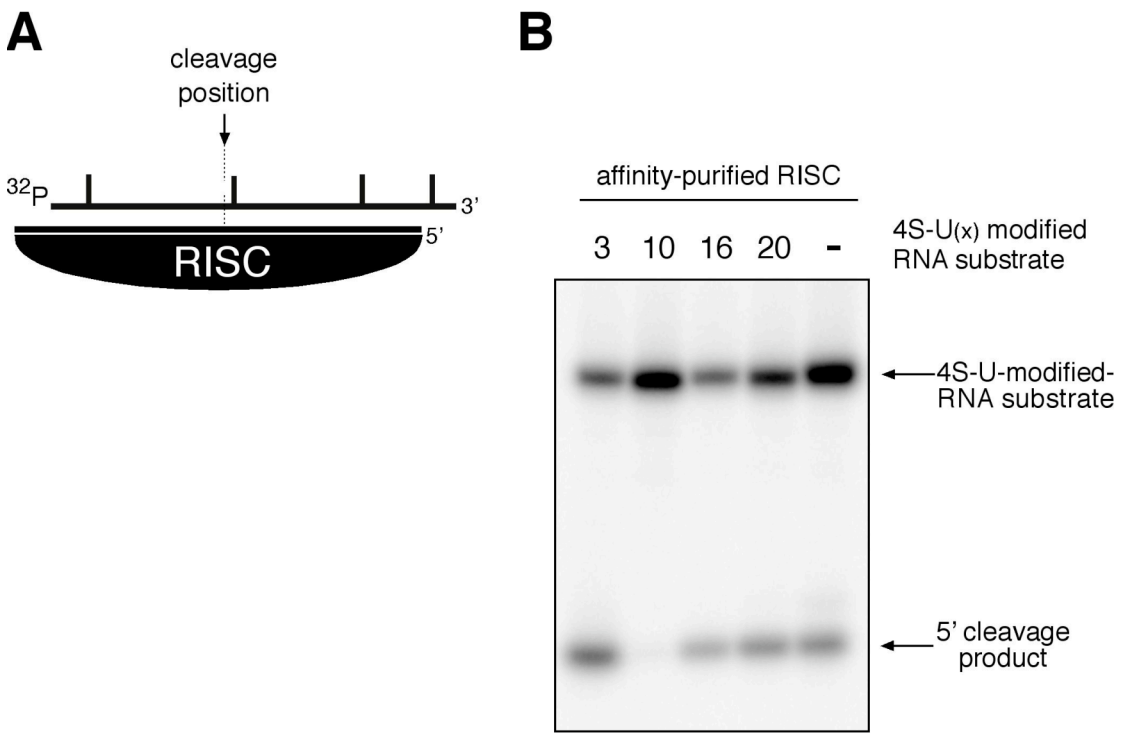
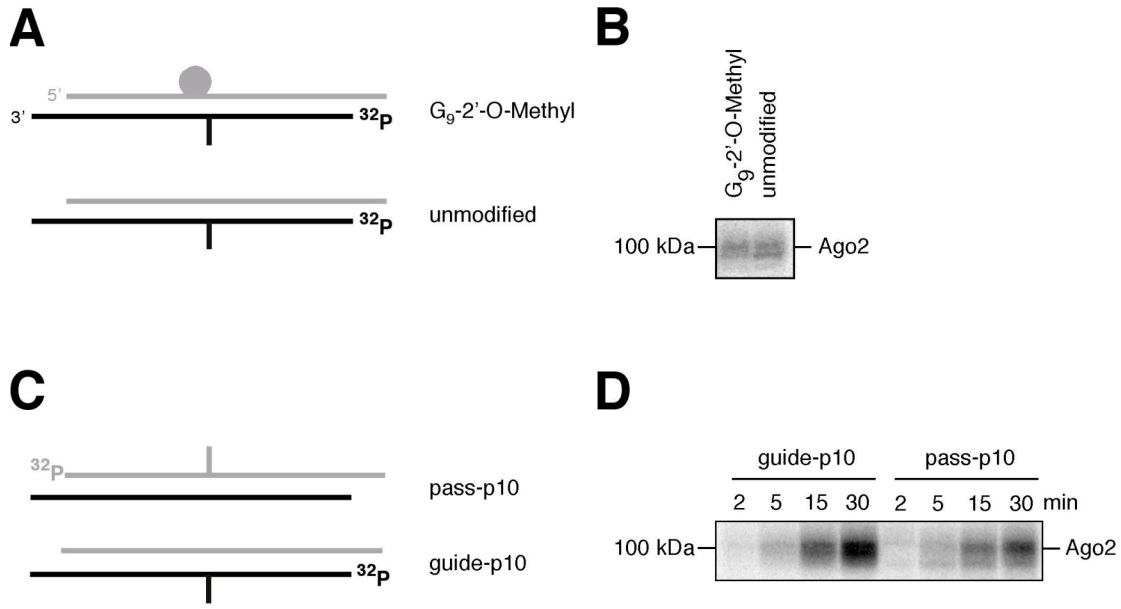
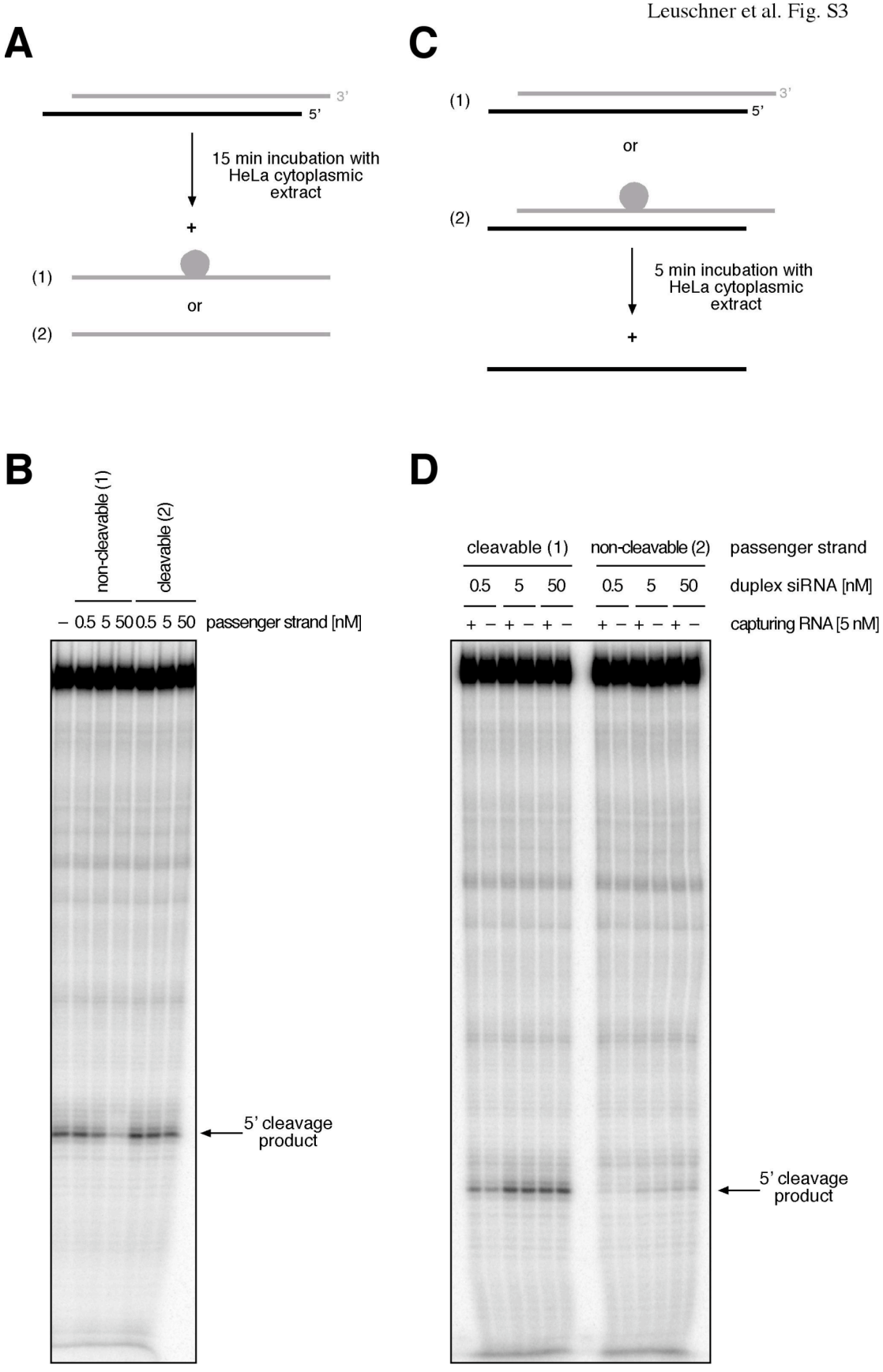
Supplementary information:

Figures:

Leuschner et al. Fig. S1




A
5'
3'
32P
G9-2'-O-Methyl
32P
unmodified
B
G9-2'-O-Methyl
unmodified
100 kDa
Ago2
C
32P
pass-p10
32P
guide-p10
D
guide-p10
pass-p10
2 5 15 30 2 5 15 30 min
100 kDa
Ago2


A
3'
5'
15 min incubation with HeLa cytoplasmic extract
+
(1)
or
(2)
C
(1)
3'
5'
or
(2)
5 min incubation with HeLa cytoplasmic extract
+
B
non-cleavable (1)
cleavable (2)
– 0.5 5 50 0.5 5 50 passenger strand [nM]
5' cleavage product
D
cleavable (1)
non-cleavable (2)
passenger strand
0.5 5 50 0.5 5 50
duplex siRNA [nM]
+ – + – + – + – + – + –
capturing RNA [5 nM]
5' cleavage product

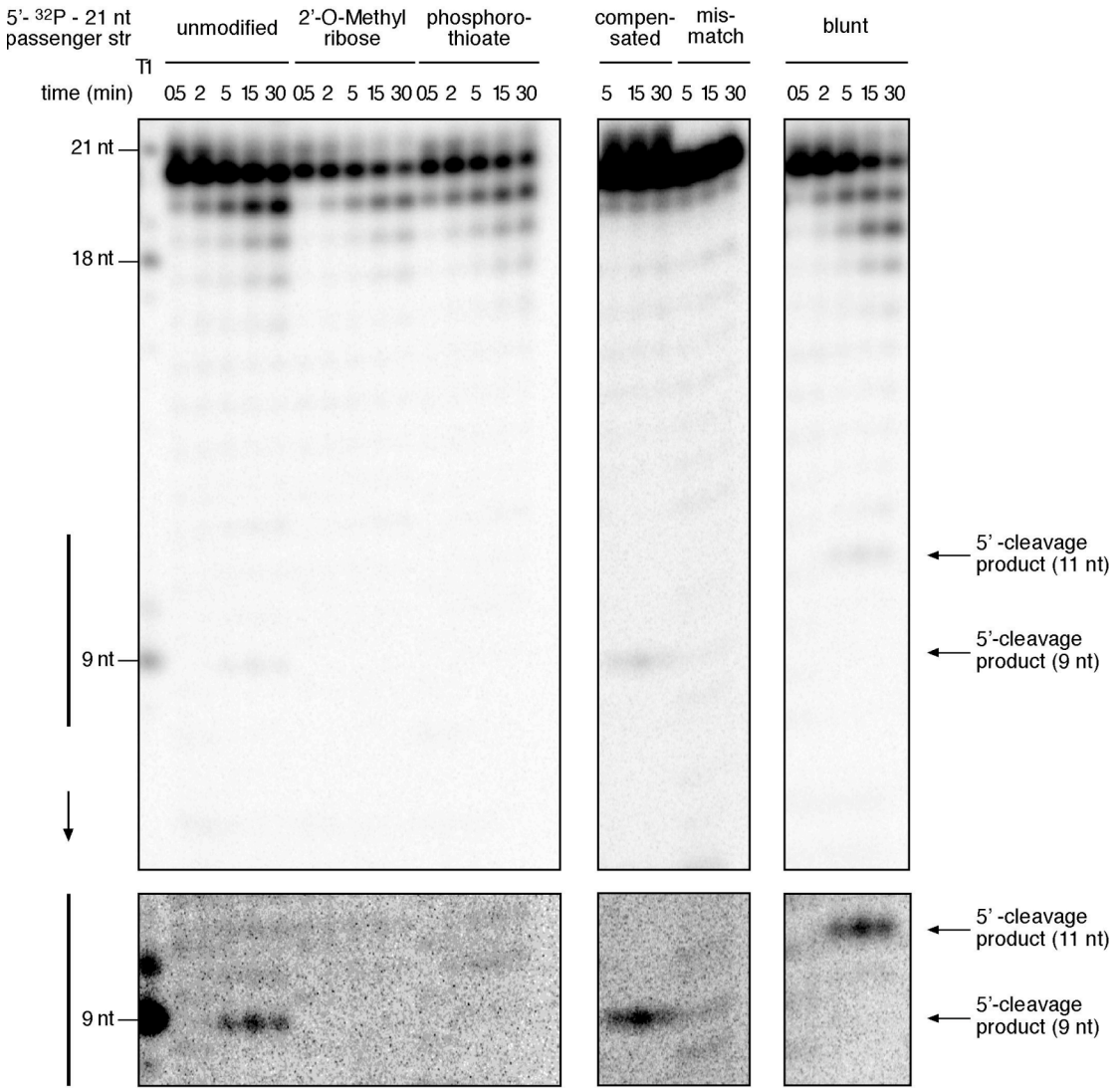

5'- 32P - 21 nt passenger str
unmodified
2'-O-Methyl ribose
phospho-ro-thioate
compen-sated
mis-match
blunt
T1
time (min)
0.5 2 5 15 30 0.5 2 5 15 30 0.5 2 5 15 30 5 15 30 5 15 30 0.5 2 5 15 30
21 nt
18 nt
9 nt
5' -cleavage product (11 nt)
5'-cleavage product (9 nt)
9 nt
5' -cleavage product (11 nt)
5'-cleavage product (9 nt)

Figure legends:

Figure S1. Affinity purified RISC is not able to cleave a short RNA substrate displaying a 4S-U modification adjacent to the cleavage site (4S-U_{10}).

(A) Graphic representation of 4S-U-modified short RNA substrates. 4S-U-modifications are depicted as bars. Affinity-purified RISC (purified as previously described (Martinez et al., 2002)) is depicted in black below the guide strand. RNA substrates were 5′ phospho-radiolabeled.

(B) Phosphorimaging analysis of a cleavage reaction resolved in a 15% denaturing gel electrophoresis. Arrows point to the 4S-U-modified RNA substrates and the labeled 5′ cleavage product.

Figure S2. Initial assembly of RISC is not disturbed by modifications on the passenger strand that impair target RNA cleavage and occurs on a double-stranded siRNA.

(A) Graphic representation of duplex siRNAs containing chemically modified or unmodified passenger strands (2´-O-methyl groups are depicted as circles), and a 5′ phospho-radiolabeled guide strand modified at position 10 with 4S-U (4S-U_{10}; depicted as a bar) to allow the cross-linking of proteins in close proximity to the center of the siRNA.

(B) Phosphorimaging of a cross-linking reaction using siRNAs depicted in (A) resolved on a 7.5% SDS-polyacrylamide gel.

(C) Graphic representation of siRNAs composed of a 5′ phospho-radiolabeled passenger strand modified with 4S-U_{10} and an unmodified guide strand, or an

unmodified passenger strand annealed to a 5′ phospho-radiolabeled guide strand modified with 4S-U_{10}.

(D) Time course analysis on the gradual recruitment of Ago2 to the siRNA, as monitored by cross-linking to both the guide and the passenger strands, resolved on a 7.5% SDS-polyacrylamide gel.

Figure S3. Non-cleavable passenger strands are not released from the siRNA.

(A) Graphic representation of the experiment. HeLa cytoplasmic extract was incubated together with 5 nM unmodified siRNA. After 15 min, when RISC assembly has already occurred (Martinez et al., 2002), different amounts of both non-cleavable or cleavable passenger strands were added. At 30 min, the complementary target-RNA (10 nM) was introduced and the cleavage reaction was continued for 2 h (Martinez et al., 2002).

(B) Phosphorimaging analysis of the cleavage reaction described in (A) resolved in a 6% denaturing gel electrophoresis showing the effect of the addition of a single-stranded, non-cleavable or cleavable passenger strands on target-RNA cleavage. Since addition of 5 nM single stranded, non-cleavable passenger strand caused no significant reduction in target-RNA cleavage, re-binding of a potentially released non-cleavable passenger strand from the siRNA duplex (also 5 nM) could not account for the drastic inhibition of target-RNA cleavage. Only when added in large excess (50 nM), enough non-cleavable passenger strand molecules managed to escape unspecific degradation in the extract and acted as ‘suicide targets’. An unmodified passenger strand did not impair cleavage, not even at 50 nM, most probably because it was immediately cleaved by functional RISC and then released.

(C) Graphic representation of the experiment. HeLa cytoplasmic extract was incubated together with different concentrations of siRNAs containing either cleavable or non-cleavable passenger strands. The capturing RNA (complementary to the passenger strand) was added after 5 minutes of initiating the reaction, to prevent premature degradation.

(D) Phosphorimaging analysis of the cleavage reaction described in (C) resolved in a 6% denaturing gel electrophoresis showing the effect of adding a capturing RNA, complementary to the passenger strand, to a target-RNA cleavage reaction. The addition of the capturing strand to cleavage reactions programmed with siRNAs featuring a cleavable passenger strand did not affect target-RNA cleavage. The reduction in target-RNA cleavage observed when using 0.5 nM siRNA is due to sub-saturated levels of RISC. Importantly, addition of the capturing strand to siRNAs featuring a non-cleavable passenger strand never rescued target-RNA cleavage, not even when the capturing strand was in a 10-fold excess over the siRNA. Taken these data together, we conclude that a non-cleavable passenger strand is never released from the original duplex, probably because its cleavage does not take place, resulting in inhibition of target-RNA cleavage through impairment of RISC assembly.

Figure S4. The passenger strand is cleaved during RISC assembly.

Picture of the whole gel depicted in Fig. 2D showing the concomitant 3′–5′ exonucleolytic degradation of the passenger strand. This degradation leads to the steady accumulation of <21 nt species during the course of the reaction irrespective of the passenger being cleaved during RISC assembly. The region of

the gel corresponding to sizes between 8 and 12 nt has been enhanced for an optimal visualization of the cleavage products.

Materials and methods:

Cross-linking reactions:

Cross-linking reactions were performed as cleavage reactions, the difference being the absence of ^{32}P-cap-labeled substrate RNA, and the use of 5 nM duplexes labeled at the 5' end of the modified guide strands (Martinez and Tuschl, 2004). Reactions were incubated at 30ºC for 30 min, transferred to a 96-well plate on ice and irradiated at 1 cm distance for 10 min under 365 nm UV light. Samples were boiled in the presence of loading buffer and resolved by 7.5% SDS-PAGE. The gel was dried and exposed, typically over-night, and analyzed by phosphorimaging.

References:

Martinez, J., Patkaniowska, A., Urlaub, H., Lührmann, R. and Tuschl, T. (2002) Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell*, **110**, 563-574.

Abbreviations:

Ago2 Argonaute 2

RNAi RNA interference

siRNA small interfering RNA

G_9 guanosine 9

A_{10} adenosine 10

A_{11} adenosine 11

4S-U 4-thio-uridine