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## Cross-cohort analysis identifies a TEAD4 ↔ MYCN positive-feedback loop as the core regulatory element of high-risk neuroblastoma

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## Abstract

High-risk neuroblastomas show a paucity of recurrent somatic mutations at diagnosis. As a result, the molecular basis for this aggressive phenotype remains elusive. Recent progress in regulatory network analysis helped us elucidate disease-driving mechanisms downstream of genomic alterations, including recurrent chromosomal alterations. Our analysis identified three molecular subtypes of high-risk neuroblastomas, consistent with chromosomal alterations, and identified subtype-specific master regulator (MR) proteins that were conserved across independent cohorts. A 10-protein transcriptional module – centered around a TEAD4 ↔ MYCN positive-feedback loop – emerged as the regulatory driver of the high-risk subtype associated with *MYCN* amplification. Silencing of either gene collapsed *MYCN*-amplified (*MYCN*<sup>Amp</sup>) neuroblastoma transcriptional hallmarks and abrogated viability *in vitro* and *in vivo*. Consistently, TEAD4 emerged as a robust prognostic marker of poor survival, with activity independent of the canonical Hippo pathway transcriptional co-activators, YAP and TAZ. These results suggest novel therapeutic strategies for the large subset of *MYCN* deregulated neuroblastomas.

## Introduction

Neuroblastoma is a malignancy arising from the developing sympathetic nervous system that typically affects very young children. About half of the cases present with widespread metastatic disease and/or extraordinarily focal amplification of the *MYCN* gene, often with >100 copies. These high-risk patients have poor 5-year survival probability (<40%), despite intensive multimodal treatment regimens (1). Diagnostically, neuroblastomas show relatively few recurrent somatic point mutations (2–5). In contrast, it has been known for decades that high-risk neuroblastomas harbor complex and recurrent somatic structural copy number alterations (CNAs), affecting large chromosomal regions, as well as focal amplification of the *MYCN* oncogene (6). Identification of the causal driver genes associated within these CNAs remains a challenge. Despite significant understanding of the genetic landscape of high-risk neuroblastoma, all newly diagnosed patients are empirically treated with intensive cytotoxic chemo-radiotherapy to achieve disease remission. Thus, further elucidation of the molecular mechanisms responsible of high-risk neuroblastoma pathogenicity is required for guiding novel, more effective and less toxic precision-oncology strategies.

Following on recent results from the assembly and interrogation of regulatory network models (*interactomes*) in human cancers, using systems biology approaches (7), we focused on the discovery of more universal tumor dependencies in high-risk neuroblastomas. This approach has been highly successful in elucidating small regulatory modules, dubbed *tumor checkpoints*, comprising Master Regulator (MR) proteins that mechanistically regulate the transcriptional state of the tumor. MR proteins have been shown to elicit either essentiality or synthetic lethality in several cancers (8–16). Critically, aberrant activity of tumor checkpoint MRs arises from one or more genetic alterations in their upstream pathways (14, 17), thus providing a rationale for the heterogeneous mutational landscape of tumors with highly similar transcriptional states (15). MR proteins have been shown to represent critical molecular tumor vulnerabilities that can be effectively targeted pharmacologically in several human malignancies (10, 11, 16, 18, 19), thus providing novel therapeutic opportunities. Here, we sought to interrogate regulatory networks of high-risk neuroblastomas to identify

and validate MR proteins representing highly conserved, tumor-specific vulnerabilities for specific subtypes of this malignancy.

## Results

Our approach represents the integration of computational inference and experimental validation, both *in vitro* and *in vivo*, to characterize the tumor checkpoint MRs that mechanistically control the transcriptional state of distinct, high-risk neuroblastoma subtypes.

### Characterization of molecular subtypes of high-risk neuroblastoma

We first dissected the heterogeneity of high-risk neuroblastoma gene expression profiles (GEPs) to classify them into molecularly-consistent tumor subtypes. Transcriptionally distinct subtypes were identified using consensus clustering (20) on GEPs of 219 high-risk tumors assembled by the NCI TARGET (Therapeutically Applicable Research to Generate Effective Treatments) initiative, excluding good-prognosis samples (stage 1) (21, 22). Optimal cluster selection identified three distinct high-risk subtypes (Supplementary Table S1), as determined by the relative area under the cumulative distribution function (Supplementary Figure S1A, S1B and S1C). This result was then validated in an independent cohort comprising 97 high-risk samples assembled by the European Neuroblastoma Research Consortium (NRC; Supplementary Figure S1D, S1E and S1F). Cross-cohort analysis of differentially expressed genes in each subtype, compared to the respective low-risk stage 1 samples, revealed a 1:1 correspondence between TARGET and NRC-derived high-risk subtypes (Figure 1A, 1B).

Further clinical and biological characterization of these subtypes identified key distinguishing features (Supplementary Table S1 and S2, respectively). A first cluster co-segregated with *MYCN* amplification status and chromosome band 1p36 deletions (78% and 83% of TARGET and NRC samples, respectively); (Figure 1C, Supplementary Figure S1G, S1H). This predominantly *MYCN*<sup>Amp</sup> (*MYCNA*) subtype showed a highly undifferentiated phenotype and high mitosis-karyorrhexis index (MK; Supplementary Figure S1I and S1J, respectively), potentially resulting from down-regulation of differentiation programs and up-regulation of cell proliferation and cell growth pathways, respectively (Figure 1E, 1F). While the majority of *MYCNA*-subtype samples harbored *MYCN* amplifications resulting in *MYCN* over-expression, a few samples appeared to compensate for low *MYCN* mRNA levels by significant *c-MYC* (*MYC*) over-expression. Consistent with previous findings that *MYCN*/*MYC* deregulation in neuroblastoma may occur via transcriptional deregulation mechanisms, independent of their genomic amplification status (23, 24). All samples in this subtype showed high *MYCN*/*MYC*-specific signature activity (25) (Supplementary Figure S1M and S1N). While samples in other high-risk subtypes presented broad 2p gains, the *MYCNA* subtype is the only one showing highly focal amplification of the *MYCN* locus (Figure 1C), consistent with the fact that non-focal 2p gain is not associated with *MYCN* overexpression (26, 27).

A second cluster was characterized by hemizygous deletions of 11q (90% and 89% of cases in TARGET and NRC respectively) and was thus referred to as the 11qLOH subtype (Figure

1C, and Supplementary Figure S1G and S1H). Overall, the clinical profile (Supplementary Figure S1I and S1J) and pathway enrichment pattern was similar to the MYCNA subtype (Spearman correlation = 0.79; Figure 1E), including enrichment of proliferation and cell cycle categories and negative enrichment of differentiation pathways. Yet, this subtype exhibited inverse association with MYCN expression and activity (Supplementary Figure S1M, S1N and S2I), as well as with activation of immune related pathways (Figure 1E).

In contrast, the third subtype did not appear to be strongly associated with specific genomic alterations (Figure 1A, 1C and Supplementary Figure S1G, S1H) and did not present with hyper-proliferative programs activation (Figure 1F and 1G). Similar to the 11qLOH subtype, however, it displayed activation of immune related pathways. Interestingly, this subtype presented a strong mesenchymal signature, highly similar to the one previously reported for high-grade glioma (Figure S2 E-H) (28) and will thus be referred to as the mesenchymal (MES) subtype.

To further disentangle tumor specific signatures from those of tumor infiltrating compartments in 11qLOH and MES subtypes, we used the ESTIMATE algorithm, which allows inferring both the stromal and immune fractions in each sample (29) (Supplementary Figure S2 A–D). Integration of immune and stromal components by ESTIMATE suggested that the MES and a subset of the 11q-LOH subtypes are characterized by lower purity, likely due to either a larger Schwannian stromal and/or immune reactive cellular infiltrate (Supplementary Figures S2 A–D, S2I). This is consistent with the previous reports of higher fraction of tumor infiltrating lymphocytes in *MYCN*<sup>WT</sup> high-risk neuroblastomas (30). The stromal component enriched in these subtypes was confirmed by high fraction of stroma-rich ganglioneuroblastomas (Supplementary Figure S1K) (31). Conversely, MYCNA subtype samples showed minimal immune signature contamination, confirming known MYCN-related mechanisms of tumor immune suppression (32–34).

To assess whether the signatures emerging from cluster analysis represent cell-autonomous or microenvironment-related mechanisms, we tested whether they could be recapitulated in a panel of high-risk human neuroblastoma-derived cell lines. Interestingly, the MES signature was strongly conserved in cell lines (Supplementary Figure S2F). Single sample GSEA (35) identified SKNAS as the neuroblastoma cell line with strongest MES signature enrichment (NES = 4.54,  $P = 5.5E-6$ ) (Supplementary Figure S2G and S2H). This confirms the tumor-cell autonomous mesenchymal nature of this subtype, since cell line cultures lack stromal or immune cell contamination.

In summary, MYCNA and 11q-LOH subtypes displayed high activity of proliferative programs; the 11q-LOH and MES subtypes showed high immune and stromal infiltration while the MES subtype showed cell-autonomous activation of mesenchymal programs (Supplementary Figure S2I). Overall, all three subtypes were associated with poor survival in both TARGET and NRC datasets (Supplementary Figure S1O and S1P), suggesting fundamentally different mechanisms leading to disease metastasis and ultimate therapy resistance.

## Inference of subtype-specific master regulators of high-risk neuroblastoma

Next, we inferred subtype-specific candidate master regulator (MR) proteins by independent analysis of TARGET and NRC cohort datasets. We first assembled TARGET and NRC specific interactomes from cohort-specific neuroblastoma GEPs, using ARACNe-AP (36), the latest version of ARACNe – an established tool for the reverse engineering of transcriptional targets of regulatory proteins (37). The TARGET and NRC interactomes comprised 205,271 and 359,846 transcriptional interactions respectively (Supplementary Table S3), 81,035 of which were overlapping ( $P < 1E-16$  by Fisher's Exact Test, odds ratio = 65.02, Supplementary Figure S3A).

Candidate MR proteins for each of the high-risk subtypes were then prioritized based on the enrichment of their transcriptional target genes in the subtype-specific signature, using the VIPER algorithm (19). Specifically, we used signatures representing the differential gene expression of each high-risk subtype compared to stage 1 samples (good-prognosis), which showed significant overlap between the TARGET and NRC datasets (Supplementary Figure S3B). Furthermore, despite completely independent MR analysis in the TARGET and NRC cohorts, top-ranking MR proteins for each molecular subtype were remarkably consistent. Indeed, overlap computed by one-sided Fisher's Exact Test of the first 50 MRs of each subtype was highly significant ( $P_{MES} = 5.18E-11$ ;  $P_{11qLOH} = 4.73E-35$ ;  $P_{MYCNA} = 4.34E-33$ ) (Supplementary Figure S3C and S3D). This confirms the robustness of the analysis, independent of cohort bias and composition. This was further confirmed by Gene Set Enrichment Analysis (38) ( $|NES| > 4$ ;  $P < 1E-4$ ) for the MYCNA subtype (Figure 2A). Given the exceedingly high congruence of the MRs identified from the two cohorts, we finalized candidate MR rank by Fisher's integration of their cohort-specific  $p$ -values (Figure 1D and Supplementary Table S4).

## Experimental validation of MYCNA subtype specific master regulators

We then proceeded to experimentally validate candidate MRs of the MYCNA subtype. We focused on this subtype for several reasons: (a) it is strongly associated with the most recurrent neuroblastoma-specific genetic alteration (39) (b) it is associated with aggressive disease and poor prognosis (40) (c) it shows the highest purity in terms of immune and stromal infiltration (Supplementary Figure S2), thus resulting in high-quality GEPs for MR predictions, and (d) it has the largest number of established cell lines (as matched by both MR and genetic analysis) for validation purposes (Supplementary Figure S4). The 25 most significant candidate MRs of the MYCNA subtype were prioritized for experimental validation (Figure 2B).

To identify optimal subtype representatives and appropriate negative controls for validation purposes, we analyzed a panel of 25 well-characterized neuroblastoma cell lines. Cell lines harboring *MYCN* amplifications as well as having high MYCN expression and signature activity (Supplementary Figure S4 A–D) were selected as candidate representatives of the MYCNA subtype and further refined based on the activity of the top 25 VIPER-inferred MRs selected for validation (Supplementary Figure S4E) (19). Most *MYCN*<sup>Amp</sup> cell lines showed high activity of these proteins, with BE2 identified as the model with the most statistically significant MR-activity match by enrichment analysis ( $P = 3.7E-24$ ;



Supplementary Figure S4E). Negative controls were prioritized based on low MYCN/MYC expression and low VIPER-inferred activity of selected MRs, including MYCN.

To validate MYCNA-subtype MRs, we performed loss-of-function assays by silencing each candidate MR in both MYCNA and control cell lines. Multiple RNAi screenings were used to mitigate false discovery resulting from off-target effects and technology specific biases. We tested a panel of MYCN-amplified and control cell lines for transduction efficiency and only cells exhibiting high transduction efficiency were chosen for validation studies (Supplementary Figure S4F).

Pooled, *in vitro* shRNA screens were performed in BE2 and IMR5 cells (MYCNA subtype) and NLF and SKNAS cells (negative controls). NLF was included as an informative negative control because, despite its *MYCN*<sup>Amp</sup> status, it showed both low MYCN expression (Z-score = -0.74) and MYCN activity (NES = -3.31; Supplementary Figure S4D), as well as no enrichment of MYCNA MRs (Supplementary Figure S4E).

MRs representing critical MYCNA-subtype specific dependencies were assessed by evaluating the depletion of shRNA hairpin representation at 28-day post-infection. We then further confirmed the results from these assays *in vivo*, to ensure a more physiologic environment for MR validation, using BE2 and SK-N-AS xenografts, based on their known tumorigenicity in immune deficient mice (Supplementary Experimental Procedures). Overall, genes were prioritized as *bona fide* MRs if (a) >2-fold decrease in hairpin representation was observed in MYCNA cells ( $p < 0.05$ ,  $z$  score  $< -1.96$ ) and (b) the ratio between MYCNA and control group hairpin representation was >2-fold, in both the *in vitro* and the *in vivo* screen (Supplementary Table S5). Using this very conservative criterion, we identified MYCNA-specific MR dependencies, common to both *in vitro* and *in vivo* screens, including: *MYCN*, *TEAD4*, *HNRNPAB*, *HMGB2*, *PRDM8*, *E2F3*, and *ECSIT* (Figure 2C, 2D).

To further validate key MRs, we next performed lentivirus-mediated shRNA silencing of each candidate MR in an extended panel of cell lines, using the two shRNA hairpins with the highest silencing efficiency (Supplementary Figure S5A, Supplementary Table S5), and assessed their cell viability (Supplementary Table S5, Supplementary Experimental Procedures). These shorter-term assays (at 72–96h post transduction) show that MYCNA cells were more sensitive to *TEAD4*, *TAF1D*, *HNRNPAB* and *ECSIT* silencing, compared to control cell lines (Figure 2E, Supplementary Table S5). We did not detect *MYCN* as a significant hit in this screen, at the selected time point, likely due to the very high *MYCN* copy number in MYCNA cells and technical difficulty to achieve sustained silencing. However, we confirmed that *MYCN* silencing in BE2 cells induced differentiation and viability reduction, at 7-day post-transduction (Supplementary Figure S5B and S5C). We reasoned that shorter-time assays (<96h) are better suited for detecting MRs producing direct effect on proliferation, while longer-term assays (>96h) are optimally suited for elucidating multifunctional dependencies. Consistently, additional MYCNA specific MR dependencies were detected in long-term (28-day) pooled shRNA screening (Figure 2C, 2D).

To reduce the effect of potential off-target shRNA effects, we used an orthogonal silencing mechanism with ON-TARGETplus siRNA pools containing a mix of four siRNAs against each of the 25 MRs (Supplementary Experimental Procedures). By comparing the average relative cell viability upon siRNA-mediated MR silencing in MYCNA cell lines and control cell lines at 96h post-transfection (Supplementary Figure S5D, Supplementary Table S5), we confirmed *TFAP4*, *HNRNPAB*, *MYBL2*, *TEAD4* and *ZNF219* as MYCNA specific dependencies (Figure 2F, Supplementary Table S5).

Taken together, comparative analysis of the three screens identified eleven potential MYCNA-specific MR dependencies (Figure 2G), which included both novel and previously reported TFs associated with the MYCNA tumors, including MYCN (41), MYBL2 (42) and E2F3 (43).

### **TEAD4 and MYCN control the MYCNA subtype master regulatory module**

We have proposed that the stability of tumor-related phenotypes is controlled by tightly auto-regulated MR protein modules (tumor checkpoints) that mechanistically regulate the transcriptional state of the cancer cell (9, 15). To test this hypothesis, we assessed the ability of MYCNA-specific MRs to mechanistically regulate each other, as well as the MYCNA-subtype transcriptional signature. To elucidate the causal control architecture of the corresponding tumor checkpoint, we performed lentiviral-mediated shRNA silencing of each of the eleven MYCNA-specific MRs that emerged from the *in vitro* and *in vivo* RNAi screens (Figure 2G), followed by qRT-PCR analysis of all other MRs (Supplementary Figure S6A).

To select the most likely transcriptional interactions, we focused on MRs whose transcripts were strongly down-regulated (> 1.5-fold) following silencing of another MR (Figure 3A). This analysis revealed a highly modular and hierarchical 10-MR tumor checkpoint architecture (Figure 3B). Note that the ECSIT protein was eliminated since it was neither significantly regulating nor was regulated by any other MR. TEAD4 emerged as the most critical MYCN effector, strongly regulating 5 of the remaining 8 MYCNA tumor checkpoint MRs. Taken together, MYCN and TEAD4 emerged at the top of the regulatory hierarchy and were jointly responsible for regulating all but one (HNRNPAB) of the other MRs, while the latter was regulated indirectly by both MYCN and TEAD4 via MYBL2. MYCN and TEAD4 thus emerged as the core control unit of the MYCNA tumor checkpoint and thus *bona fide* MRs of the MYCNA subtype.

Immunoblotting assays confirmed that MYCN silencing in BE2 cells down-regulated TEAD4 at the protein level (Supplementary Figure S5E). Furthermore, chromatin immunoprecipitation (ChIP) assays in BE2 cells, using a MYCN-specific antibody, confirmed MYCN binding to the promoter region of 3 of 4 predicted targets, including *TFAP4*, *MYBL2* and *TEAD4* (Supplementary Figure S5F).

To gain further insights into the regulatory role of TEAD4, we performed chromatin immunoprecipitation with a TEAD4-specific antibody, followed by next generation sequencing (ChIP-seq) in BE2 cells. Phantom peak quality assessment (44) of the libraries confirmed a strong signal-to-noise ratio in the experiment (Supplementary Figure S6B,



S6C). Additional quality checks after peak calling indicated a high TF-binding sequence motif affinity (Supplementary Figure S6D), as well as cell type specificity (Supplementary Figure S6E, S6F). A remarkable similarity of BE2 and SKNSH (both neuroblastoma derived cell lines) was observed, compared to other cell types, supporting the consistency of ChIP-assay results with tissue of origin.

Significant TEAD4 peaks were identified in the promoters or enhancers of 2 of 5 predicted target MRs from the perturbational analysis (Figure 3B), including *HMGB2*, *PRDM8*, as well as of *MYCN* (Supplementary Table S6), further supporting the mechanistic nature of the tumor checkpoint architecture predicted by our perturbational analysis and suggesting a possible TEAD4 ↔ MYCN autoregulatory loop. Consistently, the strong effect of TEAD4 silencing on the remaining three genes (*ZNF219*, *TFAP4*, and *MYBL2*) can be explained as being mediated by indirect interactions via *HMGB2* and *PRDM8* (Figure 3B).

Taken together, this analysis suggests a highly interconnected tumor checkpoint module, rich in autoregulatory loops, with TEAD4 representing the key effector of aberrant MYCN activity and the two proteins jointly regulating all other tumor checkpoint MRs, either physically or indirectly, via another MR.

### TEAD4 and MYCN drive MYCNA subtype gene expression signature

To further validate the functional role of the TEAD4 ↔ MYCN feedback loop as the core regulatory element of the MYCNA tumor checkpoint, we assessed the global effect of TEAD4 and MYCN silencing on the MYCNA signature. Lentiviral-mediated shRNA silencing of TEAD4 and MYCN was performed independently in BE2 cells, followed by RNA-seq profiling. To enrich for direct targets, we analyzed the results at 48hrs post-transduction and confirmed that their silencing was not yet affecting the other protein levels (Supplementary Figure S6G). MYCN and TEAD4 silencing both significantly reversed the MYCNA-signature towards a stage 1 tumor signature (Figure 3C) as confirmed by GSEA analysis ( $P_{shMYCN} = 1.87E-5$ , Figure S6H;  $P_{shTEAD4} = 1.81E-6$ ; Figure S6I). Taken together, MYCN and TEAD4 differentially expressed genes comprise ~70% of MYCNA subtype differentially expressed genes (Figure 3C, 3D). However, individually, MYCN and TEAD4 regulated a highly complementary set of targets, with only a small fraction of common targets, thus suggesting a complementary role (Figure 3D).

Next, to evaluate the biological programs controlled by these two TFs, we performed REACTOME pathway and Gene ontology (GO) biological processes enrichment analysis of the overlap between genes differentially expressed following their silencing and the MYCNA gene expression signatures. MYCN silencing significantly reversed key activated (cell growth and proliferation) and inactivated (differentiation) programs in the MYCNA signature (Figure 3E, 3F and Supplementary Table S7). In contrast, TEAD4 silencing specifically reversed key activated programs in the MYCNA signature (proliferation and DNA damage response) (Figure 3E, 3F and Supplementary Table S7). Consistent with previous reports, this suggests that, while MYCN acts as both an activator and a repressor (45, 46), TEAD4 acts mainly as a transcriptional activator (47). This was confirmed by the dramatic difference in the overlap of TEAD4 ChIP-seq binding sites and TEAD4-activated targets ( $P = 4.05E-42$ ) vs. repressed targets ( $P = 1.9E-2$ ) (Figure S6J). Consistently, the

ARACNe-AP-inferred MYCN regulon had an equivalent number of positively regulated and repressed targets, while the TEAD4 regulon consisted mostly of positively regulated targets (Supplementary Figure S6K, S6L); GSEA analyses confirmed that their ARACNe-AP-inferred regulons were highly enriched in genes differentially expressed following their RNAi-mediated silencing (Supplementary Figure S6M, S6N), thus confirming the overall validity of the regulatory model.

Consistent with its established role, MYCN-specific activated genes were highly enriched in cell-growth/metabolism programs, including protein biosynthesis, ribosomal biogenesis, rRNA processing, RNA processing and splicing as well as cell proliferation programs (Supplementary Table S7). In contrast, genes repressed by MYCN were enriched in neuronal differentiation, actin cytoskeleton organization, axon guidance and cell adhesion molecules, possibly mediated by miR-17-92 cluster activation (48) or by MIZ-1 mediated repression (49) (Supplementary Table S7). In line with these findings, we observed neurite outgrowth and neuronal differentiation upon MYCN silencing in these cells (Supplementary Figure S5C). On the other hand, TEAD4 induced highly significant activation of proliferative and DNA damage response (DDR) programs but only in cells where MYCN was also active (Supplementary Table S7). Remarkably, both proliferative and DDR-related genes were strongly enriched in a subset of 125 genes that were directly activated by TEAD4 in the MYCN subtype up-regulated signature (Figure 3G and Supplementary Table S6 and S7). Potential involvement of TEAD4 in regulating DDR-related genes was especially intriguing as it was not previously associated to its canonical role as transcriptional effector of the Hippo pathway, suggesting an aberrant role in neuroblastoma; specifically, we observed activation of genes involved in ATR-mediated response to replication stress (Supplementary Figure S7A), whose inhibition has been shown to reduce MYCN-driven neuroblastoma viability (50, 51). Consistently, we observed increase in  $\gamma$ -H2Ax following TEAD4 silencing in MYCN cell lines, BE2 and LAN1 (Supplementary Figure S7B, S7C). Functional validation of regulation of proliferation by TEAD4 will be discussed in the following sections. Finally, we assessed the regulatory mechanism between TEAD4 and other TEAD proteins and between MYCN and TEAD family proteins. TEAD4 silencing induced significant TEAD1 and TEAD2 downregulation (Supplementary Table S7), suggesting that, within this subtype, TEAD4 is the dominant driver. In addition, the regulation of TEAD family members by MYCN and TEAD4 varied (Supplementary Table S7), suggesting complex regulatory mechanisms between these proteins.

### **TEAD4 positively regulates MYCN, both transcriptionally and by inhibiting its proteasomal degradation, resulting in a positive-feedback loop structure**

Next, following up on the ChIP assays showing binding of MYCN in the TEAD4 proximal region and vice-versa (Supplementary Figure S5F, Supplementary Table S6 and Figure 3B), we proceeded to further elucidate the regulatory interaction between these two proteins. Specifically, we performed time-dependent assessment of MYCN expression and protein abundance following TEAD4 silencing in BE2 cells. This study showed small but significant MYCN mRNA downregulation following TEAD4 silencing (Figure 4B), supporting TEAD4-mediated MYCN transcriptional regulation and confirming the functional nature of

the ChIP-Seq findings. Yet, down-regulation of MYCN at the protein level was far more prominent (Figure 4A), suggesting a strong post-translational regulatory interaction.

No evidence of direct TEAD4 ↔ MYCN protein-protein interaction could be detected by co-immunoprecipitation assay. Thus, to identify additional proteins that may mediate the interaction, we assessed gene expression changes of established modulators of MYCN protein turnover, such as AURKA, FBXW7, HUWE1, TRPC4AP and CDK1 complex (52–56), following TEAD4 silencing. Both AURKA and CDK1 emerged as significantly down-regulated (AURKA: 2-fold;  $P = 2.48E-07$ ; CDK1: 2-fold;  $P = 1.38E-07$ ) (Supplementary Table S7). We further confirmed that TEAD4 regulates AURKA and CDK1 both at the protein and at the gene expression level (Figure 4A and 4B), with TEAD4 bound to CDK1's proximal region by ChIP-seq (Supplementary Table S6), thus confirming TEAD4-mediated regulation of established MYCN modulators. These interactions were confirmed in an additional MYCNA cell line, LAN-1, albeit to a lesser degree (Supplementary Figure S7D, S7E), in line with its lower enrichment in MYCNA MR signature proteins (Supplementary Figure S4E). Consistent with these findings, treatment with cycloheximide increased MYCN turnover by 2-fold following TEAD4 silencing, compared to control cells (Figure 4D, 4E), which was rescued by the proteasome inhibitor MG-132 (Figure 4F, 4G).

Stabilization and degradation of both MYCN and MYC proteins requires sequential phosphorylation at serine 62 and threonine 58, and the sequence around this region is conserved in both (54, 57). It has been reported that the expression of *MYCN* and *MYC* occurs in a mutually exclusive fashion by repressing each other in neuroblastoma cells (58). Therefore, we examined regulation of MYC by TEAD4 in *MYCN*<sup>WT</sup> cells (SY5Y). Our data confirmed that TEAD4 also regulates MYC (Supplementary Figure S7F, S7G). While there are conflicting data in the literature regarding regulation of MYC by AURKA (53, 59, 60), CDK1 has been shown to regulate both MYCN and MYC (61). Furthermore, it has been reported that TEAD4 binds to the enhancer region of *MYC* (62), indicating transcriptional regulation as well.

To gain further insight into the interplay between TEAD4 and MYCN/MYC, we assessed their correlation in neuroblastoma patient GEPs. We observed that both *TEAD4* expression and activity were increased only in MYCNA tumors or in tumors presenting over-expression of either *MYCN* or *MYC* (Figure 5A and Supplementary Figure S7H). This pattern is consistent across tumors from multiple histologies where TEAD4 positively correlates with MYC target hallmark activity (Supplementary Figure S7I). TEAD4 expression correlates positively with MYCN in tumors from neuronal origin (Supplementary Figure S7J) whereas in most other histologies TEAD4 positively correlates with MYC (Supplementary Figure S7K).

Taken together, these data show the existence of a strong TEAD4 ↔ MYCN positive-feedback loop in MYCNA subtype samples. This loop is mediated by both transcriptional and post-translational interactions and decouples these proteins from their physiologic regulatory controls, thus inducing aberrant activity of a 10-protein tumor checkpoint identified by our analysis (Figure 4C). Thus, aberrant TEAD4 activity is necessary to stabilize and complement the tumorigenic role of MYCN and MYC in neuroblastoma.

## TEAD4 promotes cellular proliferation

The observation that TEAD4 regulates MYCN/MYC and cell cycle related programs implicates an aberrant, context-specific role of TEAD4 in high-risk neuroblastoma. To evaluate the phenotypic consequences of TEAD4 silencing in *MYCN*<sup>Amp</sup> and *MYCN*<sup>WT</sup> cell lines, we chose cell lines with varying degree of expression of MYCN or MYC, yet expressing comparable levels of TEAD4 protein (Figure S8A). TEAD4 silencing led to decrease in tumorigenic potential of MYCNA cell lines BE2 and LAN1, as shown by dramatic colony count reduction in long term colony formation assays (Figure 5B, 5C). In contrast, there was no change in colony formation in MYCN-NA cell line, SKNFI and only a modest decrease in SKNAS cell lines (Figure 5B, 5C), which critically present with higher MYC levels than SKNFI (Figure S8A).

To further elucidate the role of TEAD4 in regulating cell proliferation, we performed GSEA analysis of differentially expressed genes following *TEAD4* silencing in BE2 cells and observed significant enrichment of cell cycle specific genes ( $P = 6.7E-05$ ; Figure 5D). The most down-regulated genes (i.e., in the GSEA leading-edge) contained almost half (49/128) of the KEGG cell cycle pathway genes. In particular, we observed repression of several genes involved in cell cycle progression and DNA replication. Several of these genes have a reported role in high-risk neuroblastoma pathology, often associated with *MYCN*<sup>Amp</sup> tumors. These include cyclin-dependent kinases (CDK2, CDK1, CDC25B) (54, 63, 64), cyclins (CCND1) (65), E2Fs (E2F1, E2F2, E2F3) (43), DNA replication (PCNA, MCM7, CDC6) (66–68), checkpoint kinases (CHEK1, CHEK2, WEE1) (2, 50, 69) and ubiquitin-proteasome system (SKP2) (70). In addition, 19 of the 49 cell cycle genes were shown to bind TEAD4 in their proximal region (Figure 5D, Supplementary Table S6), suggesting direct transcriptional regulation. Further investigation of the phenotypic influence of TEAD4 on cell cycle and proliferation of MYCNA cells showed that TEAD4 silencing induced significant accumulation of cells in G0/G1 with concomitant decrease of cells in S phase (Figure 5E). Consistent with this, we observed decrease in proliferating cells by EdU staining (Figure 5F). We did not observe induction of apoptosis by Annexin-V staining and only a modest increase in PARP cleavage upon TEAD4 silencing (Figure S8B, S8C). Collectively, these findings suggest TEAD4 as a critical component driving cellular proliferation of MYCNA cells and a novel MYC/MYCN mediated tumor dependency in neuroblastoma.

## TEAD4 activity in neuroblastoma is independent of YAP/TAZ modulation

YAP and TAZ are established TEAD4 co-transcriptional activators in the Hippo pathway. They are known to bind TEAD family proteins, including TEAD4, to promote cell proliferation, growth and survival (71). We thus explored the potential role of YAP/TAZ in TEAD4-mediated regulation of MYCNA signature genes. First, we assessed whether differential activity of YAP/TAZ could be detected in neuroblastoma samples. Surprisingly, YAP and TAZ activity, as defined by the expression of their target genes – based on REACTOME (72) and perturbation assays (73) – was not correlated with the TEAD4 expression or *MYCN*<sup>Amp</sup> status in neuroblastoma samples (Supplementary Figure S9A). Similarly, we observed that the active form of YAP/TAZ, as indicated by their nuclear

localization, was not correlated with TEAD4 protein expression or MYCN-amplification status in a panel of neuroblastoma cell lines (Supplementary Figure S9B).

To further validate these findings, we performed lentiviral-mediated shRNA silencing of TAZ in YAP-null cells, BE2 (Supplementary Figure S9C), and compared the gene expression signature of TAZ and TEAD4. First, we observed only minimal overlap in genes differentially expressed following their silencing, supporting independent transcriptional regulatory activity in MYCNA cells (Supplementary Figure S9 D–F). Furthermore, ARACNe-inferred TEAD4 transcriptional targets, which displayed strong enrichment in the TEAD4 knockdown signature (Figure S6N), showed no significant enrichment in the TAZ silencing gene expression signature (Figure S9G). We also confirmed that bona-fide targets of YAP/TAZ such as CTGF and CYR61 (74) were significantly down-regulated following TAZ silencing (Limma test,  $P_{CTGF} = 5.28E-5$ ;  $P_{CYR61} = 2.1E-4$ , Supplementary Table S7); and yet, the same genes were significantly up-regulated following TEAD4 silencing (Limma test,  $P_{CTGF} = 4.5E-5$ ;  $P_{CYR61} = 9.5E-6$ , Supplementary Table S7), suggesting orthogonal TAZ and TEAD4 regulatory programs. Finally, TAZ silencing in BE2 and LAN1 cells showed minimal effect on cell viability compared to TEAD4 (Supplementary Figure S9H, S9I). Taken together, our results show that aberrant TEAD4 activity in MYCNA neuroblastoma is largely independent of YAP/TAZ expression and nuclear localization.

### TEAD4 expression is prognostically relevant in neuroblastoma patients

To further investigate the clinical relevance of TEAD4 in neuroblastoma tumors, we assessed whether its expression was associated with neuroblastoma tumor progression and outcome. Indeed, TEAD4 expression and VIPER-inferred activity were both higher in the MYCNA subtype compared to other high-risk neuroblastoma subtypes, while stage 1 samples exhibited the lowest levels (Kruskal-Wallis test,  $P = 3.05E-11$ ; Figure 6A, 6B). Similarly, TEAD4 protein staining in tumor microarrays (TMA) from 116 neuroblastoma cases showed that the high-risk group expressed significantly higher levels of the protein compared to low-risk and normal tissues. This was assessed by computing the final score (intensity of staining (0–3) by percentage of cells stained (0–100)) (Supplementary Table S8, Figure 6C). Among the high-risk group, MYCNA tumors had higher expression of TEAD4 protein compared to non-MYCNA tumors (Figure 6D).

We then performed Cox proportional hazards analysis on NRC-cohort samples and the SEQC 498NB independent cohort (75). Both unbiased cohorts comprise all tumor risk groups and stages. We observed that TEAD4 expression alone was a strong predictor of patient survival ( $P_{NRC} = 5.64E-11$ ;  $P_{SEQC} = 8.99E-15$ ; Supplementary Figure S10A, S10B). Multivariate Cox regression analysis concluded that TEAD4 is a predictor of survival independent of currently used clinical and biological variables for risk stratification (76), stage ( $P_{NRC} = 8.97E-06$ ,  $HR_{NRC} = 5.36$ ;  $P_{SEQC} = 1.14E-06$ ,  $HR_{SEQC} = 2.11$ ), MYCN amplification ( $P_{NRC} = 9.05E-04$ ,  $HR_{NRC} = 4.54$ ;  $P_{SEQC} = 1.35E-06$ ,  $HR_{SEQC} = 1.65$ ), age ( $P_{NRC} = 1.87E-08$ ,  $HR_{NRC} = 9.73$ ;  $P_{SEQC} = 1.67E-06$ ,  $HR_{SEQC} = 2.43$ ) and a combination of all three covariates in NRC ( $P_{NRC} = 3.69E-03$ ,  $HR_{NRC} = 3.69$ ) but not in SEQC (Supplementary Figure S10C). We further confirmed TEAD4 as a predictor of survival independently of a meta-PCNA proliferation signature (77) ( $P_{NRC} = 6.26E-3$ ,  $HR_{NRC} = 3.3$ ;



$P_{SEQC} = 1.10E-06$ ,  $HR_{SEQC} = 1.54$ ) and at least in the NRC cohort TEAD4 expression predicts survival independently of a MYCN functional signature capturing the combined activity of MYCN and MYC of neuroblastomas (25) ( $P_{NRC} = 6.26E-03$ ,  $HR_{NRC} = 3.3$ ) (Supplementary Figure S10D).

## Discussion

Greater understanding of the molecular mechanisms downstream of the genetic alterations that drive high-risk neuroblastoma subtypes is critically required to facilitate development of novel therapeutic strategies and to improve overall patient survival. Consistent with the recently proposed *tumor checkpoint* model (15), we elucidated a modular and hierarchical 10-protein architecture, responsible for the implementation of an aggressive neuroblastoma subtype (MYCNA) associated with aberrant activity of MYCN/MYC proteins. Experimental validation of these MR proteins confirmed their enrichment in MYCNA-subtype specific essential genes and their ability to regulate each other, as well as MYCNA-signature genes, through multiple autoregulatory loops, thus establishing their role as *bona fide* master regulators. A novel TEAD4  $\leftrightarrow$  MYCN positive feedback loop, mediated by both transcriptional and post-translational interactions, emerged as the dominant regulatory structure at the top of the tumor checkpoint hierarchy and was shown to represent a critical tumor dependency.

Critically, these MR proteins were conserved in independent neuroblastoma cohorts and aberrant activation of the tumor checkpoint they comprise was found to be consistent across the entire MYCNA subtype, independent of MYCN amplification status. This is significant in light of recent findings suggesting that progression of malignant neuroblastoma with the most aggressive phenotype is driven by aberrant MYCN or MYC activity (23, 24). We show that aberrant interaction between TEAD4 and MYCN/MYC allows cells to undergo rapid proliferation and replication stress, while simultaneously activating complementary DDR pathways, thus providing mechanistic support for this observation. Consistently, inhibition of Chk1 and Wee1, both of which emerged as TEAD4 regulated, was previously shown to exhibit strong synthetic lethality in MYC driven tumors – including neuroblastomas with high MYCN/MYC activity – by inducing potent cytotoxic response (50, 51, 69). As MYC proteins are capable of mediating oncogene-induced replication stress and genomic instability (78), the positive feedback loop between TEAD4 and MYCN, and possibly MYC, further supports this tumor initiation and maintenance mechanism.

Surprisingly, our data show that TEAD4 activity in MYCNA subtype neuroblastoma is not mediated by its canonical co-transcriptional effectors in the Hippo pathway, YAP and TAZ (74, 79). This is likely because the positive feedback loop with MYCN decouples it from its normal physiologic control mechanisms by inducing saturation of its expression. While prior studies have indicated TEAD4 driving previously identified YAP/TEAD targets in neuroblastoma cells (62, 80), these studies were performed in  $MYCN^{WT}$  cells, further confirming that different regulatory mechanisms are at play in  $MYCN^{Amp}$  versus  $MYCN^{WT}$  cells. Interestingly, studies in mammary tumors have shown that MYC represses YAP/TAZ activity, while also showing that MYC induction decreases binding of YAP/TAZ to its *bona fide* targets, CTGF and CYR61, but not of TEAD4 (81); additional studies have shown YAP/



TAZ-independent regulatory activity of TEAD4 (82). In contrast to the canonical view that TEAD4 lacks independent transcriptional activation (83), TEAD4 was also recently reported to have a transcriptional activation domain, supporting transcriptional regulation independent of the YAP/TAZ DNA binding domain (84). This is consistent with the regulatory activity detected by our studies when aberrant TEAD4 protein expression is achieved due to its interaction with MYCN and MYC.

While validation was restricted to the MYCNA subtype, our analysis identified identically conserved MR protein architectures for two additional subtypes, which largely overlapped with previous classification based on genetic alterations in neuroblastomas (85), including a subtype presenting a strong mesenchymal signature but lacking hallmark genomic alterations. MR proteins for this subtype were found to be highly overlapping with those previously reported for the mesenchymal subtype of high-grade glioma (8). Additionally, conservation of these proteins in cell lines implies their role as subtype MRs independent of stromal/immune infiltration. However, high rates of stromal and immune cell infiltration in a subset of these tumors suggests that single cell analysis may be required to further elucidate the interaction between tumor cells and stroma, including the presence of mesenchymal cells within neuroblastoma tumors highlighted by recent studies (86). Perhaps most importantly, identification of master regulator proteins responsible for the implementation and stability of high-risk neuroblastoma subtypes, which are conserved in cell-line models, provides the opportunity for the systematic identification of subtype-specific therapeutic vulnerabilities using methodologies such as OncoTreat, which were recently validated in neuroendocrine tumors (16). Since OncoTreat is NYS CLIA certified, this may further support the design clinical trials enriched for patients representative of specific molecular subtypes.

Taking these results together, TEAD4 emerged as a highly conserved, mutation-independent tumor vulnerability for the MYCNA subtype of high-risk neuroblastoma, as well as a highly significant prognostic factor. As such, it may represent an ideal novel target for therapeutic intervention in this high-risk subtype. The results of this study are also highly consistent with our proposed model of a recurrent tumor architecture responsible for canalizing the effect of multiple genomic alterations to implement critical programs necessary to tumor initiation and stability (15). While MR proteins were discovered in multiple prior study (10, 13, 87, 88) for instance, their modular regulatory architecture and the existence of core autoregulatory loops had not been previously reported. These findings, combined with previous MR protein studies, suggest that similar regulatory architectures may be responsible for the implementation and stability of other cancers.

## Methods

### Subtype identification, neuroblastoma interactome assembly and master regulator analyses

Expression profile datasets on neuroblastoma from TARGET and NRC cohorts are described in Supplementary Table S1. Further characterization of the subtypes by pathway enrichment analyses (Supplemental Experimental Procedures) are provided in Supplementary Table S2. Detailed description of the approach, clustering analyses (20) and master regulator analyses (19) is provided in Supplemental Experimental Procedures. Details of the subtypes are

provided in Supplementary Table S1, neuroblastoma interactomes in Supplementary Table S3, and subtype specific MRs in Supplementary Table S4.

### Cell lines and cell culture

All neuroblastoma cell lines were obtained from the Children's Hospital of Philadelphia cell line bank, the Children's Oncology Group, or ATCC. They were maintained in DMEM or RPMI-1640 supplemented with 10% FBS, 20 mM L-glutamine and antibiotics. 293FT cells were maintained in DMEM supplemented with 10% FBS and antibiotics. We received the cell lines in 2012 and the experiments were performed between 2012 and 2017. The genomic identity of each line was routinely tested and last confirmed 2015 using the AmpFISTR Identifier kit (Applied Biosystems). In addition, lines were routinely tested to confirm lack of mycoplasma contamination.

### Functional validation of MRs

Experimental validation of top ranked MRs of MYCNA subtype was performed in neuroblastoma cell lines by lentivirus mediated pooled shRNA and individual shRNA screening (Sigma); and siRNA screening (Dharmacon), using cell viability as a readout (Supplemental Experimental Procedures). For the respective shRNA and siRNA sequences, see Supplementary Table S5. RNA based analyses were performed by RT-PCR (See Supplementary Table S9 for oligonucleotide sequences) or RNA-seq analyses; ChIP assays using kits from Millipore and ChIP-seq analyses were performed as described previously (Supplemental Experimental Procedures). Details of ChIP-seq and RNA-seq analyses are provided in Supplementary Table S6 and Supplementary S7 respectively. Details of other molecular, biochemical and cellular assays are provided in Supplemental Experimental Procedures.

### Clinical validation of TEAD4

Analyses of TEAD4 protein expression in neuroblastoma tumors were performed using TMAs from CHOP (Supplemental Experimental Procedures). Available histopathological features are summarized in Supplementary Table S8. Kaplan-Meier curve analyses and Cox proportional hazards regression analyses were performed using R "survival" package (<http://cran.r-project.org/web/packages/survival/index.html>) (Supplemental Experimental Procedures).

### High-throughput data availability

The tumor genomics data from the TARGET cohort is available through the data matrix portal (<https://ocg.cancer.gov/programs/target/data-matrix>); The NRC expression data is available in Gene Expression Omnibus with accession codes GSE85047; Data generated through the ENCODE project including TEAD4 ChIP-seq data was obtained from (<http://genome.ucsc.edu/ENCODE/downloads.html>); Additional data generated in this study including RNA-seq profiles from BE2 cells and ChIP-seq data in BE2 cells using TEAD4 antibody is available in Gene Expression Omnibus with accession GSE84389.

## In-vivo mouse models

Mice were housed in a pathogen-free animal facility. All animal studies were approved by the IACUC at Columbia University (#AAAQ2459). Mice used for subcutaneous xenograft experiments were 4–6 week old male and female athymic nude (Nu/Nu, Charles River Laboratories).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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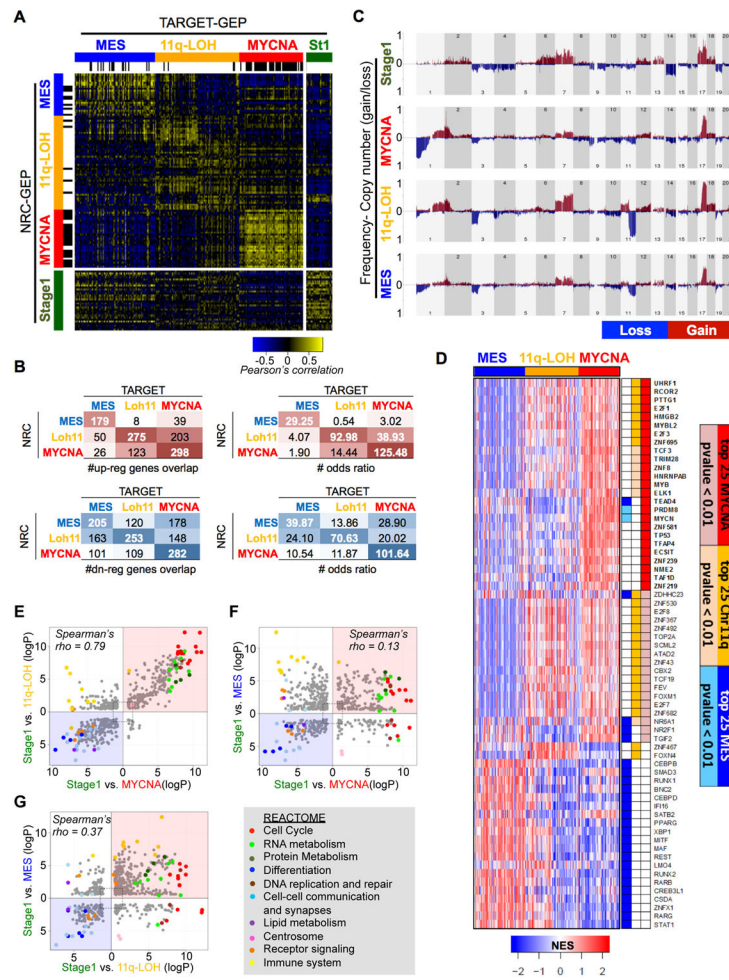


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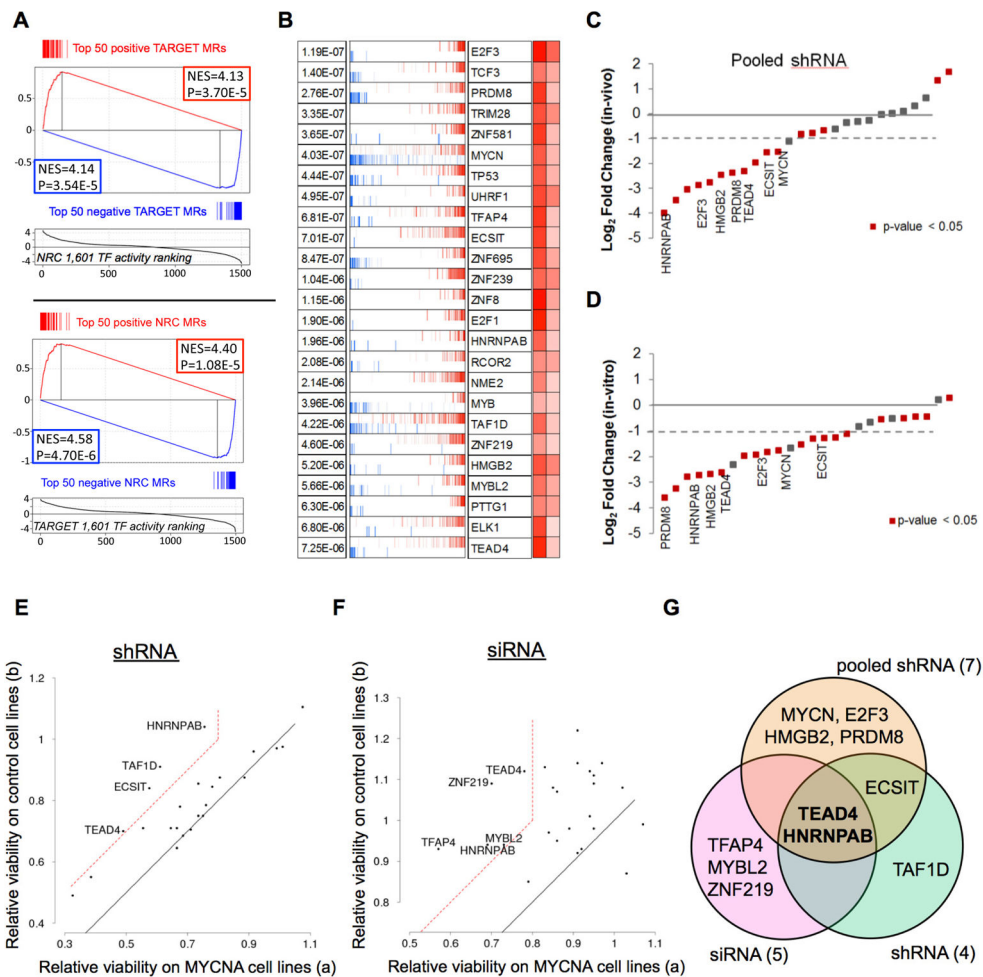
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### Significance

Despite progress in understanding of neuroblastoma genetics, little progress has been made toward personalized treatment. Here, we present a framework to determine the downstream effectors of the genetic alterations sustaining neuroblastoma subtypes, which can be easily extended to other tumor types. We show the critical effect of disrupting a 10-protein module centered around a YAP/TAZ-independent TEAD4 ↔ MYCN positive-feedback loop in *MYCN*<sup>Amp</sup> neuroblastomas, nominating TEAD4 as a novel candidate for therapeutic intervention.

**Figure 1.**

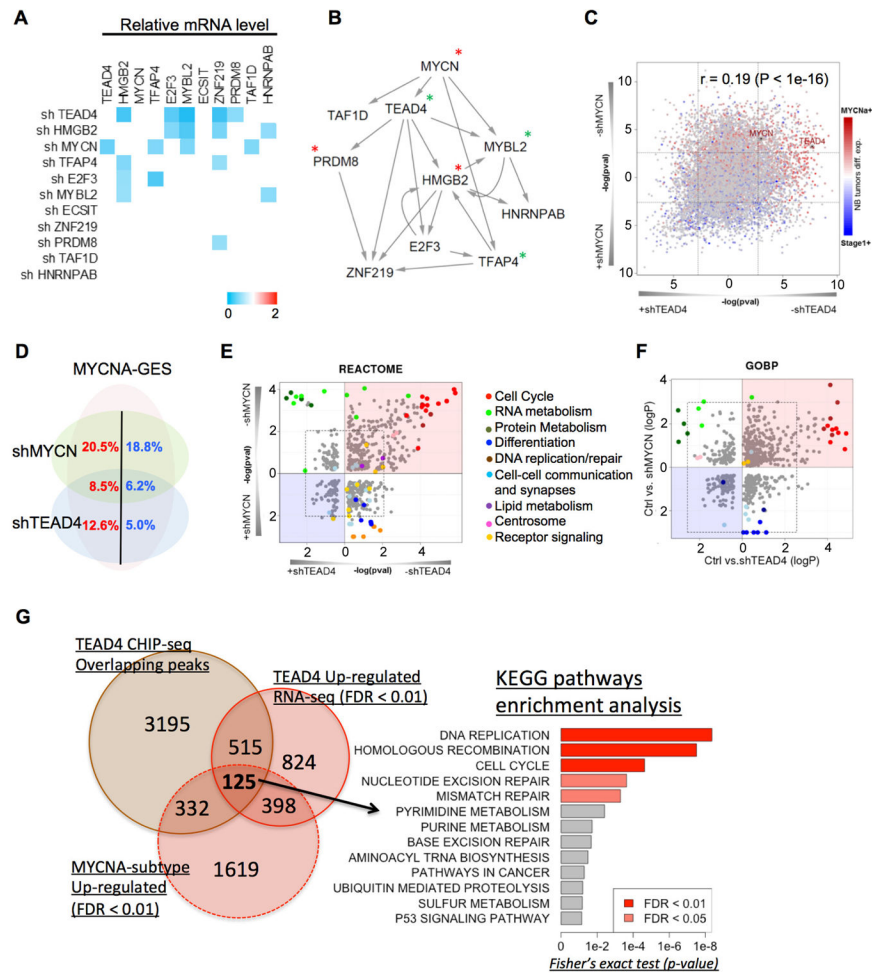
High-risk neuroblastoma molecular subtypes classification and inference of master regulators. (A) Unsupervised consensus clustering of high-risk neuroblastoma GEPs was performed to establish molecular subtypes. Three subgroups were identified according to robustness of clustering and consistency between two cohorts, TARGET and NRC. (B) The overlap of top 500 up-regulated (red) and down-regulated (blue) genes for each subtype in TARGET and NRC dataset using Stage 1 GEPs as reference, with its corresponding odds ratios. (C) Copy number frequency per genomic location of individual molecular subtypes showing segregated pattern of 11q, 3p and 1p loss. Gains are considered when the  $\log_2$  ratio between tumor and blood  $> 1.1$ , while losses are considered for  $\log_2$  ratios  $< 0.9$  (D) Top activated MRs (red) of high-risk subtypes are represented using VIPER inference of TF activity using stage 1 samples as a control group. (E, F and G) REACTOME pathway enrichment analysis of MYCNA, 11q-LOH and MES subtype gene expression signatures. Axis represents  $-\log_{10}$  of the p-value while retaining the directionality of the enrichment score. Also see Figure S1 and supplementary experimental procedures.

**Figure 2.**

RNAi screening identifies MYCNA subtype specific MRs. (A) Master regulator analysis of MYCNA versus Stage 1 tumors performed independently on both TARGET and NRC datasets show strong reciprocal reproducibility of both activated (red) and deactivated (blue) top 50 MRs (Supplementary Table S4). (B) The top 25 integrated MRs of MYCNA subtype selected for validation. The map shows distribution of positively (red) and negatively (blue) regulated targets of each MR ranked by differential expression between MYCNA subtype versus stage I patient samples. (C) In-vivo pooled shRNA screening in MYCNA (BE2) versus control cells (SKNAS) and (D) In-vitro pooled shRNA screening in MYCNA (BE2, IMR-5) versus control cells (NLF, SK-NAS), depicting average effect of putative MR silencing in MYCNA cells compared to control cells. For both (C, D), tumor-enriched shRNAs were amplified, sequenced and counted to identify enrichment and dropouts. shRNA abundance for a gene was integrated into a score and calculated as a ratio of  $T_{\text{final}}$  to  $T_{\text{initial}}$ . The MRs were first screened to include only the ones with  $p < 0.05$  in MYCNA group (red) and average fold change between MYCNA cells versus control cells was calculated. The grey dashed line shows the cutoff for  $-2.0$  fold change. (E) Scatter plot of average relative cell viability of MYCNA cells (BE2, IMR5, IMR32, NB1, LAN1) versus control cells (SY5Y, SKNAS, SKNFI) upon transduction with 2 shRNAs per MR,

normalized to control shRNA, measured 72 to 96hrs post transduction. (F) Scatter plot of average cell viability of MYCNA cells (BE2, IMR5, SKNDZ) versus control cells (SY5Y, SKNAS) upon transfection with ON-Target smartpool siRNA against each MR normalized to control siRNA, measured 96hrs post transfection. For both (E, F), the red dashed lines show the cutoff of  $a < 0.8$  (cell viability reduction) for MYCNA cells and  $b - a > 0.2$  (cell type specificity). Experiments were run in triplicate. Representative experiments are shown. (G) Venn diagram depicting potential MYCNA subtype specific MRs from (C, D) MRs common to both in-vitro and in-vivo negative selection pooled shRNA screening (E) individual shRNA screening (F) and siRNA screening. Additional data in Supplementary Figure S5 and Table S5.





**Figure 3.** TEAD4 is the master regulator of MYCNA subtype. (A) Heatmap representing gene expression changes of MYCNA subtype specific MRs from Figure 2G, upon transduction of BE2 cells with control or respective shRNAs against each MR, measured by qRT-PCR, 48hrs post-transduction. The genes showing >1.5 fold downregulation of transcript upon treatment with the shMR was considered to be a target and are displayed in the map. Samples were run in triplicate and representative experiments are shown. (B) The inter-regulatory network derived from the results in (A). \*MYCN binds to the promoter of the genes by ChIP assay (Figure S5F); \*TEAD4 binds to proximal region of these genes by ChIP-seq experiment (Supplementary Table S6) (C) TEAD4 (x-axis) and MYCN (y-axis) knockdown signatures compared with MYCNA versus stage1 signature (red-blue heat colors). (D) Venn-diagram showing proportion of MYCNA subtype signature up-regulated (red) and down-regulated (blue) genes by MYCN, TEAD4 or both knockdown signatures. (E) REACTOME and (F) Gene Ontology pathway enrichment analysis performed on TEAD4 (x-axis) and MYCN (y-axis) knockdown signatures. Red circle represent positive TEAD4 targets genes (down-regulated upon knockdown) while blue circle represents negative targets (up-regulated after knockdown). Fisher's exact test was used to calculate the statistical significance of both overlaps using a background list of 18,179 genes included in

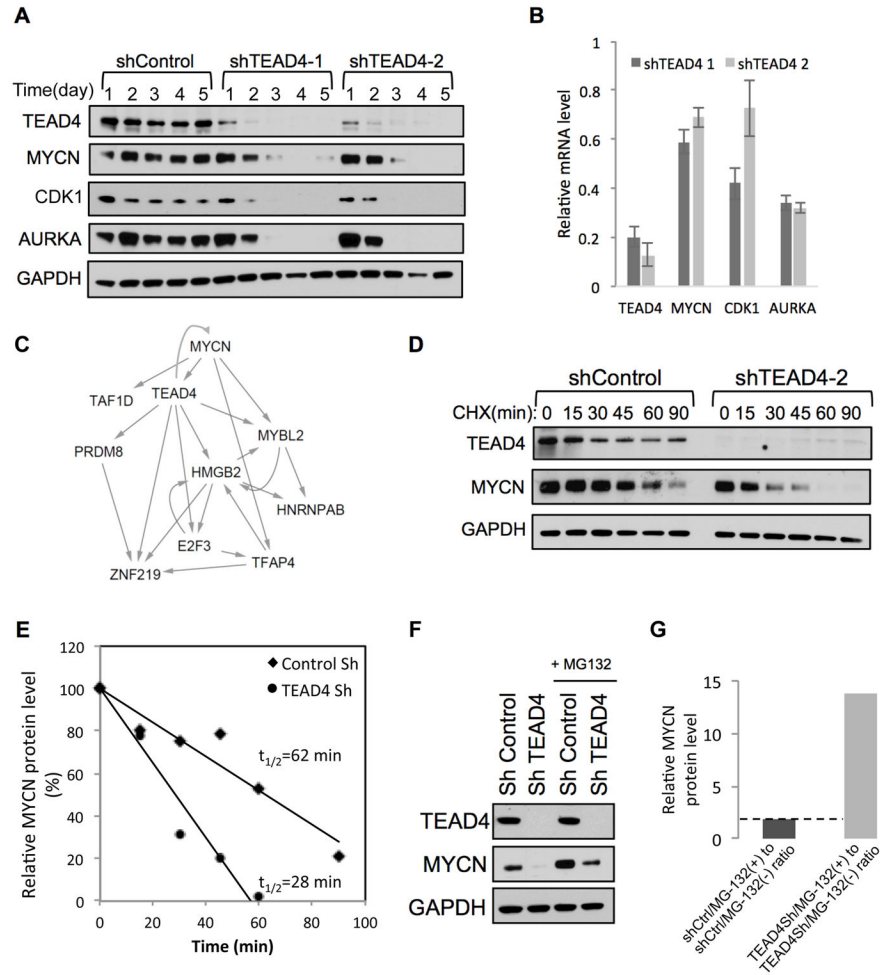
the RNA-seq signature. (G) Overlap between differentially expressed genes after TEAD4 knockdown, peak targeted genes from TEAD4-Ab ChIP-seq, and MYCNA subtype signature up-regulated genes, with the corresponding KEGG pathway enrichment analysis on the overlapping genes. See also Supplementary Figure S6 and Supplementary Tables S6 and S7.

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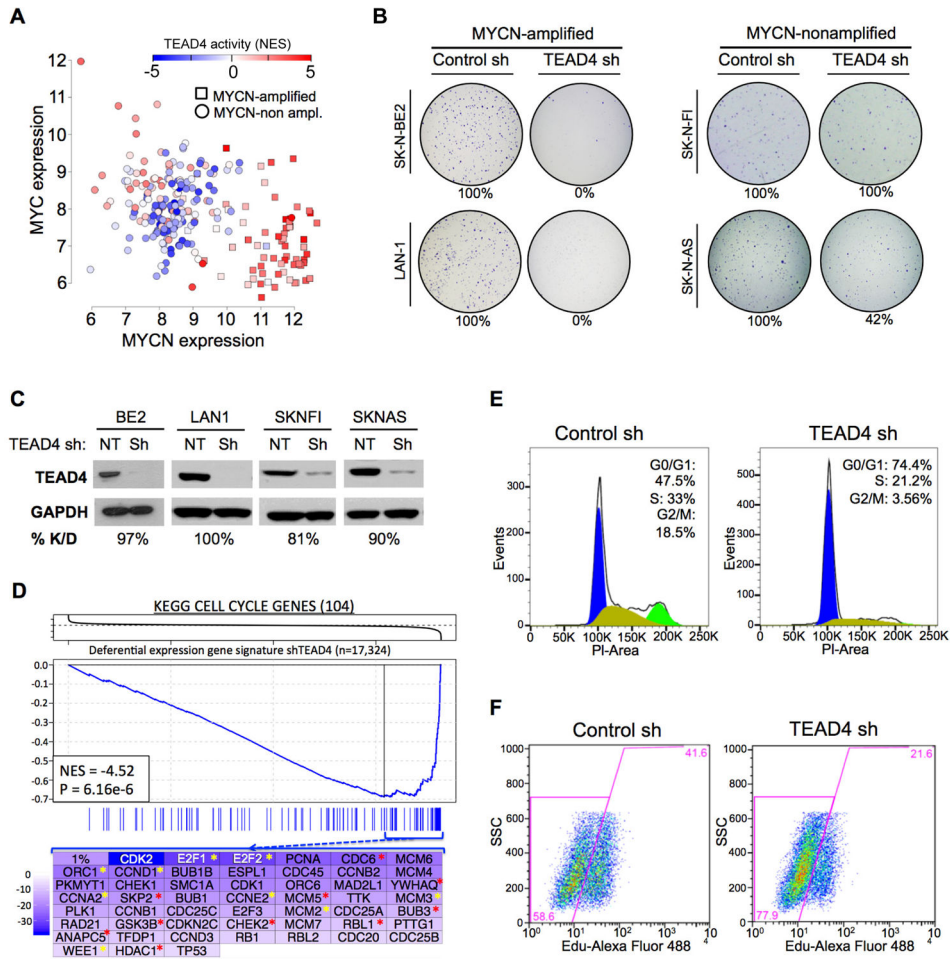
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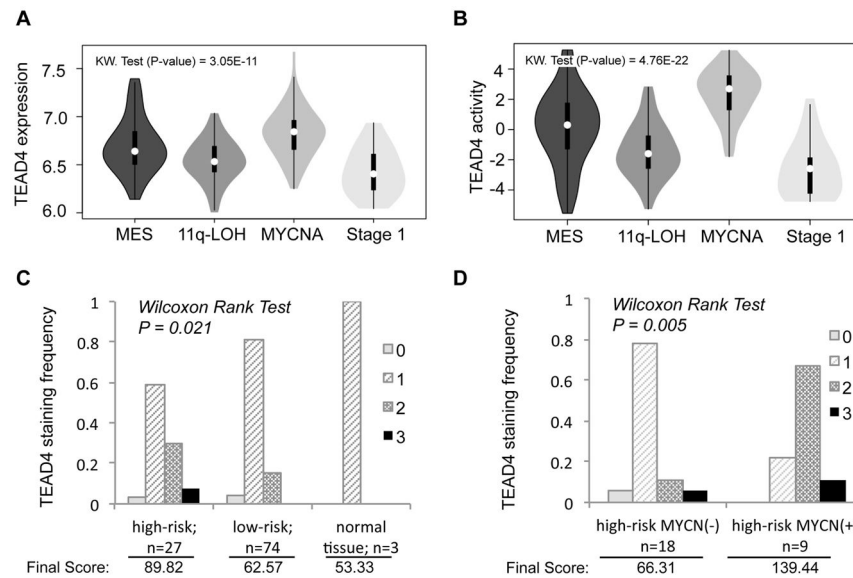
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**Figure 4.** TEAD4 promotes MYCN protein stabilization. (A) Immunoblot of TEAD4, MYCN, CDK1 and AURKA proteins in BE2 cells transduced with control or two different TEAD4 shRNAs in a time course experiment (B) qPCR analysis showing transcript levels of the corresponding genes, 2 days post-transduction. (C) Regulatory hierarchical model showing a TEAD4 ↔ MYCN positive feedback loop controlling the master regulatory module (D) Immunoblot of TEAD4 and MYCN proteins in BE2 cells transduced with control and TEAD4 shRNA, and treated with CHX for indicated times (E) Quantification of MYCN protein stability from results shown in (D) where MYCN levels were normalized to GAPDH (F) Immunoblot of TEAD4 and MYCN 72hrs post-transduction from cells treated with DMSO or MG-132, 4hrs before harvesting (G) Densitometry analysis of MYCN proteins from results shown in (F), where MYCN levels were normalized to GAPDH. Representative experiments are shown.



**Figure 5.** TEAD4 is required for cell cycle progression and cell growth of MYCNA cell lines. (A) Scatter plot representing MYCN and MYC expression in MYCNA and non-MYCNA samples from TARGET cohort. Single sample activity of TEAD4, is represented as normalized enrichment score (NES). NRC cohort results provided in Supplementary Figure S7H. (B) The effect of TEAD4 on anchorage-independent growth in MYCNA and control cell lines was evaluated by soft agar colony assays, 21 days post transduction (C) Immunoblot analysis confirming silencing of TEAD4 in the corresponding cell lines (D) GSEA plot evaluating enrichment for KEGG cell cycle gene set in shTEAD4 signature (upper) and leading edge cell cycle genes (lower) colored by their signature t-score; yellow and red asterisk indicate genes with assigned anti-TEAD4 ChIP-seq peaks by proximity and overlap criterion respectively (supplementary Experimental Procedures). (E) Cell cycle profile and (F) cellular proliferation, assessed upon treatment of BE2 cells with control or TEAD4 shRNA, 48hrs post transduction by flow cytometry. Representative experiments are shown. See also Figure S8.



**Figure 6.** TEAD4 is overexpressed in high-risk neuroblastoma tumors. (A) Expression of TEAD4 across high risk and stage 1 subtypes (B) VIPER transcriptional activity of TEAD4 across high risk and Stage 1 subtypes. (C) Histogram of primary neuroblastoma samples stained for TEAD4 protein by immunohistochemistry on a tissue microarray, segregated by risk level and (D) MYCN-amplification status in high-risk neuroblastoma, showing differential pattern of TEAD4 protein staining intensity (where 0=no staining; 1=low staining; 2=moderate staining; 3=high staining). See also Supplementary Table S8.