Conditional and Domain Specific Inactivation of the Tsc2 Gene in Neural Progenitor Cells

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Summary

Tuberous sclerosis complex (TSC) is a genetic disease characterized by multiorgan benign tumors as well as neurological manifestations. Epilepsy and autism are two of the more prevalent neurological complications and are usually severe. TSC is caused by mutations in either the TSC1 (encodes hamartin) or TSC2 (encodes tuberin) genes with TSC2 mutations being associated with worse outcomes. Tuberin contains a highly conserved GTPase activating protein (GAP) domain that indirectly inhibits mammalian target of rapamycin complex 1 (mTORC1). mTORC1 dysregulation is currently thought to cause much of the pathogenesis in TSC but mTORC1-independent mechanisms may also contribute. We generated a novel conditional allele of Tsc2 by flanking exons 36 and 37 with loxP sites. Mice homozygous for this knock-in Tsc2 allele are viable and fertile with normal appearing growth and development. Exposure to Cre recombinase then creates an in-frame deletion involving critical residues of the GAP domain. Homozygous conditional mutant mice generated using Emx1Cre have increased cortical mTORC1 signaling, severe developmental brain anomalies, seizures and die within three weeks. We found normal levels of the mutant Tsc2 mRNA though GAP-deficient tuberin protein appears unstable and rapidly degraded. This novel animal model will allow further study of tuberin function including the requirement of the GAP domain for protein stability.

Keywords
cortical development; mTORC1; mTORC2; rapamycin; Tuberous Sclerosis Complex

Results/Discussion

TSC afflicts approximately 1 in 6,000 individuals. Patients have significant morbidity due to multi-organ tumors although neurological involvement is generally more severe. Most patients present at very young ages with epilepsy and up to 90% of patients will have seizures at some point in their life (Crino et al., 2006). Additional neurologic and psychiatric manifestations including autism, attention deficit disorder and anxiety disorders are also quite common and disabling but symptom severity may vary considerably (Joinson et al., 2003; Staley et al., 2011). Inactivating mutations in either the TSC1 or TSC2 genes cause disease although TSC2 mutations are associated with greater symptom severity (Jones et al., 1997). TSC1 and TSC2 encode hamartin and tuberin respectively, which form a heterodimeric regulatory complex. Tuberin contains a highly conserved GTPase activating (GAP) domain that inactivates the G protein Rheb (Maheshwar et al., 1997). Rheb

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inactivation then inhibits mammalian/mechanistic target of rapamycin complex 1 (mTORC1), a critical regulator of protein translation and cellular growth (Benvenuto et al., 2000; Inoki et al., 2003). Loss of tuberin GAP activity is known to contribute to the molecular pathogenesis of TSC (Beaumont et al., 2012; Crino, 2004; Meikle et al., 2008); however, additional mTORC1-independent and rapamycin-resistant abnormalities likely also exist in TSC (Hartman et al., 2009; Hodges et al., 2001; Momose et al., 2007; Noonan et al., 2002; Rosner et al., 2008). Multiple mouse models of TSC have been generated through conditional inactivation of the Tsc1 gene and used to study developmental brain abnormalities (Carson et al., 2012; Fu et al., 2012; Uhlmann et al., 2002; Way et al., 2012). Constitutive mutant alleles of mouse Tsc2 have been reported (Onda et al., 1999) including a variant with a deletion involving the tuberin GAP domain and amino acid substitutions in the rapabtin-5 binding motif (Chevere-Torres et al., 2012; Govindarajan et al., 2005). A conditional mutant of Tsc2 has also been generated by targeting exons 2-4 with flanking loxP sites (Hernandez et al., 2007) that is expected to have complete loss of function when exposed to Cre recombinase.

To study the tissue specific requirement of the tuberin GAP domain in TSC pathogenesis, we designed a novel conditional allele of the Tsc2 gene that selectively targets exons within the GAP domain. A Tsc2 gene targeting vector was generated using recombineering techniques (Liu et al., 2003). Exons 36 and 37 were flanked with intronic loxP sites (Fig. 1a) designed to produce an in-frame deletion of the gene after exposure to Cre recombinase and expected to result in mutant tuberin protein absent 109 amino acids (aa1534-1642) within the GAP domain. A FRT flanked neomycin resistance cassette was used for positive selection of transfected mouse embryonic stem (ES) cells. We successfully targeted the Tsc2 gene in 6% (12 of 192) of screened ES clones as verified by Southern blot analyses and PCR (Fig. 1b). After confirming a normal karyotype, targeted ES cells were used for blastocyst injection. Multiple chimeric mice resulted and germline transmission of the Tsc2 neo.flox allele was confirmed from two independent lines by PCR (Fig. 1c) and direct sequencing of genomic DNA (data not shown).

We anticipated that the presence of the neomycin resistance cassette might interfere with Tsc2 gene transcription or translation and may result in the equivalent of a null Tsc2 allele. If so, this should lead to early embryonic lethality in homozygote mice as previously shown using conventional gene knockout approaches (Hernandez et al., 2007; Onda et al., 1999). We interbred heterozygous Tsc2 neo.flox mice and in fact did not find any homozygous Tsc2 neo.flox offspring from 10 separate litters resulting in 49 total offspring (data not shown). We conclude that homozygous Tsc2 neo.flox mice with the neomycin selection cassette are non-viable and die during embryogenesis.

We then crossed Tsc2 neo.flox mice to flippase recombinase expressing animals to remove the FRT-flanked neomycin resistance cassette in vivo, thus generating the Tsc2^36–37 conditional allele (Fig. 2a). In contrast to Tsc2 neo.flox, heterozygous Tsc2^36–37 crosses produced viable homozygous offspring at Mendelian ratios (Fig. 2b), indicating restoration of Tsc2 gene function after excision of the neomycin resistance cassette. We next assayed Tsc2 gene transcript and tuberin protein levels from brain extracts of mature homozygous Tsc2^36–37 mice and found no significant difference in expression; additionally, Tsc2^36–37 homozygotes were phenotypically indistinguishable from wild-type mice with regards to long-term growth and survival (data not shown). These findings indicate no deleterious effect from the insertion of loxP sites within the Tsc2 gene and provide further evidence that the neomycin cassette accounted for the non-viability of homozygous Tsc2 neo.flox mice.

To verify the functional integrity of the loxP sites and address the impact of this conditional allele upon neurodevelopment, we generated conditional knockout mice using Emx1Cre
expressing mice (Tsc2\textsuperscript{f36–37 Emx1 CKO}). Emx1\textsuperscript{Cre} mice are well characterized for Cre expression in dorsal neural progenitor cells. These cells give rise to excitatory neurons of the cerebral cortex as well as most astrocytes and a subset of oligodendrocytes (Gorski \textit{et al.}, 2002). We also chose Emx1\textsuperscript{Cre} to compare these results to what we have previously seen from the conditional loss of the Tsc1 gene in dorsal neural progenitors (Carson \textit{et al.}, 2012; Magri \textit{et al.}, 2011). We first determined levels of Tsc2 mRNA using quantitative PCR using RNA extracted from P5 dorsal cortex of Tsc2\textsuperscript{f36–37 Emx1 CKO.} There was no significant difference in Tsc2 mRNA expression from Tsc2\textsuperscript{f36–37 Emx1 CKO RNA extracts compared to control littermates (Fig. 3a). Despite equivalent amounts of mRNA, we found much reduced levels of tuberin protein from P5 dorsal cortex protein extracts (Fig. 3b). We sequenced a portion of the Tsc2\textsuperscript{f36–37 cDNA product surrounding the deleted region and confirmed that loss of tuberin protein did not result from use of an ectopic splice site or a frame shift after Cre recombination (data not shown). It is possible that the small amount of tuberin observed from Tsc2\textsuperscript{f36–37 Emx1 CKO brain is the mutant protein; however, we have not been able to resolve a distinct band corresponding to the predicted molecular weight of the mutant protein to support this possibility. Much more likely, rather, we think that the observed tuberin band from CKO extracts is due the minority of cells in the neocortex that are not expected to express Emx1\textsuperscript{Cre}.

\textit{Tsc2\textsuperscript{f36–37 Emx1 CKO} mice were born at expected Mendelian proportions but were obviously impaired with poor growth evident during the first few days of life (Fig. 4a). They started dying at P4 with complete mortality by P21 (Fig. 4b). Spontaneous seizures were witnessed just prior to death in multiple Tsc2\textsuperscript{f36–37 Emx1 CKO mice during routine handling, suggesting that recurrent seizures contributed to their early demise. These phenotypes of seizures and increased mortality were similar but more severe relative to Tsc1 Emx1 CKO mice (Carson \textit{et al.}, 2012) and is also consistent with other conditional knockout models of Tsc2 relative to their Tsc1 counterparts (Zeng \textit{et al.}, 2011). To see if these mice had altered mTOR signaling we determined levels of phosphorylated S6 (pS6), a well established downstream marker of mTORC1 signaling (Hay and Sonenberg, 2004; Ruvinsky \textit{et al.}, 2005). We found diffuse expression of pS6 throughout the dorsal cortex in both control and CKO sections though there was a much wider expression seen throughout the depth of the cerebral cortex in Tsc2\textsuperscript{f36–37 Emx1 CKO (Fig. 5a). To more closely quantitate mTORC1 signaling in the brain of P5 Tsc2\textsuperscript{f36–37 Emx1 CKO mice, we used dorsal cortical protein extracts for immunoblotting pS6 levels as well as phosphorylated 4E-BP1 (p4E-BP1), a direct target of mTORC1 (Schalm \textit{et al.}, 2003). p4E-BP1 was significantly increased in Tsc2\textsuperscript{f36–37 Emx1 CKO dorsal cortex; however, levels of pS6 did not differ significantly between control and CKO (Fig. 5b). The discrepancy between these two readouts may possibly reflect mTORC1 independent activation of S6-kinase (Balendran \textit{et al.}, 1999; Pullen \textit{et al.}, 1998) resulting in high basal levels of S6 phosphorylation even in control cerebral cortex at this early stage of brain development. Finally, given the well documented response of TSC mouse models to mTORC1 inhibitors (Carson \textit{et al.}, 2012; Meikle \textit{et al.}, 2008; Way \textit{et al.}, 2012), we treated several mice with rapamycin. We found that postnatal rapamycin treatment significantly improved growth and prolonged survival in Tsc2\textsuperscript{f36–37 Emx1 CKO mice (Fig. 5c and d) confirming aberrant mTORC1 signaling in Tsc2\textsuperscript{f36–37 Emx1 CKO mice.}

In conclusion, we generated a novel conditional allele of Tsc2 generating an in-frame deletion of exons 36 and 37 encoding a critical portion of the tuberin GAP domain. The conditional nature of our knock-in mutation allows study of tissue specific disease mechanisms under endogenous gene regulatory conditions making it distinct from the constitutively active, transgenic allele used by Chevere-Torres \textit{et al.} despite sharing a similar mutation. Also, in contrast the conditional Tsc2 knock-in allele reported by Hernandez \textit{et al.}, we have confirmed stability of the mutant transcript. The absence of demonstrable tuberin,
increased mTORC1 signaling and response to rapamycin does, however, indicate a loss of function allele that appears to be due to an unexpected requirement of the GAP domain for tuberin protein stability. Our novel Tsc2 allele may then better model the pathogenesis seen in patients with TSC due to GAP domain mutations. Additional investigations will be required to determine the precise mechanism of protein loss from this allele, but it is likely that the tuberin GAP domain is required for overall protein stability and that this allele can be used to study other aspects of developmental disorders in TSC.

METHODS

Knock-in vector construction

A Tsc2 gene targeting vector was generated using BAC recombineering (Lee et al., 2001; Liu et al., 2003). A BAC clone (bMQ 249d21) was used containing 3′ sequence of Tsc2 generated from 129S7/SvEv mice (Wellcome Trust Sanger Institute). Recombineering was used to isolate a region of BAC DNA encompassing Tsc2 GAP encoding exons into the PL253 retrieval vector for subsequent modifications. We used the neomycin selectable IoxP cassette from PL452 to place a 5′ IoxP site within intron 35. An EcoRI restriction site was inserted adjacent to the 5′ IoxP site to permit screening by Southern blotting. A selectable cassette from PL451 with a FRT flanked neomycin resistance gene and another IoxP site was targeted to intron 37 (Fig. 1a). All modifications were confirmed by DNA sequencing.

Generation of knock-in and conditional knockout mice

The targeting construct included left 6.8 kb homology arms and right 5.3 kb homology arms. The knock-in vector was linearized with NotI and transfected into 129S6 mouse ES cells. G418-resistant ES clones were screened by Southern blot using a 3′ external probe (Fig. 1b) with additional confirmation by PCR (Fig. 1c) using primers P1F (5′-TGG TGA GGA CTT CAA ACT GGG CAC C) and P1R (5′-AAG GCC ACG CTG CTT CCC CAC TT) that generate products of 596 base pairs (wildtype) and 2387 base pairs (knock-in). 12 of 192 (6.3%) of the drug resistant clones were successfully targeted. After confirming a normal karyotype, targeted ES cells were injected into C57BL/6 blastocysts to generate chimeric mice. Male chimeras were bred to 129S1/SvImJ mice and their offspring were genotyped by PCR to validate germline transmission. The FRT-flanked neomycin resistance gene was then deleted in vivo by breeding Tsc2neo.flox/+ mice with FLPe expressing mice (129S4/SvJaeSor-Git(Rosa)26Soro[flo])tm1(FLP1)DymJ, #3946 Jackson Laboratory, Bar Harbor, ME) (Farley et al., 2000). Tsc2 Emx1 conditional knockout mice were generated by breeding homozygous Tsc2f36–37 mice to Tsc2f36–37/++; Emx1Cre mice. Tsc2f36–37 mice were on a 129 mixed strain background and Emx1Cre mice (B6.129S2-Emx1tm1(cre)Krl/J, #5628, Jackson Laboratory, Bar Harbor, ME) were maintained on a C57BL/6J background. All procedures involving mice were approved by the Vanderbilt University IACUC. Tsc2f36–37 mice will be made available to the research community upon request.

Genotyping

Genomic DNA was isolated from tail or ear samples and analyzed using PCR. Mice carrying the wild-type and Tsc2f36–37 alleles were genotyped in a single PCR reaction using forward primer P2F (5′-TGGCAGGAGCAGGGTCATG) and reverse primer P2R (5′-TTTACAGTACCCGACGTCG) with product lengths of 388 base pairs (wildtype) and 312 base pairs (Tsc2f36–37 allele). PCR conditions: 94° C for 3 minutes, (94° C for 30 seconds, 68° C for 30 seconds, 72° C for 1 minutes) × 31 cycles, and 72° C for 10 minutes.
Immunohistochemistry

Tissues for immunohistochemistry were processed as previously described (Carson et al., 2012; Fu et al., 2012). Briefly, P5–6 pup brains were rapidly dissected and fixed overnight with 4% paraformaldehyde then cryoprotected with 30% sucrose solution for 2 days. Coronal sections at 10–20 μm were mounted on glass slides. Primary antibody: rabbit phospho-S6 (S240/244) (1:200, Cell Signaling). Secondary antibody: Alexa Fluor 568 goat anti-rabbit IgG (1:500, Life Technologies). Negative controls for each experiment were performed by omission of primary antibody. Photomicrographs were obtained using a Zeiss epifluorescence microscope.

Real time RT-PCR

Total RNA was isolated from fresh frozen dorsal cortical brain tissue using an RNeasy Mini kit (Qiagen). cDNA for use in quantitative PCR was generated using an oligo(dT) primer (Life Technologies). Age matched wildtype mouse brain tissue was used as the control. Tsc2 specific primers were used to amplify a 5′ region of the cDNA outside of the targeted region. Relative expression of Tsc2 mRNA in control and Tsc2 Emx1 CKO brain was normalized with levels of GAPDH mRNA expression. Each cDNA sample was assayed in triplicate and relative quantification was determined using the ΔΔC(t) method. qPCR reactions were quantified using a SYBR green based kit (Life Technologies) on a Bio-Rad CFX96 Real-Time PCR system using the following conditions: 95° C for 10 minutes, (95° C for 15 seconds, 60° C for 1 minute) × 40 cycles.

Immunoblotting

Immunoblots were done as previously reported (Carson et al., 2012; Fu et al., 2012). Tissues for immunoblotting were dissected and flash frozen in liquid nitrogen prior to protein extraction. Primary antibodies: rabbit tuberin N-terminus (1:500, abcam); phospho-S6 (Serine 240/244), S6, phospho-4E-BP1, (Threonine 37/46) all rabbit and used at 1:1000 (Cell Signaling). Primary antibody against β-actin was from mouse (1:2000, Sigma). Secondary antibodies: Alexa Fluor 680 goat anti-rabbit IgG (Life Technologies) and IRDye 800 goat anti-mouse IgG (Li-Cor). Fluorescence was detected using the Odyssey fluorescence imaging system (Li-Cor). Digital band density was quantified using ImageJ software (NIH, Bethesda, MD). Absolute density for each band was determined using an area-under-the curve method. Data from each sample was normalized to internal loading control proteins as indicated and expressed as fraction of the experimental control.

Rapamycin Treatment

Tsc2 Emx1 CKO mice (n=5) were treated with intraperitoneal injections of rapamycin at a dose of 0.1 mg/kg/day. Rapamycin (LC Laboratories, Woburn, MA) was dissolved as a stock at 30 mg/mL in ethanol and then diluted with vehicle consisting of 0.25% Tween-20/0.25% polyethylene glycol in PBS.

Statistical Analysis

Results were assessed for statistical significance using a Student’s t-test or Log-Rank test as appropriate with a p-value less than 0.05.

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Fig. 1.
Generation of Tsc2\textsuperscript{neo.flox} mice. (a) Schematic of targeting vector with homologous left 6.8 kb and right 5.3 kb arms. Tsc2 genomic structure and targeted allele Tsc2\textsuperscript{neo.flox} containing the PGK-neo cassette flanked by Frt sites (red arrowheads) and loxP sites flanking exons 36 and 37 that encode critical residues of the GAP domain. An additional EcoRI site to aid in genotyping was also added immediately 5' to the loxP site. Select regions have been expanded to highlight PCR primer sites. (b) Southern blot of EcoRI digested genomic DNA from mouse ES cells showing wild-type band (18.4 kb) and targeted Tsc2 allele (11.6 kb) in clone 4. C. PCR of wild-type and Tsc2\textsuperscript{neo.flox} ES cells with primers P2F and P2R confirming targeting of the Tsc2 allele with 596 bp band only in wild-type but an additional 2387 bp product in Tsc2\textsuperscript{neo.flox} mice.
Fig. 2.
Creation of Tsc2^f36–37 mice with loxP sites flanking exons 36 and 37. (a) Mice heterozygous for the Tsc2^neo.flox allele were crossed with flippase expressing animals to remove the PGK-neo cassette. (b) Summary of litter genotyping from ten sequential heterozygous Tsc2^f36–37 crosses showing Mendelian birth proportions.
Fig. 3.
Normal levels of Tsc2 mRNA but decreased tuberin protein in Tsc2^{f36–37} Emx1 CKO mice. (a) We used quantitative PCR to measure Tsc2 mRNA extracted from the dorsal cortex of P5 control littermate and Tsc2^{f36–37} Emx1 CKO mice. N=3 control and 3 Tsc2^{f36–37} Emx1 CKO mice. Inset: normalized Tsc2 expression. ns = not significant (b) Immunoblotting using an anti-tuberin antibody that recognizes an N-terminal epitope shows much decreased protein in the dorsal cortical extracts from Tsc2^{f36–37} Emx1 CKO mice compared to control littermates. *p<0.05 by Student’s t-test. N=4 control and 4 Tsc2^{f36–37} Emx1 CKO mice.
Fig. 4. *Tsc2^f36–37 Emx1* CKO mice are smaller than control littermates and die by three weeks of life. (a) Mice were weighed at postnatal day 5, *Tsc2^f36–37 Emx1* CKO were much smaller and runted appearing. N=7 control and 7 *Tsc2^f36–37 Emx1* CKO mice. (b) They started dying by the 4th postnatal day and all *Tsc2^f36–37 Emx1* CKO mice were dead by postnatal day 21. N=14 control and 11 *Tsc2^f36–37 Emx1* CKO mice. ****p<0.001, Student’s t-test for decreased body mass. The increased mortality of *Tsc2^f36–37 Emx1* CKO mice was statistically significant with a p<0.001 using a Log-Rank test.
Fig. 5. 
*Tsc2*^{f36–37}* Emx1 CKO* mice have increased mTORC1 signaling in the brain and respond to rapamycin treatment. (a) Increased expression of phospho-S6 throughout the cerebral cortex of P5 Tsc2*^{f36–37}* Emx1 CKO mice. n=4 control and 4 Tsc2*^{f36–37}* Emx1 CKO mice. Size bar equals 100 μm. CL I=cortical layer I, ML=midline, WM=white matter/corpus callosum, LV=lateral ventricle. (b) Immunoblotting of dorsal extracts from P5 Tsc2*^{f36–37}* Emx1 CKO mice shows no change in phospho-S6 but increased overall levels of phospho-4E-BP1. Two representative control and Tsc2*^{f36–37}* Emx1 CKO extracts are shown. Quantitation of immunoblotting confirms increased levels of phospho-4E-BP1. *p<0.05 by Student’s t-test. (c) Rapamycin treatment rescues small size of Tsc2*^{f36–37}* Emx1 CKO mice, *p<0.05 by Student’s t-test. (d) Rapamycin treatment of Tsc2*^{f36–37}* Emx1 CKO mice prevented early death as compared to the previous cohort of untreated CKO mice. Rescue was statistically significant using a Log-Rank test, p<0.001. N=5 Tsc2*^{f36–37}* Emx1 CKO mice.