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Rapid conditional knock-down–knock-in system for mammalian cells

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ABSTRACT

RNA interference (RNAi) is a powerful tool to analyze gene function in mammalian cells. However, the interpretation of RNAi knock-down phenotypes can be hampered by off-target effects or compound phenotypes, as many proteins combine multiple functions within one molecule and coordinate the assembly of multimolecular complexes. Replacing the endogenous protein with ectopic wild-type or mutant forms can exclude off-target effects, preserve complexes and unravel specific roles of domains or modifications. Therefore, we developed a rapid-knock-down–knock-in system for mammalian cells. Stable polyclonal cell lines were generated within 2 weeks by simultaneous selection of two episomal vectors. Together these vectors mediated reconstitution and knock-down in a doxycycline-dependent manner to allow the analysis of essential genes. Depletion was achieved by an artificial miRNA-embedded siRNA targeting the untranslated region of the endogenous, but not the ectopic mRNA. To prove effectiveness, we tested 17 mutants of WDR12, a factor essential for ribosome biogenesis and cell proliferation. Loss-off function phenotypes were rescued by the wild-type and six mutant forms, but not by the remaining mutants. Thus, our system is suitable to exclude off-target effects and to functionally analyze mutants in cells depleted for the endogenous protein.

INTRODUCTION

The nucleolus is the site of ribosomal RNA (rRNA) transcription, pre-rRNA processing and ribosome subunit assembly (1). For a long time, the nucleolus was considered to be a mere 'ribosome factory' that is solely involved in the production of ribosomes. Now, this view has been challenged, as the recent years have unraveled many essential roles in other processes. In particular, the nucleolus controls cell cycle progression by the sequestration of regulating factors and additionally serves as a site of specific maturation steps of most cellular ribonucleoprotein (RNP) particles such as small nuclear RNAs (snRNAs), transfer RNAs (tRNAs) and telomerase RNA (2–4). Interestingly, recent studies showed that the main nucleolar task, namely ribosome biogenesis itself, is linked to the mammalian cell cycle machinery. Ongoing ribosome synthesis is highly sensitive to stress signals and its disturbance induces the tumor suppressor p53 triggering cell cycle arrest and apoptosis (5–7).

Because of this remarkable connection between ribosome biogenesis and the p53 response, one may speculate that further direct links to other cellular key processes and pathways exist. Unfortunately, our knowledge of the mammalian ribosome synthesis machinery is still incomplete. Ribosome biogenesis factors have been almost exclusively studied in yeast (8). Isolation of pre-ribosomal complexes by mass spectrometry and synthetic lethal screens provided many novel candidates. The convenience of yeast genetics; in particular, the rapid generation of conditional depletion strains then allowed their functional characterization. However, depletion of a protein can interfere with the assembly of a multimolecular complex, if the presence of the factor is required for the formation of a stable complex. Further, many proteins integrate multiple roles through separate domains, and thus depletion of a single protein affects the whole functional repertoire. Therefore, missense mutations can unravel separate specific functions of a protein in cells depleted for the endogenous protein. An elegant recent study identified an essential role for the C-terminal extension of the yeast ribosomal protein rpS14 in 20S pre-rRNA processing by testing the functionality of missense mutations (9). In contrast, depletion of rpS14 affects the pre-rRNA pathway upstream

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of the 20S pre-rRNA intermediate, most likely caused by a defect in the assembly of a multimolecular complex required for the initial pre-rRNA processing steps. Therefore, reconstitution of conditional depletion strains with wild-type or mutant alleles provide a powerful genetic tool for a specific functional analysis of proteins.

Comparable genetic approaches in mammalian cells are laborious, in particular if required in a conditional manner for essential genes. For instance, the rRNA-processing factor Pes1 is essential for embryogenesis in mice which can be expected for many ribosome synthesis factors (10). RNA interference (RNAi) is a powerful tool to circumvent these obstacles and to analyze knock-down phenotypes. However, as mentioned previously, this loss-off function approach might affect multimolecular complex formation and therefore might preclude the investigation of more specific functions. Therefore, in addition to the knock-down approach, a concomitant knock-in of mutant forms is required.

In this study, we present a rapid knock-down–knock-in system for mammalian cells using two episomal constructs. The first one allows the doxycycline-dependent expression of the gene of interest, either as wild-type or mutant form. A second construct encoding a different resistance gene for stable selection in mammalian cells serves for the conditional gene knock-down. We expressed siRNA sequences embedded in an miRNA environment that target the untranslated regions (UTRs) of the endogenous mRNA. Thereby, we specifically deplete the endogenous protein without affecting the ectopic form. We performed a detailed analysis of the essential rRNA processing factor WDR12, a component of a multiprotein complex (PeBoW-complex), to show that our system is a rapid and convenient approach to assess the functionality of mutants by testing their capacity to reconstitute for depletion of the endogenous protein.

MATERIALS AND METHODS

Cloning

The 5′ or 3′ untranslated regions of human WDR12 and Pes1 were targeted by 21mer siRNA sequences designed using the Invitrogen siRNA design algorithm (BLOCK-IT RNAi Designer, https://rnaidesigner.invitrogen.com/rnaiexpress/). Selected siRNA sequences were then further adapted to the miRNA environment of the pcDNA 6.2-GW/EmGFP-miR construct (Invitrogen) that was derived from the endogenous murine miR-155 (11). We generated a subcloning construct (pMIRTOP) to allow initial functional analysis of the selected siRNA sequences, similar to the pcDNA 6.2-GW/EmGFP-miR plasmid (Invitrogen), but providing more convenient subsequent cloning into the pRTS plasmid. pMIRTOP was derived from pEGFP-C1 (Clontech). The oligos were cloned downstream of the eGFP open reading frame (ORF) into the miRNA environment. Functionality of the selected sequences was tested by indirect immunofluorescence to verify knock-down of endogenous WDR12 and Pes1 in H1299 cells. The following DNA oligonucleotides were used in this study. The number indicates the position of the target sequence within the reference mRNA of Pes1 (NM_014303) and WDR12 (NM_018256): miPes1-1903 (3′-UTR; 21mer target sequence: GCCAGAGGACCTAAGTGTGAT; miRNA-oligo: top strand TGCTGATCACACTTAGGTCCTCTGGCGTTTTGGCCACTGACTGACGCCAGAGGCTAAGTGTGAT, bottom strand CCTGATCACACTTAGCCTCTGGCGTCAGTCAGTGGCCAAAACGCCAGAGGACCTAAGTGTGATC), miPes1-308 (ORF; 21mer target sequence: TGTCAACAAGTTCCGTGAATA; miRNA-oligos: top-strand TGCTGTATTCACGGAACTTGTTGACAGTTTTGGCCACTGACTGACTGTCAACATTCCGTGAATA, bottom strand CCTGTATTCACGGAATGTTGACAGTCAGTCAGTGGCCAAAACTGTCAACAAGTTCCGTGAATAC), miWDR12-135 (5′-UTR; 21mer target sequence: TTCGTGTTGTGGGTCTGCTAA; miRNA-oligo: top strand TGCTGTTAGCAGACCCACAACACGAAGTTTTGGCCACTGACTGACTTCGTGTTGGGTCTGCTAA, bottom strand CCTGTTAGCAGACCCAACACGAAGTCAGTCAGTGGCCAAAACTTCGTGTTGTGGGTCTGCTAAC), miWDR12-1663 (3′-UTR; 21mer target sequence: CTGTGGCATACATTCTCTATA; miRNA-oligo: top strand TGCTGTATAGAGAATGTATGCCACAGGTTTTGGCCACTGACTGACCTGTGGCACATTCTCTATA, bottom strand CCTGTATAGAGAATGTGCCACAGGTCAGTCAGTGGCCAAAACCTGTGGCATACATTCTCTATAC), miLuciferase (abbreviated as miLuc; 21mer target sequence: TATTCAGCCCATATCGTTTCA; miRNA-oligo: top strand: TGCTGTATTCAGCCCATATCGTTTCAGTTTTGGCCACTGACTGACT GAAACGATGGGCTGAATA; bottom strand: CCTGTATTCAGCCCATCGTTTCAGTCAGTCAGTGGCCAAAACTGAAACGATATGGGCTGAATAC). An Eco47III/BglII fragment of pMIRTOP containing the eGFP ORF and the miRNA environment was directly cloned into the SwaI/BglII site of the pRTS construct. To obtain the DsRed-miWDR12-1663 construct, the eGFP within pMIRTOP-miWDR12-1663 was replaced by the ORF of DsRed2 (pDsRed2-N1; Clontech). An AgeI/NotI (blunt ended by T4 DNA polymerase) fragment was cloned into the AgeI/PmlI site of pMIRTOP-miWDR12-1663. Then, an Eco47III/BglII fragment containing DsRed2-miWDR12-1663 was cloned into pRTS as described above.

Generation of pRTS constructs expressing HA-tagged Pes1 and WDR12 was described previously. In general, the ORF amplified by PCR using primers that provided a CCACC Kozak sequence before the start codon and lacked the stop codon. Blunt end cloning into the EcoRV site of a subcloning construct (pSfiExpress) then provided a C-terminal HA-tag and two flanking *SfiI* sites for further cloning into pRTS constructs. pSfiExpress was generated by replacing the eGFP of pEGFP-C1 (Clontech) by a linker that was designed as outlined in Supplementary Data. Further, an additional *SfiI* site was also deleted to allow excision of the HA-tagged ORF by a *SfiI* digest. The *SfiI* fragment was then cloned into the *SfiI* sites of pRTS. Detailed information about the cloning strategies is provided in Supplementary Data.

Tissue culture

H1299 lung carcinoma cells (non-small cell lung carcinoma) were cultured in DMEM with 10% FBS at 8% CO_2. H1299 cells were transfected with the respective pRTS constructs using Polyfect (Qiagen) and polyclonal cell lines for knock-down–knock-in assays were generated by stable selection with 200 μg/ml hygromycin B and 1 μg/ml puromycin for ~10 days. Conditional gene and miRNA-embedded siRNA

expression was achieved by the addition of 0.5 μg/ml doxycycline.

Knock-down–knock-in assay

H1299 cells were treated with 0.5 μg/ml doxycycline to activate ectopic gene expression (Luciferase, WDR12 wild-type or mutant forms) together with the respective miRNA-embedded siRNAs (miLuc or miWDR12) for 4 days. Cells were trypsinized, counted and replated for functional assays. After one or two additional days, cells were harvested for metabolic labeling of nascent rRNA, western blot analysis and indirect immunofluorescence.

Metabolic labeling of nascent rRNA

H1299 were incubated in phosphate-free DMEM/10% FBS for at least 30 min and then incubated for 1 h in the presence of 15 μCi/ml [^{32}P]orthophosphate. The metabolic labeling medium was subsequently replaced by regular DMEM/10% FBS and cells were further cultivated for 4–5 h. Total RNA was then isolated using the RNeasy Mini kit (Qiagen). An aliquot of 1.5 μg of metabolically labeled total RNA was separated on a 1% agarose formaldehyde gel. The gel was then dried on a Whatman paper using a regular gel drier (Bio-Rad) connected to a vacuum pump for 2–3 h at 80°C. Metabolically labeled RNA was visualized by autoradiography. Quantification of 28S rRNA signal intensities was performed using a PhosphorImager.

Immunoblotting and immunofluorescence

Cells were directly lysed with 2× SDS loading buffer (100 mM Tris–HCl, 200 mM DTE, 4% SDS, 10 mM EDTA, 0.2% bromophenol blue and 20% glycerol). Whole cell lysates were separated by SDS–PAGE and blotted on to nitrocellulose membranes (Amersham). Immunodetection was performed with monoclonal antibodies directed against the HA-tag (3F10; Roche), WDR12 (1B8), Pes1 (8H11) and anti-tubulin (Sigma–Aldrich). The monoclonal antibodies against human WDR12 and Pes1 were described previously. For indirect immunofluorescence, cells were grown on cover slides, fixed with ice-cold methanol and air dried. Unspecific binding was blocked with PBS/10% FBS. HA-tagged forms of WDR12 were detected with the anti-HA (3F10) antibody. Primary antibodies were incubated overnight at 4°C in a humidified chamber. Cy3-labeled secondary antibodies (Dianova) were incubated for 1 h at room temperature. Nuclei were counterstained with DAPI (Sigma–Aldrich). Digital images were acquired using the Openlab acquisition software (Improvision) and a Zeiss Axiovert 200M microscope (Carl Zeiss MicroImaging) with a 63 (1.15) plan oil objective connected to a CCD-camera (ORCA-479; Hamamatsu).

RESULTS

Conditional expression of siRNAs embedded in an miRNA environment

It was previously shown that designed siRNA sequences embedded in an miRNA environment are properly processed from ectopic Pol II transcripts and efficiently confer target gene knock-down (12,13). We placed modified miRNA sequences derived from the endogenous murine miR-155 stem–loop downstream of the eGFP ORF that is controlled by a tight doxycycline-dependent bidirectional promoter (Figure 1A) (11). The Epstein–Barr virus (EBV)-derived episomal expression construct pRTS was previously described in detail and contains all features required for conditional gene expression (14). Double-stranded DNA oligonucleotides homologous to the miRNA-embedded siRNAs directed against the rRNA processing factors Pes1 and WDR12 were cloned into a subcloning plasmid (pMIRTOP) following the eGFP ORF. For convenience, we abbreviated the miRNA-embedded siRNAs as miPes1 and miWDR12. They were designed to target the UTRs of the endogenous Pes1 and WDR12 mRNAs to allow future reconstitution experiments without affecting the ectopically expressed genes that only contain the ORF but not the UTRs. Functional sequences were identified by indirect immunofluorescence verifying the knock-down of the endogenous protein in transiently transfected cells (data not shown). Fragments containing the eGFP ORF together with the miRNAs were subsequently transferred into the pRTS construct and transfected into H1299 human lung carcinoma cells by non-viral techniques. Polyclonal cultures were generated within 2 weeks by stable selection with puromycin. As recently reported, GFP expression is reduced by processing of the miRNA structure, but is still easily detected and therefore allows monitoring of conditional miRNA-embedded siRNA expression (Figure 1B). Stable polyclonal H1299 cell cultures exhibit >95% GFP positive cells upon the addition of doxycycline. Similar GFP induction rates (>95%) were also achieved in other cell lines (U2OS, TGR-1). Furthermore, we verified the knock-down of endogenous Pes1 and WDR12 protein by western blot analysis each targeted by two individual miRNA-embedded siRNA sequences (Figure 1C). Depletion of Pes1 or WDR12 resulted in impaired rRNA processing of the 32S pre-rRNA and reduced cell proliferation (data not shown), in line with previous experiments using synthetic siRNA oligos (6,15).

Development of a knock-down–knock-in system by using two individual pRTS constructs

Next, we aimed to rescue the depletion phenotype of Pes1 and WDR12 specifically by co-expression of the respective wild-type proteins. For experimental convenience, we tested whether co-transfection of two individual pRTS constructs is suitable for a knock-down–knock-in approach. One construct encodes a hygromycin resistance gene and expresses HA-tagged Pes1wt, WDR12wt or luciferase as a control (Figure 2A, upper panels, gene of interest). The other contains a puromycin resistance gene and expresses the respective miRNA-embedded siRNA together with DsRed2 and luciferase (Figure 2A, lower panels). The eGFP upstream of the miRNA region was replaced by DsRed2 to better visualize co-expression (Figure 2A). Stable polyclonal H1299 cells were generated by the simultaneous addition of puromycin and hygromycin to the culture medium for 10–14 days. Successful co-selection was verified by monitoring eGFP and DsRed2 double positive cells (Figure 2B). Further, reconstitution of Pes1- or WDR12-depleted cells with the respective HA-tagged wild-type proteins was determined by western

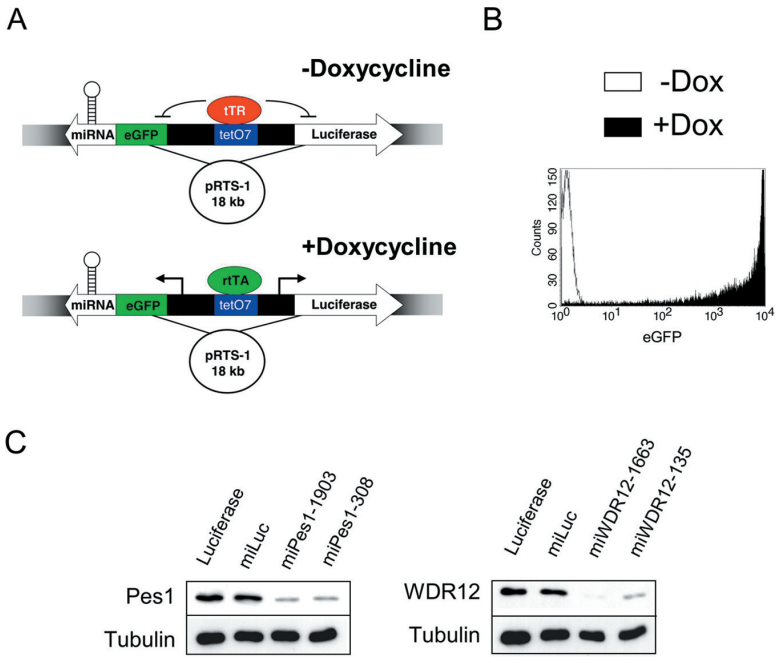

Figure 1. Conditional expression of miRNA-embedded siRNAs. (**A**) Schematic representation of the pRTS construct harboring a target gene-specific siRNA sequence embedded in the modified murine miR-155 structure behind the eGFPORF. Conditional gene activation is achieved in the presence of doxycycline by the tet-activator (rtTA), whereas active repression is mediated by the tet-repressor (rTR) in the absence of doxycycline. (**B**) Detection of eGFP positive cells by flowcytometry upon the addition of doxycycline to the culture medium. (**C**) Western blot analysis of endogenous Pes1 and WDR12 in stably transfected H1299 cells expressing either luciferase or the respective miRNA-embedded siRNAs targeting luciferase (miLuc), Pes1 (miPes1) or WDR12 (miWDR12). The numbers indicate the target site of the 21mer siRNA sequence within the Pes1 and WDR12 reference mRNAs. A tubulin blot is shown as loading control.

blot analysis (Figure 2C). The HA-tagged forms can be discriminated by their increased molecular weight and slower migration due to the C-terminal tag. An miRNA-embedded siRNA targeting luciferase served as knock-down control and likewise a luciferase expression construct as knock-in control. In particular, endogenous WDR12 is strongly depleted and hence replaced by the ectopic form. Then, we investigated whether expression of HA-tagged Pes1wt or WDR12wt rescued 32S pre-rRNA processing, a specific defect observed in Pes1- and WDR12-depleted cells (6,15). A scheme of mammalian rRNA processing pathways is given in Figure 2D. rRNA is transcribed by Pol I as a large 47S transcript that is subsequently cleaved into the mature rRNAs. The 45S and the 32S pre-rRNA are the most abundant precursor rRNAs. To analyze processing of nascent rRNA *in vivo*, we performed metabolic labeling using [^{32}P]orthophosphate. *De novo* synthesis of mature rRNAs can be visualized by autoradiography following gel electrophoresis of metabolically labeled total RNA. In control cells, mature 28S and 18S rRNA are efficiently produced (Figure 2E, lane 1). As expected, cells depleted for Pes1 and WDR12 but co-selected with an additional luciferase expression construct, exhibited a defective maturation of the 28S rRNA, whereas the synthesis of the 18S rRNA is unaffected (Figure 2E, lanes 3 and 5). Co-expression of the HA-tagged wild-type proteins completely restored processing of the 28S rRNA in Pes1- or WDR12-depleted cells (Figure 2E, lanes 2 and 4). In the case of impaired 32S pre-rRNA processing, one would also expect a strong accumulation. However, it is well known that the nuclear exosome potently degrades pre-rRNA that is blocked in its maturation (16,17). In addition, our low-activity [^{32}P]orthophosphate metabolic labeling exhibits a relatively slow uptake and incorporation kinetics. Together, this approach is very suitable to measure the endpoints of rRNA processing pathways, namely production of mature 18S and 28S rRNA, but is not very sensitive to detect accumulation of blocked precursor rRNA. Further, impaired proliferation provoked by depletion of Pes1 and WDR12 was also restored upon co-expression of Pes1 or WDR12 (Figure 2F). Cells were seeded at low density and cultured for 12 days in the presence of hygromycin, puromycin and various doxycycline concentrations. Apparently, strong ectopic gene expression by higher concentrations of doxycycline compromises cell proliferation, even in control cells (Figure 2F, row 1). However, knock-down of Pes1 and WDR12 exhibited a clear proliferation defect that could be fully restored by Pes1wt and WDR12wt co-expression. Thus, our knock-down–knock-in approach is functional, as the depletion phenotypes can be specifically rescued by co-expression of the respective wild-type protein.

Functional analysis of mutants in cells depleted for the endogenous protein

Finally we asked, whether our knock-down–knock-in approach is suitable for the functional analysis of diverse mutants in cells depleted for the endogenous protein. We have previously characterized a panel of WDR12 mutants (Figure 3A; WT, M1, M2, M6–M10). This analysis revealed only one dominant-negative mutant (M1) that blocks rRNA processing (6). We extended this set of mutants by truncation mutants (M2–M5) and various point mutations of putative CKII phosphorylation sites (M13–M17) and of a cysteine

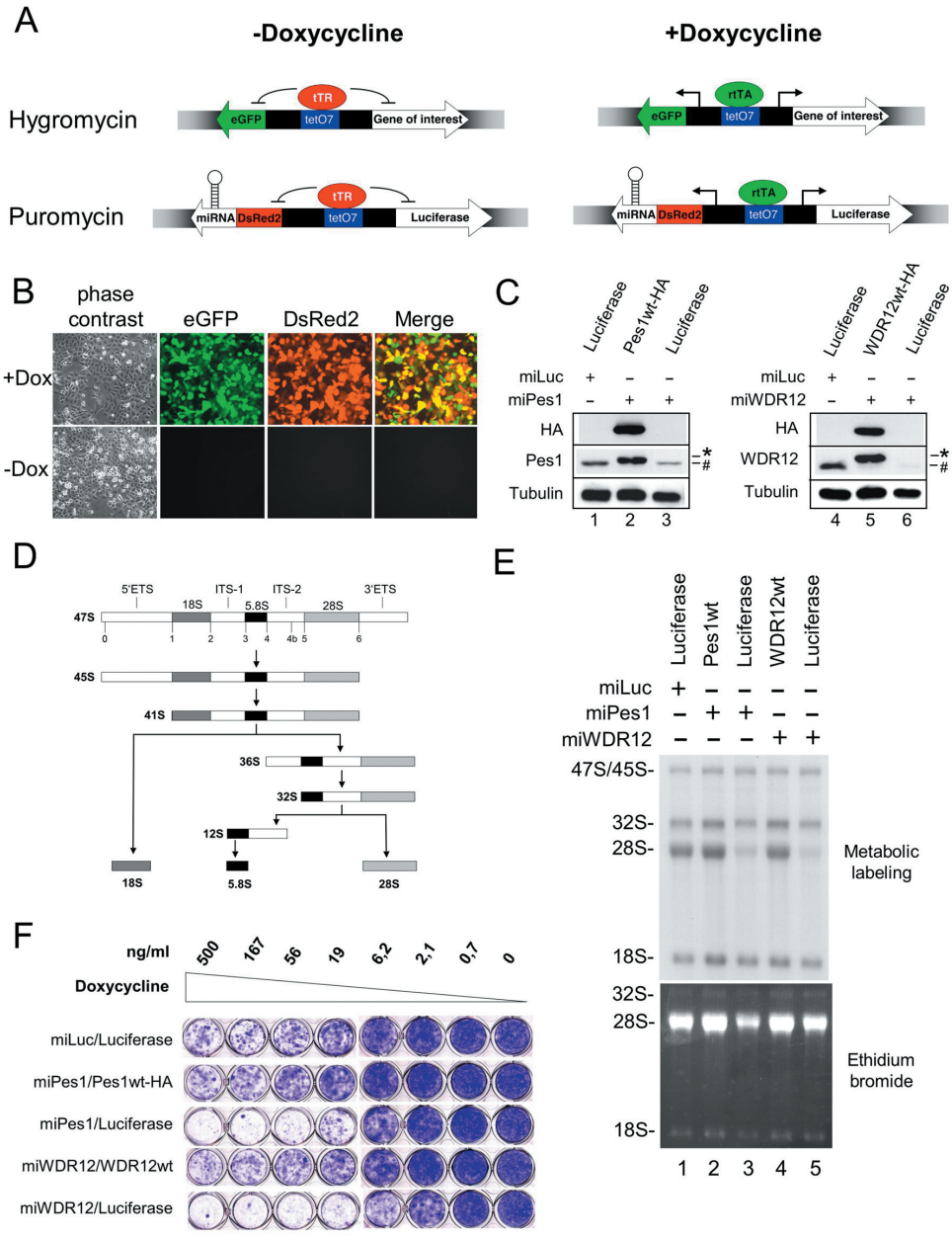

Figure 2. Development of a rapid knock-down–knock-in system for mammalian cells. (**A**) Scheme of the knock-down–knock-in approach by using two pRTS plasmids. (**B**) Verification of successful co-selection with hygromcyin and puromycin by monitoring co-expression of eGFP and DsRed2 in stably selected H1299 cells. (**C**) Western blot analysis of Pes1 and WDR12 in stably transfected H1299 cells after 5 days of doxycycline treatment. Cells co-express one of the indicated miRNA-embedded siRNAs (miLuc, miPes1-1903 or miWDR12-1663) together with either luciferase, Pes1wt-HA or WDR12wt-HA. For convenience, miPes1-1903 and miWDR12-1663 will be designated from now on as miPes1 and miWDR12. Asterisks indicate the ectopic and the hash symbols (#) indicate the endogenous forms that are only discriminated by the increased molecular weight due to the HA-tag. A tubulin blot confirms equal loading. Expression of the ectopic HA-tagged genes was also confirmed by immunodetection of the HA-tag. (**D**) Overview of mammalian rRNA processing pathways starting from the primary 47S transcript. Numbers indicate the cleavage sites within the rRNA. ETS: external transcribed spacer; ITS: internal transcribed spacer. (**E**) Metabolically labeled total rRNA was separated on a 1% agarose formaldehyde gel and visualized by autoradiography. Cell lines as described above (C). The ethidium bromide stained gel is shown as loading control. (**F**) Analysis of cell proliferation. The indicated cell lines were seeded in multiples at low density, fixed after 12 days with ice-cold methanol and stained with Giemsa. Representative wells are shown.

doublet (M12). H1299 cells were stably transfected with the indicated WDR12 expression constructs (WT, M1–M17) together with the WDR12 knock-down plasmid. Expression of the various HA-tagged forms of WDR12 and depletion of endogenous WDR12 were verified by western blot analysis (Supplementary Figure 1A).

To assess the functionality of the mutant forms, we performed metabolic labeling of nascent rRNA (Figure 3B and C). Two control cell lines (Figure 3B and C, lanes 1 and 2) were also included in our analysis to confirm the WDR12 depletion phenotype. HA-tagged WDR12wt successfully sustained the synthesis of the mature 28S rRNA in the

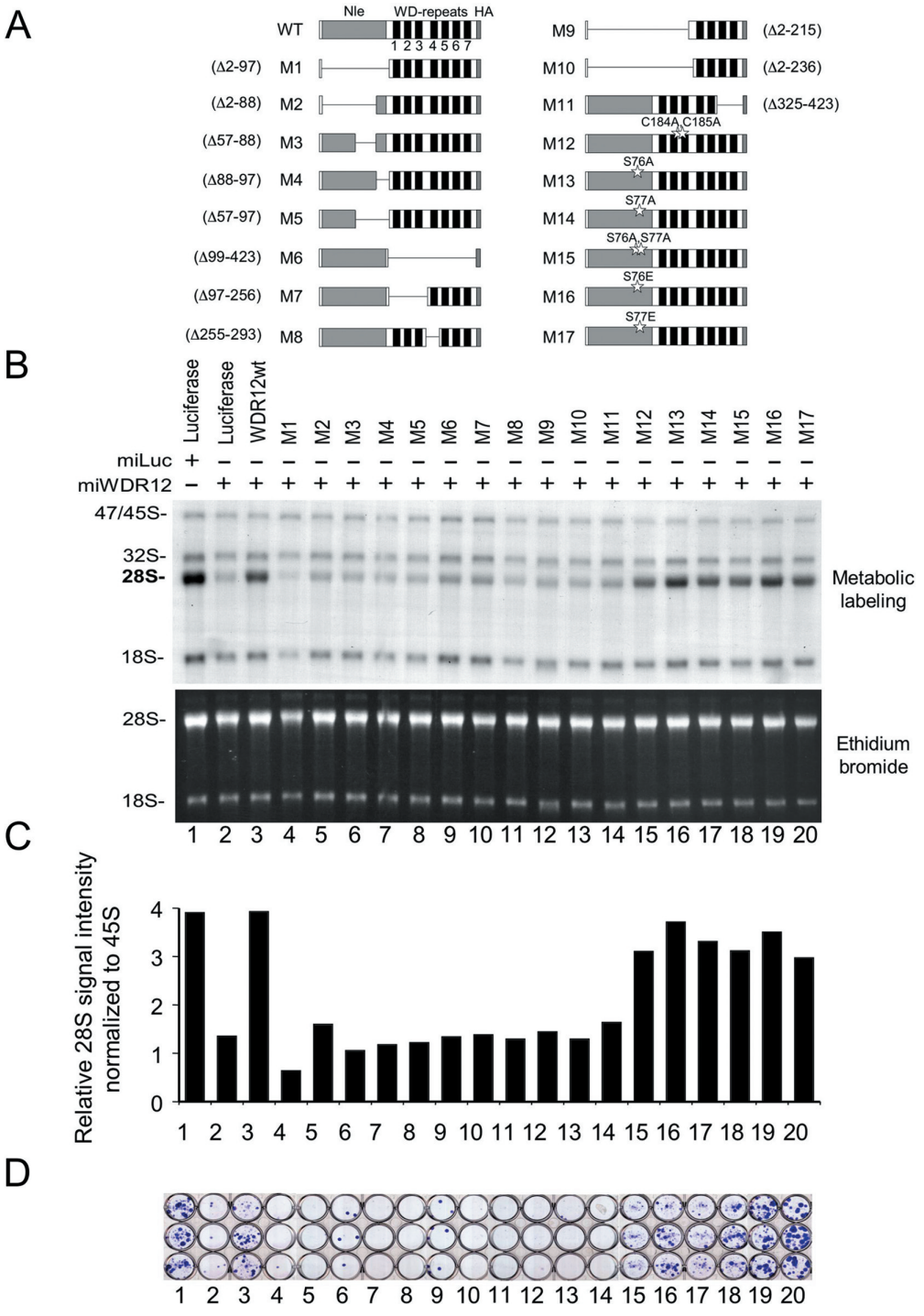

Figure 3. Functional analysis of WDR12 mutants in cells depleted for endogenous WDR12. (**A**) Overview of WDR12 mutants. Deleted amino acids are indicated. Stars depict point mutations at this site. Nle: notchless-like domain; HA: HA-tag. (**B**) Metabolic labeling of nascent rRNA as already described in this study. Stably transfected H1299 cells express either luciferase or the indicated forms of HA-tagged WDR12, in addition to one of the miRNA-embedded siRNAs targeting luciferase (miLuc) or endogenous WDR12 (miWDR12). The ethidium bromide stained gel is shown as loading control. (**C**) Experiment as described in (B). Quantification of the 28S rRNA signal intensity using a PhosphorImager. Histograms reflect arbitrary units of 28S rRNA signal intensity. (**D**) Analysis of cell proliferation. The indicated cell lines were seeded at very low density and cells were fixed and stained with Giemsa after 12 days. Cells were treated with 500 ng/ml doxycycline throughout the course of the experiment in the presence of hygromycin and puromycin.

absence of endogenous WDR12. However, all deletion mutants (M1–M11) failed to rescue rRNA processing (Figure 3B and C, lanes 4–14). Depletion of endogenous WDR12 and co-expression of the dominant-negative mutant M1 synergistically inhibited production of 28S rRNA (Figure 3B and C, lane 4). In contrast, mutants harboring point mutations (M12–M17) were functional in terms of rRNA processing (Figure 3B and C, lanes 15–20). Further,

we seeded the cells at low density to monitor colony outgrowth as a measurement of cell proliferation. Mutants, functional in rRNA processing, also promoted cell proliferation (Figure 3D, lanes 15–20). Interestingly, all functional WDR12 mutants harboring point mutations localized to the nucleolus as the wild-type protein (Supplementary Figure 1B), thus further supporting the previous results. Only the non-functional deletion mutants having deletions within the Notchless-like domain of WDR12 (M1–M5) exhibited nucleolar staining, whereas the remaining mutant forms (M6–M11) were found dispersed in the cytoplasm and nucleoplasm. In conclusion, our analysis demonstrates that the functional importance of specific regions or residues can be readily assessed by our knock-down–knock-in approach.

DISCUSSION

RNAi is a rapid and convenient approach to assess the function of mammalian genes. However, depletion of a protein not only abrogates its specific function but also can compromise the assembly of multimolecular complexes and thus results in a compound phenotype. Therefore, replacing the endogenous protein by mutant forms can preserve associated complexes and thus reveal more specific roles (9). To perform such a mutational analysis, we developed a rapid conditional knock-down–knock-in system for mammalian cells. We investigated the functionality of diverse mutants of WDR12, a factor involved in rRNA processing. Ribosome biogenesis is an essential biological process and therefore served as an appropriate example for the validation of our knock-down–knock-in approach.

Two individual episomal doxycycline-inducible pRTS constructs were used to achieve either the knock-down or the knock-in. In principle, the bidirectional promoter of the pRTS construct would allow a single plasmid strategy; however, in cell lines that are easy to transfect and suitable for double-selection, our approach is fast and convenient, as the combination of a knock-down construct with an established set of mutants does not require further cloning. In addition, knock-down of the endogenous gene was mediated by RNAi targeting the UTR of the mRNA. This strategy avoided laborious target site mutagenesis of the ectopic wild-type and mutant forms, as they only contain ORF sequences and hence lack the UTR. Even though ORF targeting is recommended in general for gene knock-down by RNAi, our UTR directed approach proved to be highly efficient in particular for knock-down–knock-in strategies. We decided to use siRNAs embedded in a modified miRNA environment, as they are processed from Pol II transcripts and could therefore be expressed from the doxycyline-dependent minimal CMV promoter of the pRTS construct. This episomal vector provides all features for doxycycline-dependent gene expression and proved to be suitable for conditional gene expression in several cell lines. Further, stable polyclonal cell lines can be established by non-viral transfection techniques. Depletion of the endogenous protein could also be achieved by synthetic siRNA oligos; however, our plasmid-based approach is more convenient and also cost effective for experiments that require large amounts of cells.

Reconstitution experiments with HA-tagged wild-type proteins demonstrated target gene specificity of the observed knock-down phenotypes induced by the miRNA-embedded siRNAs. We were able to rescue impaired rRNA processing and cell proliferation induced by depletion of endogenous Pes1 and WDR12 with the expression of HA-tagged wild-type forms. Therefore, our knock-down–knock-in approach can experimentally rule out off-target effects. Successful reconstitution experiments further provide a sensitive system to assess the functionality of mutant forms in cells depleted for the endogenous protein. This approach is advantageous over depletion experiments, as mutants can be generated that confer specific defects without affecting other functions or the assembly of associated multimolecular complexes. In respect to the tremendous recent advances in proteomics, our method provides a rapid and convenient tool for the generation of sufficient amounts of mammalian cells that have an endogenous protein replaced by a tagged mutant form. Thus, complexes can be purified and further analyzed by mass spectrometry. Moreover, the impact on a whole organelle, such as the nucleolus, can be investigated. Indeed, the nucleolar proteome was recently characterized in HeLa cells and provided novel insights into its dynamic behavior (18). Interestingly, a large group of unknown proteins was identified that had no respective yeast homologue. Our knock-down–knock-in method allows a rapid and detailed functional analysis of known and novel factors and will substantially support future proteomic approaches, not only in the field of ribosome biogenesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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