

# Tolerated wobble mutations in siRNAs decrease specificity, but can enhance activity *in vivo*

Torgeir Holen<sup>1,2,\*</sup>, Svein Erik Moe<sup>1</sup>, Jan Gunnar Sørnbø<sup>1</sup>, Trine J. Meza<sup>2</sup>,  
Ole Petter Ottersen<sup>1</sup> and Arne Klungland<sup>2,3</sup>

<sup>1</sup>Centre for Molecular Biology and Neuroscience (CMBN), and Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway, <sup>2</sup>Institute of Medical Microbiology, The National Hospital, University of Oslo, 0027 Oslo, Norway and <sup>3</sup> Department of Nutrition, Institute for Basic Medical Sciences, University of Oslo, PO Box 1046, Blindern, N-0316 Oslo, Norway

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## ABSTRACT

**RNA interference (RNAi) has become an invaluable tool for functional genomics. A critical use of this tool depends on an understanding of the factors that determine the specificity and activity of the active agent, small interfering RNA (siRNA). Several studies have concluded that tolerance of mutations can be considerable and hence lead to off-target effects. In this study, we have investigated *in vivo* the toleration of wobble (G:U) mutations in high activity siRNAs against Flap Endonuclease 1 (Fen1) and Aquaporin-4 (Aqp4). Mutations in the central part of the antisense strand caused a pronounced decrease in activity, while mutations in the 5' and 3' ends were tolerated very well. Furthermore, based on analysis of nine different mutated siRNAs with widely differing intrinsic activities, we conclude that siRNA activity can be significantly enhanced by wobble mutations (relative to mRNA), in the 5' terminal of the antisense strand. These findings should facilitate design of active siRNAs where the target mRNA offers limited choice of siRNA positions.**

## INTRODUCTION

Downregulation of mRNA transcripts by RNA interference (RNAi) and short interfering RNA (siRNA) (1) has been adopted as an invaluable research tool and holds promise as a novel principle for drug development (2). Until recently, the active agent, siRNA, has been considered as exquisitely specific (3,4). However, there is now a growing appreciation that the technique has limitations with regard to siRNA specificity (5,6) and that better procedures are needed for the identification of active siRNA positions (5,7–9).

Haley and Zamore demonstrated that in *Drosophila* extracts the RISC enzyme could tolerate many more mutations in the 3' end of the antisense strand than in the 5' end (10). Together with similar observations from microRNA investigations (11–13), and microarray investigations of off-target effects (14), this has given rise to a consensus view in the field that siRNA-target recognition is initiated (or 'seeded') by a short section of the 5' end of the antisense strand. However, the relevance of these studies for *in vivo* RNAi in mammalian cells is in some doubt. Microarray analyses and other approaches that have been used to evaluate the specificity of RNA interference have led to divergent conclusions by different groups (14–17). It is still unclear whether the siRNA and microRNA mechanisms are identical at the mechanistic level, although there are great similarities. The relevance of mutation studies performed *in vitro* in *Drosophila* lysates to the *in vivo* situation in mammals remains to be established. In particular, the mutations that were shown to be tolerated by Haley and Zamore slowed down the process considerably (from 80% target depletion in 15 s with perfect complementarity to 24 h for obtaining activity with a strand with multiple mutations at the 3' end). This raises the question whether this biochemical *in vitro* activity is irrelevant *in vivo*, where the speed of mRNA production and RNAi mRNA depletion has been shown to be finely balanced (5,18).

However, it is clear that some mutations and chemical modifications in a siRNA can be well tolerated *in vivo* (6,19). Furthermore, various siRNAs have the ability to cross-react with targets of partial sequence similarity (11) and might even non-specifically stimulate or repress hundreds of genes involved in cellular functions (12). The mechanisms of these effects are unknown, as none of the microarray experiments that exposed off-target effects have been followed up with publications validating the direct interactions between RNAi/siRNA and the target mRNA.

\*To whom correspondence should be addressed. Tel: +47 2285 1294; Fax: +47 2285 1488; Email: torgeir.holen@medisin.uio.no.

Bioinformatic approaches are being used for the design of specific and highly active siRNAs (20,21). Such approaches require knowledge of the mutation tolerance of different positions in the siRNA. Our previous studies have established that mismatches can be tolerated in certain cases (5). Furthermore, the position of the mismatch in the siRNA affects the severity of silencing loss (6).

Zamore and coworkers have performed a groundbreaking study on the architecture of the siRNA duplex in the RISC complex of cell-free *Drosophila* embryo lysates (22). The strand to be removed from the RISC complex was found to be selected on the basis of the sequence composition in the first 5 and last 5 bp of the siRNA duplex. Several algorithms for effective siRNA design have also focused on these sets of basepairs (20). Other explanation models for inactive siRNAs exist, among them mRNA restraints (23), and RISC enzyme restoration after cleavage (18). Furthermore, it is possible that the mRNA nuclease, which may be identical to Argonaute2 (24), might have preferences for certain nucleotides at the cleavage point, as is known to be the case for other RNA endonucleases (25).

In this work, we systematically introduced wobble-mutations in the siRNA, leading to G:U interactions between the siRNA antisense strand and the mRNA target. The results show that in many cases such wobble mutations are very well tolerated. In other cases there is less toleration, in particular if wobble mutations are made in central regions of the siRNA. Interestingly, wobble mutations in the terminal nucleotide of a series of low and high activity siRNAs increased the silencing capacity. This is the first demonstration of such an enhancing effect in mammalian cells *in vivo*. Our findings should offer opportunities for the development of high activity siRNAs even in cases where there are limited numbers of available positions.

## MATERIALS AND METHODS

### Preparation of siRNAs

The 21mer single-stranded RNAs were synthesized at 20 nmol scale by Ambion (Austin, TX). The RNA strands were resuspended in nuclease-free water (Ambion), and the concentration of the strands was verified by spectrometry using NanoDrop (Saveen Werner).

Annealing of the complementary strands was performed by mixing equal amounts of RNA followed by heating of the mixture to 65°C for 5 min and cooling to room temperature >5 min. Successful annealing of siRNA was verified by non-denaturing 12% PAGE, staining with SYBRgold (Invitrogen) and visualization by fluorescent scanning on a Typhoon 9410 (Amersham Biosciences).

### Cell culture and transfections

HeLa cells were maintained in DMEM supplemented with 10% foetal calf serum (FCS) (Gibco BRL). The cells were passaged at sub-confluence and  $2.5 \times 10^5$  cells plated 24 h before transfection in 6-well plates. Transfections were performed using 1.44 µg siRNA per well and 3.6 µl Lipofectamine2000 (Invitrogen), and thus a ratio of 2.5 of Lipofectamine2000 to RNA (in microliters of Lipofectamine2000 to micrograms of RNA), in a total volume of

200 µl, with a complexation time of 30 min. Thereafter, the complexes were diluted to 1 ml and added onto cells. The transfection period was 4 h, after which the transfection mixture was replaced with medium.

### Western blot analysis

Cells were harvested by trypsination and washed once in serum-containing DMEM medium, and once in PBS and the cell pellet was resuspended in a homogenization buffer [10 mM NaPi pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% SDS, COMPLETE Protease inhibitor cocktail (Roche 11 697 498 001), used according to the manufacturer's protocol]. The lysate was sonicated six times for a duration of 1 s. The lysate was then spun for 10 min at 15 000 g and the supernatant transferred to a new microcentrifuge tube. Total protein was determined by DC protein assay (Bio-Rad) based on the Lowry assay. Western blot analysis was performed using XCell SureLock Mini-Cell and XCell II Blot Module Kit (Invitrogen): equal amounts of protein were loaded on a 1.0 mm pre-cast 10% Bis-Tris gel (Invitrogen) and separated by electrophoresis at 200 V for 50 min, and thereafter electroblotted onto PVDF membranes (Invitrogen) at 30 V for 60 min. Equal loading amounts were verified after blotting by Coomassie G-250 (Bio-Rad) staining of the gels. Membranes were blocked with 5% non-fat dried milk powder (AppliChem) in TBST (0.05% Tween-20) for 60 min. Incubation with primary antibodies (rabbit-anti-Fen1 from Bethyl Laboratories, BL587, at 0.05 µg/ml, or with mouse-anti-GAPDH from Abcam ab9484, at 0.2 µg/ml) was performed in 2.5% non-fat dried milk powder and TBST at room temperature for 3 h. Membranes were washed three times for 10 min in TBST and then incubated for 1 h with alkaline-phosphatase linked anti-rabbit (Amersham RPN 5783) or alkaline-phosphatase linked anti-mouse (Amersham RPN 5781) diluted 1:10 000 in 1.25% non-fat dried milk powder and TBST. Membranes were then washed three times for 10 min in TBST. Signals were visualized by ECF (Amersham) according to the manufacturer's protocol. Membranes were scanned using a Typhoon 9410 scanner and images processed with ImageQuant TL.

### Cotransfection assays

The cotransfection assays were performed using Lipofectamine2000 (Invitrogen) in triplicate in 12-well plates with 100 ng plasmid DNA and 220 ng siRNA per well. The nucleic acids were mixed with Lipofectamine2000 at a ratio of 2.5:1 (µl Lipofectamine2000 to µg of nucleic acids) in serum-free medium. The complexation process was allowed to proceed for 30 min at room temperature before being diluted. The mixture was then added onto cells washed twice with PBS and once with serum-free medium. Luciferase activity levels were measured in 20 µl of cell lysate 24 h after transfection using the Dual Luciferase Assay (Promega) and a Turner 20/20 luminometer (Turner Biosystems).

### Isolation of mRNA and northern blot hybridizations

The mRNA was isolated at various times after transfection using Dynabeads oligo(dT)<sub>25</sub> (Dynal) and then separated by gel electrophoresis, 1.5% agarose gels containing 0.8 M formaldehyde, for 3 h at 50 V. The agarose gel was then washed two

times for 15 min in distilled water, before the RNA was blotted onto pre-wetted BrightStar-Plus nylon membranes (Ambion). The Fen1 mRNA was detected using a radioactive probe corresponding to the protein coding region (373–1515, relative to Fen1 mRNA accession number NM\_004111), using random priming with Rediprime II (Amersham Biosciences) and P-32 alpha-CTP (Amersham Biosciences). Hybridization was performed overnight using ExpressHyb (BD Biosciences), with four subsequent washes at room temperature and at 50°C with Wash Buffer I (2× SSC + 0.05% SDS), and with Wash Buffer II (0.2× SSC + 0.1% SDS), respectively. The radioactive signals were visualized with a Phosphor Screen and a Typhoon 9410 imager (Amersham Biosciences). Quantification of relative signal strength was performed with the ImageQuant software (Nonlinear Dynamics, Amersham Biosciences).

### Construction of reporter plasmids

The rat pra-Aqp4-Luc and mouse pmu-Aqp4-Luc reporter plasmids were constructed by cloning a fragment from a mouse cDNA corresponding to position 58–1023 in GenBank NM\_009700, and a rat cDNA corresponding to position 4–956 in GenBank RM\_012825, respectively, to the luciferase plasmid pGL3-Control (Promega). The sequence similarity of the two constructs in the Aqp4 cDNA region is 92% between rat and mouse sequence. The full sequence of the two plasmid constructs have been submitted to GenBank [accession numbers AY785357 (pmuAqp4-Luc) and AY785358 (praAqp4-Luc)].

## RESULTS

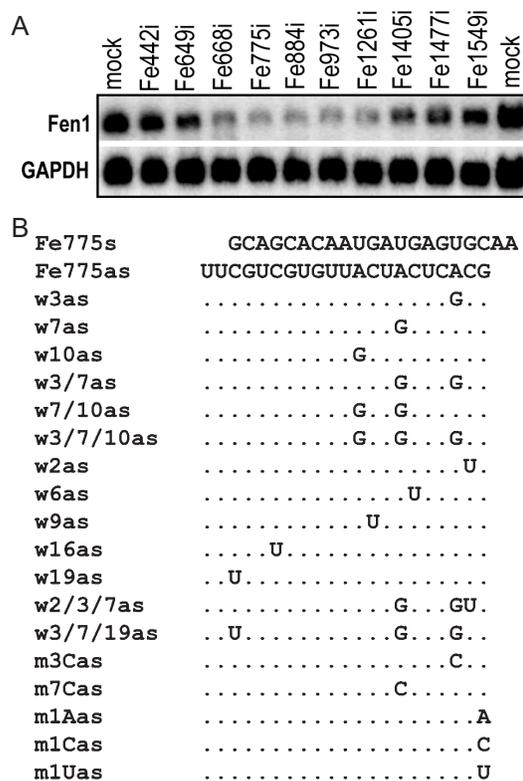
### Tolerance of wobble mutations

As part of an investigation of the biological relevance of Fen1, the gene encoding the DNA repair and replication Flap Endonuclease 1 (26), we screened 10 different siRNAs (Supplementary Table 1) and identified several candidates that depleted >70% of target mRNAs at 24 h post-transfection (T. Holen, unpublished data and Figure 1A). To identify the superior of these good candidates, we measured the activity as early as 12 h post-transfection (data not shown). One particular siRNA, Fe775i, caused maximum downregulation (~80%) at this time point.

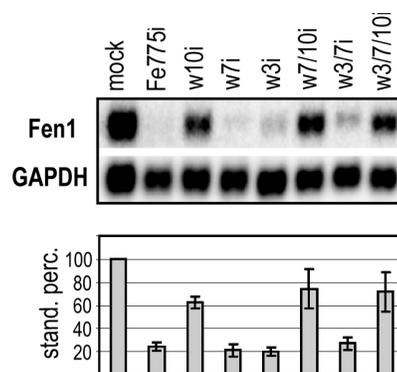
Fe775i was chosen for investigation of G:U wobble mutations. Wobble mutations can be directed to an A:U pair or a C:G pair. These conceptually mild mutations entail the loss of one or two hydrogen bonds, respectively, and retain the spatial geometry of the double helix. In contrast, inversions of G:C pairs (leading to a loss of three hydrogen bonds) are hard mutations that compromise the helical geometry (5,6). The mutated siRNAs discussed below are shown in Figure 1B.

The first series of mild wobble mutations targeted U:A pairs (mRNA:siRNA) at positions 3, 7 and 10 (Figure 1B). The results were striking. Whereas no significant loss of activity was found after wobble mutations at position 7 and 3, a single wobble mutation in position 10 resulted in a dramatic loss of activity (Figure 2). This is close to the cleavage point of the RNAi nuclease (27).

These single wobble mutations were compared with two siRNAs with double-wobble mutation (w3/7i and w7/10i)

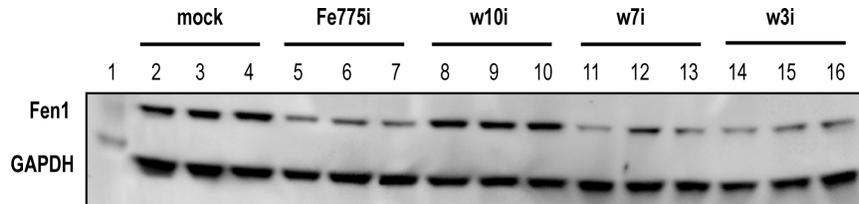


**Figure 1.** Impact of wobble mutations and hard mutations on the silencing activity of the siRNA Fe775i. (A) Northern analysis of a series of siRNAs (complete sequence of all siRNAs are presented in Supplementary Table 1) against Fen1 (upper panel) at 24 h post-transfection. The housekeeping gene GAPDH is used as internal standard (lower panel) to normalize Fen1 expression. The individual siRNAs used are indicated on top of the gel and the number represents the complementary location in the Fen1 mRNA sequence. (B) Summary of Fe775i mutations. Changes from the Fe775i wild-type sequence are indicated. Full sequences of all 18 mutated siRNAs are available in Supplementary data, Table 1.



**Figure 2.** Tolerance of A:U to G:U wobble mutations. One representative northern blot (above) is shown. Silencing of Fen1 expression by siRNA was quantified by ImageQuant (Typhoon 9410), standardized to a fraction of GAPDH expression and standard deviation calculated from three independent experiments (below).

and one siRNA with a triple-wobble mutation (w3/7/10i) (Figure 1B). Consistent with the single-wobble data, w3/7i only showed slight loss of activity, while the two siRNAs that included a mutation in position 10 (w7/10i and w3/7/10i) demonstrated a considerable activity loss (Figure 2).



**Figure 3.** Western blot analysis of Fen1 48 h after transfection. Shown are the western signals, in triplicate, from Fen1 and the control GAPDH. The band in lane 1 is a 40 kDa size marker.

### Verification of silencing capacity of mutated siRNA by western blot

To verify the silencing capacity of the mutated siRNAs w3i and w7i in an independent assay, we tested these siRNAs for silencing of protein expression. A time-series of western blot analysis for FEN1 protein demonstrated that full silencing was established at 48 h post-transfection (data not shown). This result also indicates a half-life of the FEN1 protein of <24 h. At 48 h post-transfection, both w3i and w7i revealed silencing comparable with the wild-type Fe775i sequence, while w10i showed almost no activity (Figure 3). Thus the tolerance of wobble-mutated siRNAs could be verified at the protein level.

### Effects of moderate wobble mutations

Mutating an A to a G in the siRNA antisense strand leads to the loss of only one hydrogen bond in the siRNA:mRNA interaction. An intermediate type of mutation is formed by mutating G:C-pairs (mRNA:siRNA), replacing three-hydrogen bond G:C pairs with single-hydrogen bond G:U pairs (Figure 1B). The mutations in the central part, that is, in the second and third quartile of the FE775i antisense strand, w9i and w16i, caused sharp drops in activity similar to w10i (Figure 4). Again, wobble mutations at the 5' end of the antisense (w6i and w2i) and the 3' end (w19i) were well tolerated (Figure 4).

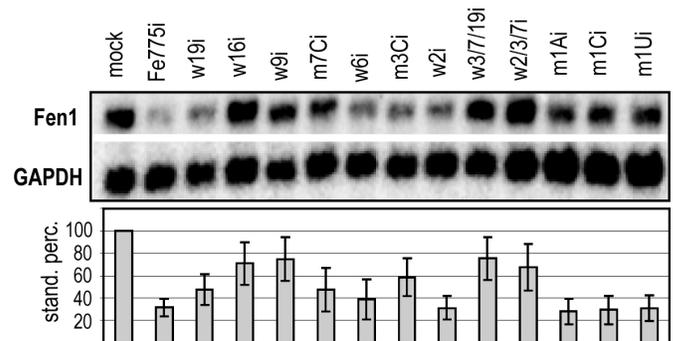
### Effects of hard mutations in positions tolerating wobble mutations

Introduction of hard mutations (resulting in C:U pairs) in position 3 and 7 led to a pronounced loss of activity, compared with the tolerance for wobble mutations in these positions (Figure 4). Interestingly, hard mutations to the terminal nucleotide of the antisense strand, m1Ai, m1Ci and m1Ui, were on the other hand well tolerated (Figure 4).

Thus, different positions in siRNAs tolerate different mutations to different degrees. This phenomenon might reflect the enzymatic and structural characteristics of the RISC complex. Statistical studies of large data-sets of siRNA experiments have revealed that high-activity siRNA duplexes seem on average to have lower pairing energy in the 5' end of the antisense strands than have corresponding siRNA duplexes with medium or low activity (8,9). The observed effect of our wobble mutation (which reduces the pairing energy) is consistent with these statistical studies, and represents the first direct experimental verification of this phenomenon *in vivo*.

### Triple mutations represent a limit to tolerance

The limit of tolerance to siRNA mutations remains to be established and incorporated into siRNA design algorithms.



**Figure 4.** Tolerance of G:C to G:U wobble mutations and hard mutations. One representative northern blot and quantification for three independent experiments (as above) for Fen1 are presented and the individual siRNA used for targeting indicated above. Silencing of Fen1 was quantified as above.

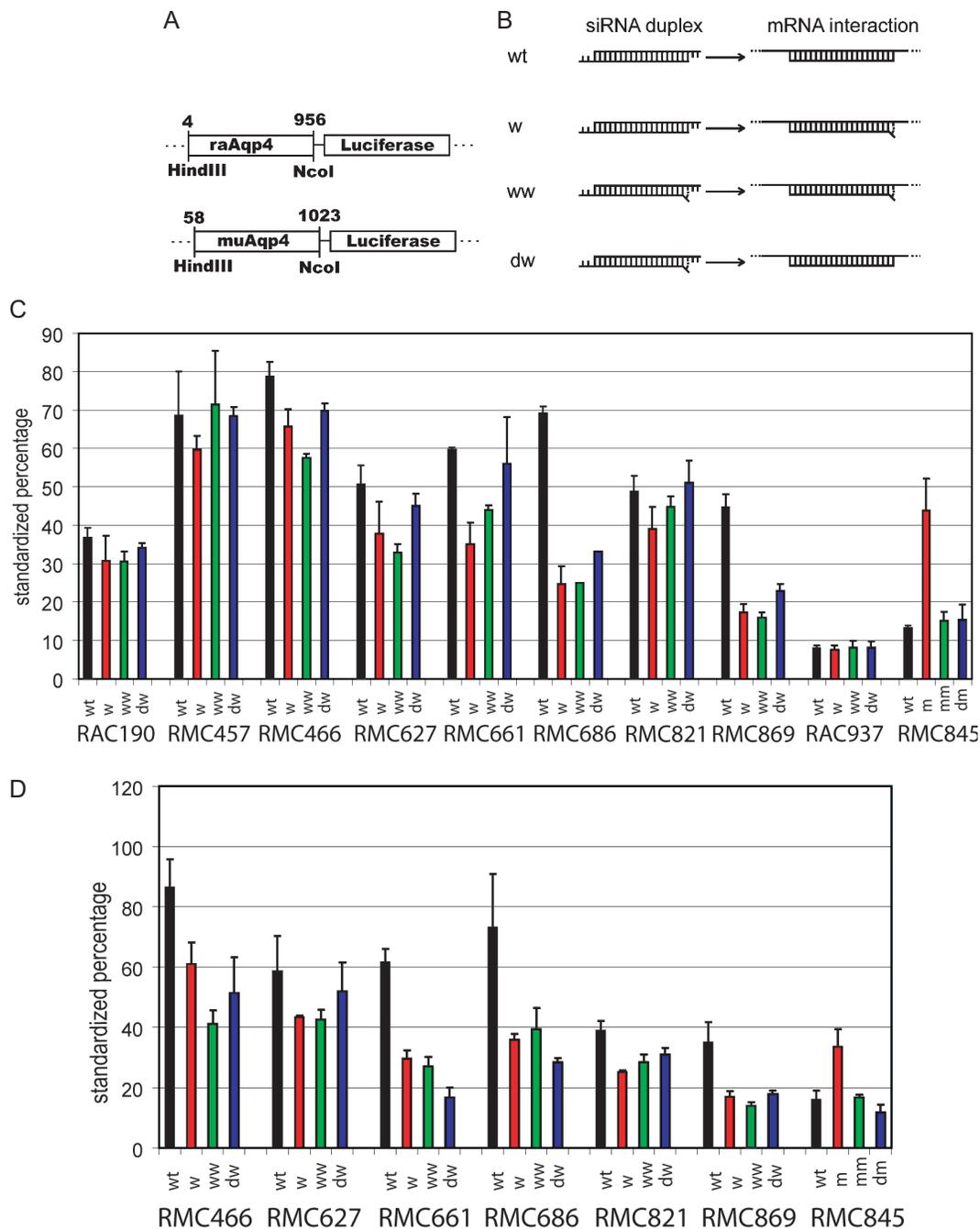
Triple-mutations seemed to exhaust most of the activity of Fe775i (Figure 2), even when combining positions with good tolerance (w3/7/19i and w2/3/7i) (Figure 4). That siRNAs tolerate a maximum of two mismatches before they lose activity may be a property common to all or most siRNAs. If so, specific siRNAs exist for most mRNAs, as a previous bioinformatics study demonstrated a specificity window between 3 and 5 mismatches for most genes (20).

### Wobble mutations to the 5' terminal antisense position can enhance siRNA activity

Our findings of weak but significant stimulatory effect of wobble mutations (Figure 2) and high tolerance of hard mutations to the antisense 5' terminal position (Figure 4) raised the possibility that wobbles in the 5' end of the antisense strand actually could enhance siRNA activity.

In order to test this idea, and to further expand the scope of our study, we decided to mutate the terminal 5' nucleotide in a series of different siRNAs targeting Aquaporin-4 (Aqp4), a water transport protein, the disruption of which protects against the development of brain edema (28). From a set of 31 different siRNAs tested against Aqp4 (T. Holen, unpublished data), we selected siRNAs that had a 5' C in the antisense strand and a range of different intrinsic activities, from very low (RMC466, RMC627i, RMC661i and RMC686i), through intermediate (RAC190i, RAC427i, RMC821i and RMC869i) to very high activity (RAC937i). Each of these siRNAs was wobble-mutated in the 5' terminal nucleotide of the antisense strand (Figure 5B).

The results, using a cotransfection assay of siRNAs and luciferase-fusion reporter constructs (Figure 5A), were



**Figure 5.** Enhancement of siRNA activity due to wobble mutations in a series of nine different siRNAs against Aqp4. **(A)** A diagram of the fusion-luciferase constructs. Shown are the inserted cDNA of mouse-Aqp4 (muAqp4) and rat-Aqp4 (raAqp4) in the pGL-Control plasmid. **(B)** Summary of siRNA duplex annealing and interaction with mRNA. The wobble mutation positions are indicated. All siRNAs were mutated in the 5' terminal nucleotide on the antisense strand (C-to-U) and on the complementary nucleotide (G-to-A). The four RNA oligo strands from the mutated version and the wild-type versions were then annealed in the four possible combinations possible: wild-type sense with antisense (wt), wobble sense with antisense (w), wild-type sense annealed with wobble antisense siRNA (ww) and wobble sense with wild-type antisense (dw). The control siRNA RMC845i was mutated (U-to-G) in the 5' terminal of the antisense strand, causing a G:A mutation versus the mRNA). See Supplementary Table 1 for a full list of all 40 siRNA duplexes. **(C)** Cotransfection assay. The rat pra-Aqp4-Luc plasmid was cotransfected with the different siRNA versions: wild-type (wt, black bars), wobble (w, red bars), wobble in both duplex and mRNA (ww, green bars) and wobble in duplex (dw, blue bars). Each siRNA was tested at least nine times, in three different experiments. Shown is a representative experiment, with standard deviation, with luciferase signal standardized to a percentage fraction of an inactive control siRNA. **(D)** Cotransfection assay. The mouse Aqp4 plasmid pmu-Aqp4-Luc was cotransfected with the indicated siRNAs (RMC-siRNAs), all of which have 100% identity (for the wild-type) to mouse cDNA.

unambiguous. In all cases, a wobble mutation (C-to-U, resulting in U:G wobble) in the 5' terminal siRNA:mRNA interaction (w-version) improved activity compared with the wild-type siRNA (wt-version) (Figure 5C).

Zamore's group recently demonstrated, in cell-free assays in *Drosophila*, that mutations at the ends of a siRNA duplex can skew the loading of the active strand into the RISC enzyme complex (22). To take this finding into consideration, and to

assess the *in vivo* response, each RNA strand from the new wobble siRNAs was annealed to the corresponding strand of the wild-type siRNA, creating two new siRNAs with mismatches in the duplex. One of these siRNAs had a wobble only in the siRNA duplex (dw) while the other also had a wobble against the mRNA target (ww) (Figure 5B).

This strategy should allow for individual evaluation of the contribution from wobbles in the siRNA duplex and the wobbles in the siRNA:mRNA interaction. In three cases (RMC661 dw, RMC686 dw and RMC869 dw), a wobble in the siRNA duplex (dw) improved the silencing activity (Figure 5C). In several other cases (RAC190 dw, RAC457 dw, RMC627 dw and RMC821 dw), there was little or no effect. In all cases, the siRNA:mRNA wobble (w) resulted in improved activities as compared with the dw. Thus, there is a significant, and independent, siRNA:mRNA wobble improvement effect.

We further expanded our study by including siRNAs characterized with a wobble both in the duplex and against the mRNA (ww, Figure 5B). Consistent with wobble versions and duplex wobble versions, these ww-versions were in some cases stronger than wobble versions and in other cases weaker, again arguing for two independent effects (Figure 5C).

A hard mutation at the ultimate 5' antisense end of RMC845i (wt) was included to further test the alternative explanation of duplex mismatch stimulation. The A:G mismatch introduced at the siRNA:mRNA interaction (RMC845 m) resulted in a considerable loss of activity (Figure 5C). The corresponding mismatch in the siRNA duplex (dm) was tolerated without gain or loss of activity. This supports our conclusion that duplex mismatches do not consistently improve an siRNA.

However, the version with a mismatch both in the duplex and against the mRNA target (RMC845 mm), was improved compared with the mutated version (RMC845 m) (Figure 5C), thus balancing the damage caused by the hard mutation. We note that the wobble improvement of our best candidate RAC937i was very modest, and speculate that already superior siRNA candidates might not be further improved by this approach.

The murine *Aqp4* gene has 92% sequence identity to the siRNA target area of rat *Aqp4*. This allowed us to further test wobble-stimulation in another experimental system as six of the siRNAs had perfect complementarity to the mouse sequence. These six siRNAs were tested against a mouse pmuAqp4-Luc construct. Again, the terminal siRNA:mRNA wobble improved the siRNAs in all experiments (Figure 5D). Significantly, wobble mutations (w, ww and dw) of one rather weak siRNA candidate (RMC686i), and of one intermediate candidate (RMC869i), improved their activity to the level of our best mouse siRNA candidate RMC845i.

## DISCUSSION

RNAi shows tremendous potential as a tool for targeted gene silencing and this mechanism was initially considered to be sequence specific (17). Recent studies have questioned the requirement of absolute sequence complementarity between the siRNA and the target mRNA. These studies have been based on the introduction of a series of mutations in the siRNAs (5,6) or on genome-wide transcription profiling by

microarray analysis (14,16). However, the microarray studies have not been entirely consistent (15,17), calling for further investigations of the mechanisms underlying tolerance of siRNA mutations.

Risk assessment for off-target siRNA silencing has been evaluated by computational tools, with respect to commonly used siRNAs and transcriptome data in humans (15) and in *Caenorhabditis elegans* and *Schizosaccharomyces pombe* (29). That mismatches between the siRNA and the mRNA target can be tolerated by the RNAi machinery is now generally accepted. Our present study shows that wobble mutations in the central part of the antisense strand cause a pronounced decrease in activity, while mutations in the 5' and 3' ends are well tolerated. This has implication for algorithms used in design of specific and highly active siRNAs. Notably, the present study adds to previous evidence suggesting that there is no absolute specificity of siRNAs. This implies that general rules for assessing siRNA activity may have to be abandoned in favor of a case-to-case analysis of individual siRNAs.

A major finding in the present study is that wobble mutations, relative to the mRNA target, not only can be tolerated, but can enhance activity if directed to the 5' end. These results were consistent with nine different siRNAs against rat *Aqp4*, and replicated with mouse *Aqp4*. The mechanistic basis of this phenomenon is unknown, but might reflect the preferences and structural characteristics of the RISC complex.

During the revision of this work, a study was published that estimated the mismatch vulnerability of siRNAs (30). This study was based on a systematic analysis of the silencing potential of an active siRNA on target mRNA carrying all possible single-nucleotide mismatches. It was concluded that the silencing potential was influenced both by the position of the mismatched base and the identity of the nucleotides forming the mismatch. The finding that central mutations give decreased silencing activity, while peripheral mutations are well tolerated, agrees well with the conclusion of the present study.

Our findings have implications for future siRNA design with respect to specificity and activity and may assume particular importance when the target mRNA offers a limited choice of siRNA positions. Examples of such targets are transcripts with point mutations, specific splice variants and junctions in fusion oncogenes. In such cases, the introduction of wobble mutations could provide a means to enhance the activity of the limited set of available siRNAs and thus extend the siRNAs technology to a broader range of targets.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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