Increased mitogenic signaling by positive effectors such as Ras or Myc can trigger senescence in normal cells, a response believed to function as a tumor-suppressive mechanism. We report here the existence of a checkpoint that monitors hypoproliferative signaling imbalances. Normal human fibroblasts with one copy of the c-myc gene inactivated by targeted homologous recombination switched with an increased frequency to a telomere-independent senescent state mediated by the cyclin-dependent kinase inhibitor p16INK4a. p16INK4a expression was regulated by the Polycumb group repressor Bmi-1, which we show is a direct transcriptional target of c-Myc. The Myc–Bmi circuit provides a mechanism for the conversion of environmental inputs that converge on c-Myc into discrete cell-fate decisions coupled to cell-cycle recruitment. A mechanism for limiting the proliferation of damaged or otherwise physiologically compromised cells would be expected to have important consequences on the generation of replicatively senescent cells during organismal aging.

M ost normal somatic cells possess a limited proliferative lifespan after which they enter into a state of terminal growth arrest known as replicative senescence. Telomere shortening is a well studied senescence trigger and is mediated primarily by a pathway involving the DNA damage sensor ataxia-telangiectasia mutated (ATM) kinase, the tumor suppressor p53, and the cyclin-dependent kinase inhibitor (CKI) p16INK4a. Telomere-independent senescence occurs in response to a variety of cellular stresses and signaling imbalances. For the most part, these pathways seem to involve the CKI p16INK4a (p16) and the retinoblastoma tumor suppressor (Rb) as the terminal effectors (3), but the events leading to the up-regulation of p16 are not well understood. The p16-Rb pathway has strong antiproliferative effects, and once engaged, seems to be irreversible (4). A well documented example of “premature” or “induced” senescence is hyperproliferative signaling elicited by activated Ras, which is believed to constitute a tumor-defense mechanism (5, 6). Whereas entry of a culture into senescence occurs gradually over many population doublings, at the single-cell level, both p16 and p21 are up-regulated with relatively rapid kinetics (1–2 days) (7, 8). Thus, p16-Rb responses are mixtures of senescent and proliferating cells, and the onset of senescence is determined by the frequency with which p16- and/or p21-positive cells are generated (4, 8, 9).

The c-Myc transcription factor can exert both activating and repressive effects by distinct biochemical mechanisms (10) and has recently been documented to regulate the expression of an unusually large number of target genes (11, 12). c-Myc activity is causally correlated with both accumulation of cell mass and cell division, and inappropriate activation is strongly tumorigenic (13). c-Myc sensitizes cells to apoptotic stimuli, and, in some contexts, its overexpression can induce senescence, both of which may constitute cancer defense mechanisms (5, 14, 15). Despite its central role in coordinating cellular metabolism and growth, the consequences of reduced c-Myc signaling on senescence mechanisms have not been investigated.

Results and Discussion

We used gene targeting to knock out one copy of c-myc in normal human diploid fibroblasts (HDF; Fig. 7A, which is published as supporting information on the PNAS web site). The strain of HDF used, LF1 (8), does not express other Myc family members (Fig. 8, which is published as supporting information on the PNAS web site). We obtained two targeted clones; the clone used for all subsequent experiments expressed ~50% less c-Myc mRNA as well as protein (Fig. 8 B and C). We introduced into the c-myc−/− cells a retrovirus vector expressing human telomerase reverse transcriptase (hTERT) to immortalize them. Although hTERT clearly extended their lifespan (Fig. 1A), several attempts with different vectors failed to elicit long term immortalization, whereas the same vectors readily immortalized c-myc−/− cells in parallel experiments.

To investigate the cause of the increased propensity for senescence, we examined the expression levels of p16, p21, and p14ARF (Arf). p21 and Arf mRNA levels were elevated ~2-fold in middle passage c-myc−/− cells relative to c-myc+/+ cells, whereas p16 expression was increased almost 4-fold (Fig. 1B). Late passage c-myc−/− cells expressing hTERT had further elevated p16 levels (7-fold), whereas, as expected, the presence of hTERT significantly reduced p21 levels. As previously noted (8), individual cells expressed either low (undetectable) or high levels of p16 protein, and the increased expression of p16 in c-myc−/− cells was characterized by the increased frequency of p16-positive cells (Fig. 9, which is published as supporting information on the PNAS web site). We proceeded to test the effects of reducing p16 or Arf expression in c-myc−/− cells by stably introducing short hairpin RNA (shRNA)-expressing retrovirus vectors. p16 mRNA levels were knocked down by 90% (Fig. 1C), the frequency of p16-positive cells was reduced from 60% to 15% (Fig. 1D and E), and cultures could be readily immortalized with hTERT (Fig. 1F). In contrast, Arf knockdown did not affect either proliferation or immortalization (data not shown).

We examined the promoter region of the Polycumb group (PcG) gene bmi-1, a known repressor of p16 transcription (16),
and found a canonical c-Myc binding site (E-box) at position −182 relative to the transcriptional start site. Quantitative real-time RT-PCR (qPCR) showed that Bmi-1 mRNA levels were reduced ~2-fold in c-myc−/− cells (Fig. 2A). To ascertain that this effect was not specific to the c-myc−/− cell strain, we acutely knocked down c-Myc mRNA expression by ~50% in normal HDF by using small interfering RNA (siRNA) oligonucleotides, and also found a 2-fold reduction in Bmi-1 expression 48 h after transfection (Fig. 2B). As expected, retrovirus-mediated overexpression of c-Myc in normal HDFs resulted in Bmi-1 mRNA induction (Fig. 2C).

To further test the mechanism by which reduced c-Myc activity leads to increased expression of p16, we knocked down c-Myc along with ectopically expressing Bmi-1. In the absence of ectopic Bmi-1, lentivirus-vector-expressed c-Myc shRNA elicited a 2-fold up-regulation of p16 mRNA within 3 days of infection. Ectopic Bmi-1 expression alone resulted in repression of p16 mRNA levels, which remained low after c-Myc knockdown (Fig. 2C). In all cases throughout this investigation, we observed a tight coupling between p16 expression at the mRNA and protein levels [the latter measured by immunohistochemistry (IHC)]. Finally, we demonstrated direct binding of c-Myc protein to the E-box in the bmi-1 promoter by chromatin immunoprecipitation (ChIP) analysis (Fig. 2D). We thus conclude that the bmi-1 gene is a direct transcriptional target of c-Myc.

To ascertain that the senescence of hTERT-expressing c-myc−/− cells was due to decreased expression of c-Myc, and hence Bmi-1, we reconstituted c-myc−/− cells with c-Myc and Bmi-1 in conjunction with hTERT in multiple combinations using retrovirus vectors (Fig. 3A). In all cases, we verified the ectopic expression of the c-myc and bmi-1 transgenes, and the presence of telomerase enzymatic activity, as appropriate (Fig. 10, which is published as supporting information on the PNAS web site). c-myc−/− cells expressing hTERT, c-Myc, or Bmi-1 alone soon senesced (Fig. 3B). In contrast, c-myc−/− cells expressing hTERT along with either c-Myc or Bmi-1 bypassed senescence and readily immortalized (Fig. 3C). The senescence of hTERT-expressing c-myc−/− fibroblasts can thus be rescued by c-Myc as well as by Bmi-1.

To investigate the generality of the c-Myc–Bmi-1–p16 regulatory pathway, we acutely knocked down c-Myc expression by using lentivirus-expressed c-Myc shRNA in a variety of primary human cells: BJ foreskin fibroblasts, IMR90 lung fibroblasts, and AG10770 endothelial cells (Fig. 4A and Figs. 11–13, which are published as supporting information on the PNAS web site). In all cases, down-regulation of c-Myc caused the down-regulation of Bmi-1 and the concomitant up-regulation of p16. Notably, in all cases, down-regulation of c-Myc caused the down-regulation of p16 protein at the single cell level was “all-or-none,” such that a decrease in c-Myc activity resulted in an increased frequency of p16-positive cells.

Increased p16 expression has been associated with aging in the mouse, and caloric restriction delays its up-regulation (17, 18). p16 is largely absent during embryogenesis but is up-regulated with age in many tissues at both the mRNA and protein levels. Given that c-Myc is not expressed in nonproliferating cells, its absence cannot be the sole switch for turning on p16. Indeed, quiescence induced by serum withdrawal or contact inhibition in either primary human fibroblasts or endothelial cells does not result in the up-regulation of p16, although in all cases c-Myc is strongly down-regulated (data not shown). We hypothesized that, similar to well documented Ras-induced senescence (6), the Myc–Bmi–p16 circuit may function to monitor signaling imbalances, except that, in this case, the purpose would be to sense hypoproliferative effects.
One prediction of this hypothesis is that the p16-inducing effects of hypoactive c-Myc signaling would require cell-cycle recruitment. We used a lentivirus vector to introduce c-Myc shRNA into contact-inhibited AG10770 endothelial cells, scratch-wounded the monolayers to allow migration into the denuded area and cell cycle entry, and monitored p16 expression at the single-cell level (Fig. 4 B and C). Although expression of the shRNA had a marginal, if any, effect on the monolayer, the frequency of p16-positive cells was significantly increased at the wound edge (Fig. 5 A). Moreover, scratch-wounding of contact-inhibited, H2O2-treated AG10770 monolayers resulted in an increased frequency of p16-positive cells at the wound edge (Fig. 5 B and C). Mock-treated control cells did not up-regulate p16 in response to wounding.

Previous studies reported that c-Myc overexpression in normal HDFs induces p16 expression (5, 15), which we confirmed (Fig. 14.4, which is published as supporting information on the PNAS web site). Because c-Myc seems to act only as a positive effector of Bmi-1, we further investigated its biphasic regulation of p16. None of the known transcriptional regulators of p16 were affected by c-Myc overexpression (Fig. 1A). The p16 promoter, however, contains two canonical E-boxes: one at −1156 and another at +1315 relative to the transcriptional start site. ChIP revealed no apparent occupancy of these sites in normal HDF, but binding became apparent (especially to the intronic site) upon c-Myc overexpression (Fig. 6). Our findings thus indicate that c-Myc does not regulate p16 in its physiological range of expression, but both hypo- and hyper-active c-Myc signaling is inducing: the former by an indirect physiological range of expression, but both hypo- and hyper-active c-Myc signaling is inducing: the former by an indirect effect on the p16 promoter.

Bmi-1 is the mammalian ortholog of Drosophila Posterior sex combs (Psc), a member of the PcG transcriptional silencers that act as multiprotein complexes to control chromatin accessibility.
Bmi-1, together with Polycomb (Pc) and Polyhomeotic (Ph) form the core of the Polycomb Repressive Complex 1 (PRC1), which binds to chromatin and directly antagonizes the ATP-dependent remodeling of nucleosome arrays by the SWI/SNF complex (20). In addition, PRC1 interacts with the Enhancer of zeste [E(z)] and Extra sex combs (Esc) complex, which contains histone deacetylase activity.

Bmi-1 is down-regulated during senescence of HDF (9). Bmi-1−/− mouse embryonic fibroblasts (MEF) express elevated levels of p16 and Arf and undergo premature senescence (16), and expression of dominant-defective Bmi-1 shortens the replicative lifespan of HDF (9). Bmi-1 overexpression results in reduced levels of p16 and Arf. Myc cooperates with Bmi-1 in promoting murine lymphomas (21). This cooperation involves the transcriptional activation of bmi-1 by proviral insertion and the consequent repression of p16 and Arf, which is believed to antagonize the growth-inhibitory and proapoptotic effects of Myc overexpression (22). However, a direct regulatory interaction between c-Myc and bmi-1 has not been hitherto appreciated.

The role of PcG is the maintenance of established gene expression states to achieve an epigenetic memory of cell identity. The initial signals that determine transcriptional patterns may be transient, but the resulting differentiation states are long-lived. Dividing cells must preserve epigenetic memory in the face of disruptions such as DNA replication or mitosis, where regulatory factors may be disassembled from promoters. PcG is thus also involved in the competence for switching (23), with every cell-cycle transition providing an opportunity to either maintain the repressed state or to switch to a derepressed state. We propose that decreased expression of Bmi-1, caused by...
reduced c-Myc expression, increases the probability of a cell switching from a p16-off to a p16-on state, and that this switch necessitates cell cycle entry and progress. The Myc–Bmi circuit thus provides a mechanism for the conversion of environmental inputs that converge on c-Myc into discrete cell fate decisions. In addition, a hyposignaling checkpoint provides a plausible explanation to link the diverse “culture-shock” senescence phenomena (3) with the up-regulation of p16 during organismal aging.

Materials and Methods

Cell Culture. LF1 is an embryonic lung HDF cell strain (8). HDF cell strains BJ and IMR90 were obtained from W. Hahn (Dana-Farber Cancer Institute, Boston) and the American Type Culture Collection, respectively. Venous endothelial cells AG10770A, isolated from a normal 21-year-old female, were obtained from the Aging Cell Culture Repository of the National Institute on Aging. Culture conditions were as follows: for LF1 and BJ, Ham's F10 nutrient mixture, 15% FBS; for IMR90, DMEM, 10% FBS; for AG10770A, gelatin-coated plates, Medium 199, 15% FBS, 0.02 mg/ml heparin. All media were supplemented with glutamine (2 mM) and penicillin/streptomycin. Incubation was at 37°C in an atmosphere of 93% N2, 5% CO2, and 2% O2.

Viral Vectors. pBABE and pWZL vectors were packaged in Amphotropic Phoenix packaging cells (24) and 293T cells were inactivated FBS. The ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) was used according to the supplier’s instructions. Immunoprecipitations were performed on 4 ml of lysate by using 8 μg of the indicated antibody and 160 μl of protein A beads. Eluted DNA was resuspended in 30 μl of 0.1× TE (1 mM Tris/0.1 mM EDTA, pH 8.0), and qPCR was performed in duplicate by using 2 μl of DNA in a 50-μl SYBR green reaction (Applied Biosystems). All reactions were performed in triplicate; actin or glyceraldehyde phosphate dehydrogenase (GAPDH) were used as internal standards.

ChIP. The ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) was used according to the supplier’s instructions. Approximately 4×106 HDF cells were used per assay. Immunoprecipitations were performed on 4 ml of lysate by using 8 μg of the indicated antibody and 160 μl of protein A beads. Eluted DNA was resuspended in 30 μl of 0.1× TE (1 mM Tris/0.1 mM EDTA, pH 8.0), and qPCR was performed in duplicate by using 2 μl of DNA in a 50-μl SYBR green reaction (Applied Biosystems). Data are expressed as fold-enrichments relative to the 0 h or empty vector controls. E-box primers: bmi-1 promoter, CTACCCGACACTAATTCCCGG, ACGTGTCCTCCCTCAT-TCTT; p16 promoter, CTGAGTAGCTGGAATTACA-CACGGT, GTGAGGTGTCGCGCCATGGT, p16 intron, GTTACATGCAGTGAAAGCCA, ATACATTATGG.

Transient RNA Interference (RNAi). c-Myc mRNA knockdowns were performed by using the SMARTpool c-myc siRNA and control nonspecific siRNA oligonucleotides (Dharmacon Research, Lafayette, CO). HDF cells were transfected by using RNAiFect (Qiagen, Valencia, CA), and RNA was harvested 48 h after transfection. Knockdown of mRNA levels was assayed by qPCR.

Immunological Procedures. Immunoblotting (25) and p16 immunohistochemistry (8) were performed as described. Antibodies were as follows: anti-HA tag (MMS-101P; Covance, Richmond, CA); anti-actin (N-350; Amersham Pharmacia); p16 (ab24190), 05-340 (Upstate Biotechnology); anti-actin (N-350; Amersham Pharmacia); p16 (ab24190). We thank W. Hahn, R. Agami (Netherlands Cancer Institute, Amsterdam), and J. Campisi for vectors and other reagents. We thank S. McMahan for communicating unpublished information. This work was supported by National Institutes of Health (NIH) Grants R01 GM41690 and R01 AG16694 (to J.M.S.). Core facilities used throughout this work were supported in part by Center of Biomedical Research Excellence (COBRE) Award P20 RR15578 from the NIH.

FIGURE 7

A

\[\text{c-myc} \quad \text{exon1} \quad \text{exon2} \quad \text{exon3} \]

\[\text{SwaI} \quad \text{ATG} \quad \text{TAA} \quad \text{XbaI} \]

\[\text{vector} \quad \text{EYFP} \quad \text{IRE} \quad \text{Neo} \quad \text{pA} \quad \text{exon3} \]

B

\[\text{Myc expr.} \quad \text{mRNA abundance} \]

\[\begin{array}{c}
\text{Cells: myc}^{+/+} \\
\text{myc}^{+/–} \\
\end{array} \]

C

\[\text{myc}^{+/+} \quad \text{myc}^{+/–} \]

\[\text{Myc} \quad \text{DAPI} \]
FIGURE 8

A

N-Myc

relative mRNA abundance

Y79  NCI-H378  LF-1

B

L-Myc

relative mRNA abundance

Y79  NCI-H378  LF-1

C

c-Myc

relative mRNA abundance

Y79  NCI-H378  LF-1
FIGURE 9

\textbf{myc}^{++} \quad \textbf{myc}^{+-}

![cell images](image-url)
**FIGURE 10**

**A**

![Graph showing relative telomerase activity](image)

**KEY:**
- **BP:** pBABE-puro
- **WB:** pWZL-blast
- **BH:** pBABE-hygro
- **EV:** empty vector

**B**

![HA and actin Western blots](image)

**C**

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FIGURE 11

A

p16 IHC

Empty vect.  Myc shRNA

B

p16 IHC

% positive cells

Empty vect.  Myc shRNA
FIGURE 12

A

![Bar graph showing mRNA abundance for c-Myc shRNA](image)

B

![p16 IHC images](image)

C

![p16 IHC percentage positive cells](image)
FIGURE 13

A

c-Myc shRNA

mRNA abundance

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B

p16 IHC

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C

p16 IHC

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FIGURE 14

A

\begin{center}
\includegraphics[width=\textwidth]{c-Myc_overexpression}
\end{center}

B

\begin{center}
\includegraphics[width=\textwidth]{mRNA_abundance}
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