Competition between Dicer mRNA, pre-miRNA, viral RNA for exportin-5 binding strikes a new regulatory mechanism for Dicer expression

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microRNA (miRNA) are a class of small non-coding RNAs that function by regulating gene expression posttranscriptionally. Alterations in miRNA expression can dramatically influence cellular physiology and are associated with human diseases including cancer. Here, we demonstrate crossregulation between two components of the RNA interference machinery in human cells. Specific inhibition of exportin-5, the karyopherin responsible for pre-miRNA export, downregulates Dicer expression, the RNase III required for pre-miRNA maturation. This effect is posttranscriptional and results from an increased nuclear localization of Dicer mRNA. In vitro assays and cellular RNA immunoprecipitation experiments show that exportin-5 directly interacts with Dicer mRNA. Titration of exportin-5 by over-expressing either pre-miRNA or the adenoviral VA1 RNA results in loss of Dicer mRNA/exportin-5 interaction and reduction of Dicer level. This saturation also occurs during adenoviral infection and enhances viral replication. Our study reveals an important crossregulatory mechanism between pre-miRNA or viral small RNAs and Dicer through exportin-5.

miRNAs are single stranded RNA of 19-24 nucleotides, predicted to regulate up to 30% of protein encoding genes. miRNA have been implicated in a vast array of cellular processes including cell differentiation, proliferation and apoptosis. miRNA repertoires are highly cell-type specific and change dramatically during development or upon activation. Changes in miRNA expression profile have been linked to human pathologies such as cancer and neurodegenerative diseases. In the nucleus, primary RNA polymerase II transcripts (pri-miRNA) containing imperfect hairpin structures are cleaved by the microprocessor complex composed of the RNase III Drosha and its RNA binding partner, DGCR8. The resulting processed stem-loop structure of ~65 nucleotides, called pre-miRNA, is recognized by exportin-5 (XPO5) and transits to the cytoplasm where it is then cleaved by the ATP-dependent RNase III Dicer. Dicer generates a small duplex of 19 to 24 bp containing mismatches, called miRNA/miRNA*. The guide miRNA strand is incorporated into the RNA-induced silencing complex (RISC) containing Argonaute protein as a core component (Ago1-4) whereas the miRNA* is degraded. Ago-bound miRNA serves as a guide to specifically recognize cellular mRNA to either induce their degradation and/or inhibit their translation.
Dicer is a key component of the miRNA pathway. Even though it is well known that Dicer is ubiquitously expressed among many cell types, deregulation of its expression may result in miRNA biogenesis defects in specific cellular contexts. Recently, several studies reported a modulation of Dicer expression in cancer cells. This regulation can be transcriptional: upon melanocyte differentiation, Dicer expression is induced through the binding of master transcriptional regulator MITF on Dicer promoter. Dicer regulation can also be post-transcriptional, Dicer mRNA can be targeted by specific miRNA: in human lung cancers, let-7 miRNA is upregulated and targets Dicer 3’UTR region; in breast cancers, miR103/107 family expression is associated with metastasis and a poor outcome in patients, these miRNA target and inhibit Dicer expression thus causing a global miRNA downregulation.

Here, we describe a new regulatory mechanism for Dicer expression in human cells involving competition for XPO5 binding between Dicer mRNA and short hairpin RNA such as premiRNA, shRNA or viral RNA.

RESULTS:

Exportin-5 controls Dicer expression posttranscriptionally

In an attempt to deplete the cellular miRNA content, we knocked down separately three key components of miRNA pathway in HeLa cells: XPO5, Dicer and Drosha using sequence specific siRNA. As expected, targeted proteins were specifically inhibited by their respective siRNA (Fig.1a); but surprisingly, Dicer expression was also abolished upon transfection of siRNA targeting XPO5 (siXPO5; Fig.1a, lane 3). We ruled out an off target effect since Dicer levels were similarly reduced following transfection of three additional siRNA designed against other regions of XPO5 messenger (Fig. S1a). Moreover, this effect was specific to Dicer because siXPO5 had no effect on Drosha or on the P-body component RCK/p54 (Fig.1a). Conversely, XPO5 overexpression increased the level of cellular Dicer (Fig.1b), consistent with previously reported enhancement of RNA interference in XPO5 overexpressing cells. To assay if XPO5 controls Dicer expression at the mRNA level, the transcript was analyzed by qRT-PCR (Fig. 1c) and northern blot (Fig. S1b) in samples shown in figure 1a. Dicer mRNA quantification was assessed using 3 sets of primers covering different regions of the messenger (Fig.S1c). As expected, Dicer mRNA levels were reduced in cells treated with Dicer specific siRNA. However,
they remained unaffected upon siXPO5 treatments (Fig. 1c and Fig.S1c). Together these results show that XPO5 regulates Dicer expression posttranscriptionally.

XPO5 is a member of the karyopherin family required for the transport of specific classes of small RNA from nucleus to cytoplasm and dsRNA binding proteins. XPO5 docks and translocates specific RNA cargoes through the nuclear pore complex, an export process dependent on a RanGTP-RanGDP gradient across the nuclear membrane. Only three classes of cellular RNA, tRNA, Y-RNA and miRNA precursors (pre-miRNA), are known to transit through XPO5. Small nuclear RNA (U1 snRNA to U5 snRNA) and some cellular RNAs containing ARE motifs depend on CRM1 for nuclear export or maturation, while tRNA is mainly exported through exportin-t (reviewed in ). In contrast, cellular mRNAs transit as ribonucleoprotein complexes through the TAP-p15 complex (also called NXF1-NXT1) in a RanGTP-independent manner. To test if XPO5 knockdown compromises the nucleocytoplasmic distribution of Dicer mRNA, total mRNA were extracted from the cytoplasm or the nucleus. Levels of Dicer transcripts were assayed by qRT-PCR in siXPO5 treated cells whereas scrambled siRNA treated cells were used as control. The analysis revealed that knockdown of XPO5 causes accumulation of Dicer mRNA in the nucleus (Fig.2). By contrast, the subcellular distribution of GAPDH mRNA, which transits through the Tap-p15 pathway, remained unchanged.

**Dicer mRNA interacts with exportin-5 in vivo and in vitro**

We therefore hypothesized that XPO5 could be the cellular karyopherin responsible for Dicer mRNA nuclear export. To analyze whether Dicer mRNA interacts with XPO5 in vivo, we developed an immunoprecipitation assay to specifically recover RNA associated with each of the three major cellular export pathways (Fig.S2). XPO5 interactions reported to date are Ran-GTP-dependent. Thus, in order to increase and stabilize specific interactions, non-hydrolysable RanQ69L-GTP was added to the immunoprecipitation buffer. Selected RNA associated with CRM1, XPO5 or TAP-p15 immunoprecipitates (Fig.3a) were then subjected to qRT-PCR analysis. As expected, U3, a snoRNA whose intranuclear transport to the nucleolus depends on CRM1, was specifically immunoprecipitated with CRM1 (Fig. 3a left top panel), pre-miR-16 with XPO5 (Fig.3a right top panel) and GAPDH mRNA with TAP-p15 (Fig.3a left lower panel). Dicer mRNA was specifically found in XPO5 immunoprecipitates (Fig.3a right lower panel) indicating that Dicer mRNA interacts with XPO5 in vivo. Direct Dicer mRNA/XPO5 interaction
was confirmed in vitro using electrophoretic mobility shift assays (EMSA) \(^{20}\). In vitro synthesized radiolabelled Dicer mRNA was incubated with increasing amounts of recombinant XPO5 and RanQ69L-GTP and subjected to RNaseT1/A treatment. Analysis by non-denaturing acrylamide gel electrophoresis showed that Dicer mRNA was protected in a XPO5 dose-dependent manner (Fig.3b, lane 1 to 6). Interestingly, pre-incubation of XPO5 with increasing amounts of unlabelled pre-miR-30, a known substrate for XPO5, decreased the amount of protected Dicer mRNA in a dose dependent manner (Fig.3b, lanes 8 to 10), showing the specificity of Dicer mRNA and XPO5 interaction. Conversely, preincubation of XPO5 with increasing amounts of unlabelled Dicer mRNA inhibits pre-miR-30/XPO5 complex formation in a dose dependent manner (Fig.3c, lanes 1 to 6). This effect is specific to Dicer mRNA since the same amounts of GAPDH mRNA have no effect (Fig.3c, lanes 7 to 10). As previously described for XPO5 partners, the interaction between Dicer mRNA and XPO5 depends on the presence of RanQ69L-GTP both in vivo (Fig.S3a) and in vitro (Fig.S3b). Taken together these experiments strongly suggest that XPO5 is the karyopherin responsible for the nuclear export of Dicer mRNA.

Overexpression of pre-miRNA or adenoviral VA1 RNA decreases Dicer protein level in cells

The above data show that Dicer mRNA binds specifically to XPO5 as previously reported for pre-miRNA \(^{23}\) and adenoviral VA RNA I (VA1) \(^{14}\). Because intracellular expression levels of XPO5 are limiting, it can be saturated by overexpression of its substrates in vivo \(^{24}\) and in vitro \(^{25}\). We therefore tested whether pre-miRNA or VA1 could outcompete Dicer mRNA for endogenous XPO5 amounts and, consequently, decrease Dicer protein levels. For this purpose, HeLa cells were transfected with increasing amounts of plasmids expressing either pre-miR-30 (Fig.4a) or VA1 (Fig.4b) or the corresponding empty plasmids (pSuper or pVV2 respectively). Dicer expression was then analyzed by western blot. Both pre-miR-30 (Fig.4a left panel) and VA1 (Fig.4b left panel) overexpression decreased Dicer levels in a dose-dependent manner. At the highest saturating dose of pre-miRNA or VA1, cell extracts were immunoprecipitated using anti CRM1, XPO5, TAP antibodies or a control isotype IgG, and Dicer mRNA was quantified by qRT-PCR. Pre-miR-30 and VA1 overexpression decreased the levels of XPO5-bound Dicer mRNA and pre-miR-16 (Fig. 4a and 4b right panels, Fig.S4). However, overexpression of pre-
miR-30 and VA1 had no effect on the amounts of U3 snoRNA and GAPDH mRNA recovered from CRM1 and TAP immunoprecipitates, respectively (Fig.S4). Thus, saturation of XPO5 by pre-miRNA or VA1 diminished its association with Dicer mRNA, and consequently decreased Dicer protein levels in cells. Taken together, our data show that Dicer levels are tightly regulated by XPO5, through direct interaction between XPO5 karyopherin and Dicer mRNA.

Exportin-5 inhibition enhances viral infection

Modulating the available level of XPO5 offers a simple and efficient cross-regulatory mechanism in which high expression of pre-miRNA could outcompete Dicer mRNA, preventing its export and translation, thereby regulating miRNA levels in the cell. This feedback mechanism might thus contribute to balance out Dicer and pre-miRNA levels. We thus assessed if the level of Dicer protein would be affected in a pathologic situation such as adenoviral infection. During adenoviral replication, VA1 and VA2 RNA are produced in large amounts and accumulate in infected cells reaching up to 20% of cellular RNA. We thus compared the effect of adenovirus infection using a wt virus (Ad5) or a mutant in which both VA structures had been deleted (Ad720) on Dicer protein level (Fig.5a). Ad5 mediates a reduction of Dicer protein level (Fig.5a), without affecting Dicer mRNA levels (Fig.5b, left panel). This downmodulation is correlated with increased amounts of VA RNA (Fig.5b right panel). In contrast, Ad720 had no effect on Dicer levels (Fig.5a,b). Together, our results clearly show that viral associated RNAs, massively produced at the late stage of adenovirus infection, are able to knockdown Dicer expression by saturating XPO5 and preventing nuclear export of Dicer mRNA. In order to evaluate if this function provides an advantage for the wt adenovirus, a kinetic of replication of both viruses was also realized. After infection, genomic DNA was purified and adenoviral DNA was quantified by qRT-PCR and normalized to GAPDH DNA. As previously described, the virus deleted for both VA replicates slowly compared to wild type virus (Fig.5c). We thus asked whether Ad720 replication could be rescued by reducing XPO5 expression. HeLa cells were transfected with siXPO5 for 48 hours and infected with Ad720. As a control, cells transfected with scrambled siRNA were infected with Ad720 or Ad5. Adenoviral replication was quantified after 12, 24, 36 and 48 hours (Fig 5c). XPO5 knockdown enhanced Ad720 replication to levels comparable to wt virus. Inhibition of XPO5 could thus complement ΔVA deficiency. Interestingly, knockdown of Dicer also complements Ad720 replication to wt level (Fig 5d). Taken together, these
experiments show that adenovirus targets the regulation of Dicer expression by XPO5 for optimal replication.

**DISCUSSION**

In this study we describe a novel cross-regulation between two essential mediators of small RNA-mediated silencing: pre-miRNA and Dicer. Three salient points emerge from our study. First, the observation that overexpression of pre-miRNA decreases Dicer level (Fig.4a), suggests that this cross-regulatory mechanism may contribute to the homeostatic control of miRNA biogenesis, similarly to the recently described post-transcriptional cross-regulation between the two subunits of the microprocessor complex, Drosha and DGCR8. Saturation of XPO5 has been described *in vivo* in mice treated with high doses of shRNA vector. They show signs of severe toxicity and ultimately die within a month. Morbidity was associated with downregulation of liver-derived miRNA. This observation was attributed to saturation of XPO5 resulting in reduced pre-miRNA export. We provide evidences that XPO5 saturation decreases not only pre-miRNA export as previously described, but also Dicer expression. Second, regulation of Dicer mRNA by XPO5 may be exploited by viruses to modulate cellular miRNA expression and overcome RNA interference. Indeed, viruses that produce small highly structured RNA able to bind and saturate XPO5 may inhibit Dicer expression, thus preventing maturation of viral RNA to small non-coding RNA (miRNA or siRNA). Targeting mRNA export pathway upon viral infection has been documented. Influenza A virus, through its NS1 viral protein, inhibits TAP-p15 export pathway. The reduction of mRNA export leads to a higher permissivity of cells to influenza virus replication. Third, the finding that XPO5 interacts with and regulates Dicer mRNA expression opens the possibility that other cellular mRNAs could interact with XPO5. Although structural determinant responsible for the recognition of short hairpin RNAs by XPO5 have been precisely defined, XPO5 is able to mediate the nuclear export of some unspliced mRNA lacking such a structural motif. This may uncover a new gene regulatory mechanism involving mRNA export through the limiting XPO5 pathway.
METHODS:

Plasmids

Myc-exportin-5, His-exportin-5 and His-RanQ69L encoding plasmids were provided by Ian Macara. pSuper and pSuper-premiR30 were provided by Bryan Cullen.

siRNA cell transfection

HeLa cells were cultured in DMEM medium complemented with 10% fetal calf serum. Cells are transfected with siRNA using INTERFERin reagent following manufacturer’s instruction (Polyplus). siRNA were synthesized by MWG with following sequences to target Exportin-5: siXPO5 UGUGAGGAGGCAUG CUUGU, siXPO5#2 GCCCUCAAGUUUUGUGAGG, siXPO5#3 CUCGAUUGGAGAAGGUGUA, siXPO5#4 GGAUAUUACAGACCAACUA; and to target Dicer: siDicer UGCUUGAAGCAGCUCUGGA. Expression level of knock down proteins was analysed by western blotting 48 hours after transfection. Briefly, cell-extracts were resolved on SDS-PAGE gels. Proteins were transferred to PVDF membrane by semi-dry electroblotting and probed overnight at 4°C with the primary antibody (anti-Dicer, anti- XPO5, anti TAP-p15 (Abcam), anti-CRM1 and anti-p54 (Bethyl)), washed and incubated with the appropriate secondary antibody (Amersham) for 1 hr. Proteins were visualized by chemiluminescence according to the manufacturer’s protocol (Pierce).

RNA immunoprecipitation, Quantitative RT-PCR

HeLa cells were grown in 60mm dishes and transfected with the indicated plasmids using jetPEI reagent (Polyplus). Cells were harvested 48 hours after transfection, lysed for 15 min in 2 mL of RIP buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 2.5 mM MgCl2×6H2O, 250 mM Sucrose, 0,05% NP40, 0,5% Triton X-100) containing RNasin (Promega) and 1mM DTT, and centrifuged to pellet debris. Supernatants were split in 4 equal parts and incubated overnight with XPO5, anti TAP-p15, anti-CRM1 antibodies or an IgG control in the presence of 10 µg of recombinant RanQ69L-GTP at 4°C followed by 1 hour incubation with protein G/A-Sepharose. Flow through were recovered and analysed at the protein and RNA level. Immunoprecipitates were washed 5 times with RIP buffer and nucleic acids were extracted with phenol/chloroform/isoamyl alcohol,
isopropanol-precipitated, ethanol-washed and resuspended in RNase-free water. Total RNA was DNase I treated. After heat inactivation, RNA was reverse-transcribed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). RT products were PCR-amplified successively using GAPDH (GAPDH forw CTGGCGTCTTCACCACCATGG; GAPDH rev CATCACGCCCACAGTTTCCCCCCG); U3 (U3 forw TTCTCTGAGC GTGAGAGCAAGCA; U3 rev GATCATCAATGGCTGACGGCAGT); VA (VA forw GTCCGGCCTGATCCATGC; VA rev CGTTGTCTGACGTGACAC) or Dicer specific primers (Dicer 190-292 forw: GCAGTAAGCTGTGCTAGAAC, Dicer 190-292 rev: ATTTGAGGAAGCAGG; Dicer 5662-5811 forw: TGGAGACAGCTGTGAGAGCAGTTA, Dicer 5662-5811 rev: TCCCGTCGTAAGTTCTCTCAGC; Dicer 7460-7639 forw: TCCCCATCACATACCAGTAGAG; Dicer 7460 rev: CAGATAAAGCAGGAAAGGCAC); To detect premiRNA, an additional step was added. RNA was polyadenylated with ATP by poly(A) polymerase at 37 °C for 1 h using the RNA tailing kit (Ambion) and reverse transcribed using 0.5 µg of poly(dT) adapter primer (Invitrogen). RT products were amplified using U3 forward primer; pre-miR-16 forward primer (GTCAGCAGCTGTGCTAGAAC), pre-miR-30 forward (GCGACUGUAACCAUCCUCGACUGGA) and reverse primer (based on the adapter sequence). Amplification was done using a Lightcycler 480 (Roche).

**Nuclear and cytoplasmic mRNA fractionation and quantification**

Nuclear and cytoplasmic RNA were purified following Wang et al protocol 19. Briefly, cells were harvested, resuspended in 1 ml of buffer (Tris10 mM Tris, pH 7.4; 10 mM NaCl; 3 mM MgCl2) and incubated on ice for 3 minutes followed by centrifugation. The volume of swelled cell pellet was estimated and resuspended by slow pipetting with 4 times its volume of buffer A (Tris10 mM Tris, pH 7.4; 10 mM NaCl; 3 mM MgCl2; 10 % glycerol, 0.5 % NP-40, 0.5 mM DTT, 100 U/ml RNasin). Nuclei were immediately pelleted by centrifugation at 4500g for 3 minutes and supernatant was saved as cytoplasm extract. Nuclei were further resuspended in buffer A supplemented with detergent (3.3 % sodium deoxycholate, 6.6 % Tween 20) and incubated on ice for 5 minutes. Nuclei were collected by centrifugation at 10 000g for 5 minutes. Nuclei integrity was monitored by microscopy after trypan blue staining and resuspended in buffer A. Cytoplasmic and nuclear RNA were extracted by Trizol reagent following
manufacturer’s instructions (Invitrogen). 2 µg of RNA was reverse transcribed and used as template for qPCR. Fractionation was assessed by checking nuclear U6 quantification.

**Northern blot**

RNAs were isolated by using mirVana miRNA Kit (Ambion) and analyzed following NorthernMax-Gly Protocol. Briefly, RNAs were separated on 1 % acrylamide-glyoxal gel and transferred for 3 hr to nylon membrane. After UV crosslink, membranes were prehybridized for 1 hr at 68°C in Ultrahyb (Ambion) and incubated overnight at 56°C with α³²P[UTP] radiolabeled RNA probe complementary to Dicer or Actin RNA. Membranes were washed at room temperature and autoradiographed.

**Electrophoretic Mobility Shift Assay**

Recombinant Exportin-5-his and RanQ69L-his were produced as previously described. Purified proteins were further dialyzed against buffer A (PBS, pH 7.5, 14 mM β-mercaptoethanol and 10% glycerol). Protein expression and structures were verified by western blot and by exportin-5 ability to specifically bind VA1 RNA more efficiently in the presence of RanQ69L-GTP and not bind to the mutant VA Mut10 (data not shown). Dicer mRNA was radiolabelled in vitro by cotranscribing the complete ORF of 5.6 kb and the 3’UTR of 4.27 Kb in the presence of α³²P[UTP] using T7 AmpliScribe kit following manufacturer’s protocol (Epicentre). PremiR-30 was radiolabeled in vitro using α³²P[UTP] as well. RNA-binding reactions were carried out in binding buffer (20 mM Hapes, pH7.9; 50 mM KCl, 5 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 10 % glycerol) for 20 minutes complemented with 50 nM of Mut10VA to reduce nonspecific binding and 10 ng of RanQ69L-GTP to increase specificity. In the case of Dicer mRNA, because of its length, 0.01µl of RNase A/T1 cocktail mix (Ambion) was added for 10 minutes at room temperature to degrade unprotected RNA. 1 µl of loading buffer (0.6 mg/ml heparin, 1mg/ml bromophenol blue) was added to the samples for 5 additional minutes. The complexes were resolved on a pre-run 5% non-denaturing acrylamide/TBE gel. Gel were dried and exposed by autoradiography.

**Adenovirus infection:**
HeLa cells were infected with 100 particles per cell of wt Ad5 or dlsub720 (a derivative Ad5 strain deleted for VA RNAI and II, provided by Goran Akusjärvi) at 4°C for 4 hours. Cells were washed with PBS twice and incubated at 37°C with DMEM 10% FCS medium. Infection was thus followed for 48 hours. Cells were harvested every 8 hours, and protein, RNA and DNA were extracted. Proteins were analyzed by western blot, RNA were reverse transcribed and Dicer, GAPDH and VA1 RNA were quantified by qRT-PCR. DNA was extracted by HIRT method to check infection. In another set of experiments, HeLa cells were transfected with a scrambled siRNA as control or siXPO5. 48 hours later, cells were infected with 0.1 particle per cell of Ad5 or Ad720 respectively. Cells were harvested every 12 hours up to 48 hours post-infection. DNA was extracted and adenoviral DNA was quantified by qRT-PCR using primers amplifying DBP viral gene, forward: CAGGGACACGTTGCGATACT and reverse: GCGGAGGCCCCAACTGCGACTTC. Results were normalized by quantification of cellular GAPDH DNA. Amplification was done using a Lightcycler 480 (Roche).

REFERENCES


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**FIGURES LEGEND**
Figure 1: Regulation of Dicer protein level by XPO5. a) HeLa cells were transfected with siRNA as indicated. Forty-eight hours post transfection, cells were analyzed for XPO5, Dicer, Drosha, and RCK/p54 expression by western blot. b) HeLa cells were transfected with either Myc-XPO5 or empty expression vectors. Forty-eight hours post-transfection, cells were analyzed for Dicer, Myc-XPO5 and Tubulin expression by western blot. c) Total RNA was extracted from samples described in a and Dicer mRNA levels were analyzed by quantitative RT-PCR.

Figure 2: XPO5 knockdown results in accumulation of Dicer mRNA in the nucleus. HeLa cells were transfected with XPO5 specific siRNA or control scrambled siRNA. Nuclear and cytoplasmic RNA were purified and Dicer mRNA, U6 small nuclear RNA and GAPDH mRNA were quantified by qRT-PCR. Cytoplasmic RNAs were normalized to GAPDH mRNA while nuclear RNA were normalized to U6 small RNA. Results are expressed in arbitrary units and representative of three independent experiments.

Figure 3: Dicer mRNA specifically interacts with Exportin-5 in vivo and in vitro. a) HeLa cell extracts were prepared and subjected to immunoprecipitation using IgG (control), anti-CRM1, anti-exportin-5, or anti-TAP/p15 antibodies in the presence of RanQ69L-GTP. After washing, a fraction of the unbound (FT) material and immunoprecipitates (IP) were analyzed by western blot using anti-CRM1, anti-exportin-5, or anti-TAP/p15 antibodies (Figure S2) and the rest of the IPs were used for RNA extraction. Purified RNA were reverse transcribed and subjected to qRT-PCR using specific primers for U3 snRNA, pre-miR-16, GAPDH mRNA and Dicer mRNA. b) Electrophoretic mobility shift assay was performed using a radiolabelled Dicer mRNA probe as described in methods section. Briefly, complex was formed in the presence of increasing amounts of recombinant exportin-5 and RanQ69L-GTP to increase specificity of binding (lane 1 to 7). Following incubation, complexes were subjected to RNase A/T1 treatment and analyzed on non denaturing 5% TBE/acrylamide gel. Complex specificity was evaluated by pre-incubating 200 ng of exportin-5 with increasing amounts of unlabelled pre-miR-30 (10, 50, 250 ng), following the same protocol (lane 7 to 9). Both binding assays were realized in the presence of RanQ69L-GTP to increase specificity (Fig.S3). c) Radiolabeled pre-miR-30 was incubated with 200 ng of XPO5 in the presence of RanQ69L-GTP as described in materials and methods. Complexes were formed in the absence (-) or in the presence of increasing amounts
(0.5, 1, and 2 µg) of the competitor RNAs indicated above the lanes: Dicer mRNA (lanes 4 to 6) or GAPDH (lanes 8 to 10) as a control. Note that this EMSA is done in the absence of RNase treatment. Following incubation, complexes were analyzed on non denaturing 5% TBE/acrylamide gel.

**Figure 4: Overexpression of pre-miRNA or adenoviral VA1 RNA affects Dicer protein levels in cells.** a) HeLa cells were transfected with XPO5 or Dicer specific siRNA, 2 µg of empty vector pSuper or increasing amounts of a vector expressing Pre-miR-30 (0.5 or 2 µg). 48 hours post transfection, a fraction of cell extracts was analyzed for Dicer and tubulin expression by western blot (left panel) and the rest was subjected to immunoprecipitation using, anti-IgG, CRM1, XPO5 or TAP-p15 antibodies. Recovered RNAs were reverse transcribed and subjected to qRT-PCR to quantify U3, U6, pre-miR-16 and GAPDH as controls (Supplemental figure 4) and Dicer mRNA. b) Experiment was performed as in a except that cells were transfected with either empty vector (pVV2) or increasing amounts of plasmid expressing VA1 (pVA1). 48 hours post transfection, cell extracts were prepared and analyzed as in a.

**Figure 5: XPO5 inhibition enhances adenovirus replication.** HeLa cells were infected with Ad5 or Ad720 mutant (dlsub720). Infected cells were harvested at different times post-infection. A fraction of cell extracts was analyzed for viral capsid, Dicer, XPO5 and tubulin by western blot (a) and the remainders were subjected to RNA purifcation. Extracted RNAs were reverse transcribed and subjected to qRT-PCR to quantify Dicer, GAPDH (b left panel), U6 and VA1 RNA (b right panel). c) HeLa cells were transfected with a scrambled siRNA as control or siXPO5. 48 hours later, XPO5 knock down was analyzed by western blot (upper panel) while cells were infected with 0.1 particle per cell of Ad5 or Ad720. Cells were harvested every 12 hours up to 48 hours post-infection. DNA was extracted and adenoviral DNA was quantified by qRT-PCR using primers amplifying DBP viral gene present in both viruses. d) HeLa cells were transfected with a scrambled siRNA as control or siDicer. 48 hours later, XPO5 knock down was analyzed by western blot (upper panel) while cells were infected with 0.1 particle per cell of Ad5 or Ad720. Cells were harvested 48 hours post-infection. DNA was extracted and adenoviral DNA was quantified by qRT-PCR using primers amplifying DBP viral gene present in both viruses. Results are expressed after normalization with GAPDH.
Figure S1: XPO5 siRNA inhibits Dicer protein expression but has no effect on Dicer mRNA levels. a) HeLa cells were transfected with three supplemental siRNA directed against three additional regions of XPO5 mRNA. 48 hours post transfection, cells were analyzed for Dicer, XPO5 and tubulin expression by western blot. b) Dicer mRNA level was analyzed by northern blot following Dicer or XPO5 siRNA transfection. c) Total RNA was extracted from samples described in figure 1a and Dicer mRNA levels were analyzed by quantitative RT-PCR using 3 sets of primers recognizing different regions of Dicer mRNA: primers 1 amplifies region 190-292, primers 2 region 5662-5811 (also used in figure 1c) and primers 3 region 7460-7639. Results are represented as Dicer mRNA levels compared to cells transfected with a control scramble siRNA.

Figure S2: Karyopherin immunoprecipitation. HeLa cell extracts were prepared and subjected to immunoprecipitation using IgG (control), anti-CRM1, anti-exportin-5, or anti-TAP/p15 antibodies. After washing, a fraction of the unbound (FT) material and immunoprecipitates (IP) were analyzed by western blot using anti-CRM1, anti-XPO5, or anti-TAP/p15 antibodies.

Figure S3: Dicer mRNA/XPO5 specific interaction requires RanGTP. a) Karyopherin immunoprecipitation analysis were realized as described in figure 3, in the absence (grey bars) or presence of RanQ69L-GTP (black bars). b) EMSA was realized as described in Figure 2 in the absence (lanes 1 and 2) or presence of RanQ69L-GTP (lanes 3 and 4).

Figure S4: Pre-miR-30 or VA1 overexpression does not affect amounts of U3 snRNA or GAPDH mRNA recovered from CRM1 and TAP immunoprecipitates. HeLa cells were transfected with either 2 µg of empty vector (pVV2 or pSuper) or plasmid expressing pre-miR-30 (A) or VA1 (B). 48 hours post transfection, cell extracts were prepared and subjected to immunoprecipitation using IgG (control), anti-CRM1, anti-exportin-5, or anti-TAP/p15 antibodies. Purified RNA were reverse transcribed and subjected to qRT-PCR using specific primers for U3 snRNA, pre-miR-16, GAPDH mRNA, pre-miR-30, VA and Dicer mRNA as in figure 2.
Figure 1

(a) Western blot analysis of XPO5, Dicer, Drosha, and p54 after siRNA treatment with scramble, Dicer, XPO5, and Drosha. Bands are shown for each siRNA condition.

(b) Western blot analysis of Dicer, myc, and Tubulin with myc-XPO5 as a control. Bands are shown for each protein.

(c) qRT-PCR analysis of Dicer mRNA levels with scramble, XPO5, and Dicer siRNA treatment. Bars show the expression levels with error bars for each condition.
Figure 2

Dicer mRNA

GAPDH mRNA

Relative mRNA (arbitrary unit)

Scramble Si XPO5

cytoplasm nucleus cytoplasm nucleus

Scramble Si XPO5

cytoplasm nucleus cytoplasm nucleus
Figure 3

a) 

GAPDH mRNA

Dicer mRNA

b) 

Exportin-5 /pre-miR 30 complex

Exportin-5 /Dicer RNA RNase resistant complex

free digested Dicer RNA

pre-miR-30

+ XPO5+RanQ69L-GTP

+ RanQ69L-GTP

cold Dicer RNA

cold GAPDH RNA

Exportin-5 /pre-miR 30 complex
Figure 4

(a) 

Dicer mRNA

IgG CRM1 XPO5 TAP IgG CRM1 XPO5 TAP

pSuper (2 μg) 

pre-miR30 (2 μg)

Dicer mRNA

IgG CRM1 XPO5 TAP IgG CRM1 XPO5 TAP

pVV2 (2 μg) 

pVA1 (2 μg)

Dicer mRNA

IgG CRM1 XPO5 TAP IgG CRM1 XPO5 TAP

pVV2 (2 μg) 

pVA1 (2 μg)
**Figure 5**

(a) Western blot analysis showing the expression levels of Dicer, viral capsid, Tubulin, and Exportin-5 for wt Adenovirus and ΔVA Adenovirus samples at different time points (0, 8, 16, 24, 36, and 48 hours post-infection).

(b) Graph showing the Dicer mRNA/GAPDH mRNA ratio over time for wt adenovirus Ad5 and ΔVA adenovirus dl-sub720. The y-axis represents the Dicer mRNA/GAPDH mRNA ratio, and the x-axis represents the hours post-infection.

(c) Graph showing changes in XPO5 and Tubulin levels after siRNA treatment.

(d) Bar graph comparing Adenoviral DNA copies normalized to GAPDH DNA for scramble, scramble + siDicer, wt adenovirus Ad5, and ΔVA adenovirus dl-sub720.
Supplemental Figure 1

(a) XPO5 siRNA

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</thead>
<tbody>
<tr>
<td>Dicer</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Exportin-5</td>
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<td></td>
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</tr>
<tr>
<td>Tubulin</td>
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</tbody>
</table>

(b) siRNA

<table>
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<tr>
<th></th>
<th>Mock</th>
<th>XPO5</th>
<th>Dicer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dicer</td>
<td>10.3 kb</td>
<td>5.7 kb</td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td>1.7 kb</td>
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</tr>
</tbody>
</table>

(c) Primers

- Primers 1: Dicer 190-292
- Primers 2: Dicer 5662-5811
- Primers 3: Dicer 7460-7639

Dicer mRNA (relative amount)
Exportin-5

100200

free digested Dicer RNA

Exportin-5 /Dicer RNA RNase resistant complex

1           2            3             4

+ RanQ69L-GTP

buffer alone
+ RanQ69L-GTP

Dicer mRNA (arbitrary units)

0          500         1000         1500         2000         2500         3000         3500

IgG       XPO5

b)

Supplemental Figure 3