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# Probing tumor phenotypes using stable and regulated synthetic microRNA precursors

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**RNA interference is a powerful method for suppressing gene expression in mammalian cells. Stable knock-down can be achieved by continuous expression of synthetic short hairpin RNAs, typically from RNA polymerase III promoters. But primary microRNA transcripts, which are endogenous triggers of RNA interference, are normally synthesized by RNA polymerase II. Here we show that RNA polymerase II promoters expressing rationally designed primary microRNA–based short hairpin RNAs produce potent, stable and regulatable gene knock-down in cultured cells and in animals, even when present at a single copy in the genome. Most notably, by tightly regulating Trp53 knock-down using tetracycline-based systems, we show that cultured mouse fibroblasts can be switched between proliferative and senescent states and that tumors induced by Trp53 suppression and cooperating oncogenes regress upon re-expression of Trp53. In practice, this primary microRNA–based short hairpin RNA vector system is markedly similar to cDNA overexpression systems and is a powerful tool for studying gene function in cells and animals.**

The sequence specificity of RNA interference (RNAi) allows experimental gene inhibition by introduction of synthetic double-stranded RNA into cells. Stable suppression can be achieved by genomic integration, often through retroviral transduction, of expression cassettes producing short hairpin RNAs (shRNAs) that are processed into small RNA duplexes[1,2]. Early-design shRNAs have stems of 19–29 nucleotides (nt) with minimal flanking RNA sequence (**Fig. 1a**) and are traditionally expressed from strong RNA polymerase III (Pol III) promoters, such as those that drive expression of the endogenous U6 small nuclear RNA (snRNA) or the H1 RNA. This stem-loop style of shRNA can also be expressed from high-copy polymerase II (Pol II) promoters, but only in the context of a minimal RNA[3–6].

In contrast to early-design shRNAs, naturally occurring primary microRNA precursors fold extensively beyond the core stem-loop structure (**Fig. 1a**). Using the entire miR-30 precursor RNA as a template, Cullen and colleagues substituted miR-30 stem sequences and showed effective and regulated target gene inhibition in transient transfection assays[7,8]. Furthermore, miR-30–based shRNAs inhibit gene expression more potently than traditional stem-loop shRNAs, again in transient assays[9,10]. These observations prompted the construction of genome-wide libraries of rationally designed shRNAs based on the primary miR-30 RNA, which currently target more than 28,000 human genes and 25,000 mouse genes[10] (RNAi Codex; see URL in Methods). In this study, we found that miR-30–based shRNAs (called shRNA-mirs) could stably suppress gene expression when driven by Pol II promoters present at a single copy in the genome. Furthermore, we used tetracycline-responsive promoters to conditionally express shRNA-mirs *in vitro* and *in vivo*, illustrating the potential of this knock-down system for studying gene function.

## RESULTS

### Different styles of shRNA have distinct vector preferences

To determine whether shRNA-mirs could stably suppress gene expression in mammalian cells, we recovered a shRNA-mir called p53.1224 (the predicted small interfering RNA (siRNA) begins at nt 1,224 of the mouse *Trp53* cDNA) from the shRNA-mir library[10]. We subcloned a U6 promoter-p53.1224 cassette into a murine stem cell virus (MSCV) and a self-inactivating (SIN) retroviral vector, generating MSCV/LTR-U6miR30-PIG (LUMP)-p53.1224 or SIN-U6miR30-PIG (UMP)-p53.1224 constructs (**Fig. 1b**). We also constructed similar vectors expressing a standard stem-loop shRNA targeting *Trp53* (p53C)[11], producing MSCV/LTR-U6shRNA-PIG (LUSP)-p53C or SIN-U6shRNA-PIG (USP)-p53C constructs (**Fig. 1b**). We introduced all four constructs into early-passage mouse embryonic fibroblasts (MEFs). We assessed the resulting cell populations for knock-down of Trp53 expression after treatment with adriamycin (a DNA-damaging agent that stabilizes Trp53) and for the ability to form colonies when plated at low density (a functional read-out of Trp53 loss).

The MSCV-based p53.1224 shRNA-mir (LUMP-p53.1224) was more effective at suppressing Trp53 than its SIN-based counterpart (UMP-p53.1224), producing nearly undetectable Trp53 levels as assessed by immunoblotting (**Fig. 1c**). Conversely, the SIN-based p53C shRNA (USP-p53C) was more effective at suppressing Trp53 than its MSCV-derived counterpart (LUSP-p53C), verifying that the

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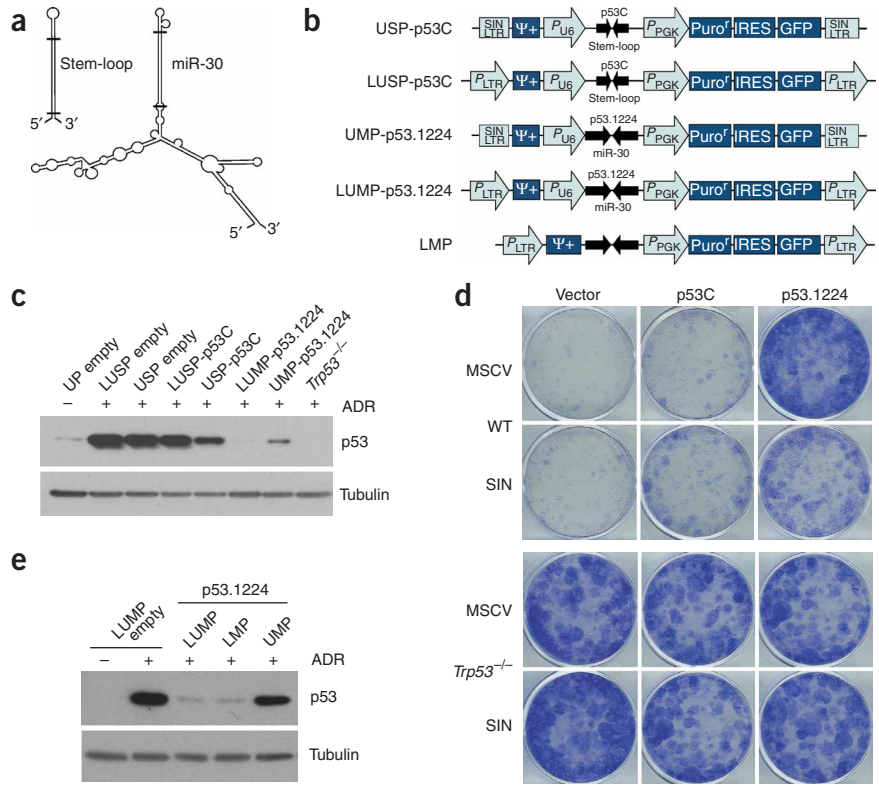


**Figure 1** Effective knock-down by single-copy expression of miR-30–based shRNAs from a retroviral LTR promoter. (**a**) Schematic representation of predicted RNA folds for simple stem-loop and miR-30–based shRNAs. Folds are based on the 29-nt stem-loop p53C shRNA[9] and the miR-30–based p53.1224 shRNA. Note extensive predicted folding for the ~300-nt miR-30–based RNA. Approximate Drosha and Dicer cleavage sites are indicated by horizontal lines. Folds were generated using mfold[35]. (**b**) Retroviral vectors used to deliver shRNAs to mammalian cells. Provirus layouts are shown to indicate promoter activity of the integrated virus. Active promoters are shown as open arrows, with two inverted black arrows representing shRNA stem sequences. (**c**) Western-blot analysis for Trp53 expression of NIH3T3 cells transduced with the retroviral vectors shown in **b** and selected in puromycin. A tubulin blot is shown as a loading control. ADR, adriamycin. (**d**) Colony formation assay for the cells shown in **c**. Cells were seeded in six-well plates at 2,500 cells per well and allowed to grow for 10 d before fixation. (**e**) Western-blot analysis for Trp53 expression in NIH3T3 cells transduced at less than 5% efficiency (assessed by GFP FACS; data not shown) with the retroviral vectors shown in **b**. A tubulin blot is shown as a loading control. Similar results were obtained in other cell types, including wild-type (WT) and *Cdkn2a*(p19ARF)-null MEFs (data not shown). ADR, adriamycin.



U6 promoter is sufficient for stem-loop shRNA expression (**Fig. 1c**). The ability of each vector to suppress Trp53 correlated precisely with its ability to stimulate colony formation at low density, with cells expressing the MSCV-based p53.1224 vector producing as many colonies as *Trp53*-null cells (**Fig. 1d**). This vector preference was also observed for several other shRNA-mirs and stem-loop shRNAs targeting diverse gene products (data not shown). As noted for stem-loop shRNAs expressed from Pol III promoters, however, not every shRNA-mir was effective at knocking-down target gene expression in the MSCV-based context (data not shown). Nevertheless, miR-30–based shRNAs can be potent when stably expressed from retroviral vectors, particularly those with a functional 5′ long terminal repeat (LTR).

### Effective shRNA-mir expression from a Pol II promoter

The more potent knock-down produced by shRNA-mirs expressed from the MSCV vector compared with the SIN vector implies that the 5′ LTR contributes to optimal shRNA-mir expression. To determine whether the LTR promoter, a strong Pol II promoter, is sufficient for effective gene knock-down using shRNA-mirs, we introduced the p53.1224 shRNA into an MSCV vector lacking a U6 promoter (MSCV/LTRmiR30-PIG (LMP); **Fig. 1b**). To facilitate comparison, we introduced this vector and its LUMP- and UMP-based counterparts into NIH3T3 cells at a low multiplicity of infection (<5% efficiency) such that most transduced cells contained single- or low-copy proviral integrations, as confirmed by Southern blotting (data not shown). Viral titers were similar for empty vector and shRNA-mir constructs (data not shown). Notably, both vectors carrying the MSCV LTR (LUMP-p53.1224 and LMP-p53.1224) suppressed Trp53 expression extremely efficiently and were far superior to UMP-p53.1224, which expresses p53.1224 from the U6 promoter alone (**Fig. 1e**). Therefore, transcription of shRNA-mir cassettes from Pol II promoters is sufficient for highly effective knock-down of a target gene, even when expressed at a single copy. This feature is essential for knock-down screens using complex libraries where infected cells are unlikely to contain multiple copies of a given shRNA vector. Because Pol II transcribes endogenous primary microRNAs[12,13], the improved performance of these vectors seems to derive from natural mechanisms of RNA-dependent gene inhibition.

Stable expression of stem-loop shRNAs can produce loss-of-function phenotypes in mice[11,14]. To determine whether shRNA-mirs expressed from Pol II promoters can efficiently modulate gene expression *in vivo*, we targeted genes for which the null phenotype was known. For example, inactivation of the BH3-only protein Bcl2l11 (also called Bim, a proapoptotic member of the Bcl2 family) accelerates lymphomagenesis in Eμ-Myc transgenic mice[15]. Therefore, we tested whether shRNA-mir–mediated suppression of Bim would also cooperate with Myc during lymphomagenesis. Mice reconstituted with Eμ-Myc hematopoietic stem cells (HSCs) transduced with two independent shRNA-mirs targeting Bim (collectively called shBim and expressed from the LTR of MSCV/LTRmiR30-SV40-GFP (LMS)) formed tumors much more rapidly than mice reconstituted with HSCs expressing a control vector ($P < 0.05$; **Fig. 2a**). Lymphomas arising in mice transduced with shBim were green fluorescent protein (GFP)-positive, expressed low levels of Bim (**Fig. 2b**) and had a mature ($IgM^+$) B-cell phenotype uniquely characteristic of Bim-deficient lymphomas[15] (data not shown). Therefore, *in vivo* loss-of-function phenotypes can be recapitulated using miR-30–based shRNAs expressed from Pol II promoters.

siRNAs have been used to identify modulators of drug action but are not suitable for long-term assays or *in vivo* systems. In principle, miR-30–based vectors should enable the identification and characterization

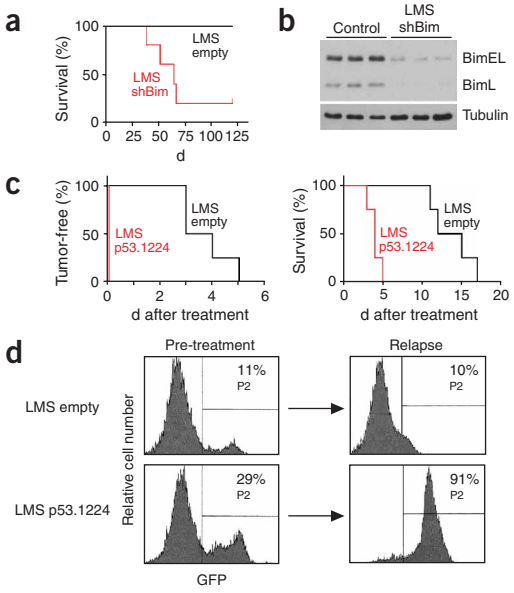


**Figure 2** RNA Pol II–driven shRNAs can effectively promote tumorigenesis and chemotherapy resistance *in vivo*. (**a**) Kaplan-Meier curve showing mouse survival after adoptive transfer of Eμ-Myc HSCs infected with LTR-driven Bim shRNAs. (**b**) Western blot showing reduced expression of two Bim protein isoforms, BimEL and BimL, in Eμ-Myc lymphomas expressing Bim shRNAs. Archived tumors arising from Eμ-Myc HSCs (on a wild-type, *Cdkn2a*(p19ARF)$^{+/-}$ or *Trp53*$^{+/-}$ background; data not shown) were used as controls for Bim expression. (**c**) Kaplan-Meier curves showing tumor-free survival (left) and overall survival (right) for mice carrying *Cdkn2a*(p19ARF)-null lymphomas infected with either the LMP-p53.1224 retrovirus or vector control. Tumor-bearing mice were given a single dose of 10 mg adriamycin per kg body weight at day zero. (**d**) Flow cytometry analysis of GFP expression in lymphoma cells from the mice in **c**. Representative histograms show the percent of GFP-positive cells at the time of treatment (left) and after tumor relapse (right).

of genes that modify drug responses *in vivo*. To test this possibility, we examined the ability of a Trp53 shRNA-mir to promote chemoresistance in Eμ-Myc lymphomas, which respond poorly to therapy in the absence of *Trp53* (ref. 16). We introduced LMS-p53.1224 (coexpressing GFP) into chemosensitive Eμ-Myc lymphoma cells at ~10% infection efficiency and transplanted the mixed cell populations into syngeneic recipient mice. Upon lymphoma manifestation, we treated mice with the chemotherapeutic drug adriamycin and monitored their tumor response. Notably, mice carrying lymphomas expressing the p53.1224 shRNA-mir did not regress after treatment with adriamycin and had significantly lower overall survival than control tumor-bearing mice (**Fig. 2c**). Furthermore, the percentage of GFP-positive cells markedly increased in lymphomas carrying p53.1224 but not the control vector, indicative of a selective advantage for cells expressing p53.1224 (**Fig. 2d**). Despite extensive previous efforts with stem-loop shRNAs (data not shown), this is the first time to our knowledge that sufficient Trp53 knock-down to promote chemoresistance *in vivo* has been

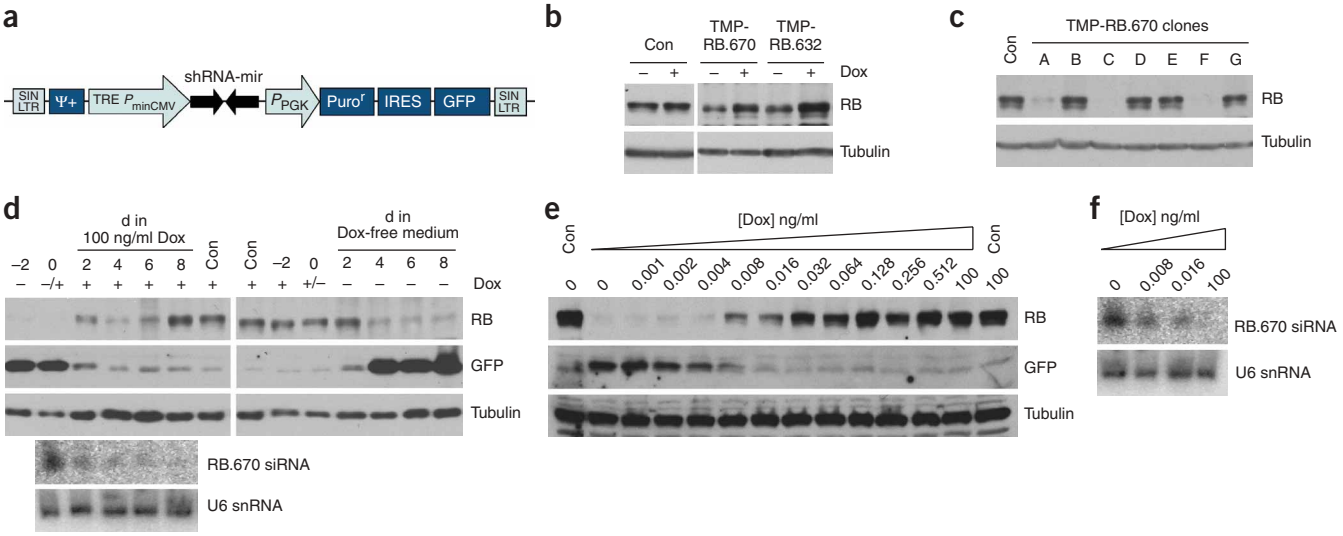


**Figure 3** Stable and regulatable shRNA expression from a tetracycline-responsive RNA Pol II promoter. (**a**) Provirus layout of the SIN-TREmiR30-PIG (TMP) retroviral vector. (**b**) Western-blot analysis of RB1 expression in HeLa-tTA cells infected with TMP-RB.670. Cells were treated with 100 ng ml$^{-1}$ doxycycline (Dox) for 4 d before collection. Control uninfected HeLa-tTA cells (Con) treated with doxycycline are also shown. (**c**) RB1 expression in homogeneous cultures derived from single-cell clones of HeLa-tTA cells infected at single-copy with TMP-RB.670. Cells were cultured in normal, doxycycline-free medium before collection. Con, control uninfected HeLa-tTA cells. (**d**) RB1 and RB.670 siRNA expression in HeLa-tTA clone RB.670C cells over time in response to shifting into or out of doxycycline (Dox). Cells were cultured without doxycycline (left panels) or in 100 ng ml$^{-1}$ doxycycline (right panels) for 8 d before being shifted into 100 ng ml$^{-1}$ doxycycline or doxycycline-free medium, respectively. Note the presence of a faint nonspecific band in the GFP immunoblot (longer exposures showed weak GFP expression, probably produced by the PGK-driven transcript). Similar results were observed for all RB.670 clones showing good RB1 knock-down in doxycycline-free medium (**c**, above), with some clonal variation in kinetics. In addition, similar results were obtained using other shRNA-mirs targeting *RB1* and *Pten* (data not shown). Lower panels show small RNA northern blots detecting the RB.670 siRNA, correlating with RB1 knock-down. U6 snRNA was used as a loading control. Con, control uninfected HeLa-tTA cells. (**e**) Doxycycline (Dox) dose-response analysis of RB1 expression in HeLa-tTA clone RB.670C. Cells were cultured for 8 d in the indicated doxycycline concentration before collection. Control uninfected HeLa-tTA cells (Con) cultured with or without doxycycline are also shown. Note the presence of a nonspecific band in the GFP immunoblot, running just below GFP. (**f**) RB.670 siRNA production in HeLa-tTA clone RB.670C cells cultured in the indicated concentration of doxycycline (Dox), determined by small RNA northern blotting with U6 snRNA as a loading control.

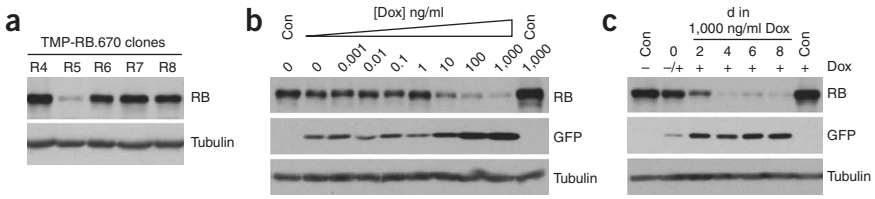


**Figure 4** Regulated shRNA expression using a tetracycline-on system. (**a**) RB1 expression in homogeneous cultures derived from single-cell clones of U2OS-rtTA cells infected at ~1% efficiency with TMP-RB.670. Cells were cultured in 1,000 ng ml$^{-1}$ doxycycline for several days before collection. (**b**) Doxycycline (Dox) dose-response analysis of RB1 expression in U2OS-rtTA clone RB.670R5 cells. Cells were cultured for 8 d in the indicated doxycycline concentration before collection. Control uninfected U2OS-rtTA cells (Con) cultured with or without doxycycline are also shown. (**c**) RB1 expression in U2OS-rtTA clone RB.670R5 cells in response to doxycycline treatment. Cells were cultured without doxycycline (Dox) for 8 d before being shifted into 1,000 ng ml$^{-1}$ doxycycline. RB.670R5 cells express some GFP in doxycycline-free medium, and RB1 levels are slightly decreased compared with controls, indicating slightly leaky expression from the TRE-CMV promoter in this particular clone. Con, control uninfected U2OS-rtTA cells.

achieved. Taken together, these data indicate that miR-30–based shRNAs expressed from Pol II promoters are suitable for a variety of *in vivo* applications, perhaps including tissue specific gene knock-downs and *in vivo* forward genetic screens.

### Tetracycline-regulated shRNA-mir expression

Inducible expression of protein-coding cDNAs in cultured cells and animals has revolutionized analysis of gene function, and reversible loss-of-function systems using regulated shRNA expression hold similar promise. Simple stem-loop shRNAs can be expressed conditionally from modified Pol III promoters[17–24], but the inducibility of these systems is variable and has not been shown *in vivo*. Given that low-copy Pol II promoters can effectively drive expression of shRNA-mirs, we reasoned that the widely used tetracycline-regulated Pol II promoter TRE-CMV[25] could be adapted to stably express shRNA-mirs. Using a SIN vector backbone, we cloned a shRNA-mir targeting human *RB1* (RB.670) downstream of the TRE-CMV promoter, producing SIN-TREmiR30-PIG (TMP-RB.670; **Fig. 3a**). We transduced HeLa cells stably expressing the tetracycline transactivator protein tTA (tetracycline-off) with TMP-RB.670 at a low multiplicity of infection (<10% efficiency) and expanded them in the absence of the tetracycline analog doxycycline. Although RB1 levels were only modestly

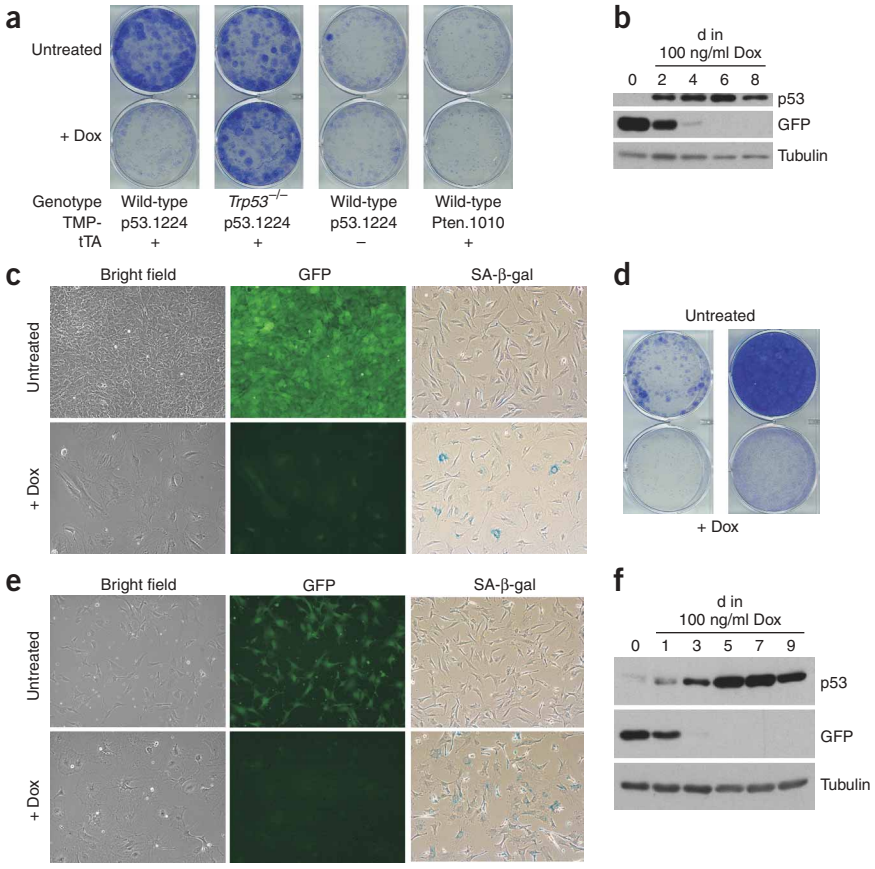


**Figure 5** Reversible Trp53 knock-down in primary MEFs. (**a**) Colony formation assays of wild-type MEFs doubly infected with TMP-p53.1224 and tTA. Cells were seeded in six-well plates at 5,000 cells per well and grown for 8 d before collection. Upper wells contained doxycycline-free medium, whereas lower wells contained 100 ng ml$^{-1}$ doxycycline (Dox). Positive control *Trp53*-null MEFs are shown, as are negative control wild-type MEFs infected with TMP-p53.1224 alone or with TMP-Pten.1010 plus tTA. (**b**) Western-blot analysis of Trp53 and GFP expression in cells expanded from a single-cell clone of wild-type MEFs infected with TMP-p53.1224 and tTA (WtT cells). Cells were cultured in 100 ng ml$^{-1}$ doxycycline (Dox) for various times before collection. (**c**) Morphology and GFP fluorescence of WtT-p53.1224 cells originally plated at colony formation density and cultured in doxycycline-free medium (upper panels) or 100 ng ml$^{-1}$ doxycycline (Dox; lower panels). Right, SA-β-gal staining of WtT-p53.1224 cells cultured in doxycycline-free medium (upper panel) or 100 ng ml$^{-1}$ doxycycline (lower panel). (**d**) Left, colony formation assay for WtT-p53.1224 cells cultured for 8 d in 100 ng ml$^{-1}$ doxycycline (Dox) then seeded in doxycycline-free medium (upper well) or 100 ng ml$^{-1}$ doxycycline (lower well). Right, colony formation assay of cells equivalent to those in the upper well of the left panel (formerly doxycycline-treated, dormant WtT-p53.1224 cells after extended culture in doxycycline-free medium). Cells were seeded and collected as described in **a**. (**e**) Morphology, GFP fluorescence and SA-β-gal staining of WtT-p53.1224 cells infected with Kras and cultured in normal medium (upper panels) or 100 ng ml$^{-1}$ doxycycline (Dox; lower panels). (**f**) Western-blot analysis of Trp53 and GFP expression in WtT-p53.1224 cells infected with Kras. Cells were cultured in 100 ng ml$^{-1}$ doxycycline (Dox) for various times before collection.

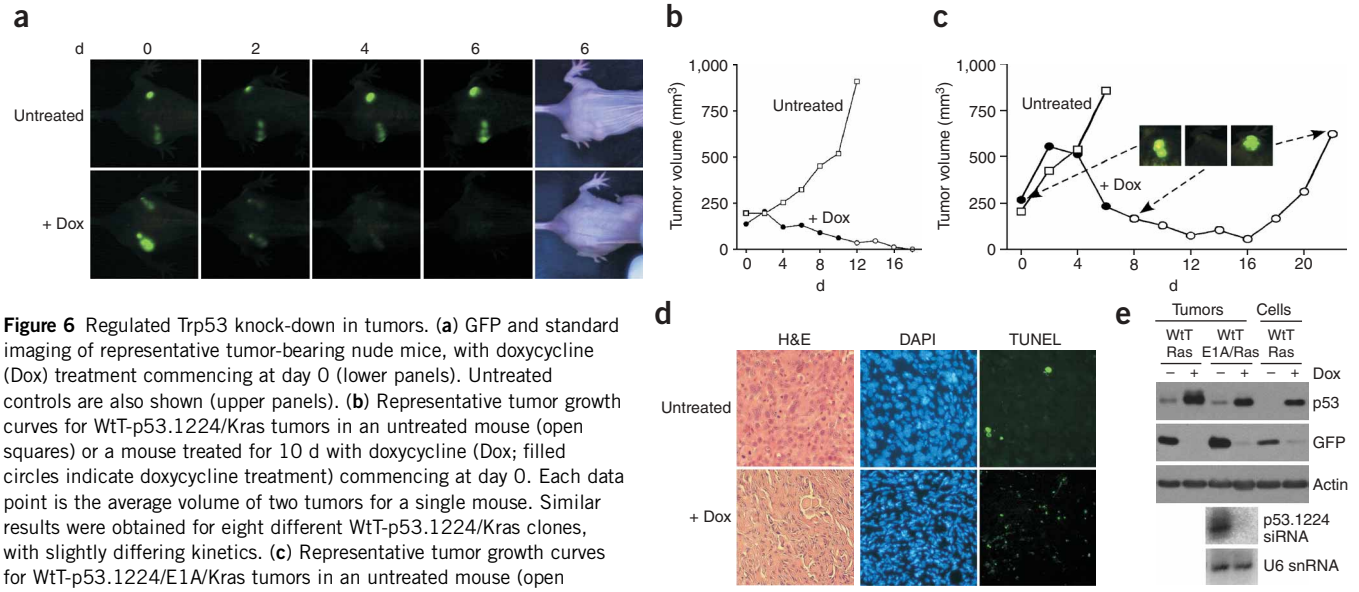


**Figure 6** Regulated Trp53 knock-down in tumors. (**a**) GFP and standard imaging of representative tumor-bearing nude mice, with doxycycline (Dox) treatment commencing at day 0 (lower panels). Untreated controls are also shown (upper panels). (**b**) Representative tumor growth curves for WtT-p53.1224/Kras tumors in an untreated mouse (open squares) or a mouse treated for 10 d with doxycycline (Dox; filled circles indicate doxycycline treatment) commencing at day 0. Each data point is the average volume of two tumors for a single mouse. Similar results were obtained for eight different WtT-p53.1224/Kras clones, with slightly differing kinetics. (**c**) Representative tumor growth curves for WtT-p53.1224/E1A/Kras tumors in an untreated mouse (open squares) or a mouse treated for 7 d with doxycycline (Dox; filled circles indicate doxycycline treatment) commencing at day 0. Each data point is the average volume of two tumors for a single mouse. Insets show GFP status of a single tumor at various times. (**d**) Histological analysis of cell morphology and apoptosis in representative nude mouse tumors from untreated mice or mice treated with doxycycline (Dox) for several days. H&E, hematoxylin and eosin. (**e**) Western-blot analysis of Trp53 and GFP expression in representative WtT-p53.1224/Kras and WtT-p53.1224/E1A/Kras tumors from untreated mice or mice treated with doxycycline (Dox) for several days. Cultured WtT-p53.1224/Kras cells treated with doxycycline are shown as a control.



suppressed in cell populations (**Fig. 3b**), 6 of 13 single-cell clones isolated from these populations showed excellent RB1 knock-down (**Fig. 3c** and data not shown), showing that the TRE-CMV promoter can effectively drive shRNA-mir expression at low copy number.

For all clones showing RB1 knock-down in the absence of doxycycline, RB1 levels returned to normal after approximately one week of treatment with doxycycline (**Fig. 3d** and data not shown). RB1 expression was dose-dependent, with maximum RB.670 siRNA production and RB1 knock-down achieved in low doxycycline concentrations and vice versa (**Fig. 3e**,**f**). In all cases, GFP and RB.670 siRNA production were positively correlated (**Fig. 3d**,**f**), whereas GFP and RB1 levels were inversely correlated (**Fig. 3d**,**e**), implying that, in this system, GFP expression serves as a surrogate marker of shRNA production. As expected, when we introduced TMP-RB.670 into U20S cells stably expressing the reverse tTA (rtTA; tetracycline-on) protein[26], doxycycline concentration and RB1 knock-down were positively correlated in a subset of single-cell clones (**Fig. 4**). Therefore, expression of shRNA-mir cassettes from the TRE-CMV promoter allows regulated gene knock-down in tetracycline-on or -off configurations.

We went on to test regulated gene knock-down in primary cells. Inactivation of the tumor suppressor *Trp53* immortalizes wild-type MEFs and transforms MEFs overexpressing oncogenic Kras[27]. We cotransduced early-passage MEFs with TMP-p53.1224 and a retrovirus expressing the tTA (tetracycline-off) protein. We reasoned that many doubly infected MEFs (called wild-type/tTA/TMP-p53.1224 or WtT-p53.1224) would show stable Trp53 knock-down when cultured in doxycycline-free medium. Indeed, WtT-p53.1224 cells plated at low density formed colonies as efficiently as *Trp53*-null MEFs (**Fig. 5a**), suggesting that Trp53 was functionally inactivated in most cells. In stark contrast, colony formation of WtT-p53.1224 cells cultured in doxycycline in parallel was similar to that of control cells (**Fig. 5a**). Growth of *Trp53*-null MEFs was unaffected by doxycycline, ruling out nonspecific effects (**Fig. 5a**). We observed similar results in single-cell clones, where doxycycline treatment produced a rapid reactivation of Trp53 and loss of GFP expression and clonogenic potential (**Fig. 5b**,**c**). The cell cycle arrest produced by Trp53 reactivation was associated with a flattened morphology and senescence-associated β-galactosidase (SA-β-gal) staining (**Fig. 5c**), indicating that loss of Trp53 is required for maintaining the immortalized state.

Because clonogenic assays are highly sensitive to 'leakiness' in the system, the data described above indicate that the shRNA-mir construct was under very tight control. Accordingly, WtT-p53.1224 clones maintained in doxycycline remained dormant for many weeks, retaining high Trp53 and low GFP expression (data not shown). Nevertheless, these senescent cells reacquired GFP expression and resumed proliferating shortly after removal of doxycycline[28] (**Fig. 5d**), a state that could be completely reversed by readdition of doxycycline (**Fig. 5d**). Even WtT-p53.1224 cells transformed by coexpression of oncogenic Kras (**Fig. 5e**) became morphologically senescent and SA-β-gal–positive when treated with doxycycline (**Fig. 5e**), with Trp53 and GFP expression changes similar to those observed in parental WtT cells (**Fig. 5f**). These data indicate that cells can be reversibly switched between cycling and senescent states simply by regulating Trp53 knock-down and that ongoing Trp53 inactivation is required for immortalization and transformation of MEFs *in vitro*. Because complete *Trp53* inactivation is strongly selected for in these models, our ability to re-establish senescence in entire cell populations using the tetracycline-controlled p53.1224 vector highlights the efficiency and tight regulation of the TRE-CMV shRNA-mir system.

### Control of tumor growth by regulating *Trp53* knock-down

Tetracycline-regulated systems expressing protein-coding cDNAs have been used to study the requirement for continued oncogene expression in tumor maintenance[29]. To test whether tetracycline-controlled expression of shRNA-mir cassettes might enable a similar analysis of

tumor-suppressor genes, we examined the requirement for Trp53 inactivation in tumors derived from WtT-p53.1224/Kras MEFs. We injected these transformed cells subcutaneously into the flanks of nude mice, where they formed visible, rapidly growing and strongly GFP-positive tumors after ~2 weeks (**Fig. 6a**). In mice treated with doxycycline in their drinking water, however, tumor GFP intensity was markedly and rapidly diminished compared with untreated mice, and after 4 d, most tumors were GFP-negative (**Fig. 6a**). Notably, tumor growth slowed upon doxycycline treatment, and mice treated with doxycycline for 10 d showed tumor regression and eventually became tumor-free (**Fig. 6b**).

Tumors obtained from doxycycline-treated mice contained cells with unusually compact nuclei and had widespread apoptosis compared with doxycycline-free controls (**Fig. 6d**). Their regression was Trp53-dependent, as Trp53 levels were rapidly restored after doxycycline addition (**Fig. 6e**), and tumors derived from *Trp53*-null MEFs infected with tTA, TMP-53.1224 and Kras lost GFP expression but continued to grow upon doxycycline administration (data not shown). Northern-blot analysis of RNA isolated from tumors showed tight regulation of the p53.1224 siRNA by doxycycline *in vivo* (**Fig. 6e**). In many cases, often when the initial tumors were small, doxycycline-treated mice became tumor-free and remained so for weeks, even upon doxycycline removal (data not shown). But removing doxycycline from mice with larger tumors or after brief doxycycline treatment often allowed renewed GFP expression and tumor growth (**Fig. 6c**). We obtained similar results for several WtT-p53.1224/Kras clones and WtT-p53.1224 cells transformed by adenovirus E1A and Kras, with variable tumor growth rates and regression kinetics (data not shown). Therefore, despite the genomic instability that accompanies *Trp53* loss[30], ongoing Trp53 inactivation is a requirement for sustained tumor growth in these models. These findings highlight the potential of this technology for the study of many aspects of biology, including identification or validation of potential drug targets in animal models.

## DISCUSSION

We generated an integrated system that exploits synthetic primary microRNAs expressed from Pol II promoters to stably and conditionally knock-down genes of interest in mammalian cells. This potent gene knock-down system can be used to study a variety of *in vitro* and *in vivo* processes, including cancer phenotypes such as tumorigenesis and treatment response. Furthermore, extremely tight conditional gene knock-down can be achieved by expressing shRNA-mir cassettes using tetracycline-responsive systems, including lentiviral systems[31]. As shown here, this facilitates analysis of the role of tumor-suppressor gene expression in the maintenance of immortalized or transformed states and in continued tumor growth *in vivo*. Finally, stable and regulatable gene knock-down can occur when a single copy of the Pol II promoter–shRNA-mir cassette is present in the genome, which is essential for complex library screens and should facilitate many *in vivo* applications.

Our data suggest that gene knock-down by expression of shRNA-mirs may, in practice, be very similar to overexpression of protein-coding cDNAs. Therefore, expression systems allowing targeted, regulated and tissue-specific expression, which have traditionally been limited to gene overexpression studies, may now be adapted for loss of function analyses. As all vectors described in this work are compatible with genome-wide, sequence-verified banks of miR-30–based shRNAs targeting human and mouse genes[10], they create a important resource for diverse, large-scale RNAi studies in mammalian systems.

## METHODS

**Vectors.** The retroviral vector MSCV-PIG[11] has an *Eco*RI site in the polylinker and another between the PuroR cassette and the IRES sequence. To facilitate cloning into the polylinker, we destroyed the second site using a PCR-based strategy. We generated a PCR product using MSCV-PIG template (primer sequences available on request), digested it with *Bgl*II and *Mfe*I and cloned it into MSCV-PIG digested with *Bgl*II and *Eco*RI, yielding MSCV-PIGdRI. We made MSCV-U6miR30-PIG by ligating the 762-bp *Bam*HI-*Mfe*I U6-miR30 context cassette from pSM2 (refs. 10,32) into MSCV-PIGdRI digested with *Bgl*II and *Eco*RI. We made MSCV-LTRmiR30-PIG by ligating the 256-bp *Sal*I-*Mfe*I miR30 context cassette from pSM2 into MSCV-PIGdRI digested with *Xho*I and *Eco*RI. We made MSCV-LTRmiR30-SV40GFP (LMS) in two steps. First, we ligated an *Eco*RI-*Cla*I SV40GFP fragment of ~1.2 kb from pBabeGFP into MSCV-puro (Clontech) digested with *Eco*RI and *Cla*I, forming MSCV-SV40GFP. We then digested this with *Xho*I and *Eco*RI and inserted the 256-bp *Sal*I-*Mfe*I miR30 context cassette from pSM2, forming MSCV-LTRmiR30-SV40GFP. We made SIN-PIGdRI by ligating the 2,524-bp *Eco*RI-*Sal*I fragment from MSCV-PIGdRI into pQCXIX (Clontech) digested with *Eco*RI and *Xho*I. We made SIN-TREmiR30-PIG in two steps. First, we generated a PCR product spanning the TRE-CMV promoter using template plasmid pQTXIX (a gift from A. Malina, McGill University, Montreal, Canada), generated by cloning the *Xba*I-*Eco*RI TRE-CMV promoter fragment from pUHD10.3 (ref. 25) into pQCXIX (Clontech) digested with *Xba*I and *Eco*RI (primer sequences available on request). We then digested this PCR product with *Bgl*II and *Mfe*I and ligated it into SIN-PIGdRI digested with *Bgl*II and *Eco*RI (removing its CMV promoter), forming SIN-TRE-PIG. We completed SIN-TREmiR30-PIG by ligating the 256-bp *Sal*I-*Mfe*I miR30 context cassette from pSM2 into SIN-TRE-PIG digested with *Xho*I and *Eco*RI. We generated DNA fragments encoding various shRNA-mir folds using oligonucleotide template PCR as described previously[32] or subcloned them as 110-bp *Xho*I-*Eco*RI fragments from the pSM2 shRNA-mir library[10]. Template sequences are available on request. We designed oligonucleotides using RNAi Central. We digested PCR products with *Xho*I and *Eco*RI and ligated them into the unique *Xho*I and *Eco*RI sites in the miR30 context in the vectors described above.

**Cell culture and expression analysis.** We grew cells in Dulbecco's modified Eagle medium containing 10% fetal bovine serum at 37 °C with 7.5% $CO_2$. We dissolved doxycycline (Clontech) in water and generally used it at a final concentration of 100 ng $ml^{-1}$. We refreshed medium containing doxycycline every 2 d. We carried out infections and colony formation assays as previously described[11]. We detected SA-β-gal activity as previously described[27], with sample equilibration and X-gal staining done at pH 5.5. For western-blot analysis, we separated Laemmli buffer protein lysates by SDS-PAGE and transferred them to Immobilon PVDF membrane (Millipore). We used antibodies against p53 (1:1,000 IMX25, Vector Laboratories), Pten (1:1,000 486, a gift from M. Myers, Cold Spring Harbor Laboratory, New York), GFP (1:5,000, Clontech), tubulin (1:5,000 B-5-1-2, Sigma), actin (1:5,000, Sigma) and RB (1:1,000 G3-245, Becton Dickinson with 1:100 XZ-55 and C36 hybridoma supernatants). To isolate total RNA, we lysed cultured cells in 1 ml of Trizol (Invitrogen). We separated 30 μg of RNA on a denaturing acrylamide gel, transferred it to Hybond N+ membrane (Amersham) and probed it with end-labeled oligonucleotides complementary to predicted siRNAs.

**Lymphoma studies.** We carried out Eμ-Myc lymphomagenesis and drug treatment studies as previously described[11,33]. We isolated chemosensitive lymphoma cells from tumors arising in mice transplanted with Eμ-Myc *Cdkn2a*(p19ARF)$^{+/-}$ HSCs, which invariably lose the wild-type *Cdkn2a* allele but retain wild-type *Trp53* (ref. 34). The Cold Spring Harbor Laboratory Animal Care and Use Committee approved all mouse experiments described in this work.

**Nude mouse tumor analysis.** We injected ~$10^6$ transformed cells subcutaneously into the two rear flanks of nude mice. We treated mice with 0.2 mg $ml^{-1}$ doxycycline in a 0.5% sucrose solution in light-proof bottles, refreshed every 4 d. We calculated tumor volume ($mm^3$) as length × width$^2$ × π/6. For analysis of protein expression, we snap-froze tumors and pulverized them in liquid nitrogen using a mortar and pestle. We lysed powdered tumor in Laemmli buffer or Trizol for western or northern blotting, respectively. For

histology, we fixed tumor tissue for 24 h in 4% formaldehyde in phosphate-buffered saline before embedding and sectioning it. We measured apoptosis by TUNEL assay (In situ Cell Death Detection Kit, POD; Roche).

**URLs.** RNAi Codex is available at http://codex.cshl.edu/. Oligonucleotides were designed using RNAi Central (http://katahdin.cshl.org/siRNA/RNAi.cgi?type=shRNA).

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**COMPETING INTERESTS STATEMENT**

The authors declare that they have no competing financial interests.

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