Non-targeting control for MISSION shRNA library silences SNRPD3 leading to cell death or permanent growth arrest

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In parallel with the expansion of RNA interference (RNAi) techniques, accumulating evidence indicates that RNAi analyses might be seriously biased due to the off-target effects of gene-specific short hairpin RNAs (shRNAs). Our findings indicated that off-target effects of non-targeting shRNA comprise another source of misinterpreted shRNA-based data. We found that SHC016, which is one of two non-targeting shRNA controls for the MISSION (commercialized TRC) library, exerts deleterious effects that lead to elimination of the shRNA-coding cassette from the genomes of cultured murine and human cells. Here, we used a lentiviral vector with inducible SHC016 expression to confirm that this shRNA induces apoptosis in murine cells and senescence or mitotic catastrophe depending on the p53 status in human tumor cells. We identified the core spliceosomal protein, small nuclear ribonucleoprotein Sm D3 (SNRPD3), as a major SHC016 target in several cell lines and confirmed that CRISPRi knockdown of SNRPD3 mimics the effects of SHC016 expression in A549 and U251 cells. The overexpression of SNRPD3 rescued U251 cells from SHC016-induced mitotic catastrophe. Our findings disqualified non-targeting SHC016 shRNA and added a new premise to the discussion about the sources of uncertainty in RNAi results.

INTRODUCTION

Gene functions can be determined by a loss-of-function approach using RNA interference (RNAi). The technique is based on the natural mechanisms of post-transcriptional gene silencing by short RNA species such as microRNA (miRNA) derived from endogenous precursors (pre-miRNAs) and small interfering RNA (siRNA) derived from exogenous long double-stranded (ds)RNA of viral origin.1

Primary miRNAs (pri-miRNAs) are processed in the nuclei of mammalian cells by the microprocessor complex to produce hairpin pre-miRNAs, which are further processed by the cytosolic endonuclease Dicer to miRNA duplexes with two nucleotide overhangs at the 3’ end of each RNA strand. Argonaute protein is loaded with one strand of the miRNA duplex to create an RNA-induced silencing complex (RISC), which interacts with a target mRNA leading to inhibition of its translation and/or degradation.3

miRNA-like siRNA duplexes that did not require endonucleolytic processing were initially designed and transfected into cells to silence genes of interest. However, inefficient transfection of many cell lines as well as the transience of siRNA activity have led to the development of short hairpin RNA (shRNA) expression vectors including retroviral and lentiviral vectors.3 An expression cassette coding for a given shRNA is stably integrated into the DNA of transduced cells, and the transcribed shRNA, which mimics pre-miRNA, is processed by Dicer to an siRNA duplex.4

The need for unified research tools has triggered the development of shRNA libraries of sequences that silence individual genes in the same shRNA backbone. Engineered shRNA libraries have facilitated the development of high-throughput methods using arrayed or pooled RNAi screens to identify proteins involved in different cellular processes or novel specific therapeutic targets.3–8

A popular lentiviral TRC shRNA library has been developed by the RNAi Consortium at the Broad Institute and marketed by Merck (Darmstadt, Germany; previously Sigma-Aldrich, St. Louis, MO, USA) under the trade name MISSION. The library contains shRNAs targeting ~15 × 103 human and ~15 × 103 mouse transcripts, each of which is covered by an average of five shRNAs. The library was generated using the pLKO.1 backbone containing the puromycin-resistance gene and shRNA sequences flanked by Pol III-recognized elements: the human U6 promoter and the T-stretch termination signal.3

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Figure 1: Effects of doxycycline on cell viability, caspase activity, and cell cycle distribution in SHC002 and SHC016 cells.

A) Cell viability was measured at different time points (days) after doxycycline treatment.
B) Cell cycle distribution was analyzed at different time points (days) after doxycycline treatment.
C) Caspase activity was determined at different time points (days) after doxycycline treatment.
D) Cell cycle distribution was analyzed at different time points (days) after doxycycline treatment.

Figure 2: Western blot analysis of p53 expression in SHC002 and SHC016 cells.

E) Western blot analysis of p53 expression in MC38CEA and MEF cells.

Figure 3: mRNA expression levels of various genes in SHC002 and SHC016 cells.

F) mRNA expression levels of various genes in MC38CEA and MEF cells.

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All experiments involving RNAi, including high-throughput screening and analyses of individual transcripts, require appropriate controls. An empty vector control does not contain any shRNA sequences and allows evaluation of the effects of transduction and of lentiviral vector elements (other than shRNA) on cells. A non-targeting shRNA control contains an shRNA-coding sequence that does not target any gene of a studied species. Cells transduced with a control lentiviral vector produce shRNAs that influence miRNA processing machinery and possibly other cellular processes according to non-specific effects triggered by gene-targeting shRNAs. This control should be used as a reference for all experimental results.

The MISSION library provides two non-targeting shRNA controls. One (SHC002) does not target mammalian transcripts, but it does target the turboGFP transcript and is therefore not recommended for cells expressing turboGFP. Supplier information states that the other non-targeting shRNA control (SHC016) does not target any transcript in any species according to bioinformatic data.

We applied the SHC016 as a control in our studies and found that it had detrimental effects on transduced cells. We ruled out the possibility that the high rate of cell death was due to the puromycin effect on the cells that remained non-transduced. We accomplished this by generating vectors with the tetracycline/doxycycline-inducible expression of either SHC002 or SHC016, which enabled us to separate the processes of transduction/selection and shRNA expression. We then confirmed that SHC016 shRNA was cytotoxic using the vectors, Tet-on-SHC002 and Tet-on-SHC016. We also found that SHC016 induces different death pathways in murine and human cells and elucidated the mechanism of its action.

RESULTS

We considered that the MISSION non-targeting control shRNA, SHC016, is deleterious to transduced cells due to unusually high mortality of the cells after transduction. We therefore compared the effects of the non-targeting shRNAs, SHC002 and SHC016, on MC38CEA cells transduced with the same amounts of either Tet-on-SHC002 or Tet-on-SHC016; then we used puromycin to select cells with incorporated transgenes. The cells were then cultured for 5 days with the expression of the non-targeting shRNAs switched on for the last 4, 3, or 2 days. Unlike SHC002, SHC016 significantly impacted transduced MC38CEA. The viability of the cells was measured using the colorimetric thiazolyl blue tetrazolium bromide (MTT) assay, in which MTT is reduced by metabolically active cells to purple formazan. The viability of MC38CEA cells decreased to ~30% on day 4 after inducing SHC016 expression (Figure 1A). The increased exposure of phosphatidylserine on the external leaflet of the plasma membrane detected by annexin V binding (Figures 1B and S1A) and the enhanced activity of the executioner caspase-3 and -7 (Figure 1C) indicated that the expression of SHC016 induced apoptosis in MC38CEA cells. This process was accompanied by an increase in the number of cells in the G2/M and a decrease in the number of cells in the G1 phase of the cell cycle, indicating cell-cycle arrest at the G2/M phase (Figures 1D and S1B).

Because p53 accumulation is believed to be the major trigger of G2/M arrest, we analyzed the influences of SHC002 and SHC016 on p53 protein levels and the expression of p53 target genes including Mdm2, the apoptosis inducers Bbc3 (also known as Puma) and Pmaip1 (Noxa), as well as those involved in inhibition of G2/M transition such as Cdkn1a (coding for p21), Gadd45a, Sfn (stratifin; 14-3-3σ), and Rprm (reprimo) in MC38CEA cells and MEFs.10,11 We also analyzed Ccnb1, which encodes mitotic cyclin (cyclin B1), and Mdm4, the product of which, apart from MDM2, binds p53 and inhibits its transcriptional activity.12

The expression of SHC016 resulted in substantially increased p53 levels in MC38CEA cells, indicating that SHC016 induced cellular stress (Figure 1E). Levels of p53 were high in all MEF cell lines regardless of the status of SHC002 or SHC016 expression (Figure 1E), which most likely reflected the activity of SV40 large T antigen (LT) that was used to immortalize MEF cells.10 A typical p53 response was not switched on in either MC38CEA or MEF; levels of p53 target genes were mostly unaffected (Bbc3, Pmaip1; data not shown) or reduced
Changes in the expression of genes related to cell-cycle control were found in MC38CEA cells and MEFs with induced expression of SHC016, but not SHC002, and the profiles of changes only partially overlapped between the cell lines (Figure 1F). The expression of Mdm4 and Sfn was decreased, and that of Ccnb1 was decreased to a small extent in both cell lines. The expression of Gadd45a was augmented only in MC38CEA cells. Levels of Rprm, which is undetectable in MC38CEA, were upregulated in MEFs expressing SHC016. The expression of both Gadd45a and Rprm might be upregulated by other transcription factors in addition to p53. 12,13,16 GADD45A is believed to affect cell-cycle progression via interactions with various molecules, such as PCNA, CDK1, and p21, and Rprm inhibits formation of the active cyclin B-CDK1 complex. Consequently, the expression of SHC016 induced distinct changes in the expression of genes related to cell-cycle control in the two mouse cell lines. Although these changes might affect the G2/M transition in MC38CEA and MEF cells, they did not imply the mechanism of SHC016 action.

We further analyzed the effects of the non-targeting sequences SHC002 and SHC016 on the human tumor cells A549, U251, HeLa, PC3, and MCF7 to determine whether the deleterious effects of SHC016 are limited to cells of murine origin or involve human cells. In contrast to SHC002, SHC016 potently impacted the viability of all cell lines (Figures 2A and S6).

We excluded the possibility that the negative effects of SHC016 were a matter of high transgene levels because qPCR analysis indicated that the levels of a pLKO cassette encoding SHC016 were comparable to or lower than those encoding SHC002 in all analyzed cell lines (Figure S7). The interferon response also did not occur in either A549 or U251 cells (Figure S8).

Unlike murine cells, apoptosis, evaluated using annexin V/propidium iodide (PI), did not seem to play a primary role in SHC016-mediated effects in human cells (data not shown). Cell-cycle analysis did not yield conclusive results. Therefore, we investigated whether the SHC016 sequence affects the proliferation or survival of human cells by simultaneously analyzing proliferation and viability after inducing non-targeting shRNAs expression; incorporation of bromodeoxyuridine (BrdU) indicated ongoing DNA synthesis associated with proliferation, and staining of dead cells indicated loss of viability (Figure 2B). The induction of SHC016 expression almost completely inhibited the incorporation of BrdU in A549 cells. The ratios (%) of U251, PC3, and HeLa cells that ceased to synthesize DNA did not increase or increased only slightly, whereas BrdU incorporation declined but to a lesser degree in MCF7 than in A549 cells. The type of response correlated with the p53 status of the cell lines. Only A549 cells express fully active p53, and the induction of SHC016 expression resulted in its accumulation in these cells (Figure 3A). Both U251 and PC3 express only mutated variants of p53, which cannot activate p53 target gene transcription, and wild-type (WT) TP53 is transcribed in HeLa and MCF7 cell lines, but the activity of p53 protein is reduced. 11 The papilloma virus protease E6 degrades p53 in HeLa cells, and MDM4 amplification impairs p53 functions in MCF7 cells. 11 Accordingly, SHC016 induced an increase in p53 levels in HeLa and MCF7 cells, although to a lesser extent than in A549 cells (Figure S9). However, growth arrest was irreversible independently of p53 status in cells with induced SHC016 expression; the results of colony formation assays showed that A549, U251, and HeLa cells lost the capacity to produce colonies (Figure 2C).

We speculated that SHC016 exerts deleterious effects via different mechanisms, one of which involved the activation of p53 in A549 cells. We then confirmed this notion and further elucidated the possible mechanism of SHC016-induced effects in A549 cells as follows. We used qRT-PCR to analyze potential changes in the expression of genes (1) activated by p53, including those encoding cell-cycle inhibitors and mediators of apoptosis (CDKN1A, MDM2, BBC3, PMAIP1, GADD45A, and Sfn); (2) encoding central players in cell-cycle progression including CDK2, CDK1, CCNB1, AURKA, and PLK1, components of chromosomal passenger complex (AURKB, BIRC5, and CDC28); and (3) encoding proteins of cell-cycle checkpoints (CHEK1, CHEK2, BUB1, BUB1B, BUB3, and MAD2L1). Switching on SHC016 but not SHC002 expression resulted in stimulation of the p53-regulated genes CDKN1A, MDM2, and BBC3, 24 h after adding doxycycline to the culture medium. This effect was further enhanced after 48 h (Figure 3B). The most stimulated gene was CDKN1A, which encodes p21, a universal inhibitor of cyclin-dependent kinases, and activates formation of the DREAM complex, which acts as a transcriptional repressor of genes involved in cell-cycle progression, including all genes in groups (2) and (3) above. 12 This explains the diminished levels of most of analyzed transcripts 48 h after inducing SHC016 expression (Figure S10) and halting A549 proliferation.

Analysis of CDKN1A expression in all studied cell lines revealed the absence of p53 transcriptional activity in U251, PC3, and MCF7 cells and slight activity in HeLa cells (Figure S11).

The expression of three potential p53 targets did not increase but rather decreased in A549 cells in response to SHC016: PMAIP1, GADD45A, and Sfn (Figure 3B). These genes might be regulated by other mechanisms. Expression of the Sfn (mouse ortholog of Sfn) was diminished in both murine cell lines expressing SHC016, and that of Gadd45a was diminished in MEF. Thus, we speculated that expression of SHC016 might impact the regulation of these transcript levels.

In addition to growth arrest, p21 might trigger a cellular senescence program, 3 in which cells permanently cease proliferation and have
significantly altered morphology but remain metabolically active.\textsuperscript{14–16} The key senescence marker, senescence-associated \(\beta\)-galactosidase (SA-\(\beta\)-gal), was active in 59.5% of A549 cells with induced SHC016 expression. In contrast, the ratios of SA-\(\beta\)-gal-positive cells among A549 cells without SHC016 induction or transduced with Tet-on-SHC002 were negligible (<1%) (Figure 3C). Enlarged and flattened areas of cells and irregular shapes are also characteristic of senescence (Figure 3C). The expression of SA-\(\beta\)-gal in PC3, HeLa, and U251 remained low despite SHC016 induction, whereas MCF7 cells expressed high levels of SA-\(\beta\)-gal independently of transduction with Tet-on vectors and doxycycline induction (data not shown).

SHC016 exerted harmful effects via different mechanisms in \(TP53\) mutant U251 and PC3 cells. Images of U251 and PC3 cells expressing SHC016 show hallmarks of mitotic catastrophe (MC) (Figures 4A and S12A–S12D). MC is a mechanism that senses aberrant mitosis...
and drives cells to an irreversible fate (death or senescence). This process occurs when defective checkpoints do not arrest cell-cycle progression in response to critical conditions such as genotoxic stress, delayed DNA replication, or aberrant spindle formation.

Giant multinucleated cells that are typical of MC comprised a considerable proportion of the U251 and PC3 cells expressing SHC016 (Figures 4A and 4B and S12). Some giant nuclei continued to synthesize DNA at 5 days after switching to SHC016 expression. The findings of fluorescence microscopy showed that BrdU was incorporated into DNA when added at the end of U251 culture (Figure S13). This agreed with the cytometric analysis of cell proliferation and viability, which showed that ~32% of U251 cells expressing SHC016 incorporated BrdU (Figure 2B). However, BrdU was not always evenly distributed within DNA, indicating unsynchronized DNA replication in some U251 cells expressing SHC016 (Figure S13).

The images of interphase nuclei of cells expressing SHC016 also revealed irregular patches of heterochromatin (another trait of necrosis and senescence), numerous micronuclei, and occasional supernumerary centrosomes (Figures 4C and S14). Multipolar spindles were formed during metaphase more often in U251 cells expressing SHC016 than SHC002 and also in these cell lines when cultured without doxycycline. Almost 25% of mitotic cells expressing SHC016 formed multipolar spindles, whereas in other groups, such a defect was observed in ~5% of mitotic cells. Chromosome alignment was also occasionally defective in U251 cells with induced SHC016 expression (Figures 4D and 4E).

We analyzed the expression of genes encoding proteins involved in cell-cycle execution and control, including those responsible for correct spindle formation. The results showed that SHC016 expression in U251 cells did not significantly affect the levels of most transcripts for at least up to 48 h after doxycycline induction (Figure S15). Like the other cell lines examined, levels of both SFN and GADD45A transcripts decreased in response to SHC016 expression in U251 cells. The effects of SHC016 expression in studied cells are summarized in Table S2.

To verify the hypothesis that the type of cell death induced by SHC016 expression depends on p53 status, we knocked down TP53 in A549 cells (Tet-on-SHC002 and Tet-on-SHC016 transduced) using the CRISPR-Cas system. We then compared the effects of SHC016 expression in p53-WT and p53-knockdown (p53-KD) A549 cells. Western blots showed that the accumulation of p53 in response to SHC016 expression was impaired in p53-KD, compared with p53-WT A549 cells (Figure 5A). The substantial decrease in p53 inducibility did not change the SHC016-mediated decline in A549 viability (Figure 5B). However, the appearance of nuclei of SHC016-expressing p53-WT and p53-KD cells significantly differed

expressing SHC002 but common among cells expressing SHC016, are indicated by arrowheads. The scale bar represents 100 μm. Three independent experiments were performed.
Figure 4. Expression of SHC016 in U251 cells results in mitotic catastrophe

Fluorescence microscopy analyses of nuclear morphology and mitosis in U251 cells. The cells were left untreated, or the expression of SHC002 or SHC016 was induced with dox 5 days prior to DNA staining. (A) Exemplary merged transmitted light and fluorescence images representing U251 with regular nuclei typical for SHC002-expressing cells or U251 with catastrophic nuclei prevailing among cells expressing SHC016. DNA was stained with DAPI (blue). Original images are available in Figure S12A. More examples are presented in Figure S12B. (B) Quantitative analysis of nuclear morphology in U251 with uninduced or induced expression of SHC002 or SHC016. Data are from two

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The fraction of cells with altered nuclear morphology (irregularly shaped nuclei or micronuclei) was substantially greater in p53-KD A549 cells with induced SHC016 shRNA expression than in p53-WT cells expressing SHC016 (49% versus 26%). Moreover, unlike p53-WT A549 cells, 20% of p53-KD A549 cells were driven toward MC after switching on SHC016 expression with doxycycline (Figures 5C, 5D, and S17). The morphological abnormalities were similar among p53-KD A549 cells and U251 and PC3 p53 mutant cells expressing SHC016, supporting the notion that p53 status influences the mode of SHC016-induced death or growth arrest in human cell lines.

Changes in the expression of genes important for cell-cycle progression differed among cell lines. Therefore, we rejected the notion that these genes are responsible for the SHC016-induced effects. We conducted a preliminary RNA sequencing (RNA-seq) analysis of RNA from two cell lines to identify the primary mediator(s) of these effects. We analyzed RNA isolated from A549 and U251 cells transduced with Tet-on-SHC016 and incubated for 24 h with or without doxycycline. Levels of cyclic AMP (cAMP)-regulated phosphoprotein 19 (ARPPI9), vacuolar protein sorting B (VPS4B), and small nuclear ribonucleoprotein Sm D3 (SNRPD3) transcripts were substantially diminished in both cell lines after inducing SHC016 expression. Analysis using gProfiler and published results indicated that the products of these transcripts regulate cell-cycle progression and cell division (ARPPI9 and VPS4 proteins) or when downregulated, trigger activation of the p53 pathway or abnormal mitosis (SNRPD3). The activity of ARPPI9, which is an inhibitor of PP2A phosphatase, is required to maintain high levels of cyclin B1-CDK1 complexes during mitosis.18 VPS4B is a part of the ESCRT III complex involved in cytokinesis,19 and SNRPD3 is a component of U1, U2, U4, and U5 Sm RNP (snRNP) complexes.20

To verify the importance of RNA-seq results, we analyzed the influence of SHC016 on ARPPI9, VPS4A, VPS4B, and SNRPD3 expression in the human cell lines and in murine MC38CEA cells. Although RNA-seq analysis revealed decreased VPS4A expression only in A549 cells, we included this gene because VPS4A is essential for proper spindle formation and cytokinesis,19 processes that are seriously disturbed in U251 cells. The levels of ARPPI9, VPS4B, and SNRPD3 transcripts were decreased in all human cell lines in response to SHC016, but not to SHC002, and the decrease in SNRPD3 was the most significant (Figures 6A and S18). The levels of VPS4A were diminished in response to SHC016 in all human cells except MCF7. Levels of Arpp19 and Vps4b were not affected, Vps4a was moderately reduced, whereas Snrpd3 expression was reduced by ~75% in murine MC38CEA cells expressing SHC016 (Figure S18).

To determine whether the decrease in the expression of any of these genes mimics the effects of SHC016, we applied a doxycycline-inducible CRISPRi system to A549 and U251 cells using two single guide RNAs (sgRNAs) to silence the transcription of each gene. We analyzed the effects of CRISPRi later than those of shRNAs because CRISPRi inhibits de novo RNA synthesis, whereas shRNA affects existing RNAs. Levels of specific mRNAs were measured at 72 h, and the effects on cell viability were assessed at 6 days after inducing dCas-KRAB-MeCP2 expression.

SNRPD3, VPS4A, VPS4B, and ARPPI9 transcript levels were moderately decreased when dCas9-KRAB-MeCP2 expression was induced in U251 cells by 100 ng/mL of doxycycline (Figure S19), whereas were decreased to ~50%, 40%, 25%, and 35%, respectively, of their initial levels by 1 µg/mL of doxycycline (Figures 6B and S19). Decreasing SNRPD3 expression by 50% in U251 cells resulted in decreased cell viability (Figure 6B) associated with the hallmarks of MC found in U251 cells expressing SHC016 (Figure S20). Silencing VPS4A, VPS4B, or ARPPI9 did not affect the viability of U251 cells (Figure 6B).

The A549 cells were resistant to transfection, and attempts to efficiently silence the expression of all genes of interest via cell transfection with CRISPRi plasmids bearing one sgRNA sequence failed (Figure S21A). Simultaneous transfection of A549 cells with two CRISPRi plasmids encoding different gene-specific sgRNAs improved the outcome, but levels of SNRPD3 and VPS4A transcripts were diminished by only ~30% (Figure S21A) when transfection was replaced by electroporation levels of silencing SNRPD3, and VPS4A reached ~50%, and VPS4B and ARPPI9 reached ~75% (Figure S21A). The decrease in the level of SNRPD3 mRNA, but no other studied transcript, resulted in a substantial decline in A549 viability and an increase in CDKN1 expression (Figure 6C). Even small reductions in SNRPD3 mRNA levels after transfection of A549 cells with single or double CRISPRi plasmids were accompanied by diminished cell viability (Figure S21B).

We concluded that reduced levels of SNRPD3 in response to SHC016 expression accounted for the processes that led to death or at least to the permanent growth arrest of various cells transduced with the
MISSION non-targeting SHC016 vector. Because SNRPD3 is a subunit of the Sm protein complex involved in splicing, we speculated that a deficiency results in defective VPS4A, VPS4B, or ARPP19 transcript splicing and a decrease in the levels of these mRNAs. However, CRISPRi-diminished expression of SNRPD3 was accompanied by slightly increased levels of VPS4B and ARPP19 transcripts in U251 cells. In contrast, the levels of VPS4A were moderately reduced upon SNRPD3 silencing (Figure S22).

Figure 5. TP53 knockdown (KD) in A549 cells promotes mitotic catastrophe in response to SHC016 expression
(A) WB analysis of p53 levels in the lysates of p53-wild-type (WT) and p53-KD A549 cells in which SHC002 or SHC016 expression was induced by dox (100 ng/mL) 2 days prior to cell lysis. Right panel: quantification of normalized WB signals; p53-linked luminescence signals of the samples from p53-WT, dox-untreated cells were set as 1. Bars represent MV ± SD from 3 independent experiments. The uncropped WB image and the picture of Ponceau S-stained membrane are available in Figure S16. (B) Cell viability assessed by MTT assay. The expression of SHC002 or SHC016 was induced by dox for the last 5, 4, 3, or 2 days of the 6-day culture. Bars represent MV ± SD from 4 independent experiments. (C) Quantitative analysis of nuclear morphology of p53-WT and p53-KD A549 cells visualized by fluorescence microscopy. The cells were left untreated, or the expression of SHC002 or SHC016 was induced with dox 5 days prior to DNA staining with Hoechst 33342. The population described as altered includes cells with irregularly shaped nuclei, those with nuclear blebs or micronuclei, and binucleated ones. Data are from one experiment representative of three independent experiments with similar results. At least 150 cells were analyzed for each experimental group. (D) Exemplary merged transmitted light and fluorescence images of p53-WT and p53-KD A549 cells. Induction of SHC016 expression resulted in mitotic catastrophe only in p53-KD cells (last image, lower panel). DNA was stained with Hoechst 33342 (blue). The scale bar represents 10 μm. Original images are available in Figure S17A. More examples are presented in Figure S17B.
Figure 6. Deleterious effects of SHC016 are caused by diminished SNRPD3 transcript levels

(A) qRT-PCR analysis of transcripts identified by RNA-seq as potentially affected by SHC016 performed 24 and 48 h after inducing expression of SHC002 or SHC016 shRNAs in A549 and U251 cells. The relative levels of the transcripts in uninduced cells were taken as 1. The data concerning other cell lines are available in Figure S18. (B)
We next investigated the mechanism through which SHC016 caused the decrease in SNRPD3 transcript levels. In contrast to the diminished levels of a mature SNRPD3 transcript, SHC016 expression did not affect its pre-mRNA levels in U251 and MC38CEA cells, indicating that SHC016 did not interfere with SNRPD3 transcription but rather acted at the post-transcriptional level (Figure 6D). We applied the luciferase reporter assay to determine whether the susceptibility of the SNRPD3 transcript to SHC016-mediated effects depended on its 3’ UTR. We cloned the human SNRPD3 (hsNRPD3) 3’ UTR (Figure 6E) into the pmirGLO vector, which enabled estimation of the effect of SNRPD3 3’ UTR on luciferase activity after switching on SHC002 or SHC016 expression. In U251 cells transfected with an empty vector (without SNRPD3 3’ UTR) or with a vector carrying the SNRPD3 3’ UTR, luciferase activity did not change after inducing SHC002 expression. The induction of shRNA expression reduced the luciferase activity in the U251 transduced with SHC016 and transfected with pmirGLO containing SNRPD3 3’ UTR. The absence of changes in the cells transfected with the empty vector indicated that the SNRPD3 3’ UTR was responsible for the diminished luciferase activity after inducing SHC016 expression (Figure 6F). Analysis of SHC016-mediated inhibition of luciferase activity in U251 cells transfected with vectors encoding luciferase CDS with SNRPD3 3’ UTR-derived fragments of various lengths revealed that a sequence comprising nucleotides (nt) 100–200 of the 3’ UTR might be involved in the SHC016-mediated downregulation of SNRPD3 mRNA (Figures 6F and S23B and S23C).

U251 cells transfected with pmirGLO vector containing nt 96–198 of the SNRPD3 3’ UTR showed almost the same reduction in luciferase activity after switching on SHC016 expression as the cells transfected with pmirGLO containing full-length SNRPD3 3’ UTR (Figure 6F). In contrast, luciferase activity was only slightly decreased when pmirGLO carried the SNRPD3 3’ UTR lacking nt 101–198 (Δ101–198). This indicated that SHC016 targets a sequence that lies within nt 101–198 of the SNRPD3 3’ UTR. We then performed analysis of potential interactions between sequences possibly derived from precursor SHC016 shRNA and the fragment of SNRPD3 3’ UTR (nt 101–198) using RNA-hybrid.23 Because shRNAs derived from pLKO1 vectors are known to undergo Dicer-independent processing, we analyzed every possible 21 nt sequence derived from SHC016. We then deleted fragments in the pmirGLO-SNRPD3 3’ UTR that were the most probable sites of interaction with shRNA-derived sequences based on the lowest free energy of binding (the sequences and locations of the deleted regions are shown in Figure 6E). Deletions of sites A and B (ΔA and ΔB, respectively) considerably diminished the negative effects of SHC016 expression on luciferase activity. Possible interactions between SHC016-derived sequences and SNRPD3 3’ UTR are presented in Figure S23D. Luciferase activity in U251 cells transfected with Δ101–198 of the SNRPD3 3’ UTR instead of the full-length 3’ UTR. Therefore, we suspect that SNRPD3 3’ UTR might have additional, low-affinity sites of interaction with sequences derived from SHC016 shRNA. The same sequences derived from SHC016 might bind to the mouse Snrpd3 (mSnrpd3) transcript mediating its downregulation (Table S3).

To verify the hypothesis that silencing SNRPD3 is the key mediator of SHC016-deleterious effects, we transfected U251 cells carrying dox-inducible SHC002 or SHC016 shRNAs with vectors carrying mSnrpd3, hSNRPD3, and optimized (opt-SNRPD3) variants of SNRPD3 CDS. All variants encoded the same amino acid sequence. Silent mutations were introduced at all possible sites in opt-SNRPD3 to ensure that the new sequence was resistant to possible SHC016-mediated silencing. Transfected U251 cells with either mouse or opt sequences allowed discrimination between endogenous and overexpressed SNRPD3 transcripts, whereas transfection with the human sequence enabled evaluations of endogenous and total SNRPD3 mRNA levels. The expression of SHC016 induced by doxycycline (100 ng/mL) for 48 h diminished levels of endogenous SNRPD3 mRNA in U251 cells transfected with a control vector encoding EGFP and in U251 cells transfected with vectors carrying any of the SNRPD3-coding sequences (Figure 7A). The effects of 10 ng/mL of doxycycline were delayed but comparable after 96 h to those of a 2-day high dose of doxycycline. In contrast to endogenous SNRPD3 mRNA, the expression of SHC016 did not affect levels of SNRPD3 mRNAs transcribed from any of the introduced vectors (Figure 7A), implying that SNRPD3 CDS is not a target of SHC016. Despite strong SNRPD3 overexpression at the transcript levels judged by a comparison of ΔCq values in qPCR analysis (for example, ΔCq [SNRPD3-3’UTR] = −1.18, −2.87, and −3.07 for mouse, human, and opt sequences, respectively), the increase in protein levels was moderate even in the absence of SHC016. This was most probably due to a
Figure 7. Restoring the expression of SNRPD3 in U251 cells reduces the deleterious effects of SHC016

(A) qRT-PCR analysis of SNRPD3 mRNA levels in U251 cells transfected with a vector coding for EGFP (control) or a vector carrying one of the SNRPD3-coding sequences: mouse (mSnrpd3), human (hSNRPD3), or optimized (opt-SNRPD3) and treated with dox to induce SHC016 expression. The relative levels of the transcripts in uninduced cells were taken as 1. Data are shown as MV ± SD from two independent experiments. (B) WB analysis of SNRPD3 levels in U251 cells described in (A) incubated for 48 or 96 h with dox. (C) MTT assay of U251 cells transfected with EGFP (ctr) or a vector carrying one of the SNRPD3-coding sequences and treated with dox to induce SHC016 expression. (D) Phase contrast images of U251 cells transfected with EGFP (ctr) or a vector carrying one of the SNRPD3-coding sequences and treated with dox to induce SHC016 expression.
mechanism that ensures a balanced supply of subunits of heteromeric complexes and involves the degradation of overproduced subunits relative to other proteins of the complex.\textsuperscript{22,23}

The decrease in SNRPD3 protein in response to SHC016 expression in U251 cells transfected with a control vector reflected changes in mRNA levels. Doxycycline (100 ng/mL for 2 days or 10 ng/mL for 4 days) caused a substantial decrease of SNRPD3 in cells transfected with the control vector (Figure 7B). The level of endogenous SNRPD3 protein that was reduced in U251 cells expressing SHC016 was fully restored by the expression of exogenous hSNRPD3 and opt-SNRPD3 but only partially complemented by that of mSnrdp3 (Figure 7B).

Cell viability determined using MTT assays was considerably improved in cells in which SHC016 expression was accompanied by exogenous SNRPD3 expression. The level of improvement correlated with that of SNRPD3 expression (Figures 7B and 7C). The restoration of efficient SNRPD3 synthesis abrogated the effect of a 24-h induction of SHC016 expression (Figure S25). Transfecting U251 with a vector encoding the mSnrdp3, hSNRPD3, or opt-SNRPD3 variants rescued the cells from MC. Nearly all U251 cells transfected with the control EGFP-coding vector underwent MC after expressing SHC016 for 7 days. In contrast, MC was not evident in U251 with restored levels of SNRPD3 (Figures 7D and S26).

How is it possible that scientists around the world widely use the MISSION system without noticing the effects we describe here? Usually, commercially available vectors with non-inducible expression of shRNAs are used with the MISSION system. Therefore, the initial massive death of cells is attributed to the effects of puromycin on non-transduced cells. We believe that with subsequent cell divisions, only cells with low SHC016 expression survive, even when cells transfected with doxycycline-inducible SHC016 discontinue the selective antibiotic puromycin, which is in line with generally accepted protocols, resulted in less deleterious effects of SHC016 compared with cells in which SHC016 was induced with doxycycline in the presence of puromycin. The viability of MCF7 and HeLa cells, evaluated using MTT assays, was further reduced by \textasciitilde 20\% points, when cells expressing SHC016 were cultured for 5 days with puromycin (Figure 8A). Adding puromycin to the culture medium of HeLa cells also resulted in the increase in the population of dead cells, increasing from 5\% to 40\% in cells expressing SHC016 but not SHC002, according to the results of the proliferation and viability determinations (Figure 8B).

To verify the hypothesis that only cells with low SHC016 expression can persist in long-term cultures, we transduced MC38CEA and U251 cells, with undetectable, original versions of MISSION control vectors: namely, the empty vector SHC001 and two vectors with constitutive expression of the non-targeting shRNA sequences, SHC002 and SHC016. We isolated DNA from the cultured cells 2 days after transduction and then at 1-week intervals and analyzed levels of the transgenes by PCR using a primer pair that amplified the sequence comprising the PGK promoter and puromycin-resistance gene and another that amplified the area flanking the shRNA cloning site. Puromycin was added 48 h after transduction, and the cells were maintained under selective pressure for 7 days. The high mortality rate of U251 cells transduced with pLKO-SHC016 did not allow analysis within 1 month after transduction. Levels of SHC002 and SHC016 cassettes were similar at 2 days after transduction in both cell lines. However, in contrast to the empty vector and pLKO-SHC002, levels of the transgene containing SHC016 decreased dramatically during cell propagation (Figure 8C).

DISCUSSION

The initial enthusiasm accompanying the increasing application of RNAi techniques for exploring gene functions has recently declined with the emergence of publications highlighting their drawbacks and limitations, such as insufficient silencing of target gene expression, which could lead to false-negative results and false-positive off-target effects that are much more dangerous in terms of the further use of research results.\textsuperscript{24} Jackson et al.\textsuperscript{25}, in the already “classic” publication, demonstrated that different siRNAs targeting the same transcript have unique global gene-expression profiles with only a few genes regulated in common. This could have far-reaching, disastrous consequences because RNAi-based studies are often the cornerstones for the development of novel therapies. The excellent study by Sheltzer’s group\textsuperscript{26} provided crushing evidence for misdirected drug development based on erroneous RNAi data that have led to failed clinical trials. They analyzed five proteins (HDAC6, mitogen-activated protein kinase 14 [MAPK14], PAK4, PKB, and PIM1) that had been selected based mostly on RNAi data, as those required for the survival and/or proliferation of cancer cells and for which small molecule drugs that specifically block their effects were already in clinical trials or preclinical studies. Using CRISPR-based techniques, the authors proved that neither knockout (via CRISPRko) nor reducing expression levels (via CRISPRi) of genes encoding these proteins affected the viability of 32 cancer cell lines. These genes were apparently wrongly selected, probably due to RNAi off-target effects. An OTS964 inhibitor selected as an inhibitor of one of these targets, namely PKB, exerted potent antimitotic effects but inhibited the kinase CDK11B. Thus, a completely different group of cancer patients should comprise the targets for treatment with this compound than was apparent according to RNAi-based studies.\textsuperscript{26,27}
These examples and other critical analyses suggest that off-target effects might be erroneously attributed to the analyzed genes. Our work points to yet another overlooked and possible cause of discrepancies between various genotype-to-phenotype data and the frequent failure of verification of RNAi results by competitive or complementary techniques. The RNAi results might also be misinterpreted because of the off-target effects related to the non-targeting shRNA control, which, by definition, should not significantly affect the expression of any gene. We considered the consequences of the silencing activity of a non-targeting shRNA for RNAi data interpretation.

The first is that the inhibition of gene expression caused by a control shRNA, which functions as a reference, would be erroneously interpreted as the stimulation of the expression of that gene by experimental shRNAs.

However, the outcome of the control shRNA used in our study and probably in many others using MISSION shRNAs was more complex because SHC016 reduces the expression of the SNRPD3 gene that is essential for cell survival. During post-transduction antibiotic treatment, the cells that were most likely to survive would have had strongly reduced expression of this toxic shRNA. Therefore, levels of non-targeting and targeting shRNAs are incomparable, and cells expressing non-targeting shRNA cease to function as appropriate controls.

Because shRNAs use miRNA processing machinery and compete with pre-miRNA for Dicer and Ago proteins, disparate loads of shRNAs in control and experimental cells might distinctly influence endogenous miRNA functions, which would be manifested as differences in gene-expression profiles unrelated to specific shRNA effects. Moreover, during puromycin treatment, the cells that survive in the presence of antibiotic despite reduced occurrence of puromycin-resistance cassette associated with reduced levels of SHC016-coding sequence are selected. This functional phenotype can be attributed to cells overexpressing multidrug resistance gene(s) encoding ABC transporters that expel puromycin from cells. Apart from differences in the expression levels of endogenous gene(s) rendering puromycin resistance, unmatched intracellular levels of transgenic puromycin N-acetyltransferase and/or puromycin itself might distinctly affect cell transcriptomes.

Applying more restrictive rules for the design of control- and gene-specific shRNAs might limit off-target effects. The problem, however,
is quite complex due to the fact that the processing of the shRNA molecules, the choice of the guide strand from the siRNA duplex, and profiles of siRNA-mRNA interactions are not fully predictable.26–29

The recently discovered process of death induced by survival gene elimination (DISE) involves a group of siRNA/shRNAs that target the 3′ UTR of numerous essential survival genes via seed sequences as short as 6 nt.30,31 This phenomenon might reflect the natural mechanism of action of tumor-suppressor miRNAs.32 A similar mechanism has recently been proposed for viral miRNA-induced cell death.33 However, SHC016 shRNA does not fit this scenario, as its sequence does not have the characteristics of those identified as the most toxic, and the RNA-seq analysis showed that the expression levels of the survival genes reduced in DISE were not substantially changed in response to SHC016 expression.

Our results indicated that SNRPD3 silencing is a major culprit responsible for SHC016-induced effects. snRNP D3 is one of core proteins of Sm, and it plays a pivotal role in all splicing steps. Given that >95% of human protein-coding transcripts are spliced, it is reasonable to consider the key role of SNRPD3 in splicing, many transcripts, including SNRPD3, also influence the profile of alternative splicing.43,44 We did not find a global reduction in transcript levels in response to SNRPD3 silencing. Therefore, we suspected that the gradual decrease in this protein level primarily affects the expression of a few proteins crucial for cell-cycle progression.

SNRPD3 was selected in genomic screenings as one of the genes, depletion of which cause lethal defects in key steps of mitosis, including metaphase chromosome alignment45 and sister chromatid cohesion.46 However, little is known about possible transcripts encoding proteins crucial for cell-cycle progression, splicing of which might depend on SNRPD3 levels and whose defective splicing might explain the effects of silencing SNRPD3 expression either unintentionally via SHC016 or purposely using CRISPRi.

One candidate is the centromere protein E (CENP-E) transcript. CENP-E, a plus-end-directed kinesin-7 motor protein participates in chromosome congression and microtubule-kinetochore conjugation as well as in activation of the spindle assembly checkpoint. A deficiency in this protein might lead to mitotic arrest, followed by cell death. Silencing SNRPD3 in DLD-1 and HCT116 colorectal cancer cell lines led to diminished CENPE expression.37 We found that CENPE expression was diminished by ~80% in A549 cells but only by ~15% in U251 cells 48 h after inducing SHC016 expression (Figure S28). As CENPE is a DREAM target, the reduction of its mRNA level in A549 cells, the timing and extent of which resembles those of other DREAM repressed genes, most probably results simply from p53 activation. The lack of a substantial decrease in CENPE mRNA in U251 cells 48 h after inducing SHC016 expression suggests that CENPE is not responsible for the early manifestations of MC triggered by SNRPD3 silencing in U251 cells. However, because the estimated half-life of SNRPD3 (protein) is >27 h,48 CENPE mRNA levels might become gradually reduced over time. Indeed, at early time points (48 h after induction), centrosome amplification seemed to be the culprit for MC in U251 cells, because defects in chromosome congression accounted for only a small proportion of all mitotic defects. However, centrosome staining 5 days after inducing shRNA expression revealed that only a portion of cells that had undergone MC had supernumerary centromeres (Figures 4C and S14), indicating yet another mechanism that drives aberrant mitosis and in consequent MC.

SNRPD3 has also been identified in genome-wide screening of genes, silencing of which influences the viability of p53-WT cancer cells.19 The authors showed that silencing several splicing factors, including SNRPD3, induces prominent p53 activation in A549 non-small cell lung cancer cells. Our findings of p53-p21 axis activation in A549 cells after SHC016 expression agree with these results. We found that SHC016- or CRISPRi-mediated silencing of SNRPD3 resulted in deleterious effects in cells with or without active p53, which also agreed with the finding that cytotoxicity mediated by SNRPD3 silencing does not depend on p53 activity. However, changes in MDM4 splicing and expression postulated as a reason for deleterious effects of SNRPD3 silencing39 do not account for cytotoxicity in A549 and U251 in our studies, as we identified substantially diminished Mdm4 mRNA levels in murine but not in human cells expressing SHC016 (Figure 1; data not shown). Thus, we postulate that at least in these cells, the altered splicing of transcripts other than MDM4 plays crucial roles in mediating the effects of SHC016 expression and SNRPD3 silencing. The transcript of structural proteasome subunit β3 (PSMB3) is defectively spliced in SNRPD3-deficient A549 cells, which might be a cause of cell lethality.50 Additionally, aberrant splicing of the pre-mRNA coding for sororin, the regulator of sister chromatid cohesion, in cells depleted in SNRPD3 might be a key factor affecting mitosis in triple-negative breast cancer cells.51 Considering the key role of SNRPD3 in splicing, many transcripts, including those encoding proteins important for cell survival and proliferation, are probably affected by incorrect alternative splicing.

MATERIALS AND METHODS

Cell line cultures

The following cell lines were used: MC38CEA (murine colon adenocarcinoma52 expressing human carcinoembryonic antigen53), MEF (immortalized with SV40 LT, a gift from Prof. Paul Saftig, Christian-Albrechts University Kiel, Germany),54 U251 MG (human glioblastoma, verified by Eurofins Medigenomics, Ebersberg, Germany), A549 (human lung carcinoma; ATCC CCL-185), PC3 (human prostate adenocarcinoma; ATCC CRL-1435), MCF7 (human breast adenocarcinoma; ATCC HTB-22), and HeLa (human cervical adenocarcinoma; ATCC CCL-2). HeLa cells were grown in MEM (Lonza, Basel, Switzerland) and all other cell lines in DMEM (Corning, Corning, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) and tetracycline negative (Capricorn Scientific, Ebsdorf, Germany) at standard conditions. The cell cultures were routinely tested by PCR for mycoplasma contamination using mycoplasma rDNA-specific primers.
Lentiviral vectors
The following vectors from the MISSION library were used: MISSION pLKO.1-puro Empty Vector Control Plasmid DNA, which does not contain any shRNA insert (SHC001); MISSION pLKO.1-puro Non-Mammalian shRNA Control Plasmid DNA (SHC002), containing an shRNA insert, which, according to the description, does not target any known mammalian genes; and MISSION pLKO.1-puro Non-Target shRNA Control Plasmid DNA (SHC016), containing an shRNA insert, which, according to the description, does not target any genes from any species. SHC002 and SHC016 controls are available exclusively in the MISSION library. To produce doxycycline-inducible shRNA expression vectors, oligonucleotides coding for non-targeting shRNAs, identical to those in MISSION SHC002 and SHC016, 5′-CCGGCAACAAGATGAAGAGCACCAACTCGAGTT GGTGCTCTCATCCTGTTGTTT7TTT-3′ and 5′-CCGGCCGGCGAT AGCGCTATAATTTCGAGAAATTATTAAGCCTATCGCGC TTTTT-3′, respectively, were annealed and cloned into AgeI/EcoRI-linearized tet-pLKO-puro (Addgene plasmid #21915, a gift from Dmitri Wiederschain).55 Resulting vectors are referred to as Tet-on-shRNA plasmids.

Production and titration of lentiviral vectors
2 days prior to transfection, 293T cells were plated on 10 cm plates. The cells were transfected with plasmids: 1.3 pmol psPAX2 and 0.72 pmol pMD2.G (Addgene plasmids #12260 and #12259; both were gifts from Didier Trono) and 1.64 pmol respective pLKO plasmid using PEI MAX 40K (Polysciences, Warrington, PA, USA) at a ratio of DNA to PEI 1:3. Medium was changed 4 h after transfection. Cell culture supernatants containing pseudoviral particles were collected 48 h later, filtered through 0.45 μm PES filters, and concentrated by overnight centrifugation at 8,500 × g, 4°C. Pellets containing pseudoviral particles were resuspended in equal volumes of serum-free DMEM. Initially, vectors were titrated using the Quick-Titer Lentivirus Titer Kit (lentivirus-associated HIV p24; Cell Biolabs, San Diego, CA, USA). The viral titers for SHC002 and SHC016 were similar; therefore, in subsequent experiments, equal volumes of concentrated viruses were used.

Cell transduction
The cells were grown in a 12-well plate. An optimal volume of viruses was determined experimentally by transducing each cell line with several dilutions of a concentrated viral stock. Aliquots of 2 μL of stocks, which resulted in 20%–60% of puromycin-resistant cells, were eventually used for transduction of all cell types via spinoculation (30 min, 1,150 × g, room temperature) in the presence of polybrene (8 μg/mL). After 48 h, the transduced cells were selected for 7 days with puromycin (Bioshop Canada, Burlington, ON, Canada) at a concentration of 1 μg/mL for human cell lines, 5 μg/mL for MC38CEA, and 8 μg/mL for MEF.

PCR analysis of stability of transgene integration
MC38CEA and U251 MG (further referred to as U251) cells transduced with lentiviral vectors SHC001, SHC002, and SHC016 were cultured for several weeks. DNA was isolated from the cells at weekly intervals. The cells were lysed in guanidinium thiocyanate solution, and DNA was isolated by phenol–chloroform extraction.

Equal amounts of DNA samples (100 ng) were subjected to PCR using Taq Master Mix (Vazyme Biotech, Nanjing, China) to amplify the puromycin-resistance gene, puromycin N-acetyltransferase, and a pLKO fragment comprising an shRNA-coding sequence, as well as an actin-coding sequence (ACTB/Actb) as a control. The primers are listed in Table S4. The PCR program included 30 cycles of 30 s at 94°C, 30 s at 60°C (human and mouse actin) or 52°C (puro and pLKO), and 30 s at 72°C. PCR products were visualized on a 1% agarose gel.

Stimulation of shRNA expression
The cells MC38CEA, MEF, U251, A549, PC3, MCF7, and HeLa transduced with Tet-on-SHC002 or Tet-on-SHC016 were seeded at a density appropriate for each cell line (between 500 and 1,500 cells/well in a 96-well plate or 10,000 and 20,000 cells/well in a 12-well plate). The cells were cultured for 5 or 6 days, and the cells of each line were divided into several experimental groups. Starting 1 day after plating of the cells, doxycycline aliquots were added every 24 h to one experimental group to a final concentration of 100 ng/mL (unless stated otherwise). One group (negative control) was left untreated. In some experiments, etoposide (2 μM) was added to one group for the last 48 h of culture as a positive control of apoptosis induction.

MTT assay
The viability of the cells was determined by MTT assay. The cells in 96-well plates were incubated in 100 μL of serum-free medium containing MTT (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 0.5 mg/mL for 1–3 h. Formazan crystals were solubilized in 200 μL of acidified isopropanol. The absorbance was measured at 570 nm using a microplate reader (VersaMax; Molecular Devices, San Jose, CA, USA). The absorbance of control cells (cultured without doxycycline) was taken as 100% viability.

Colonies formation assay
The cells were seeded at 100 cells per well in a 6-well plate. On the following day, doxycycline was added at 100 ng/mL. The cells were cultured for 7–10 days, and fresh doxycycline was added every other day. At the 5th day, puromycin was added to select the cells that retained the expression cassette. The colonies were stained with crystal violet dissolved in methanol, destained with tap water, and photographed with a Fusion FX imaging platform (Vilber Lourmat, Collégien, France).

Annexin V assay
The cells were analyzed by an annexin V/PI double-staining method.56 The cells with inducible SHC002 or SHC016 expression were grown in 12-well plates, and shRNA expression was induced with doxycycline as described above. The cells were trypanized, washed with PBS and then with annexin V binding buffer (ABB; 10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2), and incubated in ABB containing annexin V conjugated with APC (1% v/v; Exbio, Prague, Czech Republic). The cells were analyzed by an annexin V/PI double-staining method.56
Vestec, Czechia) and PI (100 µg/mL) for 20 min in the dark. Next, the cells were washed twice with ABB, resuspended in ABB, and analyzed on a FACS Calibur (BD Biosciences, San Jose, CA, USA). The percentages of apoptotic cells (annexin V positive, PI negative) and late apoptotic and necrotic cells (PI positive, annexin V positive and negative) were determined using FlowJo version (v.10.0.7) (FlowJo,ashland, OR, USA).

**Measurement of caspase activity**
MC38CEA and MEF cells with inducible SHC002 or SHC016 expression were seeded in 96-well plates, and shRNA expression was induced with doxycycline as described above. Caspase activity was determined using Caspase-Glo 3/7 Assay Systems (Promega, Madison, WI, USA) according to the manufacturer’s specifications. The luminescence was measured using a microplate reader, Synergy H1 Hybrid Reader (BioTek, Winooski, VT, USA). The luminescence values of control cells, non-treated with doxycycline, were taken as 100%.

**Cell-cycle analysis**
The cells with inducible SHC002 or SHC016 expression were plated in 12-well plates at a density of 10,000 cells/well, and shRNA expression was induced with doxycycline as described above. The cells were trypsinized, washed with PBS, fixed with 70% ethanol, and incubated at 4°C for at least 4 h. Equal amounts of cells (1 x 10^5), were permeabilized with 0.1% Triton X-100 in PBS for 15 min, washed with PBS, and incubated in 200 µL of ribonuclease A (10 µg/mL; A&A Biotechnology, Gdynia, Poland) for 15 min at 37°C. Then 200 µL aliquots of PI dissolved in PBS containing 0.1% Triton X-100 were added to the cells to a final concentration of 250 µg/mL. The cell-cycle analysis of 10,000 cells/sample was performed by flow cytometry using FACSCalibur (BD Biosciences) and the FlowJo cell-cycle Watson (Pragmatic) model (FlowJo v.10.0.7 software; FlowJo). Both debris and doublets were removed from the analysis.

**Nuclei imaging**
The cells were washed twice with PBS, fixed with ice-cold methanol for 5 min, washed thoroughly with PBS, incubated with DAPI (1 µg/mL) or Hoechst 33342 (1 µg/mL) for 15 min, and washed 4 times with PBS. The nuclei were imaged using a Leica DM6 B microscope equipped with a Leica DMC5400 camera or a DM IL LED fluorescence microscope equipped with a Leica DFC450 C (Leica Microsystems, Wetzlar, Germany) camera, and the images were processed using ImageJ software v.1.53 (National Institutes of Health).^{37,58}

**Immunofluorescence**
The cells were plated on #1.5 glass coverslips in 12-well plates. On the following day, doxycycline was added to a final concentration of 100 ng/mL. The cells were fixed with ice-cold methanol for 5 min, then washed three times with PBS, permeabilized with 0.5% Triton X-100 in PBS for 15 min at room temperature, blocked with 2% BSA and 5% normal goat serum in PBST (blocking solution) for 1 h, and incubated with mouse anti-γ-tubulin antibody (clone C-11, 2 µg/mL; Santa Cruz Biotechnology, Dallas, TX, USA), diluted in blocking solution overnight at 4°C. After washing with PBST, the coverslips were incubated with Fab fragment of goat anti-mouse immunoglobulin G (IgG; H+L) secondary antibody conjugated to Alexa Fluor 594 (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. After extensive washing with PBST, the coverslips were subjected to mild fixation with 0.4% methanol-free formaldehyde in PBS for 10 min, washed with PBS, and incubated with CoraLite-488-conjugated mouse anti-β-tubulin antibody (clone 1D4A4, 10 µg/mL; Proteintech, Rosemont, IL, USA) in blocking solution overnight at 4°C. After washing with PBST, the coverslips were stained with Hoechst 33342 and mounted onto microscopic slides with ProLong Glass (Thermo Scientific). When the cells were stained for γ-tubulin only, the post-staining fixation step was omitted. The images were acquired with a Leica DM6 B microscope equipped with a Leica DMC5400 camera using a 63 x 1.3 NA oil objective. Images shown are maximum intensity projections of z stack planes.

**BrdU labeling**
The cells were seeded at a density of 20,000 cells/well (all but A549) or 60,000 cells/well (A549) in a 6-well plate. On the following day, doxycycline was added to a final concentration of 100 ng/mL. The cells (all but A549) were cultured for 5 days or 3 days (A549). Fresh doxycycline was added every other day. BrdU (Sigma-Aldrich), at a final concentration of 20 µM, was added to the medium for the last 6 h, and then the cells were collected, washed twice with PBS, and stained with Fixable Viability Dye eFluor 520 (Thermo Scientific, Waltham, MA, USA) in PBS for 30 min at 4°C with occasional mixing. To stop the reaction, the cells were washed once in 1% BSA/PBS and once in PBS. Then, the cells were fixed dropwise with ice cold 70% ethanol and incubated overnight at 4°C. On the next day, the fixed cells were subjected to DNA denaturation in 2 M HCl containing 0.5% Triton X-100 for 30 min at room temperature; HCl was neutralized with 0.1 M sodium tetraborate, pH 8.5. After extensive washes in PBS, the cells were incubated in blocking solution (1% BSA in PBST) for 20 min and then with Alexa Fluor 647-conjugated anti-BrdU antibody at 1:50 (clone MoBU-1; Thermo Scientific) for at least 3 h at room temperature. Next, the cells were centrifuged, suspended in PBS, and analyzed on a FACSCalibur (BD Biosciences). The percentages of BrdU-positive, BrdU-negative, and dead cells (Fixable Viability Dye eFluor 520 positive) were determined using FlowJo v.10.6.1 (FlowJo). In some experiments, U251 cells were also seeded on #1.5 glass coverslips and stained as described above with minor modification (viability dye was omitted; staining was performed with Alexa Fluor 647- or Alexa Fluor 488-conjugated anti-BrdU antibodies). The samples were analyzed via fluorescence microscopy using a Leica DM6 B microscope equipped with a 63 x 1.3 NA oil objective and Leica DMC5400 camera (Leica Microsystems).

**SA-β-gal staining**
The cells were seeded 5 days prior to staining at 5,000 cells/well in 12-well plates, and shRNA expression was induced with doxycycline as described above. SA-β-gal staining was performed according to a standard protocol.^{37} Briefly, the cells were washed twice with PBS,
fixed for 5 min with 2% formaldehyde and 0.2% glutaraldehyde in PBS, then washed in PBS, and incubated overnight at 37°C in staining solution containing 40 mM citric acid/sodium phosphate buffer, pH 6.0, 1 mg/mL X-Gal (Bioshop Canada), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM MgCl₂. On the following day, the cells were washed with PBS, dehydrated in methanol, counterstained with hematoxylin, and washed extensively with water. The cells were imaged using a DM IL LED microscope equipped with a Leica DFC450 C camera. The percentage of SA-β-gal-positive cells was calculated. About 300 cells were analyzed per experiment in three independent experiments.

RNA isolation, reverse transcription, and qRT-PCR
Total RNA was isolated using the modified phenol/chloroform extraction method using a Fensolol reagent (A&A Biotechnology) and then treated with Turbo DNase (Thermo Scientific) followed by column purification with the Clean Up RNA Concentrator (A&A Biotechnology). Equal amounts of RNA (1 μg) were reverse transcribed with a mixture of oligo(dT)₁₅ and random hexamer primers using M-MLV polymerase (Promega). qRT-PCR gene expression quantifications were performed using AceQ qPCR SYBR Green Mix (Vazyme Biotech) on an Eco Real-Time PCR System (Illumina, San Diego, CA, USA). RNA expression was normalized to a geometric mean of two reference genes: Eef2 and Polr2b for mouse cell lines and EEF2 and TBP for human cell lines. All sequences of primers are listed in Table S4.

Preparation of samples for RNA-seq
Cells were seeded on 10 cm plates 2 days before doxycycline addition. After 24 h of doxycycline treatment, cells were lysed in RNA Extrakol (EurX, Gdańsk, Poland). Total RNA was isolated using the Direct-zol RNA Mini (Zymo Research, Irvine, CA, USA); the procedure was described with minor modifications, such as three (instead of two) rounds of RNA binding, washing, and elution and an additional wash in detergent-free buffer B before the final elution step. Library preparations and sequencing on an Ion Torrent platform were performed at the Genomics Centre at the Malopolska Centre of Biotechnology (Kraków, Poland). The RNA-seq data presented in this article have been deposited in the GEO database (GEO: GSE178458). Reads per million mapped read values of samples treated with doxycycline have been deposited in the GEO database (GEO: GSE178458). Reads per million mapped read values of samples treated with doxycycline have been deposited in the GEO database (GEO: GSE178458).

Construction of tet-inducible CRISPRi vector
dCas9 repressor was PCR amplified with primers containing SfiI sites from dCas9-KRAB-MeCP2 (a gift from Alejandro Chavez and George Church; Addgene plasmid #110821).63 digested with SfiI, and cloned into an SfiI-linearized pSBtet-Pur plasmid (a gift from Eric Kowarz; Addgene plasmid #60507).64

In order to clone the U6 promoter-sgRNA scaffold cassette containing Sapi restriction sites instead of BbsI sites, oligonucleotides containing Sapi sites were annealed and cloned into the BbsI-digested pX330-U6-Chimeric_BB-CBh-hSpCas9 (a gift from Feng Zhang; Addgene plasmid #42230).65 The U6-sgRNA scaffold was then PCR amplified with primers containing KpnI sites and cloned into KpnI-digested pSBtet-Pur-dCas9-KRAB-MeCP2. The resulting vector was termed pSBtet-Pur-dCas9-KRAB-MeCP2-hU6-Sapi.

sgRNA sequences targeting hSNRPD3, VPS4A, VPS4B, and ARPP19 genes were designed using the GFP sgRNA Designer (Broad Institute). Oligonucleotides containing pSBtet-Pur-dCas9-KRAB-MeCP2-hU6-Sapi-compatible ends were annealed and assembled with the vector in one restriction-ligation reaction with Sapi and the T4 DNA ligase. Sequences of oligonucleotides used for sgRNA cloning are available in Table S5.

Generation of p53-KD A549 cells
Cas9 mRNA was transcribed from the Xbal-linearized pJET1.2-SpCas9 vector62 using the HiScribe T7 ARCA mRNA Kit (with tailing) (NEB, Ipswich, MA, USA); mRNA was purified using LiCl precipitation. Oligonucleotides corresponding to the human TP53- or GFP-targeting portion of sgRNAs were cloned into pX330-U6-Chimeric_BB-CBh-hSpCas9.62 sgRNA-coding sequences were amplified with primers containing a T7 promoter sequence at the 5’ end of the forward primer, purified using the Clean-Up Concentrator kit (A&A Biotechnology), in vitro transcribed using the TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific), and then purified by LiCl precipitation. A549 cells were electroporated with 5 μg of Cas9 mRNA in electroporation buffer using Gene Pulser II (Bio-Rad Laboratories) under the following conditions: one million cells in 400 μL electroporation buffer in a 0.4-cm gap cuvette and pulse voltage and capacitance: 400 V and 700 μF. After 4 h, electroporation was repeated using the same procedure, but with 1 μg of TP53- or GFP-targeting sgRNAs instead of Cas9 mRNA. The cells were allowed to regenerate in culture (for about 3 days), and the electroporation was repeated with another TP53-targeting sgRNA (three in total). Sequences of oligonucleotides used for sgRNA cloning and for amplification of sgRNA for in vitro transcription are available in Table S5.

U251 and A549 were seeded onto 12-well plates 1 day prior to transfection. The cells were transfected with 475 ng of a respective CRISPRi plasmid together with 25 ng of Sleeping Beauty transposasase-encoding vector pCMV(CAT)77-SB100 (a gift from Zsuzsanna Izsvak; Addgene plasmid #34879) using jetOPTIMUS (A549 cells; Polyplus-transfection, Illkirch, France) or TransIT-LT1 (U251 cells; Mirus Bio, Madison, WI, USA), according to the protocols provided by the manufacturers. Alternatively, A549 cells were electroporated with 4.9 μg of CRISPRi plasmid and 100 ng of pCMV(CAT)77-SB100 in electroporation buffer (100 mM sodium phosphate,
10 mM MgCl₂, 5 mM KCl, 20 mM HEPES, 50 mM sodium succinate, pH 7.2) using Gene Pulser II (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions: two million cells in 400 µL electroporation buffer in a 0.4-cm gap cuvette and pulse voltage and capacitance: 300 V and 1,000 µF. After 24 h of transfection, the medium was changed, and the transfected cells were selected for 7 days with puromycin at a concentration of 1 µg/mL for U251 cells and 5 µg/mL for MC38CEA cells.

Construction of SNRPD3 expression vectors

The sequences coding for mSNRPD3 or hSNRPD3 were amplified from cDNA synthesized from RNA isolated from mouse brain tissue or U251 cells, respectively. cDNAs were generated using Maxima H Minus Reverse Transcriptase (Thermo Scientific). The sequence coding for SNRPD3 with introduced, at every possible site, silent mutations (opt-SNRPD3) was synthesized as a dsDNA fragment (GeneArt Strings DNA Fragments; Thermo Scientific). EGFP was PCR amplified from LeGO-iG2 (a gift from Boris Fehse; Addgene plasmid #27341), mSNRPD3 and hSNRPD3 were cloned into an SfiI-linearized pSBbi-bla vector (a gift from Eric Kowarz; Addgene plasmid #60526) using the NEBuilder HiFi DNA Assembly, whereas opt-SNRPD3 and EGFP were digested with SfiI and ligated into SfiI-linearized pSBbi-bla.

U251 and MC38CEA were seeded onto 12-well plates 1 day prior to transfection. The cells were transfected with 950 ng of a respective pSBbi-bla-SNRPD3 plasmid together with 50 ng of transposase-encoding vector pCMV(CAT)T7-SB10065 using ViaFect (U251 cells; Promega) at a ratio of DNA to reagent of 1:3 or jetPRIME (MC38CEA cells; Polyplus-transfection) at a ratio of DNA to reagent of 1:2. After 24 h, the medium was changed, and the transfected cells were selected for at least 10 days with blastcidin S (InvivoGen, San Diego, CA, USA) at a concentration of 12 µg/mL for U251 cells and 5 µg/mL for MC38CEA cells.

Western blotting

The cells were incubated with or without doxycycline for 48 h and then lysed in ice-cold RIPA buffer enriched with 5 mM EDTA and Halt Protease Inhibitor Cocktail (Thermo Scientific). After brief sonication, protein samples (25 µg for 10-well gels or 15 µg for 15-well gels) were subjected to Tris-glycine SDS-PAGE (for the detection of SNRPD3) and then lysed in ice-cold RIPA buffer enriched with 5 mM EDTA and Halt Protease Inhibitor Cocktail (Thermo Scientific) and then sonicated. The sequence codon for SNRPD3 with introduced, at every possible site, silent mutations (opt-SNRPD3) was synthesized as a dsDNA fragment (GeneArt Strings DNA Fragments; Thermo Scientific). The sequence coding for SNRPD3 with introduced, at every possible site, silent mutations (opt-SNRPD3) was synthesized as a dsDNA fragment (GeneArt Strings DNA Fragments; Thermo Scientific). The sequence coding for SNRPD3 with introduced, at every possible site, silent mutations (opt-SNRPD3) was synthesized as a dsDNA fragment (GeneArt Strings DNA Fragments; Thermo Scientific).

Western blotting

The cells were incubated with or without doxycycline for 48 h and then lysed in ice-cold RIPA buffer enriched with 5 mM EDTA and Halt Protease Inhibitor Cocktail (Thermo Scientific). After brief sonication, protein samples (25 µg for 10-well gels or 15 µg for 15-well gels) were subjected to Tris-glycine SDS-PAGE (for the detection of SNRPD3) and then transferred onto 0.45 µm nitrocellulose membranes (Immobilon; Merck and Amersham, respectively). The membranes were stained with Ponceau S to ensure equal protein loading. After destaining and blocking in 5% non-fat dried milk in TBST, they were probed with goat-anti p53 at 0.25 µg/mL (AF1355; R&D Systems) or rabbit anti-SNRPD3 at 0.2 µg/mL (NBP1-80735, Novus Biologicals; or HPA001170, Sigma-Aldrich) and then with horseradish peroxidase (HRP)-conjugated secondary antibody (rabbit anti-goat or goat anti-rabbit, both at 1:10 000; Sigma-Aldrich). Bands were developed with Immobilon Western Chemiluminescent HRP Substrate (Merck) and visualized using Fusion FX (Vilber Lourmat). The exposition time was set to “auto.” Band intensities were quantified using Fiji software; chemiluminescent signal was normalized to the total protein amount in each lane visualized by Ponceau S staining.

Luciferase reporter assay

The sequence of hSNRPD3 3′ UTR was PCR amplified from cDNA synthesized from RNA isolated from U251 cells with primers containing XbaI restriction sites. The PCR product was cloned into pJET1.2/blunt (Thermo Scientific) and then subcloned into XbaI-linearized pmirGLO (Promega). Small deletions comprising potential shRNA-targeted sites within SNRPD3 3′ UTR were introduced using inverse PCR and pJET1.2-hSNRPD3 3′ UTR as a template; mutated versions of 3′ UTR were then subcloned into pmirGLO. Primers used for cloning and mutagenesis are available in Table S6.

U251 cells were seeded onto 96-well plates at 7,500 cells/well and transfected with 50 ng pmirGLO vectors using ViaFect (Promega) at a DNA to reagent ratio of 1:3. On the following day, the medium was changed, and doxycycline was added to a final concentration of 100 ng/mL. After 48 h, medium was removed, and activities of firefly and Renilla luciferases were determined using the Dual-Glo Luciferase Assay System (Promega).

Additional information

Data analysis was performed using Microsoft Excel (Excel 2016) or GraphPad Prism v.5, and all graphs were created using GraphPad Prism v.6 (GraphPad Software).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.09.004.

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AUTHOR CONTRIBUTIONS

M.C. and J.B. designed the study and wrote the article. M.C., K.S., and A.K. performed the experiments. M.C., K.S., J.K., and J.B. analyzed the data. All authors reviewed the results and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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