Egr1 mediates p53-independent c-Myc–induced apoptosis via a noncanonical ARF-dependent transcriptional mechanism

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c-Myc is frequently deregulated in human cancers. Although deregulated c-Myc leads to tumor growth, it also triggers apoptosis in partnership with tumor suppressors such as ARF and p53. Apoptosis induced by c-Myc is a critical fail-safe mechanism for the cell to protect against unrestrained proliferation. Despite the plethora of information on c-Myc, the molecular mechanism of how c-Myc induces both transformation and apoptosis is unclear. Oncogenic c-Myc can indirectly induce the expression of the tumor suppressor ARF, which leads to apoptosis through the stabilization of p53, but both c-Myc and ARF have apoptotic activities that are independent of p53. In cells without p53, ARF directly binds to c-Myc protein and inhibits c-Myc–induced hyperproliferation and transformation with a concomitant inhibition of canonical c-Myc target gene induction. However, ARF is an essential cofactor for p53-independent c-Myc–induced apoptosis. Here we show that ARF is necessary for c-Myc to drive transcription of a unique noncanonical target gene, Egr1. In contrast, c-Myc induces another family member, Egr2, through a canonical mechanism that is inhibited by ARF. We further demonstrate that Egr1 is essential for p53-independent c-Myc–induced apoptosis, but not ARF-independent c-Myc–induced apoptosis. Therefore, ARF binding switches the inherent activity of c-Myc from a proliferative to apoptotic protein without p53 through a unique noncanonical transcriptional mechanism. These findings also provide evidence that cofactors can differentially regulate specific transcriptional programs of c-Myc leading to different biological outcomes.

oncogene | cell death

Deregulation or overexpression of the transcription factor c-Myc causes hyperproliferation and tumorigenesis and is a driving factor in the majority of human cancers (1). Although c-Myc regulates hundreds of downstream target genes involved in many different cellular processes, it is unclear which target genes mediate specific c-Myc functions (2). Apoptosis in response to deregulated c-Myc is a major fail-safe mechanism that is essential to prevent the proliferation of tumorigenic cells (3). Apoptosis induced by oncogenic c-Myc occurs through both p53-dependent and independent mechanisms that are not well understood (4, 5). A prevailing model for a p53-dependent mechanism is that the tumor suppressor ARF, which is induced by oncogenic c-Myc, causes the stabilization of the p53 protein by inhibiting its E3 ubiquitin ligase Mdm2 (6, 7). Additionally, ARF, independently of p53, binds to c-Myc directly and blocks the ability of c-Myc to activate transcription of examined canonical target genes containing a CACGTG E-box Myc binding site (EMS) and also inhibits c-Myc–induced hyperproliferation and transformation (8, 9). Despite this inhibition of canonical c-Myc activity, ARF has been shown to be essential for c-Myc to induce p53-independent apoptosis in mouse embryo fibroblasts (MEFs) (9, 10). However, the mechanism of how ARF regulates c-Myc induced p53-independent apoptosis is unknown. In this report, we examined the transcriptional consequences of the c-Myc/ARF interaction and discovered that in contrast to the ability of ARF to block c-Myc canonical target gene up-regulation, ARF is necessary for the direct transcriptional induction of Egr1 through a unique noncanonical mechanism.

The early growth response family of proteins, which are zinc finger transcription factors, have been shown to play roles in multiple pathways and processes, including differentiation, proliferation, apoptosis, and tumorigenesis, in a variety of tissues (11–14). Numerous studies suggest that Egr1, the best-characterized family member, is a tumor suppressor. For example, studies showed that the expression of Egr1 was undetectable in a majority of human breast and nonsmall cell lung carcinomas and deleted in 50% of acute myeloid leukemias (15, 16). Additionally, Egr1-null mice are more prone to skin cancer than their littermates when challenged with the two-step carcinogenesis model (17). Furthermore, several studies suggest Egr1 is an important mediator of apoptosis. The MEFs from the Egr1-null mice, as well as several other cell types with experimentally lowered levels of Egr1, are resistant to radiation-induced apoptosis (18–20). Also, Egr1 overexpression is sufficient to induce or enhance p53-dependent and p53-independent apoptosis in several different cell types (14, 21, 22). Here we show that Egr1 is essential for p53-independent c-Myc–induced apoptosis, but not ARF-independent c-Myc-induced apoptosis. Therefore, the differential transcriptional induction of Egr1 by c-Myc depending on the presence of ARF provides a mechanism for the ability of ARF to switch the inherent activity of c-Myc from a proliferative to apoptotic protein without p53.

Results

Activated c-Myc Directly Induces Egr1 in the Presence of ARF. To examine whether there are c-Myc target genes that are differentially regulated as a result of ARF, we performed microarray analyses comparing c-Myc estrogen receptor (c-MycER)-inducible gene expression between p53/ARF double KO (DKO) MEFs having no ARF with p53$^{-/-}$ MEFs having high levels of endogenous ARF. We used genetically defined MEFs without p53 to avoid effects caused by ARF activation of p53. Gene expression was analyzed 2 h following activation of the chimeric c-MycER protein by hydroxytamoxifen (OHT) to enrich for direct targets. Interestingly, through these microarray analyses we identified all the Egr transcription factor family members as c-Myc–responsive genes (Table S1). Two of the family members (Egr2 and Egr3) were up-regulated by c-Myc in DKO MycER MEFs, but not in p53$^{-/-}$ MycER MEFs, which was previously observed for the canonical c-Myc target genes nucleolin, eIF4E,

and htert (9). Surprisingly, the other two family members (*Egr1* and *Egr4*) were up-regulated by c-Myc in $p53^{-/-}$ but not DKO-MycER MEFs, suggesting that their inductions are dependent on ARF expression. The differential regulation of *Egr1* and *Egr2* in $p53^{-/-}$ and DKO-MycER MEFs following c-Myc activation was verified by real-time RT-PCR (Fig. 1*A*). Further, time course analyses of *Egr1* induction by c-MycER demonstrated that *Egr1* mRNA levels were induced by c-Myc within 1 to 2 h in the $p53^{-/-}$ MycER MEFs, but not in the DKO-MycER MEFs (Fig. 1*B*). Conversely, Egr2 mRNA levels were induced in the DKO, but not the $p53^{-/-}$ MycER MEFs (Fig. 1*C*).

Differential regulation of *Egr1* and *Egr2* by c-Myc in the presence of ARF suggests different mechanisms of induction and biological outcomes. As ARF is essential for p53-independent c-Myc–induced apoptosis, the ARF-dependent *Egr1* putative target gene was further characterized. To determine the necessity of ARF for induction of *Egr1* by c-Myc, ARF protein expression was silenced by siRNA, as confirmed by immunoblot analysis, in $p53^{-/-}$ MycER MEFs (Fig. 1*D Lower*). *Egr1* induction by c-Myc activation was reduced in the ARF siRNA treated cells compared with cells treated with control siRNA (Fig. 1*D Upper*), suggesting that ARF is necessary for c-Myc–driven *Egr1* induction. Additionally, we confirmed the induction of *Egr1* mRNA levels by c-Myc in another cell line, *c-myc*$^{-/-}$ Rat1 fibroblasts (H016) expressing c-MycER, which has low levels of ARF expression (9). Again, real-time RT-PCR confirmed that *Egr1* levels are increased by OHT activation of c-MycER, but not by mock treatment (Fig. S1). Further, we also examined the effects of c-Myc activation on Egr1 protein expression. Immu-

noblot analyses revealed that activated c-MycER induced the expression of Egr1 protein in $p53^{-/-}$ MycER MEFs within 3 to 4 h, with maximal induction by 8 h (Fig. 1*E*), but failed to induce Egr1 levels in $ARF^{-/-}$ MycER MEFs (Fig. 1*F*). Egr1 levels were also not induced by OHT treatment alone in $p53^{-/-}$ MEFs with vector (Fig. S2*A*), nor by activated c-MycER in DKO MEFs (Fig. S2*B*), indicating that Egr1 protein levels are increased by activated c-Myc only in the presence of ARF.

The relatively rapid induction of *Egr1* mRNA in $p53^{-/-}$ MycER MEFs by activated c-MycER suggests that it is a direct target. To test if c-Myc can induce *Egr1* mRNA levels in the absence of protein synthesis, $p53^{-/-}$ MycER MEFs were treated with cycloheximide to inhibit translation before c-MycER activation. Cycloheximide treatment alone caused *Elf1a*, a gene not regulated by c-Myc (23), and *Egr1* mRNA levels to increase (Fig. S3), as previously observed with many transcripts (24). However, activation of c-MycER in the presence of cycloheximide increased *Egr1* levels by approximately fourfold in 2 h versus cycloheximide treatment alone, suggesting that c-Myc can induce *Egr1* without de novo protein synthesis (Fig. 1*G*), even with relatively modest levels of c-MycER remaining (Fig. 1*G Lower*). In contrast, activated c-MycER did not enhance levels of *Elf1a* in the presence or absence of cycloheximide (Fig. 1*G*). This suggests that *Egr1* is a direct transcriptional target of c-Myc. However, c-Myc may also control the levels of *Egr1* by other mechanisms, such as inhibition of mRNA degradation. To determine whether c-Myc influenced *Egr1* mRNA degradation, real-time RT-PCR analysis was performed following transcriptional inhibition with actinomycin D treatment in H016 cells
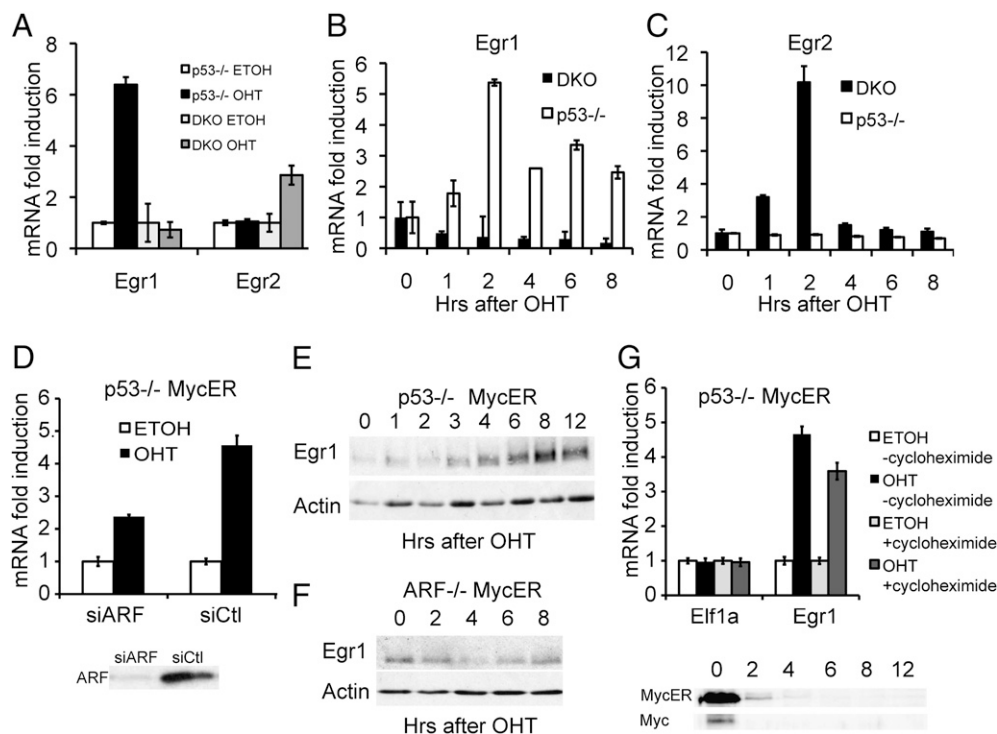

Fig. 1. c-Myc differentially induces *Egr1* and *Egr2* depending on the presence of ARF. (*A*) Microarray verification by real-time RT-PCR of *Egr1* and *Egr2* mRNA levels following 2 h of OHT treatment in $p53^{-/-}$ and DKO MycER MEFs. (*B* and *C*) Time course analyses following MycER activation of mRNA levels of *Egr1* (*B*) and *Egr2* (*C*) in $p53^{-/-}$ and DKO MycER MEFs (± OHT) as measured by real-time RT-PCR. Results are reported as the mean of the relative mRNA levels of *Egr1* or *Egr2* to β-actin at each time point normalized to time 0 to give the relative fold induction ± SD. (*D*) *Upper*: Real-time RT-PCR analysis of *Egr1* mRNA levels following c-Myc activation with OHT in $p53^{-/-}$ MycER MEFs treated first with siARF or control SMART pool. (*D*) *Lower*: Immunoblot shows ARF protein levels following siRNA treatment. (*E* and *F*) Immunoblot analyses of Egr1 protein levels following OHT activation in $p53^{-/-}$ MycER MEFs (*E*) and $ARF^{-/-}$ MycER MEFs (*F*). (*G*) c-Myc induces *Egr1* mRNA in the absence of protein synthesis. Real-time RT-PCR analysis of *Egr1* or *Elf1a* in $p53^{-/-}$ MycER MEFs (± OHT, 2 h) treated first with cycloheximide or mock treated with DMSO for 30 min to inhibit protein synthesis. Results are graphed as the mean of the relative mRNA fold induction over mock-treated (ETOH) controls ± SD. Immunoblot shows c-Myc and c-MycER protein following cycloheximide addition.

expressing c-MycER. The decay of *Egr1* mRNA levels was the same with c-MycER activation or mock treatment ([Fig. S4](#)). Therefore, activation of c-MycER primarily increases transcription of *Egr1* in cells with ARF expression, rather than enhancing *Egr1* mRNA stability.

Endogenous c-Myc Regulates *Egr1* Levels. To determine whether endogenous c-Myc regulates *Egr1* expression, *c-myc*$^{-/-}$ (HO16) and parental WT (TGR) Rat1 cells were harvested and the relative expression of *Egr1* and a negative control *Elf1a* was determined with real-time RT-PCR. *Egr1* was expressed substantially higher in the cells with c-Myc, unlike *Elf1a*, which was expressed equally in the two cell lines (Fig. 2*A*), suggesting that *Egr1* expression is controlled by endogenous c-Myc. As the *Egr* genes are also known to be immediate early genes like c-Myc that are induced by serum (25), we examined the influence of endogenous c-Myc expression on the serum induction of *Egr1*. The TGR and HO16 cells were made quiescent by serum deprivation and then the expression of *Egr1* was followed after serum stimulation. *Egr1* levels were substantially increased and sustained to a greater extent in the cells with c-Myc compared with cells without c-Myc (Fig. 2*B*). These results suggest that *Egr1* gene expression is controlled by endogenous c-Myc and that c-Myc is necessary for the full serum induction of *Egr1*.

c-Myc and ARF Are Recruited to and Regulate the *Egr1* Promoter. If *Egr1* is a direct target as suggested by the preceding data, then c-Myc must be recruited to the *Egr1* promoter. However, as the proximal promoter of *Egr1* ($\geq$15 kb in both directions) does not contain any canonical E-box Myc sites (CACGTG), we used a scanning ChIP approach to identify the c-Myc binding region. ChIP was performed using *p53*$^{-/-}$ MycER MEFs with partially overlapping primers spanning 5 kb upstream of the start site. We found that activated c-Myc was recruited to one region of the *Egr1* promoter (−904 to −1,319; Fig. 3*A*). As a positive control, primers for the c-Myc canonical target gene *eIF4E* promoter were used (Fig. 3*A*). As *Egr1* induction by c-MycER is dependent on the presence of ARF, we determined whether ARF is also recruited to the *Egr1* promoter. ChIP analysis revealed that ARF was recruited to the same region as c-Myc on OHT activation, but not to other regions of the promoter (Fig. 3*B Right*). However, ARF was not detected at the *Egr1* promoter without OHT activation (Fig. 3*B Left*), suggesting that ARF is recruited to the *Egr1* promoter only upon c-Myc activation, as previously observed with canonical target genes (9). This observation agrees with the finding that ARF is mostly nucleolar in *p53*$^{-/-}$ MEFs until c-Myc activation causes ARF to be translocated to the nucleoplasm (9, 10). Conversely, to determine whether c-Myc can be recruited to the *Egr1* promoter without ARF, we used DKO-MycER MEFs. ChIP analysis revealed that activated c-MycER was recruited to
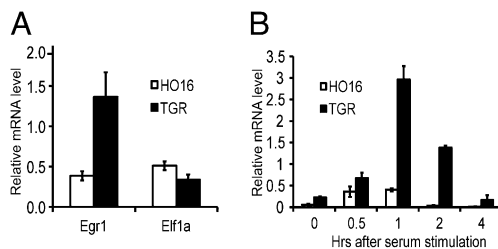

Fig. 2. Endogenous c-Myc is necessary for the full expression and induction of *Egr1*. (*A*) Real-time RT-PCR analysis of *Egr1* mRNA levels in logarithmically growing *c-myc*$^{-/-}$ (H016) and parental (TGR) rat1 cells. (*B*) Real-time RT-PCR analysis of *Egr1* mRNA levels following serum stimulation of serum-starved H016 and TGR cells. Results are graphed as the mean of the relative mRNA levels of *Egr1* to *Tbp* ± SD.
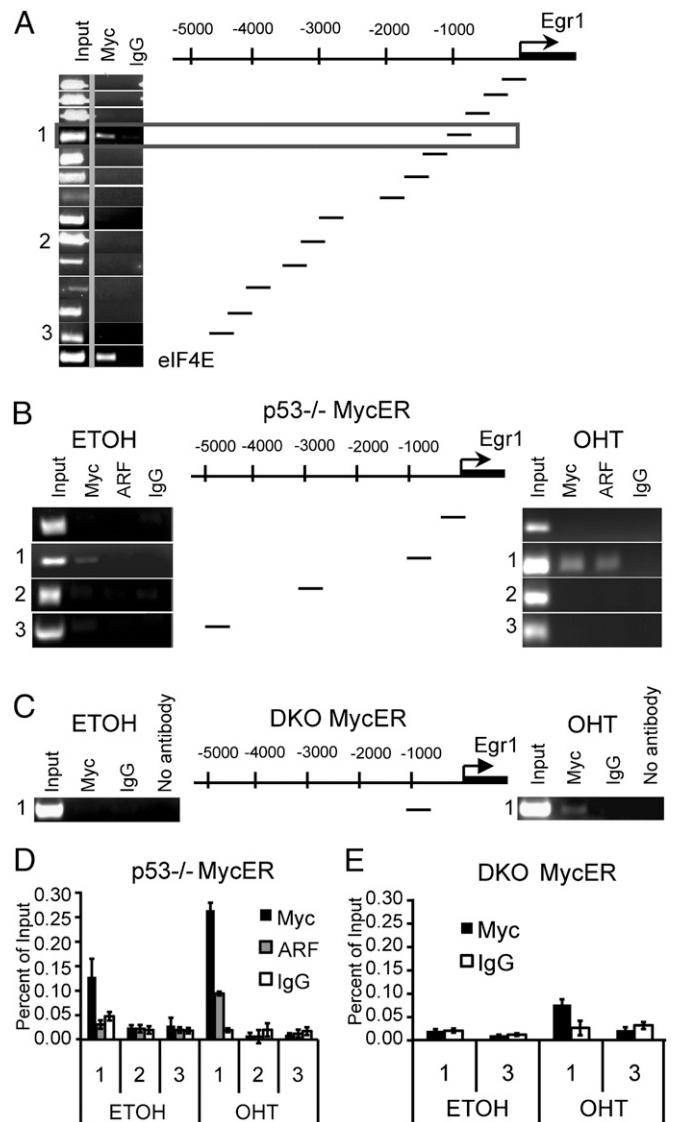

Fig. 3. c-Myc and ARF are recruited to the *Egr1* promoter at a noncanonical binding site. (*A*) Chromatin prepared from *p53*$^{-/-}$ MycER MEFs (+ OHT) was subjected to immunoprecipitation using anti-Myc or IgG followed by PCR by using the indicated panel of partially overlapping primers spanning 5 kb of the *Egr1* promoter. The box indicates a region (−904 to −1,319) to which c-Myc is recruited. *eIF4E* primers spanning an established EMS binding sequence were used as a positive control. (*B*) ChIPs performed as in *A* except MycER was either activated with OHT treatment (*Right*) or mock treated with ethanol (*Left*) for 6 h and chromatin was subjected to immunoprecipitation with anti-Myc, anti-ARF, or IgG. (*C*) Chromatin prepared from DKO MycER MEFs (± OHT) was subjected to immunoprecipitation using anti-Myc, IgG, or no antibody followed by PCR using the indicated primers that span the binding site in the *Egr1* promoter. (*D*) ChIPs as in *B* in p53$^{-/-}$ MycER MEFs ± OHT except purified DNA was subjected to real-time PCR. Results are reported as the mean of the percent of input ± SD. The numbers below the x axis indicate the region of the *Egr1* promoter being amplified as demonstrated in *A* and *B*. (*E*) Quantitative ChIPs as in *D* but in DKO MycER MEFs ± SD.

the *Egr1* promoter without ARF (Fig. 3*C Right*). In contrast to *Egr1*, *Egr2* is induced by c-MycER in DKO-MycER MEFs and the *Egr2* promoter contains three putative canonical CACGTG sites. ChIP analysis using DKO-MycER MEFs revealed that activated c-Myc was recruited to the canonical CACGTG in the *Egr2* promoter located at −2,400 ([Fig. S5*A*](#)), but not to the other two CACGTG sequences. As a positive control, primers for the *eIF4E* promoter were used ([Fig. S5*B*](#)).

To quantitatively determine the relative amounts of c-Myc and ARF recruited to the *Egr1* promoter under different conditions, we used real-time PCR to analyze the ChIP assays. We verified that c-Myc and ARF were both recruited to the *Egr1* promoter after MycER activation, but ARF was not detected without activation of c-Myc in *p53*$^{-/-}$ MycER MEFs (Fig. 3*D*). Without ARF, activated c-MycER was still recruited to the *Egr1* promoter in DKO-MycER MEFs (Fig. 3*E*), but at lower levels compared with *p53*$^{-/-}$ MycER MEFs (Fig. 3*D*), suggesting that ARF enhances the recruitment of c-Myc to the *Egr1* promoter. Without OHT activation we detected low levels of c-Myc at the *Egr1* promoter in *p53*$^{-/-}$ MycER MEFs (Fig. 3*D*), but not in DKO-MycER MEFs (Fig. 3*E*), suggesting that c-MycER is partially active without OHT in the *p53*$^{-/-}$ MycER MEFs. Taken together, the results suggest that the interaction of activated c-Myc with ARF enhances the recruitment of c-Myc to the *Egr1* promoter, but that ARF alone cannot be detected.

To further examine the regulation of the *Egr1* promoter by c-Myc and ARF we performed luciferase assays with a 2.5-kb fragment of the *Egr1* promoter containing the putative c-Myc binding site identified earlier. The *Egr1* promoter was induced in *p53*$^{-/-}$ MEFs overexpressing c-Myc (Fig. 4*A*), demonstrating that c-Myc activates the *Egr1* promoter. In contrast, the *Egr1* promoter was slightly inhibited in DKO MEFs expressing c-Myc (Fig. 4*B*), confirming the dependence on ARF for the c-Myc activation of the *Egr1* promoter. As previously reported (9), the *htert* promoter and the 4XEMS promoter were both induced by c-Myc in DKO MEFs (Fig. 4*B*), but the *htert* promoter induction was blocked in *p53*$^{-/-}$ MEFs with high ARF expression (Fig. 4*A*). In addition, the *Egr1* promoter was induced by c-Myc in Rat1a cells (Fig. 4*C*), confirming that this regulation is not cell type-specific. To determine whether the *Egr1* promoter is regulated by endogenous c-Myc, the activity of the *Egr1* luciferase promoter was compared between TGR (*c-myc* WT) and HO16 (*c-myc*$^{-/-}$) Rat1 cells. The *Egr1* promoter was more active in TGR cells than in the HO16 cells, similar to the levels observed with the artificial canonical 4XEMS promoter (Fig. 4*D*). In comparison with the
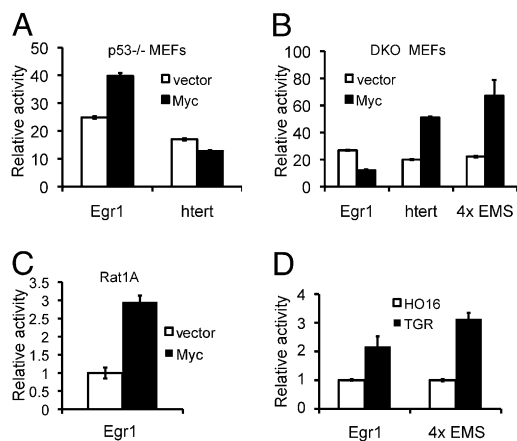
endogenous *Egr1* expression induced by c-MycER (Fig. 1*B*), the activity of the *Egr1* promoter induced by c-Myc in transient luciferase assays is relatively modest, suggesting that the chromatin environment and/or the different assay conditions influence the regulation of the *Egr1* promoter by c-Myc. Taken together these results suggest that exogenous and endogenous c-Myc induce the *Egr1* promoter by a unique ARF-dependent noncanonical transcriptional mechanism, unlike other c-Myc target gene promoters containing the canonical CACGTG binding site that are inhibited by ARF.

Egr1 Is Necessary for c-Myc–Induced p53-Independent Apoptosis. As c-Myc induces both apoptosis and Egr1 expression in an ARF-dependent manner and because Egr1 is necessary for apoptosis in several cell types (13, 14), we examined whether Egr1 might be involved in p53-independent c-Myc–mediated apoptosis. We obtained *Egr1*$^{-/-}$ MEFs and parental WT MEFs and generated lines that express comparable levels of c-MycER (Fig. 5*A Lower*). Activated c-MycER induced apoptosis in the WT MEFs, but failed to induce apoptosis in the *Egr1*$^{-/-}$ MEFs, suggesting that Egr1 expression is necessary for c-Myc–induced apoptosis (Fig. 5*A Top*). To confirm this result using another approach, we treated *p53*$^{-/-}$ MycER MEFs with Egr1 siRNA or control siRNA. Egr1 protein expression was effectively inhibited by Egr1 siRNA for up to 5 d with or without OHT treatment (Fig. S6*A*). Inhibiting Egr1 expression significantly reduced c-Myc–induced apoptosis in *p53*$^{-/-}$ MycER MEFs using a pool of Egr1 siRNA oligomers (Fig. 5*B*) or individual Egr1 siRNA oligomers (Fig. S6*B*). In addition, to confirm the necessity of ARF for p53-independent c-Myc–induced apoptosis, we inhibited ARF protein expression by using ARF siRNA (Fig. 1*D*). The reduction of c-Myc–induced apoptosis by inhibition of ARF expression was comparable to the reduction observed with inhibition of Egr1 expression (Fig. 5*B*). The combined inhibition of both ARF and
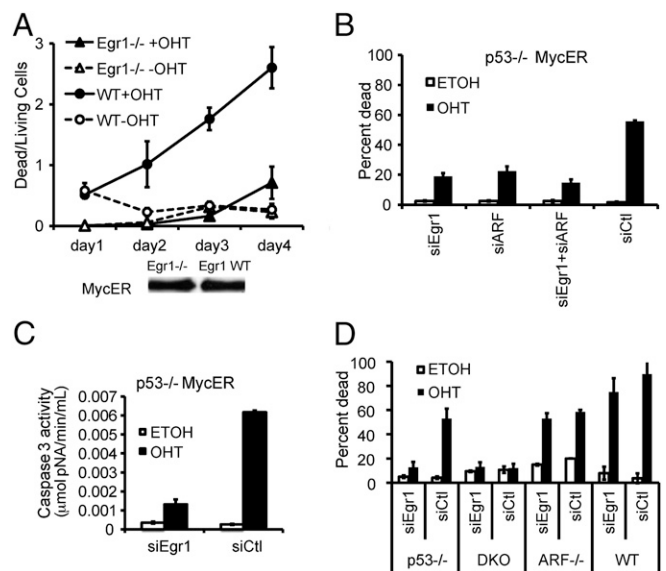

Fig. 4. c-Myc activates the *Egr1* promoter in the presence of ARF. (*A* and *B*) Reporter constructs for *Egr1* (2.5 kb upstream), *htert*, and 4XEMS promoters were transiently transfected into *p53*$^{-/-}$ MEFs (*A*) and DKO MEFs (*B*) with exogenous c-Myc or vector control and a thymidine kinase renilla luciferase (pRL-TK) transfection control. The mean of the relative luciferase reporter activity to pRL-TK is reported ± SD. (*C*) The *Egr1* reporter construct and pRL-TK were transfected into Rat1A cells that constitutively express c-Myc or vector. Relative luciferase activity was determined as described earlier and then normalized to the vector control. (*D*) The *Egr1* reporter construct or 4XEMS were transfected into c-Myc–null (H016) and WT (TGR) cells and relative luciferase activity was determined and results were normalized to the activity in *c-myc*$^{-/-}$ cells.

Fig. 5. Egr1 and ARF are necessary for c-Myc–induced p53-independent apoptosis. (*A*) *Egr1*$^{-/-}$ and parental WT MEFs expressing equal levels of c-MycER (*Lower*) were assayed for apoptosis (± OHT) in low serum by counting living and dead cells. (*B*) *p53*$^{-/-}$ MycER MEFs (± OHT) treated with siEgr1, siARF, siEgr1, and siARF, or control SMART pool no. 1 (siCtl) for 24 h were assayed for apoptosis in low serum 3 d after OHT activation. (*C*) Caspase-3 activity was determined in siEgr1 or siCtl-treated *p53*$^{-/-}$ MycER MEFs ± OHT for 3 d as described in *Materials and Methods*. (*D*) *p53*$^{-/-}$ MycER, DKO-MycER, ARF$^{-/-}$ MycER, and WT-MycER MEFs treated with siEgr1 or siCtl were assayed for apoptosis as in *B*.

Egr1 expression did not further reduce c-Myc–induced apoptosis (Fig. 5B), suggesting that ARF and Egr1 function in the same pathway. Reduced apoptosis resulting from the loss of Egr1 expression was confirmed by a decrease in caspase-3 activation (Fig. 5C).

To compare the effects of Egr1 inhibition on c-Myc–induced apoptosis in MEFs with different genetic backgrounds, we treated p53$^{-/-}$, DKO, ARF$^{-/-}$, and WT-MycER MEFs with Egr1 siRNA. Confirming the results shown in Fig. 5B, the inhibition of Egr1 expression by siRNA significantly reduced c-Myc–induced apoptosis in p53$^{-/-}$ MycER MEFs (Fig. 5D). Also, as previously shown, activation of c-MycER did not induce apoptosis in MEFs lacking p53 and ARF, and inhibition of Egr1 expression had no effect on the cells (Fig. 5D). However, activation of c-MycER in both ARF$^{-/-}$ and in WT MycER MEFs did cause apoptosis (Fig. 5D). This confirms that c-Myc can induce apoptosis independently of ARF in cells with p53, which has been previously shown (26). Importantly, inhibition of Egr1 by siRNA had no effect on p53-dependent, ARF-independent c-Myc–induced apoptosis observed in ARF$^{-/-}$ MEFs (Fig. 5D) or apoptosis caused by staurosporine treatment (Fig. S7), suggesting that reducing Egr1 levels does not cause a general defect in apoptosis, but rather Egr1 is specifically necessary for c-Myc–induced, p53-independent apoptosis. Finally, to determine whether Egr1 is capable of inducing apoptosis without activated c-Myc or p53, we generated p53$^{-/-}$ MEFs expressing an Egr1-ER fusion protein (Fig. S8 Lower). Upon 4 d of activation with OHT, Egr1-ER efficiently induced apoptosis in low serum (Fig. S8 Upper). Overall, these results suggest that Egr1 is necessary and sufficient for mediating p53-independent and ARF-dependent c-Myc–induced apoptosis.

Discussion

Our results suggest that there is a unique mechanism of c-Myc transcriptional regulation, whereby ARF binds with c-Myc at promoters and selectively and differentially induces c-Myc target genes. We propose that the differential regulation of c-Myc transcriptional activity by ARF allows for different biological outcomes. Previously, we demonstrated that ARF inhibits well established target genes with canonical Myc binding sites (9), as exemplified by Egr2 shown here. In contrast, we have now established that ARF is necessary for c-Myc to directly induce transcription of a noncanonical target gene, Egr1. In support of this finding, Egr1 was recently found to be induced by c-Myc in a microarray using B cells (27). Several previously identified canonical c-Myc target genes can induce apoptosis, including ODC and MT-MC1 (28, 29); however, it has not been shown conclusively that loss of a canonical direct target gene disrupts c-Myc–induced apoptosis independently of p53. In this report we identify Egr1 as a c-Myc target gene that mediates p53-independent, ARF-dependent c-Myc–induced apoptosis.

As ARF-dependent noncanonical induction of Egr1 is essential for c-Myc to induce apoptosis independently of p53, ARF binding essentially switches the inherent activity of c-Myc to an apoptotic protein through transcriptional regulation. As c-Myc is recruited to the Egr1 promoter without ARF, albeit at lesser amounts than with ARF, we propose that ARF may not only enhance recruitment of c-Myc to the Egr1 promoter, but also influences the transcriptional activity of c-Myc after DNA binding. Substantial control of c-Myc target genes after DNA binding is supported by the previous observation that c-Myc was directly recruited to approximately 3,000 genes in human B cells, but only 406 were induced by activated c-Myc (30).

As summarized in Fig. 6, our model is that under normal physiological low ARF conditions, c-Myc induces canonical target genes, such as cyclin D2, cdk4, nucleolin, eIF4E, and Egr2, which stimulate cell cycle progression and cell growth. Under normal conditions, p53 levels are also low as a result of Mdm2-
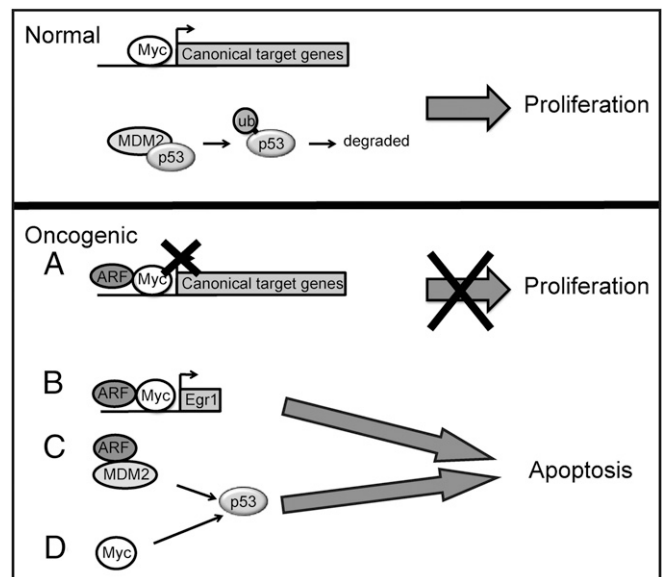

Fig. 6. Mechanisms mediating c-Myc–induced apoptosis. *Upper*: During normal physiological low-ARF conditions c-Myc drives expression of canonical target genes, leading to proliferation. Further, without ARF expression, Mdm2 mediates degradation of p53, thereby preventing a block in cell cycle progression. *Lower*: Expression of ARF is induced by oncogenic stress such as deregulated c-Myc. (*A*) ARF then directly interacts with c-Myc and inhibits canonical target gene expression necessary for proliferation. (*B*) Conversely, ARF is necessary for c-Myc to induce the noncanonical target Egr1, which then mediates c-Myc–induced apoptosis independently of p53. (*C*) ARF induced by oncogenic c-Myc also binds to and inhibits Mdm2 activity, leading to p53 stabilization and p53-dependent apoptosis. (*D*) c-Myc–induced apoptosis can also occur independently of ARF through alternate p53-dependent mechanisms. Failure to trigger ARF or p53 pathways by oncogenic c-Myc leads to unregulated proliferation and tumorigenesis.

mediated degradation of p53 protein. Upon oncogenic activation, c-Myc causes both an increase in ARF expression and a relocalization of ARF from the nucleolus to the nucleoplasm, independently of p53 (9). In a direct feedback mechanism, ARF binds with c-Myc to inhibit canonical c-Myc target gene induction and proliferation while inducing noncanonical expression of Egr1 and Egr1-mediated apoptosis. In cells with WT p53, elevated ARF also inhibits Mdm2 activity, leading to p53 protein stabilization and p53-induced apoptosis. In cells that lack functioning ARF, but have a WT p53, c-Myc induces p53-dependent apoptosis through less defined mechanisms (7, 31). Considering that p53 expression is lost in half of all human tumors, this unique c-Myc-ARF-Egr1 apoptosis pathway has the potential to be exploited for future therapeutic agents as an alternative to p53 reactivation.

Materials and Methods

Cell Culture, Transfection, and Retroviral Infection. Cos-7, p53$^{-/-}$ MEFs, DKO MEFs, and ARF$^{-/-}$ MEFs were cultured in DMEM with 10% calf serum (CS). H016, TGR, Egr1$^{-/-}$, and WT MEFs were maintained in DMEM with 10% FBS. All cells were transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol with the indicated plasmids. Cells were subjected to analysis approximately 48 h after transfection. The p53$^{-/-}$, DKO, ARF$^{-/-}$, H016 MycER, and vector cells were generated using the retroviral expression vector pBabehygro-MycER and the Egr1 WT and Egr1$^{-/-}$ MEFs were generated using pBabepuro-MycER as described previously (9).

RNA Interference. p53$^{-/-}$, ARF$^{-/-}$, DKO, and WT MEFs expressing MycER were seeded at 2 to 4.5 × 10^6 cells per 10-cm dish and treated with a final concentration of 100 nM of Egr1 SMARTpool, CDKN2A (ARF) SMARTpool, or control Non-Targeting Pool no. 1 siRNA purchased from Dharmacon using

Dharmafect Reagent 4 according to the manufacturer's instructions. Approximately 24 h later, cells were trypsinized and seeded.

Immunoblot Analysis. Cell lysates were prepared in antibody buffer (20 mM Tris, pH 7.4, 50 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholic acid (DOC), 0.5% SDS, 1 mM EDTA, 0.1 M PMSF, 10 mg/mL aprotinin, 2 mg/mL leupeptin). The proteins were resolved by 10 or 15% SDS/PAGE, subjected to immunoblot analysis using anti-MycfI (Millipore), anti-Egr1 (C-19; Santa Cruz Biotechnology), anti-ARF (Millipore), and monoclonal anti–β-actin (Sigma-Aldrich) using enhanced chemiluminescence for detection.

Quantitative Real-Time RT-PCR. MEFs expressing c-MycER were harvested at the indicated times following treatment with 2 μM hydroxytamoxifen (OHT). Lysates, RNA, and cDNA were prepared, real-time PCR was performed, and results were calculated as detailed previously (32). For mRNA level analyses in HO16 and TGR cells, the cells were shifted to media containing 0.1% FBS for 48 h before stimulation with 20% FBS. Cells were harvested at the indicated times and the mRNA levels were determined as described earlier. All real-time RT-PCR analyses were performed in triplicate with the primers listed in *SI Materials and Methods* and the results are reported as mean ± SD relative to actin levels.

ChIP. Sixteen hours after $p53^{-/-}$ MycER and DKO MycER MEFs were plated at 6×10^6 cells per 150-mm dish, they were treated with 2 μM OHT or ethanol for 6 h. Cross-linking and ChIP was performed as detailed by Farnham et al. (http://www.genomecenter.ucdavis.edu/farnham/protocol.html) with anti–c-Myc (N-262x; Santa Cruz Biotechnology), anti-ARF (CDKN2A; GeneTex), and normal rabbit IgG (Upstate). Purified DNA was subjected to PCR amplification by using specific primer sets listed in *SI Materials and Methods*. PCR products were subjected to analysis on a 1.5% agarose gel. Quantitative ChIP analyses were performed as described earlier except the purified DNA was subjected to real-time PCR using the primers listed in *SI Materials and Methods*. A standard curve was used to calculate the relative starting quantity of each sample. The percent of input was calculated by dividing the relative starting quantity from each immunoprecipitation (Myc, ARF, IgG) by the relative starting quantity of the input and then by multiplying by 100. The results are reported as the mean ± SD from triplicate samples.

Luciferase Assays. $p53^{-/-}$ MEFs and DKO MEFs were transfected with 2 μg of Myc expression vector or empty vector, 1.9 μg of reporter plasmid, and 0.1 μg of pRL-TK internal control. Luciferase assays were carried out 48 h after transfection according to the manufacturer's instructions (dual-luciferase reporter assay system; Promega). Results were normalized for expression of pRL-TK and are reported as the mean ± SD from triplicate samples. For the assays in HO16, TGR, and Rat1a cells, 3.9 μg of reporter plasmid and 0.1 μg of pRL-TK or pRL-SV40 were transfected into the cells. Luciferase assays were performed, calculated, and reported as described earlier.

Apoptosis Assays. Two days after plating $Egr1^{-/-}$ MycER and WT MycER MEFs at 1×10^5 cells per well in six-well dishes, the cells were shifted into media containing 0.5% FBS with or without 2 μM OHT (added daily). The numbers of floating (apoptotic) and attached (living) cells were determined in triplicate at the indicated times with a hemacytometer. Results are reported as a ratio of dead to living cells over time. For apoptosis assays performed on RNAi-treated cells, $p53^{-/-}$ MycER, $ARF^{-/-}$ MycER, DKO-MycER, and WT-MycER MEFs were treated with either siGENOME SMARTpool targeting $Egr1$ or siGENOME control SMARTpool no. 1 (Dharmacon). After 24 h the cells were seeded at 2×10^5 cells per well in six-well dishes in media containing 2% CS with or without 2 μM OHT (added daily). The numbers of living and dead cells were determined as described earlier in triplicate in at least three different experiments with two different polyclonal cell lines and reported as the number of dead cells divided by number of total cells multiplied by 100 (i.e., percent dead). Apoptosis was confirmed with an activated caspase-3 colorimetric assay (Sigma-Aldrich) performed according to manufacturer instructions.

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