Reduced c-Myc signaling triggers telomere-independent senescence by regulating Bmi-1 and p16INK4a

Isil Guney*, Shirley Wu†, and John M. Sedivy‡

Department of Molecular Biology, Cell Biology and Biochemistry, and Center for Genomics and Proteomics, Brown University, 70 Ship Street, Providence, RI 02903

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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-suppressor 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 Polycomb 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 repensively senescent cells during organismal aging.

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-suppressor 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 Polycomb 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 repensively senescent cells during organismal aging.

Most 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 by the tumor-suppressing 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 Polycomb 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 repensively senescent cells during organismal aging.

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. 1 D 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 Polycomb group (PcG) gene bmi-1, a known repressor of p16 transcription (16), by regulating Bmi-1 and p16INK4a.
Finally, we demonstrated direct binding of c-Myc protein to the E-box in the bmi-1 gene (16). 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–p16 regulatory circuit, 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. 4 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 Bmi-1 and p16. We hypothesized 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. 2A). 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.
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. Cells infected with a control empty virus did not up-regulate p16 in response to wounding. Pull downs were quantified by qPCR.

One case where a hyposignaling checkpoint could be of clear relevance would be to prevent cell cycle recruitment of damaged or otherwise physiologically compromised cells. Our recent understanding of c-Myc’s function as an integrator and regulator of metabolism, mass accumulation, and cell division would make it a prime candidate for such a surveillance function. Indeed, recent reports indicate that cell division makes cells more prone to senescence (19). To investigate the effects of a stress associated with aging on the Myc–Bmi–p16 circuit, we treated contact-inhibited AG10770 cells with low, sublethal concentrations of the oxidant H2O2, and subsequently trypsinized and replated the cells at subconfluent density to promote cell-cycle entry. qPCR showed that H2O2 treatment resulted in reduced c-Myc and Bmi-1 mRNA levels within 3 h of cell cycle entry (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 A, 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. 14 B). 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 hyperactive c-Myc signaling is inducing: the former by an indirect effect on the p16 promoter site, (in primers) to the bim-1 promoter (Bim-1), primers to a known Bmi-1 promoter. ChIP was performed by using LF1 HDF cells, either serum-deprived to turn off c-Myc expression (0 h), or treated with c-Myc or nonspecific siRNAs, RNA was extracted 48 h after transfection, and c-Myc and Bmi-1 mRNA levels were quantified by qPCR. (C) Ectopic expression of Bmi-1 prevents the up-regulation of p16 elicited by c-Myc knockdown. Mid-passage c-myc (LF1) cells were infected with pBabe-puro expressing Bmi-1 (BP-Bmi) or empty vector (BP). Drug-resistant pools of cells were subsequently infected with c-Myc shRNA-expressing lentivirus (shMyc) or empty vector control (EV). RNA was extracted 3 days after infection, and p16 mRNA levels were quantified by qPCR. The c-Myc shRNA used resulted in ~70% knockdown of c-Myc mRNA. (D) c-Myc binds directly to the bim-1 promoter. ChIP was performed by using LF1 HDF cells, either serum-deprived to turn off c-Myc expression (0 h), or serum-stimulated to induce c-Myc expression (4 h). Immunoprecipitating antibodies (IP-Ab) were against c-Myc, and GST as a negative control. In addition to primers (Prim) to the bim-1 promoter (Bmi-1), primers to a known c-Myc target (nucleolin) and to a promoter without E-boxes (No E-box) were used as positive and negative controls, respectively (29). Pull downs were quantified by qPCR.

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Psc/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−/− mice 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 progression. 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. L1F1 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, gelatin-coated plates, Me) and BJ, Ham’s F10 nutrient mixture, 15% FBS; for IMR90, obtained from the Aging Cell Culture Repository of the National Culture Collection, respectively. Venous endothelial cells (Dana-Farber Cancer Institute, Boston) and the American Type cell strains BJ and IMR90 were obtained from W. Hahn. Packaging was in 293T cells by using helper vectors (26, 27). c-Myc shRNA (ATGTCAAGAGGCGAACATGGTGCGCAGGTTC) were expressed in the pRetroSuper vector (GTGCTCGGAGTTAATAGCA) and Arf shRNA (GAA-500; Abcam, Inc., Cambridge, MA); anti-c-Myc (06-340; Upstate Biotechnology, Lake Placid, NY) was used according to the supplier’s instructions. Antibodies were as follows: anti-HA tag (MMS-101P; Covance, Richmond, CA); anti-actin (N-350; Amersham Pharmacia); p16 (ab2419-500; Abcam, Inc., Cambridge, MA); anti-c-Myc (06-340; Upstate Biotechnology); anti-GST (PC53; Calbiochem); anti-Bmi-1 (05-637; Upstate Biotechnology).

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FIGURE 7

A diagram showing the genetic construct with exons and restriction sites.

B Graph comparing Myc expression levels between myc+/+ and myc+/– cells.

C Image showing the localization of Myc and DAPI staining in myc+/+ and myc+/– cells.
FIGURE 8

A. N-Myc

B. L-Myc

C. c-Myc
FIGURE 9

myc\(^{++}\)  

myc\(^{+/-}\)
FIGURE 10

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

A

![Bar chart showing relative telomerase activity for different genotypes.]

- (+) control
- myc+/+
- myc+/
- myc+/-/WB-Ev
- myc+/-/WB-myc/BP-tert

B

![Western blot images showing HA and actin expression for different genotypes.]

- myc+/+
- myc+/
- myc+/-/WB-Ev
- myc+/-/WB-myc/BP-tert

C

![Immunostaining images for Bmi and DAPI for different genotypes.]

- myc+/+
- myc+/
- myc+/-/WB-myc/BP-tert
- myc+/-/BH-bmi/BP-tert

pBABE-puro
pWZL-blast
pBABE-hygro
empty vector
A

p16 IHC

Empty vect.  Myc shRNA

B

p16 IHC

% positive cells

Empty vect.  Myc shRNA
FIGURE 12

A

**c-Myc shRNA**

<table>
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<th>mRNA abundance</th>
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<tr>
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<tr>
<td></td>
<td>Myc shRNA</td>
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<tr>
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<td></td>
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<tr>
<td></td>
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B

**p16 IHC**

Empty vect. | Myc shRNA

C

**p16 IHC**

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<td>Myc shRNA</td>
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</table>

FIGURE 12

A

**c-Myc shRNA**

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<tr>
<td>p16</td>
<td>Empty vect.</td>
</tr>
<tr>
<td></td>
<td>Myc shRNA</td>
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B

**p16 IHC**

Empty vect. | Myc shRNA

C

**p16 IHC**

<table>
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<tr>
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<td>Myc shRNA</td>
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</table>
FIGURE 13

A

**c-Myc shRNA**

<table>
<thead>
<tr>
<th>mRNA abundance</th>
<th>Myc</th>
<th>Bmi-1</th>
<th>p16</th>
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<tr>
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<td>1</td>
<td>2</td>
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B

**p16 IHC**

Empty vect.  
Myc shRNA

C

**p16 IHC**

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<th>Myc shRNA</th>
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<tr>
<td></td>
<td>10</td>
<td>50</td>
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FIGURE 14