Journal of Alzheimer's Disease 20 (2010) 1–9

IOS Press

Supplementary Material

Grb2-Mediated Alteration in the Trafficking of AβPP: Insights from Grb2-AICD Interaction

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Supplementary Movie legend. http://www.saha.ac.in/sg/www/movie.htm. The movie is showing fusion of Grb2-DsRed containing smaller vesicles leading to the formation of enlarged late endosome in Neuro 2A cells. After 3 h of transient transfections, individual frames were collected using confocal microscope at 7 min interval for first 3 snaps and then after each 3 min interval for one and half hour.

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ISSN 1387-2877/10/$27.50

Supplementary Table 1. Primer sequence for SDM of $AICD^{Y682A}$

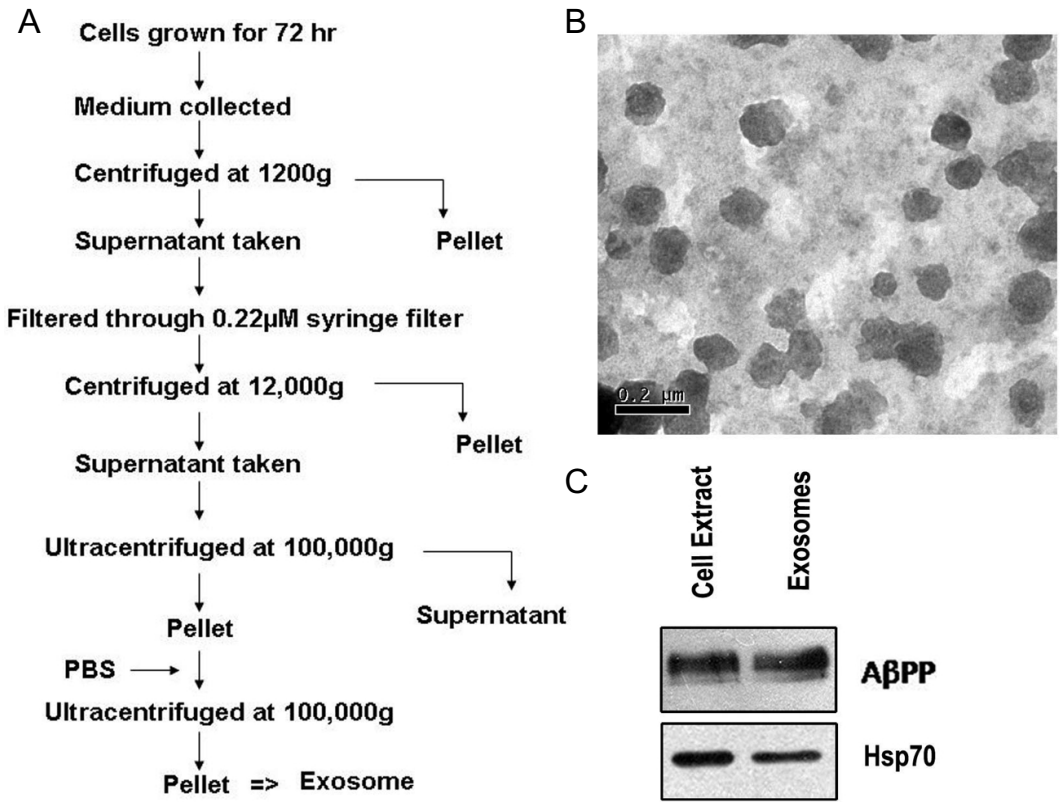
Gene	Primer sequence
$AICD^{Y682A}$	Forward: 5' CAAGATGCAGCAGAACGGCGCCGAAAATCCAACCTACAAG 3' Reverse: 5' CTTGTAGGTTGGATTTTCGGCGCCGTTCTGCTGCATCTTG 3'

Supplementary Table 2. Sequence of oligonucleotide for cloning of siRNA

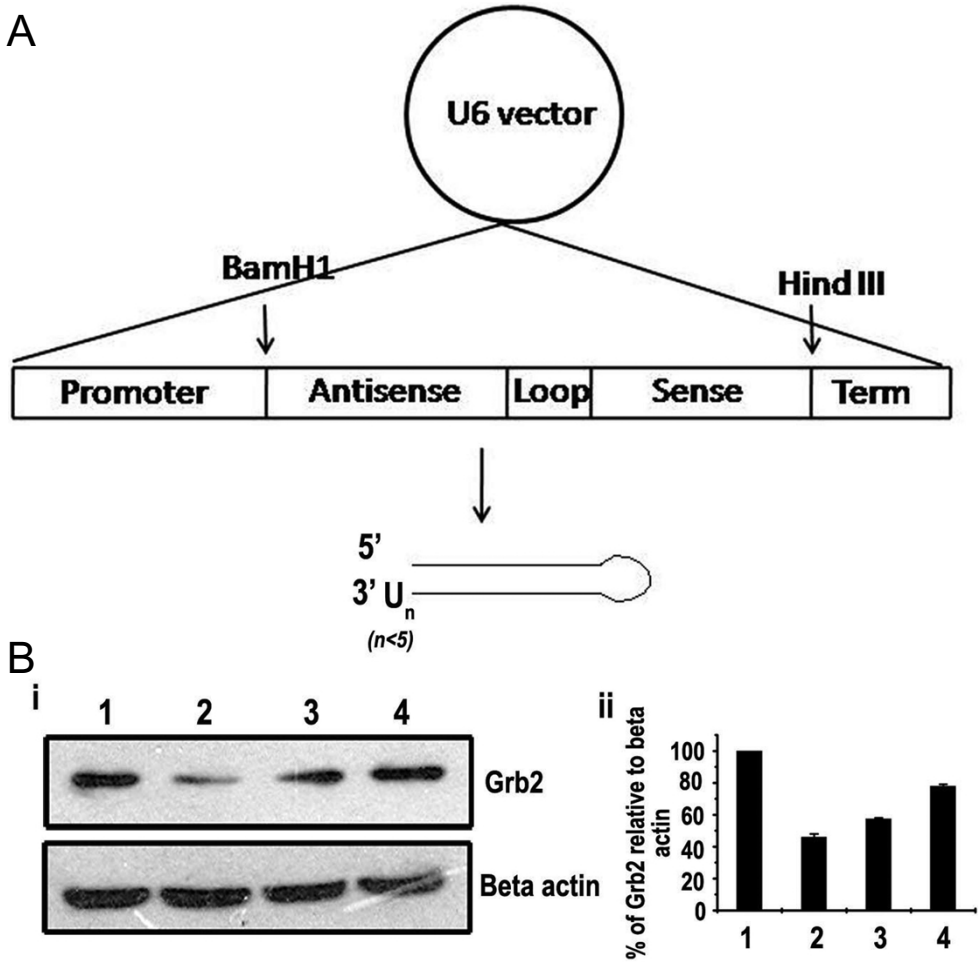
siRNA sequence for Grb2	5 -CGGGATCCATAATTGCGGGGAAACATGTTCAAGAGACATGTTTCCCCGCAATTATTTTTTCCAAAAGCTTGGG-3 RE Antisense Loop Sense dT(Termination) RE 3 -GCCCTAGGTATTAACGCCCCTTTGTACAAGTTCTCTGTACAAAGGGGCGTTAATAAAAAAGGTTTTCGAACCC-5
Negative control	5 -CGGGATCCGTCTGCGATCGCATACAATTTCAAGAGAATTGTATGCGATCGCAGACTTTTTTCCAAAAGCTTGGG-3 RE Antisense Loop Sense dT(Termination) RE 3 -GCCCTAGGCAGACGCTAGCGTATGTTAAAGTTCTCTTAACATACGCTAGCGTCTGAAAAAGGTTTTCGAACCC-5

Supplementary Table 3. Primer sequence and PCR condition of Real Time PCR

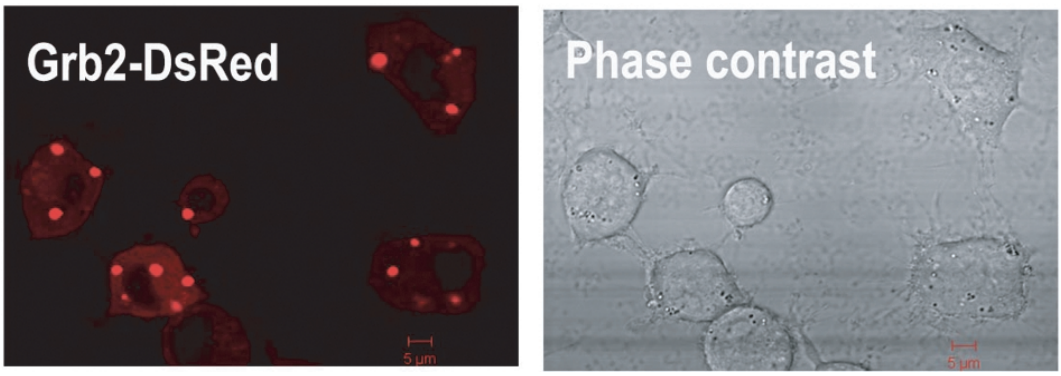
Name of the genes	PCR condition	PCR Cycle	Primer sequences (5'-3')	Size (bp)
AβPP	95°C→ 10min [95°C 30 sec, 50°C 30 sec, 60°C 1 min] 72°C →10 min	35	AGTGGAAGCCATGCTCAATGA TTCTGTTCTGCGCGGACATAC	129
β-actin	95°C→ 10min [95°C 30 sec, 55°C 30 sec, 60°C 1 min] 72°C →10 min	35	TCCTGTGGCATCCACGAAACT GAAGCATTTGCGGTGGACGAT	315



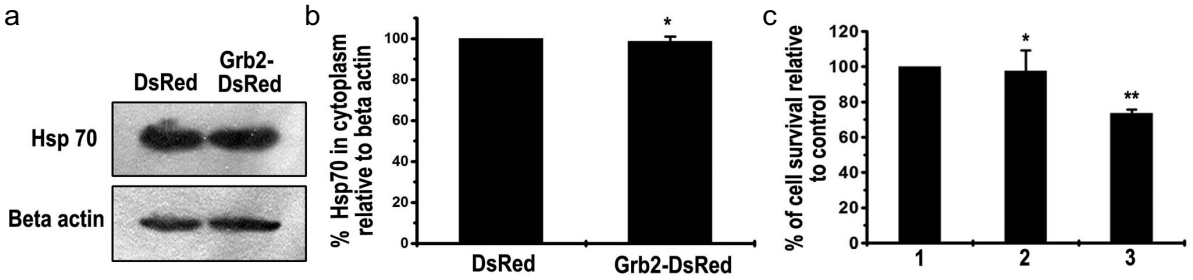
Supplementary Figure 1. Exosome preparation, visualization by electron microscopy and detection of exosomal proteins and AβPP in this preparation. Exosomes were prepared from the medium of Neuro 2A cells grown for 48 h (A). Morphology of the exosomes was analyzed by electron microscopy of the 100,000 g pellet obtained from the 48-h cell culture supernatant. A drop of exosome pellet diluted 1:50 in PBS were put on 400 mesh carbon grids for 5 min. After blotting, the grids were counterstained with 1% uranyl acetate in H_2O for 20 sec. After blotting of excess stain the grids were dried in a desiccator for 2 days and then visualized by Transmission Electron Microscope. The pellet was composed of 50–100-nm vesicles. Scale bar $= 0.2$ μm (B). The 100,000g pellet (exosomes), obtained after differential centrifugation from medium (C, second lane) or from the cell lysate (C, first lane) were analyzed by Western blotting with anti-HSP70 and anti AβPP (N-terminus specific) antibodies (C).



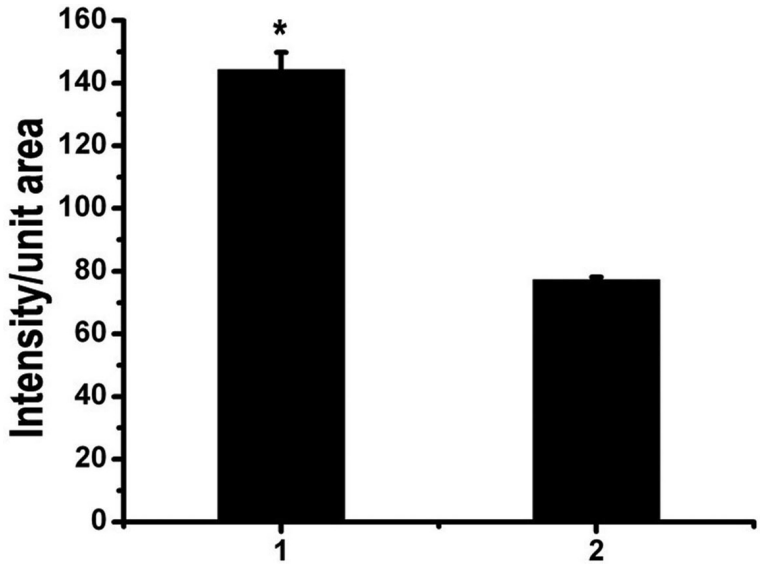
Supplementary Figure 2. Schematic representation of cloning of siRNA of Grb2 and preparation of stable knock down cells. The synthetic oligoneucleotides for the knock down of Grb2 contains an antisense region and a sense region separated by a loop. Both strands were synthesized and hybridized to form a double stranded oligoneucleotide. These synthesized oligoneucleotide contain restriction enzyme (RE) site for BamH 1 and Hind III. Then both double stranded oligoneucleotides and U6 vector were digested by the RE and ligated to form U6-Grb2 and U6-mock clone (A). Neuro 2A cells were transfected with U6-Grb2 and after 48 h of transfections cells were selected by hygromycin. After 7 days, colonies were picked up and grown under selection pressure. Proteins were prepared from three such colonies separately and analyzed by western blot against Grb2 antibody (lane 2, 3, and 4) along with wild type Neuro 2A cell extract (lane 1) (B [i]). In lane 2, there was about 60% decrease in the expression of Grb2 relative to β-actin (B [ii]). This clone was propagated and stocked for future use.



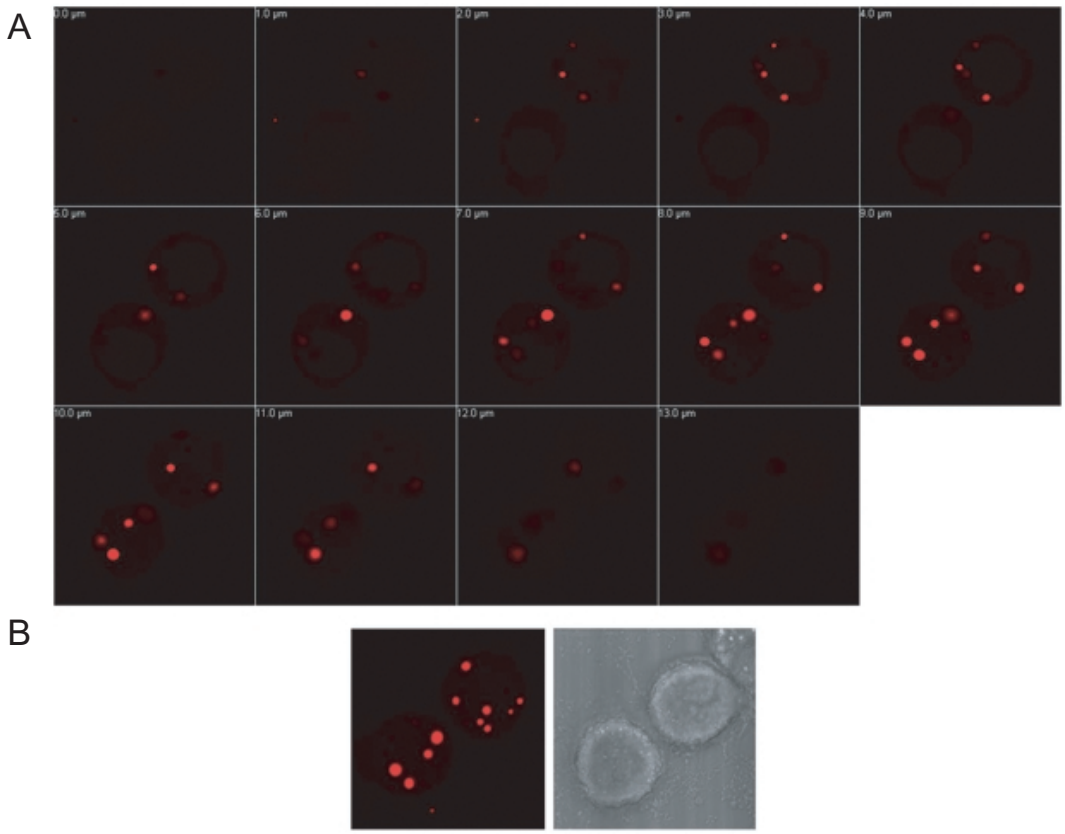
Supplementary Figure 3. Grb2-DsRed is also present predominantly in the vesicles. Grb2-DsRed transfected cells were visualized by confocal microscopy. The protein was mainly localized in vesicular structures but it is also present in the cytoplasm and is detectable at higher detector gain of the microscope.



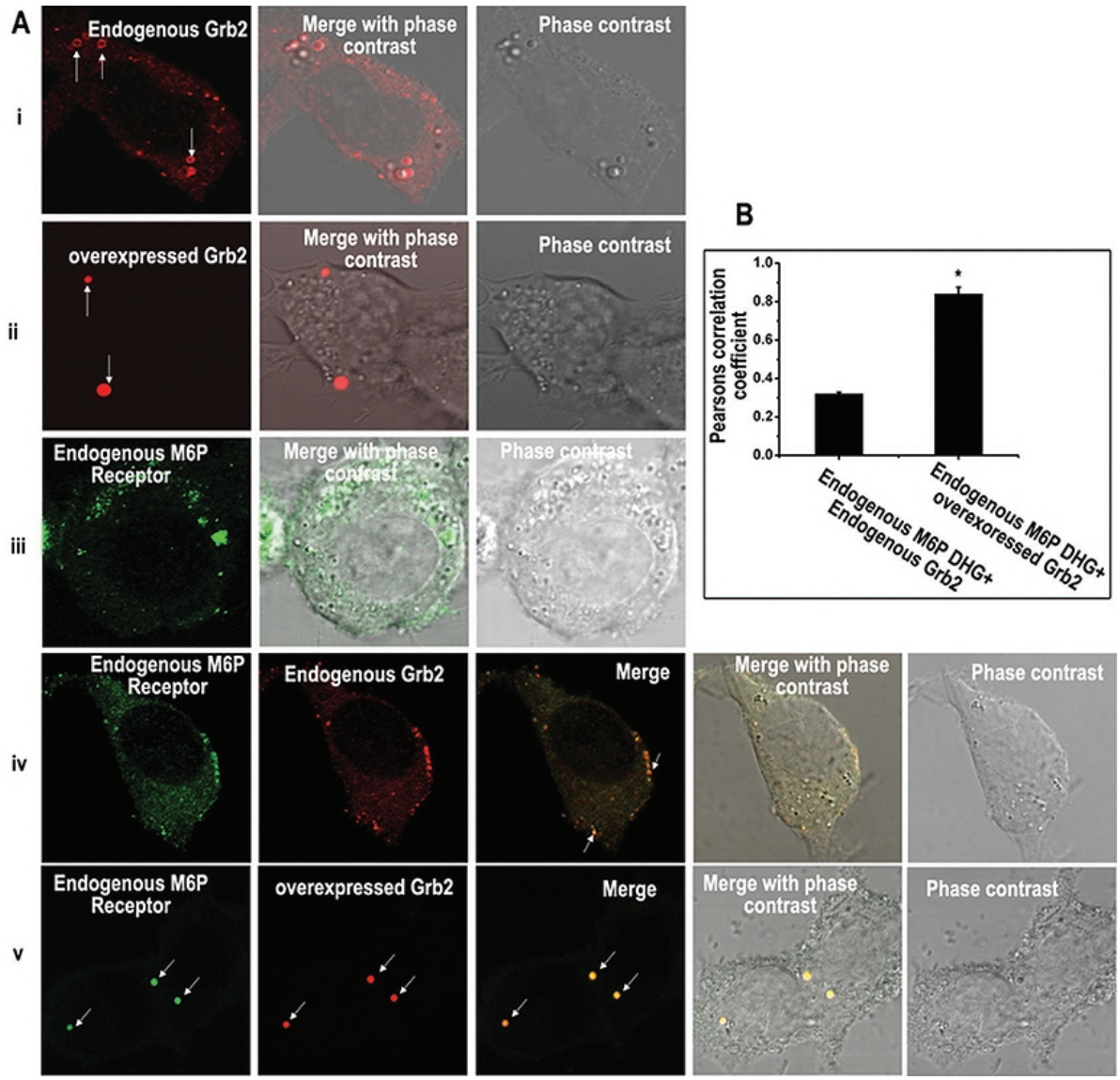
Supplementary Figure 4. No stress is activated upon Grb2 overexpression in Neuro 2A cells. Proteins were prepared from both DsRed and Grb2-DsRed transfected cells after 48 h of transfection, run on SDS-PAGE and western blot was done with antibodies against Hsp70 and β-actin [a]. Fold change was calculated by densitometry analysis taking β-actin as loading control [b]. $^{*}p$ value $= 0.59$ ($n = 3$). [c] Cell survival was determined by MTT assay considering the survival of only DsRed transfectted cells as 100% (1). Survival of Grb2-DsRed transfected cells was found to be $\sim$97% after 24 h of transfection (2). But the p value ($^{*} = 0.744$) indicated that the change was not significant. Whereas the cells treated with 50 μM H_2O_2 for 30 min showed significant ($^{**}p = 0.0001$) decrease in the survival ($\sim$73%) (3) after 24 h of the treatment.



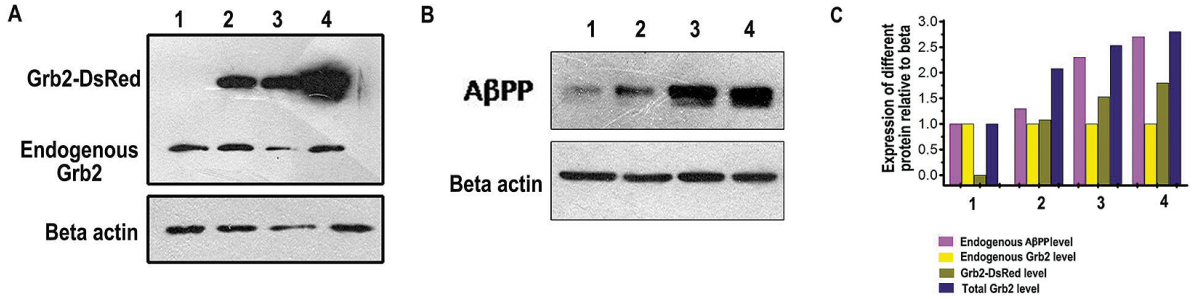
Supplementary Figure 5. Intensity difference of AICD-CFP in colocalized area *vis-a vis* outside. In case of AICD-CFP and Grb2-DsRed transfected cells, the intensity of AICD-CFP/unit area (μm^2) was measured within the co-localization area (1) and outside the co-localization area (2). $^{*}p$ value between 1 and 2 is < 0.0005.



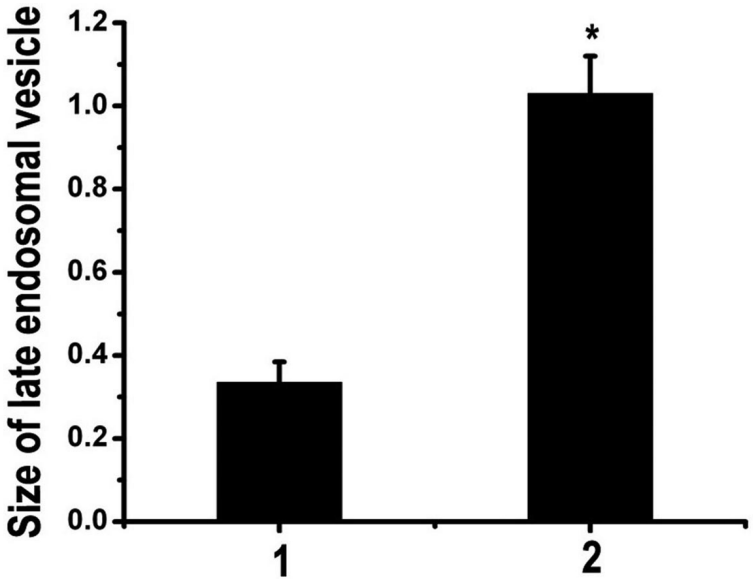
Supplementary Figure 6. Grb2-DsRed containing vesicles at different Z-sections of the cell. Grb2-DsRed transfected Neuro 2A cells were scanned in different Z plane by confocal microscope (A) and all the Z sections were merged to have a single plane view of the 3D image (B).



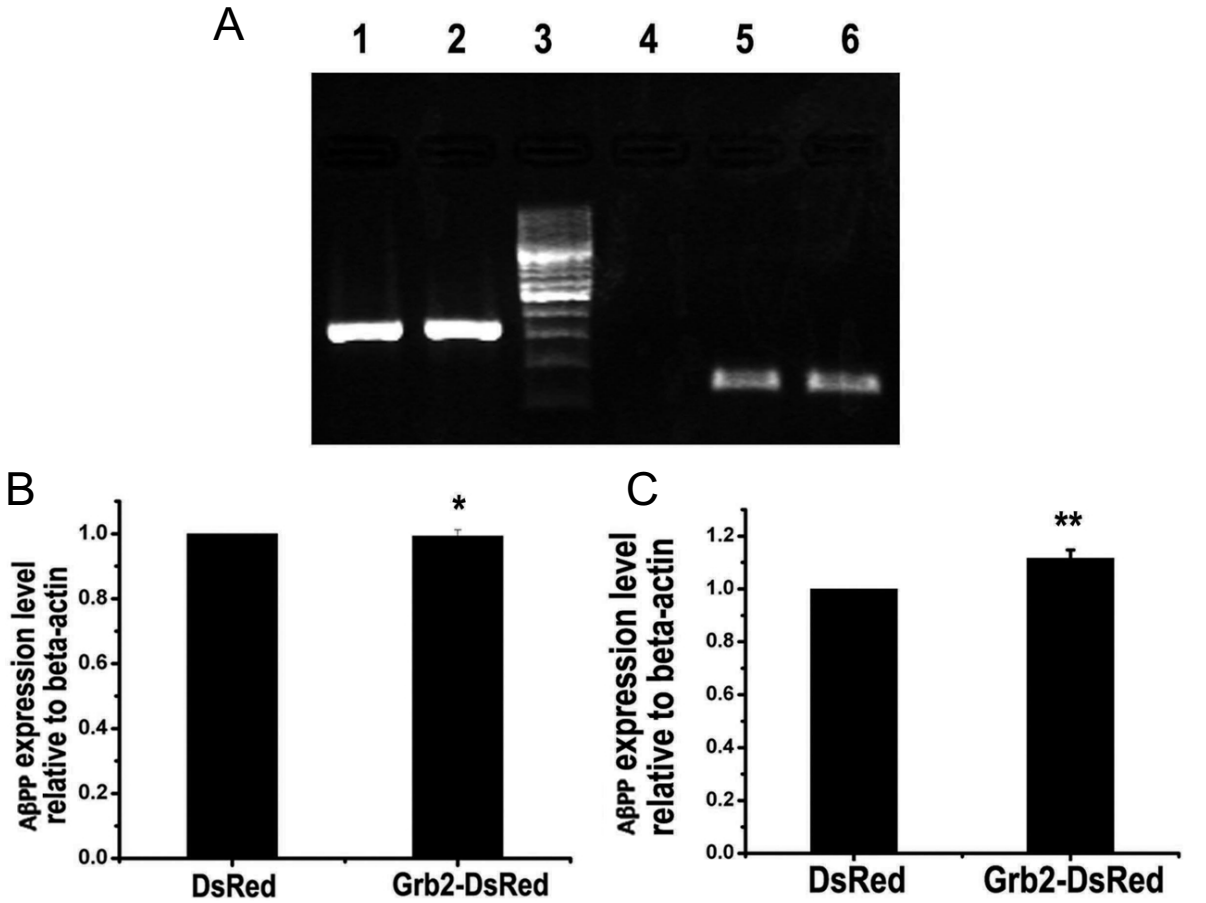
Supplementary Figure 7. Increasing the concentration of Grb2 gradually by transfections of Grb2-DsRed at increasing concentration and its effect on cellular AβPP level. Neuro 2A cells were transfected with control vector DsRed C1 and with 0.5 μg, 1 μg, and 2 μg of Grb2-DsRed constructs. Proteins were prepared from all the cell types and loaded in the above mentioned order in lane number 1, 2, 3, and 4, respectively. The proteins were immunoblotted with anti-Grb2 antibody (A) and antibody against N-terminus of AβPP (B). The relative level of endogenous Grb2 (Yellow), Endogenous AβPP (Magenta), and Grb2-DsRed (Green) were calculated relative to β-actin and total Grb2 level (endogenous + overexpressed) was designated in blue bar (C). The level of endogenous AβPP and Grb2 in DsRed transfected cells was taken as 1 fold (C1) to calculate the changes in other condition (C 2, 3, & 4).



Supplementary Figure 8. Co-localization of both endogenous and overexpressed Grb2 with late endosomal marker Receptor for Mannose 6-phosphate (M6P). [i] Neuro 2A cells were immunostained for endogenous Grb2 (red). [ii] Localization of overexpressed Grb2 in Grb2-DsRed transfected cell. [iii] Neuro 2A cells were immunoblotted with late endosomal marker receptor for mannose 6-phosphate (green). [iv] Neuro 2A cells were immunostained with marker for late endosome (green) and endogenous Grb2 (red). [v] Grb2-DsRed transfected cells were immunostained with late endosomal marker (green). B) Calculation of co-localization: The degree of co-localization between late endosomal marker and endogenous Grb2 & late endosomal marker and transfected Grb2-DsRed are illustrated in terms of 'Pearson's correlation coefficient'. $^*p$ value < 0.0001 ($n = 4$).



Supplementary Figure 9. Increase in the size of late endosomal vesicles in Grb2-DsRed transfected cells. The size of late endosome that contain endogenous Grb2 (1) and overexpressed Grb2-DsRed (2) was measured in μm^2 by the analysis software of confocal microscope LSM 510 META (Carl Zeiss, Jena, Germany). *p value between 1 and 2 is $= 0.0001$ ($n = 12$).



Supplementary Figure 10. Expression of endogenous AβPP remains unchanged in Grb2-DsRed transfected cells versus control cells. To check the expression level of AβPP, RNA was isolated from Neuro 2A cells transfected with either DsRed or Grb2-DsRed by RNeasy Mini Kit (Qiagen, Hilden, Germany). The first strand cDNA was synthesized using random hexamer primers and reverse transcriptase (Invitrogen). Using that c-DNA as template, Reverse Transcriptase PCR of β-actin from DsRed c-DNA (a, lane1), from Grb2-DsRed c-DNA (a, lane2) and AβPP from DsRed c-DNA (a, lane5), from Grb2-DsRed c-DNA (a, lane6) was done. 100bp DNA ladder was run on lane 3 (A). PCR products were visualized by running in 1.5% agarose gel by standard methods. The change in the expression of AβPP was calculated relative to beta actin and the expression of AβPP in DsRed transfected cells was taken as 1 to calculate the fold change (B). Visualization of DNA gel and calculation of band intensity was done by Quantity One software of BioRad. $^{*}p$ values $= 0.5987$. The expression of AβPP in both the cell lines was also checked by Real Time PCR using Power Syber green 2X Universal PCR Master Mix (Applied Biosystems) in ABI Prism 7500 sequence detection system. Each reaction was performed in triplicate using corresponding primers. Non-template controls, both for AβPP and β-actin, were used at the same condition to ascertain the baseline and threshold value for the analysis. The relative quantification of AβPP in Grb2-DsRed transfected cell compared to only DsRed transfected cell is expressed in terms of $2^{-\Delta\Delta Ct}$ values after normalization with respect to internal control (β-actin gene) (C). $^{**}p$ value $= 0.3010$. So at 95% confidence interval these differences were considered to be not statistically significant.