

WDR5 Supports an N-Myc Transcriptional Complex That Drives a Protumorigenic Gene Expression Signature in Neuroblastoma

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Abstract

MYCN gene amplification in neuroblastoma drives a gene expression program that correlates strongly with aggressive disease. Mechanistically, trimethylation of histone H3 lysine 4 (H3K4) at target gene promoters is a strict prerequisite for this transcriptional program to be enacted. WDR5 is a histone H3K4 presenter that has been found to have an essential role in H3K4 trimethylation. For this reason, in this study, we investigated the relationship between WDR5-mediated H3K4 trimethylation and N-Myc transcriptional programs in neuroblastoma cells. N-Myc upregulated WDR5 expression in neuroblastoma cells. Gene expression analysis revealed that WDR5 target genes included those with MYC-binding elements at promoters such as *MDM2*. We showed that WDR5 could form a protein complex at the *MDM2* promoter with N-Myc, but not p53, leading to histone

H3K4 trimethylation and activation of *MDM2* transcription. RNAi-mediated attenuation of WDR5 upregulated expression of wild-type but not mutant p53, an effect associated with growth inhibition and apoptosis. Similarly, a small-molecule antagonist of WDR5 reduced N-Myc/WDR5 complex formation, N-Myc target gene expression, and cell growth in neuroblastoma cells. In *MYCN*-transgenic mice, WDR5 was overexpressed in precancerous ganglion and neuroblastoma cells compared with normal ganglion cells. Clinically, elevated levels of WDR5 in neuroblastoma specimens were an independent predictor of poor overall survival. Overall, our results identify WDR5 as a key cofactor for N-Myc-regulated transcriptional activation and tumorigenesis and as a novel therapeutic target for *MYCN*-amplified neuroblastomas. *Cancer Res*; 75(23); 5143–54. ©2015 AACR.

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Introduction

Neuroblastoma is the most common solid tumor in early childhood (1, 2). It accounts for approximately 15% of all childhood cancer-related death despite the use of combination chemotherapy, radiotherapy, and bone marrow transplantation (1, 3). Amplification of the *MYCN* oncogene strongly correlates with an aggressive tumor behavior and is currently used as an indicator for poor patient prognosis (1, 3, 4).

Myc oncoproteins N-Myc and c-Myc induce tumorigenesis by binding to target gene promoters and consequently activating the transcription of target genes such as the E3 ubiquitin-protein ligase MDM2 (5–7), which induces p53 protein degradation (8–10). Paradoxically, N-Myc oncoprotein directly activates gene transcription of p53 by binding to its gene promoter (6). By analyzing 35 histone marks after genomic binding by Myc, Guccione and colleagues have revealed that histone H3 lysine 4 (H3K4) trimethylation at Myc-responsive elements of target gene promoters is a strict prerequisite for Myc-induced transcriptional activation (11). However, the mechanism through which histone H3K4 is trimethylated during Myc-induced transcriptional activation, is unknown.

WDR5 is a core subunit of the MLL, ASH2L, and RBBP5 histone H3K4 methyltransferase complex (12). WDR5 directly associates with transcription factors, and is required for the binding of the histone methyltransferase complex to histone H3K4, H3K4

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trimethylation, and transcriptional activity of the transcription factors (13–15). By direct binding to Oct4 and causing H3K4 trimethylation at Oct4 target gene promoters, WDR5 activates the transcription of Oct4 target genes and is required for the formation of induced pluripotent stem cells (16). By direct binding to MLL and causing histone H3K4 trimethylation at MLL target gene promoters, WDR5 induces the transcription of MLL target genes and promotes leukemia (13–15).

In the current study, we showed, for the first time, that WDR5 formed a protein complex with N-Myc at N-Myc target gene promoters, leading to histone H3K4 trimethylation, transcriptional activation of the N-Myc target genes, including *MDM2*, and reduction in wild-type p53 protein, that repression of WDR5 resulted in neuroblastoma cell growth inhibition and apoptosis, and that high levels of WDR5 expression in human neuroblastoma tissues independently predicted poor patient prognosis.

Materials and Methods

Cell culture

BE(2)-C human neuroblastoma, RAT fibroblasts and HEK293 primary embryonic kidney cells were cultured in DMEM supplemented with 10% FBS. CHP134, SK-N-BE(2), and SHEP Tet-21/N human neuroblastoma cells were cultured in RPMI1640 medium supplemented with L-glutamine and 10% FBS. BE(2)-C, HEK293, SK-N-BE(2), and RAT1 were obtained from ATCC, and CHP134 and Kelly cells from the European Collection of Cell Cultures. The identity of all cell lines was verified by small tandem repeat profiling conducted by at Garvan Institute or Cellbank Australia.

Chromatin immunoprecipitation assays and dual cross-linking ChIP assays

Chromatin immunoprecipitation (ChIP) assays and dual cross-linking ChIP assays were performed with a control, anti-N-Myc, anti-WDR5, anti-trimethyl H3K4 antibody (all from Merck Millipore) or anti-p53 antibody (Cell Signaling Technology) and PCR with primers targeting negative control regions, the *WDR5* or *MDM2* gene promoter regions with the protocol we described (17–19). Fold enrichment of the *WDR5* and *MDM2* gene core promoters was calculated by dividing PCR products from samples immunoprecipitated by experimental antibodies by PCR products from samples immunoprecipitated by control antibody.

Luciferase assays

Modulation of *MDM2* gene promoter activity by WDR5 was analyzed by luciferase assays. pGL3 construct expressing wild-type or E-Box mutant *MDM2* gene promoter was obtained from Dr. Jason Shohet (7), and pGL3 construct expressing the *MDM2* gene P4 promoter, which contained the p53-binding sites but not the Myc-responsive E-Box (20), was obtained from Addgene. Luciferase reporter activity was measured using the Dual Luciferase Assay System (Promega) as we described previously (17–19).

Protein coimmunoprecipitation assays

Nuclear protein extract from SK-N-BE cells was immunoprecipitated with control IgG, anti-N-Myc or anti-WDR5 antibody. Alternatively, HEK293 cells were cotransfected with pShuttle Flag-empty vector or pShuttle-Flag-N-Myc expression construct, together with pCMV6-Myc-DDK-empty vector or pCMV6-Myc-

DDK-WDR5 expression construct (Origene) with Lipofectamine 2000. In separate experiments, cells were cotransfected with a pCMV6-Myc-DDK-empty vector or pCMV6-Myc-DDK-WDR5 expression construct, together with a pcDNA3 Flag-empty vector or pcDNA3-Flag-p53 expression construct (Addgene). Thirty-six hours after transfections, cellular protein was extracted and incubated with a control, anti-N-Myc, anti-WDR5 or anti-p53 antibody. Eluted protein was immunoblotted with an anti-N-Myc, anti-WDR5 or anti-p53 antibody.

Animal experiments and immunohistochemistry

Animal experiments were approved by the Animal Care and Ethics Committee of University of New South Wales Australia (Sydney, Australia), and the animals' care was in accord with institutional guidelines. Wild-type and *TH-MYCN* transgenic 129/SvJ mice were bred and euthanized at the age of 1, 7, 14, and 28 days. After fixation in formalin and paraffin-embedding, mouse tissue sections containing celiac and paravertebral ganglia were subjected to immunohistochemistry analysis of WDR5 protein expression with a rabbit anti-WDR5 monoclonal antibody (1:600, Merck Millipore). Positively stained cells were quantified using ImageJ software (NIH, Bethesda, MD).

Patient tumor sample analysis

Fifty-nine untreated primary neuroblastoma tumor specimens were granted after patient consent and ethics approval from the Cologne tumor bank and the 'Universitätsklinikum' Essen, Germany. The International Neuroblastoma Staging System criteria was used (21). Patient characteristics are outlined in Supplementary Table S1. Protein was extracted from the tumors with the AllPrep DNA/RNA/protein Mini Kit (Qiagen), and subjected to immunoblot analysis of WDR5 and N-Myc. In addition, WDR5 and N-Myc mRNA expression was analyzed in 88 (Versteeg dataset; ref. 22) and 476 (Kocak dataset; refs. 23, 24) human neuroblastoma samples in the publicly available gene expression databases at the R2: Genomics Analysis and Visualization Platform website (25).

Statistical analysis

Experiments were performed at least three times. Data were analyzed with Graphpad Prism 6 program and expressed as mean \pm SE. Differences were analyzed for significance with ANOVA among groups or two-sided unpaired *t* test for two groups of numerical variables, or analyzed by the χ^2 test for categorical variables.

Correlation between N-Myc and WDR5 expression in human neuroblastoma tissues was examined with Pearson's correlation. Survival analyses were performed according to the method of Kaplan and Meier and two-sided log-rank tests (26). Multivariable Cox regression analyses were performed. Probabilities of survival and HRs were provided with 95% confidence intervals (CI). Proportionality was confirmed by visual inspection of the plots of $\log(2\log(S(\text{time})))$ versus $\log(\text{time})$, which were observed to remain parallel (27). A probability value of 0.05 or less was considered statistically significant. All statistical tests were two-sided.

Supplementary information

Supplementary Information includes Supplementary Materials and Methods, four figures, three tables, and five datasets.

Results

N-Myc upregulates WDR5 expression by binding to the WDR5 gene promoter

N-Myc activates gene transcription by binding to E-Box motifs at target gene promoters (28, 29). Our bioinformatics analysis revealed noncanonical (CACGCG; -13 to -18 bp) and canonical (CACGTG; +85-+90 bp) E-boxes at the WDR5 gene promoter. We therefore examined whether N-Myc modulated WDR5 expression in the MYCN-amplified human BE(2)-C and CHP134 neuroblastoma cell lines. Transfection with N-Myc siRNA-1 or

siRNA-2 efficiently knocked down N-Myc mRNA and protein expression, and reduced WDR5 mRNA and protein expression (Fig. 1A and B). Consistently, ectopic overexpression of N-Myc in RAT1 fibroblasts led to considerable WDR5 upregulation (Fig. 1C).

We next performed chromatin immunoprecipitation assays in BE(2)-C and CHP134 cells with a control or anti-N-Myc antibody. As shown in Fig. 1D, E, and F, the anti-N-Myc antibody significantly immunoprecipitated the WDR5 gene promoter regions containing the canonical and noncanonical E-Boxes, and

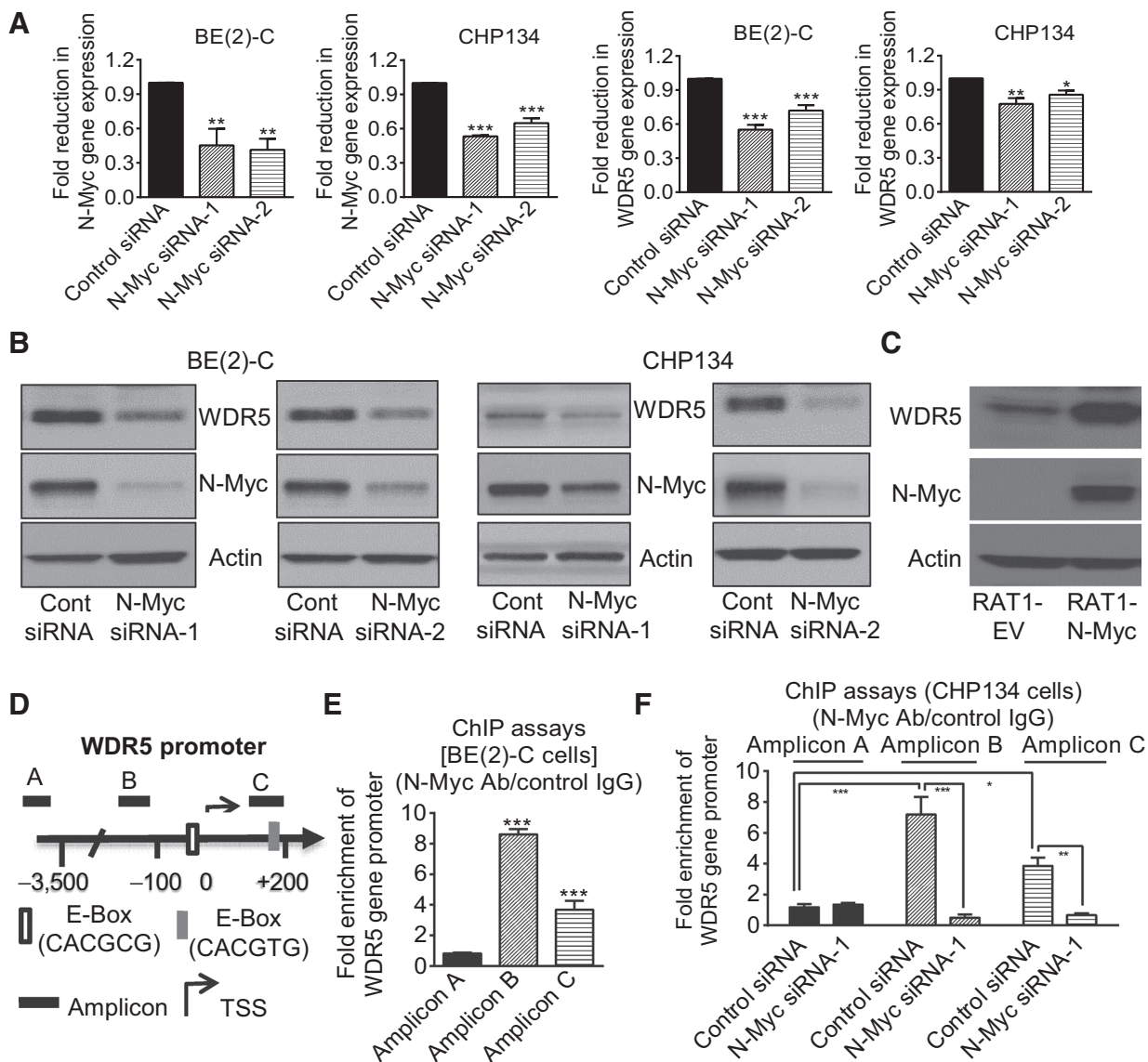
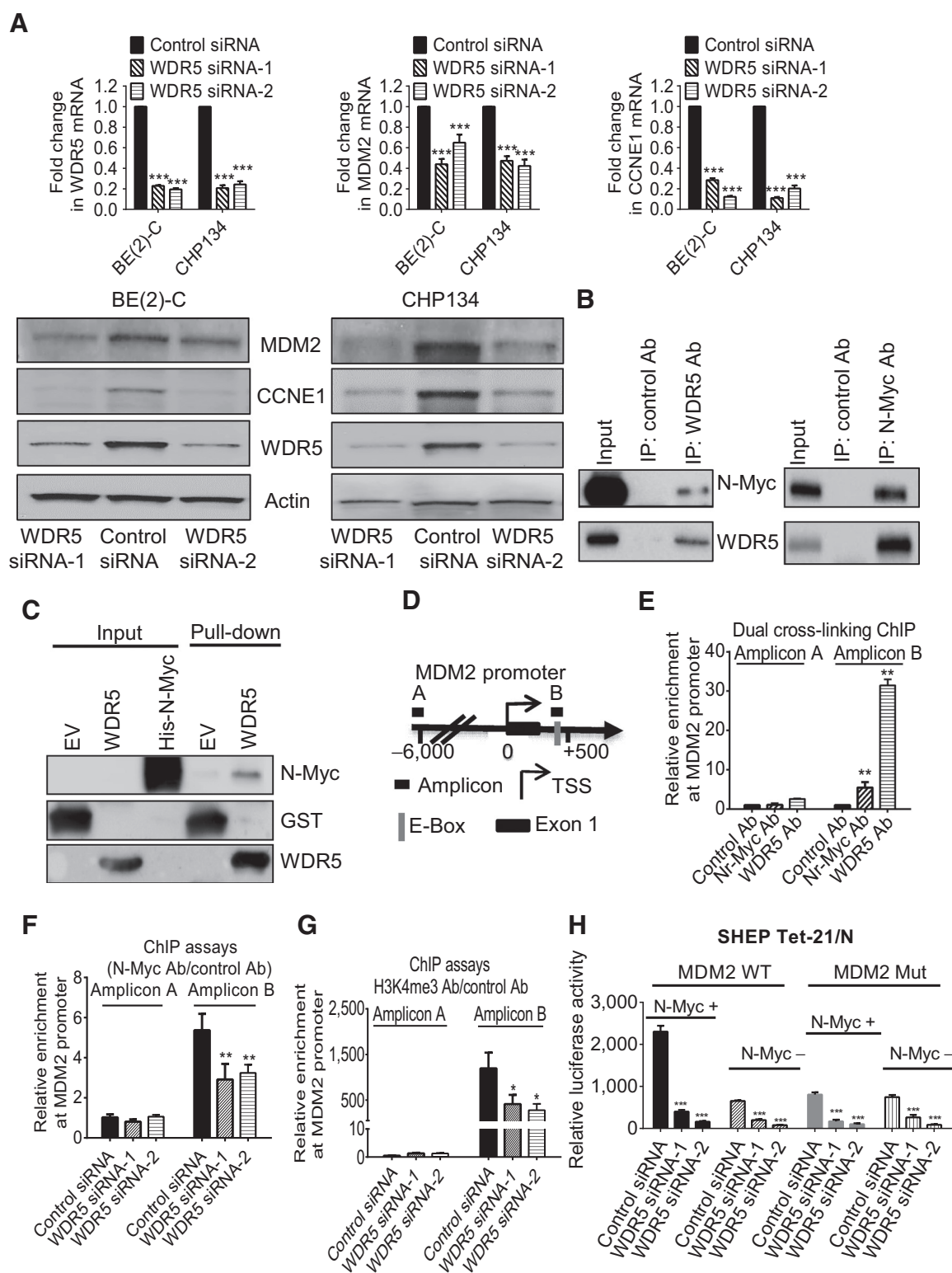


Figure 1.

N-Myc upregulates WDR5 expression in neuroblastoma cells by binding to the WDR5 gene promoter. A and B, BE(2)-C and CHP134 cells were transfected with control siRNA, N-Myc siRNA-1, or N-Myc siRNA-2 for 72 hours, followed by RT-PCR (A) and immunoblot (B) analyses of N-Myc and WDR5 expression. C, protein was extracted from RAT1 fibroblasts stably transfected with an empty vector (RAT1-EV) or full-length N-Myc expression construct (RAT1-N-Myc), and subjected to immunoblot analysis of WDR5 and N-Myc protein. D, schematic representation of the WDR5 gene promoter. TSS, transcription start site. E and F, ChIP assays were performed with a control or anti-N-Myc antibody (Ab), followed by PCR with primers targeting the negative control region (Amplicon A) or the WDR5 gene promoter containing the E-Boxes (Amplicons B and C) in BE(2)-C cells without siRNA transfection (E) or in CHP134 cells 24 hours after transfection with control siRNA or N-Myc siRNA-1 (F). Fold enrichment of the WDR5 gene promoter was calculated as the difference in cycle thresholds obtained with the anti-N-Myc antibody and with the control antibody. Error bars represent SE. **, $P < 0.01$; ***, $P < 0.001$.

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**Figure 2.**

WDR5 forms a protein complex with N-Myc at N-Myc target gene promoters, leading to histone H3K4 trimethylation and transcriptional activation of N-Myc target genes. A, BE(2)-C and CHP134 cells were transfected with control siRNA, WDR5 siRNA-1, or WDR5 siRNA-2. WDR5, CCNE1, and MDM2 mRNA and protein expression was analyzed by RT-PCR and immunoblot. B, nuclear protein extracted from SK-N-BE(2) neuroblastoma cells was immunoprecipitated (IP) overnight with 2 μ g of control IgG, anti-N-Myc or anti-WDR5 antibody (Ab). (Continued on the following page.)

N-Myc siRNA completely blocked the effect. The data indicate that N-Myc upregulates *WDR5* gene expression by directly binding to the *WDR5* gene promoter.

WDR5 forms a protein complex with N-Myc at N-Myc target gene promoters, leading to histone H3K4 trimethylation and transcriptional activation of N-Myc target genes

As WDR5 exerts biologic function by modulating gene transcription, we performed differential gene expression studies with Affymetrix microarray in BE(2)-C cells 40 hours after transfection with control or WDR5 siRNAs. The analyses showed that well-known N-Myc target genes cyclin E1 (CCNE1) and MDM2 (7, 28, 30), were among the genes significantly downmodulated by WDR5 siRNAs (Supplementary Dataset S1). Gene set enrichment analysis (GSEA) showed that genes with E-Boxes at promoters were highly enriched among those downregulated by WDR5 siRNAs (Supplementary Table S2). Consistently, GSEA analysis of microarray data from BE(2)-C cells after treatment with vehicle control or the BET bromodomain inhibitor JQ1 (31) also showed that genes with E-Boxes at promoters were highly enriched among those downregulated by JQ1 (Supplementary Table S3). RT-PCR and immunoblot validated the microarray data and confirmed that both WDR5 siRNAs and N-Myc siRNAs decreased mRNA and protein expression of CCNE1 and MDM2 in neuroblastoma cell lines (Fig. 2A and Supplementary Fig. S1).

We next performed ChIP sequencing (ChIP-Seq) with a control IgG or anti-trimethyl H3K4 (H3K4me3) antibody with DNA-protein complex from BE(2)-C cells 24 hours after transfection with control siRNA or WDR5 siRNA-1, and extracted anti-N-Myc antibody versus control IgG ChIP-Seq data from embryonic stem cells (32), as well as DNaseI hypersensitivity by Digital DNaseI datasets from ENCODE/University of Washington from BE(2)-C neuroblastoma cells for analyzing gene enhancers. Data analysis showed that knocking down WDR5 reduced H3K4me3 at 93.2% of N-Myc-binding promoters, but only at 53.5% of N-Myc nonbinding promoters (χ^2 : 1527.82, $P < 0.001$; Supplementary Fig. S2A; Supplementary Datasets S2–S5), and that H3K4me3 signal was very low at enhancers, compared with N-Myc-binding and N-Myc nonbinding gene promoters (Supplementary Fig. S2B). The data indicate that WDR5 preferentially modulates H3K4 trimethylation at N-Myc target gene promoters. We next examined whether N-Myc and WDR5 formed a protein complex. Protein coimmunoprecipitation assays showed that an anti-N-Myc antibody efficiently coimmunoprecipitated WDR5 protein, and conversely, an anti-WDR5 antibody efficiently coimmunoprecipitated N-Myc protein (Fig. 2B and Supplementary Fig. S3A). Moreover, GST pull-down assay with *in vitro* translated WDR5 and

N-Myc proteins showed that WDR5 protein specifically pulled down N-Myc protein (Fig. 2C), demonstrating that the two proteins form a protein complex.

The small-molecule OICR9429 has recently been shown to block the interaction of WDR5 with MLL and its protein-protein interaction network (33). We performed protein coimmunoprecipitation assays and confirmed that treatment with OICR9429, compared with its negative control compound OICR0547, blocked the formation of WDR5–N-Myc protein complex (Supplementary Fig. S3B). RT-PCR analysis showed that treatment with OICR9429 reduced the expression of the WDR5 and N-Myc target genes MDM2 and CCNE1 (Supplementary Fig. S4A), suggesting that OICR9429 blocks WDR5 protein binding to N-Myc protein and WDR5/N-Myc target gene expression.

We next sought to determine whether WDR5–N-Myc complex bound to the *MDM2* promoter in neuroblastoma. Dual cross-linking ChIP assays were performed in BE(2)-C cells with control, anti-N-Myc, and anti-WDR5 antibodies, followed by PCR with primers targeting a negative control region or the *MDM2* gene promoter (Fig. 2D). Results showed that the anti-N-Myc and the anti-WDR5 antibodies efficiently immunoprecipitated the *MDM2* gene promoter region containing the E-Box, compared with the negative control region (Fig. 2E).

To understand whether WDR5 is essential for histone H3K4 trimethylation and N-Myc protein binding to the *MDM2* gene promoter, we transfected BE(2)-C cells with control or WDR5 siRNAs, followed by ChIP assays with a control, anti-N-Myc, or anti-trimethylated H3K4 (H3K4me3) antibody. PCR analyses showed that knocking down WDR5 expression significantly reduced the presence of N-Myc and H3K4me3 at the *MDM2* gene promoter in BE(2)-C cells (Fig. 2F and G), suggesting that WDR5 is required for N-Myc protein binding to, and histone H3K4 trimethylation at, the *MDM2* promoter. To determine whether WDR5 binding to the *MDM2* E-box activates transcription of the *MDM2* gene, we next conducted luciferase reporter assays. SHEP Tet-21/N neuroblastoma cells were treated with vehicle control or tetracycline to induce or not to induce exogenous N-Myc expression, respectively (34). The cells were then cotransfected with control or WDR5 siRNAs, together with a pGL3 luciferase report construct expressing wild-type or E-Box mutant *MDM2* gene promoter (7). Results showed that N-Myc induction resulted in a significant increase in luciferase activity in cells transfected with the wild-type, but not the E-Box mutant, *MDM2* promoter construct. In addition, WDR5 siRNAs considerably reduced N-Myc-mediated wild-type *MDM2* promoter activity (Fig. 2H). Taken together, the data suggest that WDR5 forms a protein complex with N-Myc at

(Continued.) Immunoprecipitated protein was immunoblotted with anti-WDR5 or anti-N-Myc Ab. C, glutathione beads coated with GST and GST-WDR5 were incubated with purified 6×His-N-Myc protein (imidazole elution) and pull-down complexes were eluted and resolved into gels, followed by immunoblot analysis with antibodies against N-Myc, GST, and WDR5. Input samples for GST, GST-WDR5 and 6×His-N-Myc were loaded in the first three lanes, GST and GST-WDR5 pull-down complexes in the last two lanes. D, schematic representation of the *MDM2* gene promoter containing the N-Myc binding E-Box. E, dual cross-linking ChIP assays were performed in BE(2)-C cells with control, anti-N-Myc, and anti-WDR5 antibodies, followed by PCR with primers targeting the negative control region (Amplicon A) and the N-Myc binding site (Amplicon B) of the *MDM2* gene promoter. F and G, BE(2)-C cells were transfected with control siRNA, WDR5 siRNA-1, or WDR5 siRNA-2 for 48 hours, followed by ChIP assays with a control IgG, anti-N-Myc (F) or anti-trimethyl H3K4 (H3K4me3) antibody (G), and PCR with primers targeting the negative control region or the E-Box of the *MDM2* gene promoter. Fold enrichment of the *MDM2* promoter region was calculated as the difference in cycle thresholds obtained with the specific antibody and with the control IgG. H, SHEP Tet-21/N cells were cultured with tetracycline to not induce, or without tetracycline to induce, N-Myc expression, respectively. The cells were cotransfected with a luciferase reporter construct expressing wild-type or E-box mutant *MDM2* gene promoter, together with control siRNA, WDR5 siRNA-1, or WDR5 siRNA-2. Luciferase assays were performed, and relative luciferase activity of the wild-type and the mutant *MDM2* promoter constructs under the N-Myc (+) condition was normalized by the luciferase activity of the same reporter construct under the N-Myc (–) condition. Error bars represent SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

the N-Myc target gene promoter, leading to histone H3K4 trimethylation and N-Myc target gene transcription.

WDR5 reduces wild-type p53 protein expression by inducing histone H3K4 trimethylation at the *MDM2* gene promoter and *MDM2* gene transcription

N-Myc induces p53 gene transcription by direct binding to the p53 gene promoter (6). As MDM2 induces p53 protein degradation (8–10), we examined whether WDR5 reduced p53 protein expression through modulating MDM2 expression. Transfection with WDR5 siRNAs dramatically upregulated total wild-type p53 protein and wild-type p53 protein phosphorylated at serine 20 or 15 in CHP134 and Kelly cells, but did not significantly increase the abundance of mutant p53 protein in BE(2)-C cells (Fig. 3A). In comparison, WDR5 siRNAs did not show clear effect on p53 mRNA expression (Supplementary Fig. S5). We next cotransfected CHP134 cells with control siRNA or WDR5 siRNAs, together with a construct expressing empty vector, wild-type MDM2, or RING-finger-domain mutant MDM2 (C464A), which lacks the ability to facilitate p53 protein ubiquitination and degradation (35, 36). Immunoblot results showed that overexpression of wild-type MDM2, but not mutant MDM2, blocked WDR5 siRNA-mediated p53 protein upregulation (Fig. 3B). The data demonstrate that WDR5 downregulates wild-type p53 protein expression by upregulating MDM2.

Wild-type p53 protein directly induces *MDM2* gene transcription through binding to two p53-binding sites at the *MDM2* gene intron 1 (Fig. 3C; refs. 37, 38). Because WDR5 siRNAs reduced MDM2 mRNA expression to similar extents in p53 wild-type and mutant neuroblastoma cells but considerably upregulated wild-type p53 protein expression, we examined whether WDR5 is essential for histone H3K4 trimethylation and p53 protein binding at the two p53-binding sites at the *MDM2* gene intron 1. ChIP assays with a control, anti-H3K4me3, or anti-p53 antibody in CHP134 cells showed that transfection with WDR5 siRNAs reduced the presence of H3K4me3 and p53 at the p53-binding sites (Fig. 3D and E). However, protein coimmunoprecipitation assays with a control or anti-WDR5 antibody showed that the WDR5 antibody efficiently immunoprecipitated WDR5 protein, but not p53 protein (Fig. 3F). Importantly, luciferase assays showed that transfection with the pGL3 construct expressing the *MDM2* gene P4 promoter, which contained the p53-binding sites but not the N-Myc-binding site (20), resulted in considerable increase in luciferase activity, and that WDR5 siRNA-1 and WDR5 siRNA-2 significantly reduced the effect (Fig. 3G). Taken together, the data indicate that WDR5 enhances p53-mediated *MDM2* gene expression by inducing H3K4 trimethylation and p53 protein binding to the *MDM2* gene promoter without forming a protein complex with p53, and thus playing an important role in maintaining the p53-MDM2 negative feedback loop.

WDR5 induces neuroblastoma cell proliferation and survival through p53-dependent and -independent mechanisms

We next examined whether WDR5 induces neuroblastoma cell proliferation and survival. Alamar blue assays showed that depleting WDR5 expression with siRNAs moderately decreased the number of p53-mutant BE(2)-C cells, and severely decreased the number of p53 wild-type CHP134 cells (Fig. 4A). In agreement, WDR5 siRNAs increased the percentage of CHP134 cells positively stained by Annexin V, and cotransfection with p53 siRNA

significantly blocked this effect (Fig. 4B). In addition, treatment with the WDR5 antagonist OICR9429 reduced the numbers of viable BE(2)-C and more significantly CHP134 cells (Supplementary Fig. S4B), and combination therapy with OICR9429 and the BET bromodomain inhibitor JQ1 synergistically reduced the numbers of viable neuroblastoma cells (Supplementary Fig. S4C). Taken together, the data demonstrate that WDR5 induces neuroblastoma cell proliferation and survival through p53-independent and -dependent mechanisms, and WDR5 antagonists are potential novel anticancer agents.

WDR5 expression is upregulated in precancerous and neuroblastoma tissues in *MYCN* transgenic mice

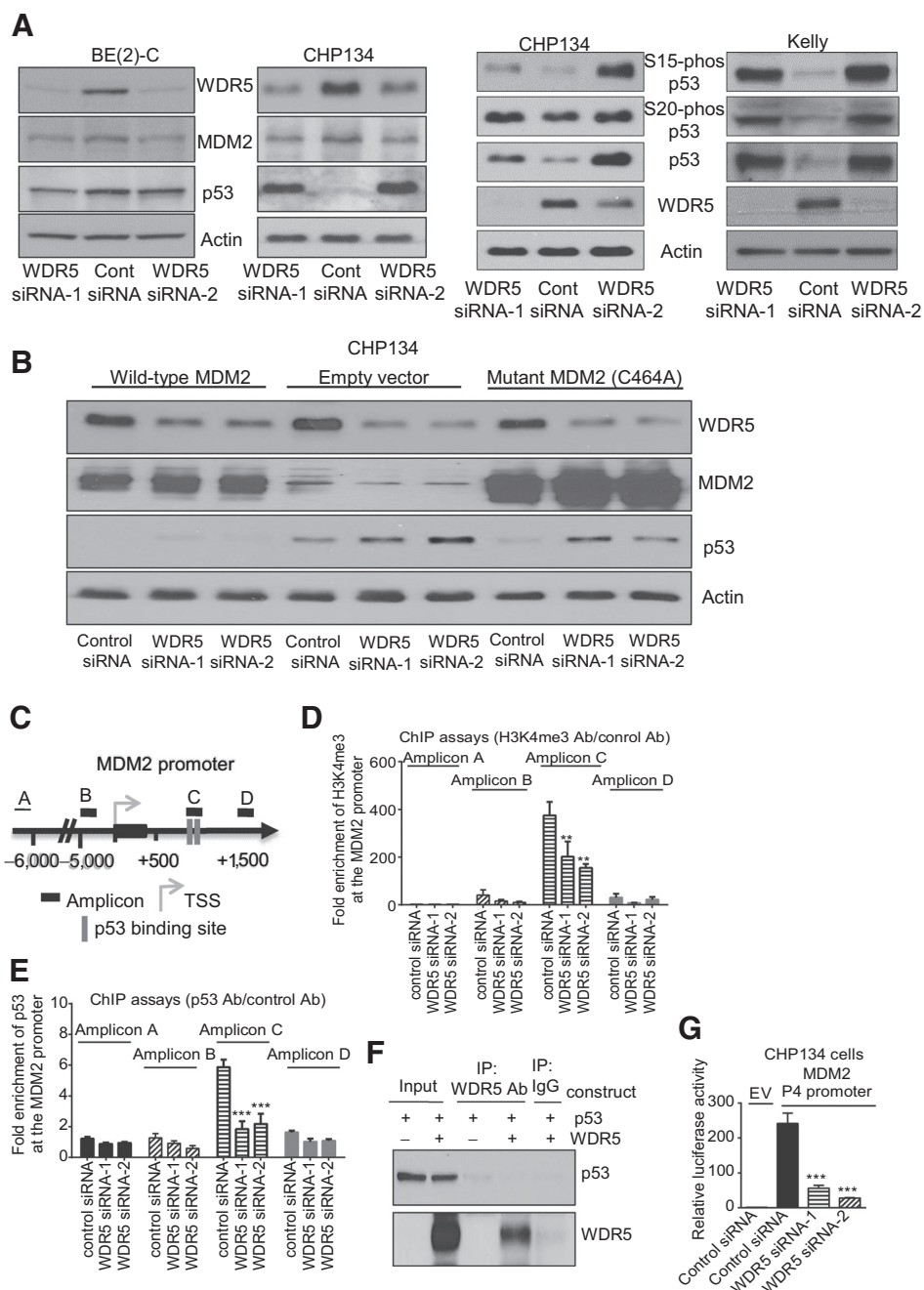
Tyrosine hydroxylase (*TH*)-*MYCN* transgenic mice develop precancerous celiac and paravertebral ganglia hyperplasia at 2 weeks old, and this hyperplasia develops into neuroblastoma at 4 weeks old (39). Immunohistochemistry studies showed that WDR5 protein was highly expressed in ganglia tissues from both newborn homozygous *TH-MYCN* transgenic and wild-type mice (Fig. 5A and B). The expression of WDR5 in ganglia tissues was maintained at high levels in 2-week-old *MYCN* transgenic mice, but decreased considerably in 2-week-old wild-type mice, which do not develop tumors (Fig. 5A and B). In addition, WDR5 protein expression remained at high levels in resulting neuroblastoma tumors in 4-week-old *TH-MYCN* transgenic mice (Fig. 5A and B). These results suggest that WDR5 may play a role in neuroblastoma initiation and progression.

High levels of *WDR5* gene expression in human neuroblastoma tissues independently predict poor patient prognosis

To assess the clinical relevance of WDR5 expression in neuroblastoma, we performed immunoblot analysis of WDR5 and N-Myc protein expression in neuroblastoma tissues from 59 patients (Fig. 6A). Two-sided Pearson correlation study showed that WDR5 protein expression correlated with N-Myc protein expression in human neuroblastomas (Fig. 6B). Using the upper quartile of WDR5 expression as the cutoff point, Kaplan–Meier analysis showed that high levels of WDR5 protein expression in tumor tissues was associated with reduced overall survival (Fig. 6C). Consistently, analyses of the publicly available R2: Genomics Analysis and Visualization Platform (25) Versteeg (22), and Kocak (23, 24) microarray gene expression datasets revealed that high levels of WDR5 mRNA expression in neuroblastoma tissues correlated with N-Myc mRNA expression (Fig. 6D) and poor overall survival rates (Fig. 6E). In addition, high levels of WDR5 expression in the 72 *MYCN*-amplified and 404 *MYCN*-nonamplified neuroblastoma tissues were positively associated with poor patient overall survival in the large Kocak dataset (Fig. 6F and G). Importantly, multivariable Cox regression analysis showed that high levels of WDR5 expression in neuroblastoma tissues was strongly associated with reduced overall survival, independent of disease stage, age at diagnosis, and *MYCN* amplification status, the current most important prognostic markers for neuroblastoma patients (Table 1; ref. 1).

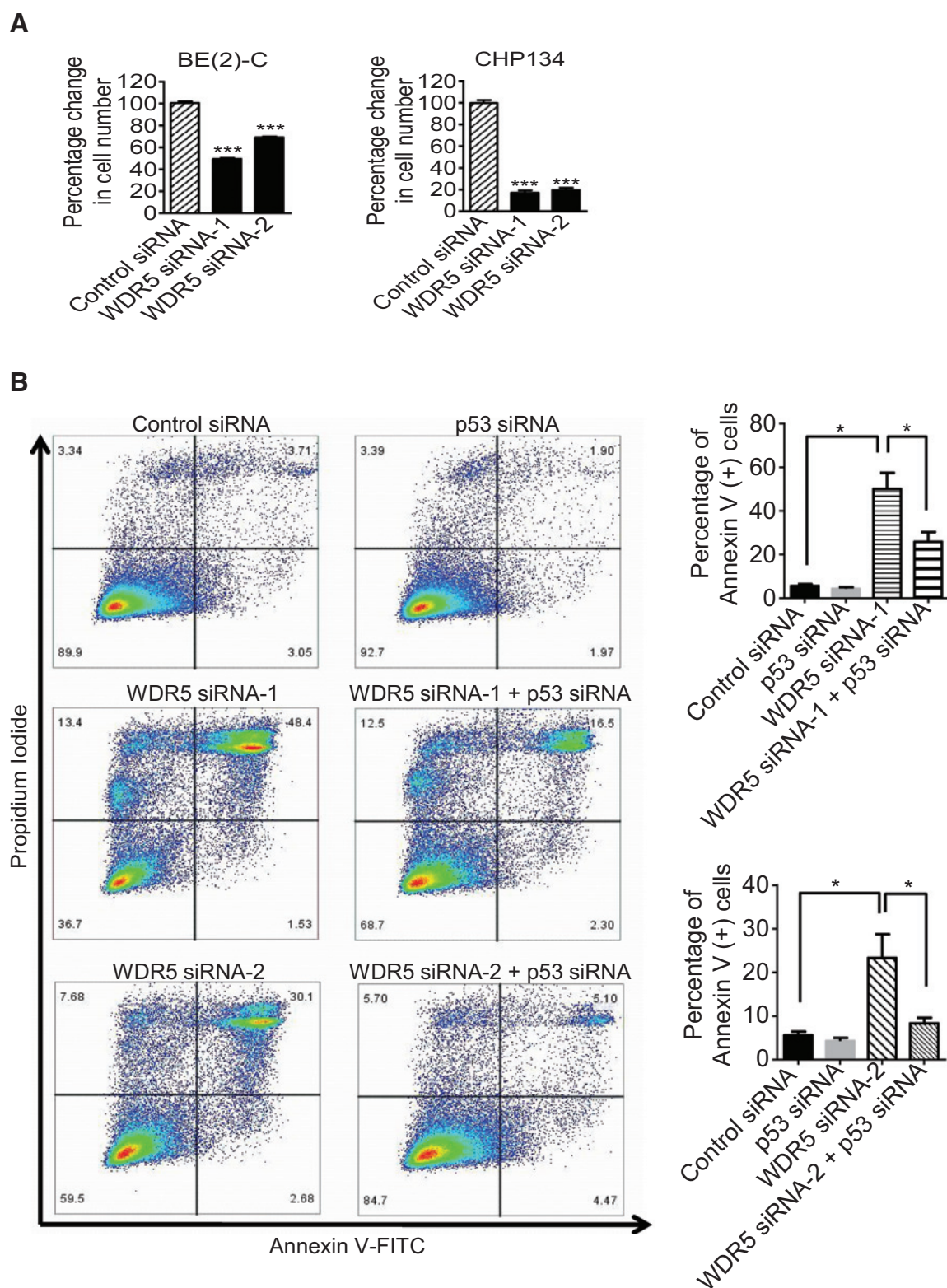
Discussion

N-Myc exerts oncogenic effects in part by binding to Myc-responsive element E-boxes at target gene promoters, leading

**Figure 3.**

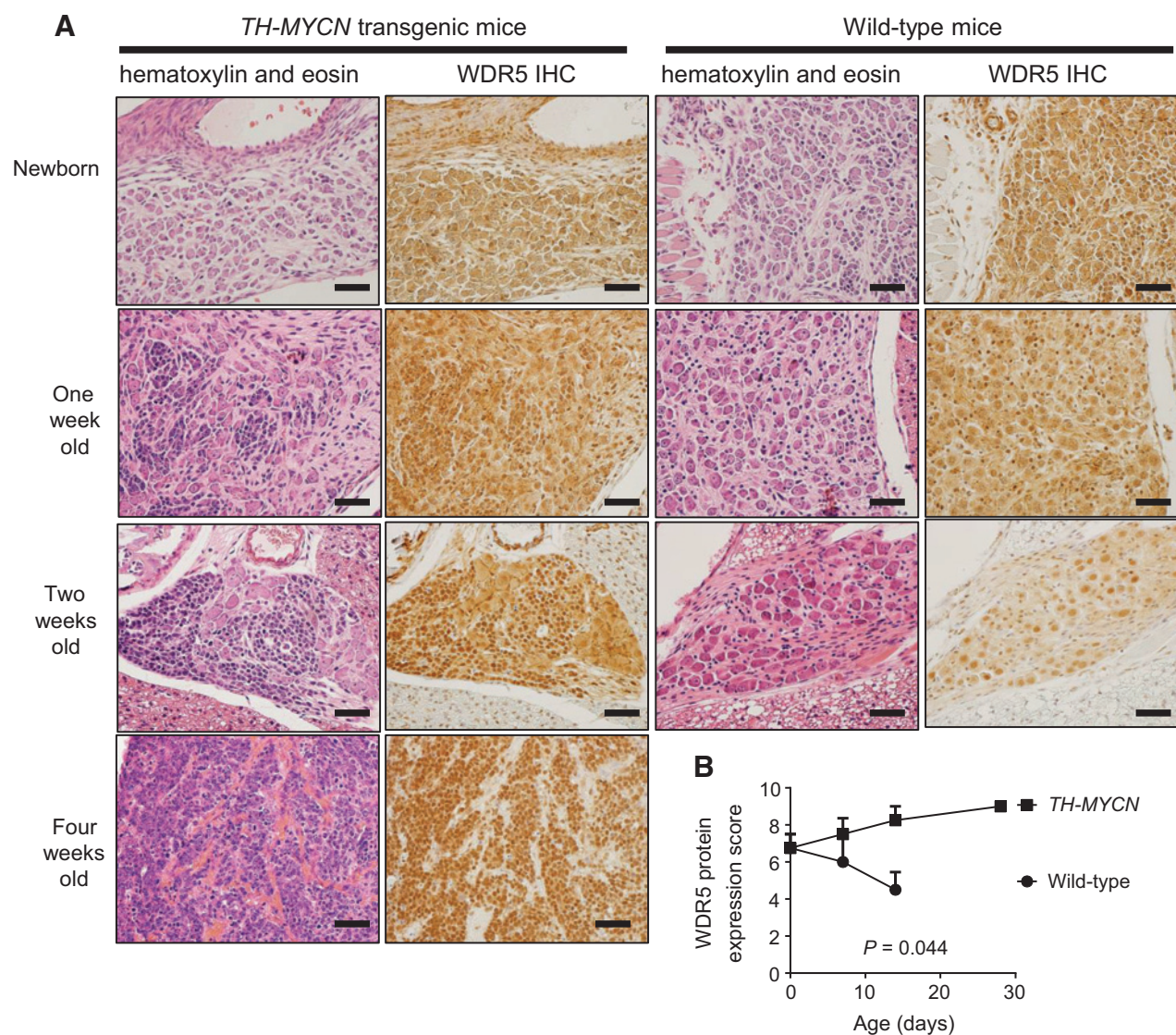
WDR5 reduces wild-type p53 protein expression by inducing histone H3K4 trimethylation at the *MDM2* gene promoter and *MDM2* gene transcription. A, p53 mutant BE(2)-C and p53 wild-type CHP134 and Kelly cells were transfected with control siRNA, WDR5 siRNA-1, or WDR5 siRNA-2, followed by immunoblot analyses of WDR5, MDM2, total p53, p53 phosphorylated at serine 20 or serine 15 proteins. B, CHP134 cells were cotransfected with control siRNA, WDR5 siRNA-1, or WDR5 siRNA-2, together with a construct expressing empty vector, wild-type MDM2, or mutant MDM2 for 48 hours, followed by immunoblot analyses of WDR5, MDM2, and p53 protein expression. C, schematic representation of the *MDM2* gene promoter containing the p53-binding sites. TSS represented transcription start site. D and E, CHP134 cells were transfected with control siRNA, WDR5 siRNA-1, or WDR5 siRNA-2 for 48 hours. ChIP assays were performed with a control, anti-H3K4me3 (D), or anti-p53 (E) antibody (Ab), followed by PCR with primers targeting negative control regions (Amplicon A, B, and D) or the p53-binding sites (Amplicon C) at the *MDM2* gene promoter. Fold enrichment of the *MDM2* promoter regions was calculated as the difference in cycle threshold obtained with the specific antibody and with the control antibody. F, HEK 293 cells were transfected with constructs expressing empty vector, p53 and/or WDR5. Protein from the cells was immunoprecipitated (IP) with a control or anti-WDR5 Ab, and coimmunoprecipitated products were analyzed by immunoblot with an anti-p53 or anti-WDR5 antibody. G, CHP134 cells were cotransfected with a luciferase reporter construct expressing the *MDM2* gene P4 promoter or empty vector (EV), together with control siRNA, WDR5 siRNA-1, or WDR5 siRNA-2. Luciferase assays were performed, and relative luciferase activity of the *MDM2* gene P4 promoter construct was normalized by the luciferase activity of the empty vector construct. Error bars represent SE. **, $P < 0.01$; ***, $P < 0.001$.

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**Figure 4.**

WDR5 induces neuroblastoma cell proliferation and survival. A, BE(2)-C and CHP134 cells were transfected with control siRNA, WDR5 siRNA-1, or WDR5 siRNA-2 for 96 hours, followed by Alamar blue assays. The numbers of cells were expressed as percentage changes. B, CHP134 cells were transfected with control siRNA, WDR5 siRNA-1, WDR5 siRNA-2, p53 siRNA, WDR5 siRNA-1 plus p53 siRNA, or WDR5 siRNA-2 plus p53 siRNA for 72 hours, followed by staining with propidium iodide and FITC-conjugated Annexin V. Cells were then subjected to flow cytometry analysis of Annexin V positively stained cells. Error bars represent SE.

*, $P < 0.05$; ***, $P < 0.001$.

**Figure 5.**

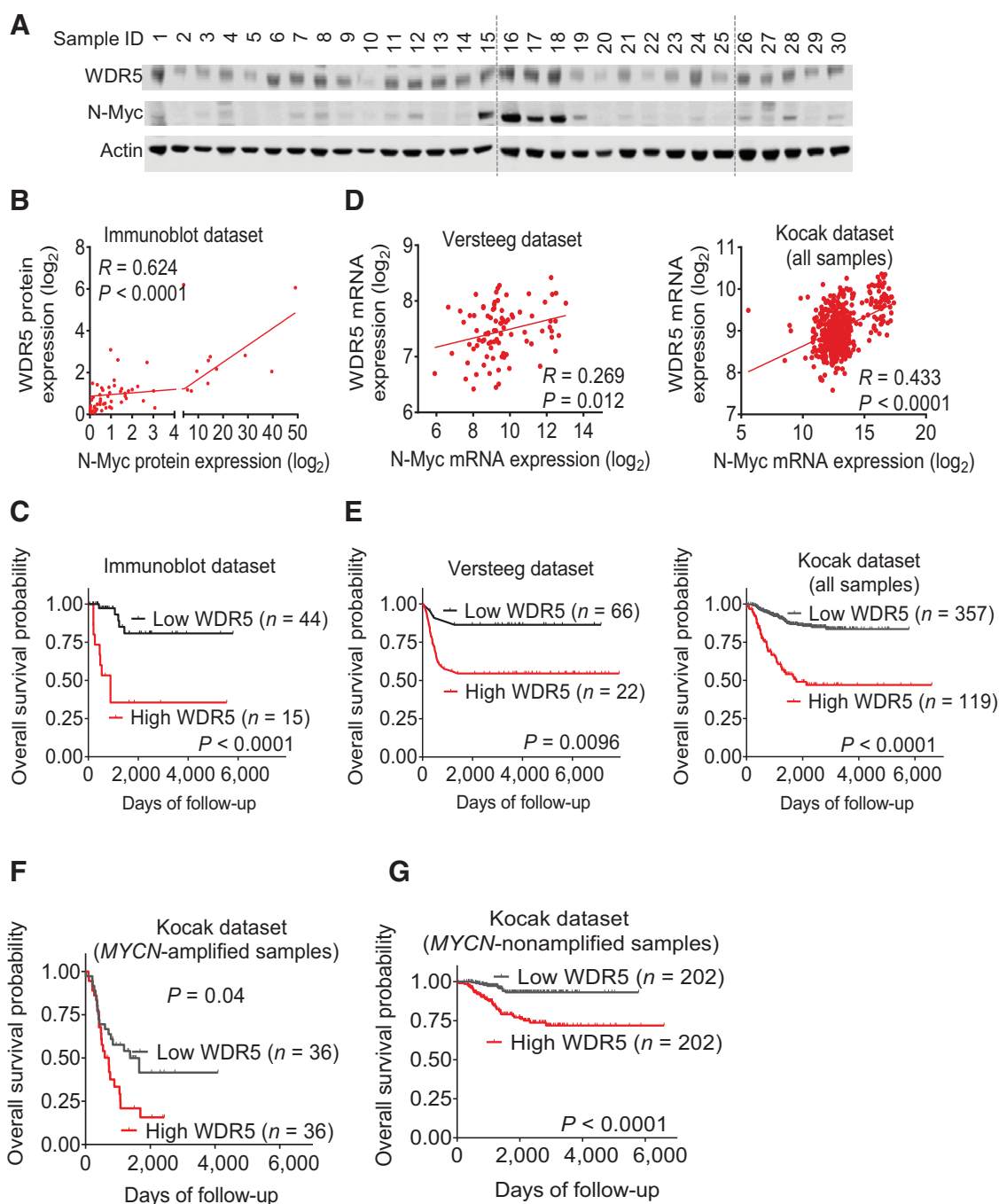
WDR5 expression is upregulated in precancerous and neuroblastoma tissues in *MYCN* transgenic mice. A, immunohistochemistry (IHC) staining with an anti-WDR5 antibody as well as staining with hematoxylin and eosin were performed in ganglia tissues from *TH-MYCN* transgenic mice and wild-type mice at the age of 0, 1, 2, or 4 weeks. Scale bar, 40 μ m. B, semiquantitative histology scoring was used to determine relative WDR5 protein expression.

to transcriptional activation (40–42). In this study, we have identified both canonical and noncanonical E-Boxes at the *WDR5* gene core promoter, and confirmed that N-Myc directly binds to the *WDR5* gene core promoter and upregulates *WDR5* mRNA and protein expression in neuroblastoma cells.

WDR5 regulates gene transcription via binding to transcription factors and inducing histone H3K4 trimethylation at target gene promoters (13–16, 43, 44). Guccione and colleagues have revealed that histone H3K4 trimethylation at target gene promoters is a strict prerequisite for Myc-induced transcriptional activation (11). However, the mechanism through which histone H3K4 is trimethylated during Myc-induced transcriptional activation is unknown. Our genome-wide differential gene expression study with Affymetrix microarray shows that *WDR5* siRNAs reduce the expression of N-Myc target genes, GSEA analysis shows that *WDR5* siRNAs prefer-

entially downregulate the expression of genes with N-Myc/c-Myc responsive element E-Boxes at promoters, and ChIP-Seq data reveal that knocking down *WDR5* preferentially reduces H3K4me3 at Myc-binding gene promoters. Protein coimmunoprecipitation and GST pull-down assays demonstrate that N-Myc protein directly binds to *WDR5* protein. Importantly, ChIP and luciferase assays show that *WDR5* and N-Myc bind to the same site of the N-Myc target *MDM2* gene promoter, and that knocking down *WDR5* expression reduces histone H3K4 trimethylation, reduces N-Myc protein binding to the *MDM2* gene promoter, and reduces the activity of the wild type, but not the E-Box mutant, *MDM2* gene promoter. Taken together, our data indicate that *WDR5* and N-Myc form a protein complex at N-Myc target gene promoters, resulting in H3K4 trimethylation and transcriptional activation of N-Myc target genes, including *MDM2*.

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**Figure 6.**

High levels of *WDR5* gene expression in human neuroblastoma tissues correlate with *MYCN* gene expression and poor patient prognosis. A, immunoblot was performed with anti-*WDR5*, anti-N-Myc, and anti-actin antibodies in 59 human neuroblastoma samples. Representative results for 30 samples were shown. B, correlation between *WDR5* and N-Myc protein expression in the 59 tumor tissues was analyzed. C, Kaplan-Meier curves showed the probability of overall survival according to the level of *WDR5* protein expression in the 59 patients. D, two-sided Pearson correlation was employed to analyze correlation between *WDR5* and N-Myc mRNA expression in 88 and 476 human neuroblastoma samples in the publicly available microarray gene expression Versteeg dataset and Kocak dataset downloaded from R2 platform. E, Kaplan-Meier curves showed the probability of overall survival of patients according to the levels of *WDR5* mRNA expression in the 88 and 476 neuroblastoma patients in the Versteeg and Kocak datasets. F and G, Kaplan-Meier curves showed the probability of patient overall survival according to the levels of *WDR5* mRNA expression in the 72 *MYCN*-amplified and 404 *MYCN*-nonamplified neuroblastoma samples in the Kocak dataset.

N-Myc directly upregulates p53 gene transcription (6). On the other hand, wild-type p53 and MDM2 form a negative feedback loop (37, 38). Wild-type, but not mutant, p53 binds to the p53-

binding sites at the *MDM2* gene promoter, leading to *MDM2* gene transcriptional activation. Conversely, *MDM2* protein targets wild-type, and to a lesser extent, mutant p53 protein for

Table 1. Multivariable Cox regression analysis of factors prognostic for outcome in 476 neuroblastoma patients^a

Factors	Event-free survival		Overall survival	
	HR (95%CI)	P	HR (95%CI)	P
High <i>WDR5</i> expression (median level as the cutoff)	2.64 (1.76–3.95)	<0.0001	2.49 (1.39–4.47)	0.0022
<i>MYCN</i> amplification	1.82 (1.25–2.65)	0.0019	3.39 (2.16–5.33)	<0.0001
Age > 18 months	1.18 (0.81–1.72)	0.3835	2.98 (1.53–5.78)	0.0013
Stages 3 and 4 ^b	2.48 (1.65–3.74)	<0.0001	3.87 (1.95–7.66)	0.0001
High <i>WDR5</i> expression (lower quartile as the cutoff)	3.27 (1.82–5.89)	<0.0001	3.01 (1.26–7.18)	0.013
<i>MYCN</i> amplification	1.98 (1.36–2.87)	0.0003	3.60 (2.31–5.61)	<0.0001
Age > 18 months	1.25 (0.85–1.84)	0.2525	3.10 (1.58–6.08)	<0.0001
Stages 3 and 4 ^b	2.72 (1.81–4.10)	<0.0001	4.02 (2.02–7.98)	<0.0001
High <i>WDR5</i> expression (upper quartile as the cutoff)	1.82 (1.26–2.6)	0.0013	1.66 (1.05–2.64)	0.0316
<i>MYCN</i> amplification	1.91 (1.29–2.84)	0.0013	3.54 (2.22–5.66)	<0.0001
Age > 18 months	1.08 (0.74–1.57)	0.6881	2.73 (1.41–5.30)	0.003
Stages 3 and 4 ^b	2.95 (1.97–4.40)	<0.0001	4.55 (2.32–8.94)	<0.0001

^aThe level of *WDR5* expression was considered high or low in relation to the median, lower quartile, or high quartile level of expression in all tumors analyzed. HRs were calculated as the antilogs of the regression coefficients in the proportional hazards regression. Multivariable Cox regression analysis was performed following the inclusion of the four above-listed factors into the Cox regression model, and *P* value was obtained from the two-sided log-rank test.

^bTumor stage was categorized as favorable (International Neuroblastoma Staging System stages 1, 2, and 4S) or unfavorable (International Neuroblastoma Staging System stages 3 and 4).

ubiquitination and degradation (8–10, 37). However, the p53 tumor suppressor gene is mutated in only 2.5% of primary human neuroblastoma tissues (45), and the mechanism through which p53 protein is kept at low levels in human neuroblastoma tissues is unknown. In this study, we have found that knocking down *WDR5* significantly reduces *MDM2* mRNA and protein expression in p53 wild-type and mutant neuroblastoma cells, shows little effects on p53 mRNA expression, and considerably upregulates wild-type but not mutant p53 protein expression. Notably, while *WDR5* protein does not form a complex with p53 protein, suppression of *WDR5* reduces histone H3K4 trimethylation at, and p53 protein binding to, the p53-binding sites of the *MDM2* gene promoter, and reduces promoter activity of the p53-binding sites. Our data suggest that *WDR5* reduces wild-type p53 protein expression by inducing histone H3K4 trimethylation and active chromatin status at the p53-binding sites of the *MDM2* gene promoter, leading to *MDM2* overexpression and p53 protein degradation, and that *WDR5* is important for maintaining the p53-*MDM2* negative feedback loop.

Inhibition of *MDM2* or induction of p53 blocks neuroblastoma tumorigenesis in *MYCN* transgenic mice (46, 47). In this study, we have found that *WDR5* is highly expressed in pre-cancer ganglia cells and neuroblastoma cells from *MYCN* transgenic mice. A high level of *WDR5* expression in primary human neuroblastoma tissues correlates with poor patient survival, independent of disease stage, diagnosis age, and *MYCN* amplification status, the current most important prognostic markers for neuroblastoma patients (1, 48). In addition, *WDR5* siRNAs induce growth inhibition in both p53 wild-type and mutant neuroblastoma cells, and cosilencing p53 blocks *WDR5* siRNA-mediated apoptosis in p53 wild-type neuroblastoma cells. Importantly, treatment with a novel small-molecule *WDR5* antagonist blocks *WDR5* protein binding to N-Myc protein, N-Myc target gene expression, and neuroblastoma cell proliferation and survival. Taken together, *WDR5* induces neuroblastoma cell proliferation and survival, and is likely to play a critical role in neuroblastoma initiation and progression. As a high level of *WDR5* expression in human neuroblastoma tissues is an independent marker for poor patient survival, suppression of *WDR5* with small-molecule antagonists represent a novel therapeutic strategy for neuroblastoma patients.

Disclosure of Potential Conflicts of Interest

C.H. Arrowsmith reports receiving a commercial research grant from Structural Genomics Consortium. No potential conflicts of interest were disclosed by the other authors.

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Other (performed a few experiments): A.E. Tee
Other (supplied key compounds OICR-9429 and OICR-0547): P.J. Brown

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WDR5 Supports an N-Myc Transcriptional Complex That Drives a Protumorigenic Gene Expression Signature in Neuroblastoma

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