Published in final edited form as:

Neurobiol Dis. 2018 December ; 120: 165–173. doi:10.1016/j.nbd.2018.08.025.

Cystatin C prevents neuronal loss and behavioral deficits via the endosomal pathway in a mouse model of Down syndrome

Gurjinder Kaur¹, Sebastien A. Gauthier¹, Rocio Perez-Gonzalez¹, Monika Pawlik¹, Amol Bikram Singh¹, Benjamin Cosby¹, Panaiyur V. Mohan¹, John F. Smiley^{1,2}, and Efrat Levy^{1,2,3,4}

¹Nathan S. Kline Institute, Orangeburg, NY, USA 10962

²Department of Psychiatry, NYU Langone School of Medicine, New York, NY, USA 10016.

³Department of Biochemistry and Molecular Pharmacology, NYU Langone School of Medicine, New York, NY, USA 10016.

⁴Neuroscience Institute, NYU Langone School of Medicine, New York, NY, USA 10016.

Abstract

Cystatin C (CysC) plays diverse protective roles under conditions of neuronal challenge. We investigated whether CysC protects from trisomy-induced pathologies in a mouse model of Down syndrome (DS), the most common cause of developmental cognitive and behavioral impairments in humans. We have previously shown that the segmental trisomy mouse model, Ts[Rb(12.1716)]2Cje (Ts2) has DS-like neuronal and behavioral deficiencies. The current study reveals that transgene-mediated low levels of human CysC overexpression has a preventive effect on numerous neuropathologies in the brains of Ts2 mice, including reducing early and late endosome enlargement in cortical neurons and decreasing loss of basal forebrain cholinergic neurons (BFCNs). Consistent with these cellular benefits, behavioral dysfunctions were also prevented, including deficits in nesting behavior and spatial memory. We determined that the CysC-induced neuroprotective mechanism involves activation of the phosphatidylinositol kinase (PI3K)/AKT pathway. Activating this pathway leads to enhanced clearance of accumulated endosomal substrates, protecting cells from DS-mediated dysfunctions in the endosomal system and, for BFCNs, from neurodegeneration. Our findings suggest that modulation of the PI3/AKT pathway offers novel therapeutic interventions for patients with DS.

Keywords

cystatin C; Down syndrome; spatial memory; early endosome; late endosome

Corresponding and contact author: Efrat Levy, Nathan S. Kline Institute, 140 Old Orangeburg Road, Orangeburg, NY 10962. Phone: (845) 398 5540; Fax: (845) 398 5422; efrat.levy@nki.rfmh.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

DS is a genetic disease caused by trisomy of human chromosome 21 (HSA21), associated with developmental brain abnormalities (Wisniewski et al., 1984) and early intellectual disabilities (Rueda et al., 2012). DS individuals develop age-related cholinergic degeneration (Casanova et al., 1985; Head et al., 2016), similar to Alzheimer's disease (AD) patients (Casanova et al., 1985; Holtzman et al., 1992; Yates et al., 1983), a cause of memory impairments (Browne et al., 2001; Das et al., 2014; Lin et al., 1999; Velazquez et al., 2013). Mouse models of DS with chromosome 16 (MMU16) segmental trisomy, orthologous to HSA21 (Davisson et al., 1990; Granholm et al., 2000; Holtzman et al., 1996; Villar et al., 2005) have been an important source of information on DS-related dysfunctions and characteristic phenotypes responsible for DS-specific changes. Previous reports have shown that, similar to DS patients, the trisomic mouse models Ts65Dn (Davisson et al., 1990) and Ts[Rb(12.17¹⁶)]2Cje (Ts2) (Villar et al., 2005) exhibit progressive degeneration of the cholinergic system (Jiang et al., 2016; Powers et al., 2016; Seo and Isacson, 2005) and further cognitive deficits (Kaur et al., 2014; Powers et al., 2016; Seo and Isacson, 2005).

The DS related characteristic progressive degeneration of the cholinergic system (Fodale et al., 2006) has been attributed to endosomal dysfunctions (Jiang et al., 2016; Salehi et al., 2006; Xu et al., 2016). While the etiology of DS-related neuronal early endosomal dysfunction is likely to be multifactorial, various studies have shown that triplication of the gene coding for the amyloid- β precursor protein (APP), located on HSA21 (Kang et al., 1987), that results in the overexpression of APP and APP metabolites, contributes to endosomal disruption in neurons (Cataldo et al., 2001; Choi, 1988; Jiang et al., 2016; Nixon, 2017; Xu et al., 2016) and non-neuronal cells (Jiang et al., 2010).

Multiple *in vitro* and *in vivo* studies have demonstrated that CysC plays protective roles via various pathways that depend upon inhibition of cysteine proteases, induction of cellular autophagy, induction of cell proliferation, or inhibition of A β aggregation [reviewed in (Gauthier et al., 2011; Kaur and Levy, 2012; Mathews and Levy, 2016)]. We have investigated whether enhanced CysC expression has beneficial effects in DS by crossing the Ts2 trisomic mouse model of DS with transgenic mice expressing human CysC (TgCysC) (Pawlik et al., 2004). Here we demonstrate prevention of endosomal pathology, degeneration of basal forebrain cholinergic neurons (BFCNs) in the median septal nucleus (MSN), and behavioral deficiencies, in trisomic mice overexpressing CysC (Ts2/TgCysC). We further demonstrate that the mechanism of protection by CysC in the DS model involves a previously not identified mechanism, activation of the phosphatidylinositol kinase (PI3K)/AKT pathway.

2. Materials and methods

2.1 Animals

A breeding colony of Ts2 mice was maintained on B6EiC3SnF1/J background. Ts2 mice were crossed with transgenic CysC overexpressing mice (TgCysC) on C57BL/6 background. Trisomic mice overexpressing CysC (Ts2/TgCysC) were studied together with their littermates including trisomic Ts2, CysC transgenic (TgCysC), and diploid control (2N)

mice at 8 months of age. Both females and males were used for all analyses. All animal procedures were performed following the National Institutes of Health guidelines with approval from the Institutional Animal Care and Use Committee at the Nathan S. Kline Institute for Psychiatric Research.

2.2 Nesting behavior

Mice were individually housed for at least 24 hours in clean plastic cages with approximately 1 cm of corn cob bedding lining the floor and identification cards coded to render the experimenter blind to the genotype of each subject. Two hours prior to the onset of the dark phase of the lighting cycle, each cage was supplied a pressed cotton square nestlet (Ancare, UK agent, Lillico). The next morning (approximately 16 hours later) cages were inspected for nest building. Pictures were taken prior to evaluation for documentation. Nest construction was scored using the established system of Deacon (Deacon, 2006) with a 5 point system: 1. Nestlet not noticeably touched. 2. Nestlet partially torn up (50–90% remaining intact). 3. Mostly shredded nestlet but often no identifiable nest site. 4. An identifiable, but flat nest. 5. A (near) perfect nest with clear nest crater. One-way ANOVA comparison of nesting behavior was made across genotypes to determine statistical significance of variance.

2.3 Object Placement behavior

A hippocampal-dependent object placement task was adapted for mice from previously described procedures (Luine et al., 2003; Macbeth et al., 2008) testing memory capabilities of the animals. Mice were acclimated to an open Plexiglas arena (20 cm×40 cm×20 cm) lined with fresh corn cob bedding twice a day for 5 min each with inter-trial intervals of 5 min on day 1, and 4 hours on day 2. On the third day mice were placed in the same arena with two identical objects (A and B) (amber glass vials, 2 cm diameter, 5 cm tall) placed equidistant to adjacent corners of the arena. Investigation duration was quantified over a 5-min time period (trial 1) then mice were returned to their cage. Investigation was scored as time of nose pointing toward the objects within 2 cm. Objects and arena were wiped with 70% ethanol between each session. The alcohol odor was evaporated before each trial. Four hours after training, one object (B) was moved longitudinally to a position diagonal from the object retaining its original position. The side of the chamber with the displaced object (B) was alternated between animals in order to avoid any possibility of side preference. Investigation time for each object was quantified as a percentage of total investigation time. An increased percentage of time spent exploring the displaced object (B) compared to the total time spent exploring both objects during testing was considered an index of enhanced performance in this task. Investigation times were compared within each condition between training and test trials using a Student's *t*-test to confirm the behavioral task effectiveness within the group. One-way ANOVA comparison of moved object investigation times during the test trial (trial 2) were made across the genotypes to determine statistical significance of variance.

2.4 Immunostaining

For all immunohistological procedures, mice were anesthetized with ketamine (50 g/kg)/xylazine (5 mg/kg) and transcardially perfusion-fixed with 4% paraformaldehyde in 0.1 M

sodium cacodylate buffer. Brains were removed and post-fixed overnight in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4°C, transferred to PBS the next day, and subsequently cut into 40 µm-thick coronal sections with a vibratome. Free-floating sections from all the mouse groups were concurrently processed for immunohistochemical examination (Kaur et al., 2017). Control sections were processed with the omission of the primary or secondary antibodies to exclude non-specific reactions. Labeling conditions and exposure times were identical throughout.

Fluorescent labeling of early and late endosomes and lysosomes was performed using antibodies to rab5a (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), rab7a (1:1000; Millipore, Billerica, MA) and anti-lysosomal marker protein 2 (LAMP2) (1:100; Developmental Studies Hybridoma Bank, Iowa City, Iowa), respectively. Following binding of fluorophore-conjugated secondary antibody (1:500; Invitrogen, Grand Island, NY), immunofluorescence was observed and captured using an LSM 510 Meta confocal microscope (Zeiss, Thornwood, NY). For quantification, approximately 30 neurons per animal were measured ($n = 4$ per genotype) randomly by a genotype-blinded observer. Appropriate anatomical regions were identified at low-power magnification. Switching to the 100× objective, the center-field neuron(s) were analyzed. Each neuron sampled was at least two cell-lengths from the closest sampled neuron, and 10–12 neurons from three sections throughout the region were analyzed. Quantification of rab5a, rab7a and LAMP2 signal was performed using ImageJ (NIH, USA) after thresholding the density of rab5a, rab7a or LAMP2 signal over background; the average endosomal area fraction was calculated as the ratio of positive-pixels per rab5a-, rab7a- or LAMP2 positive endosomes or lysosomes in a neuron to the total area of the neuron. The ratio of rab5a-, rab7a - or LAMP2 positive endosomal/lysosomal area per neuron was further normalized to 2N control mice. Double immunolabeling was performed to determine CysC endosomal localization using anti-CysC (1:1000; Millipore, Billerica, MA) with rab5a, rab7a or LAMP2 antibodies.

The number of ChAT BFCNs in the MSN was stereologically counted, using the optical fractionator method as previously described (Kaur et al., 2017; Smiley et al., 2012). Cells were immunolabeled with goat anti-ChAT (1:500, Millipore, Bellerica MA) and was visualized with anti-sheep fluorophore conjugated secondary antibody (1:500, Invitrogen, Grand Island, NY). The MSN was sampled in every third consecutive 40 µm-thick vibratome section rostral to the anterior commissure. A grid of optical dissector sampling sites with 0.25 mm spacing was placed on each section. At each sampling site, a 40× oil-immersion objective with 1.3 numerical aperture was used to collect a z-stack of fifteen 3-µm spaced images starting at a focal plane just beneath the tissue surface. Cell counting was done on 198×143 µm counting boxes drawn onto each z-stack, using the top 2 slices as the upper guard zone, the next 10 slices as a 30 µm deep counting box, and the subsequent slices as the lower guard zone. Section thickness (40.5 ± 1.7 µm mean $\pm$ S.D.), measured in triplicate on each section, showed no evidence of shrinkage from the original 40-µm section thickness. On average, 191 ± 69 cells were counted per MSN, and the coefficient of error (Dorph-Petersen et al., 2001) was 0.08 ± 0.01 (mean $\pm$ S.D.). Oneway ANOVA followed by post-hoc multiple comparisons Bonferroni's tests were used to assess the differences between genotypes; level of statistical significance was set at $p < 0.05$.

2.5 Western blot analysis

Mice were euthanized as described above, hemi-brains were dissected and flash frozen on dry ice. Tissues were homogenized in lysis buffer: 20 mM Tris-HCl (pH 7.4), 250 mM sucrose, 1 mM EDTA, and 1 mM EGTA with protease inhibitors (5 µg/ml Leupeptin-Antipain-Pepstatin A mix and 1 mM PMSF). Protein concentration was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL). Equal amounts (20 µg) of total proteins were boiled in sample buffer (1% SDS, 3% glycerol, 1.5% β-mercaptoethanol and 20 mM Tris-HCl, pH 6.8) and separated by 4-20% tris-HCl gel electrophoresis (Criterion precast gel, Bio-Rad, Hercules, CA). To blot mechanistic target of rapamycin (mTOR) protein, samples were separated by 10% tris-HCl gel (Criterion precast gel, Bio-Rad, Hercules, CA). The proteins were electrophoretically transferred onto a 0.45 µm polyvinylidene fluoride membrane (Bio Rad, Hercules, CA) in 2.5 mM Tris, 19.2 mM Glycine, 20% methanol transfer buffer. The membrane was blocked in 5% milk (Bio Rad, Hercules, CA) or 5% BSA in 10 mM Tris, 150 mM sodium chloride, pH 7.5, 0.1% Tween-20 (TBST), incubated with primary antibody overnight at 4°C and with secondary antibody for 1-2 hours at room temperature. The membranes were incubated in chemiluminescent fluid (Pierce, Rockford, IL) for 5 min, and chemiluminescence was visualized on Reflection Autoradiography film, β-tubulin was blotted on the same, stripped membrane as an internal control for loading. The protein bands were scanned, optical density was calculated using the Image J, and the ratio of protein intensity to β-tubulin in the same lane was calculated. Two corresponding blots for each protein were run at the same time to calculate the ratios of PI3K, AKT, mTOR and p70 ribosomal S6 kinase (P70S6K) phosphorylated proteins relative to their respective total proteins.

Primary antibodies used for Western blot analysis were: anti-CysC antibody (1:1000; Millipore, Billerica, MA), antibodies to PI3K, mTOR, P70S6K and AKT, phosphorylated and total proteins (1:1000, Cell Signaling Technology, Inc., Danvers, MA), anti-(LAMP2 (1:1000; Developmental Studies Hybridoma Bank, Iowa City, Iowa), anti-cathepsin D (1:1000, Scripps laboratories, San Diego, CA), anti-cathepsin B (1:1000, Millipore, Billerica, MA), anti-Beclin (1:1000, BD Life Sciences, San Jose, CA), anti-ATG5 (1:1000, EMD Millipore, Billerica, MA), anti-LC3, (1:1000; Novus Biological, Littleton, CO), anti-α-synuclein (1:1000; Sigma, St. Louis, MO), and anti-β-tubulin (1:10000; Sigma, St. Louis, MO). Secondary antibodies used were: HRP conjugated anti-rabbit or mouse antibodies (1:5000; GE Healthcare, Pittsburgh, PA).

Data are presented as mean ± SEM. One-way ANOVA followed by post-hoc multiple comparisons Bonferroni's tests were used to evaluate the differences between genotypes; level of statistical significance was set at $p < 0.05$).

3. Results

3.1 Overexpression of CysC prevents altered early endosome morphology in neurons in the brain of Ts2 mice

Confirming CysC overexpression in the Ts2/TgCysC mouse crosses, Western blot analysis of cerebral homogenates of 8 months-old littermate mice using an anti-CysC antibody

revealed 20% increased CysC expression in addition to the normal expression of mouse CysC in Ts2/TgCysC and TgCysC mice when compared to 2N diploid controls and Ts2 mice (Fig. 1A & B). Immunofluorescent labeling of CysC in cortical neurons of the frontoparietal region of the brain showed both cytosolic and punctate staining.

For investigation of the localization of the punctate staining of CysC within early endosomes, double-immunolabeling was performed with antibodies to CysC and to the small GTPase rab5a, a regulator of endocytosis and early endosome fusion, and a specific marker for early endosomes (Simpson and Jones, 2005). This double staining shows the localization of CysC within early endosomes (Fig. 1C). Double-immunolabeling with antibodies to CysC and to the small GTPase rab7a, a regulator of vesicular trafficking in the late endocytic pathway and a late endosomal marker [reviewed in (Guerra and Bucci, 2016)] showed localization of CysC within a small number of late endosomes (Fig. 1D).

For examining early and late endosomal size, mouse brain tissue sections were stained as previously described (Choi et al., 2013) either with an antibody against the early endosomal marker rab5a (Fig. 2A) or with an antibody to rab7a (Fig. 2C). Rab5a- and rab7a-immunolabelling in randomly chosen frontoparietal cortical neurons was quantified to compare the average endosomal area fraction of early and late endosomes among the four genotypes. Rab5a-positive early endosomes in cortical neurons of Ts2 mice were found to be enlarged as compared to 2N mice, consistent with previous reports (Jiang et al., 2016) (Fig. 2A & B). Similarly, immunostaining with anti-rab7a antibody revealed enlarged neuronal late endosomes in the frontoparietal region of Ts2 mice when compared to 2N mice (Fig. 2C & D). No differences in both early and late endosomal area were observed between neurons in brains of TgCysC mice and 2N controls ($p < 0.06$ and $p < 0.32$ respectively) (Fig. 2A-D), showing that increased CysC expression alone does not impact endosome size. A rescue of the Ts2 endosomal changes was observed in the Ts2/TgCysC double transgenic mice (Fig. 2A-D).

Only a few lysosomes labeled with an antibody to the lysosomal marker protein 2 (LAMP2) contained CysC staining (Fig. 1D). Moreover, morphometric measurements of LAMP-2 labeled lysosomes in frontoparietal cortices did not show lysosomal changes in the brain of Ts2 mice (supplementary figure). Western blot analyses of hemibrain homogenates using antibodies to the lysosomal markers cathepsin D, cathepsin B and LAMP2 as well as assays of cathepsin B and D activity did not show any changes across the mice groups (supplementary figure), suggesting that the CysC rescue mechanism does not involve cathepsin inhibition in Ts2 mice (supplementary figure).

3.2 CysC prevents trisomy-induced cholinergic cell loss in the basal forebrain

BFCNs depend upon trophic support mediated by the retrograde transport of signaling endosomes, a process that fails in DS (Salehi et al., 2006; Xu et al., 2016). Compared to ChAT positive cells, a marker of cholinergic neurons, in the MSN of diploid 2N mice, ChAT-positive cells in the MSN of Ts2 mice were pyknotic and displayed dystrophic neurites, with fiber deafferentation and retracted neurites, as previously reported (Jiang et al., 2016) (Fig. 3A). Using quantitative unbiased stereology, we found that Ts2 mice had significantly fewer BFCNs compared to 2N mice (Fig. 3B). Consistent with the CysC rescue

of early endosomes in the Ts2 mice, increased CysC expression prevented the loss of BFCNs, restoring cell numbers to the same number as in the brain of diploid 2N mice (Fig. 3A & B).

3.3 CysC overexpression prevents nesting and spatial memory dysfunctions in Ts2 mice

Given that increased CysC expression in Ts2 mice protects BFCN in the brain of Ts2/TgCysC mice, we examined whether CysC also prevents trisomy-induced memory deficits in Ts2 mice. Ts2 mice performed poorly in nest formation as compared to their 2N diploid littermates, a measure of hippocampus dependent non-learned innate behavior (Kaur et al., 2014) (Fig. 4A). Ts2 mice overexpressing CysC performed better in nest formation as compared to Ts2 mice, similar to the performance of 2N mice (Fig. 4A). CysC overexpression by itself did not affect the mice's nesting behavior (Fig. 4A).

Similarly, Ts2 mice are dysfunctional in the hippocampus-dependent object placement spatial memory task (Kaur et al., 2014). To investigate whether CysC overexpression in trisomic mice protects the hippocampus-dependent spatial memory, investigation times were compared among the groups. Analysis of differences between training and test trials within each group confirmed the spatial memory dysfunction in Ts2 mice and behavioral task effectiveness in 2N control mice (Fig. 4B). One-way ANOVA comparison of moved object investigation times during the test trial (trial 2) made across the conditions showed a deficit in Ts2 mice for this spatial memory task by exhibiting lower exploration time for the displaced object as compared to 2N mice, whereas Ts2/TgCysC mice spent more time exploring the displaced object than the non-displaced object, as did 2N controls and singly TgCysC mice, revealing improved functional spatial memory task (Fig. 4B).

3.4 CysC activates the PI3K pathway in the Ts2 mouse brain

It was previously suggested that the PI3K/AKT/mTOR axis controls several key pathways involved in AD (Perluigi et al., 2014) and that individuals with DS have hyperactivation of the PI3K/AKT/mTOR axis (Perluigi et al., 2014). Earlier reports suggested that mTOR pathway deregulation may occur during early brain development in patients with DS (Iyer et al., 2014). In order to explore the mechanism utilized by CysC to prevent endocytic pathway abnormality and neurodegeneration in the brains of Ts2 mice, we evaluated the PI3K/AKT/mTOR axis. Western blot analysis of hemibrain homogenates of 8 month-old littermate mice with antibodies to PI3K, AKT and P70S6K proteins and the activation of phospho-sites on each of these proteins showed higher phosphorylation of each of the proteins in the brain of Ts2/TgCysC mice as compared to the non-transgenic control and the two single transgenic groups (Fig. 5A, B, C & D). Western blot analysis showed decreased mTOR phosphorylation in the brains of Ts2/TgCysC mice as compared to Ts2 mice and 2N mice (Fig. 5 A & E). None of these changes were mediated by CysC alone, in the absence of Ts2 chromosomal triplication. The decrease in mTOR phosphorylation in the brain of Ts2/TgCysC mice is accompanied by increased p70S6K phosphorylation, a direct downstream target of mTOR activity and an indirect measure of mTOR phosphorylation. These data reveal that CysC overexpression activates the PI3K/AKT pathway in the brain of Ts2 mice.

Earlier reports have shown the accumulation of neurodegeneration-related protein α -synuclein in the brain of one-third of AD and DS patients, and its accumulation in dystrophic neurites associated with senile plaques (Wirths and Bayer, 2003). Formation of α -synuclein Lewy neurite-like aggregates in axons is associated with dysfunction of transport of endosomes (Volpicelli-Daley et al., 2014). Disrupted protein trafficking and degradation via the endosomal pathway has been shown to contribute to α -synuclein accumulation (Perrett et al., 2015). Moreover, intracerebral injection of CysC in Parkinson's mouse model downregulated α -synuclein levels and protected against α -synuclein accumulation in the brain by inducing autophagic clearance (Zou et al., 2017). Consistently, Western blot analysis of hemibrains of 8 month-old mice showed higher levels of α -synuclein in the brain of Ts2 as compared to 2N (Fig. 5A). We further observed that Ts2/TgCysC mice that displayed normal endosomal function, exhibited normal α -synuclein levels similar to 2N mouse brains (Fig. 5A & F) indicating that CysC prevented the impairment of cellular clearance in the brain of Ts2 mice.

4. Discussion

Multiple *in vitro* and *in vivo* studies have demonstrated that CysC plays protective roles in neurodegenerative conditions (Acikgoz et al., 2014; Fang et al., 2017; Hu et al., 2013; Kaur et al., 2010; Liu et al., 2013; Liu et al., 2014; Martinez-Vargas et al., 2014; Mi et al., 2007; Tizon et al., 2010; Yang et al., 2015). *In vitro* studies have shown that the effects of CysC are biphasic, with lower levels of CysC being protective and higher concentrations toxic (Tizon et al., 2010). Similarly, high concentration of CysC in an *in vivo* model of post-traumatic brain injury is associated with neuronal injury (Martinez-Vargas et al., 2014; Nagai et al., 2002). Thus, the fact that our TgCysC mice have a slightly higher level of CysC expression compared to controls and the finding that this level of CysC expression is protective in Ts2 mice are consistent with the data that low levels of CysC expression are protective. Our data also suggest that low level of CysC overexpression over a lifetime might be sufficient to prevent DS-induced pathologies.

Various CysC-induced neuroprotective mechanisms have been shown under different stress and pathogenic conditions [reviewed in (Gauthier et al., 2011; Kaur and Levy, 2012; Mathews and Levy, 2016)]. We have investigated the involvement of these diverse mechanisms in order to determine the rescue mechanism activated in the Ts2/TgCysC mice:

i) Protection by inhibition of cysteine proteases: CysC was originally identified as an endogenous inhibitor of cysteine protease activity [reviewed in (Bernstein et al., 1996; Turk et al., 2008)]. *In vivo* protection by inhibition of cathepsin B and D activity was found in a mouse model of the inherited neurodegenerative disorder, progressive myoclonic epilepsy type 1 where loss-of-function mutations in the cystatin B gene results in enhanced cathepsin B and D activities, reduced to normal levels by CysC overexpression (Kaur et al., 2010). We did not find differences in the cathepsin B and D expression levels and activity between Ts2, Ts2/TgCysC, TgCysC, and 2N control littermate mice, suggesting that the CysC rescue mechanism does not involve cathepsin inhibition in Ts2 mice (supplementary figure).

ii) Protection by reducing APP metabolism: it was previously shown that the levels of APP and the β -secretase-cleaved APP carboxyl-terminal fragment contribute to endosomal disruption in neurons (Cataldo et al., 2001; Cataldo et al., 2003; Choi et al., 2013; Jiang et al., 2010; Jiang et al., 2016; Nixon, 2017; Xu et al., 2016). Western blot analysis did not find an effect of CysC overexpression on the levels of APP and its metabolites in the brain of Ts2/TgCysC mice, revealing that the CysC prevention of the endosomal pathology does not involve changes in APP expression or processing (data not shown).

iii) Protection by inhibition of A β aggregation: it was demonstrated that CysC binds soluble A β and prevents A β aggregation into oligomeric and fibrillar forms of the peptide (Kaesler et al., 2007; Mi et al., 2007; Sastre et al., 2004; Selenica et al., 2007; Tizon et al., 2010). Given the absence of A β aggregation in the brain of Ts2 mice, this is not the mechanism of protection.

iv) Protection by induction of autophagy: CysC induces autophagy, preventing cell death under various conditions (Gauthier et al., 2011; Tizon et al., 2010; Watanabe et al., 2014). Moreover, CysC treatment recovered autophagy function displayed by macrophages isolated from CysC knockout mice (Li et al., 2016). *In vivo* data also showed that CysC protects against early brain injury and associated learning deficits under experimental hemorrhage conditions by inducing autophagic mechanisms (Liu et al., 2013; Liu et al., 2014). It was also shown that CysC promotes neuronal survival through autophagic clearance of α -synuclein aggregation (Zou et al., 2017). However, we didn't observe an effect of CysC overexpression on the autophagic markers Beclin ATG5, and LC3-II levels in Ts2/TgCysC mice (supplementary figure).

In this study we identified a novel neuroprotective mechanism activated by CysC, via the PI3K/AKT/mTOR pathway. Earlier data suggested the impairment of PI3K/AKT/mTOR axis in the brain of DS patients as a key-contributing factor to the neurodegenerative process that culminates in Alzheimer-like dementia (Perluigi et al., 2014). Associated with abnormal hippocampal synaptic plasticity, altered PI3K/AKT activities were observed in the brain of the Ts65Dn mouse model of DS (Siarey et al., 2006). Downstream the PI3K/AKT axis, disturbance in mTOR signaling has been implicated in AD (Tramutola et al., 2015) and DS (Di Domenico et al., 2017; Perluigi et al., 2015). mTOR alterations were found to cause early hippocampal developmental abnormalities and functional impairment in the DS brain (Iyer et al., 2014). mTOR hyperactivation and reduced autophagy were further shown to contribute to accumulation of protein oxidative damage in Ts65Dn mice (Tramutola et al., 2016) and the mTOR inhibitor rapamycin was shown to improve cognition in Ts1Cje mice, another DS model (Andrade-Talavera et al., 2015). Several findings support the role of mTOR inhibitors as potential therapeutic agents to reduce AD hallmarks and recover cognitive performances [reviewed in (Tramutola et al., 2017)].

In addition, PI3K/AKT pathway activation was suggested to play an important role in synaptic development and neuronal survival and as a potential treatment moderator in neurodegenerative damage [reviewed in (Heras-Sandoval et al., 2014; Huang et al., 2017)]. It was suggested that PI3K/AKT pathway activation can be induced by multiple growth factors via receptor tyrosine kinases [reviewed in (Zhang et al., 2013)]. Elevation of

PI3K/AKT activity as a result of different treatment modalities induced neuronal survival in motoneurons (Llado et al., 2013), in the hippocampus (Dong et al., 2016), and in the brain of Zebrafish (Chen et al., 2017). It was also shown that neuroprotection by carbenoxolone against ischemia injury involves the PI3K/AKT pathway (Wang et al., 2015). Similarly, cellular stress-induced up-regulation of fragile X linked mental retardation protein promoted cell survival by modulating the PI3K/AKT phosphorylation cascade (Jeon et al., 2011). Blocking PI3K with the inhibitor LY294002 caused a significant reduction in brain size in addition to growth retardation during Zebrafish embryogenesis, recovered by the overexpression of myristoylated AKT, a constitutive form of AKT (Chen et al., 2017). Moreover, activation of PI3K/AKT has been linked to survival of neurons exposed to amyloid β 25-35 neurotoxicity (Kwon et al., 2015). Consistent with these reports, our data reveal that PI3K/AKT pathway activation by CysC overexpression in Ts2 mice induces neuronal survival.

An additional axis activated by PI3K is the endocytic pathway, functioning as an rab5 effector protein (Christoforidis et al., 1999). It was shown that the p85 α subunit of PI3K binds directly to and activates rab5 (Anderson and Chamberlain, 2005). In addition to the regulation of the early endosomal protein rab5 (Vieira et al., 2003), PI3K regulates rab7 and late endocytic trafficking (Jaber et al., 2016). Rab5 recruitment in phagosomes is essential for rab7 recruitment and for the progression to phagolysosomes (Vieira et al., 2003).

5. Conclusions

Our data show that CysC overexpression in the Ts2 mouse model of DS stimulates the intracellular PI3K pathway signaling, activating the early and late endocytic pathways, leading to a normal flux throughout the endosomal system, and preventing the trisomy-induced behavioral dysfunctions. The neuroprotective mechanism activated by CysC in Ts2 mice suggests novel prospective therapeutic candidates for drugs developments targeting the PI3K/AKT pathway in order to prevent neurodegeneration and cognitive changes in DS and AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Research reported in this publication was supported by the National Institutes of Health (AG017617 and AG056732).

Grant numbers and resources: National Institutes of Health (AG017617, AG056732). The authors declare no competing financial interest.

References

- Acikgoz S, Can M, Guven B, Edebali N, Barut F, Buyukuysal C, Tekin IO, Acikgoz B, 2014 Cystatin C and neuropeptide Y levels in brain tissues after experimental subarachnoid hemorrhage. *Acta Biochim Pol* 61(4), 825–828. [PubMed: 25566547]

- Anderson DH, Chamberlain MD, 2005 Assay and stimulation of the Rab5 GTPase by the p85 alpha subunit of phosphatidylinositol 3-kinase. *Methods Enzymol* 403, 552–561. [PubMed: 16473619]
- Andrade-Talavera Y, Benito I, Casanas JJ, Rodriguez-Moreno A, Montesinos ML, 2015 Rapamycin restores BDNF-LTP and the persistence of long-term memory in a model of Down's syndrome. *Neurobiol Dis* 82, 516–525. [PubMed: 26388397]
- Bernstein HG, Kirschke H, Wiederanders B, Pollak KH, Zipress A, Rinne A, 1996 The possible place of cathepsins and cystatins in the puzzle of Alzheimer disease: a review. *Mol Chem Neuropathol* 27(3), 225–247. [PubMed: 9147410]
- Browne SE, Lin L, Mattsson A, Georgievskaya B, Isacson O, 2001 Selective antibody-induced cholinergic cell and synapse loss produce sustained hippocampal and cortical hypometabolism with correlated cognitive deficits. *Exp Neurol* 170(1), 36–47. [PubMed: 11421582]
- Casanova MF, Walker LC, Whitehouse PJ, Price DL, 1985 Abnormalities of the nucleus basalis in Down's syndrome. *Ann Neurol* 18(3), 310–313. [PubMed: 2932050]
- Cataldo A, Rebeck GW, Ghetti B, Hulette C, Lippa C, Van Broeckhoven C, van Duijn C, Cras P, Bogdanovic N, Bird T, Peterhoff C, Nixon R, 2001 Endocytic disturbances distinguish among subtypes of Alzheimer's disease and related disorders. *Ann Neurol* 50(5), 661–665. [PubMed: 11706973]
- Cataldo AM, Petanceska S, Peterhoff CM, Terio NB, Epstein CJ, Villar A, Carlson EJ, Staufenbiel M, Nixon RA, 2003 App gene dosage modulates endosomal abnormalities of Alzheimer's disease in a segmental trisomy 16 mouse model of down syndrome. *J Neurosci* 23(17), 6788–6792. [PubMed: 12890772]
- Chen S, Liu Y, Rong X, Li Y, Zhou J, Lu L, 2017 Neuroprotective Role of the PI3 Kinase/Akt Signaling Pathway in Zebrafish. *Front Endocrinol (Lausanne)* 8, 21. [PubMed: 28228749]
- Choi DW, 1988 Glutamate neurotoxicity and diseases of the nervous system. *Neuron* 1(8), 623–634. [PubMed: 2908446]
- Choi JH, Kaur G, Mazzella MJ, Morales-Corraliza J, Levy E, Mathews PM, 2013 Early endosomal abnormalities and cholinergic neuron degeneration in amyloid-beta protein precursor transgenic mice. *J Alzheimers Dis* 34(3), 691–700. [PubMed: 23254640]
- Christoforidis S, Miaczynska M, Ashman K, Wilm M, Zhao L, Yip SC, Waterfield MD, Backer JM, Zerial M, 1999 Phosphatidylinositol-3-OH kinases are Rab5 effectors. *Nat Cell Biol* 1(4), 249–252. [PubMed: 10559924]
- Das D, Phillips C, Hsieh W, Sumanth K, Dang V, Salehi A, 2014 Neurotransmitter-based strategies for the treatment of cognitive dysfunction in Down syndrome. *Prog Neuropsychopharmacol Biol Psychiatry* 54, 140–148. [PubMed: 24842803]
- Davisson MT, Schmidt C, Akeson EC, 1990 Segmental trisomy of murine chromosome 16: a new model system for studying Down syndrome. *Prog Clin Biol Res* 360, 263–280. [PubMed: 2147289]
- Deacon RM, 2006 Assessing nest building in mice. *Nat Protoc* 1(3), 1117–1119. [PubMed: 17406392]
- Di Domenico F, Tramutola A, Foppoli C, Head E, Perluigi M, Butterfield DA, 2018 mTOR in Down syndrome: Role in Ass and tau neuropathology and transition to Alzheimer disease-like dementia. *Free Radic Biol Med* 114, 94–101. [PubMed: 28807816]
- Dong XZ, Wang DX, Yu BY, Liu P, Hu Y, 2016 Kai-Xin-San, a traditional Chinese medicine formulation, exerts antidepressive and neuroprotective effects by promoting pCREB upstream pathways. *Exp Ther Med* 12(5), 3308–3314. [PubMed: 27882154]
- Dorph-Petersen KA, Nyengaard JR, Gundersen HJ, 2001 Tissue shrinkage and unbiased stereological estimation of particle number and size. *J Microsc* 204(Pt 3), 232–246. [PubMed: 11903800]
- Fang Z, Deng J, Wu Z, Dong B, Wang S, Chen X, Nie H, Dong H, Xiong L, 2017 Cystatin C Is a Crucial Endogenous Protective Determinant Against Stroke. *Stroke* 48(2), 436–444. [PubMed: 27999137]
- Fodale V, Mafra F, Caminiti V, Grasso G, 2006 The cholinergic system in Down's syndrome. *J Intellect Disabil* 10(3), 261–274. [PubMed: 16916850]
- Gauthier S, Kaur G, Mi W, Tizon B, Levy E, 2011 Protective mechanisms by cystatin C in neurodegenerative diseases. *Front Biosci (Schol Ed)* 3, 541–554. [PubMed: 21196395]

- Granhölm AC, Sanders LA, Crnic LS, 2000 Loss of cholinergic phenotype in basal forebrain coincides with cognitive decline in a mouse model of Down's syndrome. *Exp Neurol* 161(2), 647–663. [PubMed: 10686084]
- Guerra F, Bucci C, 2016 Multiple Roles of the Small GTPase Rab7. *Cells* 5(3).
- Head E, Lott IT, Wilcock DM, Lemere CA, 2016 Aging in Down Syndrome and the Development of Alzheimer's Disease Neuropathology. *Curr Alzheimer Res* 13(1), 18–29. [PubMed: 26651341]
- Heras-Sandoval D, Perez-Rojas JM, Hernandez-Damian J, Pedraza-Chaverri J, 2014 The role of PI3K/AKT/mTOR pathway in the modulation of autophagy and the clearance of protein aggregates in neurodegeneration. *Cell Signal* 26(12), 2694–2701. [PubMed: 25173700]
- Holtzman DM, Li YW, DeArmond SJ, McKinley MP, Gage FH, Epstein CJ, Mobley WC, 1992 Mouse model of neurodegeneration: atrophy of basal forebrain cholinergic neurons in trisomy 16 transplants. *Proc Natl Acad Sci U S A* 89(4), 1383–1387. [PubMed: 1741393]
- Holtzman DM, Santucci D, Kilbridge J, Chua-Couzens J, Fontana DJ, Daniels SE, Johnson RM, Chen K, Sun Y, Carlson E, Alleva E, Epstein CJ, Mobley WC, 1996 Developmental abnormalities and age-related neurodegeneration in a mouse model of Down syndrome. *Proc Natl Acad Sci U S A* 93(23), 13333–13338. [PubMed: 8917591]
- Hu Y, Hung AC, Cui H, Dawkins E, Bolos M, Foa L, Young KM, Small DH, 2013 Role of cystatin C in amyloid precursor protein-induced proliferation of neural stem/progenitor cells. *J Biol Chem* 288(26), 18853–18862. [PubMed: 23671283]
- Huang H, Liu H, Yan R, Hu M, 2017 PI3K/Akt and ERK/MAPK Signaling Promote Different Aspects of Neuron Survival and Axonal Regrowth Following Rat Facial Nerve Axotomy. *Neurochem Res* 42(12), 3515–3524. [PubMed: 28993995]
- Iyer AM, van Scheppingen J, Milenkovic I, Anink JJ, Adle-Biassette H, Kovacs GG, Aronica E, 2014 mTOR Hyperactivation in down syndrome hippocampus appears early during development. *J Neuropathol Exp Neurol* 73(7), 671–683. [PubMed: 24918639]
- Jaber N, Mohd-Naim N, Wang Z, DeLeon JL, Kim S, Zhong H, Sheshadri N, Dou Z, Edinger AL, Du G, Braga VM, Zong WX, 2016 Vps34 regulates Rab7 and late endocytic trafficking through recruitment of the GTPase-activating protein Arp2/3. *J Cell Sci* 129(23), 4424–4435. [PubMed: 27793976]
- Jeon SJ, Seo JE, Yang SI, Choi JW, Wells D, Shin CY, Ko KH, 2011 Cellular stress-induced up-regulation of FMRP promotes cell survival by modulating PI3K-Akt phosphorylation cascades. *J Biomed Sci* 18, 17. [PubMed: 21314987]
- Jiang Y, Mullaney KA, Peterhoff CM, Che S, Schmidt SD, Boyer-Boiteau A, Ginsberg SD, Cataldo AM, Mathews PM, Nixon RA, 2010 Alzheimer's-related endosome dysfunction in Down syndrome is Aβ-independent but requires APP and is reversed by BACE-1 inhibition. *Proc Natl Acad Sci U S A* 107(4), 1630–1635. [PubMed: 20080541]
- Jiang Y, Rigoglioso A, Peterhoff CM, Pawlik M, Sato Y, Bleiwas C, Stavrides P, Smiley JF, Ginsberg SD, Mathews PM, Levy E, Nixon RA, 2016 Partial BACE1 reduction in a Down syndrome mouse model blocks Alzheimer-related endosomal anomalies and cholinergic neurodegeneration: role of APP-CTF. *Neurobiol Aging* 39, 90–98. [PubMed: 26923405]
- Kaesler SA, Herzig MC, Coomaraswamy J, Kilger E, Selenica ML, Winkler DT, Staufenbiel M, Levy E, Grubb A, Jucker M, 2007 Cystatin C modulates cerebral β-amyloidosis. *Nat Genet* 39(12), 1437–1439. [PubMed: 18026102]
- Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, Grzeschik KH, Multhaup G, Beyreuther K, Müller-Hill B, 1987 The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* 325(6106), 733–736. [PubMed: 2881207]
- Kaur G, Levy E, 2012 Cystatin C in Alzheimer's disease. *Front Mol Neurosci* 5, 79. [PubMed: 22783166]
- Kaur G, Mohan P, Pawlik M, DeRosa S, Fajicula J, Che S, Grubb A, Ginsberg SD, Nixon RA, Levy E, 2010 Cystatin C rescues degenerating neurons in a cystatin B-knockout mouse model of progressive myoclonus epilepsy. *Am J Pathol* 177(5), 2256–2267. [PubMed: 20889561]
- Kaur G, Pawlik M, Gandy SE, Ehrlich ME, Smiley JF, Levy E, 2017 Lysosomal dysfunction in the brain of a mouse model with intraneuronal accumulation of carboxyl terminal fragments of the amyloid precursor protein. *Mol Psychiatry* 22(7), 981–989. [PubMed: 27777419]

- Kaur G, Sharma A, Xu W, Gerum S, Alldred MJ, Subbanna S, Basavarajappa BS, Pawlik M, Ohno M, Ginsberg SD, Wilson DA, Guilfoyle DN, Levy E, 2014 Glutamatergic transmission aberration: a major cause of behavioral deficits in a murine model of Down's syndrome. *J Neurosci* 34(15), 5099–5106. [PubMed: 24719089]
- Kwon SH, Ma SX, Hwang JY, Lee SY, Jang CG, 2015 Involvement of the Nrf2/HO-1 signaling pathway in sulfuretin-induced protection against amyloid beta25–35 neurotoxicity. *Neuroscience* 304, 14–28. [PubMed: 26192096]
- Li W, Sultana N, Siraj N, Ward LJ, Pawlik M, Levy E, Jovinge S, Bengtsson E, Yuan XM, 2016 Autophagy dysfunction and regulatory cystatin C in macrophage death of atherosclerosis. *J Cell Mol Med* 20(9), 1664–1672. [PubMed: 27079462]
- Lin L, Georgievska B, Mattsson A, Isacson O, 1999 Cognitive changes and modified processing of amyloid precursor protein in the cortical and hippocampal system after cholinergic synapse loss and muscarinic receptor activation. *Proc Natl Acad Sci U S A* 96(21), 12108–12113. [PubMed: 10518584]
- Liu Y, Cai H, Wang Z, Li J, Wang K, Yu Z, Chen G, 2013 Induction of autophagy by cystatin C: a potential mechanism for prevention of cerebral vasospasm after experimental subarachnoid hemorrhage. *Eur J Med Res* 18, 21. [PubMed: 23816364]
- Liu Y, Li J, Wang Z, Yu Z, Chen G, 2014 Attenuation of early brain injury and learning deficits following experimental subarachnoid hemorrhage secondary to Cystatin C: possible involvement of the autophagy pathway. *Mol Neurobiol* 49(2), 1043–1054. [PubMed: 24203677]
- Llado J, Tolosa L, Olmos G, 2013 Cellular and molecular mechanisms involved in the neuroprotective effects of VEGF on motoneurons. *Front Cell Neurosci* 7, 181. [PubMed: 24155688]
- Luine VN, Jacome LF, Maclusky NJ, 2003 Rapid enhancement of visual and place memory by estrogens in rats. *Endocrinology* 144(7), 2836–2844. [PubMed: 12810538]
- Macbeth AH, Gautreaux C, Luine VN, 2008 Pregnant rats show enhanced spatial memory, decreased anxiety, and altered levels of monoaminergic neurotransmitters. *Brain Res* 1241, 136–147. [PubMed: 18823955]
- Martinez-Vargas M, Soto-Nunez M, Tabla-Ramon E, Solis B, Gonzalez-Rivera R, Perez-Arredondo A, Estrada-Rojas F, Castell A, Molina-Guarneros J, Navarro L, 2014 Cystatin C has a dual role in post-traumatic brain injury recovery. *Int J Mol Sci* 15(4), 5807–5820. [PubMed: 24714089]
- Mathews PM, Levy E, 2016 Cystatin C in aging and in Alzheimer's disease. *Ageing Res Rev* 32, 38–50. [PubMed: 27333827]
- Mi W, Pawlik M, Sastre M, Jung SS, Radvinsky DS, Klein AM, Sommer J, Schmidt SD, Nixon RA, Mathews PM, Levy E, 2007 Cystatin C inhibits amyloid-beta deposition in Alzheimer's disease mouse models. *Nat Genet* 39(12), 1440–1442. [PubMed: 18026100]
- Nagai A, Ryu JK, Kobayash S, Kim SU, 2002 Cystatin C induces neuronal cell death in vivo. *Ann N Y Acad Sci* 977, 315–321. [PubMed: 12480767]
- Nixon RA, 2017 Amyloid precursor protein and endosomal-lysosomal dysfunction in Alzheimer's disease: inseparable partners in a multifactorial disease. *FASEB J* 31(7), 2729–2743. [PubMed: 28663518]
- Pawlik M, Sastre M, Calero M, Mathews PM, Schmidt SD, Nixon RA, Levy E, 2004 Overexpression of human cystatin C in transgenic mice does not affect levels of endogenous brain amyloid Beta Peptide. *J Mol Neurosci* 22(1–2), 13–18. [PubMed: 14742906]
- Perluigi M, Di Domenico F, Butterfield DA, 2015 mTOR signaling in aging and neurodegeneration: At the crossroad between metabolism dysfunction and impairment of autophagy. *Neurobiol Dis* 84, 39–49. [PubMed: 25796566]
- Perluigi M, Pupo G, Tramutola A, Cini C, Coccia R, Barone E, Head E, Butterfield DA, Di Domenico F, 2014 Neuropathological role of PI3K/Akt/mTOR axis in Down syndrome brain. *Biochim Biophys Acta* 1842(7), 1144–1153. [PubMed: 24735980]
- Perrett RM, Alexopoulou Z, Tofaris GK, 2015 The endosomal pathway in Parkinson's disease. *Mol Cell Neurosci* 66(Pt A), 21–28. [PubMed: 25701813]
- Powers BE, Velazquez R, Kelley CM, Ash JA, Strawderman MS, Alldred MJ, Ginsberg SD, Mufson EJ, Strupp BJ, 2016 Attentional function and basal forebrain cholinergic neuron morphology

- during aging in the Ts65Dn mouse model of Down syndrome. *Brain Struct Funct* 221(9), 4337–4352. [PubMed: 26719290]
- Rueda N, Florez J, Martinez-Cue C, 2012 Mouse models of Down syndrome as a tool to unravel the causes of mental disabilities. *Neural Plast* 2012, 584071. [PubMed: 22685678]
- Salehi A, Delcroix JD, Belichenko PV, Zhan K, Wu C, Valletta JS, Takimoto-Kimura R, Kleschevnikov AM, Sambamurti K, Chung PP, Xia W, Villar A, Campbell WA, Kulnane LS, Nixon RA, Lamb BT, Epstein CJ, Stokin GB, Goldstein LS, Mobley WC, 2006 Increased App expression in a mouse model of Down's syndrome disrupts NGF transport and causes cholinergic neuron degeneration. *Neuron* 51(1), 29–42. [PubMed: 16815330]
- Sastre M, Calero M, Pawlik M, Mathews PM, Kumar A, Danilov V, Schmidt SD, Nixon RA, Frangione B, Levy E, 2004 Binding of cystatin C to Alzheimer's amyloid beta inhibits in vitro amyloid fibril formation. *Neurobiol Aging* 25(8), 1033–1043. [PubMed: 15212828]
- Selenica ML, Wang X, Ostergaard-Pedersen L, Westlind-Danielsson A, Grubb A, 2007 Cystatin C reduces the in vitro formation of soluble Abeta1–42 oligomers and protofibrils. *Scand J Clin Lab Invest* 67(2), 179–190. [PubMed: 17365997]
- Sengupta S, Peterson TR, Sabatini DM, 2010 Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. *Mol Cell* 40(2), 310–322. [PubMed: 20965424]
- Seo H, Isacson O, 2005 Abnormal APP, cholinergic and cognitive function in Ts65Dn Down's model mice. *Exp Neurol* 193(2), 469–480. [PubMed: 15869949]
- Siarey RJ, Kline-Burgess A, Cho M, Balbo A, Best TK, Harashima C, Klann E, Galdzicki Z, 2006 Altered signaling pathways underlying abnormal hippocampal synaptic plasticity in the Ts65Dn mouse model of Down syndrome. *J Neurochem* 98(4), 1266–1277. [PubMed: 16895585]
- Simpson JC, Jones AT, 2005 Early endocytic Rabs: functional prediction to functional characterization. *Biochem Soc Symp*(72), 99–108. [PubMed: 15649134]
- Smiley JF, Konnova K, Bleiwas C, 2012 Cortical thickness, neuron density and size in the inferior parietal lobe in schizophrenia. *Schizophr Res* 136(1–3), 43–50. [PubMed: 22304984]
- Tizon B, Sahoo S, Yu H, Gauthier S, Kumar AR, Mohan P, Figliola M, Pawlik M, Grubb A, Uchiyama Y, Bandyopadhyay U, Cuervo AM, Nixon RA, Levy E, 2010 Induction of autophagy by cystatin C: a mechanism that protects murine primary cortical neurons and neuronal cell lines. *PLoS One* 5(3), e9819. [PubMed: 20352108]
- Tramutola A, Lanzillotta C, Arena A, Barone E, Perluigi M, Di Domenico F, 2016 Increased Mammalian Target of Rapamycin Signaling Contributes to the Accumulation of Protein Oxidative Damage in a Mouse Model of Down's Syndrome. *Neurodegener Dis* 16(1–2), 62–68. [PubMed: 26606243]
- Tramutola A, Triplett JC, Di Domenico F, Niedowicz DM, Murphy MP, Coccia R, Perluigi M, Butterfield DA, 2015 Alteration of mTOR signaling occurs early in the progression of Alzheimer disease (AD): analysis of brain from subjects with pre-clinical AD, amnesic mild cognitive impairment and late-stage AD. *J Neurochem* 133(5), 739–749. [PubMed: 25645581]
- Turk V, Stoka V, Turk D, 2008 Cystatins: biochemical and structural properties, and medical relevance. *Front Biosci* 13, 5406–5420. [PubMed: 18508595]
- Velazquez R, Ash JA, Powers BE, Kelley CM, Strawderman M, Luscher ZI, Ginsberg SD, Mufson EJ, Strupp BJ, 2013 Maternal choline supplementation improves spatial learning and adult hippocampal neurogenesis in the Ts65Dn mouse model of Down syndrome. *Neurobiol Dis* 58, 92–101. [PubMed: 23643842]
- Vieira OV, Bucci C, Harrison RE, Trimble WS, Lanzetti L, Gruenberg J, Schreiber AD, Stahl PD, Grinstein S, 2003 Modulation of Rab5 and Rab7 recruitment to phagosomes by phosphatidylinositol 3-kinase. *Mol Cell Biol* 23(7), 2501–2514. [PubMed: 12640132]
- Villar AJ, Belichenko PV, Gillespie AM, Kozy HM, Mobley WC, Epstein CJ, 2005 Identification and characterization of a new Down syndrome model, Ts[Rb(12.1716)]2Cje, resulting from a spontaneous Robertsonian fusion between T(171)65Dn and mouse chromosome 12. *Mamm Genome* 16(2), 79–90. [PubMed: 15859352]
- Volpicelli-Daley LA, Gamble KL, Schultheiss CE, Riddle DM, West AB, Lee VM, 2014 Formation of alpha-synuclein Lewy neurite-like aggregates in axons impedes the transport of distinct endosomes. *Mol Biol Cell* 25(25), 4010–4023. [PubMed: 25298402]

- Wang YK, Deng F, Miao J, Xie H, Feng JC, 2015 Neuroprotection by Carbenoxolone Against Ischemia Injury Involves PI3K/Akt Pathway. *Clin Lab* 61(10), 1561–1568. [PubMed: 26642720]
- Watanabe S, Hayakawa T, Wakasugi K, Yamanaka K, 2014 Cystatin C protects neuronal cells against mutant copper-zinc superoxide dismutase-mediated toxicity. *Cell Death Dis* 5, e1497. [PubMed: 25356866]
- Wirths O, Bayer TA, 2003 Alpha-synuclein, Abeta and Alzheimer's disease. *Prog Neuropsychopharmacol Biol Psychiatry* 27(1), 103–108. [PubMed: 12551731]
- Wisniewski KE, Laure-Kamionowska M, Wisniewski HM, 1984 Evidence of arrest of neurogenesis and synaptogenesis in brains of patients with Down's syndrome. *N Engl J Med* 311(18), 1187–1188.
- Xu W, Weissmiller AM, White JA, 2nd, Fang F, Wang X, Wu Y, Pearn ML, Zhao X, Sawa M, Chen S, Gunawardena S, Ding J, Mobley WC, Wu C, 2016 Amyloid precursor protein-mediated endocytic pathway disruption induces axonal dysfunction and neurodegeneration. *J Clin Invest* 126(5), 1815–1833. [PubMed: 27064279]
- Yang B, Zhu J, Miao Z, Zhou B, Ge W, Zhao H, Xu X, 2015 Cystatin C is an Independent Risk Factor and Therapeutic Target for Acute Ischemic Stroke. *Neurotox Res* 28(1), 1–7. [PubMed: 25697425]
- Yates CM, Simpson J, Gordon A, Maloney AF, Allison Y, Ritchie IM, Urquhart A, 1983 Catecholamines and cholinergic enzymes in pre-senile and senile Alzheimer-type dementia and Down's syndrome. *Brain Res* 280(1), 119–126. [PubMed: 6228286]
- Zhang L, Zhou F, ten Dijke P, 2013 Signaling interplay between transforming growth factor-beta receptor and PI3K/AKT pathways in cancer. *Trends Biochem Sci* 38(12), 612–620. [PubMed: 24239264]
- Zou J, Chen Z, Wei X, Chen Z, Fu Y, Yang X, Chen D, Wang R, Jenner P, Lu JH, Li M, Zhang Z, Tang B, Jin K, Wang Q, 2017 Cystatin C as a potential therapeutic mediator against Parkinson's disease via VEGF-induced angiogenesis and enhanced neuronal autophagy in neurovascular units. *Cell Death Dis* 8(6), e2854. [PubMed: 28569795]

Highlights:

- Cystatin prevents trisomy-induced pathologies in a Down syndrome mouse model
- Cystatin C neuroprotection is via activation of the PI3K/AKT signaling pathway
- PI3K/AKT stimulation leads to normal flux through the endosomal-lysosomal system
- Restoration of normal endosomal function prevents memory dysfunction
- Modulation of the PI3/AKT pathway, a novel therapy for neurodegenerative disorders

Figure 1. CysC expression level and its endosomal localization within cortical neurons. (A) Western blot analysis of cerebral homogenates of brains of 8 month-old littermate mice using an anti-CysC antibody shows a higher CysC expression in Ts2/TgCysC and TgCysC mice as compared to 2N diploid controls and Ts2 mice. (B) Densitometric analysis of the ratio of the CysC to the β -tubulin bands, normalized to the ratio in 2N mice. (C) Immunofluorescent labeling of CysC in cortical neurons in the frontoparietal region of the brain shows both cytosolic and punctate staining. Double-immunolabeling with antibodies to CysC and Rab5a in cortical neurons shows colocalization with early endosomes. (D) Double-immunolabeling with antibodies to CysC and Rab7a in cortical neurons shows localization within a small number of late endosomes. (E) Double-immunolabeling with antibodies to

CysC and LAMP2 shows CysC colocalization with a few lysosomes in cortical neurons. Nuclear boundaries in the immunofluorescent images are shown using a dotted line. Scale bars, 5 μm .

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2. CysC overexpression protects against altered endosomal morphology in the brain of Ts2 mice.

(A) Neurons in the frontoparietal cortex of Ts2 mice labeled with anti-rab5a antibody show enlarged early endosomes and increased immunoreactivity compared to age-matched 2N controls. Human CysC expression in Ts2/TgCysC mice prevented the early endosomal abnormality. (B) Enlargement of rab7a-positive late endosomes in the frontoparietal cortex in Ts2 mouse brains compared to age-matched 2N controls is prevented in Ts2/TgCysC mice. Morphometric measurements of rab5a-(C) and rab7a-(D) labeled endosomes reveal higher percentage of endosomal area per neuron in Ts2 mice as compare to 2N controls. The ratio of rab5a- and rab7a -positive endosomal area per neuron is normalized to 2N control mice in Ts2/TgCysC mice. Scale bars, 10 μ m (A), 5 μ m (B). Measurements are presented as the mean $\pm$ s.e.m. (n=4/5). Differences are significant at * p <0.05, ** p <0.01 and *** p <0.001.

Figure 3. BFCNs loss in the MSN induced by trisomy is prevented by CysC overexpression. (A) Ts2 mice exhibit a loss of choline acetyltransferase (ChAT)-immunopositive neurons in the MSN compared to 2N controls, whereas human CysC expression prevents the cholinergic cell loss in Ts2/TgCysC mice. (B) Unbiased stereological quantification of ChAT-immuno-positive cell number is depicted as cell number $\pm$ SEM. (n=5). Scale bar, 50 μ m. Differences are significant at $*p < 0.05$.

Figure 4. Deficits in nest construction and object recognition behavior in the Ts2 mice are prevented by CysC overexpression.

(A) 2N mice chewed the nestlets and organized them into well-defined nests whereas Ts2 mice exhibited a significant deficit in nest building, leaving the nestlets partially torn or poorly designed nests as compared to 2N mice. CysC overexpression in Ts2 mice alleviated the nesting dysfunction in Ts2/TgCysC mice. (B) Ts2 mice are deficient in object recognition as measured by the object-placement task. 2N mice showed a significant increase in sniff investigation of the moved object compared to the unmoved object. Ts2 mice spent the same time sniffing both objects, whereas Ts2/TgCysC mice behaved normally in this spatial memory task as shown by the differences between training and testing time, similar to 2N and TgCysC mice. All data are presented as mean $\pm$ SEM (n=10–12). The differences between the groups and between the moved and the unmoved object are significant at * p <0.05, *** p <0.001.