TBX2 is a neuroblastoma core regulatory circuitry component enhancing MYCN/FOXM1 reactivation of DREAM targets

Bieke Decaesteker,1,2 Geertrui Denecker,1,2 Christophe Van Neste,1,2 Emmy M. Dolman,4 Wouter Van Loocke1,2 Moritz Gartlgruber,5 Carolina Nunes,1,2 Fanny De Vloed,1,2 Pauline Depuydt,1,2 Karen Verboom,1,2 Dries Rombaut,1,2 Siebe Loontiens,1,2 Jolien De Wyn,1,2 Waleed M. Kholosy,4 Bianca Koopmans,4 Anke H.W. Essing,4 Carl Herrmann,6,7 Daniel Dreidax,5 Kaat Durinck,1,2 Dieter Deforce,2,8 Filip Van Nieuwerburgh,2,8 Anton Henssen,9,10,11 Rogier Versteeg,12 Valentina Boeva,3 Gudrun Schleiermacher,13 Johan van Nes,12 Pieter Mestdagh,1,2 Suzanne Vanhauwaert,1,2 Johannes H. Schulte,9 Frank Westermann,5 Jan J. Molenaar,4 Katleen De Preter,5,10,11 & Frank Speleman1,2

Chromosome 17q gains are almost invariably present in high-risk neuroblastoma cases. Here, we perform an integrative epigenomics search for dosage-sensitive transcription factors on 17q marked by H3K27ac defined super-enhancers and identify TBX2 as top candidate gene. We show that TBX2 is a constituent of the recently established core regulatory circuitry in neuroblastoma with features of a cell identity transcription factor, driving proliferation through activation of p21-DREAM repressed FOXM1 target genes. Combined MYCN/TBX2 knockdown enforces cell growth arrest suggesting that TBX2 enhances MYCN sustained activation of FOXM1 targets. Targeting transcriptional addiction by combined CDK7 and BET bromodomain inhibition shows synergistic effects on cell viability with strong repressive effects on CRC gene expression and p53 pathway response as well as several genes implicated in transcriptional regulation. In conclusion, we provide insight into the role of the TBX2 CRC gene in transcriptional dependency of neuroblastoma cells warranting clinical trials using BET and CDK7 inhibitors.

1 Center for Medical Genetics, Ghent University, Ghent 9000, Belgium. 2 Cancer Research Institute Ghent (CRIG), Ghent 9000, Belgium. 3 Institut Cochin, INSERM U1016, CNRS UMR 8104, Paris Descartes University UMR-S1016, Paris 75014, France. 4 Princess Máxima Center for Pediatric Oncology, Department of Translational Research, Utrecht 3584, The Netherlands. 5 Neuroblastoma Genomics, German Cancer Research Center (DKFZ), Heidelberg 69120, Germany. 6 Institute of Pharmacy and Molecular Biotechnology, and Biquant Center, University of Heidelberg, Im Neuenheimer Feld 267, Heidelberg 69120, Germany. 7 Division of Theoretical Bioinformatics, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, Heidelberg 69120, Germany. 8 Laboratory of Pharmaceutical Biotechnology, Ghent University, Ghent 9000, Belgium. 9 Department of Pediatric Oncology and Hematology, Charité-Universitätsmedizin Berlin, Berlin 13353, Germany. 10 German Cancer Consortium (DKTK), Heidelberg 69120, Germany. 11 Berlin Institute of Health, Berlin 13353, Germany. 12 Department of Oncogenomics, Academic Medical Center, Amsterdam 1105 AZ, Netherlands. 13 Institut Curie, PSL Research University, Equipe Labellisée Ligue contre le Cancer, Laboratoire Recherche Translationale en Oncologie Pédiatrique (RTOP), Laboratoire “Gilles Thomas”, Institut Curie, Department of Translational Research, Institut Curie, SIREDO: Care, Innovation and Research for Children, Adolescents and Young Adults with Cancer, Institut Curie, Paris 75248, France. These authors jointly supervised this work: Katleen De Preter, Frank Speleman. These authors equally contributed: Geertrui Denecker, Christophe Van Neste. Correspondence and requests for materials should be addressed to B.D. (email: bieke.decaesteker@ugent.be) or to F.S. (email: Franki.speleman@ugent.be)
Neuroblastoma (NB) is a cancer of the developing sympathetic-adrenergic nervous system and is the most common malignancy diagnosed in children during their first years of life. Sequencing revealed a relatively silent mutational landscape with only ALK activating mutations being identified in up to 10% of primary cases as well as de novo secondary or emerging subclonal ALK mutations in relapsed cases. Further, in relapsed cases additional RAS-MAPK pathway driving mutations are enriched. In contrast to mutations, DNA copy number alterations are remarkably recurrent in NB, including focal amplification of the MYCN oncogene in approximately half of the high-stage patients and large 17q segmental gains occurring in the majority of both MYCN amplified and non-amplified high stage tumors. The finding of recurrent gains of the syntenic human 17q region in MYCN driven NB mouse tumors further supports the putative functional importance of this genomic aberration in NB. Investigating dosage-sensitive genes affected by recurrent copy number alterations can offer new insights into tumor biology as was illustrated in ependymoma where multiple dosage-affected genes located within large chromosomal regions of recurrent gains and losses, were shown to act as oncogenes or tumor suppressors through installing a so-called cellular state driven through one or more altered cellular functions.

Given the recently proposed role of a core regulatory circuitry (CRC) consisting of several super-enhancer (SE) marked transcription factor constituents in NB, we decided to search for dosage-sensitive SE marked transcription factors encoding genes residing on chromosome 17q. The 'T-box 2 transcription factor' (TBX2), hitherto not reported to be implicated in NB, was prioritized as transcription factor with top-ranked SE score in NB cell lines and with expression levels highly correlated with survival outcome in NB tumors. TBX2 is a member of the T-box family of transcription factors with an important role during embryogenesis and morphogenesis and is overexpressed in several cancer entities including melanoma, breast, and pancreatic cancer. The oncogenic effect of TBX2 overexpression has been attributed to its role in proliferation as well as inducing epithelial-to-mesenchymal transition (EMT) and senescence bypass. Based on integrated analysis of TBX2 occupancy as determined by ChIP-sequencing and transcriptome analysis upon knockdown (KD), we propose TBX2 as a novel bona fide constituent of the recently reported CRC in NB.

To investigate the role of TBX2 in this CRC, functional analyses were performed showing the implication of TBX2 in cell cycle, proliferation, and downstream E2F-FOXM1 signaling. Finally, we demonstrate that combined pharmacological targeting of transcriptional addiction using a BET and CDK7 inhibitor, yields synergistic effects on TBX2 downregulation leading to massive apoptosis.

Results

TBX2 is a super-enhancer marked transcription factor on 17q. CRCs consisting of SE marked master transcription factors were recently shown to be dysregulated in NB through MYCN-dependent transcriptional amplification causing transcriptional addiction. Given the highly recurrent chromosome 17q gain in high-risk human NBs and MYCN-driven mouse NBs, we hypothesized that one or more dosage-sensitive CRC transcription factors map to 17q thus rendering a selective advantage to tumors cells exhibiting 17q gain. To identify such transcription factors, we determined SE scores using the LILY algorithm based on the intensity of H3K27ac marks in 26 NB cell lines with 17q gain, two non-malignant neural crest cell lines and the breast cancer cell line MCF-7 as non-embryonal control (gene prioritization strategy is depicted in Fig. 1a, b and Supplementary Fig. 1a, b). We identified a total of 176 SE clusters on 17q of which six were present in at least 20 NB cell lines (Supplementary Fig. 1c). These six SE clusters are located in the vicinity of 86 candidate genes of interest, including 11 transcription factors, of which 5 are actively transcribed in NB cells, i.e. TBX2, RARA, SP2, NFE2L1, and VEZF1.

Next, we assessed the expression levels of these transcription factors in relation to patient survival in two independent NB tumor cohorts (GSE85047 n = 276, GSE62564 n = 498) and observed the strongest association with overall and progression-free survival for TBX2 (Fig. 1c and Supplementary Fig. 1d, Kaplan–Meier analysis). Moreover, TBX2 is marked by a SE in all investigated NB cell lines, but not in the human neural crest line (hNCC) and the MCF-7 breast cancer cell line (Fig. 1b and Supplementary Fig. 1b).

Of further interest, the highest expression levels for TBX2 were observed in NB cell lines and primary tumors compared to other tumor entities, based on the online pan-cancer analysis in the CCLE database (cancer cell lines) and R2 platform (primary tumors and normal tissues) (Fig. 1d). TBX2 expression levels were also high in normal embryonic tissues in keeping with the established role of TBX2 in early development. Taken together, our data suggest a possible important role for TBX2 as hitherto unrecognized transcriptional regulator in NB tumor development.

4C-seq defines TBX2 promoter—super-enhancer interactions.

To provide further evidence for a functional role of the assigned SE for TBX2 gene regulation, three different viewpoints residing in the SE region (20 kb and 260 kb up TSS) or the promoter site of TBX2 (4.5 kb up TSS) were selected for 4C-sequencing in NB cell lines SK-N-AS and CLB-GA (Fig. 2a and Supplementary Fig. 2c). In addition to the potential regulatory gene which maps to the CCLE database and found that NB was the tumor entity exhibiting the most frequent gains for the TBX2 locus, the highest expression and lowest methylation levels (Figs. 1d and 2b, c).

TBX2 is a copy-number affected dosage-sensitive gene. Next, we investigated in more detail the genomic aberrations that account for the high TBX2 expression in NB (Fig. 1d). We first analyzed the CCLE database and found that NB was the tumor entity exhibiting the most frequent gains for the TBX2 locus, the highest expression and lowest methylation levels (Figs. 1d and 2b, c).

Next, we assessed the effect of DNA copy number alterations on TBX2 expression levels using an ANOVA analysis in the NRC tumor dataset (n = 218, GSE85047). Only in high-stage disease...
Fig. 1 TBX2 is a super-enhancer marked 17q transcription factor in NB. a Prioritization strategy to find SE-driven candidate oncogenes on chr17q. b H3K27ac activity in a region upstream of TBX2 in 26 NB cell lines (blue), non-malignant neural crest cell lines (green) and the non-embryonal breast cancer cell line MCF-7 (green). The Lilly annotated SE regions are indicated in red. The cluster (out of 276 clusters on chr17q) containing the overlapping SE used in the prioritization process is annotated at the bottom. c Kaplan-Meier analysis (overall survival) of 276 neuroblastoma patients (NRC NB tumor cohort, GSE85047) with high or low expression (using median as cut-off) of the five prioritized candidate oncogenes (TBX2, NFE2L1, SP2, RARA, and VEZF1). d TBX2 is highly expressed during embryogenesis as compared to normal adult tissue. TBX2 is highly expressed in NB tumors and NB cell lines as compared to tumors or cell lines from other entities. Boxplots are drawn as a box, containing the 1st quartile up to the 3rd quartile of the data values. The median is represented as a line within the box. Whiskers represent the values of the outer two quartiles maximized at 1.5 times the size of the box. If one or more values outside of the whiskers are present, then this is indicated with a single mark ‘x’ next to the implicated whisker.
**Fig. 2** TBX2 is targeted by rare focal amplifications and marked by a SE.  

**a** 4C-seq analysis showing reciprocal interaction between the promoter site and SE upstream of TBX2 in the NB CLB-GA and SK-N-A5 cell lines using three different viewpoints. **b** Copy number ratio of the TBX2 locus in a pan-cancer dataset (CCLE) with high copy number for TBX2 in NB (green arrow) as compared to cell lines from other tumor entities. Boxplots are drawn as a box, containing the 1st quartile up to the 3rd quartile of the data values. The median is represented as a line within the box. Whiskers represent the values of the outer two quartiles maximized at 1.5 times the size of the box. If 1 or more values outside of the whiskers are present, then this is indicated with a single mark dot next to the implicated whisker. Number of samples for every entity is depicted below the boxplot. **c** Genome-wide methylation profile evaluated by reduced representation bisulfite sequencing (RRBS) of the TBX2 locus in a pan-cancer dataset (CCLE). TBX2 is covered by a low methylation profile in NB (green arrow) as compared to cell lines from other entities. Boxplots are drawn as a box, containing the 1st quartile up to the 3rd quartile of the data values. The median is represented as a line within the box. Whiskers represent the values of the outer two quartiles maximized at 1.5 times the size of the box. If one or more values outside of the whiskers are present, then this is indicated with a single mark dot next to the implicated whisker. Number of samples for every entity is depicted below the boxplot. **d** Significantly increased TBX2 expression levels in cases with both high stage disease (stage 3 and 4) and increased TBX2 copy number due to 17q gain (ANOVA, \( n = 218 \), \( p = 6.004e^{-5} \), chr17 gain log2 ratio \( > 0.3 \)). **e** Log2 copy number ratio of a region on 17q with a focal amplification encompassing the protein-coding genes PPM1D, BCAS3, TBX2, C17orf82, TBX4, and NACA2, in a primary NB tumor case (58,654 Mb—59,730 Mb—hg19—log2 ratio 2.78).
(stage 3 and 4), we observed significantly increased TBX2 expression levels due to increased TBX2 copy number (p = 6.004e−5, log2 ratio > 0.3) (Fig. 2d). We specifically looked for rare TBX2 encompassing amplifications in a series of 556 high-risk NB cases and detected a single MYCN-amplified case with an additional 1.076 Mb focal 17q23.2 amplification (Fig. 2e and Supplementary Fig. 2e) encompassing only six protein-coding genes including the transcription factors TBX2 and TBX4. Of further note, a previously reported focal high level 1.8 Mb gain of a chromosome 17q23 segment in the NB cell line MP-N-T5 also encompasses the TBX2 locus. Taken together, our data indicate that TBX2 is a dosage-sensitive transcription factor affected by the common segmental 17q gains and rare amplification events in NB.

TBX2 is a core regulatory circuitry constituent in NB. To gain further insight into the TBX2-controlled regulatory network, we assessed TBX2 DNA occupancy by ChIP-sequencing and ATAC-sequencing in the NB cell line IMR-32. A total of 557 significant (adj.P.val < 0.05) TBX2 binding sites were identified and motif analysis confirmed enrichment for a TBX motif (AGGTTGTA, p = 1e−41), supporting the validity of our ChIP-seq data (Supplementary Data 1). In total, 81, 28, and 94% of TBX2 binding sites in IMR-32 respectively overlap H3K27ac, H3K4me3, and ATAC-sequencing peaks (Fisher test p < 2.2e−16, Fig. 3a, b), which confirms the binding of TBX2 to active promoter and enhancer regions. Moreover, respectively 41 and 30% of the TBX2 ChIP-seq peaks are found intergenic or are annotated to IncRNAs (Supplementary Fig. 3a, b), and 19% overlap with the SEs annotated in the cell line IMR-32 (Fisher test p < 2.2e−16, Supplementary Fig. 3c).

The recent reports on distinct CRCs in NB prompted us to investigate the possible involvement of TBX2. In line with the recent finding of invasion of MYCN into non-canonical E-boxes at enhancers, motif analysis of TBX2-bound regions showed that the non-canonical MYCN E-box motif CANTNG was found to be highly enriched (Binomial test p = 1e−63) as well as motifs for GATA1(1/2/3/4), PHOX2(A/B), HAND(1/2) and neuronal lineage-specific marker genes such as ASCL1, ISL1 and MEIS(1/2/3), (Supplementary Data 1). We integrated the ChIP-seq tracks for TBX2 with those reported for GATA3, PHOX2A/B, HAND2, PHOX2B, MYCN (this study) in NB cell lines and observed overlap of TBX2 peak sums with binding sites of these CRC transcription factors (Fig. 3b), thus supporting the notion that TBX2 is indeed actively taking part in this CRC. Overlap of PHOX2B, HAND2, and GATA3 binding with the TBX2 peaks was predominantly observed in enhancer regions (Fig. 3b and Supplementary Fig. 3d). The integration of TBX2 into this CRC is further confirmed by the observation of auto-regulation by binding of the TBX2 transcription factor to its own SE constituent and binding of at least three CRC members including GATA3, HAND2 and PHOX2B within this SE constituent (Fig. 3c).

In addition, TBX2 is binding the SE constituents of the other CRC members PHOX2B, GATA3, and HAND2, amongst others, as shown in Supplementary Fig. 3e. Finally, TBX2 expression is positively correlated with GATA3, HAND2, and PHOX2B expression levels as well as with those of other potential CRC genes important in development in a NB tumor cohort (n = 283, Supplementary Fig. 3f). Taken together, our data suggest that TBX2 is part of the recently described CRC together with HAND2, GATA3, and PHOX2B.

TBX2 controls E2F-FOXM1 driven cell cycle and proliferation. To unravel the role of TBX2 within the CRC in NB cells, we performed TBX2 KD with two shRNAs and a non-targeting control and subsequent gene expression profiling in the NB cell line IMR-5/75 (Supplementary Fig. 4a). A total of 1055 and 1326 genes were differentially down and upregulated, respectively (adj. p.val < 0.05, Supplementary Data 2), including the upregulated gene CDKN1A, which is a known target gene repressed by TBX2. Gene set enrichment analysis (GSEA) on the downregulated genes upon TBX2 KD in IMR-5/75 cells revealed enrichment (FDR < 0.01) for the hallmark and gene ontology gene sets involved in cell cycle including G2/M checkpoint, E2F, MYC(N) targets, mitosis, and DNA replication (Fig. 4a, Supplementary Data 3) and enrichment was shown for TP53 pathway among the upregulated genes. Using iRegulon, designed to detect transcription factors, targets and motifs/track from a set of genes, we identified motif enrichment (FDR < 0.01) for FOXM1, E2F, and E2F binding partners TFDP1/TFDP5 in nearly half of all downregulated genes (Fig. 4b, Supplementary Data 4). Interestingly, an E2F motif was also enriched in the TBX2 binding sites in IMR-32 cells (Supplementary Data 1). The role of E2F and the MuvB core component FOXM1 was further supported by significant enrichment for published gene sets containing FOXM1, DREAM, E2F, and CCND1/CDK4 (the latter is known to phosphorylate FOXM1) activity/target genes (Fig. 4c). In contrast, almost half of upregulated genes upon TBX2 KD showed enrichment for a motif with high similarity to the MuvB core component MYBL2, REST (transcriptional repressor implicated in neuronal differentiation) and EP300 (histone acetyltransferase) ChIP binding sites (Fig. 4b, Supplementary Data 4). In addition to FOXM1 target genes, FOXM1 itself is also downregulated upon TBX2 KD in the IMR-5/75 and CLB-GA cell lines (Fig. 4d).

Using a similar approach as for MYCN amplified IMR-5/75 cells, we also investigated the TBX2 transcriptional regulatory network in the non-MYCN amplified CLB-GA NB cells and observed similar downstream targets and enriched gene sets as those observed in IMR-5/75 upon TBX2 KD (Supplementary Fig. 4a–d, Supplementary Data 3 and 5), with the E2F/FOXM1 axis being most prominent.

To further validate these results, we performed correlation analysis in publicly available transcriptome data of 283 primary NB tumors (NRC, GSE85047). Supporting the TBX2 KD data described above, GSEA showed enrichment for cell cycle, DNA repair and DNA replication as well as chromatin architecture among the genes positively correlated with TBX2 expression levels in the NB tumor data set (FDR < 0.01, R > 4, Fig. 4e). In addition, we found a significant positive correlation for expression levels of TBX2 versus FOXM1, E2F core members, and other DREAM complex members (Supplementary Fig. 4e). Furthermore, the TBX2 KD signature score (generated in both CLB-GA and IMR-5/75 cells) was negatively correlated with the expression of these DREAM complex members (Supplementary Fig. 4e, f).

As these data collectively suggest a role for TBX2 in control of a FOXM1/E2F-driven gene regulatory network driving proliferation, we explored the phenotypical effects of TBX2 KD in NB cells following prolonged lentiviral short hairpin RNA mediated KD of TBX2 in IMR-32, CLB-GA and SK-N-AS NB cell lines with high TBX2 expression. KD of TBX2 with four different hairpins for 5 days (Supplementary Fig. 4a) leads to a decrease in colony formation capacity (Fig. 5a and Supplementary Fig. 5a) and proliferation as measured with time-lapse microscopy (every 2–3 h) (Fig. 5b). Furthermore, we also show a significant G1 growth arrest in the CLB-GA cell line upon TBX2 KD (Fig. 5c) in keeping with the above-reported effect of TBX2 KD on cell cycle genes. Importantly, no effect on proliferation or colony formation capacity was detected in the TBX2 non-expressing SH-EP cell line, indicating limited off-target effects with these four hairpins (Supplementary Fig. 5b). In summary, we have shown that TBX2...
is implicated in cell cycle and proliferation through regulating an E2F-FOXM1 driven cellular state.

**Combined TBX2-MYCN signaling targets the FOXM1/E2F network.** While CRC transcription factors bind their own enhancers as well as those of the other CRC partners, the individual contribution of each factor to the malignant phenotype of the cancer cells is largely unexplored. A recent report suggested that TWIST and HAND2 would cooperate with MYCN through binding of enhancers of a set of developmental genes. In view of these findings, we also decided to further study the TBX2–MYCN interrelationship. We first obtained indirect evidence for a functional relationship between MYCN and TBX2 from several datasets: (1) significant enrichment for publicly available MYCN (N) target/signature gene sets in the MYCN amplified IMR-32 and MYCN non-amplified cell line CLB-GA upon TBX2 KD (Figs. 4a, 6a and Supplementary Fig. 4d); (2) significant correlation of TBX2 and MYCN mRNA and protein expression in NB tumors (Supplementary Fig. 3f) and NB cell lines (Supplementary Fig. 6a), respectively; (3) correlation between MYCN activity and TBX2 shRNA signature scores (established in both IMR5/75 and

Fig. 3 TBX2 is part of a core-regulatory circuitry. **a** Significant (Fisher test p-value < 2.2e−16) overlap (min. overlap = 20 bp) of the TBX2 (qval < 0.05), H3K27ac (qval < 0.05), H3K4me3 (qval < 0.05) ChIP-seq and ATAC (qval < 10−6) peaks in the IMR-32 cell line. **b** Heatmap profiles −4 kb and +4 kb around the summit of TBX2 peaks. The heatmaps represent the ChIP-seq overlap of TBX2, H3K27ac, ATAC, H3K4me3, and MYCN peaks in the IMR-32 cell line, the histone mark H3K4me1 and the CRC genes GATA3, PHOX2B, and HAND2 in the CLB-GA cell line, and GATA3 in the SH-SY5Y, BE2, NGP, and BE2C cell lines, grouped for promoters or enhancers (homer annotation), and ranked according to the sums of the ChIP-seq peak scores across all ChIP-seq peaks in the heatmap. **c** TBX2 region (59.1 Mb–59.65 Mb) with the TBX2, ATAC, H3K27ac, and H3K4me3 ChIP profiles in IMR-32 and GATA3, HAND2 and PHOX2B ChIP profiles in CLB-GA. At the bottom, the Lilly called SEs are annotated for the two replicates in IMR-32 as well as the cluster of SEs used for the prioritization (Fig. 1c)
CLBGA) in NB tumors (CLB-GA: Spearman Correlation \( p = 1.28e^{-83}, R = -0.684 \), IMR-5/75: Spearman Correlation \( p = 7.91e^{-26}, R = -0.576 \) (Supplementary Fig. 6b) and NB cell lines (CLB-GA: Spearman Correlation \( p = 0.00179, R = -0.564 \), Spearman Correlation IMR-5/75: \( p = 6.67e^{-08}, R = -0.825 \); (4) 62% of differentially expressed genes upon TBX2 KD (FDR < 0.05, \( n = 2381 \)) in IMR5/75 overlapped (Fisher test, \( p = 0.006 \) with the differentially expressed genes upon (dox inducible) MYCN KD in the same cell line (FDR < 0.05, \( n = 4409 \)), indicating that these transcription factors co-regulate the same gene...
Fig. 4 TBX2 controls a FOXM1/E2F gene regulatory network. a Top enriched MsigDB hallmark genesets among the downregulated genes upon shTBX2 in IMR-5/75 (FD < 0.01) and the upregulated genes (FDR < 0.01) upon shTBX2 in IMR-5/75. Normalized enrichment score (NES) and false discovery rate (FDR) is depicted on the barcode plot. b Regulon motif search for the downregulated and upregulated genes (FDR < 0.01) upon TBX2 knockdown in IMR-5/75. Genes connected to a gene in a white circle do have a motif enrichment or ChIP-seq binding for the respective gene in the circle. c GSEA results for genesets from literature (FOXM1 and E2F targets, and the downregulated genes upon CCND1 and CDK4 knockdown) which are enriched in the TBX2 downregulated genes in IMR-5/75. Normalized enrichment score (NES) and false discovery rate (FDR) is depicted on the barcode plot. d Western blot of TBX2, MYCN, and FOXM1 levels upon TBX2 knockdown, in the CLBGA MYCN single copy and IMR-5/75 MYCN amplified cell lines. e Clustering of genesets (MsigDB c5.bp.v4.0) correlated with the presence of TBX2, MYCN, and FOXM1 levels upon knockdown, in the CLBGA cell line. Significance Induction of G1 cell cycle phase arrest upon TBX2 knockdown measured with time-lapse microscopy (every 2-3 h) using the IncuCyte device (one out of three biological replicates is shown). c Cell cycle analysis upon TBX2 knockdown in the CLBGA cell line. Significant induction of G1 cell cycle phase arrest upon TBX2 knockdown (statistical test is based on G1 phase percentage). Data-points were mean-centered (a,c) and error bars represent the s.d. of three biological (a,c) or five technical (b) replicates for every cell line. Mann-Whitney test *p < 0.05, **p < 0.01

sets and finally (5) significant dynamical downregulation of the shTBX2 signature was noted during Tg(TH-MYC) driven NB formation in transgenic mice, at one, two and six weeks after birth38 (Supplementary Fig. 6c).

To further experimentally explore this presumed cooperation between TBX2 and MYCN, we assessed the effects of TBX2 KD in the presence of high versus low MYCN levels. Using this approach, we observed a stronger decrease in cell proliferation and increased G1-phase arrest when combining TBX2 and MYCN KD together (Fig. 6b, c). Next, we performed RNA-sequencing to further explore the transcriptional effects of individual versus combined MYCN and TBX2 KD and confirmed synergistic effects on expression levels of gene sets implicated in cell cycle and the DREAM-E2F-FOXM1 complex, as well as publicly available MYCN signatures (Fig. 6d). Using iRegulon analysis on the enforced affected genes, motif enrichment of DREAM complex core members, such as FOXM1, E2F4, and MYB2 was observed in the additively downregulated genes, while ChIP-seq targets for EP300 and NANOG (public datasets) were enriched in the upregulated genes (Supplementary Fig. 6d, Supplementary Data 4).

MYCN protein levels were decreased upon TBX2 KD in the MYCN non-amplified CLB-GA cell line, while in the MYCN amplified IMR-5/75 cell line this was not the case (Fig. 4d). While we show that both transcription factors have an effect on each other’s transcriptional activity, the reciprocal effect on expression seems to be complex and might be regulated via feedback loops. The study of these interactions is beyond the scope of this study.
**Fig. 6** Combined TBX2-MYCN signaling targets the FOXM1/E2F network. **a** Heatmap showing GSEA enrichment scores (with FDR < 0.1) for an in house compiled gene set collection containing all MYC target genesets from the "Hallmark V5.1" catalog (MsigDB) as well as publically available MYC(N) activity or target signatures upon knockdown of TBX2 in IMR-5/75, CLB-GA, knockdown of MYCN in IMR-5/75 or the combination of TBX2 and MYCN knockdown in IMR-5/75. Size of the circles indicate FDR value and the color indicates the NES score. Positive values (red) point at enrichment among the upregulated genes while negative values (blue) indicate enrichment among the downregulated genes. **b** Synergistic reduction of cell proliferation upon combined knockdown of MYCN and TBX2 in the IMR-5/75 cell line measured with time-lapse microscopy (every 2–3 h) using the IncuCyte device (one out of three biological replicates is shown). Error bars represent the s.d. of five technical replicates. Bliss independence score was used to calculate potential synergism at 120 h for both the shTBX2 #2 and #4 compared with the double MYCN and TBX2 knockdown. Excess over bliss score for shTBX2 #2 is 0.092, excess over bliss score for shTBX2 #4 is 0.342. Excess over Bliss scores > 0 indicates synergy, < 0 indicates antagonism. **c** Combined TBX2 and MYCN knockdown in the IMR-5/75 cell line results in an enforced G1-cell phase arrest with almost no cells in S-phase or G2/M phase left (one replicate out of 3 shown). **d** Cumulative distribution plot of the absolute log fold changes of shTBX2, shMYCN or shTBX2+shMYCN up- and downregulated genes. Dashed line represents the sum of the log fold changes of the shTBX2 and shMYCN condition for the respective genes in the geneset. The difference between the blue line and the dashed line indicates synergism effect on logFC for the genes in the genesets implicated in cell cycle and MYCN regulation (two-sided paired t-test: \( p < 2.2e^{-16} \))

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Although FOXM1 is not regulated directly by TBX2, MYCN did show binding to the FOXM1 promoter in several NB cell lines (data not shown).

In summary, our data support the cooperation of TBX2 and MYCN regulating the NB driven proliferative cellular state mediated by FOXM1.

**Drugging transcriptional addiction to TBX2 and CRC genes.**

To test the possibility to drug the transcriptional addiction of NB cells to the highly expressed TBX2 gene and CRC genes, we combined the CDK7 inhibitor THZ1, which was previously shown to affect transcription of lineage-dependency genes in NB39, with the bromo-domain inhibitor JQ1, which causes transcriptional repression of SE associated oncogenes40. We observed a significant synergistic effect on cell viability upon evaluation of a concentration range of JQ1 (5.1 nM–33.3 μM) and THZ1 (0.051 nM–0.333 μM) in 5 NB cell lines (i.e. 2 MYCN amplified cell lines Kelly and SK-N-NE(2c) and 3 MYCN non-amplified cell lines CLB-GA, SH-SY5Y, and SK-N-AS) (Fig. 7a), after 72 h treatment. In addition, we expanded this analysis for two primary patient-derived tumor cell lines (one MYCN amplified and one MYCN non-amplified organoid grown in stem cell medium) for which we tested viability for drug combinations after 5 days of treatment and observed even stronger synergism (Fig. 7b). Based on these findings, we selected concentrations of 35 nM for THZ1 and 1 μM for JQ1 to evaluate treatment response over time in eight cell lines (cell lines above and IMR-32 and IMR-5/75), and observed an increasing synergistic effect on cell proliferation and apoptosis in all tested NB cell lines, while MCF-7 remained unaffected (Fig. 7c, Supplementary Fig. 7a, b).

Given that TBX2 was previously implicated in HDAC1 controlled repression of CDKN1A expression and cell cycle arrest in different cancer types20 and the strong observed effects of HDAC inhibitors in combination with other anti-cancer drugs41, we also decided to combine HDAC1 inhibitor Panobinostat together with the CDK7 inhibitor THZ1 (Supplementary Fig. 7c). We observed a significantly synergistic effect over time on cell proliferation and apoptosis albeit only in four out of eight NB cell lines (Supplementary Fig. 7d, e).

Based on these data, we identify combined BET and CDK7 inhibition as a potent synergistic drug combination to target NB cells. To gain deeper insight into the molecular basis of the observed JQ1/THZ1 drug synergism, we performed gene expression profiling after a 10 h treatment with 35 nM THZ1 and 1 μM JQ1 in cell line IMR-5/75. Treatment with the single and combined compounds resulted in a more than two-fold reduction in steady-state mRNA levels with 4.3, 3.1 and 7.9% respectively as compared to the control (Supplementary Fig. 8a). First, we evaluated synergistic effect of combined JQ1 and THZ1 treatment on transcriptional dependency of the NB cells for TBX2 and other CRC genes and confirmed strong reduction in expression levels (Supplementary Fig. 8b). More specifically, all predefined CRC genes in NB cell lines with high TBX2 expression16 or in the (nor)adrenergic module14,15 were significantly downregulated upon combination treatment (Fig. 8a, Supplementary Fig. 8c). Downregulation of TBX2 expression levels was confirmed by qPCR analysis after single compound treatment while combination of THZ1 and JQ1 treatment yielded further downregulation in the high TBX2 expressing IMR-32, IMR-5/75 and Kelly cells (Fig. 8b). Furthermore, PHOX2B, FOXM1, and the LIN28B (implicated in a MYCN-LIN28B regulatory axis)42 mRNA and protein expression levels were also strongly affected (Fig. 8b, c, Supplementary Fig. 8d).

In addition to the CRC genes, we also observed dramatic altered gene expression patterns for FOXM1/E2F/DREAM complex core genes upon combination treatment. While these effects are robust, the direct of regulation does not allow an unequivocal interpretation of the effects of drug synergism. For example, MYBL2 is strongly induced and acts, together with FOXM1 as an activator of genes driving cell cycle progression. One possible explanation is the existence of a feedback loop as reported13 (Fig. 8d, Supplementary Fig. 8e). In addition to the observed effects on CRC genes and FOXM1/E2F/DREAM complex core genes, the drug synergism also effects TP53 pathway response (Fig. 8e), in keeping with the finding that TP53 activation may sensitize transcriptionally addicted cancer cells to THZ1 inhibition44.

Further scrutinizing of the top differentially and synergistically downregulated genes (Supplementary Data 6) contains several important regulators of transcription including several chromatin remodelers such as BPTF, IWS1, and INO80 which can be assumed to be implicated in the drug synergism. In the top upregulated genes upon treatment with JQ1 (p = 0.05), THZ1 (p = 0.05) and the combination (p = 0.01), we noticed HEXIM1, a presumed tumor suppressor which forms an inhibitory complex with P-TEFb and implicated in cell cycle progression and TP53 response45, the two major phenotypic effects observed upon TBX2 KD (Supplementary Fig 80).

Taken together, we propose that the MYCN-TBX2 CRC represents an important novel therapeutic vulnerability for high-risk NB, warranting future clinical trials to assess available BET and CDK7 inhibitors.

**Discussion**

The high-risk NB genome is dominated by DNA copy number alterations, the most prominent being MYCN amplification occurring in roughly half of these cases. We and others have previously shown that chromosome 17q gain is the most frequent alteration in both MYCN amplified and non-amplified high-risk NB7–9. In addition, the syntenic region in MYCN-driven mouse NB also undergoes copy number increase10. Recently, SE marked master transcription factors were identified in NB that co-occupy most enhancers and form an auto-regulatory loop what has been called a CRC14–16.

We hypothesized that expression levels and activity of one or more CRC constituents could be affected through chromosome 17q copy number gains. To test this hypothesis, we ranked transcription factors on chromosome 17q based on H3K27ac mark and patient survival, and identified TBX2 as top candidate. TBX2, which has thus far not been studied in NB, is most highly expressed in this tumor entity as compared to other tumor entities and also strongly upregulated in mouse neural crest-derived MYCN overexpressing NB46. In addition to the common MYCN amplification, rare amplicons have been shown to impact on activity of important NB genes as has been illustrated for ALK and LIN28B47,48. We also reported recently that such rare amplicons have strong negative impact on survival in keeping with an important role of target genes in these rare amplicons in the general NB tumor biology and clinical behavior19. In the light of these observations, the finding of TBX2 amplification in two NB cases further supports the biological relevance of this gene.

Analysis of the present TBX2 and MYCN occupancy data and available data for CRC constituents such as GATA3, PHOX2B, and HAND2 provided compelling evidence that TBX2 also functions as a CRC gene in NB. Dysregulation of these CRCs has been proposed to result from oncogenic master transcription factors, altered transcription of one or more CRC downstream signaling genes or a more global downstream perturbation due to invasion of a transcriptional amplifier30. While our current understanding of the specific mode of action and contribution of
Fig. 7 Combined CDK7-BET inhibition as a novel therapeutic approach. a Heatmap with the percentage of cell viability for serial dilutions of JQ1 (5.1 nM–33.3 μM) in combination with THZ1 (0.051 nM–0.333 μM) in Kelly, SK-N-BE(2c), SK-N-AS, IMR-32, CLB-GA, and SH-5YSY), 72 h upon treatment; and respective 3D representation of Excess over Bliss scores. Excess over Bliss scores >0 indicates drug synergy, <0 indicates drug antagonism. Data points in the screen represent mean of two technical replicates. b Heatmap with the percentage of cell viability for serial dilutions of JQ1 (5.1 nM–33.3 μM) in combination with THZ1 (0.051 nM–0.333 μM) in two organoids (one MYCN amplified and one MYCN non-amplified, 5 days upon treatment); and respective 3D representation of Excess over Bliss scores. Excess over Bliss scores >0 indicates drug synergy, <0 indicates drug antagonism. Data points in the screen represent mean of two technical replicates. c Synergistic effect on proliferation (% confluency) over time for the cell lines CLB-GA and IMR-5/75 upon treatment with 35 nM THZ1 and 1 μM JQ1. One biological replicate out of three is shown, error bars represent the s.d. of the three technical replicates. Microscopic pictures of the cells 60 h upon treatment are depicted at the right (scale bar is 100 μm). Red staining of the cells indicates apoptosis (AnnexinV positivity). Bliss score was used to calculate potential synergism for 7 NB cell lines and the negative control MCF-7 breast cancer cell line. Excess over Bliss scores >0 indicates drug synergy, <0 indicates drug antagonism.
**Fig. 8** Downregulation of the CRC upon combined CDK7-BET inhibition. **a** Significant downregulation of the Module 1 CRC gene signature upon treatment with 1µM JQ1, 35 nM THZ1 and the combination in the IMR-5/75 cell line for 10 h. Kruskal–Wallis followed by a post-hoc Dunn’s multiple comparisons test (four biological replicates per condition). **b** log10 mRNA expression of various genes in IMR-5/75 and Kelly cell lines upon treatment with 1µM JQ1, 35 nM THZ1 and the combination of JQ1 and THZ1. **c** Heatmap showing the expression levels for the E2F-Dream complex core members upon treatment with 1µM JQ1, 35 nM THZ1 and the combination of JQ1 and THZ1 in the IMR-5/75 cell line. **d** Significant upregulation of the TP53 Hallmark geneset (MsigDB) upon treatment with JQ1, THZ1 and the combination in the IMR-5/75 cell line for 10 h. Kruskal–Wallis followed by a post-hoc Dunn’s multiple comparisons test (four biological replicates per condition). *p < 0.05, **p < 0.01, ***p < 0.001
the CRC to the complex NB phenotype is limited, some insights are emerging. First, the work by Zeid et al.16 revealed MYCN invasion of enhancers with canonical and non-canonical MYC (N) recognition sites in a dose-dependent fashion as one major driving event for the oncogenic CRC16. Secondly, GATA3 has been shown to act in de novo enhancer establishment as a pioneering transcription factor51. Thirdly, PHOX2B and HAND2 are typical lineage identity genes52 for which it can be assumed MYCN hyperactivity perturbs their normal epigenetic dynamic regulation leading to differentiation arrest. Here, we add TBX2 as a novel actor in this complex circuitry. Although TBX2 has some properties of a lineage dependency factor, overexpression and amplification of TBX2 has been associated with poor prognosis in various other cancer entities and TBX2 is also a well-known developmental gene acting in various tissues including neural crest53,54. TBX2 has been described as a potent growth-promoting factor, partly due to its ability to bypass senescence and to repress key negative regulators of the cell cycle such as p2120, which was confirmed as a repressed TBX2 target gene in the present study.

In keeping with this finding, TBX2 repressed genes in both CLB-GA and IMR-5/75 NB cells were found to be enriched for TP53 targets while common activated genes were enriched for FOXM1 targets, more specifically p53–p21–DREAM–E2F/CHR pathway known to control G2/M transition55. A number of the FOXM1 G2/M target genes are of particular interest, i.e. BRIP1 and RRM2 which are implicated in control of replicative stress in NB (unpublished data), RAD51 implicated in homologous DNA repair and FANCI, FANC2 and FANC CG involved in replication fork stability and interstrand cross-link DNA repair. Together with data from previous reports, we propose that TBX2 repression of p21 enforces MYCN controlled p21 repression56,58 which is followed by activation of CDK2/cyclin A, p107/p130 phosphorylation and finally DREAM complex repression. More directly, both MYCN and TBX2 activate FOXM1 expression and activity. In summary, we speculate that TBX2 plays a crucial role within the NB CRC, in a cooperative MYCN/TBX2 regulated p53–p21–DREAM–CDE/CHR pathway controlling G2/M cell cycle genes. Together with data from previous reports, we propose that TBX2 repression of p21 enforces MYCN controlled p21 repression56 which is followed by activation of CDK2/cyclin A, p107/p130 phosphorylation and finally DREAM complex repression. More directly, both MYCN and TBX2 activate FOXM1 expression and activity. In summary, we speculate that TBX2 plays a crucial role within the NB CRC, in a cooperative MYCN/TBX2 regulated p53–p21–DREAM–CDE/CHR pathway controlling G2/M cell cycle genes.57–59. Finally, a function in epithelial-mesenchymal transition (EMT) has been attributed to TBX222. While we found no evidence for a direct role for TBX2 in transition from adrenergic towards mesenchymal/neural crest-like phenotype of NB cells, in NB tumors TBX2 expression levels were highly negatively correlated with those for ZEB2, known to be implicated in cell fate switch and EMT60.

In this study, we also identified TBX2-AS1 as a long noncoding RNA tightly co-expressed with TBX2 suggesting cis-regulation, but its expression is unexpectedly increased upon TBX2 and MYCN KD, possibly due to a compensation mechanism in response of attenuated TBX2 and MYCN expression levels. Of further notice, this lncRNA has been recently proposed as a bidirectionally transcribed putative tapRNAs. Whether this is a common component of the complex regulation of CRC constituents remains to be studied.

Transcriptional addiction is emerging as an important novel drug vulnerability in cancer. Targeting multiple CRC constituents and SE driven downstream genes may prevent resistance or enhance sensitivity to BRD4 inhibitors, as JQ1 blocks the binding of BRD4 with acetylated histones, rather than targeting BRD4 itself, which is still recruited to SEs and contributing to phase separation of chromatin domains50. Based on this rationale, we tested transcription addiction as a possible “Achilles heel” for the highly active transcribed genes in NB59 and observed strong synergistic effects on cell growth and apoptosis for combined BET and CDK7 inhibition. By combining JQ1 and THZ1, we were able to target and induce a collapse of the SE-driven transcriptome including TBX2 and MYCN. Another potential mechanism explaining the observed synergism could be modulating the p53 transcriptional program by JQ1 or TBX2 inhibition, which sensitizes the cells for CDK7 inhibition, as described recently in colon cancer43, rather than targeting a dependency on transcriptional activation by TBX2 itself. In accordance with the strong TBX2 downregulation, JQ1/THZ1 combination drugging affected the FOXM1–DREAM regulated target genes. Finally, several additional downregulated genes could also be envisioned to contribute to the observed drug synergism. The chromatin remodeler BPTF is a MYC interactor required for MYC chromatin recruitment and transcriptional activity62. The chromatin remodeler IWS1 is the binding partner of SPIT6, an interaction which is induced by P-TEFB phosphorylation of RNAPII CTD and results in binding of the ALYREF mRNA export adaptor through binding nascent mRNA63. ID1 is a member of helix-loop-helix (HLH) family of proteins that regulate gene transcription through inhibitory binding to basic-HLH transcription factors and is involved in the repression of cell differentiation and activation of cell growth64. INO80 is an ATP-dependent chromatin remodeling complex involved in transcriptional regulation, DNA repair, replication fork stabilization, and restart57. In the list of upregulated genes one of the most differentially regulated ones is HEXIM1. This is intriguing given the well-established role of HEXIM1 in the switch of transcriptional programs through inhibiting P-TEFB and as sensitizer for BET inhibition. In addition, overexpression of HEXIM1 upregulates expression levels of p53 and p53 target genes by blocking p53 ubiquitination mediated by MDM2.

Of further notice, the observed drug synergism was particularly strong in primary tumor-derived cell lines (organoids) established prior to therapy. This further supports the importance for expanding in vitro testing with cell lines closer resembling the real clinical situation for optimal assessment of drugging effects. In conclusion, we identified TBX2 as a novel CRC constituent in high-risk NB contributing to the proliferative cellular state of these cells and offering opportunities for novel drugging strategies. Our data pave the way for more in-depth studies towards understanding the effects of drugging of transcriptional addiction as a guide towards novel therapies for NB.

Methods

Cell culture and generation of stable cell lines. All patient specimens and samples were used in accordance with institutional and national policies, with appropriate approval provided by the relevant ethical committees at the respective institutions. All patient-related information was anonymized. All NB cell lines used in this manuscript (genotype and mutation status in Supplementary Table 1), the HEK-293TN and MCF-7 cell line were grown in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-Glutamine and 100 IU/mL penicillin/streptavidin (referred further as complete medium) at 37 °C in a 5% CO2 humid atmosphere. The IMR-5/75 shMYCN cell line was grown as previously described58 with Tetracyclin-free FBS to avoid leakage in experiments where KD of MYCN was not desired; as such when we refer to IMR-5/75 in some experiments, this means that the IMR-5/75 shMYCN cells were used and grown in Tetracyclin-free medium. Short tandem repeat (STR) genotyping was used to validate cell line authenticity prior to performing the described experiments and Mycoplasma testing was done on a monthly basis. Patient-derived neuroblastoma tumor organoids (Kholosy et al., unpublished) were grown in Dulbecco’s modified Eagle’s medium (DMEM)-GlutaMAX containing low glucose and supplemented with 20% (v/v) Ham’s F-12 Nutrient Mixture, B-27 Supplement minus vitamin A, N-2
Supplement, 100 IU/ml penicillin, 100 µg/ml streptomycin, 20 mg/ml epidermal growth factor (EGF), 40 ng/ml fibroblast growth factor-basic (FGF-2), 200 µg/ml insulin (IGF-1), 10 µM 2-mercaptoethanol (ME). Human sodium butyrate (5 µM) was added to the culture medium. In case of differentiation, DMSO was added to the concentration range experiments of SH-SY5Y and SK-N-BE(2c) to fulfill the criteria for classical NB cell lines and patient-derived NB organoids was determined in a checkerboard fashion. Cell lines and organoids were seeded in 384-well plates in a total volume of 5 µl per well in DMEM/F12 medium and were left untreated for 10–14 days in a humid incubator at 37 °C. After an initial evaluation under the microscope, the colonies were stained with 0.005% crystal violet and digitally counted using ImageJ. The IncuCyte® Live Cell imaging system (Essen BioScience) was used for assessment of proliferation after TBX2 KD. Briefly, 17.5 × 10⁴ viable CLB-GA and 15 × 10⁴ IMR-32 were seeded in 96-well tissue culture plates (Corning costar 3596) containing complete medium. Cell viability was measured in real-time using the IncuCyte by taking photos every 2 h of the whole well (×4). Masking was done using the IncuCyte® ZOOM Software. For cell cycle analysis, 7 × 10⁴ cells were used in a checkerboard fashion with the HP D300 Digital Dispenser (Tecan). Control cells were treated with DMSO. Cell viability was determined after 72 h (classical cell lines) or 120 h (organoids) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (classical cell lines) or the CellTiter-Glo luminescent assay from Promega (organoids). Cell viabilities of DMSO-treated cells were set to 100%. Excess over Bliss scores (score = 0 indicates drug synergy, >0 indicates drug antagonism) were calculated and subsequently visualized using the R package synergist. Values more than 100% were replaced by 100% for the concentration range experiments of SH-SY5Y and SK-N-BE(2c) to fulfill the criteria for Bliss score calculation.

IncuCyte® assay for assessment of drug synergy. Neuroblastoma cells (CLB-GA, IMR-32, IMR-575, SH-SY5Y, SK-N-BE(2c) and SK-N-AS) and MCF-7 cells were seeded in complete medium and a 1:200 dilution of IncuCyte® Annexin V Red Reagent for apoptosis (Essen Bioscience) in 96-well tissue culture plates (Corning costar 3596) in triplicate at 30% confluence and allowed to recover overnight. The next day, cells were treated with the indicated concentrations of QJ1 (BPS Bioscience), TH2Z1 hydrochloride salt (Medchemexpress) and/or combinations thereof. Cell proliferation and cell death (AnnexinV positivity) were assessed continuously for 68–96 h after treatment by using the IncuCyte® Live Cell imaging system (Essen BioScience), by taking photos every 4–9 h of the whole well. Masking was done using the IncuCyte® ZOOM Software. Possible synergism was calculated using the Bliss method.

Western blot analysis. Proteins were isolated using a RIPA lysis buffer (5 mg/ml sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.1% SDS solution, 0.5% Triton X-100) supplemented with protease inhibitors, and sodium butyrate was added to a final concentration of 50 mM. The cell lysate was loaded onto an SDS-PAGE gel (10% Pre-cast, Bio-Rad), run for 1 h at 150 V and subsequently blotted onto a nitrocellulose membrane. The membranes were probed with the following primary antibodies: anti-TBX2 antibody (SC-17880, Santa Cruz, 1:1000 dilution and sc-514291, with or without anti-Myc antibody (5436 S, Cell Signalling, 1:10,000 dilution), anti-PH0X2B antibody (sc-376997,Santa Cruz, 1:500 dilution), anti-MYC antibody (SC-59939, Santa Cruz, 1:1000 dilution), anti-FoxM1 antibody (5436 S, Cell Signalling, 1:10,000 dilution). As secondary antibody, we used HRP-labeled anti-rabbit (7074 S, Cell Signalling, 1:10,000 dilution) and anti-mouse antibody (7076 S, Cell Signalling, 1:10,000 dilution). As secondary antibodies with the membrane was evaluated using the SuperSignal West Dura Extended Duration Substrate (Thermo Scientific). Pictures were taken with the ChemiDoc It Imaging System (UVP) using the WorksVision software (UVP), quantification of the blots was performed using ImageJ. Uncropped scans of the blots used in the main figures can be found in the Supplementary Figure 9.

Phenotypic assessment of cell lines. For colony formation assays, 2000 viable CLB-GA, IMR-32 and SK-N-AS cells with or without TBX2 KD were seeded in a 6-cm dish in 4 ml complete medium and allowed to grow for 48 h. After the supernatant was removed, cells were reseeded with 2.5 ml complete medium and were left untreated for 10–14 days in a humid incubator at 37 °C. After an initial evaluation under the microscope, the colonies were stained with 0.005% crystal violet and digitally counted using ImageJ. The IncuCyte® Live Cell imaging system (Essen BioScience) was used for assessment of proliferation after TBX2 KD. Briefly, 17.5 × 10⁴ viable CLB-GA and 15 × 10⁴ IMR-32 were seeded in a checkerboard fashion with the HP D300 Digital Dispenser (Tecan). Control cells were treated with DMSO. Cell viability was determined after 72 h (classical cell lines) or 120 h (organoids) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (classical cell lines) or the CellTiter-Glo® luminescent assay from Promega (organoids). Cell viabilities of DMSO-treated cells were set to 100%. Excess over Bliss scores (score = 0 indicates drug synergy, >0 indicates drug antagonism) were calculated and subsequently visualized using the R package synergist. Values more than 100% were replaced by 100% for the concentration range experiments of SH-SY5Y and SK-N-BE(2c) to fulfill the criteria for Bliss score calculation.

Monitoring of synergistic effects of drug combinations. The combined effect of QJ1 (Medchemexpress) and TH2Z1 hydrochloride salt (Medchemexpress) in classical NB cell lines and patient-derived NB organoids was determined in a checkerboard fashion. Cell lines and organoids were seeded in 384-well plates and incubated overnight. Next, cell lines and organoids were co-treated with threefold dilution series of QJ1 (0.1 nM–33.3 µM) and TH2Z1 (0.051 µM–33.3 µM), respectively, in a checkerboard fashion with the HP D300 Digital Dispenser (Tecan). Control cells were treated with 0.25% DMSO. Cell viability was determined after 72 h (classical cell lines) or 120 h (organoids) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (classical cell lines) or the CellTiter-Glo® luminescent assay from Promega (organoids). Cell viabilities of DMSO-treated cells were set to 100%. Excess over Bliss scores (score = 0 indicates drug synergy, >0 indicates drug antagonism) were calculated and subsequently visualized using the R package synergist. Values more than 100% were replaced by 100% for the concentration range experiments of SH-SY5Y and SK-N-BE(2c) to fulfill the criteria for Bliss score calculation.

Real-time quantitative PCR. Total RNA was extracted using miRNeasy kit (Qiagen) according to the manufacturer’s instructions, including on-column DNAse treatment, and concentration was determined with the Nanodrop (Thermo Scientific). cDNA synthesis was performed using the ScriptScript Advanced cDNA synthesis kit from BioRad. PCR mix contained 5 ng of cDNA, 2.5 µl SoLoAdvanced SYBR qPCR supermix (Bio-Rad) and 0.25 µl forward and reverse primer (to a final concentration of 250 nM, IDT) and was analysed on the LC-480 device (Roche) for RT-qPCR. mRNA expression levels were quantified using the ΔΔCt method with the 3 stable reference genes out of 5 reference genes tested (B2M, TBP, YWHAZ, RPLP0 and GUSB). Primer pair sequences can be found in Supplementary Table 2.

Analysis of RNA-sequencing data. RNA quality was determined with the Experion automated electrophoresis system (BioRad) prior to profiling. 250 ng of RNA was purified from CLB-GA upon proteinase K (three biological replicates per condition) and from cell line IMR-575 upon KD of TBX2 and/or MYCN (six biological replicates per condition) was used as input for library preparation with the TruSeq Stranded mRNA Sample Prep Kit from Illumina. 100 ng of RNA from the THZ2, QJ1 and combined druging was used to perform an Illumina sequencing library preparation using the QuantSeq 3' mRNA-Seq Library Prep Kit (Lexogen, Vienna, Austria) according to manufacturer’s protocol. During library preparation 15 PCR cycles were used. Size distribution and quality was evaluated with a high sensitivity DNA Chip on the bio-analyzer (Agilent) and qPCR quantification of the libraries using the Illumina Kapa Library quantification kit (Lightcypher 480 qPCR mix Kapa). RNA-seq libraries were sequenced on the Nextseq500 platform (Illumina) using the Nextseq 500 High Output Kit v2 KIT 72 cycles single-end (Illumina). Sample and read quality was checked with FastQC (v0.11.3). The QuantSeq generated reads were trimmed using cutadapt version 1.11 to remove the "Quant Seq FWD" adapter sequence. Reads were subsequently aligned to human genome GRCh38 with STAR (v2.5.2a) aligner (v2.5.2a). Final gene count values were obtained with RSEM (v1.2.31), which takes read mapping uncertainty into account. Non-locus strand specific read counts were filtered. To explore if the samples from different groups clustered together and to detect outlier samples, Principal Component Analyses (PCAs) on rlog transformed counts were performed using the R statistical computing software. Genes were only retained if they were expressed at counts per million (cpm) one above in at least four, eight or twelve samples for the drugging, shTBX2 in IMR-575 and shTBX2 in CLB-GA datasets respectively. Counts were normalized with the TMM method (R-package edgeR), followed by z-score transformation and differential expression analysis using limma (R-package limma). A general linear model was built with the treatment groups (drugging or KD) and the replicates as a batch effect. Statistical testing was done using the empirical Bayes quasi-likelihood F-test. GSEA was performed on the genes ordered according to differential expression statistic value (t). Signature scores were conducted using a rank-scoring algorithm. Limma volcano plots and heatmaps were used for visualization of gene set enrichment. In order to identify enriched functional classes and (upstream) co-regulators, iRegulon2 and enrichr were used with the default settings.
and sonicated with the M220 Covaris for 15 min to obtain 200–300 bp long fragments. Chromatin fragments were immunoprecipitated overnight using 1 μg for every 2 μl of cell lysate following the appropriate antibody procedure (H3K27ac (SC-78800), H3K4me3 (Ab8850), anti-H3K4me1 (Ab8895), anti-H3K27ac (Ab4729) and anti-MYC (SC-39933), 20 μl protein A UltraLink® Resin beads was added per 107 cells. Beads were eluted and split into two subsamples, one was used for western blot adding 2x laemmli buffer and incubation at 95 °C for 10 min at 1500 rpm to the proteins, the other subset was used for DNA isolation after resuspension in elution buffer and incubation for 22 min at 65 °C while vortexing every 2 min. Reverse crosslinking was done at 65 °C for 1 h. The chromatin for DNA purposes was resuspended in TE-buffer to dilute SDs in the elution buffer to an incubation of 2 h at 37 °C with 0.2 μg/ml RNase and followed by an incubation of 2 h at 55 °C with 0.2 μg/ml proteinase K. DNA was isolated using 400 μl phenol:chloroform:isoamylalcohol (P: C: I) in phase lock gel tubes (5Prime). Upon centrifugation, the aqueous layer was transferred to a new tube with 200 mM NaCl, 30 μg glycerol and 800 μl 100% ethanol, and incubated for 30 min at −20 °C. Upon centrifugation, the pellet was washed with 80% Ethanol and resuspended in RNase/DNase free water. Concentration was measured using the Qubit® dsDNA HS Assay Kit (Thermo Scientific). Library prep was done using the NEBNext Ultra DNA library Prep Kit from Illumina with 50–500 ng starting material and using 8–12 PCR cycles according to the manufacturer’s instructions. Libraries were evaluated on the bio-analyzer using the Agilent high-sensitivity kit, Output kit V2 75 cycles single-end (Illumina) or HiSeq SBS Kit V4 50 cycles. The NextSeq 500 or HiSeq 2000 platform (Illumina) using the Nextseq 500 High Output kit V2 50 cycles single-end (Illumina) or HiSeq SBS Kit V4 50 cycles.

Assay for transposase-accessible chromatin using sequencing. ATAC-seq ( assay for transposase-accessible chromatin using sequencing) was performed as previously described with minor changes61. In short, 50,000 cells were lysed and fragmented using digitonin and TN5 transposa (Illumina). Next, the samples were purified using the MinElute kit (Qiagen). The transposed DNA fragments were amplified and purified using Agencourt AMPure PX beads (Beckman Coulter). The library concentrations were measured with the Illumina Kapa Library quantification kit (Lightcycler 480 qPCR mix) and ChiP-seq libraries were sequenced on the NextSeq 500 or HiSeq 2000 platform (Illumina) using the Nextseq 500 High Output kit V2 75 cycles single-end (Illumina) or HiSeq SBS Kit V4 50 cycles.

ChiP-seq and ATAC-seq data-processing and analysis. Prior to mapping to the human reference genome (hg19) with bowtie2 (v2.3.1), quality of the raw sequencing data was evaluated using FastQC and adapter dimers were removed using cutadapt when necessary. Peak calling was performed using MACS2 taking a q value of 0.05 as threshold. IGV was used for visualization purposes with the IGVtools generated tdf files. Bedgraph files generated by MACS2 were converted to bigwig files and used as input for the ChiP-plots in the figures, further processed using the R package SlepiPlot. Homer was used to perform motif enrichment analysis with 2000 windows (w = 1000 base pairs). Domainograms showing the signal intensity of different ranges are plotted using a custom R-script. Bigwig files for visualization are generated using a smoothing over 21 consecutive fragments, and normalized to the total library size.

4C-sequencing and data analysis. Preparation of 4C samples was performed as described previously62, starting with 106 separated single cells that were cross-linked with 2% formaldehyde. DNA was digested using DpnII as a primary and CspII as a secondary restriction enzyme. 50 ng of the final 4C template of two NB cell lines (CLB-GA and SK-N-AS) was used for the amplification step with three different primer sets (Supplementary Table 2) designed for the TBX2 locus. PCR products were purified and pooled for multiplexed sequencing after adding 40% PhiX to increase sequence complexity. Sequencing was performed on the HiSeq 2000 platform (50 bps single reads). Sequencing reads from the 4C library were demultiplexed using the demultiplex.py script (https://gist.github.com/meren/7632168) and aligned using bowtie2 (v2.3.1) with parameters—sensitivity—time—end-to-end -q -p 2. Aligned data were stored together with the metadata into a FourSeq object64. The aligned libraries were subjected to a QC according to criteria published elsewhere65. Cis- and trans-interactions were determined using a custom script implementing the method described in57,58. Briefly, a z-score is computed on the binned signal (i.e., counting all fragments covered by reads) within a defined window (w = 1000 fragments, W = 3000 fragments), and an FDR is computed from this z-score to identify significant interactions. We use a FDR of 1% for cis-interactions and 0.5% for trans-interactions. Domainograms showing the signal intensity of different ranges are plotted using a custom R-Script. Bigwig files for visualization are generated using a smoothing over 21 consecutive fragments, and normalized to the total library size.

DNA copy number analysis. The presence of focal gains encompassing the TBX2 locus was investigated in published copy profiling data of 556 high-risk tumors66,40. TBX2 focal gains were identified as copy number segments overlapping with the TBX2 locus with log2 ratio > 0.3 and a maximal size of 5 Mb. Visual inspection of the segmented profiles was done using Vizarr40 and R (http://r2.amic.nl/). The coverage whole genome sequencing sample was performed following library construction using the NuGen kit (Ovation® Ultralow Library Systems V2). In brief, 100 ng was used to prepare a Library and 150 bp paired sequenced in a flow cell using the MiSeq V3, to achieve an average of approximately 2 x. Copy number analysis was performed using the FREEC tool48.

Quantification and statistical analysis. Statistical significance of differences between conditions for the functional analysis colony forming assay and cell cycle was determined by a non-parametric Mann–Whitney test using R package (version 3.3.3) on the differential quantifications. The ANOVA test was used to assess how much of the variability in the TBX2 expression levels can be explained by the patient stage or/and TBX2 copy number status, while the non-parametric Kruskal–Wallis followed by a post-hoc Dunn’s multiple comparisons test was used to determine differences in gene expression and signatures scores between 4 different groups with 4 biological replicates per condition. Statistical significance of overlap between conditions was determined by Fisher test using R package. The non-parametric Spearman or parametric Pearson test was used for correlation analysis depending on the homoscedasticity assumption (Pearson if the assumption is met). Kaplan–Meier analysis with log-rank statistics was used for survival analysis. All assumptions for statistical analysis (performed using R) are met and as such justified. For qPCR experiments, reference genes were excluded if Genom M value was greater than five and/or Coefficient of Variation greater than two, according to the qBaseplus software. For all experiments, at least three reference genes were used for the normalization according to good qPCR practice.

The details of quantification and statistical methods used can be found in each figure legend.
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Additional information

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