Histone chaperone CHAF1A inhibits differentiation and promotes aggressive neuroblastoma.

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No potential conflicts of interest were disclosed.

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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 pro-differentiation agents improves survival modestly. In this study, we show the histone chaperone and epigenetic regulator CHAF1A functions in maintaining the highly de-differentiated state of this aggressive malignancy. CHAF1A is a subunit of the chromatin modifier CAF1 and it regulates H3K9 tri-methylation 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 (1). The clinical and biological 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 NB with targeted overexpression of the MYCN oncogene also demonstrate that blocked neural crest differentiation leads to the malignant transformation of neuroectodermal precursors into NB (2). Efforts to define the mechanism(s) 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 (RA) therapy is an important component of treatment for residual disease of stage IV neuroblastoma after multimodal therapy (3). Nevertheless, arising resistance and treatment toxicity represent relevant limiting factors and overall response rate to RA in neuroblastoma patients is low, suggesting that only a subgroup of patients benefits from the treatment. Therefore, a better understanding of the molecular mechanisms which 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 regulator genes, 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 tri-methylation, 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.
Methods

Clinical Patient Cohort Groups.

Discovery set 1: Versteeg (n= 88). This data set of 88 unique tumors was profiled on the Affymetrix HGU133 plus2.0 platform and normalized using MASS5.0 algorithm. Expression data were freely downloaded from the R2 website (http://r2.amc.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 histological diagnosis of neuroblastoma) was available and of sufficient quality (17). Almost all patients were treated according to 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 1 disease, 18% stage 2, 18% stage 3, 23% stage 4, and 9% stage 4s. MYCN amplification was present in 17% of all patients, and in 45% of stage 4 patients. Median age at diagnosis was 7.4 months for stage 1 and 2, and 23.5 months for stage 3 and 4. Expression of CHAF1A was evaluated using quantitative RT-PCR.

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

Quantitative RT-PCR in primary samples: A qPCR assay was designed for CHAF1A and five reference genes by PrimerDesign and went through an extensive in silico validated analysis using BLAST and BiSearch specificity, amplicon secondary structure, SNP presence, and splice
variant analysis. The mean amplification efficiency was 98%. Primer design and quantitative RT-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).

ShRNA constructs and Antibodies: For p53 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 TRE promoter drives also the expression of a TurboRFP reporter. To repress CHAF1A expression, doxycycline was added at a final concentration of 1ug ml⁻¹. 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 pLSLPw, 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 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) (1:1000 dilution) were used for Western Blotting. Anti-H3K9me3 antibodies (22-442 Millipore) were used at a dilution 1:1000 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 0.1mL of PBS. Tumors where evaluated at necropsy five weeks after inoculation.
Oligonucleotide Microarray Data Analysis: Total RNA was isolated using RNAeasy kit (Quiagen) from IMR32 cells transduced 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 MAS5 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 knock down. GSEA was then performed for each of the three time points using gene permutation alternative (24). Gene Set Enrichment Analysis software v2.0.1 was used for the analysis. Default parameters were used and gene sets that met the FDR ≤ 0.25 criterion were ranked by nominal P value. Gene Ontology analysis was performed as described using the DAVID bioinformatic database (25). Details regarding cell lines, tissue culture, and quantitative RT-PCR assays and primers are found in Supplemental 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 repression of p53 functions is critical to neuroblastoma tumorigenesis, 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 neuroblastoma patients (discovery set 1) using the R2 microarray database and showed that increased CHAF1A expression strongly correlates with poor overall survival (OS p<0.0001) (**Figure 1a**) and higher stage of disease (p<0.0001) (**Supplementary Figure 1**). We further confirmed CHAF1A prognostic value in a large independent cohort of neuroblastoma patients. Quantitative RT-PCR analysis of CHAF1A expression in tumor samples from 348 patients enrolled in the International Society of Paediatric Oncology Europe Neuroblastoma Group (SIOPEN) and the Gesellschaft fuer Paediatrische Onkologie und Haematologie (GPOH) clinical trials (validation set 2) identified neuroblastoma patients with poor overall survival (OS p<0.0001) and progression-free survival (PFS p<0.001) (**Figure 1b**). In addition, multivariate logistic regression analysis showed that CHAF1A expression level is able to predict survival, independently of *MYCN* status (amplified versus non-amplified), age at diagnosis (< or >12 months), and stage (stage 4 versus other stages) with a hazard ratio of 2.37 and 2.22 for OS and EFS respectively (p<0.05 and p<0.005) (**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. Quantitative RT-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 knock down (LAN5 si p53) cells, confirming that CHAF1A repression is indeed p53-mediated (Figure 1c and Figure 1d).

**CHAF1A silencing promotes neuroblastoma differentiation in vitro.**

To assess the biological 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 co-expressed RFP. We found that silencing of CHAF1A leads to a distinct morphologic change consistent with the morphologic change observed upon retinoic acid (RA) induced differentiation in sensitive neuroblastoma cell lines (26). Silencing of CHAF1A in two neuroblastoma cell lines, LAN5 and IMR32 (Figure 2a) gradually induces the development of long dense neurite-like processes over a 7-14 day time span. In contrast, no apparent change in morphology was observed in scramble control transduced cells (Figure 2b). To define the observed morphology 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 (NGFR), tyrosine hydroxylase (TH) and growth-associated protein (GAP43). Silencing of CHAF1A is associated with significantly increased expression levels of these neuronal markers compared with non-induced and non-targeting siRNA controls (p<0.005) (Figure 3a). In addition, as CHAF1A is known to promote tri-methylation 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 (Figure 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 morphological differentiation (Supplementary Figure 2) is associated with a significant (p<0.005) down-regulation of CHAF1A expression levels after 7-10 days of RA treatment (Figure 3c). Lastly, we compared the expression of CHAF1A in a small cohort of less aggressive ganglioneuroblastoma (GNB, n=11) with more aggressive, undifferentiated neuroblastoma samples (NB, n=53) (discovery set 3) (18). As shown in Figure 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 Figure 3). To assess the role of CHAF1A in promoting tumorigenesis, loss-of-function studies using three distinct neuroblastoma cell lines (LAN-5, IMR-32 and NGP) with stable CHAF1A knock down were performed in vivo in an orthotopic neuroblastoma model (sub-renal 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 (Figure 4a and 4b). Tumors with CHAF1A knock down grossly
appeared less vascular and Western Blotting analysis suggested that tumor growth is proportional to CHAF1A levels (Supplementary Figure 4).

Detailed histological analysis shows that the control tumors have a more undifferentiated phenotype with closely apposed neuroblasts, decreased neuropil as highlighted on S100 protein immunostaining, and high mitotic karyorrhectic index (MKI) (525 ± 37 per 5,000 tumor cells). In contrast, CHAF1A knock down 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 (EM) confirmed the presence of frequent cell processes with well developed neuropil and increased dense core neurosecretory granules (Figure 4c) in CHAF1A knock down tumors compared to controls. Overall, the histological 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.

In order 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 five and ten days after CHAF1A silencing. Clustered heat map of the differentially expressed genes is shown in Figure 5a (GEO series accession number GSE51978). We then examined the occurrence of Gene Ontology (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-value<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 Table 1 and Supplementary Table 2).

In addition, Gene Set Enrichment Analysis (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) (Figure 5b) and suppresses pathways with known oncogenic function in neuroblastoma (KRAS, ALK, AKT, and BMI1) (nominal p-value <0.05 and FDR q-value <0.25) (Figure 5c). A complete list of the significant pathways is shown in Supplementary Table 3. Quantitative RT-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 activities, is markedly upregulated upon CHAF1A silencing (Figure 5d).

In summary, we demonstrate that the expression of CHAF1A is regulated by p53 and positively correlates with a more un-differentiated aggressive neuroblastoma phenotype in vitro and in vivo. In addition, silencing of CHAF1A leads to up-regulation of genes controlling neuronal differentiation, normalized glucose metabolism, as well as down-regulation of major oncogenic pathways. As discussed below, these data suggest that CHAF1A or downstream pathways may represent novel therapeutic targets which could sensitize neuroblastoma to differentiation in vivo.
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 several large cohorts of neuroblastoma patients 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’s 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 1 and 2). 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 is strongly implicated in tumorigenesis and survival of cancer stem populations (38). H3K9 tri-methylation 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 (HP1) which directs DNMT1-dependent DNA methylation (40).

Of note, previous studies demonstrate that CHAF1A acts independently of CAF1 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), while 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). Lastly, the definition of the roles of novel chromatin regulators in neuroblastoma, such as CHD5, highlights the importance of these histone posttranslational modifications in controlling gene expression during neuronal differentiation (45).

In further support of an oncogenic role of CHAF1A, our GSEA analysis demonstrates that RAS as well as AKT, BMI and ALK pathways are strongly repressed upon CHAF1A knockdown (Figure 5 and Supplementary Table 3). Ras signaling networks drive cellular proliferation and restrict differentiation and previous studies have also suggested a role for RAS-MEK signaling in regulation of responses to retinoic acid in different cellular systems (46). Activation of NRAS appears to be critical in neuroblastoma tumorigenesis, considering its function in stabilizing MYCN, promoting MYCN-dependent cell cycle progression, and blocking p53-mediated cell cycle check points and pro-apoptotic effects (22, 47).

In addition, bioinformatic analyses of gene expression changes also suggest that CHAF1A may alter metabolic pathways to promote the Warburg effect (increased glucose consumption and decreased oxidative phosphorylation). The most enriched KEGG and Biocarta pathways after CHAF1A knock down were pathways involved in cell metabolism (valine leucine and isoleucine degradation and glutamate metabolism among others, Figure 5 and Supplementary Table 3). Valine, isoleucine and leucine are three essential amino acids, whose catabolism, together with glutamate, support ATP production. The up-regulation of these Kreb cycle components together with the down-regulation of the insulin-Akt signaling upon CHAF1A silencing, suggests that suppression of CHAF1A may have a role in shifting the cell metabolism to oxidative phosphorylation. While these observations suggest CHAF1A over-expression may force
neuroblastoma cells toward the aerobic glycolysis, detailed metabolic studies will be required to formally link CHAF1A to modulation of neuroblastoma metabolism.

Taken together, our findings in both neuroblastoma patient cohorts and tumor models implicate this histone chaperone molecule in multiple oncogenic pathways in neuroblastoma. CHAF1A primarily restrains differentiation which helps 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.
Acknowledgements

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Literature Cited


47. Ho C, Wang C, Mattu S, et al. AKT (v-akt murine thymoma viral oncogene homolog 1) and N-Ras (neuroblastoma ras viral oncogene homolog) coactivation in the mouse liver promotes...
Figures Legends

Figure 1: CHAF1A expression accurately predict neuroblastoma outcome and is regulated by p53.
(a) Kaplan–Meier and log-rank analysis for overall (OS) survival of discovery set 1 (88 neuroblastoma patients) based on CHAF1A expression. (b) Independent validation of CHAF1A expression in a large cohort of neuroblastoma patients (SIOPEN/GPOH). CHAF1A expression as measured by quantitative RT-PCR versus overall (OS) and progression-free (PFS) survival in validation set 2: survival of 348 neuroblastoma patients in the 4 quartiles of the signature score is shown. (c) p53 activation represses CHAF1A expression. Quantitative RT-PCR demonstrates significant decrease of CHAF1A mRNA levels after Nutlin-3a treatment (10μM for 8 hours) in multiple p53 wild-type neuroblastoma lines (shown here LAN5, IMR32 and SY5Y). However, this effect is completely abrogated when the effect of Nutlin-3a is tested in a p53 mutant neuroblastoma cell line (LAN1) or in a p53 wild-type line LAN5 transduced with a shp53 lentivirus (d). Each error bar represents two biological replicates.

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 on SYBR Green quantitative RT-PCR and Western Blotting. (b) Conditional siRNA mediated knockdown of CHAF1A is compared to 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 to RA treatment in LAN5 cells. By contrast, IMR32 cells did not show the same morphological changes and underwent marked apoptosis after 5-7 days of RA
treatment. However, silencing CHAF1A strongly promotes neurite extension in this same cell type.

**Figure 3: CHAF1A silencing promotes molecular neuronal differentiation in vitro.**

(a) CHAF1A silencing upregulates molecular markers of neuronal differentiation. Quantitative RT-PCR confirms induction of neuron-specific marker genes (NGFR, TH, GAP43, and TUBB3) 10-14 days after doxycycline-inducible CHAF1A SiRNA expression. (b) CHAF1A knock down reduces the level of global H3K9me3 in IMR32 cells 10 days after CHAF1A SiRNA expression. Western blot data were analyzed by densitometry. (c) Quantitative RT-PCR reveals decreased CHAF1A mRNA levels after 7-10 days of treatment with retinoic acid (10uM) in LAN5, CHLA255, and NGP neuroblastoma cells. (d) CHAF1A expression in ganglioneuroblastoma (GNB, n=11) versus undifferentiated neuroblastoma (NB, n=53) in discovery set 3.

**Figure 4: CHAF1A silencing opposes tumor growth and promotes differentiation in vivo.**

(a) Western Blotting confirms knockdown of CHAF1A expression in LAN5, IMR32 and NGP cell lines. (b) Average tumor weight for each cohort ± SEM. Tumors with CHAF1A knock down are significantly smaller than control (LAN5 xenografts: *, Kruskal–Wallis method P=0.0033, mean ± SEM, n=8 in each group; IMR32 xenografts: *, Kruskal–Wallis method P=0.035, mean ± SEM, n=10 in each group; NGP xenograft: *, Kruskal–Wallis method P=0.028, mean ± SEM, n=5 in control group, n=8 in siRNA group). (c) Representative tumor samples in control and CHAF1A shRNA group for histological comparison. H+E staining, S100 protein immunostaining, electron microscopy (EM) and mitotic karyorrhectic index (MKI) quantification are shown.

**Figure 5: Gene expression profiling and gene set enrichment analysis (GSEA) in IMR32 cells upon CHAF1A silencing.**
(a) Clustered heat map of the differentially expressed genes (DEGs) upon CHAF1A silencing in IMR32 cells over time course (0, 5, and 10 days). GSEA analysis identifies differentially expressed pathways upon CHAF1A silencing. Pathways related to cell metabolism (b) and oncogenic signatures (c) are among the top differentially expressed pathways. (d) Change in expression by quantitative RT-PCR of selected transcripts involved in cell metabolism upon CHAF1A silencing in IMR32 and LAN5 cells. Each error bar represents two biological replicates.

**Table 1: Multivariate logistic regression analysis in the SIOPEN/GPOH cohort.**

p-values, risk factors, and 95% CI are shown for disease stage (stage 4 vs. others), age (<1 yrs), MYCN status (amplified vs. non-amplified), and CHAF1A expression.

**Table 2: CHAF1A silencing modulates genes associated with neuronal differentiation.**

Gene Ontology (GO) enrichment analysis. The significant enriched GO terms based on biological processes are shown. Functional categories terms, number of genes within each functional category, and corrected p-value are indicated.
Table 1

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**Figure 1**

(a) Kaplan-Meier survival curves showing overall survival probability with low and high expression of CHAF1A.

(b) Comparison of overall survival and progression-free survival by CHAF1A expression quartiles. The survival curves are indicated by different line styles:
- 1. CHAF1A expression in first quartile
- 2. CHAF1A expression in second quartile
- 3. CHAF1A expression in third quartile
- 4. CHAF1A expression in fourth quartile

(c) Bar graph showing fold change of CHAF1A mRNA expression in different cell lines: IMR32, LAN5, SH-SYSY, and LAN1.

(d) Bar graph showing fold change of CHAF1A mRNA expression in LAN5 and LAN5 Si p53. Western blot images show protein expression levels for CTRL, Sip53, N3a, p53, and Cyclophilin B.
Figure 4

**LAN5 Xenografts**

- **LAN5**
  - CHAF1A
  - CYPB
- IMR32
  - CHAF1A
  - CYPB
- NGP
  - CHAF1A
  - CYPB

**Tumor Weight (gm)**

- **LAN5**
  - CTRL
  - CHAF1A KD
- **IMR32**
  - CTRL
  - CHAF1A KD
- **NGP**
  - CTRL
  - CHAF1A KD

**H&E**

- **Control ‘undifferentiated’**
  - H+E
  - S100
  - EM

**H+E**

- **CHAFT1A shRNA – poorly differentiated**
Figure 5

(a) Heatmap showing relative Log2 signal (row mean centered) for SICAFA1A expression in CTRL and SICAFA1A Day 5 and Day 10.

(b) Metabolic Pathways
- GLUTAMATE METABOLISM
- VALINE LEUCINE AND ILEUCINE DEGRADATION
- KETOACID METABOLISM

(c) Oncogenic Pathways
- RAF1, BCR-ABL1, NRAS, PIK3CA
- AKT1, PIK3R2, PTEN, PPARGC1A

(d) Fold Change in Expression for IMR32 and LAN5
- CTRL
- CHAF1A KD
Histone chaperone CHAF1A inhibits differentiation and promotes aggressive neuroblastoma.

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