4. Chapter I: The essential role of Trib3 in neuron death in AD model

4.1 Introduction

Alzheimer's Disease (AD) is a progressive neurodegenerative disease which accounts for 60-80% of dementia today. It leads to a gradual failure in several cognitive domains resulting in loss of memory and other cognitive abilities. Extracellular plaques of β-amyloid (Aβ) and intraneuronal neurofibrillary tangles of hyperphosphorylated tau are the two major hallmarks of AD [1, 2]. Aggregation of misfolded proteins, anomalous autophagy, upregulated oxidative stress and other metabolic dysregulations are also some of the characteristic features of the disease [3-5]. Increased Endoplasmic Reticulum (ER) sensitivity occurring due to perturbations in the cellular homeostasis is also observed in AD. Excessive ER stress is deleterious to neurons as it not only has the ability to trigger inflammatory responses but it can initiate an apoptotic program in these cells [6]. Trib3 has been reported to be involved in killing cells via apoptosis [7]. Mounting evidence reveals the presence of apoptotic cells and fragmented nuclei in the brain of AD patients [8, 9].

Recent studies indicate the active role of ER stress in upregulating autophagy and promoting apoptosis of tumour cells, wherein Trib3 leads the series of events by inhibiting Akt and mTORC1 which leads to increased autophagy and ensuing apoptotic death of hepatocarcinoma cells and glioma cells [10, 11]. The endoplasmic reticulum (ER) is regarded primarily as the site of synthesis of proteins. It is responsible for protein folding of all types of proteins, be it secreted, membrane-bound or some organelle-targeted proteins. ATP, Ca2+ and an optimum oxidizing environment are among the several factors that are required for proper protein folding to facilitate disulphide-bond formation [12]. As a consequence of prevalence of this exquisite environment, the ER emerges to be highly sensitive to stresses that possess the potential to disturb the cellular energy levels, the physiological redox state or Ca2+ concentration. Such stresses have an adverse effect on the ER, they impair the protein folding capacity of the ER, as a result of which there occurs accumulation and aggregation of unwanted unfolded proteins. This condition is referred to as ER stress. Aggregation of unwanted proteins within the cell is toxic to cells and, as a consequence, several pathophysiological conditions become associated with ER stress. These include ischaemia, neurodegenerative diseases and diabetes [13]. In order to cope up with the ill effects of ER stress, cells have come up with various protective strategies, collectively

referred to as the unfolded protein response (UPR). This compact and complex cellular response is mediated through three of the ER transmembrane receptors, namely pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1). In control conditions, all three ER stress receptors remain in association with the ER chaperone, GRP78 and hence remain in an inactive state. When there occurs an accumulation of unfolded proteins, GRP78 disrupts the association with the three receptors and dissociates from them. This leads to their activation and upregulation of the UPR. The main aim of the UPR is to reduce the accumulation of shoddy unfolded proteins and restore to normalcy the ER functioning. Hence the UPR qualifies as the prosurvival response [14]. However, if there occurs a persistent aggregation of proteins and the stress turns out to be unresolvable; there occurs a switch from pro-survival signaling to a pro-apoptotic signaling. The molecular mechanisms involved in these events are not quite well studied and are only emerging now. It is in this scenario that the emerging role of Trib3 comes to the fore, and it is expected to play an important role.

Trib3 is a mammalian ortholog of the Drosophila *Tribbles* gene and is also known as neuronal death-inducible putative kinase/ Sink1/ Skip3 [15]. Trib3 is responsible for a plethora of functions ranging from glucose regulation, migration of tumour cells, suppressing differentiation of adipocytes and cell cycle control [16-19]. It was identified as a novel ER stress inducible gene which when upregulated activated several genes involved in cell death during ER stress [20]. Trib3 is also shown to be elevated by several stresses including hypoxia, 6-hydroxy dopamine, growth factor deprivation, anoxia and ethanol exposure [15, 21-27]. It has also been shown that Trib3 is elevated in Parkinson's disease (PD) brains and mediates death of neurons in various models of PD which include MPTP+ and 6-OHDA models [26]. Trib3 is a pseudokinase as bioinformatic analysis of the protein reveals that it does not possess the catalytic residues which are essential for its kinase function [28, 29]. Further analysis of the Trib3 protein reveals presence of numerous conserved domains which attribute to its ability to physically interact with several protein binding partners [24, 30-32].

Since AD has been characterized as a disease caused by several factors, here a therapeutic strategy to tackle the disease involving a single unwavering approach might prove to be ineffective. A treatment strategy which could combine several approaches could prove to be beneficial in this regard. Several approaches have been looked into,

yet, at the stage of clinical trials, most of these approaches have met with failure. Since the Aβ cascade hypothesis occupies a pivotal position in the pathogenesis of the disease, an approach which appears to be promising in this scenario could be the one which targets Aβ centrally [33, 34]. Apart from this, a complementary therapy is required to prevent the occurrence of toxicity due to Aβ, as the complete removal of Aβ is arduous. Hence, a comprehensive understanding of the molecular mechanism of death induced by Aβ is essential. In this study, we have investigated the role of Trib3 in inducing neuronal death evoked by Aβ. From our studies it appears that Trib3 is upregulated due to Aβ and promotes death of neurons by apoptosis in response to Aβ.

It is important for neuronal apoptosis to occur in the developing nervous system, as it is required for shaping the structure of the system properly and for the elimination of neural precursor cells which harbor mutations that are damaging [35]. During the late stage of development the factors which play important roles are the target-derived neurotrophic factors. They cause the removal of half the population of post-mitotic neurons because the availability of these factors decreases drastically during this stage. Only about the rest half of the best fitting neurons are allowed to survive and establish neural connectivities [36, 37]. The most essential neurotrophic factor in this regard is nerve growth factor (NGF), it promotes the survival of sympathetic neurons and a group of sensory neurons, where the neurons have to compete for the limited availability [38, 39]. This competition results in programmed cell death. A characteristic feature of it is the synthesis of the pro-apoptotic proteins dependant in a transcriptional manner [40]. Neuronal apoptosis is a complex process which involves various proteins and pathways. It is still not completely elucidated. That the BH3-only proteins of Bcl-2 family are important, has been revealed by RNAi and genetic knockout studies. But the studies also reveal that they are not indispensable in inducing apoptosis and that other pro-apoptotic molecules must also exist [41-43]. Furthermore, the process by which the intracellular machinery gets deregulated only due to a limited supply of NGF also remains a mystery. It is most likely that deactivation of the pro-survival regulatory head, Akt, is an integral part of this process. The pseudokinase Trib3 has been reported to negatively regulate Akt and inhibit it’s activation in non-neuronal cells under several stressful conditions [44].

4.2 Results

4.2.1 Aβ treatment induces Trib3 mRNA and protein levels *in vitro* and *in vivo*

Studies implicate Aβ oligomers as central to AD pathogenesis and the principal cause of it [45, 46]. Oligomeric Aβ at a concentration of 1.5 μM leads to significant death of primary cortical and hippocampal neurons after 24 h of exposure (Fig. 1A, B, C & D) [47]. We determined the levels of Trib3 in neurons after Aβ exposure. We found that Trib3 levels were increased in cultured cortical neurons following Aβ treatment. Trib3 transcript levels were significantly increased as early as after 4 h and about 3 fold increased after 8 h of Aβ treatment as detected by semi-quantitative (Fig. 2A) and real time PCR (Fig. 2B). Protein levels of Trib3 were also significantly increased within 4 h and they were about 3 fold and 3.5 fold increased after 8 h and 16 h of Aβ treatment, respectively (Fig. 2C & D). Thus, Trib3 expression was elevated well before cell death became apparent.

Next we investigated whether this increase of Trib3 *in vitro* is also reflected in *in vivo* conditions. Reports reveal that oligomeric Aβ when infused into adult rat brains results in deposition of Aβ, activation of caspase-3 and neuronal cell loss occurring in the vicinity of Aβ infusion [47]. In our study adult rats were infused with Aβ or PBS, on the right hemisphere of their brains. 21 days later these animals were sacrificed and their brains were fixed, cryosectioned and then co-immunostained with Trib3 and NeuN (a neuronal marker) antibodies. Nuclei were stained with Hoechst dye. Results revealed marked upregulation of Trib3 in Aβ infused rat brains as compared to PBS infused rat brains (Fig. 2F & G). Presence of Aβ in the infused sections was also checked (Fig. 2E). Since synthetic Aβ may behave in a manner disparate from naturally secreted Aβ, brain sections of APPswe-PS1de9 (Swedish mutation in APP and PS1 mutation) transgenic mice which naturally secrete Aβ were also examined for Trib3 expression. Presence of Aβ plaques in the transgenic mice brain was checked by Congo red staining (Fig. 2H). Transgenic and control littermate mice brains were cryosectioned and co-immunostained with Trib3 and NeuN antibodies. Hoechst dye was used to stain the nuclei. It was observed that there was a significant increase in Trib3 levels in transgenic mice as compared to the control littermates (Fig. 2I & J). Our study therefore indicates that Trib3 expression is increased in neurons upon exposure to Aβ *in vitro* and *in vivo*.

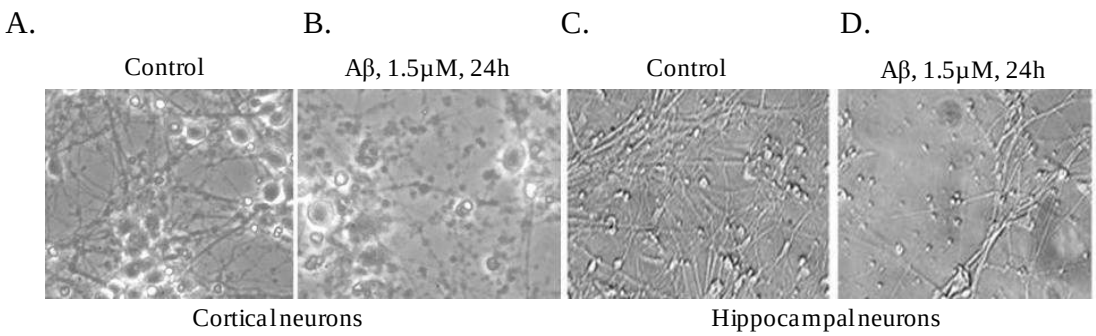
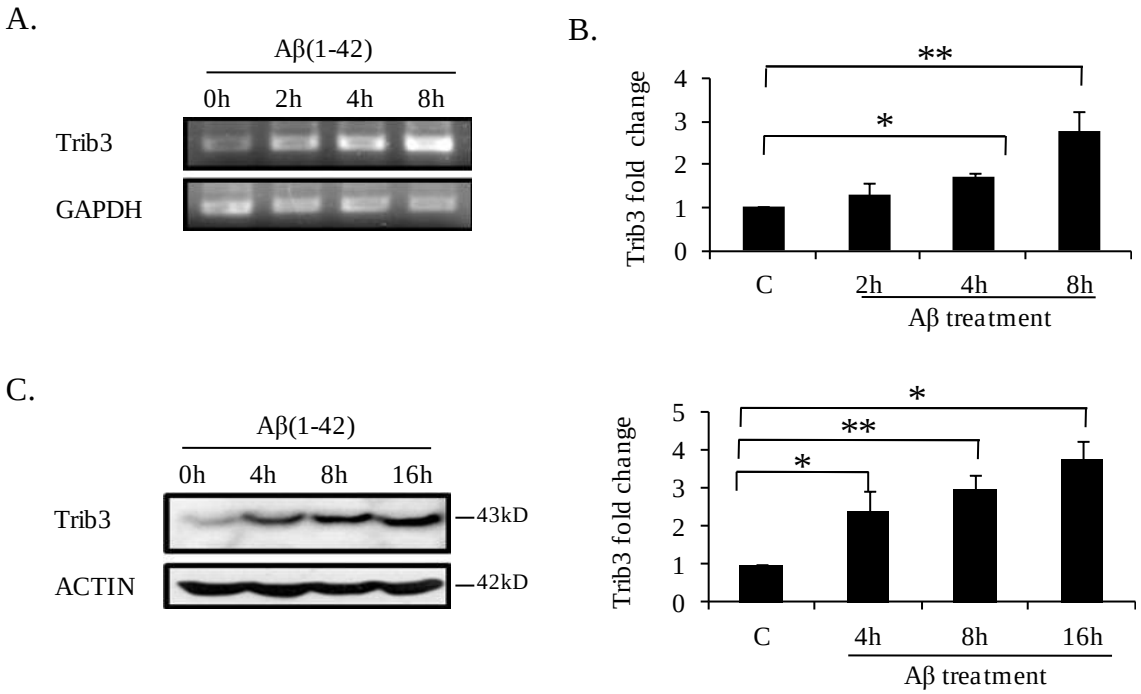
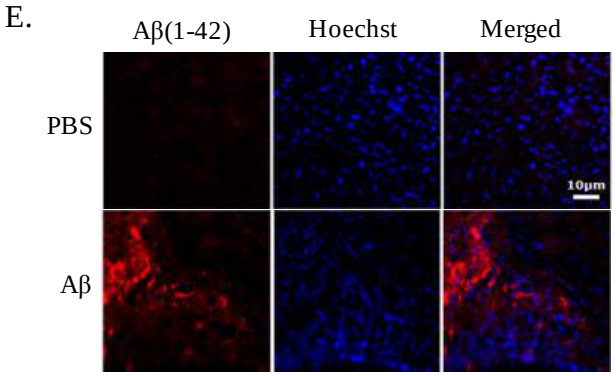


Figure C.1.1. Rat cortical neurons and hippocampal neurons die in response to Aβ. *Phase-contrast photograph of rat cortical neurons (5 DIV)* ***(A),*** *with 1.5 μM of oligomeric β-amyloid treated for 24h* ***(B).*** *Phase-contrast photograph of rat hippocampal neurons (21 DIV)* ***(C),*** *with 1.5 μM of oligomeric β-amyloid treated for 24h* ***(D).***





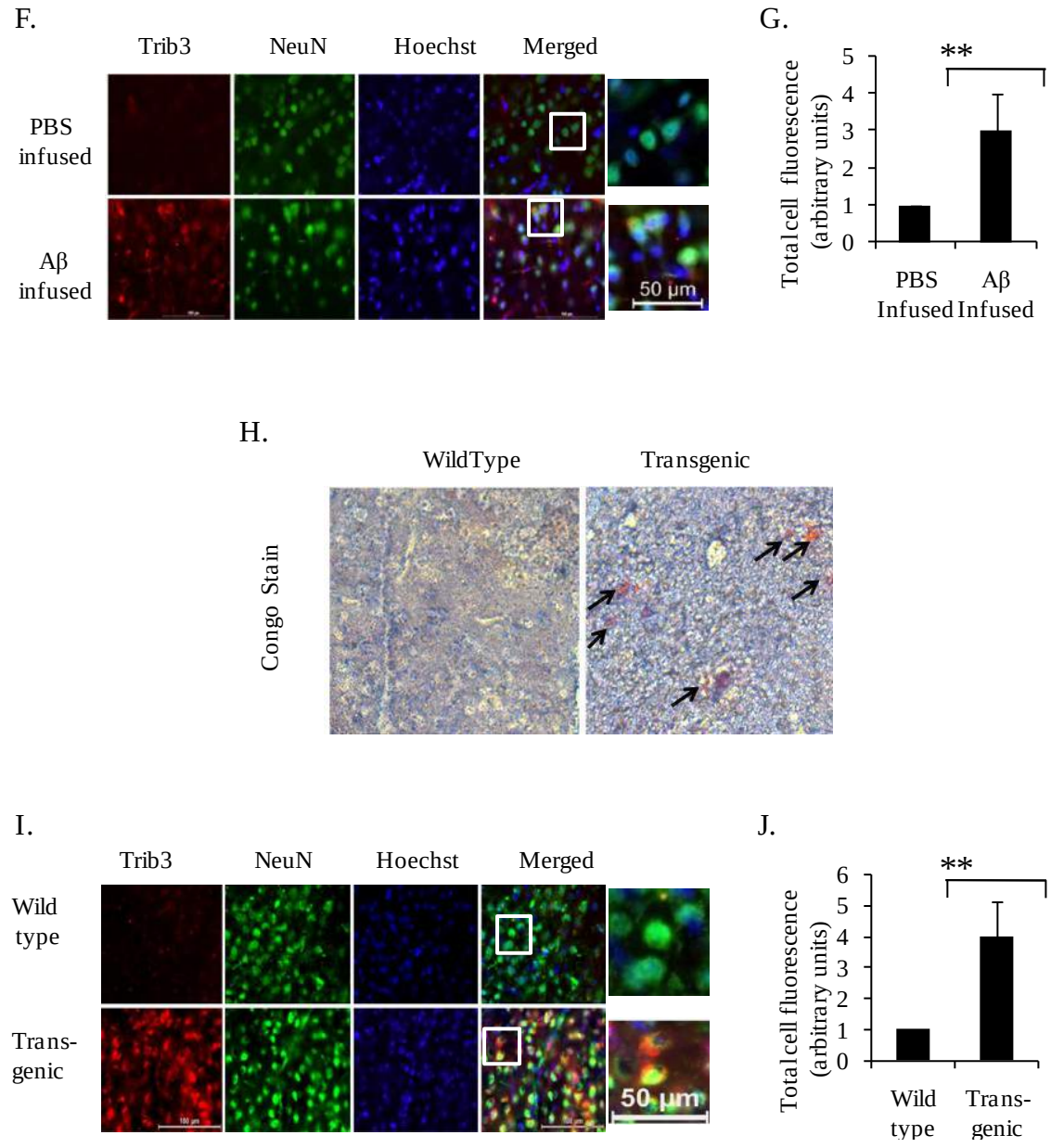


Figure C.1.2: Trib3 is induced in cortical neurons and in vivo following Aβ treatment. *Primary rat cortical neurons (7 DIV) were treated with oligomeric Aβ 1.5µM for the indicated times.* ***(A)*** *Total RNA was isolated, subjected to reverse transcription and analysed by semi-quantitative PCR using Trib3 primers. α-tubulin was used as loading control.* ***(B)*** *Graphical representation of fold changes in Trib3 transcript level upon Aβ treatment to rat cortical neurons for the indicated times by quantitative Real Time PCR. GAPDH was used as loading control. Data represents mean ± SEM of three independent experiments *p ‹ 0.05; **p ‹ 0.01.* ***(C)*** *Primary cultured rat cortical neurons were treated with Aβ for the times indicated. Total cell lysates were subjected to western blotting analysis for Trib3 levels. A representative immunoblot of three independent experiments with similar results is shown. Actin was used as loading control.* ***(D)*** *Graphical representation of the Trib3 protein levels as quantified by densitometry of western blots in cortical neurons subjected to Aβ treatment for different time points. Data are expressed relative to untreated control. Data represents mean ± SEM of three*

*independent experiments *p ‹ 0.05; **p ‹ 0.01* ***(E)*** *Presence of Aβ was analysed in brain sections obtained from rat brains infused with Aβ antibody.* ***(F)*** *Level of Trib3 expression was analysed in Aβ infused rat brain sections.* ***(G)*** *&* ***(J),*** *Graphical representation of corrected total cell fluorescence of Trib3. Difference in the intensity of Trib3 staining is quantified by NIH imageJ as described in experimental procedures. Data represent mean ± SEM; **p ‹ 0.01.* ***(H)*** *Congo red staining of brain slices from transgenic mice.* ***(I)*** *Level of Trib3 expression was analysed in brain sections obtained from AβPPswe-PS1de9 transgenic mice and control littermates.*

4.2.2 Trib3 plays an essential role in neuronal death evoked by Aβ

We next checked whether Trib3 is required for evoking neuronal death in response to Aβ. Inorder to analyse this we interfered with the expression of Trib3 using previously described shRNA constructs [22]. The Trib3 shRNA construct efficiently blocked induction of Trib3 by Aβ in neuronally differentiated (primed) PC12 cells (Fig. 3A & B). Primary cultured cortical neurons were then transfected with this shRNA construct (shTrib3) or a control shRNA construct (shRand) and maintained for 48 h followed by oligomeric Aβ treatment 1.5 μM. Transfected live green cells were monitored and counted under fluorescence microscope at different time interval. We observed that downregulation of Trib3 by shRNA blocked neurodegeneration as seen from the retention of neuronal processes and also led to significant survival of these neurons compared to shRand transfected neurons even after 72 h of Aβ treatment (Fig. 3C & D).

A similar experiment with cultured hippocampal neurons revealed that downregulation of Trib3 also provided significant protection of hippocampal neurons from Aβ induced neurotoxicity (Fig. 3E & F). Hippocampal neurons expressing shTrib3 displayed increased viability as compared to shRand transfected neurons but also showed enhanced retention of neurites and overall neuronal morphology. Further, we quantitatively assessed the retention of neurites and neuronal networks in shTrib3 transfected hippocampal neurons after Aβ treatment by Sholl analysis as described [47, 48]. Single hippocampal neurons transfected with either shTrib3 or shRand were analyzed by NIH-ImageJ as described in experimental procedures. Results revealed that the number of crossings, implicating arborizations, remained mostly same before and after treatment of Aβ in case of shTrib3 transfected neurons; whereas, there was a huge decrease in the number of crossings in shRand transfected neurons (Fig. 3G). We also found similar protective effect of shTrib3 on neuronal PC12 cells upon Aβ treatment.

Taken together, these results suggest that Trib3 plays a necessary role in mediating neuron degeneration and death evoked by Aβ toxicity.

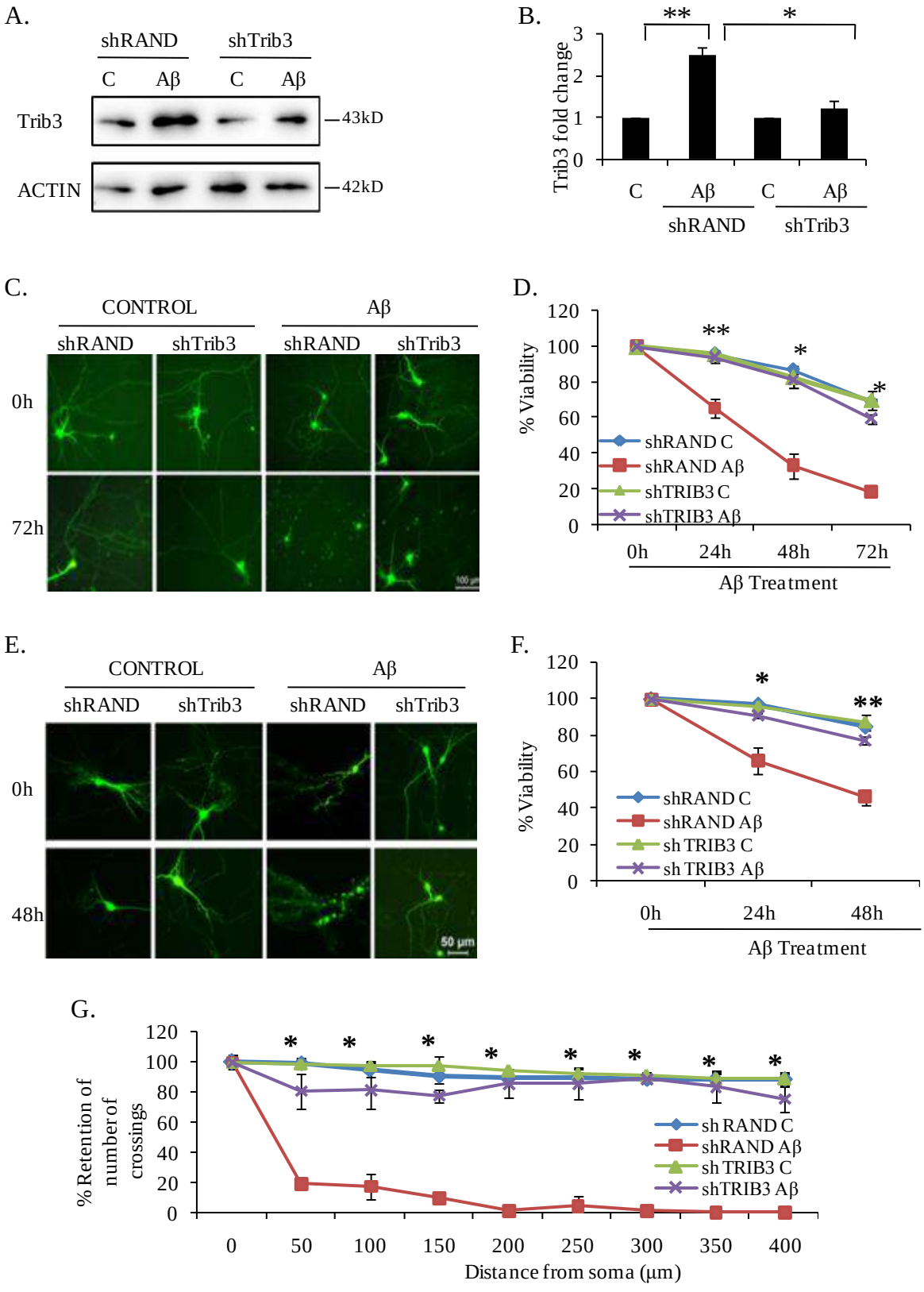
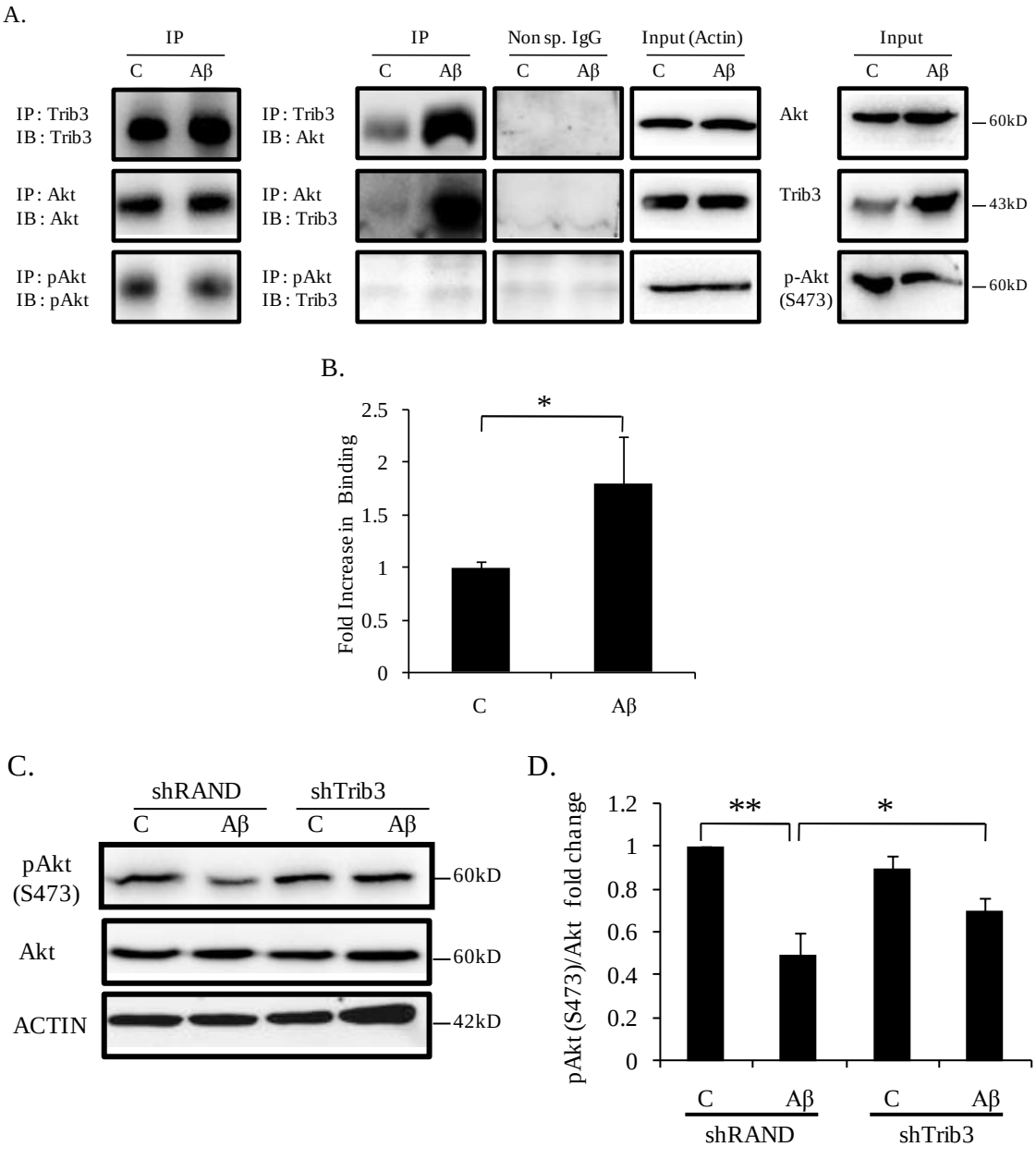


Figure C.1.3: Downregulating Trib3 by shRNA provides protection to cultured cortical and hippocampal neurons. ***(A)*** *PC12 cells were transfected with shTrib3 or shRand, primed and then treated with and without Aβ (5μM). Levels of endogenous Trib3 levels were assessed by western blot analysis using anti-Trib3 antibody.* ***(B)*** *Graphical representation of fold change of Trib3 levels by densitometric analysis of western blots. Data represents mean ± SEM of three independent experiments *p < 0.05, **p < 0.005.***(C)*** *Primary cultured rat cortical neurons (5 DIV)* ***& (E)*** *primary cultured hippocampal neurons (19 DIV) were transfected with pSIREN-shTrib3-zsgreen (shTrib3) or control pSIRENshRand-zsgreen (shRand) and maintained for 48 h and then subjected to Aβ (1.5 μM) treatment for 72 h. Representative pictures of transfected neurons that were maintained in presence or absence of Aβ for indicated time periods are shown. Images were taken using an inverted fluorescence microscope.* ***(D) & (F)*** *Graphical representation of percentage of viable green cells after each time point. Data are from three independent experiments, each with comparable results and are shown as mean ± SEM, performed in triplicates.*p< 0.05; **p<0.001.* ***(G)*** *Sholl analysis of single imaged neurons by using NIH ImageJ was done as described in 'experimental procedures'. Data represent mean ± SEM of six different neurons from three independent cultures for each class.*p<0.001*

4.2.3 Trib3 negatively regulates Akt upon Aβ treatment:

Studies reveal that Trib3 inhibits Akt activity by physically interacting with it as seen in non-neuronal cells [44, 49, 50]. It has been reported that phospho-Akt level is markedly reduced in AD brain [51], we tested whether Trib3 also negatively regulates Akt in neuronal cells in response to Aβ. Primary cultures of cortical neurons were treated with Aβ, cell lysates were immunoprecipitated with Trib3 antibody and western blotted with Akt antibody. Results revealed increased binding of Trib3 with Akt in Aβ-treated neurons compared to control neurons (Fig. 4A & B). We then checked the phosphorylation status of Akt upon Aβ treatment. We observed that the decrease in phosphorylation at S473 of Akt caused by Aβ insult was rescued when Trib3 was downregulated (Fig. 4C & D). We also observed that when PI3K is inhibited by a specific PI3K inhibitor in presence or absence of Aβ, the protein levels of Trib3 increased even in absence of Aβ (Fig. 4E & F). This intrigued us and we wanted to see whether Trib3 is regulated by some downstream target of PI3K/Akt, which may be activated upon PI3K inhibition and in turn regulated levels of Trib3. Of the plethora of downstream targets of Akt, transcription factor FoxO, downstream to PI3K/AKT and an entrenched substrate of it, is reported to translocate from cytosol to nucleus and induce its target genes transcriptionally in Aβ-treated neurons [47, 52]. Previous reports reveal the presence of several FoxO binding sites on the Trib3 promoter [53-56]. We therefore contemplated that Trib3 may be regulated by FoxO transcription factors in Aβ-treated

neurons. We performed chromatin immunoprecipitation assay to check the direct binding of FoxO1 with the Trib3 promoter. The result showed that FoxO1 occupancy of the Trib3 promoter was markedly increased in response to Aβ (Fig. 4G). These results indicate that Trib3 directly binds and inactivates Akt, this in turn activates the transcription factor FoxO1 and FoxO1 can occupy Trib3 gene promoter upon Aβ treatment.



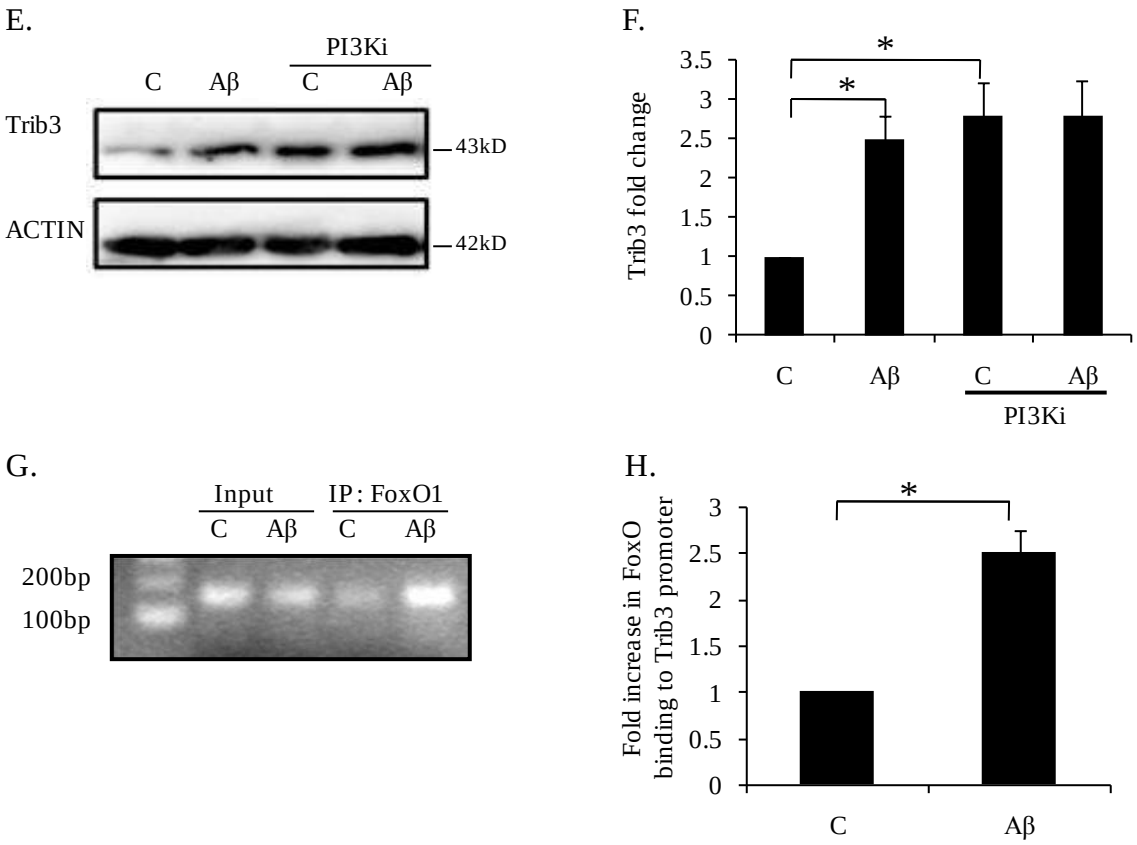


Figure C.1.4: Trib3 negatively regulates Akt. (A) *Cultured rat cortical neurons were treated with 1.5 μM Aβ for 16h; cell lysate was immunoprecipitated with Trib3 antibody and immunoblotted with Akt antibody, reverse immunoprecipitation was also performed to check interaction of Trib3 with Akt; cell lysate was also immunoprecipitated with p-Akt and immunoblotted with Trib3 (2nd column). Immunoprecipitation with non specific antibody was done to check specificity of the reactions (3rd column). Actin was used as loading control (4th column). Levels of the specific proteins in cell lysates (inputs) were also checked (5th column). Immunoblot of the respective immunoprecipitated protein was also performed (1st column).* ***(B)****Graphical representation of the Akt protein levels as quantified by densitometry of western blots. Data represents mean ± SEM of three independent experiments *p < 0.05.* ***(C)*** *PrimedPC12 cells were transfected with shTrib3 or shRand, treated with and without Aβ 5μM. Levels of endogenous p-Akt (S473) were assessed by western blot analysis using anti-p-Akt (S473) antibody.* ***(D)*** *Graphical representation of fold change of p-Akt (S473) levels by densitometric analysis of western blots. Data represents mean ± SEM of three independent experiments *p < 0.05, **p < 0.005.* ***(E)*** *Cultured cortical neurons were treated with Aβ 1.5μM for 16h, with and without LY294002 (PI3Ki). Total cell lysates were subjected to western analysis with Trib3 antibody. A representative immunoblot of three independent experiments with similar results is shown.* ***(F)*** *Graphical representation of fold change of TRIB3 upon Aβ treatment with and without PI3Ki. Data represents mean ± SEM of three independent experiments *p < 0.05* ***(G)*** *Primary cultures of rat cortical neurons were treated with or without Aβ for 8h. An equal number of cells were processed for ChIP assay using anti-FoxO1 antibody for immunoprecipitation. The immunoprecipitated materials were subjected to PCR using primers against the region of TRIB3 promoter that contains the putative FoxO1-binding site. PCR products were verified by agarose gel electrophoresis. Templates were DNA from cells before ChIP (input) or DNA from immunoprecipitated (IP) materials. PCR assays were conducted after ChIP using samples from cells that were either left untreated (control) or treated with Aβ.ChIP assay indicates that FoxO1 occupancy of rat TRIB3 promoter regions enhances after Aβ exposure.* ***(H)*** *Graphical representation of fold changes in FoxO1*

*association with the Trib3 promoter upon Aβ treatment. Data represents mean ± SEM of three independent experiments. *p ‹ 0.05.*

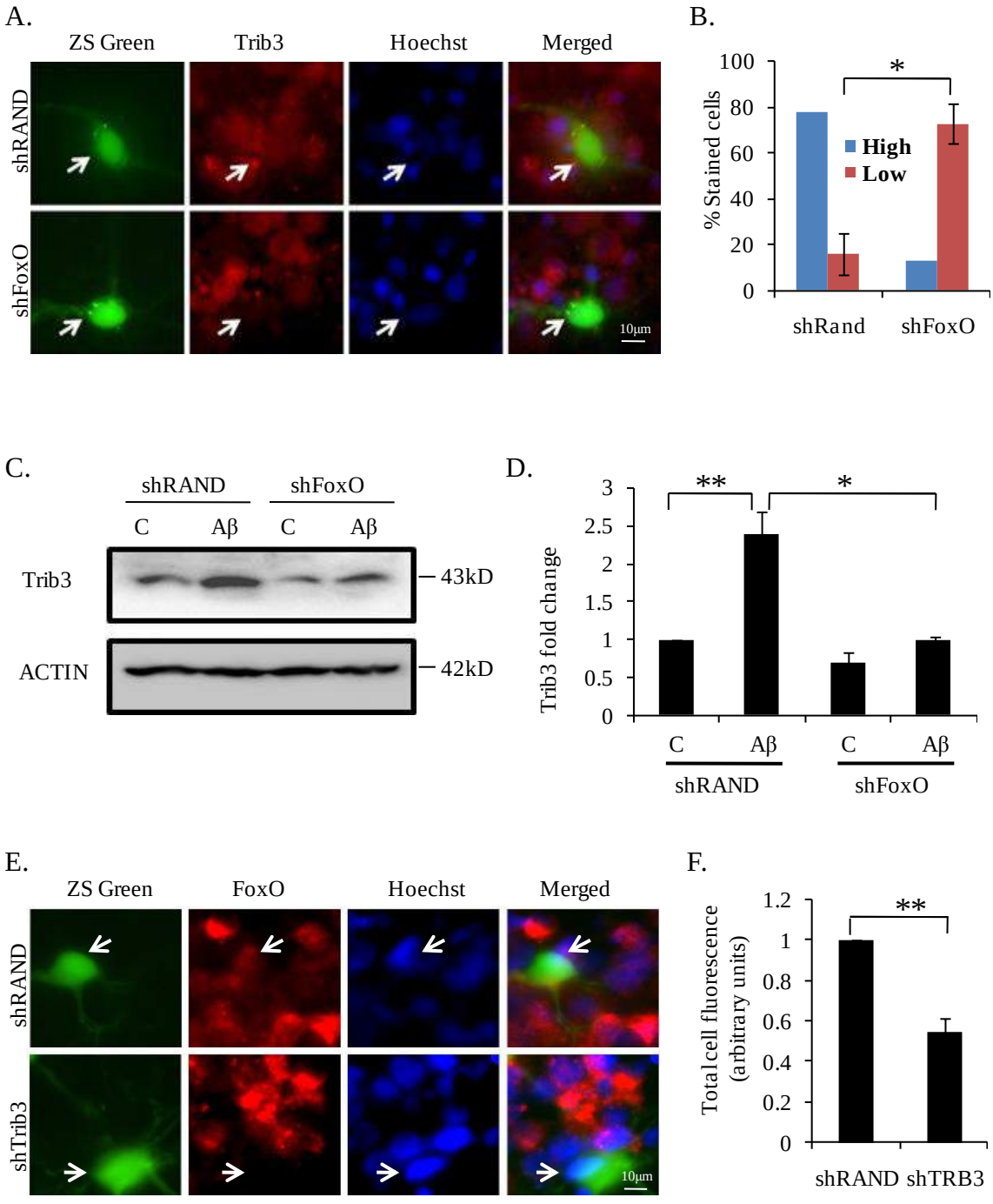
4.2.4 Feed forward regulatory mechanism acts between Trib3 and FoxO1

Our findings intrigued us to check whether a feed-forward regulatory mechanism was active between Trib3 and FoxO in neuronal cells in response to Aβ insult. To study the regulation of Trib3 by FoxO, we knocked down the FoxOs in neuronally differentiated PC12 cells by a previously reported shRNA that targets all FoxO isoforms [57] and exposed them to Aβ. Immunocytochemistry was performed to see the endogenous Trib3 expression. Results revealed that the shRNA mediated knockdown of FoxOs showed reduced expression of Trib3 in most of the transfected cells compared to non-transfected neighbouring cells upon exposure of Aβ (Fig. 5A). In contrast, no such reduction on Trib3 expression was found in shRand transfected cells (Fig. 5A). A quantitative analysis showed that about 80% shFoxO and about 20% shRAND transfected neurons had reduced Trib3 levels upon Aβ treatment (Fig. 5B). To confirm this result, we performed a western blot analysis of Aβ-treated shFoxO and shRand transfected cells, and observed the Trib3 protein levels. We observed that downregulating FoxOs blocked induction of Trib3 protein caused by Aβ toxicity (Fig. 5C & D). This indicates that FoxOs act as regulator of Trib3.

Next, we investigated whether Trib3 could regulate FoxO1 in our Aβ model of neurodegeneration. Neuronally differentiated PC12 cells were transfected with shTrib3, treated with Aβ and immunostained to study FoxO1 expression. It has already been previously reported that nuclear translocation of FoxO1 is controlled by Trib3 in NGF deprived neurons [22]. In our study we found that downregulating Trib3 not only blocked translocation of FoxO1 but also reduced its protein levels (Fig. 5E & F). To confirm our result we transfected neuronal PC12 cells with shTrib3 and shRand, treated with Aβ for 16 h and performed western blot analysis for FoxO1 protein. Results confirmed that downregulation of Trib3 led to reduced level of FoxO1 in presence or absence of Aβ (Fig. 5G & H). Thus, our findings indicate that Trib3 regulates both level and activity of FoxO1 following Aβ treatment.

Collectively, these results suggest a feed-forward loop that acts between Trib3 and FoxO1 that involves inhibition and inactivation of Akt by Trib3, activation of

transcription factor FoxO1, and transactivation of Trib3 by FoxO1 in Aβ-treated neurons.



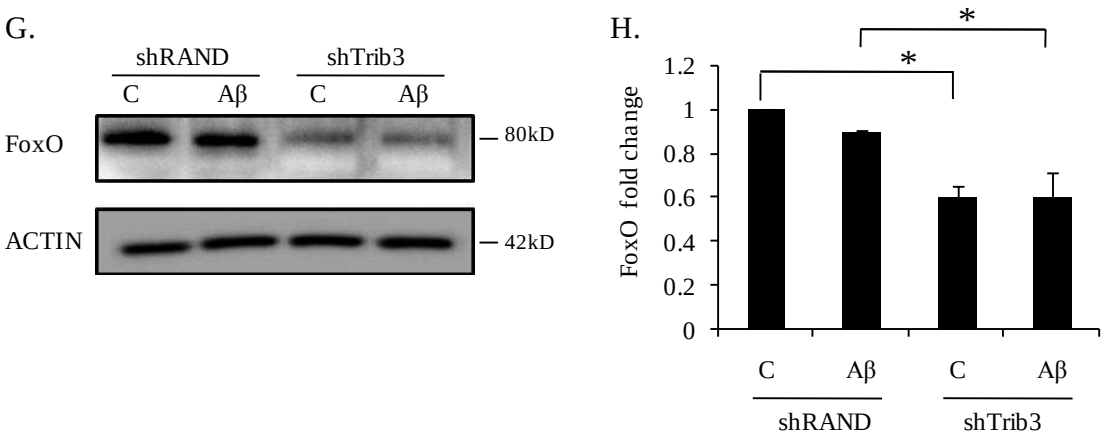


Figure C.1.5: Feed forward loop acts between Trib3 and FoxO1*. **(A)** Cultured cortical neurons at (5 DIV) were transfected with shFoxO or shRand, treated with Aβ 1.5μM for 16 h, and immunostained with Trib3 antibody. **(B)** The percentage of stained cells indicate the proportions of transfected cells (green) with high (more or equal than the neighbouring non-transfected cells) or low (less than the neighbouring non transfected cells) Trib3 immunoreactivity levels after treatment with Aβ. Data represents mean ± SEM of three experiments. Number of cells evaluated per culture are 50 (approx). *p < 0.01. **(C)** PC12 cells were transfected with shFoxO or shRand, primed and then treated with 5μM Aβ for 16 h. The downregulation of endogenous Trib3 was analyzed by western blotting with anti-Trib3 antibody**. (D)** Graphical representation of fold change of Trib3 levels by densitometric analysis upon transfection with shFoxO or shRand in presence or absence of Aβ 5μM. Data represents mean ± SEM of three independent experiments.*p < 0.05 **(E)** Cultured cortical neurons were transfected with shRand and shTrib3, maintained for 48 h then treated with Aβ 1.5μM. Immunocytochemical staining was performed with FoxO1 antibody. **(F)** Graphical representation of corrected total cell fluorescence of FoxO1 in neurons transfected with shRand or shTrib3 following Aβ exposure. Difference in intensity of FoxO1 staining is quantified by NIH imageJ as described in experimental procedures. Data represent mean ± SEM of thirty different cells from three independent experiments. *p < 0.05. **(G)** PC12 cells were transfected with shTrib3 or shRand, primed and then treated with 5μM Aβ for 16 h. Downregulation of endogenous FoxO1 was assessed by western blot analysis with anti-FoxO1 antibody. **(H)** Graphical representation of fold change of FoxO1 levels by densitometric analysis upon transfection with shTrib3 or shRand in presence or absence of Aβ. Data represents mean ± SEM of three independent experiments.*p < 0.05.*

4.2.5 Trib3 further downstream regulates pro-apoptotic gene bim in neuronal cell death induced by Aβ

Reports suggest that *bim* is a transcriptional target of FoxO, and it regulates regulates upon NGF deprivation in sympathetic neurons [58]. This laboratory has recently showed that *bim* is directly regulated by FoxO in neurons following Aβ treatment [47]. We therefore determined whether Bim is under the control of Trib3 in this death paradigm. We transfected cultured cortical neurons with shTrib3 or shRand, exposed

them to Aβ followed by immunocytochemical analysis for Bim expression. Results revealed that downregulating Trib3 resulted in a marked reduction of Bim expression, as evident from reduced Bim staining in shTrib3 transfected cells as compared to shRand transfected cells (Fig. 6A & B). To further corroborate our findings we performed western blotting analysis with Trib3 knockdown PC12 cells for Bim level following Aβ treatment. We observed a significant upregulation of Bim following Aβ exposure in shRand transfected cells as expected. But interestingly this upregulation was significantly blocked in shTrib3 transfected cells even after Aβ treatment (Fig. 6C & D). These findings thus indicate that Trib3 induces neuronal death upon Aβ exposure by activating Bim, which could be via the Akt-FoxO1 pathway.

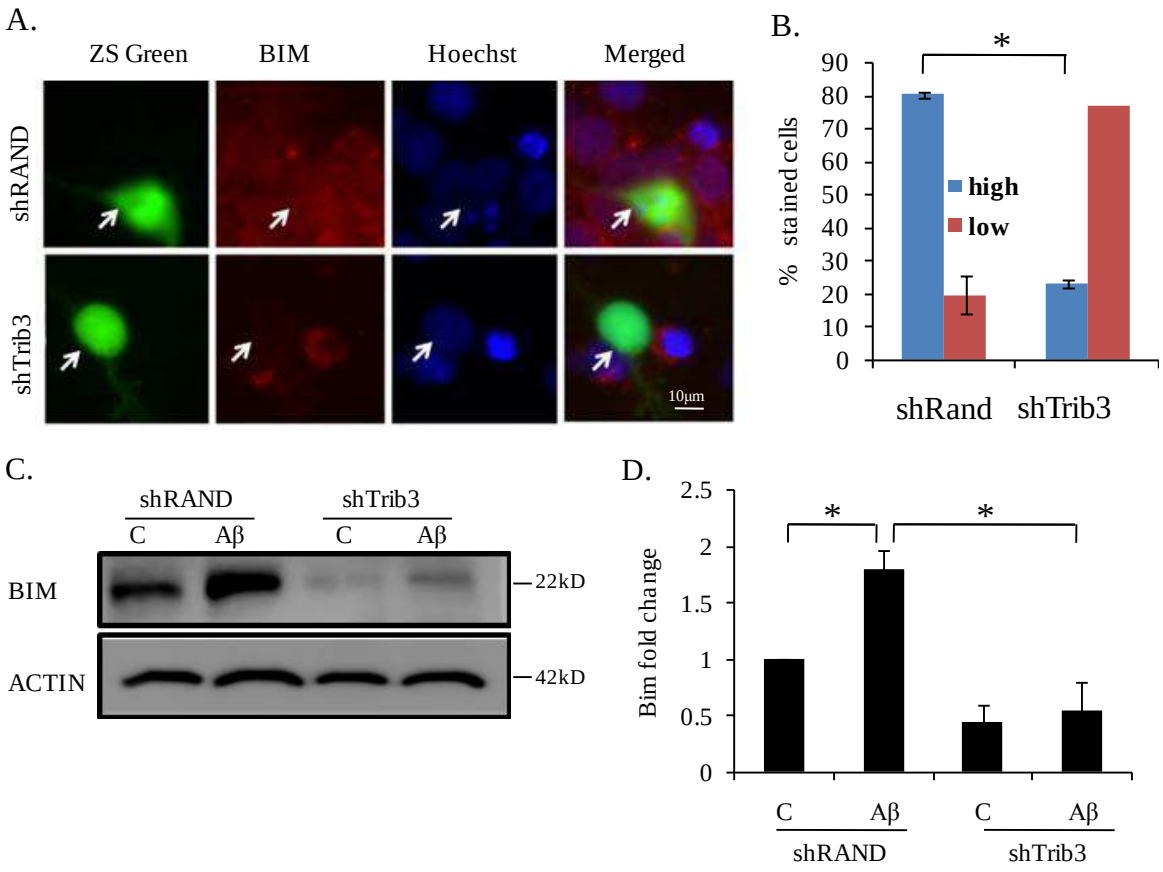


Figure C.1.6: Trib3 regulates the pro-apoptotic gene bim. (A) *Cultured cortical neurons (5 DIV) were transfected with shTrib3 and shRand, the cells were maintained for the next 48 h and then treated with Aβ 1.5µM for 16 h, after which they were immunostained with bim antibody (Red).* ***(B)*** *The Percentage of stained cells indicate the proportions of transfected cells (green) with high (more or equal than the neighbouring non-transfected cells) or low (less than the neighbouring non transfected cells) bim immunoreactivity levels after treatment with Aβ. Data represents mean ± SEM of three experiments. Number of cells evaluated per culture are 50 (approx). The asterisks denote statistically significant differences between low staining cells*

*and high staining cells: *p<0.01.* ***(C)*** *PC12 cells were transfected with shTrib3 or shRand, primed and then treated with 5μM Aβ. Downregulation of endogenous bim levels was analysed by western blotting using bim antibody.* ***(D)*** *Graphical representation of fold change of bim levels by densitometric analysis upon transfection with shTrib3 or shRand in presence or absence of Aβ 5μM . Data represents mean ± SEM of three independent experiments.*p < 0.05.*

4.3 Discussion

In this study, we looked into the role of Trib3 in Aβ induced neuron death. Our experimental observations indicate that Trib3 promotes neuronal death by apoptosis in response to Aβ. We found that Trib3 expression is induced in neuronal cells both at transcriptional and translational levels following Aβ treatment. Similarly upregulation of Trib3 was also found in the Aβ infused rat model. Since synthetic Aβ used for our experiments could have effects differing from the naturally occurring Aβ, we confirmed our results of induction of Trib3 in transgenic mice brain which overexpresses human Aβ. Our observations from these transgenic mice also revealed an elevated level of Trib3 protein which was similar to our previous *in vitro* results. Further, knocking down Trib3 by using shRNA in primary cultures of cortical or hippocampal neurons protect cells from Aβ induced cell death. By performing Sholl analysis we further observed the retention of neuronal processes and preservation of the overall neuronal morphology in neurons in which Trib3 was downregulated, even after treatment with Aβ.

Our observations on mechanistic studies reveal that Trib3 interacts with Akt and negatively regulates it. Previous reports indicate overexpression of Trib3 reduced phosphorylation of Akt at S473 and T308 [22] and knocking down of Trib3 restored phosphorylation of Akt in tunicamycin treated PC12 cells [27]. Interestingly, recently it has been shown that induction of Trib3, inhibition of Akt and activation of FoxO are all linked in a self-amplifying, feed forward loop that ends in neuron death occurring by NGF deprivation [22]. We also observed that inhibition of PI3K/Akt signalling by LY294002 led to upregulation of Trib3. Recent studies reveal that, FoxO transcription factors play an important role in neurodegeneration [47, 52]. Studies suggest presence of putative FoxO binding sites on the Trib3 promoter [53-56]. Our results revealed an enhanced occupancy of FoxO1 on the Trib3 gene and that FoxO regulated the expression of Trib3 upon Aβ treatment. These observations implicate that a feed-forward regulatory mechanism is active and occurs between Trib3, Akt and FoxO1 in Aβ-treated neuronal cells as well. Next, we wanted to analyse the downstream target of Trib3 that was involved in mediating neuronal death. Our findings revealed that downregulating Trib3 blocked the upregulation of Bim upon Aβ exposure, going onto suggest the potential role of Bim in the orchestration of apoptotic death of neurons via

Trib3. Bim is a transcriptional target of FoxO and studies reveal of its probable role in neuronal apoptosis in AD [59-62].

As first reported by Mayumi-Matsuda in 1999, Trib3 mRNA levels were significantly upregulated in PC12 cells as well as in sympathetic neurons. Apart from this, there were several other reports which suggested a wide array of functions of Trib3 in response to stress conditions, which included its role in inducing apoptosis in non-neuronal cells. Another report suggested the role of Trib3 in inducing apoptotic death of neuronal cells subjected to NGF deprivation. This intrigued our interest and we wanted to observe the effect of Trib3 in an AD model. It was reported that depriving neuronal PC12 cells and primary cultures of sympathetetic neurons of NGF over a few time points revealed that there was an increase in Trib3 mRNA and protein levels [63].These initial reports helped us to streamline our work and we were able to create our well defined objectives to look into the role of Trib3 in neuronal cell death in Aβ model. Neonatal primary cortical and hippocampal cultures were used because these areas are the specific regions involved in memory and learning. In AD, learning and memory are mostly affected. Neuronal PC12 cells were used because they mimic primary neurons and are ideal for performing preliminary experiments.

Whether Trib3 was actually required for neuronal death was determined by endogenous knockdown of Trib3 by using short hairpin RNA. In these experiments it was observed that there was significant protection to neurons in which Trib3 was downregulated in Aβ treated condition in both cortical and hippocampal cultures. Interestingly, it was observed that there was strong retention of neurites and processes of the neurons as compared to the Aβ treated shRand transfected neuronal cultures. The overall morphologies of these neurons were also maintained for a prolonged period of time. Usually in pathological neuron death the initiation occurs by degeneration of the axons. Thus, the ability of Trib3 to provide protection to neurons is significant, in that it goes on to indicate that it might also be arresting some death mechanism within these neurons [64]. A question which may arise here is whether knocking down Trib3 provided 100% protection, if it didn't then why was it not able to? As observed knocking down Trib3 did not provide 100% protection to the neurons, a possible answer to this could be that the knocking down of endogenous levels of Trib3 did not confer full knock down, with some possible residual activity still prevailing. Another reason could be the involvement of other apoptotic proteins which are also

transcriptionally upregulated upon Aβ treatment. These proteins could include, Hrk/DP5, PUMA or SM20 can participate in apoptosis [42, 65-67]. We found that when we inhibited Akt using an inhibitor there was an upregulation of Trib3. This intrigued our interest and we hypothesized that there could be some molecule downstream of Akt which gets activated when Akt is inhibited. This activated molecule could in turn lead to the upregulation of levels of Trib3. Of the plethora of downstream targets of Akt we zeroed in onto FoxO. If Akt inhibits FoxO activation and Trib3 is induced transcriptionally by FoxO, then Akt inactivation must lead to induction of Trib3. As expected, treatment of primary cultures of cortical neurons with Akt inhibiting drug upregulated Trib3, showing that Trib3 acted in a negative feedback loop with Akt. Interestingly, it was observed that Trib3 induction resulting from Akt inhibition was much greater compared to the induction observed after NGF deprivation. This is not an unusual fact because Akt phosphorylation at its active sites and activation are not solely dependent on NGF/TrkA activity. A number of growth factors and trophic factors can phosphorylate Akt, by binding to their respective receptors [68, 69]. A recent transcriptome analysis revealed an upregulation of a number of several genes upon NGF deprivation. These genes had potential role in inducing cell death [63]. Among the several BH3-only proteins that were upregulated , Bim (Bcl2 interacting mediator of apoptosis), has been studied extensively in the model of NGF deprivation. Bim is a pro apoptotic protein that is upregulated upon Aβ treatment [59, 61]. It was observed that downregulating Trib3 lessened the expression of Bim as compared to its upregulation upon Aβ treatment. Mayumi-Matsuda *et al*., reported that there was an elevation in Trib3 cDNA levels in cortical neurons in response to Ca2+ ionophore excitotoxicity [70]. The present data indicate that Trib3 targets Akt in neurons. Trib3 is necessary for Akt dephosphorylation in the presence of Aβ. These experimental data from neuronal cells are in agreement with previous reports in which Trib3 has been shown to interact with Akt and result in its dephosphorylation in non-neuronal cell [44]. Phosphorylation of Akt at Ser473 and Thr308 residues is necessary for complete activation of Akt and survival of the neuron [71]. Binding of NGF stimulates the TrkA receptors, active Akt suppresses various pro-apoptotic molecules, such as FoxO transcription factors which are phosphorylated by Akt and thus clustered in the cytoplasm [72]. It was observed that FoxO1 is regulated by Trib3 upon Aβ treatment. This must be mediated via dephosphorylation of Akt by Trib3. Downregulation of Trib3 rescued FoxO1 from dephosphorylation upon Aβ treatment. It was also observed that downregulation of

Trib3 also prevented the translocation of FoxO1 from cytosol to nucleus as seen upon Aβ treatment. Thus, the findings regarding Trib3, Akt and its substrate FoxO1 correlate with the observations that downregulation of Trib3 is protective for neuronal cells. The results regarding Trib3 regulation in NGF deprived cells conform to this model and as predicted, show that indeed there occurs a feed forward loop between Trib3 and FoxO transcription factors. The findings can be summarized as upon Aβ treatment there occurs dephosphorylation of Akt, which in turn leads to dephosphorylation of FoxO transcription factors, leading to its nuclear localization. In the nucleus FoxO transcriptionally activates Trib3 and upregulated Trib3 further binds to Akt, leading to more dephosphorylation and deactivation of Akt, thus amplifying the effect. Due to the further binding of Trib3 and Akt and inactivation of Akt, sufficient FoxO is activated which induces other pro-apoptotic genes, such as Bim that plays an important role in mediating the mitochondrial intrinsic pathway of apoptotic death.

4.4 References

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