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## ABSTRACT

## REGULATION OF MTORC1 BY HOMOCYSTEINE AND ITS EFFECTS ON AUTOPHAGY IN HUMAN AND MOUSE NEURONAL TISSUES

**By**
**Khoosheh Khayati**

The molecular mechanisms leading to and responsible for age-related, sporadic Alzheimer's disease (AD) remain largely unknown. It is well documented that aging patients with elevated levels of the amino acid metabolite homocysteine (Hcy) are at high risk of developing AD. The impact of Hcy on molecular clearance pathways in mammalian cells, including *in-vitro* cultured induced pluripotent stem cell-derived forebrain neurons and *in-vivo* neurons in mouse brains is investigated in this research project. Exposure to high Hcy levels results in up-regulation of the mechanistic target of rapamycin complex 1 (mTORC1) activity, one of the major kinases in cells that is tightly linked to anabolic and catabolic pathways. Moreover, Hcy-mediated mTORC1 activity is only specific to Hcy and not to Hcy metabolites such as Hcy-thiolactone and cysteine. Homocysteine is sensed by a constitutive protein complex composed of leucyl-tRNA-synthetase (LeuRS) and folliculin (Flcn), which regulates mTOR tethering to lysosomal membranes. In hyper-homocysteinemic human cells and cystathionine β-synthase-deficient mouse brains, an acute and chronic inhibition of autophagy, the molecular clearance pathway is detected. In Hcy-treated cells, mTORC1 mediates phosphorylation of ULK1 which consequently reduces LC3 lipidation. mTORC1 also dampens TFEB-driven transcription of autophagy-related genes resulting in a buildup of abnormal proteins, including β-amyloid and phospho-Tau. Formation of these protein aggregates leads to AD-like neurodegeneration. This pathology can be prevented

by inhibition of mTORC1 or by induction of autophagy, through TAT-Beclin1 treatments. Here it is discussed that an increase of intracellular Hcy levels predisposes neurons to develop abnormal protein aggregates, which are hallmarks of AD and its associated onset and pathophysiology with age.

# REGULATION OF mTORC1 BY HOMOCYSTEINE AND ITS EFFECTS ON AUTOPHAGY IN HUMAN AND MOUSE NEURONAL TISSUES

**by**
**Khoosheh Khayati**

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**APPROVAL PAGE**

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*This dissertation is dedicated to my family: my dear husband (Mohammad Hesarbiharas) for being so patient and supportive during all my busy and tense days, my parents (Khalil Khayati and Mehrvash Kaveh Haghighi) for their love and support throughout my life and education. I would like to thank my grandmother (Pari Fadaee) and lovely sisters (Khorvash and Khorshad) for being in touch with me whenever I needed sympathy. I would also thank my parents in law (Mehrdad Hesarbiharas and Pari Afsan) for their thoughtful support.*

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# CHAPTER 1

# INTRODUCTION AND BACKGROUND

## 1.1 mTOR is a Major Integrator of Signaling Pathways in the Cell

As a serine-threonine kinase and a member of phosphoinositide-3-kinase (PI3K) related kinase family, the mechanistic target of rapamycin (mTOR) is a central hub for various anabolic and catabolic pathways in the cell (Laplante and Sabatini 2012; Wullschleger, Loewith, and Hall 2006; Loewith and Hall 2011). Both synthetic pathways such as protein and lipid production as well as break-down processes such as autophagy are influenced by mTOR activity (Laplante and Sabatini 2012) (Figure 1.1). Being observed along with other components in two different complexes, mTOR is the catalytic subunit in both mTORC1 and C2. Nutrient and growth factor signaling are mainly attributed to mTORC1 while cytoskeleton regulation, cell growth, and death are known to be controlled by mTORC2 (Laplante and Sabatini 2012; Guertin et al. 2006; Sarbassov et al. 2004).

## 1.2 mTORC1 and Amino Acid Signaling

Mechanistic target of rapamycin complex 1 has been identified in two different states, either active or inactive. mTORC1 exists in diffused form throughout the cytoplasm in its inactive phase while localizes on the lysosomal membrane as an

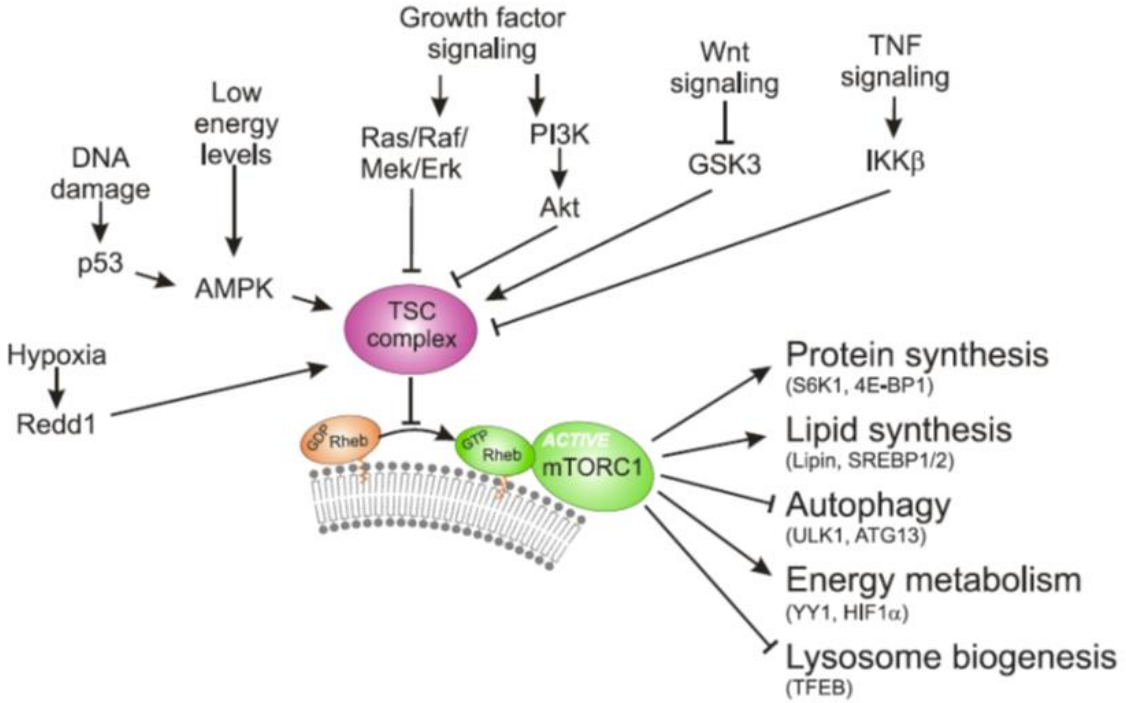



**Figure 1.1** Cellular signaling regulates mTORC1 activity through the TSC complex. The TSC complex represents a hub for incoming cellular signals ranging from hypoxia, DNA damage, and energy levels to growth factors, Wnt, and TNF signals. Signaling mediators and/or kinases modulate the GAP activity of the TSC complex to inhibit or activate Rheb. An inactive TSC complex results in activation of mTORC1 thereby promoting protein and lipid synthesis, energy metabolism, and inhibition of lysosomal biogenesis and autophagy.

Source: Khayati, K., Nnah, I. C., and Dobrowolski, R., 2015. “Cellular Metabolism and Lysosomal mTOR Signaling.” Cell Death in Therapy, 11-22.

active kinase along with other lysosomal membrane proteins which form the lysosome nutrient sensing (LYNUS) machinery (Settembre et al. 2013). LYNUS machinery is comprised of Rags (small GTPases in form of heterodimer) which act as docking sites for mTORC1 to tether on the lysosomal surface (Sancak et al. 2010), v-ATPase, a proton pump that acidifies the lysosomal lumen and plays prominent role in sensing amino acids availability inside the lysosome (Ohkuma 1982), Ragulator, a guanine nucleotide exchange factor (GEF) for Rag A and B

GTPases (Sancak et al. 2010; Huang and Fingar 2014) and finally endosomal ATP-sensitive sodium ion-permeable channel which responds to ATP levels and regulates lysosomal membrane potential and pH stability (Cang et al. 2013). The reason for mTORC1 translocation on the lysosomal membrane as part of the LYNUS machinery is for being in close proximity with its activator, Ras homolog enriched in brain (Rheb) which is known as a small GTPase residing on the lysosomal membrane (Inoki et al. 2003; Tee et al. 2003). This mTORC1 recruitment to lysosomal membrane largely depends on the availability of amino acids which are sensed by the LYNUS machinery, a process that is critical for mTORC1 to be able to regulate its downstream pathways (Sancak et al. 2010). This way, amino acids regulate mTORC1 activity by regulating its proximity to Rheb. So, amino acids are not only considered as building blocks of proteins but also play important roles in the maintenance of cellular homeostasis through activation of mTORC1 (Laplante and Sabatini 2012); among them, leucine (Leu) is known as the most potent one in regards to mTORC1 activation (Bar-peled and Sabatini 2014).

### 1.3 Inside-Out Mechanism of Amino Acid Signaling through v-ATPase

Vacuolar $H^+$- adenosine triphosphatase (v-ATPase) is known as a proton pump which acidifies lysosomal lumen by hydrolyzing ATP and production of a proton gradient. This proton gradient is usually coupled with symport/antiport mechanism of amino acid transporters (Russnak, Konczal, and Mcintire 2001). V-ATPase consists of V0 and V1 domains. V1, the cytoplasmic domain is

responsible for ATP hydrolysis that induces a conformational change in the integral membrane domain, V0. This integrated action of both domains promotes influx of protons across the endolysosomal membrane (Forgac 2007). In addition to its prominent role in vacuolar pH maintenance, v-ATPase is the main factor in sensing amino acid availability in the lysosome in order to regulate mTORC1 activity (Zoncu 2011). By inhibiting the expression of genes encoding for v-ATPase components and assessing mTORC1 activity, Zoncu et al. showed a strict dependence of mTORC1 on v-ATPase function. Furthermore, cells treated with Concanamycin A, a v-ATPase inhibitor, failed to recruit mTORC1 to their lysosomal membranes even in presence of amino acids. Therefore, it was proposed that upon amino acid availability, v-ATPase relays the signal to Ragulator, the Rag A/B activator, through which active Rags are bound on lysosomal surface which further facilitate tethering of mTORC1 to the complex (Zoncu 2011). Later, Stransky and Forgac showed that amino acid availability influences v-ATPase assembly and function as well, which in turn is crucial for culmination in mTORC1 activity (Stransky et al. 2015).

### 1.4 Amino Acids can be Sensed and Edited through Interaction with Aminoacyl-tRNA Synthetases in the Cytoplasm

Aminoacyl-tRNA synthetases (AARS) are enzymes generally known to facilitate binding of amino acids to their cognate tRNA (Serre et al. 2001). The so-called tRNA charging with amino acids is a two-step process consisting of binding of amino acid to AARS and being transferred to their cognate tRNAs; the later step

is called tRNA aminoacylation (Giegé 2008). During tRNA charging, amino acid-bound AARS conjugates with AMP (an intermediate with high energy level) which later favors a high-energy ester bond formation between an amino acid and 3'-terminal of the tRNA (Jakubowski 2012). Among AARSs, leucyl-tRNA synthetase (LeuRS) is the enzyme that promotes the transfer of Leu to its counterpart tRNA and is proposed to act as an important player in protein translation as well as being an mTORC1 activator by sensing available Leu in the cytoplasm (Han et al. 2012).

In addition to their regular function in amino acid selectivity for tRNAs, AARSs are found to be able to edit non-proteinogenic amino acids such as homocysteine (Hcy). Inaccurate amino acids need to be edited in order to inhibit their incorporation into our proteins. Among AARSs, isoleucyl-tRNA synthetase (IleRS), valyl-tRNA synthetase (ValRS), lysyl-tRNA synthetase (LysRS) and LeuRS are known to edit Hcy (Jakubowski 2011). In general, amino acid editing by AARSs takes place in two ways: pre-transfer editing (edition of amino acids while they are bound to AARSs prior to tRNA binding) and post-transfer editing (amino acid editing after transfer to tRNAs) (Cvetesic, Perona, and Gruic-sovulj 2012).

Weak interaction between the side chain of the amino acid, which is recognized by an AARS, and the active site of the enzyme triggers editing process of non-proteinogenic amino acids. In the case of methionyl-tRNA synthetase (MetRS), amino acid charging and editing happen at the same active site of the enzyme which recognizes both methionine (Met) and Hcy. Methionine

binding to MetRS induces rearrangement in the active site of the enzyme through which aminoacylation is triggered, while Hcy fails to induce such rearrangements (Serre et al. 2001).

In general, in process of editing, an amino acid conjugated with AMP or t-RNA, undergoes hydrolysis reaction which gives rise to the production of a free amino acid. But if amino acid has a nucleophile group such as γSH in Hcy, it will be converted to a cyclized form of amino acid (thiolactone in the case of Hcy) (Lincecum et al. 2003). Homocysteine-thiolactone formation through MetRS is highly conserved in bacteria, yeasts, plants and humans and prone to aggregation and excretion in urine because of the low dissociation constant (pK = 6.65) of its amino group (Jakubowski 2011). Methionyl-tRNA synthetase has a thiol-binding sub-site through which editing of Hcy occurs. There is competition between the side chain of amino acid and tRNA in binding to the carboxyl group of amino acid. Methyl group of Met binds firmly to specificity sub-site of MetRS through hydrophobic and hydrogen bonds, renders its carboxyl group free to bind to tRNA; while thiol group of Hcy is deficient in binding to specificity sub-site of MetRS, though wins the competition over tRNA in binding to carboxyl group of Hcy and produces cyclized Hcy-thiolactone (Serre et al. 2001). It is noteworthy that Hcy-thiolactone is very reactive and prone to damage protein structures by binding to amine groups of lysines incorporated into proteins leading to the formation of N-Hcy-proteins (Jakubowski, Boers, and Strauss 2008). Two enzymes which specifically hydrolyze Hcy-thiolactone back to Hcy, are bleomycin hydrolase (Blmh) and paraoxonase1 (Pon1). Bleomycin hydrolase-deficient mice

exert 1.8 fold more Hcy-thiolactone in their urine as compared to normal mice (Borowczyk, Tison, and Jakubowski 2012).

### 1.5 Leucyl-tRNA Synthetase or Folliculin; Which One Acts as a GTPase Activating Protein for Rag C/D?

Upon introduction of LeuRS as a sensor for Leu availability in the cytoplasm, a further investigation was carried out to unveil the mechanism through which LeuRS relays the signal to mTORC1. Immunoprecipitation analyses between LeuRS and Rags along with *in-vitro* GTPase assays implicated LeuRS as a GTPase-activating protein (GAP) for Rag D (Han et al. 2012). Follow-up analyses, however, failed to confirm LeuRS as the active GAP of RagD but identified Folliculin (Flcn) to be the protein to carrying out these function. Being first identified as a tumor suppressor whose loss of function leads to Birt-Hogg-Dube syndrome with characteristics including formation of benign tumors in hair follicles, lung and renal system (Nickerson et al. 2002), Flcn has been related to various pathways in the cell such as mTOR signaling, transforming growth factor β (TGF-β) signaling, AMP-activated protein kinase (AMPK) signaling, JAK-STAT signaling, cell adhesion, membrane traffic and cilia function (Petit, Roczniak-Ferguson, and Ferguson 2013). Notably, Petit et al could shed light on a new function of Flcn in translocation of transcription factor EB (TFEB) between nucleus and cytoplasm; in that nuclear localization of TFEB was observed in Flcn knockdown cells. They also found Flcn function necessary for re-activation and lysosomal localization of mTORC1 after deprivation and replenishment of amino

acids consequently (Petit et al. 2013). In the search for implicating the significance of Rag C and D in amino acid signaling machinery, Tsun et al could substantiate GAP activity of Flcn towards Rag C and D (Tsun et al. 2013). Amino acid deprivation induces Flcn interaction with the lysosomal membrane, specifically to Rag C/D which is diminished by replenishment of amino acids. Accordingly, Flcn needs to detach from the lysosomal membrane to be replaced by mTORC1 in presence of amino acids (Tsun et al. 2013). Similar to Flcn, Folliculin interacting protein (FNIP) indirectly regulates mTORC1 activity and TFEB localization as suppression of FNIP induces nuclear localization of TFEB (Petit et al. 2013). Taking into account that FNIP is necessary for interaction of Flcn and Rags and Flcn is also necessary for FNIP-Rags interaction, Flcn and FNIP are considered as components of a complex in which both proteins should be active in order for Flcn to show GAP activity towards Rag D (Petit, Roczniak-Ferguson, and Ferguson 2013; Tsun et al. 2013); since neither Flcn nor FNIP shows the activity, separately (Tsun et al. 2013).

The notion that suppressing both LeuRS and Flcn expression inhibits translocation of mTORC1 to the lysosomal membrane in response to amino acid availability (Han et al. 2012; Tsun et al. 2013), inspired us to assess the possibility of LeuRS interaction with Flcn in a complex.

## 1.6 Homocysteine; a Non-Proteinogenic Amino Acid

In addition to essential and non-essential amino acids that are used for protein synthesis, there are amino acid metabolites some of which are detrimental to the cells (Dasuri et al. 2011). These side products, the so-called non-proteinogenic amino acids, need to get removed from the protein translation pathway to inhibit error insertion in proteins and formation of misfolded proteins that ultimately lead to cell death (Lee et al. 2006). Homocysteine, a known sulfur-containing non-proteinogenic amino acid results from demethylation of Met (Prudova et al. 2006) and has been identified as a risk factor for cardiovascular disease (Garcia and Zanibbi 2004; Zhuo, Wang, and Pratico 2011). When plasma Hcy concentration exceeds 14 μM, we encounter hyperhomocysteinemia (HHcy), a pathological state which is linked to cardiovascular disease, neuronal cell death along with the loss of short and long-term memory (Ataie, Sabetkasaei, and Haghparast 2010; Zhuo et al. 2011; Kamat et al. 2013). Hyperhomocysteinemia has also been associated with Alzheimer's disease (AD), schizophrenia and depression (Folstein et al. 2007).

In process of losing a methyl group, Met converts to S-adenosine methionine (SAM) and eventually Hcy. Homocysteine needs to get re-methylated in order to convert to Met. 5-methyltetrahydrofolate, known as folic acid and SAM are main methyl donors to Hcy. Notably, methylenetetrahydrofolate reductase (MTHFR) and methionine synthase (MS) are the enzymes driving the re-methylation pathway, though are important in the elimination of Hcy from the body (Chiang et al. 1996). The alternative enzyme proposed to have a positive

effect on the removal of Hcy is cystathionine β- synthase (CBS); the enzyme which demands vitamin B6 as an efficient cofactor to function optimally. The product of CBS activity is cystathionine which will ultimately be converted to cysteine and α-keto butyrate through a second enzymatic step (Jakubowski 2012).

Given that Hcy is a modified form of Met, it is possible that Hcy can be sensed by mTORC1 through AARS. The impact this Hcy may have on mTORC1 activity was evaluated in this dissertation.

### 1.7 mTORC1 Negatively Regulates Autophagy

The transcription factor EB is a member of the basic helix-loop-helix leucine zipper family of transcription factors and shown to be a substrate of mTORC1 (Sardiello et al. 2009). Upon mTOR-mediated phosphorylation of TFEB at serine 211, TFEB interacts with the 14-3-3 protein which blocks TFEB's nuclear localization signal. This way, phosphorylated TFEB remains in the cytoplasm (Martina et al. 2012; Roczniak-ferguson et al. 2012). Unphosphorylated TFEB translocates into the nucleus where it binds to a 10-base pair DNA motif (GTCACGTGAC), the so-called Coordinated Lysosomal Expression and Regulation (CLEAR). The CLEAR element is part of the promoter of genes encoding lysosomal and autophagosomal proteins (Sardiello et al. 2009) (Figure 1.2). Thereby, TFEB induces lysosomal biogenesis and autophagy, a cellular self-eating process for degradation of protein aggregates and old organelles in

the cytoplasm (Glick, Barth, and Macleod 2010). Two of the crucial autophagy-related TFEB target genes are ubiquitin-binding protein P62, (an adaptor protein that detects ubiquitinated protein aggregates and recruits them to the degradation initiation site, autophagosomes) (Lamark et al. 2009), and microtubule-associated protein 1 Light Chain 3 (LC3), which is necessary to detect targets selected for macro-autophagy degradation. LC3 is a member of Atg proteins which is either cytosolic (LC3-I) or membrane-bound (LC3-II). The transition between the two forms of protein occurs by cleavage of LC3 on its C-terminal domain through a cysteine protease (Atg4) that is believed to expose it to further post-translational modification known as covalently phosphatidyl ethanolamine conjugation (Marino et al. 2002). The later modification renders LC3-II capable of binding to inner and outer part of autophagosomal membranes. Since LC3 detects ubiquitinated proteins and sequesters them in autophagosomes through the help of adapter proteins, ubiquitination could be considered as a signal for both proteasomal degradation and selective autophagy. The distinguishing factor deciding whether proteins should undergo proteasomal degradation or selective autophagy is binding of proteins to UBD (ubiquitin-binding domain)-containing autophagy receptors (such as P62) that simultaneously bind to Atg8 (LC3) proteins (Slobodkin and Elazar 2013). However, P62 is not dependent on LC3 to be recruited to autophagosomes; the PB1 (Phox1 & Bem1p) domain of p62 has been shown to be sufficient for its recruitment to autophagosomes, and starvation indeed boosts this process (Itakura and Mizushima 2011).

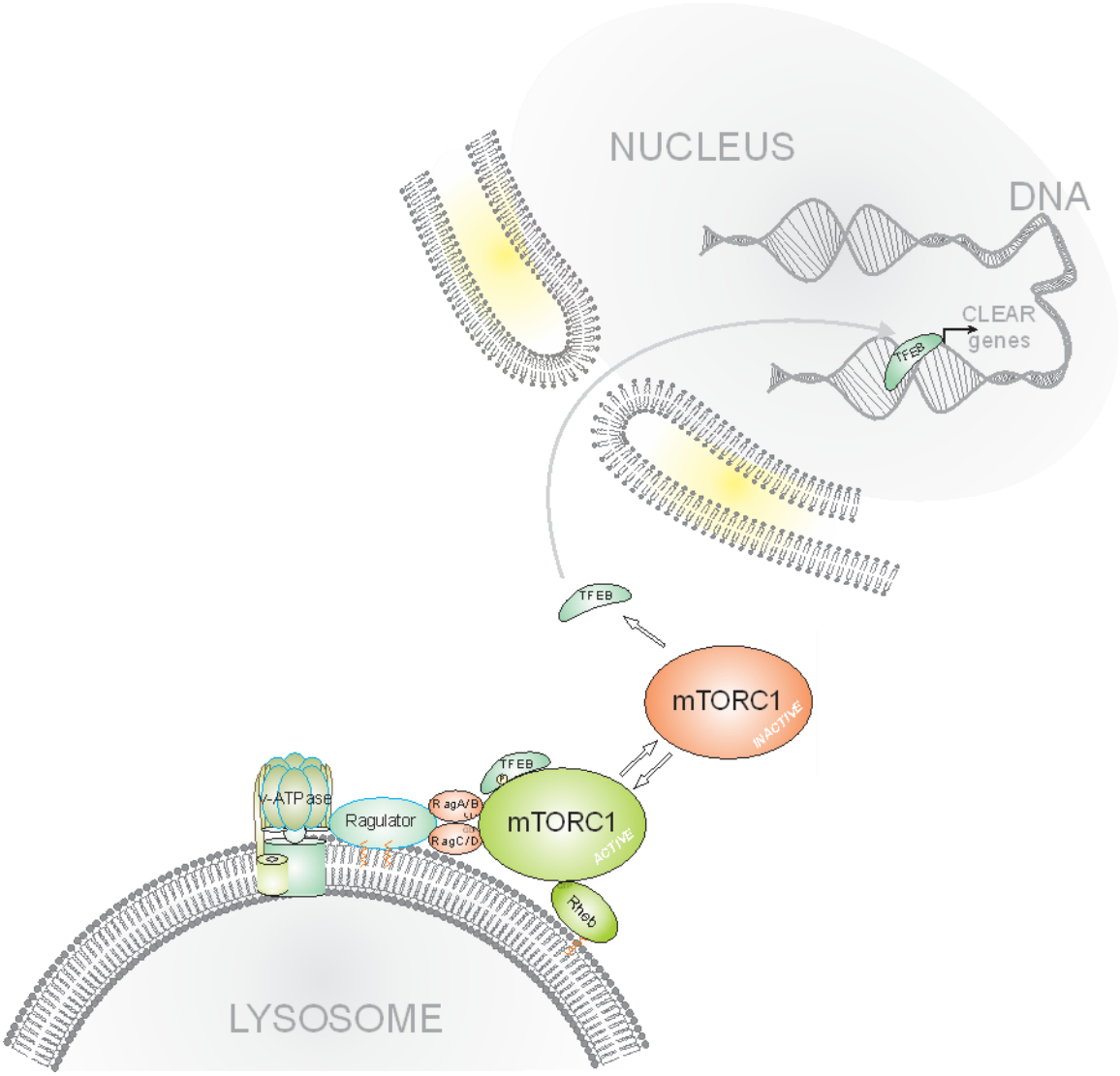



**Figure 1.2** TFEB translocation in and out of the nucleus. Phosphorylation of TFEB by mTORC1 determines its localization; as phosphorylated TFEB is kept in the cytoplasm while unphosphorylated form enters the nucleus to render its role on transcription of CLEAR-gene network.

The alternative factor which is influenced by mTORC1 activity and consequently affects autophagy initiation is unc-51 like autophagy activating kinase-1 (ULK1) which contains several phosphorylation sites that regulate its activity. Having a dual function in autophagy regulation, ULK1 is phosphorylated on various sites which determine its role in autophagy. Phosphorylation of ULK1

on serine 317 and serine 777 by AMP-activated protein kinase (AMPK) are crucial for induction of autophagy, while mTORC1 kinase activity on serine 757 of ULK1 hampers AMPK interaction to ULK1 and inhibits autophagy pathway (Kim et al. 2011).

### 1.8 Low Rates of Autophagy in Alzheimer's Disease Patients' Brains

As a protective mechanism, autophagy is initiated in response to abnormal conditions in the cells such as hypoxia, low energy level due to lack of amino acids and growth factors, aggregation of proteins, DNA damage (Mathew, Karantza-wadsworth, and White 2007) and metabolic stress (Levine and Kroemer 2008). Observation of enlarged autophagic vacuoles in patient cells of AD (Nixon and Yang 2011), Parkinson's and Huntington's diseases raised the speculation as whether autophagy is the cause for initiation of these diseases or can be used as a strategy to ameliorate the pathology of these conditions (Rubinsztein et al. 2007; Williams et al. 2006; Martinez-vicente and Cuervo 2007; Levine and Kroemer 2008). As autophagy is shown to remove paired helical filaments of tau protein aggregates (Williams et al. 2006), it is more compelling that autophagy has a positive impact on hampering of neurodegeneration and clearance of abnormal protein accumulations. On the other hand, AD is discussed by some researchers as a Lysosomal Storage Disease (LSD) due to lysosomal enzymes malfunction in degradation of cargos transported there (Nixon, Yang, and Lee 2008). Since aggregated proteins are large and can be cleared only by specific pathways in the cytoplasm such as macroautophagy, it is

significant for the pathway to run efficiently (Martinez-vicente and Cuervo 2007). To date, there is no evidence why autophagy is impaired in AD neurons. Here we introduce Hcy as one of the risk factors of AD and a possible inhibitory agent of autophagy.

### 1.9 Significance and Introduction to Dissertation Project

Dysregulation of mTORC1 activity has been correlated with the pathology of several human diseases such as cancer (Bar-peled et al, 2014), metabolic diseases, neurodegeneration, and aging process (Laplante and Sabatini 2012). Especially in the case of neurodegeneration, since intact autophagy and proteasomal protein degradation are known to have an important impact on inhibition of the disease onset (Rubinsztein 2006), mTORC1 signaling as a regulator of autophagy pathway has received attention and been the center of study in the field. By assessing mTORC1 activity in postmortem brains of human AD patients, we found up-regulation of mTORC1 kinase activity in AD samples as compared to controls (Figure 3.1 A). In parallel, high plasma levels of Hcy has been recorded for those AD patients as a highly plausible risk factor (Zhuo, Wang, and Pratico 2011; Li, Chu, and Barrero 2014; Kamat et al. 2015). Studies on *Cbs* deficient mice mated with double transgenic APP/PS1 mouse model of amyloidosis show that Hcy changes the ratios of the produced βA42 and βA40 peptides to drive the amyloidogenic pathway (Pacheco-quinto et al. 2006). Moreover, a group of B vitamins has implicated inhibitory effects on brain shrinkage and cerebral atrophy in patients with HHcy, diagnosed with AD

(Douaud et al. 2013). Additionally, substantiated data show that vitamin B12 deficiencies lead to cognitive impairment by induction of HHcy which is also linked to methylation deficiencies of myelin basic protein leading to neuronal defects (Sponne et al. 2000).

Taken together, all these data support the notion that Hcy might elicit AD phenotype through different pathways, from activation of NMDA transporters (Lipton et al. 1997), to increase in activity of β-secretase (BACE) (Fuso et al. 2005) and γ-secretase (Li et al. 2014). Yet, there is no link provided between Hcy-mediated mTORC1 activity and generation of AD phenotype. Here, we assess whether and how Hcy can be sensed by mTORC1 and explore the correlation between high plasma Hcy levels, mTORC1 hyperactivity, and autophagy inhibition in human and mouse neurons.

### 1.10 The Hypothesis of the Dissertation

Given that Hcy and mTORC1 activity, both are elevated in AD patients, it is worthwhile to evaluate Hcy contributions to mTORC1 activity and consequently on autophagy. In this dissertation we examined this hypothesis: **Leucyl-tRNA synthetase (LeuRS) (an enzyme is known to load tRNA with leucine) regulates the activity of Folliculin (Flcn) (an mTORC1 activator) in a homocysteine/leucine-dependent manner; mTORC1 hyperactivity inhibits autophagy by phosphorylation of TFEB (a transcription factor) or ULK-1 (an autophagy inducer) and leads to neurodegeneration.** To test this hypothesis

we utilized mainly HEK293T cells and iPSC-derived human neurons for *in-vitro* studies, as well as *Cbs* knockout mice for *in-vivo* analyses, and accomplished the following:

1. Elucidated Hcy effect on mTORC1 activity in cells treated with Hcy and *Cbs* knockout mouse brain samples.

2. Elucidated how LeuRS and Flcn cooperatively sense levels of cellular amino acids and their metabolites to activate mTORC1 in the *Cbs* deficient brain and cultured cells.

3. Determined rate of autophagy induction in HEK293T cells and human neurons treated with Hcy.

4. Determined TFEB activity in Hcy-treated cells and *Cbs* knockout mice brain samples.

5. Evaluated the role of Hcy in neurodegeneration and cell viability.

# CHAPTER 2

# MATERIAL AND METHODS

## 2.1 Materials

### 2.1.1 Cell Lines

**2.1.1.1 HEK293T Cells.** HEK293T cells are human embryonic kidney cells cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal Bovine serum (FBS), L-glutamine (Glu) and penicillin/streptomycin (Pen/Strep).

**2.1.1.2 Human iPS Cells.** Human neural stem cells (also called neuroprogenitor cells) were generated in our lab using previously established human iPSC lines and following dual-SMAD inhibition protocols using SB431542 (10μM, Stemgent) and LDN193189 (250nM, Stemgent) drugs for at least three weeks and until the formation of neural rosettes was obvious. Neural rosettes were micro-dissected under a microscope in sterile environment (cell culture hood), trypsinized to dissociate into single cells, and maintained in neuroprogenitor basal media (#05834 Stem Cell Technologies) including supplement A (#05836) and supplement B (#05837) and differentiated to human forebrain neurons in neurobasal media supplemented with B27 without vitamin A (#12587-010), Glutamax and Pen/Strep, for at least three weeks. Successful differentiation has

been confirmed in immunofluorescence analyses showing loss of progenitor marker expression (Nestin) and gain of neuronal marker expression (Tuj1, MAP2, Synapsin1).

**2.1.1.3 CAD cells.** CatecholAminergic Differentiated (CAD) cells are mouse neuroblastoma cells. CAD cells stably expressing TFEB under a doxycycline (DOX) sensitive promoter were established in our lab and cultured in normal DMEM 10% FBS similar to HEK293T cells.

**2.1.1.4 MEF cells.** Mouse Embryonic Fibroblasts (MEFs) were cultured and maintained in DMEM supplemented with 10% FBS.

### 2.1.2 Animals

Transgenic Tg-I278T $Cbs^{-/-}$ mice on C57BL/6J genetic background and their $Cbs^{+/-}$ littermates were bred and housed at the New Jersey Medical School Animal Facility. In these animals, the human CBS-I278T variant is under control of the zinc-inducible metallothionein promoter, which allows rescue of the neonatal lethality phenotype of $Cbs^{-/-}$ by supplementing the drinking water of pregnant dams with 25 mM zinc chloride. Zinc water is replaced by plain water after weaning. The mice were fed a normal rodent chow (LabDiet5010, Purina

Mills International, St. Louis, MO). 12-month-old animals were used for immunohistochemistry studies.

### 2.1.3 DNA constructs

Plasmids were kindly provided by following laboratories: pRK5-HA GST RagC plasmids (Addgene #19304, 19305, 19306, Cambridge, MA, USA) and Flag-Leucyl tRNA synthetase pRK5 (Addgene #46341) were gifts from Dr. David Sabatini (Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA, USA), ptfLC3 (Addgene #21074) was a gift from Dr. Tamotsu Yoshimori (Osaka University, Osaka, Japan), and ptdTomato-N1-FLCN (Addgene #49174) was a gift from Dr. Shawn Ferguson (Yale School of Medicine, NewHaven, CT, USA). The CLEAR-Luciferase construct was a generous gift from Dr. Andrea Ballabio (Telethon Institute of Genetics and Medicine (TIGEM), Pozzuoli, Italy).

### 2.1.4 Antibodies

The following were used as primary antibodies for immunostaining and immunoblotting: AKT (#4691P, Cell Signaling), Amyloid (#D54D2, Cell Signaling), β-Actin (#JLA20, DSHB), Flag (#F3165, Sigma), Flcn (#D14G9, Cell Signaling), HA (#024M4773, Sigma), mouse LAMP2 (#ABL-93, DSHB), human LAMP2 (#H4B4), LC3 a/b (#4108, Cell Signaling), LeuRS (#sc-130801, Santa Cruz), MAP2 (#ab5392, Abcam), mTOR (#7C10, Cell Signaling), P-4E-BP1

(#236B4, Cell Signaling), p62/SQSTM1 (GP62-C, Progen), P-Akt (Ser473, #4060P, Thr308, #2965P, Cell Signaling), P-p70S6K (Thr389, #9205, Cell Signaling), P70S6K (#2708, Cell Signaling), PHF pTau (#MN1020, ThermoFisher), PULK1 (Ser 757 #D706U, Cell Signaling), RagC (Rag antibody sampler kit, #9778, Cell Signaling), Rheb (#E1G1R, Cell Signaling), Sestrin2 (#10795-1-AP, ProteinTech), TFEB (#MBS120432, Abcam for immunostaining; #ab2636, Abcam, for immunoblotting, and #A303-673A, Bethyl Biosci. for immunohistochemistry), Tuberin/TSC2 (#D93F12, Cell Signaling), Tubulin (#CP06, Calbiochem). Secondary antibodies were coupled to Infrared Dyes (LICOR, IRDye 680 and IRDye 800) or to HRP (Jackson IR) for immunoblotting, or to fluorochromes (Jackson IR) for immunostaining.

### 2.1.5 Chemicals

RPMI media without leucine/glutamine was supplied by US Biological. Amino acid starvation experiments were performed in the same media supplemented with L-glutamine (#R899912) and 10% dialyzed FBS (Reddy et al. 2016). RPMI complete media was prepared by addition of L-leucine (#L800) to the starvation media. DMEM provided by Sigma were supplied with glutamine, Fetal Bovine Serum (gibco), penicillin and streptomycin (gibco). Rapamycin was provided by Sigma (#R0395), and TAT-Beclin1 by EMD Millipore/Sigma (#5060480001). Amino acids (Sigma-Aldrich) were reconstituted in water. L-Hcy (#69453) and L-Leucinol (#CDS005275) were supplied from Sigma.

### 2.1.6 Microscope

Spinning disc confocal microscope (Zeiss, Oberkochen, Germany) with lasers emitting 488, 536/561, 639 and 405 nm laser lines and 63X oil immersion objective was used for immunofluorescent imaging of this study.

## 2.2 Methods

### 2.2.1 Immunofluorescence

Cells were plated on coverslips coated with fibronectin, for one hour and fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature. Cells were incubated with primary and fluorophore-tagged secondary antibodies and subjected to imaging in confocal microscopy.

### 2.2.2 Immunohistochemistry

Mice were perfused transcardially under deep pentobarbital anesthesia. Brains were collected, post-fixed in 4% PFA, and cryoprotected in 30% sucrose in PBS solution prior to sectioning. Thirty µm thick hippocampal brain sections from PFA perfused mice were rehydrated with PBS and permeabilized with 1% Triton X-100 in PBS for 30 mins. The sections were washed 3 times for 10 minutes with PBS-Triton buffer (0.05% Triton X-100 in PBS), incubated in blocking buffer (50% NGS, 50% PBS-Triton buffer) for 2 hours, and in indicated primary antibody solution (50% blocking buffer, 50% PBS-Triton X-100) overnight at 4°C in

humidity chambers. The sections were washed with PBS-Triton buffer and incubated with fluorescently-labeled secondary antibodies for 2 h at room temperature and mounted. Sections were imaged with a Zeiss spinning disc confocal microscope, taking a Z-stack of 29 sections with an interval of 0.48µm and range of 13.45µm.

### 2.2.3 SDS-PAGE and Immunoblot

Cells were resuspended in lysis buffer (10% glycerol, 1% NP40, 20mM Tris (pH7.4), 2.5mM EDTA (pH8), 2.5mM EGTA (pH8) containing Roche Protease inhibitor cocktail (#04693116001) diluted according to manufacturer's instructions) and cell lysates were centrifuged at 14000 rpm for 15 minutes at 4°C. The cleared supernatants were collected and prepared for SDS-PAGE on 10 or 12% mini gels; protein concentrations were determined using BCA reagent. Resolved proteins were transferred onto PVDF (Immobilon) membranes followed by a 45-minute room temperature incubation in western blocking solution (5% milk, 0.1% Triton X-100 in TBS). The membrane was incubated with primary antibodies overnight and with secondary antibodies for 45 minutes. After washes in TBS-T, the membranes were analyzed using either an LI-COR Odyssey system or film exposures of luminescence generated by ECL (Pierce) for HRP.

### 2.2.4 Co-Immunoprecipitation

HEK293T cells were transfected with HA-RagC (wild type, constitutively active and dominant negative), LeuRS-Flag and Flcn-dTomato plasmids, separately followed by overnight culturing to reach appropriate confluency. IP-buffer (50mM Tris, 150 mM NaCl, 10mM EDTA, 1% NaF, 1% Na3VO4, 1% NP40, 0.5 mM DTT, 1x Complete Protease Inhibitor Cocktail (Roche)) was used for lysing the cells. Lysates were cleared by centrifugation and incubated overnight with anti-HA/Flag antibodies at 4°C followed by overnight incubation with MagnaBind goat anti-rabbit/mouse beads (ThermoScientific) on a rotating mixer. After incubation, the beads were washed 3X with IP-buffer and then resuspended in Laemmli buffer and boiled for 10 mins. The IP protein samples and protein input were analyzed by SDS-PAGE and immunoblotting to examine endogenous LeuRS, Flcn, and RagC in all cell lines under different feeding conditions.

### 2.2.5 EGFP-mRFP-LC3 Assays

To assess the maturation and amount of active autophagosomes, ptfLC3 constructs were transiently transfected into iPSC-derived human forebrain neurons. After 48 hours, some cultured cells were incubated with 100 uM Chloroquine for up to 1 hour to inhibit autophagy flux. Cells were fixed with 4% PFA, mounted and imaged. Images from approximately 100 cells per treatment were collected and quantified for the number of red puncta (mRFPLC3 signal) per cell; the GFP signal was recorded blindly, using same exposure times and

laser strength settings as for the RFP channel. A yellow signal indicates not acidified, immature autophagosomes.

### 2.2.6 Luciferase assays

4xCLEAR Luciferase construct containing four times repeated CLEAR element upstream of Firefly-luciferase gene has been used to assess the activity of TFEB and CLEAR gene network. In this assay, TFEB binding to CLEAR promoter drives the transcription of TFEB-regulated genes and luciferase. Luciferase transcription is assessed in Dual-luciferase Reporter assays (Promega) for which cells were seeded in a 24 well plate and co-transfected with 4xCLEAR Luciferase and SV40 Renilla luciferase plasmids. SV40 Renilla luciferase was used as a transfection control. Twenty-four hours after transfection, cells were harvested in passive lysis buffer (Promega) and, after short centrifugation separating cellular membranes and DNA, 10 µl of pre-cleared lysate were used to assay luciferase activity using the Promega Dual-Luciferase Reporter kit and GloMax Multi+ plate reader.

### 2.2.7 Aβ Assays

Fully differentiated human forebrain neurons or mouse brain tissues were resuspended in the standard diluent buffer supplied by the manufacturer (Wako). To quantify Aβ levels human/mouse Aβ 1-40 and 1-42 ELISA kits (Wako) were

used according to the manufacturer's instructions. All assays were repeated 3 times and averaged for each triplicate.

### 2.2.8 Quantification and Statistics

All immunoblot signals were quantified using ImageJ software. To assess the number or co-localization of autophagosomes, lysosomes, and protein aggregates their specific, punctuate staining was quantified using ImageJ software and plugins for measuring intensity maxima from images based on individual parameters, or individually counted. Statistical evaluation of the collected data was performed using the student t-test and analysis of variance (ANOVA) as appropriate. Significant differences of means are indicated as $*p < 0.05$, $**p < 0.01$, and $***p < 0.005$.

# CHAPTER 3

## HOMOCYSTEINE ACTIVATES mTORC1 SIGNALING THROUGH INTERACTION WITH LeuRS-Flcn COMPLEX

Amino acid sensitivity of mTORC1 is very well accepted in the field in that availability of amino acids activates mTORC1, provides its localization on the lysosomal membrane, and further increases protein synthesis (Hara et al. 1998); although how amino acid metabolite as modified forms of amino acids affect mTORC1 activity is yet to be assessed. Homocysteine (de-methylated form of methionine) is known as a risk factor for AD (Zhuo et al. 2011; Kamat et al. 2015). Mutations in MTHFR, methionine synthase (MS) and CBS are known factors for AD pathogenesis (Garcia and Zanibbi 2004; Beyer et al. 2003; Zhuo et al. 2011). All three enzymes mentioned above are functional to remove excess Hcy from the body; MTHFR provides carbon group to folate which later donates it to Hcy and facilitates remethylation of Hcy to Met. MS is the main enzyme acting on Hcy methylation and Met production; whereas CBS is the enzyme that converts Hcy to cysteine in a two-step transsulfuration pathway. While MS needs vitamins B12 and B9, CBS needs vitamin B6 to function properly (Jakubowski 2012). Here we elucidate the impact Hcy might have on mTORC1 activity by assessing the phosphorylation level of mTORC1 substrates and localization of mTORC1 in the cell.

The amino acid sensitivity of mTORC1 is known to be fulfilled by multiple amino acid sensors in the cell. While v-ATPase detects the luminal content of

lysosomal amino acids (Zoncu et al. 2011), LeuRs (Han et al. 2012) and Sestrin2 (Wolfson et al. 2016) are potent Leu sensors in the cytoplasm. In this chapter, we show a new mechanism for Hcy to be sensed and further regulate mTORC1 activity. We show that Flcn which is known as a GAP for RagC/D (Tsun et al. 2013), interacts with LeuRS and its activity is dependent on amino acid (including Hcy) availability.

## 3.1 Results

### 3.1.1 Homocysteine Treatment Increases Phosphorylation Level of mTORC1 Substrates

It is well known that Leu is a strong mTORC1 activator which translocates mTORC1 on the lysosomal membrane where it is in close proximity to Rheb, its activator (Bar-Peled and Sabatini 2014). Given that mTOR is a kinase, its activation corresponds to increase in phosphorylation levels of its related substrates. Thus, to assess mTORC1 activity, the phosphorylation level of P70/S6K, 4EBP-1, and TFEB were analyzed in western blot analyses of cells treated with Hcy for three hours. HEK293T cells and iPSC-derived human neurons were main cell lines utilized for completion of this research project. Cells were cultured in RPMI media supplemented with Leu, Glu and 10% dialyzed FBS in which mTORC1 is fully active. Leucine withdrawal from the above media renders mTORC1 inactive and diffused in the cytoplasm; thus, we used it as starvation media for our experiments. In Hcy-treated conditions, Leu was substituted by Hcy; in this manner, we were able to evaluate and compare

mTORC1 activity in presence of Leu or Hcy. Homocysteine-treated cells showed increased mTORC1 activity compared to starved cells as was detectable by measuring the phosphorylation levels of P70/S6K, 4EBP-1, and TFEB (Figure 3.2 A, B, and C). The activity of mTORC1 was up-regulated in presence of Leu and Hcy as compared to Leu alone (Figure 3.3 A, B, and C). These data showed Hcy ability to activate mTORC1 in absence or presence of Leu. Of note, immunoblot analyses of P70/S6K in human brain lysates of AD versus control patients showed increase of mTORC1 activity in AD patients (Figure 3.1 A), which were diagnosed with a high level of plasma Hcy (Zhuo et al. 2011; Li, Chu, and Barrero 2014; Kamat et al. 2015). However, levels of other mTORC1-related factors such as TSC2 and Rheb which inhibit and activate mTORC1 respectively did not show a significant difference between control and AD samples (Figure 3.1 B and C).

To elucidate the pathway through which Hcy leads to mTOR activation, and to rule out the possibility of Akt signaling activation by Hcy, Akt (T308) phosphorylation level was measured in immunoblot analyses of cells treated with ascending concentrations of Hcy. Although phospho-p70/S6K levels increased proportionally to increased concentrations of Hcy, phospho-Akt (T308) did not change significantly (Figure 3.4). Similarly, phospho-Akt (T308) and (S473) were probed in *Cbs* deficient and wild-type mice brain lysates; neither phospho-Akt (T308) (as a marker for growth factor signaling) nor phospho-Akt (S473) (as a readout for mTORC2 signaling) changed significantly between mutant and

normal mice (Figure 3.5 A and B). Interestingly, we did not observe significant changes of P-AKT (S473) in AD patients, as well (Figure 3.6).

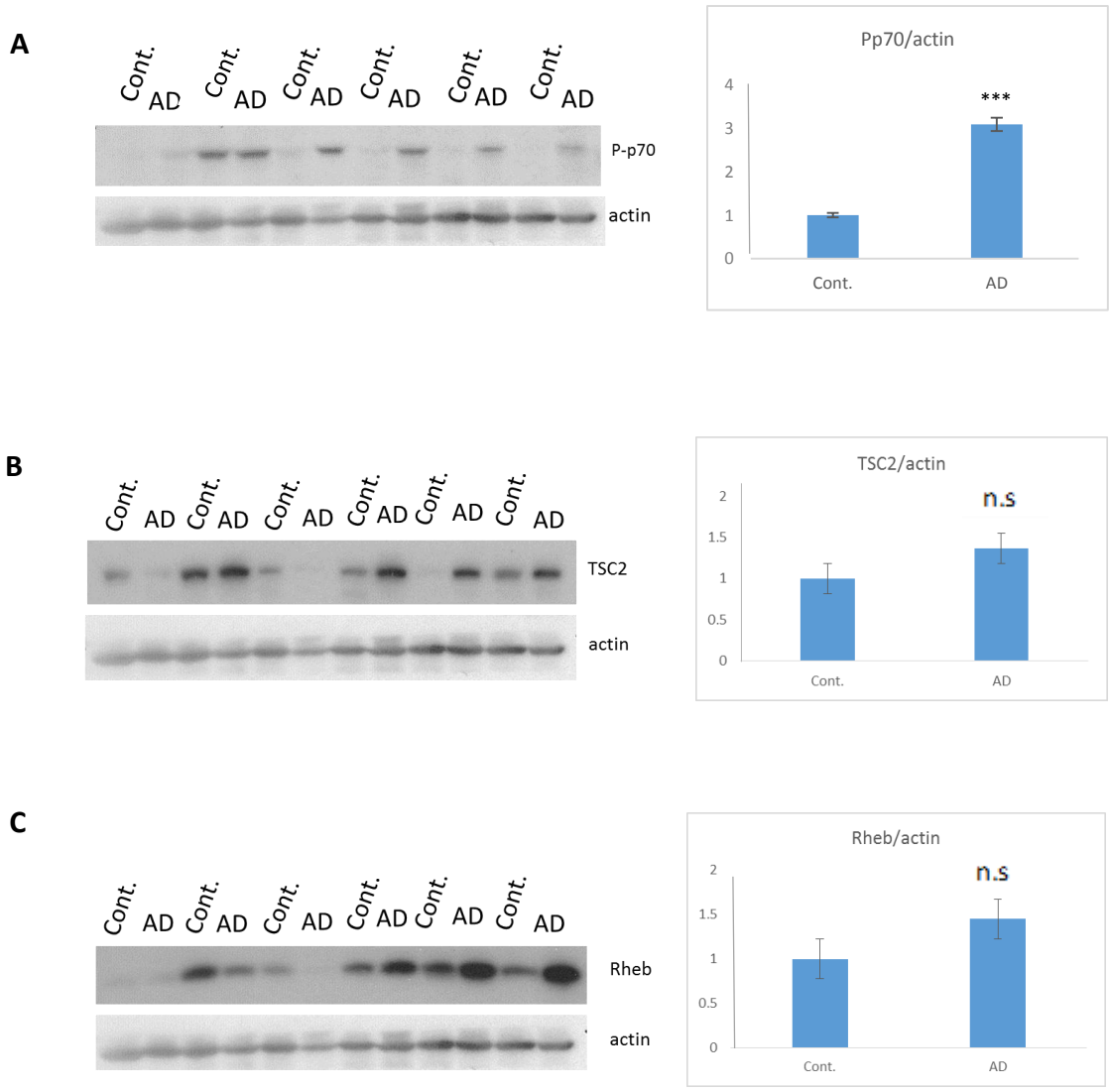



**Figure 3.1** mTORC1 is activated in postmortem brain samples of Alzheimer's disease patients. A) The phosphorylation level of P70S6Kinase increased significantly compared to control patient samples. B and C) TSC2 (mTOR inhibitor) and Rheb (mTORC1 activator) protein levels did not change significantly between control and AD patients. Bar graphs represent a quantitative analysis of protein levels assessed by immunoblot analyses. Data are means +/- SEM (n = 3 independent experiments). ***P ≤ 0.005.

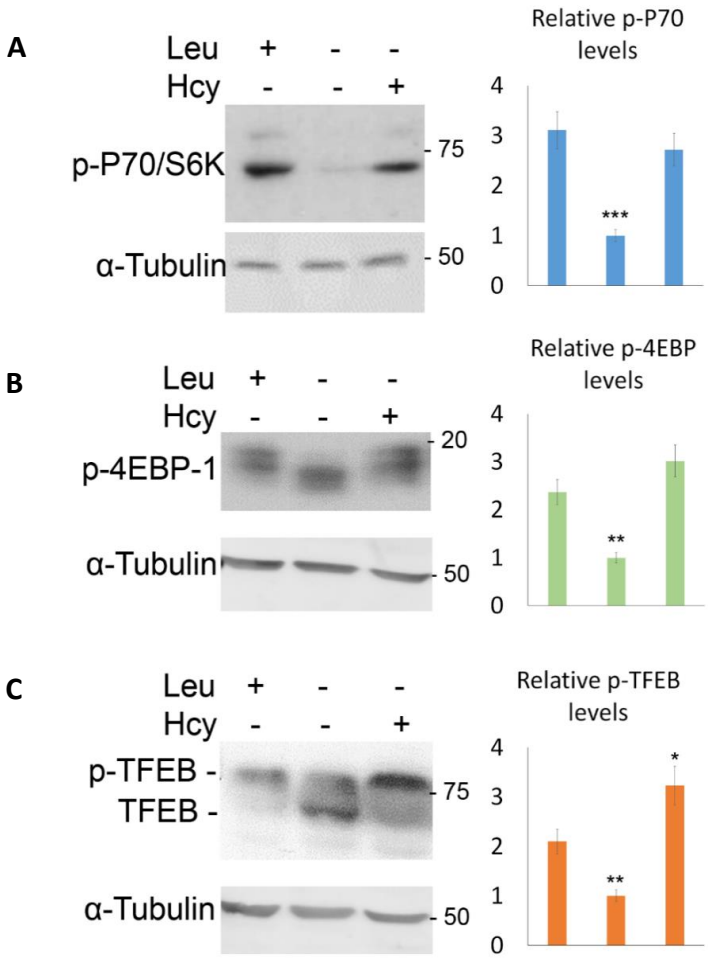



**Figure 3.2- A, B, C** Homocysteine up-regulates mTORC1 activity in absence of leucine. HEK293T cells were treated with RPMI media supplemented with Glu, dialyzed FBS and Hcy in absence of Leu (RPMI starvation media). A) Phosphorylation level of P70/S6kinase increased in Hcy-treated HEK293T cells which was indicative of mTORC1 hyperactivation (Leu: 3.1 +/- 0.4 -fold; Hcy: 2.7 +/- 0.3-fold; P = 0.004). B) Phospho-4EBP-1 was measured in western blot analyses as an indicator of mTORC1 activity. Hcy increased P-4EBP-1 level in HEK293T cells (Leu: 2.4 +/- 0.3-fold; Hcy: 3 +/- 0.4-fold; P < 0.01). C) TFEB phosphorylation level was assessed to determine mTORC1 activity. Phospho-TFEB appeared upon Hcy treatment while it was not present in starvation media without Hcy (Leu: 2.1 +/- 0.3-fold; Hcy: 3.2 +/- 0.4-fold; P = 0.014). Bar graphs represent a quantitative analysis of protein levels assessed by immunoblot analyses. Data are means +/- SEM (n = 6 independent experiments). *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.005.

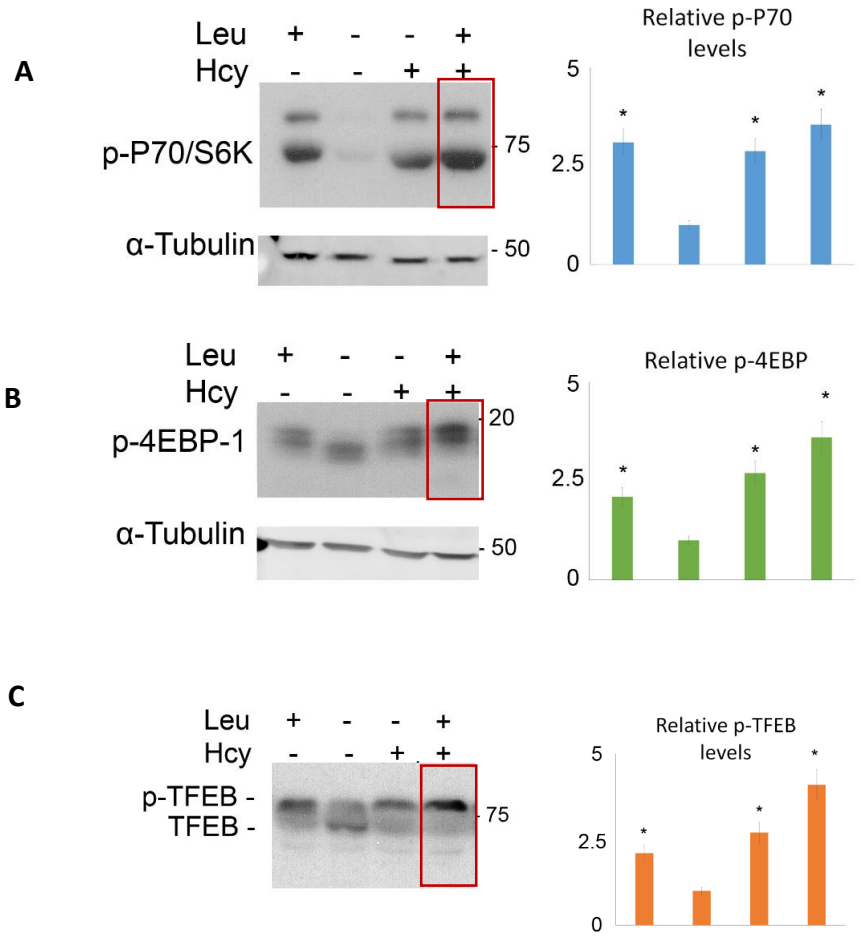



**Figure 3.3- A, B and C** Homocysteine and leucine collaboratively activate mTORC1. HEK293T cells were treated with RPMI media supplemented with Leu, Glu, dialyzed FBS (RPMI complete media) and Hcy. A) Phospho-P70/S6K increased in media contained both Hcy and Leu. B) Phospho-4EBP1 increased in presence of Hcy and Leu in the media. C) Homocysteine and Leu together gave rise to the formation of a more phosphorylated form of TFEB. Bar graphs represent a quantitative analysis of protein levels assessed by immunoblot analyses. Data are means +/- SEM (n = 6 independent experiments). *P ≤ 0.05.

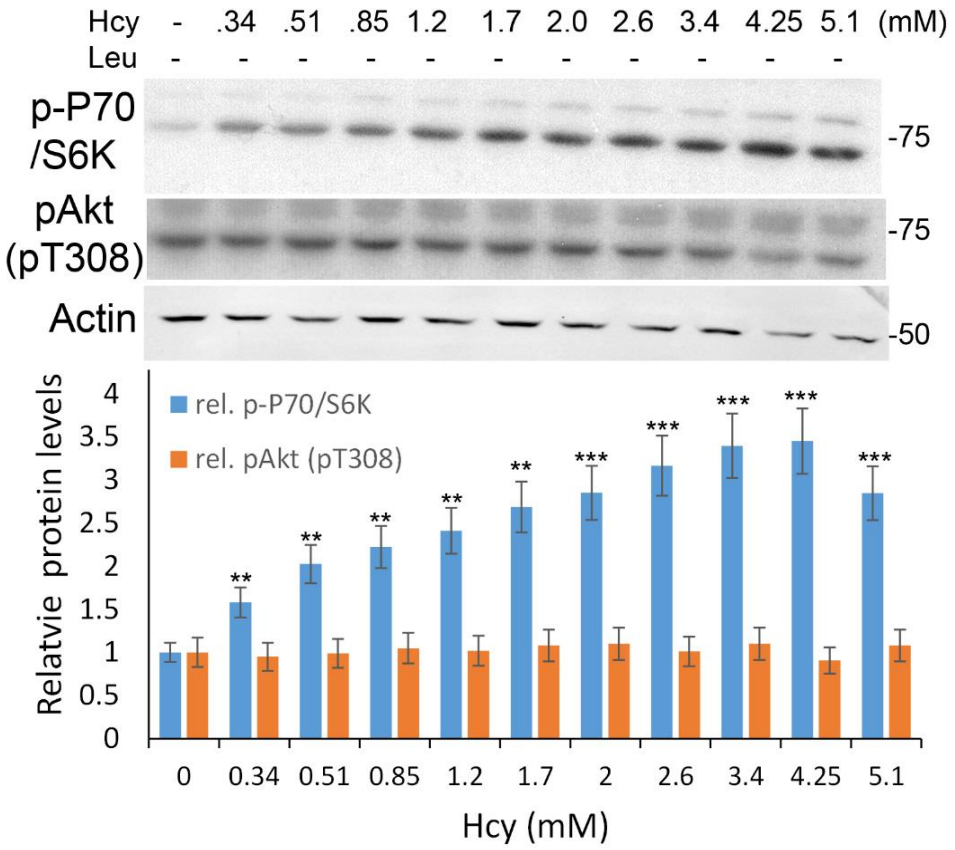



**Figure 3.4** Homocysteine regulates mTORC1 activity without changing the phospho-Akt pathway. HEK293T cells were treated with increasing concentrations of Hcy added to RPMI starvation media. Cell lysates were subjected to western blot analyses followed by probing with P-AKT (pT308) antibody. Homocysteine did not change phosphorylation levels of AKT(T308) while it did change phospho-P70/S6kinase levels, significantly (0.34 mM: 1.5 +/- 0.2-fold; 4.25 mM: 3.5 +/- 0.4-fold). Bar graphs represent a quantitative analysis of protein levels assessed by immunoblot analyses. Data are means +/- SEM (n = 6 independent experiments). **P ≤ 0.01, and ***P ≤ 0.005.

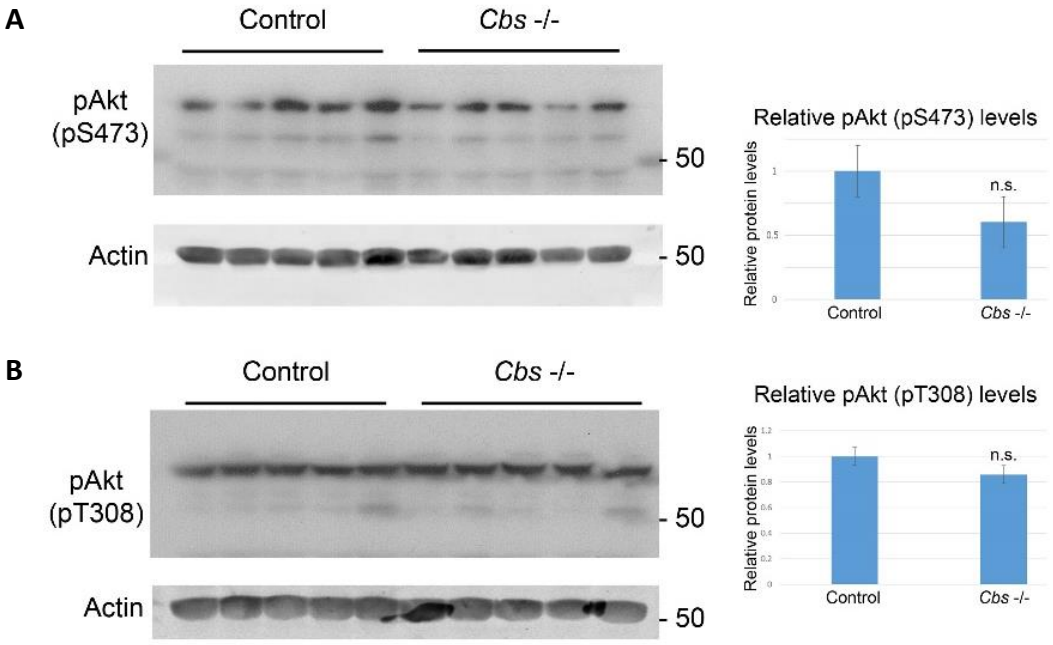



**Figure 3.5- A and B** Homocysteine does not impact phospho-AKT pathway in $Cbs^{-/-}$ mice. A) Relative phospho-Akt protein levels associated with mTORC2 signaling (phospho-Serine 473), as well as B) phospho-Akt indicative of growth factor receptor signaling (phospho-Threonine 308) did not significantly change in $Cbs^{-/-}$ mouse brains compared to control littermates. Immunoblots represent separate runs of protein samples from five control and five knockout brains. Bar graphs represent quantitative analysis of protein levels assessed by immunoblot analyses. Data are means +/- SEM (n = 3 independent experiments).

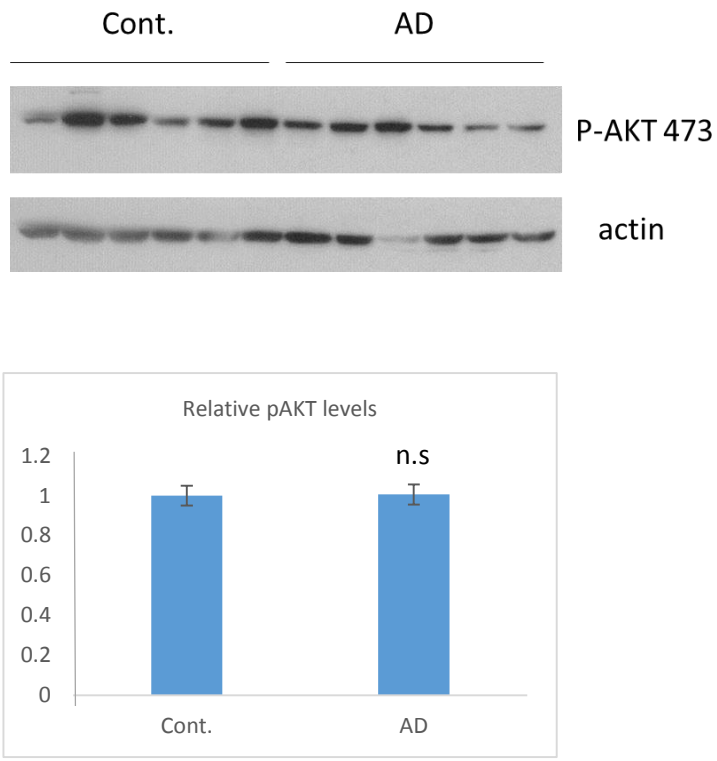



**Figure 3.6** Phosphorylated levels of AKT473 do not change in Alzheimer's disease patients. Human brain lysates of Alzheimer's and control patients were assessed in western blot analyses for determination of P-AKT473 levels. No significant changes were detected between our experimental groups. Bar graphs represent a quantitative analysis of protein levels assessed by immunoblot analyses. Data are means +/- SEM (n = 3 independent experiments).

### 3.1.2 Mechanistic Target of Rapamycin is not Activated by Homocysteine-Thiolactone

Hcy-thiolactone, a reactive Hcy metabolite, has been detected in patients with high serum Hcy (HHcy). The reason for Hcy conversion to Hcy-thiolactone arises from Hcy editing process which happens by AARS through which Hcy takes a cyclized conformation. Homocysteine-thiolactone (a cyclic thioester) is known as a reactive component which attacks free amino groups of protein lysine residues

(Jakubowski 2015). The hyperactivity of Hcy-thiolactone leads to protein damage and precipitation, hence is proposed to have pathological effects (Jakubowski 1999; Akchiche et al. 2012); however, in our study, we showed that Hcy-thiolactone did not regulate mTORC1 activity. HEK293T cells were treated with same concentrations of Hcy-thiolactone as Hcy in media with or without Leu. The activity of mTORC1 did not change in presence and absence of Hcy-thiolactone which was assessed by measuring the phosphorylation level of p70/S6K (Figure 3.7).

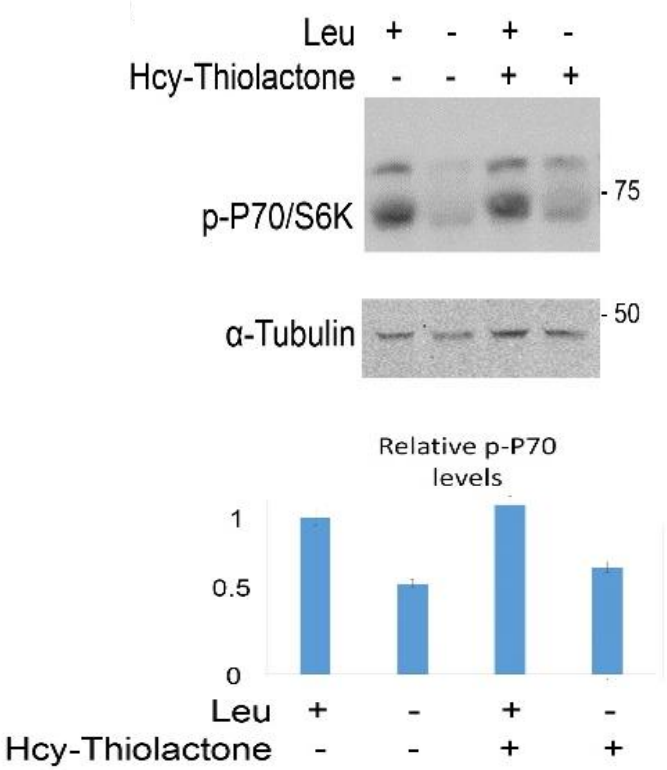



**Figure 3.7** Homocysteine-thiolactone does not affect mTORC1 activity. HEK293T cells were cultured in RPMI complete and starvation media with or without Hcy-thiolactone. Phospho-P70/S6kinase was assessed by western blot analysis. No significant changes were observed in Hcy-thiolactone versus control treatments. Bar graphs represent a quantitative analysis of protein levels assessed by immunoblot analyses. Data are means +/- SEM (n = 3 independent experiments).

To confirm the data that identified Hcy-thiolactone as not incorporating into mTORC1 activation, Blmh/Pon1 deficient mice which lack both important enzymes to hydrolase Hcy-thiolactone were fed with Met, as a Hcy precursor, and brain lysates were applied to western blot analyses. It has been shown that these transgenic mice have a high concentration of Hcy-thiolactone in their plasma (Borowczyk et al. 2012). Normal mice fed with Met were used as a control that demonstrated higher mTORC1 activity compared to normal mice fed with water. However, Met-treated mice which were deficient in Blmh/Pon1 enzymes did not show significant changes in mTORC1 activity compared to normal mice fed with methionine (Figure 3.8).

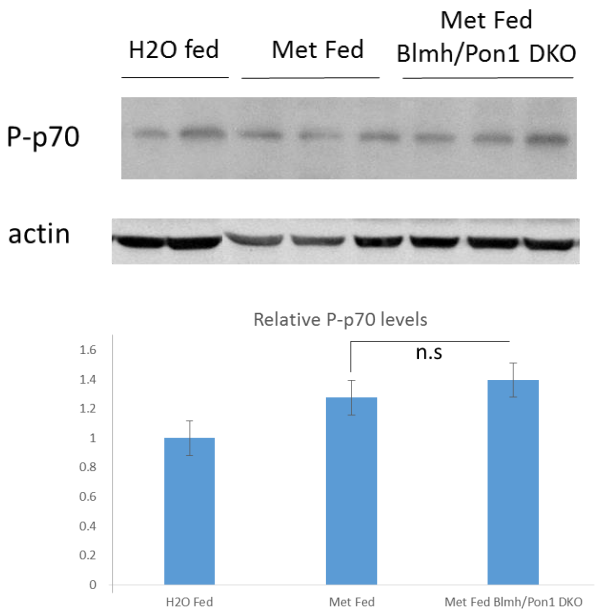



**Figure 3.8** mTORC1 activity is not affected by homocysteine-thiolactone *in-vivo*. Brain lysates of normal mice fed with water or methionine and mice deficient in Bleomycin/Paraoxonase1 which was fed with methionine were subjected to western blot analyses. No significant changes in P-p70S6kinase activity were assessed between the normal and deficient mice fed with methionine. Bar graphs represent a quantitative analysis of protein levels assessed by immunoblot analyses. Data are means +/- SEM (n = 3 independent experiments).

Since Hcy is converted to the amino acid cysteine upon accumulation in the cells, the impact cysteine might have on mTORC1 activity was measured to specify the effect Hcy has on mTORC1, specifically. HEK293T cells were treated with RPMI complete and starvation media with or without cysteine. Cells were lysed further and subjected to western blot analysis. Phospho-p70S6kinase level was assessed and showed no significant impact on mTORC1 activity (Figure 3.9).

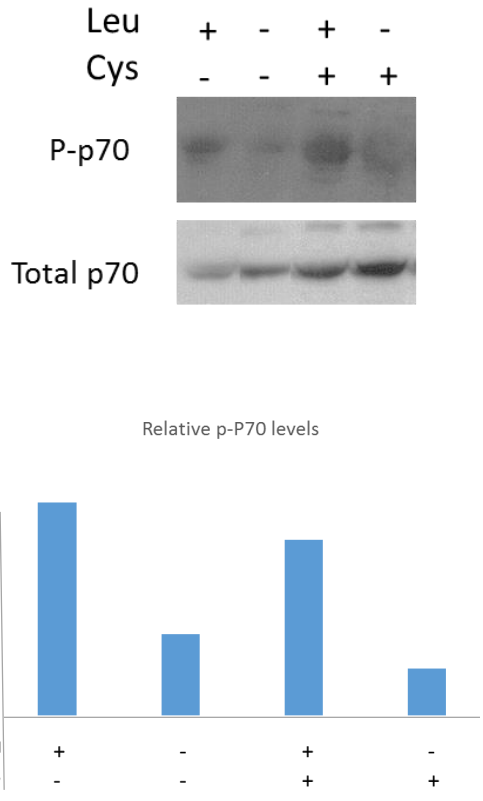



**Figure 3.9** Cysteine does not affect mTORC1 activity. The effect of cysteine on mTORC1 activity was assessed in HEK293T cells by treating with cysteine in complete and starvation media and performing western blot analyses. No significant changes were observed in mTORC1 activity between our cysteine-treated versus control cells. Bar graphs represent a quantitative analysis of protein levels assessed by immunoblot analyses.

### 3.1.3 Homocysteine Induces mTORC1 Localization on the Lysosomal Membrane in Absence of Leucine

mTORC1 localization plays an important role in its activation in that the kinase needs to tether to lysosomal membranes to become activated (Sancak et al. 2010), so localization of mTORC1 was monitored in immunostaining experiments by applying mTORC1 and lysosome-associated membrane glycoprotein 2 (LAMP2) antibodies to cells treated with Hcy. In RPMI complete media virtually all of the mTORC1 co-localized with LAMP2; whereas, in RPMI starvation media which is deprived of Leu, mTORC1 diffused in the cytoplasm. The addition of Hcy to RPMI starvation media re-tethered mTORC1 back to the lysosomal membranes; hence indicating that Hcy can be detected by the amino acid sensing machinery that binds mTORC1 to LAMP2-positive membranes. Data were representative of two different cell lines; HEK293T cells (Figure 3.10 A) and mouse embryonic fibroblast (MEFs) (Figure 3.10 B).

To further investigate the role of Hcy *in-vivo* we performed immunohistochemistry on *Cbs* deficient and wild type hippocampal sections. *Cbs* deficient mice showed increased co-localization levels of mTORC1 and LAMP2. Interestingly, overall protein levels of mTORC1 increased in these mice, whereas the number of their lysosomes decreased significantly compared to control littermates (Figure 3.11).

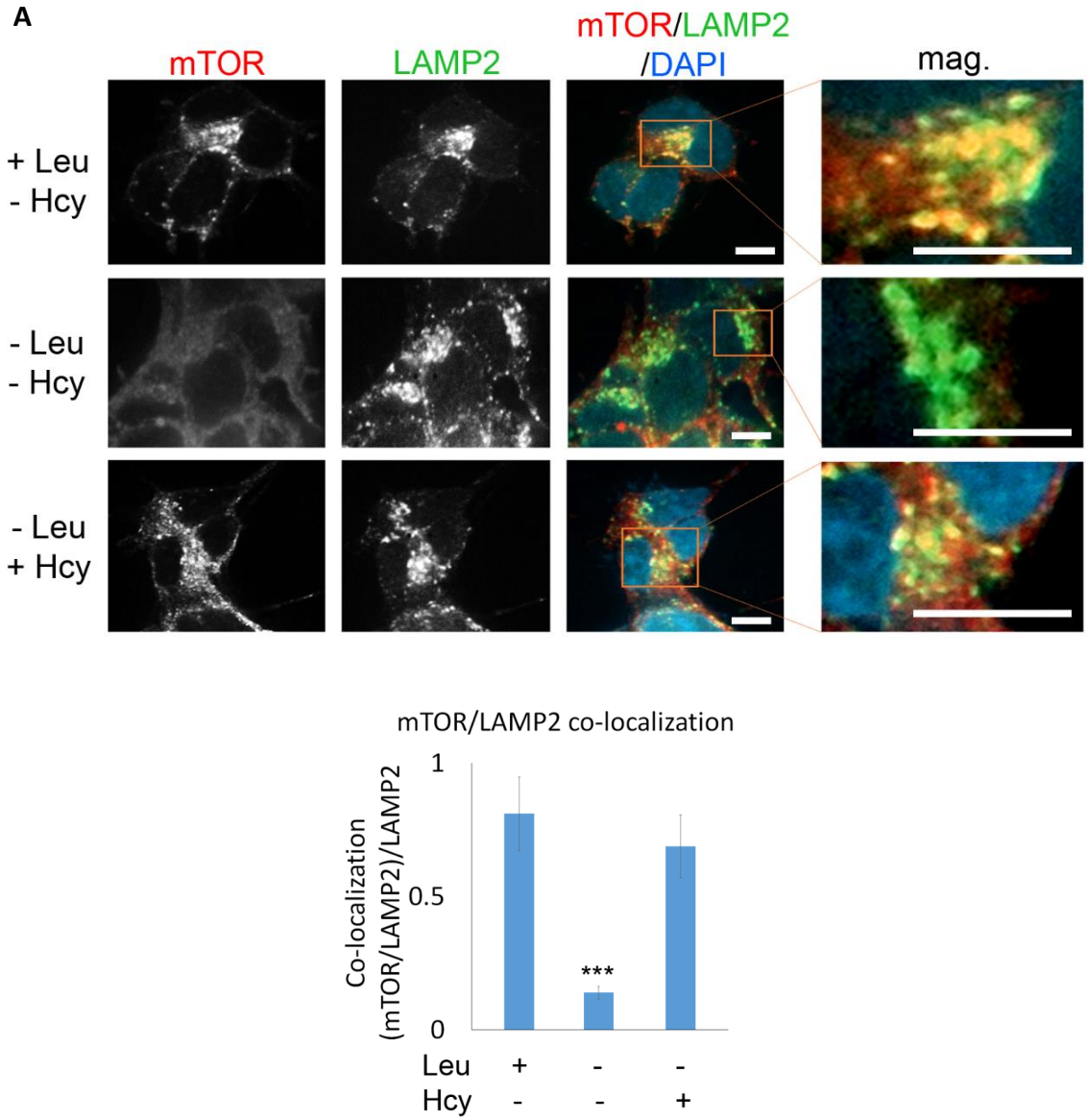



**Figure 3.10-A** Homocysteine drives mTORC1 localization on lysosomal membrane. HEK293T cells were treated with RPMI complete and starvation media with or without Hcy. mTORC1 and LAMP2 antibodies were used to probe mTORC1 and lysosomes, respectively. DAPI stained nucleus. While mTORC1 was diffused in the absence of Leu in starvation media, it localized on the lysosomal membrane in media which contained Hcy as a substitution for Leu. The inset shows magnification of selected fields. Scale bars are 10 μm. A bar graph is representative of mTOR/LAMP2 co-localization divided by the number of LAMP2 positive puncta. Data are means +/- SEM (n = 6 independent experiments). ***P ≤ 0.005.

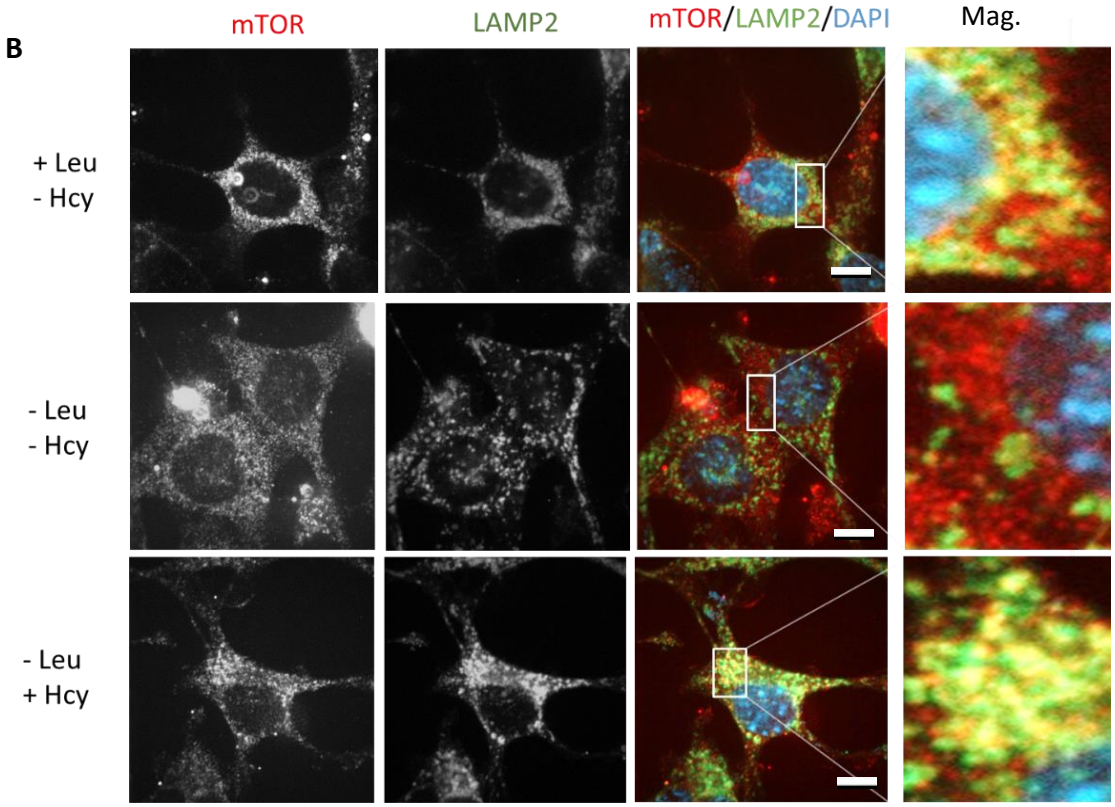



**Figure 3.10-B** Homocysteine drives mTORC1 localization on lysosomal membrane. MEFs were treated with RPMI complete and starvation media with or without Hcy. mTORC1 and LAMP2 antibodies were used to probe mTORC1 and lysosomes, respectively. DAPI stained nucleus. While mTORC1 was diffused in the absence of Leu in starvation media, it localized on the lysosomal membrane in media which contained Hcy as a substitution for Leu. The inset shows magnification of selected fields. Scale bars are 10 µm.

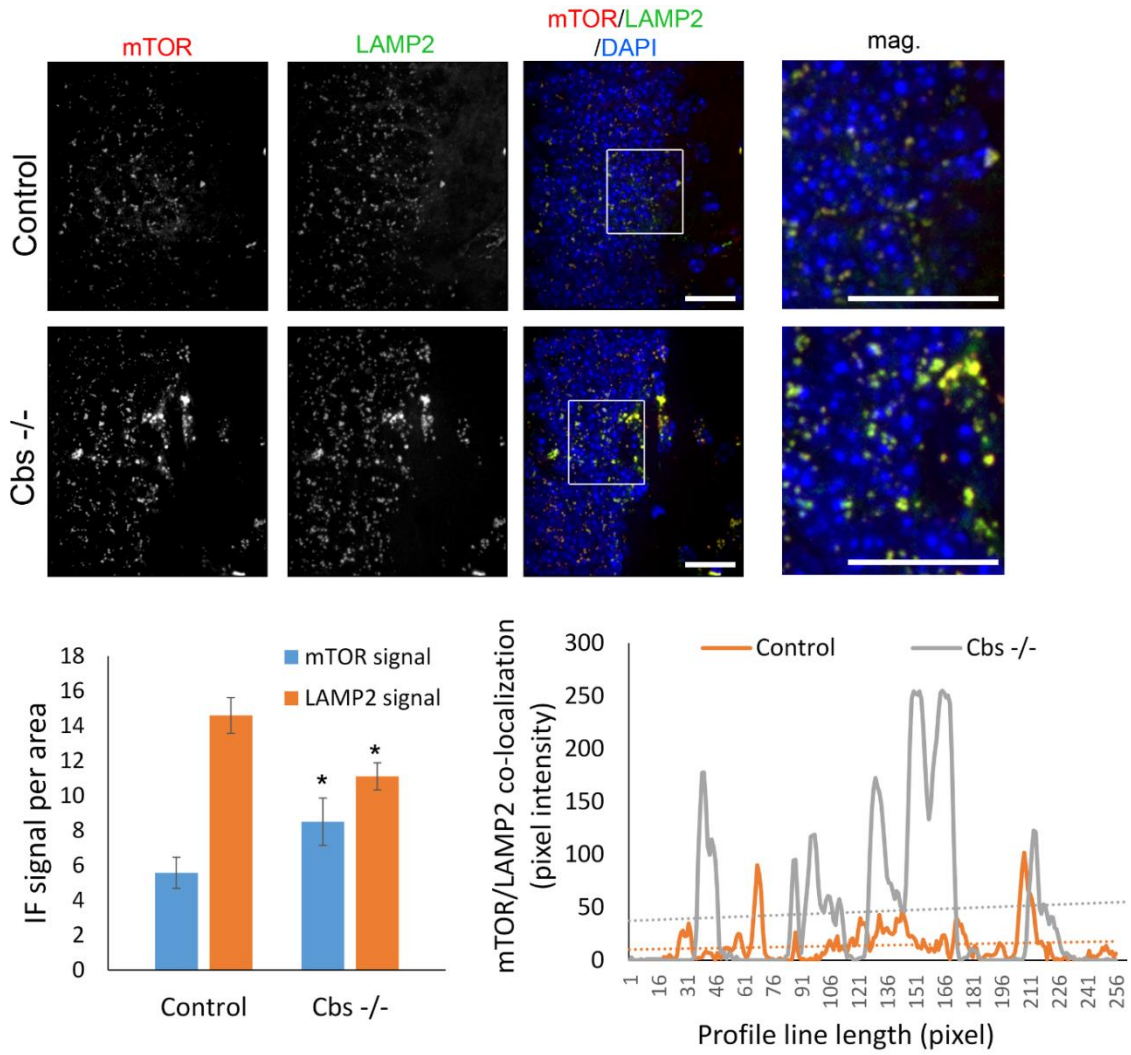



**Figure 3.11** mTORC1 localizes on lysosomal membrane in $Cbs^{-/-}$ mouse brains. Control and $Cbs^{-/-}$ mouse hippocampal dentate gyrus sections were stained with mTOR and LAMP2 antibodies as markers for mTOR and lysosomes, respectively. DAPI stained nucleus. $Cbs^{-/-}$ mice hippocampi showed higher mTORC1 and LAMP2 co-localization compared to their control littermates (3.7 +/- 0.5-fold vs. controls; P = 0.045). The bar diagram is representative of the number of mTOR and LAMP2 in control and knockout mice. The representative plot profile shows co-localization of mTOR and LAMP2 in $Cbs^{-/-}$ and control hippocampi; trendline indicates the average co-localization in the given image. Data are means +/- SEM (n = 5 mice per group). Scale bars are 20 μm. *P ≤ 0.05.

### 3.1.4 Leucyl-tRNA Synthetase and Folliculin Interact as a Permanent Complex

Leucyl-tRNA synthetase is not only known as a facilitator of tRNA-amino acid binding but also is recognized as a Leu sensor in the cell (Han et al. 2012). The affinity of Hcy in binding to LeuRS during the editing process of Hcy by LeuRS (Jakubowski 2012) raised the possibility that Hcy relays the signal to mTORC1 through LeuRS. Although GAP activity of LeuRS toward Rag C was also proposed by Han et al (Han et al. 2012), the results could not be reproduced by other research groups in the field. Shortly after, Tsun et al introduced Flcn as a potent GAP for Rag C and D (Tsun et al. 2013). During this dissertation we tested the hypothesis that LeuRS and Flcn might interact with each other in an amino acid-dependent manner. To check this hypothesis we performed co-immunoprecipitation assays testing whether Flcn would bind to LeuRS under certain metabolic conditions. For this purpose, HEK293T cells were transfected with Flcn-dTomato construct and treated in RPMI complete and starvation media with or without Hcy. Following immunoprecipitation, immunoblot analyses were performed to detect endogenous levels of LeuRS. Notably, we found the constitutive interaction between LeuRS and Flcn but did not detect a change in such interaction upon different amino acid treatments or in the starvation media (Figure 3.12 A).

To confirm our data, reciprocal immunoprecipitation was performed in which HEK293T cells were transfected with LeuRS-Flag and endogenous Flcn was probed on western blot analyses. The same constitutive interaction was

detected between LeuRS and Flcn in all treatments which indicated that Flcn and LeuRS indeed associate in a complex (Figure 3.12 B).

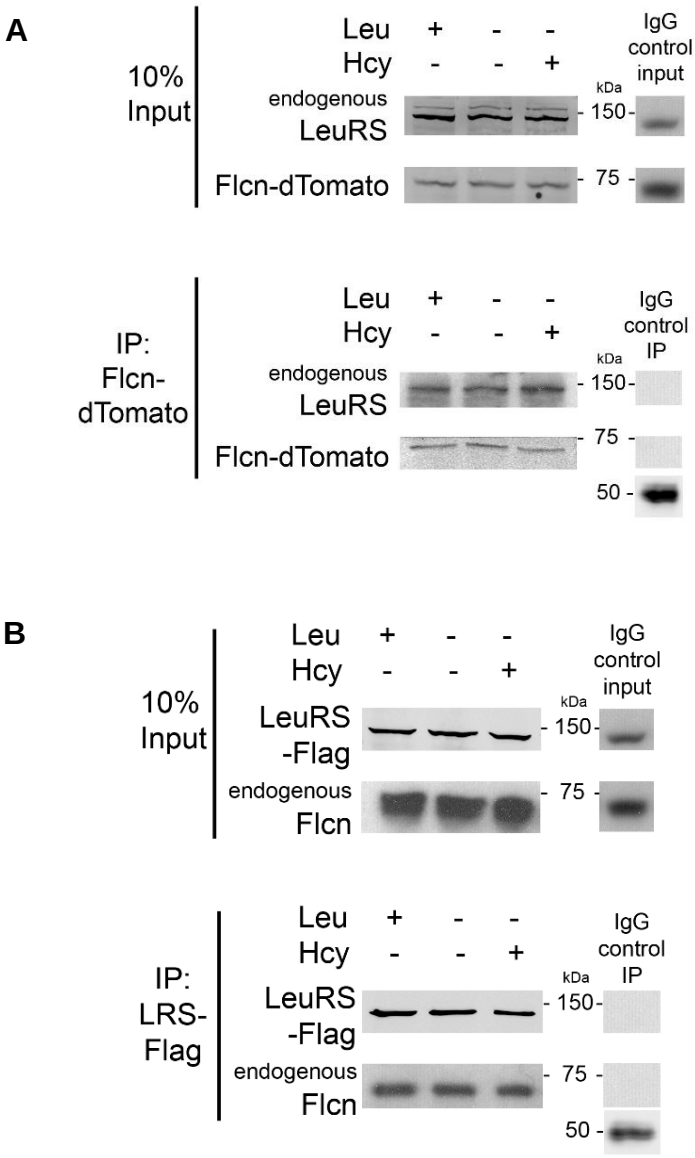



**Figure 3.12-A and B** LeuRS interacts with Flcn to form a complex, permanently. A) HEK293T cells were transfected with LeuRS-Flag plasmid and treated with RPMI complete and starvation media with or without Hcy. Cell lysates were subjected to Immunoprecipitation analyses followed by probing with Flcn antibody in western blotting. B) Whereas, reverse immunoprecipitation were applied by over-expression of Flcn-dTomato followed by LeuRS antibody staining in western blot analyses. Data is indicative of LeuRS and Flcn interaction in presence and absence of Leu or Hcy, constitutively. IgG pull-down was done as a control for immunoprecipitation. (n = 5 independent experiments).

### 3.1.5 Leucyl-tRNA Synthetase-Folliculin Complex Interacts with Rag C Conditionally

Folliculin, a tumor suppressor has been implied to have GAP activity toward Rag C/D; in that Flcn switches GTP-bound form of Rags for GDP and detaches from the lysosomal membrane as amino acids are replenished in the cytoplasm (Tsun et al. 2013). Here we provided evidence that Flcn localization is dependent on Hcy or Leu availability in the cell through two different experimental approaches. First, we examined the possibility of LeuRS-Flcn complex conditional interaction with Rag C. We performed immunoprecipitation analyses by transfecting HEK293T cells with RagC-HA construct and probing western blots with Flcn and LeuRS antibodies. The potent interaction between the complex and Rag C was observed only in absence of Leu or Hcy (Figure 3.13 A). Rag C constitutively active and dominant negative mutants were used as controls for the experiment. Reciprocal immunoprecipitation assays were conducted by transfecting the cells with LeuRS-Flag following by evaluation of endogenous Rag C levels in immunoblot analyses. Intense interaction between LeuRS-Flag and Rag C was detected in cells treated with starvation media. Whereas, LeuRS-Flag and Rag C interaction was virtually lost in presence of Leu, Hcy or both (Figure 3.13 B).

In an alternative approach, localization of Flcn was assessed in confocal immunofluorescence analyses. We used Flcn antibody to check endogenous levels of Flcn (Figure 3.14 A) or transfected HEK293T cells with Flcn-dTomato construct to investigate the localization of exogenous overexpressed Flcn (Figure 3.14 B). Cells were treated with RPMI complete and starvation media with or without Hcy. Folliculin and LAMP2 antibodies were applied to monitor Flcn and

lysosomes, respectively (Figure 3.14 A). In order to inhibit LeuRS-driven mTORC1 activity, leucinol, a Leu derivative, was applied which competes with Leu in binding to LeuRS (Han et al. 2012). Data depicted increased co-localization of Flcn and lysosomes in starvation media and in leucinol treated cells even in presence of Hcy; while Flcn was diffused in the cytoplasm of cells treated with Leu or Hcy (Figure 3.14 A). We observed the same pattern of overexpressed Flcn localization in the cells according to amino acid and Hcy availability (Figure 3.14 B).

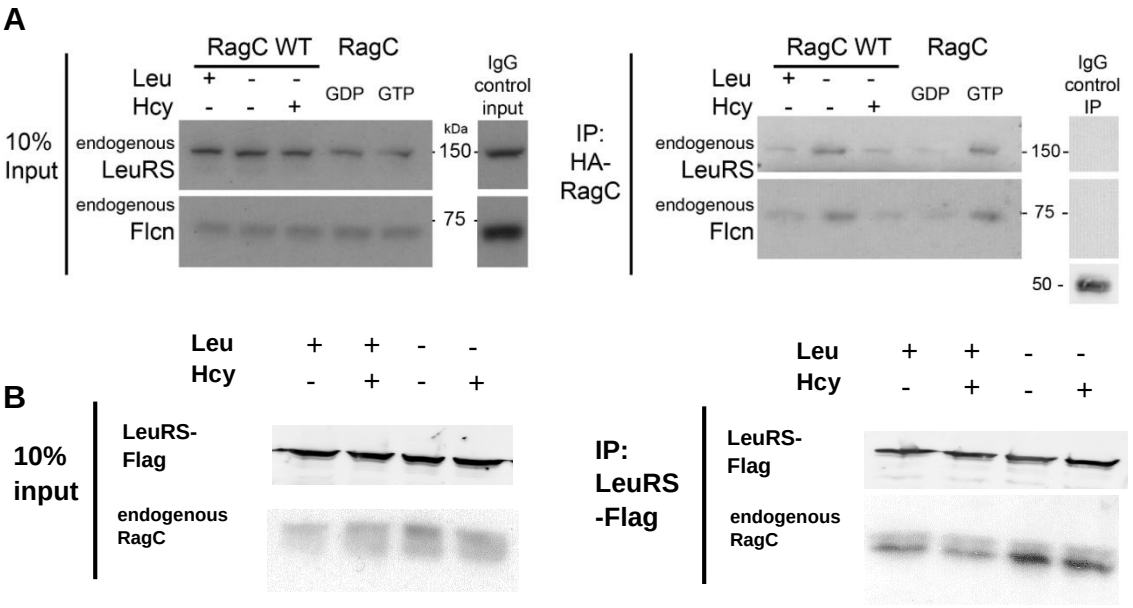



**Figure 3.13-A and B** LeuRS-Flcn complex binds to RagC conditionally. A) HEK293T cells were transfected with RagC-HA plasmid and treated with RPMI complete and starvation media with or without Hcy. Co-IP samples were probed for endogenous LeuRS and Flcn in immunoblot analyses. Data indicates that LeuRS and Flcn interact with RagC in absence of Leu or Hcy (starvation media). Cells were also transfected with RagC-GTP-bound (dominant negative) and RagC-GDP-bound (constitutively active) as negative and positive controls, respectively. IgG pull-down was done as a control for immunoprecipitation. (n = 3 independent experiments). B) HEK293T cells were transfected with LeuRS-Flag in complete and starvation media with or without Hcy. Further, immunoblots were probed for endogenous RagC. RagC showed the highest interaction with LeuRS in absence of both Leu and Hcy.

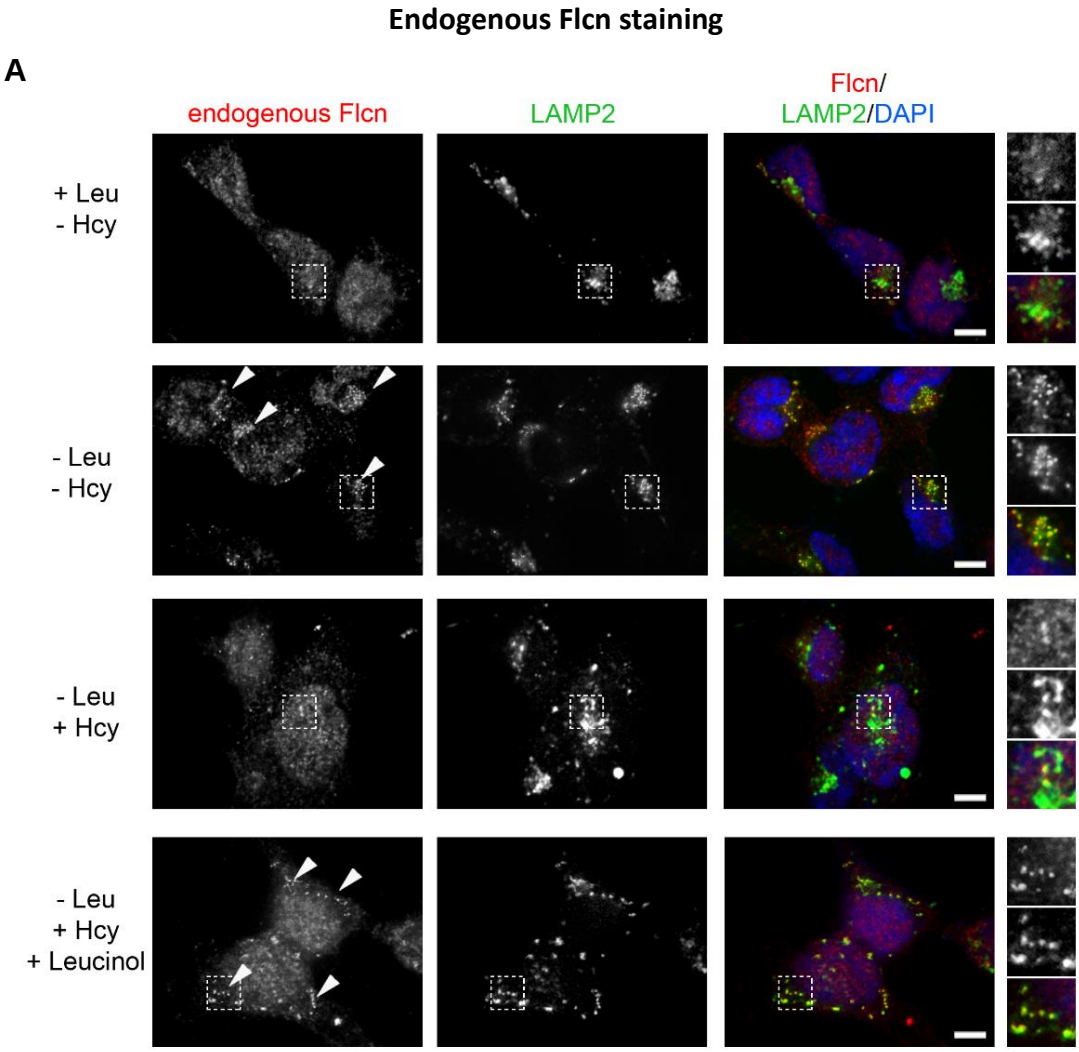
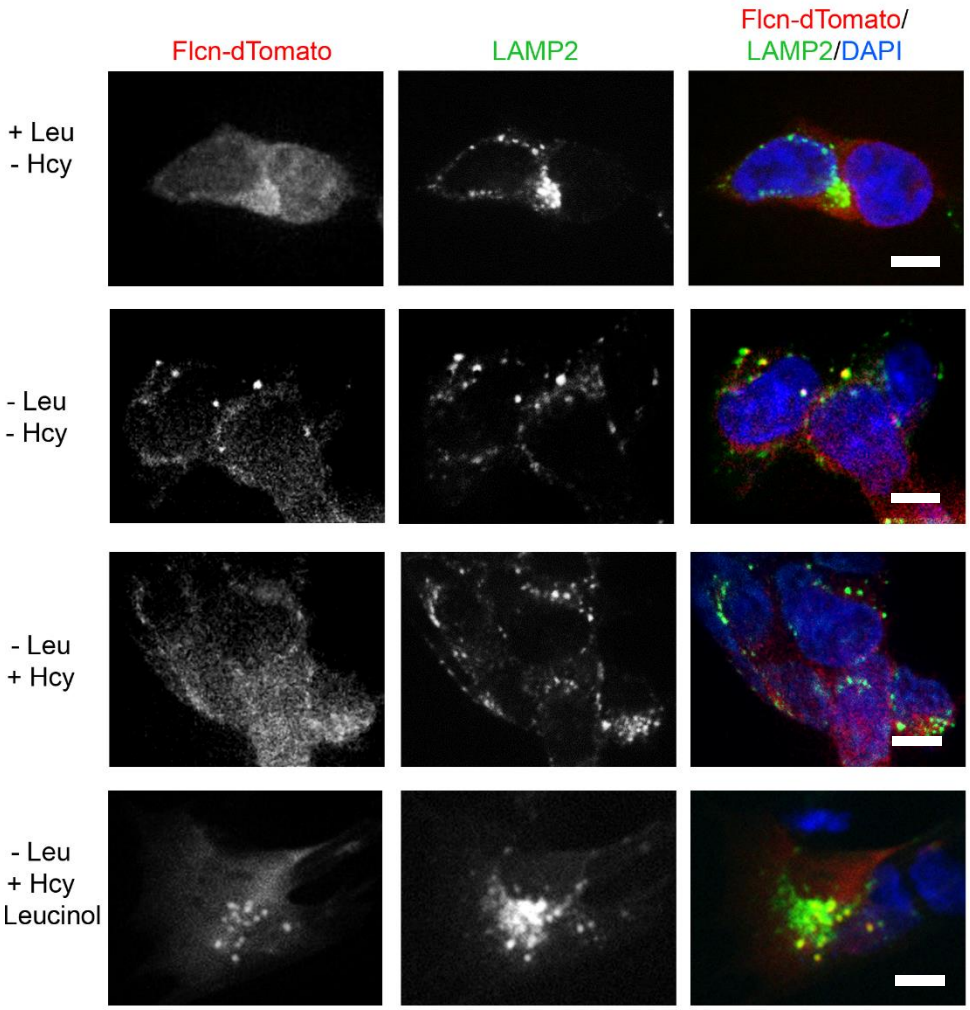



**Figure 3.14-A** Folliculin diffuses in the cytoplasm in presence of homocysteine. HEK293T cells were examined for endogenous Flcn. Cells were cultured in RPMI complete and starvation media with or without Hcy. Leucinol was used as an mTORC1 inhibitor in presence of Hcy. Flcn and LAMP2 (as a lysosomal marker) antibodies were used in immunostaining experiments. DAPI stained nucleus. Flcn (white arrowheads) resided on lysosomal membrane in absence of Leu or Hcy, but diffused in the cytoplasm in presence of either of them. Magnifications of selected areas are shown on the right. Scale bars are 10 µm.

**Exogenous Flcn staining**

**B**



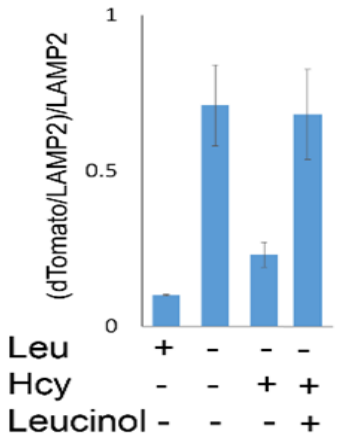



**Figure 3.14-B** Folliculin diffuses in the cytoplasm in presence of homocysteine. HEK293T cells were transfected with Flcn-dTomato. Cells were cultured in RPMI complete and starvation media with or without Hcy. Leucinol was used as an mTORC1 inhibitor in presence of Hcy. LAMP2 (as a lysosomal marker) antibodies were used in immunostaining experiments. DAPI stained nucleus. Flcn resided on lysosomal membrane in absence of Leu or Hcy, but diffused in the cytoplasm in presence of either of them (Hcy starvation conditions: 0.7 +/- 0.1 vs. Leu: 0.1 +/- 0.002 and Hcy: 0.2 +/- 0.04; P = 0.01). Bar diagram is quantitative analyses of Flcn-LAMP2 co-localization divided by the number of LAMP2-positive puncta. Data are means +/- SEM (n = 6 independent experiments). Scale bars are 10 µm. **P ≤ 0.01.

### 3.1.6 Sestrin2 Protein Levels are not Affected by Homocysteine

Sestrin2 has been implicated as a Leu sensor which is capable of binding to Leu and activating mTORC1 (Wolfson et al. 2016). In general, Sestrin2 is an interacting partner for GATOR2 (an mTOR activator) and prevents mTORC1 activity by disrupting GATOR2 function (Chantranupong et al. 2014). In presence of Leu, Sestrin2 loses interaction with GATOR2, renders induction of GATOR2-GATOR1 interaction and attenuation of GATOR1-inhibitory effects on mTORC1 which leads to increase in mTORC1 activity; thus the binding ability of Sestrin2 to Leu is necessary for it to activate mTORC1 (Chantranupong et al. 2014; Wolfson et al. 2016).

In order to evaluate if the Hcy regulates Sestrin2 expression level in the cell, the protein levels of Sestrin2 were assessed in HEK293T cells upon treatment with Hcy. Data showed no significant change in Sestrin2 protein levels when cells were exposed to Hcy (Figure 3.15 A). Similarly, brain lysates of *Cbs* deficient mice did not show alterations in Sestrin2 levels compared to wild-type mice (Figure 3.15 B).

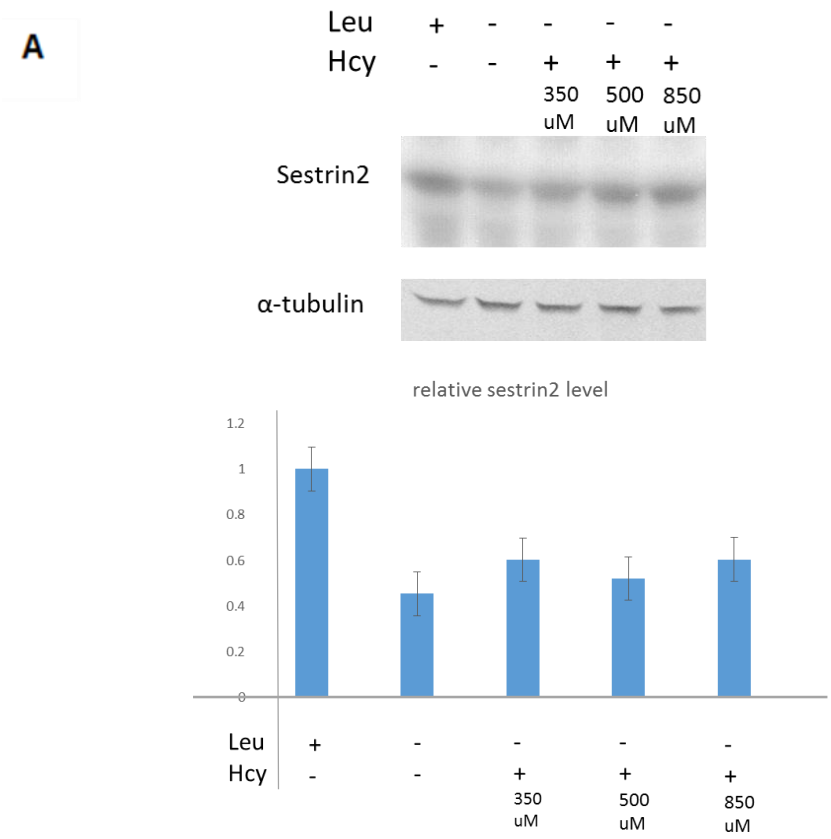



**Figure 3.15-A** Homocysteine does not influence Sestrin2 protein expression levels. HEK293T cells were treated with different concentrations of Hcy. Sestrin2 protein levels did not change with Hcy treatments. Bar graphs represent a quantitative analysis of protein levels assessed by immunoblot analyses. Data are means +/- SEM (n = 3 independent experiments).

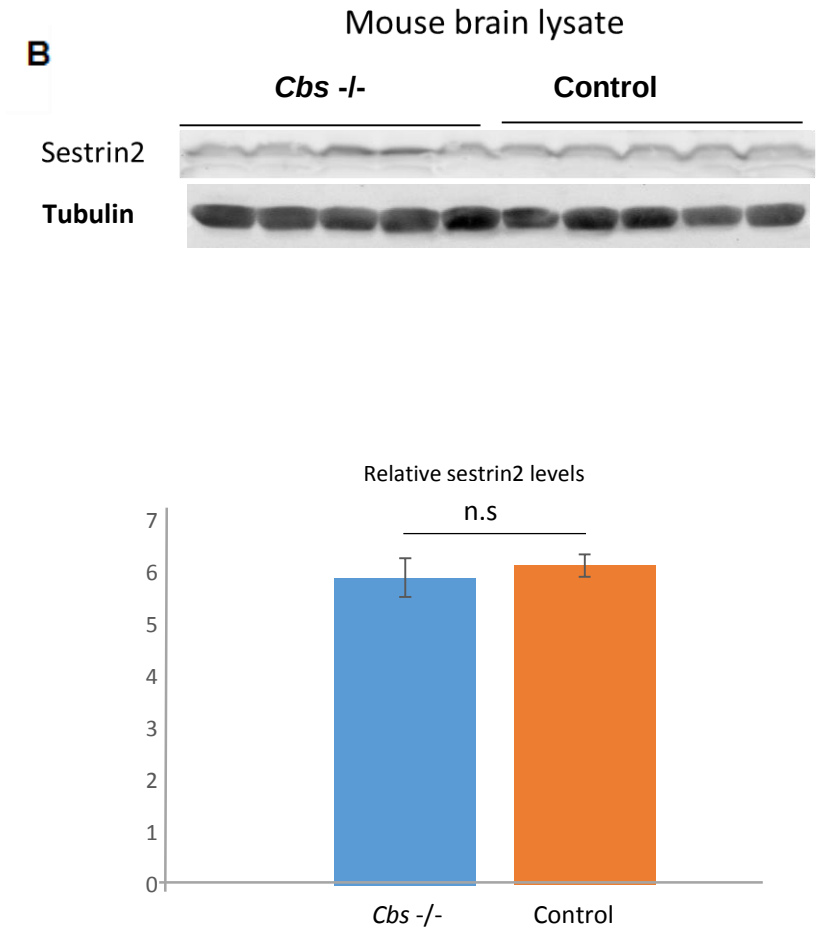



**Figure 3.15-B** Homocysteine does not influence Sestrin2 protein expression levels. *Cbs* knockout brain lysates were assessed for the levels of Sestrin2 protein. Sestrin2 protein levels did not change in hyperhomocysteinemic mice brain samples. Bar graphs represent a quantitative analysis of protein levels assessed by immunoblot analyses. Data are means +/- SEM.

## 3.2 Conclusion

mTORC1 is capable of detecting the availability of amino acids in the cytoplasm through the complex machinery residing on the lysosomal membrane (Settembre et al. 2013). In this chapter, it was shown that mTORC1 is capable of sensing

cellular Hcy. Additionally, we proposed the interaction between LeuRS and Flcn in a constitutive complex to which Hcy bound and induced cellular translocation of the complex; LeuRS and Flcn lost binding to RagC when Hcy was present. This notion lent support to the hypothesis that LeuRS-Flcn complex acts as an alternative machinery which substitutes mTORC1 on the lysosomal membrane in starvation conditions. Further, we showed that Hcy-mediated mTORC1 activity was only specific to Hcy and not to Hcy metabolites in the cell such as Hcy-thiolactone and cysteine. Finally, the protein levels of Sestrin2, a Leu sensor in the cytoplasm did not significantly change in response to Hcy. Overall, in this chapter, we showed that the amino acid metabolite Hcy is a potent inducer of mTORC1 activity and presented a molecular complex that could sense its availability.

# CHAPTER 4

## ACUTE AND CHRONIC HOMOCYSTEINE TREATMENTS INHIBIT AUTOPHAGY INDUCTION THROUGH INACTIVATION OF ULK1 AND TFEB

Autophagy is a cellular "self-eating" process that clears cytosolic protein aggregates and defective organelles and begins by the formation of a double membranous phagophore. In this process, proteins such as LC3 and P62 play role in phagophore maturation and autophagosome formation. Further, autophagosomes fuse with lysosomes to lyse their contents and provide amino acids and energy available to the cells (Glick, Barth, and Macleod 2010). Mechanistic target of rapamycin as a master regulator of nutrient signaling controls autophagy through phosphorylation of TFEB (Settembre et al. 2012; Martina et al. 2012) and ULK1 (Kim et al. 2011). During starvation conditions, mTORC1 is inactive and TFEB is no further phosphorylated; during these conditions, phospho-TFEB moiety is dephosphorylated by calcineurin, a required step for TFEB translocation into the nucleus. Consequently, active TFEB enters the nucleus and transcribes genes encoding autophagosome and lysosomal biogenesis. On the other hand, ULK1 interaction with AMPK is necessary for autophagy initiation which is impinged by mTORC1-mediated phosphorylation of ULK1 at Ser 757 (Kim et al. 2011). Since we showed that Hcy regulates mTORC1 activity, we investigate the consequences of Hcy-mediated mTORC1 activation on the autophagy pathway in acute Hcy-treated or chronic hyperhomocysteinemic cells and mouse tissue.

## 4.1 Results

### 4.1.1 Acute Homocysteine Treatments Impinge Autophagy Induction

Unc-51 like autophagy activating kinase-1 (ULK1) and TFEB are two known autophagy regulators and mTORC1 substrates (Kim et al. 2011; Settembre et al. 2012). We assessed the rate of autophagy induction in short term Hcy-treated cells. Cells co-transfected with LC3-RFP and LAMP1-GFP constructs were treated with Hcy for three hours and imaged searching for LC3-positive organelles. Homocysteine-treated cells showed a reduced number of LC3 punctal staining compared to cells treated with control complete media. Torin, a specific mTOR inhibitor, and potent autophagy inducer, was added to Hcy-containing medium and could reverse the diffused LC3 by increasing the number of LC3-positive organelles which co-localized with LAMP1 (late endosome and lysosomal marker) (Figure 4.1).

Since mTORC1 phosphorylates ULK1 relatively quickly, we analyzed levels of phospho-ULK1 along with a lipidated form of LC3 (LC3II) in western blots. To decide what time point is the best for short-term Hcy treatments, HEK293T cells were treated with the same concentration of Hcy over different periods of time (1h, 2h, 3h, 4h, and 5h). Evaluation of cell lysates on western blot identified three hours of treatment with Hcy as a time point showing a reliable increase of mTORC1 activity *in-vitro*, as was assessed by evaluation of P-p70S6/kinase level relative to protein loading control (Figure 4.2). To assess the true amount of LC3II formation in the autophagosomes prior to its lysosomal fusion and degradation, we performed autophagy flux assays. In these assays, a

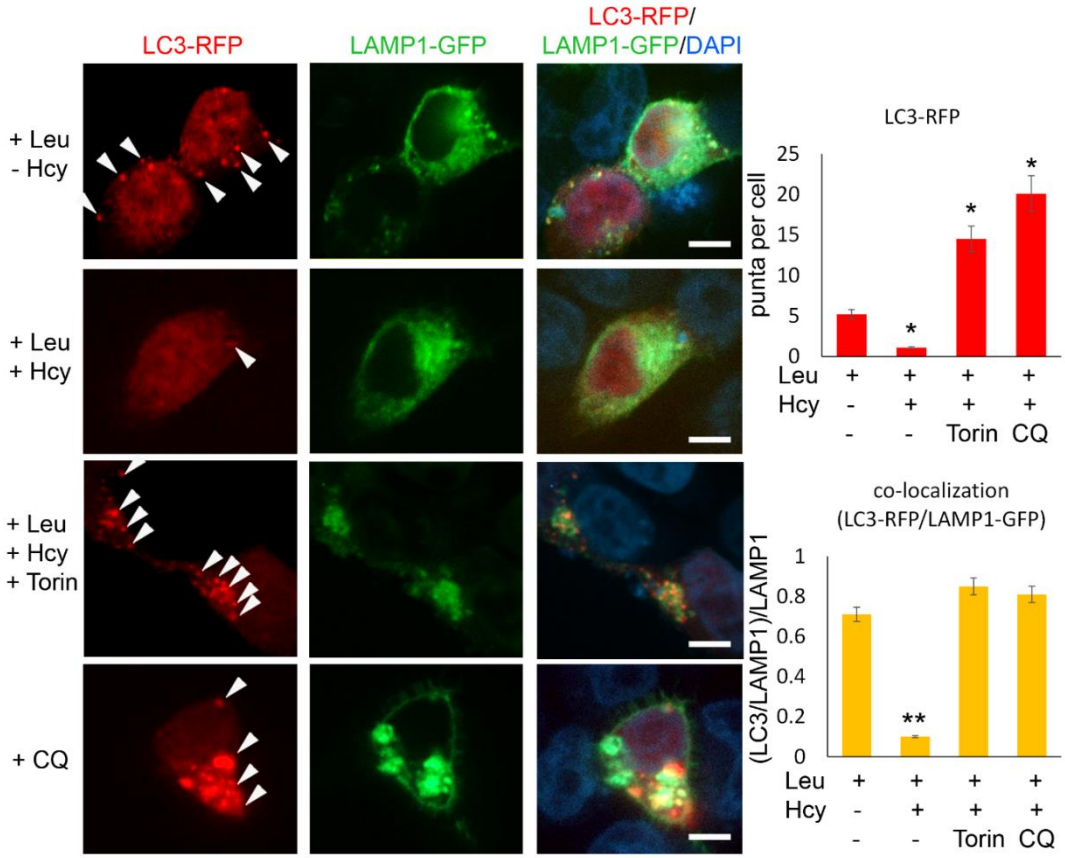



**Figure 4.1** Acute treatments of homocysteine impinges autophagy induction. HEK293T cells were co-transfected with LC3-RFP and LAMP1-GFP constructs and treated with RPMI complete media with or without Hcy. Torin was used as a specific mTOR inhibitor. Chloroquine was utilized as a positive control for malfunctioned lysosomes. DAPI stained nucleus. Homocysteine reduced formation of LC3-positive autophagosomes (white arrowheads), while Torin rescued this phenotype and increased the formation of LC3 puncta (Leu, control: 5.2 +/- 0.6 and Leu+Hcy: 1.1 +/- 0.1; P = 0.05), and (Torin in Hcy-treated cells: 14.5 +/- 1.6; P = 0.04). Bar diagram represents quantitative analyses of LC3-positive organelles per cell (red) and LC3-LAMP1 co-localization divided by the number of LAMP1-positive puncta (yellow). Data are means +/- SEM (n = 3 independent experiments). Scale bars are 10 μm. *P ≤ 0.05 and **P ≤ 0.01.

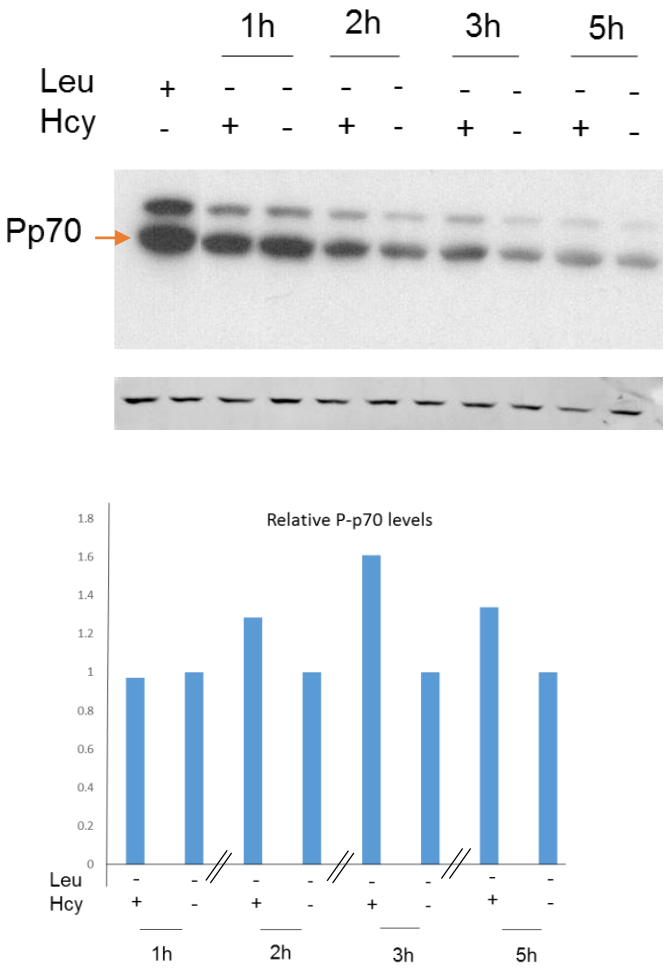



**Figure 4.2** Three hours of homocysteine treatment show the strongest impact on mTORC1 activity. Treatment of HEK293T cells with same concentrations of Hcy at different time points showed that 3 hours of Hcy-treatments had the highest impact on mTORC1 activity which was measured by evaluation of relative P-p70S6kinase protein levels. Bar graphs represent a quantitative analysis of protein levels assessed by immunoblot analyses.

lysosome inhibitor such as Chloroquine was combined with experimental treatments to let us compare the overall amount of LC3II generated by cells and becomes degraded in lysosomes (Mizushima, Yoshimori, and Levine 2010). Interestingly, cells exposed to Hcy for three hours showed considerably decreased levels of LC3II and relatively increased levels of phosphorylated ULK1

on Ser 757 (Figure 4.3). The connection between the two comes from the fact that phosphorylation of ULK1 on Ser 317 & Ser 777 by AMPK is necessary for induction of autophagy whereas ULK1 phosphorylation on Ser 757 inhibits autophagy induction, LC3I lipidation, and conversion to LC3II (Kim et al. 2011).

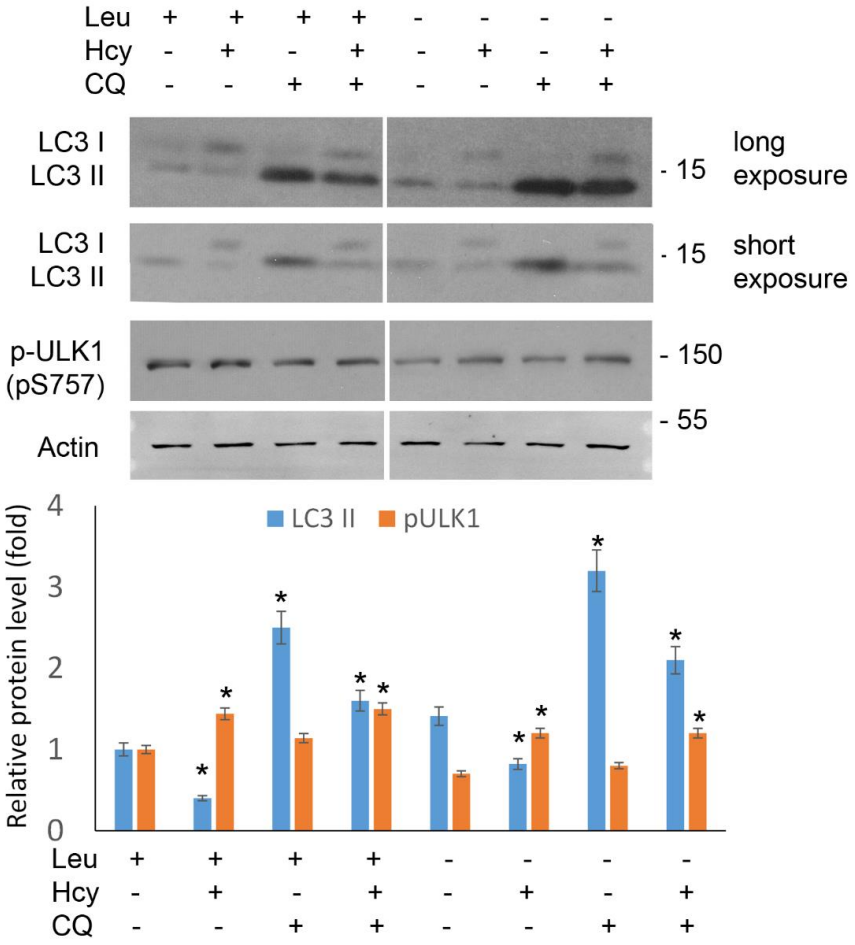



**Figure 4.3** Acute homocysteine treatments inhibit autophagy induction by increasing the level of phospho-ULK1. Human neuroprogenitor cells were treated with Hcy, Leu (for 3 hours) and Chloroquine (for 1 hour). Homocysteine clearly reduced the formation of LC3II (0.4 +/- 0.03-fold; P = 0.04) which was in parallel with an increase in the formation of P-ULK1 (1.5 +/- 0.03-fold; P = 0.05) which was indicative of less autophagy induction in the cells. Chloroquine treated cells showed a thicker LC3II band in absence of Hcy compare to its presence. Bar graphs represent a quantitative analysis of protein levels assessed by immunoblot analyses. Data are means +/- SEM (n = 3 independent experiments). *P ≤ 0.05.

An alternative approach to validate the hypothesis of autophagy inhibition by Hcy was to employ ptf-LC3 constructs, a tandem-fluorescent LC3 sensor as established by Kimura et al (Kimura, Noda, and Yoshimori 2007). ptf-LC3 has been constructed as a fusion protein of LC3, mRFP, and GFP, as such both red and green fluorescents are monitored in neutral pH environment whereas GFP is quenched in acidic organelles and only red is visible. Thus, in this experiment red organelles in merge channel are representative of functional lysosomes. Human neuroprogenitor cells were transfected with the construct and treated with RPMI complete and starvation media with or without Hcy and Chloroquine. Media containing Hcy reduced number of both red and green LC3-positive organelles which were indicative of a decline in both LC3 lipidation and autophagosomal maturation. Chloroquine which interferes with lysosomal acidification was used as a control for this experiment (Figure 4.4).

### 4.1.2 Chronic Homocysteine Treatments Interfere with Autophagy Induction through Modulation of TFEB-Regulated Gene Expression

Autophagy is known to be regulated by mTORC1 also through translation modulation of the TFEB-regulated genes (Martina et al. 2012). We took different approaches to test if TFEB is affected by the Hcy-driven mTORC1 activity. We performed autophagy flux assays to monitor LC3I and II protein levels in prolonged treatments with Hcy (16-24 h). Both LC3I and II significantly decreased in Hcy-treated cells as compared to cells treated with control media. The combination of treatments with Chloroquine depicted the reduced formation

of LC3II compared to Chloroquine treatments alone which were indicative of a reduction in autophagy induction (Figure 4.5). To analyze further the impact of long-term Hcy treatment on cells, we tested the protein levels of TFEB-target genes in wild type and *Cbs* deficient mouse brain lysates. Analyses showed reduced protein levels of both LC3I and p62 in mutant versus normal mice (Figure 4.6). Both of these experiments delineated the Hcy possible impacts on TFEB activation which are further assessed in this chapter.

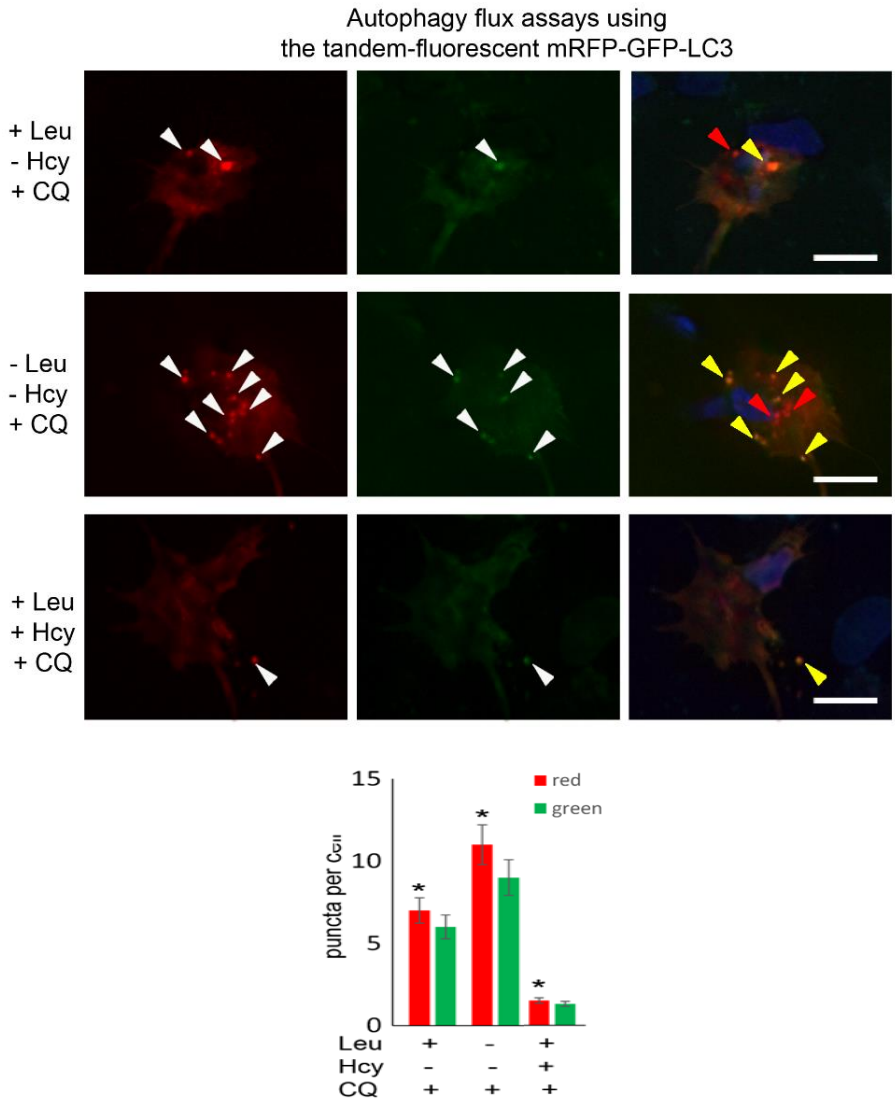


**Figure 4.4** Homocysteine interferes with autophagy induction. Human neuroprogenitor cells were transfected with tandem-fluorescent mRFP-GFP-LC3 constructs and treated with RPMI complete and starvation media with or without Hcy. DAPI stained nucleus. Red arrowheads showed acidified autophagosomes while yellow ones were indicative of immature autophagosomes; both red and yellow puncta were reduced in presence of Hcy in the media which was indicative of inhibition of autophagy induction and maturation of autophagosomes (Leu (red): 3.1 +/- 0.3, Leu (green): 1.1 +/- 0.1; Hcy (red): 1.4 +/- 0.1, Hcy (green): 1.3 6 0.1; P = 0.04). Bar diagram is quantitative analyses of LC3 red and green signals per cell. Data are means +/- SEM (n = 3 independent experiments). Scale bars are 10 µm. *P ≤ 0.05.

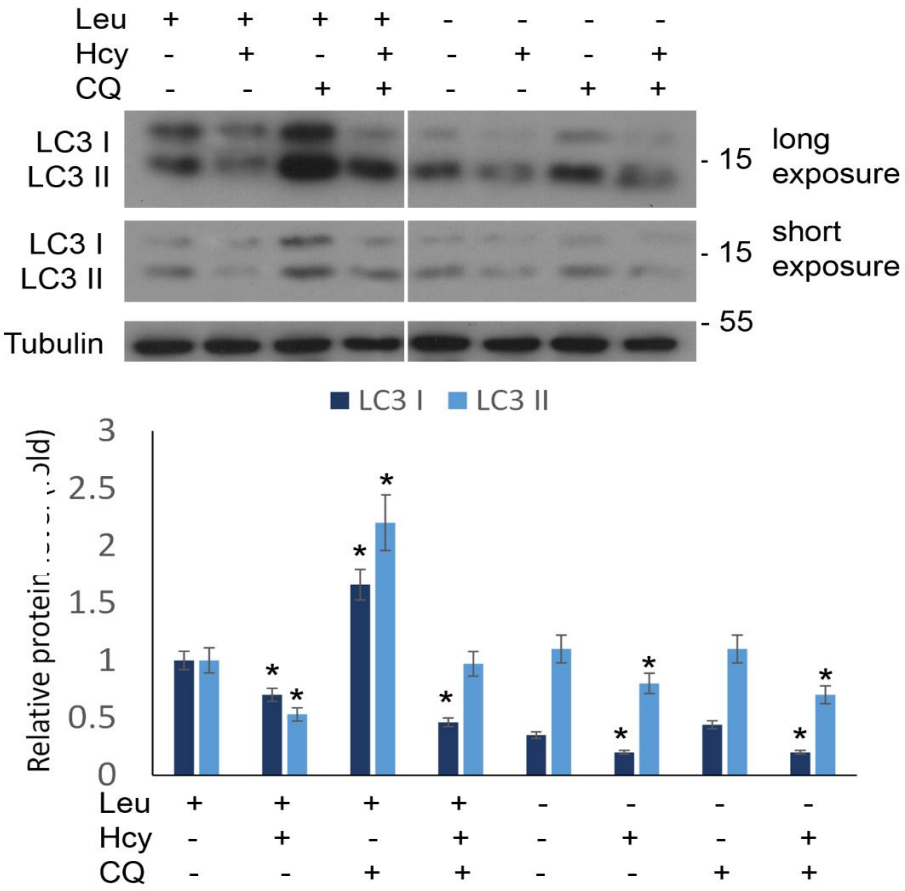



**Figure 4.5** Autophagy is inhibited by homocysteine, chronically. Human neuroprogenitor cells were treated with Leu, low concentration of Hcy (overnight) and Chloroquine (for 1 hour). Homocysteine clearly reduced the formation of LC3I (Leu/Hcy/CQ: 0.46 +/- 0.01-fold vs. Leu/CQ: 1.7 +/- 0.2-fold; P = 0.03) and LC3II (Leu/Hcy/CQ: 0.9 +/- 0.1-fold vs. Leu/CQ: 2.2 +/- 0.2-fold; P = 0.04) which were indicative of inhibition in autophagy induction and flux over longer period of Hcy-treatments. Chloroquine treated cells showed accumulation of LC3II in absence of Hcy. Bar graphs represent a quantitative analysis of protein levels assessed by immunoblot analyses. Data are means +/- SEM (n = 3 independent experiments). *P ≤ 0.05.

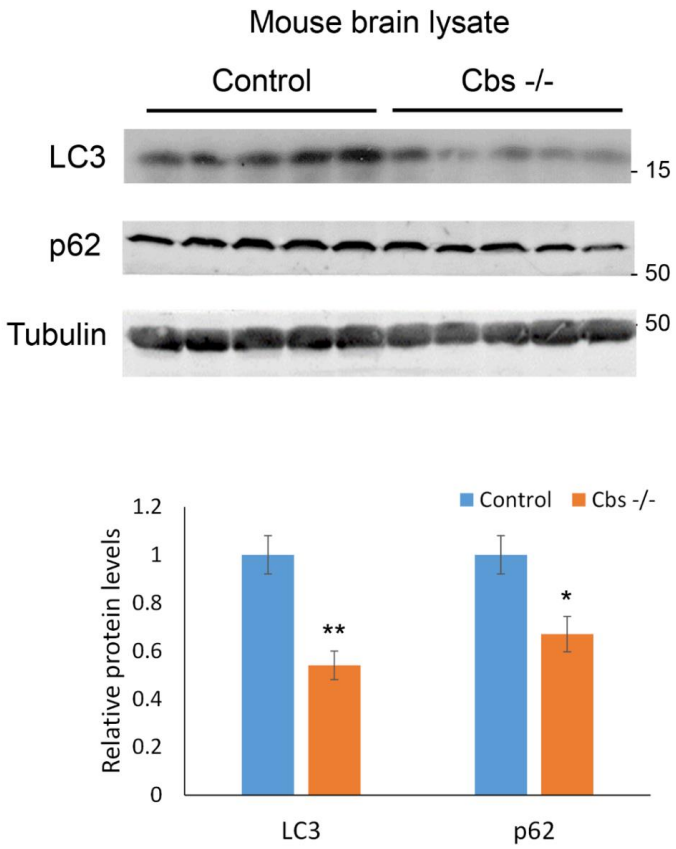



**Figure 4.6** TFEB-target gene protein levels decreased in $Cbs^{-/-}$ mouse samples. Brain lysates of *Cbs* deficient and wild type mice were subjected to western blot analyses. TFEB target genes (P62 and LC3) decreased in $Cbs^{-/-}$ mice samples compare to control ones (LC3, 0.5 +/- 0.06, P = 0.01; p62, 0.6 +/- 0.07 vs. 1.0 +/- 0.01 in controls, P = 0.04). Bar graphs represent quantitative analysis of protein levels assessed by immunoblot analyses. Data are means +/- SEM (n = 4 independent experiments). *P ≤ 0.05 and **P ≤ 0.01.

### 4.1.3 Transcription Factor EB Activity is Impaired in Homocysteine-Treated Cells

As a transcription factor, TFEB functions in the nucleus; mTORC1 kinase activity inhibits TFEB activation by maintaining TFEB in the cytoplasm far from its target genes (Martina et al. 2012). To assess TFEB activity we used four times CLEAR

element upstream of the Firefly-luciferase gene to which promoter TFEB bound and drove transcription of the CLEAR gene network and luciferase, concomitantly. Human neuroprogenitor cells were transfected with the 4xCLEAR luciferase reporter construct and treated with ascending concentrations of Leu or Hcy overnight, followed by measurement of luciferase activity, representing CLEAR network activity. Data revealed a reduction in the network activity coordinated with increased concentrations of Leu and Hcy (Figure 4.7 A). Leucine showed stronger inhibitory effects on TFEB activity since the activity was attenuated more potently with Leu compared to same concentrations of Hcy (Figure 4.7 B). We also tried to remove Leu and Hcy after overnight treatments to see how fast TFEB activity was rescued. Our data showed that Leu removal retained TFEB activity faster compared to Hcy (Figure 4.7 C).

### 4.1.4 Transcription Factor EB Localizes in the Cytoplasm of Cells Treated with Homocysteine

Given that our data showed a reduction in TFEB activity of cells exposed to Hcy, we tested the localization of this transcription factor in Hcy-treated cells and hyperhomocysteinemic mice brains. Mouse neuroblastoma cells stably expressed TFEB-Flag were treated with Hcy and stained with Flag antibody. Transcription factor EB is known to shuttle back and forth from cytoplasm to the nucleus upon presence and absence of amino acids, respectively (Settembre et al. 2012); we observed a similar TFEB translocation in presence and absence of Leu. Interestingly, TFEB localized to the cytoplasm of cells starved for Leu but

treated with Hcy, while leucinol combination translocated TFEB back to the nucleus (Figure 4.8).

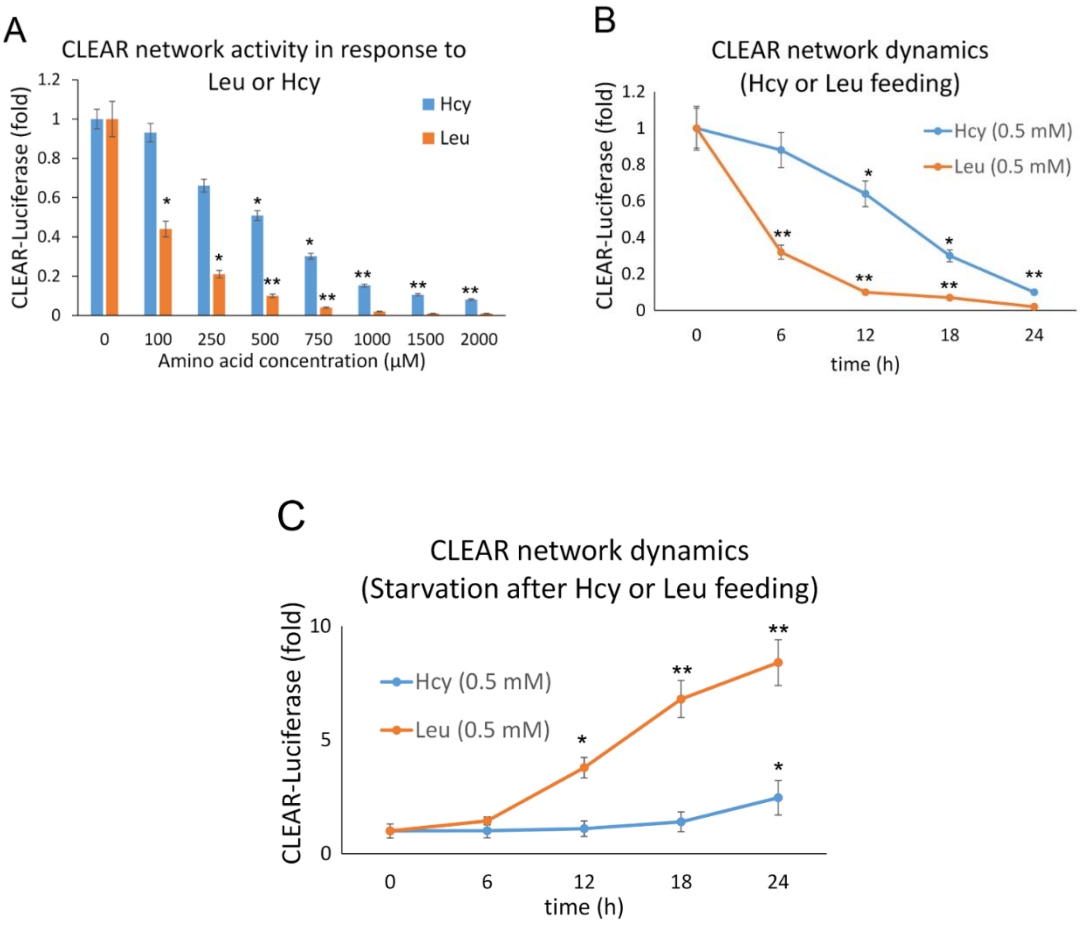



**Figure 4.7- A, B and C** CLEAR network activity is attenuated in response to homocysteine treatments. A) TFEB-driven CLEAR network activity was measured following transfection of human progenitor neurons with CLEAR-luciferase construct and treatment with Leu or Hcy overnight. Data revealed a reduction in the network activity coordinated with increased concentrations of Leu and Hcy. It also showed that Leu had a stronger effect on inhibition of CLEAR activity compared to Hcy. B) Equal concentrations of Leu and Hcy were used to measure their inhibitory effect on CLEAR network activity over time. Data showed that Leu inhibited the CLEAR network activity faster than Hcy. C) After feeding the cells with Leu or Hcy overnight, cells were washed off Leu and Hcy followed by CLEAR activity measurement. Data indicated that Hcy potently inhibited CLEAR network activity compared to Leu since Leu deprivation showed a faster increase in the activity of the CLEAR network. Data are means +/- SEM (n = 4 independent experiments). *P ≤ 0.05 and **P ≤ 0.01.

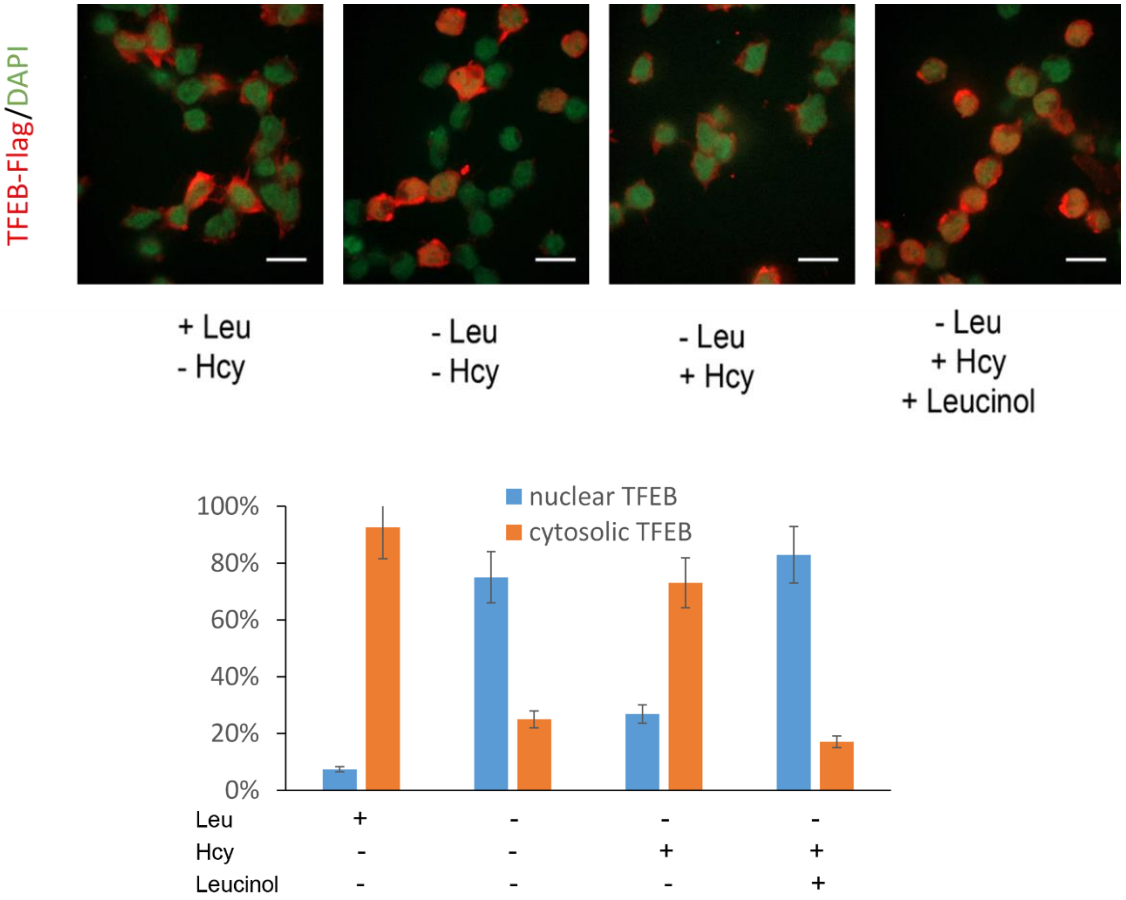



**Figure 4.8** Homocysteine drives TFEB out of the nucleus in cultured cell. Mouse neuroblastoma (CAD) cells stably expressed TFEB were treated with RPMI complete and starvation media with or without Hcy. While in absence of Leu, TFEB enters the nucleus, Hcy was able to draw TFEB back to the cytoplasm. Leucinol treatments could rescue the phenotype and translocated TFEB to the nucleus since leucinol inhibited Hcy effect on mTORC1 activity. Bar diagram shows the statistical evaluation of nuclear versus cytoplasmic localization of TFEB in the cells. Scale bars are 20 µm.

Hippocampal sections of $Cbs^{-/-}$ mice (in particular, dentate gyrus regions) were stained with TFEB and LAMP2 antibodies and compared with wild type. Most of the TFEB was observed in the cytoplasm of $Cbs^{-/-}$ mice which was

exposed to high Hcy levels in the body whereas in wild type mice TFEB localized largely in the nucleus (Figure 4.9).

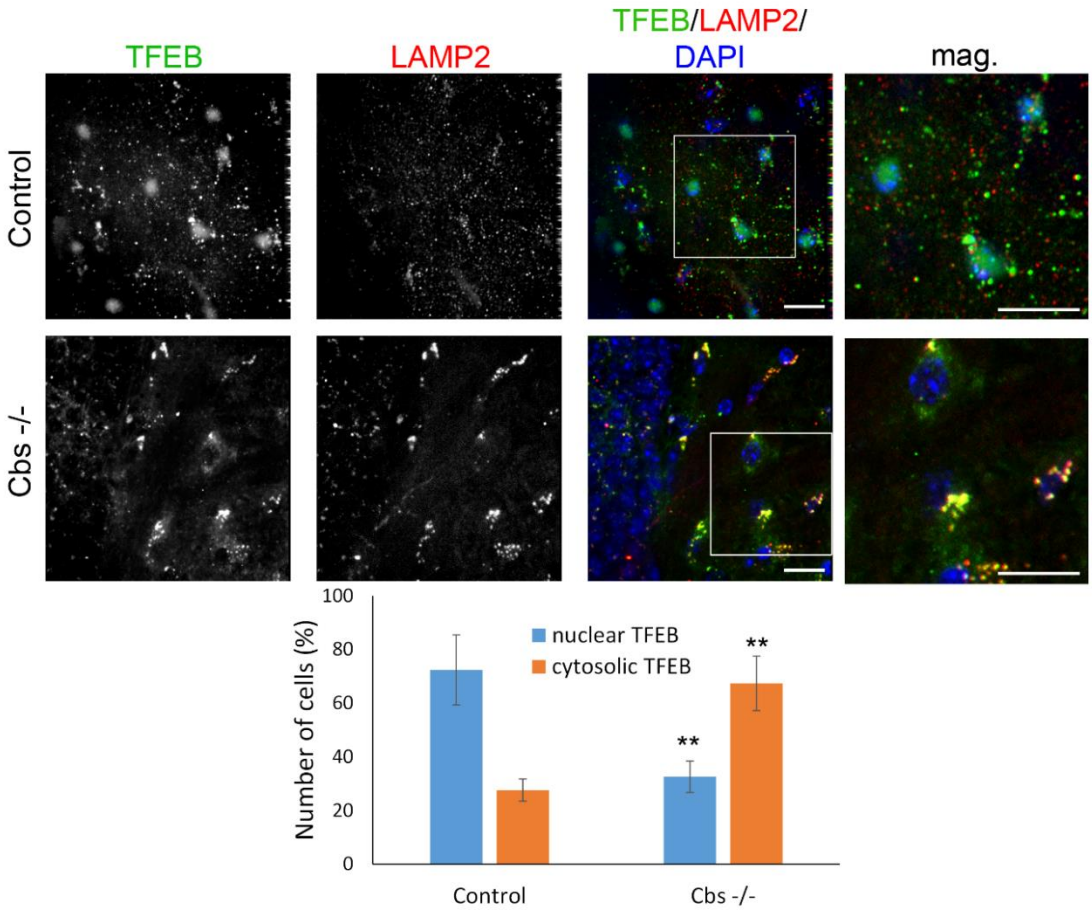



**Figure 4.9** Homocysteine inhibits TFEB translocation to the nucleus. $Cbs^{-/-}$ versus wild type mice hippocampal neurons were applied to immunofluorescent staining. TFEB and LAMP2 antibodies were used as TFEB and lysosomal markers, respectively. TFEB was mostly nuclear in control neurons (72.4 +/- 13 vs. 33 +/- 6% in $Cbs^{-/-}$; P < 0.01), while was cytoplasmic in *Cbs* deficient ones (67 +/- 10 vs. 28 6 4% in controls; P < 0.01). Bar diagram shows statistical evaluation of nuclear versus cytosolic localization of TFEB in mouse hippocampal neurons. Scale bars are 20 μm. Data are means +/- SEM (n = 5 mice per group). **P ≤ 0.01.

### 4.1.5 Leucinol Inhibits Homocysteine-Mediated mTORC1 Activity, Driving Higher Rate of Autophagy Flux

Leucinol is a modified form of Leu with high affinity to bind to LeuRS. It is able to inhibit Leu binding to LeuRS and therefore inhibit Leu-mediated activation of mTORC1 (Han et al. 2012). Here, we show that leucinol is a potent inhibitor of Hcy-mediated effects on amino acid-mTORC1 signaling pathway. Of note, leucinol relocalized Flcn to the lysosomal membranes (Figure 3.14 A and B) and translocated TFEB to the nucleus as a possible consequence of mTORC1 inhibition (Figure 4.8). We did further investigation to follow what leucinol implicated in autophagy pathway. As expected, leucinol inhibited mTORC1 activity in presence of Hcy and induced autophagy which was detectable as a decrease in LC3I and increase in LC3II formation (Figure 4.10). However, in order to distinguish if the LC3II increase was due to lysosomal inhibition or high conversion rate from LC3I to LC3II, we included Chloroquine to inhibit lysosomal function and assessed the difference in LC3II formation. Data showed that LC3II accumulation in leucinol treatments combined with Chloroquine was higher compared to leucinol-treatment alone (Figure 4.11), which implicated that leucinol did not affect lysosomal function but increased autophagy flux.

Furthermore, we observed a lower rate of proliferation in leucinol-treated cells compared to cells treated with nutrient-rich media without leucinol (Figure 4.12 A). This effect could be due to inhibition of mTORC1 activity, although it needs further investigation to assess if leucinol interferes with cell proliferation. In order to exclude the possibility of mTORC2 inhibition by leucinol, the phosphorylated levels of AKT (S473) were assessed in cells treated with or

without leucinol. Data indicated no significant changes in protein levels of P-AKT (S473) (Figure 4.12 B).

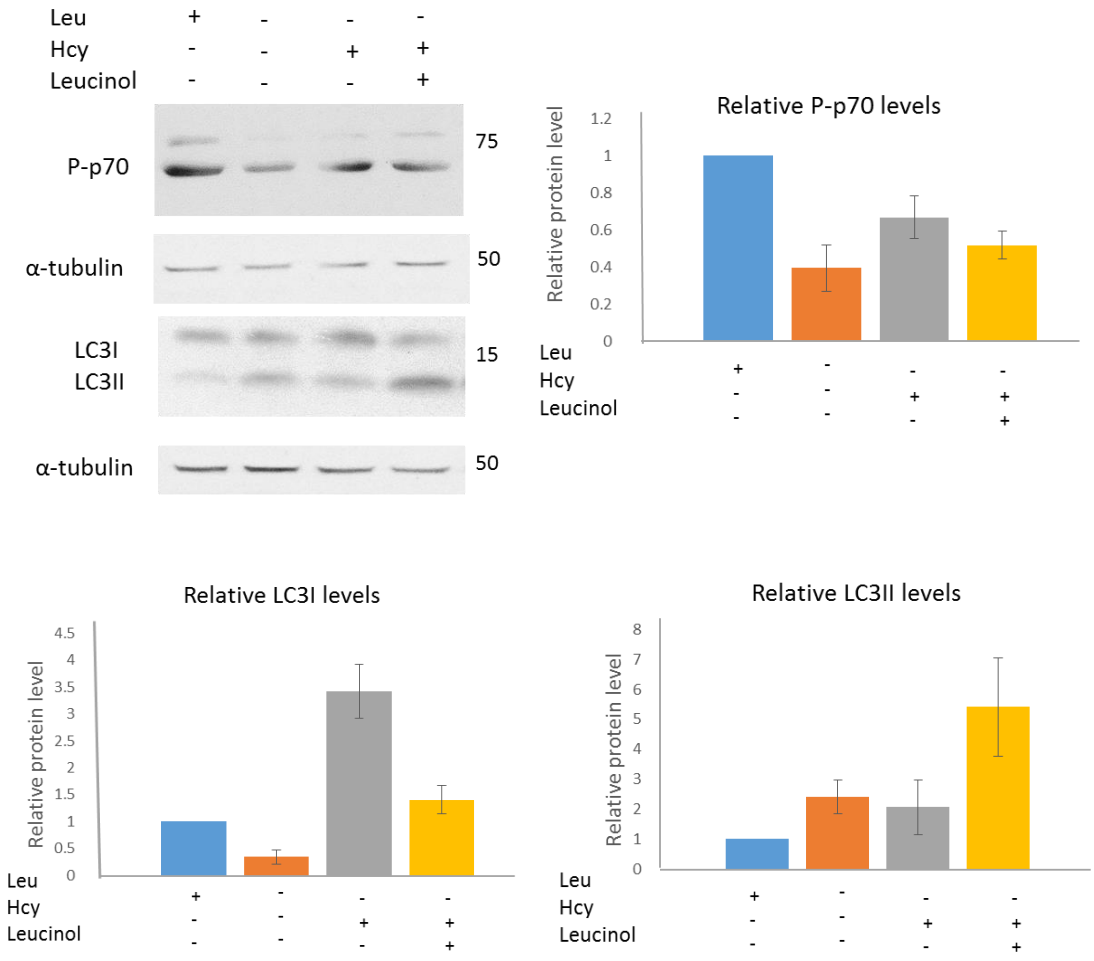



**Figure 4.10** Leucinol inhibits homocysteine-driven mTORC1 activity. HEK293T cells were treated with RPMI complete and starvation media with or without Hcy and leucinol. Leucinol inhibited mTORC1 activity in presence of Hcy as indicated by decreased level of phospho-P70/S6Kinase. This leucinol-related mTORC1 inhibition was accompanied by an increase in LC3II protein levels. Bar graphs represent a quantitative analysis of protein levels assessed by immunoblot analyses. Data are means +/- SEM (n = 3 independent experiments).

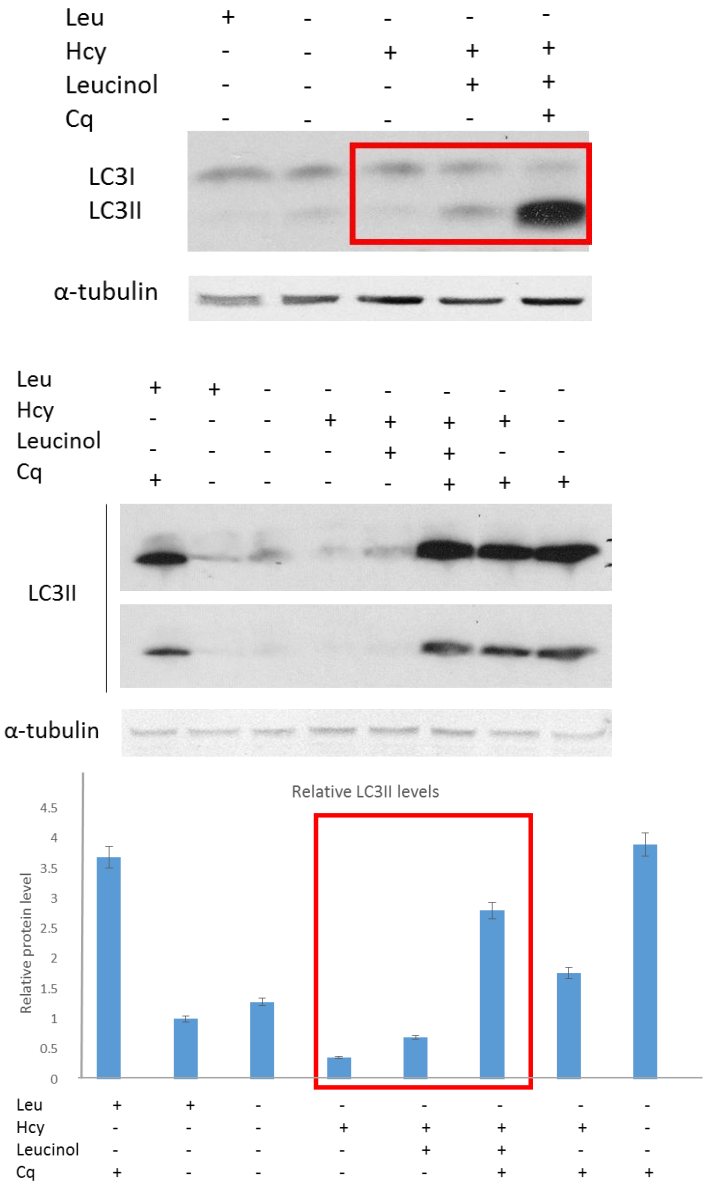



**Figure 4.11** Leucinol increases autophagy flux. HEK293T cells were treated with RPMI complete and starvation media with or without Hcy/leucinol and Chloroquine. Chloroquine and leucinol co-treatments increased LC3II formation compared to leucinol treatment alone which represented leucinol as a component which increased autophagy induction and flux but had no lysosomal inhibitory effects. Bar graphs represent a quantitative analysis of protein levels assessed by immunoblot analyses. Data are means +/- SEM (n = 3 independent experiments).

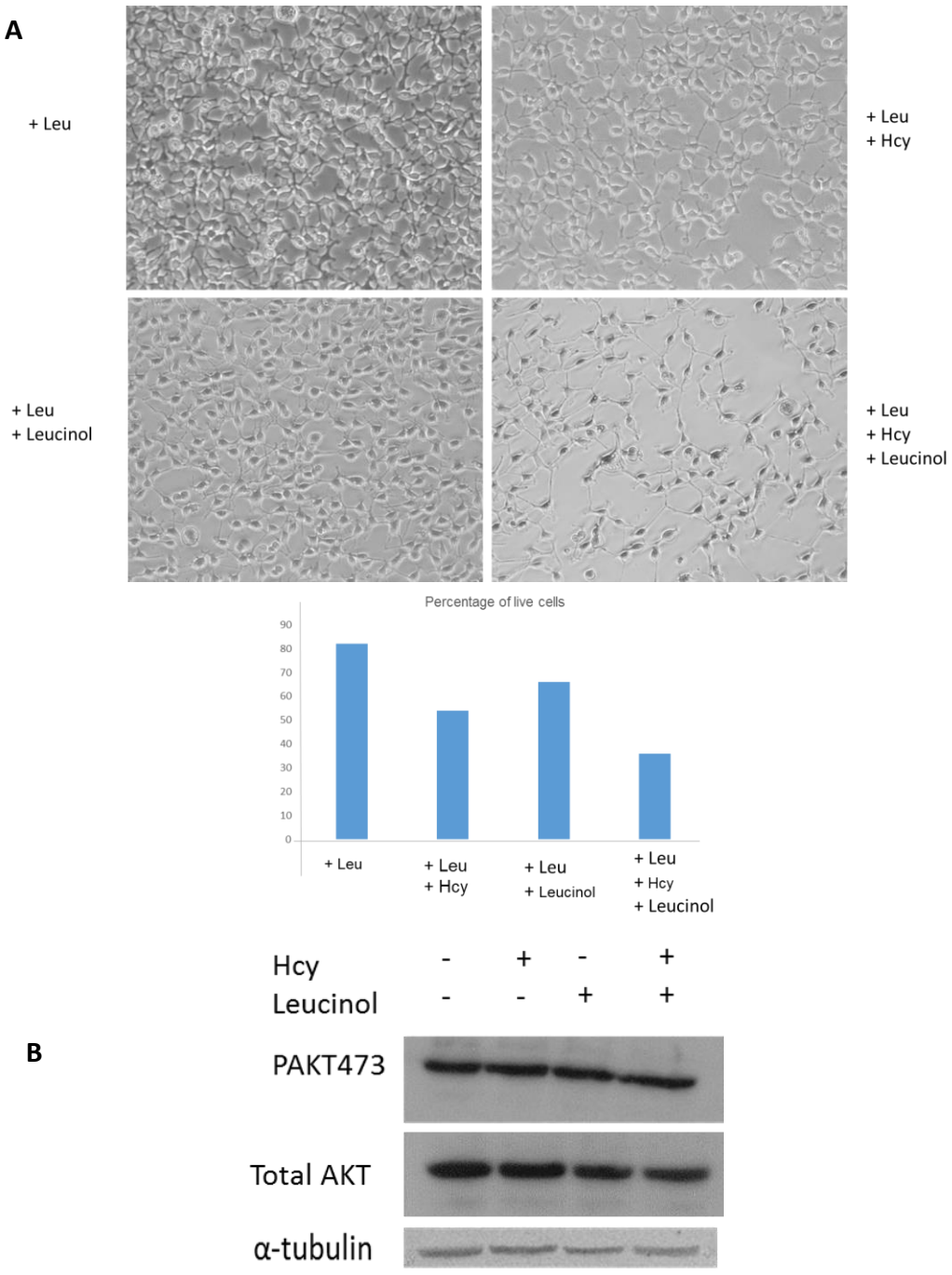



**Figure 4.12- A and B** Leucinol reduces cell proliferation without affecting mTORC2. A) HEK293T cells treated with leucinol showed a reduced rate of cell proliferation. Bar diagram represents the number of live cells in each treatment. B) P-AKT473 was assessed to measure mTORC2 activation upon leucinol treatments. No significant changes were observed between our experimental groups.

## 4.2 Conclusion

Mechanistic target of rapamycin is known to modulate autophagy as autophagy modulators have been found to be the target of mTORC1 kinase activity (Kim et al. 2011; Martina et al. 2012). Here we demonstrated that Hcy-positive impact on mTORC1 activity impinges autophagy through phosphorylation of the two major autophagy-driving factors: ULK1 and TFEB. Hyperactive mTORC1 phosphorylated ULK1 and consequently reduced LC3 lipidation. It also dampened TFEB-driven transcription of autophagy-related genes. We also showed leucinol to be a potent inhibitor of the negative Hcy-mediated effects on autophagy, thereby confirming that Hcy modulates mTORC1-TFEB pathway through LeuRS.

# CHAPTER 5

## HOMOCYSTEINE IS NEUROTOXIC BY PROMOTING FORMATION OF ABNORMAL PROTEINS

Since high levels of Hcy have been detected in sporadic AD patients (Zhuo, Wang, and Pratico 2011; Li, Chu, and Barrero 2014; Kamat et al. 2015; Coppedè 2010) a need arose to elucidate the molecular mechanism behind the correlation between high plasma Hcy concentration and disease progression, which correlates with aging (Levine et al. 2008; Tucker et al. 2005). Notably, AD hallmarks including memory loss, the formation of the amyloid deposits, and phosphorylated tau neurofibrillary tangles, have been correlated with HHcy (Li et al. 2014), identifying Hcy particularly as a risk factor for AD (Morris 2003; Pacheco-quinto et al. 2006). The reason for the notion that Hcy is a risk factor for AD and not the disease marker, arises from the epidemiological studies on AD patients which demonstrated high concentration levels of Hcy in patients' plasma prior to the development of dementia (Seshadri, 2006). Extensive research to elucidate pathways by which Hcy affects neurons is being carried out; some of which show that Hcy activates N-methyl-D-aspartate (NMDA) receptors (Lipton et al. 1997), oxidative stress (Perna, Ingrosso, and Santo 2003) and modification of DNA methylation (Fuso et al. 2005), all of which being detrimental contributions of Hcy to the brain. Additionally, Li et al. have proposed two independent pathways through which Hcy contributes to exacerbation of AD phenotype: activation of γ-secretase and CDK5 pathways which lead to the formation of β-amyloid deposition and accumulation of tau insoluble form, respectively (Li et al.

2014). Yet, there is no evidence to show whether Hcy-mediated mTORC1 activation and associated inhibition of autophagy lead to the formation of these specific protein deposits.

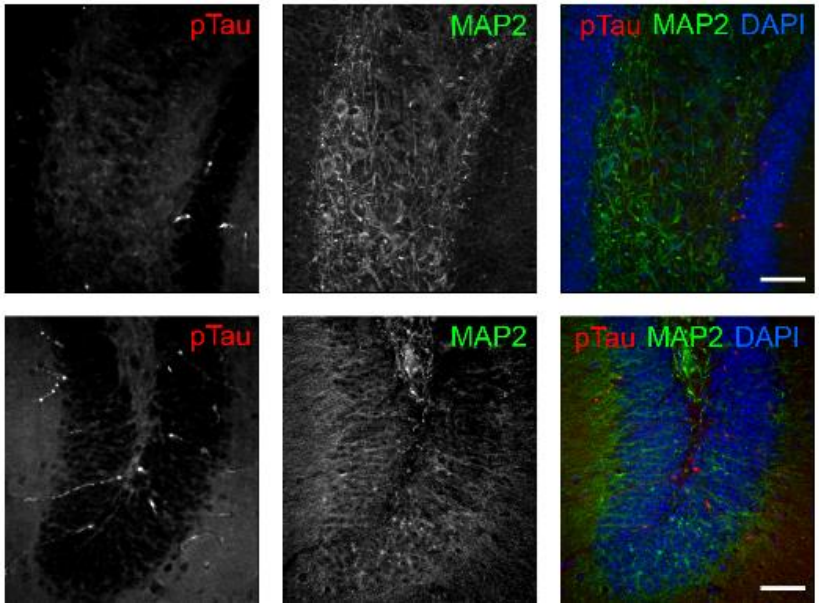
## 5.1 Results

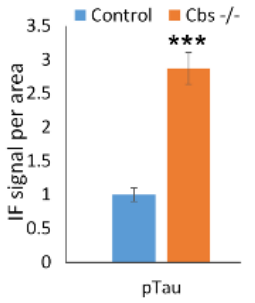
### 5.1.1 Homocysteine Induces the Formation of Abnormal Proteins Associated with Alzheimer's Disease

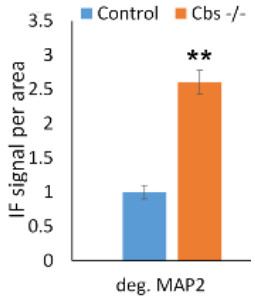
To identify future drug targets, it is crucial to elucidate the mechanism of Hcy action in AD pathophysiology, including the formation of β-amyloid and hyperphosphorylated Tau (Li et al. 2014). Hippocampal sections of $Cbs^{-/-}$ mice were assessed for paired helical filaments (PHF) of phospho-Tau which showed a significant increase compared to wild type littermates (Figure 5.1 A, B and C). Increase in phospho-Tau was associated with aggregated microtubule-associated protein 2 (MAP2) in $Cbs^{-/-}$ mice (Figure 5.1 A). Interestingly, phospho-Tau tangles partially co-localized with P62-positive autophagosomes indicating that the recognition of PHF phospho-Tau took place in $Cbs^{-/-}$ brains (Figure 5.1 B). Next interesting feature observed for phospho-Tau, was the morphology as lined up along blood vessels in normal mice, while phospho-Tau spread out in *Cbs* deficient mice brain sections, which might be another implication for abnormal clearance in $Cbs^{-/-}$ mice (Figure 5.1 C). Similarly, overall amount of intracellular β-amyloid increased by ≈2.24 fold in knockout mice as compared to wild type (Figure 5.1 C).

A

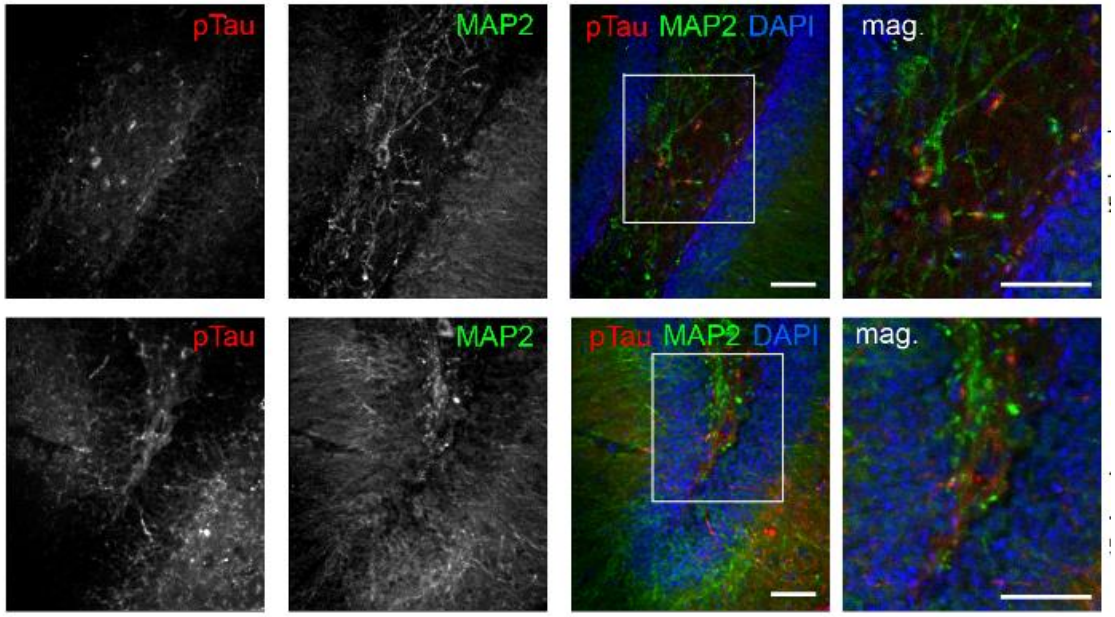
Control




Cbs -/-



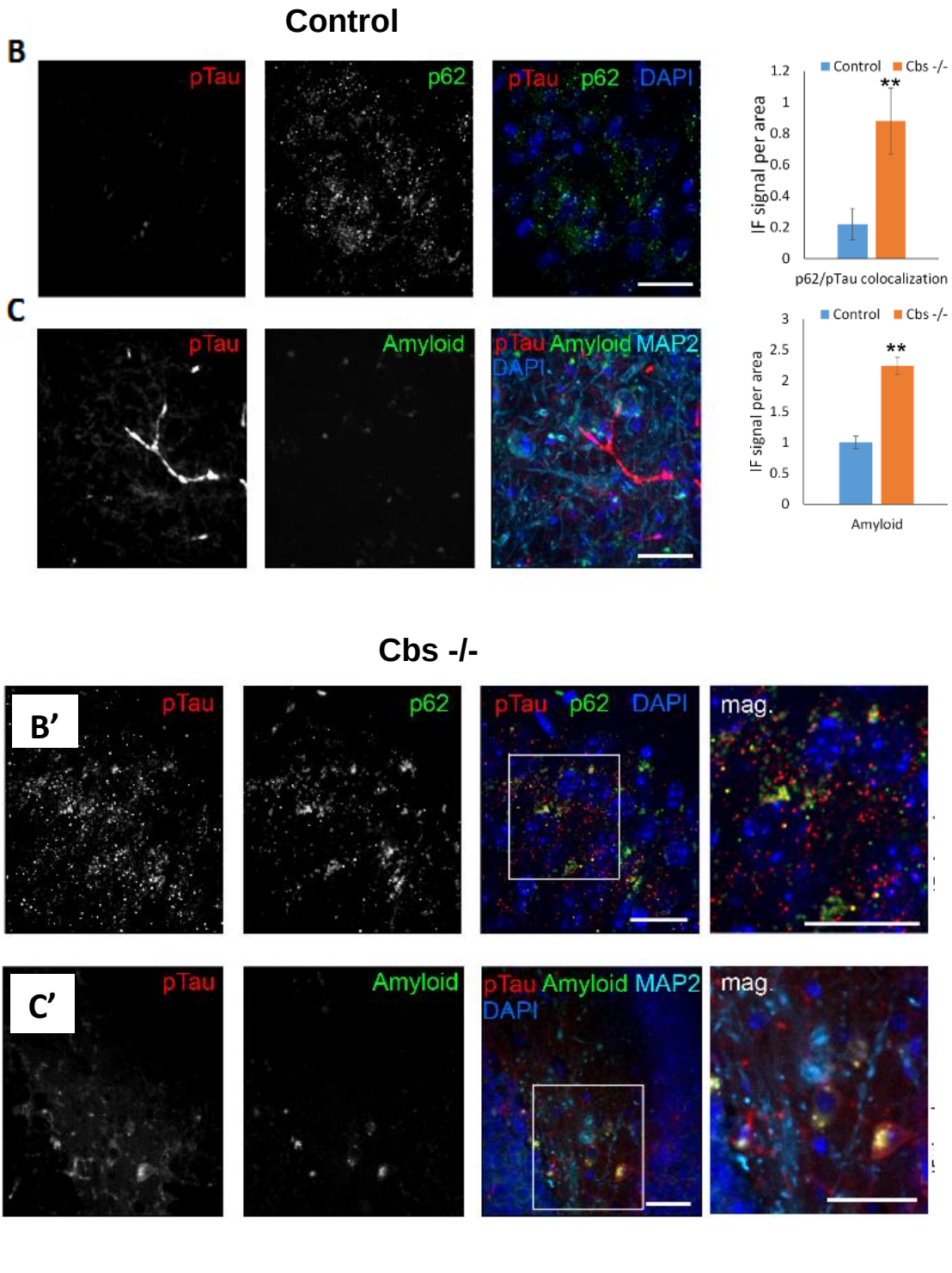

Control
B
pTau
p62
pTau p62 DAPI
C
pTau
Amyloid
pTau Amyloid MAP2 DAPI
Control Cbs -/-
IF signal per area
p62/pTau colocalization
Amyloid
Cbs -/-
B'
pTau
p62
pTau p62 DAPI
mag.
C'
pTau
Amyloid
pTau Amyloid MAP2 DAPI
mag.

**Figure 5.1- A, B and C** *Cbs* knockout mouse brains show accumulation of abnormal proteins. A) Paired helical filament phospho-Tau levels increased in hippocampal areas of $Cbs^{-/-}$ mice. Microtubule-associated protein 2 (MAP2) looked punctated and indicated with fewer number of neurons in $Cbs^{-/-}$ samples (pTau: 2.8 +/- 0.2-fold, P < 0.005; degen. MAP2: 2.6 +/- 0.2-fold; P < 0.01). B) pTau and P62 (autophagosomal marker) co-localized in *Cbs* mutated samples while overall pTau protein level increased in $Cbs^{-/-}$ compare to control sections (p62/pTau colocalization: 0.88 +/- 0.2 in $Cbs^{-/-}$ vs. 0.22 +/- 0.1 in controls; P = 0.001). C) *Cbs* knockout hippocampi showed increased levels of β-amyloid as compared to littermate controls (2.24 +/- 0.15-fold; P < 0.01). Bar diagrams show statistical evaluation of immunostained sections. Data are means +/- SEM (n = 5 mice per group). **P ≤ 0.01, and ***P ≤ 0.005.

### 5.1.2 The Homocysteine-Driven Increase in the Level of β-Amyloid is Reversible by Inhibition of mTORC1 Activity or Induction of Autophagy

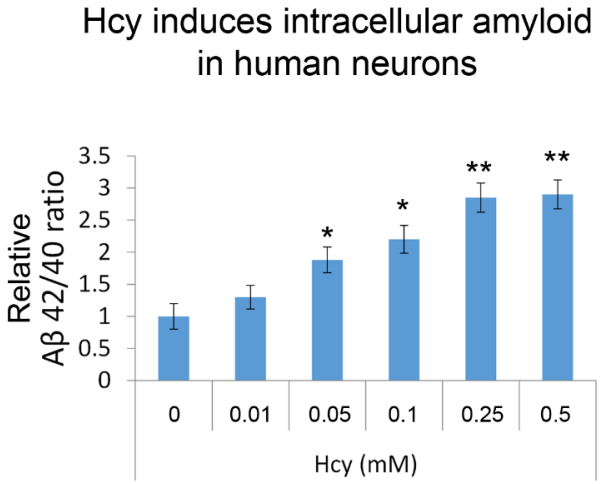
Ho et al. previously showed that Hcy exacerbates β-amyloid neurotoxicity in neurodegenerative diseases such as AD by increasing β-amyloid-mediated calcium levels in the cytoplasm and induction of apoptosis (Ho et al. 2001). Later, a link between Hcy and β-amyloid production was made through modification of γ-secretase activity, one of the enzymes responsible for cleavage of amyloid precursor protein in process of β-amyloid production (Li et al. 2014). Further studies revealed that AD phenotypes are reversible by inhibition of mTORC1 with Rapamycin in mouse models of AD (Spilman et al. 2010). Here, we show that Hcy enhances β-amyloid protein level through induction of mTORC1 activity and inhibition of autophagy. The role of autophagy in the prevention of neurodegeneration has been demonstrated before, as autophagy-deficient mice showed behavioral abnormality due to loss of neurons in their cerebral and cerebellar cortices (Komatsu et al. 2006). Employing ELISA assays to detect β-amyloid 42 versus 40 levels in cultured human neurons which were treated with

ascending concentrations of Hcy, we detected an increase in β-amyloid 42/40 levels proportional to Hcy concentrations (Figure 5.2 A). Homocysteine-treated human neurons were exposed to the mTORC1 inhibitor Rapamycin (Sabers et al. 1995) or the autophagy-inducing peptide TAT-Beclin1 (Shoji-Kawata et al. 2013) to assess the role of mTORC1 activity and autophagy in amyloid formation and survival, respectively. Our data indicated a prominent role of mTORC1 and autophagy in the regulation of β-amyloid levels; as mTORC1 inhibition or autophagy induction, both reduced the levels of β-amyloid42/40 in our quantitative analyses (Figure 5.2 B).

### 5.1.3 Homocysteine is Detrimental to Human Neurons

Autophagy has been identified to be necessary for the maintenance of neuronal homeostasis, as inhibition of autophagy led to neurodegeneration in Atg7 deficient mice (Komatsu et al. 2006). Given that activation of mTORC1 attenuates autophagy induction, we investigated the role of Hcy-mediated mTORC1 activity on the viability of human neurons. The viability of the cells was measured by employing viability assays detecting levels of cellular ATP. The viability of cultured human neurons exposed to Hcy dramatically decreased during the fourth days of treatment (Figure 5.3 A), while Rapamycin and TAT-Beclin1 rescued the neurons (Figure 5.3 B).

**A**




**B**

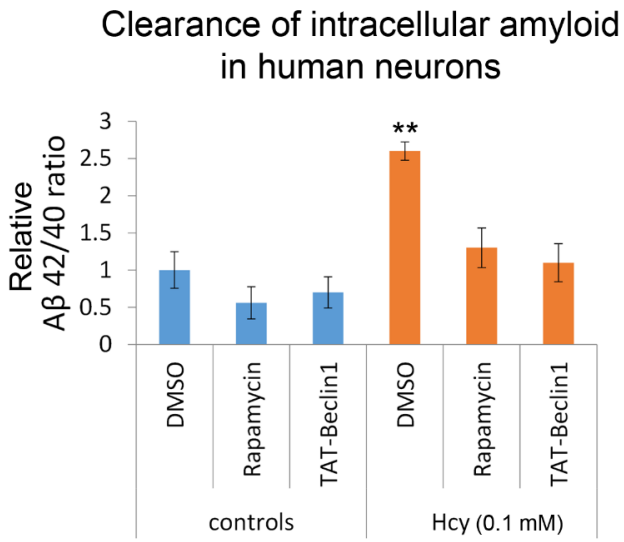
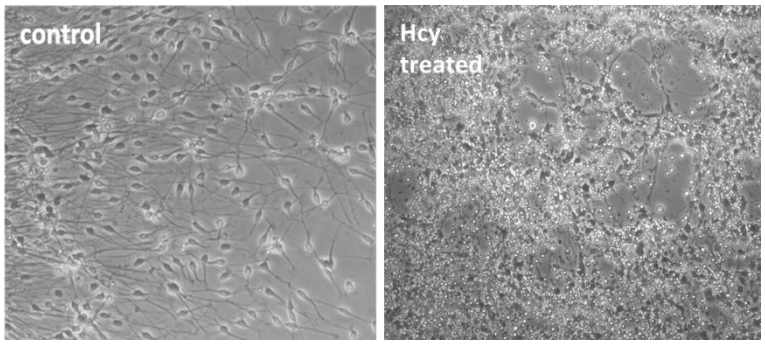
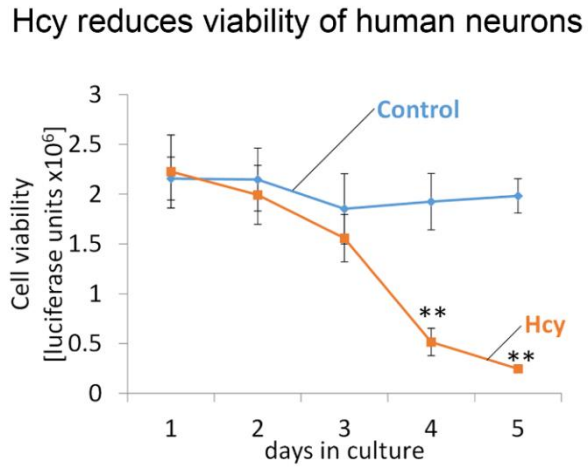



**Figure 5.2- A and B** Homocysteine increases levels of intracellular amyloid in human neurons. A) Human neuroprogenitor cells were treated with increasing Hcy concentrations and subjected to ELISA assays. Cellular β-amyloid 42/40 ratio increased proportionally to increasing levels of Hcy concentration (0.05 mM: 1.9 +/- 0.2; 0.5 mM: 2.8 +/- 0.2). B) Rapamycin (mTORC1 inhibitor) and TAT-Beclin1 (autophagy inducer peptide) decreased the ratio of β-amyloid 42/40 in HHcy conditions. Data are means +/- SEM (n = 5 independent experiments). *P ≤ 0.05 and **P ≤ 0.01.

A




B

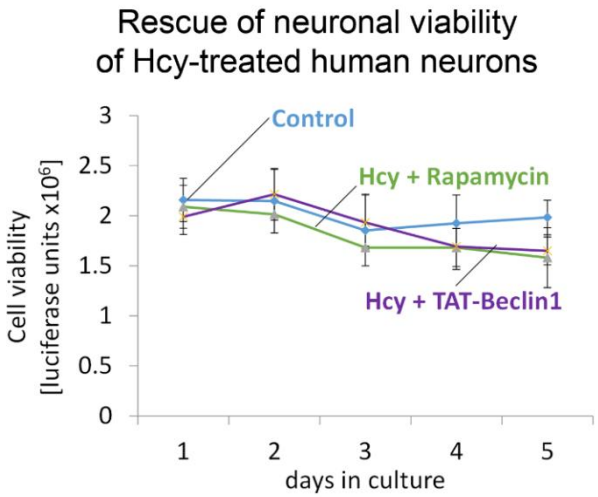



**Figure 5.3- A and B** Homocysteine proceeds degeneration of human neurons. Human neuroprogenitor cells were treated with Hcy for different periods of time and followed by luciferase-based viability assay measuring the levels of ATP. Hcy degenerated neurons drastically after four days (A), the phenotype which was rescued in Rapamycin and TAT-Beclin1 treatments (B). Data are means +/- SEM (n = 5 independent experiments). **P ≤ 0.01.

### 5.1.4 Unfolded Protein Response is Upregulated in Cbs Deficient Mice

Unfolded protein response (UPR) senses the accumulation of abnormal proteins and triggers, among other factors, phosphorylation of protein kinase RNA-like endoplasmic reticulum kinase (PERK) which was proposed to become activated through the Hcy-mediated pathway (Zhang et al. 2001). Upon PERK activation, eIF2α is phosphorylated and induces the production of ATF4 and CCAAT/enhancer-binding protein homologous protein (CHOP), a pro-apoptotic transcription factor (Babcock et al. 2013). Thereby we decided to assess the amount of CHOP protein level in our Cbs KO mouse brain sections. Mouse hippocampal sections showed increased formation of CHOP which was correlated with increased astroglial activation as detected by GFAP staining (Figure 5.4).

## 5.2 Conclusion

Formation of β-amyloid and phospho-Tau are considered as hallmarks of AD (Li et al. 2014). To evaluate neurotoxicity of Hcy, it was crucial to assess its contribution to the formation of the abnormal proteins. Here we demonstrated that Hcy indeed led to the formation of β-amyloid and phospho-Tau in hyperhomocysteinemic mouse brains. These mice had phospho-Tau tangles spread throughout the cells, while wild-type mice showed decreased levels of phospho-Tau along blood vessels in the brain. Accumulation of AD-specific protein deposits in HHcy mouse brains and decreased viability of human neurons

exposed to Hcy supports the notion that Hcy is likely a driver of AD pathophysiology. Additionally, we could rescue the phenotype by inhibition of mTORC1 or activation of autophagy. Our data underlined the importance of autophagy in neuronal homeostasis. However, future studies need to be conducted to shed light on the cell death mechanism driven by Hcy.

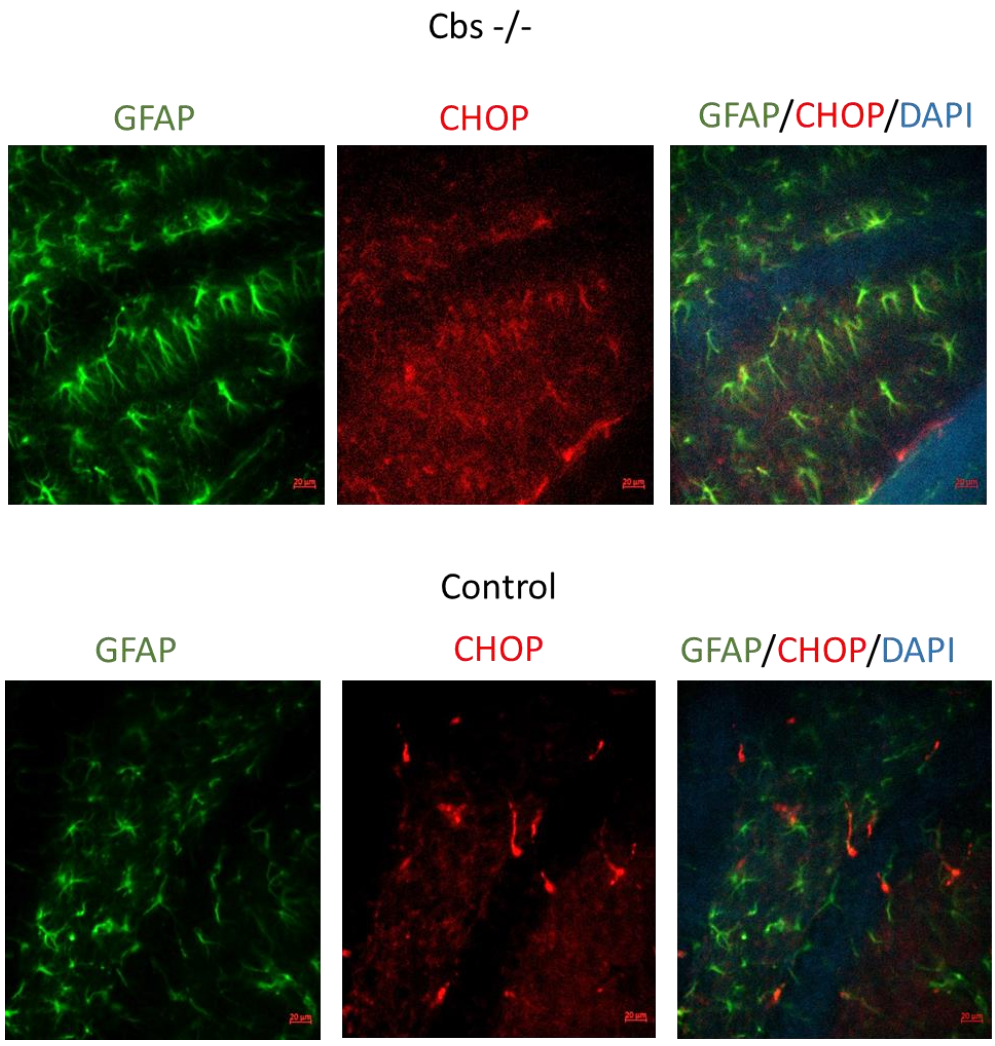



**Figure 5.4** Cbs knockout mice have higher CHOP levels and correlate with increase in activated astrocytes. CHOP staining increased in hippocampal sections of Cbs knockout mice which was in parallel with increase in GFAP staining. Scale bars are 20 um, n=2.

# CHAPTER 6

# DISCUSSION AND FUTURE DIRECTIONS

It is noteworthy to elucidate how Hcy as a common disease risk factor contributes to disease pathogenesis. Homocysteine has been correlated with cardiovascular disease (Clarke et al. 1991) and neurodegeneration by induction of apoptosis in neurovascular cells (Kamat et al. 2015; Agnati et al. 2005). The pathway by which Hcy affects apoptosis is not fully understood. In this dissertation, we could shed light on one of the possible pathways through which Hcy is sensed in the cells and leads to the phenotype observed in neurodegenerative diseases. HEK293T cells and reprogrammed human neurons were utilized in the presented *in-vitro* studies while *Cbs* deficient mice represented the genetic model with high plasma Hcy concentration levels (≈300uM) (Choumenkovitch et al. 2002) to perform *in-vivo* studies.

## 6.1 Homocysteine Increases mTORC1 Activity and Localization on Lysosomal Membrane

The relevance of high mTORC1 activity in neurodegenerative diseases has been proposed in some studies (Rubinsztein 2006; Laplante and Sabatini 2012). Having that in mind we evaluated the mTORC1 activity of brain lysates obtained from AD and control patients. Our data revealed hyperactivation of mTORC1 in disease cases while the protein levels of mTORC1 activator (Rheb) and inhibitor

(TSC2) remained equal between the experimental groups (Figure 3.1 A, B, and C). Activation of mTORC1 has been related to various upstream signaling pathways among which growth factor and amino acid signaling represented the most crucial ones (Laplante and Sabatini 2012; Khayati et al. 2015). Growth factors are necessary to inhibit TSC2 deterrent activity toward Rheb (Tee et al. 2003) & (Bar-Peled et al. 2013); whereas amino acids are main players of placing mTORC1 on lysosomal membrane where Rheb resides to activate mTORC1 (Reddy et al. 2016; Inoki et al. 2003; Zoncu 2011). Among amino acids, Leu has the strongest impact on mTORC1 activity, yet is insufficient to activate mTORC1 by its own (Bar-peled and Sabatini 2014). However, the influence of amino acid metabolites on mTORC1 activation have not been addressed yet. Here, we proposed that mTORC1 is capable of sensing Hcy to up-regulate its activity. Homocysteine-treated cells elevated mTORC1 activity in presence and absence of Leu (Figure 3.2 and 3.3 A, B and C). Likewise, we showed that Hcy is capable of inducing mTORC1 recruitment on the lysosomal membrane (Figures 3.10 A and B). Interestingly, our *in-vivo* studies in *Cbs* deficient mice resembled the phenotype seen *in-vitro,* in Hcy-treated cells. Microscopic evaluation in hippocampal regions of *Cbs* knockout and wild-type mice revealed an increase in overall levels of mTORC1 in *Cbs* deficient mice which co-localized at the lysosomal membrane. Interestingly, the number of LAMP2 positive organelles decreased which could be a likely consequence of mTORC1-mediated inhibition of TFEB and associated lysosomal biogenesis. (Figure 3.11).

### 6.2 Homocysteine-Driven mTORC1 Activity is Specific to Homocysteine

Homocysteine-driven mTORC1 activity was assumed to be independent of AKT signaling pathways, as phosphorylation levels of AKT T308 and S473 did not change in Hcy-treated cells and *Cbs* deficient mouse brain lysates (Figure 3.4 and 3.5 A and B). As such, AKT S473 phosphorylation levels were found to be the same in the analyzed post-mortem brain samples from AD and control patients (Figure 3.6). These data indicated that the growth factor signaling pathway and mTORC2 activity were not involved in Hcy-driven regulation of mTORC1 activity. Given that Hcy is unstable in cells and converts to Hcy-thiolactone (Jakubowski 1999; Jakubowski 2015; Akchiche et al. 2016), we designed experiments to assess whether mTORC1 activity can be driven by Hcy-thiolactone. We detected no significant changes in mTORC1 activity upon treatment of the cells with Hcy-thiolactone (Figure 3.7). In parallel, mTORC1 activity was measured in mouse brain lysates deficient for Bleomycin and Paraoxonase1 enzyme activities. These mice had a high level of Hcy-thiolactone (Borowczyk et al. 2012) but showed no increased mTORC1 activity compared to control mice fed with Met (Figure 3.8). As Hcy-thiolactone is harshly reactive and able to bind to lysine residues of proteins, it is known to be detrimental to cells (Jakubowski 1999; Akchiche et al. 2016). However, our data eliminated the role of Hcy-thiolactone in activation of mTORC1.

Moreover, we excluded the possibility of mTORC1 hyperactivation due to the accumulation of amino acids in the cytoplasm, which was the result of preclusion in protein synthesis, implicated by Hcy similar to cycloheximide. To

this end, we evaluated the impact of other amino acids such as cysteine on mTORC1 activity. Cysteine was the final product in process of Hcy conversion by CBS enzyme but did not exhibit a significant effect on mTORC1 activation implicating Hcy-driven mTORC1 activity being specific to Hcy (Figure 3.9).

### 6.3 Homocysteine Induces mTORC1 Activity through Interaction with LeuRS-Folliculin Complex

Molecular mechanisms through which Hcy affects mTORC1 activity were investigated in this dissertation. In addition to the regular role of LeuRS in process of protein translation (Park, Ewalt, and Kim 2005), its ability in binding to Hcy, although with lower affinity, in process of amino acid editing (Jakubowski 2012), led us to assess the possible way through which LeuRS relays the signal to mTORC1 upon binding to Hcy. Since Flcn's GAP activity on Rag C/D was investigated by Tsun et al. (Tsun et al. 2013), we assessed the possibility of LeuRS interaction with Flcn. To this end, immunoprecipitation analyses were performed and could prove the existence of such interaction between the two proteins; although LeuRs binding to Flcn turned out not to be dependent on the presence of amino acids in the culture media (Figure 3.12 A and B). Given that Flcn localization differed upon Leu availability (Petit et al. 2013; Tsun et al. 2013), Hcy impact on the localization of LeuRS-Flcn complex was assessed in this research project. Interestingly, our data showed that Hcy was able to diffuse Flcn in the cytoplasm while was resided on the lysosomal membrane in contact with Rags in absence of Leu or Hcy (Figure 3.13, 3.14 A and B). Of note, Hcy impact

on Flcn localization was hampered by employing leucinol, a Leu derivative that inhibited LeuRS activity (Han et al. 2012), suggesting a mediating role for LeuRS to activate Flcn in presence of Hcy. Further analysis needs to be conducted to investigate the mechanism through which LeuRS relays the signal to Flcn.

Another possible factor which could be functional in sensing Leu is Sestrin2. Sestrin2 was proposed to preclude GATOR2-GATOR1 interaction in absence of amino acids, releasing GATOR1 to act as a GAP, therefore an inhibitor for Rag A/B; whereas, Leu binding to Sestrin2 perturbed its interaction with GATOR2 and rendered Rag A/B active (Wolfson et al. 2016). Sestrin2 contribution to Hcy-mediated mTORC1 activation was assessed by evaluating Sestrin2 protein levels in Hcy-treated cells. There existed evidence demonstrating that prolonged amino acid deprivation promoted Sestrin2 transcription in an ATF4-dependent manner (Ye et al. 2015). Our data suggested that Sestrin2 protein levels did not change in presence of Hcy, (Figure 3.15 A and B) indicating first, Hcy was potent in precluding the starvation phenotype-related upregulation of Sestrin2, and second, there was no plausible induction of Sestrin2 production caused by Hcy.

### 6.4 Homocysteine Inhibits Autophagy Induction through Dampening TFEB Activity

Impairment of autophagy in AD patients has been considered with the observation of enlarged double membranous organelles, resembling autophagosomes (Levine et al. 2008; Nixon and Yang 2011). This phenotype is

correlated with the notion that proteinopathies arise from the accumulation of protein aggregates in the cytoplasm of neurons observed in most of the human neuronal pathologies such as AD, Parkinson's and Huntington's diseases (Williams et al. 2006). Given that autophagy can be under the control of mTORC1 activity through ULK1 (Kim et al. 2011) and TFEB phosphorylation (Martina et al. 2012; Roczniak-Ferguson et al. 2012), we investigated the impact of Hcy on autophagy. Our data obtained from autophagy flux assays revealed a role for Hcy in an increase of phospho-ULK1 which impacted LC3 lipidation (Figure 4.3). Taking these data into consideration, we concluded that Hcy inhibited autophagy induction that correlated with decreased formation of LC3-positive autophagosomes (Figure 4.1). Given that Torin reversed Hcy-mediated phenotype by increasing the number of LC3 punctual staining, we proved that Hcy effect on autophagy was mediated through the activation of mTORC1 (Figure 4.1).

Tandem fluorescent-tagged LC3 (ptf-LC3), a conjugated form of LC3 with GFP and mRFP fluorescent proteins, was utilized as a marker to distinguish autophagosomes before and after fusion with lysosomes, the final destination of autophagic organelles (Kimura, Noda, and Yoshimori 2007). Using this construct, we could confirm that Hcy inhibited LC3 lipidation and autophagosomal maturation, as indicated respectively by a reduction in the number of both green and red organelles (Figure 4.4).

The data we obtained in autophagy assays were based on short Hcy-treatments of the cells. However, since HHcy has been correlated with AD

(Coppedè 2010), it is important to assess effects of chronic exposure to high Hcy levels. Thus, we examined the rate of autophagy induction in cells exposed to Hcy, overnight. Interestingly, LC3I & II decreased in the long-term exposure of cells to Hcy (Figure 4.5), thus led us to look at the activity of the upstream effector of autophagy induction, TFEB. It is important to note that LC3I levels were indirect reporters of TFEB-mediated transcriptional activity (Karim, Kawanago, and Kadowaki 2014). Similarly, reduction in protein levels of LC3 and p62, also a TFEB-target gene (Sahani, Itakura, and Mizushima 2014), were observed in hyperhomocysteinemic mice brain samples (Figure 4.6). The data were corroborated by measuring the activity of TFEB, employing a 4xCLEAR-luciferase construct, an established reporter for TFEB activity (Sardiello et al. 2009). Cells treated with Hcy or Leu overnight showed a reduction in TFEB activity (Figure 4.7 A). These data were also confirmed by conducting experiments that showed cytoplasmic localization of TFEB in Hcy-treated cells or hyperhomocysteinemic mouse brain samples (Figures 4.8 & 4.9) where we expect to find TFEB while mTORC1 is active (Martina et al. 2012).

### 6.5 Elevation in Levels of Abnormal Proteins Correlates with Cellular Homocysteine Levels

The relevance of high levels of Hcy in plasma and the brain (HHcy) in the progression of AD has been discussed in the field. First, from the possibility of Hcy conversion to Hcy-thiolactone, it has been correlated with the pathology of the disease and production of β-amyloid (Borowczyk et al. 2012). Additionally,

Borowczyk et al. and Suszynska et al. have proposed Hcy-thiolactone as a neurotoxic agent whose phenotype was ameliorated by Bleomycin hydrolase (Borowczyk et al. 2012; Suszynska et al. 2010). Second, Hcy was able to damage vascular integrity, a phenotype which altered angiogenesis and increased membrane permeability; both of which have been observed in AD pathogenesis (Kamat et al. 2015). Li et al. also lent further support to the hypothesis that Hcy exacerbates AD phenotype; the authors introduced mechanisms by which Hcy led to the formation of β-amyloid and phospho-Tau by induction of γ-secretase and CDK5 activities, respectively (Li et al. 2014). An alternative pathway through which Hcy metabolism leads to formation of β-amyloid was discussed by Fuso et al. They showed that reduction of S-adenosine methionine (SAM) in cells deficient from enzymes converting Hcy to Met (methionine synthase), led to hypomethylation and upregulation of BACE and PS1 genes; the result is production of more β-amyloid (Fuso et al. 2005). Interestingly, what we proposed here was unique since was attributed to Hcy influence on the production of AD-related phenotype through modulation of mTORC1 activity. The increase in β-amyloid 42, the aggregation-prone form of β-amyloid, due to Hcy-treatments were reversed to a normal level by applying Rapamycin and TAT-Beclin1 (Figure 5.2 A and B); both of which modulated the mTORC1-autophagy pathway. Of note, our data supported Fuso et al. hypothesis regarding upregulation of genes related to production of β-amyloid, since mTOR is the master regulator of protein synthesis. We could also detect an increase of both β-amyloid and phospho-Tau in *Cbs* knockout mice (Figure 5.1 A,

B and C). Interestingly, the same phenotype was observed in TFEB-deficient mice (Reddy et al. 2016), unraveled the role of TFEB in the pathway.

Thus, Hcy not only inhibits clearance of protein aggregates through modulation of TFEB activity, but also increases the production of aggregation-prone β-amyloid.

### 6.6 Homocysteine Contributions to Cell Death

Homocysteine contributions to the cell death in AD has been related to induction of cerebral microangiopathy, endothelial dysfunction, oxidative stress, neurotoxicity and apoptosis (Kamat et al. 2015), while neuroinflammation has been observed prior to neuronal loss. (Walker and Sills 2012). Homocysteine oxidation due to its sulfur group has been shown in the cytoplasm and culture media. Oxidation of Hcy led to the formation of reactive oxygen species (ROS) causing neuroinflammation, apoptosis (Yan et al. 2006) and cerebrovascular dysfunction (Ataie, Sabetkasaei, and Haghparast 2010; Kamat et al. 2015). Alternatively, Hcy contribution in disruption of blood-brain barrier has been correlated with an increase of matrix metalloproteinase enzyme activity and expression observed concomitantly in *Cbs* deficient mice (Tyagi et al. 2012). Our viability assays revealed a role for the mTORC1-autophagy pathway in Hcy-driven cell death; since mTORC1 inhibition and autophagy induction could prevent neuronal death caused by prolonged exposure to Hcy (Figure 5.3 A and B).

Given that neurodegeneration was triggered by Hcy, further research needs to be implicated to decipher the mechanism of Hcy-triggered cell death. A few possibilities have been proposed earlier; such as induction of oxidative stress (Yan et al. 2006), deterioration of mitochondrial function by Hcy which led to apoptosis (Kronenberg et al. 2009), and activation of matrix metalloproteinase enzymes that perturbed extracellular matrix leading to blood-brain barrier malfunction (Rhodehouse et al. 2013). Similarly, the role of β-amyloid peptide aggregation in neuroinflammation, DNA damage, and neuronal cell death has been evaluated (Kamat et al. 2015; Kruman et al. 2002). Yet, there is no evidence showing the relevance of Hcy-mediated mTORC1-autophagy pathway in the procedure of cell death induction.

Given the results presented in this dissertation project, it is noteworthy to consider the contribution of mTORC1 hyperactivity to cell death. An accumulating body of evidence supported the notion that mTORC1 inhibition favors longer life span (Lushchak et al. 2017). mTORC1 hyperactivity increased the load of mRNA translation leading to enhanced protein production which could lead to the formation of toxic aggregations in the cell; thus, down-regulation of ribosomal S6 kinase has been shown to attenuate aging process, effectively (Selman et al. 2009). The notion that proteasomal degradation was also dampened upon mTORC1 activation by decreasing overall protein ubiquitylation (Zhao et al. 2015) and proteasomal chaperones (Rousseau and Bertolotti 2016), gives support to the hypothesis that chronic mTORC1 activation is detrimental for cells.

Moreover, the contribution of mTORC1 activity to cell death could be mediated by chronic attenuation/insufficiency of autophagic clearance; the pathway which plays a crucial role in the maintenance of neuronal homeostasis (Rubinsztein 2006; Yue et al. 2009). Other than being an active process of eating redundant proteins and old organelles, autophagy interacts with other signal transduction pathways in the cell. For example, the crosstalk between autophagy and apoptosis is implemented by Beclin1/Bcl2 interaction. While Beclin1 is essential for initiation and elongation of autophagosomes (Glick et al. 2010; Liang et al. 2006), Bcl2 inhibits such function. The interaction between Beclin1 and Bcl-2 depends upon multiple factors in the cell such as their expression level, phosphorylation level and localization where they interact (Marquez and Xu 2012). The alternative interplay of autophagy and apoptosis is given by the pro-apoptotic function of cleaved Beclin1. Following cleavage, a C-terminal fragment of Beclin1 enters mitochondria to release pro-apoptotic factors in nutrient-poor conditions (Wirawan et al. 2010).

Our data indicate that Hcy-mediated cell death requires 4 days (Figure 5.3 A). However, activation of NMDA receptors occurs quickly (Lipton et al. 1997), whereas Hcy-mediated mTORC1 activation followed by inhibition of autophagy requires more time to disturb neuronal homeostasis leading to cell death. Similar observation has been made in Atg7 knockout animals which occurs after 28 weeks of birth (Komatsu et al. 2006), suggesting that not neuronal differentiation or other developmental processes in the brain are disturbed by autophagy inhibition but the neuronal homeostasis.

## 6.7 Future Directions

Having all these cell death-promoted pathways in mind, there is an alternative pathway which was also proposed to be triggered in presence of Hcy, the unfolded protein response (UPR) (Zhang et al. 2001). Our data showed that CHOP staining is upregulated in Cbs deficient mice which correlates with higher GFAP staining (Figure 5.4). Future studies need to be done to decipher what cell types specifically express CHOP. Here we showed that inflammation was triggered in dentate gyrus of Cbs knockout mice represented by an increase in activated astrocytes (Figure 5.4). We speculate that loss of MAP2 signaling in Cbs deficient hippocampi (Figure 5.1 A) is due to induction of inflammatory response. Our future studies will shed light on the way through which Hcy initiates inflammation *in-vitro* and *in-vivo*. We are planning to do Multiplex assays in Hcy-treated neurons. Likewise, we will treat methionine-fed mice with Torin to inhibit mTOR activity and correlate with levels of activated astrocytes (GFAP staining). We assume that Hcy-mediated mTORC1/autophagy modulation increases protein accumulation in two ways: by increasing protein synthesis and decreasing the autophagic clearance of protein aggregates and other cellular debris which further increases UPR signaling and inflammation.

## 6.8 Conclusion

Taken together, data presented in this dissertation show that Hcy upregulates mTORC1 activity, inhibits autophagic clearance eventually leading to the formation of pathogenic protein aggregates. The mechanisms delineated in this work may help developing novel approaches that target components of the mTORC1 and autophagy pathways to counteract Hcy-induced pathologies.

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