p27^{Kip1} Mediates Addiction of Ovarian Cancer Cells to MYCC (c-MYC) and Their Dependence on MYC Paralogs

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The MYCC (c-MYC) gene is amplified in 30–60% of human ovarian cancers. We assessed the functional significance of MYCC amplification by siRNA inhibition of MYCC or MYC paralogs in a panel of ovarian cancer cell lines expressing varying levels of MYCC. Inactivation of MYCC inhibited cell proliferation and induced replicative senescence only in lines with amplified MYCC, indicating that these cells are addicted to continued MYCC overexpression. In contrast, siRNA knockdown of all three MYC isoforms inhibited proliferation of MYCC non-amplified ovarian cancer cells without inducing replicative senescence, and did not inhibit the proliferation of telomerase-immortalized ovarian surface epithelial cells. The arrest induced by MYCC knockdown was accompanied by an increase in the level of the Cdk inhibitor p27^{Kip1} and a decrease in cyclin A expression and Cdk2 activity, and could be reversed by RNAi knockdown of p27^{Kip1} or Rb, or by overexpression of cyclin A/Cdk2. The arrest induced by knockdown of all three MYC isoforms could similarly be reversed by p27^{Kip1} knockdown. Our findings indicate that the addiction of MYCC-amplified ovarian cancer cells to MYCC differs from the dependence of MYCC non-amplified cancer cells on MYC paralogs, but both are mediated, at least in part, by p27^{Kip1}. They also suggest that growth of ovarian cancers may be blocked by inhibition of MYCC or MYC paralogs.

One of the most common recurrent amplifications in cancer is the amplification of chromosomal region 8q24, which contains the oncogene MYCC (also referred to as MYC or c-MYC). In mammals, there are four related genes in the MYC family, MYCC, the prototype member of the family, MYCN (N-myc), MYCL (L-myc), and s-myc. Of the MYC genes, MYCC, MYCN, and MYCL have been implicated in the genesis of specific human tumors. In all cases, the amount of Myc protein is increased in the tumor tissue when compared with the surrounding normal tissues, suggesting that the elevated expression of Myc contributes to tumorigenesis (1, 2). Experiments with transgenic mice inducibly expressing the MYCC oncogene have shown that repression of MYCC expression following the induction of tumor formation can lead to tumor regression, differentiation, replicative senescence, or apoptosis (3). Such dependence of cancer cells on continued oncogene expression for proliferation or survival is frequently referred to as “oncogene addiction” (4–7). In contrast, expression of a dominant-negative form of MYCC that inhibits the function of all three isoforms of MYC induces regression of tumors initiated by activated Ras, indicating that MYC function is required for the maintenance of tumors that are not initiated by MYC (8).

MYC encodes a transcription factor that regulates the expression of a multitude of genes involved in cellular growth, proliferation, and differentiation (9, 10). In fibroblasts and human tumor cells, Myc is required at several cell cycle transitions and induced loss of Myc activity causes cells to undergo growth arrest either at the G1/S transition or at a later step in the cell cycle (11–13). In contrast, Myc does not appear to be required for the proliferation of certain normal cell lineages (14, 15). Myc accelerates cell proliferation, at least in part, through its ability to down-regulate the expression of the cyclin-dependent kinase (Cdk) inhibitor p27^{Kip1} (16, 17). Myc regulates p27^{Kip1} expression both directly, at the transcriptional level, and indirectly, by controlling the expression of two components of the SCF complex, Cul1 and Csk1 (18, 19).

We are interested in identifying the significance of MYCC overexpression in ovarian cancer. This cancer, although second in incidence as a gynecologic cancer, causes more deaths than all other gynecologic cancers combined, but the pathogenesis of the disease is poorly understood. Amplification of 8q24 was found to be very common in ovarian cancers (35–76%) (20, 21) and MYCC is overexpressed in 65% of human ovarian cancers (22). Furthermore, associations between MYCC copy number changes and the degree of malignancy of ovarian cancers have been reported (23).

To determine whether ovarian cancer cells are dependent on expression of MYCC or its paralogs, we used small interfering RNAs (siRNAs) to down-regulate the expression of MYCC and MYC paralogs in a panel of ovarian cancer cell lines expressing varying levels of MYCC and compared the responses of these...
cells to those in normal ovarian surface epithelial cells immortalized by telomerase expression. Our findings indicate that ovarian cancer cells are dependent on the expression of MYCC or MYC family members, that the critical MYC-dependent step is Cdk2 activation, and that this dependence is mediated by p27Kip1. However, MYCC-amplified cells undergo replicative senescence upon inactivation of MYC function, whereas MYCC non-amplified cells do not, suggesting that the nature of the MYC dependence differs in MYCC-amplified and non-amplified cells. These results have implications for the use of MYC antagonists in cancer therapy.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Ovarian cancer cell lines with and without amplification at 8q24, Hey, Ovca420, A2780, and Caov3, were cultured as described previously (24). Telomerase-immortalized normal ovary epithelial (TIOSE) cell lines, hTert4 and hTert6, were obtained from Michael J Birrer, NCI, NIH (25). TIOSE cell lines were maintained in a 1:1 mixture of medium 199 and MCDB 105 medium, supplemented with gentamicin. hTert4, hTert6, were obtained from Michael J Birrer, NCI, NIH (25).

**Telomerase-immortalized normal ovary epithelial** (TIOSE) cell lines, hTert4 and hTert6, were obtained from Michael J Birrer, NCI, NIH (25).

**Phenotypic Characterization**

**Myeloid Colony Formation**

**Senescence**

**Immunoblotting—Lysis of cells was carried out as described previously** (12). For immunoblotting, blots were blocked in Odyssey blocking buffer (OB; LI-COR, Inc.) and incubated (12 h; 4 °C) with primary antibodies in OB containing 0.1% Tween 20. They were then incubated (1 h, 22 °C) with secondary antibodies (0.1 μg/ml goat anti-mouse or 0.2 μg/ml goat anti-rabbit IgG coupled to IR dyes) in OB containing 0.1% Tween 20 and 0.01% SDS. Blots were quantified using an imaging system (Odyssey IR; LI-COR). Antibody against GAPDH was from Abcam, Inc. (Cambridge, MA). Antibody against phospho-Cdk2 was from Cell Signaling Technology (Danvers, MA). All other antibodies used for immunoblotting were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cdk2 Immune Complex Kinase Assays**—Cells were lysed in Cdk2 lysis buffer (50 mM Tris-Cl pH 7.4, 250 mM NaCl, 0.1% Triton X-100, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM dithiothreitol (DTT), 0.1 mM NaVO₃ containing protease inhibitors (174 μg/ml PMSF, 1.5 μg/ml benzamidine, 1 μg/ml phenanthroline, 0.5 μg/ml antipain, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.5 μg/ml aprotinin, and 0.5 μg/ml chymostatin)) for 15 min on ice. Samples containing equal amounts of protein as determined by BCA assay were used for immunoprecipitation. Lysates containing 200–500 μg protein were precleared by incubation with 1 μg of rabbit preimmune serum and 30 μl of a 1:1 slurry of protein G-Sepharose beads (Amer sham Biosciences/GE Healthcare, Piscataway, NJ) on a rocking platform at 4 °C for 30 min. After a brief centrifugation to remove precleared beads, 0.5 μg of anti-Cdk2 antibody was added to each sample and incubated on a rocking platform at 4 °C for 2 h. 30 μl of protein G-Sepharose beads were added to each sample, and the slurries were incubated on the rocking platform at 4 °C for 30 min. The beads were then washed five times with Cdk2 lysis buffer and twice with kinase buffer (50 mM HEPES, 10 mM MgCl₂, 5 mM MnCl₂, 0.1 μg/ml NaF, 10 μg/ml β-glycerophosphate, and 0.1 mM sodium orthovanadate). Samples were resuspended in 25 μl of kinase buffer containing 20 μM [γ-32P]ATP (20 Ci/mmol), 5 mM DTT, and 0.21 μg of Rb C-terminal domain protein substrate (Santa Cruz Biotechnology). Reactions were incubated for 15 min at 30 °C and stopped by the addition of an equal volume of 2× loading buffer (10% glycerol, 5% β-mercaptoethanol, 3% SDS, 6.25 mM Tris–HCl [pH 6.8], and bromphenol blue). Reaction products were boiled for 10 min and then electrophoretically resolved by SDS-PAGE and phosphorylated Rb was visualized by autoradiography.

**Bromodeoxyuridine Labeling**—The effects of siRNAs on cell cycle progression were assessed by measuring bromodeoxyuridine (BrdU) incorporation and flow cytometry analysis using a BrdU assay kit from Roche Diagnostics (Indianapolis) and the procedures specified by the manufacturer. In brief, cells were labeled with BrdU for 30 min and then fixed with 70% ice cold ethanol for 12 h, stained with mouse anti–BrdU antibody for 1 h at 37 °C, followed by staining with fluorescein-conjugated anti-mouse immunoglobulin antibody for 1 h at 37 °C. They were then incubated with RNase A (Sigma Aldrich) and stained with propidium iodide (Roche) prior to flow cytometry analysis.

**MTT Assay**—MTT assays were performed using an assay kit from ATCC (Manassas, VA) as per the manufacturer’s instructions. Cells transfected with siRNAs were plated in triplicate 48 h after transfections. After a 12-h incubation, 10 μl of MTT reagent was added, and the cells were incubated at 37 °C for another 2 to 4 h until a purple precipitate was visible. Then the detergent reagent was added, and the cell lysates left for 2 h in the dark at room temperature. Absorbance was monitored at 570 nm with a Microplate Reader (VersaMax, Molecular Devices, Sunnyvale, CA).

**Senescence-associated β-Galactosidase Assay**—The assay for the senescence-associated β-galactosidase was carried out using a Senescence Detection kit (Calbiochem, Gibbstown, NJ) as per the manufacturer’s instructions. Briefly, cells were plated in triplicate in 6-well culture plates, fixed at room temperature for 10–15 min, washed twice with PBS, and incubated with staining solution at 37 °C overnight. Cells were observed under bright-field illumination for development of blue color.

**Immunofluorescence**—Cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were then
permeabilized with 0.2% Triton X-100 (EM Science, Gibbstown, NJ) in PBS for 10 min at room temperature and incubated for 30 min in PBS containing 5% BSA and 10% normal goat serum. Samples were incubated with one primary antibody or a mixture of two primary antibodies for 1 h at room temperature. Mouse monoclonal antibody directed against HP1γ (Cell Signaling Technology, Danvers, MA), rabbit polyclonal antibody directed against trimethyl K9 histone H3 (Millipore, Billerica, MA), and rabbit monoclonal antibody directed against phospho-histone H2A.X, Ser139 (Cell Signaling Technology, Danvers, MA) were used to detect senescence markers. Signals were developed using either goat anti-rabbit or goat anti-mouse IgG for 1 h at room temperature. Samples were mounted in SlowFade antifade reagent containing DAPI (Invitrogen, Carlsbad, CA). Images were collected at room temperature using a 510 confocal laser scanning microscope (510 LSM; Carl Zeiss, Inc.) and 510 LSM software (v. 3.2 sp2; Carl Zeiss, Inc.).

Statistical Analysis—Results were compared by Student’s t test and statistical significance indicated in the figures (*, p < 0.05; **, p < 0.01). All experiments were done independently at least three times, and data are expressed as mean ± S.E.

RESULTS

MYCC-amplified Ovarian Cancer Cells Are Dependent on Overexpression of MYCC—In previous studies, a direct correlation between MYCC RNA levels and 8q24 copy number was observed (24). We selected a set of 4 ovarian cancer cell lines expressing varying amounts of Myc, with or without amplification of the MYCC gene, for further studies (Fig. 1A). As a control, we used a telomerase-immortalized normal ovary epithelial cell line (TIOSE), hTert4 (25). Based on the MYCC expression and genomic amplification data, we defined Hey and Ovca420 as MYCC-amplified ovarian cancer cells, A2780 as a normal expression line and Caov3 as a MYCC non-amplified low expression line. The expression of MYCC was then blocked by transfection with a MYCC siRNA. Quantitative RT-PCR and immunoblotting analyses confirmed that the siRNA inhibition was effective (Fig. 1A and supplemental Fig. S1). Flow cytometry following 5-bromo-2-
deoxyuridine (BrdU) incorporation and propidium iodide staining was then used to determine the effects of MYCC RNAi on cell cycle distribution. Upon MYCC RNAi of the MYCC-amplified Hey and Ovca420 cells, the percentage of S-phase cells decreased from 27.5% to 2.9% in Hey cells and from 27.5% to 3.05% in Ovca420 cells, while the percentage of G1 cells increased from 64.6% to 95.8% in Hey cells and from 63.3% to 88.8% in Ovca420 cells (Fig. 1B), indicating that MYCC knockdown leads to a G1/S block in these cells.

In contrast Caov3 cells which express low levels of MYCC RNA were not arrested upon MYCC RNAi (Fig. 1B), whereas in A2780 cells, in which there is no increase in MYCC expression, there was only a modest decrease in the fraction of S-phase cells, from 31.8% to 20.4% (Fig. 1B). This indicates that MYCC RNAi induced a G1 arrest only in MYCC-amplified cells, suggesting that MYCC-amplified ovarian cancer cell lines are specifically dependent on continued overexpression of the MYCC oncogene. Cell proliferation was also monitored by cell counting and MTT assays (supplemental Fig. S2, A and B). Consistent with the BrdU incorporation assays, the proliferation of Hey and Ovca420 cells was inhibited by MYCC knockdown, whereas the proliferation of A2780 and Caov3 was not. The inhibition of Hey and Ovca420 cell proliferation was still apparent 7 days after induction of RNAi (supplemental Figs. S2A and S6 and data not shown). These results suggest that MYCC-amplified ovarian cancer cell lines are specifically dependent on continued overexpression of the MYCC oncogene.

To further investigate the hypothesis that MYCC-dependence is specific to MYCC-amplified lines, we examined the effect of siRNA inhibition of MYCC on the proliferation of the telomerase-immortalized normal ovary epithelial cell line hTert4 (25). As shown in Fig. 1C, hTert4 cells continued to synthesize DNA upon inhibition of MYCC. In addition, the proliferation of the cells as judged by cell counting or MTT assays was not affected by MYCC knockdown (supplemental Fig. S2, A and B). These results are consistent with the hypothesis that the dependence of MYCC-amplified ovarian cancer cells on continued overexpression of MYCC results from MYCC amplification.

**MYCC Non-amplified Cells Are Dependent on MYC Paralogs**

One hypothesis to account for the resistance of the MYCC non-amplified cells to MYCC RNAi is that it is due to expression of the MYC paralogs MYCN and MYCL. We therefore quantified the levels of MYCL and MYCN RNAs by qRT-PCR (Fig. 2A). Both MYCL and MYCN RNAs were expressed in the two MYCC non-amplified cell lines A2780 and Caov3 cells. It has previously been reported that MYCL is overexpressed and in some cases amplified in many ovarian carcinomas (26, 27); however in these two lines, A2780 and Caov3, there is no...
detectable amplification of the MYCL locus.3 Interestingly, the level of \textit{MYCL} RNA was comparable or slightly lower than that in two TIOSE lines, hTert4 and hTert6, whereas the level of \textit{MYCN} RNA was severalfold lower (Fig. 2A). In the two \textit{MYCC}-amplified lines, the level of both \textit{MYCL} and \textit{MYCN} RNAs was considerably lower than in the normal immortalized lines, raising the possibility that high levels of \textit{MYCC} expression may result in reduced expression of the other two \textit{MYC} paralogs.

RNAi knockdown of \textit{MYCL} in these lines, using two different siRNAs (Fig. 2B), did not reduce BrdU incorporation, either alone or in combination with \textit{MYCC} RNAi (Fig. 2B). This indicates that \textit{MYCL} expression alone is not sufficient to account for the resistance of \textit{MYCC} non-amplified cells to \textit{MYC} RNAi. However when all three \textit{MYC} isoforms, \textit{MYCC}, \textit{MYCL}, and \textit{MYCN} were inhibited, the percentage of Caov3 and A2780 cells in \textit{S} phase was significantly reduced (Fig. 2C), suggesting that the \textit{MYCC} non-amplified lines Caov3 and A2780 are dependent on the function of \textit{MYC} family members. In parallel with this reduction in BrdU incorporation, the proliferation of these non-amplified lines was reduced upon inhibition of all three \textit{MYC} paralogs (supplemental Fig. S3). To further explore this hypothesis, we examined the effect of siRNA inhibition of all three \textit{MYC} paralogs on the proliferation of the two TIOSE cell lines, hTert4 and hTert6 (25). Both of these cell lines continued to enter \textit{S} phase after knockdown of all three \textit{MYC} family members (Fig. 2C). This difference between the behavior of ovarian cancer cells and normal ovarian surface epithelial cells indicates that ovarian cancer cells exhibit an increased dependence on the continued expression of \textit{MYC} family members for continued cell proliferation.

The Arrest of \textit{MYCC}-amplified Cells Is Accompanied by Replicative Senescence—Inhibition of \textit{MYCC} in \textit{MYCC}-inducible mouse tumor models results in tumor regression that is accompanied by signs of replicative senescence (28). To determine if this is also the case when \textit{MYCC}-amplified ovarian cancer cells are subjected to \textit{MYCC} knockdown in \textit{vitro}, we initially examined the induction of the senescence-associated \textit{\beta}-galactosidase activity in Hey and Ovca420 cells subjected to \textit{MYCC} RNAi. \textit{\beta}-gal activity was detected in both \textit{MYCC}-amplified lines upon \textit{MYCC} RNAi (supplemental Fig. S4A). Replicative senescence is also characterized by the appearance of senescence-associated (SA-) heterochromatin foci in which genes regulated by the transcription factor E2F1 are transcriptionally repressed. These foci can be detected by staining with antibodies directed against the heterochromatin protein HP1\textgamma or lysine-9 trimethylated histone H3 (H3K9me).Senescence-associated heterochromatin foci were detected in both Hey cells and Ovca420 cells upon \textit{MYCC} knockdown (Fig. 3A and data not shown). Another marker of replicative senescence is the appearance of DNA damage, which can be recognized by foci of accumulation of serine 139-phosphorylated histone H2AX (\gammaH2AX). Foci of \gammaH2AX accumulation were detected in the \textit{MYCC} -amplified lines Hey and Ovca420 upon \textit{MYCC} RNAi (Fig. 3B and data not shown), again confirming that these cells undergo replicative senescence upon \textit{MYCC} RNAi. hTert4 cells did not exhibit markers of replicative senescence when subjected to \textit{MYCC} RNAi (Fig. 3A and B).

3 P. Spellman and J. W. Gray, unpublished data.
p27Kip1 Mediates MYC Oncogene Dependence

To determine if markers of replicative senescence can be detected in MYCC non-amplified cells upon MYC inactivation, we subjected A2780 and Caov3 cells to either MYCC RNAi or knockdown of all three MYC paralogs and examined the cells for the expression of SA-β-gal, the appearance of SA-heterochromatin foci or evidence of DNA damage. The MYCC-non-amplified cells did not exhibit any signs of replicative senescence, even when proliferation was arrested by knockdown of all three MYC paralogs (supplemental Figs. S4 and S5, Fig. 3, and data not shown). Thus replicative senescence upon MYC inactivation is limited to the MYCC-amplified cells, and the non-amplified cells do not undergo replicative senescence even when their proliferation is arrested by inactivation of MYC function by knockdown all three MYC paralogs. Replicative senescence is a characteristic response to MYCC-inactivation of MYCC-induced tumors and a defining feature of oncogene addiction (28); thus the addiction of MYCC-amplified ovarian cancer cells to MYCC differs from the dependence of MYCC non-amplified cancer cells on MYC paralogs.

Cdk2 Inactivation Accompanies the Arrest Induced by MYCC Knockdown—To identify the mechanism by which MYCC RNAi leads to growth arrest, we investigated the effect of MYCC RNAi on the expression and activity of cell cycle regulators in MYCC-amplified cells and non-amplified cells. Myc promotes the induction of cyclin D2 and the activation of the cyclin D-associated kinases Cdk4 and Cdk6 during the reentry of cells into the cell cycle (11). However MYCC RNAi did not affect the expression of cyclins D1 and D2 or the activities of Cdk4 and Cdk6 in any of the four ovarian cancer cell lines tested (data not shown). Thus the sensitivity of the MYCC-amplified lines to MYCC RNAi does not appear to result from a requirement for Myc for activation of the cyclin D-dependent kinases in G1.

Cyclin D-associated Cdk4/6 and cyclin E/A-associated Cdk2 collaborate in the hyper-phosphorylation of Rb, thus promoting cell cycle progression (29). Several studies, including our own, have shown that MYCC participates in the activation of cyclin E/A-Cdk2 complexes (12, 16, 30). Interestingly MYCC RNAi resulted in down-regulation of cyclin A expression in the MYCC-amplified Hey and Ovca420 lines, but not in the non-amplified Caov3 line (Fig. 4A); cyclin E expression was not affected by MYCC RNAi (data not shown). Furthermore, Cdk2 in vitro kinase activity was down-regulated by MYCC RNAi in MYCC-amplified cells, but not in non-amplified Caov3 cells (Fig. 4B, top panel). Cdk2 is activated by phosphorylation of Thr-160 in the activation loop, a phosphorylation that is carried out by the Cdk-activating enzyme Cdk7 (31). Consistent with the down-regulation of Cdk2 activity, Cdk2 Thr-160 phosphorylation was also reduced by MYCC RNAi in MYCC-amplified cells (Fig. 4B, bottom panels). These observations suggest that a defect in the activity of Cdk2 is responsible for the proliferation arrest induced by MYCC RNAi in MYCC-amplified cells and that Myc regulation of Cdk2 activity plays an important role in the continued proliferation of MYCC-amplified cells.

p27Kip1 Mediates MYC Dependence—Two possible mediators of the proliferation arrest resulting from MYC RNAi are the Cdk inhibitors p27Kip1 and p21Cip1. p27Kip1 causes a proliferation arrest at least in part by inhibiting cyclin E/A-Cdk2 activity, and overexpression of Myc can overcome a p27Kip1-imposed cell cycle arrest (16). Both p27Kip1 and p21Cip1 have been shown to be repressed by Myc at the transcriptional level (16, 17, 32–34). Myc also regulates p27Kip1 degradation by controlling the activity of the SCF complex (18, 19). Interestingly MYCC RNAi caused a modest but reproducible increase in the
expression of the Cdk inhibitors p27Kip1 and p21Cip1 only in the MYCC-amplified Hey and Ovca420 cells, but not in the non-amplified cells A2780 and Caov3 (Fig. 4C). We therefore investigated whether the inhibition of p27Kip1 or p21Cip1 by RNAi could reverse the cell cycle block induced by MYCC inhibition. We co-transfected the cells with siRNAs targeted to MYCC and to either p27Kip1 or p21Cip1 and analyzed the cell cycle distribution by BrdU staining. As shown in Fig. 5A, p27Kip1 RNAi allowed growth-arrested MYCC-amplified Hey and Ovca420 cells to enter S phase, indicating that inhibition of p27Kip1 expression by Myc is critical for the continued proliferation of MYCC-amplified cells. Consistent with these observations, p27Kip1 RNAi blocked the replicative senescence induced in the MYCC-amplified Hey and Ovca420 cells subjected to MYCC RNAi (supplemental Fig. S4A). In contrast, p21Cip1 knockdown was not able to rescue the growth arrest induced by MYCC inhibition (Fig. 5B), suggesting that the proliferation arrest is not due to activation of a checkpoint pathway that induces p21Cip1 expression. Finally, p27Kip1 RNAi reversed the cell cycle arrest induced by inhibition of all MYC family members in the MYCC non-amplified Caov3 cells (Fig. 5C). These findings indicate that p27Kip1 mediates both the dependence of MYCC-amplified cells such as Hey and Ovca420 on continued MYCC expression and the dependence of MYCC non-amplified lines such as Caov3 on the continued expression of MYC family members. This is consist with studies (19) that have demonstrated that suppression of p27Kip1 is critical for Myc-induced lymphomagenesis, but contrasts with findings in other systems that implicate suppression of p21Cip1 or p16INK4a as mediators of oncogenesis by MYC (13, 28, 35).

**p27Kip1 Inactivation of Cdk2 and the Function of Rb Are Required for MYC Dependence**—A critical target of Cdk2 is the tumor suppressor Rb, which blocks cell proliferation by inhibiting the expression of E2F-dependent genes; this inhibition is blocked by phosphorylation by Cdk2 (36). The Rb protein has also been reported to be required for induction of replicative senescence in a number of different contexts (37). The findings reported above suggested that the MYCC-dependence of the MYCC-amplified lines results from inhibition of Cdk2 by p27Kip1 following MYCC inactivation, and the consequent activation of Rb. Consistent with this hypothesis, we observed that in the MYCC-amplified Hey cells p27Kip1 RNAi rescued the down-regulation of the expression of phospho-Thr-160 and cyclin A that resulted from MYCC RNAi inhibition (Fig. 6A), suggesting that the effect of p27Kip1 RNAi appears to result, at least in part, from its ability to rescue the defect in cyclin A expression and Cdk2 activity that results from Myc inhibition. Consistent with this model, in the same cells overexpression of either Cdk2 alone or Cdk2 and cyclin A was sufficient to rescue the inhibition of BrdU incorporation by MYCC RNAi (Fig. 6B). Finally, the replicative senescence induced by MYCC RNAi could be blocked by concomitant RNAi knockdown of Rb (Fig. 6C).

**DISCUSSION**

The results described above indicate that the MYC addiction of the MYCC-amplified lines results from a requirement for continued MYCC overexpression to repress p27Kip1, maintaining the activity of Cdk2 and inactivate Rb, thus preventing the onset of replicative senescence. Similarly in the non-amplified lines, repression of p27Kip1 by the three MYC isoforms is also required to maintain cell proliferation, although markers of replicative senescence could not be detected upon inactivation of MYC function. These findings are summarized in Fig. 6D.
The dependence of cancer cells on continued oncogene expression for proliferation or survival is frequently referred to as “oncogene addiction,” although the precise meaning of this term remains controversial (4–7). Several models have been proposed to account for oncogene addiction, including induction of feedback mechanisms that oppose the effect of the activated oncogene; differential rates of decay of prosurvival and proapoptotic signals following oncogene inhibition; induction of cellular senescence by checkpoint mechanisms activated by genome damage; and restoration of a physiological host microenvironment following oncogene inhibition.

It appears that the MYCC-amplified ovarian cancer cells are indeed addicted to continued MYCC expression. These lines arrest upon MYCC knockdown whereas telomerase-immortalized ovarian surface epithelial cells do not, even though the MYCC-amplified lines contain higher residual levels of MYCC RNA. Furthermore these lines undergo replicative senescence upon MYCC RNAi, a characteristic response to MYCC-inactivated MYCC-induced tumors and a characteristic feature of oncogene addiction (28). It is of interest that MYCC can repress telomere-independent senescence by regulating Bmi-1 and p16Ink4a (35). MYCC can also repress BRAF-induced senescence in melanoma cells (38), and Ras-induced senescence in primary rat fibroblasts (39). It has been suggested that the ability of MYCC to inhibit oncogene-induced senescence may result from its ability to facilitate DNA replication and restrain checkpoint-signaling resulting from oncogene-induced replication stress (40). The reason why the MYCC-amplified lines undergo replicative senescence upon MYCC RNAi, rather than a transient cell cycle arrest, merits further investigation.

In a recent study Shachaf et al. (41) examined the pattern of gene expression in an MYCC-inducible lymphoma model, and observed
that there is a precise threshold level of MYCC expression required to maintain a transcriptional program that supports tumorigenesis. Interestingly, CDKN1b, the gene encoding p27\textsuperscript{kip1}, was one of a set of genes showing significant changes in expression above or below this threshold. However other regulators of the G1-S transition were also found to show binary changes in expression at the MYCC threshold, as did genes involved in many other cellular processes. These observations suggest that p27\textsuperscript{kip1} is unlikely to be the sole mediator of MYCC oncogenic addiction. In the same study (41) it was shown that the MYCC threshold required to maintain tumorigenesis, below which cells undergo cell cycle arrest, differentiation or apoptosis, is well above the endogenous level of Myc. This raises the possibility that constitutive expression of the Myc protein results in some perturbation in the Myc regulatory network, for example by activating a negative feedback loop that causes desensitization of the cell to the transcriptional effects of Myc, and that this perturbation might contribute to MYCC oncogenic addiction.

Accumulating evidence suggests that MYC is an excellent target for anti-cancer therapeutics (8, 42, 43). Recently Soucek et al. (8) have used reversible expression of a dominant-negative Myc mutant in a mouse model of Ras-induced lung adenocarcinoma to study both the therapeutic impact and the side-effects of systemic Myc inhibition. In this system, Myc inhibition induced rapid tumor regression, and although it inhibited the proliferation of normal regenerating tissues, these effects were well-tolerated and rapidly reversible. These results are very encouraging, but it should be noted that the dominant-negative mutant used in these studies inhibits the function of all three MYC isoforms, and this property might be essential for its therapeutic effect. We have shown here that MYCC non-amplified ovarian cancer cell lines do undergo cell cycle arrest upon inactivation of MYC function, although the arrest is not accompanied by evidence of replicative senescence. The sensitivity of these lines to inhibition of MYC function by simultaneous knockdown of all three MYC isoforms provides further evidence in support of the concept that inhibition of overall MYC function can inhibit tumorigenesis initiated by oncogenes other than MYCC (8).

Although the studies presented here are limited to the in vitro response of cell lines, such responses are frequently effective predictors of clinical responses (44). The possibility that RNA interference could be of therapeutic value in the clinic has received considerable attention. Our finding that MYCC inhibition is sufficient to arrest the proliferation of MYCC-amplified cells, while inhibition of all three isoforms is required to arrest the proliferation of non-amplified cells, suggests that if MYC inhibition by siRNAs is to be used therapeutically, the mode of inhibition may need to be tailored to the particular tumor. If the behavior of tumors in vivo parallels their behavior in cell culture, then effective therapy may require simultaneous inhibition of all three MYC isoforms, except in those cases where MYCC has been activated by amplification or translocation. Further studies using different mouse models will be necessary to test this prediction.

Acknowledgments—We thank Dr. Michael J. Birrer for the TIOSE cells and members of the Martin laboratory for critical reading of the manuscript.

Note Added in Proof—van Riggelen et al. (45) have recently reported that Myc addiction of murine T-cell lymphomas is mediated by two other Cdk inhibitors, cdk2 nb (p15\textsuperscript{ink4b}) and cdkn1c (p57\textsuperscript{kin2}).

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