The neural crest lineage regulatory transcription factors (TFs) form a core regulatory circuitry (CRC) in neuroblastoma (NB) to specify a noradrenergic tumor phenotype. Oncogenic subversion of CRC TFs is well documented, but the role of loss of tumor suppressors plays remains unclear. Zinc-finger TF CASZ1 is a chromosome 1p36 (chr1p36) tumor suppressor. Single-cell RNA sequencing data analyses indicate that CASZ1 is highly expressed in developing chromaffin cells coincident with an expression of NB CRC TFs. In NB tumor cells, the CASZ1 tumor suppressor is silenced while CRC components are highly expressed. We find the NB CRC component HAND2 directly represses CASZ1 expression. ChIP-seq and transcriptomic analyses reveal that restoration of CASZ1 upregulates noradrenergic neuronal genes and represses expression of CRC components by remodeling enhancer activity. Our study identifies that the restored CASZ1 forms a negative feedback regulatory circuit with the established NB CRC to induce noradrenergic neuronal differentiation of NB.

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Importantly, we found that the CRC TFs in NB repress CASZ1 expression and restoration of CASZ1b suppresses expression of the noradrenergic CRC TFs. Thus, CASZ1 forms a negative feedback loop with CRC TFs and its loss of expression shapes the tumorigenic NB cell identity. These studies identify for the first time how loss of a tumor suppressor with TF activity is linked to the oncogenic subversion of a lineage specifying CRC.

RESULTS

CASZ1 is essential for neuronal differentiation of NB

SH-SY5Y is a noradrenergic type of NB cell line and our previous study showed that the restoration of CASZ1b in SY5Y cells results in decreased cell proliferation [26]. The SK-N-AS (AS) NB cell line has a mixed noradrenergic and mesenchymal phenotype [5]. Using tetracycline (Tet) inducible CASZ1b overexpressing SY5Y (SY5YtetCASZ1b) and AS (AS tetCASZ1b) cell lines, we found that the induction of CASZ1b suppressed both SY5Y and AS cell growth (Fig. 1A, B). Realtime PCR results showed that the induction of CASZ1b in both SY5Y and AS cells significantly upregulated the expression of neuronal genes including serotonin 3A receptor (HTR3A) [34], nerve growth factor receptor (NGFR) [35], and tyrosine hydroxylase (TH) [36] (Fig. 1C). Induction of CASZ1b did not induce morphologic differentiation in AS cells, but in SY5Y cells induction of CASZ1b resulted in neurite extension as detected by anti-growth associated protein 43 (GAP43) antibody staining (Fig. 1D). We have previously shown that retinoic acid (RA) treatment of SY5Y cells results in an upregulation of both CASZ1a and CASZ1b at mRNA levels [26]. Here we found that the RA treatment of SY5Y cells for 3 days resulted in an upregulation of both CASZ1a and CASZ1b at protein levels shown by western blot analysis (Fig. 1E). To understand the role of CASZ1 in RA induced cell differentiation, we silenced CASZ1 using two different short hairpin RNAs (shRNAs) in SY5Y cells (Fig. 1F). While a significant number of neurites were observed in RA treated, shRNA control vector (SY5YshCtrl) transduced cells, few neurites (Fig. 1G) and reduced neurite length per cell-body (Fig. 1H) were observed in RA treated, CASZ1 shRNA (SY5YshCASZ1) transduced cells (Fig. 1G, H). RA treatment resulted in an increase in the neuronal marker GAP43 mRNA levels in shCtrl vector transduced cells but not in shCASZ1 transduced cells (Fig. 1I). These results indicate that CASZ1 is essential for NB cell differentiation.

CASZ1 regulates sympathoadrenal lineage genes

To interrogate the transcriptional consequences of CASZ1 expression in NB cells, we performed RNA sequencing (RNA-seq) analysis of SY5Y cells and AS cells with or without restoration of CASZ1b and identified transcriptome regulated by CASZ1b in these two NB cell lines (Supplementary Table 1). Gene set enrichment assay (GSEA) of RNA-seq data showed that restoration of CASZ1b in SY5Y cells resulted in a positive enrichment of genes that positively regulate axonogenesis (Fig. 2A, top panel). Moreover, CASZ1b over-expression led to a significantly positive enrichment of genes regulating neurotransmitter transport and catecholamine secretion, which are expressed in the differentiating and differentiated noradrenergic cells. Although the transcriptome regulated by CASZ1b in AS cells was not completely the same as in SY5Y cells, GSEA showed that restoration of CASZ1b in AS cells also results in a positive enrichment of neuronal genes such as genes positively regulate axon extension, neurotransmitter receptor activity and neurotrophin signaling (Fig. 2A, bottom panel). Our results indicate that CASZ1b induces a sympathoadrenal differentiation program of NB cells.

Primary NB commonly arises in neural crest cells of the sympathoadrenal lineage. A recent study of early adrenal medulla development in mouse embryos using single-cell RNA sequencing (scRNA-seq) [37] provides an opportunity to investigate the expression pattern of CASZ1 in the normal developing murine sympathoadrenal cells at the single-cell level. By analyzing scRNA-seq data of E12.5 murine embryos, we found that CASz1 is expressed at relatively low levels in Schwann cell precursors (SCPs) and sympathoblasts yet CASz1 levels rise in transitional stage cells (bridge) and reach their highest relative levels in the developing and differentiated chromaffin cells (Fig. 2B). Adrenal medulla cells clustered based on gene expression (Supplementary Fig. 1A, top panel) were enriched in genes co-expressed with Casz1 (Supplementary Fig. 1A, bottom panel), which are similar to those clusters of cells that are enriched in neuron projection and neurotransmitter genes (Supplementary Fig. 1A, top panel). GO pathway enrichment assay of genes co-expressed with Casz1 showed a positive enrichment of synapse associated genes (Supplementary Fig. 1B). The noradrenergic NB CRC components including Phox2b, Gata3, Hnd2, Isl1, and Tbx2 are known TFs that play important roles in regulating sympathoadrenal lineage development. We found these genes and other neuronal crest regulators including Gata2, hand1, and phox2a were all expressed in sympathoblasts, transitional stage cells and developing chromaffin cells, with variable mRNA levels in SCPs (Fig. 2B, Supplementary Fig. 1C). These observations suggest that CASz1 is involved in normal adrenal medullary development and cooperates with those CRC components to regulate chromaffin cell differentiation.

We next compared the expression of CASZ1 and CRC components in human adrenal gland (AG), neuroblastoma cell lines and neuroblastoma tumors (Versteeg cohort) [38] by analyzing the R2 database (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi). We found that the relative mRNA levels of CASZ1b were lower in NB cell lines and NB tumors than in normal adrenal gland derived cells. However, the relative mRNA levels of the CRC components such as GATA3 and HAND2 were significantly higher in NB tumors than in adrenal gland derived cells (Fig. 2C). This is consistent with the function of CASZ1b as a tumor suppressor in NB [26], while CRC components are genes essential to maintain the oncogenic noradrenergic phenotype of NB cells [10]. Our results indicate a reverse correlation between CASZ1 and CRC TFs in NB cells.

CASZ1 is transcriptionally regulated by CRC components

To investigate whether the high expression of NB CRC components impacts CASZ1 expression, we first analyzed publicly available CRC TFs ChIP-seq data of noradrenergic NB cell lines BE(2)C and SY5Y [10, 39]. We found that all the CRC TFs bound to the CASZ1 gene locus with multiple peaks shown by the signal tracks (Fig. 3A). The binding sites of these TFs within CASZ1 gene locus included the promoter (Fig. 3A, black box) and the TSS and terminus (Fig. 3A, cyan box) with the strongest signal within the 2nd intron (Fig. 3A, red box). Interestingly, the active enhancer mark H3K27ac signal within the 2nd intron (Fig. 3A, red box) was lower compared to the other regions in BE(2)C and SY5Y cells, which suggests that these TFs might play a repressive role in regulating CASZ1 gene expression. Knockdown the CRC genes HAND2 or TBX2 but no other CRC components in the noradrenergic NB cell line IMR32 (MYCN-amplified), led to a significant increase in CASZ1 mRNA expression (Fig. 3B). Upon selectively silencing of HAND2 expression, followed by ChIP-seq using anti-HAND2, H3K27ac, H3K4me3 and H3K27me3 antibodies [40], we found a decrease in HAND2 binding within the CASZ1 gene locus compared to controls, and an increase in H3K27ac signal within the 2nd and 4th intron (Fig. 3C). Loss of HAND2 resulted in an increase of H3K4me3 signal and a decrease of H3K27me3 signal at the TSS region of CASZ1 gene, suggesting that the changes of H3K4me3 and H3K27me3 signal might be mediated through PRC2 complex. CASZ1 has been reported to be repressed by PRC2 complex and a bivalent mark of H3K4me3 and H3K27me3 has been found at the transcription start site (TSS) of CASZ1 gene [29].
Genome-wide binding of CASZ1 in NB cells

To gain insights into how the restoration of cellular levels of CASZ1 affects the epigenome, we performed ChIP-seq experiments using anti-CASZ1, H3K27ac, and RNA Pol II antibodies in SY5YtetCASZ1 cells treated with or without Tet for 2 days. We identified 733 CASZ1 binding sites (narrow peak calling, \( p < 10^{-7} \)) that associated with 614 genes (Supplementary Table 2) in the cells without Tet treatment (Tet\(^-\)). This represents the basal,
endogenous CASZ1 binding sites. Upon induction of CASZ1b (Tet +), we identified 13845 Tet-induced CASZ1b peaks that associate with 7525 genes (Supplementary Table 2). These sites had a dramatic increase in the CASZ1b peak centered signal compared to control cells as shown in the ChIP-seq heatmap (Fig. 4A). We further investigated changes in the chromatin landscape after CASZ1b induction to understand in more mechanistic detail how CASZ1 regulates gene transcription. ChIP-seq heatmaps showed that Tet-induced CASZ1b peak centers overlapped with H3K27ac and RNA Pol II binding sites (Fig. 4A). After the restoration of
CASZ1b in SY5Y cells, all CASZ1b-bound peaks showed a measurable increase of H3K27ac and RNA Pol II signals (Fig. 4A). Endogenous CASZ1 peak distribution analysis showed that 2.86% of CASZ1 binding sites were at promoter regions (defined by ±1 kb of transcription start site, TSS). Overlapping the promoter-distal H3K27ac peaks, which mark active enhancers, with the endogenous CASZ1 peaks, we found that 60.71% CASZ1 binding sites were within active enhancers (Fig. 4B). Tet-induced CASZ1b peak distribution analysis showed that 8% of CASZ1b binding sites were within promoter regions while 51.8% CASZ1b binding sites were within active enhancers (Fig. 4C).

To characterize the CASZ1 binding sites associated genes, Genomic Regions Enrichment of Annotations Tool (GREAT) [41] was used for gene ontology (GO) analysis of both the endogenous CASZ1 peaks and Tet-induced CASZ1b peaks. The results showed that at baseline the endogenous CASZ1 bound peak-associated genes were significantly enriched in mesenchyme development and heart morphogenesis (Fig. 4D). HOMER de novo and known motif scan of the endogenous CASZ1 binding sites identified an enrichment of GATA family TFs, ASCL1, PHOX2A binding motifs (Supplementary Fig. 2A, B). GREAT GO analysis showed that when the levels of CASZ1 expression increased, genes associated with Tet-induced CASZ1b peaks were enriched in sympathetic nervous system development and noradrenergic neuron differentiation (Fig. 4E). Like endogenous CASZ1, HOMER de novo and known motif scan of the Tet-induced CASZ1b binding sites identified an enrichment of GATA family TFs, PHOX2A, PHOX2B, HANM1 binding motifs (Fig. 4F, Supplementary Fig. 2C). These TFs are known neural crest lineage specifiers [42, 43], which suggests a role for CASZ1 in regulating normal neural crest development.

CASZ1b directly upregulates neuronal genes

To understand the regulatory consequences of CASZ1b binding, we focused on genes that were genomically bound and transcriptionally regulated by CASZ1b. To accomplish this, RNA-seq data and CASZ1b ChIP-seq data from SY5YtetCASZ1b cells with and without induced CASZ1b expression (2-day treatment with Tet) were merged. In this analysis, 1967 sites associated with 598 genes were bound and transcriptionally upregulated by CASZ1b (Fig. 5A, Supplementary Table 3), while 912 sites associated with 397 genes were bound and transcriptionally downregulated by CASZ1b (Fig. 5B, Supplementary Table 3). We found that after the restoration of CASZ1b in NB cells, there was an increase of RNA Pol II signal within the gene body of CASZ1b upregulated genes and a decrease of RNA Pol II signal within the gene body of CASZ1b downregulated genes (Fig. 5A, B). For upregulated genes, we found that there was an increase of the active enhancer mark H3K27ac signal at Tet-induced CASZ1b peak centers (Fig. 5A). For downregulated genes, we found that there was a corresponding decrease of H3K27ac signal at Tet-induced CASZ1b peak centers (Fig. 5B).

Interestingly, unlike traditional H3K27ac peaks observed in CASZ1b upregulated genes (Fig. 5A), no bimodal H3K27ac peaks (“valley” pattern) were observed at the Tet-induced CASZ1b peak center for CASZ1b downregulated genes (Fig. 5B). Ingenuity pathway analysis (IPA) revealed genes directly regulated by CASZ1b (both upregulated and downregulated) were involved in nervous system development and developmental pathways (Fig. 5C). By focusing on the genes involved in the nervous system development, IPA showed that neuronal differentiation genes were positively enriched as indicated by the positive z-score (Fig. 5D), consistent with the observation of induction of neurite extensions in SY5Y cells after the restoration of CASZ1b (Fig. 1D).

CASZ1b directly represses mesenchyme development genes, CRC components and activates neuronal genes through remodeling typical enhancers (TEs) and super-enhancers (SEs)

Enhancers play a central role in driving cell-type-specific gene expression through activating transcription of their target genes that may be several to hundred kilobases or even megabases away [44]. To investigate the pathways that are regulated by CASZ1b-bound enhancers, we focused on those peaks co-bound by both CASZ1b and H3K27ac with changes in their H3K27ac signals (>2-fold) after CASZ1b restoration. We found that 3299 H3K27ac peaks associated with 2554 genes had at least a 2-fold increased signal upon CASZ1b induction (Tet+) compared to control cells (Tet−). In contrast 1132 H3K27ac peaks associated with 1293 genes had at least a 2-fold decreased signal (Fig. 6A, Supplementary Table 4). GREAT GO analysis of the H3K27ac peaks with increased signals after CASZ1b induction showed enrichment of genes involved in nervous system development (Fig. 6A), which is consistent with RNA-seq results showing CASZ1b upregulates neuronal genes (Fig. 2A). Consistently, signal tracks showed that the induction of CASZ1b in SY5Y cells resulted in de novo CASZ1b binding, as well as an increase of H3K27ac, RNA Pol II ChIP-seq signals and RNA-seq signal on the neuronal gene NGFR and HTR3A (Fig. 6B). GREAT GO analysis of the H3K27ac peaks with decreased signals showed enrichment of genes involved in mesenchyme morphogenesis and development (Fig. 6A). Noradrenergic NB cells like SY5Y highly express a noradrenergic gene signature but not a mesenchymal gene signature [6], leading us to hypothesize that CASZ1b binds to mesenchymal gene loci to repress their expression. Indeed, GSEA showed that the restoration of CASZ1b in SY5Y cells resulted in a negative enrichment of mesenchyme morphogenesis and development genes, heart morphogenesis genes, and striated muscle tissue development genes (Fig. 6C, Supplementary Fig. 3A), consistent with an association of CASZ1 with a repressive role on genes regulating mesenchyme development. Additionally, signal tracks showed that the induction of CASZ1b in SY5Y cells resulted in a decrease of H3K27ac, RNA Pol II ChIP-seq signals and RNA-seq signals on genes regulating mesenchyme development such as TWIST1 and KITLG (Fig. 6D).

Cell-fate and cell identity are determined by super-enhancers (SEs) that are marked by extensive stretches of H3K27ac and these SEs are frequently dysregulated in cancer [45]. Our study identified 366 SEs in the control cells (Tet-) and 344 SEs in CASZ1b restored SY5Y cells (Tet+) (Fig. 6E, Supplementary Table 5). CASZ1b peaks were found in over 90% SEs in CASZ1b restored cells.
CASZ1b are enriched in regulating cell proliferation of tumor cell lines and “synthesis of norepinephrine” (Supplementary Fig. 3D, bottom panel). By focusing on the SEs that drive the CRC components and neural crest lineage specifiers, we observed that the restoration of CASZ1 resulted in a decrease of SEs associated signals on a subset of these TFs, including GATA2, GATA3, ISL1 and TBX2 (Fig. 6F, Supplementary Fig. 3E). CASZ1 also decreased the H3K27ac signals within the CDX6 gene locus (Fig. 6F). CDX6 regulates the progression of cell cycle and is a potential therapeutic target in NB [47]. In a previous study we showed that the restoration of CASZ1b in NB cells led to a decrease in the protein expression of cell cycle regulators such as CDK1, CDK6 and Cyclin D1 (CCND1), but the mechanism was unknown [27]. Here we found that CASZ1b directly repressed the expression of these cell cycle regulators by binding to the genomic loci of these genes, with the increased CASZ1b binding accompanied by decreased H3K27ac signals (Fig. 6F, Supplementary Fig. 3F). Moreover, the restoration of CASZ1 resulted in a gain of SEs on neuronal genes such as PLXNA2 and PLXNA4 (Supplementary Fig. 3G). Finally, we focused on changes in the expression of TFs that were enriched in noradrenergic NB cells [6, 10, 11] upon CASZ1b restoration. GSEA demonstrated that these noradrenergic lineage TFs were negatively enriched when CASZ1b is restored in SY5Y cells (Fig. 6G, Supplementary Table 6), with some of them being driven by SEs while others driven by typical enhancers (TEs) (Fig. 6H). Taken together, our results showed that CASZ1b cross-talks with CRC TFs, repressed cell cycle regulators, mesenchymal genes and upregulated neural differentiation genes through remodeling enhancer activity to induce NB cell differentiation (Fig. 7).

**DISCUSSION**

How genetic or epigenetic alterations subvert a normal lineage specifying core regulatory circuitry to cause cancer is an area of intense investigation. In NB, chromosomal translocations [48] and genetic polymorphisms generating neo transcription binding sites [49] have been implicated in the dysregulated activity of the CRC regulating neural crest progenitors leading to NB. In this study, we examined the molecular mechanisms by which restoration of the NB Chr1p36 tumor suppressor CASZ1 regulates gene transcription and whether CASZ1 cross-talks with the NB CRC. We find that CASZ1 is repressed by CRC components of noradrenergic NB. The restoration of CASZ1b directly represses CRC components, mesenchymal development genes, cell cycle regulators and noradrenergic NB CRC components such as HAND2, GATA3 and TBX2 (Fig. 6F, Supplementary Fig. 3G) and upregulates neuronal genes such as PLXNA2 and PLXNA4 (Supplementary Fig. 3G). Finally, we focused on changes in the expression of TFs that were enriched in noradrenergic NB cells [6, 10, 11] upon CASZ1b restoration. GSEA demonstrated that these noradrenergic lineage TFs were negatively enriched when CASZ1b is restored in SY5Y cells (Fig. 6G, Supplementary Table 6), with some of them being driven by SEs while others driven by typical enhancers (TEs) (Fig. 6H). Taken together, our results showed that CASZ1b cross-talks with CRC TFs, repressed cell cycle regulators, mesenchymal genes and upregulated neural differentiation genes through remodeling enhancer activity to induce NB cell differentiation (Fig. 7).

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**Fig. 3** CASZ1 is regulated by noradrenergic NB CRC components. A Results of analysis of publicly available ChIP-seq data of the CRC components show that the CRC components bind to the CASZ1 gene locus, with a relatively stronger signal of CRC components within the 2nd intron (red box) but a lower signal of H3K27ac. Bivalent mark of H3K27me3 and H3K4me3 is observed at the gene locus, with a relatively stronger signal of CRC components within the 2nd intron (red box) but a lower signal of H3K27ac. CASZ1b is regulated by noradrenergic NB CRC components. **B** Realtime PCR shows that the siRNA knockdown of MYCN and CRC components (left panel), and the loss of HAND2 or TBX2 results in a decrease of CASZ1 mRNA levels (right panel). **C** ChIP-seq results show that decreasing expression of HAND2 in IMR32 cells results in a decrease of HAND2 signal within the CASZ1 gene locus, which is accompanied by an increase of H3K27ac signal within the 2nd and 3rd intron (red and pink boxes), as well as an increase of H3K4me3 signal and a decrease of H3K27me3 signal within the CASZ1 promoter.
regulate and induce skeletal myogenic differentiation [33]. The common thread from these studies and our study indicates that CASZ1 regulates cell-fate decisions in different tissue types by cooperating with lineage-specific TFs.

A CRC is comprised of a small set of core TFs that form an interconnected, autoregulatory feed-forward loop that dominates and controls the expression of specific gene programs to maintain a specific cell type. Recent bioinformatic analyses generated CRC models for 75 human cell and tissue types [13]. The core TFs of a CRC are generally expressed in a lineage-specific manner and can reprogram cells from one type to another. Thus, the expression of these TFs needs to be precisely regulated during lineage specification. For example, during cell differentiation, TFs required for a differentiation state need to be upregulated, while TFs that are highly expressed to maintain a stem cell-like status need to be silenced. During normal peripheral nervous system development, a set of TFs including PHOX2B, HAND2, PHOX2A, HAND1, GATA2, GATA3 and ASCL1 interacts as a network to determine sympathetic neuronal differentiation [1, 37, 42, 43, 50, 51]. In NB, the neural crest lineage specifiers PHOX2B, HAND2, GATA3, ISL1, and TBX2 form a CRC to determine a malignant noradrenergic phenotype [10]. Recent studies showed that ISL1 regulates multiple oncogenic genes that are essential for the proliferation and differentiation of NB and sympathetic neurons [39, 52]. In this study, we found that the CRC components HAND2 and TBX2 repress CASZ1 expression but upon increasing the levels of CASZ1 there is direct repression of the expression of the CRC components PHOX2B, GATA3, ISL1 and TBX2 in NB cells. This produces a novel model in which the CRC of cell type A directly represses a cell type B lineage-specific TF. The forced expression of this B lineage TF in cell type A will repress the cell type A CRC and induce a cell type B phenotypic switching. This formation of an interconnected feedback loop between a cell type specific TF and the cell type specific CRC might be a general rule that determines the specification of a cell’s fate and its commitment.

The results of single-cell studies on normal neural crest development of the sympathoadrenal system and our results support an important role for CASZ1 in neural crest lineage differentiation. It is beyond the scope of this study to characterize the role of CASZ1 in a normal neural crest development model, but our results provide interesting insights into how loss of a tumor suppressor gene such as CASZ1 might contribute to the oncogenic dysregulation of lineage specifying CRC such as occurs in NB. Our study provides support that the loss of a key inducer or amplifier of differentiation such as CASZ1 could hamper a cell’s ability to undergo terminal differentiation, which results in increased cellular plasticity.

Our study also provides mechanisms by which CASZ1 suppresses NB growth and induces noradrenergic neuronal differentiation. We have previously shown that the restoration of CASZ1 leads to an increase in the percentage of cells in the G1 phase of the cell cycle and delays the cell cycle progression [25]. In this study, we found that CASZ1 directly represses cell cycle regulators such as CDK1, CDK6 and CCND1, which is accompanied by a decrease of H3K27ac signal and decreased transcription levels (Fig. 6F, Supplementary Fig. 3F). Dysregulation of cycle regulators such as CCND1 and CDK6 are implicated in NB, which make them therapeutic targets in NB [47, 53]. This direct
repression of cell cycle regulators by CASZ1 identifies a mechanism by which CASZ1 suppresses NB growth. The restriction in proliferative capacity and exit of NB cells from the cell-cycle after CASZ1 restoration provides opportunities for post-mitotic cellular differentiation. Indeed, the restoration of CASZ1 upregulates neuronal differentiation genes expressed in sympathoadrenal cells (Fig. 2A). Importantly, the increased expression results in CASZ1 directly activating neuronal differentiation genes. A

In summary, our results indicate that the regulation of NB differentiation programs by CASZ1 is integral to a specific feedback regulatory circuit. The loss of the CASZ1 stable knockdown SY5Y cell lines are generated by infecting SY5Y cells with either control shRNA lentiviral particles (SigmaAldrich, MISSION pLKO.1-puro. Catalog SHC002V) or CASZ1 shRNA lentiviral particles (SigmaAldrich, shCASZ1_2, catalog TRCN000129821; shCASZ1_3, catal-

MATERIALS AND METHODS

Cell culture
Neuroblastoma cell lines SH-SY5Y (SY5Y), SK-N-AS (AS), and IMR32 were obtained from the cell line bank of the Pediatric Oncology Branch of the National Cancer Institute and have been genetically verified. All the NB cells were maintained in RPMI1640 media supplemented with 10% fetal calf serum (FBS) as well as 100 µg/mL streptomycin, 100 U/mL penicillin, and L-glutamine. Cells are grown at 37 °C with 5% CO2. SY5YtetCASZ1b cells were maintained in RPMI1640 media supplemented with 10% fetal

Realtime PCR
Total mRNA was collected using the RNeasy Plus Mini Kit (Qiagen) as per the manufacturer's protocol. Quantitative measurements of total β-actin and other genes' levels were obtained using the BIO-RAD CFX Touch Realtime (RT) PCR detection system and performed in triplicate. Ct values were standardized to β-actin levels. Data from biological triplicates were shown in this study if not specifically mentioned in figure legend. Primer sequences used for realtime PCR are shown below:

Antibodies
The antibodies used for western blot, immunofluorescence staining and ChIP-seq are obtained from different companies. Antibodies from Thermo-Fisher: GAP43 (Cat. # PA1-16729, 1:250 for immunofluorescence), goat anti-mouse IgG Alexa Fluor 594 (Cat. A-11032, 1:250 for immunofluorescence staining). Antibodies from SigmaAldrich: FLAG (Cat. # F1804, 1:1000 for western blot) Antibodies from Santa Cruz: GAPDH (Cat. # sc-2778, 1:2000 for western blot), goat anti-mouse IgG HRP secondary antibody (Cat. # sc-2005,
Fig. 6  CASZ1b represses NB CRC and mesenchymal signature genes and activates neuronal genes through affecting enhancer activity. 

A Restoration of CASZ1b in SY5Y cells results an increase of H3K27ac signals (heatmap on the left) on genes that are associated with axonogenesis (GO analysis on the right) and a decrease of H3K27ac signals on genes (heatmap on the left panel) that are associated with mesenchyme development (GO analysis, right panel). B Signal tracks show the increase of CASZ1b, H3K27ac and RNA Pol II signals and RNA-seq signals on the neuronal genes NGFR and HTR3A after induction of CASZ1b (Tet+ vs. Tet−). C GSEA shows a negative enrichment of mesenchyme morphogenesis and heart morphogenesis genes upon induction of CASZ1b in SY5Y cells. D Signal tracks show the increase of CASZ1b signals, and decrease of H3K27ac, RNA Pol II and RNA-seq signals on the mesenchyme development regulators TWIST1 and KITLG after induction of CASZ1b (Tet+ vs. Tet−). E Restoration of CASZ1b in SY5Y cells re-organizes SEs. The histograms show an increase of H3K27ac signal on neuronal genes PLXNA2 and PLXNA4 and a decrease of H3K27ac signals on CRC component TBX2, as well as cell cycle progression regulator CDK6. F Signal tracks show that the restoration of CASZ1b in SY5Y cells results in a decrease of SE signals (H3K27ac), as well as RNA Pol II signals and RNA-seq reads on GATA3 and CDK6; G GSEA shows that the restoration of CASZ1b in SY5Y cells results in a significant negative enrichment of noradrenergic NB TFs coding genes. H The bar graph shows the negative regulation of noradrenergic NB TFs coding genes that either driven by SEs or typical enhancers (TEs) in SY5Y cells after restoration of CASZ1b based on the normalized RNA-seq reads (CPM: counts per million). Data represent mean ± SEM, n = 3 biological replicates. Two-sided Student's t test was used to calculate statistical difference.
1:2000), goat anti-rabbit IgG HRP secondary antibody (Cat. # sc-2004, 1:2000). Antibody from Abcam: H3K27ac (Cat. # ab4729, 4 µg/reaction for ChIP), HAND2 (Cat. # ab200040, 4 µg/reaction for ChIP). Antibody from Active Motif: RNA polymerase II (Cat. # 39097, 4 µg/reaction for ChIP). Antibody generated by collaborating with Rockland Immunochemicals Inc: CASZ1 (1:4000 for western blot, 4 µg/reaction for ChIP). Antibody from EMD Millipore: H3K27me3 (Cat. # 07-449, 4 µg/reaction for ChIP). Antibody from Cell Signaling Technology, H3K4me3 (Cat. # 9751, 4 µg/reaction for ChIP).

**Protein isolation and western blot analysis**

For assessment of protein levels, cells were lysed using RIPA buffer, and 10 µg of total protein was separated and electroblotted as described previously [28]. Protein bands were detected using a goat anti-rabbit or mouse IgG-HRP conjugated secondary antibody (200 µg/mL; Santa Cruz Biotechnology) and visualized using enhanced chemiluminescence (Amer sham Biosciences).

**Immunofluorescence**

Cells were cultured in 8-well Lab-Tek Chamber Slides (Cat. No. 177402) for indicated time. Cells were fixed, permeabilized, blocked, and stained as described previously [28]. For indirect immunofluorescent cell staining, an anti-GAP43 monoclonal antibody and an Alexa Fluor 594-conjugated goat anti-mouse antibody were used to detect GAP43. Stained cells were imaged and analyzed using a Nikon Eclipse TE300 fluorescent microscope.

**RNA-seq**

Total RNA was isolated and subjected to RNA-seq analysis from SYSYetCASZ1b cells treated with or without Tet for 48 h, and AStetCASZ1b cells that were treated with or without Tet for 72 h. Total RNA extraction was carried out using a RNeasy Plus Mini Kit (Qiagen Inc.) according to the manufacturer's instructions. Strand-specific whole transcriptome sequencing libraries were prepared using TruSeq® Stranded Total RNA LT Library Prep Kit (Illumina, San Diego, CA, USA) by following the manufacturer's procedure. RNA-seq libraries were sequenced on Illumina HiSeq 4000 of paired-end with read length of 150 bp, or Novaseq S1 of paired-end with read length of 100 bp. TheFastq files with 150 bp or 100 bp paired-end reads were processed using Partek Flow or R package Deseq2. In brief, the raw reads are aligned using STAR and the aligned reads are quantified to annotation model through Partek E/M. The normalization method used here is counts per million (CPM) through Partek Flow. The normalized counts were subject to statistic analysis using GSA or ANOVA. To get T-scores, the normalized counts acquired from Partek Flow are exported and further analyzed using Parteck Genomics Suite v7.17. To eliminate batch effect, some of the CPM from Partek Flow were analyzed using DESeq2. Statistical results of differentially expressed genes from Partek Flow, or Parteck Genomics Suite v7.17 or DESeq2 were analyzed using QIAGEN’s Ingenuity® Pathway Analysis (IPA®, QIAGEN) and gene set enrichment analysis (GSEA) (http://www.broadinstitute.org/gsea/index.jsp). By default, the false discovery rate (FDR) less than 0.25 is considered significant in GSEA.

**ChIP-seq**

ChIP was performed using the ChIP-IT High Sensitivity kit (Active Motif) as per the manufacturer’s instruction. Briefly, formaldehyde (1%, 15–15 min) fixed cells were sheared to achieve chromatin fragmented to a range of 200–700 bp using an Active Motif EpiShear Probe Sonicator. SYSYtetCASZ1b cells were sonicated at 30% amplitude, pulse for 20 s on and 30 s off for a total sonication “on” time of 15 min. Sheared chromatin samples were immunoprecipitated overnight at 4°C with antibodies targeting

**Fig. 7  Schematic diagram of CASZ1 action.** Restoration of CASZ1 directly represses CRC TFs, suppresses cell cycle regulators, mesenchymal genes and activates neural differentiation genes through remodeling enhancers.
CASZ1, H3K27ac and RNA Pol II. IMR32 cells were sonicated at 25% amplitude, pulse for 20 s on and 30 s off for a total sonication “on” time of 16 min. Sheared chromatin samples were immunoprecipitated overnight at 4°C with antibodies targeting HAND2, H3K27ac, H3K4me3, and H3K27me3. ChIP-seq DNA libraries were prepared by Frederick National Laboratory for Cancer Research sequencing facility. Libraries were multiplexed and sequenced using TruSeq ChIP Sample Prep Kit (75 cycles), cat. # IP-2-2-1012/1024 on an Illumina NextSeq machine. 25,000,000–30,000,000 unique reads were generated per sample. All the home generated ChIP-seq datasets can be found in the Gene Expression Omnibus (GEO) database.

ChIP-seq data processing

ChIP-enriched DNA reads were mapped to reference human genome (version hg19) using BWA [54]. Duplicate reads were infrequent but discarded. For IVG sample track visualization, coverage density maps (tiff files) were generated by extending reads to the average size (measured by Agilent Bioanalyzer minus 121 bp for sequencing adapters) and counting the number of reads mapped to each 25 bp window using igtools (https://www.broadinstitute.org/igv/igvtools).

ChIP-seq read density values were normalized per million mapped reads. High-confidence ChIP-seq peaks were called by MACS2 (https://github.com/taoliu/MACS) with the narrow peak calling. The peaks which overlapped with the possible anomalous artifact regions (such as high-mappability regions or satellite repeats) blacklisted by the ENCODE consortium (https://sites.google.com/site/anshulkundaje/projects/blacklists) were removed using BEDTools. Peaks from ChIP-seq of CASZ1, H3K27ac and RNA Pol II in SY5Y and CASZ1b cells were selected at a stringent p-value (p < 10^-3). Peaks within 1,000 bp to the nearest TSS were set as promoter. The distribution of peaks (as intronic, intergenic, exonic, etc.) was annotated using HOMER. Enrichment of known and de novo motifs were found using HOMER script “findMotifsGenome.pl” (http:// homer.salk.edu/homer/ngs/peakMotifs.html). Heatmaps of signal intensity of ChIP samples were also performed using ComputeMatrix function of the deepTools [55].

The enhancers were identified using the ROSE2 (Rank Order of Super-enhancers) software (https://github.com/BradnerLab/pipeline), using distal (>2500 bp from TSS) H3K27ac peaks [56, 57]. Enhancer constituents were stitched together if clustered within a distance of 12.5 kb. The enhancers were classified into typical and super-enhancers based on a cutoff at the inflection point in the rank ordered set (where tangent slope = 1) of the ChIP-seq signal (input normalized) with p < 10^-3.

Among all the CASZ1b binding sites, promoter regions were defined directly from the Homer annotated bed file within 1000 bp of the TSS. The remaining CASZ1 binding sites in the non-promoter regions were divided into active enhancer-associated regions if they overlap with the enhancers (defined by H3K27ac ChIP-seq) and others if they do not overlap with enhancers.

To determine the alterations on enhancers when CASZ1 is restored in SMS-CTR cells, signal intensity of H3K27ac ChIP-seq+/−/− CASZ1b peak centers was extracted and compared between control and Tet treated cells. Briefly, bed files of all H3K27ac that overlapped with CASZ1b peaks were extracted. ComputeMatrix function of the deepTools was used to generate a matrix of signal intensity of H3K27ac up- and down-stream of the CASZ1b peak centers, as intensity scores in 10 bp bins.

Statistics and reproducibility

The statistical analyses used throughout this paper are specified in the appropriate results paragraphs and Methods sections. Additional statistical analyses were performed using Microsoft Excel, standard two-tailed Student’s t test, one-way ANOVA and the software GraphPad Prism 8.1.0. A representative experiment such as microarrays has been repeated at least two times to three occasions.

DATA AVAILABILITY

All the home generated RNA-seq and ChIP-seq datasets can be found in the Gene Expression Omnibus (GEO) database. GEO accession number for data generated in this study is GSE182871 (ChIP-seq in SY5Y cells), GSE182872 (RNA-seq in A5 cells) and GSE182873 (RNA-seq in SY5Y cells). GEO accession number for HAND2 ChIP-seq experiment done in IMR32 cells is GSE184058 [40]. GEO accession numbers or SRA accession numbers for publicly available ChIP-seq data are: GSE94822 for ChIP-seq experiments done in BE2C; SRX4623679, GSE65664, GSE80197 for ChIP-seq experiments done in K562 cells. To evaluate CASZ1 and CRC components mRNA levels in adrenal gland, NB cell lines and patients, we queried microarray data deposited in R2 database (https://hsgevserver1.amc.nl/cgi-bin/r2/main.cgi).

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AUTHOR CONTRIBUTIONS
ZL and CJT designed the study, wrote the manuscript, and coordinated the entire study. ZL, XZ, and MX performed the experiments. ZL, XZ, and HL performed bioinformatic analyses. JS reviewed ChiP-seq and bioinformatic analyses. All the authors have reviewed the manuscript.

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The authors declare no competing interests.

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