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Loss of the Tuberous Sclerosis Complex Protein Tuberin Causes Purkinje Cell Degeneration

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Abstract

Tuberous sclerosis complex (TSC) is a neurogenetic disorder that often causes brain abnormalities leading to epilepsy, developmental delay and autism. TSC is caused by inactivating mutations in either of the genes encoding the proteins hamartin (*TSC1*) and tuberin (*TSC2*). These proteins form a heterodimer that inhibits the mammalian target of rapamycin complex 1 (mTORC1) pathway, controlling translation and cell growth. Loss of either protein results in dysregulated mTORC1 activation, an important aspect of TSC pathogenesis. About thirty percent of TSC patients have cerebellar pathology that is poorly understood. To investigate the effects of TSC on the cerebellum, we created a mouse model in which the *Tsc2* gene was selectively deleted from Purkinje cells starting at postnatal day 6 (P6). The loss of *Tsc2* caused a progressive increase in Purkinje cell size and subsequent death from apoptosis. Purkinje cell loss was predominantly cell type specific and associated with motor deficits. Immunohistochemical analysis showed that both endoplasmic reticulum (ER) and oxidative stress were increased in *Tsc2*-null Purkinje cells. The cell death and ER stress phenotypes were rescued by treatment with the mTORC1 inhibitor rapamycin. To assess whether the murine Purkinje cell loss has a correlate to the human TSC, we analyzed postmortem cerebellum samples from TSC patients and detected Purkinje cell loss is half of the samples. Our results establish a critical role for the TSC complex in Purkinje cell survival by regulating ER and oxidative stress, and reveal a novel aspect of TSC neuropathology.

Keywords

Purkinje cell; *Tsc2*; Tuberin; Tuberous sclerosis complex; Neurodegeneration

Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant, tumor predisposition disorder that causes significant morbidity and mortality in children and adults (Crino et al., 2006; Marcotte and Crino, 2006). TSC derives its name from the characteristic cerebral cortical lesions called tubers. Other brain lesions include subependymal nodules, cerebellar tubers, white matter abnormalities, and hemimegalencephaly (DiMario, 2004). The central nervous system lesions cause morbidity and mortality due to seizures, developmental delay, autism

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spectrum disorders (ASD), and psychiatric disease. Though 30% of patients have cerebellar abnormalities, the neuropathology of these lesions remains largely unknown (DiMario, 2004).

TSC is caused by germ line mutations in either one of two genes, *TSC1* or *TSC2* (Consortium, 1993; van Slegtenhorst et al., 1997). Somatic cell loss of the second allele has been demonstrated in many disease lesions, though other mechanisms of pathogenesis are likely important (Henske et al., 1997). *TSC1* encodes hamartin, a protein that biochemically interacts with the product of the *TSC2* gene, tuberlin, forming the functional TSC heterodimer complex (Plank et al., 1998; van Slegtenhorst et al., 1998). A major function of the TSC complex is to regulate the rapamycin-sensitive form of the mTOR kinase, mTORC1 (Bhaskar and Hay, 2007; Huang et al., 2008; Sarbassov et al., 2005). Activated mTORC1 phosphorylates components of the translational apparatus, thereby stimulating cap-dependent protein synthesis, cell growth and proliferation. The TSC complex inhibits the mTORC1 kinase via the carboxy-terminal GTPase activating domain of tuberlin on the small Ras-like protein Rheb (Inoki et al., 2003; Zhang et al., 2003). Loss of either member of the TSC complex causes constitutive activation of the mTORC1 kinase, leading to unregulated phosphorylation of S6 kinase, ribosomal protein S6, and 4EBP1. The dysregulation of mTORC1 is a major determinant of the pathogenesis of TSC, as this pathway is activated in many TSC lesions (Crino et al., 2006; El-Hashemite et al., 2003). The bacterial metabolite rapamycin, an mTORC1 inhibitor, has alleviated morbidity and mortality in several mouse models of TSC (Lee et al., 2005; Meikle et al., 2008; Zeng et al., 2008). Rapamycin clinical trials for the treatment of human TSC have been encouraging (Bissler et al., 2008; Franz et al., 2006).

The cerebellum was traditionally thought to primarily control motor function; however, data suggest that it is also important for mood, personality, intellect and motor learning (Gordon, 2007). While thirty percent of TSC patients have cerebellar tubers, hemisphere hyperplasia and linear migration streaks, it is unclear how these lesions form and affect brain function (DiMario, 2004). Recent studies suggest that TSC might also lead to Purkinje cell death, a previously unidentified aspect of TSC-associated cerebellar pathology. A postmortem examination of the cerebellum of a 32 year-old man with TSC due to a *TSC2* mutation showed a marked reduction in Purkinje cells (Boer et al., 2008). As the sole output of the cerebellum, Purkinje cells project to deep nuclei, cerebral cortex, thalamus, and brain stem. Their loss would have significant consequences on cerebellar-mediated function. The Allen Brain Atlas demonstrates that both *Tsc1* and *Tsc2* are highly expressed in murine Purkinje cells (Lein et al., 2007). Based on these data, we hypothesized that the TSC complex and mTORC1 regulation might be important for Purkinje cell viability and function.

In this study we demonstrate that loss of *Tsc2* in Purkinje cells causes endoplasmic reticulum (ER) and oxidative stress, leading to progressive Purkinje cell apoptosis. Purkinje cell loss was mainly cell type specific; however, a *Tsc2* haploinsufficient cellular environment accelerated Purkinje cell death. Mutant animals demonstrated motor deficits due to Purkinje cell loss. The mutant phenotype is most likely due to mTORC1 activation, as rapamycin treatment alleviated ER stress, prevented cell death and attenuated the motor deficits. We also observed a human correlate of our murine phenotype. Purkinje cell loss was detected in human cerebellum samples from TSC patients. These results underscore an important role of the TSC complex in Purkinje cell viability and suggest that the cerebellum might be a site of unappreciated pathology in TSC patients.

Materials and Methods

Murine model

All animal experiments and care were conducted according to the guidelines of the UTHSC Animal Welfare Committee. Mice were on a combined 129 and C57BL/6J background. Generation of the *Tsc2^{+/-flox}* and *Tsc2^{+/-ko}* mice has been previously described (Way et al., 2009). The expression of Cre recombinase was driven by the *Pcp2* Purkinje cell protein specific promoter (Barski et al., 2000). Mice were genotyped for the presence of the *Tsc2^{flox}* and *Tsc2^{ko}* alleles using three primers in a single PCR reaction: WT2: 5'-CAGGCATGTCTGGAGTCTTG-3', KO1: 5'-GCAGCAGGTCTGCAGTGAAT-3', and KO2: 5'-CCTCCTGCATGGAGTTGAGT-3'. Product sizes were wildtype (390bp), *Tsc2^{flox}* (434bp) and *Tsc2^{ko}* (547bp). Cre was detected as previously reported (Way et al., 2009).

Histological Studies

Mice were anesthetized with 2.5% avertin and transcardially perfused with PBS followed by 4% paraformaldehyde (PFA). Brains were removed, post fixed overnight in 4% PFA, dehydrated, embedded in paraffin and sectioned at 5µm. Sections were rehydrated and stained for routine H&E. For immunohistochemistry, rehydrated sections were subjected to antigen retrieval in a microwave with 10mM citrate buffer, pH 6. Sections were blocked with 10% serum from animal in which secondary was raised and 0.5% Triton-X-100 in 1× PBS for 20 minutes. Slides were incubated in primary antibody solution overnight at 4°C. Sections were then washed in 1× PBS and incubated with the secondary antibody for 1 hour and washed in 1× PBS. For immunofluorescence sections were stained with 1:1000 Hoechst for 10 minutes and coverslipped with Fluoromount-G (SouthernBiotech, Birmingham, AL). For DAB immunohistochemistry, slides were incubated with 0.3% H₂O₂ in methanol for 20 minutes before application of primary antibody. After incubation with biotinylated secondary, sections were washed and then incubated with the Vectastain ABC working solution (Vector Laboratories, Burlingame, CA). DAB with or without metal enhancer (Sigma-Aldrich, St. Louis, MO) was used for visualization. TUNEL was performed using the In situ cell death detection kit, POD (Roche, Indianapolis, IN), following the manufacturer's instructions. X-gal staining was performed by using a modification of a previously described protocol (Michaelidis et al., 1996). Images were taken using an Olympus IX81 microscope with a Qimaging RETIGA-2000RV digital camera and processed using Adobe Photoshop (San Jose, CA).

Antibodies

The antibodies used for immunohistochemistry were as follows: Calbindin (1:250, Sigma-Aldrich, St. Louis, MO), Calbindin (1:250 Abcam, Cambridge, MA), Cleaved Caspase-3 (1:200, Cell Signaling Technology, Bedford, MA), GADD153 (CHOP)(1:100, Santa Cruz Biotechnology, Santa Cruz, CA), Hoechst 33258 (1:1000, Invitrogen, Carlsbad, CA), Nitrotyrosine (1:500, Millipore, Billerica, MA), phospho-S6 ribosomal protein (Ser 240/244)(1:100, Cell Signaling Technology, Bedford, MA), ERp57(GRP58) (1:100, Assay Designs, Ann Arbor, MI), Superoxide Dismutase 2 (1:250, Abcam, Cambridge, MA). Secondary antibodies (1:250) (Invitrogen, Carlsbad, CA) were as follows: Alexa Fluor 488 (anti-mouse IgG₁), Alexa Fluor 488 (anti-rabbit IgG_{2a}), Alexa Fluor 594 (anti-rabbit IgG_{2a}), Alexa Fluor 555 (anti-rabbit IgG_{2a}), Alexa Fluor 594 (anti-mouse IgG₁).

Purkinje Cell Quantitation

Three to four sections from two to three mice were used for quantitation unless otherwise noted. The number of Purkinje cells was determined for folia II, VII, IX, and X. The perimeter of the Purkinje cell layer was measured using ImageJ v1.38× (W. Rasband,

National Institutes of Health, Bethesda, MD). Counts are reported as number of Purkinje cells/ distance measured (mm).

Behavioral Analysis

Motor coordination was determined by measuring latency to fall (180s cap) on an accelerating (4–40 rpm over 200s) ENV-576M Rota-Rod (Med Associates, Georgia, VT). The mice were given two trials on one day with approximately two hours between trials. The mean time of the two trials was used in the analysis. Gait was determined by using inkblot analysis. Non-toxic ink was placed on the fore (red) and hindpaw (black) of the mouse and the mouse was allowed to walk down a dark tunnel. The average length and width of the steps were measured.

RNA in situ hybridization

RNA probes were made from a Tsc2 BAC clone (ATCC 9895683). Exons 2–4 were PCR amplified and ligated into a pGEM-T Easy Vector (Promega, Madison, WI) according to manufacturer's specifications. Sense and antisense RNA probes were synthesized using T7 RNA polymerase and a digoxigenin labeling mix (Roche). In situ hybridization was performed as previously described (Sacco et al., 2010).

Rapamycin Treatment

Rapamycin (MP Biomedicals, Solon, OH) was dissolved into methanol and stored in a stock concentration of 1mg/ml at –20°C. Working solution was freshly diluted in PBS before each use. Mice were given intraperitoneal injections (IP) 3 times per week with 2mg/kg rapamycin (MP Biomedicals, Solon, OH). Injections began at post natal day 10 (P10) and continued until analysis at post natal day 90 (P90).

Human Tissue

Human tissue was obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. NICHD Brain and Tissue Bank fixed the right side of the cerebellum in 10% formalin. The cerebellum was then sectioned in 0.5cm intervals from the midline. The second of these sections were ordered for all samples. These matched folia were taken and embedded in paraffin and processed as above. TSC Patient 1' was a 56 year old female. In addition to TSC, she had diffused interstitial lung disease. Her medications were valsartan, hydrochlorothiazide, prednisone, vitamin D, calcium, avaloz and inhaled tiotropium, ipratropium and albuterol. TSC Patient 2' was a 31 year old female who developed epilepsy at nine months of age. She had lung involvement and kidney involvement as part of the manifestation of TSC pathology. Her epilepsy was initially treated with phenobarbital, but seizure frequency increased. Phenytoin and diazepam were used for many years. Several years before death, she received valproic acid and carbamazepine. TSC Patient 3' was a 47 year old female with mental retardation. She had seizures that were treated with valproic acid and phenytoin. She had multiple bilateral kidney cysts as well as angiomyolipomas of the kidneys. She also had masses in her lungs. In addition to the TSC pathology, she had chest pains, pyelonephritis (treated with IV antibiotics), hypothyroidism, recurrent pleural effusions and chylothorax, and chronic renal insufficiency. TSC Patient 4' was a 58 year old male with no additional information available.

Statistical Analyses

Statistical analyses for cell size, gait width, rotarod testing, and rapamycin rescue were performed using a two-tailed student's t-test. Alpha was set equal to 0.05 for statistical significance. Graphs are represented using standard error of the mean.

Results

Loss of *Tsc2* in Purkinje cells causes progressive cell death

To delete the *Tsc2* gene from Purkinje cells, we used a *Tsc2^{fllox}* allele and a *Pcp2-Cre* allele that expresses Cre recombinase from the Purkinje cell specific protein promoter (Barski et al., 2000; Hernandez et al., 2007). Cre expression starts on postnatal day 6 and is fully established one to two weeks later. We performed a cross to generate mice with one functional *Tsc2* allele [*Tsc2^{+/-}*; *Pcp2-Cre* × *Tsc2^{fllox/fllox}* to generate *Tsc2^{fllox/ko}*; *Pcp2-Cre* mice (*Tsc2^{f/-}*; Cre)]. We chose to start with one null allele because a *Tsc2^{fllox/ko}* (*Tsc2^{f/-}*) mouse models a TSC patient with one non-functioning *TSC2* allele. Somatic cell loss of the remaining *Tsc2^{fllox}* allele will occur primarily in Purkinje cells. Genotypes from these matings were observed in the expected Mendelian ratio. We observed spontaneous deaths in about 10% of *Tsc2^{f/-}*; Cre mice before weaning. The cause of death is unknown, but some *Tsc2^{f/-}*; Cre mice had seizures. Surviving mice were healthy and fertile. We detected Purkinje cell loss beginning at one month of age (Fig. 1A,B). Purkinje cell degeneration became progressively more pronounced over time (Fig. 1C). Folium X was somewhat resistant to cell loss, but by 7 months there was clearly some Purkinje cell degeneration (Fig. 1D). Purkinje cells of mutant mice were much larger than wild type cells, indicating activation of mTORC1 ($p=0.01$) (Fig. 1E,F,K). Molecular confirmation of mTORC1 activation was demonstrated by increased levels of phosphorylated ribosomal protein S6 (Ser 240/244) (Fig. 1G,H). Importantly, heterozygous *Tsc2^{+/-}*; Cre and *Tsc2^{f/-}* animals did not lose Purkinje cells by one year of age (data not shown). *In situ* hybridization confirmed loss of *Tsc2* message in all Purkinje cells by 6 weeks of age (Fig. 1I,J). Analysis of Cre activity in the *Pcp2-Cre* animals using the *Rosa26* reporter line demonstrated uniform X-gal staining of all Purkinje cells at 2 months of age (Supplemental Fig. 1).

Tsc2-mediated Purkinje cell death is largely cell-specific

The *Tsc2^{f/-}*; Cre animals model a TSC patient with one germline *Tsc2* null allele, and somatic, Purkinje cell specific loss, of the remaining floxed allele. This genetic scheme makes it difficult to determine whether Purkinje cell loss is mainly due to the genotype of the Purkinje cells (*Tsc2^{-/-}*), or due to the effect of neighboring *Tsc2^{f/-}* cells such as granule or molecular cells. To determine if *Tsc2*-mediated Purkinje cell loss was a cell-type specific process, we generated *Tsc2^{fllox/fllox}*; *Pcp2-Cre* (*Tsc2^{f/f}*; Cre) mice. *Tsc2^{f/f}*; Cre mice were healthy and fertile with 100% surviving past weaning. However, these animals also progressively lost Purkinje cells in a manner almost identical to *Tsc2^{f/-}* animals (Fig. 2). These data indicate that Purkinje cell death is mainly due to loss of *Tsc2* in these specific cells and not due to a *Tsc2*-haploinsufficient environment. Interestingly, by seven months of age, *Tsc2^{f/-}*; Cre mice had lost more Purkinje cells than *Tsc2^{f/f}*; Cre animals (Fig. 2C). This observation suggests that Purkinje cell death at later time points is affected by a *Tsc2* haploinsufficient environment (Fig. 2C). Because the progression of Purkinje cell death was comparable in both *Tsc2^{f/-}*; Cre and *Tsc2^{f/f}*; Cre mice, *Tsc2^{f/f}*; Cre mice were used for further studies.

Tsc2 loss in Purkinje cells causes apoptotic cell death and increased ER and oxidative stress

To assess whether the loss of *Tsc2* caused apoptotic cell death, we analyzed one month old cerebella from *Tsc2^{f/f}*; Cre mice for the expression of the apoptosis markers cleaved caspase 3 (CC3) and fragmented DNA. Many CC3 (Fig. 3A,B) and TUNEL (Fig. 3C,D) positive Purkinje cells were detected in mutant animals compared to control, indicating that apoptosis is one mechanism of cell death in *Tsc2^{f/f}*; Cre mice.

Recent data has demonstrated a role of the TSC complex in controlling endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) (DiNardo et al., 2009; Kang et al., 2010; Ozcan et al., 2008). If ER stress continues unchecked, apoptotic cell death ensues. Therefore we assessed whether ER stress and the UPR were activated in the Purkinje cells of *Tsc2f/f;Cre* mice. We examined Purkinje cell expression of CHOP, a terminal ER stress protein that marks cells for apoptotic death (Oyadomari and Mori, 2004). Purkinje cell expression of CHOP at 1 month of age was substantially increased in the *Tsc2*-null Purkinje cells compared to control (Fig. 4A,B). This observation suggests that the activation of ER stress likely contributes to Purkinje cell death. We then assessed expression of the protein disulfide isomerase (PDI), ERp57/GRP58. ERp57 is a thiol oxidoreductase of the protein disulfide isomerase family that acts as a molecular chaperone (Maattenen et al., 2006; Ni and Lee, 2007). PDIs help catalyze correct disulfide bond formation and protein folding. The expression of PDI (ERp57) was increased in mutant Purkinje cells compared to control cells. Elevated ERp57 suggests that the *Tsc2*-null Purkinje cells activate the unfolded protein response.

To better understand the time course of ER stress and cell death, we examined *Tsc2f/f;Cre* Purkinje cells at P10, P21 and 1 month of age. Both CC3 (Supplemental Fig. 2) and CHOP (Supplemental Fig. 3) were not expressed at P10 or P21, but were expressed by 1 month of age. This is consistent with complete expression of Cre by P21 (Barski et al., 2000). On the other hand, PDI (Supplemental Fig. 4) shows slight activation at P10 and P21. These data suggest that there is slight activation of the ER stress response as early as P10. We hypothesize that unabated mTORC1 activation and protein translation in *Tsc2*-null Purkinje cells overwhelms these cells, leading to CHOP induction and subsequent cell death.

Activation of mTORC1 and protein translation increases cellular energy demands on the mitochondria. Unregulated mitochondrial oxidative phosphorylation leads to detrimental increases in reactive oxygen (ROS) and nitrogen species (RNS) (Bolanos et al., 2009). These harshly oxidative metabolic byproducts can trigger apoptosis. To examine whether the redox state of the *Tsc2*-null Purkinje cells was altered, we examined levels of superoxide dismutase 2 (SOD) and nitrotyrosine adducts. Superoxide dismutases are a major cellular defense against the highly reactive superoxide radical. Protein nitrotyrosine residues are generated by several reactive nitrogen species and are a general indicator of RNS and ROS. We identified increased levels of both nitrotyrosine (Fig. 4E,F) and SOD (Fig. 4G,H) in *Tsc2*-null Purkinje cells. Taken together, these results indicate that increased ER stress, ROS, and RNS in *Tsc2*-null Purkinje cells are likely contributing to cellular degeneration.

***Tsc2f/f;Cre* mice are ataxic and display rotarod deficits**

In order to determine if *Tsc2f/f;Cre* mice suffered motor deficits, gait was analyzed at three months of age. The paws of each mouse were inked before placing it into a narrow tunnel (Fig. 5A,B). Gait width was determined by measuring the distance (cm) between the left step and the adjacent right step. *Tsc2f/f;Cre* mice were ataxic, as they took significantly ($p=0.05$) wider steps than control mice (Fig. 5C). Rotarod testing showed that *Tsc2f/f;Cre* mice tended to fall off of the rotarod sooner than the controls ($p=0.066$) (Fig. 5D). These data demonstrate that loss of *Tsc2* in Purkinje cells induces functional motor deficits in *Tsc2f/f;Cre* mice.

Rapamycin treatment rescues Purkinje cell death and alleviates ER stress

Our data indicate that loss of *Tsc2* in Purkinje cells causes an increase in mTORC1 activity, leading to ER stress and cell death. We hypothesized that if mTORC1 activation is the primary cellular insult leading to Purkinje cell degeneration, then rapamycin should rescue the cell death phenotype. Rapamycin has rescued the TSC-like pathology in many animal

models (Kenerson et al., 2005; Meikle et al., 2008; Woodrum et al., ; Zeng et al., 2008). To test this hypothesis we treated *Tsc2f/f;Cre* mice with intraperitoneal injections of 2 mg/kg rapamycin three times a week from P10 to three months of age. Purkinje cell death was rescued in treated *Tsc2f/f;Cre* mice compared to untreated animals ($p=0.004$)(Fig. 6A–E). *Tsc2f/f;Cre* Purkinje cells are normally enlarged due to mTORC1 activation. Rapamycin treatment reduced Purkinje cell size in *Tsc2f/f;Cre* mice compared to untreated *Tsc2f/f;Cre* mice ($p=0.08$). However, the Purkinje cells of the treated *Tsc2f/f;Cre* mice were still larger than the controls ($p=0.03$)(Fig. 6F). We hypothesize that rapamycin levels were not sufficient enough to reduce mTORC1 activity to control levels. Nonetheless, Purkinje cells were prevented from degeneration at 3 months. We next examined whether rapamycin rescued ER stress. As expected there were significantly fewer CHOP positive *Tsc2*-null Purkinje cells in the rapamycin-treated versus untreated cerebellum samples (Fig. 7). To assess whether the rapamycin-rescued *Tsc2 f/f;Cre* Purkinje cells were still functional, we examined the animals for motor deficits using the inked paw print test. Rapamycin treatment rescued the ataxic gait (Fig. 6G).

Purkinje cell degeneration, mTORC1 and ER stress activation in human TSC cerebellum samples

The striking phenotype of Purkinje cell loss in *Tsc2f/f;Cre* animals prompted us to investigate whether Purkinje cell loss occurs in TSC patients. We obtained four sets of anatomically matched human TSC cerebellum samples and age-matched controls from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland. Sections were stained with H&E and the density of Purkinje cells was determined (Fig. 8A–B). In two of the four TSC samples, Purkinje cell densities were lower than in age-matched control samples (Fig. 8C).

Discussion

Tuberous sclerosis complex (TSC) is a multisystemic disorder that can affect almost any organ, underscoring the importance of *TSC1* and *TSC2* in general cellular physiology. Here we present genetic data that demonstrate an important role for tuberlin in Purkinje cell viability. When tuberlin was selectively deleted from Purkinje cells in either *Tsc2f/-;Cre* or *Tsc2f/f;Cre* animals, we observed a progressive Purkinje cell degeneration due to apoptotic cell death. We believe that the difference in timing of Purkinje cell death may in part be due to the gradual expression of Cre recombinase between P6 and P21 (Barski et al., 2000). Nonetheless, the progressive pattern of Purkinje cell degeneration we observed is characteristic of many Purkinje cell degeneration mutants, such as *woozy* (*wz*), *sticky* (*sti*) and *pcd* (Kyuhou et al., 2006; Lee et al., 2006; Zhao et al., 2005). This patterned Purkinje cell loss characteristic of so many mouse models likely reflects the heterogeneous nature of these cells that represent hundreds of different topographic units in the mammalian cerebellum (Sarna and Hawkes, 2003). For reasons that are unclear, some Purkinje cells may tolerate mTORC1 activation for a much longer time than others. Purkinje cell loss in *Tsc2f/f;Cre* mice was cell type specific; however, *Tsc2f/-;Cre* animals demonstrated greater Purkinje cell loss by 7 months of age than *Tsc2f/f;Cre* animals. These data suggest that a non-cell type specific process caused by a *Tsc2*-haploinsufficient cellular environment enhanced Purkinje cell death. Similar results were observed in a Schwann cell-specific mouse model of neurofibromatosis (*Nf1*). In that *Nf1* model, a permissive *Nf1* haploinsufficient environment was important for tumorigenesis (Zhu et al., 2002). The interplay between *Tsc2* haploinsufficient cells and *Tsc2* null cells is a relatively unexplored area of TSC pathogenesis. Further analysis of these interactions might provide new avenues for pharmacologic development.

To investigate the cellular events that might have led to Purkinje cell death, we explored ER and oxidative stress. Our experiments were based upon recent data that have established an important connection between the TSC complex and normal ER stress regulation (DiNardo et al., 2009; Kang et al., 2010; Ozcan et al., 2008). The endoplasmic reticulum is a delicate organelle. Excessive translation, unfolded proteins, metabolite fluctuations and energy perturbations can trigger a cascade of events dubbed ER stress leading to the compensatory unfolded protein response (UPR) (Hotamisligil, 2010). If a cell cannot adequately cope with ER stress, programmed cell death will ensue. The TSC complex is required for a normal ER stress response, and appears to act as a feedback loop to limit translation via mTORC1 inhibition (DiNardo et al., 2009; Kang et al., 2010; Ozcan et al., 2008). We demonstrated that loss of Tsc2 in Purkinje cells caused expression of the ER stress marker CHOP and the UPR marker PDI. Our data, and those previously reported, suggest that loss of Tsc2 caused a progressive accumulation of proteins in the Purkinje cell, leading to ER stress and UPR. ER stress has also been identified in several other Purkinje cell degeneration mutants, suggesting that this may be a common mechanism leading to Purkinje cell death (Kyuhou et al., 2006; Zhao et al., 2005; Zhao et al., 2010). Purkinje cells in this *Tsc2*-based model appeared to be exquisitely more sensitive to ER and oxidative stress-mediated cell death compared to neurons in a neuron specific Tsc1 deletion mouse (DiNardo et al., 2009). Indeed the Purkinje cell appears to be a metabolically vulnerable cell (Kern, 2003; Welsh et al., 2002). The cause of this selectivity is unclear. It has been proposed that the Purkinje cell may be less efficient in clearing misfolded proteins, or more sensitive to their toxic effects (Lee et al., 2006). A more detailed molecular understanding of this differential sensitivity of Purkinje cells to ER and oxidative stress will reveal many therapeutic targets for a wide array of human diseases.

Increased cellular energy demands as well as ER stress can increase oxidative stress in the form of reactive oxygen and nitrogen molecules (Cullinan and Diehl, 2006). The TSC complex is also involved in the regulation of the cellular redox state (Alexander et al., 2010; Suzuki et al., 2008). Our data demonstrate that Tsc2-null Purkinje cells have significant elevations in ROS and RNS. It is possible that the altered Purkinje cell redox potential contributed to the initiation of the mitochondrial-based apoptotic cascade. Increased oxidative stress has been linked to many neurodegenerative disorders (Emerit et al., 2004; Lin and Beal, 2006). These observations suggest that increased oxidative stress may be an important aspect of TSC pathogenesis, paving the way for potential antioxidant treatment as another mode of therapy.

Loss of tuberlin in Purkinje cells activates the mTORC1 complex. Therefore we reasoned that rapamycin, a relatively selective mTORC1 inhibitor, would rescue the Purkinje cell loss. Intraperitoneal rapamycin treatment from P10 to 3 months of age rescued Purkinje cell death and some degree of cell overgrowth. ER stress was markedly reduced but not completely abolished. These results are opposite to what has been observed *in vitro*, where rapamycin failed to protect Tsc1^{-/-} MEFs or Tsc2^{-/-} LEFs from ER stress induced apoptosis (Kang et al., 2010). In those *in vitro* experiments, ER stress was induced with thapsigargin, tunicamycin or MG134, whereas our *in vivo* model system is based upon a genetic lesion. It is unclear what other pathways may be perturbed by these chemicals. Nonetheless, these conflicting data underscore the importance of *in vivo* model systems and may relate to the inherent differences between Purkinje cells and cultured mesenchymal or epithelial cells. As expected, the rescue of Purkinje cell death was accompanied by a functional rescue of motor deficits.

The Purkinje cell loss in Tsc2f^{-/-};Cre and Tsc2f/f;Cre animals prompted us to examine whether TSC patients might be predisposed to Purkinje cell degeneration. Two of four TSC cerebellum samples showed significant Purkinje cell loss. The variability in Purkinje cell

loss is likely multifactorial. Both of the patients had chronic seizures and had been exposed to phenytoin, two factors associated with cerebellar toxicity (Crooks et al., 2000; Ohmori et al., 1997). Based on our mouse data, we hypothesize that some Purkinje cell loss in these patients might be due to a second hit in either the *TSC1* or the *TSC2* gene. Due to the small number, we cannot yet make any generalizations about Purkinje cell loss and cerebellar dysfunction in TSC patients. While ataxia is not a commonly reported neurologic manifestation of TSC, there may be more subtle cerebellar deficits masked by typical neurologic morbidity such as seizures and developmental delay. Of particular interest is an association of cerebellar pathology and autistic features in patients with TSC (Asano et al., 2001; Eluvathingal et al., 2006; Weber et al., 2000). In particular, the most reproducible anatomic abnormality in postmortem studies of patients with autism is a loss of Purkinje cells (Kern, 2003; Palmen et al., 2004; Ritvo et al., 1986). It will be interesting to assess whether the *Tsc2f*^{-/-};Cre animals exhibit autistic-like behavior. The mouse and human data presented here provide an intriguing stimulus for future studies of cerebellar function in TSC patients and the role of the TSC complex in neurodegeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Analysis of Purkinje cells in *Tsc2* f/f;Cre and *Tsc2* f/f mice. (A–D) Calbindin immunohistochemistry shows progressive Purkinje cell loss in *Tsc2* f/f;Cre animals at different ages: (B) 1 month, (C) 3 months, and (D) 7 months of age. Arrow indicates folium X that was somewhat resistant to Purkinje cell loss. (A) 7 month old control mouse. (E–F) H&E staining shows large dysplastic Purkinje cells of a 1 month *Tsc2* f/f;Cre animal (F) compared to a control (E). (G–H) Immunohistochemistry shows increased phospho-S6 (pS6) indicative of mTORC1 activation in a 1 month old *Tsc2* f/f;Cre animal (H) compared to control (G). (I–J) In situ hybridization showing deletion of *Tsc2* mRNA in a 1.5 month old *Tsc2* f/f;Cre cerebellum (J) compared to control (I). (K) Comparison of cell area shows larger cells in 3 month old *Tsc2* f/f;Cre (n=3) compared with controls (n=3) (*p=0.01). Scale bars 50 µm.

Fig. 2. Comparison of age-dependent Purkinje cell loss between Tsc2f/-;Cre and Tsc2f/f;Cre mice. (A–C) Quantitation of Purkinje cells (Purkinje cells/mm) among control (black), Tsc2f/-;Cre (dark gray); and Tsc2f/f;Cre (light gray) mice in folia (A) II, (B) IX, and (C) X. Only in folium X at 7 months of age (C) was there a statistical difference (* p<0.05) in the number of remaining Purkinje cells between Tsc2f/-;Cre and Tsc2f/f;Cre mice

Fig. 3. Apoptotic Purkinje cell death. (A–B) Immunohistochemistry for cleaved caspase 3 (CC3) shows increased apoptotic cell death in 1 month Tsc2f/f;Cre mice (B) compared to control (A). (C–D) TUNEL analysis confirms apoptotic cell death in 1 month Tsc2f/f;Cre mice (D) compared to control (C). Arrows show TUNEL positive Purkinje cells. Purkinje cells are stained green with calbindin. antibody. Folia are shown in roman numerals. Scale bars 50 μm.

Fig. 4.

ER and oxidative stress are activated in the Purkinje cells of Tsc2f/f;Cre mice. (A–B) CHOP positive (GADD153) Purkinje cells demonstrate activation of ER stress in 1 month Tsc2f/f;Cre mice (B) compared to controls (A). (C–D) Increased expression of ERp57, a protein disulfide isomerase (PDI) in Purkinje cells of 1 month Tsc2f/f;Cre mice (D) compared to control (C). (E–F) Increased expression of nitrotyrosine residues demonstrates elevated oxidative stress in 1 month Tsc2f/f;Cre mice (F) compared to controls (E). (G–H) Elevated oxidative stress is also demonstrated by increased expression of superoxide dismutase (SOD) in 1 month Tsc2f/f;Cre mice (H) compared to controls (G). Purkinje cells are co-labeled with calbindin. Folia are shown in roman numerals. Scale bars 50 μ m.

Fig. 5. Abnormal motor function in Tsc2f/f;Cre mice. (A–C) Abnormal gait as indicated by the inked paw print analysis. Representative images are shown for three month control (A) and Tsc2f/f;Cre (B) mice. Tsc2f/f;Cre mice (n=29) had a significantly wider gait (*p=0.05) than control mice (n=21)(C). Representative gait width measurement is shown in (B). (D) Rotarod analysis showed that Tsc2f/f;Cre mice (n=6) tended to fall off rotarod sooner than controls (n=5)(*p=0.066).

Fig. 6.

Rapamycin treatment rescues Purkinje cell degeneration. (A–D) Calbindin staining shows rescue of 3 month treated Tsc2f/f;Cre mice (D) compared to 3 month untreated Tsc2f/f;Cre mice (C). Treated control mice are shown in (B) and untreated control mice are shown in (A). Scale bar 50μm. (E) Quantitation of Purkinje cell density. Untreated Tsc2f/f;Cre mice lost significantly more Purkinje cells than untreated control mice (**p=0.001). Rapamycin treatment prevented Purkinje cell loss in Tsc2f/f;Cre mice (n=4)(**p=0.004). (F) Quantitation of cell size. Untreated Tsc2f/f;Cre mice had significantly larger Purkinje cells than control mice (n=3)(*p=0.04). Rapamycin treatment partially rescued the enlarged Purkinje cell phenotype compared with untreated Tsc2f/f;Cre mice (p=0.08). This rescue, however, was only partial as treated Tsc2f/f;Cre mice still had larger Purkinje cells than treated control mice (n=4) (*p=0.03). (G) Untreated Tsc2f/f;Cre mice (n=29) also had a wider based gait than control mice (n=21) (p=0.05). Rapamycin treatment partially rescued the gait defect (n=3)(p>0.05).

Fig. 7. Rapamycin reduces ER stress in Tsc2f/f;Cre mice. (A–D) CHOP expression in a treated Tsc2f/f;Cre mouse (D) shows reduced ER stress compared to an untreated Tsc2f/f;Cre mouse (B). Note that CHOP levels in treated Tsc2f/f;Cre mouse are still higher than in control mice (A,C). White arrows indicate some CHOP positive cells. Scale bars 50 μ m. (E) Quantitation of average number of total CHOP positive cells in the cerebellum of the untreated and treated mice.

Fig. 8.

Purkinje cell pathology in TSC patients. (A–B) Human cerebellum sections were H&E stained. Representative TSC patient (2') shown in (B) compared to aged matched control patient (A). Black arrows indicate Purkinje cells. Scale bar 200μm. (C) Lower density of Purkinje cells in some of the TSC patients compared to age-matched controls.