Alternative lengthening of telomeres in childhood neuroblastoma from genome to proteome

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Telomere maintenance by telomerase activation or alternative lengthening of telomeres (ALT) is a major determinant of poor outcome in neuroblastoma. Here, we screen for ALT in primary and relapsed neuroblastomas (n = 760) and characterize its features using multi-omics profiling. ALT-positive tumors are molecularly distinct from other neuroblastoma subtypes and enriched in a population-based clinical sequencing study cohort for relapsed cases. They display reduced ATRX/DAXX complex abundance, due to either ATRX mutations (55%) or low protein expression. The heterochromatic histone mark H3K9me3 recognized by ATRX is enriched at the telomeres of ALT-positive tumors. Notably, we find a high frequency of telomeric repeat loci with a neuroblastoma ALT-specific hotspot on chr1q42.2 and loss of the adjacent chromosomal segment forming a neo-telomere. ALT-positive neuroblastomas proliferate slowly, which is reflected by a protracted clinical course of disease. Nevertheless, children with an ALT-positive neuroblastoma have dismal outcome.

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Activation of a telomere maintenance mechanism (TMM) was found to be associated with poor outcome in neuroblastoma, which is the most common extracranial solid tumor diagnosed in early childhood. Telomerase activation is one way of maintaining telomere length and results from amplified MYCN (MYCN transcriptionally activates the TERT gene) or TERT rearrangement in neuroblastoma. Activation of alternative lengthening of telomeres (ALT) presents an alternative route of telomere maintenance, which is seen in a substantial proportion of neuroblastoma tumors. Mutations in the chromatin modifier alpha thalassemia/mental retardation syndrome X-linked (ATRX) are associated with elongated telomeres and ALT in neuroblastoma. Furthermore, deletions of ATRX were associated with recurrent partial chromosomal losses in neuroblastomas without amplified MYCN. ATRX and its complex partner death associated protein 6 (DAXX) incorporate gene) or alternative lengthening of telomeres (ALT) presents an alternative mechanism that contributes to telomerase-activated tumors. Clinical practice, ALT-positive high-risk tumors are treated with a multi-omics approach including high coverage whole genome sequencing (n = 165), RNA-sequencing (n = 144), whole proteome analysis (n = 34), and ChiP-sequencing (n = 27) forming the discovery cohort. ALT tumors had a significantly higher telomere content (Fig. 2a). Telomere content and C-Circle intensity was comparable between ALT-positive tumors of the discovery and INFORM cohort (Supplementary Figs. 1–2). Overall, telomere content was positively correlated with C-Circle intensity (r = 0.76, P < 2.2e-16, Spearman, Supplementary Fig. 1c). Minimal to no TERT mRNA expression was observed for ALT-positive neuroblastomas (Fig. 2b), resulting from epigenetic silencing of the TERT locus by H3K27me3 (Supplementary Fig. 3a). In accordance with low TERT mRNA expression, ALT-positive tumors exhibited low telomerase activity (Supplementary Fig. 3b). Moreover, ALT was associated with increased expression of the polyadenylated telomeric long noncoding RNA TERRA (Fig. 2c, Supplementary Fig. 3c). Patients with ALT-positive tumors had a similar poor event-free survival compared to patients with MYCN-amplified tumors. Although the overall survival time of patients with ALT-positive tumors was significantly longer, they had a dismal outcome in the long period (Fig. 2d). No difference in event-free or overall survival was observed between patients with ALT-positive tumors stratified into high-risk and those predicted as low/intermediate risk (Fig. 2e), indicating that the current risk stratification is underestimating the poor prognosis of ALT-positive tumors. ALT-positive neuroblastomas with a higher telomere content had a significantly shorter event-free survival as compared to ALT-positive tumors with relatively lower telomere content (Supplementary Fig. 3d). There was no clear trend in telomere content gain or loss between matching primary/relapse samples of the INFORM cohort (Supplementary Fig. 4). For 3 of 7 ALT-positive relapse tumors with a matching primary, ALT activity was only observed in the relapse tumor. Two of these cases were MYCN amplified in the primary disease period and heterogeneous ALT/MNA in the relapse. Heterogeneity could be confirmed by FISH analysis (MYCN and telomere probe) of one relapsed tumor (Supplementary Fig. 5a). For the other heterogeneous relapse tumor, we found evidence of an ALT-positive subclone in FISH analysis of the primary tumor (Supplementary Fig. 5b). Note- worthy, the discovery cohort also contains three heterogeneous ALT/MNA cases, which are MYCN amplified and have a very high telomere content, but were otherwise C-Circle negative (Fig. 2a, Supplementary Figs. 1a and 5c).
survival was not significantly different between ATRX-mutated and ATRX wild-type ALT-positive cases (Fig. 3c). Mutations in the ATRX complex members DAXX and H3F3A were extremely rare (Fig. 3a). Only one ATRX wild-type tumor exhibited a large inversion event affecting DAXX, associated with low DAXX mRNA expression (66.46 TPM; range discovery cohort 50-330 TPM). Another tumor had an A92S H3F3A mutation in addition to mutated ATRX. Somatic mutations in TP53 pathway genes (TP53, CREBBP, ATM, ATR, CDKN2A, and MDM2) were significantly enriched ($P = 0.01$) in ALT-positive tumors. ALT-positive tumors had the highest prevalence of CDK4 amplifications (3/4) (Fig. 3a), associated with increased CDK4 mRNA expression (Supplementary Fig. 7a), and showed higher CCND1 mRNA expression as compared to telomerase-activated cases (Supplementary Fig. 7b). Overall, many tumors had mutations in one or another gene associated with telomere maintenance according to the TelNet database. Synaptic nuclear envelope protein 1 (SYNE1) was exclusively mutated in 7 of 60 ALT tumors (11.7%) (Fig. 3a, Supplementary Figs. 6, 9a). Furthermore, deletions in receptor-type tyrosine-protein phosphatase delta (PTPRD) were enriched in ALT-positive tumors ($P = 2.46e-05$; Fig. 3a, Supplementary Fig. 6). Structural variations affecting PTPRD were reported in neuroblastoma cohorts and low PTPRD expression was correlated with a poor prognosis. However, structural variations of PTPRD were not correlated with reduced PTPRD mRNA expression in our discovery cohort (Supplementary Fig. 9b). Noteworthy, both genes are very large and thus the high mutation frequency in ALT neuroblastomas could also reflect the general increased mutational load found in older patients or a general increase in genomic instability (Supplementary Fig. 9c–e). The mutational landscape of relapsed neuroblastomas in the INFORM cohort was

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**Fig. 1** C-Circle screening of neuroblastomas. **a** ALT frequency detected using C-Circle presence as a marker in the screening cohort ($n = 720$, left) and INFORM cohort ($n = 40$, right). Top pies illustrate the composition of the cohorts concerning INSS tumor stage. **b** Schematic overview of the analyzed patient cohorts used for C-Circle screening and high-throughput analysis using whole genome sequencing (WGS), RNA-sequencing, whole proteome analysis, and ChIP-sequencing. Sample numbers are given in brackets. Number of C-Circle positive (CC+) and C-Circle negative (CC-) patients in the subcohorts are indicated.

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comparable to the discovery cohort (Supplementary Fig. 10a, Supplementary Data 2, 3). In one matched primary relapse pair, an ATRX duplication event was retained in the relapse, further indicating that duplications can lead to nonfunctional ATRX (Supplementary Fig. 4). Notably, canonical activating RAS pathway mutations (HRAS, NRAS, KRAS, BRAF, RAF1, CDK4, CCND1, NF1) were significantly more frequent in relapsed ALT-positive tumors compared to the relapse cohort (P = 0.0013, Supplementary Fig. 10, Fig. 3a), supporting a specific impact of RAS pathway mutations in relapsed ALT-positive tumors.

**Low ATRX protein abundance in ALT tumors.** Using a mass spectrometry based whole proteome approach (n = 34) quantifying on average 6891 proteins per tumor, we found 470 proteins significantly different in ALT-positive tumors compared to the other subgroups (nominal \( P \leq 0.01 \); fold change \( \geq 2 \); Fig. 4a, Supplementary Data 5). Enrichment analysis of significantly different mRNAs and proteins for each subgroup in turn highlighted both the overall agreement of transcriptomics and proteomics as well as the distinct effects only significantly present in one or the other omics-level (Fig. 4b, Supplementary Fig. 11a, Supplementary Data 6). The Ras family proteins HRAS and NRAS, comprised in the term “prenylation”, were significantly upregulated in ALT-positive neuroblastomas (Fig. 4b, Supplementary Data 6). Among the top upregulated proteins in ALT-positive tumors were the cancer testis antigens P antigen family member 5 (PAGE5) and melanoma associated antigen 4 (MAGEA4), which may serve as potential targets for immunotherapies (Fig. 4a).

### Table 1 Clinical features of C-Circle positive tumors in the screening cohort (n = 720)\(^a\).

|                | Total cohort | C-Circle positive | C-Circle negative | \( P \) value\(^f\) |
|----------------|--------------|-------------------|-------------------|-------------------|
|                | No.\(^b\) | %\(^c\) | No.\(^d\) | %\(^d\) | No.\(^f\) | %\(^f\) | No.\(^g\) | %\(^g\) | No.\(^h\) | %\(^h\) |
| Total          | 720         |                | 66              | 9.2%        | 654        | 90.8%       |                |                |                |
| Stage 1-3\(^b\) | 416         | 57.8%          | 25              | 37.9%       | 391        | 59.8%       |                |                |                |
| Stage 4\(^b\)  | 221         | 30.7%          | 41              | 62.1%       | 180        | 27.5%       |                |                |                |
| Stage 45\(^b\) | 83          | 11.5%          | 0               | 0.0%        | 83         | 12.7%       |                |                |                |
| LR/IR\(^c\)    | 449         | 65.9%          | 24              | 36.9%       | 425        | 69.0%       |                |                |                |
| HR\(^c\)       | 232         | 34.1%          | 41              | 63.1%       | 191        | 31.0%       |                |                |                |
| Male           | 397         | 55.1%          | 39              | 59.1%       | 358        | 54.7%       |                |                |                |
| Female         | 323         | 44.9%          | 27              | 40.9%       | 296        | 45.3%       |                |                |                |
| Age\(^d\) (mean in days) | 871 |                | 2872           |              | 669        |              |                |                | <2E-16 |
| MYCN amp\(^e\) | 114         | 16.2%          | 0               | 0.0%        | 114        | 17.9%       |                |                | 5.883E-06 |
| MYCN normal\(^e\) | 588       | 83.8%          | 66              | 100.0%      | 522        | 82.1%       |                |                |                |
| Ganglieneuroma  | 11          | 1.5%           | 0               | 0.0%        | 11         | 1.7%        |                |                | 0.6115 |

\(^{a}\) No. refers to the number of tumors.
\(^{b}\) Stage 1-3 refers to tumors with ATRX LOH.
\(^{c}\) Stage 4 refers to tumors without ATRX LOH.
\(^{d}\) LR/IR c refers to tumors with LR/IR c.
\(^{e}\) MYCN amp refers to tumors with MYCN amplification.
\(^{f}\) MYCN normal refers to tumors with MYCN normal.
\(^{g}\) Fold change \( \geq 2 \).

### Table 2 Clinical features of C-Circle positive tumors in the INFORM cohort (n = 40)\(^a\).

|                | Total cohort | C-Circle positive | C-Circle negative | \( P \) value\(^f\) |
|----------------|--------------|-------------------|-------------------|-------------------|
|                | No.\(^b\) | %\(^c\) | No.\(^d\) | %\(^d\) | No.\(^f\) | %\(^f\) | No.\(^g\) | %\(^g\) | No.\(^h\) | %\(^h\) |
| Total          | 40          |                | 19              | 47.5%        | 21         | 52.5%       |                |                |                |
| Stage 1-3\(^b\) | 4           | 10.0%          | 4               | 21.1%        | 0          | 0.0%        |                |                |                |
| Stage 4\(^b\)  | 34          | 85.0%          | 14              | 73.7%        | 20         | 95.2%       |                |                |                |
| Stage 45\(^b\) | 2           | 5.0%           | 1               | 5.3%         | 1          | 4.8%        |                |                | 1.000 |
| LR/IR\(^c\)    | 7           | 17.5%          | 5               | 26.3%        | 2          | 9.5%        |                |                | 0.226 |
| HR\(^c\)       | 33          | 82.5%          | 14              | 73.7%        | 19         | 90.5%       |                |                |                |
| Male           | 32          | 80.0%          | 13              | 68.4%        | 19         | 90.5%       |                |                |                |
| Female         | 8           | 20.0%          | 6               | 31.6%        | 2          | 9.5%        |                |                |                |
| Age\(^d\) (mean in days) | 1645 |                | 2243           |              | 1104       |              |                |                | 0.003 |
| MYCN amp\(^e\) | 11          | 27.5%          | 2               | 10.5%        | 9          | 42.9%       |                |                | 0.029 |
| MYCN normal\(^e\) | 28        | 70.0%          | 17              | 89.5%        | 10         | 47.6%       |                |                |                |

\(^{a}\) No. refers to the number of tumors.
\(^{b}\) Stage 1-3 refers to tumors with ATRX LOH.
\(^{c}\) Stage 4 refers to tumors without ATRX LOH.
\(^{d}\) Age at diagnosis.
\(^{e}\) MYCN amp refers to tumors with MYCN amplification.
\(^{f}\) MYCN normal refers to tumors with MYCN normal.
\(^{g}\) Fold change \( \geq 2 \).
neuroblastomas (Fig. 4a–b, Supplementary Fig. 11b). The term DNA replication includes among others the proteins PCNA, MCM2, MCM3, MCM4, MCM5, and MCM7, which are also indicative of the proliferative capacity of a cell. Furthermore, the term "bromodomain" was significantly reduced in ALT-positive tumors, while strongly upregulated in MNA tumors. Amplified MYCN is known to be associated with increased occupancy of active promoter regions and enhancer invasion by MYCN and bromodomain proteins, leading to an increased transcriptional activity of many proliferation associated genes and downregulation of differentiation genes. Moreover, ALT-positive tumors exhibit a significantly lower fraction of cycling cells compared to MYCN-amplified tumors (Supplementary Fig. 11c). Taken together, both protein expression data and cell
cycle analysis support a low proliferative capacity of ALT-positive neuroblastomas. 96 of the 470 significantly different proteins were annotated in the TelNet database to be associated with telomere maintenance\textsuperscript{22} (Supplementary Data 5) \((P = 0.03)\). The significantly downregulated proteins in ALT-positive neuroblastomas comprised ATRX, PCNA, EXO1, GNL3, KDM1A, RIF1, SCG2, TFAP2B, and GNL3L, all functionally linked to telomere maintenance\textsuperscript{22}. Helicases and chromatin regulators, including ATRX, were less abundant at protein level in the ALT subgroup, but not at mRNA level (Fig. 4b). Importantly, ATRX itself was among the top candidates to exhibit lower protein abundance in ALT-positive tumors (Fig. 4a). Intriguingly, the reduced ATRX protein levels were independent of ATRX mRNA levels and mutation status (Fig. 5a). ATRX was among the most strongly downregulated proteins while the ATRX mRNA changes very little (Supplementary Fig. 11d), indicating that reduced ATRX protein abundance is a characteristic proteomic feature of all ALT-positive tumors (ATRX wild-type and mutated) that cannot be observed at the mRNA level. Analysis of exon-specific ATRX mRNA levels revealed that only the exons affected by deletion have reduced expression in the ATRX-deleted cases (Supplementary Fig. 12a). In summary, reduced ATRX protein level is a biomarker for ALT-positive neuroblastomas that is independent of mRNA levels and ATRX mutation. The decreased ATRX protein levels despite unchanged mRNA abundance could be due to reduced translation and/or increased degradation of ATRX in ALT-positive tumors. For example, it has been shown that unassembled subunits of multiprotein complexes (so-called orphans) are often degraded\textsuperscript{30}. Downregulating one subunit can therefore induce degradation of other subunits, which partially explains divergence of protein- and mRNA-level changes in many biological contexts\textsuperscript{31,32}. We therefore took a closer look at the ATRX binding partner DAXX. Interestingly, DAXX protein levels were specifically reduced in ALT-positive ATRX wild-type tumors (Fig. 5b). To investigate if reduced DAXX levels could explain ATRX downregulation at the protein level, we knocked-down DAXX in the ALT-negative neuroblastoma cell line NBL-S and observed that ATRX protein levels were indeed reduced after 96 h (Fig. 5c–d). Hence, the reduced ATRX protein levels in ATRX wild-type ALT positive tumors might result from downregulation of the DAXX protein. However, since DAXX mRNA levels do not differ significantly between tumors (Fig. 5b) and no recurrent mutation patterns in DAXX or ATRX/DAXX interacting proteins could be identified in ALT-positive ATRX wild-type tumors (Supplementary Figs. 12b, 13), the question what causes the downregulation of DAXX is still open. Hence, the mechanistic details behind ATRX/DAXX complex reduction remain to be uncovered.

**Heterochromatic telomeric chromatin in ALT neuroblastomas.**

Many contradictory findings were reported on the telomeric chromatin landscape of ALT-positive cells\textsuperscript{33–35}, but data on primary tumor samples was yet missing. Here, ALT-positive neuroblastoma tumors were found to be enriched for H3K9me3 at the telomeres (Fig. 6a). Overall levels of H3K9me3 at the telomeres of ALT-positive tumors were comparable to the H3K9me3 levels at SatII and SatIII alpha-satellite sequences (Supplementary Fig. 14a–b), which are known to be enriched for H3K9me3\textsuperscript{35,36}. In line with a heterochromatic enrichment, protein expression of the demethylase KDM1A responsible for removing methyl groups from H3K9me3\textsuperscript{37} was significantly lower in ALT-positive neuroblastomas compared to TERT-activated tumors (Fig. 6a). SETDB1 protein activity is required for H3K9me3 deposition at the telomeres, while the histone methyltransferases SUV39H1 and SUV39H2 catalyze H3K9 trimethylation at heterochromatic sites\textsuperscript{38–40}. Neither expression of SETDB1 nor SUV39H1/SUV39H2 could be associated with the increased telomeric H3K9me3 in ALT-positive neuroblastomas (Fig. 6c–d). H3K27ac at the telomeres was lower in ALT-positive tumors (Fig. 6a). However, this difference was not telomere specific and similarly observed at SatII and SatIII sequences indicating a general difference in H3K27ac (Supplementary Fig. 14c). Furthermore, ALT-positive tumors displayed reduced telomeric H3K27me3 (Fig. 6a, Supplementary Fig. 14d) and low protein and mRNA expression of the PRC2 component EZH2, responsible for H3K27me3 deposition (Fig. 6e). Low H3K36me3 was observed at the telomeres and satellite sequences of both ALT-positive and ALT-negative neuroblastomas (Fig. 6a, Supplementary Fig. 14e). Together, this indicates epigenetic dysregulation of repressive marks at the telomeres in ALT-positive tumors with excess of H3K9me3 and reduction of H3K27me3.

**Increased frequency of telomeric repeat loci in ALT tumors.**

ALT-positive neuroblastomas exhibited an increased frequency of telomeric repeat loci compared to the other groups (Fig. 7a), with ALT-positive tumors displaying up to 19 such events per tumor. The telomeric repeat loci frequency was significantly higher in ATRX-mutated than ATRX wild-type ALT tumors. Telomeric repeat loci are characterized by a chromosomal junction site and the presence of telomeric repeat sequences either upstream or downstream of this junction site (one-sided) (Fig. 7b). In rare cases, two telomeric repeat loci in close proximity (10 kb) with opposite orientation can form a two-sided event (Supplementary Fig. 15a). In our discovery cohort, only five such two-sided events were observed (Supplementary Fig. 15b). Two-sided events can result either from two separate events in close proximity or from telomere sequences that are spanning the region between the two junction sites forming a true insertion. Evidence for a true insertion, meaning that mates of a read pair matched to both sides of the insertion could only be identified for two of these events (Supplementary Table 1). The presence of more than three telomeric repeat loci in a tumor was found to be associated with a significantly reduced overall survival in ALT-positive neuroblastomas (Fig. 7c). Hotspot regions for recurrent telomeric
repeat loci were found on chromosomes 1q42.2, 18q23, and 19q13.43 (Fig. 7d). The increased occurrence of telomeric repeat loci in ALT-positive neuroblastomas and their enrichment on 1q42.2 was confirmed in the INFORM dataset (Supplementary Fig. 15c). 80.3% (212/264) of all t-type (TTAGGG) containing telomeric repeat loci showed at least 1 bp microhomology to the canonical telomeric repeat sequence, which is more than one would expect by chance. This enhanced microhomology at the junction sites of telomeric repeat loci indicates that telomeric sequences were added via a microhomology-dependent mechanism (Supplementary Fig. 15d-f). In general, 55.3% of all telomeric repeat loci were associated with a chromosomal copy...
Fig. 3 Mutational landscape of ALT-positive neuroblastos. a Somatic alterations in the discovery cohort. Samples ordered based on relative telomere content. Exonic single nucleotide variations (SNVs), exonic small insertions and deletions (INDELs) and structural variations in exonic and intronic regions (SVs) in selected ALT-associated genes, genes associated with telomere biology according to the TelNet database22 [https://maleone2.bioquant.uni-heidelberg.de/fma/webd/TelNet] and TP53 pathway genes are illustrated. Telomere maintenance features, clinical features, chromosomal aberrations, and ALK/RAS pathway mutations (HRAS, NRAS, KRAS, BRAF, NF1 or ALK) are given in the top panel. b Exact genetic location of mutations affecting the ATRX gene. Bars represent structural variations (deletion, duplication) and small deletions, while SNVs are illustrated as lollipops and colored by the SNV type. c Event-free and overall survival rate of patients with an ATRX-mutated ALT-positive neuroblastoma (n = 33) compared to patients with an ATRX wild-type ALT-positive neuroblastoma (n = 27). P values were calculated using a log rank test. n describes the number of analyzed tumors.

Discussion

In summary, this in-depth analysis revealed that ALT-positive neuroblastos are biologically and clinically distinct from tumors with telomerase activation.

ALT in neuroblasto was associated with characteristic ALT features, including high telomere content, low TERT expression, and increased TERRA expression, which is in line with previous findings on ALT-positive tumors44,45. ATRX mutations were identified in 55% of the ALT-positive neuroblastos. In line with this observation, an ATRX mutation frequency of 60% was reported in a parallel study46. The most frequent type of ATRX mutations were large deletions. However, we could also identify focal intragenic ATRX duplication events. Focal duplication of ATRX was also observed in a matching primary/relapse pair indicating that these events are leading to loss of ATRX. Similar events resulted in loss of ATRX function in patients with the genetic disorder alpha thalassemia X-linked intellectual disability syndrome (ATRX-syndrome)17,48 further supporting a loss of ATRX function by partial duplications.

Integrating proteomic profiling revealed reduced ATRX/DAXX protein complex abundance as a recurrent event in ALT-positive neuroblasto, which could often not be explained by mutations in these genes. The observation that all five ALT-positive tumors with wild-type ATRX depicted reduced DAXX protein levels is intriguing. It is tempting to speculate that the ALT phenotype always results from loss of the ATRX/DAXX complex activity, which is caused either by ATRX mutations or by reduced ATRX/DAXX protein levels. Future studies will show if this is indeed the case in neuroblasto and/or possibly other ALT-positive cancers. Nevertheless, we could identify a subgroup of ALT-positive neuroblastos with wild-type ATRX, but low ATRX protein abundance. Reduced ATRX protein levels in ALT ATRX wild-type neuroblasto could be explained by the reduced DAXX protein levels, which we specifically observed in this subgroup of tumors. In this scenario, reduced ATRX protein levels impair assembly of the ATRX/DAXX complex, which then causes degradation of orphan ATRX protein molecules. Consistent with this idea, we observed that knocking down DAXX reduced ATRX protein levels in neuroblasto cells. However, the cause of reduced DAXX protein levels is not yet clear, especially since mRNA levels do not change significantly. Reduced DAXX protein levels in ALT-positive tumors may result from posts transcriptional events. It is known that DAXX is regulated via various posttranslational modifications including phosphorylation, SUMOylation, and ubiquitination49. Irrespective of the mechanistic details involved, our study highlights that proteomic data is closer to phenotypes than transcriptomic data, which is especially valuable in a clinical context32.

A recent report stated that ATRX mutation and amplified MYCN are mutually exclusive50. C-Circle screening of a cohort of 720 neuroblastos also found a mutual exclusivity of C-Circle presence and amplified MYCN. However, in the INFORM cohort we identified two cases with amplified MYCN and a positive C-Circle signal. Additionally, in our discovery cohort we classified three cases as ALT/MNA heterogeneous. These cases were negative in the C-Circle assay, but exhibited a very high telomere content. FISH analysis of some of these cases could also confirm that amplified MYCN and ultra-bright telomere spots are present in the same tumor (Supplementary Fig. 3). No ATRX mutations were detected in the MNA/ALT heterogeneous tumors (for NBI26 only lcWGS and WES data was available). Taken together, heterogeneous tumors with amplified MYCN and presence of ALT markers seem to occur, but are very rare. Further analysis and larger sets of these cases would be necessary to draw meaningful conclusions on the cladality of these events and the activity of the respective telomere maintenance mechanism.

ChIP-sequencing analysis of ALT-positive neuroblasto tumors showed that telomeres as well as SatII and SatIII sequences show lower H3K27ac compared to ALT-negative tumors. Furthermore, proteins comprised in the term “bromodomain”, including EP300 responsible for H3K27ac, were reduced in ALT tumors and enhanced in telomerase-activated tumors. Moreover, the telomeres of ALT-positive neuroblasto tumors were enriched for H3K9me3. These observations confirm the results of a previous study on cell lines35 that only the telomeres of ALT-positive tumors are enriched for H3K9me3. In addition, we observed low H3K27me3 at the telomeres of ALT cases, which goes in line with a significantly...
**Fig. 4 Proteomic analysis of ALT-positive neuroblastomas.**

**a** Label free quantification (LFQ) analysis comparing ALT tumors with MNA and TERT tumors as well as tumors without a genetic telomere maintenance mechanism (OTHER). P values were calculated using a two-sided Student’s t-test. Colors indicate P value and fold change categories as well as the association to telomere biology based on the TelNet database22 [https://malone2.bioquant.uni-heidelberg.de/fmi/webd/TelNet].

**b** Differences between neuroblastoma subtypes (ALT, TERT, MNA, OTHER). UniProt keywords ([https://www.uniprot.org/keywords/]; retrieved on 2016-11-02) with significant enrichments (BH adjusted P < 0.000005, at least 2.5-fold enriched, at least 3 protein/mRNAs per keyword) in at least one group with significantly more or less abundant mRNA or proteins comparing each group to all other samples in turn (BH adjusted P < 0.05, * marks significant enrichment). Positive and negative sqrt enrichment values for enrichments in more and less abundant mRNA or protein, respectively. Enrichment P values were calculated by hypergeometric test and multiple-testing adjusted by Benjamini-Hochberg method. Exact P values and individual proteins contributing to Uniprot keywords are given in Supplementary Data 6.
lower protein and mRNA expression of EZH2 in ALT neuroblastomas compared to telomerase-activated tumors.

ALT-positive neuroblastoma tumors exhibited a high rate of telomeric repeat loci. Since telomeric repeat loci were characterized by telomeric repeat sequences either upstream or downstream of a non-telomeric junction site, these events cannot have occurred from breakage of interstitial telomeric sequences (ITS). However, telomeric repeat loci frequently overlapped with breakpoints of copy number changes or structural variations. Because terminal chromosomal breaks are in need of telomeric repeats to protect the newly formed ends from degradation51–53, we propose that ALT-positive tumors are capable of adding telomeric repeat sequences to open ends of chromosomal breaks forming neo-telomeres (Fig. 8a).

The high degree of microhomology to the telomeric repeat sequence at the junction sites indicates that telomeric repeats are added via a microhomology-dependent process like microhomology-mediated end-joining or non-homologous end-joining54. Further, we propose that the presence of microhomology at an open chromosomal break determines if telomeric sequences can be added to this site. Chromosomal loss of certain fragments might present a selection advantage leading to a selection of cells harboring the neo-telomere. We also identified a subset of copy number neutral telomeric repeat loci with no associated structural variation. This might be due to the fact that these events are subclonal and were thus not detected by the CNV/SV calling algorithm. Moreover, the detection limit of the used copy number algorithm is 50 kb and thus smaller copy number changes cannot be detected. Two-sided events, defined as two telomeric repeat loci in a 10 kb window with opposite orientation of the telomeric repeats, were very rare. These events may represent insertions of telomeric repeat sequences similar to previously described events55. Only two of five two-sided events exhibited evidence of a true insertion by mates of a read pair mapping to both sides of the insertion. Alternatively, two-sided events may result from neo-telomere formation on both sides of a breakpoint (Fig. 8b). A recurrent hotspot for telomeric repeat loci was identified on chr1q42, which was associated with 1q42.2-1qter loss forming a neo-telomere on chr1q42.2. Telomeric repeat loci were also associated with ALT and overlapped with chromosomal breakpoints in a pan-cancer cohort, but no enrichment was seen for telomeric repeat loci on 1q42.256, indicating that neo-telomere formation on chromosome 1q42.2 is neuroblastoma-specific. 1q42.2-1qter deletions retained in relapsed ALT cases indicate a selective advantage.

![Fig. 5 ATRX/DAXX complex abundance. a ATRX protein and ATRX mRNA expression in ALT ATRX-mutated (dark pink) and wild-type (light pink) tumors compared to TERT (blue), MNA (yellow), and OTHER (gray). b Boxplots of DAXX protein and DAXX mRNA expression in ALT ATRX-mutated (dark pink) and wild-type (light pink) tumors compared to TERT (blue), MNA (yellow), and OTHER (gray). a, b Boxplots indicate the median value (middle line) and the 25th and 75th percentiles (box). The upper/lower whisker spans from the hinge to the largest/smallest value (values expanding a distance of 1.5 x inter quartile range are not considered). Dots represent individual tumors. P values in boxplots were calculated with two-sided Wilcoxon rank sum tests. n describes the number of analyzed tumors. c, d ATRX protein expression in NBL-S cells (with moderate MYCN expression) after 96-h treatment with siRNAs against DAXX (black). ATRX siRNAs as well as two negative control siRNAs were used as controls (gray). Exemplary western blot (c) and quantification of three biological replicates (d) is shown. P values calculated using a two-sided Student’s t-test. Horizontal line shows median value. Dots represent biological replicates. Uncut images of all biological replicates are shown in the source data file.](https://www.nature.com/articles/s41467-021-21247-8)
ALT activity was associated with unfavorable outcome and a protracted clinical course of disease, which further substantiates previous observations. Furthermore, ALT was enriched in a population-based clinical sequencing study cohort for relapsed neuroblastomas. Multiple factors may contribute to this enrichment in the INFORM cohort. The INFORM inclusion criteria might contribute to an underrepresentation of the most aggressive neuroblastomas, which are most likely MYCN-amplified or TERT-rearranged, since patients are required to have sufficient general condition and a life expectancy of at least three months to be included in the registry trial. Furthermore, we could show risk underestimation of a substantial proportion of ALT-positive tumors, which was initially estimated by risk-prediction models based on standard clinical criteria. Additionally, we could show risk underestimation for ALT tumors with MYCN rearrangements and TERT mutations, which were initially included in the analysis based on criteria of MYCN amplification and TERT rearrangement, respectively. Therefore, we recommend including ALT tumors in the risk-prediction model to reflect the true biology of this aggressive and heterogeneous tumor type.
diagnosed as low or intermediate risk, but nevertheless had poor outcome. The current standard treatment regimens targeting strongly proliferating tumors are probably not suited to treat slowly growing ALT tumors, which might lead to a selection and enrichment of ALT-positive cases or subclones in the relapse setting. Thus, ALT activation might present a therapy resistance mechanism. Analysis of matching primary/relapse pairs revealed a gain of ALT or outgrowth of an ALT-positive subclone in the relapse tumor. Taken together, a revised therapy concept based on a better molecular understanding of this subtype and considering ALT-specific vulnerabilities is urgently needed for these children.
Methods

Patient cohorts and tumor samples. We retrospectively analyzed cohorts of primary and relapsed neuroblastoma tumors. ALT-activity was determined using C-Circle assays for 720 neuroblastoma tumors (screening cohort, Supplementary Data 1). High coverage whole genome sequencing (WGS) data was obtained for a subset of 165 tumors (discovery cohort). RNA-sequencing ($n = 144$), whole proteome analysis ($n = 34$), and ChIP-sequencing ($n = 27$) was done for subsets of the discovery cohort. A summary of the clinical information and available data for every patient in the discovery cohort is given in Supplementary Data 3. Patients were enrolled in the neuroblastoma clinical trials (NB2004-HR/NCT03042429, GPOH-NB2004/NCT00410631, GER-GPOH-NB97/NCT00017225, GER-NB95-S/NCT00002803, GER-NB90/NCT00002802, NB2016-registry) of the Society for Fig. 7 Telomeric repeat loci. a Telomeric repeat loci frequency in ATRX-mutated (dark pink) and ATRX wild-type (light pink) ALT-positive tumors compared to TERT (blue), MNA (MNA), and tumors without genetic evidence of an activated telomere maintenance mechanism (OTHER, gray) in the discovery cohort. Boxplots indicate the median value (middle line) and the 25th and 75th percentiles (box). The upper/lower whisker spans from the hinge to the largest/smallest value (values expanding a distance of 1.5 x inter quartile range are not considered). Dots represent individual tumors. $P$ values were calculated with two-sided Wilcoxon rank sum tests. b Example of one telomeric repeat locus in tumor NBD119. Dark blue color indicates sequencing coverage and light blue color illustrates clipped sequences. Reads are shown in medium gray and clipped bases are color-coded. Non-telomeric ends of a discordant read pair are labeled in dark gray. c Overall survival probability of ALT-positive tumors harboring more than three telomeric repeat loci compared to ALT-positive tumors with three or less than three events. $P$ value calculated using a log rank test.

d Chromosomal location of telomeric repeat loci in the discovery cohort. e Exact genomic location of events contributing to the hotspot on chromosome 1q42.2 in the discovery and INFORM cohort and two ALT-positive cell lines. The 1q42.2 events of NBD158 and NBD137 were added manually (see Methods). f Chr1 copy number profiles of tumors with a telomeric repeat locus on chr1q42.2. Junction sites and orientation of telomeric repeats are indicated in orange. Color-coding according to subgroup (# HET ALT/MNA). g Differential mRNA expression of genes located in the chr1q42.2-1qter deleted region comparing 1q42-1qter deleted to non-deleted tumors. Genes are colored by their mean expression normalized by exonic length or by their presence in the TelNet database22. *) Matched primary relapsed pair.

Fig. 8 Model of neo-telomere formation. Graphical abstract illustrating the hypothesis of neo-telomere formation in ALT-positive neuroblastomas. a The majority of telomeric repeat loci are one-sided. We hypothesize that ALT-positive cells are able to add telomeric sequences to open chromosomal breaks to protect them from degradation. I Microhomology to TTAGGG favors the formation of a neo-telomere at the open chromosomal break. Loss of some chromosomal regions might present a selection advantage for the cell and cells with a neo-telomere at the chromosomal breakpoint are selected. Ii Without microhomology, structural rearrangements involving other chromosomal arms (light gray) can represent an alternative route of protecting open chromosomal breaks. b Rare two-sided events can result from insertion of telomeric sequences I or from the formation of neo-telomeres on both sides of the breakpoint II. Small circles represent C-Circles. Chromosomes are shown in gray and telomeric sequences in blue.
Pediatric Oncology and Hematology (GPOH) between 1991 and 2018. All trials were approved by the Ethics Committee of the Medical Faculty, University of Cologne and collection of tumor tissue material was approved. Tumors of nine Austrian neuroblastoma patients were analyzed as part of the screening and discovery cohort. Tumor and control DNA/RNA was provided by Prof. Dr. Peter Ambros and Dr. Sabine Taschner-Mandl from the St. Anna Kinderkrebshospital at the Children’s Cancer Research Institute in Vienna, Austria. Austrian neuroblastoma patients were in part enrolled in the NB-A-87/5 or the HR-NBL-13 SIOPEN trial (NCT01704716, EudraCT number 2006-001489-17). The copy number status of MYCN using FISH analysis was assessed as a routine diagnostic marker in all neuroblastoma clinical trials. Survival data for German patients of the discovered cohort was provided by Dr. Barbara Hero from the study office of the Neuroblastoma trial in Cologne. Additionally, a relapsed neuroblastoma cohort of patients enrolled in the registry trial INFORM was analyzed (n = 40). Whole exome sequencing and low coverage whole genome sequencing was done as part of the INFORM workflow. For a subset of these (n = 31) high coverage whole genome sequencing was done. A detailed overview of all clinical parameters and available data of the INFORM cohort is given in Supplementary Data 2. All genome sequencing was done. A detailed overview of all clinical parameters and available data of the INFORM cohort is given in Supplementary Data 2. All genome sequencing was done. A detailed overview of all clinical parameters and available data of the INFORM cohort is given in Supplementary Data 2.

C-Circle assay. C-Circle assays were performed based on a previously described protocol, which is briefly described in the following. 30 ng of genomic DNA were used for every tumor sample. Prior to amplification, tumor DNA was digested with RalI (4U/μg) and Hinfl (4U/μg) and RNase (25 ng/μg) treated for 1 h at 37 °C. 0.2 mg/ml BSA, 0.1% Tween 20, 1 mM dATP, dGTP and dTTP, 2 x 20 ng and 7.5 μg per ml, were added to the PCR reaction mixture. Amplification was run at 30 °C for 8 h, followed by an inactivation at 65 °C for 20 min. For every sample, a sample without polymerase was included as control. Whole genome sequencing. High coverage whole genome sequencing libraries were sequenced on the Illumina HiSeq 2000 (100 bp paired end) or on a patterned flowcell v2.2 (150 bp paired end) with coverage of 30–60% for the tumor and whole blood control samples. Tumor samples exhibited a tumor cell content of ≥60%. Sequence alignment was performed using the NEB Next Ultra DNA kit (NEB) according to the manufacturers’ instructions. Libraries for sequencing on the patterned flowcell v2.5 were generated using the Truseq DNA Nano kit (Illumina) according to the manufacturers’ instructions. Libraries were size selected using SPIR beads (Beckman Coulter Genomics). One Touch U6 Pipelining (Yang et al. 2016, see above) was added to the protocol. The library amplification was run at 30 °C for 8 h, followed by an inactivation at 65 °C for 20 min. For every sample, a sample without polymerase was included as control. Whole genome sequencing data was aligned to the 1000 Genomes project phase 3 assembly with decoy and PhiX control DNA/RNA was isolated from whole blood using the NucleoSpin Blood DNA extraction kit (Macherey-Nagel).

Telomere length analysis. The telomere content was calculated using the software tool TelomereHunter version 1.0.1. TelomereHunter v1.0.1 is available at [https://www.dkfz.de/en/applications/telomerehunter/telomerehunter. html]. Telomere reads containing six non-consecutive instances of the four most common telomeric repeat types (TTAGGG, TCAGGG, TGAGGG, and TGGGG) were extracted. For the further analysis, only unmapped reads or reads with a very low alignment confidence (mapping quality lower than 8) were used. Telomere repeat losses that were not detected by the TelomereHunter were not considered. Any copy number losses that were not detected by the software tool were not used for normalization.

Subgrouping based on telomere maintenance mechanism (TMM group). All tumors were categorized into four groups according to the genetic evidence of an activated telomere maintenance mechanism (ALT, MNA, TERT) or the absence of such a mechanism (OTHER). Tumors being either C-Circle positive or having a log2 telomere content relative to the control sample >1 were classified as ALT. Tumors with an amplified MYCN gene were considered to use telomerase activation by MYCN (MNA). The TERT group comprises tumors with a structural variation affecting TERT or TERT promoter mutation. Tumors with a relative log2 telomere content above one and amplified MYCN were included in the heterogeneous group ALT/MNA despite being C-Circle negative.

Telomeric repeat loci. To find telomeric repeat loci we searched for tumor-specific discordant paired-end reads, where one end was an extracted telomere read and the other end was a non-telomeric read. These reads were mapped to the telomere repeat locations (mapping quality ≥30). In 1 kb regions containing at least four discordant reads the tumor sample and none in the matching control, exact junction sites were defined by at least three split reads. The split reads had to contain at least one TTAGGG repeat. Regions with discordant read pairs in at least 15 control samples were excluded. Further criteria were the use of the Integrative Genomics Viewer (IGV)62,63 to identify and remove remaining false positives. 1q42.2 hotspot region was manually inspected to add automatically excluded telomeric repeat loci (NBD158 and NBD137). The pipeline used to detect telomeric repeat loci is available via Github [https://github.com/linasverling/TelomereRepeatLoc].

RNA-seqencing. Using the Nucleo Spin RNA kit (Macherey Nagel) RNA was isolated from fresh frozen tissue according to the manufacturers’ instructions. mRNA was isolated using poly A selection with Dynabeads (Thermo Fisher). 100 bp paired-end sequencing was carried out using the Illumina Hiseq 2000 and Hiseq 4000. Alignment to the reference genome (1000 Genomes project phase 3 assembly with decoy and Phix contigs) was done using STAR version 2.5.2b66 with the parameters—sjdbOverhang 200—runThreadN 8—outSAMtype BAM SortedByCoordinate—limitBMemRam 100000000000—outBAMsortOrder N1—outSAMstrandField intronMotif—outSAMunmapped KeepWithinPairs—outFilterMultimapNmax 1—outFilterMismatchNmax 5—outFilterMismatchNoverLmax 0.3—twoPassMode Basic—twoPassReads N1—genomeLoad NoSharedMemory—chimSegmentMin 15—chimScoreMin 1–chimScoreJunctionNonGTAG 0–chimJunctionOverhangMin 15—chimSegmentReadGapMin 3—alignSizeStitchMismatchNmax 5 —1 5—alignlntronMax 1100000—alignMatesGapMax 1100000—alignSJOverhangMin 3—alignlntronMin 20—clip3pAdapterTarFile AGATCGGAAGAGCACCGCTGAC TCCGATCA-readFilesCommand gunzip -c. Sambamba version 0.6.95 was used for merging and duplication marking. Bam files were filtered with SAM tools version 1.3.167. Read counts per gene were obtained with featurecounts version 1.5.1 under counting reads over exon features using GENCODE 19 as a gene model. Both reads of a paired fragment were used for counting and only unique alignments were considered by setting the quality threshold to 255. The read counting was performed in an unstranded manner. The workflow used for RNA-seqencing is available at [https://github.com/DKFZ-ODCF/RNAseqWorkflow]. For the calculation of TPM expression values, genes on chromosomes X, Y, MT and rRNA and TRNA genes were omitted when calculating total library abundance calculations to prevent library size estimation biases. Differential expression analysis was performed using DESeq2 version 1.18.168 to find differences between samples with chipRq2.2-tqet deletion and those without a specific deletion in this region. chipRq2.2-tqet deletion was defined as reduced copy number compared to the upstream neighboring segment. Low-count genes with less than 10 reads across the entire cohort were excluded. Gender and sequencing location were included as possible confounding variables. Filtered raw count data was used for the default analysis including size factor estimation, dispersion estimation, negative binomial generalized linear model fitting, and Wald statistics. Log fold change shrinkage was applied. The results were filtered afterwards to include only genes from 230 Mb to the end of chromosome 1.

TERRA expression. To determine TERRA expression, RNA-seq sequencing reads containing telomere repeats were extracted with TelomereHunter version 1.0.1 using a threshold of 14 telomere repeats per 100 bp read length and otherwise...
default settings. All extracted telomere reads were considered as TERRA reads independently of the mapping position. To normalize for different library sizes, the default settings. All extracted telomere reads were considered as TERRA reads independently of the mapping position. To normalize for different library sizes, the default settings. All extracted telomere reads were considered as TERRA reads independently of the mapping position. To normalize for different library sizes, the default settings. All extracted telomere reads were considered as TERRA reads independently of the mapping position. To normalize for different library sizes, the default settings. All extracted telomere reads were considered as TERRA reads independently of the mapping position.
Tumoral reads containing at least 8 telomeric repeats per 100 bp read length were extracted in ChiP and input samples using TelomerHunter64 with the option -rt 8 -rl. Number of telomeric reads was normalized to the total number of mapped reads. Log2 enrichment of normalized telomeric/satellite reads in ChiP was calculated relative to input files. Samples with a fraction of reads in peaks below one were excluded from the analysis.

\[
\text{Tel}(\log 2)\text{enrichment} = \log 2\left(\frac{\text{telomeric reads in IP}}{\text{total mapped reads}} / \frac{\text{telomeric reads in input}}{\text{total mapped reads}}\right)
\]

\[
\text{SatII}(\log 2)\text{enrichment} = \log 2\left(\frac{\text{SatII reads in IP}}{\text{total mapped reads}} / \frac{\text{SatII reads in input}}{\text{total mapped reads}}\right)
\]

\[
\text{SatIII}(\log 2)\text{enrichment} = \log 2\left(\frac{\text{SatIII reads in IP}}{\text{total mapped reads}} / \frac{\text{SatIII reads in input}}{\text{total mapped reads}}\right)
\]

**Statistics** Differences between various neuroblastoma subgroups in terms of telomere content, telomeric repeat loss, enrichment of ChiP signals, TPM, and LPF expression values were tested with two-sided Wilcoxon rank-sum tests using R (R Foundation for Statistical Computing, version 3.5.1 or 3.6.1). All boxplots were generated using the function geom_boxplot() in the R package ggplot2 (version 3.3.1). Horizontal line is representing the median value. P values for Western blot quantification were calculated using a two-sided unpaired Student’s t-test in R with the function t.test. P values for differential RNA expression analysis were calculated with Wald tests within DESeq2 version 1.18.185. P values for differential protein expression analysis were calculated with two-sided Student’s t-test statistics using Perseus software version 1.5.5.3.52. Correlation analysis was performed using ggpubr (version 0.3.0) or with the function cor in R. Enrichment analysis was tested by hypergeometric test on gene symbols of significantly different proteins and mRNA determined for this analysis by SAM-modified50 (SO = 0.01) Welch t-test were performed using R. Multiple testing correction by Benjamini-Hochberg method was used with the R function p.adjust. Survival curves were generated using Kaplan Meier analysis in the R package survminer (version 0.4.7). P values for survival analysis were calculated using log rank tests. The optimal cut point to assess the prognostic impact of the number of telomeric repeat lost and telomere content was determined using Maximally Selected Rank Statistics with the criteria that at least 20% of the cases have to be in each group using the R package survminer (version 0.4.7).

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