Histone Chaperone CHAF1A Inhibits Differentiation and Promotes Aggressive Neuroblastoma

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Abstract

Neuroblastoma arises from the embryonal neural crest secondary to a block in differentiation. Long-term patient survival correlates inversely with the extent of differentiation, and treatment with retinoic acid or other prodifferentiation agents improves survival modestly. In this study, we show the histone chaperone and epigenetic regulator CHAF1A functions in maintaining the highly dedifferentiated state of this aggressive malignancy. CHAF1A is a subunit of the chromatin modifier chromatin assembly factor 1 and it regulates H3K9 trimethylation of key target genes regulating proliferation, survival, and differentiation. Elevated CHAF1A expression strongly correlated with poor prognosis. Conversely, CHAF1A loss-of-function was sufficient to drive neuronal differentiation in vitro and in vivo. Transcriptome analysis of cells lacking CHAF1A revealed repression of oncogenic signaling pathways and a normalization of glycolytic metabolism. Our findings demonstrate that CHAF1A restricts neural crest differentiation and contributes to the pathogenesis of high-risk neuroblastoma.

Introduction

Neuroblastoma arises from residual immature neural crest cells within the peripheral sympathetic ganglia of very young children. The clinical and biologic behavior of this tumor is extremely heterogeneous, encompassing fatal tumor progression, as well as spontaneous regression and differentiation into mature ganglioneuroma. Furthermore, the degree of neuronal tumor differentiation strongly affects patient outcome. Studies from transgenic mouse models of neuroblastoma with targeted overexpression of the MYCN oncogene also demonstrate that blocked neural crest differentiation leads to the malignant transformation of neuroectodermal precursors into neuroblasto
toma (2). Efforts to define the mechanisms for this blockage in neuroblast differentiation have been the focus of major research efforts over the past years, and have led to the incorporation of several differentiation strategies into neuroblastoma treatment. Retinoic acid therapy is an important component of treatment of residual disease of stage IV neuroblastoma after multimodal therapy (3). Nevertheless, rising resistance and treatment toxicity represent relevant limiting factors, and the overall response rate to retinoic acid in patients with neuroblastoma is low, suggesting that only a subgroup of patients benefits from the treatment. Therefore, a better understanding of the molecular mechanisms that restrict neuroblastoma differentiation could lead to improved therapeutic approaches for this highly aggressive malignancy.

Alterations in components of the transcriptional machinery and chromatin modifier genes are now associated with initiation and differentiation of multiple cancers (4), including neuroblastoma (5). A role for epigenetics in tumorigenesis is further supported by recent genome-wide sequencing studies revealing recurrent cancer-associated mutations in key epigenetic regulators, including histone modifiers, histone chaperones, and DNA methylation modifiers (6). In particular, methylation of histone H3 at position lysine 9 (H3K9) has been extensively studied as a major factor regulating transition between transcriptionally active euchromatin and inactive heterochromatin (7). In addition, H3K9 histone methyltransferases interact with DNA methyltransferases (e.g., DNMT1/3b) to indirectly modulate gene silencing through DNA methylation (8). The histone modifiers EZH2 (9) and LSD1 (10) are deregulated in neuroblastoma with high expression conferring worse prognosis. In addition, repression of the tumor suppressor and chromatin modifier CHD5 through loss of heterozygosity and DNA methylation negatively correlates with long-term survival (11).

CHAF1A (CAF p150) is a primary component of the chromatin assembly factor 1 (CAF-1), composed of p150, p60, and p48 subunits, which promotes rapid assembly of nucleosomes on newly replicated DNA (12). The importance of CHAF1A in cancer pathogenesis is supported by the finding that its
overexpression has been linked to tumor progression (13), cancer susceptibility (14), and more recently, epigenetic silencing (15). In addition, CHAF1A participates in a complex with MBD1 and SETDB1 during initiation of a gene-silencing program by promoting H3K9 trimethylation, heterochromatin formation, and DNA methylation (16).

We show here that CHAF1A restricts neuroblastoma differentiation using both in vitro and in vivo orthotopic models. Elevated expression of CHAF1A indeed strongly correlates clinically with an undifferentiated neuroblastoma phenotype and poor overall survival. We also demonstrate that CHAF1A promotes oncogenic signaling pathways (including RAS, AKT, BMI1, and WNT) as well as alters glycolytic metabolism pathways. Together, these data support a novel function for the histone modifier CHAF1A restricting neural crest differentiation and promoting neuroblastoma tumorigenesis.

Patients and Methods

Clinical patient cohort groups

**Discovery set 1.** Versteeg (n = 88). This dataset of 88 unique tumors was profiled on the Affymetrix HGU133 plus2.0 platform and normalized using the MASS5.0 algorithm. Expression data were freely downloaded from the R2 website ([http://r2.amel.nl](http://r2.amel.nl)).

**Validation set 2.** Vermeulen (n = 348). This cohort included 348 patients with neuroblastoma taken from the International Society of Pediatric Oncology, European Neuroblastoma Group (SIOPEN) and from the Gesellschaft fuer Paediatrische Onkologie und Haematologie (GPOH). Patients were only included if primary untreated neuroblastoma tumor RNA (at least 60% tumor cells and confirmed histologic diagnosis of neuroblastoma) was available and of sufficient quality (17). Almost all patients were treated according to the SIOPEN protocols. The median follow-up was 63 months and greater than 24 months for most patients (91%). In this cohort, 32% of the patients had stage I disease, 18% stage II, 18% stage III, 23% stage IV, and 9% stage IVs. MTCNv amplification was present in 17% of all patients, and in 45% of stage IV patients. Median age at diagnosis was 7.4 months for stage I and II, and 23.5 months for stage III and IV. Expression of CHAF1A was evaluated using quantitative real-time PCR (qRT-PCR).

**Discovery set 3.** Delattre (n = 64). This public dataset of 64 neuroblastic tumors (11 ganglioneuroblastoma and 53 neuroblastoma) was profiled on Affymetrix chips HG U133 plus 2.0. It was freely downloaded from the Gene Expression Omnibus dataset, accession number GSE12460 (18).

qRT-PCR in primary samples

A quantitative PCR assay was designed for CHAF1A and five reference genes by PrimerDesign and went through an extensive in silico validated analysis using basic local alignment search tool (BLAST) and BiSearch specificity, amplicon secondary structure, single-nucleotide polymorphism presence, and splice variant analysis. The mean amplification efficiency was 98%. Primer design and qRT-PCR analysis were performed as described previously (17). Primer sequences are available in RTPRimerDB (2). CHAF1A (ID = 8273) and reference genes: HPRT1 (ID = 5), SDHA (ID = 7), UBC (ID = 8), and HMBS (ID = 4). Data handling and calculations (normalization, rescaling, inter-run calibration, and error propagation) were performed in qBasePlus version 1.1 (19, 20).

Short hairpin RNA constructs and antibodies

For p53 short hairpin RNA (shRNA), second-generation lentiviruses expressing shp53 and shLuc control were used as described (21). To knock down CHAF1A expression, a TRIPZ lentiviral inducible shRNAmir with Tet-inducible promoter was used (Open Biosystems). The tetracycline response element (TRE) promoter also drives the expression of a TurboRFP reporter. To repress CHAF1A expression, doxycycline was added at a final concentration of 1 μg/mL. Control lines using scrambled shRNAmir were also generated. A GIPZ lentiviral stable shRNA (Open Biosystems) was instead used to transduce neuroblastoma lines for in vivo studies. Briefly, 293T cells were transfected with pLSLp, TRIPZ, and GIPZ constructs along with packaging plasmids, pVSVG, and pLV-CMV-delta 8.2 by using lipofectamine. Virus-containing supernatants were collected at 48 and 72 hours and neuroblastoma cells were transduced in the presence of 8 mg/mL polybrene (Sigma). CHAF1A rabbit monoclonal antibodies (Epitomics; #5464-1: 1:500 dilution) and p53 mouse monoclonal antibodies (Sigma; #P6874: 1:1,000 dilution) were used for Western blotting. Anti-H3K9me3 antibodies (22-442; Millipore) were used at a dilution 1:1,000 after acid extraction of the histones.

Xenograft model

Orthotopic xenografts of human neuroblastoma were generated as described previously (23) by injection under the renal capsule of an inoculum of 10⁶ tumor cells in 0.1 mL of PBS. Tumors were evaluated at necropsy 5 weeks after inoculation.

Oligonucleotide microarray data analysis

Total RNA was isolated using the RNAeasy Kit (Quiagen) from IMR32 cells transfected with inducible CHAF1A shRNA. Gene expression profiling using Affymetrix U133+2.0 arrays was performed in neuroblastoma cells upon CHAF1A silencing over time course (0, 5, and 10 days) in triplicate. Differentially expressed genes were identified by MASS detection P values ≤ 0.05, ANOVA P value ≤ 0.05, and absolute fold change ≥ 2. For each time point, genes were ranked with respect to the average expression change upon CHAF1A knockdown. Gene Set Enrichment Analysis (GSEA) was then performed for each of the three time points using gene permutation alternative (24). GSEA software v2.0.1 was used for the analysis. Default parameters were used and gene sets that met the false discovery rate (FDR) ≤ 0.25 criterion were ranked by nominal P value. Gene Ontology (GO) analysis was performed as described using the DAVID bioinformatic database (25).

Details about cell lines, tissue culture, and qRT-PCR assays and primers are found in the Supplementary Methods section.

Results

CHAF1A is repressed by p53 and highly expressed in undifferentiated neuroblastoma

Neuroblastoma is primarily a p53 wild-type malignancy, and as part of previous efforts to profile the p53 transcriptional response of neuroblastoma, we observed that increased p53
levels correlated with decreased CHAF1A expression. As the repression of p53 functions is critical to neuroblastoma tumorogenesis, and CHAF1A expression is altered in other malignancies, we proceeded to further analyze CHAF1A function in neuroblastoma.

Prognostic factors for neuroblastoma include age, stage at diagnosis, histology, and specific genetic alterations, including MYCN amplification/overexpression. We first analyzed the prognostic value of CHAF1A in a clinical cohort of 88 patients with neuroblastoma (discovery set 1) using the R2 microarray database and showed that increased CHAF1A expression strongly correlates with poor overall survival (OS; \( P < 0.0001 \); Fig. 1A) and higher stage of disease \( (P < 0.0001; \text{Supplementary Fig. S1}) \). We further confirmed CHAF1A prognostic value in a large independent cohort of patients with neuroblastoma. qRT-PCR analysis of CHAF1A expression in tumor samples from 348 patients enrolled in SIOPEN and GPOH clinical trials (validation set 2) identified patients with neuroblastoma with poor overall survival \( (P < 0.0001) \) and progression-free survival \( (\text{PFS}; P < 0.001; \text{Fig. 1B}) \). In addition, multivariate logistic regression analysis showed that CHAF1A expression level is able to predict survival, independent of the MYCN status (amplified vs. nonamplified), age at diagnosis (< or >12 months), and stage (stage IV vs. other stages) with a hazard ratio of 2.37 and 2.22 for OS and event-free survival (EFS), respectively \( (P < 0.05 \) and \( P < 0.05; \text{Table 1}) \).

To confirm the regulation of CHAF1A by p53 activity, CHAF1A gene expression was assessed in multiple p53 wild-type neuroblastoma lines treated with the MDM2 inhibitor, Nutlin-3a. qRT-PCR demonstrated significant repression of CHAF1A expression upon treatment \( (P < 0.005) \). However, this effect is totally abrogated in neuroblastoma p53 mutant (LAN1) or p53 knockdown (LAN5 si p53) cells, confirming that CHAF1A repression is indeed p53-mediated \( (\text{Fig. 1C and D}) \).

**CHAF1A silencing promotes neuroblastoma differentiation in vitro**

To assess the biologic function of CHAF1A in neuroblastoma, we first generated multiple neuroblastoma cell lines with inducible shRNA-mediated CHAF1A knockdown using a Tet-On conditional system that coexpressed red fluorescent protein (RFP). We found that silencing of CHAF1A leads to a distinct
Table 1. Multivariate logistic regression analysis in the SIOPEN/GPOH cohort

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<th>Risk factor</th>
<th>0.95 Confidence interval</th>
</tr>
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<tr>
<td>Stage (IV vs. others)</td>
<td>5.46E−08</td>
<td>10.33</td>
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<td>Age (&lt; or &gt;1 year)</td>
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<td>MYCN status (± amplification)</td>
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<td>2.37</td>
<td>1.05</td>
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EFS

<table>
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<tr>
<th>OS</th>
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<th>Risk factor</th>
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<td>Stage (IV vs. others)</td>
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<td>Age (&lt; or &gt;1 year)</td>
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<td>MYCN status (± amplification)</td>
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<tr>
<td>CHAF1A expression (&lt; or &gt; median)</td>
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<td>2.22</td>
<td>1.37</td>
</tr>
</tbody>
</table>

NOTE: P values, risk factors, and 95% confidence interval are shown for disease stage (stage IV vs. others), age (< or >1 year), MYCN status (amplified vs. nonamplified), and CHAF1A expression. Significant values for CHAF1A expression are shown in bold.

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morphic change consistent with the morphologic change observed upon retinoic acid-induced differentiation in sensitive neuroblastoma cell lines (26). Silencing of CHAF1A in two neuroblastoma cell lines, LAN5 and IMR32 (Fig. 2A), gradually induces the development of long dense neurite-like processes over a 7 to 14 day time span. In contrast, no apparent change in morphology was observed in scramble-control–transduced cells (Fig. 2B). To define the observed morphologic change as neuronal differentiation, we measured the gene expression of several well-characterized markers of terminal neuronal differentiation: β3 tubulin (TUBB3), nerve growth factor receptor (NGBR), tyrosine hydroxylase (TH), and growth-associated protein (GAP43). Silencing of CHAF1A is associated with significantly increased expression levels of these neuronal markers compared with noninduced and nontargeting siRNA controls (P < 0.005; Fig. 3A). In addition, as CHAF1A is known to promote trimethylation of histone H3 at position lysine 9, we examined the global H3K9me3 levels and found that silencing of CHAF1A significantly reduces the level of global H3K9me3 in IMR32 cells (Fig. 3B).

To further determine the role of CHAF1A as an inhibitor of differentiation, we evaluated CHAF1A expression levels in neuroblastoma cells treated with retinoic acid. In all three lines tested (LAN5, CHLA255, and NGP), the morphologic differentiation (Supplementary Fig. S2) is associated with a significant (P < 0.005) downregulation of CHAF1A expression levels after 7 to 10 days of retinoic acid treatment (Fig. 3C). Finally, we compared the expression of CHAF1A in a small cohort of less aggressive ganglioneuroblastoma (n = 11) with more aggressive, undifferentiated neuroblastoma samples (n = 53; discovery set 3; ref. 18). As shown in Fig. 3D, CHAF1A expression is significantly (P < 0.05) higher in the undifferentiated neuroblastoma group.

CHAF1A promotes tumorigenesis and opposes differentiation in vivo

We then generated neuroblastoma lines with stable CHAF1A knockdown and found that CHAF1A silencing markedly inhibited in vitro proliferation of LAN5 and IMR32 neuroblastoma cell lines by day 4 (MTT assay; Supplementary Fig. S3). To assess the role of CHAF1A in promoting tumorigenesis, loss-of-function studies using three distinct neuroblastoma cell lines (LAN5, IMR-32, and NGP) with stable CHAF1A knockdown were performed in vivo in an orthotopic neuroblastoma model (subrenal capsule injection). This model closely recapitulates the highly angiogenic and invasive growth characteristics of undifferentiated human neuroblastoma (27). We found that CHAF1A silencing significantly reduces tumor growth in all the three cell lines tested (Fig. 4A and B). Tumors with CHAF1A knockdown grossly seemed less vascular and Western blot analysis suggested that tumor growth is proportional to CHAF1A levels (Supplementary Fig. S4).

Detailed histologic analysis shows that the control tumors have a more undifferentiated phenotype with closely apposed neuroblasts, decreased neurolip as highlighted on S100 protein immunostaining, and high mitotic karyorrhectic index (MKI; 525 ± 37 per 5,000 tumor cells). In contrast, CHAF1A knockdown tumors display more neuronal differentiation with increased well-developed neuropil-separating neuroblasts, and a much lower MKI (193 ± 43 per 5,000 tumor cells). Finally, electron microscopy confirmed the presence of frequent cell processes with a well-developed neurupil-separating neuroblasts, and a much lower MKI (193 ± 43 per 5,000 tumor cells). Overall, the histologic differences are consistent with a change in grade from "undifferentiated" to "poorly differentiated," which correlates with the reduced growth observed in vivo.

CHAF1A silencing induces neuronal differentiation pathways and inhibits major oncogenic pathways

To unveil changes in gene expression associated with changes in neuroblastoma phenotype induced by CHAF1A, we performed gene expression profiling (Affymetrix U133+2.0 arrays) in IMR32 cells 5 and 10 days after CHAF1A silencing. Clustered heat map of the differentially expressed genes is shown in Fig. 5A (GEO series accession number GSE51978). We then examined the occurrence of GO terms of genes associated with changes in CHAF1A expression using the DAVID online analysis platform (28). Notably, the most significantly enriched...
functional categories (\( P < 0.05 \)) upon CHAF1A silencing are associated with multiple processes involved in neuronal differentiation (axonogenesis, synaptic transmission, cell-cell signaling, catecholamine biosynthesis, and nervous system development; Table 2), validating CHAF1A as a potential critical regulator of neuronal differentiation. Furthermore, these functional categories were distinct from the ones described to be enriched in neuroblastoma-cell differentiation upon Cyclin D1 and Cdk4 silencing (29) or retinoic acid treatment (30), suggesting a distinctive mechanism for CHAF1A in inducing cell differentiation (Supplementary Tables S1 and S2).

In addition, GSEA revealed that genes regulated by CHAF1A were associated with major metabolic and oncogenic pathways. CHAF1A silencing significantly enriches for cell metabolism pathways (valine, leucine, and isoleucine degradation, glutamate metabolism, and insulin pathways; Fig. 5B) and suppresses pathways with known oncogenic function in neuroblastoma (KRAS, ALK, AKT, and BMI1; nominal \( P < 0.05 \) and FDR \( q < 0.25 \); Fig. 5C). A complete list of the significant pathways is shown in Supplementary Table S3. qRT-PCR confirmed that CHAF1A affects the expression of selected metabolic genes with important roles in insulin, type 2 diabetes, valine, leucine, and isoleucine degradation pathways both in IMR32 and LAN5 cells. Notably, DHRS2, an enzyme with crucial oxidoreductase activity, is markedly upregulated upon CHAF1A silencing (Fig. 5D).

In summary, we demonstrate that the expression of CHAF1A is regulated by p53 and positively correlates with a more undifferentiated aggressive neuroblastoma phenotype \textit{in vitro} and \textit{in vivo}. In addition, silencing of CHAF1A leads to upregulation of genes controlling neuronal differentiation.

![Figure 2](attachment:image.png)

**Figure 2.** CHAF1A silencing induces a differentiated neuronal phenotype. A, knockdown of CHAF1A expression by CHAF1A siRNA but not siRNA control in LAN5 and IMR32 cells. CHAF1A mRNA and protein level after knockdown was determined by SYBR Green qRT-PCR and Western blotting. B, conditional siRNA-mediated knockdown of CHAF1A is compared with conditional siRNA control and retinoic acid (RA) treatment over time (Tet-on cells visualized by fluorescent microscopy for RFP). CHAF1A silencing induces long neurite extension comparable with retinoic acid treatment in LAN5 cells. By contrast, IMR32 cells did not show the same morphologic changes and underwent marked apoptosis after 5 to 7 days of retinoic acid treatment. However, silencing CHAF1A strongly promotes neurite extension in this same cell type.
Discussion

Currently, the predictive risk factors used for neuroblastoma risk stratification are age, stage, tumor histology, and MYCN gene amplification status. We observed that elevated expression of one such chromatin chaperone, CHAF1A, significantly correlates with poor survival in severe large cohorts of patients with neuroblastoma independently of these clinical features. CHAF1A expression is also much lower in spontaneously regressing infant neuroblastomas and in ganglioneuroblastomas (a highly differentiated form of neuroblastoma), and markedly elevated in the most undifferentiated aggressive metastatic cases.

These clinical observations suggest that CHAF1A plays an important role in neuroblastoma biology. Oncogenic functions of deregulated CHAF1A and the CAF-1 histone chaperone complex continue to be defined. Expression of CAF-1 has been associated with cell proliferation in breast cancer (31), deregulation of DNA repair in squamous cell carcinoma (32), and genomic instability and cancer susceptibility in the recessively inherited Bloom syndrome (14). Furthermore, activating single nucleotide polymorphisms within the CHAF1A gene strongly correlate with glioma tumorigenesis (33).

Mirroring the clinical observations, using an orthotopic xenograft model of neuroblastoma, we show that CHAF1A expression drives a more undifferentiated neuroblastoma phenotype in vivo. Suppression of CHAF1A strongly induces neuroblastoma to differentiate, suggesting that CHAF1A restricts innate differentiation pathways and may modulate resistance to differentiation-based therapies. Of note, we present gene expression data suggesting that CHAF1A may act through mechanisms independent of previously characterized Cyclin D1/Cdk4 or retinoic acid-driven pathways (Supplementary Tables S1 and S2). However, neuroblastoma differentiation is a complex and poorly understood process involving multiple networks of genetic and epigenetic pathways. Understanding the regulatory mechanisms of neuroblastoma differentiation is important for obtaining insight into basic biology and for developing novel therapies that may overcome the resistance to retinoids.
Epigenetic changes, including histone modifications, play a central role in controlling differentiation and defining the pluripotent state of embryonic and cancer stem cells (34). Recent comprehensive genome-wide studies define distinct patterns of histone modifications and DNA methylation during multilineage differentiation of stem cells (35, 36). These and other studies point to a complex interaction of DNA histone modifications and DNA methylation controlling cellular differentiation barriers (37), and disruption of these epigenetic mechanisms may strongly implicate in tumorigenesis and survival of cancer stem populations (38). H3K9 trimethylation has been characterized as a major factor regulating transitions between transcriptionally active euchromatin and inactive heterochromatin (39). Binding of UHRF1 to methylated H3K9 is required for DNA methylation maintenance (8). Methylated H3K9 serves as a binding platform for heterochromatin protein 1, which directs DNMT1-dependent DNA methylation (40). Of note, previous studies demonstrate that CHAF1A acts independently of CAF-1 as an epigenetic-silencing factor (15), regulating H3K9me3 epigenetic marking of heterochromatin domains in pluripotent embryonic cells (41). CHAF1A also modulates DNA methylation, forming a complex with MBD1 and SETDB1, and modulating DNA methylation (16).

As with other aggressive embryonal malignancies, histone modification and DNA methylation alterations are implicated in the pathogenesis of neuroblastoma. High expression of the class II HDAC SIRT1 stabilizes MYCN and promotes tumorigenesis (42), whereas aberrant DNMT3B transcripts are expressed in high-risk neuroblastoma with globally altered DNA methylation (43). In addition, altered EZH2 expression (polycomb histone methyltransferase) leads to repression of...
multiple tumor suppressor genes in neuroblastoma (9), and genome-wide DNA methylation studies identified candidate DNA methylation markers with important prognostic value in neuroblastoma (44). Finally, the definition of the roles of novel chromatin regulators in neuroblastoma, such as CHD5, highlights the importance of these histone posttranslational

Table 2. CHAF1A silencing modulates genes associated with neuronal differentiation

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<tr>
<td>0007286</td>
<td>Synaptic transmission</td>
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<td>Response to organic nitrogen</td>
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<td>Anatomical structure development</td>
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NOTE: GO enrichment analysis. The significant enriched GO terms based on biologic processes are shown. Functional categories terms, number of genes within each functional category, and corrected P values are indicated.
CHAF1A primarily restrains differentiation, which helps in explaining its high expression in the most aggressive neuroblastoma cases. Loss-of-function studies suggest that targeting CHAF1A or its downstream pathways would provide a novel therapeutic approach to high-risk neuroblastoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Development of methodology: E. Barbieri, E.S. Kim, J.M. Shohet
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Barbieri, K.D. Preter, D.M. Hsu, J. Hicks, B. Versteeg, A. Pession
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Barbieri, K.D. Preter, M. Capasso, Z. Chen, D.M. Hsu, G.P. Tonini, F. Speleman, E.S. Kim, J.M. Shohet
Writing, review, and/or revision of the manuscript: E. Barbieri, K.D. Preter, M. Capasso, F. Speleman, E.S. Kim, J.M. Shohet
Study supervision: E.S. Kim, J.M. Shohet

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References


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