Overexpression of *MYC* causes *p53*-dependent G_2 arrest of normal fibroblasts

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Overexpression of the proto-oncogene *MYC* has been implicated in the genesis of diverse human cancers. One explanation for the role of *MYC* in tumorigenesis has been that this gene might drive cells inappropriately through the division cycle, leading to the relentless proliferation characteristic of the neoplastic phenotype. Herein, we report that the overexpression of *MYC* alone cannot sustain the division cycle of normal cells but instead leads to their arrest in G_2. We used an inducible form of the *MYC* protein to stimulate normal human and rodent fibroblasts. The stimulated cells passed through G_1 and S but arrested in G_2 and frequently became aneuploid, presumably as a result of inappropriate reinitiation of DNA synthesis. Absence of the tumor suppressor gene *p53* or its downstream effector *p21* reduced the frequency of both G_2 arrest and aneuploidy, apparently by compromising the G_2 checkpoint control. Thus, relaxation of the G_2 checkpoint may be an essential early event in tumorigenesis by *MYC*. The loss of *p53* function seems to be one mechanism by which this relaxation commonly occurs. These findings dramatize how multiple genetic events can collaborate to produce neoplastic cells.

The *MYC* proto-oncogene encodes a transcription factor whose activity has been implicated in diverse cellular functions, including proliferation, differentiation, and apoptosis (1–3). Overexpression of *MYC* has been found in numerous human tumors and is thought to play a role in tumorigenesis (1). Excessive activity of *MYC* might contribute to tumorigenesis in at least three ways: by driving cells inappropriately through the division cycle (1, 2), by creating a mutator phenotype consequent to destabilization of the cellular genome (4–8), and by impeding cellular differentiation (1, 2).

The effect of *MYC* on the cell-division cycle has been examined mainly in established lines of rodent cells, which are readily transformed by oncogenes and presumably carry multiple genetic lesions. In this study, we examined the effect of *MYC* on the division cycle of normal rodent and human cells. To perform these studies, we used a molecular construct in which *MYC* is fused to the hormone-binding domain of the human estrogen receptor (*MYCER*; ref. 9). The chimeric gene product is active only in the presence of estradiol (E2) or hydroxytamoxifen.

Stimulation with *MYC* caused normal rodent and human cells to traverse the G_1 and S phases of the division cycle, but the cells then arrested in G_2 and frequently became aneuploid, apparently as a result of endoreduplication. Absence of the tumor suppressor protein p53 or its downstream effector p21 reduced the frequency of both G_2 arrest and aneuploidy, presumably by compromising the G_2 checkpoint. Thus, relaxation of the G_2 checkpoint may be an essential early event in tumorigenesis by *MYC*. Loss of p53 function is among the most common genetic lesions in cancer cells and represents a means by which such relaxation could occur (10, 11).

Methods

Cell Culture. Normal human fibroblasts (NHF) were derived from newborn foreskin. Mouse embryonic fibroblasts (MEF) were isolated by using conventional techniques from day 14 embryos. Cells were cultured in DMEM supplemented with 10% (vol/vol) FCS and penicillin/streptomycin. Fibroblasts were infected with preparations of the pBABE-puro retrovirus containing *MYCER* as described (7).

Induction of *MYC* Activity. NHF or MEF with *MYCER* were treated with E2 at a concentration of 1 μM prepared as a 1,000× stock in 100% (vol/vol) ethanol.

Proliferation Assays. Cells were plated into 6-well tissue culture plates in tissue culture medium in the absence or presence of E2 (2×10^3). At each time point, wells in triplicate were trypsinized, and the live cells were enumerated by counting the number of Trypan-blue-negative cells.

Cell Death. Apoptosis was measured by video microscopy as described (12). At least 25 cells were examined per experiment for 60 h in the absence or presence of E2. The cumulative total numbers of cells, numbers of mitoses, and numbers of apoptoses were measured.

DNA Content. Fluorescence-activated cell sorting analysis of propidium-iodide-stained cells was performed as described (7). Quantitative image analysis of Feulgen-stained cells was performed by plating cells into 4-chamber tissue culture slides (Tissue Tek, Becton Dickinson) in growth medium in the absence or presence of E2 for 2 days. In some cases, cells were first synchronized in G_0 by putting the cells in medium containing low serum (0.05%) for 2 days. Cells were fixed in 10% (vol/vol) formalin for 2 h and then stained with Feulgen. Nuclear Feulgen staining was measured in at least 100 cells at a wavelength of 550 nm (AHRENS system, Bargteheide, Hamburg, Germany).

Results

Excess *MYC* Causes Proliferative Arrest in Normal Cells. We overexpressed *MYCER* in NHF and MEF as described (7). Activation of *MYCER* with E2 immediately suppressed the proliferation of normal cells that had been proliferating asynchronously, whereas *MYCER* in the absence of E2 had no effect (Fig. 1 *a* and *b*). In an additional measure of proliferation, cloning efficiency of NHF was reduced by the sustained activation of *MYCER* (Fig. 1*d*). Transient activation of the fusion protein reduced the proliferation of normal cells (data not shown) but did not reduce their cloning efficiency (Fig. 1*e*). Thus, the inhibition of cellular proliferation by *MYCER* seems to be reversible.

Under certain circumstances, the activation of *MYC* can lead

Abbreviations: E2, 17-β-estradiol; NHF, normal human fibroblasts; MEF, mouse embryonic fibroblasts.

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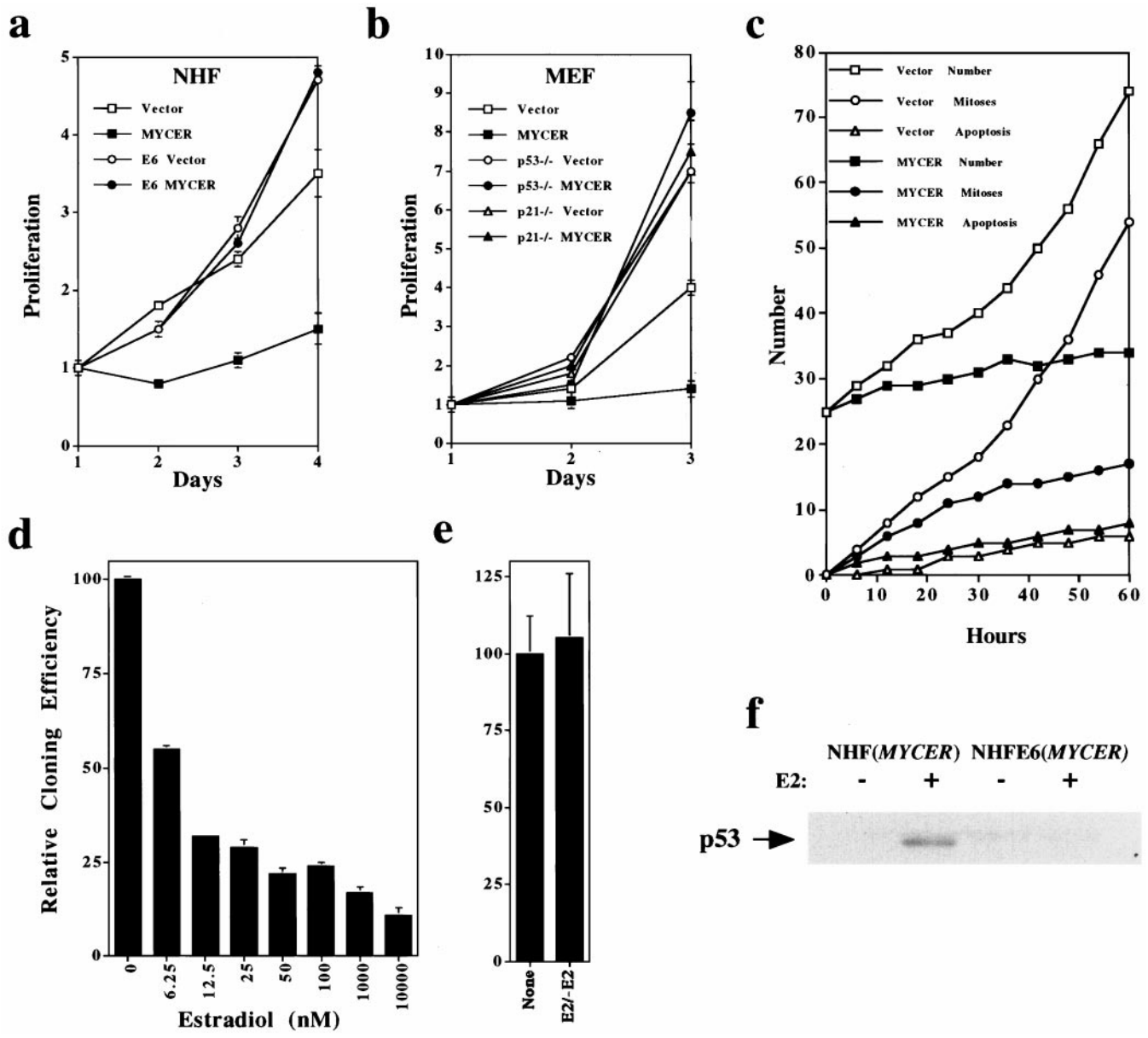

Fig. 1. Excess of *MYC* activity inhibits the proliferation of NHF and MEF. To activate *MYC* activity conditionally, NHF, NHF-E6, or MEF infected with the retroviral vector alone (vector) or the retroviral vector containing *MYCER* were treated with E2 (1 μM or as otherwise specified). (*a*) Proliferation of NHF or NHF-E6 in the absence (Vector) or presence (MYCER) of excess of *MYC* activity. (*b*) Proliferation of MEF that are wild-type, *p53*$-/-$, or *p21*$-/-$ in the absence (Vector) or presence (MYCER) of excess activity of *MYC*. For *a* and *b*, proliferation is expressed as the number of cells counted after a given time in culture, divided by the initial number of cells plated. (*c*) Video microscopy of NHF in the absence (Vector) or presence (MYCER) of excess of *MYC* activity. NHF ($n = 25$) with vector or *MYCER* were followed for 60 h after E2 treatment and examined for cumulative total number of cells, mitoses, and apoptosis. (*d*) Cloning efficiency of NHF in the presence of increasing levels of activity of *MYC*. NHF ($n = 200$) with *MYCER* were plated into 10-cm tissue culture plates and exposed to the indicated concentrations of E2. Colonies of over 50 cells were counted after 1 month of *in vitro* culture. (*e*) Cloning efficiency after 2 days of E2 NHF or NHF after 2 days of excess of *MYC* activity was measured by plating cells into 10-cm tissue culture plates in the absence of E2; colonies of over 50 cells were counted after 1 month. (*f*) *p53* protein expression in NHF or NHF-E6 in the absence ($-$) or presence ($+$) of excess activity of *MYC*. Protein was measured by Western analysis with the monoclonal antibody pAB421 (Calbiochem). (*a*–*e*) Samples were obtained in triplicate. Mean values $\pm$ SD are shown. One of at least three experiments is shown.

to cellular apoptosis (3). Therefore, we examined the possibility that apoptosis might account for the failure of NHF and MEF to proliferate in response to activated *MYCER*. We examined cells for apoptosis by phase microscopy, trypan blue exclusion, fluorescence-activated cell sorting analysis, and video microscopy. We did not detect appreciable apoptosis of either NHF or MEF in response to activation of *MYCER* (data not shown and Fig. 1*c*). By video microscopy, we observed that, on activation of *MYCER*, the number of mitoses did increase during the first 24 h, but cells did not divide. We conclude that *MYCER* arrests the cell cycle by preventing the completion of cellular division.

Excess *MYC* Causes Cell-Cycle Arrest in G_2. We analyzed at what point the cells had arrested in the cell cycle by measuring their DNA content by fluorescence-activated cell sorting analysis of propidium-iodide-stained cells (data not shown) or by quantitative image analysis of Feulgen-stained cells (Fig. 2). Under normal growth conditions, greater than 80% of the NHF were

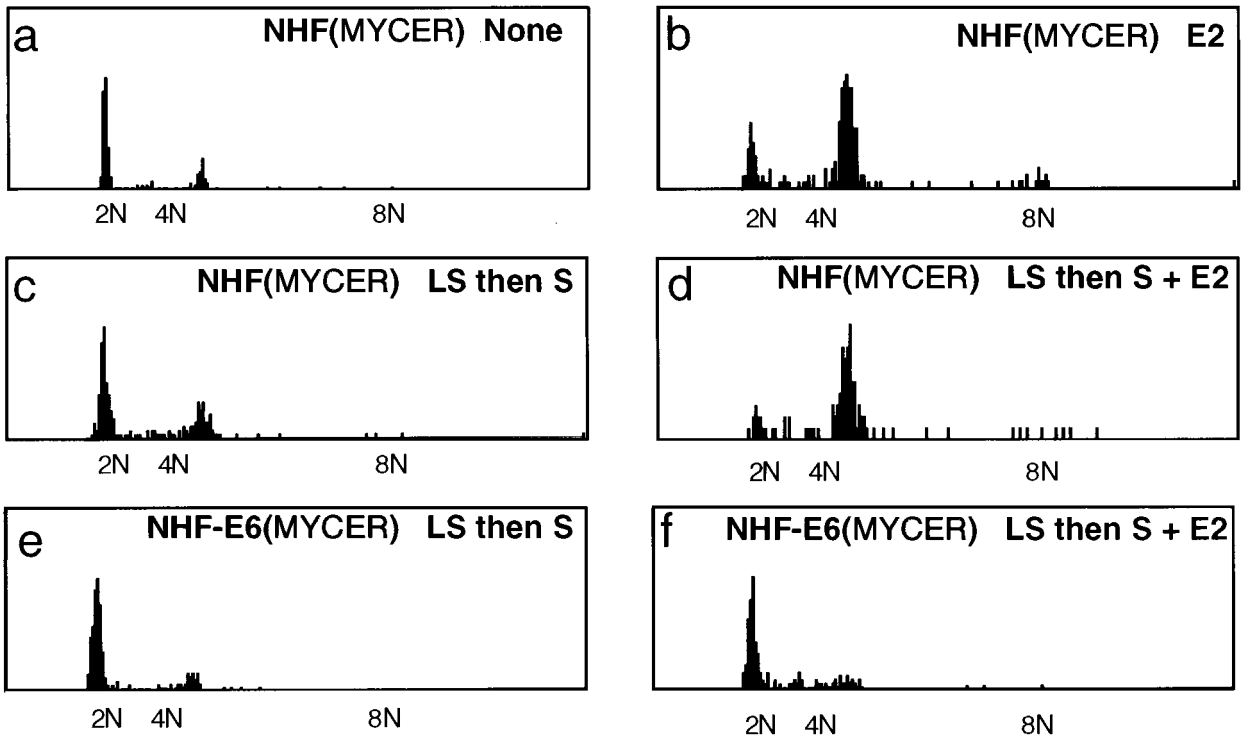

Fig. 2. Excess of *MYC* activity causes G_2 arrest. NHF or NHF-E6 containing *MYCER* were either not treated or treated with E2 (1 μM) for 2 days. As outlined below, in some cases, NHF were first synchronized in low serum (0.05%) for 2 days (LS) and then treated with serum (10% vol/vol) (S) or S and E2. The histograms display DNA content as measured by quantitative image analysis of Feulgen-stained preparations of 10% (vol/vol) formalin-fixed cells. (*a*) Untreated NHF with *MYCER*. (*b*) E2-treated NHF with *MYCER*. (*c*) NHF with *MYCER* treated with LS and then S. (*d*) NHF with *MYCER* treated with LS and then S and E2. (*e*) NHF-E6 with *MYCER* treated with LS and then S. (*f*) NHF-E6 with *MYCER* treated with LS and then S and E2. Representative data are shown from one of at least five experiments. Identical results were obtained by measuring DNA content by fluorescence-activated cell sorting analysis of propidium-iodide-stained cells.

in G_1 (Fig. 2*a*). In contrast, over 70% of the cells were in G_2 after 2 days of sustained activation of *MYCER* (Fig. 2*b*).

Because *MYC* is thought to stimulate entry into the cell cycle, we speculated that the activation of *MYC* may have greater consequences if cells were synchronized in G_0 through serum starvation. Indeed, we found that when cells were first synchronized in G_0, the concurrent addition of serum and activation of *MYCER* now resulted in over 90% of the cells accumulating in G_2 (Fig. 2*d*). Thus, cells in G_0 may be more sensitive to the effects of *MYC* activity. Note that the serum treatment alone of cells previously synchronized in G_0 in low serum for 2 days resulted in less than 20% of cells accumulating in G_2 (Fig. 2*c*). We conclude that *MYC* caused cells to arrest in G_2.

Arrest in G_2 Is *p53*-Dependent. We presumed that the arrest in G_2 could be attributed to activation of a checkpoint control (13). The tumor suppressor protein *p53* can play a role in implementing the G_2 checkpoint (14–16). We found that the activation of *MYCER* in NHF led to an increase in the amount of *p53* (Fig. 1*f*).

We pursued the role of *p53* in the G_2 arrest by expressing the E6 oncogene of the human papilloma virus in NHF. The E6 protein facilitates the proteolytic destruction of *p53* (17) and, accordingly, reduced the amounts of *p53* in NHF, even when *MYCER* had been activated (Fig. 1*f*). The absence of *p53* allowed NHF to proliferate normally even when *MYCER* had been activated with E2 (Fig. 1*a*). Similarly, activation of *MYCER* had no effect on the proliferation of MEF that were genetically deficient in either *p53* or the cell-cycle kinase inhibitor *p21*, which is a downstream effector of *p53* (ref. 17; Fig. 1*b*). Accordingly, activation of *MYCER* in the *p53*-deficient cells did not cause an abnormal accumulation of cells in G_2 (Fig. 2 *d* versus *f*). We conclude that *p53* is involved in the implementation of the G_2 arrest elicited by the activation of *MYCER*.

Excess *MYC* Causes Aneuploidy. Overexpression of *MYC* causes aneuploidy (4–8). Our results described above suggest a possible mechanism. *MYC* seems to cause cells to arrest in G_2 and then may cause the reinitiation of DNA replication, resulting in endoreduplication. If this supposition were the case, we predicted that if we prevented cells from arresting in G_2, then we would reduce the ability of *MYC* to cause aneuploidy.

Indeed, in NHF deficient in p53, the activation of *MYC* failed to arrest the cells in G_2 (as described above), and the frequency of aneuploidy was reduced by 10-fold compared with NHF with intact p53 (Fig. 2 *d* versus *f*; see below Fig. 4*a*). However, when we examined mitotic cells, we found that *MYC* caused a comparable frequency of aneuploidy in NHF intact or deficient in p53 (Figs. 3 and 4*a*). The frequency of aneuploidy among cells in G_2 was also similar regardless of the status of p53 (Fig. 4*a*). We infer that the absence of p53, rather than preventing *MYC* from causing aneuploidy, reduces the pool of cells in G_2 that could become aneuploid. In fact, the frequency of aneuploidy caused by *MYC* correlated with the number of cells that were in G_2 (Fig. 4*b*).

Discussion

Excess Activation of *MYC* Prohibits Proliferation. The overexpression of *MYC* is generally thought to cause tumorigenesis by constitutively promoting cellular proliferation (1, 2). Our results demonstrate that, instead, excess *MYC* activation causes the proliferative arrest of NHF and MEF. The overexpression of *MYC* cannot of itself elicit neoplastic proliferation of otherwise

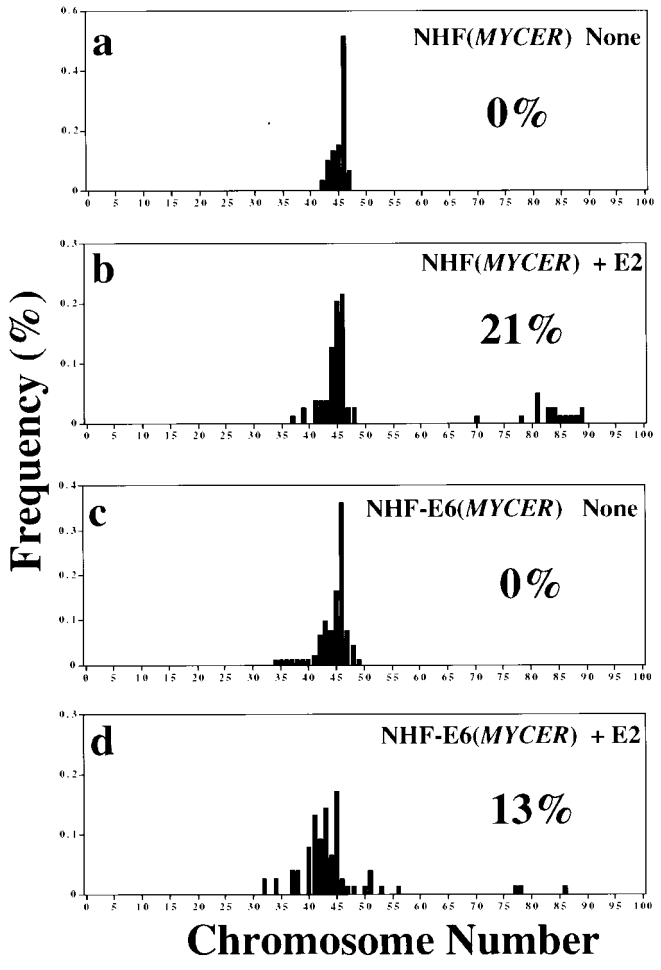

Fig. 3. Aneuploidy caused by excess of *MYC* activity. To activate *MYC* conditionally, NHF or NHF-E6 with *MYCER* were not treated or treated with E2 (1 μM) for 2 days. In each panel, the percentage of aneuploid metaphase spreads with >50 chromosomes is indicated. The histograms reflect the percentage of metaphases found to have a particular number of chromosomes. Metaphases were prepared and analyzed as described (7). (*a*) NHF with *MYCER* that were not treated with E2. (*b*) NHF with *MYCER* that were treated with E2. (*c*) NHF-E6 *MYCER* that were not treated with E2. (*d*) NHF-E6 with *MYCER* that were treated with E2.

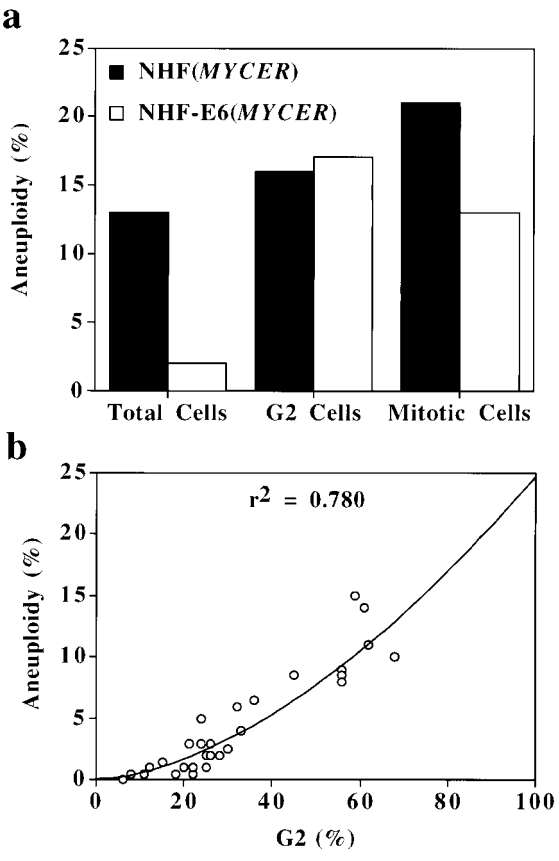

Fig. 4. Aneuploidy caused by excess of *MYC* activity seems to require G_2 arrest. To activate *MYC*, NHF with *MYCER* or NHF-E6 with *MYCER* were treated with E2 for 2 days. Aneuploidy was measured as the percentage of the cells with a >4 N DNA content (Total Cells), the percentage of cells with a >4 N DNA content divided by the number of cells in G_2 (G_2 Cells), or the percentage of metaphases with >50 chromosomes (Mitotic Cells). (*a*) Aneuploidy caused by *MYC* in NHF or NHF-E6. (*b*) Aneuploidy versus the number of cells in G_2.

normal cells (1). Our results provide a possible explanation. When a normal cell undergoes a genetic event that causes the overexpression of *MYC*, the cell may be incapable of further proliferation.

Normal cells express *MYC* when they transit the cell-division cycle (1, 2). Indeed, *MYC* has been shown to induce cells to proliferate (1, 2). A possible explanation for the discordance with the results that we report herein is that physiological levels of *MYC* promote cellular proliferation, whereas high levels of *MYC* activation cause proliferative arrest. This arrest may not have been appreciated previously in experiments in which *MYC* was constitutively overexpressed, because cells that expressed high levels of *MYC* would be incapable of proliferating.

Substantial evidence suggests that *MYC* may be restrained from causing the malignant transformation of cells, because *MYC* activation induces apoptosis (3). Our results suggest that the activation of *MYC* in NHF and MEF causes a proliferative arrest without augmented apoptosis. Whether the activation of *MYC* causes proliferative arrest or induces apoptosis may depend on the particular cellular lineage, the differentiative state, and the mitogenic stimulation provided by the local microenvironment (16). In this regard, it is notable that the conditional activation of *MYC in vivo* was not observed to induce apoptosis in the skin (18).

Several reports document that *MYC* induces telomerase activity, which in turn may contribute to cellular immortalization (19–21). Our results suggest that although *MYC* may promote immortalization, cells are prevented from further proliferation unless cell-cycle checkpoints are first abrogated.

***MYC* Causes *p53*-Dependent G_2 Arrest.** *MYC* activation has been shown to cause genomic destabilization (4–8). Oncogenes may cause genomic damage by accelerating transit through the G_1 and S phases of the cell cycle (13). Normal cells may withdraw from the cell-division cycle after the activation of *MYC*, because they suffer from DNA damage. This damage may trigger a checkpoint response. Cells may arrest in G_2 rather than G_1, because *MYC* seems to be capable of overriding the cell-cycle inhibition caused by gene products that normally would be responsible for causing cells to arrest in G_1 (7). Apparently, *MYC* is incapable of overriding a G_2 checkpoint response.

We observed that the implementation of the G_2 checkpoint requires the activity of *p53* as well as its downstream effector *p21*, because the loss of either gene product permitted the proliferation of normal cells in the presence of excess *MYC* activity. The absence of *p53* function has been shown to permit cells arrested in G_2 to adapt and re-enter the cell cycle (14–16).

Tumorigenesis caused by *MYC* overexpression is greatly accelerated by the loss of *p53* (11, 22). The loss of *p53* accelerates tumorigenesis associated with *MYC* activation by preventing apoptosis (10, 11). Our results suggest the additional mechanism that the loss of *p53* permits the continued proliferation of cells that are overexpressing *MYC*.

***MYC* Causes Aneuploidy.** Previously, we described that *MYC* activation induces aneuploidy in NHF (7). Our results presented herein provide a possible explanation. *MYC* activation seems to cause the arrest of cells in G_2. Cells arrested in G_2 may be enforced by *MYC* to reinitiate DNA replication resulting in aneuploidy. This model is supported by the observation that the proportion of cells that become aneuploid on *MYC* activation correlates with the frequency of cells in G_2 of the cell cycle (Fig. 3*f*). Furthermore, when the activation of *MYC* was prevented from causing G_2 arrest through the inactivation of p53, the number of cells that became aneuploid was decreased 10-fold (Fig. 3*e*).

The loss of the function of p53 causes genomic destabilization (17); thus, it seemed paradoxical that its loss seemed to reduce the frequency of aneuploidy caused by *MYC*. However, on closer analysis, we found that the absence of p53 did not prevent *MYC* from causing aneuploidy but rather reduced the pool of cells accumulated in G_2/M that could become aneuploid (Fig. 3*e*). Thus, within the subset of cells that are in the G_2 or M phases of the cell cycle, the activation of *MYC* causes the same proportion of these populations to become aneuploid regardless of the status of p53 function (Fig. 3*e*). Indeed, because the loss of p53 prevents the arrest of the cell cycle, aneuploid cells would now be capable of proliferatively expanding.

Cell-Cycle Checkpoints as Surveillance Against Oncogenes. We have shown that *MYC* overexpression causes the proliferative arrest of normal cells. This arrest may be an important mechanism by which *MYC* overexpression is prevented from causing tumorigenesis. Several other oncogenes have been shown to induce the arrest of the cell-division cycle. These include *RAS*, *RAF*, *MAPK*, and *E2F1* (23–26). In contrast to what we observed with *MYC*, these oncogenes induce a G_1 cell-cycle arrest and cellular senescence. One reason for this difference may be that *MYC* is capable of overriding the checkpoints that operate during G_1 but incapable of overriding the checkpoints during G_2.

In addition to monitoring cells for DNA damage, cell-cycle checkpoints may generally function as surveillance mechanisms to prevent a single oncogene from initiating tumorigenesis, as has been suggested (27). Cancer is a multistep process, possibly because cells that acquire individual oncogenic events are suppressed from further malignant progression unless multiple cell-cycle checkpoints are compromised, presumably by other genetic events. Our results specifically suggest that for the activation of *MYC* to cause tumorigenesis, the G_2 checkpoint may first have to be relaxed. The loss of *p53* function may be one mechanism by which this relaxation commonly occurs (10, 11).

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