Self-duplexing CUG repeats selectively inhibit mutant huntingtin expression

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ABSTRACT

Huntington's disease (HD) is a neurodegenerative genetic disorder caused by the expansion of the CAG repeat in the translated sequence of the *HTT* gene. This expansion generates a mutant huntingtin protein that contains an abnormally elongated polyglutamine tract, which, together with mutant transcript, causes cellular dysfunction. Currently, there is no curative treatment available to patients suffering from HD; however, the selective inhibition of the mutant allele expression is a promising therapeutic option. In this study, we developed a new class of CAG repeat-targeting silencing reagents that consist of self-duplexing CUG repeats. Self-duplex formation was induced through one or several U-base substitutions. A number of self-duplexing guide-strand-only short interfering RNAs have been tested through transfection into cells derived from HD patients, showing distinct activity profiles. The best reagents were highly discriminatory between the normal and mutant *HTT* alleles (allele selectivity) and the *HTT* transcript and other transcripts containing shorter CAG repeats (gene selectivity). We also demonstrated that the self-duplexing CUG repeat short interfering RNAs use the RNA interference pathway to elicit silencing, and repeat-targeting reagents showed similar activity and selectivity when expressed from short hairpin RNA vectors to achieve more durable silencing effects.

INTRODUCTION

MicroRNAs (miRNAs) are natural regulators of gene expression that guide the RNA-induced silencing complex (RISC) to partially complementary sites in the 3′-UTR of mRNAs, thereby causing the deadenylation and subsequent degradation or translational inhibition of mRNAs in animal cells (1,2). Exogenous short interfering RNAs (siRNAs), which are used in RNA interference (RNAi) technology, use the miRNA pathway to silence the expression of selected genes (3). Effective siRNAs, unlike miRNAs, have perfect or nearly perfect complementarity to the target sequence and are located in either the ORF or UTR of the gene. The siRNA targets are cleaved through the 'slicer' activity of the Argonaute 2 (AGO2) protein in the RISC complex (4,5). The miRNA- and siRNA-mediated gene silencing is primarily governed by the type of AGO protein involved and the level of complementarity between the target gene and short RNA sequence (6). siRNAs exhibited behavior similar to miRNAs when their complementarity with their target was decreased, and miRNAs behaved similarly to siRNAs when their sequence mismatches with their target were replaced with perfect matches (7–9).

RNAi technology is typically used to silence a gene of interest through targeting sequences specific to that gene. However, RNAi might also be used to target repetitive sequences, such as the CAG repeats that cause Huntington's disease (HD) and other polyglutamine disorders (10,11). In the search for potential therapies against these diseases, several attempts have been made to selectively silence the mutant alleles that contain expanded CAG repeats in the presence of the normal alleles of these genes and other genes containing shorter tracts of CAG repeats (12–19). However, selective silencing is particularly challenging because the siRNA duplex that targets the expanded CAG repeat is composed of a CUG repeat sequence and a complementary CAG repeat strand, which is also active in RNAi and downregulates transcripts containing CUG repeats (13).

To increase discrimination between normal and mutant huntingtin alleles containing repeat sequences of different lengths, specific mutations were introduced into the repeat-targeting siRNA duplexes (13,15). A number of different approaches have been proposed to increase gene selectivity and reduce the off-target effects of the passenger strand in typical RNAi applications (20). In the most straightforward approach, the passenger strand

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was completely eliminated, and guide-strand-only siRNAs were reported as active in gene silencing (5,21–23).

In addition, the guide-strand-only siRNAs, which targeted specific sequences and showed ability to form imperfect duplexes, were reported to silence the expression of genes nearly as effectively as regular duplexes (24).

In this study, we first compared the *HTT* silencing activity of single-stranded siRNAs and their corresponding duplexes, and showed that the duplexes have a much higher activity. To increase the guide strand activity, we introduced mutations into specific positions of the CUG repeat strand, which enhanced its ability to form self-duplex structures. We showed the selective silencing activities of several self-duplexing siRNA reagents (sd-siRNAs) targeting mutant CAG repeats. We also provided evidence that sd-siRNAs use RNAi pathway for their activity and demonstrated that active reagents can be expressed in cells from genetic vectors.

MATERIALS AND METHODS

Cell culture and transfection

Fibroblasts from HD patients (GM04281–17/68 CAG, GM04208–21/44 CAG and GM01187–18/47 CAG) were obtained from the Coriell Cell Repositories (Camden, New Jersey, USA) and grown in minimal essential medium (Lonza; Basel, Switzerland) supplemented with 8% fetal bovine serum (Sigma-Aldrich; St. Louis, USA), antibiotics (Sigma-Aldrich) and non-essential amino acids (Sigma-Aldrich). Cell transfections were performed using Lipofectamine 2000 (Life Technologies; Grand Island, NY, USA) according to the manufacturer's instructions. The transfection efficiency was monitored using BlockIT fluorescent siRNA (Life Technologies).

All RNA oligonucleotides were synthesized at Metabion (Martinsried, Germany) and annealed according to the manufacturer's instructions. Briefly, RNAs were combined in annealing buffer (Metabion) to a 20-μM duplex concentration and incubated at 90°C for 1 min, followed by additional incubation at room temperature for 45 min. Polymer p(I:C) was purchased from Sigma-Aldrich. The sequences of the synthetic RNAs used in this study are presented in the figures or specified in Supplementary Table S1. The 5′-O-methylo oligonucleotides were synthesized at Future Synthesis (Poznan, Poland).

Western blot

The western blot analysis for *HTT* protein (17/68Q tract) was performed as previously described (13). Briefly, 25 μg of total protein was run on a Tris-acetate sodium dodecyl sulphate (SDS)-polyacrylamide gel (1.5 cm, 4% stacking gel/4.5 cm, 5% resolving gel, acrylamide:bis-acrylamide ratio of 35:1) in XT Tricine buffer (Bio-Rad; Hercules, CA, USA) at 140 V in an ice-water bath. For the analyses presented in Supplementary Figure S7, which displays the separation of *HTT* proteins that differ in the length of the relatively short polyglutamine tracts (21/44Q and 18/47Q), an 8% stacking/10% resolving Tris-acetate SDS-polyacrylamide gel with a ratio of acrylamide:bis-acrylamide of 250:1 was run at 180 V in an ice-water bath. The resolution of ATXN3 (∼45 kDa), TBP (∼40 kDa), FOXP2 (∼80 kDa), EIF2AK3 (∼125 kDa), RPL14 (∼25 kDa), LRP8 (∼105 kDa) and GAPDH (∼40 kDa) proteins for simultaneous analysis on a single gel was performed on Novex 4–20% Tris-Glycine Mini Gels (Life Technologies). Subsequently, the proteins were wet-transferred to a nitrocellulose membrane (Sigma-Aldrich). All of the immunodetection steps were performed using a SNAPid system (Millipore; Billerica, MA, USA) in a PBS/0.9% NaCl/0.1% Tween-20 buffer containing 0.25% non-fat milk. The immuno-reaction was detected using an ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA), WesternBright Quantum HRP Substrate (Advansta; Menlo Park, CA, USA) or BCIP/NBT Substrate (Sigma-Aldrich). The protein bands were scanned directly from the membrane using a camera and were quantified using Gel-Pro Analyzer. A list of all antibodies used is provided in Supplementary Table S2.

RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was isolated from fibroblast cells using TRI Reagent (BioShop; Burlington, Canada) according to the manufacturer's instructions. The RNA concentration was measured using a NanoDrop spectrophotometer. A total of 500 ng of RNA was reverse transcribed at 55°C using Superscript III (Life Technologies) and random hexamer primers (Promega; Madison, WI, USA). The quality of the reverse transcription (RT) reaction was assessed through polymerase chain reaction (PCR) amplification of the GAPDH gene. The RT-PCR primer sequences are listed in Supplementary Table S3. The reaction products were separated on 1.5% agarose gels in 0.5× TBE buffer and stained with ethidium bromide.

Cell viability assay

The effect of RNA reagents on cell mortality was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) based on the quantification of ATP.

Plasmids and viral vectors

The short hairpin RNA (shRNA) expression cassettes were generated from DNA oligonucleotides (Sigma-Aldrich, see sequences in Supplementary Table S4) based on an A2 sd-siRNA reagent (shA2_R and shA2_R1). The sd-siRNA A2 strand was placed into the 3′ arm of the shRNA stem. shRNAs, under an H1 promoter, comprised a 22-bp stem and 10-nt miR-23 loop. We used shLuc, which targets the luciferase gene, and sh2.4, which targets exon 2 of *HTT* (25) as negative and positive controls of huntingtin silencing, respectively. Pairs of oligonucleotides were annealed and ligated into the pGreenPuro (System Biosciences) expression plasmid and verified through sequencing. For lentivirus production, the plasmids were cotransfected with the packaging plasmids pPACKH1-GAG, pPACKH1-REV and pVSV-G (System Biosciences) in HEK293TN cells. The medium was collected at days 2 and 3, and the viral supernatants

were passed through 0.45-μm filters and concentrated using PEGit Virus Precipitation Solution (System Biosciences). The lentiviral vectors were resuspended in Opti-MEM (Gibco), and the virus titers (TU/ml) were determined through flow cytometry (Accuri C6, BD Biosciences) based on copGFP expression.

Lentiviral transduction

The transduction of fibroblasts (GM04281) was performed at a multiplicity of infection of 10 in the presence of polybrene (4 μg/ml). Total RNA and protein were harvested 7 days post-transduction.

Northern blotting

The detection of silencing effectors expressed from the vectors was performed using northern blotting as previously described (26,27). Briefly, total RNA was isolated from HEK293T cells using TRI Reagent (BioShop) according to the manufacturer's instructions. Total RNA (35 μg) was resolved on denaturing polyacrylamide gels (12% PAA, 19:1 acrylamide/bis and 7.5 M urea) in 0.5× TBE. The RNAs were transferred to a GeneScreen Plus hybridization membrane (PerkinElmer) using semi-dry electroblotting (Sigma-Aldrich). The membrane was probed with a specific DNA probe complementary to the A2 sequence and labeled with $[\gamma^{32}P]$ ATP (5000 Ci/mmol, Hartmann Analytics) using OptiKinase (USB) according to the manufacturer's instructions. The hybridization was performed at 37°C overnight in a buffer containing 5× SSC, 1% SDS and 1× Denhardt's solution. The radioactive signals were quantified through phosphorimaging (Multi Gauge v3.0, Fujifilm).

Statistical analysis

All experiments were repeated at least three times. The statistical significance of silencing was assessed using a one-sample t-test, with an arbitrary value of 1 assigned to the cells treated with control siRNA (C). Selected data were compared using an unpaired t-test. The two-tailed P-values of <0.05 were considered significant. The IC_{25}, IC_{50} and IC_{75} values were obtained from fitting curves based on the results of dose-response experiments.

RESULTS

siRNA duplexes outperform single-stranded RNAs in targeting specific and repeated sequences

To directly compare the activities of siRNA duplexes and their corresponding single antisense strands, we used reagents of both types, targeting either sequences specific to the *HTT* transcript (siRNA HD ds and siRNA HD as) or the CAG repeat region (siRNA CAG/CUG and siRNA CUG). All reagents were transfected into HD fibroblast cells (GM04281, 17/68 CAG in *HTT*) at concentrations of 5 and 200 nM. The siRNA duplexes outperformed the single strands in silencing *HTT* expression by targeting both types of sequences (Supplementary Figure S1A and Supplementary Text S1). Based on studies suggesting that the 5′ nucleotides of RNAi reagents might be selected

through AGO2 (28), we examined the performance of three CAG/CUG siRNAs using different 5′-end nucleotides in each strand of each duplex (Supplementary Figure S1B). We observed that the d7-1, d7-2 and d7-3 siRNAs transfected into HD fibroblasts at 20 nM did not exhibit significantly different activities at the *HTT* protein level. Therefore, the 5′-end nucleotide was not considered in the further design of the CAG repeat-targeting reagents.

Single CUG repeat strands may be forced to self-duplex and become active

Considering that only duplexes are highly efficient in gene silencing (Supplementary Figure S1) and that each strand of the CAG/CUG siRNA exerts potent silencing activity, we designed silencing reagents composed of single antisense strands capable of forming duplexes. Thus, passenger strands composed of CAG repeats were excluded, and the duplex was formed from two identical CUG repeat strands. Based on the preliminary results of studies of CUG repeat self-duplexing (see Supplementary Text 2 and Supplementary Figure S2A), we designed 16 A-series reagents composed of CUG repeats containing one or more U>A substitutions, generating A–U base pairs in place of U–U mismatches in the predicted duplexes (Figure 1A). These duplexes were formed from 20-, 21- or 22-nt guide strands and contained single nucleotide 3′ overhangs. The structural features of the sd-siRNAs were assessed using a non-denaturing gel mobility assay and computational methods (Supplementary Text S3 and Figure S2B). Each of these reagents was introduced into HD fibroblasts at a 200 nM concentration, and the subsequent silencing of mutant and normal protein was analyzed. Four reagents significantly inhibited mutant huntingtin, and none of the reagents tested markedly inhibited normal huntingtin expression. The most active and allele-selective reagents were A2, A4, A5 and A15, all of which downregulated mutant protein to 15–35% of the control levels (Figure 1B and Supplementary Figure S3).

We also examined a set of sd-siRNA reagents containing one or more U>G substitutions, resulting in the formation of U•G wobble base pairs in the predicted duplexes (Figure 1C). The reagents of this G-series mimicked the properties of the reagents of the previously tested A-series in their tendency to form duplex structures (Supplementary Figure S2C). Strikingly, the profile of huntingtin inhibition for G-series reagents in the same HD fibroblasts was much different from that of A-series reagents (compare Figure 1D and B). The reduction of mutant huntingtin was more prevalent and varied from 50% to 90% of the control levels. However, considerable activity against the normal *HTT* protein was also observed, causing a reduction of up to 50% of the normal *HTT* levels (Figure 1D and Supplementary Figure S3).

Considerable differences in the observed activities of A-series and G-series reagents prompted us to design 'mixmers'. These reagents were derivatives of A6 and G6 oligonucleotides that contained single A and G substitutions in positions 13 and 16 (AG 6 and GA 6)
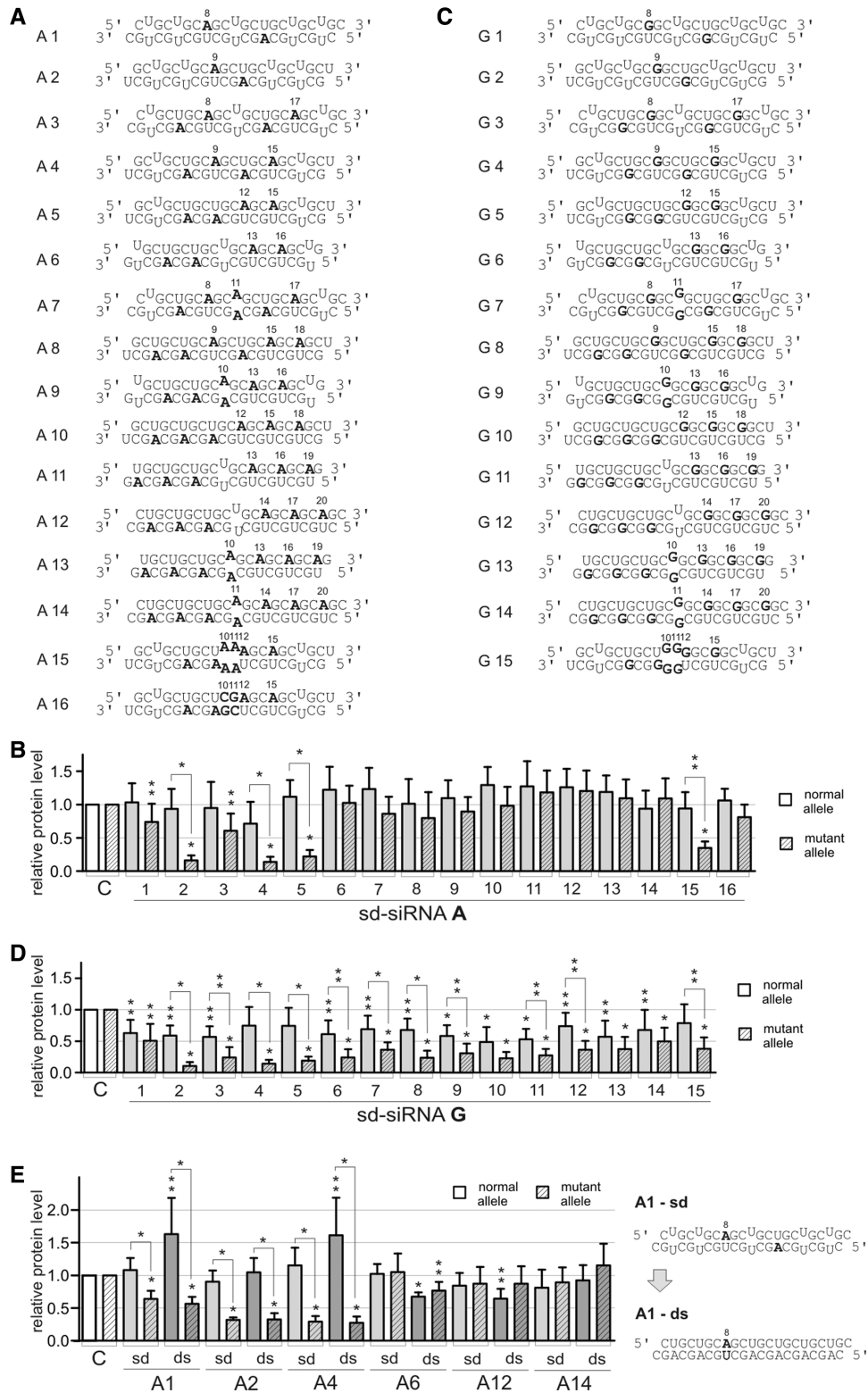
Figure 1. Activity of sd-siRNAs containing U>A and U>G substitutions. (**A**) The nucleotide sequences and predicted structures of sd-siRNA duplexes containing U>A substitutions at the indicated positions. A16 also has C and G substitutions. (**B**) Western blot analysis of huntingtin levels in HD fibroblasts (17/68 Q) at 72 h after transfection with 200 nM sd-siRNAs containing U>A substitutions. (**C**) The nucleotide sequences and predicted structures of the sd-siRNA duplexes containing U>G substitutions at the indicated positions. (**D**) Western blot analysis of huntingtin levels in HD fibroblasts (17/68 Q) at 72 h after transfection with 200 nM sd-siRNAs containing U>G substitutions. (**E**) Western blot analysis of huntingtin levels in HD fibroblasts (17/68 Q) at 72 h after transfection with 50 nM sd-siRNAs A1, A2, A4 (sd) or their corresponding fully complementary duplexes (ds). An example of sequences tested is shown for A1. We observed upregulation of normal *HTT* level, up to 1.8-fold, by some perfect duplexes, consistent with our earlier observations (13). C–reference bar indicates the *HTT* expression level in the cells transfected with control siRNA. In the graph, signal intensities were normalized to plectin protein levels and compared using a one-sample *t*-test. Error bars represent standard deviations. The *P*-values are indicated by asterisk (*$P < 0.001$; **$0.001 < P < 0.05$). Examples of raw data are shown in Supplementary Figure S3.

(Supplementary Figure S4A). The mixmers showed activity intermediate between those of A6 and G6 reagents (Supplementary Figure S4B).

Determinants of sd-siRNAs activity

The silencing activity of sd-siRNAs cannot be fully explained by their tendency for duplex formation, as revealed by gel mobility test (see Supplementary Text S3 for more details). To determine whether the activity of sd-siRNAs is associated with their cellular stability, we analyzed the stabilities of selected sd-siRNAs (active A2 and G2, much less active A1) and the *HTT*-specific siRNA siHTT as reference (Supplementary Figure S5). For this purpose, we used northern blot analysis of siRNAs transfected into cells, which we considered more relevant than testing the stability of siRNAs incubated in serum (29). We observed that 20–50% of transfected siRNAs remained in cells 1 day after transfection, and ∼5% of transfected siRNAs remained 7 days after transfection (Supplementary Figure S5). However, the small differences in the stability of sd-siRNAs could not explain the large differences in silencing activity.

To obtain further insight into other factors potentially involved in the disparate activity of sd-siRNAs, we tested additional reagents. These reagents were fully complementary duplexes that contained selected sd-siRNAs, namely, active A1, A2 and A4, and not active, A6, A12 and A14, as guide strands. However, the direct comparison of the silencing activities of these guide-strand-only siRNAs and their fully paired counterparts revealed their similar efficiencies for the inhibition of mutant huntingtin expression (Figure 1E). Therefore, we proposed the interaction with target sequence as the critical factor for sd-siRNAs activity. We thought that mismatches between the sd-siRNA reagent and target sequence apparently exert diverse effects, depending on the mismatch type, number and location.

Six reagents were selected based on their silencing characteristics

We further characterized several reagents exhibiting considerable activity and a strong preference for the silencing of the mutant *HTT* allele. Four reagents from the A-series and 11 reagents from the G-series were selected for transfection into HD cells at concentrations of 2, 10 and 50 nM. Most of these sd-siRNAs significantly reduced the levels of mutant protein at 2 nM (up to a 50% decrease); stronger silencing effects were achieved at 50 nM and similar effects were observed at 200 nM (Figure 2 and Supplementary Figure S6). Based on these results, the doses required for *HTT* silencing to 25%, 50% and 75% of control level (values of inhibitory concentration: IC_{25}, IC_{50} and IC_{75}, respectively) were calculated (Supplementary Table S5). For further analyses, we selected six reagents (A2, A4, A5, G2, G4 and G5) that fulfilled the following criteria: (i) significant activity
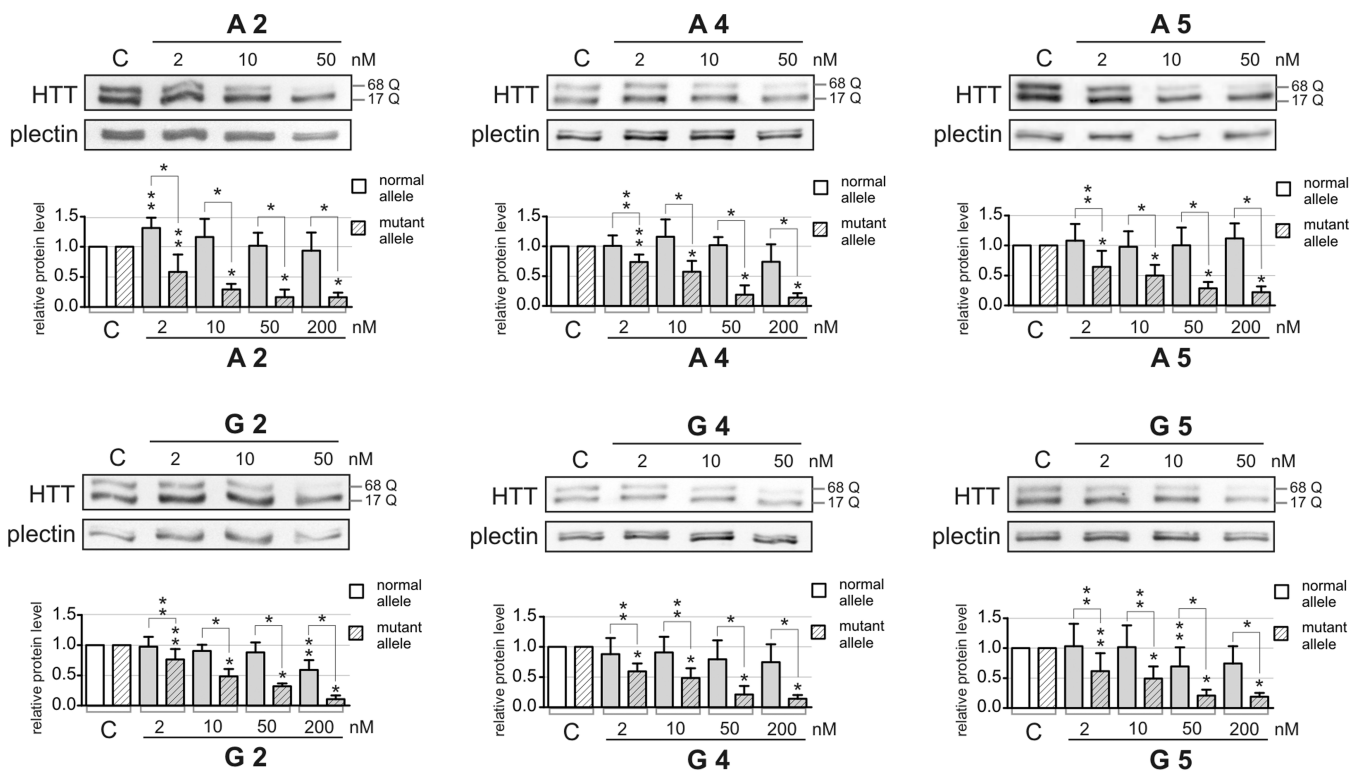

Figure 2. Silencing activity of selected sd-siRNAs at lower concentrations. Western blot analysis of huntingtin levels in HD fibroblasts (17/68 Q) at 72 h after transfection with 2, 10 or 50 nM RNA containing A-substitutions (A2, A4 and A5) or G-substitutions (G2, G4 and G5) (the 200 nM results from Figure 1 are included on the graph). C–the reference bar indicates *HTT* expression levels in the cells transfected with control siRNA. In the graphs, signal intensities were normalized to plectin protein levels and compared using a one-sample *t*-test. Error bars represent standard deviations. The *P*-values are indicated by asterisk (*$P < 0.001$; **$0.001 < P < 0.05$).

toward mutant huntingtin, i.e. an IC_{25} M (for mutant *HTT*) of <150 nM, and (ii) high allele-discriminating properties, i.e. IC_{75} N to IC_{75} M ratio higher than 20, indicating considerable preference for silencing of the mutant allele.

The selected reagents were also tested in two other HD patient-derived cell lines containing shorter CAG repeat tracts in the mutant *HTT* alleles (21/44 and 18/47) (Supplementary Figure S7). We demonstrated that reagents from both series significantly discriminated between the normal and short mutant alleles typically expressed in patients suffering from HD. However, the allele-discriminating properties of the tested reagents were lower than those observed for longer mutant *HTT* alleles.

Different sd-siRNAs show different silencing kinetics

To obtain insight into the type of silencing mechanism involved in *HTT* inhibition, we monitored the time course of huntingtin silencing using selected sd-siRNAs at the mRNA and protein level (Figure 3 and

Supplementary Figure S8). RT-PCR was used to measure transcript silencing because this method facilitates the separate assessment of the cellular levels of the normal and mutant *HTT* alleles based on differences in the repeat tract length. The results were confirmed through an analysis of the total *HTT* mRNA level (Supplementary Figure S8).

A significant reduction of mutant huntingtin protein was observed at 24 h for all reagents used at a concentration of 50 nM (Figure 3). The maximum decrease, up to 20% of the control levels of mutant *HTT* protein, was observed between 48 and 72 h for the majority of the tested reagents. The maximum difference in the silencing of mutant and normal huntingtin was achieved at the 72-h time point. The reduction of mutant huntingtin remained substantial at ∼50% of the protein control level at 7 days post-transfection. The maximum silencing of both normal and mutant *HTT* transcripts and normal *HTT* protein generally occurred earlier, at ∼24 h, and these levels decreased faster than those of the mutant *HTT* protein. The A-series reagents only slightly affected the levels of
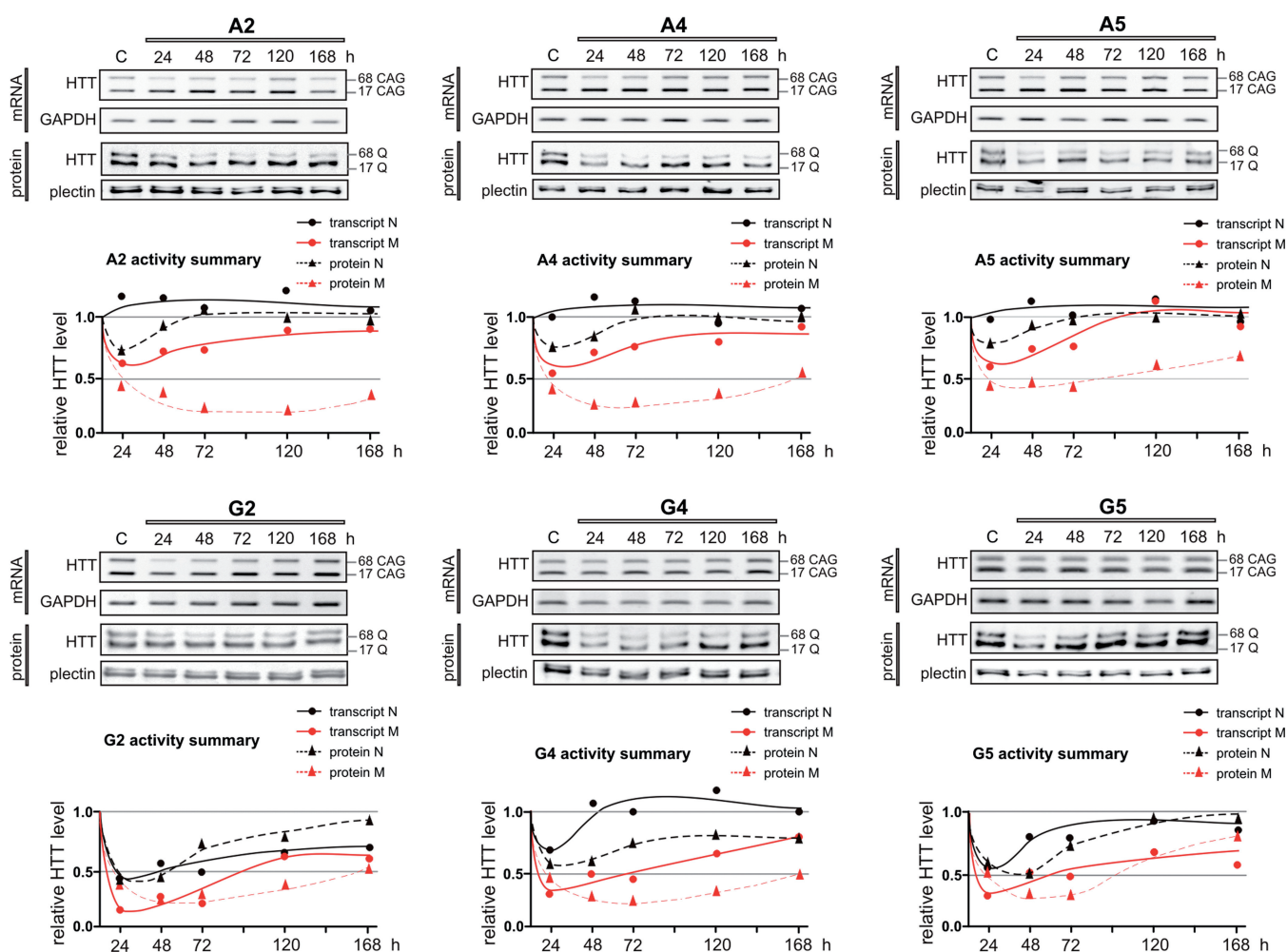

Figure 3. Time-course analysis of *HTT* transcript and protein silencing by sd-siRNAs. RT-PCR and western blot analyses of *HTT* expression at the transcript and protein levels in HD fibroblasts (GM04281) transfected with 50 nM sd-siRNA (A2, A4, A5, G2, G4 or G5) at the indicated time points. C–the reference point indicates the *HTT* expression levels in the cells transfected with control siRNA. The mean signal intensity values are presented in the graphs; for statistical analysis, see Supplementary Figure S7.

normal and mutant transcripts and showed a strong allele-selective effect in the silencing of the mutant protein. In contrast, the G-series reagents inhibited transcript levels more effectively but showed somewhat lower allele selectivity at the protein level. In each case, however, the reduction of the protein was greater than the reduction of the corresponding transcript. Enhanced allele selectivity for mutant *HTT* protein silencing was achieved with A-series reagents, which inhibited translation without a substantial decrease in transcript levels (Figure 3).

RNAi-based mechanism of sd-siRNAs

As short RNA duplexes, sd-siRNAs are expected to be active in the mechanism involving RISC complex. We tested specifically modified oligonucleotides to verify the RNAi-based mechanism of sd-siRNA activity. The modifications included (i) base substitutions in seed region, critical for target sequence recognition through RISC-loaded siRNAs or miRNAs (1) and (ii) 5′-end chemical modification by introducing a 5′-*O*-methylo (5′OMe) group, which blocks 5′-end phosphorylation in cells required for oligonucleotide-activated RISC (30). Seed region modifications were applied to two active sd-siRNAs, A5 and G6, in which the introduced substitutions were relocated from the 3′ to the 5′ half of the oligonucleotide (Figure 4A). The activity of the respective reversed siRNAs, A5-rev and G6-rev, was strongly decreased. In a second approach, the most active sd-siRNAs, A2 and G2, were modified through the addition of a 5′OMe (A2-5′ and G2-5′) and also showed

strongly reduced activity (Figure 4B). These results indicate the AGO-dependent activity of sd-siRNAs.

The high gene selectivity of sd-siRNAs is correlated with low toxicity

To characterize the gene selectivity of the sd-siRNAs, we analyzed the cellular levels of several other proteins encoded by genes containing pure or interrupted CAG repeats (ATXN3, TBP and FOXP2) and CTG repeats (EIF2AK3, RPL14 and LRP8) in HD fibroblasts (Figure 5). The silencing activities of repeat-targeting duplexes, i.e. d7 (CAG/CUG siRNA) (13,18), W13/16 (13) and P9b (15), were tested as controls. The silencing profiles of individual sd-siRNAs against different proteins differed only slightly. Generally, the silencing activity was low, and a significant decrease in protein levels was observed in only a few cases. For all reagents tested, the smallest decreases were observed for the FOXP2 and TBP proteins, which contain transcripts with CAA-interrupted CAG repeats. The presence of numerous CAA interruptions within the CAG repeats of the target sequences inhibited gene silencing, even by the d7 duplex comprising pure CUG/CAG repeats. In addition, for most of the proteins analyzed, the sd-siRNAs were less efficient for downregulation than RNAi duplexes.

We next evaluated the toxicity of each of these reagents by measuring cell viability at 72 h post-transfection (Figure 6). The sd-siRNAs caused significantly less cell death than d7, which was used as a reference. Even at a high concentration (200 nM), the sd-siRNAs caused <50% loss of cell viability, whereas only ~30% of cells
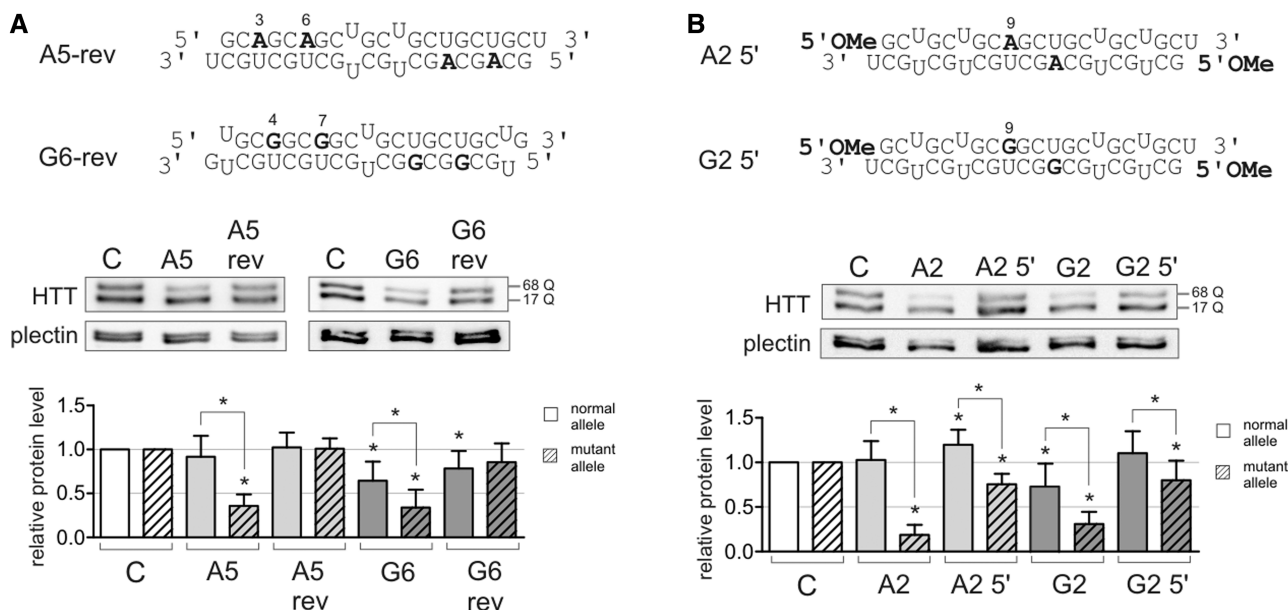

Figure 4. Evidence for AGO-dependent activity of sd-siRNAs. (**A**) The nucleotide sequences and predicted structures of A5-rev and G6-rev sd-siRNAs with substitutions at the indicated positions are shown, together with the results of the western blot analysis of huntingtin protein levels in HD fibroblasts (17/68 Q) at 72 h post-transfection with 50 nM sd-siRNAs A6 and G6 or their corresponding reversed versions with substitutions in the seed sequence. (**B**) Western blot analysis of huntingtin levels in HD fibroblasts (17/68 Q) at 72 h post-transfection with 50 nM sd-siRNAs containing 5′-*O*-methylo groups: A2-5′ and G2-5′ and their standard versions. C–the reference bar indicates *HTT* expression levels in cells transfected with control siRNA. In the graphs, the signal intensities were normalized to plectin protein levels and compared using a one-sample *t*-test. The error bars represent standard deviations. The *P*-value is indicated with an asterisk (*$P < 0.05$).
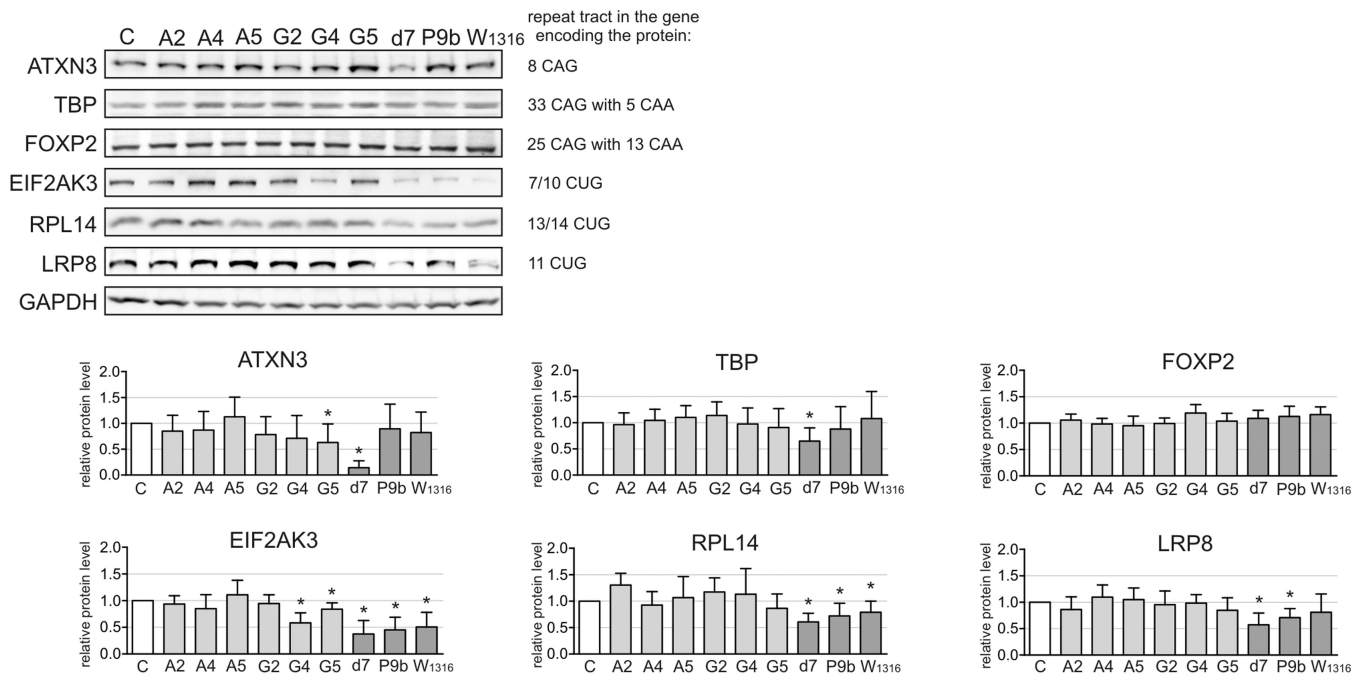
Figure 5. Analysis of the gene selectivity of *HTT* silencing using sd-siRNAs. Western blot analysis of ATXN3, TBP, FOXP2, EIF2AK3, RPL14 and LRP8 levels in HD fibroblasts (GM04281) at 72 h post-transfection with 50 nM RNAs (A2, A4, A5, G2, G4 or G5; reference reagents include d7, P9b and W13/16). The nucleotide sequences of the repeat tracts in the genes encoding the analyzed proteins include TBP tract (CAG)3(CAA)3(CAG)9(CAA)(CAG)(CAA)(CAG)20 and FOXP2 tract (CAG)4(CAA) (CAG)4(CAA)2(CAG)2(CAA)2(CAG)3(CAA)5(CAG)2 (CAA)2(CAG)5(CAG)(CAG)5. C–the reference bar indicates the expression levels in the cells transfected with control siRNA. In the graphs, the signal intensities were normalized to the GADPH protein level and compared using a single-sample *t*-test. The error bars represent standard deviations. The *P*-value is indicated with an asterisk (*$P < 0.05$).

remained viable after transfection with 50 nM d7. Generally, the toxicity profiles of the sd-siRNAs did not differ greatly, and the profiles were similar to those observed for control siRNA (C) (Figure 6). In addition, the transcript levels of the three genes involved in the interferon response (MX1, IFIT1 and IFNβ) (31) were assessed using RT-PCR (Supplementary Figure S9). Different gene activation profiles were observed for the different sd-siRNA and reference reagents, but these differences were associated with low interferon activation. None of the reagents tested triggered the interferon induction characteristic of the positive controls containing specific molecular patterns recognized by the cellular sensors of foreign RNA [long RNA containing 5′-triphosphate (3pRNA), long dsRNA (UAA/AUU17) or poly(I:C)].

Vector-expressed silencing reagents are also active and allele-selective

One of the drawbacks of therapeutic approaches using synthetic siRNA is the need for repeated administration due to the low stability of short RNAs in cells. Long-term silencing could be achieved using vector-based RNAi. Therefore, we designed shRNA expression cassettes based on the most active reagent, A2 (Figure 7A). We examined two variants of shRNAs to increase the likelihood that the potent A2 sequence will be released from shRNA (Supplementary Figure S10).

High-resolution northern blotting showed that H1-shRNA transcripts are heterogeneous in length, likely reflecting the variable length of the 3′U tail. These heterogeneous transcripts are processed through Dicer into a pool of 19–24 nt siRNAs (Figure 7B). To examine the silencing efficacy of shRNAs, HD fibroblasts were transduced with lentiviral particles. Western blot analysis demonstrated that shRNAs A2R and A2R1 efficiently reduced mutant huntingtin protein expression to ~10% of the control level and left the normal huntingtin gene intact (Figure 7C).

DISCUSSION

The selective targeting of expanded CAG repeats is a promising approach for the curative treatment of neurodegenerative polyQ diseases. The advantage of this approach over targeting SNPs linked to repeat expansions is its universality; i.e. a single drug could potentially be used to treat a number of polyQ diseases (32,33). Various RNAi reagents have recently been tested to achieve selectivity in targeting the disease-causing alleles and genes, and promising results were obtained with miRNA-like functioning duplexes (13,15). However, the abundance of transcripts containing either CAG repeats or CUG repeats in human cells (34) and the short-lived silencing effect remain a major challenge for this approach; thus, a continued search for more selective and effective reagents is necessary. Recently, single-stranded CUG repeat siRNAs that contained specific chemical modification patterns and base substitutions were shown to be effective silencing tools for
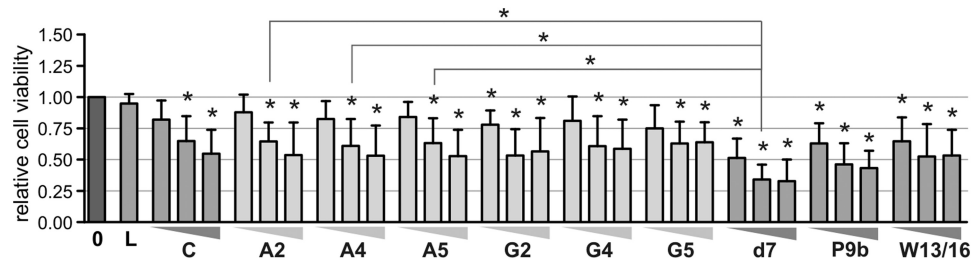
Figure 6. Cell viability after treatment with sd-siRNA and control reagents. The results from the cell viability test performed at 72 h post-transfection of HD fibroblasts (GM04281) with the indicated reagents at 10, 50 or 200 nM. The luminescent signal intensities were normalized to the signal from untreated cells (0) and compared using a one-sample *t*-test. L–cells treated with lipofectamine only; C–cells transfected with control siRNA. The error bars represent standard deviations. The *P*-value is indicated with an asterisk (*$P < 0.05$).
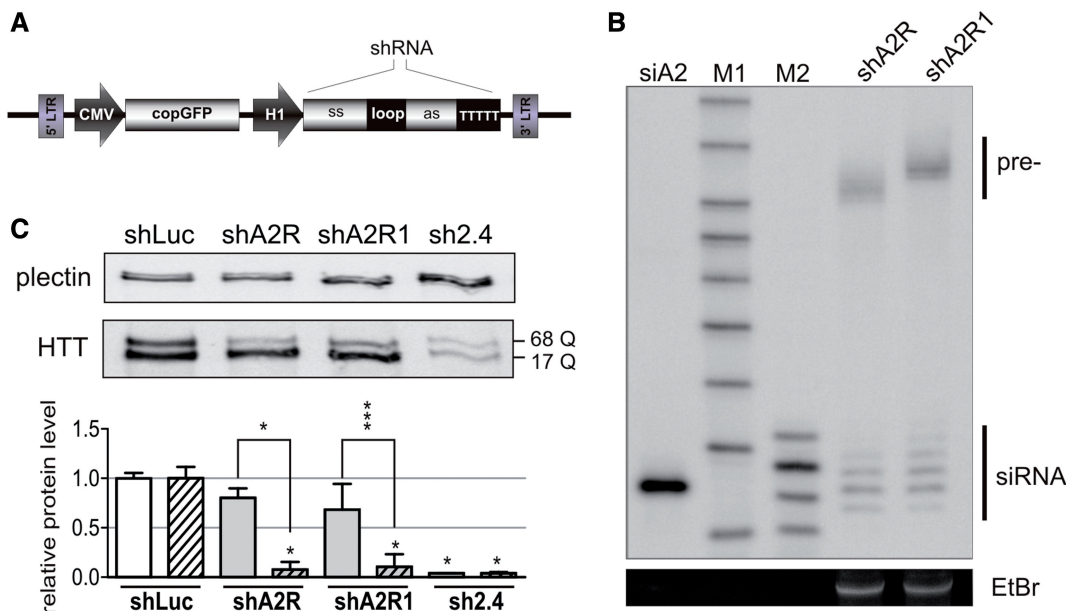

Figure 7. Selective inhibition of mutant huntingtin expression by vector-based RNAi triggers. (**A**) Lentiviral vector containing copGFP and shRNA expression cassettes. (**B**) High-resolution northern blot analysis of shRNA processing in HEK293T cells; pre denotes unprocessed precursor, M1 denotes RNA low molecular weight marker (USB), M2 denotes end-labeled 19-, 21-, 23- or 25-nt RNA oligonucleotides and siA2 denotes 0.4 pmoles of 21-nt synthetic A2 RNA oligonucleotide. Ethidium bromide staining is shown as the loading control. (**C**) Lentiviral vectors expressing shRNAs were used to transduce human fibroblast cell line containing 17/68CAG repeats (GM04281). The cells were infected at multiplicity of infection of 10 and evaluated 7 days post-transduction. shLuc, negative control, shRNA targeting Luc gene; sh2.4, positive control, targeting specific sequence of *HTT* gene; shA2R and shA2R1, sd-siRNA A2-based shRNA reagents targeting CAG repeats. In the graph, the signal intensities for huntingtin were normalized to shLuc and plectin protein levels and compared using a one-sample *t*-test. The error bars represent standard deviations. The *P*-values are indicated with an asterisk (*$P < 0.001$, ***$0.001 < P < 0.05$).

the mutant huntingtin gene in cell culture and the HD mouse model (35).

In our attempt to identify silencing reagents, we used the natural tendency of long CUG repeats to form hairpin structures containing double-stranded stem portions with periodic U–U mismatches (36). We used this property to obtain self-annealing guide-strand-only duplexes through the complexation of short CUG repeats 20–22 nt in length into duplexes after introducing U to A or U to G substitutions at one or more positions. The base substitutions were introduced in the 3′ portion or central portion of the CUG repeat oligomer, leaving the seed region intact. As a result, A–U or G–U base pairs were formed in place of some U–U mismatches. Thus, the single CUG repeat strands with low activity were transformed into potent silencing agents.

We demonstrated that sd-siRNAs use the RNAi pathway for *HTT* silencing through the reduced efficiency of sd-siRNA after the modification of the seed sequence or 5′-end. We also showed that sd-siRNAs, converted to full duplexes and released from shRNAs, are as active as standard sd-siRNAs, further indicating the involvement of RNAi machinery.

In the case of sd-siRNAs, the discrimination between active and inactive reagents might occur during at least three steps: duplex formation, which is the prerequisite for efficient RISC entry; duplex unwinding and single-strand selection; and RISC interaction with its target sequence (Figure 8). Our experiments revealed that nearly all of the G-series reagents and only some A-series reagents were active in gene silencing. We also showed that the low activity of most A-series reagents
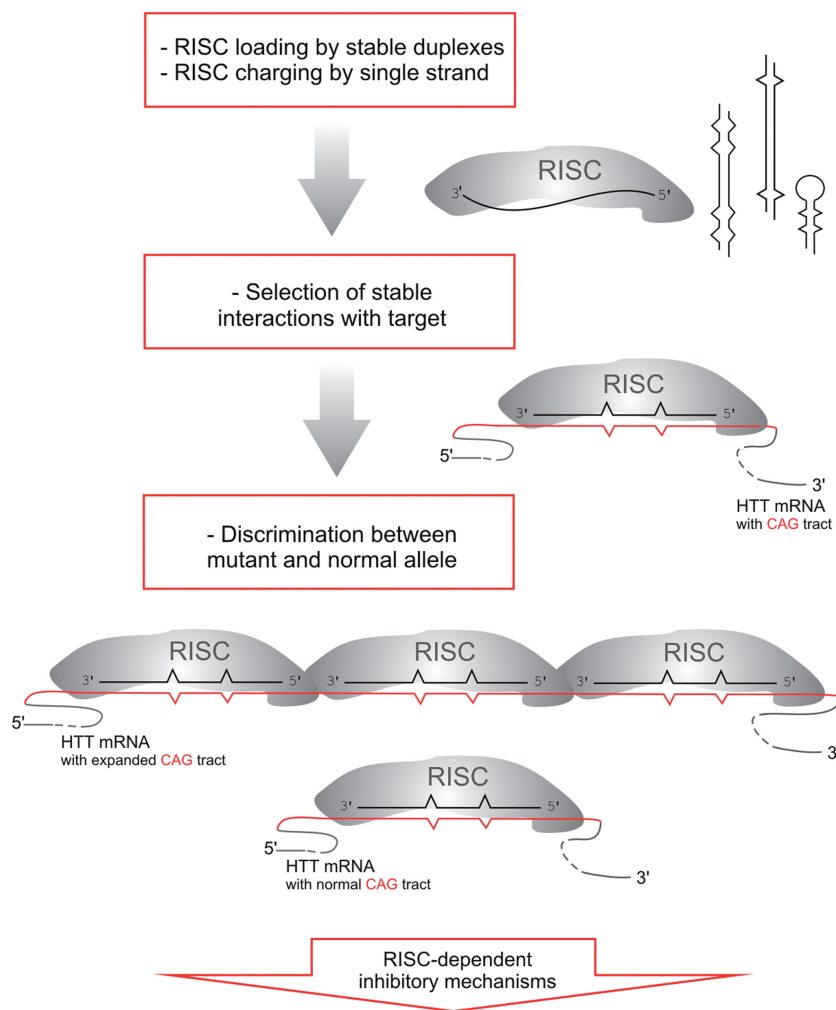
Figure 8. Schematic representation of the steps at which the determination of sd-siRNAs activity may occur. See Discussion for more details.

does not result from the failure to form duplexes. The strongest discrimination between active and inactive reagents occurs during the RISC interaction with the target sequence. A–A mismatches destabilize the structure of RNA duplexes more than G–A mismatches (37,38). Therefore, it is likely that A-series reagents trigger interactions between RISC and the target less efficiently than G-series reagents.

The picture that emerges from the analysis of silencing kinetics shows that the predominant mechanism of *HTT* silencing through the most efficient A-series sd-siRNAs (A2, A4 and A5) is translational inhibition, whereas the best G-series reagents also induce significant transcript downregulation. Based on our present and previous results (13) and the results of other researchers (15), we note that repeat-targeting reagents that induce substantial transcript degradation, e.g. d7 and G2, show lower allele selectivity than reagents that induce weaker or no transcript degradation (W13/16, P9b, A2 and A4); i.e. the difference in the number of RISC-binding sites between normal and expanded repeats is more important for the miRNA-like silencing mechanism than the siRNA mechanism. Consistent with the approximate AGO2 50-nt footprint on RNA (39), the tandemly repeated CAG motifs in a typical normal *HTT* allele bind a single RISC, whereas the mutant allele accommodates two, three or more silencing complexes (Figure 8). This difference likely determines the allele-selectivity of *HTT* silencing, making sd-siRNAs highly efficient for longer repeat tracts. The CAG repeat in *HTT* mRNA is located in the ORF, and the silencing complexes bound to these sequences must experience collisions with the translation machinery. It is likely that translating ribosomes easily deplete the mismatched RISC from normal transcripts but are less efficient in displacing the more stable complexes formed by multiple RISCs on the mutant transcripts.

The sd-siRNA reagents showing different activities in *HTT* gene silencing offer different silencing options. The A-series reagents selectively reduce mutant protein levels without decreasing transcript levels. However, G-series reagents, which are slightly less selective at the protein level, are also active in reducing transcript levels. This property might be advantageous considering the growing number of reports showing the direct involvement of mutant RNA in HD pathogenesis (10,40,41). The

advantageous features of sd-siRNAs include high gene selectivity and low toxicity. In addition, these silencing effectors might be released in cells from shRNAs to achieve long-term silencing effects.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online, including Supplementary References [42,43].

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