpha subunit (SCN8A, HsSCN8A_1_SG); Si100 calcium binding protein B (Si100B, Hs_SI00B_1_SG); and stathmin-like 3 (STMN3, Hs_STMN3_1_SG).
value as a cutoff) to select genes with quantifiable expression in at least 2 measurements. A relaxed threshold for the interquartile range (IQR) filter was selected (0.3) and applied to exclude genes with low variability. Only probes which showed a positive Pearson correlation between biological replicates ($r > 0.8$) were included in analyses. The remaining intersection of filtered probes for both biological replicates was used in unsupervised hierarchical clustering of Pearson correlation distances ($1 - Pearson correlation coefficients$) to obtain clusters with common expression profiles. All arrayed probes are henceforth referred to as genes for simplification. Gene Ontology Tree Machine (GOTM) was used for functional annotation of expression data (16). GOTM compares the distribution of GO terms within a gene set (defined here as gene clusters with common expression profiles) to that in a reference gene set (defined here as all genes represented on the G4112 array). To test for a statistically significant enrichment of GO terms within gene sets, a hypergeometric test was used with a significance level of 0.01 (16).

Survival analysis

CAMTA1 expression was derived from expression profiling data from a cohort of 251 neuroblastomas (17). Of 251 tumors, 70 were previously analyzed for CAMTA1 expression by cDNA microarray or QPCR (4). All patients were enrolled in the German Neuroblastoma Trial and diagnosed between 1989 and 2004 ($n = 68$ stage 1, $n = 46$ stage 2, $n = 39$ stage 3, $n = 67$ stage 4, $n = 31$ stage 4S; $n = 31$ MYCN amplified, $n = 220$ MYCN nonamplified; $n = 168$ age at diagnosis $< 1.5$ years, $n = 83$ age at diagnosis $\geq 1.5$ years). Criteria for sample selection were availability of sufficient amounts of tumor material, 60% or more tumor content, and RNA integrity number more than 7.5. The composition of the cohort in terms of tumor stage, MYCN status and age at diagnosis was in agreement with the composition of an unselected cohort of 940 patients diagnosed between 1995 and 2001 in Germany (data not shown). Univariate survival analysis was done to validate established prognostic variables as described previously (4). Multivariate Cox regression was used to investigate the prognostic power of CAMTA1 expression adjusting for established prognostic variables as described previously (4). The cutoff value for dichotomization of CAMTA1 expression was estimated by maximally selected log-rank statistics (18). Parameter estimate shrinkage was applied to correct for potential overestimation of the hazard ratio estimate due to cutoff selection (19). Bootstrap resampling, together with a shrinkage procedure, was used to correct confidence limits and $P$ values (20). Event-free survival (EFS) was measured from date of diagnosis until occurrence of disease progression, relapse, or death from disease. EFS times of patients who experienced no events within the follow-up time were censored.

Results

Low CAMTA1 expression predicts poor neuroblastoma outcome

Low CAMTA1 expression was previously identified as a predictor of poor outcome (4). To validate the prognostic value of CAMTA1 in a larger set of patients, CAMTA1 expression was derived from expression profiling data from a cohort of 251 neuroblastomas (17) and analyzed. Multivariate survival analysis confirmed low CAMTA1 expression as a predictor of poor outcome, independent of established risk markers, including 1p status, MYCN status, tumor stage and age of the patient at diagnosis (Table 1). Even within the cohort of 1p nondeleted tumors, CAMTA1 expression emerged as an independent prognostic factor (Table 2).

CAMTA1 suppresses growth of neuroblastoma cells in vitro and in vivo

The effect of CAMTA1 on neuroblastoma cell growth was explored in stable clones allowing tetracycline-inducible CAMTA1 expression in the SH-EP cell line, which has low endogenous CAMTA1 expression (21) (validated by QPCR, data not shown). Induction of CAMTA1 in SH-EP cells significantly decreased colony formation ability and growth rate

<table>
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<th>Factor</th>
<th>Effect</th>
<th>Hazard ratio (95% confidence limits)</th>
<th>$P$</th>
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<td>3, 4 vs. 1, 2, 4S</td>
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<td>Age</td>
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<tr>
<td>MYCN amplification</td>
<td>Yes vs. no</td>
<td>0.85 (0.41–1.76)</td>
<td>0.66</td>
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Table 1. Cox proportional hazards regression for event-free survival (251 neuroblastomas)

NOTE: CAMTA1 expression was derived from array expression data from a cohort of 251 neuroblastomas (17). Results specific for oligo probe A_32_P4981 are shown. Two other independent CAMTA1-specific probes (A_32_P4985 and A_24_P220921) revealed similar results. Established risk factors included in the model were all associated with decreased event-free survival in univariate survival analysis: 1p deletion (HR 4.05, $P < 0.001$), higher stage ($3$ and $4$; HR $3.36$, $P < 0.001$), age $\geq 1.5$ years (HR $3.8$, $P < 0.001$), and MYCN amplification (HR $3.55$, $P < 0.001$).

$^a$To correct for potential hazard ratio overestimation due to cutoff selection, parameter estimate shrinkage was applied. To correct confidence limits and $P$ values, bootstrap resampling, together with a shrinkage procedure, was used.
CAMTA1, a 1p36 Candidate Tumor Suppressor in Neuroblastoma

Table 2. Cox proportional hazards regression for event-free survival in patients without 1p deletion (195 neuroblastomas)

<table>
<thead>
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<th>Factor</th>
<th>Effect</th>
<th>Hazard ratio (95% confidence limits)</th>
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<td>1.14 (0.5–2.63)</td>
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<td>1.74 (0.76–3.99)</td>
<td>0.19</td>
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<tr>
<td>MYCN amplification</td>
<td>Yes vs. no</td>
<td>2.38 (0.27–20.99)</td>
<td>0.43</td>
</tr>
</tbody>
</table>

NOTES: CAMTA1 expression was derived from array expression data from 195 neuroblastomas (17). Results specific for oligo probe A_32_P4981 are shown. Two other independent CAMTA1-specific probes (A_32_P4985 and A_24_P220921) revealed similar results.

\*To correct for potential hazard ratio overestimation due to cutoff selection, parameter estimate shrinkage was applied. To correct confidence limits and \( \hat{P} \) values, bootstrap resampling, together with a shrinkage procedure, was used.

CAMTA1 induces markers of neuronal differentiation and is upregulated during neuroblastoma cell differentiation

Microscopic inspection of CAMTA1-induced SH-EP cells revealed a higher degree of morphological differentiation, including acquisition of neurite-like processes (Fig. 2A). To investigate whether this morphology is associated with induction of neuron-specific markers, we measured expression of genes encoding the early neuronal marker β3 tubulin (TUBB3), and the later neuronal markers microtubule associated protein 2 (MAP2) and neurofilament light chain (NEFL). CAMTA1 induction in SH-EP cells significantly increased the expression levels of all 3 neuronal markers compared with noninduced controls (Fig. 2B). To test whether CAMTA1 is upregulated during neuronal differentiation, we assessed CAMTA1 expression in 4 established neuroblastoma in vitro differentiation models that were extensively characterized in previous studies (22, 23): (i) Be(2)-C treated with retinoic acid, (ii) SH-EP treated with valproic acid, (iii) Be(2)-C treated with HC-toxin, and (iv) SH-EP treated with HC-toxin. Morphological differentiation and induction of the neuronal marker MAP2 were associated with a significant increase of CAMTA1 expression levels in all tested neuroblastoma differentiation models (Fig. 3). Together, these data support that CAMTA1 regulation is part of the response to differentiation signals and induces genes characteristic of neuronal differentiation.

CAMTA1 induces genetic programs mediating neuronal functions and growth inhibition

We investigated time-resolved genome wide transcription profiles of CAMTA1-induced SH-EP cells to analyze the global molecular changes induced by the transcription factor CAMTA1 and to elucidate the biological basis of the observed CAMTA1-associated phenotype. We identified a total of 683 genes regulated on CAMTA1 induction (Supplementary Table S1). Unsupervised clustering resulted in 5 clusters comprising genes with common dynamic expression patterns (Fig. 4). Of these, 2 clusters (A, 368 genes and B, 133 genes) contained genes that were time-dependently upregulated by CAMTA1 induction, and whose expression was unchanged in the 12 hours noninduced control. Cluster E comprised 88 genes downregulated on CAMTA1 induction (expression unchanged in noninduced control). All CAMTA1-regulated genes from clusters A, B, and E were associated to GO annotations. This categorization of genes into functional classes was used to provide insight into the molecular processes contributing to the CAMTA1-induced phenotype. We also tested whether specific GO terms were enriched among CAMTA1-induced or CAMTA1-repressed genes. A given GO term was considered enriched when the observed number of genes from that category was significantly greater than the number expected by chance (\( P < 0.01 \)). GO terms enriched among CAMTA1-induced genes (Fig. 4, clusters A and B) reflected the CAMTA1-associated differentiation phenotype.

(76x254) in vitro both shifts the cell cycle away from proliferation and suppresses together, these data show that higher CAMTA1 expression negative controls had no significant effect (Fig. 1E). Taken together, these data support that CAMTA1 regulation is part of the response to differentiation signals and induces genes characteristic of neuronal differentiation.

CAMTA1-induced differentiation is achieved through both in vitro and in vivo growth of neuroblastoma cells.

We investigated time-resolved genome wide transcription profiles of CAMTA1-induced SH-EP cells to analyze the global molecular changes induced by the transcription factor CAMTA1 and to elucidate the biological basis of the observed CAMTA1-associated phenotype. We identified a total of 683 genes regulated on CAMTA1 induction (Supplementary Table S1). Unsupervised clustering resulted in 5 clusters comprising genes with common dynamic expression patterns (Fig. 4). Of these, 2 clusters (A, 368 genes and B, 133 genes) contained genes that were time-dependently upregulated by CAMTA1 induction, and whose expression was unchanged in the 12 hours noninduced control. Cluster E comprised 88 genes downregulated on CAMTA1 induction (expression unchanged in noninduced control). All CAMTA1-regulated genes from clusters A, B, and E were associated to GO annotations. This categorization of genes into functional classes was used to provide insight into the molecular processes contributing to the CAMTA1-induced phenotype. We also tested whether specific GO terms were enriched among CAMTA1-induced or CAMTA1-repressed genes. A given GO term was considered enriched when the observed number of genes from that category was significantly greater than the number expected by chance (\( P < 0.01 \)). GO terms enriched among CAMTA1-induced genes (Fig. 4, clusters A and B) reflected the CAMTA1-associated differentiation phenotype.
Figure 1. A. tetracycline-inducible CAMTA1 expression and detection via custom-made polyclonal antibody (here in SH-EP). B. CAMTA1 expression suppresses growth of SH-EP cells as determined by colony formation assay and Alamar Blue viability assay (mean ± SD, 3 replicates). C. CAMTA1 expression in SH-EP cells results in an increased proportion of cells in G1/G0 phase 48 hours after induction as determined by FACS analysis (1 of 3 replicates is shown). D. CAMTA1 suppresses anchorage-independent growth of IMR5–75 cells in soft agar. E. CAMTA1 induction suppresses growth of subcutaneous IMR5–75 tumors in nude mice. Sample size: 8 mice inoculated with IMR5–75-CAMTA1 and 6 mice inoculated with IMR5–75-LacZ negative control cells. Doxycycline was administered via drinking water (2 mg/mL) and orogastric lavage (2 mg/mouse) when all tumors were progressive and reached a volume of at least 100 mm³. RT-PCR was performed on total RNA isolated from 1 CAMTA1 ON and 1 CAMTA1 OFF tumor, respectively, to validate CAMTA1 induction via doxycycline administration. Cells allowing inducible LacZ expression were used as negative controls.
A large fraction of enriched GO terms related to neuronal differentiation or function (e.g., “nervous system development,” “transmission of nerve impulse,” “voltage-gated sodium channel activity,” and “neurofilament”). Overrepresentation of the GO term “kinase inhibitor activity” among CAMTA1-induced genes was in line with the observed inhibitory effect on cell cycle progression. The enrichment of the GO term “Ca\textsuperscript{2+}/calmodulin-dependent protein kinase complex” was of particular note considering the Ca\textsuperscript{2+}/calmodulin-dependent activity of CAMTAs (24). Among CAMTA1-repressed genes (Fig. 4, cluster E), the majority of enriched GO terms related to cell cycle associated processes. General inhibition of the cell cycle was indicated by overrepresentation of the GO term “regulation of cyclin-dependent kinase activity.” Mitotic inhibition was reflected by enrichment of GO terms, such as “mitosis” and “spindle organization and biogenesis.” Inhibition of DNA synthesis was indicated by overrepresentation of GO terms, such as “DNA replication initiation.” We chose 5 CAMTA1 targets that are representative of the functional classes “neuronal differentiation” and “cell cycle inhibition” for validation by QPCR in an independent SH-EP-CAMTA1 clone (Fig. 5): CDKN1C (p57 Kip2) that is involved in G1 phase arrest and is a critical terminal effector of pathways controlling differentiation, TMOD2, encoding a neuron-specific member of the tropomodulin family of actin-regulatory proteins, SCN8A, encoding a subunit of voltage gated sodium channels, S100B, encoding a Ca\textsuperscript{2+} binding protein involved in neurite extension and axonal proliferation and STMN3, a paralog of STMN2 (SCG10), which is implicated in terminal differentiation of sympathetic neurons (25). The consistent CAMTA1-dependent regulation of the tested genes in this independent setting supports the robustness of our approach. Overall, time-resolved expression profiling in CAMTA1-induced cell models and functional classification using GO term analysis indicate that CAMTA1 induces differentiation programs and inhibits effectors of cell cycle progression.

Discussion

CAMTA1 is pinpointed by a 1p36.3 smallest region of consistent deletion in neuroblastoma (2–4). In the absence of somatic mutations (8), low CAMTA1 expression is an independent predictor of poor survival as determined by QPCR and multivariate survival analysis in a cohort of 102 neuroblastomas (4). Here, we further confirmed this result in an extended cohort of 251 neuroblastomas employing oligonucleotide array expression data (17), supporting the robustness of this prognostic marker independent of the technical platform used. CAMTA1 is also included in most of the recently reported prognostic neuroblastoma expression classifier gene sets, highlighting its predictive power (17, 26–28). The consistently low expression of CAMTA1 in aggressive
neuroblastomas led us to hypothesize that (i) downregulation of CAMTA1 mediates a selective advantage of malignant neuroblastoma cells and (ii) reexpression of CAMTA1 in neuroblastoma cells with low endogenous CAMTA1 levels may inhibit features of malignancy. In line with this hypothesis, CAMTA1 induction in SH-EP cells suppressed colony formation and growth rate and induced accumulation of cells in the G1/G0 phase of the cell cycle. In IMR5-75 cells, CAMTA1 inhibited anchorage independent growth and in vivo growth in nude mice, further strengthening the role of CAMTA1 as a tumor suppressor candidate.

The induction of neurite-like processes and neuronal marker genes (TUBB3, MAP2, and NEFL) on CAMTA1 induction in SH-EP points to a role of CAMTA1 in neuroblastoma cell differentiation. This is further supported by CAMTA1 upregulation in different in vitro models of neuroblastoma differentiation. The histone deacetylase inhibitors used here, valproic acid and HC-toxin, exhibit antineuroblastoma activity (23, 29) and are candidates for future clinical use. Retinoic acid is already implemented in the postconsolidation therapy of stage 4 neuroblastomas (30). Whether upregulation of CAMTA1 contributes to the antineuroblastoma properties of these drugs needs to be addressed in further studies.

CAMTA1 acts as a transcription activator (6). Our results from integrating CAMTA1-induced transcription profiles and corresponding GO annotations are consistent with the idea that CAMTA1 regulates effectors of neuronal function and cell cycle inhibition. High expression of neuron-specific genes is a feature of localized tumors (stages 1 and 2) (31) and, despite poor histological differentiation, disseminated 4s tumors (32). The high expression of CAMTA1 in stages 1, 2, and 4s tumors (4) may indicate that the genetic programs induced by CAMTA1 in vitro contribute to the favorable phenotype of this subgroup in vivo. Intracellular Ca\(^{2+}\) fulfills a pleiotropic role in both the physiology and differentiation of neuronal cells, and neuritic outgrowth can be induced in neuroblastoma cells by promoting Ca\(^{2+}\) influx (33). CAMTA family members respond to Ca\(^{2+}\) signaling by binding to calmodulin (24), and the GO term "Ca\(^{2+}\)/calmodulin-dependend protein kinase complex" was enriched among CAMTA1-induced genes. This suggests that CAMTA1 acts as both integrator and effector of Ca\(^{2+}\)-dependent processes in neuronal differentiation. GO terms enriched among CAMTA1-repressed genes indicate that processes of mitosis and DNA replication are inhibited by CAMTA1. Together with the previous observation that CAMTA1 is

Figure 3. CAMTA1 is induced in neuroblastoma differentiation models. QPCR reveals induction of CAMTA1 and the neuronal marker MAP2 in Be(2)-C cells treated with all-trans retinoic acid or Helminthosporium carbonum (HC)-toxin, and SH-EP cells treated with valproic acid or HC-toxin (mean ± SD, 3 replicates). Incubation time was 5 days for valproic acid or HC-toxin and 7 days for all-trans retinoic acid. Morphological differentiation was confirmed microscopically.
expressed in a cell cycle dependent manner with highest levels in S and M phase (21), this may indicate that CAMTA1 acts as a negative regulatory factor during DNA synthesis and mitosis.

A variety of regulatory mechanisms could be responsible for the downregulation of CAMTA1 in unfavorable neuroblastomas. In line with a haploinsufficiency model, CAMTA1 expression is lower in 1p deleted neuroblastomas (4). However, low CAMTA1 expression predicts poor outcome also within the subgroup of 1p nondeleted neuroblastomas, which calls for additional negative regulators of CAMTA1 expression. A common mechanism mediating transcriptional repression of growth-regulating genes in tumors is methylation of cytosine residues in gene-associated CpG islands. However, we found no evidence for CAMTA1 promotor methylation using methylation specific PCR on bisulfite treated neuroblastoma DNA samples (data not shown). Further epigenetic factors may play a role. It has been reported that histone deacetylase inhibitors reexpress silenced tumor suppressors including p21	extsuperscript{WAF1/CIP1}, p16, p53	extsuperscript{Rb}, and p19	extsuperscript{INK4d} (34). The induction of CAMTA1 on treatment with the histone deacetylase inhibitors valproic acid and HDAC-toxin is in line with a similar regulatory model. Whether CAMTA1 induction by HDAC inhibitors involves chromatin remodeling at the CAMTA1 locus or whether factors upstream of CAMTA1 are activated, remains to be investigated.

To pinpoint 1p36 tumor suppressor genes, a previous study used chromosome engineering generating mouse models with gain and loss of a region corresponding to human 1p36 (35). Gain of this region inhibited proliferation, whereas loss of the same region rendered cells sensitive to oncogenic transformation. In search of the gene(s) mediating this phenotype, several candidates, including CAMTA1, were knocked down to test whether their depletion could reverse the proliferation defect.

Figure 4. Genetic programs induced by CAMTA1 in SH-EP cells as determined by time-resolved whole-genome microarray expression analysis. RNA harvested at 3, 6, 12, and 24 hours after CAMTA1 induction and at 12 hours without induction (control) was hybridized against RNA harvested at time point 0 hour (uninduced). Experiments were done in 2 biological replicates. Gene clustering based on Pearson correlation coefficients revealed 5 clusters containing genes with common time-dependent expression profiles on CAMTA1 induction (Clusters A–E). Left panel, GO terms significantly enriched among CAMTA1 induced genes (clusters A + B) according to GOTM analysis (P < 0.01). Right panel, GO terms significantly enriched among CAMTA1 repressed genes (cluster E) according to GOTM analysis (P < 0.01).
of mouse embryonic fibroblasts with gain of the 1p36 homologous region. In this assay, knockdown of most tested genes, including CAMTA1, had no significant effect, whereas knockdown of another gene (CHD5), functionally rescued the proliferation defect. The failure of CAMTA1 to show an effect in this context is likely to be due to tissue specificity. CAMTA1 is predominantly expressed in neuronal tissues and, in light of the data presented here, its growth suppressive effect in neuroblastoma cells is closely linked to its ability to induce effectors of neuronal differentiation. In a mouse embryonic fibroblast background, both the expression of CAMTA1 and the potential to induce differentiation are likely to be limited.

Together, our data suggest that CAMTA1 is a 1p36 tumor suppressor candidate that inhibits key features of malignant cells and is involved in neuronal differentiation. Understanding the function of CAMTA1 may help develop diagnostic tools and/or effective therapeutic strategies for children with unfavorable neuroblastoma. Further dissection of CAMTA1 downstream signaling and identification of mechanisms regulating CAMTA1 will be the points of departure to reach this goal.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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