Transcriptional Repression of SKP2 Is Impaired in MYCN-Amplified Neuroblastoma

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Introduction

The cell cycle regulator, SKP2, is overexpressed in various cancers and plays a key role in p27 degradation, which is involved in tumor cell dedifferentiation. Little is known about the mechanisms leading to impaired SKP2 transcriptional control in tumor cells. We used neuroblastoma as a model to study SKP2 regulation because SKP2 transcript levels gradually increase with aggressiveness of neuroblastoma subtypes. The highest SKP2 levels are found in neuroblastomas with amplified MYCN. Accordingly, we found 5.5-fold (range, 2–9.5) higher SKP2 core promoter activity in MYCN-amplified cells. Higher SKP2 core promoter activity in MYCN-amplified cells is mediated through a defined region at the transcriptional start site. This region includes a specific E2F-binding site that makes SKP2 activation largely independent of mitogenic signals integrated through the SP1/ELK-1 site. We show by chromatin immunoprecipitation that SKP2 activation through the transcriptional start site in MYCN-amplified cells is associated with the low abundance of pRB-E2F1 complexes bound to the SKP2 promoter. Transcriptional control of SKP2 through this regulatory mechanism can be re-established in MYCN-amplified cells by restoring pRB activity using selective small compound inhibitors of CDK4. In contrast, doxorubicin or nutlin-3 treatment—both leading to p33-p21 activation—or CDK2 inhibition had no effect on SKP2 regulation in MYCN-amplified cells. Together, this implies that deregulated MYCN protein levels in MYCN-amplified neuroblastoma cells activate SKP2 through CDK4 induction, abrogating repressive pRB-E2F1 complexes bound to the SKP2 promoter. Cancer Res; 70(9); 3791–802. ©2010 AACR.

Materials and Methods

Cell culture. SK-N-SH, SH-EP, IMR-32, IMR5-75, and Kelly cells were cultured in RPMI 1640 (10% FCS) and SH-SY5Y and BE(2)-C were cultured in DMEM (10% FCS). Culture and induction of SH-EP-E2F1 and Tet21N was previously described (7, 8). Drugs were 100 μg/mL cycloheximide (Sigma),...
0.1 μg/mL doxorubicin (Sigma), and 10 μg/mL nutlin-3 and CDK4 inhibitors (RO505124 and RO506220, Roche).

Inducible stable CDK4 and MYCN knockdown cell lines. Stable IMR5-75 neuroblastoma cell line expressing small hairpin RNAs (shRNA) against CDK4 and MYCN under control of the Tet-repressor were generated stepwise. In a first step, we generated IMR5-75 cells stably expressing the tetracycline repressor protein (pcDNA/6TR, Invitrogen) using Lipofectamine 2000 (Invitrogen). In a second step, CDK4 and MYCN shRNA–expressing vectors were generated: oligonucleotides targeting MYCN and CDK4 (for sequences see Supplementary Table S1) were generated and cloned into pTER as described (9). The resulting pTER/MYCN and pTER/CDK4 knockdown constructs were transfected into IMR5-75-pcDNA/6TR. Individual clones were selected, expanded, and assayed for knockdown upon tetracycline addition (1 μg/mL in 70% ethanol) using Western blotting. Selection drugs were Blasticidin (7.5 μg/mL) and Geneticin/Genetin.

Figure 1. Deregulated SKP2 expression in MYCN-amplified neuroblastoma tumors and cell lines. A, box plots of SKP2 and E2F1 gene expression in MYCN nonamplified (NA; n = 219) and amplified (AMP; n = 32) tumors are given. Horizontal boundaries of the box represent the 25th and 75th percentile. The 50th percentile (median) is denoted by a horizontal line in the box. Whiskers above and below extend to the most extreme data point that is no >1.5 times the interquartile range from the box. B, schematic representation of truncated SKP2 promoter-luciferase constructs including locations of E2F-binding sites and TSSR. C and D, luciferase activity (24 h posttransfection of 0.3 μg) of promoter constructs normalized to CMV-Renilla -luciferase (1:10 transfection ratio) in SH-EP (C) and Kelly (D) cells. Experiments performed in triplicate twice. Negative control, pGL3-basic vector.
G418 sulfate (200 μg/mL). Small interfering RNAs (siRNA) targeting CDK4, CDK2, and two nontarget controls (Supplementary Table S1) were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturers’ instructions.

**Microarray analysis.** Generation of tumor gene expression profiles was previously described (10). All tumor samples were collected before any cytoreductive treatment. They were checked for at least 60% tumor cell content. The clinical characteristics of the neuroblastoma patients (n = 251) have been previously described (ref. 11; see additional data file 12). Raw and normalized microarray data are available at ArrayExpress (accession: E-TABM-38).

**SKP2 promoter cloning.** Cloning of a 1.7-kb fragment of the human SKP2 promoter was done by using PCR from human blood DNA (bases −1469 to +248) and ligation into XhoI sites of pGL3 firefly luciferase vector (Promega). Truncated SKP2 promoter fragments were generated by PCR using the pGL3-SKP2-full as template, KpnI and XhoI, digested, and religated into pGL3-basic (for primer sequences, see Supplementary Table S1). E2F-binding site mutations in the SKP2 promoter were generated by using the QuickChange II Site-Directed Mutagenesis kit (Stratagene) as described by the manufacturer (for primer sequences, see Supplementary Table S1).

**SKP2-luciferase reporter assays.** Cloning of SKP2 promoter fragments and E2F-binding site mutations is described in Supplementary Materials and Methods. Fragment gene location is relative to the transcriptional start. Luciferase reporter assays were performed as previously described (11). For iCDK4- and nutlin-3–treated cells, as well as IMR5-75-shMYCN cells, luciferase activity was normalized against total protein concentration (BCA-assay, Pierce Biotechnology).

**Flow cytometry.** Cell cycle distribution analysis was performed as described (12). In brief, cells were stained with 50 μg/mL 4′,6-diamidino-2-phenylindole for DNA content analysis.

**Western blotting.** Immunoblots were performed as previously described (13). Antibodies were anti-pRB (BD-Biosciences), anti-p21 (Upstate), anti-p27 (Dianova), anti-SKP2 (Invitrogen), anti-E2F1 (Medac), anti-CDK4 (DCS-31, Dianova), anti-MYCN (sc-53993), anti-CCNE1 (sc-248; both Santa Cruz), anti-pSer780-RB (Cell Signaling #9307), and anti-β-actin (Sigma).

**Quantitative PCR.** Quantitative PCR (qPCR) (primer sequences in Supplementary Table S1) was performed as described (6, 14).

### Table 1. Differential effect of positive and negative regulatory regions within the SKP2 core promoter in MYCN-amplified and nonamplified neuroblastoma cells

<table>
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<th>Cell line</th>
<th>MYCN status</th>
<th>SKP2 activation (full)*</th>
<th>SKP2 core activation (del#1)*</th>
<th>Deletion TSSR and E2F sites</th>
<th>Deletion TSSR (including E2Fbdg#1)</th>
<th>Deletion SP1/ELK-1</th>
<th>Deletion E2Fbdg2/3</th>
<th>Confirmed by point mutations</th>
<th>PTMA activation</th>
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<td>SH-EP</td>
<td>Non-AMP</td>
<td>2.5 (0.1)</td>
<td>8.0 (0.6)</td>
<td>0.45</td>
<td>0.10</td>
<td>0.51</td>
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<td>SH-SY5Y</td>
<td>Non-AMP</td>
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<td>17.2 (1.5)</td>
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<td>0.06</td>
<td>0.55</td>
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<td>SK-N-SH</td>
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<td>0.09</td>
<td>0.59</td>
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Abbreviation: Nd, not determined.

*Luciferase activity; mean and SD.
Figure 2. MYCN influences SKP2 expression. A, SKP2 core promoter activity in different neuroblastoma cell lines. PTMA-promoter-luciferase reporter served as a positive control for MYCN transcriptional activity. B, mRNA expression analyses of MYCN, SKP2, and PTMA using qPCR upon MYCN induction in MYCN inducible Tet21N cells. Western blot analyses show MYCN protein levels at the respective time points. Luciferase assay shows SKP2 promoter activity 24 hours after MYCN induction. C, Western blot and promoter analyses of SKP2 in IMR5-75 cells after targeted knockdown of MYCN using shRNA.
**Chromatin immunoprecipitation.** Chromatin immunoprecipitation (ChIP) was performed as described (15) with 10 μg of anti-E2F1 (sc-193), anti-pRB (sc-50), and anti–normal mouse IgG (sc-2025; all Santa Cruz). Precipitated DNA from SH-SY5Y and Kelly cells was quantified using the Taqman PCR mix (Applied Biosystems) and the ΔΔct method using input sample as reference and sample without antibody as calibrator or as described (16). Primers for a region 5.2 kb downstream of the SKP2 promoter and the ALU control region (17) served as negative controls (primer/probe sequences in Supplementary Table S1).

**Results**

**High SKP2 expression in MYCN-amplified neuroblastomas.** We previously showed by qPCR that high SKP2 expression is associated with amplified MYCN and poor outcome in a cohort of 117 primary neuroblastomas (6). Here, mRNA analyses of a large cohort of 251 neuroblastoma patients using a customized 11K oligonucleotide microarray revealed that SKP2 significantly correlated with E2F1 (Pearson's correlation coefficient = 0.46; 95% confidence interval, 0.36-0.55; P < 0.001). SKP2 and E2F1 expression were also significantly higher in MYCN-amplified tumors (P < 0.001 for both; Fig. 1A). These in vivo findings are consistent with previous in vitro observations that showed that E2F transcription factors can induce SKP2 expression (4).

**The SKP2 core promoter contains activating and repressing elements.** Evidence for high E2F activity in high-risk neuroblastomas also comes from large-scale gene expression profiles, indicating that a set of cell cycle genes regulated by activating E2Fs is expressed at higher levels in high-risk, including the MYCN-amplified cases, than in low-risk neuroblastomas (6, 18). This led us to focus on the analysis of SKP2 transcriptional regulation in neuroblastoma cells. To investigate the mechanisms leading to high SKP2 expression in neuroblastoma cells, we initially performed an in silico analysis of the SKP2 promoter region previously described as the minimal region for transcription in human cells (4, 19). Using MatInspector (20), we identified one DNA binding motif for SP1/ELK-1 and three for E2F transcription factors (mean matrix similarity score = 0.92). To assess the influence of each regulatory region on SKP2 promoter activity, we cloned different SKP2 promoter deletion constructs (Fig. 1B) and performed luciferase reporter assays in MYCN-nonamplified SH-EP cells (Table 1; Fig. 1C). Del#1, containing −133 to +248 relative to the transcriptional start, was chosen as reference for the other deletion constructs and as the putative SKP2 core promoter because its luciferase activity in different neuroblastoma cells closely correlated with that of a 1.7-kb full-length SKP2 promoter construct (Pearson's correlation coefficient = 0.97; 95% confidence interval, 0.89-0.99; P < 0.001; Table 1). Del#5, lacking the SP1/ELK-1 binding region and harboring the transcriptional start site and the three-predicted E2F-binding sites, accounted for 51% of del#1 activity (del#5/del#1=0.51; Table 1), indicating that the SP1/ELK-1 binding region mediates approximately half of the SKP2 core promoter activity in these cells. This is in line with previous reports showing that the SP1/ELK-1 regulatory element mediates mitogenic signals (19, 21). We found that the SKP2 promoter region downstream of SP1/ELK-1, harboring the transcriptional start and the three predicted E2F-binding sites, integrates both positive and negative signals. Del#4, lacking the transcriptional start site and the three-predicted E2F-binding sites and only harboring the SP1/ELK-1 binding region, accounted for 45% of the luciferase activity observed for del#1 (del#4/del#1=0.45; Table 1; Fig. 1C). In addition, del#6 showed a 10-fold lower activity than del#5 (del#6/del#5=0.10; Table 1; Fig. 1C), indicating that the transcriptional start site region (TSSR), including E2F-binding sites #1 (E2Fbdg#1), missing in del#6, mediates SKP2 activation downstream of the SP1/ELK-1 site. Intriguingly, deletion of E2Fbdg#2 and E2Fbdg#3 (del#3) increased activity 2.8-fold (del#3/del#1=2.8; Table 1; Fig. 1C), indicating that these regions mediate SKP2 promoter repression. Similar results, which are activation through the SP1/ELK region (mitogen response) and the TSSR, as well as repression through the regions harboring E2Fbdg#2 and E2Fbdg#3, were observed in other nonamplified neuroblastoma cell lines (SH-SY5Y and SK-N-Sh; Supplementary Fig. S1; Table 1). Taken together, the region including −133 to −248 (del#1) is important for SKP2 regulation in neuroblastoma cells and contains elements for activation and repression. In addition, the core promoter region we describe here is smaller and maps within a previously identified minimal promoter region (4, 19).

**High SKP2 core promoter activity in MYCN-amplified cells.** To gain insight into SKP2 regulation in a MYCN-amplified genetic background, we compared SKP2 core promoter activity in MYCN-amplified and nonamplified neuroblastoma cell lines. We measured the activity of the SKP2 core promoter-luciferase construct del#1 in relation to a prohyinosin (PTMA) promoter-luciferase construct. PTMA is a bona fide direct target gene of MYCN (8) and a good marker gene for direct MYCN transcriptional activity in neuroblastoma cells in vitro and in vivo (15). The mean activity of the SKP2 core promoter was 5.5-fold (range, 2–9.5) higher in MYCN-amplified versus nonamplified cells (Fig. 2A). SKP2 core promoter activity, however, did not strictly correlate with PTMA promoter activity in MYCN-amplified cells (BE-2-C, 50- to 70-fold MYCN amplification mutant TP53 < IMR-32, 15- to 20-fold amplification < IMR5-75, 75-fold amplification < Kelly, 100- to 120-fold amplification; Table 1; Fig. 2A), suggesting that SKP2 activation is not directly linked to MYCN activity. To further assess MYCN involvement in SKP2 regulation, we used the cell line Tet21N, which contains a tetracycline-inducible MYCN transgene (8). SKP2 mRNA expression and core promoter activity increased 2-fold upon MYCN induction (Fig. 2B). Moreover, time-resolved analysis of SKP2 mRNA expression upon MYCN induction in Tet21N cells revealed a delayed SKP2 mRNA increase (~4 h) compared with the PTMA mRNA increase, which is in line with an indirect regulation of SKP2 by MYCN. We also analyzed SKP2 regulation in IMR5-75-sMYCN cells, allowing tetracycline-inducible expression of small hairpin RNAs specifically targeting MYCN (shMYCN) in a MYCN-amplified genetic background. SKP2 expression and promoter activity was reduced...
2-fold after MYCN knockdown (MYCN protein reduction following conditional shMYCN expression was $\sim 65\%$; Fig. 2C). Taken together, these data suggest that dysregulation of MYCN contributes to induction of the SKP2 core promoter in MYCN-amplified cells. However, they also suggest that the SKP2 core promoter is indirectly influenced by deregulated MYCN and also integrates MYCN-independent signals.

**SKP2 activation in MYCN-amplified cells is primarily mediated through the TSSR.** To test through which of the identified SKP2 core promoter regulatory elements deregulated MYCN activates SKP2 in MYCN-amplified cells, we compared the activation pattern of SKP2 promoter-luciferase deletion constructs in different MYCN-amplified versus non-amplified cells. We observed that the activity of the SKP2 promoter deletion constructs differed in MYCN-amplified and non-amplified cells (Supplementary Fig. S1; Table 1; Fig. 1C and D). SKP2 promoter activation in MYCN-amplified cells was primarily mediated through the TSSR, including E2Fbdg#1: del#4, lacking the TSSR and the E2F sites and only harboring the SP1/ELK-1 binding region, accounted in average for only 16% (del#4/del#1 ranged from 0.02–0.41) of the luciferase activity observed for del#1 in MYCN-amplified cells, whereas del#4 accounted in average for 66% (del#4/del#1 ranged from 0.37–0.88) of del#1 in MYCN nonamplified cells (Table 1). In addition, the activity changed drastically between the del#6 and del#5 constructs, differing only by the TSSR, including E2Fbdg#1, in MYCN-amplified cells, further supporting the prominent role of the TSSR, including E2Fbdg#1, on SKP2 activation in these cells (Supplementary Fig. S1; Table 1). The SP1/ELK-1 binding region, which

**Figure 3.** Influence of point mutations in E2F-binding sites on SKP2 promoter activity. Luciferase analyses of the SKP2 promoter harboring individual or combined mutations in the three predicted E2F-binding sites in (A) MYCN nonamplified SH-SY5Y, (B) MYCN-amplified Kelly, and (C) SH-EP-E2F1 cells with and without induced E2F1 expression. Arrow in the schematic representation of E2F-binding sites point mutation constructs indicates position of primers used for ChIP-qPCR.
mediates mitogenic signals, had less effect on SP1/ELK-1 binding region (del#5/del#1 did not differ in MYCN-amplified and nonamplified cells; Table 1), but due to the strong relative effect of the TSSR, including E2Fbdg#1, on SKP2 core promoter activation. We could largely exclude the possibility that the SKP2 promoter repressor sites, encompassing E2Fbdg#2 and #3, turn into...
activator sites, a mechanism previously described for at least some E2F-binding sites (22): the del#6 and del#7 deletion constructs, containing E2Fbdg#2 and #3, respectively, had no intrinsic activity (Supplementary Fig. S1; Fig. 1C and D). In addition, deletion of the repressor sites, encompassing E2Fbdg#2 and #3, decreased SKP2 core promoter activity in only one MYCN-amplified cell line (Kelly, del#3/del#1=0.5), but predominantly increased SKP2 core promoter activity in MYCN-amplified cells (del#3/del#1 for IMR5-75=1.4, IMR32=1.9, SK-N-Be2(c)=1.5; Table 1).

PTMA promoter activation, a proxy measure for MYCN activity in MYCN-amplified cells, was positively correlated with the relative effect of the TSSR, including E2Fbdg#1, and negatively correlated with the relative effect of the SP1/ELK-1 binding region on SKP2 core promoter activity (Table 1), suggesting that mechanisms induced by deregulated MYCN might be involved in this characteristic SKP2 activation mechanism. In line with this, targeted expression of the MYCN transgene in Tet21N cells increased, whereas targeted knockdown of MYCN in IMR5-75-shMYCN cells reduced the effect of the TSSR on SKP2 core promoter activation (Supplementary Fig. S1; Table 1). SKP2 core promoter deletion constructs produced an intermediate activation pattern in SK-N-Be2(c) cells, which have amplified MYCN and also mutant TP53. These cells exhibited high SKP2 core promoter activity and a comparable effect of the SP1/ELK-1 binding site and TSSR (del#4/del#1=0.41 and del#5/del#1=0.59), despite the lowest PTMA promoter activity among all tested MYCN-amplified cells (Supplementary Fig. S1; Table 1). This indicates that the SKP2 core promoter integrates not only indirect signals related to the activity of amplified MYCN but also signals related to mutant TP53 most likely through the SP1/ELK-1 binding site (del#4 showed the highest activity in SK-N-Be2(c) cells; Supplementary Fig. S1). Together, this suggests that further induction of the SKP2 core promoter in MYCN-amplified cells, which usually have wild-type TP53 (23), is strongly dependent on signals that promote activation through the TSSR, including E2Fbdg#1, but largely independent of mitogenic signals mediated through the SP1/ELK-1 binding region.

To measure the direct influence of the predicted E2F-binding sites on SKP2 promoter activity, we mutated the three sites individually and in combination (Fig. 3A) and performed luciferase reporter assays (Fig. 3B and C). Independent of amplified MYCN, E2Fbdg#1 mediated the activation and E2Fbdg#2 and #3 repression of the SKP2 promoter, confirming our findings with SKP2 core promoter deletion constructs. Mutation of all predicted E2F-binding sites resulted in SKP2 reporter activity levels similar to that of the intact construct (del#1), suggesting that mutation of the repressing sites can compensate for the loss through the mutation of the activating site. However, these results do not explain the quantitative SKP2 core promoter activation differences between MYCN-amplified and nonamplified cells, and point toward a complex interplay between the different E2F-binding sites that might differentially bind transcriptional activator or repressor complexes.

Targeted E2F1 activation in MYCN-nonamplified cells favors SKP2 repression at the TSSR. To further analyze the interplay between the different putative E2F-binding sites (4), we stably expressed an ER-E2F1 fusion protein in MYCN-nonamplified SH-EP cells to allow conditional E2F1 activation by 4-OHT (7). SKP2 mRNA was induced 2-fold following E2F1 activation (Fig. 4A). SKP2 mRNA induction did not require protein biosynthesis as activation also occurred in the presence of the translation inhibitor, cycloheximide. In addition, 8-fold mRNA induction of CCNE1, a well-known direct E2F target (24), was observed upon E2F1 activation and was independent of protein synthesis (Fig. 4A). The SKP2 core promoter (del#1) was moderately induced following E2F1 activation in SH-EP-E2F1 cells, in line with SKP2 mRNA induction in these cells (Fig. 4B). Intriguingly, the different E2F-binding regions had an inverse effect on SKP2 core promoter activity in SH-EP-E2F1 cells after E2F1 activation compared with MYCN-amplified and nonamplified cells. The TSSR was involved in SKP2 promoter activation in MYCN-amplified cells (Fig. 1D), but mediated SKP2 core promoter repression in SH-EP-E2F1 cells following E2F1 activation because the activity of del#3, harboring the TSSR including E2Fbdg#1 but no other E2F-binding sites, decreased upon E2F1 activation (Fig. 4B) and del#3/del#1 decreased from 2.4 to 0.7 upon E2F1 activation (Table 1). In addition, the region including E2Fbdg#2 and #3, which was involved in SKP2 promoter repression in MYCN-amplified and nonamplified cells, mediated SKP2 core promoter activation following E2F1 induction: del#6 and del#7 deletion constructs, containing E2Fbdg#2 and #3, respectively, showed increased activity upon E2F1 activation (Fig. 4B) and del#6/del#5 increased from 0.11 to 0.69. Reporter constructs containing point-mutated E2F-binding sites corresponding to the respective deletion constructs showed that SKP2 core promoter activity in SH-EP-E2F1 cells after E2F1 activation was primarily dependent on E2Fbdg#2: mutation of E2Fbdg#2 abrogates activation of the SKP2 core promoter upon E2F1 activation (Fig. 3D).

Analyses of the cell cycle showed that E2F1 activation in SH-EP-E2F1 cells led to cell cycle arrest at the G1-S phase transition (Supplementary Fig. S2A; Fig. 4C), which is in line with cell cycle arrest after E2F1 overexpression in other cell types than neuroblastoma (25, 26). The observed cell cycle arrest was associated with stably hypophosphorylated pRB and elevated SKP2, p21, p27, and CCNE1 protein levels (Fig. 4C). Induction of p27 despite increasing levels of SKP2 in SH-EP-E2F1 cells can be explained by E2F1-mediated activation of the CDKN1Bpp27 gene (26) together with reduced activity of the SCF<sup>Skp2</sup> ubiquitin ligase, which requires displacement of pRB from the SKP2 protein (27) and CDK2-mediated p27 phosphorylation for p27 degradation (2). Intriguingly, the observed protein changes were associated with the repression of the TSSR (primarily activated in MYCN-amplified cell lines). Therefore, we conclude that SKP2 activation in neuroblastoma cells may not only depend on E2F1 abundance but also on active, hypophosphorylated pRB.

SKP2 activation in MYCN-amplified cells is associated with reduced pRB-E2F1 complex bound to the SKP2 promoter. To test whether an increased presence of transcriptional repressive complexes is involved in the differential promoter
activity between MYCN-amplified and nonamplified cell lines, we analyzed the abundance of E2F1 and pRB at SKP2 promoter regulatory sites using ChIP-qPCR (Fig. 4D). E2F1 induction in SH-EP-E2F1 cells resulted in 15- and 2.2-fold increases of E2F1 and pRB, respectively, at the SKP2 promoter. In MYCN-amplified cells, 7-fold more E2F1 bound the SKP2 promoter, whereas 5-fold less pRB was bound. In summary, we suggest that repressive pRB-E2F1 complexes bound to the SKP2 promoter may assist the differential SKP2 control in MYCN-amplified versus nonamplified cells.

**CDK4 inhibition restores transcriptional control of SKP2 in MYCN-amplified cells.** E2F1 overabundance in the presence of active, hypophosphorylated pRB still did not result in full SKP2 activation in SH-EP-E2F1 cells. Therefore, we analyzed SKP2 core promoter activation upon G1 cell cycle arrest by either p53 or pRB activation in a MYCN-amplified background. We have previously shown that deregulated MYCN directly activates both CDK4 and the TP53-MDM2 axis while it suppresses CDKN1A/p21Cip1 in MYCN-amplified cells (15, 28). Together, this leads to synergistic suppression of pRB functions and increased E2F1 abundance and activity. High CDK4 increases pRB phosphorylation, and high MDM2 and MYCN suppress p53-p21 functions, subsequently leading to further pRB phosphorylation through CDK2, resulting in E2F1 release from pRB and further transcriptional activation of E2F1, a prototypic E2F target.

**Figure 5.** Control of SKP2 expression in MYCN-amplified cell lines is restored after CDK4 inhibition. Neuroblastoma cells treated with (A) doxorubicin (doxo), (B) nutlin-3, or (C) iCDK4. SKP2, pRB/pRB780, and E2F1 expression is shown by Western blot. Luciferase activity of transiently transfected SKP2 promoter constructs is shown. Numbers next to bars, the ratio of treatment to mock. C, Western blot analyses of CDK4 and SKP2 expression after conditional knockdown of CDK4 using tetracycline-inducible shRNA in MYCN-amplified IMR5-75 cells. SKP2 promoter expression was analyzed using luciferase assay. D, ChIP-qPCR analysis after iCDK4 treatment.
We used either doxorubicin, which induces p53 through DNA damage (29) or nutlin-3, which disrupts MDM2-p53 interaction leading to p53 stabilization (30), to activate p53. Two selective CDK4 inhibitors, RO0505124 and RO0506220 (31), were used to reactivate pRb inactivated downstream of MYCN (15). RO0505124 and RO0506220 have IC50 values of 20 nmol/L for recombinant CDK4-cyclin D in vitro that were >100-fold more potent for CDK4 compared with CDK1 and CDK2 in vitro (31). In line with this, CDK4 inhibitor treatment led to decreased pRb phosphorylation at Serine 780 (Fig. 5B), specific for CDK4 (32), in SH-SY5Y cells (Fig. 5B). Intriguingly, doxorubicin, nutlin-3, and CDK4 inhibitors all led to a prominent reduction of SKP2 protein levels in nonamplified cells, in contrast to MYCN-amplified cells, in which only iCDK4 reduced SKP2 protein levels (Fig. 5A and B). SKP2 was also reduced in a MYCN-amplified genetic background by tetra-cycline-inducible, targeted knockdown of CDK4 in stable IMR5-75 cell clones, or after transient transfection of CDK4 cycline-inducible, targeted knockdown of CDK4 in stable MYCN–amplified cells, in which only iCDK4 suppressed these constructs in MYCN-amplified cells (Fig. 5A and B). RO05124 performed similarly in other MYCN-amplified cell line (SK-N-Be2C) also gave strong SKP2 reduction in small compound inhibitors or siRNAs targeting the specific usage of distinct E2F-binding sites within the MYCN-promoter (4, 5, 19). Taken together, these data support a characteristic SKP2 activation mechanism in MYCN-amplified cells through the TSSR, which is abrogated through pRB activation in most neuroblastoma cells, particularly in those with amplified MYCN. However, our results suggest, at least for human neuroblastoma, that active induction of SKP2 through E2Fbdg#2 is unlikely because deletion/mutation of E2Fbdg#2 did not reduce but in contrast increase SKP2 promoter activity in most neuroblastoma cells, further suggesting that E2Fbdg#2 acts as an actively repressing site in neuroblastoma cells. It is important to note that we also describe an experimental condition in a neuroblastoma cell line in which E2Fbdg#2 turned into an activating site, which is E2F1 overabundance in the context of hypophosphorylated, active pRb (SH-EP-E2F1, stably expressing an ER-E2F1 fusion protein activated upon treatment with 4-OHT). Under this artificial condition, mutation/deletion of E2Fbdg#2 reduced SKP2 promoter activity, which would be in line with active induction of SKP2 by E2F1 through E2Fbdg#2. Intriguingly, E2F1 overabundance in SH-EP-E2F1 cells is also associated with SKP2 repression at the TSSR, including E2Fbdg#1, and high abundance of repressive pRB-E2F1 complexes bound to the SKP2 core promoter region. The TSSR, including E2Fbdg#1, is usually important for SKP2 activation in neuroblastoma cells, particularly in those with amplified MYCN. In contrast, E2Fbdg#2 and E2Fbdg#3 mediate SKP2 repression in most neuroblastoma cells. Our results are in contrast to previous studies reporting controversial results on the role of the different E2F-binding sites within the SKP2 promoter (4, 5, 19). Zhang and colleagues showed (4) that E2Fbdg#2 is important for SKP2 activation in transformed cells, derived from cervical carcinoma (HeLa), osteosarcoma (MG63), and glioma (U343) cells. SKP2 activation in mouse embryonic fibroblasts (5) or repression in HeLa (19) dependent on the third E2F-binding site (E2Fbdg#3) has been reported as well. This argues in favor of the cell type–specific usage of distinct E2F-binding sites in the SKP2 promoter either as activating or repressing sites. Several studies of a large number of well-characterized E2F target genes suggest that, in general, E2F regulates cell cycle-dependent gene expression by three modes: active induction, derepression, and active repression (reviewed in ref. 22). Zhang and colleagues (4) suggested that E2F regulates SKP2 promoter function through an active induction mechanism through E2Fbdg#2, in which gene transcription is induced upon replacement of repressive E2Fs (e.g., E2F4 and E2F5) by activating E2Fs (e.g., E2F1-3). However, our results suggest, at least for human neuroblastoma, that active induction of SKP2 through E2Fbdg#2 is unlikely because deletion/mutation of E2Fbdg#2 did not reduce but in contrast increase SKP2 promoter activity in most neuroblastoma cells, further suggesting that E2Fbdg#2 acts as an actively repressing site in neuroblastoma cells. It is of major importance for SKP2 activation in neuroblastoma cells, particularly in those with amplified MYCN. We used either doxorubicin, which induces p53 through DNA damage (29) or nutlin-3, which disrupts MDM2-p53 interaction leading to p53 stabilization (30), to activate p53. Two selective CDK4 inhibitors, RO0505124 and RO0506220 (31), were used to reactivate pRb inactivated downstream of MYCN (15). RO0505124 and RO0506220 have IC50 values of 20 nmol/L for recombinant CDK4-cyclin D in vitro that were >100-fold more potent for CDK4 compared with CDK1 and CDK2 in vitro (31). In line with this, CDK4 inhibitor treatment led to decreased pRb phosphorylation at Serine 780 (Fig. 5B), specific for CDK4 (32), in SH-SY5Y cells (Fig. 5B). Intriguingly, doxorubicin, nutlin-3, and CDK4 inhibitors all led to a prominent reduction of SKP2 protein levels in nonamplified cells, in contrast to MYCN-amplified cells, in which only iCDK4 reduced SKP2 protein levels (Fig. 5A and B). SKP2 was also reduced in a MYCN-amplified genetic background by tetra-cycline-inducible, targeted knockdown of CDK4 in stable IMR5-75 cell clones, or after transient transfection of CDK4 cycline-inducible, targeted knockdown of CDK4 in stable MYCN–amplified cells, in which only iCDK4 suppressed these constructs in MYCN-amplified cells (Fig. 5A and B). RO05124 performed similarly in other MYCN-amplified cell line (SK-N-Be2C) also gave strong SKP2 reduction in small compound inhibitors or siRNAs targeting the specific usage of distinct E2F-binding sites within the MYCN-promoter (4, 5, 19). Taken together, these data support a characteristic SKP2 activation mechanism in MYCN-amplified cells through the TSSR, which is abrogated through pRB activation (hypophosphorylated) following CDK4 inhibition, but not p53-p21 induction. ChIP-qPCR showed that CDK4 inhibition reduced E2F1 bound to the SKP2 promoter independent of MYCN amplification (Fig. 5D). Despite strongly reduced total pRB protein levels (Fig. 5D), CDK4 inhibition increased or did not affect the pRb bound to the SKP2 core promoter in SH-SY5Y or Kelly cells, respectively (Fig. 5D). Taken together, our data indicate that CDK4 suppression by selective small compound inhibitors abrogates SKP2 expression by restoring transcriptionally repressive pRB-E2F1 complexes at the SKP2 core promoter region including the TSSR and E2Fbdg#1.

**Discussion**

**SKP2** is a prototypic E2F target, harboring three E2F-binding sites in the vicinity of the transcriptional start site, one site, E2Fbdg#1, directly upstream and two other sites, E2Fbdg#2 and E2Fbdg#3, more downstream of the transcriptional start site (4). Here, we show that the TSSR, including E2Fbdg#1, is
E2F3) from a repressor to an activator resulting into pronounced SKP2 transcription. This SKP2 activation mechanism seems to be relevant particularly in MYCN-amplified neuroblastoma cells and resembles E2F target gene activation in Rb-deficient cells (33). Because RBI gene mutations are rare in neuroblastoma, functional inactivation of pRB by deregulated cyclin-dependent kinases (CDKs) downstream of amplified MYCN is most likely in these tumor cells. In support of this, inhibition of CDK4, a direct target of MYCN, reduces E2F1 and favors pRB binding to the SKP2 core promoter resulting in reduced SKP2 promoter activity.

SKP2 is part of an autoinduction loop, comprising of pRB-E2F, SKP2, p27, and Cyclin E-CDK2, in which the stimulatory effect of E2F on SKP2 gene expression feeds back to sustain pRB inactivation, E2F release, and further induction of the SKP2 gene (5). Once activated, this autoinduction loop ensures cell cycle progression through the restriction point even without mitogenic stimulation. Our results define CDK4 as an entry point into the SKP2 autoinduction loop downstream of deregulated MYCN in MYCN-amplified cells. CDK4, which is transcriptionally activated by deregulated MYCN in neuroblastoma cells (15), is important for SKP2 activation in MYCN-amplified cells, making SKP2 activation largely independent of mitogenic signals integrated through the SP1/ELK-1 site. CDK4 knockdown by using shRNAs, siRNAs, or selective small molecule inhibitors efficiently suppressed SKP2 activation through the TSSR, including E2Fbdg#1, in MYCN-amplified cells. Intriguingly, we observed that neither p53 activation by chemotherapeutic drugs or nutlins, disrupting the MDM2-p53 interaction—both leading to p21 induction—nor direct CDK2 inhibition was capable of reducing SKP2 expression in MYCN-amplified genetic background. Collectively, we propose that this CDK4-dependent SKP2 activation mechanism induced by deregulated MYCN may contribute to perpetual cell cycle entry in MYCN-amplified cells even under growth factor-depleted conditions. In addition, our results establish selective small-molecule inhibitors for CDK4 as compounds specifically targeting deregulated SKP2 in neuroblastoma cells with amplified MYCN.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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