eIF3c: A potential therapeutic target for cancer

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Cancer cells are rapidly evolving due to their unstable genome, which contributes to the development of new cancer clones with different gene expression profile (GEP). Manipulating the expression of the genes vital for the progression of the disease is essential to overcome its heterogeneity. However, targeting overexpressed genes, retrieved from GEP analysis, would be efficient for a specific kind of a malignancy. Alternatively, manipulating the expression of genes that are part of a fundamental mechanism in the cell would be effective against a wide range of malignancies. To test this hypothesis we characterized, using RNAi approaches, the therapeutic potential of the housekeeping eIF3c gene in five different cancer cell lines NCI-ADR/RES (NAR), HeLa, MCF7, HCT116 and B16F10. eIF3c is one of the core subunit of the eukaryote translation initiation factor (eIF) 3 complex, which has a crucial role in the translation initiation process. In this study, we demonstrated that eIF3c is vital to translation initiation in vivo, as its down-regulation decreases the global protein synthesis and causes a polysome run-off. In addition, reducing the expression of eIF3c mediates G0/G1 or G2/M arrest in a tissue dependent manner, which leads to a reduction in cell proliferation and eventually to cell death. Moreover, we demonstrated the efficiency of the hyaluronan (HA)-coated lipid-based nanoparticles (LNPs) platform to deliver eIF3c-siRNAs to mouse melanoma cells. Taking together, our results emphasize the importance of seeking ubiquitously expressed housekeeping genes such as eIF3c rather than tumor associated overexpressed genes as therapeutic targets for the heterogeneous malignancies.

1. Introduction

Cancer cells are characterized by the alteration of gene expression profile, which supports the progression of the disease. Translation is one of the fundamental apparatuses that control the expression of genes in the cells. The translation machinery has a crucial role in maintaining homeostasis in addition to modulating permanent changes in cell physiology or fate, due to rapid changes in cellular concentration of protein encoded from existing mRNAs [1]. Therefore, inhibiting translation is expected to have destructive effects on the cells. The translation process can be divided into initiation, elongation, termination and ribosome recycling. Most regulation of translation occurs at the initiation stage, which begins with the recruitment of the 40S ribosomal subunit to the Cap region of the mRNA by different eukaryotic translation initiation factors (eIFs) [1]. The internal mRNA ribosome binding mechanism is alternative translation initiation machinery that is activated when the general protein synthesis is inhibited. This machinery involves the recruitment of the ribosome inside the 5′ untranslated region of the mRNA (5′ UTR) close to the AUG via the recognition of a complex element termed the internal ribosome entry site (IRES). IRESes first discovered in some viral mRNAs and also found in many cellular transcripts with structured 5′ UTR, which are generally involved in growth control, cell cycle and apoptosis [2]. One of the eIFs that have a key role in both translation initiation machineries is eIF3, which stabilizes the binding of the 40S subunit to the mRNA and is essential for the recognition of the starting AUG codon [3,4]. Thus omitting the eIF3 from the translation initiation complex abolishes general translation. The mammalian eIF3 comprises 13 subunits, designated eIF3a to eIF3m, only few of them are responsible for its core activity [5]. It has been shown using reconstitution assay that the eIF3c subunit is essential for the activity of eIF3 and for the integrity of translation initiation [6]. Mass spectrometry analysis [7] and in vivo reconstitution assays done in a bacterial system [8] have revealed that the eIF3c subunit is crucial for the assembly of the core eIF3 complex. In addition eIF3c was found to mediate the interactions between eIF3 and other components of the translation initiation complex [9].

It has been shown that individual overexpression of several subunits of eIF3, including eIF3c, promote malignant transformation of the fibroblast NIH3T3 cell line. This phenomenon
might occur due to the alteration in the expression of growth-regulating genes [10]. eIF3c was found to be overexpressed only in few malignancies, testicular seminomas [11] and in meningiomas bearing mutation in the tumor suppressor protein neurofibromatosis 2 (NF2). In normal conditions NF2 decreases cell proliferation through interaction with the N-terminus region of eIF3c thus interrupting with its translational activity [12].

Due to the fundamental role of eIF3c in translation regulation, which has not been shown to be compensated by other cellular factors, and its ubiquitous expression in all tissues, we aimed in this study to evaluate the therapeutic potential of this factor. We manipulated, using RNAi strategy, the expression of the eIF3c subunit in different cancer cell lines. We were able to demonstrate that reducing the expression of eIF3c attenuated translation at the initiation stage, which eventually caused cell death. Thus, our
results imply the potential of eIF3c as a therapeutic target for a wide range of malignancies.

2. Materials and methods

2.1. Cell lines

NCI-ADR/RES (NAR), HCT116 and MCF7 cells were grown in RPMI medium containing 10% FBS, 1% L-Glutamine and 1% antibiotics. B16F10 cells were grown in RPMI medium supplemented with 1% sodium pyrovate. HeLa cells were grown in DMEM medium containing 10% FBS, 1% L-Glutamine and 1% antibiotics. All cell culture supplies were from biological industries, Israel.

2.2. siRNA sequences

Human eIF3c siRNA cocktail (Santa cruz sc-40545), designated sieIF3c_A, sieIF3c_B, sieIF3c_C. Mouse eIF3c siRNA (Dharmacon, siGENOME D-052317), designated simeIF3c_A, simeIF3c_B, simeIF3c_C, simeIF3c_D. Unless differently indicated, the human cell lines were transfected with the cocktail anti eIF3c siRNA and the mouse B16F10 melanoma cell line was transfected with the simeIF3c_D sequence. Luciferase gene as a control sequence (siLuc, sense strand: CUUACGCUGAGUACUUCGA) were designed and screened by Alnylam Pharmaceuticals (Cambridge MA, USA).

2.3. Real-time analysis

2.5/C2 10³ cells/well were seeded onto 6-well plate. 24 h later cells were transfected with either 20 nM of Luciferase or eIF3c siRNA (Santa Cruz, sc-40545) using Oligofectamine transfection reagent (Life Technologies, Carlsbad, CA, USA), according to the manufacturer instructions. B16F10 cells were transfected with siRNA targeting the simeIF3c_D gene using RNAi-Max transfection reagent (Life Technologies, Carlsbad, CA, USA), according to the manufacturer instructions. Cells were harvested 24 h following transfection and total RNA was extracted using EZ-RNA kit (biological industries, Israel). The concentration of the RNA was measured and its quality was determined by electrophoresis on agarose gel. 1 µg of RNA was used to generate cDNA with high capacity cDNA kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturers’ protocols. qRT-PCR was performed with Fast SYBR® Green Master Mix and the ABI StepOnePlus device (Life Technologies, Carlsbad, CA, USA), for eIF3c, eIF1 and GAPDH were used as controls. The following primers were used: Human eIF3c (NM_001037808) Forward: 5’ ACCAGAGAGTGTGCGAACAGT G, Reverse: 5’ CTATGGCCATCCGGAATATGGAG, Human eIF1 (NM_005801) Forward: 5’ TTGAGCATCCGGAAATATGGAG, Reverse: 5’ CAAA TCTCTACGAGGAACTGGCA; Human GAPDH (NM_002046.4) Forward: 5’ ATGGCATTACGGATG GTCC; Mouse eIF3c (NM_146200) Forward: 5’ GTATCTGACCCCTTTATTTT; Mouse eIF1 (NM_011508) Forward: 5’ CAGAACCTCCACTCTTTCGACC, Reverse: 5’ TCAGTGCCAGCAGGAAGCAG; Mouse GAPDH (NM_008084) Forward 5’ TTGTGGAAGGGCTCATGACC, Reverse 5’ TCTTCTGGGTGGCAGTGATG.

2.4. Cell proliferation assay

About 3 × 10⁵ cells/well were grown in 96-well plate. 24 h post cell seeding, cells were transfected with either siRNA against the Luciferase gene as a control or with different concentrations of eIF3c as described earlier. 72 h post transfection or 48 h in the case of B16F10 cell line, cell proliferation was measured using the cell proliferation kit (XTT, biological industries, Israel) as recommended by the manufacturer.

2.5. Protein synthesis rate assay

About 2.5 × 10⁵ cells/well of B16F10 and NAR cells were seeded onto 6 well plate. 24 h post seeding cells were transfected with either 20 nM siLuc or sif3c. 48 h or 72 h post transfection cells were starved for 15 min with cysteine free medium, then labeled with (250–1000 µCi/ml 35S-met/cys for 30 min. The plates were washed with PBSx1, scrape and lysed (100–400 µl) with 1% triton X-100 and counted in a Beckman LS6000SE liquid scintillation counter. The percentage of translation initiation was calculated by normalizing the labeling rate of each siRNA transfected treatment to the corresponding untransfected control. The data was collected in triplicate for each siRNA transfected group and error bars represent the standard deviation between the experiments.
NAR cells transfected with siIF3c cocktail and B16F10 cells with siIF3c-D. (B) NAR cells transfected with siIF3c_A and siIF3c_B individually. (C) B16F10 cells transfected with siIF3c_A, B and C individually 48 h, 72 h and 6 days post transfection cell cycle profile was determined by propidium iodide. Representative cell cycle analysis histograms are described and the graphs summarize the results of three independent experiments ± SD. p-Value was calculated for the deference significance between the siLuc and siIF3c treatment. "One-way ANOVA p < 0.001," t-test p < 0.005.

2.6. Polysome profile analysis

Polysomal profiles were performed according to Johannes and Sarnow [13] with modifications. $1.5 \times 10^5$ NAR cells were grown in 10 cm tissue culture dishes. 24 h later cells were transfected with either 20 nM siLuc or siIF3c as previously described. 72 h post transfection the cells were incubated for 5 min with 100 μg/ml of cycloheximide (CHX), harvested, and stored at −70°C. To analyze the cells, the pellets were resuspended in 0.4 ml of LBA buffer (18 mM Tris, pH 7.5, 50 mM KCl, 10 mM NaF, 10 mM α-glycerophosphosphate, 1.4 μg/ml pepstatin, 2 μg/ml leupeptin, EDTA-free protease inhibitor cocktail (Complete; Roche), 70 μg/ml CHX, 1.25 mM dithiothreitol, and 200 μg/ml heparin), and Triton X-100 and deoxycholate were added to a final concentration of 1.2% each for lysis of 5 min on ice. The same amount of extracts, determined by measuring their optical density units (OD = 260 nm), were loaded on each sucrose gradient. Following centrifugation of the sucrose gradients, the samples were analyzed.

2.7. Cell cycle analysis

$2.5 \times 10^5$ cells/well or $1.5 \times 10^5$ cells/well of NAR and B16F10 cells, respectively, were seeded onto 6 well plates. 24 h later cells were transfected with 20 nM of either a control or eIF3c siRNAs as described above. 48 h, 72 h and 6 days post transfection cells were harvested, washed with PBSx1, resuspended with 0.3 ml PBSx1. Prior to FACS analysis cells were permeabilized with 0.1% Triton and propidium iodide was added to a final concentration of 15 μg/ml (Sigma, St. Louis, MO, USA). Fluorescence was measured by flow cytometry. Data from at least 10^5 cells were acquired using BD FACS calibur™ and the CellQuest™ software. Analyses were done with Flowjo™ software. The Dean–Jett–Fox model was applied on at least 10,000 gated cells.

2.8. Cell apoptosis analysis

$5 \times 10^4$ Cells/well of B16F10 or NAR cells were seeded onto 6-well plate. 24 h later cells were transfected with 20 nM of Luc siRNA or eIF3c siRNA. 4 days or 7 days post transfection cells were harvested and apoptosis was evaluated by flow cytometry using Annexin V and PI apoptosis detection kit (eBioScience, San Diego, CA, USA) as recommended by the manufacturer. Data from at least 10^4 cells were acquired using BD FACS calibur™ and the CellQuest™ software.

2.9. Preparation and characterization of the HA-coated LNPs entrapping siRNAs and transfection into B16F10 mouse melanoma cells

DOTAP, Cholesterol and DLPE were dissolved in ethanol at mole ratio of 60:30:10, respectively then evaporated to dryness under reduced pressure in a rotary evaporator (Buchi Rotary Evaporator Vacuum System Flawil, Switzerland). The dry lipid film was hydrated in 10 ml of 0.1 M Borate buffer pH 9. The MLV were extruded through a Lipex extrusion device (Northen lipids, Vancouver, Canada), operated at 65°C and under nitrogen pressures of 200–500 psi. The extrusion was carried out in stages using progressively smaller pore-size polycarbonate membranes (Whatman Inc., UK), with several cycles per pore-size, to achieve unilamellar vesicles in a final size range of ~100 nm in diameter. Lipid mass was quantified as previously reported [14] with an average size of ~150 nm and a zeta potential of ~−30 mV as assayed by a Malvern Zetasizer Nano ZS. For siRNA encapsulation, 100 μM siIF3c or siLuc was mixed with the lipids at weight ratio of 1:1 and the complex was incubated at 37°C overnight. Hyaluronan (HA) (700 KDa Lifecore) was activated by the addition of EDC at a Hyaluronan:HA weight ratio of 1:1 and the complex was incubated at 37°C overnight. Hyaluronan (HA) (700 KDa Lifecore) was activated by the addition of EDC at a weight ratio of 1:20 respectively in 0.1 M Acetate buffer pH 4.5 at 37°C for 1.5 h while shaking at 150 rpm. 24 h before transfection 5 × 10^5 B16F10 cells were seeded onto 6-well plates. The medium was replaced with 1 ml serum-free medium.
and 100 μl of the siRNA entrapping HA-coated LNPs were added in each well. 4 h later 0.5 ml of medium with 30% serum was added and 24 h post transfection the medium was replaced with a fresh medium containing 10% serum.

2.10. Flow cytometry analysis

CD44 was surface labeled with a pan-CD44 monoclonal antibody clone IM7 and the staining was done as previously reported [15] and analyzed using a FACS calibur CellQuest (Becton Dickinson, Franklin Lakes, NJ). 10⁴ cells were analyzed at each experimental point. Data analysis was performed using FlowJo software (Tree Star, Inc., Oregon, USA).

2.11. Statistical analysis

The results are represented as the mean ± SD of at least three independent experiments. One tailed student t-test or one-way ANOVA was performed to estimate the significance strength of the results.

3. Results

3.1. Down-regulating the expression of elf3c reduces cell proliferation of cancer cells

To test whether elf3c could serve as a therapeutic target in cancer, we studied the effect of reducing its expression using siRNA. Several cancer cell lines were used: NAR (NCI-ADR/RES, Adriamycin resistance ovarian carcinoma), MCF7 (breast ductal carcinoma), HeLa (cervical carcinoma), HCT116 (colon carcinoma) and mouse melanoma B16F10 cells. In the case of the human cell lines we used a siRNA cocktail of three sequences. According to our results, only two of the three sequences caused reduction in the mRNA levels of elf3c as assayed by Real-Time PCR in NAR cells (data not shown). In the case of the B16F10 melanoma cell line we...
used a single siRNA sequence. To estimate the knockdown efficiency on the expression of eIF3c at the mRNA level in the different cell lines, the cells were seeded onto 6-well plates and transfected with either 20 nM of siEIF3c or siLuc, as a control. The mRNA level of eIF3c was monitored 24 h post transfection (Fig. 1A). The results show a reduction in the mRNA level of eIF3c in all cell lines ranging between ~70% in the case of HCT116 to more than 90% in the case of MCF7 and B16F10 cell lines, while no effect observed on the expression of eIF3c when the cells were treated with siLuc. In addition, to estimate the knockdown specificity of sieIF3c we measured the mRNA level of the eIF1 gene, a component of the translation initiation complex, which shows either no change or a slight induction relative to the cells treated with siLuc. These results indicate that the silencing effect is specific to eIF3c. To study the effect of sieIF3c on cell proliferation, the cell lines mentioned above were seeded onto 96-well plates and 24 h later we transfected the cells with sieIF3c at concentrations ranging between 0.6 nM and 20 nM or with 20 nM of siLuc as a control. 48 h or 72 h post transfection, XTT cell proliferation assay was performed (Fig. 1B). According to the results the siEIF3c has inhibitory effect on the proliferation rate of all cell lines relative to the siLuc treatment, with an IC50 values ranging between 1.2 nM and 5 nM.

### 3.2. eIF3c is essential for protein synthesis in vivo

Previous reconstitution studies, performed in vitro, have shown that eIF3c is a core subunit for the function of the eIF3 complex. Eliminating the eIF3c subunit causes dramatic reduction in translation initiation [6]. To confirm that the inhibitory effect on the cell proliferation following sieIF3c treatment is caused by eIF3c subunit knockdown, we studied the effect of down-regulating the expression of the eIF3c gene on global protein synthesis and on polysome profile in vivo (Fig. 2). NAR and B16F10 cells were transfected with 20 nM of either siRNA targeting the eIF3c gene or siRNA targeting the Luciferase gene (siLuc), as a control. 48 h or 72 h post-transfection (B16F10 and NAR cells, respectively) we measured the incorporation of 35S labeled Methionine (Met) and Cysteine (Cys) into newly synthesized proteins. According to the results described in Fig. 2A and B, reducing the expression of the eIF3c gene caused reduction of approximately 70% in the level of global proteins synthesis.

To demonstrate that the effect on the translation machinery was at the initiation stage, we analyzed the polysomes profile following down-regulation of eIF3c gene expression in NAR cells (Fig. 2C). According to the results, relatively to the siLuc sieIF3c caused a polysome runoff associated with an increase in the amount of free 80S ribosomes seen as a monosomal peak in a polysome profile. These findings indicate that the translation machinery was inhibited at the initiation stage.

### 3.3. eIF3c regulates cell cycle progression

To further characterize the inhibitory effect on cell proliferation following the knock down of eIF3c subunit, we performed a cell cycle analysis in B16F10 and NAR cells as described above. B16F10 cells were transfected with a single sieIF3c sequence (sieIF3c-D), while NAR cells were transfected with the cocktail siRNA. Cell cycle profile was assayed in different time points as early as 48 h post transfection and up to 6 days post transfection (Fig. 3A). According to Fig. 3, in the case of B16F10 cells, 48 h post transfection 90% of the cell population was arrested at G0/G1 phase in the case of sieIF3c compared to 50% in cells transfected with siLuc (p-value < 0.005). In NAR cells, 72 h post transfection, the cells showed an increase of ~12% in the G2/M fraction and a decrease of ~13% in G0/G1 fraction relative to the cells treated with siLuc (p-value < 0.005). In both cases, the cells showed an increase of ~12% in the G2/M fraction and a decrease of ~13% in G0/G1 fraction relative to the cells treated with siLuc (p-value < 0.005). In NAR cells, 72 h post transfection, the cells showed an increase of ~12% in the G2/M fraction and a decrease of ~13% in G0/G1 fraction relative to the cells treated with siLuc (p-value < 0.005).
of the cells treated with siLuc (p-value < 0.0001). In addition, while in the control ~70% of the cells were in the G0/G1 phase less than 20% of the cells treated with si elf3c are in this phase (p-value < 0.0001). The difference in the cell cycle arrest phase obtained between B16F10 and NAR cells could be explained by either cell dependent or sequence dependent effect. To address this question we transfected NAR cells with two out of three siRNA sequences of the cocktail (si elf3c-A and elf3c-B) that were able to reduce the mRNA levels of the mammalian gene (data not shown). B16F10 cells were individually transfected with three additional siRNA sequences that are efficiently targeting the mouse elf3c subunit (data not shown). As shown in Fig. 3B and C, the same effect on the cell cycle of B16F10 and NAR cells obtained when different siRNA sequences targeting the elf3c subunit were used. Therefore we reasoned that the phase of the cell cycle arrest is tissue specific.

3.4. elf3c knockdown leads to cell death

Our main goal is to find out whether elf3c gene is an effective anticancer target. We assessed its ability to lead to cell death in both B16F10 and NAR cells. The cells were transfected with either 20 nM of si elf3c or siLuc as a control then we assayed for programmed cell death using Annexin V and Propidium Iodide (PI) to detect cells both in early and late stages of apoptosis (Fig. 4). Reducing the expression of elf3c subunit in B16F10 induced apoptosis in more than 50% of the cells as early as 4 days post transfection In NAR cells almost 50% of the cells treated with si elf3c were either in the early or late stages of apoptosis 7 days post transfection, while only ~4% of apoptotic cells observed in the siLuc control. The delay in cell death observed in NAR cells relative to B16F10 cells (7 days and 4 days, respectively) is in agreement with the rapid inhibitory effect observed on the progression of the cell cycle in B16F10 cells relative to NAR cells (2 and 6 days respectively, Fig. 3). Thus, these results strongly suggest that elf3c can serve as a potential anticancer target.

3.5. Hyaluronan (HA)-coated lipid-based nanoparticles (LNPs) downregulate the expression of elf3c in the B16F10 cells

To utilize the use of elf3c as an anti cancer target, the therapeutic cargoes should be selectively targeted to the cancer cells. We have previously demonstrated that coating lipid-based nanoparticles with high molecular weight HA, which bind to the CD44 receptor which is highly expressed on many types of cancer cells, increases the transfection efficiency of siRNAs into cancer cells [15]. Here, we utilized this selective strategy to test if it
can also be used to carry RNAi to downregulate the expression of eIF3c in the mouse melanoma B16F10 cells (Fig. 5). Flow cytometry analysis has shown that B16F10 cells highly express CD44 (Fig. 5A). We encapsulated fluorescently labeled, Cy5-siRNA, in the HA-coated LNPs. These complexes were incubated with B16F10 cells then imaged by fluorescent microscopy (Fig. 5B). According to the results the nanoparticles were able to penetrate the cell membrane and the Cy5 labeled siRNA molecules (pink dotes) accumulated in the cytoplasm of the cells. Moreover, the HA-coated LNPs were used to transfect the B16F10 cells with eIF3c-siRNA. The results described in Fig. 5C show that these particles achieved a specific reduction of ~60% in the expression of the eIF3c mRNA relative to the untreated cells and cells transfected with siRNA targeting the Luciferase gene. Thus, these results together with previously published data [15] suggest that the HA-coated LNPs might be a potential platform for studying the effect of manipulating the expression of eIF3c in vivo.

4. Discussion

Genes that might be essential for the progression and viability of malignancy can be predicted using Gene expression profiling (GEP). The expression of these genes could be manipulated by means of RNAi strategies and/or small molecules, in order to achieve remission of the disease. However, to find these genes of interest, GEP data will need to be integrated with genomic, epigenomic and proteomic data and to be correlated with clinical data [16]. This raises the complexity to attain optimal treatment for different malignancies. Therefore, in this study we targeted, using RNAi strategy, the ubiquitously expressed housekeeping gene, eIF3c. This gene is considered a candidate for a therapeutic target because it has a vital role in both Cap dependent and IRES mediated translation initiation, no other factor is known to compensate for the activity of eIF3c and it is ubiquitously expressed in all tissues. Thus, we believed that interrupting with the function of the translation apparatus via the manipulation of the expression of eIF3cs would have destructive effects on the cells regardless their GEP. Our findings demonstrated that reducing the expression of eIF3c attenuated the proliferation of different cancer cell lines. This is done through the inhibition of translation initiation. In agreement with a study performed in vitro [6] we were able to show that knocking-down the expression of eIF3c inhibited the rate of newly synthesized protein and caused a polysome run off (Fig. 2). Moreover, we found that eIF3c regulated cell cycle progression, and reducing its expression caused either G0/G1 arrest or G2/M arrest, in a tissue specific manner. These results could be explained by the existence of different mutations in cell cycle checkpoints. Eventually manipulating the expression of eIF3c led to cell death. The effect of eIF3c down-regulation in the B16F10 cell line was obtained earlier than in the NAR cell line (Figs. 3 and 4), which could be explained by a higher silencing efficiency, ~98% vs. ~80% respectively (Fig. 1), of the siRNA sequence used in each case. These results suggest that eIF3c is indeed an anti cancer target and emphasize the importance to further study and design more efficient molecules that would reduce its activity to gain a rapid and stronger effect on cell viability.

To exploit the use of ubiquitously expressed housekeeping genes as therapeutic targets, delivery vehicles [17–19] should be developed to increase the specificity of the treatment to cancer tissues. In this study, we demonstrated that HA-coated LNPs, which have previously been shown to increase the transfection efficiency of siRNAs into cancer cells [15], could penetrate into the B16F10 cells and reduce the expression of eIF3c mRNA. We expect that the advantage of HA-coated LNPs would be further emphasized in vivo, since cancer cells express several types of CD44 splice variants that have higher affinity to HA molecules than normal cells [20,21], thus would promote the retention of the nanoparticles to the tumor vicinity and would increase the specificity of the treatment. Utilizing such a delivery strategy will enable us to study the potential of genes such as eIF3c to serve as therapeutic targets in different malignancies.

Conflict of Interest

Dan Peer has financial interest in Quiet Therapeutics. The rest of the authors declare no financial interest.

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