Fluorescence correlation spectroscopy and fluorescence cross-correlation spectroscopy reveal the cytoplasmic origination of loaded nuclear RISC \textit{in vivo} in human cells

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ABSTRACT

Studies of RNA interference (RNAi) provide evidence that in addition to the well-characterized cytoplasmic mechanisms, nuclear mechanisms also exist. The mechanism by which the nuclear RNA-induced silencing complex (RISC) is formed in mammalian cells, as well as the relationship between the RNA silencing pathways in nuclear and cytoplasmic compartments is still unknown. Here we show by applying fluorescence correlation and cross-correlation spectroscopy (FCS/FCCS) \textit{in vivo} that two distinct RISC exist: a large ~3 MDa complex in the cytoplasm and a 20-fold smaller complex of ~158 kDa in the nucleus. We further show that nuclear RISC, consisting only of Ago2 and a short RNA, is loaded in the cytoplasm and imported into the nucleus. The loaded RISC accumulates in the nucleus depending on the presence of a target, based on an miRNA-like interaction with impaired cleavage of the cognate RNA. Together, these results suggest a new RISC shuttling mechanism between nucleus and cytoplasm ensuring concomitant gene regulation by small RNAs in both compartments.

INTRODUCTION

Small RNAs have emerged as key regulators of gene expression, acting in an evolutionary conserved group of gene-silencing pathways found in eukaryotes (1–3). The short ~19–24 nucleotide (nt) long dsRNA silencing triggers are produced from endogenous or exogenous dsRNA substrates of various secondary structure (4–7). Short interfering RNAs (siRNAs) consist of a dsRNA with a stem of ~19 nt containing a 5'-phosphate and 2 nt overhangs at both 3'-ends (5,7). To function as targeting cofactors, siRNAs are bound by the RISC-loading complex (RLC) consisting of Dicer, TRBP and a member of the Argonaute (Ago) family (6,8,9). The siRNA-duplexes are separated into single strands as they assemble into an Argonaute protein, the core of the RNA-induced-silencing complex (RISC) (10). The RLC specifically incorporates the strand with the lowest thermodynamic stability at the 5'-end of the duplex, termed ‘guide’ strand, into Argonaute, thereby forming activated RISC whereas the ‘passenger’ strand is removed from the complex and degraded (11–13). Depending on the extent of complementarity between the guide RNA and the target mRNA sequence, in conjunction with the associated Argonaute family member, RISC can silence gene expression by endonucleolytic cleavage, translational repression, deacetylation, decapping and/or by translocation into P-bodies (14–16). Furthermore, it has been shown that siRNAs as well as the nuclear encoded miRNAs, another class of small dsRNA silencing triggers, depend on Exp5 mediated nuclear export for cytoplasmic localization and efficient silencing (17,18).

Nuclear functions for Ago proteins have also been reported, such as in \textit{Schizosaccharomyces pombe}, where Ago is involved in heterochromatin formation to promote transcriptional gene silencing and in other organisms including plants, \textit{Drosophila} and \textit{Caenorhabditis elegans} (19–21). Although Ago proteins have been found in cytoplasmic and nuclear extracts from mammalian cells, it could not be excluded experimentally that the nuclear extracts were contaminated by Ago proteins associated with the nuclear envelope (22,23). Further indications

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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for the presence of Ago proteins in the nucleus are that the small nuclear RNAs (snRNAs) 7SK and U6 could be knocked down with siRNAs (23). Also, it has been shown that exogenously introduced siRNAs complementary to target sequences within gene promoters (agRNAs, antigenic siRNAs) can either inhibit (transcriptional gene silencing, TGS) or activate gene transcription in an Argonaute protein dependent manner in mammalian cells (24–26). The Argonaute proteins bind to an antisense transcript or extended 5'-untranslated region of the mRNA that overlaps the gene promoter thereby mediating the formation of complexes with proteins and chromosomal DNA necessary for the activation of transcription or TGS in mammalian cells (27,28). However, endogenous, small dsRNA triggers involved in transcription regulation still need to be identified. In addition, it is unknown where the nuclear RISC is assembled and how the cell ensures the nuclear localization of agRNA-loaded RISC. The knowledge of the localization of its assembly, e.g. whether nuclear RISC is specifically loaded inside the nucleus or originates from cytoplasmic loaded RISC is important to better understand RNAi-mediated gene regulation and the interconnection between the cytoplasmic and nuclear RNAi pathways.

In this study, we used fluorescence correlation spectroscopy (FCS) and fluorescence cross-correlation spectroscopy (FCCS) to quantitatively analyze siRNA incorporation levels in Ago2 proteins and RISC-complex sizes concomitant in the cytoplasm and nucleus in vivo. FCS and FCCS are highly sensitive and specific optical techniques used for the study of dynamics and interactions of individual fluorescently labelled molecules in solution or living cells (for details see Methods section and Supplementary data) (29–31).

We provide the first direct characterization of nuclear RISC and show that cytoplasmic RNA-induced silencing complex (crISC) and nuclear RNA-induced silencing complex (nrISC) are two, clearly distinguishable RISC complexes in vivo. Analyzing the asymmetric incorporation of fluorescently labelled siRNAs into nrISC and crISC, we found that nrISC originates almost predominantly from loaded crISC. Consequently, nrISC and crISC are interconnected by the dynamic exchange of RISCs between the cytoplasm and the nucleus.

MATERIALS AND METHODS

Cell culture

ER293 cells stably transfected with the pERV3 vector (Stratagene) were cultured at 37°C in DMEM (high glucose, Sigma) with 10% FCS (PAA Laboratories GmbH), 2 mM glutamine (Gibco) and 0.3 mg/ml G418 (50 mg/ml, Gibco) for 3 weeks. Cells were diluted to 1 cell/0.2 ml and transferred onto 96-well plates for recloning. Wells with single colonies were cultured, resulting in EGFP-Ago2 positive clones that were confirmed via western blot, immunofluorescence and PCR. The generated cell line 10G stably expressing EGFP-Ago2 was cultured at 37°C in DMEM (high glucose, Sigma) with 10% fetal calf serum, 2 mM glutamine, 0.3 mg/ml G418 and 0.4 mg/ml Hygromycin B. All cells were regularly passaged at subconfluency and were plated with 1–5 × 10⁶ cells/ml density.

siRNA sequences and calculation of the 5'-end free energy

All siRNA strands were obtained from IBA GmbH. The RNA oligonucleotides were synthesized with a 5'-phosphate and a 3'-amino group on a C6-carbon linker and were labelled with Cy5 succinimidyl ester (Cy5-NHS, Amersham Biosciences) as described previously (17). The nucleotides 18–21 were 2'-O-methyl modified to protect the label from degradation except for silencing assays were the siRNAs were not modified. Target-RNA and siRNA sequences are listed in the Supplementary data.

For siRNA control experiments, we used Silencer™ Negative control #2 (NegsiRNA) from Ambion.

The free energies of the first 4 bp of each siRNA strand of the duplex at the 5'-end was calculated in kcal/mol by using the nearest-neighbour method and the mfold algorithm (32,33).

Microinjection

For microinjection, 7–9 × 10⁴ ER293/10G cells were transfected onto MatTek chambers coated with Fibronectin (25 μg/ml in PBS including CaCl₂ and MgCl₂, Roche) 24 h before microinjection. The micropipette (Femtotip 2, Eppendorf) is loaded with 1.5–4 μl labelled siRNAs (WGA 2.25 μg/μl, Biomeda) in 110 mM K-gluconate; 18 mM NaCl; 10 mM HEPES pH 7.4 and 0.6 mM MgSO₄. The micromanipulator consists of a FemtoJet and InjectMan NI2 which is mounted directly on a microscope. Working pressure for injection was between 25 and 80 hPa for 0.1 s and a holding pressure of 15 hPa.

Fluorescence correlation spectroscopy setup

Fluorescence correlation spectroscopy and laser scanning microscopy (LSM) were carried out on a commercial system consisting of a LSM510 and a Confocor3 (Zeiss, Jena, Germany). The 488 nm line of a Ar-Ion laser and the 633 nm line of a HeNe laser were attenuated by an acousto-optical tunable filter to 3.5 and 1.05 kW/cm² and directed via a 488/633 dichroic mirror onto the back aperture of a Zeiss C-Apochromat 40×, N.A. = 1.2, water immersion objective. Fluorescence emission light was collected by the same objective and split into two spectral channels by a second dichroic (LP635). To remove any residual laser light, a 505–610 nm bandpass or 655 nm longpass emission filter, respectively, was employed. The fluorescence was recorded by avalanche photodiode detectors (APDs) in each channel. For EGFP-Ago2 autocorrelation measurements, a mirror substituted the second dichroic and a 505-nm longpass emission filter was used.
in a one channel setup. Out-of-plane fluorescence was reduced with a 70-μm pinhole. The fluorescence signals were software-correlated and evaluated with MATLAB (Mathworks, Natick, MA, USA) by using weighted Marquardt non-linear least-square fitting routine.

Laser scanning microscopy was performed using the APD’s of the Confocor3 on the same setup. Cell measurements were performed in air-buffer (150 mM NaCl, 20 mM HEPES pH 7.4, 15 mM glucose, 15 μg/ml BSA, 10 mM trehalose, 5.4 mM KCl, 0.85 mM MgCl$_2$, 0.7 mM CaCl$_2$) at RT.

**Preparation and fractionation of cell extracts**

Fractionated cell extracts were prepared from 10G, 293 and ER293 cells from four confluent T175 chambers essentially performed as previously described by Robb et al. (23). Sucrose gradients were exactly performed as has been described in (34).

**Immunoprecipitation**

ER293 and 10G cells were lysed with IP buffer (0.5% NP-40, 150 mM KCl, 25 mM Tris–HCl pH 7.5, 2 mM EDTA, 1 mM NaF, 0.5 mM DTT, protease inhibitors, Roche) and centrifuged at 10,000g for 10 min. Extracts were incubated with anti-GFP (Roche) or anti-p53 for 2 h at 4°C. Antibodies were pulled down with protein G Sepharose 4 fast flow beads (GE Healthcare) for 1 h at 4°C. Beads were subsequently washed with buffer (0.1% NP-40, 1 mM NaF, 0.5 mM DTT, protease inhibitors, Roche) and with PBS.

**Western blotting**

For western blot analysis, the cells were lysed with ×1.5 loading buffer for denaturing polyacrylamide gel electrophoresis. For immunoblotting, proteins were run on 7.5–10% PAGE and transferred onto Protran Nitrocellulose Transfer Membrane (Schleicher & Schuell). Antibodies: α-EF1 (Abcam ab290, 1:2000), α-Ago2 clone 11A9 (1:50), α-GAPDH (Abcam 9484, 1:2000), α-Actin (Abcam AC15). Immunoreactive signals were detected using ECL Western Blotting Detection Reagents according to the manufacturer’s protocol (Amersham Biosciences).

**qPCR**

Total RNA was isolated using the Prep Ease kit (USB, USA), according to the manufacturer’s instructions, using DNaseI digestion on column. cDNA was synthesized with random hexamer primers from 2 μg of total RNA using the First Strand cDNA synthesis kit (Fermentas, Canada), according to the manufacturer’s protocol. qPCR was performed on a MyiQ cycler (BioRad, USA) using the Mesa Green qPCR mastermix (Eurogenetec, Belgium) with primers at 100 nM final concentration and a two-step PCR protocol (5 min initial denaturation at 95°C, 40 cycles with 10 s at 95°C and 1 min elongation at 60°C). The primers were (forward, reverse): GAPDH, 5′ TGTTATCGTGGAAGACTCATGAC, 3′ ATGCCAGTGGACCTTCCCGT, TCAGC, 5′ CCTGCTAGAACCTCAAAACAG, 5′ GCCTCATTGGATGGTGTCGT. Data were evaluated using the ΔΔCt method.

**RESULTS**

To investigate RISC by FCS/FCCS in vivo, we created a 293 based cell line, in which hAgo2 is tagged with the enhanced green fluorescent protein (EGFP). For this study, hAgo2 was chosen, because it has been demonstrated that it is involved in nuclear RNAi, is associated with siRNAs and miRNAs, endonucleolytically active and that tagging hAgo2 on the N-terminus with EGFP had no effect on hAgo2 RNAi activity and sub-cellular localization (35,36). The characterization of the newly generated cell line 10G revealed that the EGFP-Ago2 protein reproduces the enzymatic activity, protein/RNA interactions, sub-cellular localization, expression level and function of endogenous hAgo2 protein (Supplementary Figure S1A–D).

**Nuclear RISC differs from cytoplasmic RISC**

Although the human RISC complex has been intensely studied, the protein composition is still not entirely clear, with reported molecular weights from ~160 kDa to ~2 MDa (37,38). The huge deviation in molecular mass might depend on extract generation or on the experimental procedure for their estimation. To avoid preparation artefacts, we measured hAgo2 containing RISC complexes in thermal equilibrium in vivo in 10G cells and determined the molecular mass of the individual RISC in different cellular compartments by FCS (for details see Supplementary materials). The autocorrelation curves of EGFP in 293 cells resulted in almost identical diffusion times in the cytoplasm and the nucleus of $r$EGFPCytopl = (354 ± 13) μs and $r$EGFPnuc = (368 ± 8) μs, corresponding to a diffusion coefficient of $D$EGFPCytopl = (25.5 ± 0.9) μm$^2$/s and $D$EGFPnuc = (24.5 ± 0.5) μm$^2$/s, indicating that the viscosity and therefore the motility is approximately the same in both compartments (Figure 1A) (39). In contrast, measurements in 10G cells displayed a significant difference in diffusion time for EGFP-Ago2 between the cytoplasm ($r$EGFP-Ago2Cytopl = (1678 ± 58) μs, $D$EGFP-Ago2Cytopl = (5.4 ± 0.2) μm$^2$/s) and the nucleus ($r$EGFP-Ago2nuc = (657 ± 22) μs, $D$EGFP-Ago2nuc = (13.7 ± 0.5) μm$^2$/s). With respect to EGFP, these diffusion times correspond to a molecular weight of (3.0 ± 0.6) MDa for cRISC and (158 ± 26) kDa for nRISC. While the obtained molecular weight for cRISC is in the range of a previously isolated RISC of about 2 MDa (37), the molecular weight of nRISC resembles almost the distinct EGFP-Ago2 alone. It should be noted that the molecular mass for cRISC could be overestimated due to a higher effective viscosity a larger molecule experiences in a crowded environment. Yet, due to the ~20-fold difference in molecular mass, we conclude that cRISC represents a different complex compared to nRISC. Further analyses of the measurements indicate that within the accuracy of the FCS technique no distinct
EGFP-Ago2 is present in the cytoplasm (Supplementary data).

Western blot analysis of nuclear and cytoplasmic 10G-extracts with an EGFP-specific antibody yielded a single specific band for EGFP-Ago2 at 130 kDa, demonstrating the absence of contaminating EGFP-Ago2 fragments in the nucleus (Figure 1B). Therefore, nRISC is not formed by a truncated version of EGFP-Ago2. To corroborate the FCS data, sucrose gradients with nuclear and cytoplasmic extracts were performed with α-Cyclin T1 and α-GAPDH antibody. (C) HEK 293 cytoplasmic and nuclear extracts were separated by sucrose density centrifugation under conditions that allow the separation of different hAgo2 containing mRNPs that were analysed by western blot with specific α-hAgo2 antibody.

To characterize the sub-cellular distribution of hAgo2 containing complexes, we quantified the amount of EGFP-Ago2 in the nucleus and the cytoplasm by FCS and imaging. EGFP-Ago2 preferentially localized to the cytoplasm with a cytoplasmic to nuclear ratio of (4.2 ± 0.5) : 1 determined by FCS and (4.7 ± 0.5) : 1 determined by imaging (for details see Supplementary materials). The higher value for imaging might be caused by the accumulation of EGFP-Ago2 into P-bodies, which are not accessible with FCS.

We could directly characterize RISC sizes in the nucleus in thermal equilibrium by FCS in vivo without preparation artefacts and contaminations by eRISC. Our data demonstrate that cytoplasmic EGFP-Ago2 is part of a large complex and differs from nRISC which most probably represents the discrete EGFP-Ago2 protein. We obtained a cytoplasmic to nuclear EGFP-Ago2 concentration of ~4–5, indicating that hAgo2 is not as underrepresented in the nucleus as estimated by previous imaging studies. This might result from the high expression levels in transient systems in previous reports, compared to the almost endogenous expression levels of EGFP-Ago2 in 10G cells (35,36).

Asymmetric guide strand incorporation into nuclear and cytoplasmic RISC

The incorporation of siRNAs into nRISC and the effects of target interactions on the distribution of activated RISC in the cytoplasm and the nucleus in mammalian cells are largely unknown. To assess siRNA incorporation levels and asymmetric loading of the guide strand into RISC by FCCS in vivo (for details see Supplementary materials), we covalently labelled siRNAs on the 3'-end of the guide or passenger strand with Cy5 (illustrated in Figure 2C and D top) and delivered them directly into 10G cells via microinjection to avoid siRNA segregation within endocytic compartments. This resulted in silencing active and homogeneous localized cytoplasmic siRNAs required for FCCS (17). The siRNA siTK3 targets the mRNA of Renilla luciferase encoded on the plasmid pRL-TK. The 5’-end hybridization energy of siTK3 was calculated as described (12) to define the guide and passenger strand of the siTK3 duplex (Figure 2A). The siTK3 siRNA was used as it displayed high levels of Renilla luciferase silencing, demonstrating the incorporation of the guide strand into endogenous RISC (Figure 2B).

Microinjection of the passenger strand labelled siTK3 resulted in cross-correlation amplitudes between 0% and 5% in the cytoplasm and nucleus up to 12 h, indicating the exclusion of the passenger strand from RISC (Figure 2C). On the contrary, guide strand labelled siTK3 lead to significant cross-correlation amplitudes in the nucleus and the cytoplasm gradually increasing for up to 6 h with a slight decline thereafter (Figure 2D). As the relative amplitude of the cross-correlation function is directly proportional to the concentration of double labelled species, this result demonstrates the specific incorporation of the guide strand into nuclear and cytoplasmic RISC whereas the passenger strand is excluded during the loading process. The specific incorporation of the guide strand could be...
observed down to a 5'-hybridization energy difference of 0.5 kcal/mol (siGL2) (data not shown). Interestingly, the incorporation levels in the nucleus and the cytoplasm synchronistically increased and declined, in the nucleus however to a lower level, reaching saturation after 6 h (Figure 2D, bottom panel). Similar results were obtained with other siRNAs: siTK2 (Supplementary Figure S3), siRNA targeting Firefly luciferase (siGL2) and siGAPDH (data not shown). Guide strand labelled siRNAs could be examined for longer time periods compared to passenger strand labelled siRNAs, due to the loss of the Cy5 signal in passenger strand containing cells. This most probably results from a stabilization of the guide strand caused by the interaction with RISC whereas the passenger strand gets degraded. Control experiments with EGFP supplemented with guide strand labelled siTK3 exhibited no cross-correlation amplitude, whereas a double labelled control resulted in 80% cross-correlation amplitude in vitro (Supplementary Figure S4A and B).

For the first time, we could monitor the asymmetry dependent incorporation into nRISC and show that nuclear levels of guide strand incorporation correlate with cytoplasmic incorporation levels in time in vivo. The specific guide strand incorporation into nRISC provides additional evidence for the specific loading and function of nRISC.

**nRISC loading levels are affected by target RNA interactions**

The snRNA 7SK can be specifically silenced with an siRNA (23). In the previous section, we showed that siRNAs can be detected in nRISC independently of endogenous targets. To elucidate the effect of a nuclear target, we microinjected guide or passenger strand labelled si7SK1 (23) targeting 7SK snRNA. In conformity with our previous experiments, the asymmetric incorporation of the guide strand into cytoplasmic and nuclear RISC was observed, whereas the passenger strand was excluded from both complexes (Figure 3A). Interestingly, the si7SK1 loaded RISC accumulated much stronger in the nucleus between 1 h and 6 h after microinjection compared to all siRNAs tested (Figure 3A and B). Knock down of 7SK snRNA with two unrelated siRNAs 48 h before microinjection resulted in a decrease in cross-correlation amplitude that resembles the values of siTK3 (Figure 3C) and other siRNAs (data not shown) indicating a target dependent si7SK1-loaded RISC accumulation in the nucleus. A control transfection with NegsiRNA did not affect si7SK1-RISC accumulation in the nucleus (Figure 3C). Interestingly, another siRNA targeting 7SK snRNA (si7SK2) did not result in increased cross-correlation amplitudes in the nucleus, suggesting that the presence of a nuclear target per se is not sufficient for target dependent nRISC accumulation (Figure 3B and C). The target sites of the two siRNAs within 7SK snRNA structure are different (40), si7SK1 hybridizes with its seed sequence in a single stranded loop whereas si7SK2 binds to a relatively unstructured part of the 7SK snRNA to its perfect matching bulge (si7SK3-bulge) with the target site in an unstructured part of the 7SK snRNA (Figure 3D). By analyzing the silencing efficiency of si7SK1 and 2, we found that si7SK2 silences 7SK snRNA down to 20% whereas si7SK1 resulted in ~60% of relative 7SK levels (Figure 3E). It has been shown that dsRNA structures in close proximity to the cleavage site from the 3'-end inhibit cleavage (41). Furthermore, a target site in a small loop structure leads to steric problems resulting in reduced cleavage rates. These results are consistent with our silencing data (Figure 3E). To test the idea that binding of RISC to its target RNA without endonucleolytic cleavage leads to accumulation of loaded RISC in the nucleus, we compared an siRNA forming a central bulge (si7SK3-bulge) with the target site in an unstructured part of the 7SK snRNA to its perfect matching siRNA (si7SK3). The silencing efficiencies of the two siRNAs were analysed by qRT-PCR. Si7SK3 efficiently
silenced 7SK down to 16% whereas a central mismatch at position 9–11 completely impaired silencing (si7SK3-bulge; Figure 3E). Evaluation of the incorporation levels in the cytoplasm and the nucleus also displayed the accumulation of silencing impaired si7SK3-bulge loaded RISC in the nucleus, whereas the perfect match siRNA resulted in a 2-fold lower cross-correlation amplitude inside the nucleus. The cytoplasmic incorporation levels were not affected (Figure 3F). Additionally, the inhibition of target RNA cleavage by the si7SK3-bulge resulted in higher incorporation levels in the nucleus compared to the si7SK1 for incubation times longer than 6 h (Supplementary Figure S5A). The si7SK1 nuclear cross-correlation amplitude decreased after 6 h, a result of reduced levels of 7SK RNA due to the marginal silencing activity of si7SK1 during the time course compared to si7SK3-bulge with impaired cleavage. The analysis of the cytoplasmic to nuclear ratio of EGFP-Ago2 concentration did not show significant changes that can be related to the accumulation of EGFP-Ago2 loaded with si7SK3 and si7SK3-bulge siRNA in the nucleus (Supplementary Figure S5B).

We also analysed the sub-cellular localization of si7SK1 in living cells by quantifying fluorescence intensity. We could not detect increased levels of si7SK1 RNA labelled on either strands in the nucleus of 10G or ER293 cells compared to other siRNAs (Supplementary Figure S5C). The analysis of siRNA sub-cellular localization by imaging does not correlate with target RNA localization, since the majority of siRNAs is not bound by the RLC/RISC and therefore the sub-cellular localization is mainly affected by Exp5 mediated export illustrated by siRNA incorporation levels of <35%.

Taken together, our data show that nRISC loaded with a specific guide RNA can accumulate in a target and duration of the interaction dependent fashion, indicating that this process is dynamic and does not result from a stable fraction of RISC in the nucleus. The nuclear hAgo2 protein concentration seems to be highly regulated, since a 2-fold increase of specifically loaded nRISC did not change the overall cytoplasmic to nuclear concentration ratio of EGFP-Ago2.

**Cleavage impaired RISC-target interactions mediate RISC accumulation in the nucleus**

To directly investigate the effect of RISC-target-RNA interactions in the nucleus and cytoplasm by FCCS, we microinjected a 50 nt long RNA into 10G cells that is labelled with Cy5 on the 5’-end, contains the siTK3 target site and is modified with 2’-O-methyl on each end.

*Figure 3.* nRISC loading levels are affected by target RNA interactions. (A) Cross-correlation amplitude in the cytoplasm (filled boxes) and nucleus (open circles) of the nuclear targeting siRNA 7SK1 for different incubation times. Values were obtained by either labelling the guide strand (blue) or passenger strand (grey) and measuring in at least 10 cells. The sequence and free energies of the siRNA is illustrated. (B) Cross-correlation experiments of the nuclear targeting siRNA 7SK1 as shown in (A), graphed as ratio of the cross correlation in the nucleus over the cross-correlation in the cytoplasm. In addition, the ratios of other tested siRNAs are plotted. (C) Cross-correlation experiments of the nuclear targeting siRNA 7SK1 as shown in (A), plotted as ratio of the cross correlation in the nucleus over the cross-correlation in the cytoplasm. In addition, the ratios of other tested siRNAs are plotted. (D) Target specificity of the nuclear accumulation of the siRNA 7SK1. Plotted are the cross-correlation ratios nucleus to cytoplasm after 3 h of incubation for 7SK1 and for 7SK1 after the transfection (48 h) of a NegsiRNA, the siRNA 7SK2 and 7SK3. Additionally, the ratios for si7SK2 and siTK3 are depicted for comparison. (E) Schematic overview of the target sites of the used 7SK siRNAs used to analyse the influence of target interaction. (F) Cross-correlation amplitude in the cytoplasm (filled bars) and nucleus (open bars) after 3 h of incubation for the siRNAs 7SK1, 7SK3-bulge, 7SK3, 7SK2 and for comparison GAPDH. Mean values ± SEM.
to protect the target-RNA molecule against cellular RNases. The interaction of the EGFP-labelled RISC containing an unlabelled siRNA with the target-RNA can then be visualized by increased cross-correlation amplitudes (Figure 4A). The target-RNA localized to the cytoplasm and displayed a slight accumulation of fluorescence signal in the nucleus and even stronger in nucleolar structures (Figure 4B). FCCS measurements in non-transfected and NgsiRNA transfected 10G cells after 1 and 3 h of target-RNA delivery showed no interaction of RISC with the target-RNA illustrating assay specificity and the absence of miRNA target sites within the target-RNA (Figure 4C). Longer incubation times were not tested as already 30% of target-RNA was degraded after 3 h in S20 extracts as determined by PAGE (data not shown). Despite that, transfection of perfectly matching siTK3 resulted in very low levels of EGFP-Ago2-target-RNA interactions (Figure 5C). In contrast, transfection of siTK3-B2 which forms a central bulge with the siTK3-target site yielded high levels of RISC-bound target-RNA in the cytoplasm. In the nucleus however, levels were 3-fold lower after 1 h with an increase after 2 and 3 h almost reaching cytoplasmic interaction levels (Figure 4C). The complete inactivation of the siTK3-B2 cleavage activity was verified by a dual luciferase assay (Figure 4D).

These results demonstrate (i) the localization of functional guide strand loaded RISC to the nucleus, (ii) the short interaction time of RISC loaded with a perfectly matching guide strand with the target-RNA and that (iii) bulges in the centre of the guide-target-RNA hybridizations increase the lifetime of RISC-target-RNA interaction, resulting in the accumulation of the complex in the nucleus over time. This is in strong agreement with our previous results showing the accumulation of specifically loaded RISC in a target- and duration of the interaction dependent fashion. Therefore, the target-dependent accumulation of nRISC is caused by the stable interaction of RISC with its target and not by the nuclear localization of

Figure 4. Cleavage impaired RISC-target interactions mediate RISC accumulation in the nucleus. (A) An outline of the experiment is illustrated. EGFP-Ago2 serves as a fluorescent label for RISC that can interact with the Cy5 labelled target-RNA. The interaction with the target-RNA is determined by FCCS in vivo. (B) LSM images of a target-RNA microinjected 10G cell (green: EGFP-Ago2, red: target-RNA, right panel: overlay). Scale bars indicate 10 μm. (C) Cross-correlation measurements were performed 1 or 3 h after microinjection. Data are represented as mean ± SEM, filled bars indicate measurements in the cytoplasm, open bars in the nucleus. (D) ER293 cells were transfected with the indicated amounts of NgsiRNA, siTK3 and siTK3-B2 together with the fixed concentration of the pGL2-Control and pRL-TK reporter plasmids. After 48 h, the ratios of target to control luciferase concentrations were normalized to the NgsiRNA control (black), siTK3-B2 (grey) and siTK3 (white). The plotted data were averaged from three different experiments ± SD.

Figure 5. Nuclear activated RISC originates from the cytoplasm. The incorporation of the guide strand of 7SK1 into RISC was measured for a cytoplasmic microinjection, a cytoplasmic injection together with wheat germ agglutinin (WGA) to inhibit active transport through the nuclear pore and a nuclear injection of 7SK1 and WGA. LSM images (red: Cy5, green: EGFP, yellow: overlay) and cross-correlation measurement were taken 3 h after incubation. Scale bars indicated 10 μm, data are represented as mean ± SEM; filled bars indicate measurement in the cytoplasm, open bars in the nucleus.
7SK snRNA, again underlining the dynamic levels of specifically loaded nRISC.

Nuclear activated RISC originates from the cytoplasm

The fact that we could detect a target dependent accumulation of nRISC raises the question of the origination of nRISC. Two mechanisms are possible: First, RISC is loaded both inside the nucleus and the cytoplasm or second, RISC is loaded in the cytoplasm and can than be imported into the nucleus to regulate gene expression/function. The first hypothesis is less likely for several reasons. The nRISC that was characterized by FCS in vivo displayed a molecular mass of ~158 kDa which is too small for the RLC (containing EGFP-Ago2, TRBP and Dicer which should result in a complex of approximately 380 kDa). In addition, siRNAs are too short to contain localization signals, therefore the RLC is incapable to locate the appropriate cellular compartment for individual silencing triggers until RISC interacts with its target RNA. And last, miRNAs and siRNAs depend on their cytoplasmic localization, mediated by Exp5, for effective silencing to occur (17,18,42). To elucidate the cytoplasmic origin of nRISC, we microinjected si7SK1 into the cytoplasm of 10G cells and allowed for exchange between nucleus and cytoplasm. This resulted in a preferential cytoplasmic localization of the siRNAs with 24% of EGFP-Ago2 loading levels in the cytoplasm with the corresponding nuclear levels of 21% after 3 h (Figure 5). In the second experiment, we microinjected siRNAs into the cytoplasm together with wheat germ agglutinin (WGA), an inhibitor of nuclear pore-mediated active transport. The subcellular localization of the siRNAs was still preferentially in the cytoplasm, but due to their small size could still be detected in the nucleus (Figure 5). The cytoplasmic incorporation levels were unaffected compared to WGA-free microinjected cells showing that WGA did not interfere with the RISC loading process. The nuclear incorporation level decreased 3-fold down to 7%, indicating that most of nRISC is originating from the cytoplasm. The same decrease in EGFP-Ago2 nuclear incorporation levels were detected by nuclear microinjection of si7SK1 together with WGA. The drop of loaded EGFP-Ago2 with si7SK1 in the cytoplasm results from reduced siRNA levels in the cytoplasm mediated by WGA. The preferential delivery of siRNAs via microinjection into the nucleus could be detected by the higher fluorescent intensity in the nucleus in comparison with the cytoplasm (Figure 5). The same results were obtained for si7SK3-bulge (Supplementary Figure S6). The inhibitory function of injected WGA on import and export processes was verified with the stress kinase Mk2 tagged with EGFP. Upon stress this predominantly nuclear protein shuttles into the cytoplasm (43). We could show that EGFP-MK2 shuttles into the cytoplasm after treatments with Anisomycin for 3 h that could be stopped with the addition of Leptomycin B which is an inhibitor of the exporter of MK2 (Supplementary Figure S7A, upper panel and S7B). The microinjection with WGA inhibited the export of EGFP-MK2 very efficiently (Supplementary Figure S7A, bottom panel and S7B). Treatment with cycloheximide did not change incorporation levels of EGFP-Ago2 in the nucleus, thereby excluding that the accumulation of specifically loaded RISC in the nucleus results from de novo synthesis of EGFP-Ago2 which is transported in the nucleus and subsequently loaded (Supplementary Figure S8).

Our results show that RISC is loaded in the cytoplasm and is imported into the nucleus. Therefore, the RLC is strictly localized in the cytoplasm. The accumulation of the si7SK1 and si7SK3-bulge siRNA indicates a dynamic exchange of RISC between the nucleus and the cytoplasm. Furthermore, the interaction with the target RNA seems to interfere with the export of RISC leading to the accumulation of this particularly loaded RISC in the nucleus. RISC shuttling between the cytoplasm and the nucleus represents a mechanism to access targets in both cellular compartments.

DISCUSSION

To summarize our findings, we propose the following model for human RISC loading and shuttling between the cytoplasm and the nucleus (Figure 6). (i) We could show for the first time that the RLC is localized and interacts with siRNAs/miRNAs exclusively in the cytoplasm. This is supported by Exp5 mediated nuclear export of small dsRNAs that actively reduces the concentration of silencing triggers in the nucleus and hence would lower the loading efficiency of nRISC (17,18). Consequently, miRNAs as well as siRNAs depend on the cytoplasmic localization for efficient silencing activity. The RLC senses the asymmetry of the duplex and specifically incorporates the guide strand into hAgo2, thereby forming cRISC. This process seems to be regulated, as FCCS measurements immediately after microinjection yielded low...
levels of loaded RISC. Most of the guide strands were incorporated within the first 3–12 h, followed by a decline for longer incubation times. (ii) We proved that the majority of activated cRISC remains in the cytoplasm as a complex of ∼3 MDa to regulate gene expression of mRNAs. (iii) In case of perfectly priming guide strands to target RNAs, eRISC catalyses RNA degradation. For miRNAs, cRISC remains bound to the mRNA to regulate its expression and/or translocation to P-bodies. (iv) Our results indicate a partial translocation of activated cRISC into the nucleoplasm thereby forming nRISC of ∼158 kDa, most likely composed of only hAgo2. We were not able to monitor whether EGFP-Ago2 loses the RLC components Dicer, TRBP and other factors before or during the translocation, but RLC disassembly after guide strand incorporation is supported by previous reports in vitro (44,45). The partial translocation of loaded hAgo2 is in agreement with the findings by Robb et al. (23) who observed cleavage activity in cytoplasmic and nuclear extracts. (v) In contrast to Robb et al., we were able to directly compare guide strand incorporation levels with hAgo2 concentration and intracellular localization as well as the interaction dynamics of RISC with its target RNA. We therefore could demonstrate that perfectly priming guide-target RNA hybridizations lead to target RNA cleavage, immediately causing RISC-target RNA-complex disassembly, resulting in free nRISC which is in agreement with the kinetic analysis of minimal RISC in vitro (41). (vi) On the contrary, cleavage impaired nRISC, e.g. caused by dsRNA regions or bulges, results in the accumulation of this particularly loaded RISC in the nucleus, facilitated by its prolonged interaction with the target RNA. Due to the artificially induced target-RNA-RISC accumulation, we can exclude that the target dependent accumulation of nRISC is facilitated by the strict nuclear localization of 7SK snRNA and therefore results from the stable interaction of RISC with its target. Since we never detected a concentration increase of nRISC, we reason that a stable equilibrium between n- and cRISC exists that is shifted for a particularly loaded nRISC depending on the target interaction.

Takken together, our results propose a RISC exchange/shuttling mechanism between the cytoplasm and the nucleus. Due to the absence of nuclear localization signals in siRNAs and miRNAs, this shuttling mechanism ensures the accessibility of activated RISC to target containing cellular compartments. The shuttling of hAgo2 into the nucleus also explains why agRNAs can function in the nucleus. Due to the identical localization patterns of hAgo1 and hAgo2 (23), we propose that nuclear hAgo1 also originates from the cytoplasm to mediate transcription activation or TGS. In agreement with our model, the findings that also let-7 miRNA is incorporated into nuclear hAgo2 and that computational approaches showed that many identified miRNAs have substantial complementarity to sequences within gene promoters provide further evidence for the existence of an endogenous RNA-mediated transcription regulation machinery originating from cytoplasmic activated Argonaute proteins (23,28).

In a previous report, Berezhna and colleagues observed by confocal imaging that siRNAs targeting 7SK and U6 snRNAs localize into the nucleus as duplexes whereas an siRNA targeting cytoplasmic hepatitis C virus replicon RNA dissociates into the cytoplasmic localized guide strand and a degraded passenger strand after transfection. From this Berezhna et al. (46) concluded the existence of a mechanism by which the RISC-loading machinery orchestrates a target-determined localization of siRNAs and provide evidence for RISC-loading in the nucleus. In contrast to their findings, we showed that the analysis of siRNA sub-cellular localization by imaging is mainly affected by Exp5 mediated export, since the majority of siRNAs is not bound by the RLC/RISC thereby excluding a target-determined localization. Further arguing against the target-determined siRNA localization hypothesis is the stable hAgo2 concentration that we observed in the nucleus independent of the incorporated guide strand and our direct evidence for exclusive cytoplasmic RISC-loading. In addition, RISC has been shown to identify its target and defines the silencing mode after the removal of the passenger strand depending on the complementarity to its target, supporting our observations (47). The localization of the complete siRNA duplex in the nucleus, as observed by Berezhna et al., would imply that the RLC is able to sense whether the bound siRNA duplex has a nuclear or cytoplasmic target. However, siRNAs are only bound briefly to the RLC in form of a duplex as we could not detect significant levels of incorporated passenger strand in neither RISCs. Findings by Robb et al. (23) also showed the target independent localization of guide RNAs in nuclear extracts. In addition, we showed that nRISC consists of a distinct hAgo2 protein that cannot bind dsRNA (48).

Underlining our model, it was shown that miR-138 is restricted to distinct cell types, whereas its precursor premiR-138-2 is ubiquitously expressed and cytoplasmically localized, thus showing the regulation on the post-transcriptional level in the cytoplasm (49). In combination with our results that the RLC is restricted to the cytoplasm, this post-transcriptional control of miRNA maturation in the cytoplasm applies to the regulation of guide strand incorporation into c- and nRISC.

As the interaction of nRISC with its target seems to prevent its export, this mechanism might prevent specific RNA transcripts from entering the cytoplasm and associating with the translation machinery. Further studies on the effect of nRISC-target RNA interactions and the sequencing of miRNAs or other classes of small RNAs in nRISC are needed to unravel the biological role of the nuclear RNAi machinery.

The advantage of our newly established experimental platform is the detailed real-time monitoring of the incorporation process into RISC in vivo. It represents a unique tool to address so far inaccessible questions from a new perspective. Our FCCS assay is superior in studying the impact of siRNA sequence and structure on RISC loading and strand asymmetry compared to standard silencing readouts that are normally used in high-throughput assays. Therefore, our technique can contribute to the development of improved siRNA duplexes with different
functions suitable for the application as therapeutic agents. It can be used for screening chemicals that affect the activity of the RLC or the interaction of RISC with the target RNA which was not possible in vivo until now. Furthermore, the possibility of studying RISC-target interactions can lead to the engineering of RNAs that can inhibit specific RNA functions, structures or affect for example alternative splicing and other RNA dependent mechanism.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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