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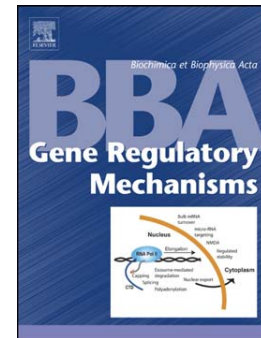
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Patrick Connerty, Sarah Bajan, Judit Remenyi, Frances V. Fuller-Pace, Gyorgy Hutvagner

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**The miRNA biogenesis factors, p72/DDX17 and KHSRP regulate the protein level of Ago2 in human cells.**

Patrick Connerty<sup>2</sup>, Sarah Bajan<sup>1</sup>, Judit Remenyi<sup>3</sup>, Frances V. Fuller-Pace<sup>3</sup>, Gyorgy Hutvagner<sup>1,4</sup>

1: Faculty of Engineering and Information Technology, Centre of Health Technologies, University of Technology, Sydney, Australia

2: Faculty of Science, University of Technology, Sydney, Australia

3: Division of Cancer Research, Jacqui Wood Cancer Centre, University of Dundee, Ninewells Hospital and Medical School, Dundee, UK

4: Correspondence to be sent: [gyorgy.hutvagner@uts.edu.au](mailto:gyorgy.hutvagner@uts.edu.au)

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**Abstract:**

MicroRNAs (miRNAs) are short (21-23nt long) RNAs that post-transcriptionally regulate gene expression in plants and animals. They are key regulators in all biological processes. In mammalian cells miRNAs are loaded into one of the four members of the Argonaute (Ago) protein family to form the RNA-induced silencing complex (RISC). RISCs inhibit the translation of mRNAs that share sequence complementarity with their loaded miRNAs. miRNA processing and miRNA-mediated gene regulation are highly regulated processes and involve many RNA-binding proteins as auxiliary factors.

Here we show that the two RNA-binding proteins, p72 and KHSRP, both with known roles in promoting miRNA biogenesis, regulate the protein level of human Ago2 in transformed human cells. We determined that p72 and KHSRP influence Ago2 stability by regulating miRNA levels in the cell and that loss of p72/KHSRP results in a decrease of unloaded Ago2.

## Introduction

miRNAs are key post-transcriptional regulators of gene expression. Mature miRNAs undergo several processing steps before they are loaded onto the RNA induced silencing complex (RISC) and are engaged in gene repression. In general, primary miRNAs (pri-miRNA) are processed in the nucleus by the Microprocessor complex into a precursor miRNA (pre-miRNA) [1]. The precursor is then transported to the cytoplasm [2], where Dicer cleaves it into a double stranded miRNA complex [3]. One strand of the miRNA is then loaded onto an Argonaute protein which uses the bound strand as a guide for the sequence-specific targeting of mRNAs [4–7].

miRNAs control highly regulated biological pathways such as cell differentiation, proliferation and the immune response; therefore, it is not surprising that miRNA-mediated gene regulation itself is also tightly regulated. Transcription of miRNAs, miRNA processing and miRNA maturation are all influenced by auxiliary factors that modulate general miRNA biosynthesis and function or regulate the biogenesis of a subset of miRNAs [8].

p72 (DDX17) and KH-type splicing regulatory protein (KHSRP), are two RNA-binding proteins that are necessary for the production of subsets of miRNAs [9–11]. p72 is a DEAD-box helicase subunit of the Drosha complex which is required for altering pri-miRNA structure to facilitate Drosha processing [12]. KHSRP associates with pre-miRNA loops and promotes the recruitment of Dicer to pre-miRNAs [13]. KHSRP interacts with single-strand AU-rich-element-containing mRNAs and plays a vital role in mRNA decay, and binds to RNA-degrading enzymes and thus can promote rapid RNA degradation [14]. Additionally, KHSRP auto-regulates *let-7* production and in turn regulates Dicer expression through a negative feedback regulatory mechanism, as Dicer mRNA is a target of *let-7* [8,15,16].

An increasing amount of evidence shows that miRNA-mediated gene regulation is

regulated through post-translational modification of Ago2, the main effector protein of the miRNA pathway in mammals [17]. Inhibition of HSP90 (Heat Shock Protein 90), a chaperone required for the loading of miRNAs into Argonaute proteins, leads to the degradation of unloaded Ago2 protein. This mechanism is mediated by the proteasome and therefore likely involves the ubiquitination of Ago2 [17,18]. Recent studies have shown that decreasing miRNA levels induces the reduction of Ago2 protein levels post-transcriptionally [19,20]. In these studies the roles of both the proteasome [20] and the lysosome were demonstrated [19].

Here we report that inhibiting auxiliary factors to the miRNA pathway, p72 and KHSRP, which promote the processing of abundant miRNAs at distinct steps of miRNA maturation, also leads to reduced levels of Ago2 protein in human cells. We also demonstrate that the loss of Ago2 is regulated post-transcriptionally and the lowered Ago2 expression is a direct consequence of the decreased miRNA levels in the cell. We also have evidence that this mechanism affects the pool of Ago2 that is not loaded with miRNAs.

**Material and methods*****siRNAs, PCR primers, RNA mimics,***

siRNA targeting p68: CUCUAAUGUGGAGUGCGAC. siRNAs targeting p72 used in this study: CAAGGGUACCGCCUAUACC, ON-TARGETplus SMARTpool DDX17 siRNA (L-013450-01-0005), siGenome Smartpool Anti-DDX172 (Dharmacon), siRNA targeting KHSRP: ON-TARGETplus SMARTpool KHSRP (L-009490-00-0005).

Taqman probes used are as follows: EIF2C2 (Hs01085579\_m1), DDX17 (Hs00428757\_m1), KHSRP (Hs01100867\_g1), 18S (Hs03003631\_g1), GAPDH (Hs02758991\_g1).

Taqman miRNA assay probes used were as follows: U75 (001219), hsa-miR-21(4427975), hsa-let-7e (4427975), hsa-let-7a(4427975), hsa-miR-17-3p (4427975). hsa-miR-19 (4427975) .RNA mimic used are as follows: hsa-miR-17-3p mirVana miRNA mimic (4464066).

***Plasmids:***

pCMV5 FLAG::Ago2 plasmid (Johnson et al, 2010).

psiCHECK2-let-7 X8 was a gift from Yukihide Tomari [21] psiCHECK2 -let-7 3x [22]

***Cell culture:***

HeLa, U2OS and HEK293 cells were grown in DMEM (Gibco) supplemented with 2mM L-glutamine, 10% fetal calf serum and 1% Penicillin Streptomycin. Cells were grown in T75 flasks at 37°C in 5% CO<sub>2</sub>

***siRNA and miRNA transfection:***

Cells were transfected with appropriate smartpool siRNAs (20 nM) (Dharmacon) and miRNA mimic (Life Technologies) using RNAiMax transfection reagent (Invitrogen) following the manufacturer's instructions. Unless stated, a second transfection of 20 nM siRNA was administered to each well 24 hours after initial transfection. Cells were harvested 24 hours after second siRNA treatment. Each transfection was performed in a six well plate.

***Plasmid transfections:***

HeLa cells were co-transfected with 20nM siRNA and increasing amount of FLAG tagged Ago2 expressing plasmid using Lipofectamine 2000 (Invitrogen) following the manufacturers instruction. After 48 hours post initial transfection cells were harvested followed by the purification of RNA and proteins. HeLa cells were transfected with KHSRP siRNA as outlined above. 24 hours after initial transfection cells were re-transfected with KHSRP siRNA as well as luciferase plasmids at 160 ng plasmid per well using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. 24 hours later cells were harvested for RNA and protein purification or lysed for luciferase assay.

***Geldanamycin treatment:***

HeLa cells were transfected with p72/KHSRP siRNA as described above. 48 hours after transfection Geldanamycin was diluted in DMSO and added to cells for 18 hours at a final concentration of 10 uM. Cells were then harvested for RNA and protein purification.

***MG132 treatment:***

HeLa cells were transfected with p72/KHSRP siRNA as described above. 30 hours after transfection MG132 was diluted in DMSO and added to cells for 18 hours at a final concentration of 20  $\mu$ M. Control cells were treated with DMSO. Cells were then harvested for RNA and protein purification.

***Protein Purification:***

Protein samples were collected by lysing cells in 200 $\mu$ L of NP-40 Lysis Buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (v/v) NP-40, protease inhibitors). The lysate was centrifuged for 5 minutes at 12,000 x g at 4°C and the supernatant was saved and resuspended in 30 $\mu$ L of 1M DTT and 270 $\mu$ L of 2 X SDS loading dye (125mM 1M Tris (pH 6.8), 4% v/v SDS, 20% v/v Glycerol, 0.1% w/v Bromophenol Blue). The concentration of the protein was determined using Pierce BCA protein Assay Kit (Thermoscientific) following manufacturer's instructions. The samples were then heated at 95°C for 10 minutes and stored at -20°C.

***Dual Luciferase Assay:***

Cells transfected with luciferase plasmid with Lipofectamine 2000 (Invitrogen) transfection reagent following the manufacturer's instruction and 24 hours after transfection were lysed in 100 $\mu$ L of passive lysis buffer (Promega) per well of a 24 well plate. Luciferase activity was quantified using a dual luciferase reporter assay system (Promega). Luminescence was measured on a Microlumat Plus LB96V microplate Luminometer. Each sample was run in triplicates and averaged for analysis.



**Western blot:**

Protein samples were run in 10% SDS-polyacrylamide Tris-glycine gels. Antibodies used are as follows: Ago2 Rat monoclonal Anti-Ago2 Clone 11A9 (Sigma Aldrich), Rabbit Monoclonal Anti-KHSRP (Cell Signaling Technology), Mouse Monoclonal-Anti- $\alpha$ -Tubulin Clone DM1A (Sigma Aldrich), all at a 1:1000 dilution. Donkey Polyclonal Secondary Antibody to Rat IgG (Abcam), Donkey Polyclonal Secondary Antibody to Mouse IgG (Abcam), Anti-Rabbit IgG, HRP-linked Antibody (Cell Signaling Technology) all at a 1:5000 dilution. Images were visualized using LAS-3000 Imaging System (Fuji). Western blots were quantified using ImageJ software

**Quantitative real time PCR:**

All qPCRs to measure the level of miRNAs and mRNAs were completed with the ABI's Taqman® system. Total RNA was purified from cells using Direct-zol RNA miniPrep kit (Zymo) following the manufacturer's instructions and diluted to 200ng/ $\mu$ L in RNase free water. For mRNA quantification cDNA was synthesised using TaqMan reverse transcription reagents (Applied Biosystems) following the manufacturer's instructions. For small RNA quantification cDNA was synthesized from TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). cDNA samples were diluted 1:4 with RNase free water. cDNA samples were then prepared for qPCR using Taqman 2X PCR Master Mix (Applied Biosystems) followed the manufacturer's instructions. Samples were run in a PCR-7500 Real Time PCR System (Applied Biosystems). Individual biological samples were run in technical triplicates and averaged for analysis. Experiments repeated in triplicates were collectively averaged for the purposes for

calculating changes in expression.

***Statistical analysis:***

All statistical analysis was calculated using Microsoft excel. Differences/correlations between groups were calculated with Student's t-test. A P-value of less than 0.05 was considered statistically significant.

## Results

### *The RNA-binding proteins p72 and KHSRP stabilise the protein level of human Ago2*

For an unrelated study, we tested the consequences of down-regulating three RNA-binding proteins; p68, p72 and KHSRP, in HeLa cells using gene specific siRNAs. Surprisingly, we observed that the loss of both p72 and KHSRP reduce Ago2 protein level (Supplementary Figure 1A). We decided to further study this unexpected finding.

First, we used gene-specific siRNAs to knock down p72 and KHSRP expression by carrying out sequential transfection of siRNAs. qPCR analysis of the mRNA level of p72 and KHSRP (Figure 1A and Figure 2A) and Western blotting of KHSRP (Figure 2C) confirmed that the knockdown was successful and resulted in more than an 80% reduction of gene expression in both cases.

Both RNA-binding proteins have a well-described role in the regulation of processing of miRNAs. p72 is required for the production of subsets of miRNAs, which includes miR-21, by facilitating pri-miRNA processing [12]. KHSRP is also involved in the regulation of the processing of certain miRNAs including *let-7a* [16,23]. Therefore, to test if the downregulation of p72 and KHSRP impairs miRNA processing we used qPCR to quantify miR-21 and *let-7a* miRNA levels in the p72 and KHSRP targeted samples respectively. We observed a strong reduction in the steady-state level of both miRNAs (Figure 1B and Figure 2B) in the samples where p72 and KHSRP were knocked down. As a negative control we assayed the level of miR-19 which production do not require the activity of p72 or KHSRP. miR-19 level was undisturbed in both the p72 and the KHSRP knocked down cells (Figure 1B and Figure 2B). These data suggest that we were able to successfully impede the function of p72 and KHSRP.

After the initial characterization of the p72 and KHSRP knockdowns we tested the effects of their impaired functionality on endogenous Ago2. In both cases we confirmed our

initial observation that the reduction of p72 or KHSRP decreases the protein level of Ago2 (Figure 1C and Figure 2C).

Next, we tested if this phenomenon is specific in HeLa cells or if p72 and KHSRP knockdowns affect Ago2 level in other immortalized human cells. Knocking down the expression of p72 and KHSRP in HEK 293 and U2OS cells also resulted in a marked decrease in the protein level of Ago2 (Supplementary Figure 1B and C).

### ***P72 and KHSRP regulate Ago2 post-transcriptionally***

The first step in investigating the mechanism of p72 and KHSRP influence on the protein level of Ago2 was to determine if the two proteins alter the transcription of Ago2 or they regulate its expression post-transcriptionally. We used qRT-PCR to quantify the mRNA levels of Ago2 from p72 and KHSRP knocked down cells that displayed reduced Ago2 protein expression. We could not measure a significant difference in the steady state level of Ago2 mRNA between the control and p72/KHSRP targeted samples suggesting that these two proteins control Ago2 expression post-transcriptionally (Figure 1 D and Figure 2 D).

To further confirm that Ago2 expression is post-transcriptionally regulated by p72 and KHSRP we transfected an increasing amount of FLAG tagged Ago2 [17] expressing plasmid into HeLa cells in which p72 and KHSRP were targeted with siRNAs. We used qPCR and Western blotting to quantify p72 mRNA (Figure 3A) and KHSRP protein levels (Figure 3B) respectively to confirm successful knockdowns. Western blot analysis revealed that when both KHSRP and p72 expression was knocked down, FLAG::Ago2 levels were also decreased despite being independently transcribed from endogenous Ago2 (Figure 3A and B). This data confirms that p72 and KHSRP affect the expression of Ago2 at a post-transcriptional level. We have also noted that the regulatory effect of p72 and KHSRP could be saturated since

HeLa cells transfected with the highest concentration of the FLAG::Ago2 plasmid showed no change in Flag tagged Ago2 expression between the p72/KHSRP knocked down and control cells (Figure 3A and B).

***P72 and KHSRP regulate Ago2 protein level through influencing the level of cellular miRNAs***

An increasing amount of evidence suggests that, in addition to auxiliary factors such as p72/KHSRP, the miRNA pathway is also modulated through post-translational modification of the effector protein Ago2 [17]. In addition, unloaded Argonaute is degraded by the proteasome [20] and a decrease of miRNA production via the inhibition of key miRNA processing proteins such as Drosha, DGCR8 and Dicer, results in the drop of the level of Ago2 protein [19,20]. Since knocking down both p72 and KHSRP resulted in a decrease of miRNA abundance it is a possibility that the reduced expression of Ago2 is a response to the decline in miRNA levels. To test this hypothesis we transfected an increasing amount of mature miRNA into HeLa cells followed by knocking down p72 and KHSRP. In this experiment we used a mature miR-17 mimic because production of this miRNA is not regulated by either of these two RNA-binding proteins. Also, by providing the miRNA in its mature form we circumvent all processing steps that may be impaired by the downregulation of p72 and KHSRP prior to miR-17 incorporation into Ago2. Our results clearly show that increasing the level of available miRNA restores the level of the Ago2 protein in both experiments (Figure 4A, 4B and Supplementary Figure 2).

***KHSRP and p72 regulate the level of unloaded Ago2***

Next, we wanted to determine which pool(s) of Ago2 are regulated by KHSRP and/or p72. Ago2 is a part of both low (LMW-RISC) and high molecular weight (HMW-RISC) Ago2 containing protein complexes. LMW-RISC consists of unloaded Ago2 and Ago2 loaded with a small RNA (minimal RISC) which performs RNAi [24]. HMW-RISC contains miRNA loaded

Ago2 that is possibly associated with the translational machinery [25].

To investigate this, in the case of KHSRP, we conducted functional assays to test whether the Ago2 affected in the KHSRP knocked down cells is involved in translational repression or RNAi. First, we transfected a Luciferase plasmid that carries eight *let-7a* target sites in its 3'UTR [21] into HeLa cells. *let-7a* regulates luciferase expression via translational repression. We observed that in KHSRP knocked down cells *let-7a* mediated inhibition was significantly relieved (Supplementary Figure 3A). These results are consistent with the original study that first described the role of KHSRP in *let-7* processing. However, from this assay it is not possible to conclude if this effect is a consequence of the declined level of miRNA and/or Ago2. To test if the minimal RISC is affected by the decrease of the level of KHSRP we transfected a Luciferase reporter that is regulated by endogenous *let-7a* using RNAi into HeLa cells [22]. *Let-7a* mediated RNAi did not show any difference between KHSRP knocked down and control cells (Supplementary Figure 3B). This result could suggest that Ago2 that forms the minimal RISC but is not yet associated with translational repression is not subjected to the regulation mediated by KHSRP. The alternative interpretation of this data is that the ~ 50% decrease of *let-7a* level in the KHSRP knocked down cells is not enough to detect changes in the enzymatic activity of the RISC that performs RNAi.

We and others have previously shown that the HSP90 machinery has a crucial role in the loading of Argonaute with small duplex RNAs and its activity is required for the stabilization of unloaded Ago1 and 2 [22,26]. In order to test if KHSRP and p72 regulate Ago2 that is not associated with miRNAs we knocked down these proteins in HeLa cells followed by a treatment with Geldanamycin (GD), a potent inhibitor of HSP90 activity. As expected, inhibition of HSP90 in the control cells resulted in a significant 70% drop in Ago2 protein level (Figure 5A). However, GD induced a more subtle decrease of Ago2 level in the p72 and KHSRP knocked down cells (50 % and 30% respectively, Figure 5). This suggests

that in cells with impaired p72/KHSRP function there are less available unloaded Ago2. When we compared the expression level of Ago2 between the control and the KHSRP/p72 knocked down cells after GD treatment we observed no difference in the steady state level of Ago2 (Figure 5A). This strongly suggests that KHSRP and p72 influence the level of unloaded Ago2 and it is very unlikely that they have an additional effect on the amount of Ago2 that binds to small RNAs and are engaged in gene regulation.

Multiple studies have previously shown that unloaded Argonautes are degraded by the proteasome as degradation of Agos that are not bound to small RNAs can be prevented by the addition of known proteasome inhibitors such as MG132 [18,22]. To further strengthen our hypothesis that p72 and KHSRP regulate the level of unloaded Ago2 we knocked down the expression of both proteins followed by treating the cells with MG132 for 18 hours. The inhibition of proteasome activity clearly restored Ago2 level in both the p72 and KHSRP knocked down cells suggesting that both proteins are involved in the stabilization of Ago2 via a proteasome dependent mechanism (Figure 5B).

## Discussion

In this study we have shown that impairing the function of KHSRP and p72, two RNA-binding proteins with auxiliary functions in diverse steps of miRNA maturation, results in a significant decrease of Ago2 protein in several transformed human cell lines. We have demonstrated that p72 and KHSRP regulate Ago2 post-transcriptionally. Our data support the notion that the reduction of Ago2 protein level is very likely a consequence of the decrease of abundant miRNAs in the cells due to insufficient miRNA processing. We have presented supporting evidence that the p72/KHSRP mediated regulatory mechanism targets unloaded Ago2 and they unlikely influence the quantity of small RNA associated Ago2 complexes.

Our data support the recent findings that investigated the effects of miRNA abundance on the stability of mammalian and fly Ago2 [19,20]. Those studies demonstrated that interfering with essential factors of miRNA biogenesis, by inhibiting the function of DGCR8/Dicer in mouse MEF and ESCs and Drosha in fly S2 cells, results in a drop in the level of Ago2 protein by the lysosome and proteasome respectively [19,20]. Here we further demonstrate that a similar mechanism exists in the highly transformed HeLa cell line. Our finding that the miRNA level regulates the level of the unloaded Ago2 in HeLa cells supports the hypothesis that the proteasome is a key factor regulating Ago2 that are not bound to small RNA [20,22]. However, we cannot exclude the possibility that Ago2 level is regulated differently in embryonic stem cells [19].

One of our key results here indicates that auxiliary proteins, not essential to the processing of all miRNAs, can also influence miRNA levels to a degree that the key effector protein is destabilized. Increasing numbers of auxiliary proteins have been identified in the miRNA pathway that either regulate the processing of all miRNAs or influence the maturation of a subset of miRNAs [8]. Most of these proteins have well described roles in diverse signaling pathways and are regulated by various cellular and environmental stimuli [27]. Therefore, the



regulation of auxiliary proteins of the miRNA pathway could alter the functionality of the whole miRNA pathway by modulating the level of unloaded Ago2 that would, in time, affect Ago2 complexes that are engaged in gene regulation. This could be applied to proteins that specifically regulate the processing of a subset of miRNAs since characteristically, only a few miRNAs contribute dominantly to the total miRNA level in mammalian cells [28].

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**Figure legends**

**Figure 1:** p72 stabilises human Ago2 protein. HeLa cells were treated with non-targeting (Control) siRNA and siRNA targeting p72 (p72) in triplicate experiments. (A) The p72 knockdown was confirmed by measuring the relative p72 mRNA/18S RNA levels using Taqman qPCR. Each graph represents the averaged qPCR values of triplicate experiments. (\* =  $p < 0.05$ ) (B) Knocking down p72 resulted in a decrease of the level of mature miR-21 (left panel) but not miR-19 (right panel). The relative miR-21/U75 and miR-19/U75 RNA levels were quantified using Taqman small RNA qPCR. (\*\*\*) =  $p < 0.001$ ). (C) The reduction of p72 destabilizes the human Ago2 protein. Human Ago2 was visualized by Western blotting and the relative Ago2/Tubulin protein level was calculated. The numbers above the panels indicate relative Ago2/Tubulin protein levels compared to the first control lane. (D) Knocking down p72 does not decrease the level of human Ago2 mRNA. RNA levels were quantified using Taqman qPCR and the relative Ago2 mRNA/18S RNA levels were compared.

**Figure 2:** KHSRP stabilises human Ago2 protein. HeLa cells were treated with non-targeting (Control) siRNA and siRNA targeting KHSRP (KHSRP) in triplicate experiments. (A) The KHSRP knockdown was confirmed by measuring the relative KHSRP mRNA/18S RNA levels using Taqman qPCR and Western blotting of KHSRP protein. Each PCR graph represents the averaged qPCR values of triplicate experiments (\*\* =  $p < 0.01$ ) (C top panel) (B) Knocking down KHSRP resulted in a decrease of the level of mature *let-7a* (left panel) but not miR-19 (right panel). The relative *let-7a*/U75 RNA and miR-19/U75 levels were quantified using Taqman small RNA qPCR. (\*= $p < 0.05$ ) (C) The inhibition of KHSRP destabilizes the human Ago2 protein. Human Ago2 was visualized by Western blotting and the relative Ago2/tubulin protein level was calculated. The numbers above the panels indicate the relative Ago2/tubulin protein levels compared with the first control. (D) Knocking down KHSRP does not decrease

the level of human Ago2 mRNA. RNA levels were quantified using Taqman qPCR and the relative Ago2 mRNA/18S RNA levels were compared.

**Figure 3:** p72 and KHSRP regulate Ago2 via a post-transcriptional mechanism. p72 (A) and KHSRP (B) was knocked down in HeLa cells using siRNAs followed by the transfection of increasing amount of FLAG tagged human Ago2 (FLAG::Ago2). The efficiency of p72 knockdown was verified with qPCR by calculating the relative level of the p72 between the knockdown and control cells. 18S RNA was used to normalize expression (A). The efficiency of KHSRP knockdown was verified with Western blotting. The relative KHSRP expression levels are indicated at the bottom of the panel. Tubulin was used as a loading control (B). The recombinant FLAG tagged Ago2 was detected by Western blotting using a FLAG antibody. The

FLAG::AGO2 protein levels were quantified and their relative expression in the knocked down cells compared to the control cells were calculated. The relative FLAG::Ago2 expressions are indicated on the top of the panels. Tubulin was used as a loading control. (-): cells transfected with non-targeting siRNA.

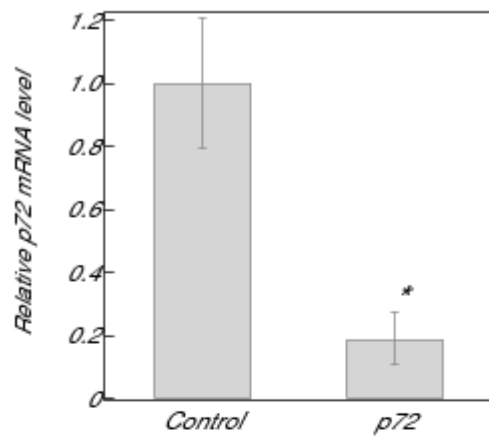
**Figure 4:** miRNA abundance regulates Ago2 protein level. p72 (A) and KHSRP (B) were knocked down in HeLa cells using siRNAs followed by the transfection of increasing amount of a miR-17 mimic. The efficiency of p72 knockdown was verified with qPCR by calculating the relative level of the p72 between the knockdown and control cells. 18S RNA was used to normalize expression (A). The efficiency of KHSRP knockdown was verified with Western blotting. Tubulin was used as a loading control (B). miR-17 expression was measured using Taqman qPCR. U75 was used to normalize expression. The relative miR-17/U75 levels compared to the cells that were not transfected with miR-17 mimic were plotted. Endogenous

Ago2 level was quantified with Western blotting using Tubulin as a loading control. The relative Ago2 levels between the knocked down cells and cells transfected with non-targeting siRNAs (-) are indicated above the panels.

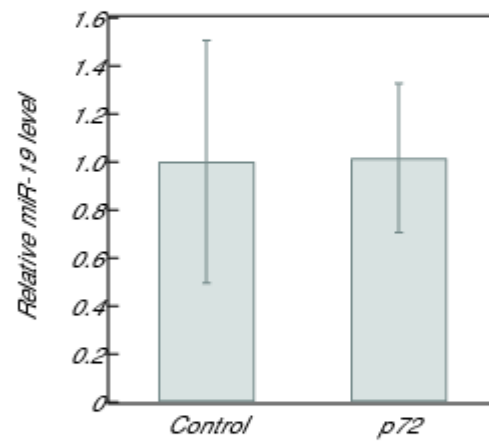
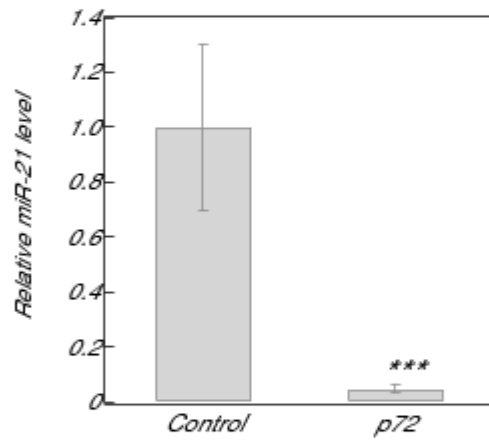
**Figure 5:** p72 and KHSRP regulate the level of unloaded Ago2. (A) p72 and KHSRP were knocked down in HeLa cells using siRNAs followed by mock (M) and Geldanamycin (GD) treatments (10 $\mu$ M for 18 hours). The efficiency of p72 knockdown was verified with qPCR by calculating the relative level of the p72 between the knockdown and control cells. 18S RNA was used as a loading control (\*\*p<0.001). The efficiency of KHSRP knockdown and Ago2 level were verified with Western blotting. Tubulin was used as a loading control. The top set of values above the panel show the relative Ago2 level between the knockdown cells and the cells that were transfected with non-targeting siRNA (c). The second set of values above the panel show the relative Ago2 levels comparing the mock treated and GD treated cells. (B) p72 and KHSRP knockdown promotes Ago2 degradation via the proteasome. p72 (P) and KHSRP (K) were knocked down in HeLa cells using siRNAs followed by mock (-) and MG132 (+) treatments (20 $\mu$ M for 18 hours). The efficiency of p72 knockdown was verified with qPCR by calculating the relative level of the p72 between the knockdown and non-targeting siRNA transfected cells (C). 18S RNA was used as a loading control (right panel). The efficiency of KHSRP knockdown and Ago2 level were verified with Western blotting. Tubulin was used as a loading control. The values above the panel show the relative Ago2 level between the knockdown cells and the cells that were transfected with non-targeting siRNA (C).



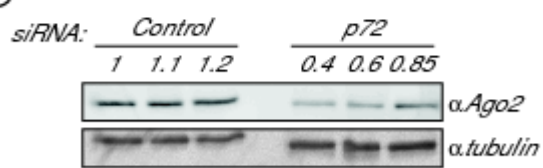
A



B



C



D

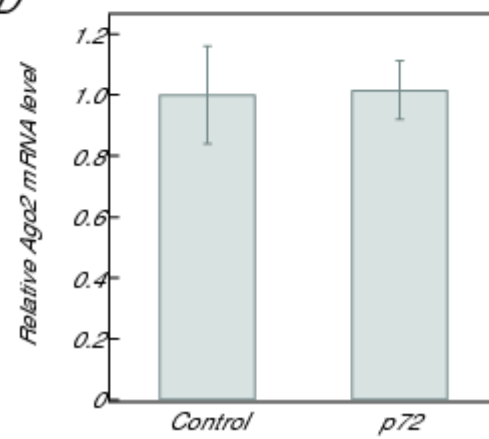


Figure 1

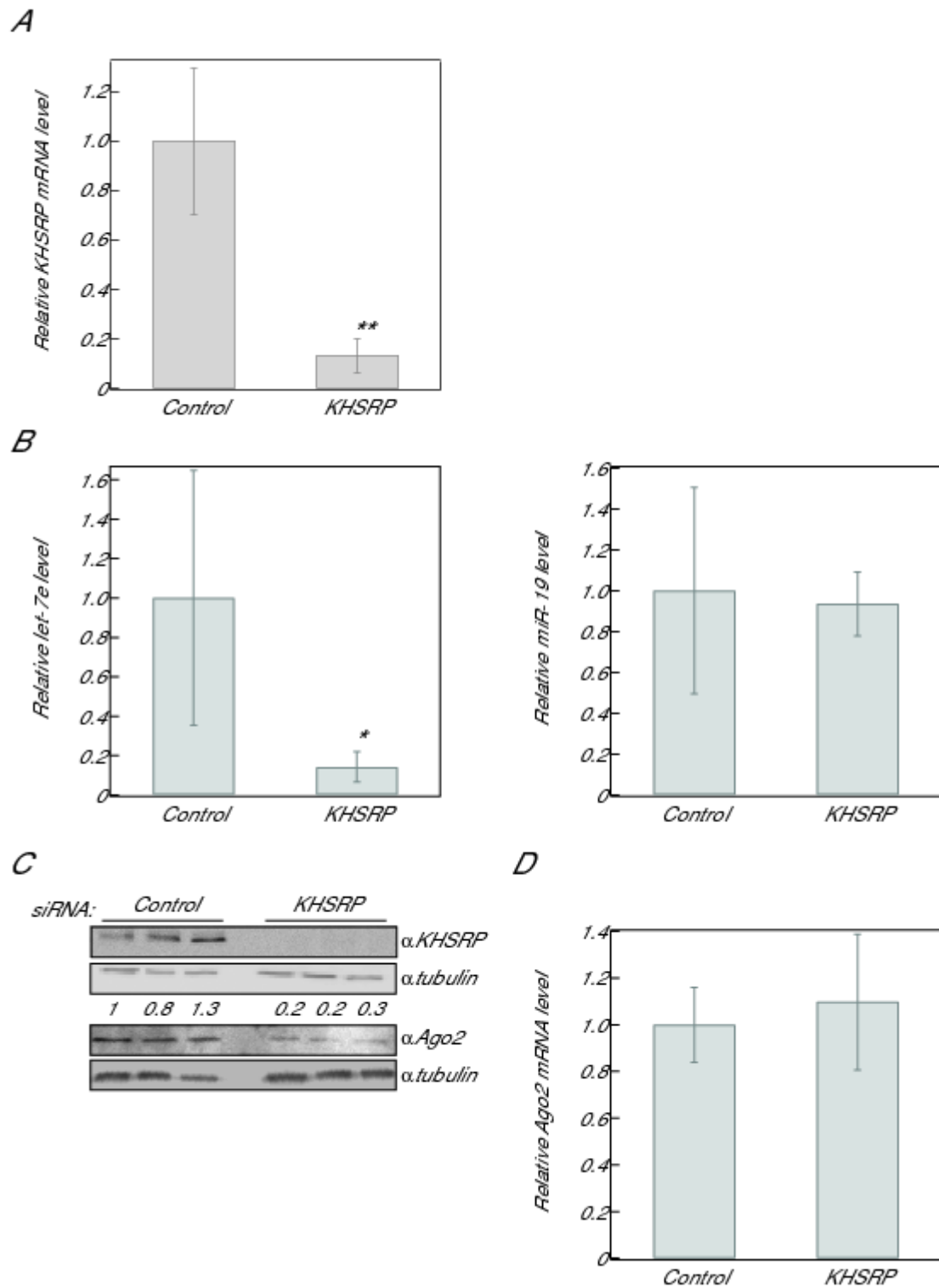


Figure 2

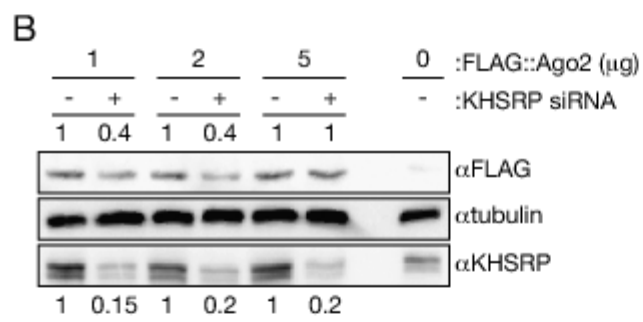
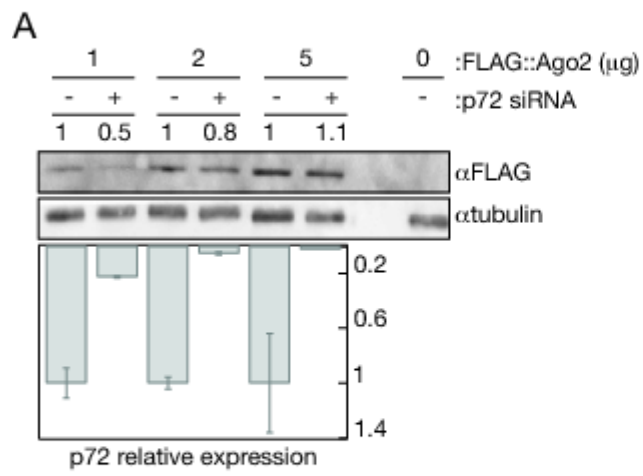


Figure 3

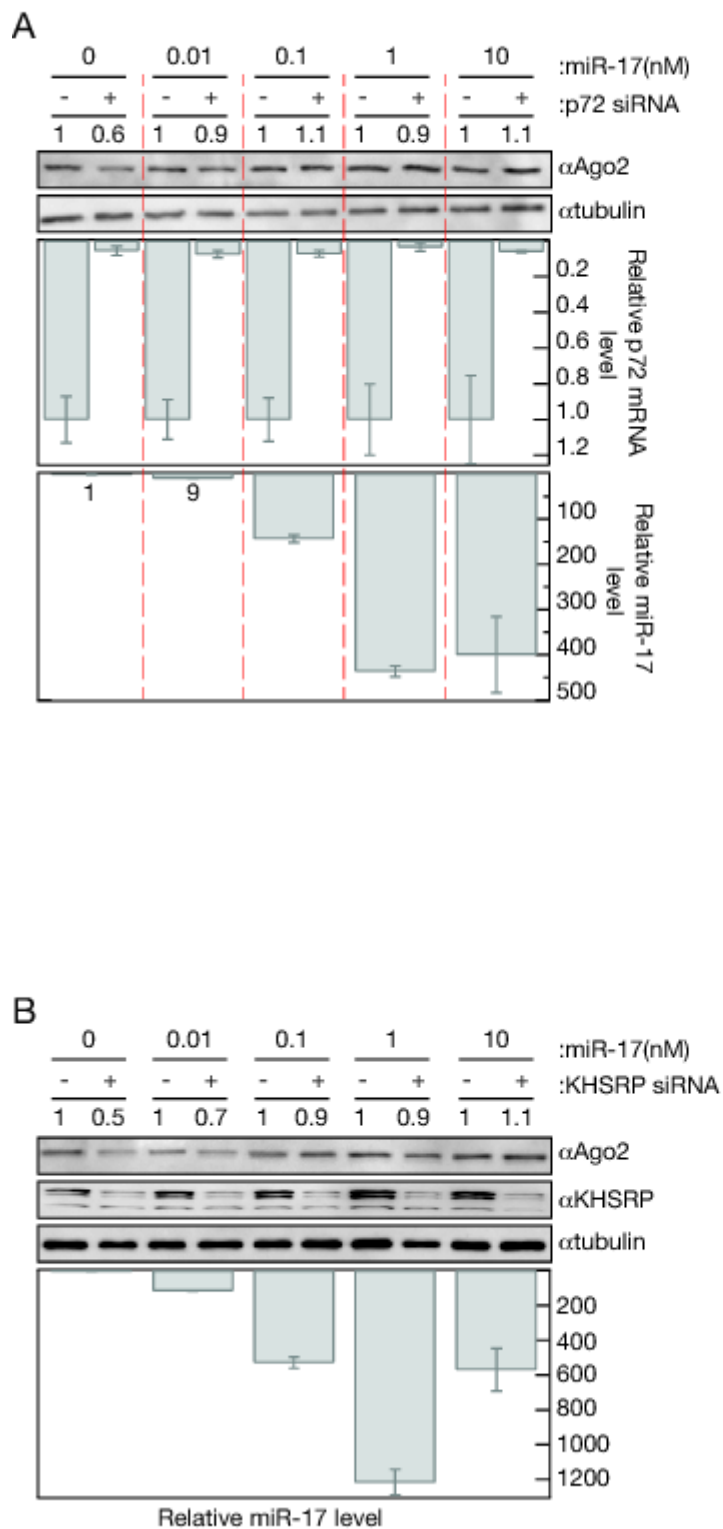


Figure 4

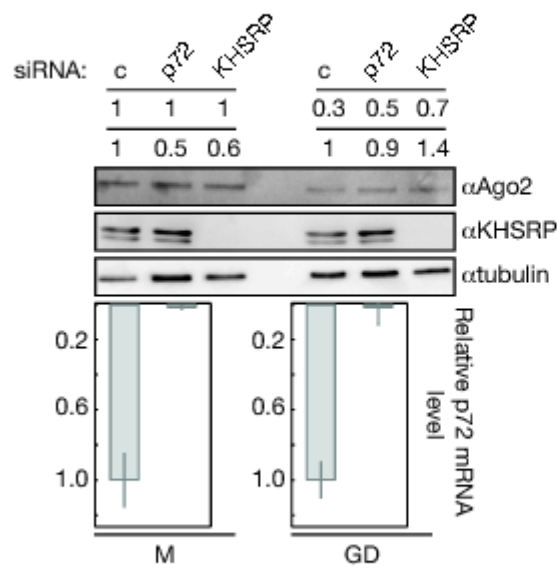
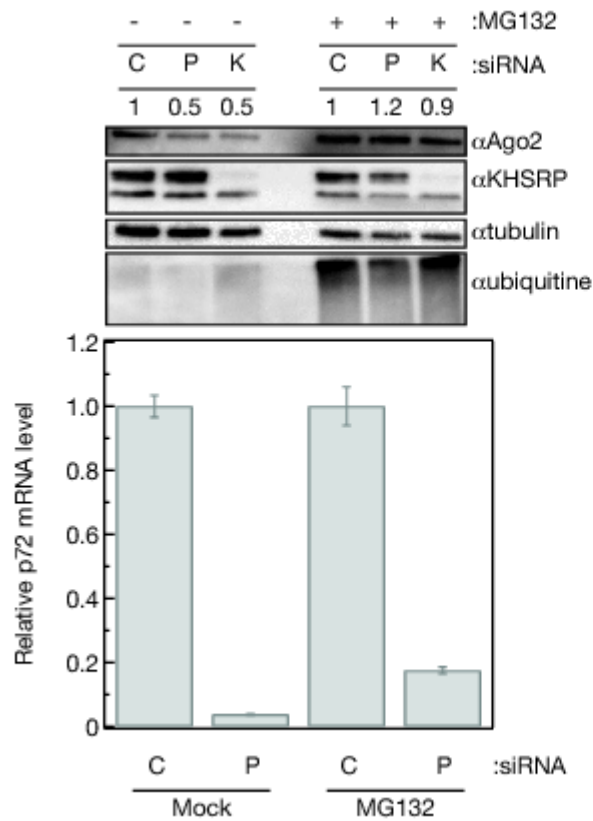
**A****B**

Figure 5

**Declaration of conflict of interest:**

The authors declare no conflict of interest!

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**Highlights:**

In this new submission we repeated the experiment (Supplementary Figure 2) that was requested by both reviewers and added a new experiment requested by Reviewer 3 (Figure 5B).

Both results are supporting our original hypothesis that two auxiliary RNA binding proteins that are involved in miRNA biogenesis regulate the level of unloaded Ago2 via the level of available/loadable miRNA pool.

We additionally clarified a few concerns were raised by both reviewers.

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