The ribonuclease DIS3 promotes let-7 miRNA maturation by degrading the pluripotency factor LIN28B mRNA

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ABSTRACT

Multiple myeloma, the second most frequent hematologic tumor after lymphomas, is an incurable cancer. Recent sequencing efforts have identified the ribonuclease DIS3 as one of the most frequently mutated genes in this disease. DIS3 represents the catalytic subunit of the exosome, a macromolecular complex central to the processing, maturation and surveillance of various RNAs. miRNAs are an evolutionarily conserved class of small noncoding RNAs, regulating gene expression at post-transcriptional level. Ribonucleases, including Drosha, Dicer and XRN2, are involved in the processing and stability of miRNAs. However, the role of DIS3 on the regulation of miRNAs remains largely unknown. Here we found that DIS3 regulates the levels of the tumor suppressor let-7 miRNAs without affecting other miRNA families. DIS3 facilitates the maturation of let-7 miRNAs by reducing in the cytoplasm the RNA stability of the pluripotency factor LIN28B, an inhibitor of let-7 processing. DIS3 inactivation, through the increase of LIN28B and the reduction of mature let-7, enhances the translation of let-7 targets such as MYC and RAS leading to enhanced tumorigenesis. Our study establishes that the ribonuclease DIS3, targeting LIN28B, sustains the maturation of let-7 miRNAs and suggests the increased translation of critical oncogenes as one of the biological outcomes of DIS3 inactivation.

INTRODUCTION

DIS3 encodes a ribonuclease endowed with two different RNase activities: a 3′–5′ exonucleolytic activity via the RNaseII/R(RNB) domain and an endonucleolytic activity via the PiT N-terminal (PIN) domain (1). DIS3 is the catalytic subunit of exosome, a multiprotein complex present in both the nucleus and the cytoplasm, which plays a crucial role in processing, quality control and turnover of a large number of cellular RNAs (2,3). The core of the eukaryotic exosome is composed of nine subunits that form a cylindrical, barrel-like structure with a prominent central channel (2,4,5). Six of the subunits share overall structural similarity with the bacterial phosphorolytic nuclease RNase PH and assemble into a hexameric ring. The other three subunits are positioned on one side of the ring and are characterized by S1/KH domains often found in RNA-binding pro-
teins. The eukaryotic core-exosome displays RNA-binding properties but lacks enzymatic activity, which is provided by DIS3. DIS3 binds at the distal end of the barrel relative to the S1 and KH ring and provides catalytic activity to the entire ten-subunit complex (4,5).

Recent next-generation sequencing efforts have identified somatic mutations in DIS3 in the hematological cancers multiple myeloma (MM) and acute myeloid leukemia. In MM, in particular, up to 18% of MM patients present mutations affecting this gene. DIS3 mutations are located for the most part within the major ribonuclease domains of the protein, suggesting the mutations impair the enzymatic activity of DIS3 (6,7). Indeed, mutant strains in yeast harboring nucleotide substitutions corresponding to the mutations detected in human patients show growth inhibition and changes in nuclear RNA metabolism consistent with the dysfunction of the exosome (8). In addition in vitro assays have shown that yeast DIS3 mutants have a reduced ability to exonucleolytically digest RNA substrates (8). The reduced activity of yeast DIS3 mutants alongside genomic data, that show how tumor cells usually retain only the mutated copy of DIS3, suggest a role for DIS3 as a tumor suppressor in multiple myeloma (7). However, how loss-of-function DIS3 mutations are tumorigenic and how they contribute to multiple myeloma pathogenesis remains largely unknown.

MicroRNAs (miRNAs) are an evolutionarily conserved class of small (18–22 nucleotides) noncoding, single stranded RNAs that regulate gene expression at post-transcriptional level by binding the 3′-untranslated (3′ UTR) region of mRNAs and inducing translational inhibition and/or degradation of their targets (9). miRNAs are transcribed by RNA polymerase II that produces long primary miRNA transcripts (pri-miRNAs) that subsequently undergo post-transcriptional modifications. In the nucleus, the RNase III enzyme Drosha crops the pri-miRNA into a 70 nucleotide (nt) hairpin-structured precursor (pre-miRNA). Pre-miRNA is then exported to the cytoplasm where it is cleaved by another RNaseIII enzyme, Dicer, that removes the ‘terminal loop region’, finally yielding the mature 22 nt miRNA (10). The tight control of miRNA biogenesis, both at the transcriptional and post-transcriptional level, is critical for the proper functioning of a variety of central biological processes including development, differentiation (11) and hematopoiesis (10). Deregulation of miRNA expression has been associated with multiple diseases including cancer (12,13) where a global reduction of miRNAs has been often observed as a general trait (14–16). Notably, this repression is not due to a reduction in the transcription of primary miRNA species, suggesting a critical role of miRNA processing in tumorigenesis (17).

Among miRNAs, let-7 represents an important family. It includes 12 members residing in various locations throughout the genome that are frequently deleted in human cancers. The let-7 miRNA family members act as tumor suppressors. They negatively regulate the translation of oncogenes and cell cycle regulators including RAS, MYC and HMGAl2 (11,18,19). let-7 miRNAs present decreased expression in several cancer types, and low let-7 levels correlate with poor prognosis (20,21). Conversely, overexpression of let-7 blocks cellular proliferation, inhibits cell growth and impairs tumor development in a xenograft model of non-small cell lung cancer (22).

The pluriptency factor LIN28 reduces let-7 expression, blocking its maturation (22,23). Reducing let-7, LIN28 maintains the undifferentiated and proliferative state of stem cells (24). LIN28 is highly expressed during embryogenesis and then silenced in adult somatic cells. There are two paralogues of LIN28 (LIN28A and LIN28B) structurally similar but distinguishable in expression patterns, subcellular localization and mechanism leading to let-7 inhibition (25–27). LIN28A acts in the cytoplasm, recruiting a TUTase, ZCCHC11 to let-7 precursors and hampering their processing by Dicer (28). On the contrary, LIN28B sequesters immature let-7 transcripts in the nucleus, inhibiting their further processing by Microprocessor (27). LIN28A and LIN28B exert prominent roles in stem cell biology, tissue development, and also in tumorigenesis (29). LIN28A and B untimely and inappropriate re-expression has been reported in several cancer type (29). They are often overexpressed in a mutually exclusive manner, and behave as onco-genes largely through their repression of let-7 miRNAs (30).

In this study, we aimed to explore and define the role of DIS3 on miRNA biogenesis. Our findings point to a crucial role for the ribonuclease DIS3 in promoting the maturation of the let-7 miRNA tumor suppressor family, through a DIS3-mediated control of the pluriptency factor LIN28B.

MATERIALS AND METHODS

Cell culture and transfections

U2OS, HEK-293T and NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% of Penicillin and Streptomycin antibiotics. RPMI-8226 and KMS12-BM cells were cultured in RPMI medium with 10% FBS and 1% of Penicillin and Streptomycin. To silence SKI2 and MTR4 we used Dharmacon SMART pool siRNAs (Dharmacon, Lafayette, CO, USA) specific for SKI2 (cat. no. L-013435-01-0010), for MTR4 (cat. no. L-031902-02-0010) or a SMART pool of scrambled siRNAs (cat. no. D-001810-01-20) as control. To overexpress miRNA let-7a U2OS cells were transfected with miRNA mimic of let-7a (Ambion, Naugatuck, CT, USA; cat.no. 4464066) or a miRNA mimic negative control (cat. no. 4464058). siRNAs and miRNA mimics were transfected in U2OS cells by lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) at a final concentration respectively of 30 and 33 nM according to the manufacturer’s instructions.

Lentiviral vectors

To knockdown DIS3 expression, pLKO.1 lentiviral vectors carrying short hairpin RNAs targeting human or murine DIS3 were used in infection experiments. Non-targeting, scrambled shRNA (ctrl shRNA) was used as negative control. For further details see supplementary methods.

Western blotting

The protocol, the antibodies and working dilutions are described in details in the supplementary methods.
RNA extraction, fractionation and qRT-PCR

Total RNA was extracted from cells using TRIzol® RNA Isolation Reagent (Invitrogen, Carlsbad, CA, USA) and transcripts were quantified by q-RT-PCR using comparative threshold cycle method. Further details are provided in the supplementary methods.

mRNA and miRNA expression profiling

Total RNA from RPMI-8226 and KMS12-BM cell lines knocked-down with a scrambled shRNA (control sh) or DIS3 shRNA (DIS3 sh) was collected 72 h after infection and extracted with TRIzol® RNA Isolation Reagent. Quality assessment was performed using a Bioanalyzer Agilent 2100 (Agilent, Santa Clara, CA, USA). Total RNA samples were processed using the FlashTag labeling kit and then hybridized to the GeneChip® miRNA arrays v1.0 (Affymetrix Inc., Santa Clara, CA, USA), according to the Affymetrix recommended protocol. Expression values for 847 human miRNAs were extracted from CEL files using Affymetrix miRNA QC tool software (RNA normalized and log2-transformed (31)). Data were analyzed using the software dCHIP (http://biosun1.harvard.edu/complab/dchip). The microarray data have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE55246 (available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=orydwgsrvmnbaz&acc=GSE55246). Gene set enrichment analysis (GSEA) was performed as previously described (GSEA v2.0 at http://www.broad.mit.edu/gsea, (32)) using gene set as permutation type and 1000 permutations and signal to noise as metric for ranking genes. In line with GSEA default settings, only miRNA families including at least five members were included in the analysis.

RNA stability assay

U2OS infected with a scrambled shRNA or a shRNA specific for DIS3 were puromycin selected and, 72 h after infection, were treated with 100 µg/ml of cycloheximide for 0.5, 1, 2, 3 h. Cell lysates were blotted for MYC and LaminB. Reverse transcription was performed with random primers and mRNA levels were measured by qRT-PCR. Primers sequences are provided in Supplemental Table S3.

Cycloheximide treatment

U2OS infected with a scrambled shRNA, as control, or with a shRNA specific for DIS3 were puromycin selected and, 72 h after infection, were treated with 100 µg/ml of cycloheximide for 0.5, 1, 2, 3 h. Cell lysates were blotted for MYC and LaminB.

Focus formation assay

Focus formation assay of NIH3T3 cells infected with scrambled shRNA (as control) or with shRNA specific for DIS3 were plated at 60,000 cells for six-well, grown for 21 days, fixed and stained with crystal violet.

RESULTS

Loss of DIS3 specifically decreases let-7 miRNA levels, without affecting other miRNA families

To investigate the potential role of DIS3 in miRNA regulation, we first explored miRNA changes resulting from DIS3 inactivation. To this end, we tested five shRNAs targeting DIS3 in HEK-293T cells and obtained a robust knockdown with two hairpins, shRNA2 and shRNA4 (Figure 1A). We then infected two different MM cell lines, KMS12-BM (KMS12) and RPMI-8226 (RPMI), with DIS3 shRNA4 or a scrambled shRNA, as control (Figure 1B). The RNA obtained from these cells was then hybridized to miRNA arrays, where their differential expression was assessed upon DIS3 down-regulation. Remarkably, only a small fraction of the miRNAs present on the platform was impacted by DIS3 knockdown (Figure 1C). Also, to our surprise, the number of miRNAs up-regulated after DIS3 inactivation was more than compensated by the miRNAs that were down-regulated, suggesting that DIS3 might not directly control miRNA levels. In all, four miRNAs were up-regulated and eight were down-regulated 1.5-fold or more in both cell lines. Intriguingly, among the miRNAs down-regulated, there were several members of the let-7 family (Figure 1D). To obtain a more rigorous assessment of the miRNA families impacted by DIS3, a Gene Set Enrichment Analysis (GSEA) was performed (32). Remarkably, the only pathway that was significantly dysregulated as a result of DIS3 inactivation was indeed the let-7 miRNA family (NES = 1.48, P < 0.05, FDR = 0.099, Figure 1E), with almost all the members of this family affected by DIS3 knockdown. Intriguingly, however, let-7 family members were down-regulated, and not up-regulated, as it would have been predicted given the ribonuclease activity of DIS3 (Figure 1F). To validate the array data, we chose four members of the let-7 family (let-7a, let-7b, let-7f, let-7g) and we evaluated the effect of DIS3 silencing on their expression by quantitative PCR (Figure 1G). Additionally, to further confirm the miRNA array data, we performed a northern blot to visualize the levels for one member of the let-7 family (let-7g), in the same cells (Figure 1H). Consistent with the miRNA array results, probe for let-7g detected a reduction after DIS3 silencing. To rule out that let-7 reduction was an off target effect of shRNA4, we evaluated let-7 levels upon DIS3 knockdown with the second shRNA (shRNA2). ShRNA2-mediated silencing of DIS3 reduced the levels of all let-7 tested, as with shRNA4 (Supplementary Figure S1). All together, these data suggest that DIS3 does not pervasively regulate miRNAs, but rather impacts only on a very limited set of miRNAs, specifically affecting the miRNA family of tumor suppressor let-7.

We next ascertained whether let-7 modulation by DIS3 was a mechanism specific for MM or could be extended to unrelated cellular systems. We hence knocked down DIS3 in human osteosarcoma U2OS cells, in human kidney embryonic HEK-293T cells and in mouse embryonic fibroblast NIH3T3 cells, using an shRNA targeting respectively the DIS3 human (shRNA4) and DIS3 mouse sequence (Figure 2A). qPCR demonstrated a strong reduction for let-7a, let-7b, let-7f and let-7g in U2OS and NIH3T3 cells, compa-
Figure 1. DIS3 knockdown in MM cells selectively affects let-7 miRNA family. (A) Western blot to test the efficiency of five different shRNAs to silence DIS3 in HEK-293T cell line. (B) Western blot analysis of DIS3 expression in MM cell lines. KMS12-BM (KMS12) AND RPMI-8226 (RPMI), infected with a scrambled shRNA (ctrl) or DIS3 shRNA4 (shRNA). Lamin B was used as loading control. (C) Pie charts summarizing the number of miRNAs deregulated >1.5-fold in KMS12 and RPMI, and in both cell lines after DIS3 knockdown. (D) Heat map showing the expression levels of miRNA varying >1.5-fold in both KMS12 and RPMI upon DIS3 silencing. The color scale bar in the heat map represents the relative miRNA expression with red representing up-regulation and blue representing down-regulation. (E) GSEA ES plot showing the enrichment of let-7 miRNA family gene set after DIS3 silencing. (F) Heat map of let-7 miRNA family levels in KMS12 and RPMI cell lines after infection with a scrambled shRNA (ctrl) or DIS3-specific shRNA4 (sh). (G) Expression of the let-7 miRNA members (a, b, f, g) assayed by qRT-PCR in KMS12 and RPMI cell lines infected with scrambled (ctrl) or DIS3-specific shRNA4 (sh), 72 h after infection. Results are normalized over RNU6B. Bars represent SDs (n = 2 independent experiments). *P < 0.05; **P < 0.005 using two-tailed Student’s t test. (H) Northern blot analysis of endogenous let-7g in KMS12 and RPMI cell lines infected with scrambled (ctrl) or DIS3-specific shRNA4 (sh), 72 h after infection. RNU6B was used as loading control.
Figure 2. *let-7* levels after *DIS3* knockdown in human and mouse cell lines. (A) Western blot analysis of *DIS3* expression in human U2OS and HEK293T cells, and in mouse NIH3T3 cells, 72 h after infection with scrambled (ctrl) and human or mouse *DIS3*-specific shRNAs (sh). Results are normalized over loading control Lamin B and RAN. (B) Expression of the *let-7* miRNA members was assayed by qRT-PCR. Results are normalized over *RNU6B* and *miR-16* respectively for human and mouse cells. Bars represent SDs (*n* = 2 independent experiments). *P* < 0.05 using two-tailed Student’s *t* test.

**DIS3** knockdown increases MYC and RAS protein levels through *let-7*.

*Let-7* miRNAs act as tumor suppressors by reducing the levels of oncogenes including RAS, MYC, and HMGA2 (24). We therefore tested the hypothesis that *let-7* reduction, mediated by *DIS3* knockdown could lead to an increase in MYC and RAS levels. The depletion of *DIS3* was indeed associated with an increase in MYC and RAS protein levels in MM cells as well in NIH3T3 cells and U2OS cell lines (Figure 3A and Supplementary Figure S2A). Similar results were obtained using the second shRNA, shRNA2 (Supplementary Figure S3A). We then tested whether the mRNA levels of MYC and RAS were similarly affected by *DIS3* down-regulation. Notably, no significant changes in MYC and RAS mRNA levels were detected, suggesting that *DIS3* controls indirectly MYC and RAS protein expression levels, not through RNA modulation (Figure 3B, Supplementary Figures S2B and S3B). To gain insight on the mechanism responsible for this phenotype, we explored the possibility that *DIS3* could affect the stability of the proteins. To this end, RPMI cells were treated with cycloheximide to abolish mRNA translation, and MYC protein levels were determined by western blotting at increasing time points after the addition of the drug, with or without *DIS3* knockdown. The kinetic of MYC degradation was similar between cells infected with *DIS3* shRNA to that observed in cells infected with a control shRNA (Figure 3C). All together, these results imply that *DIS3* does not affect the stability of the protein but rather promotes the translation of *MYC* mRNA. It has been previous shown that *let-7* regulate the translation of their targets, more than the mRNA levels (33–35). Indeed, our results obtained on HEK-293T cells support this notion. In these cells, where *let-7* levels did not change after *DIS3* knockdown (Figure 2), both the protein and the mRNA levels of MYC and RAS similarly remained unchanged (Supplementary Figure S4).

To conclusively demonstrate that the increase in MYC protein levels arising from *DIS3* knockdown cells was due to *let-7* down-regulation, we over-expressed a *let-7a* mimic in silenced *DIS3* cells and measured MYC levels by western blot (Figure 3D). *let-7a* mimic abrogated the increase in MYC protein levels induced by *DIS3* knock-down, demonstrating that *DIS3* affects MYC through *let-7*.

Reduced *let-7* miRNAs correlate with increased transformation capacity in vitro (36). We then ascertained whether *DIS3* silencing could promote transformation. To this end, a focus formation assay was performed in NIH3T3 cells. We found that *Dis3* knockdown robustly induced focus formation, suggesting that *Dis3* silencing is able to transform...
Figure 3. **DIS3** silencing increases MYC and RAS proteins and induces transformation. (A) Representative blot and quantification of **DIS3**, MYC and RAS proteins in RPMI, KMS12 and NIH3T3 cells, 72 h after infection with scrambled (ctrl) or human and mouse **DIS3**-specific shRNAs (sh). Results are normalized over loading control lamin B and RAN, respectively for MM cells and NIH3T3 cells. The error bars represent SD of two independent experiments. (B) **MYC** and **RAS** mRNA levels normalized over GAPDH in the same cells of panel (A). Bars represent SDs (n = 2 independent experiments). *P < 0.05, **P < 0.005. (C) U2OS infected with scrambled (ctrl) or **DIS3**-specific (sh) shRNAs were treated with 100 μg/ml cycloheximide for the time indicated and lysates were immunoblotted for **DIS3** and MYC. Lamin B represents a loading control. (D) Western blot and quantification of levels for endogenous **DIS3** and MYC in U2OS cells knocked-down with a scrambled shRNA (ctrl) or a **DIS3** specific shRNA (shRNA4, sh), 48 h after transfection with let-7a (+) or control RNA (−) mimics. Lamin B was used as loading control. (E) Western blot of **DIS3** levels and focus formation assay of NIH3T3 cells infected with scrambled shRNA (ctrl) or with murine **DIS3** shRNA4 (sh). Colonies were counted from three independent platings. The error bars represent SD. **P < 0.005 using two-tailed Student’s t test. RAN represents the loading control.
Figure 4. DIS3 affects let-7 processing regulator LIN28B. (A) Measurement by qRT-PCR of the pri-miRNAs let-7-a/f/d (left panel) and mature miRNA let-7-a (right panel) in RPMI, KMS12 and U2OS cells, 72 h after infection with scrambled (ctrl) or DIS3-specific shRNA (sh). pri-miRNAs let-7-a/f/d expression data were normalized over GAPDH. let-7-a expression data were normalized over RNU6B. Bars represent SDs (n = 2 independent experiments). * P < 0.05; ** P < 0.005 using two-tailed Student’s t test. (B) LIN28A and LIN28B mRNA levels assessed by qRT-PCR with respect to GAPDH expression 72 h after infection. Bars represent SDs (n = 2 independent experiments). ** P < 0.005 using two-tailed Student’s t test. (C) Blot (left panel) and quantification (right panel) representative for two experiments showing DIS3 and LIN28B protein levels in RPMI, KMS12 and U2OS 72 h after infection with shRNA targeting DIS3. Lamin B was used as loading control.
DIS3 controls let-7 through LIN28B. LIN28B mRNA (left panel) and let-7-a and let-7-g (right panel) levels of one representative experiment in which RPMI cells infected with a scrambled shRNA (ctrl) or with a DIS3 shRNA (DIS3 sh) underwent, after 3 days, a second infection with a scrambled shRNA (ctrl) or with a LIN28B shRNA. LIN28B and let-7 levels were measured 4 days after the second infection and normalized over GAPDH and RNU6B respectively. Bars represent SDs (n = 2 replicas of one representative experiment).

Figure 5. DIS3 controls let-7 through LIN28B. LIN28B mRNA (left panel) and let-7-a and let-7-g (right panel) levels of one representative experiment in which RPMI cells infected with a scrambled shRNA (ctrl) or with a DIS3 shRNA (DIS3 sh) underwent, after 3 days, a second infection with a scrambled shRNA (ctrl) or with a LIN28B shRNA. LIN28B and let-7 levels were measured 4 days after the second infection and normalized over GAPDH and RNU6B respectively. Bars represent SDs (n = 2 replicas of one representative experiment).

fibroblasts in vitro (Figure 3E). This result was confirmed with a second shRNA for DIS3 (Supplementary Figure S5).

DIS3 controls let-7 processing through LIN28B.

Transcriptional and post-transcriptional mechanism, including processing and maturation from the pri- to pre- and mature forms, regulate miRNA levels (10). We next sought to define whether DIS3 directly affects let-7 transcription or is instead involved in its maturation. To this end, we compared and contrasted the levels of immature pri-let-7 with mature let-7 after DIS3 knockdown. We did not detect any reduction in let-7 primary transcripts upon DIS3 down-regulation, indeed even an increase, especially in KMS12 cells (Figure 4A, left panel). On the contrary, qPCR analysis demonstrated a consistent reduction in mature let-7, in DIS3 silenced cell lines, including RPMI, KMS12 and U2OS cells (Figure 4A, right panel). The discrepancy between reduced let-7 mature levels and unchanged or even increased let-7 immature forms strongly suggests a role for DIS3 on let-7 maturation. These results further imply the existence of a negative regulatory factor inhibiting let-7 processing in DIS3 knocked down cells.

Previous studies have demonstrated that let-7 cleavage and maturation is under the control of the pluripotency factor LIN28 (29). We then asked whether DIS3 down-regulation alters the levels of LIN28A and LIN28B. qPCR and western blot analysis revealed that the expression levels of LIN28A and LIN28B were variable among the cell lines tested, with KMS12 and U2OS showing the highest, and RPMI and NIH3T3 with low basal levels (Figure 4B and C and Supplementary Figures S6 and S7). Notwithstanding, we found a specific increase of LIN28B both at the RNA and at the protein level upon DIS3 knockdown, with both shRNAs, in all cell lines where let-7 was reduced upon DIS3 knock-down (Figure 4B and C and Supplementary Figures S6–S8). Intriguingly, LIN28A, expressed at very low levels also in basal conditions, was not affected by DIS3 silencing suggesting that DIS3 controls let-77 through a selective modulation of LIN28B levels (Figure 4B). Accordingly, LIN28B levels did not change in HEK-293T cells, where let-7 levels did not decrease upon DIS3 knockdown (Supplementary Figure S9).

To conclusively demonstrate that LIN28B is required for the repression of let-7 as a result of DIS3 knockdown, the concomitant silencing of LIN28B and DIS3 was carried out in RPMI and U2OS cells (Figure 5 and Supplementary Figures S10 and S11). We found that in both cell lines knockdown of LIN28B completely abrogated the reduction of mature let-7 mediated by DIS3 silencing, confirming that LIN28B is required for DIS3-mediated let-7 reduction (Figure 5 and Supplementary Figure S11, right panel).

DIS3 affects RNA stability of LIN28B.

We next investigated the relationship between DIS3 and LIN28B. As DIS3 is the catalytic subunit of the exosome, a complex playing a crucial role in exosome-mediated RNA processing and decay (37) and since its silencing results in an increase of LIN28B mRNA levels, we speculated that DIS3 could be involved in the degradation of LIN28B RNA.

To test this hypothesis, we infected U2OS cells with a scrambled shRNA or with a shRNA specific for DIS3, treated cells with 5,6-dichloro-ribofuranosylbenzimidazole (DRB) to block RNA synthesis and then measured LIN28B RNA or GAPDH (as control) levels over a 4-h time interval. Upon transcriptional block, LIN28B RNA levels were significantly more stable after DIS3 knockdown when compared with cells infected with scrambled shRNA (Figure 6A). On the contrary GAPDH mRNA half-life was not affected. These results indicate that DIS3 specifically controls the mRNA stability of LIN28B.

It has been previously shown that exosome preferentially degrades RNA enriched in ARE sites (38,39). AREs consist of one or several AUUUA pentamers located in an adenosine and uridine rich region (40). These elements are bound by ARE-binding proteins endowed with RNA stabilizing or destabilizing functions, able to modulate mRNA stability or translation efficiency of mRNA target (40,41). Indeed, an analysis using the AREsite tool (http://rna.tbi.univie.ac.at/Aresite), revealed a significant enrichment of productive, accessible and conserved AU-rich elements in LIN28B mRNA (Supplementary Figure S12). Since mRNAs containing an AU-rich element (ARE) in the 3’ UTR undergo a rapid decay in the cytoplasm (42), we hypothesized that LIN28B degradation by the exosome occurs in the cytoplasm. The eukaryotic exosome associates with two ATP-dependent regulators, the cytoplasmic SKI complex and the nuclear TRAMP complex. Both interact with RNA substrates and thread them through the internal channel of the exosome core (43). Each complex is endowed with an helicase activity, provided respectively by SKI2 and MTR4, to unwind RNA targets. To determine in which cellular compartment LIN28B mRNA is degraded, we blocked cytoplasmic or nuclear exosome activity by transfecting U2OS with a pool of siRNAs specific for SKI2 or MTR4. Forty-eight hours after transfection we treated cells with DRB and measured LIN28B and GAPDH RNA levels over a 4-h time interval (Figure 6B and C and Supplementary Figure S13). We found that the inactivation of the cytoplasmic complex SKI increased LIN28B mRNA stability, recapitulating the effect observed upon DIS3 silencing (Figure 6B). Once again the stabilization was specific because no significant changes were observed on GAPDH mRNA half-life. On the contrary silencing of MTR4, the helicase included in the nuclear complex TRAMP, did not affect the stability of LIN28B mRNA (Figure 6C). All together these results suggest that LIN28B mRNA levels are specifically controlled
Figure 6. DIS3 affects RNA stability of LIN28B RNA. U2OS cells were infected with a scrambled shRNA (control sh) or a DIS3 shRNA (shRNA4) (A) or were transfected with a control siRNA (ctrl) or a siRNA against SKI (B) or TRAMP (C). 3 days after infection and selection or 48 h after transfection, cells were treated with DRB (100 μg/ml) to block RNA synthesis. The stability of LIN28B and GAPDH total mRNA was measured by qRT-PCR at 0, 2, 3 and 4 h after treatment and expressed as relative abundance with respect to mRNA level at 0 h. Bars represent SDs (n = 2 independent experiments in A; n = 2 replicas of one representative experiment in B and C). * P < 0.05 using two-tailed Student’s t test.

by DIS3, mostly in the cytoplasm and probably through the recognition of ARE sites element present in the 3’ UTR.

**DISCUSSION**

In this study, we provide evidences that DIS3 inactivation stimulates oncogenic signaling pathways through a down-regulation of the tumor suppressor miRNA family let-7. DIS3 loss selectively increases LIN28B levels, a negative regulator of let-7 maturation. The up-regulation of LIN28B decreases mature let-7 and de-represses of downstream let-7 targets. In particular the protein levels of two critical onco-genes, MYC and RAS, increased. Indeed, DIS3 knockdown transformed NIH3T3 cells (Figure 7).

Curiously, in cancer, DIS3 mutations have been found in MM and, at a lower percentage, in AML, while mutations in this gene have not been reported in the sequencing efforts ongoing in epithelial cancers, with hundreds of patient genomes screened as of today. Nonetheless, our findings suggest that DIS3 impacts on the let-7 pathway in cell lines of hematopoietic and non-hematopoietic heritage, raising MYC and RAS protein levels in both cellular contexts. One possible explanation for the selective presence of DIS3 somatic mutations in haematological cancers is the prominent role exerted by MYC, and to a lesser extent by RAS, in these cancers. KRAS and NRAS have been recently confirmed as the most mutated genes in MM (6,7,44,45). MYC is deeply involved in the pathogenesis of several hematological cancers. In MM, in the more advanced stages, chromosomal rearrangements juxtapose the MYC locus on 8q24 with the IgH or IgL locus, resulting in a several-fold enhancement of MYC transcription (46). Recently, a more general role for MYC has been proposed during MM pathogenesis. In fact, both mouse and human data support the notion that a moderate increase in MYC expression would be crucial for the transition from the pre-malignant condition of monoclonal gammopathy of undetermined significance (MGUS) toward frank MM (47). Herein, we demonstrate that DIS3 silencing affect MYC protein levels without any change in RNA levels. It would be tempting to speculate that MM cells empower crucial oncogenes such as RAS and MYC not only increasing their mRNA levels, but, in selected cases, further boosting their activity through increased translation rate. In line with our results a recent study showed that MMSET, the histone methyltransferase translocated in up to 15% of MM patients, specifically impacts on MYC translation, and not mRNA levels, through the down-regulation of miR-126* (48). Taken together these results suggest that
the dysregulation of MYC translation could have a strong impact on MM pathogenesis. As such, the conventional assessment of MYC RNA levels as a reliable readout of MYC activity might not be adequate, in particular for the identification of the patient subgroups endowed with enhanced translation.

DIS3 is the catalytic subunit of the exosome, a multi-protein complex present both in the cytoplasm and in the nucleus that is involved in the degradation of many RNA species. The cytoplasmic exosome is not required for viability and it targets normal and aberrant mRNAs, regulating their 3′ to 5′ turnover (49,50). The activity and the role of the nuclear exosome are less well understood. Unlike its cytoplasmic counterpart, it is required for viability, and it targets a broader set of RNA species, including pre-mRNAs, pre-tRNAs, pre-rRNAs, snRNAs and snoRNAs (3,51–58). Several studies have started to comprehensively map genes and pathways affected by the specific inactivation of various exosome components (59–64). However, the mechanistic details by which the exosome or its subunits shape cell physiology are largely unknown. In particular it remains unclear whether the inactivation of the enzymatic activity of this complex would have a pervasive impact on RNAs and ultimately on cell physiology, or whether its activity is channeled through specific targets and pathways, with clear cellular outcomes. Our findings argue in favor of this second hypothesis, given the limited influence of DIS3 on miRNAs as a whole, and its specific effect on LIN28B and let-7 family.

The human genome encodes three DIS3 homologues: DIS3, DIS3L, and DIS3L2. Unlike DIS3 and DIS3L (65), DIS3L2 is not associated with the exosome (66,67), since it lacks the conserved N-terminal PIN and CR3 domains (65,68). Surprisingly, it has been recently shown that DIS3L2 regulates the let-7 pathway, with a mechanism however that is entirely different from the one described herein for DIS3 (69). In fact, our data suggest that these ribonucleases regulate let-7 biogenesis at two different steps, in two different cellular compartments and with a different mechanism. Let-7 are initially transcribed as primary transcripts (pri-let-7) and then cleaved by the microprocessor complex of DGR8 and DROSHA into 70- to 100-nt hairpin-shaped precursors (pre-let-7) (70). These pre-let-7 are exported into the cytoplasm and processed by the RNase III enzyme Dicer to their mature form (let-7). DIS3L2 degrades uridylated pre-let-7 in the cytoplasm, partnering with LIN28A. On the contrary DIS3 modulates let-7 pathway through the regulation of LIN28B levels, thus affecting the abundance of primary let-7 transcripts available for microprocessor activity. Therefore, the inactivation of DIS3L2, as reported in Perlman syndrome and in a subset of Wilms tumors, would have entirely different functional consequences for the cell, when compared with the effect of DIS3. In fact, DIS3L2 knockdown results in the accumulation of uridylated pre-let-7 with no changes in the levels of mature let-7, consistent with the DIS3L2 preferential targets, pre-let previously modified (uridylated) and thus no more available for maturation. On the contrary DIS3 silencing reduces let-7 mature levels, ultimately affecting the whole let-7 pathway downstream, an outcome not anticipated in the presence of DIS3L2 inactivation.

In conclusion, we reveal a novel regulation pathway of let-7 miRNAs controlled by the exoribonuclease DIS3. We identified LIN28B as a direct target of the enzymatic activity of DIS3 and we demonstrated that through LIN28B DIS3 selectively regulates a subset of critical let-7 targets such as MYC and RAS. Future studies are warranted to determine the extent to which this pathway is affected in MM patients mutated for DIS3 and how it contributes to MM pathogenesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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