original reports abstract

# FOXR2 Stabilizes MYCN Protein and Identifies Non–*MYCN*-Amplified Neuroblastoma Patients With Unfavorable Outcome

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**PURPOSE** Clinical outcomes of patients with neuroblastoma range from spontaneous tumor regression to fatality. Hence, understanding the mechanisms that cause tumor progression is crucial for the treatment of patients. In this study, we show that *FOXR2* activation identifies a subset of neuroblastoma tumors with unfavorable outcome and we investigate the mechanism how FOXR2 relates to poor outcome in patients.

**MATERIALS AND METHODS** We analyzed three independent transcriptional data sets of in total 1030 primary neuroblastomas with full clinical annotation. We performed immunoprecipitation for FOXR2 and MYCN and silenced FOXR2 expression in two neuroblastoma cell lines to examine the effect on cellular processes, transcriptome, and MYCN protein levels. Tumor samples were analyzed for protein levels of FOXR2 and MYCN.

**RESULTS** In three combined neuroblastoma data sets, 9% of tumors show expression of *FOXR2* but have low levels of *MYCN* mRNA. *FOXR2* expression identifies a group of patients with unfavorable outcome, showing 10-year overall survival rates of 53%-59%, and proves to be an independent prognostic factor compared with established risk factors. Transcriptionally, *FOXR2*-expressing tumors are very similar to *MYCN*-amplified tumors, suggesting that they might share a common mechanism of tumor initiation. FOXR2 knockdown in *FOXR2*-expressing neuroblastoma cell lines resulted in cell cycle arrest, reduced cell growth, cell death, and reduced MYCN protein levels, all indicating that FOXR2 is essential for these tumors. Finally, we show that FOXR2 binds and stabilizes MYCN protein and MYCN protein levels are highly increased in FOXR2-expressing tumors, in several cases comparable with *MYCN*-amplified samples.

**CONCLUSION** The stabilization of MYCN by FOXR2 represents an alternative mechanism to *MYCN* amplification to increase MYCN protein levels. As such, *FOXR2* expression identifies another subset of neuroblastoma patients with unfavorable clinical outcome.

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## INTRODUCTION

Neuroblastoma, derived of the sympathetic nervous system, represents the most common extra-cranial solid tumor in pediatric cancer.[1,2] The clinical outcome of patients with neuroblastoma is highly variable ranging from spontaneous tumor regression to progression with fatal outcome.[3] Low-risk neuroblastoma, primarily classified in International Neuroblastoma Staging System (INSS) 1 and 2, shows 5-year overall survival (OS) rates > 95%, whereas high-risk tumors, often diagnosed in patients above 18 months showing INSS4 status and/or harboring *MYCN* amplification, indicate a poor clinical outcome of around 40%-50% 5-year OS.[4–7] Furthermore, *TERT* activation, *ALK* mutations, or alternative lengthening of telomeres (ALT) also identify subgroups of neuroblastoma with an unfavorable clinical outcome.[8–11] Here, we report on another independent prognostic group of neuroblastoma with unfavorable outcome that is characterized by expression of *Forkhead Box R2* (*FOXR2*). *FOXR2* expression has previously been associated with tumorigenesis, aberrant cell growth, and poor prognosis in, for instance, breast cancer and endometrial adenocarcinoma.[12–18] In pediatric tumors, *FOXR2* activation by enhancer hijacking is the genetic hallmark of CNS neuroblastomas with *FOXR2* activation (CNS NB-*FOXR2*), a novel distinct pediatric brain tumor entity.[19] In addition, subsets or single cases of medulloblastoma, pineoblastoma, or glioblastoma also express elevated *FOXR2* levels.[16,20,21] First insights into the function of FOXR2 revealed that FOXR2 binds to MYC and MYCN proteins.[15,22] The interaction between MYC and FOXR2 proteins seems to stabilize the short-lived MYC protein, which is broadly implicated in the oncogenesis of often aggressive, poorly differentiated tumors.[15,22,23]

ASSOCIATED CONTENT

**Data Supplement**

Author affiliations and support information (if applicable) appear at the end of this article.

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## CONTEXT

**Key Objective**

In this study, we report on a yet unidentified subset of neuroblastoma expressing *FOXR2*. What is the role of FOXR2 in neuroblastoma tumorigenesis and does it identify an independent prognostic risk factor?

**Knowledge Generated**

*FOXR2* expression identifies a subset of neuroblastoma patients with a poor outcome and proves to be prognostic independent of other well-established risk factors. Furthermore, we found that FOXR2 stabilizes MYCN, thereby presenting an alternative mechanism to *MYCN* amplifications increasing MYCN protein levels.

**Relevance**

Identification of *FOXR2* as independent prognostic factor in neuroblastoma may further improve the current risk stratification of patients with neuroblastoma. Revealing the mechanism of MYCN stabilization by FOXR2 may explain the correlation with poor outcome and generates important knowledge for therapeutic approaches.

Here, we have investigated the role of FOXR2 in neuroblastoma of the sympathetic nervous system. Using in vitro models and tumor samples, we investigated the mechanistic interaction of FOXR2 and MYCN to understand why patients with *FOXR2*-expressing tumors have a poor outcome.

## MATERIALS AND METHODS

### Neuroblastoma Cohorts

Three RNA-based data sets with full clinical annotation were analyzed using the R2 platform[24]:

1. Tumor-Neuroblastoma-SEQC-498-RPM-seqcnb1, containing RNA-seq data on 498 primary neuroblastoma[25] (Gene Expression Omnibus (GEO): gse62564)
2. Tumor-Neuroblastoma-Primary-NRC-283-rma_sketch_(bc)-huex10t, containing microarray data on 283 primary neuroblastoma (GEO:gse85047)
3. Tumor-Neuroblastoma-TARGET-Asgharzadeh-249-custom-huex10t, containing primarily high-risk neuroblastoma[26] (dbGaP_ID:phs000218.v22.p8).

### Generation of Knockdown Cell Lines

*FOXR2* knockdown cell lines were generated by lentiviral transduction of pLKO-TET-ON[27,28] with inducible *FOXR2* shRNA insert 5′-CTGGAAGAGCACCATTCATTA-3′ or control scrambled insert 5′-CCTAAGGTTAAGTCGCCCTCG-3′. Lentivirus was produced as described previously.[29] Experiments were conducted under Biosafety Level 2 conditions in accord with the National Institutes of Health Guidelines.

### Quantitative Real-Time Polymerase Chain Reaction

RNA was isolated with the Maxwell RSC using simplyRNA Tissue Kit. Quantitative real-time polymerase chain reaction was performed applying the Power SYBR Green RNA-to-CT 1-Step Kit (Applied Biosystems, Waltham, MA). The fold change was calculated using the $2^{-\Delta\Delta ct}$ method. Primers are listed in the Data Supplement (online only).

### Cell Viability

The viability of SK-N-AS was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). RealTime-Glo MT Cell Viability Assay (Promega) was used to quantify viability of SK-N-FI. RealTime-Glo dilution was added to the cells and incubated for 1 hour at 37°C before measurement.

### Cell Cycle and Cell Death

Cell death was determined using the Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich, St Louis, MO) and detected by flow cytometry using BD FACSCanto II. Cell cycle analysis was conducted by measurement of the cellular DNA content by PI staining and flow cytometry. Cells were fixed in 70% ethanol and incubated in PI staining solution (0.1% TritonX-100, 10 μg/mL PI, and 100 μg/mL DNase-free RNase) for 30 minutes at 37°C. Analysis was conducted using FlowJo, on the basis of at least 10,000 events.

### Western Blot Analysis

Western blots were performed as described previously[29] using FOXR2 (Sigma-Aldrich, HPA057358), MYCN (Sigma-Aldrich, MABE333), MYC (Abcam, Cambridge, United Kingdom, ab32072), HA-tag (Abcam, ab9110), and β-actin (Abcam, ab49900) antibodies and were quantified using ImageJ.

### Cyclohexamide Assay

300 ng/μL cyclohexamide (CHX, Sigma-Aldrich) was added to the cells stopping translation. After specific time points, cells were analyzed by western blot.

### Co-Immunoprecipitation

Cells were lysed in NETN buffer and precleared using 10 μl of magnetic A/G beads for 1 hour at 4°C. 2 μg of antibody was added to the precleared lysate for 1 hour at 4°C, before

25 μg of magnetic beads were added and incubated overnight at 4°C. Subsequently, target proteins were eluted and analyzed by western blot.

### Gene Expression Analysis

Gene expression profiles of SK-N-AS and SK-N-FI with inducible *FOXR2* knockdown were generated in triplicates on Affymetrix GeneChip Human U133 Plus2.0 arrays (GEO:GSE156092) and analyzed using the R2 platform. Pathway analyses were conducted through Gene Set Enrichment Analysis[30,31] and Ingenuity Pathway Analysis (Qiagen, Hilden, Germany).

### Statistical Analyses

*FOXR2* levels between knockdown cell lines were assessed using two-tailed *t*-tests. Differentially expressed genes in SK-N-AS and SK-N-FI upon *FOXR2* knockdown were determined using ANOVA *P* value $< .05$, corrected using false discovery rate. The value for minimal expression difference and the minimal expression value that should be met in at least one sample of the data set were set to 50 units. Chi-squared analyses were performed to determine the distribution of *FOXR2*-expressing patients with neuroblastoma between INSS stages and of *TERT*-positive patients with neuroblastoma over the expression-based

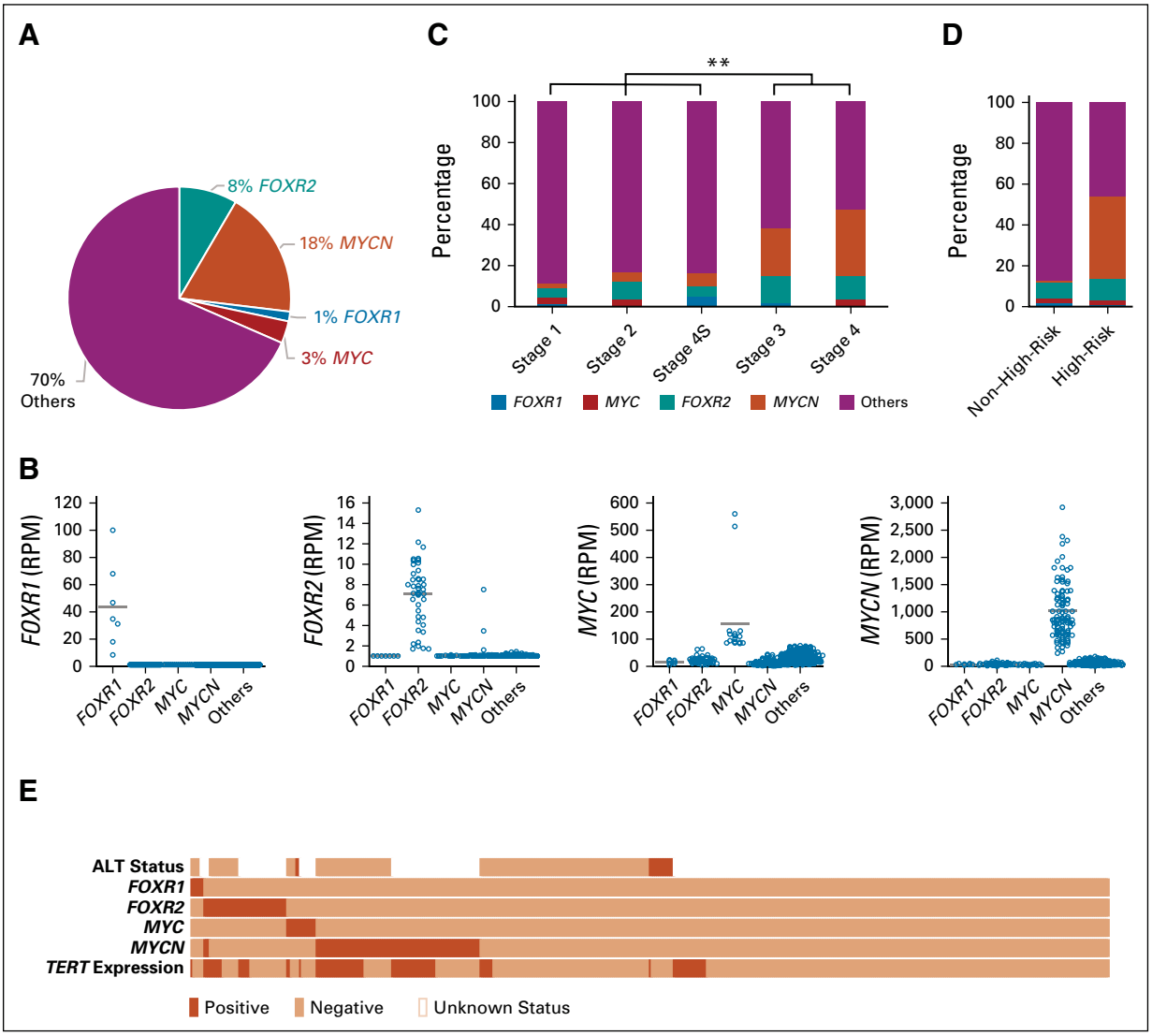
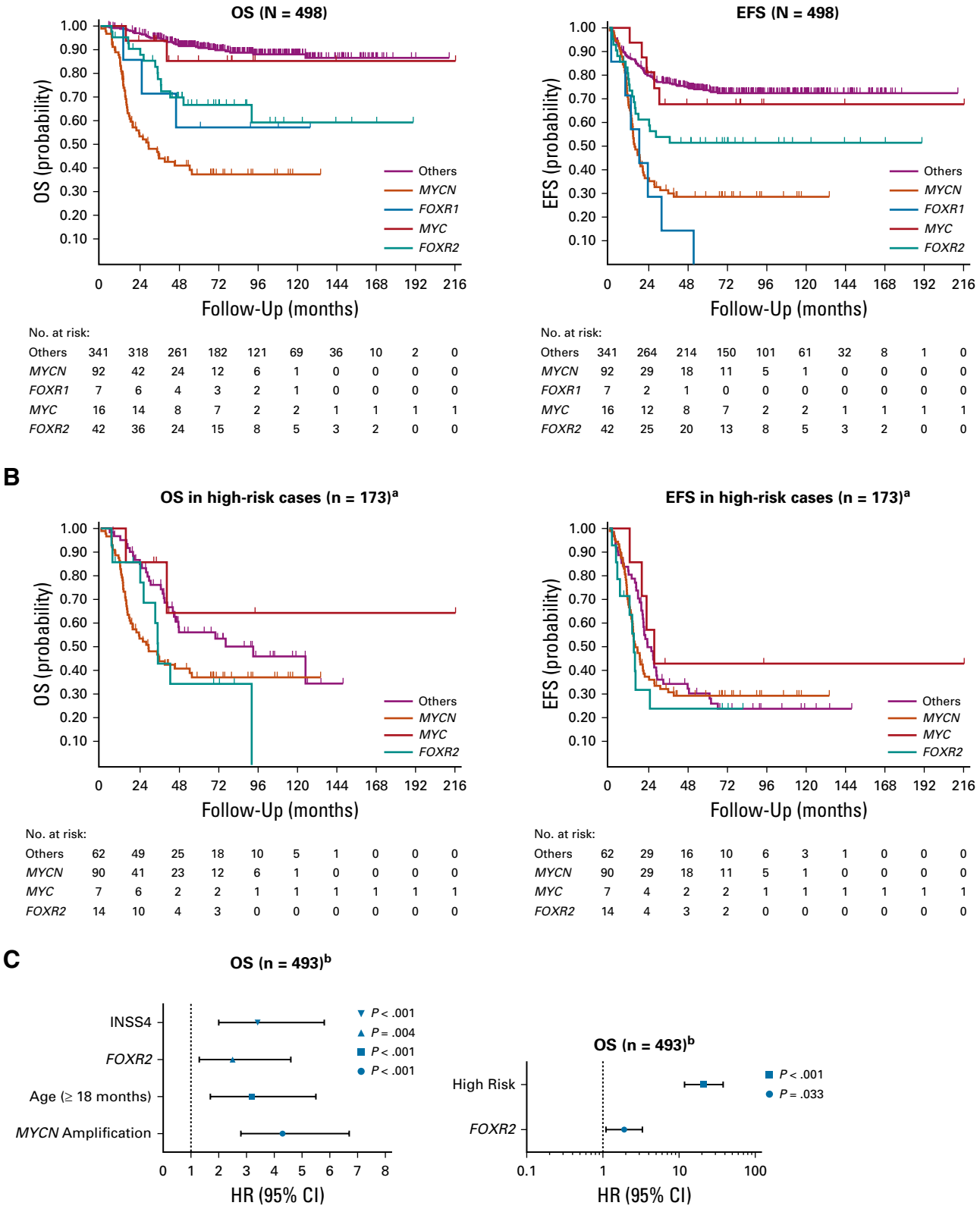



**FIG 1.** *FOXR2* is expressed in a distinct subset of neuroblastomas. (A) Cohort overview of the primary neuroblastoma RNA-seq data set (N = 498). (B) Expression of *FOXR1*, *FOXR2*, *MYC*, and *MYCN* is in nearly all cases mutually exclusive from another. (C) Percentage of *FOXR1*-, *FOXR2*-, *MYC*-, and *MYCN*-expressing cases within the INSS stages 1, 2, 3, 4, and 4S. The *FOXR2* group is significantly enriched in stages 3 and 4 when compared with stages 1, 2, and 4S. (D) Percentage of *MYCN*-, *FOXR2*-, *FOXR1*-, and *MYC*-expressing cases within the non–high-risk and the high-risk group (N = 1,016; legend of [C] applies). (E) Oncoplot of the RNA-seq data set (N = 498), showing the status of the *FOXR1*, *FOXR2*, *MYC*, *MYCN*, and *TERT* expression and the ALT phenotype. ALT, alternative lengthening of telomeres; INSS, International Neuroblastoma Staging System; RPM, Reads per million mapped reads. $^{**}P \leq .005$.


A
OS (N = 498)
OS (probability)
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0 24 48 72 96 120 144 168 192 216
Follow-Up (months)
Others
MYCN
FOXR1
MYC
FOXR2
No. at risk:
Others 341 318 261 182 121 69 36 10 2 0
MYCN 92 42 24 12 6 1 0 0 0 0
FOXR1 7 6 4 3 2 1 0 0 0 0
MYC 16 14 8 7 2 2 1 1 1 1
FOXR2 42 36 24 15 8 5 3 2 0 0
EFS (N = 498)
EFS (probability)
Others
MYCN
FOXR1
MYC
FOXR2
No. at risk:
Others 341 264 214 150 101 61 32 8 1 0
MYCN 92 29 18 11 5 1 0 0 0 0
FOXR1 7 2 1 0 0 0 0 0 0 0
MYC 16 12 8 7 2 2 1 1 1 1
FOXR2 42 25 20 13 8 5 3 2 0 0
B
OS in high-risk cases (n = 173)a
OS (probability)
Follow-Up (months)
Others
MYCN
MYC
FOXR2
No. at risk:
Others 62 49 25 18 10 5 1 0 0 0
MYCN 90 41 23 12 6 1 0 0 0 0
MYC 7 6 2 2 1 1 1 1 1 1
FOXR2 14 10 4 3 0 0 0 0 0 0
EFS in high-risk cases (n = 173)a
EFS (probability)
Others
MYCN
MYC
FOXR2
No. at risk:
Others 62 29 16 10 6 3 1 0 0 0
MYCN 90 29 18 11 5 1 0 0 0 0
MYC 7 4 2 2 1 1 1 1 1 1
FOXR2 14 4 3 2 0 0 0 0 0 0
C
OS (n = 493)b
INSS4
FOXR2
Age (≥ 18 months)
MYCN Amplification
P < .001
P = .004
P < .001
P < .001
0 1 2 3 4 5 6 7 8
HR (95% CI)
OS (n = 493)b
High Risk
FOXR2
P < .001
P = .033
0.1 1 10 100
HR (95% CI)

groups. Differences in survival (months from initial diagnosis) between groups were analyzed using log-rank tests. Multivariable Cox regression analyses were conducted, evaluating *FOXR2* expression in the context of established risk factors, using SPSS.

## RESULTS

### *FOXR2* Is Expressed in a Subset of Neuroblastoma

In healthy human tissue, *FOXR2* mRNA is usually not expressed, except for male reproductive tissues.[32] By contrast, *FOXR2* levels are elevated in subsets of various tumors, including brain, breast, prostate, or colorectal cancer, but expression of *FOXR2* in neuroblastoma has not been reported, yet.[13,16,19–21,33,34] Investigating an RNA-Seq data set of 498 primary neuroblastomas, we identified 42 cases (8%) expressing *FOXR2* (Fig 1A). *FOXR1*, a homologue of *FOXR2* and previously identified to be expressed in a small subset of neuroblastomas,[35] was expressed mutually exclusive from *FOXR2* in seven cases (1%). Interestingly, elevated *MYC* and *MYCN* levels, in 90 of 92 (98%) cases driven by gene amplifications, were identified in 14 (3%) and 92 (18%) cases, respectively, and almost mutually exclusive from each other and from the *FOXR1*- and *FOXR2*-expressing cases (Figs 1A and 1B).[36] All remaining cases (n = 341), here called others group, did not have elevated expression levels of any of these four genes.

In two other independent neuroblastoma Affymetrix expression data sets, the Neuroblastoma Research Consortium (NRC) series (N = 283) and the TARGET series (N = 249), *FOXR2* was present in 17 (6%) and 34 (14%) cases, respectively, and again almost mutually exclusive with subsets that express elevated levels of *FOXR1*, *MYCN*, or *MYC* (Data Supplement).

Investigating the proportion of the *FOXR2* group (90 of 1025 cases, 9%) within the INSS stages in all three data sets, we found *FOXR2* expression in neuroblastoma of all stages, but significantly enriched among stage 3 and stage 4 tumors ($\chi^2$-test, *P* value .001), similar to that for the *MYCN* group (Fig 1C). Subdividing by risk group, we observed 8% (39 of 509) *FOXR2*-expressing cases in the non–high-risk subset and 10% (52 of 507) in the high-risk subset within the three combined data sets (Fig 1D).

### *FOXR2*-Expressing Cases Show Elevated *TERT* Expression But No ALT Phenotype

*MYCN*-amplified cases identify a subset of high-risk neuroblastoma and are usually mutually exclusive from cases with *TERT* rearrangements or ALT phenotype, two other markers associated with poor outcome.[9,11,37,38] Analyzing cases with available ALT status (174 of 498 cases), assessed via C-Circle assay as part of another study by Hartlieb et al (submitted), indicated that *FOXR2- or FOXR1*-expressing neuroblastomas occur like *MYCN* amplifications mutually exclusively from an ALT phenotype in neuroblastoma (Fig 1E).

On the basis of a defined cutoff (lowest expression level of *TERT*- among *TERT*-rearranged cases), we found that *TERT*-activated cases are significantly enriched for the *FOXR2* group ($\chi^2$-test, $P < .001$) and, as expected, the *MYCN* group ($\chi^2$ test, $P < .001$), whereas the residual groups showed less *TERT*-activated neuroblastomas (Fig 1E, Data Supplement). This is in line with our observation on higher *TERT* expression levels in *FOXR2*-activated cases compared with cases with low *FOXR2* expression in an *MYCN*-nonamplified subset (n = 406; Data Supplement). Because of a low number of cases with status annotations on *TERT* rearrangement (62 of 498), no conclusions on distribution can be made for those cases. Altogether, *FOXR2* expression identifies a subset of neuroblastomas distinct from *MYCN*-amplified tumors or cases with ALT phenotype, but they share the activated *TERT* expression with *MYCN*-amplified tumors.

### *FOXR2* Identifies Neuroblastoma Patients With Unfavorable Clinical Outcome

Survival analyses in the RNA-seq data set revealed that patients with *FOXR2*-expressing neuroblastoma have a significantly ($P < .001$) reduced survival, showing a 5- and 10-year OS of 67% (95% CI, 49 to 79) and 59% (95% CI, 38 to 75) and a consistent 5- and 10-year event-free survival (EFS) of 51% (95% CI, 35 to 65), whereas the others group shows a comparably good clinical outcome with a 5- and 10-year OS of 91% (95% CI, 87 to 93) and 88% (95% CI, 83 to 91) and a 5- and 10-year EFS of 74% (95% CI, 69 to 78) and 72% (95% CI, 67 to 77; Fig 2A). Only the outcome of the *MYCN* group was significantly ($P = .002$) worse than that of the *FOXR2* group, showing the consistent 5- and 10-year OS and EFS of 37% (95% CI, 26 to 48) and 29% (95% CI, 19 to 39). Also in the independent NRC data set, *FOXR2*-expressing tumors have a significantly ($P < .001$) worse outcome, showing a 5- and 10-year OS of 53% (95% CI, 25 to 74) than the others group with a 5- and 10-year OS of 82% (95% CI, 74 to 87) and 80% (95% CI, 71 to 86), but a better outcome than the *MYCN* group (5- and 10-year OS of 26% [95% CI, 14 to 40]; Data Supplement). The TARGET series

**FIG 2.** Survival data of distinct molecular groups. (A) OS and EFS from initial diagnosis of patients with neuroblastoma of the RNA-seq data set (N = 498) separated by the groups *FOXR1, FOXR2, MYC, MYCN*, and others. (B) OS and EFS of exclusively high-risk patients in the RNA-seq data set (n = 173). [a]The *FOXR1* group is excluded because of low number (n = 2). (C) Multivariable Cox regression analyses of the RNA-seq data set (N = 498) for OS taking into account *FOXR2* and the established prognostic factors age above 18 months at diagnosis, *MYCN* amplification, INSS 4, and *FOXR2* and risk group in a separate analysis. HRs with 95% CIs and *P* values are indicated. [b]The *MYCN* amplification status is unavailable for five cases in the RNA-seq data set, and therefore, this multivariate analysis was conducted for 493 of 498 cases. EFS, event-free survival; HR, hazard ratio; OS, overall survival.

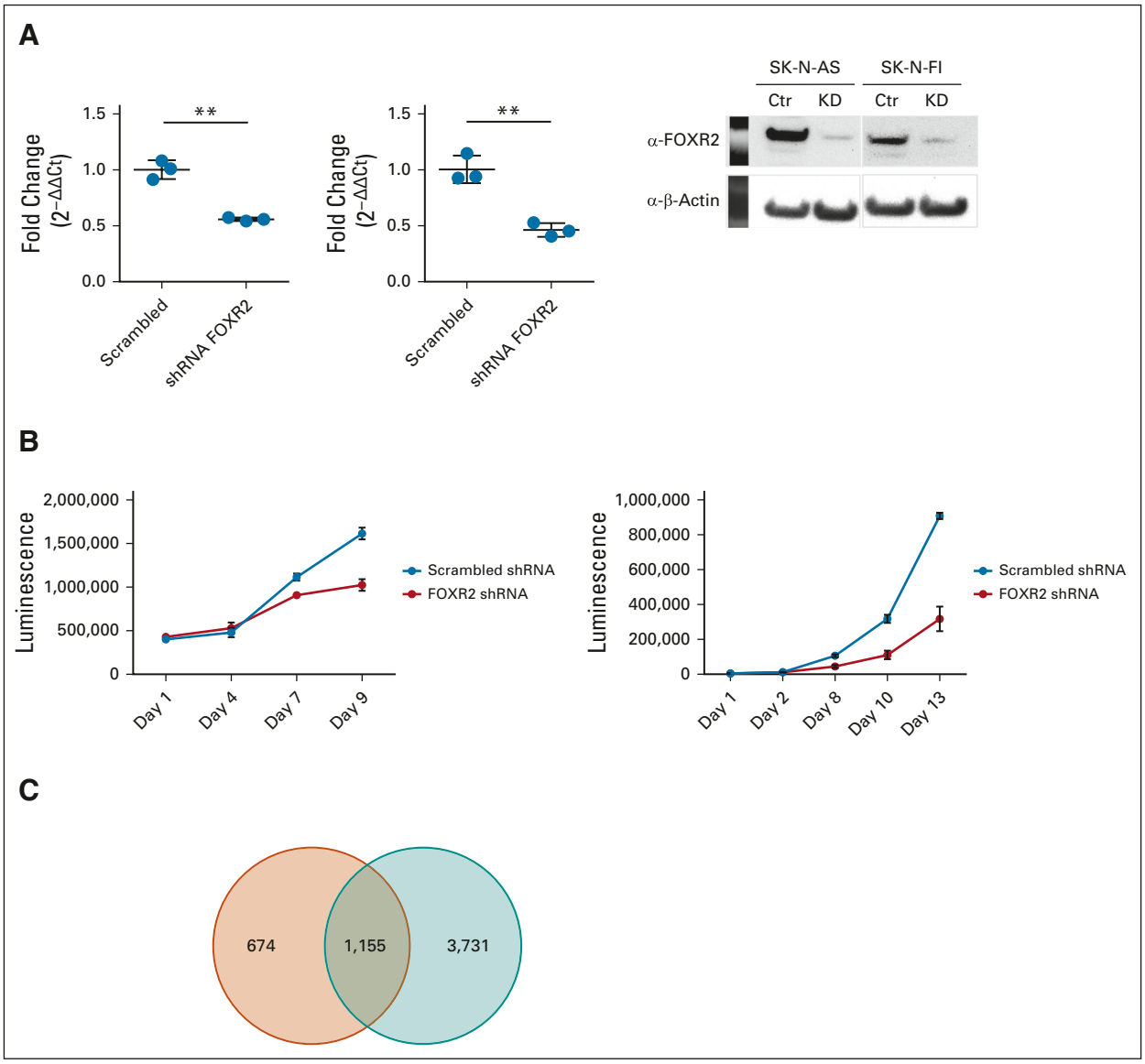
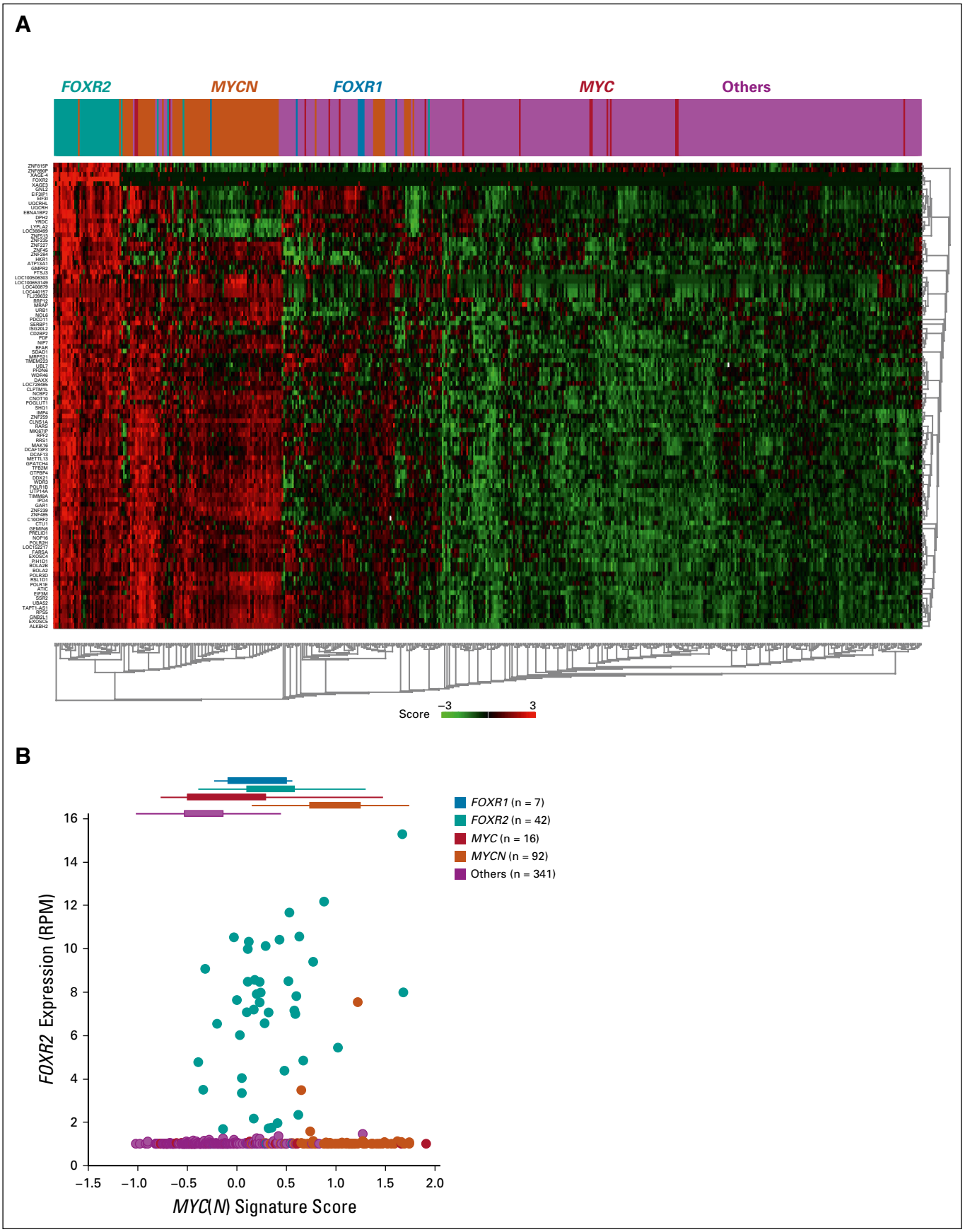



**FIG 3.** FOXR2 silencing reduces proliferation. (A) FOXR2 KD in neuroblastoma cell lines SK-N-AS (left) and SK-N-FI (right) shown by quantitative real-time polymerase chain reaction and western blot. (B) Growth curve of SK-N-AS (left) and SK-N-FI (right) upon FOXR2 KD reveals reduced cell proliferation. (C) Venn diagram indicating the FOXR2 KD signature overlap of SK-N-FI (red) and SK-N-AS (blue). Ctr, control; KD, knockdown. $^{**}P \leq .005$.

was due to its bias to high-risk tumors solely used as a reference for high-risk subsets.

Investigating high-risk neuroblastoma only in the RNA-seq, NRC, and TARGET data sets, we observed the very poor 5-year OS of 34% (95% CI, 11 to 60), 0%, and 24% (95% CI, 11 to 40), respectively, for the patients with *FOXR2*-expressing tumors, which is significantly worse than the others group in NRC and TARGET ($P < .001$) and almost significant in the RNA-seq data set ($P = .052$; Fig 2B, Data Supplement). No significant difference in outcome was observed between the *FOXR2* and *MYCN* groups in high-risk neuroblastoma in the RNA-seq ($P = .971$), NRC ($P = .063$), and TARGET ($P = .172$) data sets.

*FOXR1*-expressing tumors showed a comparable clinical outcome with the *FOXR2* group ($P = .715$; 5- and 10-year OS of 57% [95% CI, 17 to 84]), whereas *MYC*-expressing tumors revealed a relatively good outcome (5- and 10-year OS of 85% [95% CI, 52 to 96]), but numbers were small in both groups (Fig 2A). Finally, we also compared the *FOXR2* group with patients with an ALT phenotype in the ALT-annotated subset of the RNA-seq data set (n = 174), revealing the similar 5-year OS of 72% (95% CI, 41 to 88) and 61% (95% CI, 25 to 84) and the 5-year EFS of 47% (95% CI, 22 to 69) and 29% (95% CI, 9 to 53), respectively. Both the *FOXR2* and ALT group showed a significantly worse overall outcome ($P < .001$ in both cases) than the


A
FOXR2
MYCN
FOXR1
MYC
Others
Score
−3
3
B
FOXR1 (n = 7)
FOXR2 (n = 42)
MYC (n = 16)
MYCN (n = 92)
Others (n = 341)
FOXR2 Expression (RPM)
MYC(N) Signature Score
0
2
4
6
8
10
12
14
16
−1.5
−1.0
−0.5
0.0
0.5
1.0
1.5
2.0

remaining patients without ALT phenotype or elevated expression of *FOXR1*, *FOXR2*, *MYC*, and *MYCN* (5-year OS 99% [95% CI, 92 to 100] and an EFS of 74% [95% CI, 64 to 82]; Data Supplement).

### *FOXR2* Expression Is an Independent Prognostic Risk Factor

The clinical relevance of *FOXR2* expression in neuroblastoma was further evaluated by multivariable Cox regression analyses, comparing *FOXR2* expression with other well-established risk factors like INSS4 stage, *MYCN* amplification, and age above 18 months (Data Supplement). In both the RNA-seq and NRC data sets, *FOXR2* expression is an independent and significant prognostic risk factor with hazard ratios of 2.5 and 3.5 ($P = .004$ and $P = .003$, respectively; Data Supplement). Also, when comparing *FOXR2* expression with high-risk status (defined by INSS4 patients above 18 months and *MYCN*-amplified patients independent from age and INSS stage[7]) within the whole RNA-seq and NRC cohort, *FOXR2* expression sustained in both data sets an independent and significant prognostic marker in multivariable analyses (Fig 2C, Data Supplement). In line with this, within the non–high-risk subset of the NRC and RNA-seq data sets, *FOXR2*-expressing patients show a significantly reduced survival ($P = .012$ and $P < .001$, respectively) compared with patients without or very low *FOXR2* expression, indicating that *FOXR2* expression identifies patients at higher risk of recurrence among the group of children who are not currently designated as having high-risk disease (Data Supplement). *FOXR2* expression remained prognostic also when taking into account other prognostic markers like 11q status or segmental chromosome alterations in general (including 1p, 3p, and 11q deletion and 17q gain) for which data were available in a subset (n = 183) of the RNA-seq data set (Data Supplement). Because of its high-risk bias, the TARGET data set was excluded from these analyses.

### FOXR2 Knockdown Is Associated With Reduced Cellular Growth, Cell Cycle Arrest, and Cell Death in Neuroblastoma Cell Lines

To investigate the role of FOXR2 in neuroblastoma, we selected two neuroblastoma cell lines (SK-N-AS and SK-N-FI) with *FOXR2* expression for shRNA-induced knockdown experiments (Fig 3A). Interestingly, in the SK-N-AS line, *FOXR2* is activated by enhancer hijacking of an active gene *KLHL13*. How *FOXR2* is activated in SK-N-FI is unclear as no fusions could be detected. Both cell lines do not have an *MYCN* amplification, but still express relatively low levels of either *MYCN* mRNA (SK-N-FI) or *MYC* mRNA, because of a t(4;8) rearrangement (SK-N-AS).[39] FOXR2 knockdown resulted in a significantly reduced growth in both cell lines (Fig 3B). Expression analyses after *FOXR2* knockdown in SK-N-AS and SK-N-FI identified 4886 and 1829 significantly differentially expressed genes (*P* value: .05, ANOVA), with 1155 mutually affected genes (Fig 3C). Pathway analyses illustrate the oncogenic role of FOXR2 (Data Supplement), which was further validated with in vitro experiments, showing that *FOXR2* knockdown is associated with cell cycle arrest and increased cellular death (Data Supplement).

### The *FOXR2* Group Is Transcriptionally Similar to the *MYCN* Group

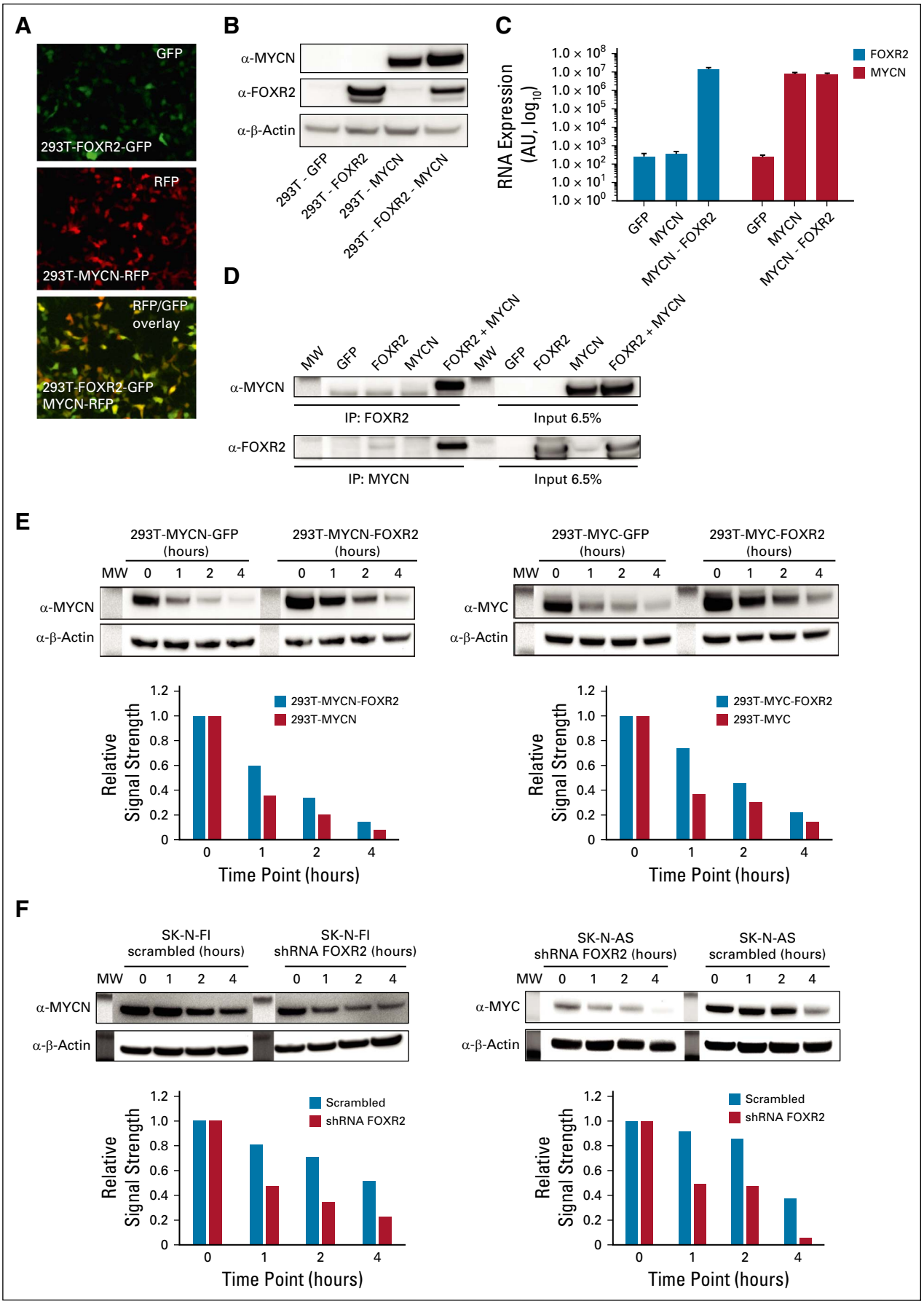
On the basis of transcriptomic data, we found that *FOXR2*-expressing cases do not form a separate distinct cluster when clustering all cases (Data Supplement), but when comparing *FOXR2*-expressing cases with the others group and using the differentially expressed genes (Data Supplement) as a gene signature to cluster all cases, we found that the *MYCN*-expressing cases shared most of the genes that are upregulated in the *FOXR2* group (Fig 4A). Also, when using our previously developed *MYC*(*N*) signature[36] to cluster all cases, we found that the *FOXR2* and *MYCN* groups are transcriptionally related (Data Supplement). Finally, we used our *MYC*(*N*) signature to calculate the *MYC*(*N*) activity score for all cases. *FOXR2*-expressing tumors have a positive *MYC*(*N*) activity score, in many cases similar in magnitude to *MYCN*-amplified tumors, indicating that FOXR2-expressing neuroblastomas also activate MYCN target genes despite low *MYCN* mRNA expression levels (Fig 4B).

### FOXR2 Binds and Stabilizes MYCN and MYC Protein Levels

To investigate FOXR2 and MYCN mechanistically, we transfected 293T cells with constructs expressing HA-tagged FOXR2 co-expressed with GFP or MYCN co-expressed with RFP (Fig 5A). Interestingly, western blot analyses showed that when FOXR2 and MYCN were expressed together, MYCN protein levels were about 2-fold higher (Fig 5B), which could not be attributed to increased expression of *MYCN* RNA (Fig 5C). Co-immunoprecipitation analyses showed that FOXR2 binds to MYCN (Fig 5D) and is thereby stabilizing the short-lived protein, as demonstrated by cycloheximide chase assays, tracking MYCN, and also MYC protein turnover in the presence and the absence of FOXR2 (Figs 5E and 5F).

Finally, to confirm stabilization of MYCN protein by FOXR2 in neuroblastoma tissue, we compared *FOXR2*-activated tumors showing minimal *MYCN* expression with low-risk

**FIG 4.** *FOXR2* and *MYCN* tumors are transcriptionally similar. (A) Applying the *FOXR2* tumor signature on the RNA-seq data set (N = 498), the *MYCN* group resembles the *FOXR2* group transcriptionally. (B) *FOXR2* expression plotted against the *MYC*(*N*) signature score reveals a positive *MYC*(*N*) activity score for the *FOXR2* group.


A
GFP
293T-FOXR2-GFP
RFP
293T-MYCN-RFP
RFP/GFP overlay
293T-FOXR2-GFP MYCN-RFP
B
α-MYCN
α-FOXR2
α-β-Actin
293T - GFP
293T - FOXR2
293T - MYCN
293T - FOXR2 - MYCN
C
RNA Expression (AU, log10)
FOXR2
MYCN
GFP
MYCN
MYCN - FOXR2
D
MW
GFP
FOXR2
MYCN
FOXR2 + MYCN
α-MYCN
α-FOXR2
IP: FOXR2
IP: MYCN
Input 6.5%
E
293T-MYCN-GFP (hours)
293T-MYCN-FOXR2 (hours)
293T-MYC-GFP (hours)
293T-MYC-FOXR2 (hours)
α-MYCN
α-MYC
α-β-Actin
Relative Signal Strength
293T-MYCN-FOXR2
293T-MYCN
293T-MYC-FOXR2
293T-MYC
Time Point (hours)
F
SK-N-FI scrambled (hours)
SK-N-FI shRNA FOXR2 (hours)
SK-N-AS shRNA FOXR2 (hours)
SK-N-AS scrambled (hours)
Scrambled
shRNA FOXR2

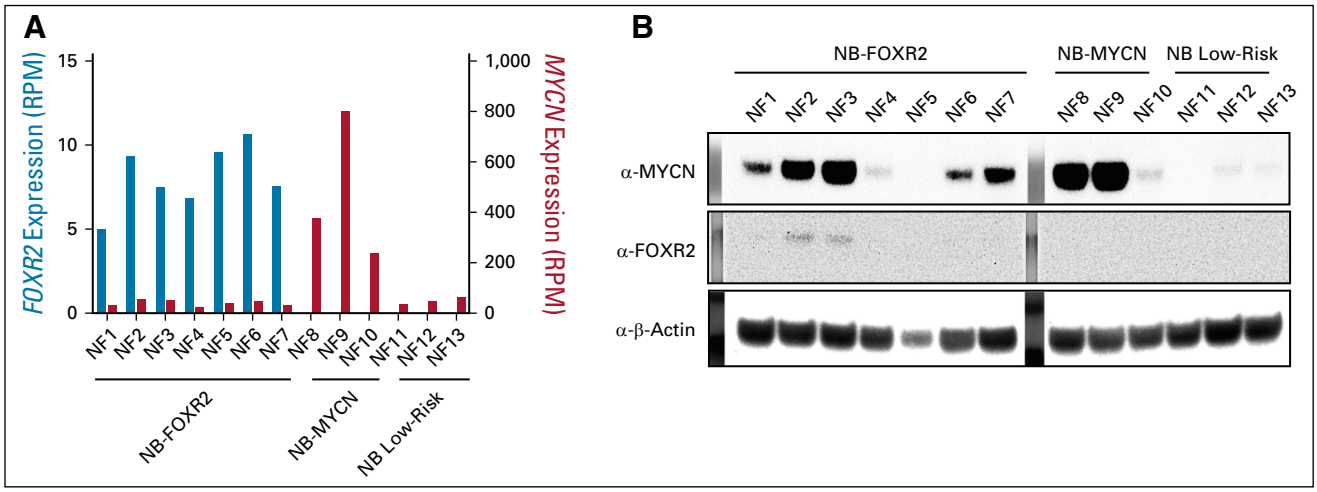



**FIG 6.** MYCN protein levels are high in *FOXR2*-expressing neuroblastoma. (A) mRNA levels and (B) western blot of seven *FOXR2*-expressing neuroblastomas, three *MYCN*-amplified neuroblastomas, and three low-risk neuroblastomas reveal that MYCN protein levels are highly increased in most *FOXR2* expressing neuroblastoma samples despite low *MYCN* expression.

samples (without *FOXR2* or low *MYCN*) and *MYCN*-amplified samples. Despite the minimal *MYCN* RNA expression, MYCN protein levels were highly increased in 5 of 7 of *FOXR2*-expressing samples compared with the low-risk tissues. In two of the *FOXR2*-expressing cases, we even observed MYCN to a level that is similar in the *MYCN*-amplified cases (Fig 6). FOXR2 protein could hardly be detected by western blot; nonetheless, the two samples that showed an FOXR2 signal were precisely the ones with the highest levels of MYCN protein (Fig 6). From these findings in vitro and in tumor tissue, we conclude that FOXR2 stabilizes MYCN in neuroblastoma on the protein level.

## DISCUSSION

We have identified *FOXR2* expression as a new prognostic marker associated with unfavorable clinical outcome in neuroblastoma, independent of other previously well-established prognostic factors.[12,13]

Moreover, we have shown that FOXR2 is essential in neuroblastoma and stabilizes MYCN protein, indicating that FOXR2 forms an alternative mechanism to *MYCN* amplification.[40,41] The mechanism of *FOXR2* activation in peripheral neuroblastoma remains elusive since in contrast to CNS neuroblastoma with *FOXR2* activation,[19] gene fusions driving *FOXR2* expression in peripheral neuroblastoma were rarely detected. Poor survival of the *FOXR2* group was similarly observed within the separately analyzed NB97 and NB2004 treatment studies of the RNA-seq data set (data not shown). Since the response of high-risk patients to different induction[42] or consolidation treatment arms was similar in the RNA-seq data set (data not shown), it is unlikely that the outcome of the *FOXR2* groups is influenced by the different protocols. Interestingly, the almost identical 5- and 10-year EFS and progression-free survival of *FOXR2*-expressing neuroblastoma indicate that primarily, only early events occur in this group of patients.

Moreover, we observed enriched *TERT* expression in the *FOXR2*-expressing group, most likely a direct consequence of stabilized MYCN, which is a transcriptional activator of TERT.[9,43,44]

In our study, we observed that high *MYC* mRNA–expressing patients show a relatively good outcome, in contrast to previous studies.[39,45] However, Zimmermann et al[39] considered exclusively high-risk patients, whereas in the RNA-seq data set analyzed here, 8 of 16 *MYC*-expressing cases belong to the non–high-risk group, which might explain varying findings. Moreover, Wang et al[45] analyzed MYC protein levels, which are not directly translatable to mRNA levels used in our study.

Limitations of this study, including its retrospective nature and potential confounding related to variability in treatment,

**FIG 5.** FOXR2 protein binds to and stabilizes MYCN protein. FOXR2, MYCN, and a combination of both overexpressed in HEK 293T cells as shown by (A) fluorescence immunostainings, (B) on protein level, and (C) on RNA level. (D) Immunoprecipitation analysis revealed that FOXR2 binds to MYCN. (E) CHX assays of FOXR2- and MYC(N)-overexpressing HEK 293T cells show MYC and MYCN stabilization by FOXR2 on western blot (upper panels). Band intensities were quantified and signal intensities are shown for each time point, normalized to β-actin and the initial signal intensity at the time point 0 hour (lower panel). (F) CHX assays of the FOXR2 knockdown cell lines SK-N-AS and SK-N-FI indicate that MYCN and MYC degrade more rapidly upon FOXR2 knockdown (upper panel). Band intensities were quantified, and signal intensities are shown for each time point, normalized to β-actin and the initial signal intensity at the time point 0 hour (lower panel). CHX, cyclohexamide.

underline the need for further prospective studies of uniformly treated patients who are otherwise considered non–high-risk. These requirements in combination with the relatively small number of *FOXR2*-expressing neuroblastomas would likely require an international collaboration. Additionally, our study was limited by the unavailable subclassification into low risk and intermediate risk and did not allow for further characterization of *FOXR2* within these risk groups. The need for biomarkers to identify patients with poor outcome, especially in subsets such as patients age above 18 months, INSS 3, and unfavorable histology, where risk classification is not consistent between different international studies, underlines the importance of further characterization of FOXR2 within risk groups.

Altogether, our study identifies an *FOXR2*-activated subset in neuroblastoma that shows an unfavorable clinical outcome and we have identified *FOXR2* expression as a new independent prognostic factor. Our mechanistic study reveals that MYCN is stabilized by FOXR2, which may explain the poor clinical outcome of patients and provides crucial knowledge for a targeted treatment of *FOXR2*-activated neuroblastoma.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## AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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## AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

**FOXR2 Stabilizes MYCN Protein and Identifies Non–*MYCN*-Amplified Neuroblastoma Patients With Unfavorable Outcome**

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