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CASZ1, a candidate tumor-suppressor gene, suppresses neuroblastoma tumor growth through reprogramming gene expression

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Neuroblastoma (NB) is a common childhood malignant tumor of the neural crest-derived sympathetic nervous system. In NB the frequent loss of heterozygosity (LOH) on chromosome 1p raises the possibility that this region contains tumor-suppressor genes whose inactivation contributes to tumorigenesis. The human homolog of the *Drosophila* neural fate determination gene *CASZ1,* a zinc-finger transcription factor, maps to chromosome 1p36.22, a region implicated in NB tumorigenesis. Quantitative real-time PCR analysis showed that low-*CASZ1* expression is significantly correlated with increased age ($\geq$18 months), Children's Oncology Group high-risk classification, 1p LOH and *MYCN* amplification (all $P<0.0002$) and decreased survival probability ($P=0.0009$). *CASZ1* was more highly expressed in NB with a differentiated histopathology ($P<0.0001$). Retinoids and epigenetic modification agents associated with regulation of differentiation induced *CASZ1* expression. Expression profiling analysis revealed that *CASZ1* regulates the expression of genes involved in regulation of cell growth and developmental processes. Specific restoration of *CASZ1* in NB cells induced cell differentiation, enhanced cell adhesion, inhibited migration and suppressed tumorigenicity. These data are consistent with *CASZ1* being a critical modulator of neural cell development, and that somatically acquired disruption of normal *CASZ1* expression contributes to the malignant phenotype of human NB.
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Neuroblastoma (NB) is the most common extra-cranial childhood solid tumor and derives from the neural crest cells that give rise to the developing sympathoadrenal nervous system.[1] Deletion of chromosome 1p (chr1p) is found in approximately 35% of NB[1] and frequently occurs in other cancers, such as melanoma, oligodendroglioma and breast cancers.[2] Evidence indicates that the region between D1S508 and D1S244 markers on chr1p36 is one of the commonly deleted regions in NB.[3] There are 30 genes in this region and expression analysis of 4 of the 30 genes showed that two of them (*CASZ1* and *PIK3CD*) are expressed at significantly lower levels in unfavorable NB as compared with favorable ones.[3] However, the tumor-suppressor functions of *CASZ1* and *PIK3CD* have not been investigated.[3]

CASZ1 maps to Chr1p36.22 and is a recently described zinc-finger (ZnF) transcription factor whose expression is upregulated during induction of NB and myoblast cell differentiation.[4] In *Drosophila*, the *CASZ1* homolog, *Drosophila castor* (*dcas),* is expressed at a late stage of neuroblast development that precedes the cessation of proliferation and initiation of neuronal differentiation.[5–7] *Dcas* functions to regulate neural fate and loss of *dcas in Drosophila* results in impaired differentiation and alterations in glial cell number and migration.[5,7,8] In *Xeneopus CASZ1* is required for heart development and onset of cardiomyocytes differentiation at the ventral midline; the *CASZ1*-depleted midline cells overproliferate and remain a coherent population of nonintegrated cells positioned on the outer wall of the ventricle.[9] Although functional studies have not been performed in mammals to date, murine studies indicate developmental regulation of *CASZ1* in the neural crest-derived peripheral nervous system[10] cells, which are thought to be the origin of NB tumors.

NB patients whose tumors are undifferentiated have poor prognoses while those whose tumors exhibit a more differentiated histopathology or gene expression profile have less malignant tumors and better prognoses.[1] The finding that re-introduction of Chr1p into NB cells induces differentiation[11] supports the concept that loss of genes regulating developmental processes contributes to NB tumorigenicity. Developmentally regulated transcription factors are implicated in tumorigenesis in several pediatric tumors: *MYCN* in NB,[1–4] *RB* gene in retinoblastoma,[12] and *REST/NRSF* in medulloblastoma.[13] These transcription factors are also important

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Abbreviations: NB, neuroblastoma; LOH, loss of heterozytosity; Chr1p, Chromosome 1p; ZnF, zinc-finger; EFS, event-free survival; RA, retinoic acid; Tet, tetracycline; FACS, fluorescence-activated cell sorting; SRO, shortest region of overlap
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regulators of signal transduction pathways that control developmental programs. Thus, we propose that the ZnF transcription factor *CASZ1* is a candidate NB tumor-suppressor gene based on the following observations: first, *CASZ1* localizes to chr1p36 and it is underexpressed in unfavorable NB patients;[3] second, *CASZ1* is required for neuronal differentiation in *Drosophila* and is a neural fate determination gene;[5] third, *CASZ1* expression is developmentally regulated during neurogenesis in mouse and is upregulated when NB cells are induced to differentiate.[4,10]

In this study, we examine the expression of *CASZ1* in primary tumors from NB patients and determine how the reconstitution of *CASZ1* expression in NB tumor cells alters their biologic functions. We provide evidence that *CASZ1* has properties associated with tumor-suppressor gene function as it induces cell differentiation, inhibits tumor cell migration and tumor growth *in vitro* and *in vivo* in murine xenograft models.

Results

Decreased *CASZ1* expression is clinically relevant and significantly associated with poor prognosis in NB. Using fluorescence *in situ* hybridization, *CASZ1* cDNA maps to chr1p36 and loss of a *CASZ1* allele occurs in a number of NB cell lines and primary NB tumor specimens (Supplementary Figures 1a and b). *CASZ1* deletion in each of the four NB tumor samples tested, as represented by the percentage of cells with *CASZ1* deletion *versus* all cells counted vary from 60 to 88%. To investigate *CASZ1* expression in NB cell lines with and without 1p loss of heterozygosity (LOH), we performed real-time PCR to assess *CASZ1* mRNA level. We found that low-*CASZ1* is not always associated with 1p LOH in the eight NB cell lines tested, as shown in Supplementary Figure 1c, *CASZ1* level is low in SY5Y and NGP cells, which have no 1p LOH, suggesting that in addition to 1p LOH, epigenetic alterations may contribute to low expression of *CASZ1* in NB cells with or without 1p LOH. We treated AS, BE2, SY5Y, KCNR and NGP NB cell lines, which have representative genetic backgrounds characteristic of NB tumors (Supplementary Table 1) with the demethylating agent 5-Aza-20-deoxycytidine (5-Aza-dC) or the clinically relevant class I histone deacetylase inhibitor, depsipeptide (rompidepsin) and examined changes in *CASZ1* expression. Consistent with a previous report,[3] we did not see the induction of *CASZ1* by 5-Aza-dC in the AS or BE2 cell lines but 5-Aza-dC induced a three to sevenfold increase in *CASZ1* gene expression in SY5Y, KCNR and NGP (Supplementary Figure 1d). Unlike 5-Aza-dC, depsipeptide-induced *CASZ1* gene expression in all five NB cell lines tested from $\sim$2- to 20-fold (Supplementary Figure 1e). Thus, changes in DNA methylation or histone acetylation are associated with increases in *CASZ1* expression in NB cells either with or without 1p LOH.

We evaluated *CASZ1* mRNA expression in 59 primary NB patients' tumors by TaqMan real-time PCR. All samples had detectable levels of *CASZ1* mRNA. The proportion of patients by known risk factors was similar to those of the NB population in general (Supplementary Table 2). The median level of *CASZ1* expression, 0.77 was selected as the optimal cut-off in this cohort (in terms of event-free survival, EFS) to categorize each sample as either a high-*CASZ1* or a low-*CASZ1* expresser. Although the sample size is small, there is statistical evidence to support any choice of *CASZ1* expression cut-off between 0.77 and 1.5.

Low levels of *CASZ1* were detected in the majority (77%) of tumor samples in patients $\geq$18 months, only 27% of the samples in patients $<$18 months samples expressed low-*CASZ1* levels ($P=0.0002$, Table 1). The relative risk for an event in a patient $\geq$18 months expressing low-*CASZ1* is 2.9-fold higher than that for a patient $<$18 months. Similarly, low-*CASZ1* expression was statistically significantly associated with unfavorable Shimada histology, 1p LOH, no 11q LOH and MYCN amplification, and having a Children's Oncology Group (COG) high-risk profile[14] (all $P<0.004$, Table 1). Although *CASZ1* mRNA level is higher in stage

Table 1 Association of high-*CASZ1* expression level with prognostic factors ($n=59$)

Factor[a]	*N*	Number (%) of patients with hCas expression $\geq$0.77	*P*-value[b]
Age			
$<$18 months	33	24 (73)	0.0002
$\geq$18 months	26	6 (23)	
INSS stage			
1, 2, 3, 4s	32	20 (63)	0.0692
4	27	10 (37)	
MYCN			
Not amplified	39	29 (74)	$<$0.0001
Amplified	20	1 (5)	
Shimada histology			
Favorable	27	23 (85)	$<$0.0001
Unfavorable	29	7 (24)	
Unknown	3		
Ploidy			
Hyperdiploid	39	23 (59)	0.1033
Diploid	20	7 (35)	
1p			
No LOH	39	26 (67)	$<$0.0001
LOH	16	2 (13)	
Unknown	4		
11q			
No LOH	38	15 (39)	0.0039
LOH	17	14 (82)	
Unknown	4		
Risk group			
Low/intermediate	30	24 (80)	$<$0.0001
High	29	6 (21)	
Grade			
Differentiating/poorly differentiated	41	22 (54)	0.1085
Undifferentiated	4		
Unknown	14	0 (0)	

[a]From the International Neuroblastoma Risk Group (INRG) classification system
[b]From two-sided Fisher's exact test

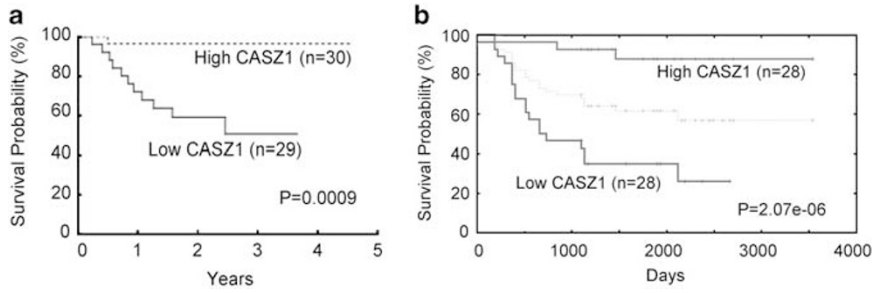

Figure 1 Loss of *CASZ1* expression is correlated with poor prognosis in NB. (**a**) Kaplan–Meier curves of overall patient survival for low (<0.77) *versus* high (>0.77) *CASZ1* expression in 59 NB patients. The association of high-*CASZ1* expression with good prognosis is highly statistically significant ($P=0.0009$). *CASZ1* mRNA level was determined on Taqman real-time PCR. (**b**) Kaplan–Meier curves of overall patient survival for low *versus* high-*CASZ1* expression in 56 other NB patients (using median *CASZ1* level as cutoff) from NB Prognosis Database (http://home.ccr.cancer.gov/oncology/oncogenomics/). The association of high-*CASZ1* expression with good prognosis is highly statistically significant ($P=0.00000207$). *CASZ1* mRNA level was determined on microarray

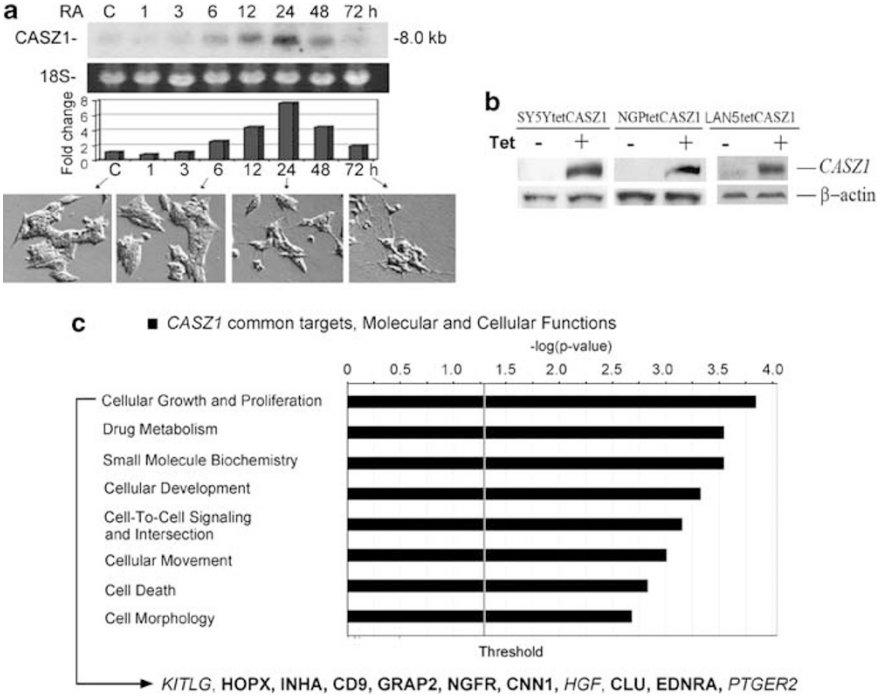

Figure 2 Transcriptome regulated by *CASZ1*. (**a**) Northern analysis of *CASZ1* expression in RA-induced KCNR differentiation. Top: the *CASZ1* expression in KCNR after RA treatment at the indicated hours (shown as h), 18S RNA stained with ethidium bromide before transfer was used as loading control. Middle: the *CASZ1* expression level as normalized to 18S RNA. The *CASZ1* level in the control cells (shown as c) is defined as one unit. Bottom: the morphological differentiation of KCNR after RA treatment of indicated amount of time. (**b**) Full-length FLAG-tagged *CASZ1* in Tet-on vector was stably transfected into SY5Y, NGP and LAN5 NB cell lines that express Tet repressor. The stable clones obtained were named SY5YtetCASZ1, NGPtetCASZ1 and LAN5tetCASZ1. Induction of *CASZ1* expression by Tet (1 μg/ml) at 24 h was visualized by immunoblotting whole cell lysate with anti-FLAG antibody. (**c**) IPA of 125 common targets of *CASZ1* in SY5YtetCASZ1 replicates, NGPtetCASZ1 and LAN5tetCASZ1 determined by microarray. Threshold represents $P=0.05$

1/2/3/4S than in stage 4 disease, the difference is not statistically significant ($P=0.0692$, Table 1). NB patients whose tumors expressed low-*CASZ1* generally had a poor overall survival rate ($P=0.0009$) (Figure 1a). Consistent with this finding, NB patients whose tumors express low-*CASZ1* have a poor overall survival rate ($P=0.0000027$) was also found in NB Prognosis Database from Dr. Javed Khan's lab (http://home.ccr.cancer.gov/oncology/oncogenomics/), which was based on microarray analysis using 56 patient tumor samples (Figure 1b). Similarly, in Dr. Rogier Versteeg's R2 database (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi), in a study of 88 NB patients, those patients whose tumors express low-*CASZ1* have a poor overall survival rate ($P=0.0011$) (Supplementary Figure 2).

***CASZ1* regulates the expression of genes that are important for cell growth and developmental processes.** Retinoids are known to induce growth arrest and differentiation of NB tumor cells, and in the KCNR cell line treated with 5 μM all-trans retinoic acid (RA), *CASZ1* expression increased within 6 h, peaking at 24 h (Figure 2a, top). At this time, the KCNR cells showed obvious morphological changes marked by the loss of cellular aggregation, increased cell adherence and neurite extension

(Figure 2a, bottom). The induction of *CASZ1* by RA suggests a role for *CASZ1* in the regulation of NB cell differentiation.

As the transcriptome regulated by *CASZ1* has not been investigated in any species to date, we evaluated the global transcriptome regulated by *CASZ1* to gain insight into potential targets and mechanisms of action. We used Agilent whole human genome oligo microarray analyses (G4112F, Agilent, Santa Clara, CA, USA) to investigate the genome-wide transcriptional consequences of expression of *CASZ1* in three NB cell lines in which *CASZ1* gene expression is regulated by tetracycline (Tet) (SY5YtetCASZ1, NGPtet-CASZ1 and LAN5tetCASZ1). Tet-induced FLAG-*CASZ1* expression was visualized by immunoblotting (Figure 2b) and immunocytochemical staining revealed that *CASZ1* predominantly localizes in the nucleus within 12 h of Tet treatment (Supplementary Figure 3). After Tet-induction (24 h) 125 genes were commonly regulated by *CASZ1* in SY5YtetCASZ1 replicates, the NGPtetCASZ1 and LAN5tet-CASZ1 cell line (Supplementary Table 3, increased/de-creased >1.5-fold in all cell lines). Bio-function assay of these 125 differently expressed genes using Ingenuity Pathways Analysis (IPA) tool, indicated that the top 8 molecular and cellular function classes of the altered genes involved cell growth and proliferation, cell death, cellular movement, as well as cellular development (Figure 2c).

There are 11 common *CASZ1* target genes involved in cell growth and proliferation, the upregulated genes include *HOPX*, *INHA*, *CD9*, *GRAP2*, *NGFR*, *CNN1*, *CLU* and *EDNRA*; and the downregulated genes include Kit ligand (*KITLG*), hepatocyte growth factor (*HGF*) and *PTGER2*. Among those upregulated genes, CLU is a recently reported NB tumor- and metastasis-suppressor gene,[15] while *NGFR* also suppresses NB tumor-igenicity.[16] To validate the microarray data, we performed real-time PCR focus on those genes that involved in tumor growth and developmental processes. *CASZ1* induces a 2- to 40-fold increase in mRNA level of these genes including tumor-suppressor gene *CD9*, *CLU* and *NGFR* (Figure 3a). This induction is not due to Tet because there is no change in the level of mRNA expression of these genes after Tet treatment of the empty vector-transfected NB cell lines (unpublished data).

The following studies support NGFR being a transcriptional target of *CASZ1*. Consistent with the *CASZ1*-induced increases in *NGFR* mRNA, induction of *CASZ1* increases membrane *NGFR* expression in SY5YtetCASZ1 cells, as detected by fluorescence-activated cell sorting (FACS) analysis (Figure 3b). Conversely, in the SK-N-BE2 NB cell line that expresses relatively high endogenous *CASZ1* mRNA, siRNAs targeted to *CASZ1* cause an 80% decrease in *CASZ1* that is accompanied by a 50% decrease in NGFR mRNA expression (Figure 3c). The finding that induction of *CASZ1* expression increased *NGFR* promoter reporter constructs p-3724 luciferase activity (3.5-fold) is consistent with *CASZ1* transcriptionally regulating *NGFR* promoter (Figure 3d). The upregulation of NGFR by *CASZ1* is

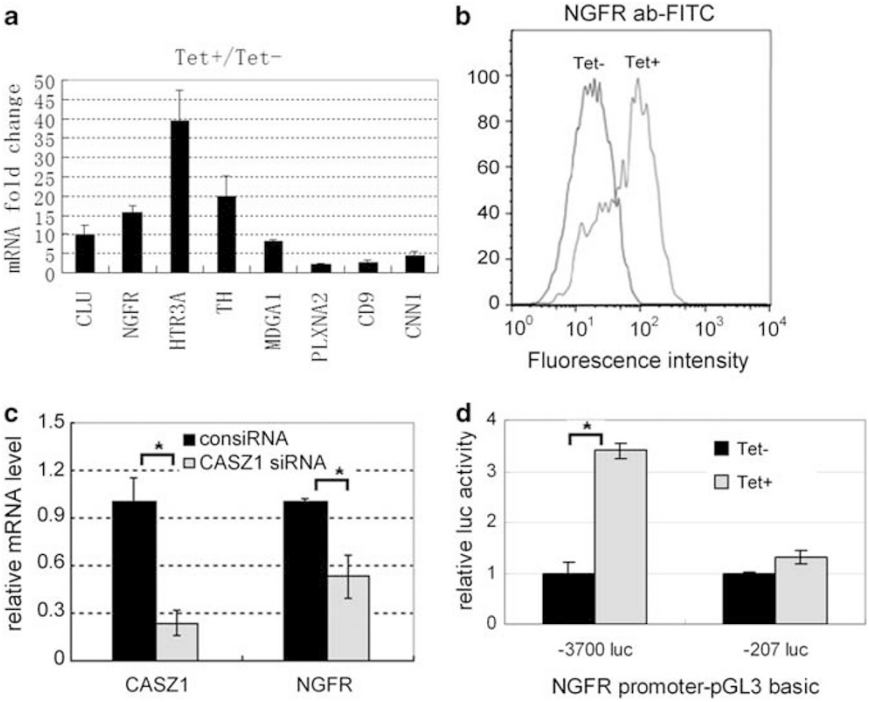

Figure 3 *CASZ1* regulates tumor-suppressor and developmental gene expression. (**a**) The genes upregulated by *CASZ1* in SY5YtetCASZ1 cells discovered by microarray were confirmed by real-time PCR (all $P<0.05$). (**b**) The upregulation of NGFR protein expression in SY5YtetCASZ1 cells by *CASZ1* was detected by FACS analysis using FITC-labeled NGFR antibody. (**c**) Knock down of *CASZ1* expression by *CASZ1* siRNA but not control siRNA downregulated NGFR mRNA expression in SK-N-BE2 cells (detected by real-time PCR, data are shown as mean ± S.D., $^*P<0.05$). (**d**) Two regions of the NGFR promoter representing -3724 to $+13$ bp ($+1$ marks transcription start site) (p-3724luc) and -207 to $+13$ bp (p-207 luc) coupled to a luciferase reporter gene expression vector [38] were transiently transfected into SY5YtetCASZ1 cells. Transfected SY5YtetCASZ1 cells were kept in the presence or absence of Tet for 2 days, and proteins were harvested to evaluate NGFR promoter activity. Relative activity of NGFR promoter analyzed using dual-luciferase assay kit show that induction of *CASZ1* expression significantly increases p-3724 luc activity ($^*P<0.0002$)

consistent with a role for *CASZ1* in inducing NB cell differentiation and suppressing tumor growth.

***CASZ1* induces NB cell differentiation.** Consistent with our finding that *CASZ1* regulates developmental programs and neural differentiation, our biologic assessment of *CASZ1* function indicated that within 3 days of induction of *CASZ1,* neurite extensions are detected in SY5YtetCASZ1 (Figure 4a top, left panel). By day 12, there is more overt morphological differentiation with the formation of dense cellular aggregates and the anastamoses of neuritic extensions to form thick fasciles (Figure 4a bottom, left panel). This morphologic differentiation is accompanied by increases in MAP2 and GAP43 levels, biochemical markers of neural differentiation (Figure 4a, right panel). *CASZ1* strongly upregulates expression of tyrosine hydroxylase (TH) at protein level (Figure 4a, right panel), implying *CASZ1* induces dopaminergic differentiation in SY5Y. Moreover, *CASZ1* induction of SY5YtetCASZ1 cell differentiation under serum-free conditions is even more dramatic with marked neurite extensions and growth cone varicosities, and increases in the growth cone associated protein GAP43 (Supplementary Figure 4, green arrow). *CASZ1*-induced differentiation is accompanied by downregulation of c-Myc expression (Supplementary Figure 4), which frequently occurs on induction of differentiation in a number of cell lineages. Induction of *CASZ1* also induces differentiation in LAN5tetCASZ1 cells (Figure 4b). Although *CASZ1* does not induce morphologic differentiation in NGPtetCASZ1, analysis of the 125 *CASZ1* common target genes using BibiloSphere Pathway Edition tool, revealed that *CASZ1* induced 22 genes that are also expressed in mature neurons (Supplementary Table 4) including HTR3A, NGFR and TH mRNA (Figures 3a and 4c) in all three cell lines. Thus *CASZ1* induces neural gene expression in all tested cell line models and morphologic differentiation in a subset of NB cell line models.

***CASZ1* enhances adhesion and inhibits motility.** *CASZ1* upregulates gene expression of a number of cell–cell adhesion molecules, such as *COL13A1*, *CD9*, *ICAM2* and *PCDH1* in all of the NB cell lines tested (Supplementary Table 3). So we functionally evaluated the effect of *CASZ1* expression on cell adhesion and motility in NB cells. Cells expressing increased levels of *CASZ1* are 2–3-fold more adherent based on their relative resistance to trypsinization (Figure 5a). Moreover increased expression of *CASZ1* results in inhibition of cell movement in a scratch wound assay. We found a significant inhibition of cell movement on induction of *CASZ1* expression in both SY5YtetCASZ1 (2.7-fold) and NGPtetCASZ1 (3.2-fold) (Figures 5b and c). The inhibition of wound healing was not a consequence of slower proliferation rates in the *CASZ1* overexpressing cells,

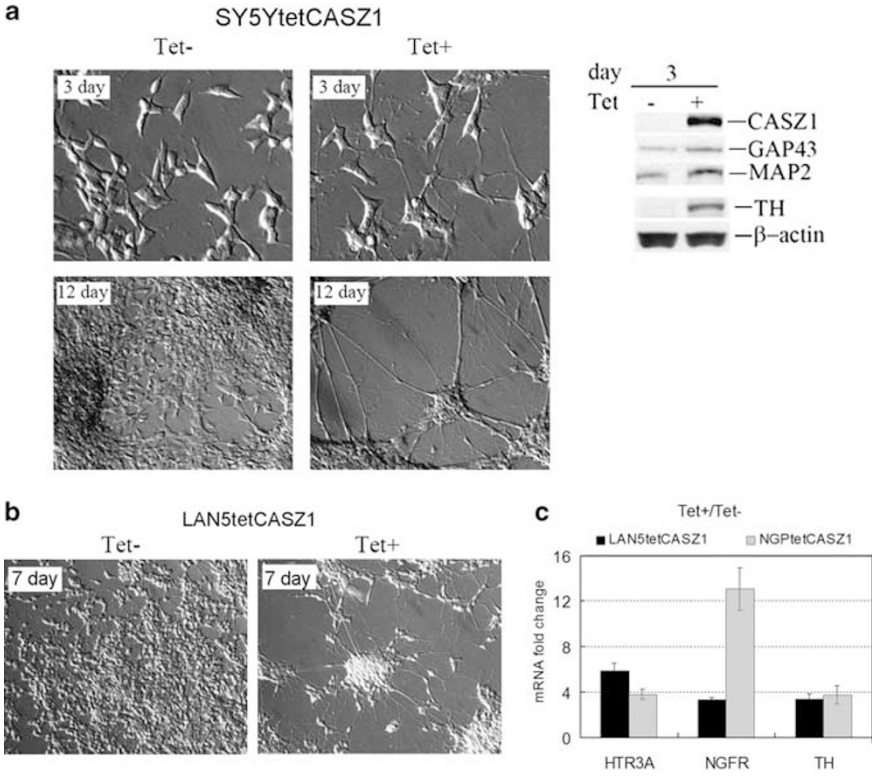

Figure 4 *CASZ1* induces NB cell differentiation. (**a**) In SY5YtetCASZ1 cells (left, top panel), neurite extension is seen within 3 days after induction of *CASZ1* expression with more overt morphological characteristics of differentiation including dense cellular aggregates and fasciculation of neurites after 12 days (left, bottom panel). GAP43, MAP2 and TH, biochemical markers of neural differentiation were upregulated by *CASZ1*. The upregulation was visualized by immunoblotting with antibodies against GAP43, MAP2 and TH. The induction of *CASZ1* expression was visualized by immunoblotting with anti-Flag antibody (right panel). (**b**) In LAN5tetCASZ1 cells, morphological characteristics of differentiation including dense cellular aggregates and fasciculation of neurites after 7 days induction of *CASZ1* expression. (**c**) The upregulation of neural genes expression in LAN5tetCASZ1 and NGPtetCASZ1 cells by *CASZ1* were confirmed by real-time PCR (all $P<0.05$)

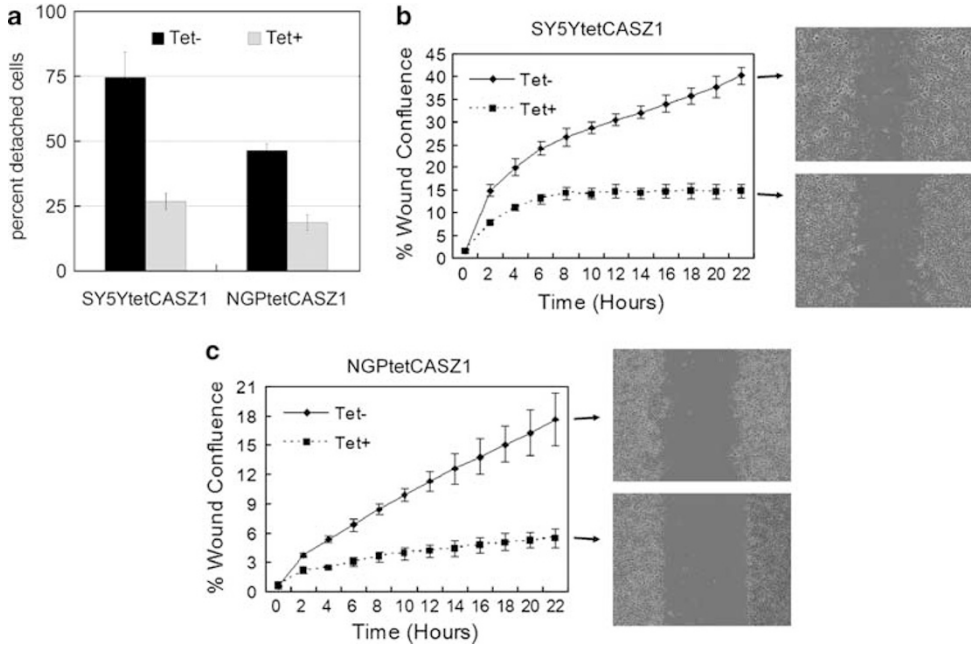

Figure 5 *CASZ1* regulates NB cell adhesion and migration. (**a**) Detachment assay was performed by trypsin treatment of cells for 2–3 min; the ratio of the number of detached cells to the total cell number was calculated. The NB cell lines adhered more strongly to the substratum after induction of *CASZ1* expression for 3 days (*$P<0.005$). (**b**) Wound confluence was graphed over time to quantitatively evaluate the characteristics of wound closing. The IncuCyte scratch wound assay software was used to analyze the data. Induction of *CASZ1* expression caused a statistically significant reduction in migration in SY5YtetCASZ1 cells compared with the migration of control cells in a scratch wound assay (all time point $P<0.00001$ after wounds generated for 2.5 h). The cells photographed under phase-contrast microscopy at 22 h were showed at the right. (**c**) Wound confluence on induction of CASZ1 expression caused a statistically significant reduction in migration in NGPtetCASZ1 cells compared with the migration of control cells in a scratch wound assay (all time point $P<0.0005$ after wounds generated 2.5 h). The cells photographed under phase-contrast microscopy at 22 h were showed at the right

because *CASZ1* inhibited cell movement in cells, which had been growth inhibited by the cell proliferation inhibitor mitomycin C before the scratch assay. These data indicate that *CASZ1* expression increases cell adhesion and decreases cell motility function.

***CASZ1* suppresses NB tumorigenicity.** Upon *CASZ1* induction, NB cell growth was inhibited 80% in SY5Y cells and 50% in NGP cells at day 8 (Figure 6a). MTS assays showed similar results while the growth of the empty vector control cell lines were not affected by Tet treatment (Figure 6b). FACS analysis showed that in NBtetCASZ1 cell lines, overexpression of *CASZ1* leads to a modest increase in the percentage of cells in the G1 phase of the cell cycle (5–8%) at day 4 (Supplementary Figure 5a, b). The overexpression of *CASZ1* functions to delay progression of SY5YtetCASZ1 cells through the cell cycle (Supplementary Figure 5c).

To test the effects of *CASZ1* on tumorigenicity of NB cells, anchorage-independent growth was first assessed by soft agar clonogenicity. *CASZ1*-expressing cells showed a 90–50% decrease in soft agar clonogenicity compared with the controls in SY5YtetCASZ1 cells and NGPtetCASZ1 cells (Figure 6c).

To assess *in vivo* tumorigenicity, SY5YtetCASZ1 and NGPtetCASZ1 cells were implanted at a subcutaneous site into mice that had received placebo or Tet in the drinking water during the previous week. Tet-treated animals showed

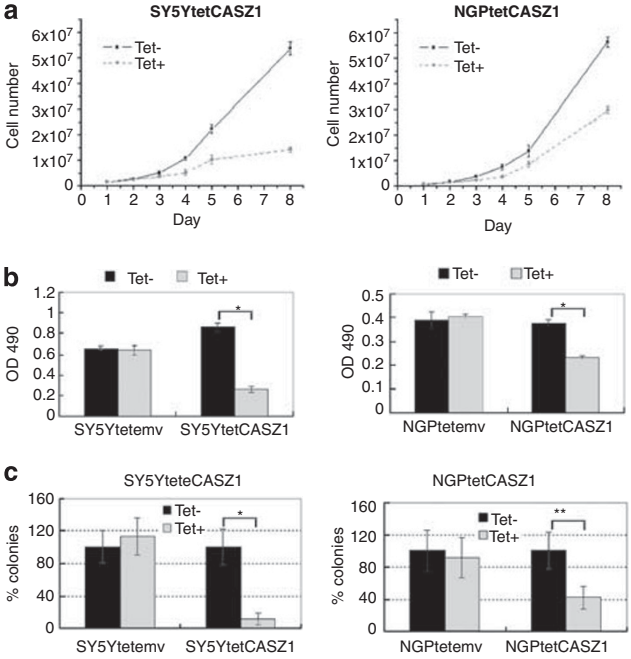

Figure 6 *CASZ1* inhibits NB cell growth *in vitro*. (**a**) Cell number was counted at different time point using hemocytometer (all time point $P<0.05$ since day 3). (**b**) Cell proliferation was assessed using MTS assay after NB cells treated with or without Tet for 7 days (*$P<0.005$). (**c**) SY5YtetCASZ1 and NGPtetCASZ1 cell soft agar clonogenicity decreased when *CASZ1* was induced to express by Tet (*$P<0.005$; **$P<0.05$)

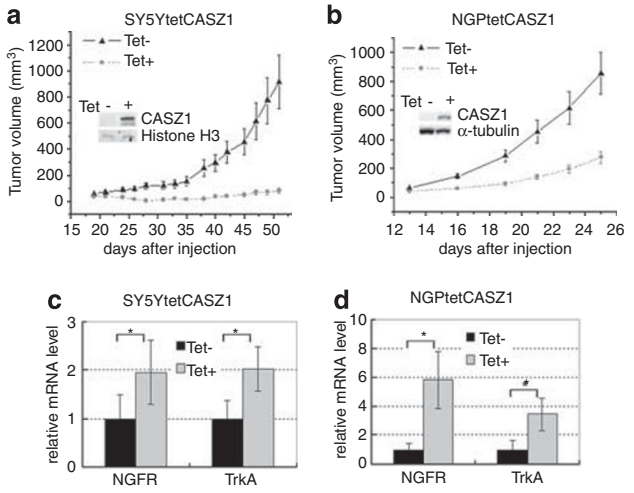

Figure 7 *CASZ1* inhibits NB cell growth *in vivo*. (**a**) SY5YtetCASZ1 cells were subcutaneously injected into nude mice. The induction of *CASZ1* expression significantly inhibited tumor growth in the xenograft model ($n=8$ mice per group, total 16 mice; Volume = long $\times$ short^2/4; data are shown as mean $\pm$ S.E.M., all time point $P<0.02$ since day 24). (**b**) NGPtetCASZ1 cells were subcutaneously injected into nude mice. The induction of *CASZ1* expression significantly inhibited tumor growth in the xenograft model ($n=10$ mice per group, total 20 mice; data are shown as mean $\pm$ S.E.M., all $P<0.02$ since day 16). (**c**) TrkA and NGFR mRNA levels were significantly upregulated in SY5Y xenograft tumors expressing *CASZ1* (*$P<0.05$). (**d**) TrkA and NGFR mRNA levels were significantly upregulated in NGP xenograft tumors expressing *CASZ1* (*$P<0.05$)

decreases in tumor growth and increased *CASZ1* expression was detected in their tumors (Figures 7a and b). A nearly complete inhibition of tumor growth was detected by eight weeks when *CASZ1* was expressed in the SY5Y cell background compared with placebo-treated mice (Figure 7a). A 75% decrease in tumor growth was detected by four weeks in NGP cell background compared with placebo-treated mice (Figure 7b). *CASZ1*-expressing xenograft tumors did not lead to a more differentiated histology (data not shown). However, in *CASZ1*-expressing tumors there is an increase in expression of *NGFR* (1.9-fold for SY5Y; 5.8-fold for NGP) as well as *TrkA* (2-fold for SY5Y; 3.4-fold for NGP) (Figure 7c and d), which are highly expressed in differentiated NB tumors and markers of good prognosis in NB.[16,17]

Discussion

This study demonstrates a role for *CASZ1* as a candidate NB tumor-suppressor gene based on: its localization to the chromosome 1p36 region;[1–3] the association of low-*CASZ1* expression with poor prognosis and overall survival (Table 1, Figure 1); its regulation of genes involved in tumor growth and developmental processes (Figure 2, Supplementary Table 3); and its functional ability to inhibit NB tumor cell growth *in vitro* and *in vivo* (Figures 6 and 7).

Previous studies showed that there are at least three discrete regions at chr1p are commonly deleted in NB, which indicates that these regions harbor putative tumor-suppressor genes.[1] Recent evidence indicates that *CHD5* (1p36.31) a chromatin remodeling gene,[18,19] *KIF1β* (1p36.21) a pro-apoptotic gene[20,21] and miR-34a, a micro RNA known to regulate *MYCN* expression[22,23] are candidate 1p36 NB tumor-suppressor genes. *CASZ1* (1p36.22) localizes to one of the commonly deleted region in NB on chr1p between the markers D1S508 and D1S244.[3] Although the recent study in 184 primary NB with 1p LOH showed that 1p36.3 (minimally between D1S2795 and D1S252) is the shortest region of overlap (SRO),[24] we found in their study that loss of *CASZ1* occurs in 180 of the 184 (98%) 1p LOH primary NB tumors. In this study, we provide functional evidence that *CASZ1* is a tumor-suppressor gene. The identification of multiple loci on 1p36 with tumor suppressor-like activity is consistent with the hypothesis that alterations at multiple loci within this region contribute to tumorigenesis.

Classical tumor suppressors have altered expression of both alleles resulting in a loss of gene function. Although no coding nonsynonymous tumor-specific *CASZ1* mutations have been found by sequencing 46 NB patient DNA samples,[3] we cannot exclude the possibility that one allele of *CASZ1* is deleted and the other allele is mutated in a larger NB population. Currently, we are sequencing the 160 kb regions encompassing the *CASZ1* gene to assess mutational status in NB patient samples. With respect to *CASZ1*, our unpublished studies combined with others[3] showed that although a demethylating agent induces *CASZ1* expression, bisulfite sequencing results indicate that methylation of CpG rich regions 5′ and 3′ of the *CASZ1* gene does not account for low-*CASZ1* expression. We found that depsipeptide, a clinically relevant HDAC inhibitor upregulated *CASZ1* expression even in NB tumors with an intact Chr1p. Although HDAC inhibitors result in activation of hundreds of genes, treatment with TSA induces only *CASZ1* and 3 other genes in the 30 genes that localize to the Chr1p36 SRO between the markers D1S508 and D1S244.[15] This indicates that only a subset of Chr1p36 genes may be silenced by histone deacetylation. Taken together, the above information suggests that epigenetic silencing either directly or indirectly contributes to the low expression of *CASZ1* in NB cells.

Low-*CASZ1* expression is predictive of poor outcome and is associated with other unfavorable clinical and biological features: age $\geq$18 months, unfavorable histology, 1p LOH and MYCN amplification. *CASZ1* expression and grade are not associated in this study, yet this may be due to the fact that in 14 of the 59 samples tumor grade was unknown (Table 1). It was somewhat unexpected that high-*CASZ1* expression would be associated with 11q LOH, which is a poor prognostic indicator (Table 1). This could be because *CASZ1* levels are only relevant in the 1p LOH subset of NB tumors. However, the high levels of *CASZ1* associated with 11q LOH may be due to the loss of upstream regulators of *CASZ1* on 11q that may directly or indirectly suppress *CASZ1* levels. Conversely, high levels of *CASZ1* may also be due to loss of gene(s) on 11q, which are *CASZ1* downstream targets that *CASZ1* regulates to control neuroblast cell growth and tumorigenicity, and this loss causes a negative feedback circuit. Such a model has been described in lung cancer in which inactivation of the tumor-suppressor Rb leads to increased expression of the tumor-suppressor *p16^{INK4a}* (see ref. 25). In fact, in *Drosophila*, *CASZ1* (*dcas*) is expressed at a specific time and in specific cells by both feedback and feed-forward regulation with other temporal transcription factor *Hunchback*, *Kruppel*,

Pdm and *Grainyhead* to keep the proper neuroblast sublineage development.[26] It is unknown if a similar network occurs in mammals. Clearly the elucidation of the *CASZ1*-regulated signal transduction pathways will be important in understanding its association with 11q LOH. A larger study is also needed in order to determine if *CASZ1* expression is independently prognostic in comparison with other prognostic factors.

The level of expression or *CASZ1* gene dosage is associated with NB patient prognosis. Haploinsufficiency for tumor suppression has been demonstrated in the case of $p27^{KIP1}$, *p53*, *NF1* and *PTEN*.[27] With respect to *CASZ1*, HDAC inhibitors upregulate *CASZ1* expression in the cell lines with 1p LOH, suggesting the remaining allele is epigenetic silenced. However, we also found that *CASZ1* mRNA expression was detectable in all NB patient samples and NB cell lines tested. These findings suggest epigenetic silencing of *CASZ1* leads to its low expression, instead of complete depletion of *CASZ1* in 1p LOH lines. Thus, *CASZ1* may function like haploinsufficient tumor-suppressor genes, where a partial loss of *CASZ1* may be sufficient to result in a cellular phenotype that leads to NB tumorigenesis.

Our study represents the first functional evidence in mammalian models that the highly evolutionarily conserved *CASZ1* gene regulates the expression of genes important for cell growth and neural and muscle developmental processes as proposed in *Drosophila* and *Xenopus* models.[5,7–9] Our study provides compelling functional evidence that the *CASZ1* suppresses tumor cell growth *in vitro* and *in vivo* (Figures 6 and 7). *CASZ1* induces NB cells differentiation *in vitro*; and in *CASZ1*-expressing xenograft tumors there is an increase in expression of *NGFR* and *TrkA* (Figures 7c and d), which are highly expressed in differentiated NB tumors and markers of good prognosis in NB. Although the *CASZ1*-expressing xenograft tumors did not have a more differentiated histology (data not shown), this may be due to the subcutaneous transplantation of the NB cell lines. Additional studies in an orthotopic setting will be needed to determine whether *CASZ1* restoration enables differentiation of NB tumors *in vivo*.

The transcriptome regulated by *CASZ1* in NB cell lines reveals a number of induced targets whose increased expression has been associated with tumor suppression or with metastasis suppression (*NGFR*, *CLU*, *CD9* and *ICAM2*).[15,28–31] Among the targets that *CASZ1* suppresses are the metastasis enhancers *HGF* and *KITLG*.[32,33] Preliminary studies have not identified a single critical target gene that mediates *CASZ1* functions and current studies are aimed at delineating the targets and signaling pathways by which *CASZ1* suppresses tumor growth and metastasis.

CASZ1 is expressed in a number of different tissues besides neuronal cells.[4] It is interesting that *CASZ1* in *Xenopus* is required for cardiomyocytes differentiation and loss of *CASZ1* causes over-proliferation of cardiac progenitor cells.[9] Inspection of data from available expression profiling data sets (http://www.oncomine.org),[34] reveals that in neural crest-derived melanoma, lower levels of *CASZ1* expression are found in cutaneous melanoma compared with normal skin ($P = 2.64E{-}5$, Supplementary Figure 6). Low expression of *CASZ1* is also found in gastric intestinal type adenocarcinoma, head and neck squamous cell carcinoma, and clear cell renal cell carcinoma (all $P < 0.0001$, Supplementary Figure 6). Our functional studies support a role for *CASZ1* as a tumor-suppressor in NB but it is possible that loss of *CASZ1* may interrupt the normal developmental program of other tissues types and have a role in their tumorigenesis.

Materials and Methods

Cell culture. NB cell lines LAN5, SMS-KCNR (KCNR), SK-N-AS (AS), SK-N-BE2 (BE2), NGP and SH-SY5Y (SY5Y) were cultured in RPMI 1640 containing 10% fetal bovine serum, 2 mM glutamine and antibiotics as previously described.[35] Background of these NB cell lines is shown in Supplementary Table 1. Cells were transfected using Nucleofector (Amaxa Biosystems, Gaithersburg, MD, USA) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

Cell treatment. When noted, cells were treated with 5 μM of RA dissolved in 95% ethanol. 5-Aza-dC (Sigma-Aldrich Co., St. Louis, MO, USA) was dissolved to 20 mM in 50% acetic acid in distilled water. Depsipeptide, provided by Fujisawa Pharmaceuticals (Osaka, Japan), 5 mg/ml was dissolved in 4 : 1 propylene glycol/ethanol, diluted to100 μg/ml in dimethyl sulfoxide and stored at $-20\,^{\circ}$C. For experiments, 5-Aza-dC and depsipeptide were diluted to indicate concentrations in cell culture media.

Stable clones. The full-length human *CASZ1* transcription factor gene spans 160 kb, encodes an 8.0 kb transcript with 21 exons (Genbank accession number: NM_001079843) with 11 class C2H2 ZnF motifs.[4] Full-length Flag-tagged *CASZ1* cDNA[4] was cloned in the Tet inducible vector pT-REx-DEST30. The control vector pDest-30 with an out-of-frame *CAT* gene with no start codon served as the empty vector control.

Tet-inducible expression is dependent on expression of the Tet repressor in the recipients. Recipient cells were prepared by stable transfection of the pcDNA6/TR (Tet repressor) plasmid (Invitrogen) into NGP and LAN5 cells and selection with blasticidin. Clones, LAN5tet and NGPtet, were isolated and expanded in media containing 5 μg/ml blasticidin (Invitrogen). Full-length Flag-tagged *CASZ1* in pT-REx-DEST30 or an empty vector control were transfected into SY5Ytet,[36] LAN5tet and NGPtet cells selected with G418 (300–500 μg/ml) and blasticidin (3–5 μg/ml). Antibiotic-resistant transfectants were isolated and evaluated for Tet (1 μg/ml) regulated *CASZ1* expression. Stable clones expressing *CASZ1*, are labeled SY5YtetCASZ1, NGPtetCASZ1 and LAN5tetCASZ1 while the stable clones with empty vector are labeled SY5Ytetemv, NGPtetemv and LAN5tetemv.

RNAi. RNAi was performed as described previously.[4] To target *CASZ1* expression we designed a *CASZ1* siRNA to the target sequence 5′-AACGGACT GCCCACAGATAAA-3′. Non-silencing siRNA (Qiagen, Valencia, CA, USA; cat. no. 1022076100) was used as negative control. SiRNA was transfected into NB cells by using Nucleofector (Amaxa Biosystems) following the manufacturer's protocol.

Real-time quantitative PCR. The primer sets for the *CASZ1*, NGFR and TH genes are shown in Supplementary Table 5. Expression of β-actin was used for normalization. Quantitative real-time PCR was performed on ABI Prism 7000 (PE Applied Biosystems, Poster City, CA, USA) using SYBR Green SuperMix as described previously.[4] The PCR was performed in duplicate or triplicate and repeated at least once. Taqman real-time PCR was applied for primary tumor study.

Primary tumor study. Total RNA from NB tumors from 59 patients before chemotherapy was provided by the COG NB Biology Group. RNA (2 μg) was used as template for cDNA synthesis using Superscript III (Invitrogen), and *CASZ1* levels assessed by Taqman real-time PCR. *CASZ1* (part no. 4331182, assay ID: Hs00214901_m1) and β-actin (endogenous control) (part no. 4333762F) real-time PCR primers were designed and synthesized by PE Applied Biosystems. PCR reactions were performed according to the manufacture's protocol and standard curves were made during each PCR reaction.

Northern analysis. Total RNA was isolated with RNeasy kit (Qiagen) following the manufacturer's protocol. Forty micrograms of total RNA was run on 1% formaldehyde gel blotted to Nytran filters and hybridized purified inserts of *CASZ1* DNA. The probes were labeled with ^{32}P by Rediprime II (Amersham Biosciences, Buchinghamshire, UK).

FACS analysis for the determination of NGFR expression. SY5YtetCASZ1 cells treated with Tet (1 μg/ml) for 2 days were washed with PBS twice and detached by trypsin-free detachment agents, then washed once with 5 ml of PBS. Two million cells were incubated with anti-NGFR-FITC antibody (Cedarlane Laboratories Limited, Burlington, NC, USA) or normal mouse IgG-FITC for 45 min at 4 °C and washed with PBS. FACS analysis was performed using a flow cytometer (FACScan; Becton Dickinson, Franklin Lakes, NJ, USA). NGFR expression was measured using the FlowJo software (Ashland, OR, USA).

Cell cycle analysis. Cell cycle analysis was performed using propidium iodide staining. NBtetCASZ1 cells treated with Tet (1 μg/ml) for 4 days were harvested, washed with PBS twice and stained with 50 μg/ml propidium iodide containing 5 μg/ml RNase A for 30 min at room temperature, and analyzed by flow cytometry (FACScan; Becton Dickinson) and FloJo software.

Western blot and indirect immunofluorescence assay. Nuclear protein extraction, whole cell protein extraction, western blot analysis and indirect immunofluorescence assay were performed as descried previously.[4] For western blot analyses, an anti-Flag (1 : 1000) antibody from Sigma (St. Louis, MO, USA) was used to detect Flag-*CASZ1* expression. Antibodies to MAP2 (1 : 1000) (Chemicon International, Billerica, MA, USA), GAP43 (1 : 1000) (Abcam, Cambridge, MA, USA), α-tubulin (1 : 2000), β-actin (1 : 2000), Histone H3 (1 : 200) and TH (1 : 1000) (Santa Cruz, Santa Cruz, CA, USA) was used to detect each protein expression. For indirect immunofluorescent cell staining, cells were incubated with anti- GAP43 (1 : 250) or anti- α-tubulin (1 : 200) (Molecular Probes, Carlsbad, CA, USA) for 1 h at 23 °C, washed three times, followed by incubation with goat anti-rabbit IgG-Rhodamine or goat anti-mouse IgG-FITC (1 : 200) for 45 min at 23 °C.

Cell adhesion and scratch wound assay. Cell adhesion was performed as described by Su *et al*.[37] with some modification. Briefly, cell adhesion was measured using a trypsinization assay. SY5YtetCASZ1 and NGPtetCASZ1 cells were plated onto a six-well plate overnight and then treated with Tet (1 μg/ml) for 3 days, then treated with 0.5 ml of 0.05% trypsin-EDTA for 2–3 min to stimulate cell detachment. The number of detached cells was counted using a hemocytometer and expressed as a percentage of the total number of cells on the plate. For scratch wound assay, SY5YtetCASZ1 and NGPtetCASZ1 cells were plated into Essen ImageLock 24-well plates (for NGPtetCASZ1 cells, the plate was coated with collagen I), and after 24 h incubation, the cells were treated with or without Tet (1 μg/ml) for 24 h, then the medium was changed from 10% FBS containing complete medium to 0.5% FBS containing low serum medium with or without Tet (1 μg/ml). After overnight incubation, the cells were incubated with mitomycin-C (10 μg/ml) for 2 h to inhibit cell proliferation, after three times washing with low serum medium, the confluent monolayer of the cells was scratched using the Essen 24-well WoundMaker (Ann Arbor, MI, USA) following their protocols and the cells were cultured in the complete medium with or without Tet (1 μg/ml). The wound confluence was obtained and analyzed by using the IncuCyte phase-contrast imaging and scratch wound assay system and software (ESSEN INSTRUMENTS, Ann Arbor, MI, USA).

Promoter activity assay. The NGFR promoter constructs were generously provided by Philip Barker.[38] The constructs were transiently transfected into SY5YtetCASZ1 cell line and after 24 h, cells were treated with Tet. After 2 days, cells were harvested, protein isolated and relative luciferase activity assessed using a dual-luciferase reporter assay system (Promega, Madison, WI, USA), the experiments were repeated for three times.

Cell proliferation and clonogenicity assays. To assess the effect of *CASZ1* on NB cell proliferation, SY5YtetCASZ1 and NGPtetCASZ1 cells were plated into 10 cm dishes. The next day Tet was added to the RPMI-1640 containing 10% fetal calf serum. Trypan blue diluted cell suspension was counted using hemocytometer at indicated time points. MTS assay (Promega) was also performed to assess effects of *CASZ1* on NB cell proliferation. IncuCyte (ESSEN BioSCIENCE, Ann Arbor, MI, USA) was used to monitor kinetic SY5YtetCASZ1 cell proliferation in 24-well plate using the integrated confluence algorithm as a surrogate for cell number. To assess effects of *CASZ1* on anchorage-independent cell growth, 1–2 $\times 10^4$ cells were cultured in 0.7% top agarose in media containing G418 and Blasticidin ($\pm$ Tet) plated on a layer of 1.4% bottom agar/RPMI to prevent the adhesion of cells to the culture plates. Medium was changed twice a week and visible colonies counted after 4 weeks.

***In vivo* tumorigenesis.** Suspensions of SY5YtetCASZ1 (2×10^6) or NGPtetCASZ1 (5×10^5) cells were mixed with an equal volume of Matrigel solution (Trevigen, Gaithersburg, MD, USA) and implanted subcutaneously in the dorsal flank of 16–20 SCID mice (6–8 weeks female mice) per cell line. The animal studies were approved by the Animal Care and Use Committee of the National Cancer Institute (PB-023). Control mice received 5% sucrose in their drinking water while the other half received Tet (2 mg/ml) in 5% sucrose for 1 week before tumor implantation and during the course of the experiment. The tumors were measured thrice weekly, and the mice were killed once the tumor diameter reached 20 mm.

The oligo microarray. Agilent whole human genome oligo microarray kit (G4112F, Agilent) were used to determine the *CASZ1*-induced transcriptional profiles in SY5YtetCASZ1, NGPtetCASZ1 and LAN5tetCASZ1. Gene expression after Tet induction (24 h) was analyzed in the three different NB cell lines, as well as a biologic replicate for SY5Y cell line. Array hybridization, chemiluminescence detection and image acquisition and analysis were performed by GenUs Biosystems, Northbrook, IL, USA. The differentially expressed gene list was uploaded to IPA (Ingenuity http://www.ingenuity.com/) and BiblioSphere Pathway Edition tool (Genomatix http://www.genomatix.de/), and these were used to generate unbiased molecular and cellular functional analyses and physiological system development and functions.

Statistical analyses. The method of Kaplan–Meier was used to generate survival curves, with standard errors per Peto,[39] and differences between the curves were tested with a log rank test. For EFS, time to event was calculated from the time of diagnosis until the first occurrence of relapse, progression, secondary malignancy or death from any cause, or until last contact if no event occurred. An event for overall patient survival was death. *P*-values <0.05 were considered statistically significant.

To identify an *CASZ1* expression cut-off that maximized the difference in EFS rate between two groups (low *versus* high expression), 13 Cox proportional hazards models[40] were run, one at each of 13 different *CASZ1* expression cut-offs. Each model included only one term, a binary indicator for high-*CASZ1* expression; the assumption of proportional hazards was tested, and was not violated. The value for every sixth patient (i.e, at each decile) was tested as a cut-off, and additional cut-offs was tested to gain resolution in the area of interest. A combination of minimum *P*-value and maximum relative risk were used to identify the cut-off that was the most prognostic of outcome.

The study was not powered to determine if *CASZ1* expression was independently predictive of outcome, so multivariable modeling was not performed.

IPA of microarray data was analyzed by Fisher's exact test (http://www.ingenuity.com/). Additional statistical analyses of continuous data were performed using a *t*-test with $P<0.05$ considered significant. Values in the graphs are expressed as means $\pm$ S.E.M. or S.D. The statistical tests were two-sided.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)