Exportin-5 Mediates the Nuclear Export of Pre-microRNA's and Short Hairpin RNA's

Yi, R. *et al.* (2003) *Genes and Development* 17(24): 3011-3016.

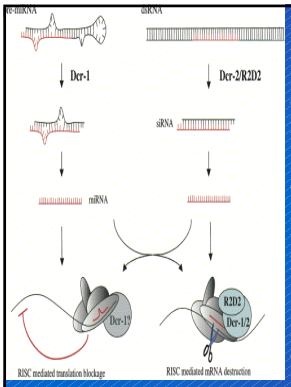
Presented by Ron Yahil
March 1, 2005

Background

•Karyopherins are proteins that work in concert with the nuclear pore complex (NPC) to regulate the traffic of molecules into and out of the nucleus. Their activity is dependent on the binding of the "cargo" by Ran-GTPase.

•Micro RNAs (miRNAs) are among the many types of molecules regulated by karyopherins. miRNAs are ~22 nt noncoding sequences whose function is to post-transcriptionally regulate mRNA expression (e.g. *let-7* and *lin-4* regulate proper larval development in *C. elegans*).

•The purpose of this study is to determine whether the karyopherin Exportin-5 (Exp5) is involved in transport of pre-miRNAs and short hairpin RNAs (shRNAs).



Tijsterman and Plasterk (2004). *Cell* 117(1): 1-3

•miRNAs are initially expressed as imperfect hairpins of ~80 nt in a longer transcript called a primary miRNA (pri-miRNA).

•These hairpins are cleaved by RNAse III (Drosha) into ~65 nt intermediate called pre-miRNA. These hairpins are usually left with very short 3' overhangs (2-3 nt).

•The pre-miRNA is then transported into the cytoplasm by an unknown mechanism, where it is further modified by Dicer into a mature 22 nt miRNA. The miRNA is then incorporated into the RNA-induced silencing complex (RISC), and then guides RISC to the target mRNA transcript.

•Dicer can also generate small interfering RNAs (siRNA) from double-stranded RNA transcripts, which signals RISC to cleave and degrade the target mRNA. This is known as RNA interference (RNAi), and is useful experimentally since artificial siRNAs can be easily incorporated into cells.

•Experimentally, the best way to make artificial siRNA is to transcribe a short hairpin RNA (shRNA) using an RNA polymerase III promoter. These transcripts generally have a 19-29 nt stems with a 3' 2 nt overhang.

•Exp5 is known to mediate the nuclear export of adenovirus VA1. Specifically, Exp5 binding requires a terminal dsRNA helix of >14 nt with a base-paired 5' end and a 3' overhang of ≥ 3 nt.

•Since adenovirus VA1 shares structural similarity to pre-miRNA and shRNA, could Exp5 be involved in the nuclear transport of miRNA and shRNA?

General Methods

•RNAi of Exp5 was achieved by transfection of 293T cells with an siRNA targeted to the ORF of human *Exp5*.

•Since RNAi is transient, transfections were done at multiple timepoints (0, 36, 60, and 96 hours). Exp5 gene expression was tested at 96 hours.

•At 60 hours, 293T cells were co-transfected with a luciferase reporter construct containing target sites of human miR-30, and either an miR-30-containing pre-miRNA, shRNA or a mature miR-30 miRNA. Some experiments use miR-21 instead of miR-30.

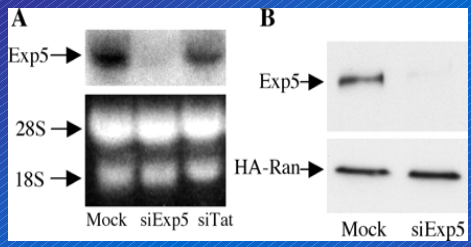


Figure 1. Knockdown of endogenous human Exp5 mRNA and protein expression by RNA interference. (*A*) 293T cells were transfected at 0, 36, and 60 h with the siExp5 RNA duplex, with the siTat duplex as a negative control, or mock-transfected. At 96 h, Exp5 mRNA expression levels were determined by Northern analysis. Ribosomal RNA served as a loading control. (*B*) Similar to panel *A*, except that 293T cells were cotransfected with pBC12/MS-HA-Ran, which expresses an HA-tagged Ran protein, at 60 h. Western analysis was performed at 96 h using a rabbit polyclonal anti-Exp5 antiserum or an HA-specific mouse monoclonal antibody.

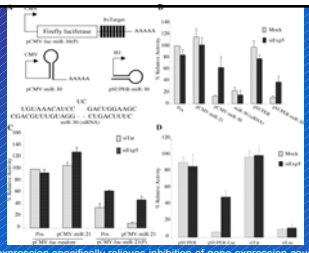


Figure 2. Loss of Exp5 expression specifically relieves inhibition of gene expression caused by pre-miRNAs or shRNAs. (*A*) Schematic representation of the pCMV-luc-miR-30(P) indicator plasmid, and of the different miR-30 RNA variants used. (*B*) At 60 h, coincident with the third siExp5 or mock transfection, 293T cells were cotransfected with the pCMV-luc-miR-30(P) indicator plasmid, a Renilla luciferase internal control plasmid, the indicated pBC12/CMV- or pSuper-derived control or miRNA expression plasmids, or the synthetic miR-30(siRNA) RNA duplex. Firefly and Renilla luciferase expression levels were determined at 96 h and adjusted for minor variations in the Renilla internal control. Pos (Positive control) refers to mock- or siExp5-transfected cultures that had been cotransfected with pCMV-luc-miR-30(P) and the Renilla and pBC12/CMV control plasmids. Data are presented relative to the firefly luciferase activity detected in the mock-transfected positive control culture, which was set at 100. Average of three experiments with standard deviation indicated. (*C*) These data were generated as described in panel *B* except that the indicator plasmids pCMV-luc-miR-21(P) and pCMV-luc-random were used. In addition, this panel used cells transfected with the siTat duplex as the control for siExp5. (*D*) These data were obtained as described in *B*, using the pCMV-luc-miR-30(P) indicator plasmid, and are presented as shown in *B*.

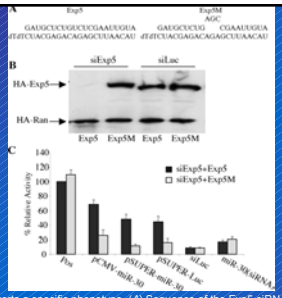


Figure 3. RNAi of Exp5 exerts a specific phenotype. (*A*) Sequence of the Exp5 siRNA (*lower* strand) and of the mRNA target (*upper* strand) in wild-type Exp5 and in the Exp5M mutant. (*B*) Coincident with the third siRNA transfection, here using either siExp5 or siLuc as a control, cells were cotransfected with plasmids expressing HA-tagged versions of wild-type or mutant Exp5 or Ran. A further 36 h later, HA-tagged protein levels were determined by Western analysis. (*C*) This transfection experiment was performed as described in Fig. 2*B*, except that at 60 h the 293T cells were also cotransfected with plasmids expressing wild-type Exp5 or the Exp5M mutant. All cultures were treated with the siExp5 RNA duplex. The Pos (Positive control) culture here refers to cells transfected with the pCMV-luc-miR-30(P) indicator, the Renilla and pBC12/CMV control plasmids, and the siExp5 RNA duplex. Data are given relative to the Pos culture cotransfected with the wild-type Exp5 expression plasmid, which was set at 100.

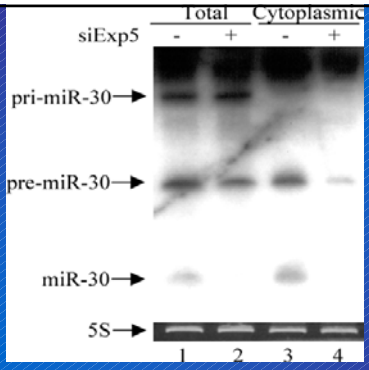


Figure 4. Analysis of miR-30 RNA expression. 293T cells were transfected with siExp5 or mock-transfected. At 60 h, coincident with the third siExp5 or mock transfection, cells were also cotransfected with pCMV-miR-30; 36 h later, total and cytoplasmic RNA fractions were isolated and subjected to Northern analysis. The location of the pri-miR-30, pre-miR-30, and mature miR-30 RNAs is indicated; 5S rRNA served as a loading control.

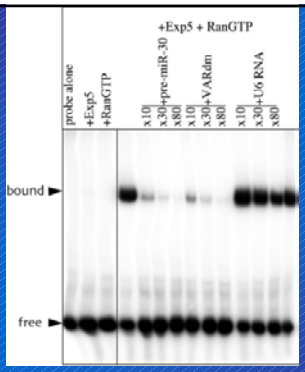


Figure 5. Specific binding of pre-miR-30 by Exp5 in the presence of Ran-GTP. A ^{32}P-labeled pre-miR-30 RNA probe was incubated with recombinant Exp5-His and/or recombinant Ran-GTP in the presence or absence of the indicated fold excess of various unlabeled competitor RNAs. Protein:RNA complexes were detected by nondenaturing gel electrophoresis and autoradiography.

Conclusions

- Exp5 is involved in the transport of miRNAs and shRNAs across the nuclear membrane.
- Based on evidence from this paper and from previous studies on adenovirus VA1, Exp5 binding seems to require a binding motif of > 14 nt RNA stem, along with a base-paired 5' end and a short 3' overhang. This motif is shared by pre-miRNAs and shRNAs.
- Exp5 is dependent on Ran-GTPase for its activity.
- Optimization of siRNA may now be possible, since the structural requirements of shRNA binding to Exp5 is now known.