A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells

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The advent of RNA interference has led to the ability to interfere with gene expression and greatly expanded our ability to perform genetic screens in mammalian cells. The expression of short hairpin RNA (shRNA) from polymerase III promoters can be encoded in transgenes and used to produce small interfering RNAs that down-regulate specific genes. In this study, we show that polymerase II-transcribed shRNAs display very efficient knockdown of gene expression when the shRNA is embedded in a microRNA context. Importantly, our shRNA expression system [called PRIME (potent RNA interference using microRNA expression) vectors] allows for the multicistronic cotranscription of a reporter gene, thereby facilitating the tracking of shRNA production in individual cells. Based on this system, we developed a series of lentiviral vectors that display tetracycline-responsive knockdown of gene expression at single copy. The high penetrance of these vectors will facilitate genomewide loss-of-function screens and is an important step toward using bar-coding strategies to follow loss of specific sequences in complex populations.

genetic screen | lentivirus | MAGIC

A new era of eukaryotic genetics began with the discovery of RNA interference (RNAi), in which dsRNA introduced into cells silences gene expression of the homologous gene (1, 2). Upon cell entry, the dsRNA is cleaved by the nuclease Dicer into double-stranded small interfering RNAs (siRNAs) of 21 nt length with a two-base 3′ overhang. These siRNAs are recognized by the RNA-induced silencing complex (RISC), and assembly of one siRNA strand into RISC is used to identify complementary mRNAs, thereby targeting them for destruction by a second nuclease in the pathway, Ago 2 (reviewed in ref. 3). In this manner, RNAi allows for the sequence-specific destruction of mRNAs expressed from both alleles in diploid organisms. Thus, mutant phenotypes can be generated and explored in diploids with a speed and precision never before possible.

The discovery of RNAi has led to the development of methods to exploit these findings for high-throughput genetic analyses (1, 2). Whereas RNAi with long dsRNAs works well for *Caenorhabditis elegans* and *Drosophila melanogaster*, it is toxic in most mammalian cells, presumably because of the activation of antiviral responses (1, 2). This toxicity was circumvented when it was discovered that the siRNAs generated by Dicer cleavage were able to target complementary cellular mRNAs for destruction but small enough to evade significant detection by the antiviral responses (4). A second important development was the finding that siRNAs could be genetically encoded in an organism by expressing a short hairpin RNA (shRNA), consisting of a sequence of 21–29 nt, a short loop region, and the reverse complement of the 21- to 29-nt region driven by a polymerase (pol) III promoter such as U6 or H1 (reviewed in refs. 3 and 5). When transcribed *in vivo*, this short transcript folds back on itself to form a hairpin structure, which is converted by endogenous nucleases (in a manner that is not yet clear) into short RNAs that are recognized by the RNA-induced silencing complex and used to target mRNAs for destruction.

The ability to genetically encode shRNAs in cells has led to the generation by our laboratories and others of collections of shRNAs expressed from retroviruses that cover large numbers of mammalian transcripts, with the ultimate goal of targeting every transcript encoded in the genome of the organism of choice (6, 7). These shRNA libraries have been used to perform genetic screens in tissue culture cells for a variety of phenotypes including cell transformation and others (6–8). Although the current shRNA expression systems show great promise, their broad applicability is often constrained by a number of limitations. First, most vector systems transcribe the shRNA constructs under the control of pol III promoters, which are constitutively expressed in all cell types. There are many examples where tissue-specific expression of shRNAs (using cell type-specific pol II promoters) would be desired. Second, many genes are essential, and therefore constitutively expressed constructs are toxic to the transduced cells. Having the ability to control the timing and levels of shRNA expression would be extremely valuable for a variety of experimental designs and would allow for strict isogenicity of control experiments. Finally, one of the key advances in the use of the vector-based shRNA technology is the ability to use bar coding to deconvolute complex libraries of virally encoded shRNA for phenotypes of interest (1). However, this ability rests on the assumption that each viral construct for a given shRNA will have high penetrance in knocking down its intended target at single copy.

A number of systems have recently been developed to attempt regulated shRNA expression from pol II or pol III promoters (9–13). Upon closer inspection, it becomes apparent that these systems relied either on transient expression of the vectors or the integration of multiple copies of the regulatable shRNA construct (ranging from 10 to >100). Importantly, none of the systems hitherto described has been shown to generate high penetrance regulatable knockdown at single copy. In this study, while attempting to use a cre–lox-based inducible shRNA system, we discovered an efficient method to express microRNA (miR)30-based shRNAs from pol II promoters. Based on this finding, we developed a series of lentiviral vectors (pPRIME, potent RNAi using miR expression) that provide high penetrance regulatable knockdown at single copy. In addition, pPRIME allows for the tracking of shRNA expression with a variety of reporter genes. The pPRIME vectors should facilitate

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Abbreviations: RNAi, RNA interference; siRNA, small interfering RNA; miR, microRNA; pPRIME, potent RNAi using miRNA expression; pol, polymerase; shRNA, short hairpin RNA; DOX, doxycycline; Rb, retinoblastoma; MOI, multiplicity of infection; Tet, tetracycline; tTA, Tet activator; TREX, Tet-repressor-based expression system; LNGFR, low-affinity nerve growth factor receptor; Neo, neomycin.

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functional genomic applications in mammals and provide sufficient penetrance for the use of bar-coding strategies to deconvolute complex libraries of virally encoded shRNAs.

Materials and Methods

Generation of Plasmids. The miR30-based retinoblastoma (Rb) targeting sequence (5′-AGCAGTTCGATATCTACTGAAA-3′) was designed by using the RNAi design algorithm at http://katahdin.cshl.org:9331/siRNA/RNAi.cgi?type=shRNA and cloned into pSM2 as described (14). To generate pSico-miR30, the PheS-containing mating recipient cassette (15) was PCR-cloned into the NotI and XhoI site of pSico, and pSM2-Rb was mated with the pSico–PheS recipient by using the MAGIC cloning protocol (15), yielding pSico–miR30–Rb. To generate the inducible pPRIME vectors, we replaced the CMV promoter (XbaI/Age1) with the tetracycline (TET) (pTRE-Tight, Clontech) or Tet-repressor-based expression system (TREX) promoter (pcDNA/TO/lacZ, Invitrogen) in pSico–miR30. The neomycin (Neo), internal ribosome entry site–Neo, and low-affinity nerve growth factor receptor (LNGFR) coding regions were PCR-cloned from pMSCVneo (Clontech), pQCXIN (Clontech), and pMACS LNGFR (Miltenyi Biotec, Auburn, CA), respectively. Detailed vector maps and sequence information are available on request.

Cell Culture. HeLa Tet-OFF and 293 TREX cells were cultivated in DMEM supplemented with 10% Tet-approved FBS. U2OS Tet-ON and U2OS Tet-OFF cells were cultured in McCoys medium supplemented with 10% Tet-approved FBS. Doxycycline (DOX) was used at a concentration of 1 μg/ml unless otherwise specified in the figure legends.

Virus Generation and Infection. Lentiviruses were generated by cotransfecting 15 μg of lentiviral vector and 7.5 μg of each packaging vector (coding for Gag, Pol, Tat, Rev, and VSVG) in 293T cells by using calcium phosphate reagent (Clontech). Supernatants were collected 48 h after transfection, filtered through a 0.4-μm membrane, and used directly to infect cells. Unless stated otherwise, cells were infected so that the percentage of GFP/dsRed-positive cells was <30% to ensure that the majority of cells contained single proviral integrants. GFP/dsRed-positive cells were sorted 4 days after infection. To isolate LNGFR-expressing cells, cells were stained with FITC-conjugated LNGFR antibody (CD271–FITC, Miltenyi Biotec) and sorted by FACS.

Western Blot Analysis. Equal numbers of cells were lysed in SDS sample buffer, boiled, sonicated, and loaded onto 4–20% gradient Tris·HCl gels (Invitrogen). Western blotting was performed by using anti-Rb (Pharmingen), anti-GFP (Santa Cruz Biotechnology), and anti-GAPDH (Santa Cruz Biotechnology) antibodies.

Results

miR30-Based shRNAs Provide Efficient Knockdown When Transcribed from the Pol II Promoter. We recently developed the pSM2 shRNA vector, which carries the short hairpins embedded within a miRNA transcript from the naturally occurring miR30 (14, 23). We chose miR30 because the processing of the larger pre-miRNA transcript to its functional miRNA has been well characterized *in vitro* and *in vivo* (reviewed in ref. 16). The pSM2 vector has been designed to allow the replacement of the mature miR30 encoding region with shRNA sequences that target any transcript of choice (Fig. 1*A*) (14, 17). In pSM2, the miR30-based shRNA is transcribed under the control of the pol III-based U6 promoter. Based on transient transfection assays, the pSM2 vector has been shown to significantly outperform standard short hairpin vectors such as pSM1 (23).

To test the efficiency of the pSM2 vector in targeting endogenous proteins when present at low copy number, we infected cells with pSM2 virus that contains a sequence targeting the transcript of the Rb protein (pSM2–Rb). Whereas cells transduced at a multiplicity of infection (MOI) of 2 showed some reduction Rb protein levels compared with uninfected cells, we found that cells containing single integrants (MOI <1) exhibited only minimal reduction of Rb protein levels (Fig. 1*C*). While attempting to establish a cre-inducible lentiviral vector based on the miR30-based shRNA expression, we fortuitously discovered

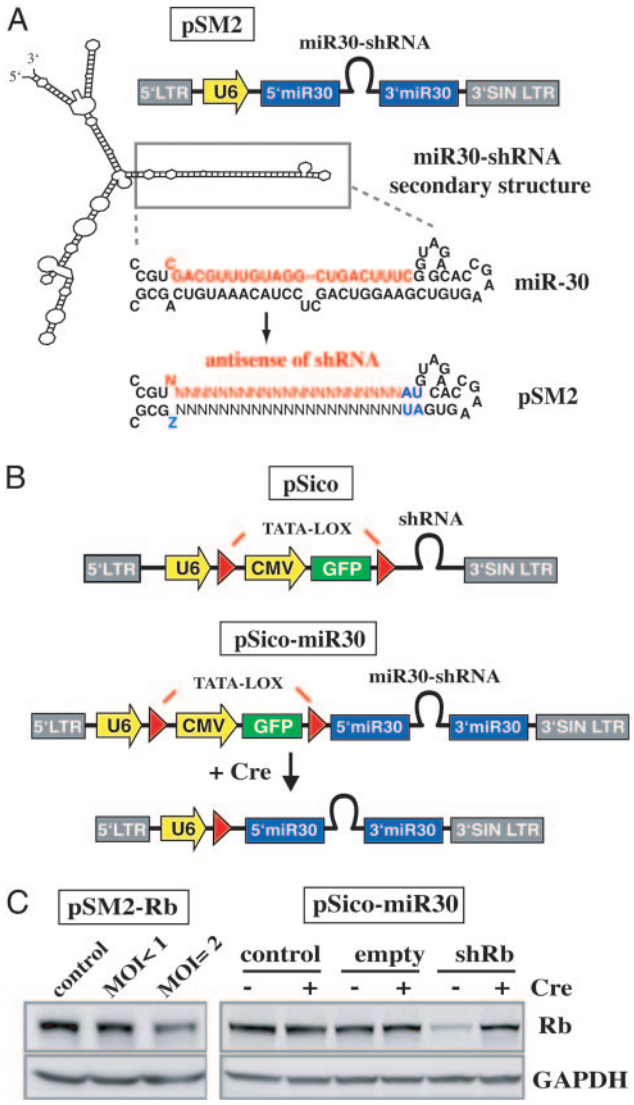

Fig. 1. Pol II-driven miR30-based shRNAs function efficiently at single copy. (*A*) Schematic representation of the pSM2 vector structure (see Fig. 6 for additional information), showing the secondary structure fold of the miR30-based shRNAs as predicted by RNAFOLD (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). In pSM2, the region coding for the mature miR30 miRNA (upper hairpin) is replaced with sequences that encode shRNAs targeting any gene of choice (shRNA, lower hairpin). The antisense strand, which encodes the targeting siRNA is shown in red. (*B*) Schematic representation of pSico and pSico–miR30 before and after Cre-mediated recombination. (*C*) U2OS cells were transduced at the indicated MOIs with pSM2 containing an shRNA that targets the Rb transcript (pSM2–Rb) and cultured for 1 week in puromycin (1 μg/ml)-containing medium. For pSico–miR30, U2OS cells were infected with the indicated lentivirus (MOI <0.3) and sorted for GFP-positive cells. These cells were infected with Ad–Cre and sorted for GFP-negative cells 4 days postinfection. Cell extracts were immunoblotted for Rb and GAPDH (loading control) protein levels. Uninfected cells serve as control.

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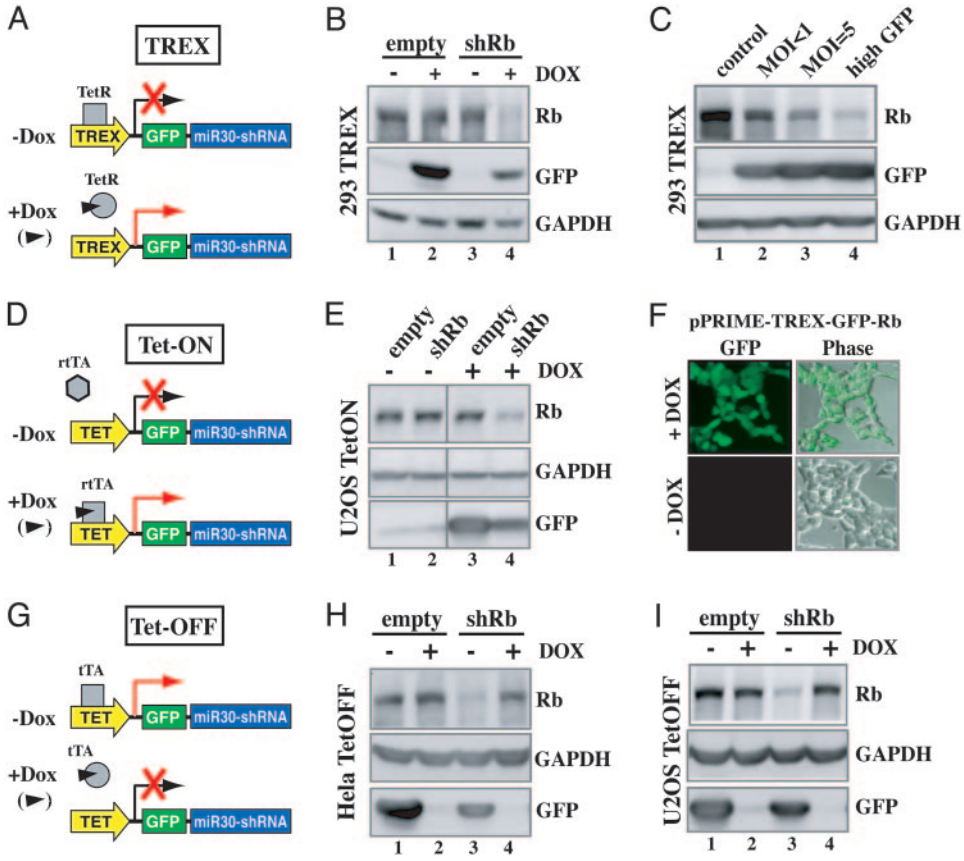

Fig. 2. Tet-regulatable pPRIME vectors generate highly penetrant knockdown at single copy. (*A*) Schematic representation of the TREX system. In the absence of DOX, the Tet repressor (TetR) binds to the Tet-operator binding sites located downstream of CMV's TATA box, thereby preventing transcription. Binding of DOX to TetR causes its displacement from the CMV promoter, thus allowing for transcription of the GFP–miR30–shRNA transcript. (*B*) 293 TREX cells were transduced with the indicated lentiviruses (MOI <0.4), grown in DOX for 3–4 days, and sorted for GFP-positive cells. Cells were split and grown either in the presence (+DOX) or absence (−DOX) of DOX for 1 week. Whole-cell extracts were immunoblotted for Rb, GFP, and GAPDH (loading control). (*C*) 293 TREX cells were transduced with the indicated lentiviruses. Cells infected at low MOI (<0.4) and high MOI (MOI = 5) were grown in DOX for 3–4 days and sorted for GFP-positive cells. Cells infected at the high MOI were also sorted for the highest 10% of GFP expression levels. Whole-cell extracts were immunoblotted against Rb, GFP, and GAPDH (loading control). (*D*) Schematic representation of the Tet-ON system. In the absence of DOX, the reverse tTA (rtTA) does not bind to the TET promoter. Binding of DOX to rtTA promotes its binding to the TET promoter, thereby initiating the transcription of the GFP–miR30–shRNA transcript. (*E*) U2OS Tet-ON cells were transduced with the indicated lentiviruses (MOI <0.4), grown in DOX for 3–4 days, and sorted for GFP-positive cells. Cells were treated and analyzed as described in *B*. (*F*) Images of 293 TREX transduced with pPRIME–TREX–GFP–Rb grown either in the absence (*Lower*) of DOX or the presence of DOX (induced for 16 h, *Upper*). (*G*) Schematic representation of the Tet-OFF system. In the absence of DOX, the tTA is bound to the TET promoter and drives transcription of the GFP–miR30–shRNA transcript. Binding of DOX to tTA leads to its displacement from the promoter and shutdown of transcription. (*H* and *I*) HeLa Tet-OFF and U2OS Tet-OFF cells were transduced with the indicated lentiviruses (MOI <0.4), treated, and analyzed as described in *B*.

a vector system that efficiently down-regulates Rb protein levels even when present at single copy.

To establish a miR30-based cre-inducible RNAi system we took advantage of a recently described conditional shRNA lentiviral vector named pSico (9). pSico contains a modified U6 promoter interrupted by a loxP-flanked cassette encoding a CMV–GFP gene (Fig. 1*B*). The presence of CMV–GFP prevents the U6 promoter from transcribing short hairpin constructs positioned after the 3′ loxP site caused by the absence of a functional U6 promoter (9). After Cre expression, recombination between the loxP sites (TATA–LOX) deletes CMV–GFP and places a functional U6 promoter (generated by realignment of the proximal sequence element and TATA box) adjacent to the shRNA-coding region, thereby activating RNAi (Fig. 1*B*). To generate an equivalent conditional construct for miR30-based hairpins, we replaced the conventional stem-loop shRNA expressed in pSico with a miR30-based shRNA targeting the Rb transcript (pSico–miR30–Rb, Fig. 1*B*). Unexpectedly, the miR30-based derivative behaved in precisely the opposite manner as conventional shRNAs expressed from pSico. Cells transduced with pSico–miR30–Rb and sorted for GFP-positive cells exhibited significant knockdown of the Rb protein before infection with Adeno–Cre (Fig. 1*C*). In contrast, deleting CMV–GFP by infection with Adeno–Cre and sorting for GFP-negative cells restored Rb expression to levels found in uninfected cells (Fig. 1*C*). Importantly, cells were infected at an MOI of 0.3 to ensure that the majority of transduced cells contain single proviral integrants. These findings suggest that single-copy miR30-based shRNA integrants are much more efficient at reducing Rb protein levels when transcribed from the CMV promoter compared with the U6 promoter. It is important to note that the CMV-driven transcript encodes GFP upstream of miR30. In fact, the physical linkage of the GFP and miR30–shRNA transcript provides a very useful feature by marking the cells that experience knockdown (see below). We named this vector pPRIME and propose the use of qualifiers following pPRIME to indicate the nature of the promoter, reporter gene, and the gene targeted by the shRNA; e.g., pPRIME–CMV–GFP–Rb indicates that the CMV promoter transcribes GFP and miR30-based shRNA targeting Rb.

Development of Tet-Regulatable pPRIME Vectors. To develop a regulated miR30-based shRNA system, the CMV promoter was

replaced with different Tet-responsive pol II-based promoters (18). First, we replaced the CMV promoter in pPRIME–CMV–GFP with a Tet-responsive promoter that contains two TET-repressor binding sites downstream of CMV's TATA box (TREX, Invitrogen, Fig. 2*A*), yielding pPRIME–TREX–GFP. 293 cells stably expressing the Tet repressor (293 TREX) were infected by using a low MOI to yield single integrants and grown under inducing conditions (+DOX). After sorting for GFP-positive cells, cultures were split and grown for 1 week either in the presence or absence of DOX. We found that the expression of the miR30-based shRNA construct was tightly regulated. Cells grown in the presence of DOX showed a dramatic increase in GFP levels compared with cells grown in the absence of DOX (Fig. 2 *B* and *F*). Importantly, cells induced to express the construct targeting Rb (miR30–shRb) showed a significant reduction of Rb protein levels (Fig. 2*B*, lane 4), whereas the same vector lacking miR30–Rb (empty) did not affect Rb levels (Fig. 2*B*, lane 2). The fact that the miR30–Rb-containing vector expresses lower GFP levels than the empty control vector (Fig. 2*B*, compare lanes 2 and 4) is probably caused by processing of the bicistronic transcript for RNAi, thereby creating a less stable GFP mRNA.

As GFP and miR30–shRNA expression are physically linked in the pPRIME–TREX–GFP vector, we hypothesized that cells with higher GFP expression will express high levels of miR30–shRNA and produce more efficient knockdown of their target gene. To test this notion, we infected cells at low MOI (MOI = 0.3) and high MOI (MOI = 5) and sorted for cells with the highest GFP levels (Fig. 2*C*, lane 4). The analysis of Rb levels in these sorted populations revealed that, as predicted, the amount of Rb knockdown positively correlates with the amount of GFP expression. We presume either that the cells in the high GFP population contain a higher number of integrants or the integration events occurred in chromosomal locations that allow for more efficient transcription. Our finding suggests that the knockdown generated by pPRIME–GFP-based vectors can be maximized by sorting for the highest GFP-expressing cells.

To explore other regulated promoters, we modified pPRIME to allow for its regulation with established Tet activator (tTA) systems. We replaced the CMV promoter in pPRIME–CMV–GFP with a minimal CMV promoter containing seven upstream Tet-operator sites (Clontech, hence referred to as TET) (18–20), yielding pPRIME–TET–GFP. The TET-ON system requires expression of the reverse tet-controlled transcriptional activator (rtTA), which activates transcription from the TET promoter in response to DOX (Fig. 2*D*) (19, 20). To test pPRIME–TET–GFP–Rb, U2OS Tet-ON cells (stably expressing rtTA) were infected at low MOI (0.3), sorted for GFP-positive cells (after a brief induction), and cultured either in the presence or absence of DOX. As expected, GFP was expressed only in cells grown in the presence of DOX (induced state, Fig. 2*E*, lanes 3 and 4). U2OS Tet-ON cells transduced with empty pPRIME–TET–GFP failed to reduce Rb levels (Fig. 2*E*, lane 3). In contrast, cells expressing miR30–shRb significantly reduced Rb levels in the induced (+DOX, Fig. 2*E*, lane 4) but not in the uninduced state (−DOX, Fig. 2*E*, lane 2).

Lastly, we probed pPRIME–TET–GFP–Rb in the TET-OFF system, which shows inverse responsiveness to DOX compared with the TET-ON system (Fig. 2*G*) (18, 19). HeLa Tet-Off cells stably expressing a WT tTA activate transcription only in the absence of DOX (Fig. 2*G*). Importantly, Rb protein levels were only reduced under inducing conditions (−DOX) and in cells containing the Rb targeting construct (Fig. 2*I*). We also observed regulatable knockdown at low MOI (<0.3) with pPRIME–TET–GFP–Rb in U2OS Tet-OFF cells (Fig. 2*I*). Furthermore, pPRIME–TET–GFP carrying a shRNA targeting PTEN was able to generate regulatable knockdown of PTEN expression (Fig. 5, which is published as supporting information on the PNAS web site), providing further evidence of the broad applicability of the pPRIME–TET–GFP system. We consistently

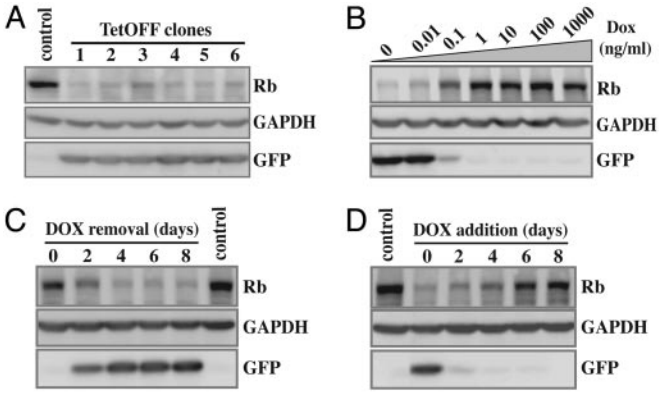

Fig. 3. Kinetics and dose–responsiveness of Rb regulation by pPRIME–TET–GFP–Rb. (*A*) Clonal isolates of the HeLa Tet-OFF cells expressing pPRIME–Tet–GFP–Rb (see Fig. 2*H*) were grown under inducing conditions (−DOX) and immunoblotted for Rb, GAPDH, and GFP protein levels. (*B*) Tet-OFF clone 1 (see *A*) expressing GFP–miR30–Rb was cultured for 1 week in medium containing the indicated concentration of DOX, and cell extracts were blotted for the indicated proteins. (*C*) Tet-OFF clone 1 was cultured for 1 week in the presence of 1 μg/ml DOX (repressing condition). At day 0, the drug was withdrawn from the culture medium, and cells were harvested at the indicated time points. Whole-cell extracts were analyzed by Western blotting for the indicated proteins. (*D*) Tet-OFF clone 1 was cultured for 1 week under inducing conditions (−DOX), followed by addition of DOX at a concentration of 1 μg/ml. Cells were harvested just before DOX treatment (day 0) and at the indicated time points. Whole-cell extracts were analyzed by Western blotting for the indicated proteins.

observed a higher degree of Rb knockdown in the TET-OFF compared with the TET-ON system, presumably because of the stronger transcriptional induction observed in the TET-OFF system compared with TET-ON (20, 21). Taken together, our results show that the pPRIME–TET–GFP system allows for the controllable suppression of cellular genes both with remarkable efficacy and without significant leakiness.

It is well established that the amount of viral transcript generated by single-copy retroviral insertions greatly depends on the site of integration within the host genome. Therefore, we generated several clonal isolates of HeLa Tet-OFF cells transduced with pPRIME–TET–GFP–Rb and compared their Rb protein levels in the induced state (−DOX, Fig. 3*A*). Although some clones clearly reduced Rb levels more efficiently than others, we were surprised to find that every clone showed a significant reduction of Rb levels, providing further evidence of the high penetrance of the pPRIME–TET–GFP system. We next defined the kinetics, reversibility, and DOX dose responsiveness of protein knockdown. We chose clone number one for this analysis, which we confirmed to contain a single proviral integrant by genomic Southern analysis (data not shown). Rb levels decreased significantly 2 days after DOX removal (Fig. 3*C*), and the knockdown reached completion 4 days postinduction. It is likely that the kinetics of knockdown depend to a large extent on the stability of the target protein, as the induction of miR30–shRNA in response to DOX removal occurs very rapidly (<12 h, unpublished observation). Furthermore, we found that it takes ≈6 days to fully recover WT Rb levels after turning off miR30–shRb transcription (DOX addition, Fig. 3*D*), indicating that the miR30-generated shRNAs remain active for several days.

The dose–response analysis revealed an extreme sensitivity to DOX control and pointed to the possibility of some modulation of the extent of gene suppression. Whereas partial Rb down-regulation was already apparent at 0.1 ng/ml DOX, maximal suppression was achieved only at doses <0.01 ng/ml (Fig. 3*B*). It is possible that a less efficient shRNA than the one used in this

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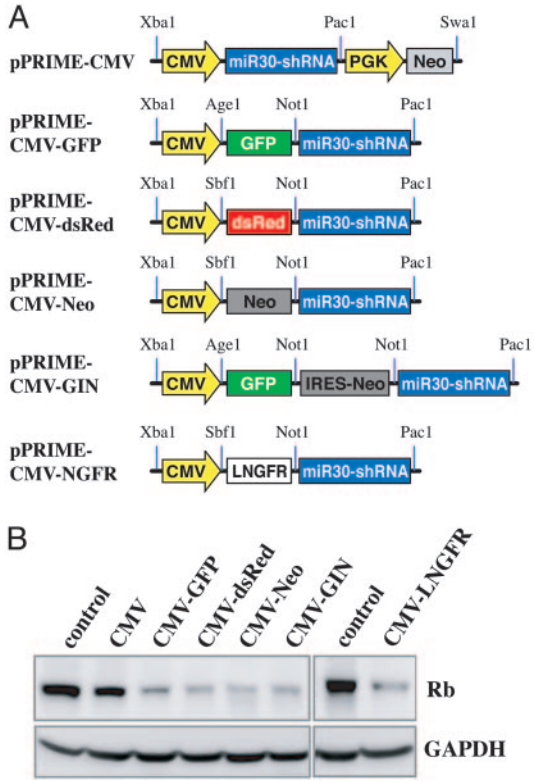

Fig. 4. pPRIME vectors can accommodate a variety of reporter genes. (*A*) Schematic representation of pPRIME–CMV derivatives. For more detailed vector information see Fig. 6. PGK, phosphoglycerate kinase promoter; IRES, internal ribosome entry site. (*B*) U2OS cells were transduced with the indicated lentiviruses (MOI <0.4, uninfected cells served as control). GFP-, dsRed-, and LNGFR-expressing cells were FACS-sorted 4 days after infection, and Neo-expressing cells (CMV, CMV-NEO, and CMV-GIN) were selected with 500 μg/ml G418 for 1 week. Drug selection was withdrawn 1.5 days before harvesting the cells. Whole-cell extracts were analyzed by immunoblotting for the indicated proteins.

experiment might allow for an even wider modulation of the degree of gene knockdown.

pPRIME Vectors Can Accommodate Various Reporter Genes. The initial pPRIME vectors rely on GFP expression as a molecular marker for the identification and isolation of shRNA-expressing cells. To increase the versatility of the pPRIME system, we examined whether GFP can be replaced with other reporters without compromising knockdown efficiency. Interestingly, when we placed the CMV promoter directly upstream of the miR30–shRNA cassette, Rb knockdown was much less efficient compared with the GFP-containing vector (Fig. 4, compare pPRIME–CMV and pPRIME–CMV–GFP). However, when we replaced GFP with the coding region for dsRed (pPRIME–CMV–dsRed), Neo (pPRIME–CMV–Neo), or LNGFR (which encodes LNGFR lacking the cytoplasmic signaling domain), the Rb knockdown efficiency was similar to the GFP-containing vector (Fig. 4). Even when we increased the spacing between CMV and the miR30–shRNA by inserting a Neo cassette (containing a 5′ internal ribosome entry site) downstream of GFP, Rb knockdown was still very efficient at low MOI (Fig. 4, pPRIME–CMV–GFP-IRES-Neo).

Discussion

The ability to interfere with gene expression in mammals has allowed the performance of genetic screens in these cells that had previously been possible only in lower eukaryotes (1, 2). Thus we can now envision mammalian gene knockout collections similar to those available in budding yeast. We, and others, have been pursuing such strategies and have generated collections of retroviral vectors constitutively expressing shRNA constructs covering thousands of genes (23). Our initial design incorporated a bar-coding feature, a 60-nt sequence unique to each vector, that allows the abundance of each shRNA vector to be monitored within a complex mixture by microarray hybridization. This strategy has been used successfully in lower eukaryotes, and we recently used it successfully in a mammalian enrichment screen (8). This strategy can also be used in relative growth assessment assays or screening for synthetic lethal relationships. However, the latter types of screens are only feasible if each shRNA integrant demonstrates high penetrance of the phenotype. Otherwise, the dynamic range of the signal change will be too low for statistical significance. While attempting to take advantage of an inducible pol III shRNA expression system to express shRNAs embedded in a miR30 context, we discovered that miR30-based shRNAs could very efficiently be expressed from pol II promoters such that single-copy proviral integrants provide high-level knockdown in polyclonal cell populations. In other words, the pPRIME vectors exhibit the high penetrance required for efficient functional genomic screens, in particular those using bar-coding strategies.

The use of pol II promoters to drive miR30 constructs had been demonstrated previously in transient assays but not in a single-copy retroviral context (12, 17). Interestingly, when we placed the CMV promoter directly upstream of miR30, high-level knockdown was not observed. Only when we inserted the GFP, dsRed, LNGFR, or Neo-coding region between CMV and the miR30–shRNA transcript did we see high-level knockdown (Fig. 4). We interpret this finding to mean that either the spacing is important or that special enhancing sequences exist in these coding regions that promote miRNA function. Our observations are consistent with the recent discovery that many endogenous miRNAs are transcribed by pol II (22). However, it is important to note that conventional stem-loop shRNAs, such as those used in pSico, are not processed into functional siRNAs when transcribed as part of a bicistronic pol II transcript (9).

The PRIME vectors described in this study have several useful features. First, replacing the CMV with cell type-specific pol II promoters provides the possibility of tissue-specific expression in animals. Second, all vectors are lentivirus based, which allows for the efficient transduction of a broad range of cell types, including nondividing cells and cells that are hard to infect by retroviruses. Finally, in contrast to the binary cre–lox system, Tet regulation allows for the modulation of gene knockdown by varying the dosage of DOX and exhibits full reversibility.

A limitation of most existing inducible vectors is that the cells experiencing knockdown are not marked. Thus, a fourth advantage of the PRIME series is that GFP (or other molecular markers) and the shRNA are part of a bicistronic transcript, thereby unequivocally marking the shRNA-expressing cells. This feature allows one to identify cells that experience knockdown within a complex population of cells, a feature particularly valuable for *in vivo* animal studies. Another important feature of the inducible PRIME vectors is the ability to sort for reporter-positive cells, which obviates the need for the time-consuming generation of individual clonal isolates in many experimental settings. Furthermore as some shRNA expression constructs are silenced after multiple passages, the direct linkage to a reporter gene would allow the selection of populations maintaining shRNA expression.

Finally, we have adapted pPRIME to the MAGIC cloning system (15), which allows for the rapid and cost-efficient transfer of our pSM2 library into different expression contexts (Fig. 6, which is published as supporting information on the PNAS web site). For example, the MAGIC system will facilitate the generation of complex tet-inducible shRNA libraries, which will provide an invaluable resource for genomewide library screens. In

a parallel study, Dickins *et al.* (24) found that miR30-based shRNAs also generate highly penetrant knockdown when driven by retroviral LTRs. Together, these efficient knockdown systems significantly extend and improve our RNAi toolbox for functional genomic studies in mammalian cells.

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