# Supporting Information

## Zhang et al. 10.1073/pnas.1208334110

### SI Materials and Methods

**Cell Culture, Transfection, and Retroviral Infection.** All cells were maintained in DMEM with 10% serum, either FBS or calf serum (CS), as designated in the methods below. Transfections were performed using oligofectamine (Invitrogen) for siRNA transfections, Lipofectamine 2000 (Invitrogen) for siRNA cotransfected with plasmids, and FuGene 6 (Roche) or Lipofectamine 2000 (Invitrogen) for plasmid-only transfections according to manufacturer's protocols with the indicated plasmids. The $p53^{-/-}$ MycER and $p53^{-/-}$ MycER$^{N6KR}$ mice embryo fibroblasts (MEFs), $ARF^{-/-}p53^{-/-}$ (DKO) MycER and DKO MycER$^{N6KR}$ MEFs, and $Egr1^{-/-}$ MycER$^{N6KR}$ and WT MycER$^{N6KR}$ MEFs were generated by retroviral infection as described previously (1) using the pBabe hygro-MycER or pBabe hygro-MycER$^{N6KR}$ vector. The $p53^{-/-}$ and DKO MycER MEFs stably expressing T7-Skp2 were generated using retroviral infection of pBabe puro-T7-Skp2 and pBabe hygro-MycER as described previously (1).

**Plasmids and Expression Vectors.** The mouse c-Myc expression vectors pRcCMV-c-Myc and retroviral pBabe hygro-c-MycER have been previously described (2). The pBabepuro-T7-Skp2 expression vector was generated by PCR amplification of T7-tagged mouse Skp2 from pCGT-T7-Skp2 and cloning into pBabe puro vector using primers with EcoRI sites at both ends: forward: 5′-GCGCGAATTCGCCACCATGGCTTCTAGGATGGCATC-3′ and reverse: 5′-GCGCGAATTCTCATCATAGACAACTGG-GCTTTTGCAGTG-3′. The EcoRI fragment was digested and inserted into the EcoRI site of the retroviral pBabe puro vector. The pBabe-hygro-MycER$^{N6KR}$ expression vector was generated using PCR-mediated mutagenesis (Clontech) with the pBabehygro-MycER vector as template and changing the c-Myc N-terminal domain 6 lysines to arginines. Mouse c-Myc167 was generated using PCR-mediated mutagenesis (Clontech) as previously described (1). All of the constructs were verified by sequencing and expression of all constructs was confirmed by immunoblot analysis.

**Cycloheximide Chase Analysis.** Cells were plated at a density of $5 \times 10^5$ cells/6-cm dish and allowed to grow overnight. Cells were treated with 50 μg/mL cycloheximide (CHX) to block protein synthesis and harvested at the indicated times. Immunoblot analysis was performed by using anti-Mycfl (Millipore), band intensities were measured by scanning densitometry (UN-Scan-It; Silk Scientific), and protein half-lives were calculated from the resultant data.

**Apoptosis Assays.** DKO MycER, DKO MycER$^{N6KR}$, $p53^{-/-}$ MycER, and $p53^{-/-}$ MycER/Skp2 MEFs were plated at $1 \times 10^5$ cells/35-mm dish in triplicate. Twenty-four hours later, cells were shifted into media containing 1% CS and treated with 2 μM hydroxytamoxifen (OHT) or ethanol; 2 μM OHT or ethanol was added daily. $Egr1^{-/-}$ MycER$^{N6KR}$ and WT MycER$^{N6KR}$ MEFs were shifted into media containing 2% FBS and treated daily with 2 μM OHT or ethanol. DKO MycER MEFs transfected with siRNA were replated at $2 \times 10^5$ cells/35-mm dish in media containing 10% CS and treated daily with 2 μM OHT or ethanol. HeLa cells were seeded at $1.5 \times 10^5$/35-mm dish and transfected the next day. Two days following transfection, the HeLa cells were either kept in 10% FBS or shifted to 0.1% FBS for 10 h. The number of floating (apoptotic) and attached (living) cells was counted at the indicated times with a hemacytometer. All results are reported as the number of dead cells divided by number of total cells multiplied by 100 (percent dead). At least three different experiments were performed with two different independently derived polyclonal cell lines.

**Quantitative Real-Time PCR Primers.**

| | |
|---|---|
| *Nucleolin* | Forward: 5′-ACACCAGCCAAAGTCATTCC-3′ |
| | Reverse: 5′-ATCCTCATCACTGTCTTCCTTC-3′ |
| *CDK4* | Forward: 5′-GCAGTCTACATACGCAACAC-3′ |
| | Reverse: 5′-TCGTCTTCTGGAGGCAATC-3′ |
| *eIF4E* | Forward: 5′-GGACGGGATTGAGCCTATGTG-3′ |
| | Reverse: 5′-CAGCAGTGTCTCTAGCCAGAAG-3′ |
| *RCL* | Forward: 5′-GGTTCCAGGTGTGGGACTACG-3′ |
| | Reverse: 5′-GAAGATAAGCCTCAAAGTACCG-3′ |
| *β-Actin* | Forward: 5′-GCTGTGCTATGTTGCTCTAG-3′ |
| | Reverse: 5′-CGCTCGTTGCCAATAGTG-3′ |
| *EGR1* | Forward: 5′-GAGGAGATGATGCTGCTGAG-3′ |
| | Reverse: 5′-TGCTGCTGCTGCTATTACC-3′ |
| Bim | Forward: 5′-CGGATCGGAGACGAGTTCA-3′ |
| | Reverse: 5′-TTCCAGCCTCGCGGTAATCA-3′ |
| Skp2 | Forward: 5′-GCTTCAGCTCTTTCCGGGTAC-3′ |
| | Reverse: 5′-CGGCAGATTCAGAAAACCCA-3′ |

**ChIP Real-Time Primers.**

| | |
|---|---|
| *Egr1* | Forward: 5′-CTAACCATCACAAGAACCAACAG-3′ |
| | Reverse: 5′-ACTAATGGCAGGGTCACTTTC-3′ |
| *Tert* | Forward: 5′-AAGAACTGAGATTGCCACCACCCA-3′ |
| | Reverse: 5′-GATGGTCACAATGCTGGTGCATGA-3′ |

**Luciferase Reporter Assays.** For transient Myc overexpression experiments, DKO MEFs were transfected with 2 μg of c-Myc or empty vector, 1.9 μg of firefly luciferase reporter plasmid having either the Egr1 promoter region described previously (3) or the canonical 4XEMS promoter (1), and 0.1 μg of pRL-TK (Renilla luciferase) internal control in triplicate. For the assays in Rat1a cells, 3.9 μg of reporter plasmid, 0.1 μg of pRL-TK or pRL-SV40, and 3 μg Skp2 or empty vector were transfected into the cells. Luciferase assays were carried out 48 h after transfection according to the manufacturer's instructions (dual-luciferase reporter assay system; Promega). For experiments involving OHT induction of DKO and $p53^{-/-}$ MEFs stably expressing c-MycER or c-MycER$^{N6KR}$, cells were plated in six-well plates and grown overnight. At ∼50% confluency, cells were transfected with 0.8 μg of reporter plasmid, 0.1 μg of pRL-TK internal control, and 0.1 μg of GFP expression plasmid in triplicate. To evaluate the effect of Skp2 on reporter activation by activated c-MycER, 1 μg of T7-Skp2 expression vector or empty vector was cotransfected with the luciferase reporters. Approximately 18 h after transfection, cells were shifted to low serum medium (DMEM with 0.1% FBS) and incubated for 24 h. Cells were then treated with 2 μM OHT or ethanol for 6 h. Cell lysates were prepared in Reporter Lysis Buffer (Promega) with protease inhibitors, and luciferase assays were carried out as described above. Firefly luciferase results were normalized for expression of Renilla luciferase from pRL-TK and are reported as the mean ± SEM from triplicate samples.

**RNA Interference.** DKO MycER, DKO-MycER$^{N6KR}$ MEFs, and HeLa cells were plated at $2 \times 10^6$ cells/10-cm dish and 24 h later were treated with 100 nM of Skp2 or Egr1 siGENOME SMARTpool or siGENOME Non-Targeting Control SMARTpool no. 1 from Dharmacon according to the manufacturer's instructions. Approximately 24 h later, cells were trypsinized and replated for specific experiments. For siRNA coupled luciferase assays in MEFs stably expressing MycER or MycER$^{N6KR}$, cells were transferred to six-well plates and allowed to grow to ~50% confluence; 50 nM siSkp2 or siCon was cotransfected with luciferase constructs for 24 h. Cells were shifted to low serum (0.1% FBS) medium for another 24 h and then analyzed for Egr1 promoter activity as described above.


1. Qi Y, et al. (2004) p19ARF directly and differentially controls the functions of c-Myc independently of p53. *Nature* 431(7009):712–717.
2. Li Z, Boone D, Hann SR (2008) Nucleophosmin interacts directly with c-Myc and controls c-Myc-induced hyperproliferation and transformation. *Proc Natl Acad Sci USA* 105(48): 18794–18799.
3. Boone DN, Qi Y, Li Z, Hann SR (2011) Egr1 mediates p53-independent c-Myc-induced apoptosis via a noncanonical ARF-dependent transcriptional mechanism. *Proc Natl Acad Sci USA* 108(2):632–637.


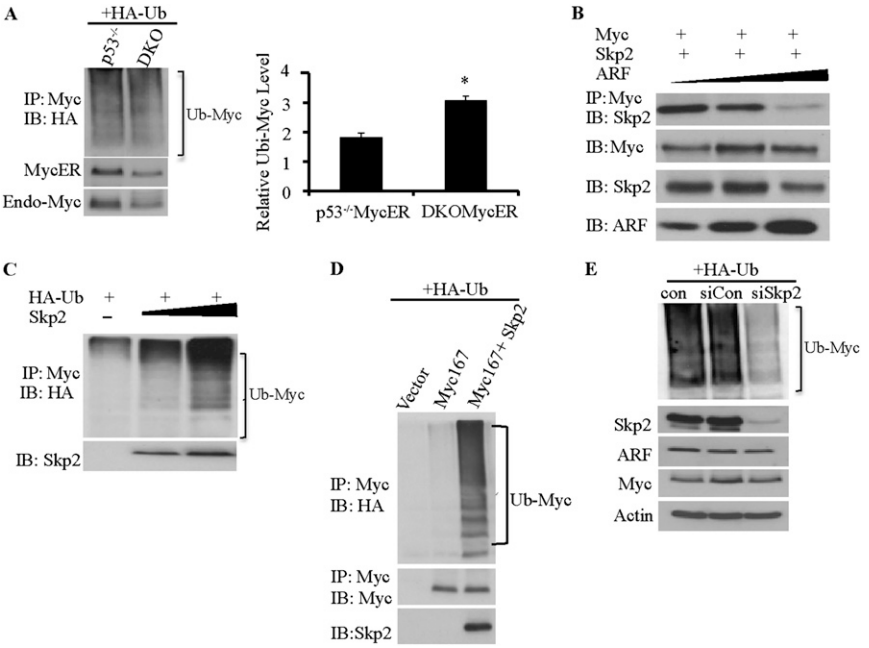


**Fig. S1.** ARF and Skp2 control c-Myc ubiquitylation. (*A*) *p53*$^{-/-}$ MycER and DKO MycER MEFs were transfected with HA-ubiquitin. One day later, the cells were treated with 2 μM OHT for 24 h, and in vivo ubiquitylation analyses of Myc were performed. Results are reported as the ubiquitilyated Myc level relative to total Myc. Asterisk indicates a statistically significant increase in ubiquitylated Myc in OHT-treated DKO MycER MEFs compared with *p53*$^{-/-}$ MycER MEFs. (*B*) Cos7 cells were transfected with c-Myc and T7-Skp2 with increasing amounts of ARF. Coimmunopreciptiation was performed using anti-Myc (C-33) followed by immunoblot analysis using anti-Skp2. Immunoblot analysis was also performed using anti-MycN100, anti-ARF, and anti-Skp2. (*C*) HeLa cells were transfected with HA-ubiquitin and increasing amounts of Skp2. Endogenous c-Myc was subjected to in vivo ubiquitylation analysis. Immunoblot analysis was performed using anti-Skp2. (*D*) Cos7 cells were transfected with HA-ubiquitin and either empty vector or c-Myc167, with or without Skp2, and in vivo ubiquitylation analyses of Myc were performed. Immunoblot analysis was performed using anti-Skp2. Immunoblot analysis of Myc was performed using anti-MycN100 following immunoprecipitation with anti-Mycfl. (*E*) HeLa cells were transfected with HA-ubiquitin and Skp2 siRNA (siSkp2) or control siRNA (siCon). In vivo ubiquitylation analyses of endogenous Myc were performed. Immunoblot analysis of ARF was performed using human p14 ARF antibody.

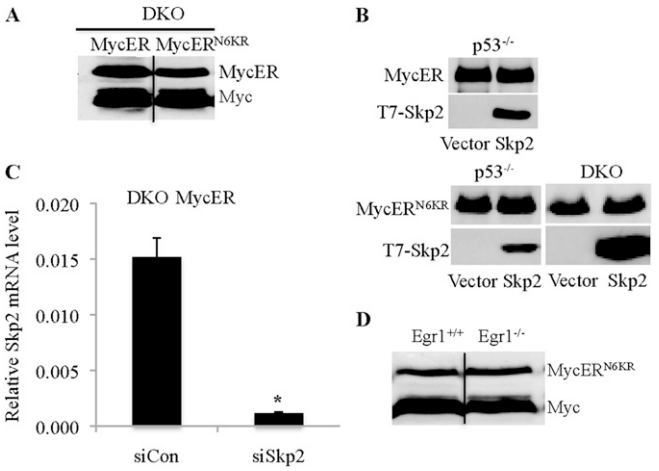


**Fig. S2.** Expression analyses of Myc and Skp2. (*A*) Immunoblot analysis of Myc expression in DKO MycER and MycER$^{N6KR}$ MEFs. (*B*) Immunoblot analysis of Myc and T7-Skp2 expression in *p53*$^{-/-}$ MycER/vector, *p53*$^{-/-}$ MycER/Skp2, *p53*$^{-/-}$ MycER$^{N6KR}$/vector, *p53*$^{-/-}$ MycER$^{N6KR}$/Skp2, DKO MycER$^{N6KR}$/vector, and DKO MycER$^{N6KR}$/Skp2 MEFs. (*C*) Real-time RT-PCR analysis of endogenous Skp2 mRNA expression in DKO MycER MEFs transfected with siCon or siSkp2. Asterisk indicates a statistically significant decrease in relative Skp2 mRNA level in cells treated with siSkp2 compared with siCon. (*D*) Immunoblot analysis of Myc expression in *Egr1*$^{+/+}$ and *Egr1*$^{-/-}$ MEFs.

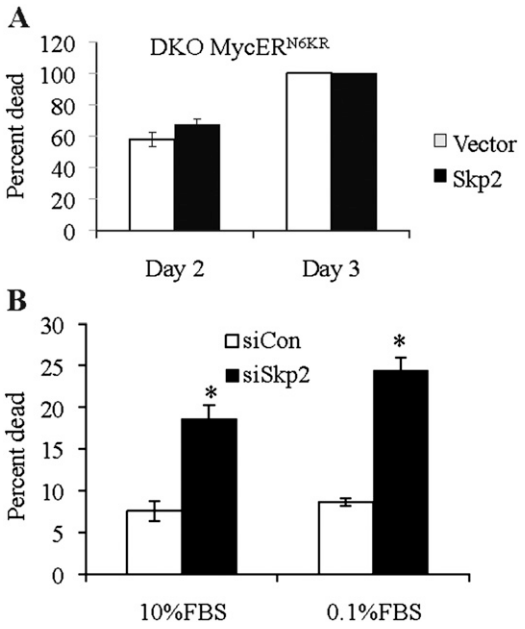


**Fig. S3.** Effects of Skp2 on apoptosis. (*A*) Apoptosis analyses of DKO MycER$^{N6KR}$/vector MEFs and DKO MycER$^{N6KR}$/Skp2 MEFs following OHT activation in low serum. (*B*) HeLa cells treated with siCon or siSkp2 for 24 h were assayed for apoptosis in both high serum and low serum 10 h after media change. Asterisks indicate statistically significant increases in cell death of HeLa cells treated with siSkp2 compared with siCon.

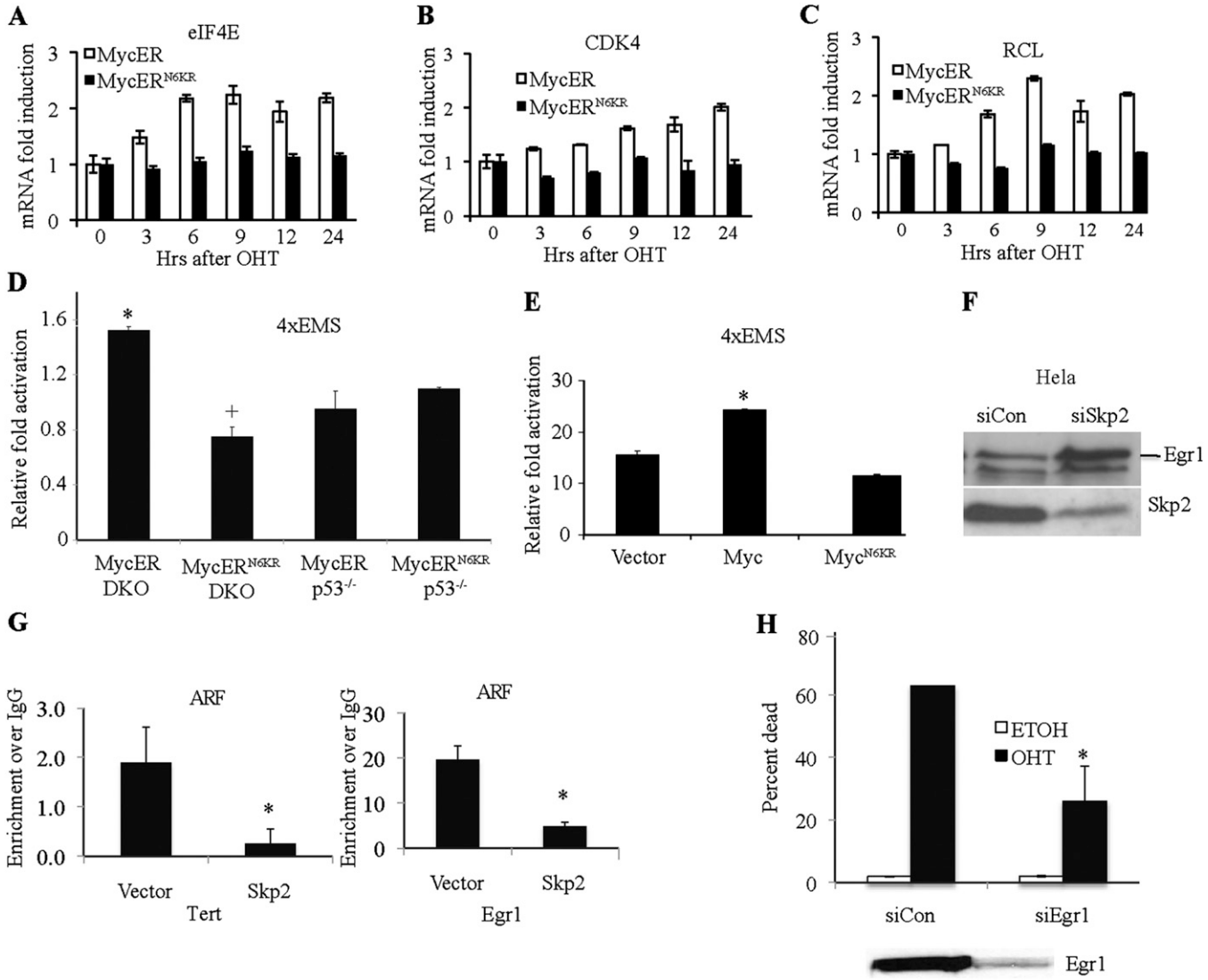


**Fig. S4.** Ubiquitylation controls c-Myc activity at canonical target promoters, as well as the noncanonical *Egr1* promoter, which is essential for MycER$^{N6KR}$-induced p53-independent apoptosis. (*A*–*C*) Real-time RT-PCR analysis of *eIF4E* (*A*), *cdk4* (*B*), and *rcl* (*C*) in DKO MycER and DKO MycER$^{N6KR}$ MEFs following OHT activation. Results are reported as the mean of the relative mRNA levels of the target genes to $\beta$-*actin* at each time point normalized to time 0 to give the relative fold induction ± SD. (*D*) Luciferase reporter analyses of 4XEMS promoter activity performed in DKO MycER, *p53*$^{-/-}$ MycER, and *p53*$^{-/-}$ MycER$^{N6KR}$ MEFs transfected with 4XEMS luciferase reporter and treated with 2 μM OHT for 6 h. Data are expressed in terms of relative fold activation (OHT/ethanol). Asterisk indicates data in which a statistically significant increase in luciferase activity was observed in OHT-treated cells compared with the ethanol control. (*E*) Luciferase reporter analyses of 4XEMS promoter activity performed in DKO MEFs transfected with 4XEMS luciferase reporter and either empty vector, Myc, or Myc$^{N6KR}$. Data are expressed as the mean of the relative luciferase reporter activity (firefly/Renilla luciferase). Asterisk indicates data in which a statistically significant increase in luciferase activity was observed in cells expressing Myc compared with the vector control. (*F*) HeLa cells were transiently transfected with siCon or siSkp2 for 48 h and then subjected to immunoblot analysis using anti-Egr1 and anti-Skp2. (*G*) ChIP analyses of ARF were performed using chromatin prepared from *p53*$^{-/-}$ MycER$^{N6KR}$/Skp2 and *p53*$^{-/-}$ MycER$^{N6KR}$/vector MEFs after activation with 2 μM OHT for 6 h. The chromatin was subjected to immunoprecipitation (IP) using anti-ARF or IgG followed by real-time RT-PCR. Results are reported as the mean of enrichment over IgG calculated by dividing the percent of input from the ARF IP by the percent of input from the IgG control ± SD. Asterisk indicates a statistically significant decrease in ARF binding at the target promoters in cells stably expressing T7-Skp2 compared with the vector control. (*H*) DKO MycER$^{N6KR}$ MEFs transfected with siCon or siEgr1 for 24 h were subjected to apoptosis analyses. Immunoblot analysis was performed using anti-Egr1. Asterisk indicates a statistically significant decrease in cell death with Egr1 siRNA (siEgr1) compared with siCon after treatment with OHT.