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Rescue of miRNA export from amyloid-exposed astroglia by Ras homolog enriched in brain (Rheb) protein

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ABSTRACT

Amyloid protein disrupts miRNA activity in astroglial cells by inducing miRNA sequestration in RNA processing bodies or P-bodies, thereby hindering extracellular vesicle-mediated intercellular communication through miRNAs. The mTOR activator Rheb facilitates miRNA recycling and export, mitigating the negative effects of amyloid on miRNA pathways. Additionally, the miRNA-binding protein Syntaxin 5 or STX5 speeds up the miRNA export process, possibly by relocating miRNPs from P-bodies. However, STX5 cannot support miRNA repressive activity or recycling in astroglia exposed to amyloid. Therefore, relocating miRNA out of P-bodies is necessary for export but not sufficient for miRNA reactivation in amyloid beta (Aβ)-affected cells. Interestingly, Rheb enhances both miRNA P-body relocalization, reactivation, and export, thereby counteracting amyloid-related disruptions.

Key words: miRNA; STX5; mTORC1; Rheb; Extracellular vesicles; C6 astroglia

INTRODUCTION

microRNAs (miRNAs) are small, non-coding RNAs that form AGO-miRNP complexes, and bind to target mRNAs, inhibiting protein production [1]. Regulation of miRNA activity is essential for controlling target mRNA expression and protein synthesis. miRNA-repressed mRNAs, along with AGO miRNPs, are often stored in phase-separated subcellular structures such as RNA processing bodies (P-bodies) [2,3], impairing miRNA function. In metazoan cells, miRNA levels are also tightly regulated through their export via extracellular vesicles (EVs), a process controlled by multiple RNA-binding proteins, including the miRNA "sponge" protein HuR, which selectively binds and facilitates the export of specific miRNAs from mammalian cells [4]. The localization of miRNAs to P-bodies appears to oppose miRNA export, and factors such as HuR, which promote export, counteract P-body localization of miRNAs [5].

In astroglial cells, elevated miRNA levels are inconsistent with reduced repressive activity of miRNAs in the presence of amyloid beta oligomers. The impaired miRNAs caused by amyloid exposure are found to be localized to P-bodies. The role of amyloid proteins in the cellular protein translation process remains unclear [6]. However, amyloid fibrils and oligomers can cause RNA metabolic defects in astroglia [7]. Oligomeric beta-amyloid impairs miRNA activity by inhibiting recycling and reducing the repression of target mRNAs, thereby increasing cytokine expression, which otherwise remains repressed by miRNAs [7,8]. Questions remain about how miRNAs can be relocalized from P-bodies in cells exposed to beta-amyloid oligomers Aβ1-42, and about the factors that might enhance miRNA recycling and export by preventing their sequestration into P-bodies. Export of miRNAs via EVs enables communication between neurons and glial cells, with glial-derived EVs being crucial for neuronal function [9]. The extracellular loading of miRNAs is tightly regulated and is thought to contribute to miRNA activity. The decision to load miRNA into endosomes for export via EVs is regulated at multiple steps, and several protein factors have been identified as contributors to miRNA export [10]. Interactions with particular RNA-binding proteins, such as the La antigen, YBX1, hnRNPs or HuR, regulate the export of miRNAs through EVs to adjacent cells [4,11–18]. These proteins specifically promote miRNA export, thereby regulating cytosolic miRNA levels.

In our previous studies, we documented that miRNA and AGO phase separation to P-bodies is consistent with increased miRNA stability in human cancer [5] and astroglial cells [7], and that phase-separated miRNAs are excluded from recycling and export, a process augmented by amyloid beta oligomers [7]. By ensuring P-body localization of miRNAs, Aβ1-42 oligomers prevent their recycling and reactivation [8]. mTORC1 activity is linked to rapid miRNA recycling in astroglia. Rheb-protein, the mTORC1 activator, can cause defective P-body targeting of miRNAs in *in vivo* and *ex vivo* conditions [7,8,19], and we posit that by preventing P-body targeting of miRNAs, Rheb protein ensures export of miRNAs from amyloid beta oligomer-exposed glial cells.

Our findings show reduced miRNA levels in extracellular vesicles from astroglia exposed to amyloid beta oligomers, indicating restricted EV-mediated miRNA export from glial cells in Alzheimer's disease. This data is consistent with increased cellular levels of miRNAs noted

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in amyloid-treated astroglial cells and in rat brains, as well as increased localization of miRNPs to P-bodies in amyloid-treated astroglia [7,8,19]. We found that Aβ1-42 oligomers prevent miRNA loading into exosomes, a process mitigated by HuR. The ELAVL protein HuR forms complexes to accelerate miRNA loading into endosomes. Other miRNA export-related factors, like RalA and Rab5 [20], did not restore miRNA import into endosomes under Aβ1-42 exposure. Rheb-Myc, an mTORC1 activator, restores miRNA function and enhances miRNA export by influencing P-body-compartmentalization of Ago2 and miRNAs [7,8]. Overall, miRNA export depends on P-body relocalization, and mTORC1 activation by Rheb-Myc that improves P-body relocalization, miRNA recycling, and export, reducing the impairment of intra- and intercellular miRNA function caused by Aβ1-42 oligomers.

MATERIALS AND METHODS

Cell culture and transfection of cells

C6 glioblastoma cells were cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco) with 10% heat-inactivated fetal calf serum (HI-FCS, Gibco) and 1% Penstrep (Gibco). They were incubated at 37°C in a 5% CO_2 environment.

Preparation of Amyloid beta oligomers

Lyophilized Aβ1-42 (American Peptide) was reconstituted in 100% hexafluoro-2-propanol (HFIP) to prepare a 1 mM stock solution. HFIP was subsequently removed via evaporation using a SpeedVac (Eppendorf). The resultant pellet was dissolved in anhydrous DMSO and sonicated in a bath sonicator for 40 minutes at 37°C. The stock solution was then adjusted to a final concentration of 5 mM and stored at -80°C. Oligomers of Aβ1-42 was generated by diluting the stock peptides in Phosphate Buffered Saline (PBS) and 0.2% Sodium Dodecyl Sulfate (SDS) to attain a peptide concentration of 400 μM. This solution was incubated at 37°C for 16 hours, then diluted in PBS to 100 μM and incubated again at 37°C for 16 hours to facilitate proper oligomerization before use. Oligomeric status was assessed using NTA.

Extracellular vesicles isolation and characterization

C6 glioblastoma cells transfected with specific plasmids were gently split into 90mm plates 24 hours post-transfection. After incubating for another 24 hours to reach 70-80% confluence, the conditioned media were collected for EV isolation using a slightly modified method from a previous report [21]. To prevent contamination with serum-derived exosomes, cells were cultured in growth medium containing EV-depleted serum. The cell culture conditioned media were clarified by centrifugation at 2,000xg for 10 minutes, then at 10,000xg for 30 minutes, and then filtered through a 0.22-μm filter. EVs were isolated via ultracentrifugation at 100,000xg for 90 minutes. For protein analysis, a different ultracentrifugation step was performed over a 30% sucrose cushion at the same speed and duration. The EV pellet was resuspended in 1× PBS and ultracentrifuged again at 100,000×g for 90 minutes. To characterize the EVs, they were diluted 10-fold in PBS, and was analyzed using a NanoSight NS-300 to determine their number, size, and other parameters or Western blotted for marker proteins [21].

Endosome enrichment via subcellular fractionation

Optiprep density gradient medium, made with 60% iodixanol (Sigma-Aldrich), was used to prepare a 3 ml step gradient with concentrations of 5%, 10%, and 15% (by layering 1 ml of the lighter solution over 1 ml of the denser gradient). This process was carried out in a buffer containing 78 mM KCl, 4 mM MgCl2, 8.4 mM CaCl2, 10 mM EGTA, and 50 mM HEPES (pH 7.0) to separate subcellular organelles. Cells were washed with phosphate-buffered saline (PBS), then homogenized in a Dounce homogenizer with a buffer supplemented with 0.25 M sucrose, 78 mM KCl, 4 mM MgCl2, 8.4 mM CaCl2, 10 mM EGTA, and 50 mM HEPES (pH 7.0), along with 100 μg/mL Cycloheximide, 0.5 mM DTT, and a 1X amount of PMSF. The lysate was clarified by centrifugation at 1,000×g for 5 minutes, then 1 ml of the supernatant was layered onto a 3 ml gradient at 4°C. After ultracentrifugation at 133,000×g for 5 hours, seven fractions were collected for endosome/MVB analysis. The top three fractions were pooled, diluted with buffer to reach a total volume of 4 ml, and then ultracentrifuged at 133,000×g for 2 hours. The resulting membrane pellet was resuspended in buffer containing cycloheximide, DTT, and PMSF, and kept on ice for subsequent *in vitro* assays as described previously [20].

Cell-free in vitro reconstitution assay

The cell-free in vitro assays using endosomes were performed according to a previously published protocol [20]. An endosome-rich suspension was gently prepared from C6 cells and then carefully divided into equal parts for the experiments. As outlined in the Results section, we used synthetic miRNA and a small RNA pool as cargo, all in the presence of ATP. The mixture was incubated for 30 minutes at 37°C, and then we carried out an RNase protection assay as described earlier [20]. Vesicles were carefully washed and then re-isolated using ultracentrifugation at 133,000 x g for 2 hours. The resulting vesicle pellets were gently processed for RNA and protein extraction, preserving their integrity for accurate analysis.

Immunoblotting

Western blotting involves preparing protein samples from lysates, fractions, or immunoprecipitated materials. These samples were then separated using SDS-PAGE and transferred onto PVDF membranes overnight at 4°C. To reduce nonspecific binding, membranes were blocked with 3% BSA for 1 hour [20]. They are then carefully probed with specific antibodies for at least 16 hours at 4°C. Subsequently, the membranes were incubated for 1 hour at room temperature with HRP-conjugated secondary antibodies (diluted 1:8000). Finally, the results were captured using the UVP BioImager 600 with VisionWorks software version 6.8.

RNA isolation and real-time PCR

Total RNA was isolated according to the manufacturer's protocol using TRIzol or TRIzol LS (Invitrogen). Samples with low microRNA content were precipitated using isopropanol and glycoblue (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized from 100-200 ng of RNA extracted from cellular samples, or from an equivalent volume of *in vitro* post-assay or extracellular vesicle (EV) samples, employing a TaqMan reverse transcription kit (Applied Biosystems). MicroRNA assays were performed by real-time PCR using specific primers, and analysis was conducted on a Bio-Rad CFX96™ system with TaqMan probes. One-third of the reverse transcription mixture was amplified in triplicate using TaqMan Universal PCR Master Mix and target miRNA-specific reagents. Cellular microRNA levels were normalized to U6 small nuclear RNA (snRNA); for *in vitro* samples containing synthetic miR-122, miR-146a served as the normalization control. For messenger RNA (mRNA) quantification, 200 ng of RNA was reverse-transcribed into cDNA and subsequently analyzed by quantitative PCR (qPCR) with SYBR Green (Eurogentec), normalized

to GAPDH, with all assays performed in triplicate using the comparative Ct method as described earlier [20].

Phosphate 5' end-labeling of synthetic miRNA

Essentially as described earlier [20], 50 pmol of synthetic miR-122 RNA (22 nt) was incubated with T4 PNK enzyme (10 units/µl) and 1 mM ATP in 1X T4 PNK buffer at 37°C for 30 minutes under static conditions. The reaction was then stopped with Tris-EDTA solution and filtered through a Mini Quick Spin Oligo column (Roche Diagnostics). RNA was extracted using TRIzol LS, precipitated with isopropanol at -20°C overnight, and co-precipitated with glycoblue. The resulting RNA pellet was resuspended in nuclease-free water to reach a final concentration of 1 pmol/µl.

Preparation of recombinant HuR

Following the published protocol [4], the recombinant HuR protein was purified. The HuR gene was cloned into the pET42a(+) expression vector and introduced into BL21(DE3) *Escherichia coli* cells. Bacterial cultures were grown to an appropriate density and then induced to express HuR protein overnight using IPTG. The cells were harvested and lysed in a buffer mixture containing Tris–HCl for pH stabilization, potassium chloride (KCl) to maintain ionic strength, magnesium chloride ($MgCl_2$) which can stabilize ribosomes and other structures, β-mercaptoethanol to reduce disulfide bonds, imidazole to prevent nonspecific binding during purification, Triton X-100 as a detergent to solubilize membranes, glycerol to stabilize proteins, lysozyme to degrade cell walls, and protease inhibitors to prevent protein degradation. The lysate was then subjected to sonication, a process that uses ultrasonic waves to lyse cells and shear DNA, thereby releasing the target protein. The resulting lysate was incubated with Ni-NTA agarose beads for 4 h at 4°C, allowing the His-tagged HuR protein to bind specifically to nickel ions on the beads. After binding, the beads were washed to remove non-specifically bound proteins, and the HuR protein was eluted using buffers containing increasing concentrations of imidazole, which competes with the His-tag for nickel binding. Finally, the purified HuR protein was concentrated and stored at -80°C to preserve its stability until subsequent use.

STATISTICAL ANALYSIS

GraphPad Prism version 5.00 (GraphPad Software, San Diego) was used to analyse the data from the experimental plots. These datasets were typically obtained from experiments conducted in triplicate to ensure accuracy and reproducibility, unless otherwise specified. To assess the statistical significance of the results, Student's t-test was used to calculate P values, with a significance threshold set at $P < 0.05$. The data are presented with error bars indicating the mean ± standard deviation, providing a visual representation of the variability within each dataset.

Details of plasmids, oligo sequence, miRNA assays, and antibody and chemicals used are part of the Supplementary Tables S1-S4.

RESULTS

Retarded miRNA export from Aβ1-42-exposed rat astroglia

We previously documented that amyloid beta-oligomers influence cellular miRNA levels, with the increased miRNPs localizing in P-bodies [7,8]. The previous report also suggests P-bodies negatively regulate miRNA export, leaving P-body-localized miRNAs non-functional due to defective recycling via LE (Late endosomes)-lysosome interaction [5]. Because miRNA export is vital for miRNA homeostasis, we investigated how the amyloid protein affects miRNA export in astroglial cells. Amyloid Aβ1-42 oligomers were prepared and used to treat C6 glioblastoma cells and observe an increase in the number of extracellular vesicles (EVs) (Figure 1A-B; and Figure S1). Interestingly, although the number of EVs increased, their size remained unchanged after Aβ1-42 oligomer treatment, accompanied by increased association of Alix and Flotillin-1, two known EV markers. Alix is ESCRT-related, and Flotillin-1 is a lipid raft protein, both of which are essential for different aspects of EV formation and cargo loading [22]. The absence of Cytochrome C and Calnexin confirmed the purity of the isolated EVs, free of contaminating ER and apoptotic bodies (Figure 1C and D). Interestingly, the second band in the Flotillin Western blot for cellular samples, which may indicate phosphorylated Flotillin 1 [23] was not found in EVs, as only one band appeared in the EV Western blot. This also suggests that the EV fraction is pure and free from cytoplasmic contamination. When measuring miRNA levels in the EVs, we observed a decrease in endogenous miR-146a and ectopically expressed miR-122, both of which showed reduced levels in EVs isolated from Aβ1-42 oligomer-treated C6 cells, supporting an inhibitory role of Aβ1-42 oligomer on miRNA export (Figure 1E and F). Interestingly, as previously reported, both miR-146 and miR-122 showed 3.8- and 2.6-fold increases, respectively, in cellular levels upon treatment of C6 glioblastoma cells with Aβ1-42 oligomers [7,8].

miRNA loading into endosomes is limited by Aβ1-42 oligomers and is restored by ELAVL protein HuR1

The miRNA export process begins with the loading of miRNAs into endosomes via diverse RNA-binding proteins [4,17,20,24,25]. We developed an *in vitro* assay using isolated endosomes from C6 glioblastoma cells to investigate miRNA packaging, a crucial step for EV export. Assuming that amyloid oligomers block miRNA entry into endosomes, we tested this hypothesis by incubating endosomes with increasing concentrations of Aβ amyloid protein, which resulted in a dose-dependent decrease in miRNA entry into endosomes (Figure 2A and B). HuR forms complexes with miRNAs, facilitating their entry into the endosomal lumen [20]. The presence of recombinant HuR bound to substrate miRNAs promotes miRNA entry and can rescue this process even in the presence of Aβ1-42 oligomers. This indicates that the HuR-miRNA complex remains unaffected by Aβ1-42 (Figure 2C and D). The HuR-miRNA complex is known to activate the RalA.

GTPase, which is crucial for miRNA entry into endosomes—a process further enhanced by the Rab5 protein [20]. Interestingly, ectopic expression of both HA-Rab5 and HA-RalA in cells used for endosome isolation increases miRNA import into the endosomal lumen during the *in vitro* miRNA import assay [20]. However, this does not prevent the inhibition caused by amyloid proteins for miRNA entry. These results suggest that amyloid proteins act upstream of both RalA and Rab5, blocking miRNA entry into endosomes (Figure 2E and F). To illustrate the impact of Rab5, RalA, or HuR overexpression on endosome-associated miR-122, imported during the in vitro assay, normalized to endogenous miR-146a in Aβ-treated conditions, the ΔCt values were plotted. This revealed a statistically significant difference among the groups (Figure 2G). The *in vitro* assay was performed with rHuR present in the assay with miRNA substrate and Aβ oligomers, and rHuR can rescue the effect of amyloid oligomers on miR-122 entry into endosomes, possibly due to pre-binding of HuR to miRNA, which amyloid oligomers cannot act on to prevent its endosomal entry, as

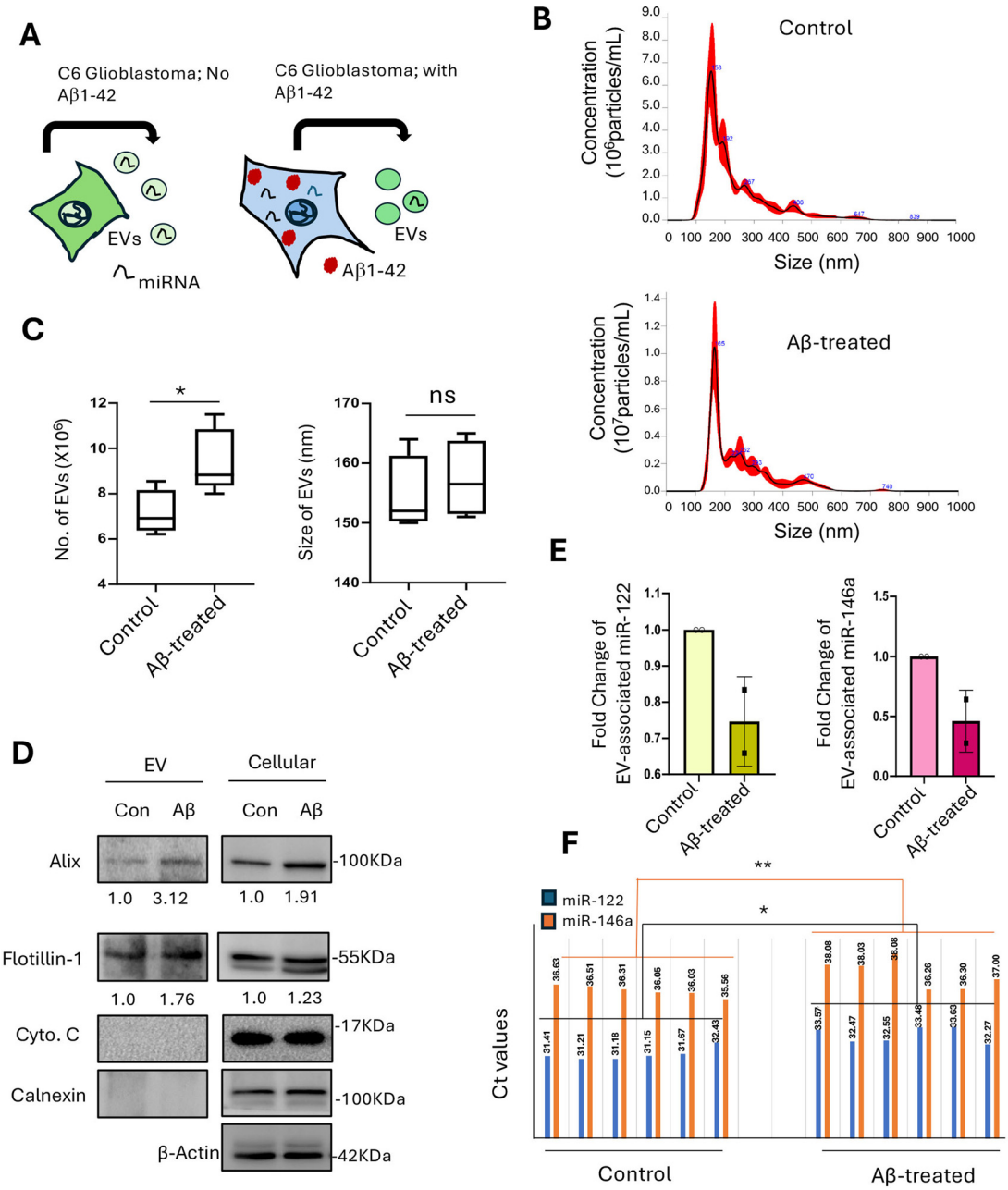

Figure 1. Amyloid beta Aβ1-42 oligomer restrict the export of miRNAs via EVs from C6 cells.

A. Schematic of the experimental procedures. C6 glioblastoma cells were treated with Aβ1-42 oligomers to assess their effects on EV miRNA content and the amount of EV released.

B. NTA profile of the EVs isolated from control and Aβ1-42 oligomer-treated cells (2.5 μm for 24 hours).

C. The impact of Aβ1-42 oligomer treatment on the number and size of exosomes or EVs secreted by C6 glioblastoma cells.

D. The influence of Aβ1-42 oligomer treatment on the expression of cellular and EV-associated marker proteins in C6 glioblastoma cells, as determined by Western blot analysis. β-Actin is used as a loading control for cellular proteins. Equal amounts (100 μg) of proteins were loaded for Western blots. Relative quantification of Alix levels was performed using ImageJ densitometry from multiple blots and is shown below the lanes.

E. The effect of Aβ1-42 oligomer treatment on the EV-associated miR-122a and miR-146a, released by glioblastoma cells expressing miR-122 from pmiR-122 expression plasmid. miRNA content was analysed by relative quantification from Ct values obtained, normalized to Alix levels.

F. Ct Values of miR-146a and miR-122 in the EVs isolated from control and Aβ1-42 oligomer treated C6 cells.

Statistical analysis was performed with n>2. Data are presented as mean ± SD. ns, non-significant; *p < 0.05; **p < 0.01. P-values were obtained using a two-tailed unpaired Student's t-test.

observed in Figure 2D. This was not observed in experiments in 2E-F, where cells expressing HA-HuR, RalA, or Rab5 ectopically in combination were used for isolation of endosomes, which were expected to be more compatible for endosomal entry of miRNAs than pCIneo control vector-expressing cell-derived endosomes. The assays were performed in the absence of additional recombinant HuR. There is no significant rescue effect on miRNA entry into endosomes in cells ectopically expressing HA-Rab5,

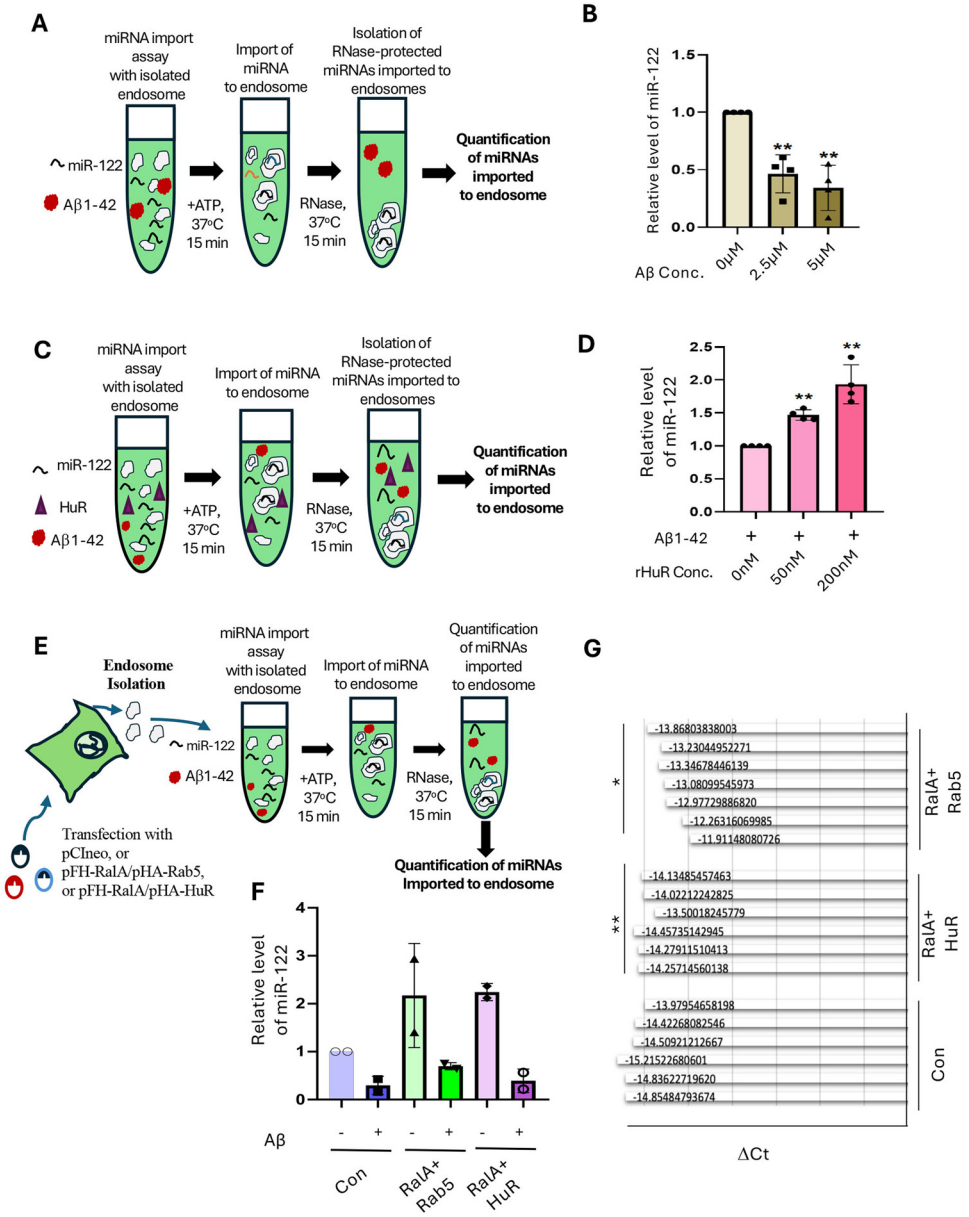

Figure 2. miRNA import into endosomes is restricted by Aβ1-42 oligomers and rescued by miRNA-binding protein HuR.

A. Schematic of the *in vitro* miRNA import assay done with endosomes isolated from C6 glioblastoma cells.

B. The import assay of miRNA into endosomes in the presence of Aβ1-42. Single-stranded (ss) synthetic miR-122 (10 nM) was incubated with increasing concentrations of Aβ1-42 oligomers in an in vitro miRNA import assay for endosomes, using 1 mg of endosomes in 50 μL of import reaction with 1 mM ATP. After import and RNase protection, the endosomes were recovered by ultracentrifugation, and RNA quantification was performed by qRT-PCR, normalized against the amount of endogenous miR-146a inside the endosomes. Data from three independent experiments.

C. Effect of recombinant HuR on miRNA import in the presence of Aβ1-42 oligomer. Increasing the concentration of HuR enhances the import level of miR-122 when 2.5 μM Aβ1-42 oligomer is present in the endosome import assay.

D. Effect of increasing concentrations of recombinant HuR on the import level of miR-122 measured in the presence of Aβ1-42. The recovered endosomes were used for RNA estimation and miRNA quantification after the assay, normalized against the endogenous miR-146a level within the endosomes. The data represent three independent experiments.

E. Assessed the potential of miRNA import into endosomes isolated from cells expressing FH-RalA, HA-Rab5, or HA-HuR ectopically in C6 glioblastoma cells, in the presence and absence of Aβ1-42 oligomer.

F. Effect of ectopic expression of FH-RalA and HA-Ra5 or FH-RalA and HA-HuR on miR-122 import levels in the absence and presence of Aβ1-42 oligomers. Endosomes from pCIneo control plasmid–expressing cells were used as a control. Relative levels of miR-122 were normalized against endogenous miR-146a present inside the isolated endosomes used for the assay.

G. Depict the effect of HA-Rab5 and FH-RalA, or FH-RalA and HA-HuR expression on endosome-associated and imported miR-122 levels after the assay normalized to endogenous miR-146a in Aβ-treated condition. ΔCt values were plotted, suggesting a statistically significant difference among groups.

Statistical analysis was performed with n>3. Data are presented as mean ± SD; ns, non-significant; *p < 0.05; **p < 0.01. P-values were obtained using a two-tailed unpaired Student's t-test.

HA-HuR, or FH-RalA. In this condition, amyloid beta oligomers may interact with miRNA before RalA or HuR present at the endosomal membrane can act on it, and thus membrane-associated RalA, HuR, or Rab5 cannot reverse the effect of amyloid oligomer on miRNA entry. This is consistent with the hypothesis that HuR acts upstream of miRNA entry into endosomes, while amyloid oligomers act on a downstream step in miRNA entry pathway to endosomes. Interestingly, the positive effect of Rab5, RalA, or HuR expression on miRNA entry was observed in Figure 2F, in the absence of amyloid beta oligomers. This is consistent with our previous observations, which showed that the effects of HA-HuR, HA-Rab5, or FH-RalA were significantly enhanced in both *in vivo* and *in vitro* endosomal miRNA entry [20].

Rheb-Myc expression restores miRNA export

Previous research has shown that Rheb-Myc expression reduces cellular miRNA levels, but increases miRNA recycling, thereby increasing repression of miRNA-targeted mRNAs and inflammatory

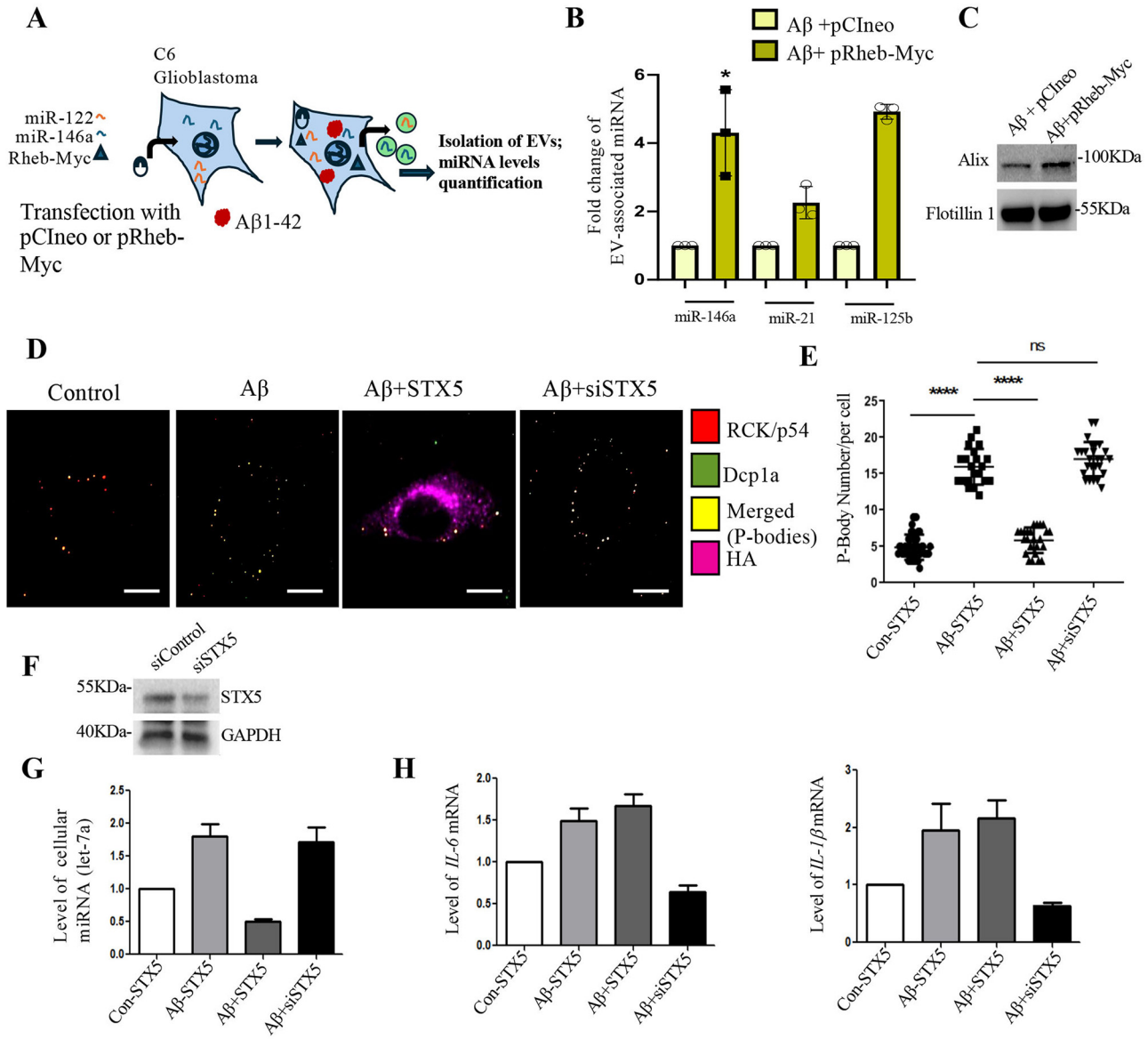

Figure 3. Rheb-Myc rescue miRNA export in Aβ1-42 oligomer-treated C6 astroglia cells.

A. Schematic of the experiment expressing Rheb-Myc expression vector or control pCIneo vector to measure the effect on EV-associated miRNA levels.

B. The relative levels of miRNAs measured in EVs isolated from control or Rheb-Myc expressing cells treated with 2.5 μM Aβ1-42 oligomers for 24 hours, before the EVs were isolated from cell culture supernatant. Measurements were performed across three independent experiments.

C. Effect of Rheb-Myc expression on EV-marker proteins present on EVs.

D-E. STX5 causes reversal of the effect of Aβ1-42 oligomers treatment on enhanced P-bodies in C6 glioblastoma cells. Cells expressing FH-STX5 or transfected with siSTX5 have a reversed effect on P-body numbers (D). For control, cells without Aβ1-42 oligomers treatment were used as control and for comparative quantitative data (E). Scale bar 10 μm.

F. Effect of siSTX5 expression on STX5 levels in C6 cells. Non-targeting siControl was used as control.

G-H. Effect of FH-STX5 expression or siRNA-mediated STX5 depletion causes reverse effect on amyloid beta induced change in cellular miRNA level (G) and cellular inflammatory cytokine mRNA levels (H) measured by qRT-PCR. Values normalized against U6 RNA and GAPDH mRNA for miRNA and mRNA estimation.

Statistical analysis was performed with n>3. Data are presented as mean ± SD; ns, non-significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. P-values were obtained using a two-tailed unpaired Student's t-test.

cytokines [7,8]. This effect helps restore cognitive function in rat brains injected with amyloid oligomers. It is linked to the relocalization of miRNA and Ago2 from P-bodies in astroglial cells exposed to Aβ1-42 [19]. How Myc-Rheb expression affects miRNA export was the question to be addressed. Results indicate that Rheb-Myc expression promotes miRNA export from astroglia exposed to amyloid beta (Figure 3A-C).

Rheb-Myc is also known to affect miRNA recycling by promoting lysosomal targeting of P-bodies/endosome-located miRNAs while relocating Ago2 miRNPs from P-bodies [19,26]. Is the relocalization from P-bodies sufficient for miRNA export and recycling? We need to test it under conditions that affect PB-relocalization but may prevent lysosomal miRNA targeting. Syntaxin 5 or STX5 is another miRNA-binding protein known to promote miRNA export *via* the HuR-RalA-endosome axis [21]. STX5 also enhances the export of miRNA by preventing lysosomal targeting of endosome or multivesicular body (MVB)-associated miRNAs [21]. Thus, STX5 is a candidate to test whether P-body relocalization is sufficient for miRNA recycling and export.

As reported for Rheb-Myc in our previous work [19], STX5 also reduces the number of P-bodies in cells ectopically expressing FH-STX5. These findings suggest that changes in PB relocalization in amyloid-treated FH-STX5-expressing C6 cells are responsible for enhanced miRNA export, but with reduced miRNA activity, as assessed by let-7a, a known regulator of IL- 6 expression [27] (Figure 3D-G and Fig. S2A-B). Interestingly, unlike Rheb-Myc, FH-STX5 failed to restore mTORC1 activity reduced by amyloid beta treatment [7,8] (Fig. S2C). siSTX5 treatment increases cellular miRNA levels and cytokine repressive activity, possibly by inhibiting miRNA export (Figure 3F-H). However, reactivation of miRNAs in FH-STX5 cells was not achieved and was accompanied by an increase in inflammatory cytokine expression (Figure 3F-H), consistent with the hypothesis that miRNP recycling was not restored by FH-STX5 but only miRNA export was augmented by HA-STX5. Depletion of STX5 accelerates recycling and reactivation of miRNPs, ultimately limiting miRNA export and enhancing miRNA-mediated suppression of inflammatory cytokines (Figure 4). Therefore, the relocation of miRNA from P-bodies alone suffices for its export triggered by Rheb, HuR, and STX5; however, the targeting of miRNA to lysosomes via Rheb-activated mTORC1 is essential for miRNA recycling [7,8].

DISCUSSIONS

We hypothesize that the RNA storage within RNA Processing Bodies or P-bodies (PBs) enhances the stability of microRNA (miRNA) in glial cells exposed to amyloid beta, thereby affecting miRNA export and function, leading to increased cytokine expression due to the absence of functional miRNPs to repress the target cytokine mRNAs [7,8]. Findings indicate that Rheb-Myc protein can reverse miRNA storage in P-bodies, possibly by activating mTOR signalling, which promotes miRNA cycling by enhancing interactions between late endosomes (LE) and lysosomes [7,8]. It also increases miRNA export, lowering cellular miRNA levels, such as miR-146a, and increasing their levels in extracellular vesicles (EVs), which may be exported to naive glial cells or neurons. This process may protect cells from the harmful effects of amyloid beta by triggering an anti-inflammatory environment that preserves glial and neuronal cells health, thereby activating anti-inflammatory pathways [28]. Rheb-Myc-driven miRNA export likely contributes to the cognitive recovery observed in rat brains after Aβ1-42 oligomer administration, by enhancing the anti-inflammatory environment in affected brain tissue.

miRNA export and recycling are interconnected processes regulated by proteins governing miRNA storage within P-bodies. Rheb-Myc inhibits miRNA targeting to P-bodies, thereby accelerating both export and recycling; however, it remains uncertain whether Rheb-Myc or mTORC1 directly influences miRNA relocalization [19]. Our investigations demonstrate that oligomeric amyloid beta accelerates

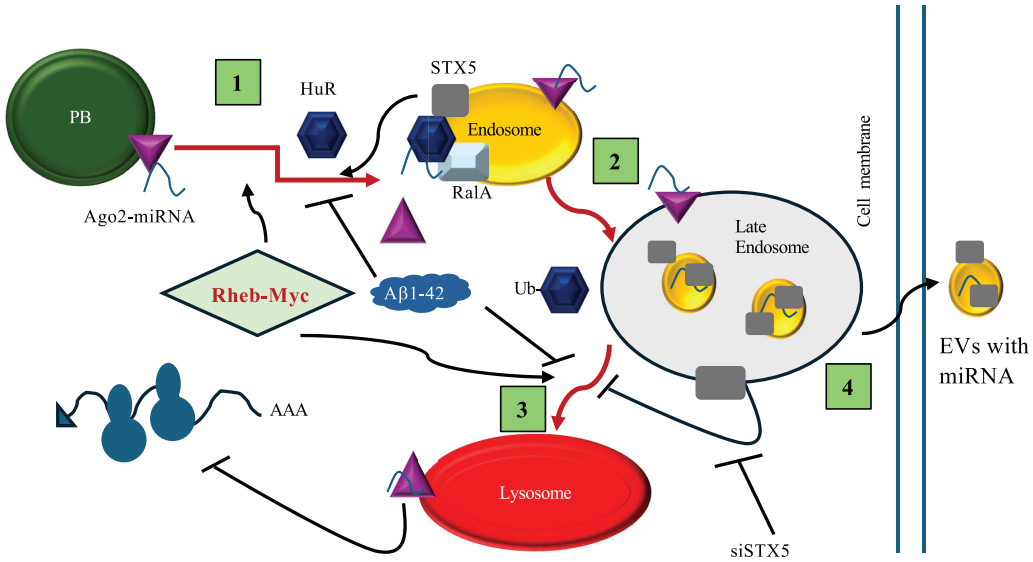

Figure 4. Rheb-induced miRNA export.

The working model of how Rheb-Myc rescues miRNA export involves affecting PB relocalization [1] and accelerating miRNA export [4]. Rheb-Myc facilitates miRNA export and also impacts late endosome-lysosome targeting [3] to speed up miRNA recycling. STX5 is known to be involved in EV loading [2] and export [4] and to promote PB localization of miRNA along with HuR [1]. STX5, by inhibiting the targeting of late endosomes to lysosomes, prevents miRNA recycling.

Argonaute 2 (Ago2)-mediated miRNA targeting to PBs [19]. Rheb-Myc may modulate miRNA export through HuR, which displaces Ago2-miRNA complexes from mRNAs and facilitates miRNA sponging and export [19].

The recycling of miRNAs depends on interactions between late endosomes and lysosomes and seems to operate independently of miRNA export. The protein STX5 helps relocate miRNAs from P-bodies and aids in miRNA export, but it does not support miRNA recycling. This is probably because it inhibits the interaction between late endosomes and lysosomes, which is crucial for miRNA recycling [8]. Conversely, Rheb-Myc promotes both the export and recycling of miRNA, creating a dual pathway of miRNA activation and communication via extracellular vesicle (EV)-mediated export and lysosomal recycling in astroglial cells exposed to amyloid beta oligomers (Figure 4).

Overall, this manuscript provides convincing evidence for a previously unknown mechanism through which Rheb-Myc influences miRNA dynamics in glial cells, suggesting new therapeutic approach to reduce amyloid-β (Aβ)-induced pathology.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

The authors agree to permit the publication of the manuscript.

AVAILABILITY OF DATA AND MATERIALS

The dataset supporting the conclusions of this article is included within the article (and its additional file).

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AUTHORS' CONTRIBUTIONS

S.N.B. and K.M. conceived the idea, designed the experiments, and analysed the data. S.G. and S.H.C. contributed to the design and planning and. performed the experiments. S.N.B. and K.M. wrote the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

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