Phosphorylation by Cdk2 is required for Myc to repress Ras-induced senescence in cotransformation

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The *MYC* and *RAS* oncogenes are frequently activated in cancer and, together, are sufficient to transform rodent cells. The basis for this cooperativity remains unclear. We found that although Ras interfered with Myc-induced apoptosis, Myc repressed Ras-induced senescence, together abrogating two main barriers of tumorigenesis. Inhibition of cellular senescence required phosphorylation of Myc at Ser-62 by cyclin E/cyclin-dependent kinase (Cdk) 2. Cdk2 interacted with Myc at promoters, where it affected Myc-dependent regulation of genes, including *Bmi-1*, *p16*, *p21*, and *hTERT*, which encode proteins known to control senescence. Repression of senescence by Myc was abrogated by the Cdk inhibitor p27Kip1, which is induced by antiproliferative signals like IFN-γ or by pharmacological inhibitors of Cdk2 but not by inhibitors of other Cdks. In contrast, a phospho-mimicking Myc-S62D mutant was resistant to these manipulations. Inhibition of cyclin E/Cdk2 reversed the senescence-associated gene expression pattern imposed by Myc/cyclin E/Cdk2. This indicates a role of Cdk2 as a transcriptional cofactor and activator of the antisenescence function of Myc and provides mechanistic insight into the Myc-p27Kip1 antagonism. Finally, our findings highlight that pharmacological inhibition of Cdk2 activity is a potential therapeutical principle for cancer therapy, in particular for tumors with activated Myc or Ras.

oncogenes | transcription | cell cycle | p27Kip1 | cyclin E

More than two decades ago, Weinberg and co-workers (1) showed that no more than two activated oncogenes, c-*myc* and H-*ras*, are sufficient to transform primary rodent cells into cancerous cells. Since then, it has become clear that H-*ras* is a member of a gene family, among which H-, K- and N-*ras* are the most well studied, encoding membrane-bound GTPases that transduce signals from growth factor receptors to signal receivers in various cell compartments (2). Amplifications of or activating point mutations in *RAS* family genes are frequently found in many types of human cancers. *MYC* and its family members, *MYCN* and *MYCL*, code for transcription factors that control the expression of many genes involved in distinct processes relevant for tumorigenesis, including cell growth, apoptosis, metabolism, immortalization, differentiation, and stem cell function (3). Deregulated expression of *MYC* family genes has been linked to the development of many types of human tumors and often correlates with poor prognosis. In addition, point mutations at or near the phosphorylation site Thr-58 are often found in lymphomas (4). Because phosphorylated Thr-58 is targeted by the $SCF^{Fbw7/Cdc4}$ E3 ligase for rapid turnover via the proteasome pathway, mutations at this site result in stabilization and accumulation of Myc (5). Phosphorylation of Thr-58 requires a priming phosphorylation at Ser-62 by proline-directed kinases, such as Erk and cyclin-dependent kinase (Cdk) 1 (6, 7).

The tumorigenic potentials of Myc and Ras are limited by the activation of an apoptotic response by Myc (3) and by the induction of premature cellular senescence by Ras (8). Cellular senescence is a state of irreversible growth arrest that normal cells undergo eventually as a result of telomere erosion, but it can be induced prematurely during inappropriate activation of oncogenes. This often involves triggering a DNA damage response as a result of replicative stress or generation of reactive oxygen species, and it is associated with increased levels of the tumor suppressor p53 and the Cdk-inhibitor $p16^{INK4a}$ (9, 10). Studies during recent years have, however, revealed that antitumor programs like differentiation, apoptosis, and cellular senescence can still exist latently in tumor cells. Potentially, these processes can be reactivated if the oncogene, which promotes tumorigenesis, is inactivated (11, 12). For instance, in mouse tumor models driven by regulatable Myc, switching off Myc was shown to be sufficient for sustained tumor regression of several types of cancer (for reviews, see refs. 11–13). Recently, Myc inhibition using a dominant-negative approach also resulted in regression of Ras-dependent tumors, although normal tissues were spared, substantiating the suitability of Myc as a therapeutical target in Myc- and Ras-driven tumors (14). This emphasizes the urge to find drugs that can target Myc and/or Ras activity.

It still remains unclear how Myc and Ras cooperate. Previous work demonstrated that Ras suppresses Myc-induced apoptosis (15). Here, we provide evidence that Myc contributes to malignant transformation by repressing Ras-induced senescence and, furthermore, we define how this is achieved and regulated mechanistically.

Results

Repression of Ras-Induced Senescence by Myc Depends on Ser-62 Phosphorylation. Using primary rat embryo fibroblasts (REFs), we confirmed that oncogenic H-Ras induced senescence as scored by senescence-associated β-Gal (SA-β-Gal) activity (Fig. 1 *A* and *B*), whereas c-Myc induced apoptosis that was antagonized by Ras (Fig. 1*B*), in agreement with previous observations (8, 15).

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Importantly, Ras-induced senescence was blocked efficiently by Myc (Fig. 1 *A* and *B*), thus providing a rationale for the strong cooperativity between Myc and Ras in transformation. Similarly, Myc antagonized senescence induced by downstream effectors of Ras, including constitutively active variants of c-Raf and MEK, and by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which stimulates MAPK signaling via activation of PKC (Fig. 1 *C* and *D*). The phosphorylation sites Thr-58 and Ser-62 have been implicated in regulating the activity and stability of Myc, including its apoptotic function (3, 5, 16) (Fig. S1*A*). We found that Myc-S62A, in contrast to WT Myc or the phospho-mimicking mutant Myc-S62D, was unable to rescue REF cells from Ras-induced senescence, whereas Myc-T58A showed an intermediate phenotype (Fig. 1 *E* and *F*). A Myc-T58A/S62A double mutant was, like the Myc-S62A single mutant, unable to repress senescence. Expression of Myc-S62A did not promote senescence in the absence of Ras. The Myc-S62A, T58A, and S62A/T58A mutants, which are all deficient in Thr-58 phosphorylation, were somewhat elevated in expression compared with WT Myc, as expected (Figs. S1 *A* and *B* and S2*A*). The ability of Myc-S62A to induce transformed foci together with Ras was comparable to that of Myc, Myc-T58A, and Myc-S62D. However, a large number of SA-β-Gal-positive cells were specifically detectable in Myc-S62A/Ras foci (Fig. 1 *G* and *H* and Fig. S1*C*), and these foci eventually regressed (data not shown), probably because the cells are unable to become immortalized. These observations suggested that Ser-62 phosphorylation is not critical for cell proliferation but is required to repress Ras-induced senescence.

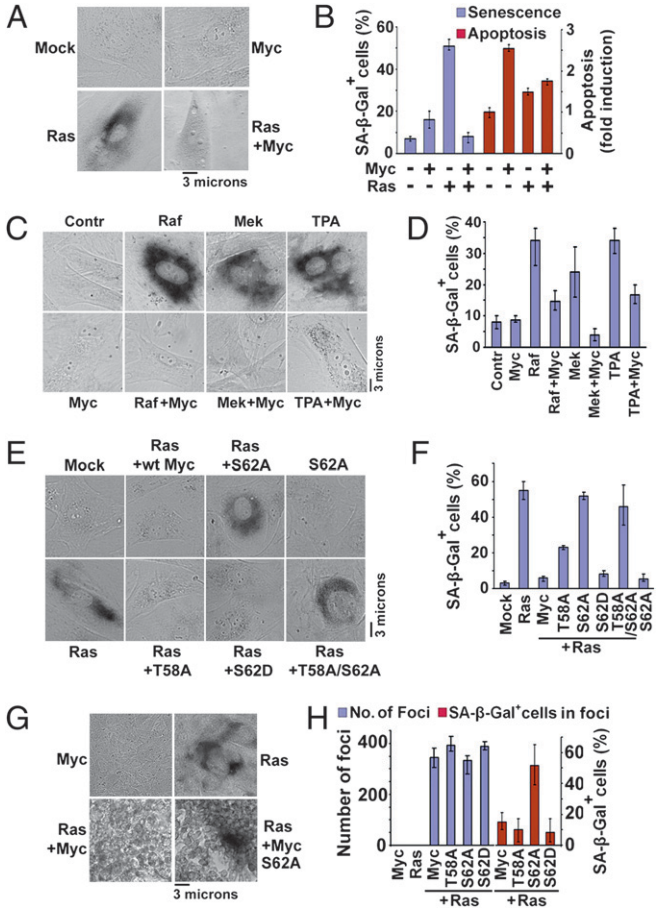

Fig. 1. Repression of Ras-induced senescence by Myc in REFs depends on Ser-62. (*A*) Primary REFs were transfected with expression plasmids for Myc and/or oncogenic Ras. The micrographs show transfected cells cultured for 5 days before analysis of senescence by SA-β-Gal staining. (*B*) Mean values and SDs of SA-β-Gal staining (blue columns) of at least three experiments are given based on analysis of 50 randomly chosen cells. Apoptosis (red columns) was analyzed as described in *Materials and Methods*. (*C–F*) REFs were transfected and analyzed as in *A* and *B* using activated c-Raf or MEK or were treated with TPA (*C* and *D*) or transfected with different Myc mutants (*E* and *F*). (*G*) Focus formation assay of REFs transfected with Ras + WT Myc or mutants. (*H*) Number of foci in *G* was counted (blue columns) and analyzed for SA-β-Gal staining (red columns) 12 days after transfection. Contr, control.

Phosphorylation of Ser-62 Is Mediated by Cyclin E/Cdk2. To identify Ser-62 kinases, U2OS cells were transfected with Myc-T58A to avoid cross-talk between the two sites (Fig. S1*A*) and treated with a panel of kinase inhibitors, after which phosphorylation of Myc at Ser-62 was determined using phospho-specific antibodies (Fig. S2*A*). The Cdk2/Cdk1 inhibitor roscovitine most efficiently reduced Ser-62 phosphorylation (Fig. 2*A*). Because the more Cdk1-specific inhibitor kenpaullone and PD98059, an Erk inhibitor, were not as effective as roscovitine, we considered Cdk2 as a candidate kinase. Indeed the Cdk2-selective inhibitor CVT-313 (CV Therapeutics, Inc.) revealed a potent effect compared with inhibition of Cdk1 or Cdk9 (Fig. 2*B*). Further, Ser-62 phosphorylation was sensitive to the knockdown of Cdk2 or cyclin E1 (Fig. 2*C*) and to the Cdk inhibitors p27Kip1 (p27) and p21Cip1 (p21) (Fig. 2*D* and Fig. S2*B*) but not to knockdown of Cdk1 (Fig. S2*C*). Moreover, overexpression of WT Cdk2 or an inactive Cdk2 kinase mutant increased and decreased Ser-62 phosphorylation, respectively (Fig. S2*B*). Of note, c-Myc phosphorylation increased in cells arrested in early S-phase by aphidicolin, and this signal was completely abolished in response to roscovitine (Fig. S2*D*). Finally, bacterially expressed Myc served as substrate of cyclin E/Cdk2 and, to a lesser extent, of cyclin A/Cdk2 complexes in vitro only when Ser-62 was present, whereas mutation of Thr-58 had no effect (Fig. S2*E*). Together, these findings define Ser-62 as a Cdk2 phosphorylation site.

Repression of Ras-Induced Senescence and Growth Arrest by Myc Is Abrogated by Cdk2-Selective Pharmacological Inhibitors and by p27Kip1. To investigate whether repression of Ras-induced senescence by Myc was dependent on Cdk2, Myc + Ras-transfected REFs were exposed to different kinase inhibitors. Indeed, as well as coexpressing p27, the Cdk2-selective inhibitors (CVT-313, CVT-2584, and CVT-2454; CV Therapeutics, Inc.) and the Cdk2/Cdk1 inhibitor roscovitine abrogated Myc-dependent inhibition of Ras-induced senescence, whereas inhibitors more specific for Cdk1, Cdk9, or Mek1/2 (upstream kinases of Erk1/2) had no effect (Fig. 2 *E* and *F*). Importantly, CVT-313 was not able to override inhibition of senescence by the phospho-mimicking Myc-S62D mutant. Further, the Cdk2-selective inhibitor CVT-2584 strongly inhibited growth of Myc + Ras-expressing REFs, similar to expression of Ras alone (Fig. 2*G*). Treatment with the kinase inhibitors or expression of p27 in the absence of Myc and Ras did not induce cellular senescence (Fig. 2 *E* and *F* and data not shown) nor did CVT-2584 inhibit proliferation in the absence of Myc and Ras (Fig. 2*G*), suggesting a specific role of Cdk2 in mediating repression of Ras-induced senescence by Myc via phosphorylation of Ser-62.

Canceling Myc Repression of Senescence in Human Leukemic Tumor Cells by IFN-γ Correlates with p27 Expression and Loss of Ser-62 Phosphorylation. We have shown previously that v-Myc blocks TPA-induced terminal differentiation and growth arrest of human U-937 leukemic monoblasts (17) (Fig. 3*A* and Fig. S3*A*). Of note and similar to the findings in REFs (Fig. 1 *C* and *D*), Myc blocked TPA-induced senescence in U-937 cells (Fig. 3 *B* and *C*). However, TPA-induced replication arrest and differentiation were restored by treatment with IFN-γ (Fig. 3*A* and Fig. S3*B*) (17) without affecting v-Myc expression (Fig. 3*D*). Importantly, IFN-γ was also able to overcome the v-Myc-dependent re-

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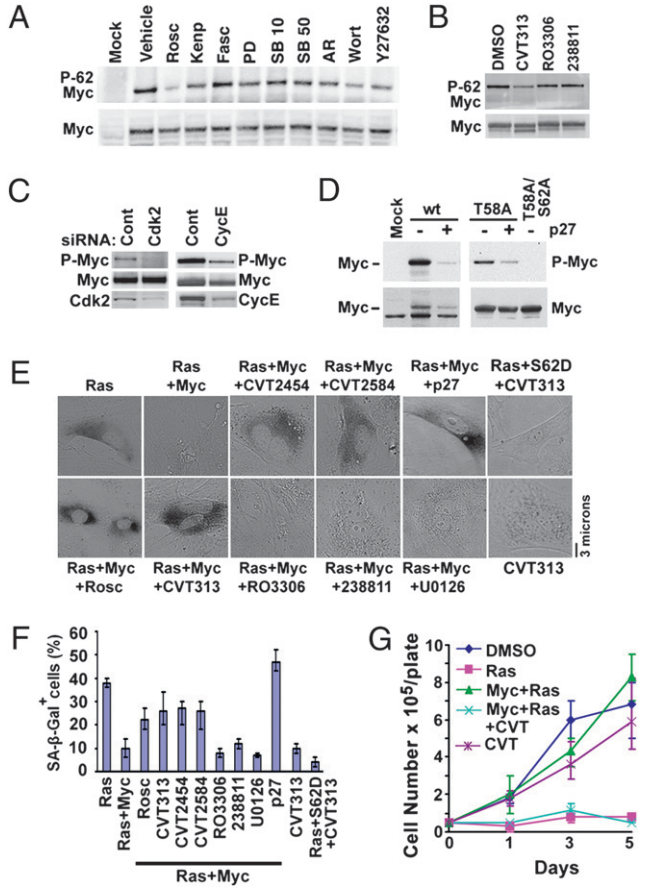

Fig. 2. Ser-62 phosphorylation and repression of Ras-induced senescence are abrogated by Cdk2-selective pharmacological inhibitors and by p27Kip1. U2OS cells were transfected with Myc-T58A and treated with the indicated kinase inhibitors (4 h) (*A* and *B*) and the proteasome inhibitor MG115 (2 h) or were cotransfected with siRNA oligos against GFP, Cdk2, or cyclin E (*C*). Myc proteins were analyzed by immunoblotting using phospho- and pan-Myc antibodies. Roscovitine (Rosc) (inhibits Cdk2/Cdk1), kenpaullone (Kenp) (inhibits Cdk1 > Cdk2), fascalypsin (Fasc) (inhibits Cdk4 > Cdk1/Cdk2), PD98059 (PD) (inhibits Mek1), SB203580 (SB) (inhibits p38 MAPK > JNK), AR-A014418 (AR) (inhibits GSK3), wortmannin (Wort) (inhibits PI3K), Y27632 (inhibits ROCK), CVT-313 (inhibits Cdk2), CVT-2454 (inhibits Cdk2), CVT-2584 (inhibits Cdk2), RO3306 (inhibits Cdk1), 238811 (inhibits Cdk9), and U0126 (inhibits Mek1/2). (*C*) To visualize knockdown efficiency, Western blot analysis of total cell lysates was performed as indicated. (*D*) WT c-Myc or Myc-T58A was cotransfected with p27 into HeLa cells, and c-Myc phosphorylation was analyzed subsequently as in *A*–*C*. A T58A/S62A double mutant was used as a negative control. (*E* and *F*) Senescence in Myc + Ras-transfected REFs in the presence of kinase inhibitors or p27 coexpression, performed as in Fig. 1 *A* and *B*. (*G*) Effect of CVT-2584 on proliferation of Myc + Ras-transfected REFs. Graphs represent cell numbers per plate. Concentrations of the inhibitors are specified in *Materials and Methods*.

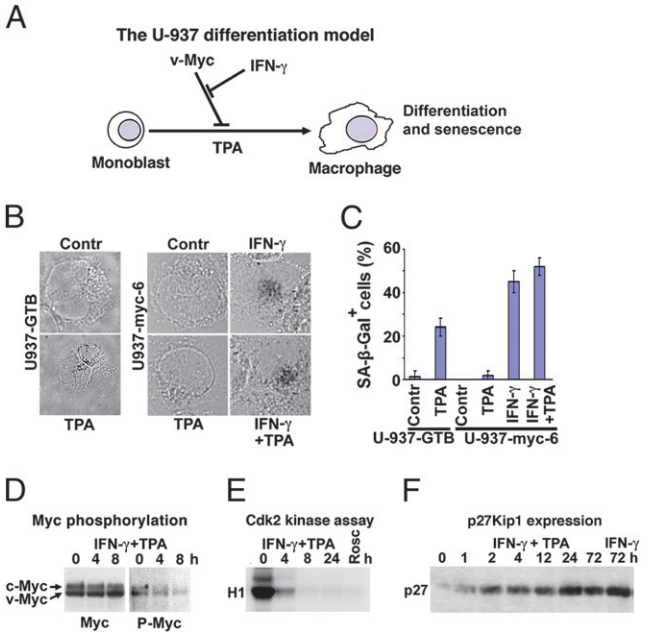

Fig. 3. IFN-γ abrogates repression of senescence in v-Myc-expressing human U-937 cells, correlating with induced p27 expression, inhibited Cdk2 activity, and reduced Ser-62 phosphorylation. (*A*) Schematic picture describing the U-937 differentiation model. IFN-γ reverses the v-Myc block of monocytic differentiation and senescence. (*B*) U-937-GTB parental cells (*Left*) and U-937-myc-6 cells (*Right*) were treated with TPA and/or IFN-γ for 6 days before SA-β-Gal staining. Contr, control. (*C*) Mean values and SDs of SA-β-Gal staining are given based on analysis of 50 randomly chosen cells. Ser-62 phosphorylation (*D*) and p27 expression (*F*) of IFN-γ + TPA-treated cells as determined by immunoblot analysis. (*E*) Cell lysates immunoprecipitated with cyclin E antibodies were assayed in vitro for histone H1 phosphorylation. Rosc, roscovitine.

pression of TPA-induced senescence, as indicated by increased SA-β-Gal activity (Fig. 3 *B* and *C*). IFN-γ also caused senescence and replication arrest in the absence of TPA, albeit at a much slower rate (Fig. 3 *B* and *C*, Fig. S3*B*, and data not shown).

These findings suggested that IFN-γ interferes with Myc function. Interestingly, IFN-γ-treatment with or without TPA led to a strong reduction of Ser-62 phosphorylation, whereas TPA treatment had much less effect (Fig. 3*D* and Fig. S3*C*, *Upper*). This pattern correlated very well with a reduction in Cdk2 activity (Fig. 3*E* and Fig. S3*C*, Cdk2 kinase assay) and reduced phosphorylation of the retinoblastoma protein (pRb), a cyclin E/Cdk2 substrate, in IFN-γ+/− TPA-treated cells (Fig. S3*C*, 4th panel). Furthermore, the decrease in Cdk2 activity within 4 h of IFN-γ treatment correlated with increased p27 protein expression (Fig. 3*F*) and increased p27/Cdk2 complex formation (Fig. S3*C*, lower panels). No changes in the activity of the serine/threonine phosphatase PP2A, which acts on c-Myc Ser-62 (18), occurred after IFN-γ + TPA treatment (Fig. S3*D*).

Ectopic p27Kip1 Expression and Cdk2-Selective Inhibitors Enforce Senescence in Myc-Expressing Human Tumor Cells. Our studies suggested a role of p27 in mediating the effect of IFN-γ in inducing cellular senescence (Fig. 3 and Fig. S3). Therefore, U-937-myc-6 cells were transduced with retroviral vectors encoding WT p27 or a more stable mutant, p27-T187A (19). Compared with controls, cells expressing p27 and more pronounced p27-T187A were positive for SA-β-Gal activity (Fig. 4 *A* and *B*). These results suggested that inactivation of Cdk2 by p27 is a key regulatory step to reduce Myc Ser-62 phosphorylation and to induce cellular senescence on IFN-γ treatment. Further, the Cdk2-selective inhibitors CVT-313 and CVT-2584 also induced senescence in U-937-myc-6 cells (Fig. 4 *C* and *D*)and strongly inhibited proliferation (Fig. S3*E*).

Cyclin E/Cdk2/p27 Targets Ser-62 at Myc Target Promoters in an IFN-γ- and Cdk2-Inhibitor-Regulated Manner. If Myc is a direct target of cyclin E/Cdk2/p27, it may be possible to measure physical interactions between Myc and cyclin E/Cdk2 and/or p27 in cells. Low but measurable amounts of Myc were specifically coimmunoprecipitated with both cyclin E and p27 (Fig. S4*A*). Myc/p27 interactions were also detected in IFN-γ- and/or TPA-treated cells (Fig. S4*B*). In addition to Myc, we detected cyclin E, Cdk2, and p27 on *cyclin D2*, a Myc target gene (3), by quantitative ChIP (Fig. S4*C*). *Cyclin D2* is a component of the p16Ink4a-Rb-pathway, one of the major pathways controlling oncogene-induced senescence (9, 10).

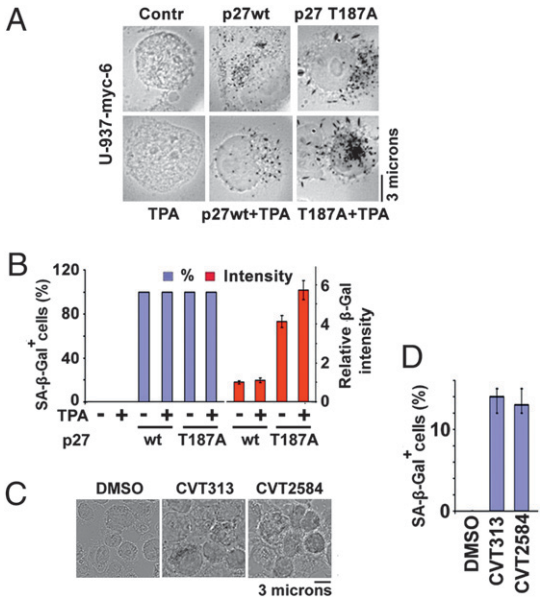

Fig. 4. Cdk2 inhibition by p27 or pharmacological inhibitors of Cdk2 causes senescence in v-Myc-expressing human U-937-myc-6 cells. (*A* and *B*) Cells were transduced with retroviral vectors encoding p27WT or p27T187A and GFP as a fluorescent marker. GFP$^+$ cells were sorted and cultured ± TPA for 6 days, followed by SA-β-Gal staining. (*B*) To estimate the relative SA-β-Gal intensity of p27-transduced cells, pixel intensities were measured for 25 cells.

In the parental U-937-GTB cells, less Myc bound to the *cyclin D2* promoter, as expected, and the Cdk2 signal was hardly above background (Fig. S4*D*), In HL-60 cells, which contain amplified *MYC* genes and express high levels of c-Myc, Cdk2 clearly associated with chromatin (Fig. S4*E*, *Left*, "U-937 scale"), correlating with the abundant presence of Myc (Fig. S4*E*, *Right*, Lower scale). Furthermore, re-ChIP experiments demonstrated that Myc colocalizes with Cdk2 and p27, indicating that Myc forms complexes with these factors on chromatin (Fig. S4*F*). In U-937-GTB and HL-60 cells, the ratios of phosphorylated Myc to total Myc as well as of Cdk2 to total Myc were lower than the corresponding ratios in v-Myc-expressing U-937-myc6 cells (Fig. S4 *C–H*). This is likely attributable to higher turnover of the WT phosphorylated c-Myc species, because Ser-62 phosphorylation primes for Thr-58 phosphorylation and subsequent degradation (Fig. S1*A*).

We next investigated whether the association of phosphorylated and total Myc with chromatin was affected in response to IFN-γ + TPA at target genes relevant for oncogene-induced and/or replicative senescence. On IFN-γ + TPA treatment, Myc association, particularly phosphorylated Myc, decreased markedly at the *cyclin D2*, *BMI-1*, *p16INK4a*, and *telomerase* (*hTERT*) promoters, which have been reported to be activated by Myc (3), and also at the *p21CIP1* promoter, which is repressed by Myc (Fig. 5, *Left*, and Fig. S4*G*). Bmi-1 is a polycomb group protein involved in transcriptional repression of the cyclin/Cdk inhibitor (CKI) *p16Ink4a*, which interferes with the catalytic activities of D-type cyclin complexes. The p53 target gene *p21Cip1*, a CKI that inhibits E- and A-type cyclin kinase complexes, affects the G1- to S-phase transition. These genes have been recognized as components of different senescence pathways, including oncogene-induced senescence (9, 10). hTert regulates telomere length and is unlikely to play a direct role in Ras-induced senescence, but it is known to regulate replicative senescence/immortalization. *hTERT* shutoff may therefore be important for long-term maintenance of oncogene-induced senescence. Moreover, the Cdk2 inhibitor CVT-313 caused reduced association of total and phosphorylated Myc at these promoters, comparable to IFN-γ + TPA treatment (Fig. 5, *Left*). This was paralleled by a loss of Cdk2 and an increase in p27 binding to these promoters in response to IFN-γ + TPA and/or CVT-313 treatment (Fig. 5, *Right*, and Fig. S4*H*). Consistent with this, acetylation of histone H4, a well-established modification associated with Myc binding to responsive sites in chromatin, decreased at the *cyclin D2* promoter after treatment with IFN-γ + TPA (Fig. S4*I*). The selectivity of CVT-313 for Cdk2 was validated by its inhibition of pRb Thr-356 phosphorylation (Fig. S4*J*). In contrast, lamin A/C phosphorylation, a Cdk1 target, was only minimally affected by CVT-313 but was substantially reduced by the Cdk1-selective inhibitor RO3306 (Fig. S4*K*).

Collectively, IFN-γ-induced p27 and pharmacological Cdk2 inhibitors target Myc-bound cyclin E/Cdk2, resulting in reduced phosphorylation and loss of Myc from promoters of Myc target genes involved in senescence.

Cyclin E/Cdk2 Possesses Myc Coactivator Functions. The above results suggested a cofactor function of cyclin E/Cdk2 for Myc. Indeed, cyclin E/Cdk2 enhanced transcription of a Myc-driven reporter gene (Fig. 6*A*). Further, activation of an *hTERT*-promoter/luciferase reporter by Myc-S62A was attenuated compared with WT Myc, Myc-T58A, or Myc-S62D mutant (Fig. 6*B*), suggesting that Ser-62 phosphorylation is important for Myc-driven transcription. In addition, a Myc-regulated reporter gene was repressed after treatment of U-937-myc-6 cells with IFN-γ ± TPA (Fig. 6*C*). In agreement with the role of S62 phosphorylation on reporter gene expression, the *hTERT* and *Bmi-1* genes were repressed by IFN-γ + TPA and by the Cdk2 inhibitor CVT-313 (Fig. 6*D*), whereas the *p21* and *p16Ink4A* genes were strongly induced (Fig. 6*E*). The changes in gene expression are consistent with the ChIP results and indicate that cyclin E/Cdk2 enhances, whereas IFN-γ, through the activation of p27, represses Myc-activated transcription (and vice versa for Myc-repressed transcription) of genes involved in cellular senescence.

Discussion

Our results provide a rationale for the cooperativity between Myc and Ras in malignant transformation. We present evidence for an important function of Myc in repressing Ras-induced senescence as well as senescence triggered by other activators of the MAPK pathway, including activated c-Raf, Mek, and TPA. This is consistent with the recent observation that c-Myc repressed $BRAF^{V600E}$- and $NRAS^{Q61R}$-induced senescence in melanocytes (20). Thus, together with the antiapoptotic role of Ras, these two oncoproteins interfere with two main barriers of tumorigenesis, apoptosis and cellular senescence (see model, Fig. 6*F*). It is clear from studies during recent years that the programs associated with these two processes can exist in latent form in tumor cells. Importantly, even on short-term inactivation of the oncogene that drives tumorigenesis, such programs can be reactivated, resulting in tumor regression (11–13). Indeed, in several mouse tumor models, switching off Myc was sufficient for sustained tumor regression (for reviews, see 11–13), often accompanied by induction of senescence (21). Recently, Myc inhibition using a dominant-negative approach also resulted in regression of Ras-dependent tumors, whereas normal tissues were spared, substantiating the suitability of Myc as a therapeutical target in Myc- and Ras-driven tumors (14). It has been proposed that regression of tumors on oncogene inactivation is the result of "addiction" of the tumor cells to abnormally high levels of a particular activated oncoprotein (11, 12). Our results suggest that oncoproteins like Myc and Ras complement each other by repressing senescence and apoptosis, respectively (Fig. 6*F*), not necessary attributable to abnormal functions of these proteins. Indeed, induction of cellular senescence has also been observed in heterozygous *MYC* "knockout" human fibroblasts expressing lower levels of c-Myc (22). Our finding that Myc represses Ras-induced cellular senescence is probably related to other well-known attributes of Myc, such as its ability to block cell cycle exit

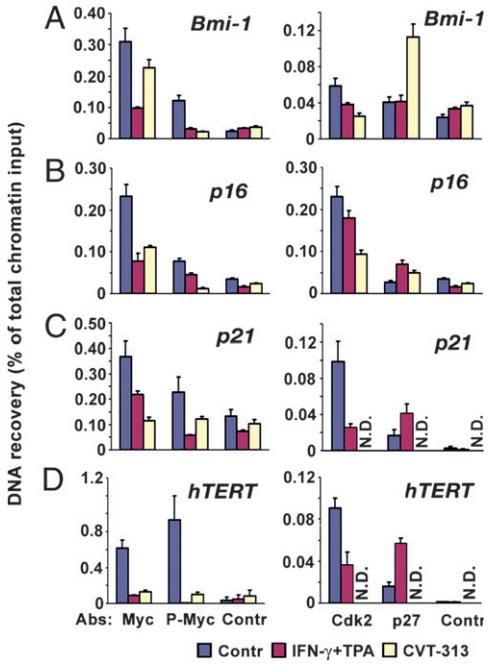

Fig. 5. Myc phosphorylation and Myc-cyclin E/Cdk2/p27 association at Myc target promoters regulated by IFN-γ and the Cdk2 inhibitor CVT-313. (*A–D*) Quantitative ChIP analysis of the association of Myc and phosphorylated Myc (*Left*) and Cdk2 and p27 (*Right*) with the *Bmi-1* (*A*), *p16* (*B*), *p21* (*C*), and *hTERT* (*D*) promoters after IFN-γ + TPA or Cdk2 inhibition in U-937-myc-6 cells. Contr, preimmune serum; N.D., not determined.

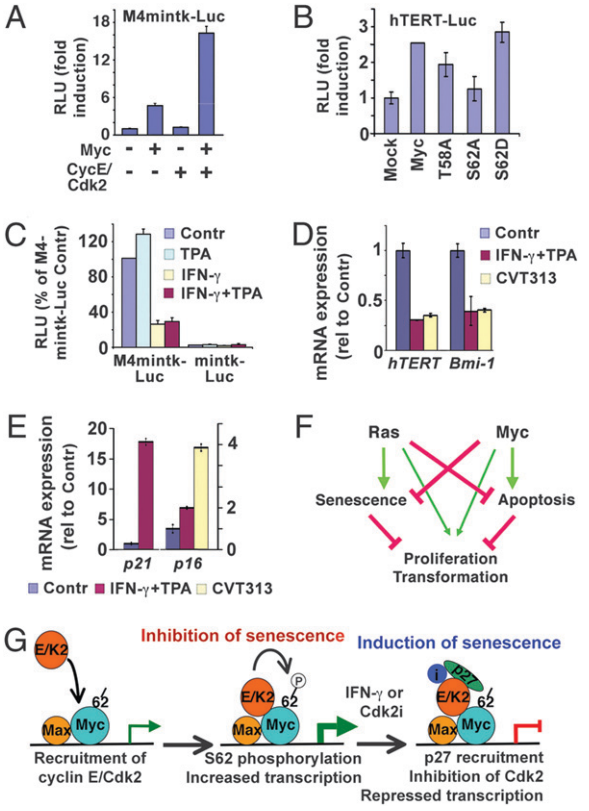

Fig. 6. Cyclin E/Cdk2 functions as a cofactor for Myc-driven transcription. (*A* and *B*) Luciferase reporter gene activities of M4mintk-Luc in U2OS cells (*A*) and of *hTERT*-Luc in REFs (*B*) after cotransfection with constructs expressing the indicated WT and mutant Myc and/or cyclin E/Cdk2. (*C*) M4mintk-Luc activity in U-937 cells after IFN-γ and/or TPA treatment. (*D* and *E*) mRNA expression of the indicated Myc target genes was analyzed by quantitative RT-PCR in U-937-myc-6 cells treated with IFN-γ + TPA (24 h) or CVT-313 Cdk2 inhibitor (48 h). Proposed models for the cooperativity between Myc and Ras in transformation (*F*) and for the regulation of senescence by Myc (*G*). E/K2, cyclin E/Cdk2. (See text and figure for further explanations.)

during terminal cell differentiation or in response to growth inhibitory signaling; to promote immortalization; and to control stem cell functions, including self-renewal (3).

Our findings further provide insight into the mechanism by which Myc represses senescence by revealing Ser-62 phosphorylation as a crucial step in this process and uncovering a unique role of cyclin E/Cdk2 as a Ser-62 kinase and transcriptional cofactor. In support of this conclusion, selective pharmacological inhibition of Cdk2 but not of other kinases abrogated repression of senescence by WT Myc. Importantly, a phospho-mimicking Myc-S62D mutant could still repress senescence under these conditions. Furthermore, we show that the cyclin E/Cdk2 coactivator function is regulated by growth inhibitory signaling, such as IFN-γ, leading to induction of p27, thereby turning cyclin E/Cdk2 off, which results in repression of transcription (Fig. 6*G*). Similar to our observation, Ser-62 phosphorylation by Erk was reported to enhance c-Myc recruitment to the γ-GCS promoter in response to oxidative stress (23). However, our data suggest that Cdk2 cannot be replaced by Cdk1 (7) or Erk (6, 23) to regulate Myc's function in senescence. The reason for this is presently unclear. Cyclin E/Cdk2 might carry out functions at Myc target promoters that cannot be performed by other Ser-62 kinases. This is an indication for additional substrates of cyclin E/Cdk2 and/or a more general cofactor function. However, we cannot rule out the possibility that Myc Ser-62 phosphorylation in combination with a function of cyclin E/Cdk2 unrelated to transcription is relevant for repression of oncogene-induced senescence.

Our results demonstrate that Ser-62 phosphorylation has a function in addition to its role in priming for Thr-58 phosphorylation by GSK3β (5). This is fully compatible with the latter function but suggests that the priming function is part of a negative feedback loop built into the system to be able to tune down the antisenescence function of Myc.

In addition to repression of Ras-induced senescence as observed here, Myc has been proposed to induce senescence under certain conditions (24, 25), as in the case of Ras (26), likely as a result of induced DNA damage (27, 28). Indeed, we observed an approximate 2-fold increase in the number of senescent REF cells in response to Myc (Fig. 1*B*), but Ras was a much more potent inducer of senescence, and under conditions of Ras activation, Myc repression of senescence predominated. Interestingly, Cdk2 suppresses senescence induced by Myc itself (24). It is at present unclear whether this mechanism is similar or distinct to the one described here; this would be a topic for future studies.

Although loss of Cdk2 and cyclin E can be compensated for by other Cdks and cyclins during murine embryonic development (29–31), our findings suggest that cyclin E/Cdk2 may have unique functions in the regulation of cellular senescence under conditions of oncogene deregulation not normally encountered during development. The observations that Cdk2$^{-/-}$ and cyclin E$^{-/-}$ MEFs are difficult to immortalize and that cyclin E$^{-/-}$ MEFs are not readily transformed by Myc together with Ras (29, 30) are compatible with our results. Indeed, Cdk2 is known to have a critical role in melanoma growth (32), and deregulated expression of cyclin E is observed in many cancers and correlates with poor outcome, for instance, in breast cancer (33). Of course this does not necessarily mean that cyclin E/Cdk2 is essential for all tumors or even for all Myc-driven tumors (34, 35), because it is possible that in addition to its role in senescence, other functions of Myc, including its stimulation of apoptosis, are rate-limiting for the development of some tumors. Goga et al. (36) recently reported that pharmacological inhibition of Cdk1 enhances Myc-induced

apoptosis by targeting the antiapoptotic protein survivin. Although both Cdk1 (7) and Cdk2 (this article) can function as Ser-62 kinases, their differences in activity during the cell cycle and in substrate specificity probably contribute to the cellular consequences of their inhibition. Thus, for therapeutical manipulations of Myc in the future, it might be important to try to define the most vulnerable aspect of Myc function in a given tumor.

The observation that IFN-γ-induced p27 acts as a strong Myc antagonist sheds light on the relation between Myc and p27 in cancer development. IFN-γ has been shown to induce growth arrest, promote differentiation, and repress Myc function also in MYCN-amplified human neuroblastoma cells (37). Because p27 is a target of the Skp2 E3 ligase, the previously defined coactivator function of Skp2 in Myc-driven transcription may relate to its role as a p27 antagonist (38). Expression of p27 is frequently downregulated in many types of cancers (39), and loss of p27 has been reported to synergize with Myc in murine lymphomagenesis (40). In addition, p27 is relocated into the cytoplasmic compartment in human tumors (19), and would therefore be unable to target Myc. Moreover, Mad1, a Myc antagonist, cooperates with p27 to promote granulocytic differentiation (41) and is repressed by cyclin E/Cdk2 (42). We recently showed that Mad1 is essential for TGF-β-induced cellular senescence in Myc-transformed U-937 cells (43). Taken together, these observations support a very close relation of the Myc/Max/Mad network with cyclin E/Cdk2/p27.

Our results therefore indicate that Cdk2 should be reevaluated as target for cancer therapy. Indeed, we show that Cdk2-selective pharmacological inhibitors push Myc-transformed cells into senescence, suggesting that inhibition of Cdk2, possibly in combination with Cdk1 inhibition, could potentially be a therapeutical principle for combating tumors with deregulated Myc or Ras. This research should be facilitated by the fact that Cdk2 inhibitors are already in clinical development.

Materials and Methods

Cells were cultured in DMEM (REF, HeLa, and U2OS) or RPMI-1640 (U-937) supplemented with 10% FCS and antibiotics. The U-937 clone myc-6 expresses the OK10 v-*myc* gene (17). U-937 cells (10^5 cells/mL) were treated with 1.6×10^{-8} M TPA (Sigma) and/or 100 U/mL IFN-γ (generously provided by G.R. Adolf, Ernst-Boehringer Institute, Vienna, Austria). Methods for gene transfer and for senescence, apoptosis, DNA synthesis, and kinase assays are given in *SI Materials and Methods*. Immunoprecipitation, Western blot, ChIP, and quantitative RT-PCR analyses were performed as described previously (17, 38). The antibodies, constructs, and primers used are listed in *SI Materials and Methods*. Kinase and protease inhibitors and concentrations used are also given in *SI Materials and Methods*.

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