Loss of nuclear envelope phosphatase CTDNEP1 drives aggressive medulloblastoma by triggering MYC amplification and genomic instability

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Abstract

MYC-driven medulloblastomas are highly aggressive childhood brain tumors, however, the genetic events triggering MYC amplification and malignant transformation remain elusive. Here we report that mutations in CTDNEP1, a CTD nuclear-envelope-phosphatase, are the most significantly enriched recurrent alterations in MYC-driven medulloblastomas, and define high-risk subsets with poorer prognosis. CTDNEP1 ablation transforms murine cerebellar progenitors into MYC-amplified medulloblastomas, resembling their human counterparts. CTDNEP1 deficiency stabilizes MYC protein by elevating MYC serine-62 phosphorylation, and triggers genomic instability with eventual MYC amplification and p53 loss. Further, phosphoproteomics reveals that CTDNEP1 post-translationally modulates the activities of key regulators for proper chromosome segregation and mitotic checkpoints including topoisomerase TOP2A and checkpoint kinase CHEK1. Co-targeting CHEK1 and MYC activities synergistically inhibits CTDNEP1-deficient MYC-amplified tumor growth and prolongs animal survival. Together, our studies identify CTDNEP1 acting as a tumor suppressor in highly aggressive medulloblastomas by maintaining homeostatic MYC levels and genomic stability, highlighting a CTDNEP1-dependent therapeutic vulnerability.
**Introduction**

Medulloblastomas (MB), which arise from cerebellar neural progenitor cells, are among the most common malignant childhood brain tumors, and feature high genomic instability\textsuperscript{1-3}. Based on gene expression and/or DNA methylation profiling MBs are classified into four major subgroups: Wingless (WNT), Sonic Hedgehog (SHH), Group 3 (G3), and Group 4 (G4), with intertumoral heterogeneity within each subgroup\textsuperscript{4-7}. Dysregulation of the WNT and hedgehog (HH) pathway has been implicated in WNT and SHH subgroup tumors, respectively\textsuperscript{8-12}. MYC-driven G3-MB, which comprises approximately 17\% of G3-MBs, has a very poor prognosis and is associated with amplification and overexpression of the \textit{c-MYC} oncogene (\textit{MYC})\textsuperscript{13-15}. Patients with G3-MB tumors often relapse following therapy, and exhibit metastases, and eventually succumb to the disease\textsuperscript{4,14,16,17}. Currently, targeted therapeutics for the G3-MB tumors are lacking in part due to the incomplete understanding of tumorigenic mechanisms and clinical correlates of genetic alterations. Although large-scale genomic studies have identified many somatically mutated genes in G3-MB tumors, the function validation and regulatory circuitry of candidate cancer genes remain poorly defined.

\textit{MYC} amplification or activation has been shown to induce genomic instability that is linked to tumor initiation\textsuperscript{18-20} including tumor initiation in murine G3-MBs from cerebellar cell types\textsuperscript{21-24}. Currently, regulatory networks that control MYC activation, genomic instability, and transformation of neural precursors into the most aggressive MYC-driven G3 MBs are poorly defined. Recent genomic studies have identified candidate mutations implicated in MB tumorigenesis\textsuperscript{5,6,10,25}, however, no presumptive driver mutations in the \textit{MYC} gene itself have been found in patients. Control of MYC protein stability and compartmentalization at the nuclear periphery are critical for its oncogenic activity, in part mediated through phosphorylation at the critical serine-62 (p62-MYC)\textsuperscript{26-28}. Recent global proteomes and phospho-proteomes indicate that post-translational modifications of MYC such as phosphorylation are associated with poor outcomes in G-3 MBs\textsuperscript{29}. However, at present, the genetic and molecular pathways that regulate MYC post-translational modification to control MYC stability and amplification during MYC-driven MB tumorigenesis remain elusive. In this study, by integrating analyses of newly diagnosed MBs and publicly available cohorts to characterize the recurrent genetic alterations, we identify that \textit{CTDNEP1}, encoding a CTD nuclear envelope-enriched phosphatase (a.k.a \textit{Dullard})\textsuperscript{30-32}, is among the
most significantly mutated genes in G3-MBs compared with other MB subgroups. Mutations or low CTDNEP1 expression levels define a subset of highly aggressive MYC-driven MBs and predict poor patient outcomes. Notably, ablation of Ctdnep1 induces the chromosome instability and Myc focal amplification and p53 loss to promote the transformation of cerebellar progenitors into aggressive MYC-amplified MBs through a stage-specific mechanism. Our studies demonstrate that tumorigenesis for aggressive MYC-driven MBs, long appreciated to be driven by oncogenes, can be initiated by loss-of-function mutations in a nuclear envelope-enriched CTDNEP1 phosphatase, which maintains MYC protein homeostasis and genomic stability, pointing to a protein phosphatase-dependent targetable vulnerability in the highly aggressive MB tumors.

Results

**CTDNEP1 mutations are most significantly enriched in aggressive G3-MBs and are correlated with MYC amplification and poor prognosis**

To identify the genetic alterations in aggressive MBs, we analyzed whole-exome sequencing data of newly diagnosed MBs (n=89) cohort from East Asia and combined with the publicly available MB cohorts (total n = 844) to catalogue recurrent somatic mutations. We identified a set of somatic mutations with high frequency among G3 MB samples (n = 209), including mutations in SMARCA4 (16 patients, 7.7%), KBTBD4 (14 patients, 6.7%), KMT2D (13 patients, 6.2%), and KMT2C (9 patients, 4.3%) (Fig. 1a), which is in keeping with previous reports. Among the recurrent somatic mutations in all available MB cohorts, we found that CTDNEP1 mutations are most significantly enriched in the G3 MB subgroup compared with other MB subgroups (Fig. 1b and Extended Data Fig. 1a).

CTDNEP1 mutations occur most frequently in MBs in a comparison with other CNS tumor types (Extended Data Fig. 1b). CTDNEP1 expression is also lower in MB tumors than normal brain and cerebellar tissues (Extended Data Fig. 1c). Among the MBs from the Asian cohort and publicly available cohorts, 19 CTDNEP1 somatic mutations were identified. The majority of patients identified with CTDNEP1 mutations (15 out of 19) had been diagnosed with G3-MBs, with no or low frequency in patients with WNT (1 out of 19), SHH (0 out 19), or G4 subgroup (3 out of 19) MBs (Fig. 1c and
Moreover, CTDNEP1 mutations were mainly distributed in the critical Dullard-like phosphatase domain or resulted in truncations of this domain (Fig. 1d), suggesting that these mutations may be associated with CTDNEP1 loss-of-function (LOF). The somatic nature of the mutations was confirmed in a set of Asian G3 MBs (Extended Data Fig. 2a,b).

We observed loss-of-heterozygosity (LOH) of the other allele of CTDNEP1 on Chr17p in the majority of the G3 MBs carrying CTDNEP1 mutations (11 out of 12; Extended Data Fig. 2c-f), while LOH was not detected in the normal apparent peri-tumoral tissue in the same patient (Extended Data Fig. 2c). In contrast, in G4 MBs, which do not have high MYC levels, CTDNEP1 mutations were detected in only three G4 MBs (3 out of 173) with one tumor showing i17q (Extended Data Fig. 2g). The CTDNEP1 expression level was lower in Chr17p-deleted MBs compared to SHH or WNT MBs (Extended Data Fig. 3a-c).

To examine the relation of MYC amplification and CTDNEP1 mutations or loss to genomic alterations in G3 MB tumors, we analyzed the copy number variation from the publicly available cohorts and the Asian cohort. In G3 MBs, we found that CTDNEP1 mutations significantly co-occurred with MYC amplification, copy number gains on Chr8p, Chr8q, Chr17q, and Chr1q, as well as the loss of Chr17p, while the mutually exclusive events included gain of Chr7p or Chr7p and loss of Chr16q or Chr10q (Fig. 1e and Extended Data Fig. 2c,d). The co-occurrence of isochromosome 17q (i17q) and Chr8q gain was observed in both CTDNEP1-mutated and MYC-amplified MBs (Fig. 1e and Extended Data Fig. 2e,f). Together, these observations indicate that CTDNEP1 mutation or loss is correlated with MYC amplification and genomic instability in G3-MB tumors.

Due to variable expression levels, we stratified the MB cohorts from publicly available datasets into patient populations with high and low CTDNEP1 expression across MB subgroups (Extended Data Fig. 3d), and found that lower CTDNEP1 expression was correlated with significantly decreased overall survival in SHH-, G3- and G4-MB cohorts (Fig. 1f and Extended Data Fig. 3e), except for the WNT subgroup, which has the best prognosis among MBs. Based on the survival data, patients carrying CTDNEP1 somatic mutations exhibited a worse prognosis than those with CTDNEP1 wildtype alleles in G3 MB tumors without MYC-amplification, but similar to those with MYC-amplification (Fig. 1g). Among G3 MB tumors, the patients with both CTDNEP1 mutation and MYC amplification showed
the poorest prognosis when compared with those with the *CTDNEP1* mutation or *MYC* amplification alone (Fig. 1g). Together, these observations suggest that the prevalence and clinical impact resulting from *CTDNEP1* low expression or mutations define a subset of highly aggressive G3-MBs.

**CTDNEP1 deficiency promotes MB tumor cell growth**

To determine the potential mechanism by which CTDNEP1 deficiency enhances tumor cell growth, we inhibited *CTDNEP1* expression in different G3-MB tumor cell lines with or without *MYC* amplification using lentiviral shRNAs. In *MYC*-amplified human G3-MB cell lines D425 and MB004 (Extended Data Fig. 4a)\(^{36}\), *CTDNEP1* knockdown resulted in an increase in cell proliferation measured as the percentage of replicating cells incorporating EdU or BrdU (Fig. 2a,b and Extended Data Fig. 4b) and cell growth rates (Fig. 2c and Extended Data Fig. 4c) compared to control cells. In addition, by using a soft agar assay, we found that silencing of *CTDNEP1* resulted in a higher clonogenic capacity in a non-*MYC* amplified G3 MB cell line (D283)\(^{36}\) (Fig 2d). Moreover, *CTDNEP1* knockdown led to significant increases in tumor sphere formation in both *MYC* amplified (D425) and non-*MYC* amplified (D283) G3-MB cell lines (Fig. 2e,f). Cell-cycle analysis using flow cytometry revealed that CTDNEP1 deficiency also increased the proportion of D283 G3-tumor cells in S phase (Fig. 2g). These results indicate that CTDNEP1 deficiency promotes the proliferation of G3-MB tumor cells *in vitro*.

To assess the *in vivo* effect of *CTDNEP1* inhibition on tumor formation, D425 or D283 MB cells with or without *CTDNEP1* knockdown were subcutaneously transplanted into NOD scid gamma (NSG) mice. The sizes of tumors derived from *CTDNEP1*-deficient cells were much larger than those from control shRNA tumor cells (Fig. 2h,i). Thus, the loss of CTDNEP1 promotes tumor growth of both *MYC*-amplified and non-*MYC*-amplified G3-MB cells in xenografts, suggesting a tumor suppressive role of CTDNEP1 in G3-MBs.

To assess the effect of CTDNEP1 overexpression on tumor cell growth, we transduced D425 MB cells with a lentivirus for expressing CTDNEP1. CTDNEP1-overexpressing cells did not proliferate as rapidly as cells transduced with control viral vector (Fig. 2j). In addition, tumors in mice xenografted with CTDNEP1-overexpressing D425 cells were smaller than tumors of mice grafted with control cells with normal CTDNEP1 levels (Fig. 2k), suggesting that CTDNEP1 overexpression inhibits tumor cell...
growth. Together, these observations suggest that CTDNEP1 has a tumor-growth suppressive activity in G3-MB cells.

**CTDNEP1 depletion leads to upregulation of the MYC signaling pathway**

To further determine the pathways that mediate the potential tumor suppressive effects of CTDNEP1, we performed transcriptome profiling of D425 MB cells transduced with non-targeting control shRNAs and shCTDNEP1 RNAs. We identified a set of genes that were significantly altered (>1.5-fold change, \( p < 0.01 \)) in CTDNEP1-depleted cells compared with control cells (Fig. 3a,b). Among the most upregulated genes were those pertinent to tumor progression, including the signature genes for G3 MBs (e.g., NRL, NR2E3, and RORB) \(^5\,^37\) and candidate MYC-targeted genes (e.g., RPL21, CAV3, GDNF, GPR27, TIMP3 and SLC35G2) \(^38\) (Fig. 3a,b and Extended Data Fig. 4d), which were co-expressed with MYC within human MB gene expression datasets (Fig. 3c). In addition, the genes associated with NOTCH signaling (e.g., HES1, GATA3, and MAGEA1) and cell migration (e.g., ICAM1, NELL2, CD40, SLIT2, and SPINT2) were also upregulated upon CTDNEP1-knockdown (Fig. 3a,b). In contrast, the downregulated genes were associated with normal neural development (Fig. 3a). Strikingly, the upregulated genes showed a strong enrichment in MYC-activation transcriptomic profiles (Fig. 3d). These observations suggest that depletion of CTDNEP1 results in the upregulation of genes associated with increased MYC protein levels or MYC upregulation.

Consistently, the analysis of human G3-MB transcriptomic profiles \(^6\) revealed that CTDNEP1-mutated MBs exhibited a similar expression pattern with MYC-high or -amplified MBs (Extended Data Fig. 4e). Similarly, gene set enrichment analysis (GSEA) \(^39\) revealed a significant upregulation of MYC target genes in CTDNEP1-mutated patients (Extended Data Fig. 4f). Together, these observations suggest that CTDNEP1 deficiency leads to MYC upregulation or activation of the MYC oncogenic pathway.

**CTDNEP1 phosphatase activity destabilizes MYC by dephosphorylating MYC at Ser62**

Despite the increase in MYC pathway gene expression in CTDNEP1-deficient cells, MYC mRNA levels were not substantially altered in D425 tumor cells treated with shRNAs targeting CTDNEP1 (Fig. 3e), suggesting that CTDNEP1 might regulate the post-translational modification and/or stability of
Western blotting analysis indicated that there was an increase in MYC protein levels in the cells treated with shRNA targeting *CTDNEP1* (Fig. 3e), raising the possibility that CTDNEP1 regulates the stability of MYC protein. Further, phosphorylation at the critical serine 62 (p-S62) has been shown to stabilize MYC\(^40\), and the enrichment and compartmentalization of MYC and p-S62-MYC at the nuclear periphery or nuclear envelope is critical for MYC oncogenic activity\(^{26-28}\). Since *CTDNEP1* encodes a nuclear envelope-enriched serine/threonine protein phosphatase \(^{30,41}\) (Extended Data Fig. 5a), we hypothesized that CTDNEP1 activity destabilizes MYC via removal of the phosphate from S62. We examined MYC expression after treatment with a protein synthesis inhibitor cycloheximide (CHX), and found that both MYC and p-S62-MYC levels remained higher in the MYC-amplified D425 and MB004 as well as non-MYC amplified D283 cells with *CTDNEP1* knockdown than in control cells (Fig. 3f and Extended Data Fig. 5b,c), suggesting that *CTDNEP1* depletion led to an increase in p-S62 MYC and MYC protein stability.

To determine whether dephosphorylation of MYC at S62 is catalyzed by CTDNEP1, we generated constructs carrying a mutation at the codon D67 (D67N) or D69 (D69N) required for CTDNEP1 phosphatase activity\(^{30}\). Three residues (D67, D69 and L72) are highly conserved in the catalytic motif DXDX(T/V) among the phosphatase protein family (Fig. 3g). Furthermore, we constructed an expression vector carrying a G3-MB patient-derived CTDNEP1 mutation near the catalytic domain, L72H, to examine the impact of the disease-relevant mutation on CTDNEP1 activity. The phosphatase activity of affinity-purified wild-type and CTDNEP1 mutant proteins was assessed using p-nitrophenyl phosphate as a substrate\(^{30}\). The wild-type enzyme catalyzed dephosphorylation of p-nitrophenyl phosphate, but none of the CTDNEP1 mutants did (Fig. 3g). We next co-expressed CTDNEP1 or its activity-deficient mutants along with MYC, and found that overexpression of CTDNEP1, but not the mutants, increased MYC degradation (Fig. 3h). Importantly, in further support of its potential role in regulating MYC levels by dephosphorylating it at S62, CTDNEP1 overexpression did not alter the stability of the non-phosphorylatable MYC-S62E mutant (Fig. 3h). To directly confirm that CTDNEP1 dephosphorylates MYC, CTDNEP1 and its mutants D69N and L72H were purified and incubated with the lysates of D425 cells, which express a high level of MYC. p-S62-MYC levels were substantially reduced in the presence of wild-type CTDNEP1 but were not altered in the presence of CTDNEP1.
mutants (Fig. 3i). In addition, co-immunoprecipitation assays indicated that CTDNEP1 was associated with MYC in a complex in 293T cells transfected with vectors expressing \textit{CTDNEP1} and \textit{c-MYC} (Fig. 3j). Furthermore, over-expression of \textit{CTDNEP1} in D425 G3-tumor cells substantially downregulated the levels of MYC and p-S62-MYC (Fig. 3k). To further determine the clinical relevance of the experimental findings of \textit{Ctdnep1}-loss-induced MYC protein stabilization by increased phosphorylation at S62, we examined human G3 tumor tissues with or without CTDNEP1 mutations. We found that the expression of MYC protein and S62 phosphorylation is higher in \textit{CTDNEP1}-mutated G3-MB tumors than non-MYC-amplified G3-MB and SHH-MB tumors (Extended Data Fig. 5d,e). These observations indicate that CTDNEP1 can interact with and dephosphorylate MYC at S62 to regulate MYC stability.

**Deletion of \textit{Ctdnep1} induces neural progenitor transformation into MYC-driven MB tumors**

To gain insight into the role of CTDNEP1 in MB tumorigenesis \textit{in vivo}, we knocked out \textit{Ctdnep1} in neural stem/progenitor cells (NPCs) in mice by breeding \textit{Ctdnep1}\textsubscript{flox/flox} mice with a Nestin-Cre line to generate \textit{Ctdnep1}\textsubscript{flox/flox}\textsubscript{; Nestin-Cre} mice referred to here as \textit{Ctdnep1}-cKO mice (Fig. 4a). Strikingly, all \textit{Ctdnep1}-cKO mice died before postnatal day 40 (Extended Data Fig. 6a), and the cortex and cerebellum of the animals lacking \textit{Ctdnep1} were significantly smaller compared with controls (Extended Data Fig. 6b,c). We observed an increase of apoptosis (marked by cleaved caspase 3) and DNA damage responses (marked by γH2AX) in the cerebellar progenitor cells during embryonic development in \textit{Ctdnep1}-cKO animals (Extended Data Fig. 6d), suggesting that \textit{Ctdnep1} deletion results in DNA damage and cell death in a population of cerebellar progenitors during development.

The smaller size and increase of cell death in the cerebellum of \textit{Ctdnep1}-deficient animals suggests that CTDNEP1 may control cerebellar NPC development. To determine the effect of \textit{Ctdnep1} deficiency on NPC growth, we isolated cerebellar NPCs from control NPCs and \textit{Ctdnep1}-cKO mice at postnatal day 4. Although \textit{Ctdnep1}-cKO NPC spheres appeared smaller than wildtype NPCs during early stages e.g., 10 days in culture (Div 10), \textit{Ctdnep1}-cKO NPC growth rapidly accelerated and exhibited substantially higher proliferation rate than control NPCs at late-stages e.g., Div 60 (Fig. 4b,c). This suggests that a population of \textit{Ctdnep1}-deficient NPCs at the later stages acquired a growth advantage.
To evaluate the capacity of *Ctdnep1*-cKO NPCs to cause tumorigenesis, we orthotopically transplanted the luciferase-expressing NPCs from control and *Ctdnep1*-cKO mice into the cerebella of NSG mice. No tumors formed in mice transplanted with control NPCs, but the animals transplanted with *Ctdnep1*-cKO NPCs at Div 60 developed tumors in the cerebellum with full penetrance (14 of 14 mice), as detected by bioluminescence analyses (Fig. 4d). The tumors had a large cell/anaplastic (LC/A) morphology (Fig. 4e) and resemble that observed in human MYC-driven G3 MB. The animals transplanted with *Ctdnep1*-cKO NPCs had a short lifespan and died around 90 days post transplantation (Fig. 4f). To evaluate the tumorigenicity of *Ctdnep1*-deficient neoplastic cells, we transplanted primary neoplastic cells from allografts at varying cell doses into secondary recipients orthotopically and generated tumors with full penetrance (Extended Data Fig. 7a,b), suggesting that *Ctdnep1*-deficient neoplastic cells are tumorigenic and enable aggressive MB formation.

Immunohistochemical characterization indicated that cells in the tumors derived from *Ctdnep1*-cKO NPCs had a significantly higher proliferative rate than normal cerebellar regions, as assayed by Ki67 (Fig. 4g). In addition, these tumors exhibited strong expression of the oncogenic factor MYC and p-S62 MYC as well as stem cell/progenitor marker Nestin, but weak staining for the astrocytic marker GFAP (Fig. 4g), which are characteristics similar to previously described G3-MB mouse models. Notably, *Ctdnep1*-deficient NPCs transplanting from early stage culture (e.g., Div 15) into nude mice propagated into the same type of tumors with MYC overexpression, although the average latency period for tumor formation was longer than that from late stage culture cells (Extended Data Fig. 7c,d). Together, these results suggest that a population of NPCs acquires the tumorigenic potential in the absence of *Ctdnep1*.

Given the loss-of-function p53 could accelerate MYC-overexpressing NPCs to form G3-like MB, we then transduced freshly isolated NPCs from *Ctdnep1*-cKO animals with retroviruses expressing dominant-negative p53 (DNp53). The *Ctdnep1*-cKO NPCs transduced with DNp53 after 24 hr were transplanted into the cerebellum of NSG mice and were able to form G3 MB-like tumors (6 out 8) in allografts orthotopically (Extended Data Fig. 7e-g), while DNp53-transduced wildtype NPCs did not form tumors, suggesting that p53 loss-of-function enhances the tumorigenic potential of *Ctdnep1*-deficient NPCs.
Transcriptomic analysis indicated that *Ctdnep1*-cKO tumor cells had higher levels of G3-MB signature genes (e.g., c-Myc and Npr3) than NPCs from normal cerebella (Fig. 4h). Principal component analysis showed that the gene profiles of *Ctdnep1*-cKO tumor cells had a closer relationship to those of murine MYC-driven G3-MB tumors from NPCs (e.g. Myc/Trp53⁻/⁻ and Myc_Gfi1 MB models) than those from astrocyte progenitors (Sox2+ Myc model), SHH-MB tumors (e.g. SmoM2 OE and *Ptch1* models) or normal cerebella (Fig. 4i). In addition, the expression profile of *Ctdnep1*-cKO-tumor cells exhibited a greater similarity to that of MYC-driven G3 MB mouse models when compared with *Ctdnep1*-cKO NPCs and control NPCs (Fig. 4i). To further define the subgroup of *Ctdnep1*-cKO tumors, we compared the signature genes of human MB subgroups to those of *Ctdnep1*-cKO tumors. The *Ctdnep1*-cKO tumors more closely resembled the human MYC-amplified G3-MB than non-MYC-amplified G3-MBs and other human MB subgroups (Fig. 4j). *Ctdnep1*-cKO-tumors exhibited a higher correlation score than *Ctdnep1*-cKO NPCs when compared with human group 3 MB (Fig. 4j). Together, these observations indicate that *Ctdnep1*-loss-induced mouse MBs resemble the aggressive human MYC-driven MBs.

To further understand how loss of *Ctdnep1* drives gene expression profiles that underlie the development of aggressive MBs, we examined the genomic landscape and chromatin accessibility by performing ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing) and observed alterations in accessible chromatin sites in the absence of *Ctdnep1* (Extended Data Fig. 8a). Strikingly, we observed strong ATAC-seq peaks in the regulatory regions of the MYC locus in *Ctdnep1*-cKO NPCs and *Ctdnep1*-cKO tumors compared to control NPCs (Fig. 4k), suggesting an increase of open chromatin accessibility for MYC expression in *Ctdnep1*-deficient tumorigenic cells. In addition, gene loci associated with MYC-driven G3-MB (e.g., *Kcnj2*, *Ccnd1*, and *Tgfβ3*) were more accessible in the *Ctdnep1*-cKO NPCs (Extended Data Fig. 8a,b), whereas the accessibility of genes that regulate normal chromosome segregation (e.g., *Kif2c*, *Kif18a*, and *Esp1l1*) was reduced compared to NPCs from wild-type mice (Extended Data Fig. 8a,c). These data suggest that *Ctdnep1* loss may promote tumorigenic programs by altering the chromatin landscape, thereby resulting in the activation of MYC-driven pathways while downregulating genes associated with normal chromosome segregation.
Sustained Ctdnep1 deletion results in MYC upregulation and p53 downregulation during malignant transformation

GSEA analysis of transcriptome profiles revealed that the expression levels of both MYC and p53 pathway genes were upregulated in Ctdnep1-cKO NPCs at Div 12 compared to wild-type NPCs (Fig. 5a,b). MYC upregulation has been shown to induce DNA damage, activation of DNA damage responses, and cell death \(^{48,49}\). Consistent with this, we observed an upregulation of DNA damage-related markers γH2A.X and p53 in Ctdnep1-cKO NPCs at the early stage (Div 12) and in developing cerebella in mice (Fig. 5c,d), as well as CTDNEP1-knockdown MB tumor cells (Extended Data Fig. 9a), suggesting that Ctdnep1 deletion induces DNA damage, and activates DNA damage responses and apoptotic programs.

When examining Ctdnep1-cKO NPC tumor-like cells at the late stage (Div 60), in contrast to the early passage, GSEA analysis indicated the MYC pathway was upregulated. Interestingly, the p53 pathway was downregulated compared with wild-type cerebellar NPCs at Div 60 (Fig. 5e). qRT-PCR analysis confirmed the downregulation of p53 pathway genes (e.g., Trp53, Noxa, and p21)\(^{50}\) and upregulation of MYC pathway genes (e.g., c-Myc, Nfkb2, and Slc6a15)\(^{51}\) in Ctdnep1-cKO NPCs (Fig. 5f). In addition, western blot analysis indicated a strong upregulation of MYC and p-S62 MYC while p53 was downregulated in the Ctdnep1-cKO NPCs at Div 60 (Fig. 5g).

To determine if MYC is essential for hyperproliferative growth in the Ctdnep1-cKO tumor cells, we knocked down Myc utilizing a lentiviral shRNA and found that depletion of c-Myc strongly reduced the growth of Ctdnep1-cKO tumor cells (Fig. 5h), suggesting that tumor cell growth mediated by Ctdnep1-deficiency is dependent upon MYC levels. Together, these observations indicate that sustained Ctdnep1 deficiency induces the activation of MYC signaling, which can further downregulate the p53 tumor suppressor \(^{52}\), leading to malignant transformation of neural progenitors.

Loss of Ctdnep1 induces chromosome instability and Myc amplification

MYC upregulation together with p53 loss induces chromosome instability and enables cell survival with DNA damage \(^{49,53-57}\), which is associated with tumorigenesis in G3 MBs \(^{22,23}\). Our transcriptome profiling analysis indicated a downregulation of mitotic sister-chromatid segregation in Ctdnep1-cKO tumor cells compared with control NPCs (Fig. 4h). We then analyzed chromosome segregation during
mitosis via DAPI staining in Ctdnep1-ablated and wild-type NPCs and observed a substantial increase of lagging or bridging chromosomes in mutant NPCs compared to control NPCs at Div 12 (Fig. 5i), suggesting that Ctdnep1 depletion leads to chromosome mis-segregation and aneuploidy. The increased aneuploidy is generally associated with a poorer prognosis\textsuperscript{58,59}, and may drive the aggressive nature of Ctdnep1-deficient MBs.

Consistent with the chromosome segregation defects in mutant NPCs observed (Fig. 5i), karyotype analysis showed that the majority of the Ctdnep1-ablated NPCs evaluated (approximately 55\%) exhibited aneuploidy, specifically triplication of chromosome 15 carrying the Myc gene at Div 45 (Extended Data Fig. 9b). To further confirm the Myc-locus-specific amplification, we performed whole genome sequencing (WGS) analysis and found that the Myc gene locus was amplified in Ctdnep1-cKO NPC and Ctdnep1-cKO-tumor cells (Fig. 5j and Supplementary Table 2), along with Trp53 gene loss-of-heterozygosity. Moreover, the fluorescent in situ hybridization (FISH) assays detected six and four Myc amplicons in the majority of Ctdnep1-cKO tumor cells (17/20) and Ctdnep1-cKO NPCs (14/20) at Div 70, respectively, compared with the normal two Myc gene copies in control NPCs (Fig. 5k). The strongest Myc amplification signals at metaphase were detected in two rearranged chromosomes in Ctdnep1-cKO NPCs and Ctdnep1-cKO tumor cells (Fig. 5k). These data suggest that Ctdnep1 deletion leads to Myc amplifications mostly through focal copy number gain, consistent with elevated MYC amplicons in human Group 3 MB\textsuperscript{60}.

To determine the potential sequence over time of MYC amplification versus p53 loss-of-function, we performed qRT-PCR analysis at different time points and found that Trp53 expression was downregulated at Div 24 in Ctdnep1-cKO NPCs (Extended Data Fig. 9c), while at a later stage, an increase in Myc expression, gene amplification, and chromatin accessibility was detected in cKO-Ctdnep1 NPCs (Fig. 4k, 5j and Extended Data Fig. 9d). These data suggest that Trp53 downregulation might occur prior to Myc amplification during the transformation of Ctdnep1-cKO NPCs, alongside the loss of Ctdnep1 leading independently to the activation of MYC activity through its phosphorylation. Thus, CTDNEP1 loss-of-function or deficiency can lead to chromosome instability and aneuploidy, which can potentially drive MB tumorigenesis via amplification or activation of MYC and loss of p53.
CTDNEP1 post-translationally modulates the activities of key regulators for proper chromosome decatenation and mitotic checkpoints

To better understand how CTDNEP1 loss may promote tumorigenesis, we sought to use proteomes to identify CTDNEP1 downstream interacting effectors through protein-protein interactions aside from the MYC protein (Fig. 3). For this purpose, we performed immunoprecipitation with an antibody to CTDNEP1 and analyzed co-precipitated proteins from HEK293 cells by mass spectrometry. We identified 195 CTDNEP1 binding proteins (Fig. 6a), which regulate cell cycle transition, RNA splicing, chromosome segregation/organization, and DNA repair processes (Fig. 6a).

While little is known of CTDNEP1 substrate specificity as a protein phosphatase, we further sought to identify CTDNEP1 effects on phosphorylation, either direct or indirect, which could be related to tumorigenesis. Towards this end, we performed label-free mass spectrometry of wild-type and Ctdnep1-cKO NPCs to identify the upregulated phosphorylated proteins. When compared with control NPCs, nearly 3,236 phosphorylated peptides, corresponding to 1,365 proteins (fold changes > 5; \( p < 0.05 \)), were detected at significantly higher levels in Ctdnep1-cKO NPCs (Fig. 6b). Gene ontology (GO) analysis indicated that the upregulated phospho-proteins are associated with cell-cycle progression and chromosome segregation (Fig. 6c). These proteins include critical regulators of chromosome decatenation and mitotic checkpoints for proper chromosome segregation such as DNA topoisomerase TOP2A and NCD80 \(^{62,63}\); MCM2, which promotes DNA replication \(^{64}\); cell-cycle regulators SRPK1 and CDK1-3, which are serine-threonine protein kinases that regulate the G2-M transition and mitotic progression \(^{65}\); and the cell-cycle inhibitor RB1, which regulates transcription and the G1-S transition and is repressed by phosphorylation \(^{66}\) (Fig. 6d and Extended Data Fig. 10).

We identified approximately 31 proteins as candidate direct targets or substrates of CTDNEP1. They were the CTDNEP1-binding proteins, and their phosphorylation were upregulated in Ctdnep1-cKO NPCs as compared to control NPCs; these included key mitosis and chromosome-segregation regulatory factors such as TOP2A (Fig. 6e). GO analysis showed the enrichment of processes critical for chromosome segregation and chromatin remodeling among the candidate proteins regulated by CTDNEP1 (Fig. 6f). These proteins interacted with CTDNEP1 and their phosphorylation was upregulated in CTDNEP1-deficient cells, suggesting that they are candidate direct substrates of
CTDNEP1. The increase in phospho-TOP2A, which regulates DNA replication and chromosome separation \(^{63,67}\) in *Ctdnep1*-cKO NPCs was confirmed by western blot analysis (Fig. 6g). The upregulation in phosphorylation of mitosis-related proteins is consistent with the increase in cell proliferation in the MB cells with depletion of CTDNEP1 (Fig. 2).

To further investigate the effect of CTDNEP1 depletion on the phosphorylation of mitosis-associated proteins, we examined the MPM2 epitopes that mark the phosphorylation of multiple M-phase-mitosis-promoting regulators during the mitotic metaphase \(^{48,68}\). Western blot analysis of control and *CTDNEP1*-knockdown in near-diploid DAOY MB cells without substantial spindle checkpoint defects revealed that CTDNEP1 depletion increased levels of mitotic phospho-proteins marked by MPM2 including TOP2A, MAP2, HSP70, and CDC25 (Fig. 6h). These results suggest that CTDNEP1 loss activates mitosis-associated checkpoint regulators to maintain mitotic homeostasis.

Consistent with activation of DNA damage responses induced by *Ctdnep1* loss (Fig. 5), we detected an increase in phosphorylation of a critical mitotic checkpoint kinase CHEK1, which responds to DNA damage, regulates cell-cycle checkpoint signaling, and genomic integrity \(^{69,70}\), in *Ctdnep1*-cKO NPCs (Fig. 6g). Similarly, we found that the level of phospho-CHEK1 was elevated in CTDNEP1-depleted MB tumor cells (Fig. 6h). Thus, our proteomics and phospho-proteomics reveal that CTDNEP1 can directly modulate key regulators of chromosome decatenation and mitotic checkpoints for maintaining proper chromosome segregation and mitotic homeostasis.

Co-targeting MYC and CHEK1 effectively inhibits the growth of MYC-driven MB tumors

Since MYC and CHEK1 activities are upregulated in response to mutation or loss of *CTDNEP1*, this may provide a vulnerability for targeting of CTDNEP1-deficient tumors. Thus, we utilized the BET inhibitor JQ1 and CDK7 inhibitor THZ1, both of which were shown to inhibit MYC expression \(^{71,72}\) and reduce cell proliferation in MB cells \(^{73}\). Indeed, treatment with JQ1 or THZ1 resulted in inhibition of *Ctdnep1*-cKO tumor cell proliferation compared to treatment with vehicle (Fig. 7a and Extended Data Fig. 11a). JQ1 treatment decreased MYC expression and elevated apoptosis marked by cleaved-caspase 3 (Fig. 7b).
In view of upregulation of DNA damage repair and activation of CHEK1 in Ctdnep1-deficient cells (Fig. 6g), we also treated Ctdnep1-cKO tumor cells with prexasertib, a selective inhibitor of the checkpoint kinase CHEK1 currently under multiple clinical trials; prexasertib disrupts DNA replication and prevents DNA damage repair, causing cell death by replication catastrophe \(^{74,75}\). This treatment similarly inhibited cell growth of Ctdnep1-deficient tumor cells (Fig. 7c). Importantly, combined JQ1 and prexasertib treatment of Ctdnep1-cKO tumor cells at reduced doses was more effective than either single agent (Fig. 7d). To determine if the effects of the two drugs on cell growth are synergistic, we applied the Bliss model to calculate synergy scores \(^{76}\) and classified the Bliss score > 10 as synergistic \(^{76}\). The combined treatment with JQ1 and prexasertib elicited synergistic effects on net cell growth in Ctdnep1-cKO tumor cells across a range of concentrations (Fig. 7e). To further determine the selectivity of the treatment on the growth of wildtype and CTDNEP1-deficient tumor cells, we treated control or CTDNEP1-knockdown D283 cells with JQ1 and prexasertib. CTDNEP1 knockdown substantially increased the sensitivity of D283 cells to the drug combination treatment compared with control D283 cells (Extended Data Fig. 11b,c), suggesting that this combined treatment more selectively inhibits the growth of CTDNEP1 deficient tumor cells.

To assess the potential of MYC, CHEK1, or both for treating Ctdnep1-deficient tumors *in vivo*, we treated NSG mice orthotopically transplanted with Ctdnep1-cKO tumor cells daily with a single dose of JQ1 (50 mg/kg), which is blood-brain barrier penetrant \(^{77}\), or in combination with prexasertib (2 mg/kg), which also exhibits CNS penetration \(^{78,79}\), from day 45 to 60 post-transplantation. Compared with vehicle-treated mice, mice treated with JQ1 had prolonged survival (Fig. 7f). Importantly, mice with Ctdnep1-cKO NPC tumors treated with JQ1 combined with prexasertib had an increased lifespan compared to the mice treated only with JQ1 or vehicle (Fig. 7f). The combined treatment also resulted in an increase in tumor cell death compared to vehicle or JQ1 only (Fig. 7g,h), suggesting that the combined JQ1 and prexasertib treatment induces cell apoptosis to inhibit tumor cell growth.

To examine the effect of inhibition of MYC and CHEK1 on the growth of human MB tumor cells, we treated MYC-driven D425 MB cells with JQ1 or prexasertib individually or in combination. Similarly, combined treatment synergistically enhanced inhibition of D425 cell growth in vitro (Fig. 7i and Extended Data Fig. 11d,e), and prolonged animal survival in orthotopic xenografts with D425 cells.
compared to vehicle or single drug treatment alone (Fig. 7j). In addition, combined MYC and CHEK1 inhibition substantially inhibited the cell growth in other MYC-amplified MB004 and murine Myc-driven G3-like MB cells (Extended Data Fig. 11f), while the non-MYC-amplified cell lines such as D283 and DAOY were less sensitive to the combined treatment (Extended Data Fig. 11g). These data suggest that combined inhibition of MYC and CHEK1 activities had a selective antitumor effect in MB cell lines with MYC amplification.

Our present analyses (Fig. 5a) and previous reports also suggested that inhibition of CTDNEP1 elevated TGF-β signaling; however, treatment with TGF-β receptor kinase inhibitors (galunisertib or LY-364947) did not significantly alter the growth of Ctdnep1-cKO NPCs relative to controls (Extended Data Fig. 11h,i).

Discussion

Nuclear envelope phosphatase CTDNEP1 functions as a novel potent tumor suppressor in highly aggressive MBs

Although recent genome-wide studies have provided insight into somatically altered genes in MBs, identification and functional validation of cancer-driving alleles remains enigmatic. The regulatory mechanisms that control MYC activity and amplification for tumorigenesis in highly aggressive MYC-driven MB have remained incompletely understood. By integrating the transcriptomic and genomic profiles from our newly diagnosed and publicly available MB cohorts, we found that CTDNEP1 mutations, which present predominantly in MYC-driven MBs, define a specific subset of aggressive MB tumors. In contrast to many mouse models of MYC-driven G3 MB, which require MYC overexpression and additional loss of p53 function, here we demonstrate that ablation of a single gene, Ctdnep1, is sufficient to trigger MYC amplification and genomic instability, and promote malignant transformation of cerebellar NPCs into MYC-driven MB tumors. The CTDNEP1-deficiency induced tumors resemble the histopathological, transcriptomic, and clinical features of the human G3 MB counterpart, suggesting that CTDNEP1 is a potent tumor suppressor in the highly aggressive MYC-driven G3 MBs.

MYC has been shown to tether to the nuclear pore specifically in cancer cells, and the enrichment of pS62-MYC at the nuclear periphery promotes its stability and oncogenic activity. We find that
clinically-identified mutations in CTDNEP1 disrupt its phosphatase activity, resulting in an increase of pS62-MYC and stabilization of the MYC oncoprotein, suggesting that the nuclear envelope-enriched CTDNEP1 phosphatase is required to curtail MYC protein levels and its oncogenic activity. Consistently, our data indicate that the growth of Ctdnep1-deficient tumor cells depends on MYC levels. Given that MYC upregulation induces proliferation and chromosome instability, this suggests that CTDNEP1 exhibits a tumor suppressive activity by antagonizing MYC-driven proliferation and genomic instability. Importantly, CTDNEP1-deficiency drives MYC amplification both in NPCs and in tumors, suggesting that low expression or mutation of CTDNEP1 can lead to MYC upregulation by increasing both MYC stability and MYC copy numbers. Thus, the loss of CTDNEP1 function not only directly leads to increased MYC levels by stabilizing it but also provides a selective advantage to cells that can express even greater levels of MYC due to increased copy numbers. Notably, since CTDNEP1 mutations or deletions have also been identified in other cancers, CTDNEP1 might have a broader role in cancer formation and serve as a molecular link through regulating MYC and genomic instability across different cancer types.

**Stage-specific mechanisms of CTDNEP1 loss for MB tumorigenesis**

We observed an initial upregulation of DNA damage response and p53-dependent apoptotic programs at the early stage, however, a population of Ctdnep1-ablated cerebellar NPCs at later stages exhibits p53 downregulation, acquires chromosomal aneuploidy, and is eventually transformed into MYC-driven MB tumors, suggesting a stage-dependent mechanism of Ctdnep1 loss in G3-MB tumorigenesis. Downregulation of p53 appears to occur during selection of clones with MYC upregulation, which represses p53 expression. Sustained CTDNEP1-depletion and upregulation of MYC can also induce genome instability, resulting in loss or downregulation of p53 that may further contribute to oncogenic transformation, which is in keeping with the timing of p53 mutations occurred often at later stages during tumor evolution. In addition, ATAC-seq analysis reveals that CTDNEP1 deficiency also leads to a decrease in chromatin accessibility at the promoter regions of the regulatory genes associated with genomic stability including Trp53. p53 target expression in CTDNEP1 mutated G3-MBs was reduced compared with wildtype G3-MBs.
Consistent with this, we show that p53 loss-of-function by overexpressing DN-p53 can accelerate the tumorigenesis of Ctdnep1-deficient NPCs in allografts.

Intriguingly, all tumors from patients with CTDNEP1 mutations appeared to exhibit loss-of-heterozygosity (LOH) for the other allele of CTDNEP1 (Extended Data Fig. 1). This suggests that CTDNEP1 may be bi-allelic inactivation, which is typical of many tumor suppressors. While the exact mechanism(s) underlying chromosome instability induced by mutations in CTDNEP1 remains to be determined, we observe increased MYC protein, MYC amplification, DNA damage and downregulation of p53, which can lead to genomic instability. Since CTDNEP1 and TP53 are tightly linked on chromosome 17p, one copy of TP53 will be lost along with an allele of CTDNEP1. CTDNEP1 loss-of-function due to mutation in the other allele could drive increased proliferation and genome instability due to MYC upregulation. Importantly, independent LOH for TP53, subsequent to CTDNEP1 loss-of-function, could further promote transformation and tumorigenesis by resulting in deficient cell cycle arrest, apoptosis, or both, in cells with increased DNA damage and genome instability.

Moreover, we find that the loss of CTDNEP1 alters the posttranslational modification of key regulators for proper chromosome decatenation and mitotic checkpoints such as TOP2A and CHEK1. Given that the dysregulation of DNA replication, chromosome segregation, or mitosis is known to result in genomic imbalance, aneuploidy, and tumorigenesis, CTDNEP1 may potentially regulate genomic stability in a MYC-independent manner by maintaining proper DNA replication and mitotic exit.

A dual role of CTDNEP1 in regulating MYC stability and mitotic checkpoint signaling

The activity of mitotic related proteins is frequently controlled by modulation of their phosphorylation levels. Our data indicate that a key direct target of CTDNEP1 is MYC, which appears to be mediated by CTDNEP1-catalyzed dephosphorylation of S62 of MYC, leading to destabilization of the MYC protein. Phospho-proteomic analysis further demonstrates that CTDNEP1 deficiency led to upregulation of phosphorylation of a set of mitotic regulators such as TOP2A, MCM2, CDK1-3, and RB1.
Our unbiased screen further identifies a set of CTDNEP1-interacting partners, including proteins critical for DNA replication, chromosome decatenation, and mitotic checkpoints. Among them, TOP2A and CHEK1 displayed increased phosphorylation in CTDNEP1-deficient cells, suggesting that they may be direct downstream effectors for the CTDNEP1 phosphatase. CTDNEP1 may normally regulate chromosomal segregation and mitotic checkpoints through dephosphorylation of factors such as TOP2A and CHEK1. Thus, our data suggest that CTDNEP1 exhibits a dual-function by inhibiting MYC activity and maintaining mitotic homeostasis and genomic stability.

**Combined targeting MYC and CHEK1 synergistically inhibits G3-MB associated tumor growth**

Our data showing upregulation of MYC and CHEK1 activities may present as a potential therapeutic vulnerability. Importantly, LOH of CTDNEP1 only in the tumor may increase the therapeutic window for combined treatment with MYC and CHEK1 inhibition. Such a dual inhibitory strategy could be especially beneficial in the treatment of MYC-driven MB; elevated MYC levels have been shown to sensitize cancer cells to the inhibition of mitosis and cell-cycle checkpoints by increasing apoptosis, while increased CHEK1 expression and activity is correlated with poor prognosis in MYC-driven MB. Notably, we find that JQ1 that suppresses MYC transcription can synergize with the CHEK1 inhibitor prexasertib to suppress the growth of human MYC-driven MB tumor cells more effectively than either single agent alone and prolongs the survival of animals bearing CTDNEP1-deficient MYC-amplified MB tumors. Since MB is a pediatric disease, it should be noted that prexasertib is currently being evaluated in clinical trials including treatment of pediatric malignancies. Patients with MB tumors such as highly aggressive MYC-driven MB commonly have DNA replication stress and pronounced DNA damage responses, resulting in upregulation of checkpoint signaling. Prexasertib treatment may induce cell apoptosis or mitotic catastrophe by inhibiting the repair of DNA damage caused by CTDNEP1 deficiency. Thus, combined targeting of MYC and the checkpoint regulator CHEK1 might serve as a therapeutic means to improve outcomes on treatment of aggressive G3 MBs with CTDNEP1-deficiency.

Further, these vulnerabilities underscore a dual role of CTDNEP1 in suppressing malignant transformation of highly aggressive MBs by inhibiting MYC and maintaining mitotic homeostasis and
genomic stability. Thus, our study uncovered clinically relevant mutations of CTDNEP1 in a cohort of highly aggressive MYC-driven G3 MBs. This is the first study to demonstrate the nuclear-envelope-enriched CTDNEP1 protein phosphatase functions as a potent tumor suppressor in the highly aggressive G3-MBs and provides new biological insights into the regulation of MYC oncogenic activity and genomic stability as well as creates opportunities for individualized therapy.

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Figure legends:

**Fig. 1. Prevalence and clinical impact of recurrent mutations of CTDNEP1 in G3 MBs**

- a. Frequency of known and recurrent genetic variants in pediatric G3 MBs.
- b. The significance enrichment plot of somatic recurrent mutated genes in G3-MB compared with other MB subgroups. *P* values were calculated based on Fisher’s exact test and were then adjusted for multiple testing by Bonferroni correction methods.
- c. Frequency of **CTDNEP1** LOF variants in different MB subgroups.
- d. Somatic **CTDNEP1** mutation profile in patients with MBs. fs, Frameshift.
- e. Association between somatic **CTDNEP1** LOF variants and somatic chromosomal alterations (*n* = 136 G3-MB). *p* values were calculated using Bayesian logistic regression analysis, likelihood ratio tests, and adjusted for multiple testing based on 5% false discovery rate (FDR) correction.
- f. Kaplan-Meier analysis of overall survival of patients with WNT, SHH, G3, and G4 MB based on the **CTDNEP1** high and low expression across subgroups in publicly available MB cohorts. Log-rank test.
- g. Kaplan-Meier analysis of overall survival of patients with **CTDNEP1** (**CTD**) mutation and no **MYC** amplification (**CTD w/o MYC**), **MYC** amplification and no **CTDNEP1** mutation (**MYC w/o CTD**), **CTDNEP1** mutation and **MYC** amplification (**CTD+MYC**) and other G3 MB patients (other G3). *p < 0.05; **p < 0.01, ***p < 0.001, Log-rank test.

**Fig. 2. Inhibition of CTDNEP1 expression promotes MB cell proliferation**

- a. qRT-PCR quantification of **CTDNEP1** in D425 cells transduced with lentiviral control (shCtrl) and shRNAs targeted against **CTDNEP1** (shCTD). Data are means ± SD. **p < 0.01, two-tailed Student’s *t* test.
- b. Left, representative images of shCtrl and shCTD-transduced D425 cells stained for EdU (scale bar, 50 μm); right, quantification of EdU+ cells. Data are means ± SD, n=6 independent experiments. *p < 0.05; **p < 0.01, two-tailed unpaired Student’s *t*-test.
- c. Growth of shCtrl and shCTD-transduced D425 cells assayed by WST-1. Data are means ± SD of 6 independent measurements. *p < 0.05; **p < 0.01, two-tailed unpaired Student’s *t*-test.
- d. Upper, the number of clones per well in a soft agar assay of Ctrl and shCTD D283 cells after 10 days culture. Lower: Representative images of clones in soft agar plates. Data represent means ± SD, n=3 independent experiments. *p < 0.05; two-tailed unpaired Student’s *t*-test.
- e,f. Left: Representative images of neuro-spheres of control and shCTD- transduced D425 (**e**) and D283 cells (**f**) (scale bars, 100 μm). Right panels: the number of spheres. *p < 0.05 (n=3 independent experiments), two-tailed unpaired Student’s *t*-test.
- g. Left and center: Representative flow cytometry of cell-cycle stages of D283 cells transfected with shCtrl or shCTD in D283 cells. Right: percentage of cells at different cell-cycle stages (n=3 independent experiments), *p <0.05; ns: no significance, two-tailed unpaired Student’s *t*-test.
- h,i. Upper: representative photographs of tumors from mice transplanted subcutaneously with 1x10^6 shCtrl and shCTD-treated D425 (**h**), or D283 (**i**) cells. Lower: Weights of tumors as means ± SD (n = 6 mice per group). *p < 0.05, **p < 0.01, two-tailed Student’s *t* test.
- j. Upper: Immunoblots of CTDNEP1 overexpression (OE) in D425 cells. Lower: Cell proliferation as monitored by WST-1 in control and CTD-OE D425 cells. Data are means ± SD. n = 3 independent
experiments, **p < 0.01, two-tailed Student’s t-test.

k. Upper: Photographs of tumors from mice transplanted subcutaneously with control or CTDNEP1-overexpressing D425 cells. Lower: Weights of tumors as means ± SD (n = 4 animals per group). **p < 0.01, two-tailed Student’s t-test.

**Fig. 3. Loss of CTDNEP1 increases MYC stability and activity.**

a, Heatmap of differentially expressed genes in D425 cells treated with shCtrl and shCTD (#1, 2 and 3) (n = 3 independent experiments).

b, Volcano plot of transcriptome profiles between control D425 cells and shCTDNEP1 (CTDNEP1-KD) treated cells. Red and blue dots represent genes significantly upregulated and downregulated in cells depleted of CTDNEP1 (p < 0.05, false discovery rate [FDR] <0.1), respectively.

c, Correlations of upregulated genes in shCTDNEP1 D425 cells with MYC expression in G3 MBs.

d, Pathway perturbation analysis of D425 cells depleted of CTDNEP1 and control cells (n=3 independent experiments) showing the most differentially regulated transcriptional factors (identified using Enrichr).

e, Left: qRT-PCR quantification of c-MYC mRNA in shCtrl and shCTD treated D425 cells. Data are means ± SD. n=3 independent experiments; n.s. not significant; *p < 0.05, two-tailed Student’s t test. Right: Representative immunoblots for total MYC protein and p-S62-MYC in shCtrl and shCTD-treated D425 cells.

f, Representative immunoblots from 3 independent experiments for total MYC protein and p-S62-MYC in shCtrl and shCTD-treated D425 cells treated or not with cycloheximide (CHX, 10 μg/ml) for 1 or 3 h.

g, Upper: Conserved residues in the conserved catalytic motif of DXDX(T/V) proteins. Lower: The phosphatase activity of CTDNEP1 wildtype and mutant (D67N, D69N, and L72H) proteins. Values are the mean ± SD. n=3 independent experiments; **p < 0.01, two-tailed Student’s t test.

h, Representative immunoblots from 3 independent experiments for flag-tagged c-MYC after treatment by CHX at indicated time points in 293T cells co-transfected with wildtype (wt) or flag-tagged c-Myc mutant (S62E), and wildtype (wt) or myc-tagged CTDNEP1 mutants (D69N or L72H) as indicated (left). Quantification of c-Myc-flag expression after 4 hrs CHX treatment as compared to untreated cells (right), **p < 0.01, two-tailed Student’s t test.

i, Representative immunoblots from 3 independent experiments for MYC protein and its Ser62 phosphorylation levels in D425 cell lysates after 1 hour incubation with wild-type and mutants of CTDNEP1-myc-tagged proteins.

j, Immunoprecipitation assay (n=3 independent experiments) for CTDNEP1 with wild-type MYC in 293T cells transfected with vectors expressing CTDNEP1-myc-tag and MYC-flag-tag after 4-hour of MG132 treatment.

k, Representative immunoblots from 3 independent experiments for MYC protein and p-S62-MYC in stable CTDNEP1 overexpressing D425 cells transduced with lenti-CTDNEP1-myc-tag. Viral vector infected D425 cells serve as the control.

**Fig. 4. Deletion of CTDNEP1 in cerebellar NPCs induces G3 MB-like tumor formation.**

a, Diagram depicting the generation of Ctdnep1-cKO mice.
**Fig. 5. Ctdnep1 ablation induces genomic instability and Myc amplification.**  

**a.** Heatmap of differentially expressed genes in Ctdnep1-cKO NPCs (n=2) compared to wild-type NPCs (Ctrl, n=2) at early and late-stage.  

**b.** GSEA plots of data from Ctdnep1-cKO NPCs and control NPCs at early-stage showing p53 pathway and MYC target genes.  

**c,d.** Representative immunoblots for MYC, p53, γH2A.X and cleaved-caspase 3 (CC3) in the early-stage NPCs (c) (from 3 independent experiments) and the cerebellum of Ctdnep1-cKO mice (n=3 animals/group) at postnatal day 4 (d).  

**e.** GSEA plots of data from Ctdnep1-cKO and control NPCs at late-stage showing p53 pathway and MYC target genes.  

**f.** qRT-PCR quantification of indicated transcripts in control and Ctdnep1-cKO NPCs at late-stage. Plotted are means ± SD relative to levels in Ctrl cells (n = 3 independent experiments). *p < 0.05, **p < 0.01, two-tailed Student’s t test.  

**g.** Representative immunoblots from 3 independent experiments for MYC and p53 in Ctdnep1-cKO and Ctrl NPCs.  

**h.** Upper, relative Myc expression in Ctdnep1-cKO NPCs transduced with non-targeting control shRNA and shMyc RNAs as indicated. Lower, cell viability of Ctdnep1-cKO NPCs transduced with non-
targeting shRNA and shMyc RNAs measured by WST-1 assay. Data are means ± SD. n=3 independent experiments; *p < 0.05, **p < 0.01, two-tailed Student’s t test.

i, Anaphase analysis of early-stage (DIV 12) control and Ctdnep1-cKO NPCs synchronized using nocodazole and released after 1 h with fresh medium. Upper: Representative maximum projection images of anaphase cells. Scale bar, 5 μm. Lower: Quantification of cells with lagging or bridged chromosomes. A total of 60 anaphase cells were counted.

j, Upper: CNV analysis of Ctrl NPCs and Ctdnep1-cKO NPCs at late-stage (DIV 60), and two independent Ctdnep1-cKO tumor cells (cKO-T) based on 30x WGS analysis. Lower: Red and blue regions represent the focal amplified or deleted segments of the indicated chromosome, respectively.

k, Representative FISH images showed the Myc gene (Red) and chromosome 15 (Green) in control NPCs at DIV 70 (16/20), Ctdnep1-cKO NPCs (cKO-NPC) at DIV 70 (17/20) and Ctdnep1-cKO tumor cells (14/20). White arrows, the Myc gene in the chromosome 15; yellow arrows, Myc amplification translocated out of chromosome 15.

Fig. 6. CTDNEP1 post-translationally modulates the activities of key regulators for chromosome decatenation and mitotic checkpoints.

a, CTDNEP1 binding proteins identified by mass spectrographic analysis in 293T cells which was overexpressing CTDNEP1.

b, Mass spectroscopy analysis of phosphorylated proteins in Ctdnep1-cKO NPCs (cKO) at passage 10 compared with Ctrl NPCs.

c, Pathway analysis of the most differentially up-regulated phospho-proteins in Ctdnep1-cKO NPCs compared with wild-type NPCs.

d, Upper, representative phosphorylated proteins involved in cell-cycle progression that are enriched in Ctdnep1-cKO NPCs compared to Ctrl NPCs. Lower panel; the phosphorylation intensity of mitosis and chromosome segregation proteins in the NPCs detected by mass spectrometry. Data represent means ± SD, n=2 independent experiments. *p < 0.05; **p < 0.01, two-tailed unpaired Student’s t-test.

e, Venn diagram of CTDNEP1 binding proteins and phospho-proteins enriched in Ctdnep1-cKO NPCs compared to wild-type NPCs.

f, GO analysis of candidate CTDNEP1 regulated phospho-proteins in Ctdnep1-cKO NPCs.

g, Representative immunoblots from 3 independent experiments for p-CHEK1 and p-TOP2A in Ctdnep1-cKO and wild-type NPCs at late-stage (Div 20).

h, Representative immunoblots from 3 independent experiments for the indicated phospho-proteins in DAOY cells transfected with control siRNA or siCTDNEP1 after treatment with nocodazole for 14 h and sampled at indicated timepoints in fresh medium. NOW; nocodazole washout.

Fig. 7. Combined targeting MYC and CHEK1 activities inhibits CTDNEP1-deficient tumor progression.

a, Cell viability of control and Ctdnep1-cKO NPCs treated with the indicated concentrations of JQ1 relative to vehicle-treated cells. Data represent means ± SD, n=5 independent experiments. *p < 0.05; **p < 0.01, two-tailed unpaired Student’s t-test.

b, Representative immunoblots from 3 independent experiments for MYC and cleaved Caspase 3 in control or Ctdnep1-cKO NPCs treated with JQ1 (0.5 μM: +; 1 μM: ++) or DMSO (-).
c, Cell viability of Ctdnep1-cKO NPCs and wild-type NPCs treated with prexasertib (Prex) for 3 days at indicated concentrations relative to vehicle-treated cells. Data represent means ± SD; n=5 independent experiments. *p < 0.05; **p < 0.01, two-tailed unpaired Student’s t-test.
d,e, Heatmap showing the percentage of growth inhibition of Ctdnep1-cKO tumors and wild-type NPCs by combined treatment with JQ1 and prexasertib relative to vehicle-treated cells (d); Bliss score for JQ1 and prexasertib double titrations (e). n=5 independent experiments.
f, Kaplan-Meier survival for Ctdnep1-cKO tumor bearing allografts treated with vehicle, JQ1, or JQ1 and prexasertib once/day for two-weeks after transplantation at day 45. **p < 0.01 and ***p < 0.001; log rank test.
g, Representative images of immunostaining for cleaved-Caspase 3 (CC3) in vehicle, JQ1, or JQ1 and prexasertib treated mice with Ctdnep1-cKO tumors 62 days after implantation. Red arrows CC3+ cells in the tumor section.
h, quantification of CC3+ cells in the tumor tissues of the indicated treatment groups. Scale bars: 50 μm. Data are means ± SD. n=3 allografts; **p < 0.01 and ***p < 0.001; two-tailed Student’s t test.
i, Viability of D425 cells treated with JQ1 (1μM), prexasertib (10nM), or the combination relative to vehicle-treated cells. Data represent means ± SD; n=5 independent experiments. *p < 0.05; **p < 0.01, two-tailed unpaired Student’s t-test.
j, Kaplan-Meier survival for MYC-driven D425 orthotopic xenografts treated with vehicle, JQ1, prexasertib or JQ1 and prexasertib once/day for two-weeks after transplantation at day 10. *p < 0.05; **p < 0.01; log rank test.
Supplementary Figure legends:

**Extended Data Fig.1. The frequency of recurrent mutations across different cohorts.**
a, Overview and merge of MB cohorts based on individual patient ID, clinical information, and genomic alterations from WES or WGS profiling show the number of MBs and CTDNEP1 mutation counts in each cohort.
b, Frequency of alterations in CTDNEP1 in different types of brain tumors from the TCGA database (www.cbioportal.org).
c, Analysis of CTDNEP1 levels in normal brain (NB), cerebella (CB) and MB tumors obtained from indicated MB cohorts. Individual data points with the means ± SD are shown. **p < 0.01, *p < 0.05, two-tailed unpaired Student’s t test.

**Extended Data Fig. 2. Alterations of nuclear envelope-expressing CTDNEP1 in brain tumor patients and co-occurrence of genetic alterations with MYC amplification.**
a, WES sequencing on tumor samples from 89 newly diagnosed patients showing representative G3-MB tumor samples carried LOF CTDNEP1 mutations, wherein wildtype signals were completely substituted by mutant signals.
b, Sanger-sequencing validations of homozygous mutations on CTDNEP1.
c, CNV analysis of one peri-tumor brain tissue and three human G3-MBs with CTDNEP1 mutations based on 850K array methylation, showing LOH for the short arm of chromosome 17p and MYC amplification.
d, Co-occurrence of somatic chromosomal aberrations in CTDNEP1 mutation-associated G3-MBs.
e, Co-occurrence between MYC amplification and somatic chromosomal alterations, n = 136 G3-MBs. P values were calculated using Bayesian logistic regression analysis, likelihood ratio tests, and adjusted for multiple testing based on FDR correction.
f,g, CNV analysis of Chr17 from nine representative human G3-MB (f) and one G4-MB (g) with CTDNEP1 mutations from publicly datasets (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi).

**Extended Data Fig. 3. CTDNEP1 expression levels correlate with MB patient survival across subtypes.**
a, CTDNEP1 and TP53 expression in G3 and G4 MBs with chromosome 17p loss (n = 63), balanced (n = 38) and 17p gain (n = 5) using the published MB cohort6.
b, CTDNEP1 expression in novel subgroups using publicly available datasets5. **p < 0.01, ***p < 0.001, two-tailed unpaired Student’s t-test.
c, CTDNEP1 expression in four subgroups of MBs using publicly available datasets (GEO: gse85217).
d, Subgroup of MBs based on population density of CTDNEP1 expression from 612 MB patients with survival information. Green and red line showed the threshold for 35% low and 35% high expression respectively.
e, Overall survival of all MB patients with high CTDENP1 expression (35% high) compared with those with low CTDENP1 expression (35% low). p value, Log-rank test.

**Extended Data Fig. 4. MYC expression and MYC target genes regulated by CTDNEP1.**
a, c-MYC and p-Ser62 MYC expression in different human MB cells including MYC amplified G3-MB lines (MB004, D458 and D425), a non-MYC amplified G3 MB line, D283, and a SHH-MB cell line, DAOY).

b, Left, representative images of control and shCTDNEP1-expressing G3 MB004 cells stained for BrdU (scale bar, 50 μm); right, quantification of BrdU⁺ cells. Data represent means ± SD, n=6 independent experiments. *p < 0.05, two-tailed unpaired Student’s t-test.

c, Cell proliferation assayed by WST-1 in control and shCTDNEP1 MB004 cells. Data are means ± SD. n = 3 independent experiments, *p < 0.05, two-tailed unpaired Student’s t-test.

d, Genome browser view of G3 signature genes (NR2E3, NRL and RORB) and MYC target genes in G3 MBs (RPL21, CAV3, GABRA5, CHRNBJ, TIMP3 and MPZL2) of MYC and H3K27ac ChIP–seq binding sites in MYC amplified G3 MBs (GSE143376). Pink highlights indicate regulatory elements.

e, Heatmap of differentially expressed genes in published datasets of G3 MBs 6. CTDNEP1 mutated (n=5), MYC amplified or high expressed (n=14, 1 overlapped with CTDNEP1 mutated patient) and MYC low expressed G3 patients (n=23).

f, GSEA plots of CTDNEP1 mutated G3-MB and CTDNEP1 wildtype G3-MB patients 6 showing upregulation of MYC target genes in CTDNEP1 mutated G3-MBs.

Extended Data Fig. 5. Deletion of nuclear envelope-enriched CTDNEP1 increases MYC and MYC (p-Ser62) expression.

a, Expression of HA-tag CTDNEP1 in murine glial progenitor cells. Arrows indicate the immunostaining signals of HA-tag CTDNEP1 in the nuclear membrane. Scale bar, 20 μm.

b, Western blotting of c-MYC and p-Ser62 expression in MB004 cells treated with control and shCTDNEP1.

c, c-MYC and p-Ser62 expression in D283 treated with control and shCTDNEP1 in the presence of cycloheximide (CHX) at the indicated time.

d,e, Immunoblots for MYC and p-Ser62 MYC (d) and the quantification (e) in three individual CTDNEP1 mutated G3 MB tissues, one SHH-MB, two non-MYC amplified and two MYC amplified G3 MB tumor tissues.

Extended Data Fig. 6. Deletion of Ctdnep1 in mouse NPCs induces apoptosis and DNA damage.

a, Kaplan-Meier survival curves of mice with Nestin-Cre-mediated knockout of Ctdnep1. N=10 animals per group; p < 0.001; log rank test.

b, Representative images of hematoxylin and eosin stains of the cortex and cerebellum from control and Ctdnep1-cKO mice at P18. Scale bars: 1 mm.

c, Bar graph shows the quantification of the relative volume of the cortex and cerebellum from control and Ctdnep1-cKO mice at P18. n = 3 animals/genotype. **p < 0.01, two-tailed unpaired Student’s t-test.

d, Immunofluorescence of cleaved-Caspase 3 and γH2A.X in control Ctrl and Nestin Cre: cKO-Ctdnep1 cerebellum at embryo 13.5 (left). Scale bars: 200 μm. Bar graph shows the quantification of cleaved-Caspase 3 and γH2A.X-positive cells (right). Data represent means ± SD (n=3 independent measurements). *p < 0.05; **p < 0.01, two-tailed unpaired Student’s t-test.
Extended Data Fig. 7. Enhanced tumorigenic potential of Ctdnep1-deficient NPCs with p53 inhibition.

b, Survival curves of Ctdnep1-cKO tumor cells transplanted into the cerebellum of NSG mice at varying doses.
c, Tumor formation in the nude mice subcutaneously transplanted with wildtype (WT) and Ctdnep1-cKO (cKO) NPCs (at Div 15) after 6-month post-transplantation. Green or red arrows show the transplantation sites of WT or cKO NPCs.
d, Hematoxylin and eosin staining of subcutaneous Ctdnep1-cKO-derived tumors and the representative images of Ki67, c-Myc, and Nestin immunostaining for Ctdnep1-cKO-derived tumors. Scale bars: 50 μm.
e, Schematic diagram showing that freshly isolated NPCs from Ctdnep1-cKO mice at postnatal day 4 were transduced with retroviruses expressing DNp53 for implantation.
f, Representative G3-MB-like tumor formation in the cerebella transplanted with Ctdnep1-cKO NPCs transduced with DNp53 in 6 out of 8 implanted NSG mice.

Extended Data Fig. 8. Chromosome access status alteration induced by Ctdnep1 deletion in mouse NPCs.

a, ATAC-seq analysis of Ctdnep1-cKO and Ctrl NPCs.
b,c, Representative ATAC-seq tracks for G3 MB signature genes (b) and genome stability regulators (c) are shown.

Extended Data Fig. 9. Ctdnep1 depletion increases DNA damage responses and induces chromosome instability.

a, Immunofluorescence of γH2A.X and DAPI in control shCtrl- and shCTD-treated DAOY cells. Right; bar graph shows the quantification of γH2A.X-positive cells. Data represent means ± SD (n=3 independent measurements). *p < 0.05; **p < 0.01, two-tailed unpaired Student’s t-test.
b, Karyotypes of control and Ctdnep1-cKO NPCs at late-stage scored from 20 spreads each. Red arrow points to the triplicated chromosome 15.
c,d, mRNA of Trp53 (c) and Myc (d) expression in cKO-Ctdnep1 NPCs (cKO-1 and cKO-2) and control NPCs (Ctrl-1 and Ctrl-2) at early-stage (Div 12), interim-stage (Div 24) and late-stage (Div 60). ns, no significant difference, ***p < 0.001, two-tailed Student’s t-test.

Extended Data Fig. 10. Identification of phospho-proteins upregulated in Ctdnep1-cKO NPCs.

a, List of the cell-cycle related phosphorylated proteins that are enriched in Ctdnep1-cKO NPCs compared to wild-type NPCs.
b, Motif analysis of phosphorylated proteins based on the phosphorylation site sequences enriched in Ctdnep1 cKO NPCs compared with Ctrl NPCs.
c, Mass spectrometry identification of phospho-proteins that are upregulated in Ctdnep1-cKO NPCs.
Extended Data Fig. 11. MB cell proliferation is inhibited by small-molecule inhibitors of c-MYC and CHEK1, but not TGF-β signaling.

a, Cell viability of control and Ctdnep1-cKO NPCs treated with the indicated concentrations of THZ1 relative to vehicle-treated cells. Data represent means ± SD, n=5 independent experiments. **p < 0.01, two-tailed unpaired Student’s t-test.

b,c Heatmap showing the percentage of growth inhibition of control and shCTD-treated D283 cells by combined treatment with JQ1 and prexasertib relative to vehicle-treated cells (b); Bliss score for JQ1 and prexasertib double titrations (c). n=5 independent experiments.

d,e Heatmap showing the percentage of growth inhibition of D425 cells with combined treatment of JQ1 and prexasertib relative to vehicle-treated cells. n=5 independent experiments (d), Bliss score for treatment with JQ1 and prexasertib double titrations (e).

f,g Heatmap showing the percentage of the growth inhibition of MYC amplified human G3 MB004 and mouse G3 (MYC+DNp53) tumor cells (f), and non-MYC amplified cells (G3 MB D283 and SHH-MB DAOY cells (g) treated with combined treatment of JQ1 and prexasertib relative to vehicle-treated cells. n=5 independent experiments.

h,i Cell viability of control and Ctdnep1-cKO NPC tumor cells treated with TGFβ pathway inhibitors LY-364947 (h) or galunisertib (i) relative to vehicle-treated controls. n=5 independent experiments. n.s; no significance, two-tailed unpaired Student’s t-test.

Extended Data Fig. 12. Alterations of CTDNEPI in brain tumor patient samples.

a, Frequency of alterations in CTDNEPI in pan-tumor types from the TCGA database (www.cbioportal.org).

b, Genome browser tracks of ATAC-seq signals at the locus of Trp53 in control and Ctdnep1-cKO NPCs. Highlight: a reduction of ATAC-seq signals on the promoter/enhancer element of Trp53.

c, Representative GSEA plots of p53 target genes between CTDNEPI mutated G3 MB (G3 CTD mut) and wildtype G3-MB (G3 CTD wt) MBs using the published datasets 6 and CBTTC cohort (https://cbttc.org/). Combined p-value was calculated using Stouffer's method.
METHODS

Animals

Mice carrying Ctdnep1 floxed alleles (Acc. No. CDB0564K) were generated as described. Ctdnep1<sup>Ff</sup> mice were crossed with mouse lines carrying Cre recombinase driven by the nestin promoter (Nestin-Cre<sup>+/−</sup>) to generate Ctdnep1-cKO (Ctdnep1<sup>Ff</sup>; Nestin-Cre<sup>+/−</sup>) and controls (Ctdnep1<sup>Ff</sup>; Nestin-Cre<sup>−/−</sup> or Ctdnep1<sup>Ff</sup>). Immunodeficient NOD scid gamma (NSG) mice were provided by Cincinnati Children’s Hospital Medical Center (CCHMC) animal core. In this study, mice of either sex were analyzed, and littermates were used as controls unless otherwise indicated. Mouse strains were generated and maintained on a mixed C57BL/6; CD-1 background and fed (4 or less mice per cage) in a vivarium with a 12 hrs light/ 12 hrs dark cycle. All studies complied with all relevant animal use guidelines and ethical regulations. The animal studies were approved by the IACUC (Institutional Animal Care and Use Committees) of the Cincinnati Children’s Hospital Medical Center, USA.

MB tumor tissues, CTNDEP1 mutation and survival analysis

All medulloblastoma tumor samples from the Asian cohort were collected after receiving informed consent according to an institutional review board approved protocol at the Children’s Hospital of Fudan University. The tumor tissue collection was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Children’s Hospital at Fudan University. The subgroup analysis (45 tumor tissues) was based on DNA methylation profiling using the Infinium CytoSNP-850K v1.2 BeadChip array (EPIC 850K array). The others (44 tumor tissues) were subgrouped based on a combination of histopathological, RNA-seq transcriptomics analysis, and immunostaining with subgroup-specific markers.

To identify independent CTNDEP1 mutations in publicly available datasets from reported cohorts, CBTTC cohort (https://cbttc.org/), and the Asian cohort, we performed quality control (QC) based on the unique patient IDs and clinical information including gender, age, tumor subgroup, and histological subtype, as well as copy number variation (CNV) features, to ensure the removal of sample duplicates among different cohorts and identify individual patients with CTNDEP1 mutations (Supplementary table 1).

For the survival analysis based on CTDNEP1 expression, in view of the unequal expression CTDNEP1 in each subgroup, we plotted the density curves of MB cohorts (total 612 MBs) from publicly available datasets for CTDNEP1 expression and survival analysis and found that the patient density across different MB subgroups is enriched between CTDNEP1 expression levels 7.67 and 7.98 (log<sub>2</sub> intensity) (Extended Data Fig. 3d). We therefore set up the threshold that 35% highest expression samples were stratified into the high group (log<sub>2</sub> intensity >7.98) and the 35% lowest expression samples as the low group (log<sub>2</sub> intensity <7.67) for all MB samples (total 612 MBs) from publicly available datasets for CTDNEP1 expression and survival analysis. Namely the highest 35% and lowest 35% patient populations are established as the cutoff to compare patient survival within two subgroups for all patients or each subgroup. Overall survival curves were plotted with the Kaplan-Meier method and compared by using a two-sided log-rank test.

DNA methylation array processing and CNV analysis
MB sample DNA methylation profiling was performed using the Infinium CytoSNP-850K v1.2 BeadChip array (EPIC 850K array) according to the manufacturer’s instructions (Illumina). The subtypes of human MB samples were defined based on published annotations\(^9,11\). All DNA methylation analyses were processed in R v.3.3.0 (R Development Core Team, 2016) and the detail information was described previously\(^6\). MB clusters were defined as WNT, SHH, G3 and G4 based on classification using a previously described 48 CpG signatures\(^9,11\). CNV analysis of the MB from 850K methylation array data was performed using the comee Bioconductor package v.1.4.0. A set of 10 control samples methylation array data displaying a balanced copy-number profile was used for normalization.

**Primary NPC Isolation and subculture**

Primary cerebellar NPCs were isolated according to the previous report\(^98\) with minor modification from Ctdnepl-cKO (Ctdnepl\(^{fl/fl}\);Nestin-Cre\(^{+/-}\)) and control mice, which carry a Rosa26:ccGFP reporter. Briefly, isolated cells were cultured in complete NPC medium (Neurobasal medium containing B27 supplement, N2 supplement, 2 mM L-glutamine, 20 ng/ml EGF, 20 ng/ml bFGF, 2 mg/ml Heparin and 50 µg/ml BSA). NPC spheres were dissociated at a diameter of 100-200 µm and were changed with complete NPC medium every 3 days to maintain cell metabolic activity. In addition, freshly isolated NPCs from Ctdnepl-cKO animals were also transduced with retroviruses expressing dominant-negative p53 and transplanted into the cerebellum of NSG mice.

**Medulloblastoma Cell Culture**

Medulloblastoma cell lines D425, D283, D458, and DAOY were obtained from American Tissue Culture Collection (ATCC, Rockville, MD, USA). MB004 and murine G3 MB lines were provided by Dr. Martine Roussel. Cells were cultured in DMEM/F12 media with 10% FBS, 2 mM L-glutamine and 1% Penicillin/Streptomycin. DAOY and HEK293T were maintained in DMEM with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/l streptomycin at 37°C in an atmosphere of 5% CO₂.

**Vectors, siRNA and Lentivirus Production**

Wild type CTDNEP1 and mutant D67N, D69N and L72H were cloned with Myc-tag into a pLVX-puro vector (Addgene). c-Myc and mutant S62E, D62A were amplified and then cloned into p3xflagcmv. shRNAs against CTDNEP1 were designed at https://rnaidesigner.thermofisher.com/rnaiexpress/ and then cloned into pGreen-puro vector. CTDNEP1 KO sgRNA were designed by https://crispr.mit.edu/ and then cloned into lenti-gRNA-puro vector. siRNA targeting CTDNEP1 or control siRNA was ordered from Sigma-Aldrich (www.sigmaaldrich.com). siRNA interfering CTDNEP1 were performed using Lipofectamine RNAiMAX Transfection Reagent (Qiagen) according to the manufacture instructions.

To produce lentiviruses, HEK293T cells were co-transfected with shRNA or GFP vector packaging using Lipofectamine 3000 reagent (Life Sciences). Supernatants were collected and filtered at 48 and 72 hr following transfection. Viral supernatant was concentrated by centrifugation at 25,000 rpm for 2 hr at 4°C and used to infect cells (MOI=5-10) overnight in the presence of 10 µg/mL polybrene. Cells were selected and maintained with puromycin (2 µg/ml). Gene expression was verified by western blot or real time PCR.

**Cell proliferation, Colony Formation and Neurosphere Formation Assays**
Cell proliferation was measured by CCK-8/WST-1 or EdU assays. For EdU assays, we used the Click-iT™ Plus EdU Cell Proliferation Kit for Imaging (Invitrogen). After EdU incubation, cells were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100, and EdU detection was performed according to the manufacturer’s instructions. Nuclei were counterstained with Hoechst 33342 reagent. At least 500 nuclei were counted in triplicate, and the number of BrdU-positive nuclei was recorded. For colony-formation assays, Ctrl and shRNA infected cells were seeded in 6-multiwell plates. After 2 weeks, cells were fixed with 4% PFA and stained with crystal violet. For neurospheres, 1,000 cells/ml were seeded in low attachment 96-multiwell plates in DMEM with neurosphere medium as previously described. The number of neurospheres was counted and captured images after 10–15 days.

Tissue Processing, Antibodies, Immunostaining and Immunoblotting

Mouse brains were dissected and fixed overnight in 4% (w/v) paraformaldehyde and processed for cryosectioning or paraffin embedding and sectioning described previously. Briefly, for the immunostaining, cryosections or pre-deparaffinized tissue sections were firstly blocked 1 hour by block solution [PBS with 5% v/v normal goat serum (Sigma-Aldrich) and 0.3% v/v Triton X-100] and incubated overnight in primary antibodies diluted in antibody dilution solution [PBS with 5% v/v normal goat serum (Sigma-Aldrich)]. After washing with PBS 5 times, sections were then either incubated overnight with the biotinylated goat anti-mouse IgG antibody (Vector Laboratories, BA-9200), followed by using the ABC avidin/biotin method to visualize staining signals under light microscopy with the peroxidase/diaminobenzidine (DAB) method, or incubated with corresponding fluorophore-conjugated secondary antibodies (Jackson ImmunoResearch) under fluorescent microscopy.

For cell immunostaining, spheres were fixed them with 4% PFA for 10 min and washed five times with PBS and dehydrated with 30% sucrose overnight, then blocked with OCT frozen embedding media (CRYO-4; Polarstat Inc.) and cryosectioned at 5 μM thickness. For adherent cells, cells were planted on the coverslips and fixed with 4% PFA for 10 min and washed five times with PBS. Then placed the sections or coverslips with cells in blocking solution for 30 min. We incubated primary antibodies in blocking solution with proper dilutions and stained cells for 1 h at room temperature. For BrdU staining, cells or tissue sections were denatured with 0.1N HCl for 1 h in 37 °C water bath. After denaturation, sections were neutralized with 0.1 M Borax, pH 8.5 (Sigma) for 10 min. Sections were washed with 1×PBS 3 times and blocked with 5% normal donkey serum (Sigma-Aldrich) in wash buffer for 1 h at room temperature. Mouse-anti BrdU (BD Bioscience, 1:500) antibody was used to label BrdU overnight at 4 °C. DAPI counterstain was included in the final washes before the samples were mounted in Fluoromount G (SouthernBiotech) for microscopy. Tissue or cell images were quantified in a blinded manner. All immunofluorescence-labeled images were captured using a Nikon C2+ confocal microscope.

Primary antibodies used in this study were as follows: Nestin (Abcam, ab22035), Ki67 (Thermo Sci, clone SP6), BrdU (BD Bioscience 347580; Abcam, ab6326), ZIC1 (Rockland, 200-401-159), Cleaved Caspase 3 (Cell Signaling, 9661), c-Myc (Cell Signaling; Cat#5605S), γH2A.X (Cell Signaling, Cat# 9718S), p53 (Cell Signaling, Cat# 2524S), p-S15 p53 (Cell Signaling; Cat#9284), GAPDH (Thermo Sci, Catalog # 39-8600), and p-S62 c-Myc (Abcam, ab51156).

For Western blot, cells were lysed with RIPA lysis buffer (Millipore) supplemented with phosphatase and protease inhibitor cocktail (Roche). Protein concentration of each sample was
determined by BCA assay using the BCA kit (Beyotime) according to manufacturer’s instructions and equal amounts were loaded and separated by 12% SDS-PAGE gel. PVDF membrane (Millipore) was used for gel transfer and the membrane was probed with primary antibodies as indicated, followed by secondary antibodies conjugated with HRP. The signal was detected with Super Signal West Pico/Femto Chemiluminescent Substrate (Thermo Scientific).

**Anaphase, Mitotic exit, Karyotype Analysis and FISH Experiment**

For anaphase analysis of the cultured NPCs, all cells were planted in the coverslips precoated by poly-L-lysine (Sigma-Aldrich, St. Louis, MO, P5899) at 100 μg/ml 30 mins and coated with laminin (Sigma-Aldrich, St. Louis, MO, L4544) at 50 μg/ml for 30 mins. For capturing the anaphase, NPCs were synchronized at the G2-M boundary by nocodazole (Sigma-Aldrich, St. Louis, MO, M1404) at 100 ng/ml for 4 h and released to a fresh medium and continued to culture 10-60 mins. Coverslips were fixed with 4% PFA for 15 mins at 5 min interval and stained with DAPI for DNA contents.

For mitotic exit assays, prometaphase-arrested cells were obtained by performing a double thymidine (2 mM; Sigma-Aldrich, St. Louis, MO) block (18 hr each, separated by a 6 hr incubation in fresh medium) followed by release into fresh medium containing nocodazole (100 μg/ml; Sigma-Aldrich, St. Louis, MO) and incubation for 12 or 14 hrs. Release from prometaphase arrest was obtained by washing detached cells twice with PBS and twice with fresh medium, followed by incubation in fresh medium. Cells in G1 were obtained after 120 min incubation from prometaphase release. The probes for mouse Myc and chromosome 15 (RP23-275E10) were purchased from Empire Genomics for Fluorescence In Situ Hybridization (FISH). FISH experiments and G-band karyotype analysis of mouse NPCs and tumors were analyzed by Cincinnati Children’s Hospital histology core (https://www.cincinnatichildrens.org/service/d/diagnostic-labs/cytogenetics).

**Western Blotting**

Tumor tissues were lysed in modified RIPA buffer (50 mM Na-Tris, pH 7.4, 150 mM NaCl, 1% (v/v) NP-40, 0.25% sodium deoxycholate, 1 mM dithiothreitol, 10 mM NaF, 1 mM active sodium vanadate, 1 mM PMSF and 1× a cocktail of cOmplete protease inhibitors (Roche Applied Science) and centrifuged at 13,000 r.p.m. for 15 min at 4 °C. After the determination of protein concentration (Bio-Rad), the lysates were separated by 4–12% SDS-PAGE. Bands were visualized with secondary antibodies conjugated to horseradish peroxidase (Bio-Rad) and ECL western blotting detection reagents (Pierce) per the manufacturer’s instructions.

**Proteomic Profiling**

CTDNEP1 binding protein was analyzed by immunoprecipitation for CTDNEP1-myc in CTDNEP1 enforced expression HEK293T cells and followed by mass spectrometry at the Fudan University core facility (http://ibs.fudan.edu.cn/about.php). Phospho-proteome were processed according to protocols adapted from previous studies and performed using label-free quantitative proteomics technology (Clinproteomics Co., Ltd).

**Transplantation of Ctdnep1-null NPC Tumor and Drug Treatment in vivo**
MB cells were subcutaneously injected into eight-week-old female athymic BALB/c nude mice or NSG mice. Tumors will be harvested after 6 to 10 weeks and quantified. $1 \times 10^5$ NPCs in a 5 μl with 2 μl matrigel were stereotactically injected into the NSG mouse cerebellum. Animals were monitored weekly and euthanized when they showed signs of brain tumor. The mouse brain tissue with tumor was embedded in paraffin and sectioned at a thickness of 5 μm for H/E and immunohistochemistry assays. The NSG mice with Ctdnep1-cKO NPCs tumor in the cerebellum, were randomized into groups and administered JQ1 (50mg/kg) or vehicle (10% DMSO in 10% HP-β-Cyclodextrin, Sigma) on alternating days via intraperitoneal injection for 14 days.

Whole Genome Sequencing (WGS) and Single Nucleotide Variant Calling and Copy Number Variations (CNV) analysis

WGS-derived raw image files were processed by DNBseq basecalling Software for base-calling with default parameters and the sequence data of each individual is generated as paired-end reads as FASTQ format. Single nucleotide variant analyses conducted using the Genome Analysis Tool Kit (GATK)(https://gatk.broadinstitute.org/hc/en-us). Briefly, the fastq data files from mouse WGS were mapped to mouse genome (mm10) by Burrows-Wheeler Aligner (BWA) in the GATK4 module. HaplotypeCaller was used to call the single nucleotide variants (SNV) and insertion deletions (Indel). All variants were removed the SNPs and then annotated by ANNOVAR. CNVs were called using SOAPcnv software. Based on the result of SOAP alignment, the depth of each base should be calculated and standardized by the mean depth of its chromosome to calculate the copy number variation. CNVs were detected by the following steps: 1) DNA sequences were separated into fragments according to the depth of each base from the alignment results; 2) The P-value was calculated for each fragment to estimate its probability to be a CNV ; 3) The fragments that passed the criteria (fragment length longer than 2 kb, P-value <= 0.35, mean depth less than 0.5 or more than 2.0) were kept as CNVs. The mapped bam files from WGS were used for CNV analysis. We followed the somatic copy number variation pipeline from GATK4 CNV (https://github.com/ding-lab/gatk4wxscnv). The final segment ratio files with CNV type annotation for all NPC and tumor samples were further annotated by AnnotSV.

Phosphatase and Cell cycle Assays

The phosphatase activity of CTDNEP1 was analyzed using p-NPP (para-nitrophenyl phosphate, Sigma) as described. Briefly, 5 μg CTDNEP1 and its mutations plasmids were introduced into $1 \times 10^6$ 293T cells and the CTDNEP1 proteins were purified by immunoprecipitation using anti-Myc-tag-beads (Sigma and Cat# A7470). The reaction mixture (20 μl) contained 50 mM Tris-acetate (pH 5.5), 10 mM MgCl₂, 0.5-50 mM p-NPP, and CTDNEP1 proteins incubated at 37°C for 20 min. 80 μl of 0.25 N NaOH was added to stop the reaction. Release of p-NP (para-nitrophenol) was determined by measuring the absorbance at 410 nm.

For the dephosphorylation of c-Myc, we added the CTDNEP1 proteins purified by myc-affinity beads to the 50μl D425 cell lysate which contained Tris-acetate and MgCl₂ incubated 1 hour and the phosphorylation of c-Myc was determined by immunoblotting.

For Cell cycle analysis, the CycleTEST™ PLUS DNA Reagent Kit (BD 340242) used to cell stain according to the manufacturer’s instructions. Flow cytometry was conducted using BD Canto machines. Raw data was analyzed using FlowJo software.
RNA-seq and Differential Gene Expression Analysis

Total RNA was extracted from fresh cells or frozen tissue using TRIzol (ThermoFisher) and purified by RNeasy kit (www.qiagen.com). Quality of total RNA for each sample was checked on an Agilent Bioanalyzer 2100 RNA Nano chip (Agilent). RNA samples with RNA Integrity Numbers of at least 7 were sent to Novogene (https://en.novogene.com/) or BGI (www.bgi.com) for library preparation (polyA enrichment) and RNA sequencing (150 base pair Paired End reads).

To examine transcriptomic differences, cDNA reads were aligned to hg19 for human cells or mm10 for mouse cells using TopHat2 alignment to generate bam files\(^1\). Unnormalized gene read counts were generated using Cufflinks (http://cole-trapnell-lab.github.io/cufflinks/). Differentially expressed genes were normalized and analyzed using the Cuffdiff.

Assay for Transposase-accessible Chromatin Using Sequencing (ATAC-Seq)

ATAC-seq assays were performed as previously described\(^1\). Briefly, we isolated nuclei of ~50,000 cells in a cold lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.1% IGEPAL CA-630). After spinning down at 500g for 10 min at 4°C, nuclei were resuspended in transposition mix containing TD (2 x reaction buffer), TDE1 (Nextera Tn5 Transposase) at 37°C for 30 min. Immediately following transposition, DNA were purified using a Qiagen MinElute PCR Purification Kit. Transposed DNA fragments were subsequently amplified and the amplified library was purified using Qiagen MinElute PCR Purification Kit. Libraries were generated using the Ad1_noMX and barcoded primers and were amplified for 11 total cycles. Libraries were purified with AMPure beads (Agencourt) to remove contaminating primer dimers. All libraries were sequenced on the Illumina HiSeq 2500 with 75 bp single-end reads.

Reads of ATAC-seq data were aligned to rn5 genome using Bowtie with the following options:--best–chunkmbs 200 (http://bowtie-bio.sourceforge.net). Peak calling was performed using Model-based analysis of MACS version 2.12 (https://github.com/taoliu/MACS) with specific parameters without the prebuilt model:--shift 75–extsize 150–nomodel–call-summits–nolambda–keep-dup all -p 0.01, to call peaks, which extend and shift the fragments to get the region cut by the Tn5 sites. We calculated the peak_RPKM, then GSEA (v2.2.0) was used to analyze the enrichment of signature gene sets.

Statistical and Survival Analysis

All analyses in this research were performed using Microsoft Excel, GraphPad Prism 6.00 (San Diego California, https://www.graphpad.com) or RStudio (https://www.rstudio.com/ and R v.3.3.0, R Development Core Team, 2016). We use the “cor” function in R to calculate the Pearson correlation coefficient. Association between CNV and somatic mutational events were performed using Fisher Exact Test (R), FDR was used to adjust multiple tests. The Fisher’s exact test was used to determine the significance of gene mutations that are specifically enriched in G3-MB compared with other MB subgroups. Statistical significance was determined using two-tailed Student’s t tests as indicated. One-way ANOVA test was performed by multiple comparisons following Turkey’s ranking tests when comparing multiple groups. Data are shown as mean ± SD (error bars). Values of p < 0.05 denoted a statistically significant difference. Quantifications were performed from at least three experimental groups in a blinded fashion. The n value was defined as the number of experiments that were repeated independently with similar results. No statistical methods were used to predetermine sample sizes, but
our sample sizes are similar to those generally employed in the field. No randomization was used to collect all the data, but data were quantified with blinding.

**Data availability**
All high-throughput data generated in the paper are deposited in the NCBI Gene Expression Omnibus (GEO). The accession number is GSE145921 (code token: ebyxogkarbgrjsd). The mass spectrometry proteomics data are available via ProteomeXchange with identifier PXD019067 (login name: reviewer13637@ebi.ac.uk; Password: A9hHhz6).

**Conflict of interest**
None of the authors have competing financial or non-financial interests.
References:


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Fig. 2

(a) D425

(b) EdU

(c) Cell Number

(d) # clones per well (1×10⁴)

(e) D425

(f) D283

(g) Cell Proportion (%)

(h) D425

(i) D283

(j) Cell Number (1×10⁶)

(k) Weight of Tumor (g)
Fig. 7

(a) Graph showing inhibition rate (%) of Ctrl (IC50: 1.5 μM) and cKO (IC50: 0.3 μM) against JQ1 (log μM).

(b) Western blot images for JQ1, c-Myc, c-Caspase3, and GAPDH controls.

(c) Graph showing inhibition rate (%) of Ctrl (IC50: 6.9 nM) and cKO (IC50: 1.9 nM) against Prexasertib (log nM).

(d) Heatmap displaying inhibition of Prexasertib (nM) against JQ1 (μM) for Ctrl and cKO.

(e) BLISS Score heatmap indicating strong synergy.

(f) Graph showing percent survival of Cld-cKO NPC allografts after treatment with Vehicle (n=6), JQ1 (n=6), and JQ1+Prex (n=7).

(g) Images of C-Casp3 (CC3) staining under Vehicle, JQ1, and JQ1+Prex treatments.

(h) Bar graph showing percentage of CC3+ cells for Veh, JQ1, and JQ1+Prex.

(i) Graph showing cell number (1x10^5) over days for G3 D425 xenografts treated with DMSO, JQ1, Prex, JQ1, and JQ1+Prex.

(j) Graph showing percent survival of G3 D425 xenografts over days for Vehicle, Prex, JQ1, JQ1, and JQ1+Prex.
Extended Data Fig. 6

(a) Kaplan-Meier survival curves showing percent survival over time. The red line represents the cKO group (n=10), and the black line represents the Ctrl group (n=10). The p-value is less than 0.001.

(b) Histological images of P18 cortex and cerebellum stained with Hematoxylin and Eosin (H&E). The images show tissue morphology in both groups.

(c) Bar graph comparing relative volume of the cortex and cerebellum between Ctrl and cKO groups. Significant differences are indicated by * (p < 0.05) and ** (p < 0.01).

(d) Immunofluorescence images of E13.5 embryos stained for c-Caspase3, γH2A.X, and DAPI. The images show the localization of these markers in Ctrl and cKO groups, with quantification of positive cells shown in the right panel. The cKO group shows increased expression compared to Ctrl, as indicated by * (p < 0.05) and ** (p < 0.01).
Extended Data Fig. 7

(a) 1st cKO tumor 2nd transplantation 3 weeks

(b) cKO 2nd tumors

- 1x1.0e4 (n=8)
- 1x1.0e5 (n=6)
- 5x1.0e5 (n=6)

% survival vs. Days

(c) WT/cKO NPC transplantation

(d) H&E  Ki67  c-Myc  Nestin

(e) Wildtype ccGFP or Cldnep1cKO ccGFP

- Isolated NPCs from cerebellum
- Transduced with DNP53 for 24 hr
- Cerebellar implantation in NSG mice
- Monitoring for tumor formation

(f) Neonatal 4 days

- WT NPCa + DNp53
- Cldnep1/cKO NPCa + DNp53

(g) MYC  pS62-MYC  Ki67
Prevalence and clinical impact of recurrent mutations of CTDNEP1 in G3 MBs. a. Frequency of known and recurrent genetic variants in pediatric G3 MBs. b. The significance enrichment plot of somatic recurrent mutated genes in G3-MB compared with other MB subgroups. P values were calculated based on Fisher's exact test and were then adjusted for multiple testing by Bonferroni correction methods. c. Frequency of CTDNEP1 LOF variants in different MB subgroups. d. Somatic CTDNEP1 mutation profile in patients with MBs. fs, Frameshift. e. Association between somatic CTDNEP1 LOF variants and somatic chromosomal alterations (n = 136 G3-MB). p values were calculated using Bayesian logistic regression analysis, likelihood ratio tests, and adjusted for multiple testing based on 5% false discovery rate (FDR) correction. f. Kaplan-Meier analysis of overall survival of patients with WNT, SHH, G3, and G4 MB based on the CTDNEP1 high and low expression across subgroups in publicly available MB cohorts. Log-rank
g. Kaplan-Meier analysis of overall survival of patients with CTDNEP1 (CTD) mutation and no MYC amplification (CTD w/o MYC), MYC amplification and no CTDNEP1 mutation (MYC w/o CTD), CTDNEP1 mutation and MYC amplification (CTD+MYC) and other G3 MB patients (other G3). *p < 0.05; **p < 0.01, ***p < 0.001, Log-rank test.

**Figure 2**

Inhibition of CTDNEP1 expression promotes MB cell proliferation. a. qRT-PCR quantification of CTDNEP1 in D425 cells transduced with lentiviral control (shCtrl) and shRNAs targeted against CTDNEP1 (shCTD). Data are means ± SD. **p < 0.01, two-tailed Student’s t test. b. Left, representative images of shCtrl and shCTD-transduced D425 cells stained for EdU (scale bar, 50 μm); right, quantification of EdU+ cells. Data are means ± SD, n=6 independent experiments. *p < 0.05; **p < 0.01, two-tailed unpaired Student’s t-test. c. Growth of shCtrl and shCTD-transduced D425 cells assayed by WST-1. Data are means ± SD of 6
independent measurements. *p < 0.05; **p < 0.01, two-tailed unpaired Student’s t-test. d. Upper, the number of clones per well in a soft agar assay of Ctrl and shCTD D283 cells after 10 days culture. Lower: Representative images of clones in soft agar plates. Data represent means ± SD, n=3 independent experiments. *p < 0.05; two-tailed unpaired Student’s t-test. e,f, Left: Representative images of neurospheres of control and shCTD- transduced D425 (e) and D283 cells (f) (scale bars, 100 μm). Right panles: the number of spheres. *p <0.05 (n=3 independent experiments), two-tailed unpaired Student’s t-test. g, Left and center: Representative flow cytometry of cell-cycle stages of D283 cells transfected with shCtrl or shCTD in D283 cells. Right: percentage of cells at different cell-cycle stages (n=3 independent experiments). *p <0.05; ns: no significance, two-tailed unpaired Student’s t-test. h,i, Upper: representative photographs of tumors from mice transplanted subcutaneously with 1x10^6 shCtrl and shCTD-treated D425 (h), or D283 (i) cells. Lower: Weights of tumors as means ± SD (n = 6 mice per group). *p < 0.05, **p < 0.01, two-tailed Student’s t test. j, Upper: Immunoblots of CTDNEP1 overexpression (OE) in D425 cells. Lower: Cell proliferation as monitored by WST-1 in control and CTD-OE D425 cells. Data are means ± SD. n = 3 independent experiments, **p < 0.01, two-tailed Student’s t-test. k, Upper: Photographs of tumors from mice transplanted subcutaneously with control or CTDNEP1-overexpressing D425 cells. Lower: Weights of tumors as means ± SD (n = 4 animals per group). **p < 0.01, two-tailed Student’s t-test.
Loss of CTDNEP1 increases MYC stability and activity. a, Heatmap of differentially expressed genes in D425 cells treated with shCtrl and shCTD (#1, 2 and 3) (n = 3 independent experiments). b, Volcano plot of transcriptome profiles between control D425 cells and shCTDNEP1 (CTDNEP1-KD) treated cells. Red and blue dots represent genes significantly upregulated and downregulated in cells depleted of CTDNEP1 (p < 0.05, false discovery rate [FDR] <0.1), respectively. c, Correlations of upregulated genes in shCTDNEP1 D425 cells with MYC expression in G3 MBs. d, Pathway perturbation analysis of D425 cells depleted of CTDNEP1 and control cells (n=3 independent experiments) showing the most differentially regulated transcriptional factors (identified using Enrichr). e, Left: qRT-PCR quantification of c-MYC mRNA in shCtrl and shCTD treated D425 cells. Data are means ± SD. n=3 independent experiments; n.s. not significant; *p < 0.05, two-tailed Student's t test. Right: Representative immunoblots for total MYC protein and p-S62-MYC in shCtrl and shCTD-treated D425 cells. f, Representative immunoblots from 3 independent experiments for total MYC protein and p-S62-MYC in shCtrl and shCTD-treated D425 cells treated or not
with cycloheximide (CHX, 10 μg/ml) for 1 or 3 h. g, Upper: Conserved residues in the conserved catalytic motif of DXDX(T/V) proteins. Lower: The phosphatase activity of CTDNEP1 wildtype and mutant (D67N, D69N, and L72H) proteins. Values are the mean ± SD. n=3 independent experiments; **p < 0.01, two-tailed Student's t test. h, Representative immunoblots from 3 independent experiments for flag-tagged c-MYC after treatment by CHX at indicated time points in 293T cells co-transfected with wildtype (wt) or flag-tagged c-Myc mutant (S62E), and wildtype (wt) or myc-tagged CTDNEP1 mutants (D69N or L72H) as indicated (left). Quantification of c-Myc-flag expression after 4 hrs CHX treatment as compared to untreated cells (right), **p < 0.01, two-tailed Student's t test. i, Representative immunoblots from 3 independent experiments for MYC protein and its Ser62 phosphorylation levels in D425 cell lysates after 1 hour incubation with wild-type and mutants of CTDNEP1-myc-tagged proteins. j, Immunoprecipitation assay (n=3 independent experiments) for CTDNEP1 with wild-type MYC in 293T cells transfected with vectors expressing CTDNEP1-myc-tag and MYC-flag-tag after 4-hour of MG132 treatment. k, Representative immunoblots from 3 independent experiments for MYC protein and p-S62-MYC in stable CTDNEP1 overexpressing D425 cells transduced with lenti-CTDNEP1-myc-tag. Viral vector infected D425 cells serve as the control.
Figure 4

Deletion of CTDNEP1 in cerebellar NPCs induces G3 MB-like tumor formation. a, Diagram depicting the generation of Ctdnep1-cKO mice. b, Representative images of Ctdnep1-cKO NPCs (cKO) and control (Ctrl) NPCs at different stage. Scale bars: 100 μm. c, Left: Representative images of EdU stained control and Ctdnep1-cKO NPCs. Scale bar: 100 μm. Right: Mean ± SD percentages of EdU+ cells (n=5 independent experiments) in Ctdnep1-cKO NPCs and WT NPC cultures at late-stage (Div 60). ***p < 0.001, two-tailed Student’s t test. d, Representative bioluminescence imaging of mice transplanted with WT and Ctdnep1-cKO NPCs that express luciferase at 65 days post-transplantation. e, Hematoxylin and eosin staining of cerebellum sections from mice transplanted with WT and Ctdnep1-cKO NPCs. Scale bars: 5 mm (up) and 100 μm (down). f, Survival curves for animals transplanted with Ctrl (n = 6 animals) and Ctdnep1-cKO (n=9 animals) NPCs. ***p< 0.001; log rank test. g, Representative immuno images and quantification of the positive cell of tumors and para-tumors from cerebella transplanted with Ctdnep1-cKO NPCs. Scale
bars: 50 μm. Data are means ± SD. n=3 allografts; *p < 0.05, two-tailed Student’s t test. NT, non-tumor tissue; T, tumor tissue. h, Heatmap of differentially-expressed genes in Ctdnep1-cKO tumors (n = 2) compared with Ctdnep1-cKO NPCs (n = 2) and Ctrl NPCs; n = 2 samples/group at the early (Div12) and late-stage (Div 60). i, Principal component analysis of transcriptomes of Ctdnep1-cKO NPCs (at late-stage, Div 60) and cKO tumors with mouse SHH (SmoM2 OE 45 and GFAP-cKO Ptc1h 94) and G3 MB (Myc_Gfi1 tumor 95, Myc/Trp53- Group3 MB 96, and Sox2+ Myc 24 models and normal cerebella 45,46. j, Correlation of transcriptomic profiles of Ctdnep1-cKO NPCs (late-stage, Div 60) and cKO tumors with human MB subgroups using MB signature genes. MYCAMP and MYCNA represent MYC amplification and non-amplification G3-MBs, respectively. k, Genome browser tracks of ATAC-seq signals at the locus of Myc in control NPCs, Ctdnep1-cKO NPCs, and cKO-tumors. Highlight: ATAC-seq signals on the promoter/enhancer of Myc.
Ctnep1 ablation induces genomic instability and Myc amplification. a, Heatmap of differentially expressed genes in Ctnep1-cKO NPCs (n=2) compared to wild-type NPCs (Ctrl, n=2) at early and late-stage. b, GSEA plots of data from Ctnep1-cKO NPCs and control NPCs at early-stage showing p53 pathway and MYC target genes. c,d, Representative immunoblots for MYC, p53, γH2A.X and cleaved-caspase 3 (CC3) in the early-stage NPCs (c) (from 3 independent experiments) and the cerebellum of Ctnep1-cKO mice (n=3 animals/group) at postnatal day 4 (d). e, GSEA plots of data from Ctnep1-cKO and control NPCs at late-stage showing p53 pathway and MYC target genes. f, qRT-PCR quantification of indicated transcripts in control and Ctnep1-cKO NPCs at late-stage. Plotted are means ± SD relative to levels in Ctrl cells (n = 3 independent experiments). *p < 0.05, **p < 0.01, two-tailed Student’s t test. g, Representative immunoblots from 3 independent experiments for MYC and p53 in Ctnep1-cKO and Ctrl NPCs at late-stage. h, Upper, relative Myc expression in Ctnep1-cKO NPCs transduced with non-targeting control shRNA and shMyc RNAs as indicated. Lower, cell viability of Ctnep1-cKO NPCs transduced with non-targeting shRNA and shMyc RNAs measured by WST-1 assay. Data are means ± SD. n=3 independent experiments; *p < 0.05, **p < 0.01, two-tailed Student’s t test. i, Anaphase analysis of early-stage (DIV 12) control and Ctnep1-cKO NPCs synchronized using nocodazole and released after 1 h with fresh medium. Upper: Representative maximum projection images of anaphase cells. Scale bar, 5 μm. Lower: Quantification of cells with lagging or bridged chromosomes. A total of 60 anaphase cells were counted. j, Upper: CNV analysis of Ctrl NPCs and Ctnep1-cKO NPCs at late-stage (DIV 60), and two independent Ctnep1-cKO tumor cells (cKO-T) based on 30x WGS analysis. Lower: Red and blue regions represent the focal amplified or deleted segments of the indicated chromosome, respectively. k, Representative FISH images showed the Myc gene (Red) and chromosome 15 (Green) in control NPCs at DIV 70 (16/20), Ctnep1-cKO NPCs (cKO-NPC) at DIV 70 (17/20) and Ctnep1-cKO tumor cells (14/20). White arrows, the Myc gene in the chromosome 15; yellow arrows, Myc amplification translocated out of chromosome 15.
Figure 6

CTDNEP1 post-translationally modulates the activities of key regulators for chromosome decatenation and mitotic checkpoints. a, CTDNEP1 binding proteins identified by mass spectrographic analysis in 293T cells which was overexpressing CTDNEP1. b, Mass spectroscopy analysis of phosphorylated proteins in Ctdnep1-cKO NPCs (cKO) at passage 10 compared with Ctrl NPCs. c, Pathway analysis of the most differentially up-regulated phospho-proteins in Ctdnep1-cKO NPCs compared with wild-type NPCs. d, Upper, representative phosphorylated proteins involved in cell-cycle progression that are enriched in Ctdnep1-cKO NPCs compared to Ctrl NPCs. Lower panel; the phosphorylation intensity of mitosis and chromosome segregation proteins in the NPCs detected by mass spectrometry. Data represent means ± SD, n=2 independent experiments. *p < 0.05; **p < 0.01, two-tailed unpaired Student’s t-test. e, Venn diagram of CTDNEP1 binding proteins and phospho-proteins enriched in Ctdnep1-cKO NPCs compared to wild-type NPCs. f, GO analysis of candidate CTDNEP1 regulated phospho-proteins in Ctdnep1-cKO NPCs. g, Representative immunoblots from 3 independent experiments for p-CHEK1 and p-TOP2A in Ctdnep1-cKO and wild-type NPCs at late-stage (Div 20). h, Representative immunoblots from 3 independent experiments for the indicated phospho-proteins in DAOY cells transfected with control siRNA or
siCTDNEP1 after treatment with nocodazole for 14 h and sampled at indicated timepoints in fresh medium. NOW; nocodazole washout.

Figure 7

Combined targeting MYC and CHEK1 activities inhibits CTDNEP1-deficient tumor progression. a, Cell viability of control and Ctdnep1-cKO NPCs treated with the indicated concentrations of JQ1 relative to vehicle-treated cells. Data represent means ± SD, n=5 independent experiments. *p < 0.05; **p < 0.01, two-
tailed unpaired Student’s t-test. b, Representative immunoblots from 3 independent experiments for MYC and cleaved Caspase 3 in control or Ctdnep1-cKO NPCs treated with JQ1 (0.5 μM: +; 1 μM: ++) or DMSO (-). c, Cell viability of Ctdnep1-cKO NPCs and wild-type NPCs treated with prexasertib (Prex) for 3 days at indicated concentrations relative to vehicle-treated cells. Data represent means ± SD; n=5 independent experiments. *p < 0.05; **p < 0.01, two-tailed unpaired Student’s t-test. d,e, Heatmap showing the percentage of growth inhibition of Ctdnep1-cKO tumors and wild-type NPCs by combined treatment with JQ1 and prexasertib relative to vehicle-treated cells (d); Bliss score for JQ1 and prexasertib double titrations (e). n=5 independent experiments. f, Kaplan-Meier survival for Ctdnep1-cKO tumor bearing allografts treated with vehicle, JQ1, or JQ1 and prexasertib once/day for two-weeks after transplantation at day 45. **p < 0.01 and ***p < 0.001; log rank test. g, Representative images of immunostaining for cleaved-Caspase 3 (CC3) in vehicle, JQ1, or JQ1 and prexasertib treated mice with Ctdnep1-cKO tumors 62 days after implantation. Red arrows CC3+ cells in the tumor section. h, quantification of CC3+ cells in the tumor tissues of the indicated treatment groups. Scale bars: 50 μm. Data are means ± SD. n=3 allografts; **p < 0.01 and ***p < 0.001; two-tailed Student’s t test. i, Viability of D425 cells treated with JQ1 (1μM), prexasertib (10nM), or the combination relative to vehicle-treated cells. Data represent means ± SD; n=5 independent experiments. *p < 0.05; **p < 0.01, two-tailed unpaired Student’s t-test. j, Kaplan-Meier survival for MYC-driven D425 orthotopic xenografts treated with vehicle, JQ1, prexasertib or JQ1 and prexasertib once/day for two-weeks after transplantation at day 10. *p < 0.05; **p < 0.01; log rank test.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplTable1.xlsx
- SuppTable2.xlsx