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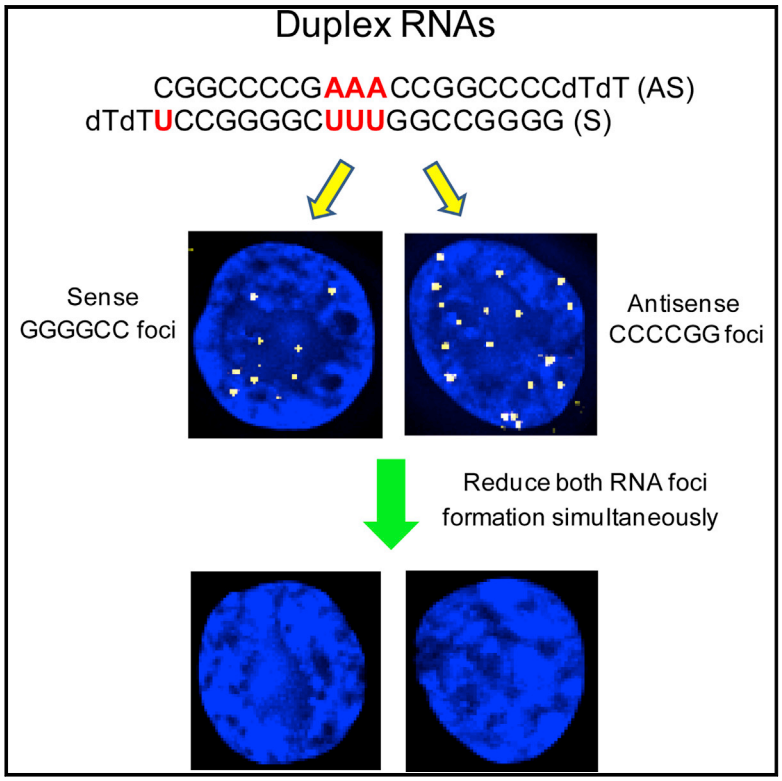
Engineering Duplex RNAs for Challenging Targets: Recognition of GGGGCC/CCCCGG Repeats at the ALS/FTD *C9orf72* Locus

Graphical Abstract



Highlights

- Duplex RNAs can be engineered to recognize RNA targets composed entirely of C/G
- Duplex RNAs inhibit sense and antisense foci at C9orf72 locus
- RNAi is active at suppressing foci in mammalian cell nuclei

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In Brief

A GGGGCC hexanucleotide expansion within the *C9orf72* gene can cause familial amyotrophic lateral sclerosis and frontotemporal dementia. Hu et al. have engineered duplex RNAs to enable them to recognize difficult C/G targets and inhibit potential disease-causing foci formed by both GGGGCC and CCCCGG RNA.

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Engineering Duplex RNAs for Challenging Targets: Recognition of GGGGCC/CCCCGG Repeats at the ALS/FTD *C9orf72* Locus

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SUMMARY

A GGGGCC expansion within an intronic region of the *C9orf72* gene forms RNA foci that are associated with one-third of familial amyotrophic lateral sclerosis and one-quarter of frontotemporal dementia. The *C9orf72* locus also expresses an antisense transcript with a CCCCGG expansion that forms foci and may contribute to disease. Synthetic agents that bind these hexanucleotide repeats and block foci would be leads for therapeutic discovery. We have engineered duplex RNAs to enable them to recognize difficult C/G targets. Recognition inhibits foci formed by both GGGGCC and CCCCGG RNA. Our findings show that a single duplex RNA can be used to recognize both disease-related *C9orf72* transcripts. More broadly, we extend RNAi to previously inaccessible C/G sequences and provide another example of target recognition in human cells by nuclear RNAi.

INTRODUCTION

An expanded hexanucleotide repeat has been implicated in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). This repeat expansion occurs in the first intron of the chromosome 9 open reading frame 72 (*C9orf72*) gene. It accounts for one-third of familial ALS and one-quarter of familial FTD (Renton et al., 2011; DeJesus-Hernandez et al., 2012). The sequence of the repeat within *C9orf72* pre-mRNA is GGGGCC. Patients with ALS or FTD typically have one mutant *C9orf72* allele that contains 700–1,600 repeats, while unaffected individuals have fewer than 24 repeats in both alleles (DeJesus-Hernandez et al., 2012). The *C9orf72* locus also expresses an antisense transcript that encodes a CCCCGG repeat that may contribute to disease (Gendron et al., 2013).

Expanded repeats may form structures that disrupt normal RNA-protein interactions, affect RNA processing, and contribute to pathogenesis (Ling et al., 2013). The expanded sense and antisense RNA transcripts are C/G rich, and the G-rich sense strand is known to form a stable G-quadruplex structure (Haeusler et al., 2014). The expanded repeats and the structures they form may sequester proteins and disrupt normal function by decreasing the effective concentrations of associated proteins within cells (Lee et al., 2013). A similar mechanism of action has been demonstrated for the expanded CUG repeats that occur with the mutant DM protein kinase gene responsible for myotonic dystrophy (Wheeler et al., 2009; Sobczak et al., 2013). Because of their potential to disrupt normal processes in cells and contribute to disease, both the sense and antisense repeat transcripts at the *C9orf72* locus are targets for inhibitors that block RNA, disrupt structure, and alter the potential for RNA-protein interactions.

Both ALS and FTD are currently incurable, leading to an urgent need for new insights into treatment. One strategy to blunt the impact of mutant *C9orf72* RNA is to inhibit expression of the gene. Antisense oligonucleotides that are complementary to intronic regions within the *C9orf72* transcript have been tested. These oligonucleotides were designed to recruit RNase H to their target sites and lead to degradation of intronic RNA. Introduction of these oligonucleotides into cells caused foci formation to decrease, and reduced RNA toxicity (Donnelly et al., 2013; Lagier-Tourenne et al., 2013; Sareen et al., 2013). In this report, we investigate whether duplex RNAs can also block foci formation and whether a single duplex RNA can interfere with foci formed by both the sense GGGGCC transcript and the antisense CCCCGG transcript. Duplex RNAs would have the advantage of using the potent RNAi mechanism and inhibiting both transcripts with one agent.

We hypothesized that duplex RNAs complementary to the expanded repeat would target the GGGGCC and CCCCGG repeats simultaneously. Previous work had shown that it was possible to use duplex RNAs to target expanded trinucleotide repeats. Our laboratory and others had previously developed engineered duplex RNAs that target genes containing expanded CAG trinucleotide repeats (Hu et al., 2010; Fiszer et al., 2011).

The *C9orf72* GGGGCC or CCCCGG repeats, however, pose novel challenges to recognition that go beyond past experience targeting CAG repeats. One challenge is that, unlike CAG repeats, the GGGGCC repeat is intronic rather than within an exon. The GGGGCC and CCCCGG foci are detected in cell nuclei, rather than the cytoplasm. A second challenge, therefore, is that recognition by duplex RNA would need to occur in cell nuclei and involve nuclear RNAi rather than the more familiar cytoplasmic RNAi mechanism.

A third challenge is that RNA duplexes with high C/G content are considered to be poor candidates for gene silencing by

Table 1. siRNAs Targeting the Hexanucleotide Repeat

No.	Sequence (AS, 5′-3′; S, 3′-5′)	Mismatch Number	Duplex T_m (°C)	% Inhibition G-Rich Foci	% Inhibition C-Rich Foci
R1	CGGCCCCGGCCCCGGCCCCdTdT (AS) dTdTGCCGGGGCCGGGGCCGGGG (S)	0	>87	NI	8
R2	CGGCCCCGG**AA**CCGGCCCCdTdT (AS) dTdTGCCGGGGCC**UU**GGCCGGGG (S)	10, 11	>87	18	–
R3	CGGCCCCG**AAA**CCGGCCCCdTdT (AS) dTdT**U**CCGGGGC**UUU**GGCCGGGG (S)	9, 10, 11	>87	73	58
R4	CGGCCCCG**AAAA**CGGCCCCdTdT (AS) dTdT**U**CCGGGGC**UUUU**GCCGGGG (S)	9, 10, 11, 12	>87	52	48
R5	CGGCCCC**AAAAA**CGGCCCCdTdT (AS) dTdT**U**CCGGGGC**UUUU**GCCGGGG (S)	8, 9, 10, 11, 12	83.7	43	–
R6	CGGCCCCG**AAAA**CG**A**CCCCdTdT (AS) dTdT**U**CCGGGGC**UUUU**GCCGGGG (S)	9, 10, 11, 12, 15	83.1	54	–
R7	CGGCCCCG**AAAA**CG**A**CC**A**CdTdT (AS) dTdT**U**CCGGGGC**UUUU**GCCGGGG (S)	9, 10, 11, 12, 15, 18	76.2	32	–
R8	CGGCCCCG**AA**CC**A**GG**A**CCCdTdT (AS) dTdT**U**CCGGGGC**UU**GG**U**CC**U**GGG (S)	9, 10, 13, 16 (AS)	>87	65	–
R9	CGGCCCCG**AA**CC**A**G**A**CCCCdTdT (AS) dTdT**U**CCGGGGC**UU**GG**U**CC**U**GGG (S)	9, 10, 13, 15 (AS)	84.0	78	–
R10	CGGCCCCG**AAA**CCG**A**CCCCdTdT (AS) dTdT**U**CCGGGGC**UUU**GGCCGGGG (S)	9, 10, 11, 15 (AS)	86.7	72	–
R11	CGGCCCCG**AA**CCCG**A**CCCCdTdT (AS) dTdT**U**CCGGGGC**UU**GGGC**U**GGGG (S)	9, 10, 15 (AS)	>87	55	–
R12	CGGCCCCG**AAA**CCG**A**CCCCdTdT (AS) dTdT**U**CCG**A**GGC**UUU**GGCCGGGG (S)	9, 10, 11, 15, 19 (S)	77.3	–	62
R13	CGGCCCCG**AAA**CCGGCCC**U**dTdT (AS) dTdTGCCG**A**GGC**UUU**GGCCGGGG (S)	9, 10, 11, 15 (S)	84.3	–	45
R14	CGGCCCCG**AAA**CCGGCCC**U**dTdT (AS) dTdTGCCG**A**G**A**CC**UU**GGCCGGGG (S)	9,10,13,15 (S)	70.8	–	46
Control siRNA					
C1	GCUAUACCAGCGUCGUCAUdTdT (AS) dTdTCGAUAUGGUCGCAGCAGUA (S)	–	–	NI	NI
C2	CGG**AAA**CGGCCCCGGCCCCdTdT (AS) dTdTGCC**UUU**GCCGGGGCCGGGG (S)	4,5,6 (AS) Seed mismatch	>87	NI	–
C3	CGG**A**C**A**CG**AAA**CCGGCCCCdTdT(AS) dTdT**U**CC**U**G**U**GC**UUU**GGCCGGGG (S)	4, 6, 9, 10, 11 (AS) Seed mismatch	>87	NI	–
C4	CCGCCGGG**AAA**CGGCCCGGdTdT(AS) dTdT**U**GCGGCCC**UUU**GCCGGGCC (S)	9, 10, 11 (AS) Scrambled/mismatch	>87	NI	–
C5	CGGCCCCGGCCCC**AAA**CCCdTdT (AS) dTdTGCCGGGGCCGGGG**UUU**GGG (S)	4, 5, 6 (S) Seed mismatch	>87	–	NI
C6	CGGCC**A**CG**AA**CCC**AAA**CCCdTdT (AS) dTdTGCCGGGGCCGGGG**UUU**GGG (S)	4, 5, 6 (S) Seed mismatch	71.9	–	NI
C7	GCAGCUGUUGCUACUGUUGdTdT (AS) dTdTCGUCGACAACGAUGACAAC (S)	–	–	NI	NI
C8	CAGACAAUGAUUCACACGGdTdT (AS) dTdTGUCUGUUACUAAGUGUGCC (S)	–	–	NI	NI
E1	UGGAAUAAUACUCUGACCCdTdT (AS) dTdTACCUUAUUAUGAGACUGGG (S)	–	–	–	–

Bases that are mismatched relative to the GGGGCC/CCCCGG repeat are in boldface and underlined. NI, no significant inhibition detected; –, not measured.

RNAi (Petri and Meister, 2013). High C/G content impairs both strand loading and target recognition. RNA duplexes that are C/G rich will not readily unwind to release the RNA guide strand. Target RNA transcripts that are C/G rich are likely to form strong secondary structures that will resist binding by complementary small RNAs.

Here, we demonstrate that these challenges can be overcome. We show that duplex RNAs can be engineered to

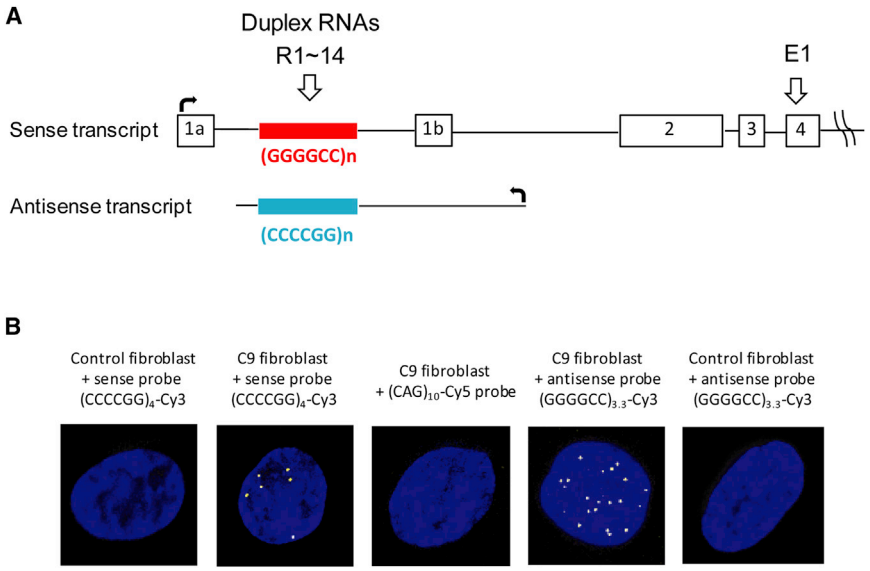

Figure 1. Sense and Antisense Nuclear RNA Foci Are Detected in *C9orf72* Patient-Derived Fibroblast Cells

(A) Scheme showing duplex RNAs targeting sense and antisense *C9orf72* transcripts and the location of the expanded CCCCGG or GGGGCC repeat regions.

(B) FISH images of expanded GGGGCC or CCCCGG RNA foci in wild-type control fibroblasts or *C9orf72* patient-derived fibroblasts. A $(CAG)_{10}$-Cy5 probe complementary to a CUG repeat was used as a control in C9orf72 fibroblasts.

overcome the barriers of high G/C content, function inside cell nuclei, recognize GGGGCC and CCCCGG repeats, and inhibit both sense and antisense strand foci.

RESULTS AND DISCUSSION

Engineering Duplex RNAs to Recognize C/G-Rich Sequences

Duplex RNAs intended for gene silencing consist of a guide strand complementary to the target RNA and a passenger strand that is complementary to the guide strand. Because duplex RNAs have two strands, a single duplex RNA has the capacity to recognize a sequence within an mRNA and a sequence within a corresponding antisense transcript. The challenge for recognition of GGGGCC/CCCCGG repeats is the likelihood that a C/G duplex will be unable to enter the RNAi-induced silencing complex (RISC) because the parent duplex will be too stable.

AGO2 is the catalytic engine of RNAi (Liu et al., 2004) that drives cleavage of target RNAs when sequences are fully complementary. AGO2 can, however, also promote recognition of mismatched sequences. For example, microRNAs that occur naturally supply an endogenous gene-silencing mechanism that typically involves duplex RNAs that are mismatched relative to their mRNA targets. The introduction of mismatches into the central region of the RNA duplex eliminates the potential for substrate cleavage by AGO2 while continuing to permit the guide RNA strand to recognize the target site (Wang et al., 2008).

We have previously tested duplex anti-CAG RNAs with central mismatches as inhibitors of huntingtin, ataxin-3, and atrophin-1 expression (Hu et al., 2014). We found that these duplexes do not promote cleavage of their targets but can be potent and allele-selective inhibitors of protein expression. We reasoned that the introduction of central mismatches into the C/G-rich duplex RNA between bases 8 and 12 would reduce the affinity of the RNA duplex, increase the potential for the two strands to dissociate from one another, and make it more likely that the strands could enter into a complex with AGO2.

Duplex RNAs Inhibit GGGGCC and CCCCGG Foci

We tested the ability of RNA duplexes (Table 1) to inhibit foci. Duplex RNA R1 (R = repeat-targeted) was fully complementary to the C/G-rich repeat. All other duplex RNAs contained A or U substitutions. RNA R1 has a measured melting temperature (T_m) of >87°C. Some modified RNAs with two, three, or four mismatches also had T_m values >87°C. RNAs with more than four mismatches had T_m values as low as 70.8°C. RNAs C1–C6 (C = control) were mismatched within their critical "seed" regions (bases 2–8) or scrambled duplexes. RNA E1 (E = exonic) was fully complementary to exon 4 and was used as a positive control for transfection efficiency.

We introduced anti-GGGGCC/CCCCGG duplex RNAs (Figure 1A and Table 1) into ALS patient-derived fibroblast cells by transfection with cationic lipid. Two days after transfection we used fluorescent in situ hybridization (FISH) followed by analysis using fluorescent microscopy to monitor both CCCCGG and GGGGCC foci (Figure 1B). Hundreds of cells were analyzed for each treatment to permit accurate quantitation of the number of cells containing at least one foci and the number of foci per 100 cells.

After establishing the FISH detection assay, we tested duplex RNAs for their ability to affect foci. Fully complementary duplex RNA R1 did not affect the total number of cells containing GGGGCC foci, nor did it reduce the number of foci per cell (Figure 2A). This result is consistent with the expectation that an entirely C/G duplex would be too stable to enter the RISC. Duplex R2, which contained two A/U substitutions, also did not significantly affect foci.

We hypothesized that two mismatches may have been insufficient, and tested duplexes with three to six mismatches relative to the target G-rich strand. Duplexes R3, R4, R5, and R6 reduced the number of cells with GGGGCC foci and the number of foci per 100 cells, demonstrating the potential for duplexes to inhibit foci (Figures 2A and 2B). Duplex R7 with six mismatches relative to the target G-rich strand did not significantly inhibit foci, suggesting a limit to the number of substitutions that could be tolerated.

We also tested duplexes R1, R3, and R4 for inhibition of foci formed by the C-rich antisense transcript. Similar to the outcome we had observed for the G-rich strand, complementary duplex R1 did not reduce foci detection (Figure 2C). Duplexes R3 and R4 reduced both the total number of cells with C-rich foci and

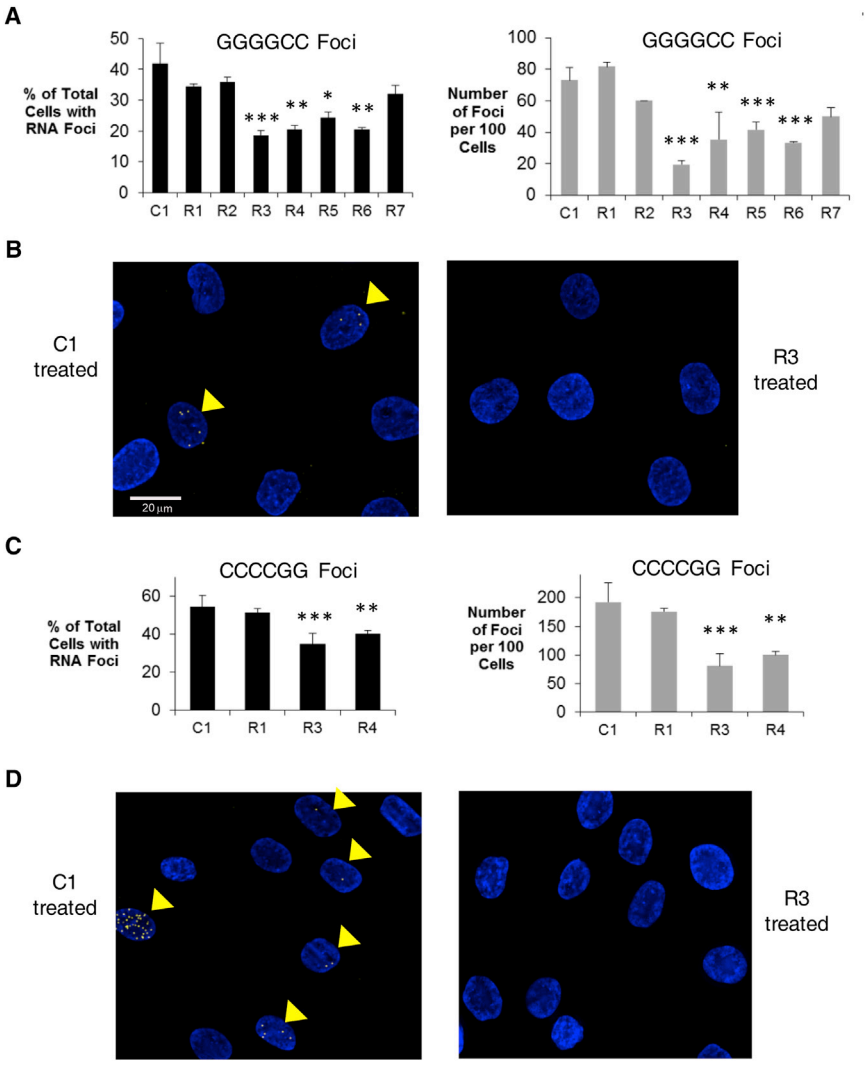

Figure 2. Inhibition of GGGGCC or CCCCGG Foci by Duplex RNAs Evaluated by Fluorescent Microscopy

(A) Effect of duplex RNAs on detection of expanded GGGGCC repeat RNA with *C9orf72* intronic RNA.

(B) Sample microscopy images used for evaluating GGGGCC *C9orf72* sense foci.

(C) Effect of duplex RNAs on detection of expanded CCCCGG *C9orf72* antisense transcript.

(D) Sample microscopy images used for evaluating CCCCGG *C9orf72* antisense foci.

Error bars represent SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with control C1. At least 100 cells were analyzed for each experiment. The yellow arrowheads point to cells containing foci.

the number of foci per cell (Figures 2C and 2D). For duplexes R3–R12 we had introduced a 3′ U substitution into the 3′-sense strand to bias loading toward the antisense strand (Schwarz et al., 2003; Malefyt et al., 2014). Duplexes R3 and R4 that contained this substitution inhibited both the G-rich and C-rich foci. It is possible that standard rules for optimizing RNA duplexes are less powerful in the context of atypical C/G-rich duplexes.

After demonstrating inhibition of both G-rich and C-rich foci by duplex RNAs, we tested whether it would be possible to improve potency by redesigning the RNA sequences. We evaluated the effect of adding mismatches outside the central region of the duplex (Table 1). Duplexes R8, R9, R10, and R11 were all potent inhibitors of G-rich foci formation (Figure 3A). We also examined duplexes R12, R13, and R14 that preserved seed-sequence complementarity toward the C-rich sequence. R13 and R14 had a 3′ U substitution to bias loading recognition toward the C-rich target. We found that each of these RNAs were effective inhibitors of C-rich foci (Figure 3B). We did not observe a correlation between reduced T_m value and efficacy, presumably because even the lowest mismatch-containing duplex possessed a value greater than 70°C.

To further evaluate inhibition of foci by RNA duplexes, we examined inhibition as a function of time. Significant reductions in sense G-rich and antisense C-rich foci were observed 8–12 days after transfection (Figure 3C). The fibroblast cells divide every 2–3 days, and reduced efficacy is similar to that typically observed in our laboratory when using duplex RNAs.

Efficient gene silencing by duplex RNAs requires complementarity between the guide strand and the RNA target at bases 2 through 8, a region known as the seed sequence. To begin to test the mechanism of anti-GGGGCC/CCCCGG duplex RNAs, we introduced mismatches into the seed sequence. We tested noncomplementary control RNAs C1, C7, and C8, seed mismatched RNAs C2, C3, C5, C6, and scrambled duplex C4. We observed that duplexes with altered seed sequences did not inhibit sense or antisense foci (Figures 4A and 4B). Preventing foci inhibition by disrupting seed-sequence complementarity is consistent with function through the RNAi machinery. We tested other noncomplementary or scrambled duplex RNAs, and these also did not affect foci formation, also consistent with inhibition being an "on-target" effect through direct Watson-Crick interaction with the expanded GGGGCC or CCCCGG targets.

Because AGO2 is a key component in RNAi (Liu et al., 2004), we used RNA immunoprecipitation (RIP) to determine whether our repeat-targeted RNAs were recruiting AGO2 for recognition of GGGGCC repeats within *C9orf72* intronic RNA. Our RIP experiment employed an antibody that recognizes endogenously expressed AGO2, and detection employed PCR primers designed to amplify intron 1 RNA downstream of the GGGGCC repeat. RIP revealed that addition of RNA R3 promoted association of AGO2 with intronic *C9orf72* RNA (Figures 4C and 4D). Sequencing confirmed that the RIP product was derived from *C9orf72* intronic RNA (Figure 4E).

Introduction of central mismatches relative to a target RNA is predicted to eliminate cleavage by AGO2 (Wang et al., 2008).

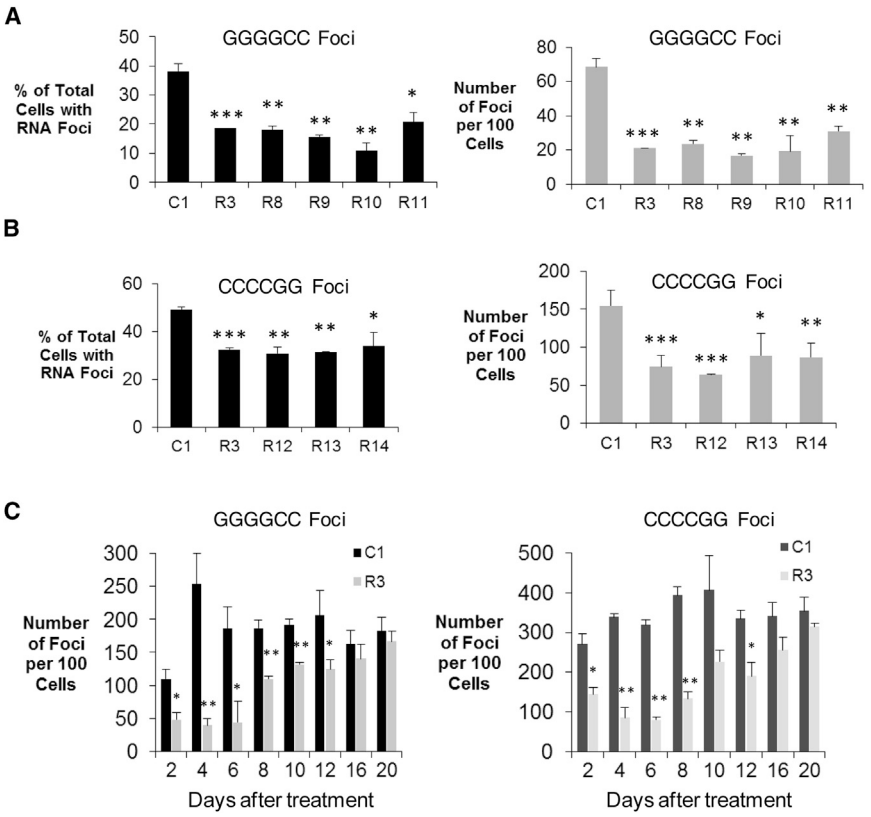

Figure 3. Optimizing Inhibition of GGGGCC and CCCCGG Foci

(A) Inhibition of GGGGCC foci by optimized duplex RNAs.

(B) Inhibition of optimized CCCCGG foci by optimized duplex RNAs.

(C) Time course showing effect of duplex RNA on foci number and foci per cell.

Error bars represent SEM. *p < 0.05; **p < 0.01; ***p < 0.001 compared with control C1. At least 100 cells were analyzed for each experiment.

To determine the effect of these mismatches on *C9orf72* mRNA and intron 1 RNA, we examined transcript levels by qPCR. Levels of both the mRNA (Figure 4F) and intron 1 RNA (Figure 4G) were unchanged after treatment with various mismatch-containing repeat-targeted duplex RNAs. By contrast, fully complementary positive control duplex E1 efficiently silenced *C9orf72* expression. These results are consistent with a mechanism of action that does not require slicer activity and with the conclusion that inhibiting foci formation does not require RNAi-mediated silencing of *C9orf72*. RNA levels remain constant, and inhibition of foci is most likely due to binding of the RNA duplexes to the C-rich or G-rich transcripts.

SIGNIFICANCE

The discovery of a linkage between the GGGGCC repeat expansion within intronic *C9orf72* RNA and ALS/FTD was important because it is the most common inherited marker for these two diseases. There are currently no curative treatments for either disease, and agents that could slow disease progress would help satisfy a major unmet medical need. Application of anti-GGGGCC or anti-CCCCGG RNAs reduced detection of RNA foci by 40%–60%. The sense and antisense transcripts derived from the mutant expanded hexanucleotide repeat have the potential to contribute to disease. Our data suggest that duplex RNAs can be designed such that a single RNA can block both strands. While we did not test all duplexes for inhibition of both G-rich and C-rich foci, the duplexes are similar and we would expect similar outcomes.

It is difficult to directly compare this work with previous studies (Donnelly et al., 2013; Lagier-Tourenne et al., 2013) because the antisense oligonucleotides used are not commercially available. Even without a direct comparison, there are two clear advantages of our approach. The first is that our duplexes function through the RNAi pathway, rather than the using ASOs that act by recruiting RNase H. This opens up the prospect of using a silencing mechanism that exploits the high potency of RNAi. The second advantage is that our duplex RNAs inhibit both G-rich and C-rich foci. For example, one of the papers (Lagier-Tourenne et al., 2013) tested ASOs for C-rich foci and found no inhibition.

RNAi is a commonly used technique, but highly C/G-rich sequences are often thought to be off limits because of the high stability of structure formation. Our results demonstrate that sequences composed entirely of C and G can be recognized by manipulating the presence of mismatched bases to tailor affinity. This finding widens the pool of cellular RNA sequences that can be accessed by RNAi and increases the potential to control gene expression. C9orf72 foci are located in human cell nuclei, and their inhibition by duplex RNAs provides another example of the power of nuclear RNAi to control gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Small Interfering RNA Transfection

Mutant expanded repeat C9orf72 patient-derived fibroblast cell line is a gift from Dr. John Ravits of UCSD. The fibroblasts were maintained at 37°C and 5% CO_2 in Eagle's minimum essential medium (MEM) (Sigma, M4655) supplemented with 15% heat-inactivated fetal bovine serum (Sigma) and 0.5% MEM nonessential amino acids (Sigma).

Small interfering RNAs (siRNAs) were obtained from IDT. Double-stranded RNAs were prepared by annealing the two RNA strands in 2.5× PBS solutions. siRNAs were transfected into cells with lipid RNAiMAX (Life Technologies) as previously described (Hu et al., 2010). For qPCR analysis, cells were plated at a density of 80,000 per well of a 6-well plate 48 hr before transfection. Cells were typically harvested 2 days after transfection.

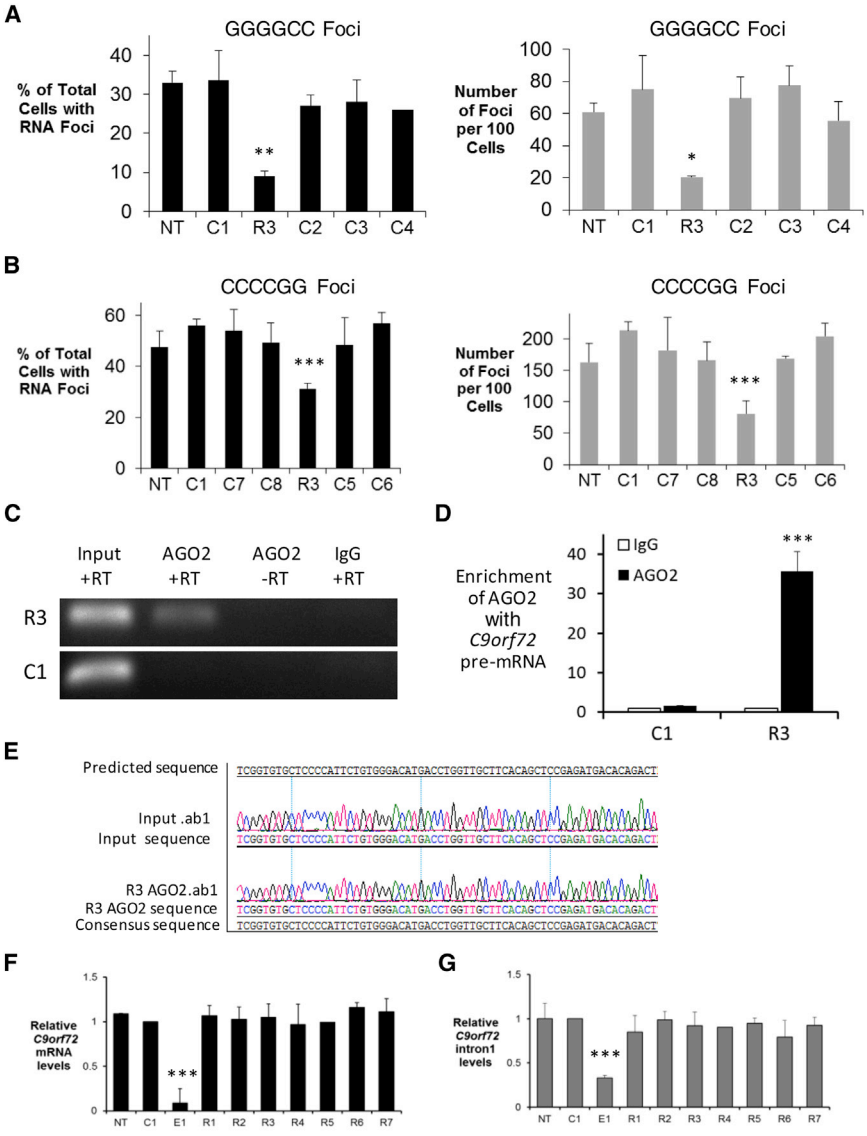

Figure 4. Involvement of RNAi

(A) Inhibition of GGGGCC foci is seed-sequence dependent.

(B) Inhibition of CCCCGG foci is seed-sequence dependent. At least 100 cells were analyzed for each experiment in (A) and (B).

(C) RNA immunoprecipitation demonstrates recruitment of AGO2 to *C9orf72* intronic RNA upon addition of duplex RNA.

(D) Quantitation of enrichment of AGO2 on *C9orf72* intronic RNA.

(E) Sequencing of amplified product from RIP confirms identity as *C9orf72* intronic RNA.

(F) qPCR showing effect of duplex RNAs on *C9orf72* mRNA levels.

(G) qPCR showing effect of duplex RNAs on levels of *C9orf72* intron 1 RNA.

Error bars represent SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ relative to treatment with noncomplementary control RNA C1. NT, no treatment.

qPCR Analysis

Total RNA from fibroblast cells was extracted using TRIzol agent (Invitrogen). Samples were then treated with DNase I at 25°C for 10 min. Reverse transcription reactions were done using a High-Capacity Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol.

C9orf72 expression was analyzed by qPCR on a 7500 real-time PCR system (Applied Biosystems) using iTaq SYBR Green Supermix (Bio-Rad). Data were normalized relative to levels of *GAPDH* mRNA. Primers specific for *C9orf72* mRNA of all three variants are as follows: F 5′-AGA AGG CAC AGA GAG AAT GGA A-3′; R 5′-TCA TCA TCA TTG AGT ACT GTA TCA GC-3′. Primers for *C9orf72* intron 1: F 5′-ACG CCT GCA CAA TTT CAG CCC AA-3′; R 5′-CAA GTC TGT GTC ATC TCG GAG CTG-3′. Primers for *GAPDH*: F 5′-GTC ATC AAT GGA AAT CCC ATC AC-3′; R 5′-TTC TCC ATG GTG GTG AAG AC-3′.

RNA Immunoprecipitation

RIP was performed as previously described with optimization (Hu et al., 2012). Fibroblasts were seeded in 150-cm^2 dishes (1,400,000/dish), and were transfected with duplex RNAs in the next day. Cells (~90% confluency) were harvested in 48 hr. Detached cells were lysed in a hypotonic lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40) with a volume about three times the cell pellet size, gently pipetted up and down to break up the pellet, then set on ice for 5 min, followed by centrifugation (500 × *g*) for 5 min to collect the nuclei. The pellet was washed again with hypotonic buffer for 5 min to remove all cytoplasmic components. The nuclei were lysed with nuclear lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM KCl, 3 mM $MgCl_2$, 0.5% NP-40, protease inhibitor [EDTA-free; Roche] and RNase inhibitor [Promega; 50 U/ml final]). The mixture was sonicated on ice for 20 s, three times, with 20% power. After centrifugation, the supernatant was isolated and stored at −80°C.

60 μl of Protein A/G agarose Plus beads and 300 μl of nuclear lysate were incubated with 5 μl of anti-AGO2 antibody (015-22031, Wako), or mouse anti-immunoglobulin G antibody in 1× PBS (pH 7.4) at 4°C with gentle agitation overnight. The beads were washed with nuclear lysis buffer twice, then eluted with elution buffer (1% SDS, 0.1 M $NaHCO_3$ and RNase inhibitor). After proteinase K treatment, and RNA extraction and precipitation, samples were treated with recombinant DNase I, followed by reverse transcription. The enrichment of *C9orf72* intron 1 levels were quantified by qPCR.

RNA FISH and Imaging

RNA FISH was performed following a Biosearch protocol with minor modifications. Fibroblast cells were plated at a density of 10,000/well into a Lab-Tek 8-well chambered coverglass slides. After 1 day, siRNA/lipid complex was added at 50 nM final concentration. 48 hr after transfection, cells were fixed with 4% formaldehyde in 1× PBS and permeabilized in 70% ethanol at 4°C overnight. Cells were washed with wash buffer (10% formamide in 2× saline sodium citrate [SSC]) for 5 min, then incubated with pre-hybridization buffer (40% formamide in 2× SSC) at 60°C for 20 min. A $(CCCCGG)_4$-Cy3 DNA probe or a $(GGGGCC)_{3.3}$-Cy3 probe in hybridization buffer (100 mg/ml dextran sulfate and 40% formamide in 2× SSC) was added. The slide was placed in a humidified chamber and incubated in the dark at 37°C overnight. On the next day, cells were washed twice with wash buffer at 37°C, then stained with mounting media with DAPI (Vector Labs, H-1500).

Cells were imaged at 60× magnification using a Widefield Deltavision microscope. Images were processed by blind deconvolution with AutoQuant X3.

Visualization of RNA foci were made using ImageJ. For quantification, at least 20 pictures were taken from randomly chosen microscopic fields, containing 100–300 cells for each treatment. Counting of foci was performed by different investigators. All data were generated by at least three independent experiments.

AUTHOR CONTRIBUTIONS

J.H. and J.L. designed and executed experiments. K.T.G. initiated the project. D.R.C. supervised experiments and wrote the manuscript.

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